For my mum and dad
I hereby declare that the work described in this thesis is the result of my own independent investigation, unless otherwise stated.

This work has not been previously submitted for any degree and is not being currently submitted in candidature for any other degree.

Chen Chen
ABSTRACT

With high affinity and specificity, antibodies are now proven biotherapeutics for a wide range of diseases, such as cancer and immunological conditions. However, antibody Fc-domain mediated cross reactivity with associated side effects has hindered the development of antibody therapy in a number of applications. The development of engineered recombinant antibody fragments to address the problems seen with whole monoclonal antibodies (mAbs). Their smaller size enables rapid antigen localisation, tissue penetration and higher specificity ratios. Whereas, poor pharmacokinetics due to fast blood clearance from the circulation is an important obstacle to these small molecules, and modification of antibody fragments is required for improved tissue exposure and uptake.

In order to improve protein pharmacokinetics whilst maintaining good tissue/tumour to blood ratios and low cross-reactivity, conjugation of antibody fragments with a natural polymer polysialic acid (PSA) composed by N-acetylneuraminic acid (Neu5Ac or NANA), has been investigated. Compared to polyethylene glycol (PEG) conjugation, which is the predominant approach, PSA appears to be a better alternative because it is biodegradable, non-toxic and highly hydrophilic.

In this project, an anti-carcinoembryonic antigen scFv (MFE-23), and an anti-HER2 scFv (C6.5) were used as model antibody fragments to conjugate with PSA. To overcome the limitations of chemical conjugations, this project was focused on the investigation of recombinant polysialylation of single chain antibodies. ScFv genes were firstly incorporated with a naturally polysialylatable (Ig5 and FN1) domains from human neuronal cell adhesion molecule. After transfections into polysialyltransferases-expressing HEK-293 cells, the biosynthesized sialic acid was enzymatically added to the N-glycosylated fusion protein Ig5 domain for elongating the attached PSA chain. Expressed by monoclonal cell secretion, polysialylated scFvs were successfully generated and purified. Based on the negative charge of PSA, an ion exchange method was developed for separating different polysialylated scFv isoforms. The degree of polymerization (DP) was detected by mass spectrometry, which showed an average of recombinant DP of up to 20 NANA residues attached to a complex N-glycan core. Affinity binding tests by ELISA confirmed the antibody $K_D$ was significantly retained at approximately 5nM after polysialylation, however BIAcore kinetic analysis demonstrated influencing change to the $K_D$ due to the strong negative charge of PSA. In vitro
experiments using both FACS and confocal microscopy suggested that recombinant polysialylation has no affect of targeting antigens expressed on the live cell surface. Molecular hydrodynamic radius increases of polysialylated scFvs were detected by size exclusion chromatography, which led to corresponding pharmacokinetic improvement from in vivo mice studies with enhanced serum half-lives.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABD</td>
<td>Albumin-binding domain</td>
</tr>
<tr>
<td>ADCC</td>
<td>Antibody dependent cellular cytotoxicity</td>
</tr>
<tr>
<td>ADCP</td>
<td>Antibody-dependent cell phagocytosis</td>
</tr>
<tr>
<td>ADEPT</td>
<td>Antibody-directed enzyme prodrug therapy</td>
</tr>
<tr>
<td>ADME</td>
<td>Absorption, distribution, metabolism and excretion</td>
</tr>
<tr>
<td>AIID</td>
<td>Autoimmune and inflammatory disorder</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myeloid leukaemia</td>
</tr>
<tr>
<td>AP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen-presenting cell</td>
</tr>
<tr>
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<tr>
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<td>Complimentary determining region</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
</tr>
<tr>
<td>CEA</td>
<td>Carcinoembryonic antigen</td>
</tr>
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<td>C_H</td>
<td>Constant heavy chain</td>
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<tr>
<td>C_L</td>
<td>Constant light chain</td>
</tr>
<tr>
<td>CLSM</td>
<td>Confocal laser scanning microscopy</td>
</tr>
<tr>
<td>CMAS</td>
<td>Cytidine monophospho-N-acetylneuraminic acid synthetase</td>
</tr>
<tr>
<td>CMP</td>
<td>Cytidine monophosphate</td>
</tr>
<tr>
<td>CMPNT</td>
<td>CMP-Neu5Ac transporter</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>cPCR</td>
<td>Colony polymerase chain reaction</td>
</tr>
<tr>
<td>C_{18}SP</td>
<td>Sep-Pak™ C_{18} cartridge</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DE</td>
<td>Delayed Extraction</td>
</tr>
<tr>
<td>dH_{2}O</td>
<td>Distilled water</td>
</tr>
<tr>
<td>DIG</td>
<td>Digoxigenin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>-------------</td>
<td>-----------</td>
</tr>
<tr>
<td>DHB</td>
<td>2,5-dihydroxybenzoic acid</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DP</td>
<td>Degree of polymerization</td>
</tr>
<tr>
<td>dsFv</td>
<td>Disulfide-stabilized Fv antibody fragment</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>E.Coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>ECD</td>
<td>Extracellular domain</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbant assay</td>
</tr>
<tr>
<td>EMEA</td>
<td>European medicine agency</td>
</tr>
<tr>
<td>Endo-N</td>
<td>Endoneuraminidase</td>
</tr>
<tr>
<td>EPO</td>
<td>Erythropoietin</td>
</tr>
<tr>
<td>Erk</td>
<td>Extracellular regulated kinase</td>
</tr>
<tr>
<td>ES</td>
<td>Electrospray</td>
</tr>
<tr>
<td>Exo-N</td>
<td>Acetyl-neuraminyl hydrolase / Exoneuraminidase</td>
</tr>
<tr>
<td>Fab</td>
<td>Fragment of antigen-binding</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>Fc</td>
<td>Fragment crystallisable</td>
</tr>
<tr>
<td>FcRn</td>
<td>Neonatal Fc-receptor</td>
</tr>
<tr>
<td>FcγR</td>
<td>Fcγ receptor</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and drug administration</td>
</tr>
<tr>
<td>FD</td>
<td>Fluorometric detection</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FL</td>
<td>Full length</td>
</tr>
<tr>
<td>FN</td>
<td>Fibronectin domain</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast protein liquid chromatography</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle stimulation hormone</td>
</tr>
<tr>
<td>FT</td>
<td>Flow-through</td>
</tr>
<tr>
<td>Gal</td>
<td>Galactose</td>
</tr>
<tr>
<td>αGalT</td>
<td>α1,3galactosyltransferase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Term</td>
</tr>
<tr>
<td>--------------</td>
<td>------</td>
</tr>
<tr>
<td>GDNF</td>
<td>Glial-derived neurotrophic factor</td>
</tr>
<tr>
<td>GF</td>
<td>Growth factor</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>Gl</td>
<td>Gastrointerestine</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-acetylglucosamine</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>GMean</td>
<td>Geometric mean fluorescence value</td>
</tr>
<tr>
<td>GNE</td>
<td>Glucosamine (UDP-N-acetyl)-2-epimerase/N-acetylmannosamine kinase</td>
</tr>
<tr>
<td>GNPNAT1</td>
<td>Glucosamine-phosphate N-acetyltransferase 1</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycophosphatidyl-inositol</td>
</tr>
<tr>
<td>h (or hr)</td>
<td>Hour</td>
</tr>
<tr>
<td>HACA</td>
<td>Human anti-chimeric antibody</td>
</tr>
<tr>
<td>HAHA</td>
<td>Human anti-human antibody</td>
</tr>
<tr>
<td>HAMA</td>
<td>Human anti-mouse antibody</td>
</tr>
<tr>
<td>HAP</td>
<td>Homo amino acid polymer</td>
</tr>
<tr>
<td>HEMB</td>
<td>Homogenized embryonic mouse brain</td>
</tr>
<tr>
<td>HER</td>
<td>Human EGF receptor</td>
</tr>
<tr>
<td>HES</td>
<td>Hydroxyethyl starch</td>
</tr>
<tr>
<td>His&lt;sub&gt;6&lt;/sub&gt;</td>
<td>Hexahistadine</td>
</tr>
<tr>
<td>HMW-MAA</td>
<td>Human high molecular weight melanoma-associated antigen</td>
</tr>
<tr>
<td>HPAEC</td>
<td>High performance anion-exchange chromatography</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HSA</td>
<td>Human serum albumin</td>
</tr>
<tr>
<td>ICH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Methyl iodide</td>
</tr>
<tr>
<td>ID</td>
<td>Injected dose</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IgC2-like</td>
<td>Homologous to the Ig C2 constant region</td>
</tr>
<tr>
<td>IgV-like</td>
<td>Ig variable homology domain</td>
</tr>
<tr>
<td>IL-2</td>
<td>Interleukin-2</td>
</tr>
<tr>
<td>IM</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>IMAC</td>
<td>Immobilised metal affinity chromatography</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>IR</td>
<td>Infusion reaction</td>
</tr>
<tr>
<td>iv</td>
<td>Intravenous</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>KBH&lt;sub&gt;4&lt;/sub&gt;</td>
<td>Potassium borohydride</td>
</tr>
<tr>
<td>kbp</td>
<td>Kilo base-pair</td>
</tr>
</tbody>
</table>
\( K_D \)  
Dissociation equilibrium constant

kDa  
Kilo dalton

KDN  
2-keto-3-deoxynononic acid

\( k_{\text{off}} \)  
Off-rate

KOH  
Potassium hydroxide

\( k_{\text{on}} \)  
On-rate

LS  
Leader sequence

M  
Molar

MAb  
Monoclonal antibody

MAC  
Membrane attack complex

MAG  
Myelin-associated glycoprotein

MALDI-TOF  
Matrix Assisted Laser Desorption Ionisation-Time of Flight

ManNAc  
\( N\)-Acetyl-D-mannosamine

MAPK  
Mitogen-activated protein kinase

MEK  
MAPK/extracellular signal-related kinase

MEKK  
MEK kinase

mTOR  
Mammalian target of rapamycin

MAPK  
Mitogen-activated protein kinase

MHC  
Major histocompatibility complex

MS  
Mass spectrometry

MW  
Molecular weight

\( MW_{\text{app}} \)  
Apparent molecular weight

m/z  
Mass to charge ratio

NaCl  
Sodium chloride

NANA  
5-\( N\)-acetyleneuraminic acid

NANP  
\( N\)-Acetyleneuraminic acid phosphatise

NANS  
\( N\)-Acetyleneuraminic acid synthase

NaOH  
Sodium hydroxide

NCA  
Nonspecific cross-reacting antigen

NCAM  
Neural cell adhesion molecule

NEU  
Neuraminidase

NeuNAc  
5-\( N\)-acetyleneuraminic acid

Neu5Ac  
5-\( N\)-acetyleneuraminic acid

Neu5Gc  
5-\( N\)-glycolyneuraminic acid

Neu5,9Ac\(_2\)  
\( N\)-acetyl-9-\( O\)-acetyleneuraminic acid

NHL  
Non-Hodgkin's lymphoma

NK  
Natural killer cell

NMR  
Nuclear magnetic resonance
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen activator inhibitor-1</td>
</tr>
<tr>
<td>PAS</td>
<td>Poly-(Pro-Ala-Ser)</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PD</td>
<td>Polydextran</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PGM</td>
<td>Phosphoacetylglucosamine mutase</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinases</td>
</tr>
<tr>
<td>PK</td>
<td>Pharmacokinetics</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLAP</td>
<td>Placental alkaline phosphatase</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PNGas F</td>
<td>N-Glycosidase F</td>
</tr>
<tr>
<td>PolyST</td>
<td>Polysialyltransferase</td>
</tr>
<tr>
<td>PrP</td>
<td>Prion Protein</td>
</tr>
<tr>
<td>PSA</td>
<td>Polysialic acid</td>
</tr>
<tr>
<td>PSMA</td>
<td>Prostate specific membrane antigen</td>
</tr>
<tr>
<td>PST</td>
<td>ST8SiaIV</td>
</tr>
<tr>
<td>PVA</td>
<td>Polyvinylalcohol</td>
</tr>
<tr>
<td>RES</td>
<td>Reticuloendothelial system</td>
</tr>
<tr>
<td>RI</td>
<td>Reflection index</td>
</tr>
<tr>
<td>rpm</td>
<td>Rotations per minute</td>
</tr>
<tr>
<td>RSA</td>
<td>Rat serum albumin</td>
</tr>
<tr>
<td>RSV</td>
<td>Respiratory syncytial virus</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>RU</td>
<td>Response unit</td>
</tr>
<tr>
<td>SA</td>
<td>Streptavidin</td>
</tr>
<tr>
<td>SC</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>scDb</td>
<td>Single-chain diabody</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>SEC</td>
<td>Size exclusion chromatography</td>
</tr>
<tr>
<td>scFv</td>
<td>Single-chain fragment variable</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immune deficient</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
</tbody>
</table>
SIP        Small immune proteins
SMP        Schwann cell myelin protein
Sn         Sialoadhesin
SPR        Surface plasmon resonance
ST3GAL     ST3 β-galactoside α2,3-sialyltransferase
ST6GAL     ST6 β-galactosamide α2,6-sialyltransferase
ST6GALNAC  ST6 (α-N-acetyl-neuraminyl-2,3-β-galactosyl-1,3)-N-acetylgalactosaminideα2,6-sialyltransferase
ST8SIA     ST8 α-N-acetyleneuraminide α2,8-sialyltransferase
STX        ST8Sialll
taFv       Tandem scFv
TAE        Tris-acetate EDTA buffer
TAG        Tumour-associate glycoprotein
TBS        Tris buffered saline
TC         Tissue culture
TCM        Tissue culturing medium
TEMED      N,N,N',N'-Tetramethylethylenediamine
TFA        Trifluoroacetic acid
TGF        Transforming growth
TMD        Transmembrane domain
TNF        Tumour necrosis factor
TPKC       L-1-tosylamido-2-phenylethyl chloromethyl ketone
TT         Triple transfection
TTBS       TBS-Tween
TT-Hc      Recombinant Hc fragment of tetanus neurotoxin
t_{1/2α}    Half-life of α-phase clearance (distribution phase)
t_{1/2β}    Half-life of β-phase clearance (elimination phase)
UAP        UDP-N-acetylgalactosamine pyrophosphorylase 1
UDP        Uridine diphosphate
UT         Untransfected
UV         Ultra violet
VEGF       Vascular endothelial growth factor
VEGFR      VEGF tyrosine kinases receptor
V_{H}      Variable heavy chain
V_{L}      Variable light chain
VIB        B-subunit of *E. Coli* verotoxin
VPA        Valproic acid
WT         Wild type
“Don't get set into one form, adapt it and build your own, and let it grow, be like water. Empty your mind, be formless, shapeless — like water. Now you put water in a cup, it becomes the cup; You put water into a bottle it becomes the bottle; You put it in a teapot it becomes the teapot. Now water can flow or it can crash. Be water, my friend.” — Bruce Lee
CHAPTER 1

Introduction
1.1 Antibodies in Therapy

1.1.1 Antibody – Immune system’s powerful weapon

Natural antibodies are immunoglobulin glycoproteins, naturally synthesized and secreted by all vertebrate B lymphocytes. They are critical components in the mammalian immune system which connects the innate and adaptive responses and are specifically produced against antigens that are mostly foreign and/or harmful to the organism. The typical immunoglobulin (Ig) isotype, IgG, is a Y-shaped molecule, with a molecular weight of 150 kDa and composed of two identical heavy chains and two identical light chains linked by disulphide bridges (Porter 1973) (Figure 1.1).

![Figure 1.1. The domain structure of IgG antibody molecule.](image)

As the most classical immunoglobulin, IgG has a Y configuration and flexible hinge region (black lines) that allows extensive movement of the Fab arms. Both identical light chains and heavy chains are indicated, and each light chain and heavy chain is interlinked by disulphide bonds (yellow lines) in order to form one half of a full-size antibody molecule. The two halves are further covalently linked by disulphide bonds. One light chain (25 kDa) consists of a C-terminal constant domain, C\_L (blue) and an N-terminal variable domain, V\_L (green), while each heavy chain preserves four compartments, one N-terminal heavy variable domain, V\_H (red), and three constant domains, C\_H1, C\_H2, and C\_H3 (purple). Hypervariable regions are associated with both V\_H and V\_L domains, which recognise the antigens and initiate the antibody-antigen binding. Each IgG molecule has a pair of Fab arms that maintain the same antigen specificity of the hypervariable regions, making the antibody bivalent. The C\_H2 domain in the Fc region contains glycosylated moieties (pink) at Asn297 that contribute to immunological responses, and modulate pharmacokinetic properties.

In humans there are two types of light chain termed kappa (κ) and lambda (λ), which are encoded on chromosome 2 (Malcolm et al. 1982) and on chromosome 22 (de la Chapelle et al. 1983) respectively. The heavy chain locus is found on chromosome 14 (Cox et al. 1982), with
its constant region genes dictating five different antibody isotypes (A, D, E, G and M). Each heavy chain and light chain are connected by disulphide bonds, forming two halves of the antibody. Proteolytic enzyme digestion with papain cleaves above the hinge region, and separates the IgG molecule into two 50 kDa Fab (fragment antigen-binding) fragments retaining the antigen recognition ability and a 50 kDa domain that can be readily crystallized termed Fc (fragment crystallisable) (Deutsch et al. 1961; Porter 1973).

IgG molecules circulate in the blood as one of the most predominant and important serum immuno-surveillant proteins to recognise non-self antigens present on various infectious agents or allergens that exogenously entered the body, or endogenous malignant targets such as cancer antigens. The Fab fragments of an antibody molecule are principally responsible for antibody-antigen binding as its name refers to. On the N-terminus of each Fab fragment, the association of the heavy and light chain variable domains leads to the antigen combining region, and each of the variable domains hosts three hypervariable sequence regions (Figure 1.1). Scattered within the frame-work of variable sequence regions at the complimentary determining regions (CDRs) which are specifically presented to accomplish the targeting purpose of the antibody to its cognate antigen epitopes (Chothia et al. 1992; Chothia et al. 1998). The Fc domain derived primarily by the antibody heavy chain constant domains (CH2 and CH3) elicits the effector functions including antibody dependent cellular cytotoxicity (ADCC) or complement dependent cytotoxicity (CDC), and innate immune responses. With the aid of the antibody Fc portion, effector cells including macrophages, neutrophils and natural killer (NK) lymphocytes are recruited, pertinently and culminantly to neutralise and eliminate the disease-causing sources (Casadevall et al. 2004). Moreover, immunoglobulins also have a natural retention system and catabolic clearance control depends on Fc-domain to neonatal Fc-receptor (FcRn, salvage receptor) binding via the reticulo-endothelial system (Ghetie and Ward 1997; Anderson et al. 2006). IgG compromises four subclasses, IgG1 to IgG4, where human IgG1 and IgG3 have the highest binding efficiencies in harnessing effector functions among all antibody classes and subclasses, while IgG2 and IgG4 are relatively poor. Base on the excellent antigen recognition, IgG1 molecules have been mostly chosen to make therapeutic antibodies (Isaacs 2009).

Arguably, as the most useful weapon in the immune system, antibodies exhibit huge variation to potential harmful antigenic entity. B-lymphocytes are the manufacturers of different antibodies. They have immunoglobulin surface receptors for the decisive engagement to its
selected antigen (Moller 1977). This interaction triggers a cascade process co-stimulated by T-cells and other immune components, and develops the B-cell with a clonal expansion and maturation, together with the secretion of soluble IgMs, which pentavalently target to a specific antigenic epitope. The matured B-cells then step into a process called somatic hypermutation, during which they may undergo various recombination events that mostly occur in the antigen hypervariable regions, and switch class to produce soluble bivalent IgG molecules that are possessed with an enhanced affinity to recognise and bind a specific antigen (Harriman et al. 1993; Neuberger and Milstein 1995; Li et al. 2004).

1.1.2 History of Antibody Based Therapies

Antibody-based therapies have experienced their historical rises and falls, abandonment and renaissance, and now are gradually entering the era of the illustrious. Discovered in the early 1890s, Behring and Kitasato found that specific antibodies were capable in being used to fight against bacterial toxins. This finding led to a vast development of using passive antibody therapy for infectious disease treatment (Casadevall and Scharff 1995; Casadevall et al. 2004). The initial approaches of antibody generation were derived from immunized animals or human donors; therefore this type of antibody therapy was also called “serum therapy”. Notwithstanding the effectiveness of serum therapy in infectious disease treatment, the associated side effects were far more severe and considerable. This is due to the large amount of other foreign proteins in the animal serum also introduced to the patients during this type of antibody administration, which caused immediate hypersensitivity reactions to serum sickness. Although, later on improved purification methods reduced the toxicity of antibody preparations, serum therapy still went into a decline, as by the late 1940s, sulphonamide drugs and other types of chemotherapies were practised clinically to have superior effects to treat microbial causing diseases, and produced in a relatively more economical way and safer in administration (Casadevall et al. 2004). This nearly pressurised antibody-based therapy into an abandonment for clinical use, however a number of viral and hard to treat diseases kept antibody therapies within a niche field, and consequently provoked a reformational renaissance of massive therapeutic antibody applications for an expanded range of disease treatments.

Tracking the time line back a hundred years ago (Figure 1.2), during the age of serum therapy, German chemist Paul Ehrlich proposed that antibodies could be used as "magic bullets" to
selectively deliver toxins to targets in order to eradicate human diseases (Ehrlich 1956). Following this concept, the revolutionary switch-over of therapeutic antibody history began from original immunized animal based polyclonal antibody production to monoclonal antibody (mAb) derived from a single cell line. The domination of monoclonal antibody based therapies initiated after the first generation of mAbs produced in 1975 from a hybridoma cell line developed by Georges Köhler and César Milstein (Kohler and Milstein 1975). This hybridoma method consisted of the selection of an immunized B-lymphocyte clone that persisted in secreting specific antibodies recognising a unique determinant antigen epitope. Several murine-originate mAbs were then developed in the early 1980s against various antigens, including the first commercialised anti-CD3 mAb, OKT3 (muromonab) from Johnson & Johnson’s Orthoclone and from others targeting cancer antigens (Bargmann et al. 1986; Drebin et al. 1986). However, several limitations of these murine mAbs have also been critically reviewed from their clinical trials, such as immunogenicity when injected into humans, also known as human anti-mouse antibodies (HAMA) responses, shortened plasma circulation half-life, and inefficient effector function recitations due to their rodent constant regions (Shawler et al. 1985; Ober et al. 2001). The initial strategy to overcome these anaphylactic problems was to construct a chimeric antibody consisting around 1/3 parental mouse antibody with all antigen recognition features reserved, joined with human constant domains for the remaining 2/3 (Boulianne et al. 1984; Morrison et al. 1984). Chimerisation reduced the murine limitation somewhat but the significant improvement of mAb activity for clinical use was greatly renovated after the development of humanisation, which was processed by grafting the murine complementary-determining regions into a human immunoglobulin framework (Riechmann et al. 1988), and this process resumed approximately 95% human protein to the mAb. Finally, fully human mAbs have been made through technologies including phage display or using human immunoglobulin transgenic mice (McCafferty et al. 1990; Bruggemann et al. 1991; Green et al. 1994; Fishwild et al. 1996; Mendez et al. 1997), both of which utilize human variable regions as repertoires in construction. Fully human mAbs are predicted to be dominant for the future antibody drug launches, as it has the lowest immune related side effects for reaching the highest therapeutic function.
Figure 1. Development of monoclonal antibodies. (a) The early timeline of mAb development for clinical applications. (b) Schematic evolution of mAbs from murine (green shading) to fully human (blue shading). Red spheres indicate glycosylation in the constant domain. Figure (b) was taken from (Deonarain 2008).
1.1.3 Approved mAbs for Disease Treatment

After more than a century of evolutionary research and development, antibodies have now proven to be very powerful and valuable biotherapeutics, with more and more candidates going into clinical development for human disease diagnosis and treatment. A spectrum of diseases, including cancer, autoimmune and inflammatory disorder (AIID), infection, ophthalmic disease and heart disease have been treated clinically with specific and innovative antibody drugs. The growing list of antibody therapeutics approved by the US Food and Drug Administration (FDA) and by the European Medicine Agency (EMEA) also attracted a great deal of attention and investment by the pharmaceutical industries (Wu and Senter 2005; Reichert 2008). The global market for antibody therapy has economically succeeded in 2006 worth approximately US $24 billion (Maggon 2007), and sales are projected to increase by 14% per year between 2006 and 2012 (Reichert 2008).

Hundreds of antibodies have been clinically tested since the 1980s, and by now, at least 36 of them have been approved world wide (Walsh 2006; Deonarain 2008; Reichert 2008). A list of approved monoclonal antibodies with details of generic formats, antigen targets, clinic applications and approval details are shown in Table 1.1 below.

Table 1.1. A to Z of therapeutic monoclonal antibodies approved for disease treatment and diagnostic purposes.

<table>
<thead>
<tr>
<th>Drug name</th>
<th>Generic name</th>
<th>Target and format</th>
<th>Major therapeutic category</th>
<th>Year approved and manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arzerra</td>
<td>Ofatumumab</td>
<td>CD20, Human IgG1</td>
<td>Cancer</td>
<td>2009, Genmab/GSK</td>
</tr>
<tr>
<td>Avastin</td>
<td>Bevacizumab</td>
<td>VEGF, Humanized IgG1κ</td>
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<tr>
<td>Bexxar</td>
<td>131I-Tositumomab</td>
<td>CD20, Murine 131-Iodine IgG2αλ</td>
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<td>Campath</td>
<td>Alemtuzumab</td>
<td>CD52, Humanized IgG1κ</td>
<td>Cancer</td>
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<td>CEA-scan</td>
<td>Arcitumomab</td>
<td>CEA, Murine technetium-99m IgG1κ Fab</td>
<td>Cancer (diagnosis)</td>
<td>1996, Immunomedics</td>
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<td>Cimzia</td>
<td>Certolizumab pegol</td>
<td>TNFα, Humanized PEGylated Fab</td>
<td>Immunological</td>
<td>2008, UCB</td>
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<td>CrotalidaePolyvalent Immune Fab (Ovine)</td>
<td>Snake venom, Ovine IgG Fab</td>
<td>Poison antidote</td>
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</tr>
<tr>
<td>Drug name</td>
<td>Generic name</td>
<td>Target and format</td>
<td>Major therapeutic category</td>
<td>Year approved and manufacturer</td>
</tr>
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<tr>
<td>Digibind</td>
<td>Digoxin-specific antibody fragment</td>
<td>Digoxin, Ovine IgG Fab</td>
<td>Poison antidote</td>
<td>2001, GSK</td>
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<td>DigiFab</td>
<td>Digoxin Immune Fabs (Ovine)</td>
<td>Digoxin, Ovine IgG Fab</td>
<td>Poison antidote</td>
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<td>Erbitux</td>
<td>Cetuximab</td>
<td>EGFR, Chimeric IgG1κ</td>
<td>Cancer</td>
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<td>Herceptin</td>
<td>Trastuzumab</td>
<td>Her2, Humanized IgG1κ</td>
<td>Cancer</td>
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<td>Votumumab</td>
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<td>Humira</td>
<td>Adalimumab</td>
<td>TNFα, Human IgG1κ</td>
<td>Immunological</td>
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<td>Igovomab</td>
<td>CA-125, Murine ¹¹¹-Indium mAb fragment (Fab₂)</td>
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<td>OncoScint CR/OV</td>
<td>Satumomab pentetide</td>
<td>TAG-72, Murine ¹¹¹-Indium IgG1κ</td>
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<td>OrthoclonelKT3</td>
<td>Muromonab</td>
<td>CD3, Murine IgG2α</td>
<td>Immunological</td>
<td>1986, Johnson &amp; Johnson</td>
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<td>ProstaScint</td>
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<td>PSMA, Murine ¹¹¹-Indium IgG1κ</td>
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<td>Raptiva</td>
<td>Efalizumab</td>
<td>CD11α, Humanized IgG1κ</td>
<td>Immunological</td>
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<td>Remicade</td>
<td>Infliximab</td>
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<td>Immunological</td>
<td>1998, Centonor</td>
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<td>ReoPro</td>
<td>Abciximab (GP II/IIIa), Chimeric IgG1κ Fab</td>
<td>Glycoprotein</td>
<td>Hemostasis</td>
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Table 1.1-Continued
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<th>Generic name</th>
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<th>Year approved and manufacturer</th>
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<td>Basiliximab</td>
<td>CD25, Chimeric IgG1κ</td>
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<td>1998, Novartis</td>
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<td>Soliris</td>
<td>Eculizumab</td>
<td>Complement-5, Humanized IgG2/4κ</td>
<td>Immunological</td>
<td>2007, Alexion Pharmaceuticals</td>
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<td>Synagis</td>
<td>Palivizumab</td>
<td>RSV, Humanized IgG1κ</td>
<td>Infection</td>
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<td>Tecnemab KI</td>
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<td>HMW-MAA, Murine mAb fragments (Fab/Fab₂ mix)</td>
<td>Cancer (diagnosis)</td>
<td>1996, Sorin</td>
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<td>Tysabri</td>
<td>Natalizumab</td>
<td>α4-integrin, Humanized IgG4κ</td>
<td>Immunological</td>
<td>2004, Biogen IDEC/Elan</td>
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<td>Vectibix</td>
<td>Panitumumab</td>
<td>EGFR, Human IgG2κ</td>
<td>Cancer</td>
<td>2006, Amgen</td>
</tr>
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<td>Verluma</td>
<td>Ofetumomab merpentan</td>
<td>CAA, Murine technetium-99m IgG1κ Fab</td>
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<td>Xolair</td>
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<td>IgE, Humanized IgG4κ</td>
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<td>Zenapax</td>
<td>Daclizumab</td>
<td>CD25, Humanized IgG4κ</td>
<td>Immunological</td>
<td>1997, Hoffmann–La Roche</td>
</tr>
<tr>
<td>Zevalin</td>
<td>Ibritumomab tiuxetan</td>
<td>CD20, Murine ⁹⁰Yttrium IgG1κ</td>
<td>Cancer</td>
<td>2002, Biogen IDEC</td>
</tr>
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</table>

Monoclonal antibody nomenclature: Generic names for complex drug molecules such as antibodies follow a nomenclature system used by the WHO and US adopted names. The name provides information about the antibody target and source. For example, a ‘tu(m)’ denotes miscellaneous tumours whereas a ‘pr(o)’ denotes prostate cancer and ‘go(v)’ denotes ovarian cancer. This is then followed by ‘o’ for murine, ‘u’ for human, ‘xi’ for chimeric or ‘zu’ for humanised followed by the suffix ‘mab’. There is a variable, specific prefix in front of the name (Deonarain 2008). Full details can be found in [http://www.amaassn.org/ama/pub/category/2956.html](http://www.amaassn.org/ama/pub/category/2956.html) (last accessed 18 January 2010).

Abbreviations:
CA-125: Carbohydrate antigen -125; CAA: Carcinoma-associated antigen; CEA: Carcinoembryonic antigen; EGFR: Epidermal growth factor receptor; Her2: Human EGF receptor 2; HMW-MAA: Human high molecular weight melanoma-associated antigen; NCA: Nonspecific cross-reacting antigen; PSMA: Prostate specific membrane antigen; RSV: Respiratory syncytial virus; TAG: Tumour-associate glycoprotein; TNFα: Tumour necrosis factor-alpha; VEGF: Vascular endothelial growth factor.

Table 1.1-Contiued
Instead of treating infectious diseases, as was the intended initial purpose of antibodies in serum therapy, the application and development of modern mAbs are predominately focused on another two therapeutic categories – cancer and immunological diseases (Reichert and Pavolu 2004). As shown in Table 1.1, nine of the approved antibodies are used for direct cancer treatment with an additional 7 products applied for cancer diagnostic purposes. For immunological disease treatment, such as autoimmune and inflammatory disorders, ten mAb approvals have already been launched successfully.

1.1.4 Therapeutic Mechanisms of Approved mAbs

In contrast to Ehrlich’s 1908 Noble Prize predictions, the most successful cases of antibody therapeutics in most of clinical disease treatment have been when the antibody was directly utilized as naked IgG without conjugation (Table 1.1). Based on the structural specificity of antibodies, antibody therapy is determined to be one of the subclasses of “targeted therapy”. The wide variety of targeted therapy can be classified into direct and indirect approaches (Schrama et al. 2006), and a summary of direct approaches are schematically showed in Figure 1.3.

![Figure 1.3. Direct actions of therapeutic antibodies.](image_url)

**Figure 1.3. Direct actions of therapeutic antibodies.** Cells expressing various antigens (green) can be targeted by corresponding mAbs (orange), which then induce effector functions including ADCC (via binding between Fc from antibody and Fcγ receptor on the effector cells) and CDC (via C1 component). Signal transductions induced cell growth, proliferation and angiogenesis can be inhibited by specific mAbs, whose bindings down-regulate the signalling proteins, such as vascular endothelial growth factor (VEGF) and other growth factors (GFs). Signalling interferences can cause subsequent apoptotic cell death to the targeted cells. Endocytosis of internalizing cell surface antigens bound with corresponding mAbs also leads to cell damage and cell death. This figure is cited from (Ben-Kasus et al. 2007).
Using medications in cancer therapy as a typical example, several approved mAbs with numerous clinical trial pipeline candidates are following direct approaches in terms of fulfilling their therapeutic purposes. The direct targets contain tumour specific or associated proteins, and the direct approaches for therapeutic mAbs executing malignancies (Figure 1.3) include alteration of the cancerous cell signalling pathways, which lead to cell death or the effectiveness of antibody Fc domain mediated immune functions, such as ADCC and CDC (Deonarain 2008; Nimmerjahn and Ravetch 2008).

1.1.4.1 Antibodies Interfering in Cell Signalling Transduction Mechanisms (Fab-mediated activities)

It is becoming clear that tumourigenesis involves a myriad of interactions and processes. Factors that maintain a tumour-favourable environment have turned out to be an important area for target therapies, and consequently, these factors are termed “tumour-associated targets”. One of the most important and well studied tumour-associated targets is vascular endothelial growth factor (VEGF). It is a heparin-binding homodimeric glycoprotein consists of two identical 23 kDa subunits. Several different VEGF gene splicings encode a number of VEGF isoforms (Ferrara 1999). Normal embryonic vessel formation and angiogenesis were reported as essential functions of VEGFs (Adams and Weiner 2005), which are achieved by binding to VEGF tyrosine kinases receptor (VEGFR). Various subsequent signalling cascades are induced by the VEGFRs, including the Phosphoinositide 3-kinases (PI3K), the Akt (RAC-α serine/threonine-protein kinase or protein kinase B) pathways that enhance the cell survival capacity, Ras/Raf/MEK/ERK signalling transduction (see legend for Figure 1.4) and mitogen-activated protein kinase (MAPK) mediates DNA replication and cell proliferations (Roy et al. 2006; Ho and Kuo 2007). Upregulated oncogenic VEGF and its receptors are frequently over produced by tumour cells to propagate tumour growth, tumour-associated angiogenesis and metastasis (Fukumura et al. 1998; Tsuzuki et al. 2000; Dvorak 2002).

**Bevacizumab (Avastin®)**

Bevacizumab is a recombinant humanized mAb against VEGF modified from its murine format mAb A4.6.1 (Kim et al. 1992; Presta et al. 1997) with a very high affinity ($k_d \sim 0.5\text{nM}$). Bevacizumab binds specifically to all human VEGF-A isoforms and bioactive fragments, but no evidence has shown its binding to other VEGF gene family members (Presta et al. 1997; Ferrara et al. 2004). As one of the well-characterized representative mAbs, bevacizumab set a presidence for the therapeutic success in signalling transduction interference. Preclinical and
clinical development showed bevacizumab significantly neutralized VEGF interaction with VEGFRs in cancer patients and restrained the subsequent downstream signalling pathways assisting tumour growth and vascularising as mentioned above (Dvorak 2002). Pericytes can be recruited to the tumour vasculature after VEGF blockage by bevacizumab, which stabilize tumour vessel formation, and enhance the delivery of oxygen to the hypoxic zones (Winkler et al. 2004). Mediated by angiopoietin-1 and matrix metalloproteinases, tumour angiogenesis can be normalized, making a solid tumour which is more sensitive to radio- and chemotherapy (Lin and Sessa 2004; Winkler et al. 2004). In combination with cytotoxic chemotherapies, bevacizumab has provided prolonged survival to patients with colorectal, lung, and breast cancer, especially for the treatment of metastatic cases. It may act as an active angiogenesis inhibitor to be singly applied in ovarian and renal cancer therapies (Ferrara et al. 2004; Adams and Weiner 2005). In terms of pharmacokinetic properties, bevacizumab has been previously described with consistent terminal half-life between 17 to 21 days (Lin et al. 1999), and no antibody-related side effects have been reported in clinical trials to date.

**Trastuzumab (Herceptin®)**

Bevacizumab is an excellent example of an antibody curtailing tumour angiogenesis by blocking the soluble signal initiating cytokine binding to its receptor, which induces the malfunction of subsequent signal transduction pathways. Another successful humanized mAb, trastuzumab demonstrates an alternative approach to interrupt the signalling mechanisms in cancer treatment. Trastuzumab targets human epidermal growth factor receptor 2 (HER2; erbB2/neu), an 185 kDa glycoprotein that belongs to the ErbB class of epithelial growth factor receptor (EGFR) family – a family of transmembrane receptor tyrosine kinases (RTKs). Other members such as HER1 (also known as EGFR), HER3 (erb3) and HER4 (erB4) are also included in the EGFR family (Adams and Weiner 2005; Spector and Blackwell 2009). The family is prominently involved in regulating signal transduction, particularly during embryogenesis, which lead to cell growth, survival, differentiation, and migration (Yarden 2001; Park et al. 2008). Except HER2, most HER proteins are activated on ligand binding, which then undergo homo- or heterodimerization among other family monomers. HER2, having no known ligand, is constitutively active, and ready to be dimerized in the absence of ligand. This makes HER2 a preferred coupling partner with other HER proteins, and these heterodimers are potent in signalling initiation (Pinkas-Kramarski et al. 1996; Graus-Porta et al. 1997). Phosphorylation of the intracellular tyrosine kinases linked to the receptors after their dimerization trigger downstream survival, differentiation and proliferation signalling.
events (Yarden 2001). Like VEGF-VEGFR induced signalling pathways, PI3K and MAPK are closely involved in the intercessions of HER2 induced signal networks (Figure 1.4).

HER2 became an attractive therapeutic target from its over-expression in a variety of human adenocarcinomas, including breast, ovary, prostate, lung and gastrointerstinal cancers (Slamon et al. 1989). The HER2 gene is located on chromosome 17, approximately 20%-25% breast cancers are currently estimated to be related to HER2 gene amplification and protein over-expression (Slamon et al. 1987; Owens et al. 2004), and HER2-positive tumours are detected in large proportions among breast cancer occurrences annually (Ferlay et al. 2007), associated with poor prognosis and decreased response to hormonal and chemotherapies in clinical treatment (Slamon et al. 1987; Gusterson et al. 1992; Leitzel et al. 1995). Following the normal signal transduction, cancer over-expressed HER2 actively accelerates homodimer formation, which deregulates subsequent signal networks and has a vital impact on tumour cell survival, expansion, metastasis and transformation (Wang and Greene 2008).

Blockage of HER2 activation has been demonstrated in preclinical models and it was shown that inhibition of the survival pathways led to the induction of tumour cell apoptosis (Yakes et al. 2002). Trastuzumab was designed with high selectivity and affinity (~5nM) to HER2 (Carter et al. 1992), and predominantly tested for HER2 over-expressed metastastic breast cancer (Cobleigh et al. 1999; Vogel et al. 2002). Like bevacizumab, trastuzumab is also a humanized IgG1 version of its murine precursor, called 4D5, that targets c-erbB2, the extracellular domain (ECD) of HER2 (Fendly et al. 1990; Carter et al. 1992). The exact antitumour mechanisms exerted by trastuzumab are not fully understood at the moment, but several possible approaches have been proposed from different studies (Figure 1.4).

As opposed to bevacizumab (which mainly blocks a tumour associated cytokine interacting with its receptor), shown in Figure 1.4 trastuzumab actively targets and down-regulates tumour cell surface expressing HER2 receptor to cease HER2-provoked intracellular survival and growth signalling messages. Similar to bevacizumab, key signalling pathways, such as
Both possible extracellular and intracellular mechanisms of trastuzumab are depicted. BAD, proapoptotic protein of the Bcl-2 family; JNK, c-Jun N-terminal kinase; MEK, MAPK/extracellular signal-related kinase; MEKK, MEK kinase; mTOR, mammalian target of rapamycin; NK, natural-killer cell; PAI-1, plasminogen activator inhibitor-1; TGF-α, transforming growth factor α. (Spector and Blackwell 2009).

PI3K-Akt and MAPK pathways were disrupted after trastuzumab-HER2 interaction, resulting in reduced tumour cell growth and survival rates, and making the cells more vulnerable to death in harsh conditions (Nagata et al. 2004). Moreover, trastuzumab recognises HER2 ECD, but does not inhibit HER2 dimerization. The HER2 heterodimer often undergoes internalization after antibody targeting, followed by ubiquitination and proteolysis (Maier et al. 1991; Gilboa et al. 1995; Qian et al. 1997). Internalized trastuzumab bound HER2 is degraded by lysosomes through clathrin-mediated endocytosis. This process also subsequently quenches the HER2 mediated tumour survival signal transductions (Hurvitz et al. 1995; Klapper et al. 2000). Signal transduction inhibitions also result in the accumulation of cyclin-dependent kinase (CDK) inhibitor p21/WAF1 in the cell nucleus that decreases the CDK2 activity, which leads to cell cycle arrest and apoptosis (Xia et al. 2004; Hudis 2007). DNA damage can also be induced in cancer cells as an outcome of blocking HER2 mediated DNA repair response, as trastuzumab promotes the cell to escape early from DNA-repair required cycle arrest (G1 and S) phases especially after combined chemo- and radiotherapies (Pietras et al. 1994; Pietras et al. 1999). Inhibition of angiogenesis was thought to be another signalling interference feature of trastuzumab. In a similar way seen with bevacizumab treatment, downregulation of intratumoral VEGF, and other pro-angiogenic factors by trastuzumab were shown from preclinical murine xenograph tumour model combined with
chemotherapeutic drug treatment. Tumour blood vessel shrinkage and reduced tumour growth were also reported (Izumi et al. 2002). Additionally, HER2 often undergoes proteolytic cleavage shedding the ECD into blood circulation, and leaving the truncated membrane-bound fragment capable of signalling (Fornier et al. 2005). Trastuzumab prevents both basal and activated HER2 ectodomain cleavage, which stops further receptor activation for signalling transduction (Molina et al. 2001).

**Rituximab (Rituxan®)**

CD20 is a cell surface anchored receptor protein found on both healthy B-cell and 99% of B-cell lymphomas and leukaemias (Coiffier 2007). No ligand has been reported for CD20, and as yet its biology is poorly understood. However, it has been found that CD20 assembles as a tetramer into lipid rafts upon antibody binding and acts as a store-operated calcium channel modulator for downstream caspase activation and signalling events (Polyak et al. 1998; Bonavida 2007). Rituximab, the first antibody approved by the US FDA, was engineered as a chimeric IgG1 that specifically targets CD20, and originally applied for indolent B-cell non-Hodgkin’s lymphoma (NHL) treatment (McLaughlin et al. 1998).

Rituximab works on the CD20 signalling pathways to cause cancer cell apoptosis by two main routes: acceleration of calcium ion flux rate, and inhibition of intrinsic signalling cascade including MAPK proliferative signalling mediated by p38-MAPK/NF-κB and extracellular regulated kinase (erk) (Ben-Kasus et al. 2007). Together with other signalling inhibition on Akt and other cancerous associated anti-apoptotic survival pathways and caspase activations, these signal alterations induced by the cross-linking of rituximab and CD20 can also greatly increase cancer cell apoptosis and make tumour cells more sensitive to chemo- and radiotherapies (Bonavida 2007).

**Certolizumab pegol (Cimzia®)**

Not only applied to the whole monoclonal Ig molecule, cell signal transduction impedances are expected to be more efficiently exhibited by the smaller derivative of antibodies – antibody fragments. In April 2008, certolizumab pegol, a recombinant humanized antibody Fab fragment conjugated to a ~40 kDa polyethylene glycol (PEG) for plasma half-life enhancement, was approved by the US FDA for the treatment of moderate-to-severe Crohn’s disease (CD) (Melmed et al. 2008). The antibody Fab targets specifically tumour necrosis factor α (TNFα), one of the most important pro-inflammatory cytokines involved in chronic
inflammatory diseases (Bouma and Strober 2003; Korzenik and Podolsky 2006). More details on certolizumab pegol as a powerful anti-inflammatory signal blocker will be discussed in the later antibody engineering sections.

As listed in Table 1.1, a number of mAbs have been approved for interfering signalling molecules or receptor proteins to achieve their therapeutic applications. Like trastuzumab, some mAbs even have several possible disease-fighting mechanisms, such mAbs include cetuximab (Erbitux®), adalimumab (Humira®), ranibizumab (Lucentis®), natalizumab (Tysabri®), panitumumab (Vectibix®) and infliximab (Remicade®).

1.1.4.2 Antibodies Eliciting Host Effector Functions (Fc mediated activities):
Apart from those targeting functions involved in alteration of cell signalling networks, a more significant and effective degree of naked full-length therapeutic mAb is mediated by Fc-based immune responses such as CDC, ADCC, and antibody-dependent cell phagocytosis (ADCP). This engagement of the host immune system has the potential to augment the efficacy for antibody direct therapeutic activities against functional antigens (i.e. signalling) and also has a further prospective to turn non-functional antigens into useful targets (Griggs and Zinkewich-Peotti 2009). In order to react with the antibody Fc domain, a range of Fcγ receptors (FcγRs) are expressed by a diverse set of immune cell populations, including B cells, dendritic cells, macrophages, mast cells, NK cells, and neutrophils (Desjarlais et al. 2007). Most FcγRs are Ig superfamily glycoproteins, and often appear in activatory and inhibitory polymorphisms (Nimmerjahn and Ravetch 2008). Activatory (FcγRI, FcγRIIA and FcγRIII) and inhibitory (FcγRIIB) Fcγ receptors are co-expressed and regulate the actions of effector cells. B cells and NK cells are exceptions, as B cells only express FcγRIIB to inhibit endogenous IgG activation, while NK cells only express FcγRIII for independent cell killing after target confirmation (Ravetch and Bolland 2001). A serial signal transduction cascade can be triggered after the antibody Fc engagement to the activating FcγRs, which leads to the final activation of ADCC and ADCP. Immune effector cells under the targeted antibody recruitment can then perform a variety of functions, including the release at local cytolytic proteins (i.e. granzymes and perforins) and phagocytosis of the target cell. The lysis of the target cell facilitates the antigen presentation, which stimulates T cell activation and production of various regulatory cytokines (Carter 2001). CDC is triggered through classical activation pathway, which is initiated by the binding of complement component C1q to the Fc
portion of antigen bound IgG molecule. The targeted antigen expressing cell can be lysed, by the consequent formation of membrane attack complex (MAC) of the complement system (Iannello and Ahmad 2005; Desjarlais et al. 2007). Variations of active IgG isotypes exist on the capacity of immune effector cell recruitment. Based on the FcγR expression profile, human IgG1 and IgG3 (and murine IgG2a) are normally present as principal functional isotypes with relatively high affinity to activate the immune responses, while extended hinge region makes IgG3 less stable, resulting IgG1 is usually more preferred for drug design also because of its longer serum half-life (Dijstelbloem et al. 2001; Nimmerjahn and Ravetch 2005; Desjarlais et al. 2007).

Containing an IgG Fc structure, both trastuzumab and rituximab also exhibit important anti-cancer advances through their Fc mediated immune responses (Lazar et al. 2006). Both in vitro and in vivo studies suggested that ADCC was induced by trastuzumab and no anti-tumour response was detected from FcγR deficient mouse models (Cooley et al. 1999; Clynnes et al. 2000; Mimura et al. 2005). Pivotal preclinical and clinical studies also provided supportive evidence showing that trastuzumab has a role in engaging cognate FcγR on the immune effector cells (predominantly NK cells) as key therapeutic mechanisms (Gennari et al. 2004; Arnould et al. 2006). There is also strong evidence supporting rituximab in utilizing Fc elicited cytotoxic and therapeutic effects. Clinical trials of NHL patients with Fc receptor polymorphisms indicated ADCC as an approach of rituximab immunotherapy (Cartron et al. 2002; Weng and Levy 2003). In addition, combinational use of NK cell promoting cytokines, including granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-2 (IL-2), was reported to enhance rituximab effect with increase NK cell development and immune activity (Olivieri et al. 2005).

CDC was also showed to be activated by trastuzumab in vitro when human serum was tested but was not activated when applied to sera from mouse origin (Spiridon et al. 2002). Investigations in animals lacking C1q and employing complementary inhibitory proteins showed a reduced efficacy of rituximab indicating the mAb has a role in activating complement lysis (Golay et al. 2001; Di Gaetano et al. 2003). Other important therapeutic mAbs supporting immune effector functions include cetuximab (Erbitux®), alemtuzimab (Campath®), tositumomab (Bexxar®) and many others.
1.1.4.3 Indirect Antibody Targeting Therapeutic Approaches:

In contrast to direct therapies, antibody indirect therapies rely on specific target delivering different kinds of effector molecules that are fused or conjugated to the antigen recognising antibodies. As a result, researchers have selected a variety of ammunition to equip antibodies, including drugs, toxins, radionuclides, and recombinant fusion proteins, and there are a whole collection of ways for mAb arming listed in Figure 1.5 below.

![Diagram of indirect therapeutic actions by antibody](image)

**Figure 1.5. Summary overview of indirect therapeutic actions by antibody.**

* a. Directed antibody targeted therapy. Antibodies fused with cytokines or ligand proteins that can modulate immune response after the antigen targeting. Bispecific single chain variable fragments (scFvs) binding the antigen and provoking the immune system are classified in the same category. In addition, antibody–ligand fusion proteins can induce apoptosis to targeted cells as well as bystander cells by.

* c. Direct cytotoxicity can be induced to the antibody target through conjugated cytotoxic drug, toxin or radionucleotides.

* d. The antibody-directed enzyme prodrug therapy (ADEPT) approach specifically aims at delivering antibody-enzyme fusion to the disease target, and the enzyme part takes part in converting the subsequently applied prodrug to a chemotherapeutic (Schrama et al. 2006).

The armed mAbs are speculated to be more powerful therapeutics compared to the naked mAbs. Rather than traditional chemo- and radiotherapies, antibody immunonjugates or fusions function by delivering lethal doses of cytotoxicity or radiation to the malignant targets, resulting in cell death (Goldenberg and Sharkey 2006; Schrama et al. 2006) while achieving diminished systemic toxicity through mAb based targeting strategies. In this regard, one antibody conjugated with an extremely cytotoxic compound, and two radiolabelled mAbs
have been approved for cancer treatment (also see Table 1.1). In order to optimise the conveyance purpose, low immunogenic chimeric or humanized mAbs, even antibody fragments with high affinities to antigens were often used for conjugation. Efficient antibody internalization into cells after target binding, site-specific modification for uniform conjugation with retention of antibody binding, linker technologies to enhance the novel cell killing agents, selection of new drugs and radioisotope with better therapeutic potencies, and optimizing pharmacokinetics and clearance are the important regulating issues in this field (Wu and Senter 2005).

**Gemtuzumab ozogamicin (Mylotarg®)**

Gemtuzumab ozogamicin is the only clinically approved mAb-drug conjugate. It consists of a humanized anti-CD33 IgG4 and the antitumour antibiotic ozogamicin (N-acetyl-γ calicheamin) that causes DNA breaks. The two parts are connected by an acid-labile hydrazone linker (Bross et al. 2001). Mylotarg has been approved to treat CD33 over-expressing acute myeloid leukaemia (AML), and the rapid internalization of CD33 after antibody targeting implied a significant breakthrough of gemtuzumab ozogamicin with positive data from previous clinical trials (Sievers et al. 1999; Sievers and Linenberger 2001). Other cytotoxic drugs include doxorubicin, DM1, monomethyl auristatin E and geldanamycin (Baloglu et al. 2004; Schrama et al. 2006), together with toxins such as denileukin diftitox (Frankel et al. 2000), are currently under investigations.

**131I-Tositumomab (Bexxar®) and 90Y-Ibritumomab tiuxetan (Zevalin®)**

According to the success of radiotherapies for disease treatment, radio-immunotherapeutic antibodies have aimed to improve this. Successfully applied in haematological cancers (i.e. NHL), two anti-CD20 antibodies, 131I-Tositumomab and 90Y-Ibritumomab tiuxetan (see Table 1.1) conjugated with two different radioisotopes have to date been approved. Like rituximab, both antibodies utilized in the immunoconjugates are good CD20 binders with high specificities to the antigen expressing cancer cells (Sharkey and Goldenberg 2005; Davies 2007). Toxic radionuclides 131I and 90Y were attached to the antibodies by either direct tyrosine residue conjugation or chemical chelation. 90Y emits a short path length beta emission with short radioactive half life, while 131I alternatively gives off both beta and gamma radiation with a longer path length and more prolonged half life (Milenic et al. 2004). Significant responses of both radiolabelled antibodies were dramatically indicated in treating rituximab-refractory NHL (Wu and Senter 2005). Other powerful isotopes such as bismuth-
213 ($^{213}\text{Bi}$) and Astatine-211 ($^{211}\text{At}$) producing alpha particles have also been tested for conjugation with mAbs for therapeutic applications (Sgouros et al. 1999; Milenic et al. 2004). Coupling of gamma-emitting isotopes, such as technetium-99m ($^{99m}\text{Tc}$) or Indium-111 ($^{111}\text{In}$) to full length mAb and antibody fragments have been extensively used for computed tomography as a major part of radioimmunoscintigraphy for preclinical and clinical imaging aspects (Wu and Senter 2005). According to Table 1.1, eight mAbs have been approved for imaging. A number of positron emitters (for example, $^{18}\text{F}$, $^{64}\text{Cu}$, $^{68}\text{Ga}$, $^{86}\text{Y}$ or $^{124}\text{I}$) have been attached to antibodies for positron emission tomography (PET) for better imaging evaluations with higher sensitivity and molecular resolution (Olafsen et al. 2005; Robinson et al. 2005).

Rather than relying on binding to internalized antigen to deliver the lethal insult into the target cell, to expand targeting non-internalized ligands, antibody-directed enzyme prodrug therapy (ADEPT) was devised. This method enzymatically turns weakly-low toxic prodrug to highly toxic agents at the antibody-antigen site (see Figure 1.5), allowing the cytotoxin to freely defuse into the targeted cell (Bagshawe 1987; Denny 2004).

As reviewed above, most conjugated antibodies achieve direct killing of the cell by the attached toxic or radioactive components. New approaches involving antibody fusion proteins and antibody fragment combinations with more than one binding specificities (see Figure 1.5) acting by various therapeutic mechanisms are evolving as promisingly new generations of indirect antibody targeting therapies.

1.2 Antibody Engineering – Fragmentation and Recombinant Fusion

As described in the section above, the historical milestones of antibodies in therapies have revealed a development of protein drugs. From the “serum therapy” which uses polyclonal antibodies from different species, to hybridoma cell line produced murine monoclonal antibodies; this nascent field has already come through its third generation, the booming stage which was fundamentally achieved by the advances of antibody engineering to characterise the mAbs with essential human properties (see Figure 1.2b) and expand mAb with broad
clinical applicabilities (Weiner 2007). Seeing the great success of generating fully human full-length mAbs with two already approved for therapies - Adalimumab (Humira) and Panitumumab (Vectibix), the phase of mAb development third generation seems to have been well established, but unfortunately it has not brought a “mission accomplished”. The occurrence of limitations and new problems, especially for the whole IgG format, in clinical practise and laboratory investigations require improvements and other perspectives for the next generation of this field.

1.2.1 Strength and Weaknesses of the Antibody Fc Domain
Generally speaking, mAbs are still expensive biopharmaceuticals to commercially produce, and the primary applications of therapeutic mAbs are introduced in treating extreme and refractory diseases. The main attention of antibody engineering has been for years focused on improving the antigen binding variable domains with the development of a number of techniques to enhance the affinity of antibody clones (Loo et al. 2008). However, effects brought out by the distal constant region Fc portion also have significant pharmacological impacts. Effective modifications to exaggerate the Fc properties have won noteworthy improvements, however undesirable consequences can be very serious, and maybe even unavoidable.

1.2.1.1 Therapeutic Efficacies and Side Effects
As powerful as it has been repeatedly proved in many different therapeutic applications such as tumour cell killing and infectious disease treatment (Wallace et al. 1994), antibodies engage immune system through Fc fragment mediated ADCC and ADCP after FcγR binding and CDC by a series of complement system constitutional blood proteins. The mechanisms of the effector system signal activation and consequential immune responses (i.e. T cell activation, and cytokine release) were described with approved mAb models in the previous section (1.1.4). In order to enhance Fc-stimulated effector functions, a number of engineering modifications have been evolved. Pioneering studies on antibody isoforms, variants and glyco-forms led to a way of variety of Fc engineerings. For improving ADCC elicitation, investigations primarily focused on amino acid sequence and oligosaccharide modifications in the Fc domain. Amino acid mutation changes of IgG1 Fc have been reported to modulate binding affinity to FcγIIIA (Shields et al. 2001). Recent mutational studies by yeast display selected an IgG1 Fc variant having ~ 10-fold increased affinity to FcγIIIA, which led to
increased an ADCC capacity (Lazar et al. 2006). Such protein engineering modifications have also been carried out on trastuzumab and alemtuzumab for therapeutic ADCC activity augmentation (Lazar et al. 2006). The N-linked oligosaccharide in the C1\(\text{H}2\) domain of Fc fragment have an important role in Fc bio-activities, especially ADCC (Wright and Morrison 1997). The addition of a bisecting N-acetylglucosamine (GlcNAc) to the core mannose residue and removal of fucose residue from IgG1 Fc glycosylation structure respectively exhibited more than \(~10\) and \(~100\)-fold respectively higher ADCC activity in various systems (Lifely et al. 1995; Shinkawa et al. 2003; Niwa et al. 2005). CDC activity has also been engineered by facilitating mAb Fc to C1q binding. Mutations on Fc C\(\text{H}2\) domain or hinge region, together with generation of mixed IgG1 and IgG3 constant region sequences have all shown different degrees of CDC enhancement (Idusogie et al. 2001; Dall'Acqua et al. 2006; Natsume et al. 2008). Financially backed by many big pharmaceutical and biotech companies, the pharmacological impacts of Fc induced immune effector functions are pivotal in certain disease treatment, and Fc-modified antibodies have shown great improvement from clinic data (Desjarlais et al. 2007; Strohl 2009). Moreover, this improvement also provides Fc-fusion proteins with more promising development.

On the other hand, unwanted drug side effects seem never to be avoidable and they are also associated with therapeutic mAbs. These problematic issues are mostly related to whole antibody Fc domain-dependent immunotoxicities, which are predominantly caused by unnecessary cross-reaction immunogenicity and immunostimulation (Swann et al. 2008).

**Immunogenicity**

Based on the preferred parenteral administration routes (e.g. intravenous [IV], subcutaneous [SC] or intramuscular [IM]) of delivery antibody products, all approved intact therapeutic mAbs to date have shown some degree of immunogenicity, especially due to their murine origin (Tang et al. 2004). Examples such as first approved murine mAb, muromonab-OKT3 have been reported with therapeutic inefficiencies due to the generation of neutralising anti-globulin or HAMA response (Schroeder et al. 1990). Although influential reductions have been made during the antibody generational development by converting its murine origin to human type, as both chimeric and humanised antibodies were both designed with human Fc domains, human anti-chimeric antibody (HACA) and human anti-human antibody (HAHA) responses were still seen in patients (Mirick et al. 2004). Despite any foreign protein sequences left in the “designer” intact mAbs, the constant regions of the immunoglobulin
have always been the direct targets for the host antiglobulin responses, where Fc fragment consists of 50% of total constant regions, typically after repeat systemic administrations (Batra et al. 2002). Although the development has already achieved to place human Fc in the antibody structure, the basis of elicitation of antibody response to therapeutic mAbs still remains as a debate by now; however, it is clear that size reduction will lead to diminishment of immunogenic side effects and the choice between eliminating size from either the antigen selecting Fab domain (more precisely the CDR regions) or pharmacological immune response eliciting Fc domain seems to be obvious but also tough in clinical applications.

$N$-linked glycosylations at asparagaine-297 of $C_H2$ domain of IgG presents another feature of antibody Fc domain, which may induce extra potential immunogenic risks. As glycosylation is a species- and cell-specific dependant characteristic, the production of these therapeutic proteins, even the fully human recombinants are not exactly the same as the natural endogenous protein (Hermeling et al. 2004; Descotes and Gouraud 2008). The maintenance of human type glycosylation consistency is very critical for mAb production (Jefferis 2005). However, different innovative eukaryotic cell expression methods (e.g. yeast, plant, mammalian expression system) with promising yield may increase the risk of producing heterogeneous glyco-profiles of the antibody (Filpula 2007). The host-specified antibody Fc fragment glycosylation portfolio can significantly influence the mechanisms of effector function activation. Even with modifications of ADCC improved glycostructure presentations, the oligosaccharide alterations of intact mAb Fc domain can lead to increased xenogenic potentials causing host hypersensitivity reactions (Jain et al. 2007).

**Immunostimulation**

Immunostimulation from Fc fragment normally involves both antigen-specific immune responses with canonical therapeutic effects and unnecessary/non-antigen-specific immuno-toxic effects. Interaction with antigen expressed on tissues other than the intended target can cause the antibody Fc part cross-linking with the Fc receptors expressed on the effector cells, leading to the same immune responses utilized to fight against malignancies to the healthy tissues. These non-specific toxicity occurrences resulting from the unexpected pharmacological effects of the mAbs may include infusion reactions (IRs) and massive cytokine release (Roskos et al. 2004).
Most IR are allergic-type reaction, and mAb induced infusion reactions can be clinically characterized as mild flu-like symptoms like fever, chills, rash, nausea and so on; however in rare cases it can be fatal such as cardiovascular collapse and respiratory failure (Chung and O'Neil 2009). The associated immunological mechanisms behind IR often vary, however there is experimental evidence indicating cross-linking of effector cells by FcγRs targeting bispecific antibodies which can elicit infusion reactions (Curnow 1997; McCall et al. 2001). This consequently designates the possibility of antibody Fc cross-reacting with the effector cells with facilitated IRs. Severe reactions seen in patients including elevated lymphocyte counts and anaphylactic activation of complement cascades have also been evidenced in acute IRs caused by unexpected Fc-Fc receptor cross-linking from various mAbs, especially reported in rituximab treatment (Winkler et al. 1999; van der Kolk et al. 2001). The immune effector responses gathered by the therapeutic Fc domain to the healthy cells after their inevitable non-specific antigen binding were estimated to cause the life-threatening IR symptoms. Such symptomatic heart failure severity was observed in patients treated with trastuzumab for HER2-overexpression metastatic breast cancer, and relevant adverse effects of trastuzumab’s ADCC and CDC on HER2-expressing cardiomyocytes were subsequently elucidated (Cook-Bruns 2001; Roskos et al. 2004).

In addition, it has been clinical confirmed that cytokine release also plays a pivotal role in the IR mechanisms. As normally dedicated, cytokines act in a much localized microenvironment as autocrine or paracrine factors at the site of an immunological synapse. During antibody therapy, as a consequence of direct activation of various immunocompetent cells by the mAb Fc region, essentially released cytokines work as an important part in immunotherapies, including differentiation and activation of more cytotoxic immunocompetent cells to the target area (Waldmann 2003). However, non-specific antigen binding of mAb to healthy tissues can lead to imbalance release of pro-inflammatory cytokines, later reported as “cytokine storm” (Suntharalingam et al. 2006), causing IR associated symptoms, which is clinically termed as the cytokine-release syndrome (Breslin 2007). Symptoms seen in monotherapy trials of different approved mAbs, initially from muromonab-OKT3 to other recent approvals including rituximab, cetuximab, alemtuzumab, and trastuzumumab, have all suggested various levels of cytokine-release associated adverse reactions to patients, which are theoretically stimulated by mAb Fc cross-linkings to FcγRs (Kang and Saif 2007; Descotes and Gouraud 2008; Wing 2008; Chung and O'Neil 2009). Cytokine release triggered by intact mAb administration seems to be unavoidable especially for those antibodies utilizing
Fc domains to fulfil their therapeutic duties. Redundant cytokines produced after treatment with muromonab-OKT3 were believed to be caused by Fc mediated therapeutic mechanisms, such as T cell opsonisation and subsequent lympholysis (Gaston et al. 1991). Inadequate/unexpected cytokine release can give significant hazards to allergic and aged patients; therefore adequate premeditation, careful patient monitoring, and prompt intervention are required when hypersensitivity signs occur. For severe IR cases, the mAb therapy needs to be discontinued, which may significantly disturb the treatment progress, especially to the patients suffering cancer or immunological diseases (Lenz 2007).

1.2.1.2 Pharmacokinetics (PK) and Biodistribution
The pharmacokinetic and biodistribution mechanisms of protein drugs, in terms of their plasma half life, transportation/localisation, as well as absorption, distribution, metabolism and excretion (ADME) profiles, are influenced by several factors including their size, shape, charge, hydrophilicity, antigen/tissue binding properties, and proteolytic sensitivities (Tang et al. 2004). Intact therapeutic mAbs exhibit specialized pharmacokinetic and biodistributional characteristics, where properties of the IgG Fc domains play a central role.

**Plasma Half Life**
Attesting to the importance and prevalence in the serum and non-mucosal tissue, the immune protective role of IgG antibodies is mediated by their extensive blood circulation periods (1 to almost 4 weeks depending on the subclasses). Research found the preservation of these long-lived molecules were closed related to neonatal Fc receptor (FcRn) (Ghetie and Ward 1997; Anderson et al. 2006). The major histocompatibility complex (MHC) class I related receptor, FcRn was initially recognised as a salvage receptor that transfer the IgGs in intact from the mother to offspring (Simister and Rees 1985). Recent studies showed the present of the FcRn also has an intricate function of maintaining the homeostasis of IgGs in the circulation (Ghetie and Ward 1997). Illustrated in Figure 1.6, with high expression level of FcRn in vascular epithelial cells and the reticuloendothelial system (RES), the elevated affinity binding of IgG Fc domain with FcRn at acidic pH (<6.5) makes the immunoglobulin internalized into these cells and recycled or delivered through transcytosis back to the basolateral side of the cells, where the neutral pH dissociate the FcRn binding and releases the IgG back to circulation, thus extending its serum half-life (Ghetie and Ward 1997; Dall'Acqua et al. 2002; Kenanova et al. 2005). The expression of FcRn has also been detected from a range of cell and tissue
types including antigen-presenting cells (APCs), adult guts, kidneys, lungs and blood-brain barriers. The precise biophysiological reasons for the existence of FcRn on such tissues are not fully understood; however, their interactions with IgG Fc, followed by internalization process, are hypothesized as a facilitate mechanism for IgGs cross-membrane transport to achieve their antigen targeting motives (Ghetie and Ward 1997; Roopenian and Akilesh 2007).

Figure 1.6. Proposed mechanism for IgG retention by FcRn. Described in the text, binding between IgG Fc and FcRn receptor is a pH dependent interaction. Cycling of FcRn salvage pathway increases IgG blood residence time by avoiding both serum proteolytic proteins and lysosomal catabolism. Figure from (Ghetie and Ward 1997).

The alteration and manipulation of this pH-dependent IgG Fc-FcRn interaction has been investigated in many antibody and Fc-fusion pharmacokinetics for various research and therapeutic purposes. Using site-directed mutagenesis, functional amino acids of the Fc region involved in the interaction of IgG and FcRn have been mapped (Medesan et al. 1997; Kim et al. 1999; Shields et al. 2001), and several researchers have introduced different mutations in the Fc region critical to the FcRn binding site of human, mouse and human-mouse chimeric IgGs. They have showed significantly prolonged antibody serum half-life with increased binding affinity to FcRn at pH 6.0 and inhibited interaction at pH 7.5 in mice (Ghetie and Ward 1997; Hornick et al. 2000; Shields et al. 2001), rhesus monkeys (Hinton et al. 2004; Hinton et al. 2006), cynomolgus monkeys (Dall'Acqua et al. 2006) and humanized mice models (Petkova et al. 2006). Along with increased serum half-life, opportunities for antigen binding and major effector functions such as ADCC and CDC can be also improved.
indicating the modification of increase Fc-FcRn affinity may prove to be an effective way of retaining antibody pharmacological purposes.

Conversely, the extended blood residence time caused by the interaction between FcRn and antibody Fc can be highly undesirable for situations such as the administration of radioisotopes, toxins, and other cytotoxic reagents conjugated with therapeutic mAbs or using mAbs for biological imaging purposes. An acute effect is therefore required, as the transcytosis mediated by FcRn also uptakes the poison doses conjugated with the mAb into the healthy tissues, prolonged exposure of these toxic doses in the circulation can be extremely harmful to patients, and revoking the concept of antibody targeted therapies. Long exposure time of imaging materials linked with mAbs significantly increase the background interference, causing enormous difficulties and inaccuracies and poor contrast for diagnosis purposes and biodistribution studies. In addition, potential immunogenic and immunostimulatory side effects brought along with the therapeutic mAbs can be consequently exaggerated by the increased exposure of the therapeutic IgG molecule enabled by the recycling process established between antibody Fc domain and cell surface FcRn receptor. Strategies to interfere with FcRn-Fc interaction involve mutations in the IgG Fc structure to reduce the level of IgG in the circulation. Kim et al. and Medesan et al. created shorter half-lived chimeric IgGs (Medesan et al. 1997; Kim et al. 1999), and they were introduced in the immunotoxins work later on, in order to minimise the toxin side effects and improve therapeutic indices (Pop et al. 2005). In the field of tumour imaging using radiolabeled antibody, both enhanced antigen localization rate and improved clearance rate were required. To tailor the pharmacokinetics and imaging properties, attenuating the IgG Fc-FcRn interaction provides a helpful approach in controlling the antibody serum half-time for optimised antigen targeting with reduced imaging background interference and side effects (Kenanova et al. 2005; Jaggi et al. 2007).

**Extravasation and Biodistribution**

The movement of molecules across tissue membranes is generally governed by active and/or passive transport processes. Active transport is mediated by specific receptor or carrier, whereas the passive transfer is more likely to happen via simple diffusion. As shown in Figure 1.7 below, the passive diffusion is generally regulated by factors such as tissue permeability, membrane surface area, concentration, and pressure gradients of drug across the membrane (Tabrizi et al. 2009).
Classified as a large, hydrophilic substance, IgG antibodies transport across the vascular endothelial cells very slowly, and diffusion and convection are believed to be the primary driven force to drag the antibodies from blood fluid to tissue interstitial fluid. The following “into-tissue” extravasation movement of antibodies through various capillary networks is determined by the rates of fluid movement from blood to lymph and the sieving effect of paracellular and lymphatic pores in the vascular endothelial beds that allow antibody permeability.

Figure 1.7. Passive diffusion processes for molecules move between blood and body tissues. In general, blood flow limited perfusion (a) and physiological structures (i.e. permeability) (b) of tissue membrane regulate the rate of drug distribution in tissues. Pressure gradient mediated molecule convection (c), and concentration difference induced diffusion (d) processes are two main mechanisms of transvascular transport that may facilitate macromolecules extravasation. Figure taken from (Tabrizi et al. 2009).

There are four types of blood capillaries that could facilitate the transport of mAbs, however, only the sinusoids having clefts (~100nm) around organs such as liver and spleen can allow mAb travel freely. Fenestrated capillaries found in the gastrointerstinal (GI) tract, various glands and renal glomeruli with clefts about 30-80nm in diameter can already be restrictive to IgG transport, and disallow the macromolecule renal clearance. Consequently, much slower diffusion of mAbs is found in structures such as skin, muscle and other connective tissues through the continuous capillaries, and nearly no IgG antibodies can be distributed into the brain due to the tight brain capillary endothelial wall, also known as the blood-brain barrier (Wang et al. 2008; Tabrizi et al. 2009). Concentrations of unbound IgG are approximately tenfold lower than plasma concentrations in many tissues, except those with leaky vasculature capillaries (Tabrizi et al. 2009).
Shown in Figure 1.8, receptor-mediated and fluid phase endocytosis may be another important pathway for antibody to distribute in body tissues (Lobo et al. 2004). Internalization of interstitial antibodies bound to membrane antigens, and FcγRs via Fc domain may initiate blood–tissue hydrostatic pressure gradient facilitating antibody convective transport into tissue fluid.

**Figure 1.8. Antibody distribution.** (a) Antibody transport to the tissue interstitium or return to the blood via fluid phase endocytosis by FcRn mediated entry into vascular endothelial cells; (b) Sieving effect of paracellular pores in the vascular endothelium regulates the entry of antibody into the tissue interstitium via convective transport, and the return of antibody to lymph fluid with much quicker rate due to larger diameter of lymphatic vessels. It is reasonable to expect lower antibody concentrations in interstitial fluid relative to blood. However, exceptional distribution can be found in organs with leaky capillary structures and high affinity/high capacity binding of antibody in tissue; (c) Binding of antibody to cellular antigens. Scheme is cited from (Lobo et al. 2004).

More importantly, fluid phase endocytosis has been suggested for antibody extravasation via vascular endothelial cell internalization contributed by FcRns, specially supported by FcRn bi-directional (apical and basolateral) manner of transporting IgG (Antohe et al. 2001; Lobo et al. 2004). The wide expression of FcRn in different cells may facilitate transport of IgG molecules, and it does not mediate the uptake or influx of IgG in the blood to tissue direction (Ghetie and Ward 1997; Roskos et al. 2004). As outlined in Figure 1.8, this antibody “distribution” phase is also termed as the α-phase when measured by the biexponential clearance model.

Clearly, the excessive large size of intact IgG molecule significantly limits its in vivo distribution, which limits quantities delivered to malignant tissues or tumours, and poor diffusion to the neoplastic blood vessels around the tumours (Batra et al. 2002). Insufficient therapeutic effects, increased side-effects and cytotoxicity, and poor bio-imaging efficiency
can all be considered due to poor extravasation and antigen localization via using whole length IgG antibodies. Particularly for treating larger tumour mass, irregular vasculature, cell density, tissue structure, extracellular matrix (ECM) composition, and most crucially, the hypoperfused tumour regions with elevated interstitial fluid pressure could make the diffusion of an IgG across half a centimetre in weeks to months (Jain and Baxter 1988; Beckman et al. 2007). Therefore, in order to deliver mAbs into brain and solid tumour masses, tailoring antigen specificity with smaller molecular size of antibody for better tissue penetration is highly required in order to achieve improved therapeutic effects of the antibody (Beckman et al. 2007; Pardridge 2008). The Fc domain that consists of one third of the total IgG structure could possibly be considered as a redundant part, especially when cytotoxic payloads or imaging materials are conjugated, while functionally, targeting specific antigens seems to be more important and valuable for mAbs in different therapeutic purposes.

**Degradation and Clearance**

Therapeutic IgG molecules can be degraded and eliminated from body circulation through a number of mechanisms, which are affected by many Fc domain related factors including affinity to FcRn receptor, immunogenicity and glycosylation degree of the mAb, susceptibility for the mAb to proteolysis, and antigen-mediated eliminations (Mascelli et al. 2007). Due to the large size of IgG molecule, other than normal urinary excretion via kidney, intact mAbs are primarily degraded by plasma proteolytic catabolism, following fluid-phase or antigen-mediated endocytosis.

Pointed out previously, binding between IgG Fc to FcRn receptors protect the IgG molecules from a variety of proteolytic enzymes and lysosomal catabolic destinies. This reinforcement for therapeutic mAb blood stability was observed in clinical trials from short half-life of murine originated mAbs to long circulation time of more human-like mAbs, due to different interspecies of IgG Fc domain affinities to human FcRn (Roskos et al. 2004). However, excessive administration of stable IgG molecules with prolonged serum half-life can be aetiological agents in certain types of autoimmune disease treatment. Strategy to modulate *in vivo* antibody levels, developed by Vaccaro *et al.*, uses administration of engineered IgGs with high affinity but reduced pH-dependent binding to FcRn. Therefore, these IgGs are not readily to be released by FcRn at the cell surface, and the excessive of this accumulation can block the FcRn of recycling other serum IgGs. They called this man-made IgG “Abdeg”, and it has the function of enhancing IgG degradation. The inability of FcRn-binding caused by
Abdegs may be useful in autoimmune disorders or inducing rapid clearance of IgG-toxin or IgG-drug complexes (Vaccaro et al. 2005). The concept was also supported by the research of Petkova et al. from their humanized mouse models (Petkova et al. 2006).

Based on the phenomenon of the concentration-catabolism effect, FcRn’s IgG restoration can be interfered or abolished by competitive occupation and saturation of FcRn receptor, which then facilitates IgG clearance. Overloading with “innocuous” exogenous IgG raises the serum concentration above the equilibrium set point and saturates the FcRn. This has been one possible way to shorten the half-life of excess IgGs with potential side effects. Losing the chance to bind to FcRn, the IgGs have to enter the degradation pathways (Yu and Lennon 1999). Monoclonal antibody directly against FcRn as well as small molecules or peptide inhibitors of the FcRn-Fc interaction could be used as alternative approaches to modulate the intact mAb clearance (DeLano et al. 2000; Marino et al. 2000; Liu et al. 2007).

Antigen-mediated antibody elimination is also very important to remove circulating IgGs via lysosomal catabolism, and significantly affect the PK of antibodies. Although three classes of FcγRs as introduced previously (section 1.1.4.2) are primarily responsible in regulating various effector cells functions after binding IgG Fc domains with varying degrees of affinity, they are also contributing impact on antibody clearance. In the case of targeting soluble antigens, therapeutic IgG molecules establish antigen-antibody complexes (or immuno complexes) after antigen binding. Recent findings highlight that FcγRs expressed on phagocytic cells capture the immuno complexes through FcγR and IgG Fc interaction, and internalize the complexes to lysosome for protein degradation and eventual clearance (Tabrizi et al. 2009). Both experimental and theoretical studies have also predicted more rapid clearance rate followed by increases in antibody-antigen binding in circulation (Johansson et al. 2002; Johansson et al. 2002). The antigen-mediated clearance mechanism is very dose dependent, and it considerably shortens the therapeutic mAb half-life when the dose level is insufficient to saturate the antigen pool (Roskos et al. 2004). An obvious deduction of trastuzumab half-life was reported in patients expressing both low and high serum levels of shed HER2 (Baselga et al. 1996).

As mentioned previously, exogenous mAb induced immunogenicity is a product specific phenomenon, where IgG Fc domain specifically highlights the immunogenic features. The presence of endogenous anti-globulin antibodies may lead to a wide range of PK effects, as
the sizes of different immune complexes formed between the endogenous anti-drug antibodies and the therapeutic mAbs can lead to various elimination rates through FcγR featured phagocytosis, and this impact on the disposition of therapeutic mAb can be complex and hard to predict (Wang et al. 2008).

1.2.2 Antibody Fragmentations and Recombinant Fusions
In the context of using antibodies as therapeutic agents the advantages and disadvantages of antibody have been compared in the text and summarized in Table 1.2.

Table 1.2. Properties of intact IgG based mAbs modulated by the Fc fragment in clinical development. The summary was partially adapted from previous reviews (Baumann 2006; Mascelli et al. 2007).

<table>
<thead>
<tr>
<th>Property</th>
<th>Intact IgG based therapeutic mAbs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physicochemical</td>
<td>large molecular weight (150 kDa); Complex and undefined properties (e.g. tertiary structure, extent and type of glycosylation); High cost of biotechnological production by host cell lines and isolated from culture media; Broad specifications which may change during development, difficult to standardize.</td>
</tr>
<tr>
<td>Toxicological</td>
<td>Antigen-mediated toxicity; Inappropriate Fc receptor (FcγR and FcRn) cross-interactions; Cytokine release; Exaggerated pharmacodynamic responses and biological toxicity.</td>
</tr>
<tr>
<td>Immunogenicity</td>
<td>Usually elicits antigenic responses</td>
</tr>
<tr>
<td>Pharmacokinetic</td>
<td>Very long half-life of a magnitude of weeks; Distribution usually limited to plasma and/or extracellular fluids; Poor extravasation for antigen localisation, and heterogeneous tissue/tumour penetration; Potential over-exposure to normal organs; Eliminated primarily by serum/lymphatic proteolytic degradation and lysosomal catabolism to endogenous amino acids; Slow blood clearance, which may not be linear (e.g. receptor mediated disposition).</td>
</tr>
<tr>
<td>Usability</td>
<td>Relatively stable in therapeutic usage; Limited therapeutic efficacies in brain and solid tumour treatments, due to restricted capillary diffusion and hypoperfusion; Limited application in imaging studies.</td>
</tr>
</tbody>
</table>

The engineering of antibodies is sometimes required to eliminate the undesirable negative effects associated with the Fc fragment. Various fragmentation approaches and different innovative structural and recombinant designs have been used to engineer a new generation of smaller antibodies without an Fc domain. Additionally, new molecular strategies have aimed to create novel antibody products with enhanced affinity, stability, reduced side effects,
modulated PK, and therapeutic efficacies. Recombinant technology has also provided a high yield and low cost lower cost method of production, allowing for the economically viable manufacture of therapeutic agents.

1.2.2.1 Design of Antibody Fragments

Initial attempts to remove the Fc domain and make smaller antibody fragments were based on the digestion of full length IgGs by the proteolytic enzymes pepsin or papain, which generates Fab$_2$ or Fab fragments, respectively (Figure 1.9). These fragments retain their specific antigen-targeting capacities, demonstrate enhanced tissue distribution and antigen localization, and have faster homogenous tumour penetration and reduced immunological side effects compared with intact IgGs (Batra et al. 2002; Holliger and Hudson 2005; Jain et al. 2007). The production of different recombinant antibody fragments has also been facilitated by advances in recombinant DNA technology.

The development of genetically engineered antibody fragments was initiated nearly 20 years ago through the engineering of a single gene encoding the variable heavy (V$_{H}$) and light (V$_{L}$) chains connected by a short flexible polypeptide linker (Figure 1.9), resulting in the production of a single-chain variable fragment (scFv) with monovalent antigen binding capability like that of the parental Fab fragment from which it was derived (Bird et al. 1988; Huston et al. 1988; Holliger and Hudson 2005). Based on the influential invention of the scFv, numerous recombinant possibilities have been explored with regards to modifying antibody valency, functionality, biodistribution, PK, and therapeutic efficacies (Hudson and Souriau 2003). A schematic illustration of versatile recombinant antibody designs that have been investigated by now is shown in Figure 1.9 below.

**Monovalent Recombinant Antibody Fragments**

In the early 70s, initial reports described studies of antibody variable domains isolated from enzymatic digestion which were non-covalently associated and retained all the antigen binding characteristics of the related Fab fragment (Inbar et al. 1972). These 26 kDa molecules termed Fv fragments (Figure 1.9) exhibited improved tissue penetration due to their smaller size. However, poor stability at low concentrations and heterogeneity during purification significantly limited the expansion of this format (Skerra and Pluckthun 1988; Ward et al. 1989). Fragment design forward and managed to resolve the problems seen in Fv
Figure 1.9. Schematic diagram of different recombinant antibody formats. Structures of three intact immunoglobulin molecules predominantly found from camel, shark and human are illustrated. They are named as camelid VhH-Ig, shark Ig-NAR and the ‘classic’ IgG respectively. The V domains are responsible for target binding, where the monomer of camelid V domain is a called VhH, and the shark variable domain monomer is known as V-NAR. Both of the monomers (~15 kDa) have monovalent binding capacities and also known as nanobodies with various functional purposes. A variety of recombinant antibody fragments are also depicted, including single-domain VhH, Fab, scFv and their multimeric formats, alongside different scFv fusion derivatives as well (sizes given in kilo Daltons are approximate). Figure was taken from (Holliger and Hudson 2005) and modified based on the reviews from (Hudson and Souriau 2003; Kontermann 2005; Carter 2006; Deonarain 2008).

fragment production. Both antibody V_H and V_L domains were commonly tethered by a 15 amino acid (Gly_4Ser)_3 linker that provides both flexibility and hydrophilicity to the more stabilized monomeric single chain antibody fragment (Bird et al. 1988; Huston et al. 1988; Maynard and Georgiou 2000). In order to investigate scFvs further for improving functionality, stability and expression levels, issues including domain orientation (V_H-linker-V_L versus V_L-linker-V_H) (Tsumoto et al. 1994; Kim et al. 2008) and the nature (i.e. length and composition) of the inter-domain linker were subjects of additional study (Stemmer et al. 1993; Turner et al. 1997). Aggregation tendency is another characteristic associated with scFvs which is primarily influenced by the intrinsic antibody sequence (Arndt et al. 1998). An
alternative monovalent format to the scFv is known as a dsFv, which stabilizes both the V\textsubscript{H} and V\textsubscript{L} domains by inserting a disulfide bond into the Fv framework region, and has been found to offer greater stability and less aggregation compared to the scFv (Kreitman et al. 2001).

With an even smaller size than Fv fragments, high affinity single variable (V)-like domains have been discovered from at least two types of organisms – the camelid (camels and llamas) and cartilaginous fish (sharks), and named as VhH in camels and V-NAR in sharks (De Genst et al. 2004; Streltsov et al. 2004; Dooley and Flajnik 2005). These single V-like domains (~15 kDa) are generally soluble and stably expressed (Figure 1.9). Nanobodies are derivatives of the VhH and V-NAR antibodies. Modifications based on their ultra-small sizes and unique antigen binding surface structures, sensitive diagnostic and therapeutic nanobodies have promised to accomplish superior tissue penetration and targeting cryptic epitopes (i.e. enzyme active sites and canyons in viral and infectious diseases) (Cortez-Retamozo et al. 2004; Stanfield et al. 2004; Stijlemans et al. 2004). Attempts to make nanobodies from human IgG single variable domain would be more preferred with reduced immunogenic side effect brought along current xenogenic nanobody formula (Colby et al. 2004; Jespers et al. 2004; Holliger and Hudson 2005).

In general, monovalent antibody fragments (i.e. Fab, scFv, Fv, dsFv, and nanobodies listed above) are easily expressed with good yields and can be manipulated by genetic engineering to produce different monovalent fusion formats and various multi-functional and multivalent polymeric designs for therapeutic applications (Holliger and Hudson 2005; Jain et al. 2007; Weisser and Hall 2009). In terms of bi-functional fusion, a vast range of molecules (see section 1.1.4 and Figure 1.5) have been fused to provide important ancillary activities after target binding (Hudson and Souriau 2003). Various scFv-based fusion proteins have utilized these formats, such as scFv-enzyme fusions for ADEPT in cancer therapies and scFv-cytokine fusions for treating immunological diseases (Kousparou et al. 2002; Gafner et al. 2006; Kanter et al. 2007; Afshar et al. 2009). In this study, rather than delivering a secondary agent to a target, the primary purpose of producing a bifunctional fusion was to use the ancillary component to modulate the pharmacokinetic modality of the antibody for therapy. (described in more detail in section 1.5).
Bivalent and Multivalent Recombinant Antibody Fragments

A major characteristic of monovalent interactions is the moderate dissociation constant ($K_D$) and modest retention time between antibody fragments and their antigen, especially in the non-equilibrium environment of body vasculature and tissues (Holliger and Hudson 2005). For many applications requiring a slower target off-rate, and longer retention times, the use of multivalent antibodies with increased avidity and optimal size that can be beneficial in providing both improved binding kinetics and increased serum half-life (Jain et al. 2007; Weisser and Hall 2009).

As illustrated in Figure 1.9, Fab, scFv fragments and nanobodies can all be engineered into di-, tri, tetrameric formats conjugated primarily by either chemical approaches or genetic linker designs. Construction of multimeric scFv’s can be easily achieved through the reduction of scFv linker length to between zero to five amino acid residues. The shortened inter-linker of scFv molecule enables the VH of one chain to non-covalently pair with the VL of another, and consequently form a dimer molecule (diabody, ~50 kDa) (Holliger et al. 1993). Linkers less than 3 amino acids can force scFv’s to associate into tri-bodies or tetrabodies (Iliades et al. 1997; Dolezal et al. 2003). A cysteine-modified diabody has also been engineered to improve the structural confirmation consolidation of the non-covalent format, and facilitate additional chemical modifications (e.g. with a radiometal for imaging) (Olafsen et al. 2004). Different strategies such as genetically engineering linkers between two or more monomeric fragments and using multimerization domains (e.g. streptavidins, p53, leucine zippers and verotoxin) fused to antibody monomer fragments have been studied to generate various divalent, such as tandem single chain variable fragment (taFv) and single chain diabody (scDb) and multivalent molecules (Batra et al. 2002; Kontermann 2005; Jain et al. 2007).

Bispecific and Multispecific Recombinant Antibody Fragments

The combination of two or more different antibody fragments with more than one antigen target can have therapeutic applications. The bi- or multispecific antibody fragment can be constructed following the same principle used to form bi- or multivalent antibody fragments with different monomer elements (see Figure 1.9 for bispecific scFv and diabodies as schematic examples) (Todorovska et al. 2001). One of the most important and useful characteristics possessed by antibody fragments with dual or multiple specificities is the direction of effector functions towards the targeted antigen. With one arm targeting a specific antigen and the other arm engaging effector targets, such as T cell CD3, NK cell CD16, and
complement C1q. Such multispecific antibody fragments can recruit effector function without needing the antibody Fc domain (Holliger and Hudson 2005; Filpula 2007; Weisser and Hall 2009); therefore overcoming the side effects associated with the Fc domain as mentioned in section 1.2.1.1. Equipped with more than one antigen targeting specificities, bi- and multispecific antibody can also be very important in the treatment of autoimmune and inflammatory diseases (van Spriel et al. 2000).

Minibodies are recombinant antibodies consisting of antigen binding antibody fragments and intact or truncated Fc domains (eg. scFv-C1_3 dimers in Figure 1.9, and scFv2-Fc). This arrangement can be considered as another bifunctional design, where the Fc domain can elicit effector functions, however, these formats can only be most useful where the negative effects of having the Fc domain present are not considered detrimental to function (Batra et al. 2002; Jain et al. 2007; Weisser and Hall 2009).

1.2.2 Advantages of therapeutic Antibody Fragments

When engineering antibodies for therapy, agents must generally exhibit high affinity with discriminating specificity, minimal immunogenicity and low cross-reactivity (Ober et al. 2001); these are the criteria for the successful development of a antibodies for therapy. Good expression yields, relatively simple construction, easy manipulation by recombinant engineering, has placed monovalent Fab or scFv fragments in the forefront of antibody engineering development. Large natural and synthetic immune repertoires have been used to create various molecular library formats for selecting specific high-affinity antibodies (Hoogenboom and Chames 2000; Krebs et al. 2001; Hudson and Souriau 2003). The established display formats including bacteriophage display (McCafferty et al. 1990), ribosome display (Hanes and Pluckthun 1997; He and Taussig 1997), cell surface display (Francisco et al. 1993) and transgenic mice (He et al. 2002). More frequently, increased and repeated selection cycles have also been used to enhance antibody affinity maturation. As required, the selected high-affinity small monovalent antibody fragments can be reassembled back to Ig or multivalent or specific variants as described previously. A wide variety of expression hosts can be used for high yield production of monoclonal antibody fragment, including prokaryotes (i.e. E. Coli and B.subtilis) and eukaryotes, including yeast, insect, plant and mammalian cells (Weisser and Hall 2009). However, the production of smaller and unglycosylated antibody fragments can be easier and less costly to produce in microbial
expression systems than in conventional mammalian cell culture systems.

The humanization of smaller formats can also reduce immunogenicity to a significant degree. Using as an example the anti-TNFα humanized recombinant Fab fragment certolizumab pegol, which was approved by the FDA for Crohn’s disease treatment, it was shown through safety and efficacy assessments that it had a lower rate of adverse-events than any other approved anti-TNF agent (Nesbitt et al. 2007; Sandborn et al. 2007), and a similar therapeutic efficacy to infliximab, a market leader for CD treatment (Melmed et al. 2008; Peyrin-Biroulet et al. 2008). Low levels of human anti-Cimzia antibodies were detected in patients with various concentrations of intravenous infusions, especially lower incidence in patients injected with higher doses after a treatment cycle (Choy et al. 2002). Furthermore, data from patients suggested Cimzia has no cross-reactivity with other therapeutic agents, and other agents induced antidrug antibodies. Thus, combinational use of the Fab fragment, especially use as a replacement for patients develop immune response to traditional IgG antibodies (i.e. infliximab) can propose further therapeutic impact for CD treatment.

Other FDA approved mAb Fab fragments with direct therapeutic functions are abciximab that prevents blood clots in angioplasty treatment, and ranibizumab for wet age-related macular degeneration treatment, CroFab™ for snake venom neutralization, and DigiFab™/Digibind™ as antidote for digitoxin poisoning, alongside one approved in China (metuximab) for cancer treatment (Holliger and Hudson 2005; Nelson and Reichert 2009). With a diversity of clinical antigen targets, a number of antibody fragment products have already been tested in different clinical and preclinical trial stages (Figure 1.10), while a huge pipeline of ongoing fragments also attracting a great deal of research and investment attentions. Among these products, novel scFv and nanobody based technologies have been added with Fab fragments to lead the new development wave of therapeutic mAbs (Nelson and Reichert 2009).
The remaining approved mAb fragments (6 in total) are applied in imaging for diagnostic usages (see Table 1.1). Most importantly, the smaller version of antibody fragments exhibit strong points such as enhanced tissue penetration, rapid vascular extravasation and antigen localization, which are the key factors to improve solid tumour antibody therapies (Jain and Baxter 1988; Holliger and Hudson 2005). Faster blood clearance rate as another major characteristic associated with small mAb fragments has been considered as ideal outcome to deliver enhanced imaging and radiotherapy applications with low background and high contrast and minimized over-exposure hazards to normal tissues (Holliger and Hudson 2005). However, this quick clearance rate can be a major concern to the antibody fragments to realize their therapeutic activity before being eliminated from the body circulation.

1.2.2.3 Pharmacokinetics of Small Antibody Fragments

One of the most important issues in antibody engineering is developing ones which exhibit optimal pharmacokinetics: appropriate dosing leading to optimal bioavailability, uptake, distribution and clearance in targeted and non-targeted tissues, leading to optimal
pharmacodynamics (Beckman et al. 2007; Constantinou et al. 2010). Due to the small size for most monovalent and bivalent antibody fragments (less than 50 kDa), renal metabolism is the major pathway for small protein and peptide elimination. Three possible routes are involved in kidney elimination, (1) glomerular filtration followed by (2) proximal tubules reabsorption and (3) hydrolytic degradation (Tang et al. 2004; Mahmood and Green 2005). The glomerular filtration selectivity is regulated by the size, molecular conformation, and charge of the protein. In general, protein sizes less than the approximate 70 kDa cut-off point are easily sieved out, which is typically small mAb fragments (Akhtar and Al Mana 2004). Studies on glomerular charge-selectivity also suggested anionic polymers are less readily to pass through the negatively charged basement membrane of the glomerulum than cationic molecules (Deen et al. 2001; Tang et al. 2004). As mAb hence have little charge influence, hydrolysis by brush border enzymes located on the luminal membrane appears to be more efficient and specific to small linear proteins (Carone et al. 1982). Lastly, rapid and feasible extravasation of size-reduced mAb fragments also facilitates the post-glomerular capillary reabsorptions through proximal tubules (Tang et al. 2004).

Despite the many benefits of being engineered into smaller versions, the biggest drawback of using small mAb fragments in therapies is its quick body clearance predominately via kidney elimination. Outlined in Figure 1.11, molecular size (often represented by proteins molecular weights) deductions of different recombinant antibody and fragments, migrated from intact IgG molecule (150 kDa) to monovalent scFv (30 kDa) and single V domain (15 kDa) antibody fragments, indicate corresponding plasma half-life (particularly drug elimination time, $t_{1/2}$) reductions in general. Comparing to full length IgG molecules, a significantly shortened half-life has been shown to size reduced antibody fragments. As most mAb fragments are Fc domain free constructs, losing the Fc structure leads to the subsequent loss of Fc mediated FcRn salvage recycles, which adds another mechanism for fragments having a curtailed blood circulation time, on top of the size-provoked rapid renal clearance (Holliger and Hudson 2005; Kontermann 2009; Weisser and Hall 2009). As depicted in Figure 1.11 (b) above, small scFvs typifying small antigen binding proteins, have an extremely shortened residence time through both blood circulation and targeting tissues (i.e. tumour). Fragments with monovalent binding property also result in a poor antigen-expressing tissue retention capacity implied by the tumour/blood ratio analysis. Together with the rapid vascular extravasation and increased blood clearance rate via kidney elimination, a decreased in vivo antigen recognising window, reduced retentive support and decreased drug concentration

- 64 -
Figure 1.11. The diversity of mAb formats. (a) A cartoon representation of different antibody formats together with their respective molecular weights (kDa) and human serum half-life (β phase). The glomerular cut-off (~70 kDa) line is also indicated. (b) Biodistribution of different Her2 targeting mAb formats in two xenograft models. Left graph: tumour (solid lines) and blood levels (dashed lines) plotted against time after injection (h) for anti-Her2 mAb IgG (blue), diabody C6.5 (black) and scFv C6.5 (red) in severe combined immune deficient (SCID) mice bearing solid Her2 expressing tumour. Right graph: Tumour-to-blood ratios of anti-CEA IgG, minibody, diabody and scFv in athymic mice with LS174 (CEA expressing) colon carcinoma xenografts plotted versus time after infusion (h). Figure adapted from (Holliger and Hudson 2005) and (Olafsen et al. 2004; Kenanova et al. 2005; Olafsen et al. 2005).

and accumulation at the site of action can be seen in small antibody fragments after injection. It has been shown by anti-tumour diabodies having bivalent antigen binding capacity but failed to show improvement in serum half-life and tumour retention times (Figure 1.11b), due to its smaller size than the renal cut-off point. These unwanted pharmacokinetic features can be disadvantageous for a range of treatments requiring prolonged blood residence time (e.g. neutralizing harmful cytokines or toxins) or enhanced target binding and tissue retention or accumulation specificities (i.e. antibody directed solid tumour and payloads delivery therapies). Without the FcRn salvage pathways mediated by Fc domain interaction, there is an increased likelihood that antibody fragments will be degraded by serum proteolytic enzymes or lysosomal catabolism. This can be considered as another cause leading antibody fragments to a reduced serum half-life. Therefore, an ideal biopharmaceutical reagent, specifically for administrative longevity needed tumour treatment or neutralization purposes, probably requires an intermediate molecular size with consistent (perhaps multivalent) binding
specificity, which provides rapid tissue penetration and antigen localization, high target retention and relatively good blood clearance rate to avoid over-exposure and immunogenicity elicitation (Holliger and Hudson 2005; Carter 2006).

1.3 Modulating the Pharmacokinetics of Therapeutic Antibodies

As previously described, the therapeutic efficacy of recombinant antibodies is strongly dependent on their pharmacokinetic properties, especially the time course within body circulation and their particular retention and concentration at the target site (Kontermann 2009). Specifically subjected to the advent of small antibody fragments and alternative binding scaffolds, that are devoid of Fc-regions, strategies to increase the half-life of small proteins are becoming increasingly important (Constantinou et al. 2010). Traditionally, in order to prevent the rapid elimination via renal filtration and degradation (Tang et al. 2004), repeated large doses of these small protein drugs have to be administrated by infusion or intravenous bolus injections for high therapeutic efficacy maintenance over a long time period, and difficult administrative approaches, such as loco-regional intravitreal injection, has to be used to preserve a good antibody concentration at the disease site (Lobo et al. 2004; Kontermann 2009). Not only with respect to therapeutic functions, can this pharmacokinetic remediation significantly increase the risks of immunogenicity and other side-effects. The huge productive expenditure, as well as the cost, convenience and compliance to patient to complement this kind of administrative consumption is another reason for the expansion of using antibody fragments in therapies (Mahmood and Green 2005; van de Weert et al. 2005; Kontermann 2009).

There are two general approaches to extending the longevity and stability of therapeutic proteins: chemical methods and recombinant/genetic techniques (Figure 1.12). Chemical methods pre-date the recombinant approaches and involve the chemical attachment of moieties which alter the physiochemical properties of the protein. Applied to antibodies, this includes the attachment of polymers, sugars and protein domains. Recombinant methods require the genetic engineering of the target protein or host cell line to produce variants with altered structure or function. The structural changes which lead to altered pharmacokinetics
include size, hydrodynamic volume, aggregation status and electric charge (pl) and the functional changes include various receptor binding abilities (Werle and Bernkop-Schnurch 2006; Kontermann 2009; Constantinou et al. 2010).

Chemical conjugation methods has been the most investigated method for modulating antibody pharmacokinetics so far, where by chemically inert moieties are strategically attached (see Figure 1.12 strategies 1-3). The main function of the conjugates is to effectively increase the apparent size or hydrodynamic volume of a given small particle, and consequently alter its pharmacokinetic properties. In general, the larger the conjugate attached the greater the half-life becomes, and this is most significant for proteins below the molecular weight cut-off threshold of glomerular filtration (Akhtar and Al Mana 2004; Tang et al. 2004; Constantinou et al. 2010).

However, a balance must be achieved for the pharmacokinetic modifications, in case the benefits of lower clearance rates are not gained at the expense of significantly poorer tissue penetration and/or poor tissue to blood ratios. This is a particularly important consideration if antibodies are used as carriers of cytotoxic agents, such as radioisotopes (Davies 2007), where high doses leading to extended systemic residency could cause damage to normal tissues. Certainly, for antibodies neutralising systemic factors such as VEGF for solid tumour

Figure 1.12. Schematic diagram illustrating the different pharmacokinetic enhancing strategies. Both chemical and recombinant methods are involved. Strategies 1–3 work by increasing hydrodynamic radius, via conjugating chemicals (i.e. PEG or polysialic acid), homo amino acid polymer (HAP) fusions and fusion with polysialic acid (PSA) carriers; whereas strategies 4–9 utilize long serum-living proteins that have binding capacities to the neonatal Fc receptor, and methods in turns are bispecific albumin binding antibody fragment, chemicals, peptides or proteins, and direct albumin fusion, along with reconstruction with Fc portion. Key: blue line connections represent recombinant fusions and grey line connections represent chemical linkages. This figure has been directly taken from (Constantinou et al. 2010).
treatment (Panares and Garcia 2007) or tissue TNFα for Crohn’s disease (Tracey et al. 2008), a long half life is desirable.

Clearly, “one size fits all” form of conjugation is not practically applicable in manipulating small antibody drug pharmacokinetics. However, one general pharmacokinetic improvement profile is commonly required to be achieved, which includes extending the longevity of the antibody, allowing a more level range of bioavailability with enhanced potency, and directing the antibody particular mechanism of action specifically at the target site (Constantinou et al. 2010).

1.3.1 Chemical Conjugation

1.3.1.1 Random (amine) versus Site-specific (thiol) Coupling

Chemical conjugation mostly happens by interacting with various reactive groups within the primary antibody sequence. Typical reactive groups are lysine, cysteine, histidine, arginine, aspartic acid, glutamic acid, serine, threonine and tyrosine, as well as the N-terminal amino group and C-terminal carboxylic acid groups of the peptide. Lysine residues are traditionally used as active conjugating points as they often have a high surface propensity. However, these functional groups are not automatically predisposed as a conjugation point normally. Reactive groups may be sterically hindered as they are often embedded within the core tertiary structure of the antibody and therefore unavailable for conjugation. Moreover, essential amino acids for the antibody’s function represent undesirable sites for conjugations. Although non-site specific random conjugation has shown some success (Lee et al. 1999; Chapman 2002), the more prevalent and preferred approach is to engineer proteins that eliminate residues where conjugation is undesired and re-engineer specific sites where conjugation is desired, particularly thiol-containing cysteines (Kaushik and Moots 2005; Junutula et al. 2008; Constantinou et al. 2009). Unlike the ubiquitous nature of amine groups, surface-available thiol (-SH) group are not frequently expressed on proteins, and this modification and manipulation of thiol groups are becoming increasingly favoured for conjugation purposes. As a remnant from the whole immunoglobulin structure, a free thiol is often found in antibody Fab fragments at the C-terminus of the light chain and it can be easily be introduced in other recombinant fragments. Three major benefits can be achieved through such engineering for chemical conjugations; firstly conjugations at undesirable sites that can be detrimental to protein activity are avoided, secondly a more homogeneous form of conjugates with specific
conjugate isoforms are established in order to accomplish more effective pharmaceutical usages, and lastly, the number of conjugation reactive sites can be engineered for potential pharmacokinetic manipulation. In comparison, random amine coupling is a more feasible and direct approach for chemical conjugation, however unprosperous, undesired and protein function detrimental results are the most significant hindrance or even stop points for this type of conjugations. Summarised in previous studies, amine-directed chemical polysialic acid conjugation (see below) was successfully used to modify the PK of an anti-tumour Fab (Constantinou et al. 2008), however the same process reduced the antigen recognising function of an anti-tumour scFv which was resolved by site-specific coupling modification (Constantinou et al. 2009). Similarly, conjugation of larger polymer chains was found to be more detrimental on antibody activity after random amine coupling, which has been observed from an anti-TAG-72 scFv conjugated with long PEG chains (Lee et al. 1999). This demonstrates that antibody bioactivity has a dependence on different residues within the complementarity-determining region (CDR) which are difficult to predict in the absence of structural information. If known, sensitive conjugation sites can be eliminated by mutagenesis. Such approach has been successfully utilized to mutate a non-essential lysine residue in the $V_H$ CDR3 of the C6.5 scFv into a non-conjugatable alanine residue to prevent unwanted chemical conjugation in the anti-HER2 scFv antigen binding site (Adams et al. 2000).

1.3.1.2 PEGylation - Hydrophilic Polymer Conjugates

As illustrated in Figure 1.12, a wide range of polymers and attachment moieties have been studied so far for their pharmacokinetic modulatory properties. For polymer conjugates, apart from their basic chemistry properties, factors including conjugation number, length and structural complexity of the polymer can be varied in order to produce a different pharmacokinetic impact. A number of polymers, such as polydextrans (PD), polyvinylalcohols (PVA), polystyrene-co-maleic acid, polysialic acid (PSA), polyglycerols, polyals, hydroxyethyl starch (HES) and polyethylene glycol (PEG) has been investigated as conjugation candidates for therapeutics pharmacokinetic modification purposes (Kontermann 2009; Constantinou et al. 2010). Typically, the residual components of these polymer molecules have a high hydrophilic ability allows their interactive attraction to water molecules in solution. Accumulatively, such hydrated polymers appear to have a much greater apparent molecular weight and hydrodynamic radius than the polymer alone after the attachment of the water molecules. Pharmacological explorations of these hydrophilic
polymers conjugated to therapeutic molecules have resulted in masking immunogenicity, solubility improvement and pharmacokinetics modulation of native proteins (Figure 1.13).

Figure 1.13. Main advantages of PEGylated protein. The figure represents a polymer-protein conjugate. PEG, as a water attracting polymer establishes a “watery shield” around the protein surface and protect the inside from degrading agents by steric hindrance. More importantly, the increased hydrodynamic volume of the conjugate exceeds the cut-off point of the glomerular filtration, leading to decreased kidney clearance of the PEGylated protein. These distinguishable advances demonstrated by PEGylation are also shared by most hydrophilic polymers (i.e. polysialic acid). This schematic diagram was taken from (Veronese and Pasut 2005).

To date, the physicochemical properties of PEG have been the most extensively studied, which serves as the most advanced carrier system technology to date. With a long acknowledgeable history as a non-toxic, inert, hydrophilic, uncharged and non-biodegradable polymer, PEG has also been approved by the US FDA for therapeutic use (Veronese and Pasut 2005). Subjecting to hydrophilicity, each ethylene glycol subunit of PEG can support up to three water molecules (Sasahara 1995) indicating PEG can carry water up to its own molecule mass, resulting in an great increase of hydrodynamic volume (Harris and Chess 2003; Fee and Van Alstine 2004). Furthermore, the pharmacokinetic behaviour of the PEG chains can be dictated by their lengths and conformations (i.e. linear or branched structure) (Yamaoka et al. 1994; Yamaoka et al. 1995; Fee 2007; Gursahani et al. 2009). It has been also noticed that the general clearance rate of the protein therapeutics is decreased when the polymer-conjugate size increases; however their permeability into potential target sites also slows (Cohen et al. 2001; Yang et al. 2003; Gursahani et al. 2009). Additionally, the charge associated with the polymer-conjugates can take credits in altering the overall isoelectric point of protein being modified, which also influences the pharmacokinetics, and sometimes the functioning properties of the conjugated therapeutics. Therefore, a number of factors, such as
altering PEG length and/or conjugation ratios, as well as charge, have to be considered to modulate according to obtain a desired pharmacokinetic effect.

A number of therapeutic agents benefiting from PEGylation including Adagen™ (PEGademase bovine), the treatment of immunocompromised individuals awaiting bone-marrow transplantation; Oncaspar™ (PEGasparaginase) for the treatment of lymphoblastic leukaemia; PEGasys™ (PEGinterferon α-2a; ribavirin) for the treatment of chronic Hepatitis C infections; PEGanone™ (ethotoin) for the control of epileptic convulsions, and Macugen™ (PEGaptanib sodium) for the treatment macular degeneration have all been approved by FDA and showed improved pharmacokinetic outcomes in therapies (Jevsevar et al. 2010).

Subjected to antibody fragments, the bioactivity and blood clearance rates of an anti-TNFα scFv conjugated by maleimide-PEG chains with various molecular weights (5, 20 and 40 kDa PEG) at one or both of two available thiol sites have been systematically investigated previously. Although some loss of bioactivity was suggested from in vitro binding studies, cytotoxicity assays showed similar neutralization IC$_{50}$ values for all conjugate variants. In terms of PK, the half-life of conjugates increased as their PEGylation content increased, and up to 100-fold prolongation of circulating half-lives has been demonstrated compared to the native scFv (Yang et al. 2003). Other than detrimental effect to the antibody bioactivities, Lee et al used different lengths of PEGs ranging from 2 to 20 kDa to conjugate with scFv for PK improvement. They found that an increase in polymer length was found to be more effective for extending serum half-life than using a corresponding increase in total PEG by using smaller polymer lengths at a higher conjugation ratio (Lee et al. 1999). Same conclusions were also indicated by non-antibody studies as well (Clark et al. 1996).

Alongside preferential site-specific PEG conjugation approaches, branched PEG chains over linear ones has been promotionally generated with promising improvement features for circulatory half-lives (Harris and Chess 2003). It is claimed that a branched PEG can act as if it were much larger than a corresponding linear PEG of the same molecule weight (Yamasaki et al. 1988). As mentioned previously, the first FDA approved PEGylated antibody fragment certolizumab pegol is marketed as a branched PEG conjugate (Melmed et al. 2008). Although it has been recently shown that there was no significant difference between the viscosity radii of branched and linear PEG-proteins having the same total molecular weight of PEG adducts (Fee 2007), branched PEGs are suggested to be better at cloaking the attached protein drugs when exposed to proteolytic cleavage. Greater resilience to cleavage than its linear polymer
conjugate counterparts was demonstrated, which can be partially considered as an reason to explain branched conjugates having a seemingly longer half-life in vivo compared to those with linear PEG chains (Monfardini et al. 1995). Since the earliest demonstration in 1977 (Abuchowski et al. 1977; Abuchowski et al. 1977), PEGylation has expanded into a major industrial biotechnology to improve therapeutic protein pharmacokinetics (Harris and Chess 2003). Antibody fragments are now emerging as favourite protein candidate from this modification pipeline, in order to realize better therapeutic efficacies.

**PEGylation Chemistries**

With a clear efficacy direction of modulating antibody PK, different chemical conjugational approaches are subjected to factors such as conjugate homogeneity and reproducibility. Summarised in Table 1.3 below, various methods of activating polymers for conjugation via certain functional groups within proteins have evolved over time to minimise problems encountered with its predecessors (Roberts et al. 2002). The process of adding PEG polymer on to therapeutic proteins, also known as PEGylation, has been through a few generations. So-called ‘first generation’ PEGylation chemistry, which preferentially react to alpha and epsilon amino acids, have used derivatives such as PEG dichlorotriazine, PEG tresylate, PEG succinimidyl carbonate, PEG benzotriazole carbonate, PEG p-nitrophenyl carbonate, PEG trichlorophenyl carbonate, PEG carbonylimidazole and PEG succinimidyl succinate (Harris and Chess 2003; Constantinou et al. 2010). Problematic limitations including unstable linkages, PEG impurities, and a lack of selectivity in modification predominately occurred when applying these PEG derivatives to low molecular weight moieties. For improvement, unifunctional methoxylated PEG (mPEG) has since been generally applied to minimize the effect of cross linking between conjugates (Monfardini et al. 1995), while the ‘second generation’ PEG chemistries that also exploit amine conjugation have been considered of taking more credits to reduce the difficulties described above. These include the use of PEG-aldehydes/hydrate (Table 1.3). Partial selective conjugation is observed preferentially at primary amine via a Schiff’s base, which is subsequently reduced to give a stable secondary amine linkage (Kinstler et al. 1996). Conditions of conjugation is also very critical for preserving the protein base integrity and PEG-carboxylic acid have been therefore extensively used of being more stable and are favoured due to their reactivity at near physiological conditions (Zalipsky and Barany 1990).
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<tr>
<th>PEG Derivatives</th>
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<td><strong>First Generation Agents</strong></td>
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<tr>
<td>PEG Carbonyl-limidazole</td>
<td>N-terminal α-arnines and lysine ε-arnine group acylation leading to formation of carbamate linkages. But reactivity to other residues such as histidines and tyrosines may also be observed depending on the reaction conditions and the pKa effects of neighbouring residues. PEG Benzotriazole carbonate and PEG Succinimidyl carbonate are particularly noted for forming imidazolecarbonate linkages with histidine residues.</td>
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<td>(Beauchamp et al. 1983; Veronese et al. 1985)</td>
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<td>PEG Succinimidyl carbonate (Zalipsky and Lee 1992; Miron and Wilchek 1993)</td>
<td>It should be noted that the polymer backbone ester of PEG succinimidyl carbonate can be readily hydrolyzed leading to possible immunogenicity towards the remaining tagged conjugate if used in vivo.</td>
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<td>PEG Succinimidyl succinate (Abuchowski et al. 1984)</td>
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<tr>
<td>PEG Dichlorotriazine (Zalipsky and Lee 1992)</td>
<td>Reacts with lysine, seine, tyrosine, cysteine and histidine residues. Remaining Chloride may allow cross-linking between conjugates.</td>
</tr>
<tr>
<td>PEG Tresylate (Francis et al. 1998)</td>
<td>May form secondary conjugates with degradable sulfamate linkage.</td>
</tr>
<tr>
<td><strong>Second Generation Agents</strong></td>
<td></td>
</tr>
<tr>
<td>PEG Aldehyde:</td>
<td></td>
</tr>
<tr>
<td>1. Acetalaldehyde; 2. Propionaldehyde (Harris and Herati 1993)</td>
<td>Reactive amination, selective for N-terminal α-arnines and ε-arnine groups found on lysine residues via a Schiff base. The hydrate form of the PEG aldehyde is more stable and therefore the preferred agent to use.</td>
</tr>
<tr>
<td>PEG Aldehyde hydrate:</td>
<td></td>
</tr>
<tr>
<td>1. Acetalaldehyde hydrate</td>
<td></td>
</tr>
<tr>
<td>2. Propionaldehyde hydrate</td>
<td></td>
</tr>
<tr>
<td>(Kinstler et al. 1996; Bently and Harris 1999)</td>
<td></td>
</tr>
<tr>
<td>PEG NHS Esters (Zalipsky and Barany 1990)</td>
<td>Active esters of carboxylic acids are the most favoured acylating agents as they allow formation of very stable bonds at near physiological conditions.</td>
</tr>
<tr>
<td><strong>Carbohydrate Specific</strong></td>
<td></td>
</tr>
<tr>
<td>PEG Amine (Zalipsky and Menon-Rudolph 1997)</td>
<td>Conjugation to oxidised residues leads to the formation amine bonds; however reactivity with other protein amine groups can lead to the formation of cross-linked aggregates.</td>
</tr>
<tr>
<td>PEG Hydrazine (Gaertner and Offord 1996; Youn et al. 2005)</td>
<td>Oxidation of carbohydrate residues or N-terminal serine or threonine residues allows conjugation by this chemistry. The resulting hydrazone bond (shown above) can be stabilized by reduction to form a more stable amine bond.</td>
</tr>
<tr>
<td><strong>Thiol Specific</strong></td>
<td></td>
</tr>
<tr>
<td>PEG Maleimide (Goodson and Katre 1990; Constantinou et al. 2009)</td>
<td>Maleimide can react under physiological conditions but is not stable under aqueous conditions.</td>
</tr>
<tr>
<td>PEG Vinyl sulfone (Morpurgo et al. 1996)</td>
<td>May also react with lysine residues</td>
</tr>
<tr>
<td>PEG Iodoacetamide (Kogan 1992)</td>
<td>Reaction must be perfomed under dark conditions to limit the generation of free iodine that may react with other residues</td>
</tr>
<tr>
<td>PEG o-pyridyl didulfide (Woghiren et al. 1993)</td>
<td>Forms stable disulfide bonds except under reducing conditions.</td>
</tr>
</tbody>
</table>

Table 1.3. Development of PEGylation chemistries and modification strategies. A variety of chemistries have been used to covalently modify antibodies with specific characteristics. Although primarily used for protein PEGylation, other agents can be attached. This table is taken from (Constantinou et al. 2010).
Increasingly, conjugations through surface thiol groups which offer greater coupling selectivity than that of lysine residues have become more desirable. Furthermore, free cysteine groups rarely play a role in biological activity unless they form structurally related disulphide bonds; in which case these do not partake in the conjugation process. Hence, no loss of biological is normally expected. As free thiols rarely appear on the surface of proteins, recombinantly engineered cysteine residues can be created to facilitate site-specific conjugations with the expression of thiol groups (Goodson and Katre 1990; Natarajan et al. 2005; Constantinou et al. 2009).

Also indicated in Table 1.3, PEG derivatives forming stable thioether linkages such as PEG-maleimide, PEG-vinylsulphone and PEG-iodoacetamide have all been investigated for site-specific conjugations (Goodson and Katre 1990; Morpurgo et al. 1996). Achieving stable disulfide linkages by PEG derivative like orthopyridyl disulphifide-PEG have shown similar site specific conjugational advances, however such linkages are fragile and unstable in reducing environments (Woghiren et al. 1993). Most recently, certolizumab pegol became the first approved antibody fragment using thiol specific PEG chemistry with enhanced serum half life and successful therapeutic effects. The use of slow-release or hydrolysable PEGylation (i.e. PEG-intron®) has also been investigated, and such innovations can be exceptionally useful to restore the protein bioactivities potentially lost upon previous conjugation (Roberts et al. 2002).

**Limitations in the use of PEG**

PEG is generally regarded as a non-biodegradable polymer, and longer PEG chains especially used for protein PEGylation, are not subjected to metabolism and feasible elimination mechanisms. Depending on their molecular mass, renal filtration is less relevant for PEGs above 20 kDa, and alternative pathways such as liver uptake or faecal excretion become more predominant for higher molecular weight PEGs (Caliceti and Veronese 2003; Veronese and Pasut 2005). The concerns of PEG accumulation with high dosages and high molecular weight PEG conjugates for life-long therapies are hard predicted at the early stage, and the accumulations in liver may increase the risk of toxicity and severe side effects to patients (Bukowski et al. 2002; Caliceti and Veronese 2003; Jevsevar et al. 2010). Additional toxicology studies also demonstrated that very high dose treatment of PEG-proteins conjugates could induce renal tubular vacuolization and this symptom was not observed by using non-PEGylated protein (Bendele et al. 1998). PEGylation normally reduces the
immunogenicity of therapeutic proteins, transforming non-self proteins into a tolerogen (Sehon 1991). However, formation of anti-PEG antibodies has been detected in some patients during phase I trial of PEGylated urate oxidase (Ganson et al. 2006) and other PEGylated proteins and drug delivery systems (Armstrong et al. 2007; Ishida et al. 2007). The antigenicity induced by PEG has been primarily speculated on the chemical structure of its derivatives that facilitate drug conjugation purposes, and more exaggerated immunogenic responses are anticipated after repeated administration and slow elimination due to the non-biodegradation nature of PEG (Gregoriadis et al. 2005; Jevsevar et al. 2010). Serum complement activation has also been reported after high levels usage of PEG as an intravenous therapeutic agent leading to unwanted inflammation and immunotoxicity to patients (Hamad et al. 2008).

Like all synthetic polymeric products, PEG’s polydispersive feature results in a composition of various polymers with different number of monomeric residues, and yield a Gaussian (normal) distribution of molecular weights to the drug conjugates (Veronese and Pasut 2005). Especially when conjugating with small protein drug (i.e. antibody fragments), the PEGylated proteins or peptides might have polydispersive biological characteristics that mainly represent in body-residence time, clearance rate and safety issues (eg. toxicity and immunogenicity). Although improved synthetic and purification procedures have been developed to formulate PEGs with less polydispersive and better PK-enhancing characteristics (i.e. branched PEG), a high cost is usually induced, which elicits another big problem for manufacturing with high demands.

**1.3.1.3 Polysialylation – The Natural Way**

Of course, the same types of chemistries described above are not unique to PEG conjugates, and the same the advantages and disadvantages can be applied to any other hydrophilic polymers activated with the same chemistry. For example polysialic acid (PSA) conjugation has been investigated using amine and thiol chemistries with similar observations made. PSA applied in various existing therapeutic usages, is a naturally-occurring biopolymer (α-2,8 or α-2,9 linked sialic acid/N-acetyl neuraminic acid) found as colominic acid (in bacteria) or PSA (in mammalian cells) (Janas and Janas 2004). Details of the PSA chain and the sialic acid monomers will be described alongside PSA biosynthesis in section 1.4.3. Furthermore, polysialic acids have a high degree of chemical versatility, which enables a wide variety of
drugs and other molecules to be covalently linked to PSA, with preservation of much of the
drug’s activity (Gregoriadis et al. 2005). Gregoriades and colleagues firstly proposed that
PSA comprises the biophysical properties similar to PEG such that its hydrophilicity could be
used to modulate the half-life of proteins (Gregoriadis et al. 1993). Initially demonstrated in
1993 with fluorescein and in 1996 with catalase (Gregoriadis et al. 1993; Fernandes and
Gregoriadis 1996), Colominic acids derived from *N. meningitidis* and *Escherichia. Coli* K1
capsular polysaccharides were covalently conjugated to achieve extended serum half-lives
with preserved protein functions. Also suggested from the immunogenicity aspect of
chemically polysialylated asparaginase, polysialylation reduced the antigenicity of
asparaginase and as a result prolongs its circulation in the blood even in the presence of anti-
asparaginase antibodies (Fernandes and Gregoriadis 2001). Other polysialylated proteins
including insulin (Jain et al. 2003), interferon α-2b, β-Galactosidase, Aprotinin, EPO
(Gregoriadis et al. 2005) have been investigated *in vitro* and *in vivo* with increased solubility
and stability, reduced immunogenicity, preserved function, and improved pharmacokinetics.
Extending this application to antibody fragment, in the first instance amine-based reductive
amination using a PSA-aldehyde, conjugation to H17E2 Fab, an antibody against the
oncofoetal tumour antigen, placental alkaline phosphatase, was shown to produce conjugates
that retain activity. Several coupling ratios were investigated, and all demonstrated increased
blood residency *in vivo* compared to the parental Fab and approaching that of the whole
immunoglobulin. Interestingly, the longest or highest PSA substitution ratio was not the most
effective (Constantinou et al. 2008). However, the same reductive amination chemistry was
applied to MFE-23, a single-chain Fv directed against another oncofoetal antigen,
carcinoembryonic antigen. This resulted in immuno-conjugates of reduced blood clearance
and high bioavailability, but poor immuno-reactivity. This was resolved using the site-specific
method with a thiol-directed maleimide activated PSA (Constantinou et al. 2009). In addition,
as described previously, anionic molecules are more reluctant to be cleared through
glomerular filtration. PSA, a highly negatively charged molecule must therefore be
desialylated by neuraminidase-like enzymes before cleared via either kidney or through the
asialoglycoprotein receptors during hepatic elimination of large molecules (Stockert 1995;
Deen et al. 2001). The additional desialylation routes consequently imply a delayed clearance
process of PSA-conjugated molecules.
1.3.1.4 HESylation

Similar to chemical polysialylation, another organic polymer called hydroxyethyl starch (HES), a modified, branched amylopectin (e.g. derived from waxy maize starch), composing of D-glucose units linked by α1,4-glycosidic bonds and α1,6-bonds for the branched points, was chemically conjugated (or HESylated) to several proteins (i.e. erythropoietin and G-CSF) with improved bioactivity and pharmacokinetic properties (Besheer et al. 2007; Kontermann 2009). Various HES derivatives can be prepared with preferable properties serving a flexible approach to modulate PKs. Although HESylation has not been applied or described for antibody fragment PK, its polysaccharide composition and biocompatibility make it an attractive alternative material to existing technologies (Kontermann 2009).

1.3.1.5 Conjugating with Modulating Proteins and Peptides

Another appropriate antibody fragment PK-modulating strategy categorized in chemical conjugation involves chemically coupling the mAb fragments with other proteins that have a long serum half-life. Similar to immunoglobulin’s 21 days and also sharing the similar salvage recycling strategy by interacting with the neonatal Fc-receptor, albumin (19-day serum half-life) is commonly considered as an ideal pharmacokinetic modulating protein partner (Anderson et al. 2006). Albumin is a 67 kDa heart-shaped monomeric molecule and is found to be the most abundant protein in the blood serum (around 45mg/ml in humans). It also has a low pI and consequently a net negative charge in the blood which hampers kidney filtration due to the anionic kidney basal membrane (Anderson et al. 2006). Using albumin for drug conjugation recovers many of the advantages of the antibody Fc domain such as prolonging small molecule blood circulation time, and outweighs the cross-reaction and side effects from Fc-mediated annoyances. The most notable example is the work of Smith et al (2001) who evaluated three different albumin-binding strategies. Chemical conjugation of rat serum albumin (RSA) with an anti-TNFα Fab2 retained immunoreactivity but increased its bioavailability 17 to 200-fold (Smith et al. 2001). The same group also chemically cross-linked an endogenous RSA specific Fab to anti-TNFα Fab2, and produced an 8-fold increase in bioavailability (Smith et al. 2001). On top of these successful pioneering studies of chemical conjugation, Smith et al investigated recombinant fusion approaches that genetic conjugate appropriate protein with antibody fragment, and indeed, most of modulating proteins and peptides entitled conjugation approaches have been focussed on recombinant fusions (see section 1.3.2 below).
1.3.1.6 Conjugates with Modulating Small Molecules

Inheriting the similar feature of the bispecific anti-RSA-anti-TNF Fab\(_2\) motif described above (Smith et al. 2001), small organic molecules that bind tightly to serum albumin have been identified and applied to increase small protein drug blood-residence time. Among most applications, a ‘portable’ small albumin-binding molecule called ‘Albu tag’ from a DNA-encoded combinatorial chemical library. This small chemical molecule, a 4-(p-iodophenyl)butyric acid derivative, interacts with serum albumin and extends the half life of imaging agents (Dumelin et al. 2008) and antibodies (Trussel et al. 2009). The portability of the ‘Albu tag’ retains the tag with a high affinity to albumin upon attachment and overcomes the problems such as loss of albumin binding upon chemical conjugation which have often seen in other similar attempts.

1.3.2 Recombinant Approaches

Unlike chemical modulations with decades of research experience, genetically engineering therapeutic proteins with refined PK characteristics is still at its infant stage. However, because of the versatility of recombinant technology and the vast selections of useful fusion materials (i.e. long-lived serum proteins and glycosylation domains), the recombinant approaches for antibody fragment PK modulation may open many new areas that are more applicable, feasible, effective, and economical.

1.3.2.1 Antibody Fragment Engineering

The proliferation of antibody fragments (Holliger and Hudson 2005), and alternative binding frameworks (Ewert et al. 2004) have led to many recombinant approaches to pharmacokinetic engineering. The selection and characterisation of scFvs, diabodies, nanobodies, DARPs and anticalins is normally followed by the re-engineering into bigger molecules such as immunoglobulins, small immune proteins (SIPs) (Borsi et al. 2002) or artificially multimerised proteins (Hanes and Pluckthun 1997). Evidence include ones described in section 1.2.2.1 and Figure 1.9, using different peptide linkers to develop divalent and multivalent scFv molecules with two or more scFvs monomers (Goel et al. 2000; Goel et al. 2000); the introduction of multimerization domains, including streptavidin, p53 and leucine zippers, which have been used for making a variety of antibody fragment fusions (Schultz et al. 2000; Willuda et al. 2001; Suntharalingam et al. 2006). A self assembling molecule known as the B-subunit of E. Coli verotoxin (VTB) was also engineered to create a pentamerized
V_{iH}, and a bispecific decavalent molecules by fusing an antibody pentamer of one specificity to the N-terminus and a different one to the C-terminus of VTB (Zhang et al. 2004; Stone et al. 2007). The association of multiple antibody fragments multiplies the size of one single monomer which enables a longer blood half-life due to the increased size, and further more improves the binding affinity in the same magnitude.

Larger proteins have a longer blood half-life as of course, immunoglobulins which have a natural retention and clearance mechanism through Fc-domain:neonatal Fc-receptor binding via the reticulo-endothelial system (Ghetie and Ward 1997; Anderson et al. 2006). There are many strategies for increasing or decreasing the affinity between immunoglobulin Fc and FcRn to increase or decrease blood serum half-life which have been well described in a number of key papers (Roopenian and Akilesh 2007; Presta 2008; Andersen and Sandlie 2009; Ward and Ober 2009). As a result of these modifications, smaller version of whole IgG was made with antibody fragments such as scFv directly fusing to the Fc region resulted in a smaller sized dimeric protein (Colcher et al. 1998; Holliger and Hudson 2005). Much more compact antibody forms such as SIPs or minibodies lead a more favoured type of fusion trend by restoring antibody fragments with modified parts of the Fc domains. Not only minimizing the side-effects brought out by the entire Fc domain, these approaches also effectively improve the protein serum half-life control. An anti-Her2 compact antibody (Erbicin) currently being evaluated in preclinical studies for cancer treatment is an excellent example of Fc portion reunion approach (De Lorenzo et al. 2002; De Lorenzo et al. 2004).

1.3.2.2 Albumin Fusion Strategies
Firstly introduced by direct chemical conjugation (section 1.3.1.4), albumin, particularly human serum albumin (HSA) which has a long half life feature in the blood circulation was considered to be one of the favourable macromolecules for therapeutic efficacy improvement of short-lived proteins (Chuang et al. 2002; Muller et al. 2007), and the genetic engineering strategies showed an easy way to produce fusion proteins having HSA in the structure. Successful fusion models include hormones e.g. insulin (Duttaroy et al. 2005), human growth hormone (Osborn et al. 2002), and cytokines e.g. interferon-α (Osborn et al. 2002), interferon-β (Sung et al. 2003), and IL-2 (Melder et al. 2005). Antibody-albumin fusions have been studied as a way to both slow down and accelerate blood clearance (Figure 1.12). ScFv-HSA fusions using a variety of linker peptides increased the blood residence times approximately
12-fold (Smith et al. 2001). These fusions expressed well in *Pichia pastoris* pointing to a way for low-cost expression. A radiolabeled anti-CEA scFv-HSA fusion protein (‘immunobulin’), expressed in mammalian NS0 cells was designed by Yazaki *et al.* for tumour biodistribution and imaging studies. This scFv-HSA fusion showed a dramatic increase in tumour uptake, persistent high tumour:blood ratios, and limited normal tissue uptake in comparison with the scFv alone (Yazaki *et al.* 2008). After 72 hr, 27% injected dose/gram was reached with a tumour:blood ratio of almost 19:1. This is significantly better than that seen with whole immunoglobulins. A series of anti CD3 (T-cell)/anti-CEA (tumour cell) retargeting bispecific antibodies were also fused to HSA in order to prolong their half-life for immunotherapy applications (Muller *et al.* 2007). These rather complex molecules were successfully expressed in HEK293 cells. Mammalian cells have been shown by the Kontermann lab and others including ourselves (unpublished) to express bispecific scFvs with yields from *E. coli* being very poor (Wright and Deonarain 2007). These constructs, bispecific scFv-, single-chain diabody- and tandem scFv-HSA fusion proteins (scFv2-HSA, scDb-HSA, and taFv-HSA) were all stable and had increased *in vivo* bioavailability, as shown by a 6-8-fold increase in the blood exposure time. *In vivo* therapeutic benefit of such increased residence time is yet to be shown. Conversely, ‘HSAbodies’ are highly glycosylated scFv-albumin fusion proteins which have accelerated blood clearance making them appropriate for 2-step drug delivery systems (Huhalov and Chester 2004).

1.3.2.3 Albumin Binding Strategies

Rather than utilising the long residence time of albumin directly, there have been many recombinant approaches that “piggy-back” on albumin, in a similar way to the albu-tag described above. Using peptide phage display, Dennis *et al.* identified a specific core sequence (DICLPRWGCLW) which bound to albumin with high affinity (Junutula *et al.* 2008). They generated a peptide called SA21 that non-covalently bound to albumin with 1:1 stoichiometry at a site distinct from known small molecule binding sites (Figure 1.12). Recombinantly fusing the peptide to an anti-tissue factor Fab (D3H44) significantly reduced the Fab *in vivo* clearance, and achieved 25-43% of the albumin half-life in mice and rabbits (Junutula *et al.* 2008). For tumour targeting, they incorporated the albumin-binding peptide to the Herceptin-derived Fab (Fab4D5), and showed a significant improvement in tumour deposition and retention, high tumour to blood ratios compared with Fab alone. This Genentech technology is known as AB.Fab, which has the advantage of cheap production in *E.*
coli. The authors also suggested that association with albumin leads to an altered route of clearance and metabolism (Junutula et al. 2008). A possible refinement of this technology is the ability to modulate the pharmacokinetics of AB.Fabs by altering the affinity of the peptide, which was demonstrated using peptide with $K_{D}$ of 4nM to 2.5mM (Nguyen et al. 2006).

Recently, another group applied the same albumin binding strategy to a bivalent anti-epidermal growth factor receptor (EGFR) nanobody. They also illustrated improved tumour uptake (as high as cetuximab), and a reduced blood clearance rate (Tijink et al. 2008). An alternative albumin-binding approach is to fuse a homologous albumin-binding domain (ABD3-46 amino acids/6 kDa) from streptococcal protein G with a recombinant antibody. ABD3, has a broad albumin species specificity, and interacts with HSA with $K_{D}$ of approximately 4nM (Johansson et al. 2002; Linhult et al. 2002). Stork et al. applied this strategy to a bispecific single-chain diabody (scDb anti-CEA/anti-CD3) developed for retargeting of cytotoxic T cells to carcinoembryonic antigen (CEA) expressing tumour cells. They successfully showed all three parts of the chimeric protein were functional with 5- to 6-fold increase of prolonged circulation time. The drawback seemed to be a decreased immunostimulatory activity compared to the diabody alone (scDb) (Muller et al. 2007). In a similar approach a radiaolabelled anti-HER2 dimeric Affibody molecule (14 kDa) was also tested with ABD fusion for improved therapeutic efficacy. Good cellular retention, and reduced renal uptake in comparison with the non-fused dimer molecule were observed (Tolmachev et al. 2007).

1.3.2.4 Recombinant Amino Acid Polymer

As described above, the use of inert, hydrophilic polymers represents the major strategy for pharmacokinetic engineering. Certain amino acid polymers bear this property and can also be advantageously attached to protein by genetic fusion. Schlapschy et al. investigated a glycine-rich homo-amino-acid polymer (HAP) that had a large hydrodynamic. They used anti-HER2 Fab 4D5 as a model system and fused 100 and 200 residues of a repetitive sequence (Gly$_4$Ser) to its light chain. By using size exclusion chromatography, they showed that the 200 residue ‘HAPylated’ Fab acquired a hydrodynamic volume more than double that of the Fab alone, and a moderate rise in half-life, but lower than the enhancement made by the ABD fusion (see above). Compared with more hydrophilic polymers such a PEG or PSA, the coiled structure of HAPs may hinder its development. However, this moderate effect could be beneficial to
specialized applications, such as in vivo imaging (Schlapschy et al. 2007). Other sequences and more extended and hydrophilic polymer chains are currently under investigation. Poly-(Pro-Ala-Ser) fusions have been developed as alternatives (PASylation) which acquire a more hydrophilic characteristic and much enhanced hydrodynamic radius, leading to better pharmacokinetic enhancement (A. Skerra, unpublished).

Recently, genetic fusion of an unstructured hydrophilic recombinant polypeptide (~864 amino acids), called XTEN, to peptide or protein provided an extraordinary plasma half-life extension. Such modified exenatide with an original half-life of 2.4 hours showed a projected time of 139 hours, and a tuneable manner of the polypeptide was also suggested by the authors for different PK requirements in practice (Schellenberger et al. 2009). Supported by data of other XTEN fusion proteins, it is very promising to see this method being used for improving antibody fragment pharmacokinetics.

1.3.2.5 Recombinantly Modified Glycosylation
As described, glycosylation has been show to influence Fc-mediated effector functions of whole IgG molecule (Wright and Morrison 1997), and it also has an effective contribution on the plasma half-life modulation (Kontermann 2009). A few studies have performed investigations of carbohydrate compositions on pharmacokinetics of recombinant antibody molecules. One study genetically engineered two or three tandem or overlapping N-glycosylation sites at an extension part of a scFv molecule C-terminal, and used yeast system to produce a mannose-rich glycosylated scFv. Faster plasma elimination was revealed after intravenous injection into mice, and the clearance was explained by the uptake of phagocytic cells with high surface expression of mannose receptors (Wang et al. 1998). Two-fold lower affinity compared to the parental scFv molecule was also reported by the authors, which further demonstrates that post-translational modification can affect antigen binding of small antibody fragments seen previously with the chemical conjugation approaches do. The introduction of new N-glycans has been found to increase the hydrodynamic radius and thus the blood-residence time of the recombinant proteins. Investigations of a bispecific single-chain diabody molecule with additional 3 to 9 N-glycosylation sites recombinantly introduced to the linker and C-terminal extension regions indicated increased hydrodynamic radius of the scDb and an approximate 3-fold increase of area under the blood concentration-time curve (AUC) from 0-24 hours (Stork et al. 2008).
Similar PK enhancing results were also suggested from other hyperglycosylated protein such as the synthetic form of EPO (darbepoetin alfa) (Egrie and Browne 2001), follicle stimulation hormone (FSH) (Weenen et al. 2004) with additional N-glycosylation site recombinantly engineered, and a moderate increase in plasma half-lives has been commonly agreed with this type of glycol-modification (Kontermann 2009). Terminal sialic acid residues appearing frequently in most protein glycosylation compositions can be considered as a very important component contributing in the recombinant glycosylation method for increasing therapeutic protein serum half-life (Byrne et al. 2007), and additional of sialic acid content resulted a moderate increase of blood residence period suggested by research on sialylation modified erythropoietin (Egrie and Browne 2001) and asparaginase (Fernandes and Gregoriadis 2001).

1.3.2.6 Recombinant Polysialylation
Currently, which will be more precisely described in this thesis, a new recombinant glycosylation method has been investigated and expected to achieve a more intensive increase for antibody fragment plasma half-life improvement through an extreme glycosylation feature – polysialylation (Constantinou 2005). Successfully introduced by chemical conjugation for PK improvement, PSA, a long chain carbohydrate, and a good organic substitute for PEG, has been found in mammals on heavily polysialylated (to 200 sialic acid residues with a unique α2-8 linkage) neural cell adhesion molecule (NCAM) (Georgopoulou and Breen 1999). With a door opened by HAPylation approach, the idea of using other recombinant approaches to obtain hydrophilic biopolymers for pharmacokinetic benefits was also examined in this PhD project. We managed to tailor the polysialylatable NCAM domains with the chemical properties of PSA to the antibody fragment, and developed recombinantly polysialylatable scFv fusion protein that could have particular improvements over chemical modification techniques for PK enhancement (following chapters of this thesis).

1.3.3 Summary of PK Modulating Approaches for Antibody Fragment
A wide range benefits were promised by improving the longevity of therapeutic proteins. From clinical to commercial aspects, benefits including better dosing regimens and delivery profiles, improved therapeutic efficacy and side effect control, as well as extended product lifecycles are distinctive. Particularly required by the emergence of antibody fragments and alternative binding frameworks, half-life improving technologies are promised to reveal the
paramount capabilities and the small size advantages in a range of disease-fighting applications. Described in the aforementioned texts, many available PK-modulating approaches have already been established or currently investigated for antibody fragments, and their overall features are compared in Table 1.4 below.

Table 1.4 Advantages and disadvantages of various pharmacokinetic enhancing strategies.

<table>
<thead>
<tr>
<th>Approach Name</th>
<th>Modulation Type</th>
<th>Advantage</th>
<th>Concerns</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEGylation</td>
<td>Chemical</td>
<td>Hydrophilic water attraction</td>
<td>Non-biodegradable</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Improved protein solubility/stability</td>
<td>Toxic accumulation</td>
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<tr>
<td></td>
<td></td>
<td>Industry-established method for extending half-life</td>
<td>Immunogenic</td>
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<td></td>
<td></td>
<td></td>
<td>Protein inactivation</td>
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<td></td>
<td></td>
<td></td>
<td>Downstream processing</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Costs and yields</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Homogeneity control</td>
</tr>
<tr>
<td>Polysialylation</td>
<td>Chemical</td>
<td>Hydrophilic water attraction</td>
<td>Protein inactivation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Improved protein solubility/stability</td>
<td>Downstream processing</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Biodegradable</td>
<td>Costs and yields</td>
</tr>
<tr>
<td></td>
<td>Recombinant</td>
<td>Hydrophilic water attraction</td>
<td>Homogeneity control</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Improved protein solubility/stability</td>
<td>Less advanced technology</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Biodegradable</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Potentially non-immunogenic</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Promising clinical data</td>
<td></td>
</tr>
<tr>
<td>Albumin conjugation or binding</td>
<td>Chemical</td>
<td>Potentially non-immunogenic</td>
<td>Protein inactivation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Biodegradable</td>
<td>Downstream processing</td>
</tr>
<tr>
<td></td>
<td>Recombinant</td>
<td>Hydrophilic water attraction</td>
<td>Costs and yields</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Improved protein solubility/stability</td>
<td>Homogeneity control</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Biodegradable</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Potentially non-immunogenic</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Easier production</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Less downstream processing</td>
<td>Less advanced technology</td>
</tr>
<tr>
<td>Unstructured Polypeptide</td>
<td>Recombinant</td>
<td>Hydrophilic water attraction</td>
<td>Low hydrophilicity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Improved protein solubility/stability</td>
<td>Long protein polymers</td>
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<tr>
<td></td>
<td></td>
<td>Biodegradable</td>
<td></td>
</tr>
<tr>
<td>Glycosylation sites</td>
<td>Recombinant</td>
<td>Hydrophilic water attraction</td>
<td>Low hydrophilicity</td>
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1.4 NCAM and Mammalian Polysialic Acid

This current PhD project focused on a recombinant method of adding polysialic acid on special recipient protein domains from neural cell adhesion molecule. As an unusual and extreme glycosylation feature, having terminal polysialic acid structure in a glycoprotein carbohydrate composition is merely naturally occurred in few proteins. NCAM is one of such protein that performs important neuronal and other cell functions which can be strictly regulated by its polysialic acid. Furthermore, the understanding of the enzymes that specifically catalyse the polysialylation, and the unique biosynthesis pathways of sialic acid, hence PSA that have been intensively studied during previous researches, are extremely useful for conducting research in recombinantly polysialylating antibody fragments, and the further manipulation of this technology for modulating the polymerization degrees. Special enzymes involved glycan recycling pathways indicate the organic bio-degradable property of PSA as a better polymeric alternative for drug conjugation.

1.4.1 Neural Cell Adhesion Molecule

Initially described as a brain-specific synaptosomal membrane glycoprotein (Jorgensen and Bock 1974), NCAM, the first identified neural cell adhesion molecule in the mammalian system, was characterised as a unique member of the immunoglobulin superfamily (Thiery et al. 1977). The extracellular domain of NCAM consists of five N-terminal immunoglobulin-like (Ig-like) domains and two fibronectin Type-III (FNIII) domains (Figure 1.13). Depending on the attachment to the cell membrane and the length of transmembrane domain (TMD), often occur as cell type-specific alternatively spliced variants, three major isoforms of NCAM; NCAM-180, NCAM-140, and NCAM-120 (named according to NCAM molecular weight in kDa) (Figure 1.13) were found predominantly in glial and neuronal cells (Cunningham et al. 1987). As a cell adhesion molecule (CAM), implications of different NCAM expression in all stages of neurodevelopment have been suggested from intensive previous studies. Containing a longer cytoplasmic domain, NCAM-180, predominately expressed in neurons late in development, and enriched at sites of cell contact and postsynaptic densities of mature neurons (Persohn et al. 1989). NCAM-140, having a distinguishable shorter transmembrane domain than NCAM-180, is expressed by both neurons and glia, and localized to migratory growth cones and axon shafts of developing neurons and mediates neurite outgrowth responses. Both isoforms having TMD connected to the intracellular cytoskeleton for signal
transduction. NCAM-120 which attaches the cell membrane with a glycoposphatidyl-inositol (GPI) anchor with no intracellular residue is predominantly found on glial cells (Maness and Schachner 2007). NCAM has been linked to a number of human brain disorders including schizophrenia, bipolar disorder and Alzheimer’s diseases. Learning and memory deficits alongside behavioural abnormalities have also been shown from NCAM-deficient mice (Stork et al. 1997; Santuccione et al. 2005; Maness and Schachner 2007)

**Figure 1.14. Schematic structure of NCAM.** All three NCAM isoforms are generated from a single gene by alternative splicing. The extracellular portion of all three isoforms consists of five Ig-like domains (Ig1-5) and two FNIII repeats (FN1 and FN2). GPI anchor and transmembrane domains are illustrated corresponding to each NCAM isoform. The cytoplasmic domains of NCAM-140 and NCAM-180 differ in length due to alternative splicing. NCAMs are important PSA carriers. Six N-glycosylation sites and two possible polysialylation are schematically indicated upon specific NCAM domains (Kleene and Schachner 2004).

Many studies have been suggested that the extracellular Ig-like domains were involved in NCAM-mediated homophilic adhesion (Soroka et al. 2010). The subsequent three-dimensional structure of NCAM Ig modules also localized the homophilic binding through a crystal structure of Ig1-2-3 dimer which was consequently suggested as primary domain orientated binding and predicted the homophilic adhesion structures of Ig4 and 5 domains (Kasper et al. 2000; Soroka et al. 2003; Atkins et al. 2004). The homophilic dimerization of NCAM (or *trans*-interaction) on the same cell surface has important signal transduction and neural development (i.e. neuroblast migration, neurite outgrowth, axon path-finding, and fasciculation) properties. NCAM *trans*-interaction activates the fibroblast growth factor (FGF)
receptor and a number of different signal transduction pathways guided by regulatory molecules such as non-receptor tyrosine kinase p59\textsuperscript{fyn}, focal adhesion kinase (FAK), phospholipase C (PLC), protein kinase C (PKC) and Ras-MARK, which have significances in altering neural cell proliferation and differentiation (Kolkova et al. 2000; Prag et al. 2002; Maness and Schachner 2007). Such NCAM dimerization also induces signalling responses via calcium influx enhancement through G protein-dependent channels (Ronn et al. 2002).

NCAM homophilic interaction has been proposed in the process of cell-cell adhesion (Kasper et al. 2000). More importantly, heterophilic coupling (or cis-interaction) between NCAM and other surface molecules provides co-signalling in specific cell types, and such adhesive ligand induced cell communication and neurotransmission stimulate specific neural cell migration, axonal growth and other neural developmental activities (Berezin et al. 2000; Rougon and Hobert 2003; Maness and Schachner 2007). These heterophilic ligands of NCAM comprise heparan sulphate and chondroitin sulphate proteoglycans (Cole and Burg 1989), L1-CAM (Kristiansen et al. 1999), FGF receptor (Kos and Chin 2002), Prion Protein (PrP) (Santuccione et al. 2005), and a subunit of glial-derived neurotrophic factor (GDNF) receptor (Paratcha et al. 2003).

The two extracellular FN\textsubscript{III} domains have also been reported with a function in receptor activation and NCAM-mediated neurite growth, signalling and learning (Anderson et al. 2005; Kiselyov et al. 2005). Alternative splicing and insertion of sequences between the two FN\textsubscript{III} domains may modulate the NCAM mediated cell interaction (Doherty et al. 2000; Johnson et al. 2004). Probably the most significant property of FN1 domain is that it contains the docking site for polysialyltransferases (PolySTs) which catalytically elongate the terminal polysialic acid structure of specific N-glycosylation sites on the neighbouring NCAM Ig5 domain (Muhlenhoff et al. 1996; Mendiratta et al. 2005).

### 1.4.2 NCAM Polysialylation

NCAM has been claimed as a pivotal regulator of synaptogenesis and synaptic plasticity (Dityatev 2006; Gascon et al. 2007; Muller et al. 2008). Together with other NCAM functions, a great influence from its extracellular carbohydrate content containing negatively charged oligosaccharide chains composed up to 90 sialic acid residues in a unique α2, 8-linked polysialic acid chain was confirmed by previous research (Rutishauser 1998; Georgopoulou
and Breen 1999; Galuska et al. 2008). Due to the polyanionic and high water possession capacities, PSA forms large watery shells that enlarge the space between interactive ligands or neighbouring cells and creating steric hindrance, making NCAM less-adhesive and facilitating the cell migration, axon growth and synaptic reorganization during brain development (Rutishauser 1998; Johnson et al. 2005; Muhlenhoff et al. 2009; Soroka et al. 2010). Evidence from both homophilic and heterophilic NCAM interactions showed that PSA worked as a de-or anti-adhesive component of NCAM and decreased both NCAM cis- and trans-interactions in vitro (Cunningham et al. 1983; Sadoul et al. 1983; Storms and Rutishauser 1998). Studies using prokaryotic originated endoneraaminidase (Endo-N) that specifically cleaves off PSA from NCAM, also suggested the NCAM adhesive feature restoration after PSA removal (Hallenbeck et al. 1987; Rutishauser and Landmesser 1996).

Based on the necessary requirement of vertebrate brain development, the expression of PSA-NCAM is strictly temporally and spatially regulated. Early, in day 8 of embryonic brain development (E8), NCAM start to appear and one day after (E9) PSA consequently emerges with corresponding PolySTs transcripts detected at E8.5 (Probstmeier et al. 1994; Kurosawa et al. 1997; Ong et al. 1998; Oltmann-Norden et al. 2008). Thereafter, NCAM expression increasingly reaches a maximum in the perinatal and early postnatal phase. Alongside the NCAM progression, from E11 onwards, the PolyST levels are exceedingly amplified over the entire embryonic development, and the upregulation of PolyST gene expression is almost identical to parallel the NCAM transcript incline steep (Ong et al. 1998; Oltmann-Norden et al. 2008). This phenomenon proves a highly efficient enzymatic polysialylation of almost all NCAM during the late embryonic brain development. Beginning from the fifth day of the postnatal stage (P5) to P11, prominent decrease of PolyST levels was shown, and on the PSA product side, a 70% reduction was reflected by the down-regulation of the enzymes during early postnatal development (Oltmann-Norden et al. 2008). Coinciding to the completion of brain developmental morphogenesis, the conversion to PSA-free NCAM dominates the neural cells surface with adhesive cellular interaction properties (Edelman and Chuong 1982; Rothbard et al. 1982). All three major NCAM isoforms consistent with structural elements can serve as PSA acceptors and heavily polysialylated in the early brain development and persist expressed in specific areas of the brain such as the hippocampus, the suprachiasmatic nucleus, and olfactory bulb precursors (Franceschini et al. 2001; Colley 2008). In addition, NCAM was also identified on the NK cell surface with another name known as CD56. Often
expressed as a polysialylated form, the impact of PSA-CD56 has not yet been fully understand regarding to NK cell activity (Moebius et al. 2007). Polysialylation is an protein-specific glycosylation event (Colley 2008). As indicated in Figure 1.15, NCAM polysialylation occurs specifically at the fifth and sixth N-glycosylation sites on the Ig5 domain (Nelson et al. 1995; Kleene and Schachner 2004). More precisely, asparagines 430 and 459 were identified with suggestions of two separate PSA chains attached on the Ig5 domain of NCAM (Nelson et al. 1995; Liedtke et al. 2001). As mentioned above, the polysialyltransferases bind to the first FNIII (FN1) domain to enzymatically catalyse the PSA elongation on NCAM (Close et al. 2003).

Typically, sialylation and polysialylation normally occurs as a terminal structure in most glycoproteins. In most cases, N-glycans contain the core portion consists of three mannose and two N-acetylglucosamine residues, and the outer chain portion (Figure 1.15). N-glycans are classified into three different groups: high mannose, complex and hybrid (Angata et al. 2000; Geyer and Geyer 2006). The diversity of N-glycans is mainly caused by tremendous variations in the side chains. Particularly highlighted by the terminal sialic acid residue numbers, sialylation (1 or 2 sialic acids), oligosialylation (between 2 to 7 sialic acid residues), and polysialylation (more than 7 sialic acid residues) are the three common sialic acid featured mammalian glycosylation variations (Janas and Janas 2004).

**Minimal NCAM Structure Required for Polysialylation**

PolySTs substrate specification feature has been described over several studies previously, and NCAM, the most studied polysialoglycoprotein was suggested as the best polysialylation substrate so far under *in vitro* conditions. Therefore special protein features within NCAM must be recognized by PolySTs to serve as efficient polysialylation glycan substrate (Kojima et al. 1997; Angata et al. 2000). Early NCAM truncation studies demonstrated Ig4-Ig5-FN1 fused to the TMD was polysialylated by endogenous PolySTs, and speculation that all three NCAM domains simultaneously recognised by PolySTs was consequently proposed (Nelson et al. 1995). Based on a series of NCAM domain deletion mutants, Close et al identified the minimal domains that are essential for PolyST to carry out polysialylation (Close et al. 2003). Agreeing with the findings from Fujimoto et al (Fujimoto et al. 2001), they suggested that the Ig4 domain was not necessary for polysialylation, while Ig5 and FN1 domains together offer the basic substrate for PolyST catalysis (Figure 1.18), and either Ig5 or FN1 single domain was not enough for polysialylation (Close et al. 2003). Additional findings from soluble
Figure 1.15. Glycosylation profile of polysialylated NCAM. (a) Scheme of PSA-NCAM. The N-glycans located at the fifth and sixth N-glycosylation sites are modified by one or more PSA chains composed of uniquely α2, 8-linked sialic acid residues. Large hydrodynamic radius recruited by PSA is illustrated as pink shaded sphere. This watery shell separates NCAM with other binding ligands and increases the synaptic plasticity during brain development. The chemical formula of α2, 8-linked PSA has also been depicted (Muhlenhoff et al. 2009). (b) Possible structures of mammalian polysialylated N-glycosylations. Possible di- and triantennary N-glycan core structures attached to the two N-glycosylation sites within NCAM Ig5 domain are presented with terminal multiple α2, 8-linked sialic acids. The PSA chain was found as an extension from one antenna initiated from a terminal sialic acid α2, 3 or α2-6-linked to a galactose (the NeuNAcα(2-3) or α(2-6)Gal linkages). These two disaccharide linkages were proposed as polysialylation epitopes for PolySTs initiating the sialic acid polymerization. Fucoses and bisecting N-acetylgalcosamine were found occasionally in some PSA-carrying N-glycans. Different monosaccharide are annotated and depicted in unique colour spheres (Kleene and Schachner 2004).
NCAM protein truncates suggested that membrane association was not necessary for polysialylation, even the Ig5-FN1 mutants, however membrane association could enhance polysialylation level in some cases (Close et al. 2003).

Common findings from all previous polysialylating NCAM domain studies noted that the FN1 domain plays an important role in positioning the Golgi membrane-associated PolySTs for NCAM recognition, and specific PolyST docking-sites exist for polysialylation of the N-glycans on the adjacent Ig5 domain (Fujimoto et al. 2001; Close et al. 2003; Angata et al. 2004; Colley 2008). Previous studies from structural modelling, domain deletion and alternative replacement as well as amino acid mutagenesis confirmed the critical aspects of NCAM FN1 for NCAM polysialylation, and a single mutated FN1 domain carrying engineered O-glycosylation sites have been suggested to initiate O-glycan based polysialylyations (Mendiratta et al. 2005; Mendiratta et al. 2006).

Other Polysialylated Mammalian Proteins

In mammals, PSA is normally presented as a homopolymeric α2, 8-linked Neu5Ac capping terminal α2, 3- or α2, 6-linked sialic acid (Figure 1,15b) on the N-linked and O-linked glycans of a small group of proteins (Colley 2008). Other than NCAM, proteins specifically modified by PSA include α-subunit of the voltage dependent sodium channel (Zuber et al. 1992), CD36 as a form of the scavenger receptor found in milk (Yabe et al. 2003), neuropilin-2 found on dendritic cells (Curreli et al. 2007) and an autopolysialylation feature from the two PolySTs (Close and Colley 1998). The limited number of polysialylated proteins found in mammals also implies the protein specificity feature of polysialylation process.

Polysialic acid was also found in a huge population of different human tumour cells (Troy 1995). The α2, 8-polysialylated NCAM with chain length around 55 sialic acid residues was firstly reported from neuroblastomas (Livingston et al. 1988; Hildebrandt et al. 1998), and nephroblastomas (Wilms’ tumour) (Roth et al. 1988), and subsequently revealed in a variety of many other types, alongside α2, 9-polysialic acid found in ovarian teratocarcinoma (Troy 1995). Corresponding to the physiochemical properties of PSA, polysialylation in tumour cells could enhance the metastatic and mobile potential, which promote the detachment and blood vessel penetration of the cancer cells (Soroka et al. 2010).
1.4.3 Mammalian Polysialic Acid

PSA has been identified on the surface of a variety of cells from microbes to vertebrates, and is covalently attached to membrane glycoconjugates (glycoproteins or glycolipids) (Troy 1992; Troy 1995). Regarding this PhD project, the biosynthesis pathway of α2,8-linked PSA specifically attached to mammalian glycoprotein will be overviewed. This covers the sialic acid monomers that form the PSA chain, mammalian PSA metabolism, and the biosynthesis of the basic N-glycan cores that are found in NCAM Ig5 domain to initiate the terminal polysialylation.

1.4.3.1 Sialic Acid Monomers

Based on a cyclic-carbon structure with a carboxylic acid group at the C1 position (Figure 1.17a), sialic acid comprises a family of about 40 derivatives due to the number of locations available for structural modification as well as core variation. Because of the carboxylic acid group, sialic acids have an inherent negative charge. The most common sialic acids are 5-N-acetylneuraminic acid (Neu5Ac, NeuNAc or NANA), 5-N-glycolyneuraminic acid (Neu5Gc), and N-acetyl-9-O-acetylneuraminic acid (Neu5,9Ac₂) (Traving and Schauer 1998; Schauer 2004). Substitutions may be made at carbons C4, C7, C8, and C9 and the amine located at C5, and multiple substitutions contribute to the variety of possible structures.

In rarer cases, selected sialic acid monomers can interlink each other and form linear carbohydrate chemical structure of PSA chains. These chains have been found to consist of Neu5Gc, Neu5Ac, or 3-deoxy-D-glycero-D-galacto-2-nonulosonic acid (or 2-keto-3-deoxynononic acid, KDN) -KDN is a 5-deamino-, 5-hydroxyneuraminic acid (Troy 1992; Traving and Schauer 1998). Therefore only three natural building-units of the sialic acid family form PSA chains, which Neu5Gc is only predominantly found in fish egg polysialylglycoproteins (Troy 1992), leaving Neu5Ac and KDN the main component in mammalian PSA compositions (Figure 1.17b) with defined intersialyl linkages shown as in Figure 1.17c. (Muhlenhoff et al. 1998; Janas and Janas 2004).

1.4.3.2 Mammalian Biosynthesis of Sialic Acid and Polysialic Acid

In the aforementioned text, bacterially produced sialic acid polymer (colominic acid) has been chemically conjugated for small protein drug PK modulation. Internal linkages between sialic acid monomers in bacterial produced PSA chains are joined through α2,8- or α2,9-ketosidic
Figure 1.16. Sialic acid. (a) Neuraminic acid, the core structure of all sialic acids. The nine-carbon backbone common to all known sialic acids is shown, in the α-configuration. The following variations can occur at the carbon positions indicated by R1, R2, and R3. Annotations of R1-3 and molecule index are also depicted (Varki and Schauer 2009). (b) Two common “primary” sialic acids that form polysialic acid chains in mammals: Neu5Ac, 5-N-acetyleneuraminic acid, and KDN, 3-deoxy-D-glycero-D-galacto-2-nonulosonic acid or 2-keto-3-deoxynononic acid. Neu5Ac is more common than KDN in most vertebrate cell types, and these are derivatives from the neuraminic acid core structure shown in (a) (Varki and Schauer 2009). (c) The most frequent intersialyl linkages found in mammalian PSA chains, which α2,8-ketosidic linkage between Neu5Ac is the homopolymeric unit forms polysialic acid chains in human NCAM (Janas and Janas 2004).

linkage, and the α2,8-linked PSA is identical to the one that found in mammalian polysialylproteins, especially NCAM PSA as mentioned above (Traving and Schauer 1998; Janas and Janas 2004). Successful innovation of a chemical polysialylation drug delivery system using α2,8-linked bacterial colominic acid with low immunogenicity (Gregoriadis et al. 1993; Fernandes and Gregoriadis 2001; Gregoriadis et al. 2005) led to a more promising prospective of introducing human PSA via a recombinant approach (introduced by this PhD project).
As a important component within certain bacterial capsule, colominic acid, having a degree of polymerization (DP) up to 200 sialic acid residues, employs a unique biosynthesis process involving the KPS gene cluster to synthesize PSA chains and transport them to the outer membrane for the regulation of distinct polysaccharide capsular expression (Bliss and Silver 1996; Swartley et al. 1996; Muhlenhoff et al. 1998). However, mammalian cells utilize a very different biosynthesis pathway of producing sialic acid and polysialic acid that attach to protein substrates. The mammalian sialic acid metabolic system is summarized in Figure 1.18 below.

Like all mammalian glycosylation, many complex parameters are involved in the biosynthesis of sialic acid. Initiated by the influx of de novo or salvage \( N \)-Acetyl glucosamine or glycolysed fructose-6-phosphate through the hexosamine pathway, which converts GlcNAc-6P to the nucleotide sugar, UDP-GlcNAc under a series of enzymatic reactions (Figure 1.17A) (Hanover 2001). Relatively minor proportion of cytosolic UDP-GlcNAc is converted to ManNAc by epimerization of the hydroxyl-group in position 2 and cleavage of UDP by the UDP-\( N \)-acetylglucosamine 2-epimerase, a part of bifunctional GNE (Stasche et al. 1997). ManNAc, the specific sugar precursor for the biosynthesis of mammalian Neu5Ac is responsible for increasing the intracellular sialic acid pool (Pels Rijcken et al. 1995). Following the sialic acid biosynthetic pathway (Figure 1.17B), almost all ManNAc are metabolized to Neu5Ac in the cytosol, and the process is initiated by the phosphorylation of ManNAc catalysed by ManNAc kinase, the other part of GNE. After reaction with pyruvate and de-phosphorylation, the synthesized primary sialic acid is either modified to other sialic acid derivatives for other purposes or transported into the nucleus and converted to CMP-sialic acid, the substrate to form sialylated glycans (Bork et al. 2009; Du et al. 2009). This sugar metabolite can give an allosteric feedback inhibition to GNE (Figure 1.17B), which keeps a tight control of ManNAc and consequent by sialic acid production (Keppler et al. 1999). CMP-Neu5Ac is then transported into the Golgi apparatus via CMP-Neu5Ac transporters (CMPNT) (Eckhardt et al. 1996), where synthesis of sialylated glycans begins (Figure 1.17C) involving sialyltransferases. This constitutes a class of glycosyltransferases that are capable of catalysing the transfer of sialic acid from CMP-NeuAc to distinct glycan acceptor structures. These enzymes are highly specific for the linkage that is formed (\( \alpha2-3 \), \( \alpha2-6 \), \( \alpha2-8 \)), as well as for the primary structure and the spatial conformation of the acceptor (Nemansky and van den Eijnden 1993). Based on the nature of different sialyltransferases and acceptor specificities, they then catalyse CMP-NeuAc in the sialylation of oligosaccharide
Figure 1.17. Overview of mammalian sialic acid and polysialic acid biosynthesis and recycling. Four major routes are involved in the sialic acid metabolic system including: (A) The hexosamine pathway that converts GlcNAc-6P into UDP-GlcNAc, which is then catalysed to ManNAc by GNE; (B) The sialic acid biosynthetic pathway that converts ManNAc, the metabolic precursor into Neu5Ac in the cytosol, which then enters the nucleus for the synthesis of CMP-Neu5Ac (in most nonhuman species, a proportion of CMP-Neu5Ac is converted to CMP-Neu5Gc); (C) Sialoglycoconjugate production, during which the nucleotide sialic acids are transported into the lumen of the Golgi apparatus where several types of sialyltransferases are assembled to produce α2,3-, α2,6-, or α2,8-linked sialoglycoproteins or gangliosides. The polysialyltransferases are among these enzymes and specifically elongate the α2,8-linked PSA chain (i.e., polysialylated NCAM). Colour indications delicately specific sialyltransferases and corresponding glycol-linkage products. (D) The glycan recycling process that enables sialo- or polysialosides degradation by neuraminidases, thereby regenerating sialic acid monomers that can be re-used by a cell. Abbreviations – GlcNAc: N-Acetyl-D-glucosamine; ManNAc: N-Acetyl-D-mannosamine; UDP: uridine diphosphate; CMP: cytidine monophosphate; GNPNAT1: glucosamine-phosphate N-acetyltransferase 1; PGM3: phosphoacetylglucosamine mutase; UAP1: UDP-N-acetylglucosamine pyrophosphorylase 1; GNE: glucosamine (UDP-N-acetyl)-2-epimerase/Nacetylmannosamine kinase; NANS: N-Acetylneuraminic acid synthase; NANP: N-Acetylneuraminic acid phosphatase; CMAS: cytidine monophospho-N-acetylneuraminic acid synthetase; CMPNT: CMP-Neu5Ac transporter; ST3GAL: ST3 β-galactoside α2,3-sialyltransferase; ST6GAL: ST6 β-galactosamide α2,6-sialyltransferase; ST6GALNAC: ST6 (α-N-acetyl-neuraminyl-2,3-β-galactosyl-1,3)-N-acetylgalactosaminidase2,6-sialyltransferase; ST8SIA: ST8 α-N-acetylgalactosaminide α2,8-sialyltransferase; NEU: neuraminidase. Figure taken from (Du et al. 2009).
chains of glycoproteins. Polysialyltransferases are distinctive members of sialyltransferases, involved in polysialylation to form long chain polymers in certain proteins (i.e. NCAM). Lastly, shown Figure 1.17D, bio-degradation of sialylated or polysialylated glycoconjugates is processed under the hydrolysis by several mammalian neuraminidases, and the liberated sialic acid can be salvaged and recycled by the cells (Du et al. 2009). In addition, a recent study indicated that free N-Acetylneuraminic acid is able to scavenge free radicals such as hydrogen peroxide and lipid hydroperoxide, and other sialic acids may also have antioxidative potentials (Iijima et al. 2009)

**Neuraminidases**

Despite the wide distribution in nature from virus to vertebrates, such as human, it has been identified that neuraminidases (or exo-α -sialidases) are localized in the cytoplasm, plasma membrane and lysosomes intracellularly, and cells expressing neuraminidases can be found broadly in a variety of tissues, including the pancreas, skeletal, muscle, kidney, placenta, heart, lung, liver and the brain (to a less extent) (Achyuthan and Achyuthan 2001; Monti et al. 2002). Belonging to the family of exoglycosidases that catalyze the hydrolytic cleavage of non-reducing sialic acid residues linked to mono- or oligosaccharide chains of glycoconjugates preferentially, human neuraminidases have high catalytic efficiencies cleaving off the terminal α2,3 and α2,6-linked sialic acids. They are also capable of hydrolysing α2-8-linked neuraminic acid residues with a relatively lower efficiencies (Achyuthan and Achyuthan 2001; Monti et al. 2002). Shown in Figure 1.17D, human neuraminidases 1 to 4 are the most important ones responsible for sialic acid recycling and have been studies in detail at the molecular level. The existence of active neuraminidases implies that PSA is a bio-degradable substance, and points to the clearance process of the sugar polymer. Dedicated to the removal from the terminal sialic acids, the exo-α –sialidases hydrolysis of PSA chains (especially ones with many neuraminic acid residues) with α2,8-linked sialic acid linkages will be a time-consuming process, leading to PSA being a sustainable material for conjugation purposes.

**1.4.3.3 Natural Receptors of Sialic Acid**

Within vertebrate species, sialic acid-containing glycans are mainly recognised by two families of lectin receptors: the Selectin family and Siglec (Ig-like lectins) family. Selectins, a well-known calcium-dependent CAMs, are single-chain transmembrane glycoproteins that
have a high affinity for sugar moieties and therefore are considered to be a C-type lectin (Varki 2007). Selectin sub-groups including L-selectin on leukocytes, P-selectins on the surface of activated platelets and E-selectins on the surface of activated endothelial cells are found to target specific sialic acids involved oligosaccharides (i.e. Sialylated Lewis X antigen), and such interactions facilitate cellular activities such as lymphocytes and endothelial cells at the sites of inflammation (Rosen and Bertozzi 1994; Weninger et al. 2000; Pavlovic et al. 2002; Varki 2007).

Siglecs are I-type transmembrane proteins, with Ig-like extracellular domains, a signal transduction transmembrane region and a cytoplasmic domain (Angata 2006). They are known as the largest vertebrate lectins for sialic acid-containing glycan recognition (Varki and Angata 2006). Also known as the sialoadhesin family, Siglecs show high selective recognition of sialic acids. Famous family members including sialoadhesin (Sn)/Siglec-1, CD22/Siglec-2, CD33/Siglec-3, myelin-associated glycoprotein (MAG)/Siglec-4a, and Schwann cell myelin protein (SMP)/Siglec-4b (Angata 2006), are predominately expressed on the cells having immunity function, such as B-cells, natural killer cells, monocytes, macrophages, neutrophils, and eosinophils. Although the exact mechanism of the coupling with sialic acid has not been fully understood yet, the signal transduction via these Siglec receptors has been shown to regulate immune cell activation (Crocker and Varki 2001; Crocker and Varki 2001; Varki and Angata 2006). In addition, the binding of sialylated glycans by the Siglec receptor reduces the likelihood of external viral attachment via hemagglutinin-sialic acid binding (Varki and Varki 2007).

With a basic recognizing structure, the Sia-Gal-HexNAc sugar sequence, features including the type of sialic acid, its linkage to galactose and the general carbohydrate composition have been specified to a high degree by a range of Siglec receptors. Preferentially, polysialylation epitopes, Neu5Ac α2-3-linked and Neu5Ac α2-6-linked Galβ1-4GlcNAc are specifically bound by Sn/Siglec-1 and CD22/Siglec-2 respectively. Regarding recognition of α2,8-linked sialic acid, two human Siglecs has been reported with Siglec-7 having a weak affinity for disialic acid (Yamaji et al. 2002), and Siglec-11 having a rapid-diminishing binding style to α2-8-linked oligosialyic acid (around 2 to 6 neuraminic acid residues) (Angata et al. 2002). Apart from this sialic acid binding, there has not yet been any natural receptor reported for polysialic acid recognition, thus making PSA an ideal polymer for drug conjugation with no obvious receptor or ligand interactions. Furthermore, a study on bacteria α2,8-linked
homopolymerized PSA suggested that PSA alone, as a complex or a conjugate, is poorly immunogenic in vivo (Devi et al. 1991).

### 1.4.4 Polysialyltransferases

Assisted by a series of glycotransferases including essential sialyltransferases (listed in Figure 1.17C) for the construction glycan cores and polysialylation epitopes, the biosynthesis of α2,8-linked PSA chains is catalysed by two polysialyltransferases, ST8SiaII (STX) and ST8SiaIV (PST), two closely-related polysialyltransferases that belong to the sialyltransferase gene family. They share two conserved amino acid sequences - sialylmotif L and S (Angata et al. 1998). Structural dissection of the natural PolySTs demonstrated the enzymes have a short cytoplasmic segment but a large intralumenal domain consisting of a stem region and catalytic domain. The homologous sialylmotif L is involved in the engagement with the active sugar substrate, CMP-NeuNac, while the sialylmotif S participates in the binding to both the sugar metabolite and the glycan acceptors (Figure 1.18) (Datta and Paulson 1995; Datta et al. 1998; Angata et al. 2004). STX is the principal enzyme that is expressed in the embryo, whereas PST is more prominent in the postnatal brain (Sandi 2004). Both enzymes are capable of working independently or cooperatively to induce NCAM polysialylation, and they have been frequently found to be expressed in the same tissue during normal development (Nakayama and Fukuda 1996; Angata et al. 1997; Angata et al. 2002). However, the differences in their biological functions, expressing regulation, and individual contribution remain to be unravelled (Seidenfaden et al. 2000). They terminate the polysialylation process when the DP reaches a certain size and the repulsion force from the new PSA inhibits the enzymes to interact with the polysialylated glycans (Angata et al. 2002).

Although PST and STX are highly homologous to each other with 59% identical amino acids sequences, they are very different to the PSA synthases found in bacteria (Troy 1992). Substrate specificities of mammalian PolySTs particularly contribute to their unique enzymatic activities. As mentioned above in NCAM polysialylation (also illustrated in Figure 1.18), the two PolySTs have a well-characterised docking-site on the NCAM FN1 domain and by sharing the same N-glycans at the fifth and sixth N-glycosylation sites on the adjacent Ig5 domain, they are generating the same product and consistently synthesize polysialic acid by adding the first α2,8-linked sialic acid to α2,3 or α2,6-linked sialic acid, followed by the multiple addition of α2,8-linked sialic acid residues (Angata et al. 1998; Angata et al. 2002;
Angata and Fukuda 2003). Autopolysialylation was also found in both polysialyltransferases, which is not required for, but does enhance, NCAM polysialylation (Close et al. 2000).

**Figure 1.18.** Schematic representation of polysialyltransferase (ST8SiaII or ST8SiaIV) in the action of polysialylating NCAM. The fourth and fifth Ig-like domains (Ig4 and Ig5) and two FNIII repeats (FN1 and FN2) of NCAM are shown on the left. ST8SiaII and ST8SiaIV share structural homology. The structure of the PolyST (right) is dominated by a large intralumenal segment held by two disulfide-bond bridges. Both sialylmotif L and S outline the catalytic domains of the enzyme, where the L motif is specifically responsible for conveying CMP-NeuNac to the S motif, which catalyses the addition of the active sugar monomer to specialized glycan, and the elongation of the PSA chain. The specific recognitions of Ig5 and FN1 domains by the PolyST are also depicted, which the PolyST docks on the FN1 domain and begins synthesize α2,8-linked sialic acid to the N-glycans attached to the Ig5 domain. Denotations: ●, ○, □ indicate sialic acid, CMP-NeuNac and CMP respectively. N-glycosylation sites in NCAM are shown as □ and thin lines in between denote cysteine and the disulfide bridges. Figure is taken from (Angata et al. 2004).

Other four α2,8-sialyltransferases have been identified and cloned so far, among which ST8SiaI and ST8SiaV are involved in ganglioside synthesis (Kono et al. 1996) and ST8SiaIII adds α2,8-linked sialic acid residues to the same terminal α2,3-linked sialic acid epitope utilized by PST and STX (Sato et al. 2000). However, all these α2,8-sialyltransferases can only transfer mono- or oligo-α2,8-linked sialic acid to the end structure of the glycan (Angata and Fukuda 2003).
1.5 Concept and Initial Study of Recombinant Polysialylation for Improving Antibody Fragment Pharmacokinetics

1.5.1 Concept of Recombinant Polysialylation

Following the concept of chemical conjugation of naturally-occurring polysialic acid (Section 1.3.1.3), an alternative polysialylation approach through recombinant fusion of minimal polysialylatable domains from NCAM was initiated to provide a chemistry-free polysialylation method for modulating protein pharmacokinetics (Constantinou 2005). Described in Section 1.4.2, NCAM polysialylation (DP extend up to ~90 α2, 8-linked sialic acid residues) has been mainly found during mammalian neonatal nerve system development and this phenomenon is strictly regulated by the expression level of specific polysialyltransferases (STX and PST) (Abuchowski et al. 1984; Ong et al. 1998; Rutishauser 1998; Oltmann-Norden et al. 2008). More defined PolyST specificity studies determined minimal NCAM structure (Ig5-FN1 domain) for polysialylation to take place, where (showed in Figure 1.18) FN1 domain offers the docking site for the enzymes to elongate the α2, 8-linked PSA chains from two sialylated N-glycans sites located of the Ig5 domain (Close et al. 2003; Angata et al. 2004).

With the demand of increased blood circulation half-life, antibody fragment scFvs have been used as exceptional models in a range of PK modulation studies outlined in an update review (Constantinou et al. 2010). The recent study by Dr. Constantinou who chemically conjugated PSA to MFE23, an anti-carcinoembryonic antigen (CEA) scFv suggested that random amine coupling had detrimental effect to the scFv-CEA binding in vitro, whilst the scFv modified with a glycine-cysteine C-terminal peptide enabling site-specific thiol coupling avoided the interruption with the scFv binding site, and increased its in vivo half-life (Constantinou et al. 2009). Based on these findings, the concept of the recombinant polysialylation approach was maturated by genetically constructing a fusion protein consisting MFE23 scFv and the polysialylatable NCAM Ig5-FN1 domains on the C-terminal (Figure 1.19) (Constantinou 2005). Mammalian cell lines carrying out PSA biosynthesis pathway are also needed to express the fusion cDNAs with intracellular polysialylation and perhaps extracellular secretion.
Figure 1.19. Concept of scFv recombinant polysialylation. The scFv-fusion protein is schematically showed in a polysialylated format, which theoretically follows the NCAM polysialylation features. The hydrodynamic volume improvement of the polysialylated scFv-fusion protein in comparison with the scFv was demonstrated schematically via PSA mediated water molecule associations.

No single chain-Fv polysialylation has been studied for PK enhancement until recent chemical conjugation of PSA to MFE23 scFv which targets CEA as a popular tumour marker (Constantinou et al. 2009). Like CD20 (described in Section 1.1.4.1 rituximab), CEA is also a cell surface expressed, slow-internalizing receptor protein (internalization half-time ranging from 10-16 hours) (Schmidt et al. 2008), which enabled an initial investigation on how PSA effects scFv antibody targeting on cell surface antigens, in addition to polysialylation mediated PK modulation. To compare with the PSA chemical polysialylation, same scFv-antigen binding model was chosen to understand the effectiveness of recombinantly polysialylated scFv-fusion protein.

1.5.2 Carcinoembryonic Antigen (CEA)

CEA belongs to the immunoglobulin superfamily, with two sub-groups differentiate to each other based on the C-terminal transmembrane (TM) domain (Olsen et al. 1994). For the purpose of this thesis, only the sub-group that contains a hydrophobic TM followed by a cytoplasmic domain will be introduced. As a highly N-glycosylated cell surface located protein (up to 50% carbohydrate composition of CEA’s total molecular weight) (Yamashita et al. 1987; Yamashita et al. 1989), CEA consists of an Ig variable homology domain (IgV-like) and six domains homologous to the Ig C2 constant region (IgC2-like) (Paxton et al. 1987; Williams and Barclay 1988; Olsen et al. 1994). As an onco-fetal antigen that normally found during fetal life and very low level in adults, a specific adult tissue expression profile of CEA,
including a number of epithelial and goblet cell types found in colon, stomach, tongue, oesophagus, sweat glands, cervix and prostate has been revealed by previous tissue distribution studies (Nap et al. 1988; Nap et al. 1992; Prall et al. 1996). It has also been suggested that CEA was only found at the apical surface (glycocalyx/micovillus region) of polarized cells (Toner et al. 1971). The exact function of CEA has not been fully understood so far, however the x-ray crystallography and neutron scattering of the CEA structure proposed potential dimerization and protein-protein adhesion interactions (Hammarstrom et al. 1989; Boehm et al. 1996). Further in vitro studies showed that CEA and CEA related proteins can undergo home- or heterophilic adhesive binding process determining CEA is capable of acting as a cell adhesion molecule (Benchimol et al. 1989; Oikawa et al. 1992; Zhou et al. 1993). Additionally, CEA has been proposed for a role in the innate immunity, including interaction between fimbriated bacteria with the heavily glycosylated carbohydrate compositions of CEA (Leusch et al. 1990; Sauter et al. 1993), and the recognition of CEA N-domain by Opa proteins in Neisseria gonorrhoeae and Neisseria meningitides (Virji et al. 1996; Bos et al. 1997). These evidences suggested that micro-organisms can be trapped by CEA enriched apical glycocalyx and prevented from invading to the epithelial cells.

Overexpression of CEA has been found in a wide variety of epithelial malignancies, especially for colorectal tumors (Minton et al. 1985; Zimmermann et al. 1988; Sheahan et al. 1990; Kim et al. 1992). In comparison with the healthy individuals, the expression of CEA is restricted on the apical surface of mature columnar cells, and its emergence in the blood is less than 2μg/l; whilst in colorectal cancer patients, CEA is expressed all over the whole unpolarized malignant cell surface and frequently shed into the blood. The increased blood and tumour level in correlation with the tumour size increment made CEA an exceptional antigen marker for therapeutic approaches (Minton et al. 1985; Wanebo et al. 1989). In addition, the continuous increase of CEA level has often reported as an indication of tumour recurrence or distant metastasis for patients after colorectal cancer surgery (Evans et al. 1978; Minton and Martin 1978)

1.5.3 MFE23 scFv
Based on different degrees of specificity, a number of CEA specific IgGs were produced in the 1980s (Hammarstrom et al. 1989), and many of which were used for clinical tumour imaging purposes (Ledermann et al. 1991; Pedley et al. 1993). Introduced in Section 1.2.1,
limitations of full length IgGs, including immunogenicity and Fc domain cross-reactive side effects alongside restricted tumour penetration leading to poor tumour/blood ratios significantly reduced the speciality of anti-CEA IgGs for targeted therapies. Antibody fragments with removed the risk of human immunogenicity and cross reactivity to normal tissues were consequently engineered, such as a recombinant Fab of monoclonal A5B7 antibody, an original anti-CEA IgG (Chester et al. 1994).

MFE23, a high affinity and specificity single chain Fv antibody raised against CEA was the first anti-tumour scFv selected by phage display technology (Chester et al. 1994). Other becoming much smaller in size, the most distinctive advantage of MFE23 scFv was that it displayed a 10-fold greater affinity for CEA than the A5B7 Fab (Chester et al. 1994; Chester et al. 1994), and this significantly enhanced the scFv tumour penetration and antigen localization potentials, which may gave credits to improved tumour/blood ratios demonstrated by mice transplanted with human tumour xenografts (Casey et al. 1995; Verhaar et al. 1995). Initially radio-labelled with I^{123} for imaging purposes in the clinic (Begent et al. 1996; Mayer et al. 2000), MFE23 scFv represented the first generation of scFv used in patients. Since then, MFE23 scFv has been largely used for targeted therapy and showed great clinical potential for ADEPT after fusing with a number of enzymes with therapeutic purposes (Bhatia et al. 2000; Kousparou et al. 2002; Francis et al. 2004; Sharma et al. 2005). Successes from in vivo tumour delivery of a recombinant MFE23 scFv::TNFα fusion protein also demonstrated its capacity for targeted therapies (Cooke et al. 2002). Moreover, the previous established recombinant MFE23 scFv enzyme or cytokine fusion proteins also suggested the capability of MFE23 scFv in forming fusion proteins with sustained antigen binding affinity. However, like all the other antibody fragments, MFE23 scFv being a small protein molecule, has a very short plasma half life which significantly limits its therapeutic applications. Predominately lost from glomerular filtration, MFE23 scFv’s rapid blood clearance also resulted a high kidney accumulation, which appears to be a common drawback when applying scFv with high doses for therapeutic use (Huston et al. 1993; Milenic et al. 2004).
1.6 Project Aims

The aim of this project is to accomplish the recombinant fusion approach for antibody fragment (i.e. scFv) polysialylation, and develop sustainable production and purification systems for the polysialylated scFv-fusion protein. Consequent protein characteristic investigations will include verifications of correct fusion protein expression, retention of scFv-antigen binding affinity as well as the structural studies of associated PSA. As a novel and alternative pharmacokinetic modulating method, evaluations of the fusion protein hydrodynamic volume and in vivo blood clearance profile will also be conducted in this research. The progression of the research will include:

(1) Understanding the requirements for polysialylating scFv-fusion protein, and create mammalian cell host producing polysialylated scFv-fusion protein after fusion protein DNA transfection;
(2) Monoclonal selection of mammalian cell lines most capable for producing polysialylated scFv-fusion protein;
(3) Establishing sustainable laboratory-scaled fusion protein production and purification system;
(4) Characterizing the scFv-fusion protein, as well as its in vitro antigen-binding profile in comparison with mammalian cell expressed scFv;
(5) Showing the degree of polymerization of recombinantly elongated PSA and other glycosylation features associated with the scFv-fusion protein;
(6) Showing the changes in hydrodynamic volume of scFv-fusion protein in comparison with the scFv, and the bioactivity improvement from in vivo PK studies;
(7) Establish another scFv-fusion protein indicating with another specialized antigen binding profile (i.e. internalization).
CHAPTER 2

Materials and Methods
2.1 Materials

Solutions needed to be degassed were bubbled by nitrogen gas for sufficient time. Filter sterilization through 0.2µm PES vacuum filtration devices (250ml and 500ml, Helana Biosciences; 1L VWR) was applied as required. Small amount solutions were syringed through 0.2µm or 0.45µm PES sterile syringe filters (VWR) for required sterilization.

All general chemicals and reagents (not specified) were purchased from Sigma-Aldrich, Merck KGaA, and VMR. All reusable glassware and plastics were detergent washed prior to use and autoclaved where necessary for sterile and molecular work.

2.1.1 General Solutions and Buffers

**Water:** distilled water (dH₂O) was obtained from Purite Select systems and sterilized by autoclaving if necessary. Nuclease Free water (Promega) was applied for molecular biology experiments. Tissue culturing specified water was used in making solutions for tissue culture applications.

**Phosphate buffered saline (PBS):** a concentrated (10x) stocked was prepared containing 80g NaCl, 2g KCl, 2.4g KH₂PO₄ and 14.4g Na₂HPO₄ per litre of dH₂O, pH 7.5. The 1x PBS was diluted from the 10x stock with distilled water. The 1x PBS-Tween was made from 1x PBS with additional 0.01% Tween 20.

**Tris buffered saline (TBS):** a 10x concentrated stock was prepared with 58.44g NaCl, 23.64g Tris-HCl and 6.06g Tris-Base per litre of dH₂O, pH 7.5. The 1x TBS and 1x TBS-Tween (TTBS) was produced the same way as described in making PBS buffers.

Others specific buffers and solutions will be explained explicitly with associated methodologies in the following content. Without further description, all solution was made in dH₂O.
2.1.2 Agarose Gel Electrophoresis

**Tris-acetate EDTA buffer (TAE):** the 50x stock solution was commercially purchased from VWR. The 1xTAE buffer contains 242g Tris-Base, 37.2g Na₂EDTA.2H₂O and 57.1ml glacial acetic acid per litre of dH₂O, and was used as the agarose gel electrophoresis running buffer.

**Agarose gel:** 1% Agarose (Bioline) in 1x TAE buffer. Ethidium bromide (10mg/ml stock) with a final concentration 0.5µg/ml was added into the microwave-heating dissolved gel for DNA visualisation.

**Others:**
Crystal 5x DNA Loading Buffer Blue (Bioline)
HyperLadder I™ (Bioline) – detection range from 200bp – 10,000bp
HyperLadder IV™ (Bioline) – detection range from 100bp – 1000bp

2.1.3 Polyacrylamide Gel Electrophoresis (PAGE)

**PAGE running buffer:** 3.03g Tris-Base, 14.4g glycine, 1g sodium dodecyl sulphate (SDS) made up to 1L with dH₂O.

**Laemmli (SDS-sample) buffer (6x):** 375mM Tris-HCl (pH 6.8), 9% SDS, 50% glycerol, 9% (fully reducing) or 5% (semi-reducing) β-mercaptoethanol, and 0.03% Bromophenol blue made up with dH₂O.

**Resolving gel:** for effective protein display and separation, resolving gels were prepared in different polyacrylamide concentrations depends on the size of protein samples.

**Table 2.1. Components of making 10ml SDS-polyacrylamide gel with four different concentration.**
ProtoGel® (National diagnostics): 30g acrylamide and 0.8g methylene bis-acrylamide per 100ml solution; APS: ammonium persulphate; TEMED: N,N,N',N'-Tetramethylethylenediamine.

<table>
<thead>
<tr>
<th>Gel</th>
<th>Tris-HCl (pH8.8)</th>
<th>ProtoGel®</th>
<th>10% SDS</th>
<th>dH₂O</th>
<th>10% APS</th>
<th>TEMED</th>
</tr>
</thead>
<tbody>
<tr>
<td>7%</td>
<td>2.5ml</td>
<td>2.33ml</td>
<td>100µl</td>
<td>4.99ml</td>
<td>80µl</td>
<td>5µl</td>
</tr>
<tr>
<td>8%</td>
<td>2.5ml</td>
<td>2.67ml</td>
<td>100µl</td>
<td>4.65ml</td>
<td>80µl</td>
<td>5µl</td>
</tr>
<tr>
<td>10%</td>
<td>2.5ml</td>
<td>3.33ml</td>
<td>100µl</td>
<td>3.99ml</td>
<td>80µl</td>
<td>5µl</td>
</tr>
<tr>
<td>12%</td>
<td>2.5ml</td>
<td>4ml</td>
<td>100µl</td>
<td>3.32ml</td>
<td>80µl</td>
<td>5µl</td>
</tr>
</tbody>
</table>
Stacking gel: 3ml dH₂O, 0.5ml 1M Tris-HCl (pH6.8), 0.5ml ProtoGel®, 40µl 10% SDS, 40µl 10% APS, 4µl TEMED.

Semi-dry PAGE transfer buffer: 5.8g Tris-Base, 3g glycine, 0.4g SDS, 200ml methanol made up to 1L dH₂O.

PAG fix buffer: 45% methanol and 10% acetic acid in dH₂O.

### 2.1.4 Bacterial Strains and Culture

**Stains:**
The XL1-Blue (Stratagene) *E. coli* (recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F’ proAB lacIqZΔM15 Tn10 (Tetr)]) tetracycline resistant were used in DNA cloning by mini-and midi-prep.

The HB2151 (GE Healthcare) *E. coli* (nal r thi-1 ara Δ(lac-proAB) [F’ proAB + lacI q lacZΔM15]) were used for the expression of soluble scFv.

**Culture Media:**
2TY broth: 16g bacto tryptone, 10g bacto yeast, 5g NaCl made up to 1L with dH₂O. 2TY Agar: 15g bacto agar mixed with 1L 2TY broth (above).

### 2.1.5 Plasmids

pcDNA™4/TO/myc-His A, B, and C (Invitrogen) (Appendix 1) were the main vectors utilized in this research for both recombinant fusion construction in molecular level and *in vitro* cell transfection. Modified pcDNA™4/TO/myc-His A vectors incorporated with NCAM extracellular expression leader sequence from mammalian cell line as well as NCAM polysialylatable domains (Ig5 and FN1) or FN1 domain only (Figure 2.1a) were created by Dr. Antony Constantinou in our lab previously (Constantinou, A. PhD thesis, 2005). He has also constituted and donated all four recombinant DNA constructs in pcDNA™4/TO/myc-His (FL-NCAM, MFE23-Ig5-FN1, MFE23-FN1, and LS-MFE23) (Figure 2.1b) for the
investigation of recombinant polysialylation of MFE23 scFv (Constantinou, A. PhD thesis, 2005). The MFE23 scFv sequence is outlined in Appendix 6 and the NCAM leader sequence as well as the Ig5 and FN1 domain sequences are showed in Appendix 8. Full length NCAM140 sequence was originally obtained from pIg vector (Appendix 2) provided by Dr. Jane Saffell (Imperial College London).

Figure 2.1. The DNA constructs for recombinant polysialylation of scFvs. (a) Intermediate constructs containing NCAM extracellular expression leader sequence with Ig5-FN1 domains or only FN1 domain in pcDNA™4/TO/myc-His A vector. The gap sequences between PciI and NotI are cleavable for incorporation with scFv sequences. (b) Four DNA constructs used in the study for MFE23 scFv recombinant polysialylation. LS-MFE23 sequence is contained in pcDNA™4/TO/myc-His C vector, while others are in pcDNA™4/TO/myc-His A vector. Figure taken and modified from (Constantinou 2005).

pUC119 vector (Appendix 4) containing C6.5 scFv sequence (Appendix 7) was a kind gift from Professor James Marks (University of California, San Francisco), and was used in the study of recombinant polysialylation of C6.5.

PST and STX sequences were inserted in the pcDNA3.1 V5/His B vector (Invitrogen) (Appendix 3), and provided by Professor Karen Colley (University of Illinois, USA).
pEGFP-C1 plasmid vector (Appendix 5) containing engineered green fluorescent protein (GFP) was purchased from Clontech.

### 2.1.6 Molecular Biology Enzymes and PCR Primers

**Restriction endonucleases:** all restriction enzymes and associated reaction buffers were purchased from Fermentas and New England Biolabs.

**DNA Polymerases:**
- **TAQ:** purchased from Fermentas
- **PFU-Turbo:** purchased from Stratagene

Both polymerase enzymes were supplied with respective buffers and nucleotide mixtures.

**DNA ligase:** T4 DNA ligase and reaction buffer (New England Biolabs).

**Oligonucleotide PCR Primers:** all primers were designed in-house and ordered from Thermo Fisher Scientific (supplied as 100 pmol/µl stock concentration).

Table 2.2. Primers used in construction of recombinant polysialylatable C6.5 scFv. The sequences include NcoI and NotI (red), and C6.5 scFv backbones (blue).

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Direction</th>
<th>Primer Sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>C6.5 NcoI</td>
<td>Forward</td>
<td>CCT ATT TCC ATG GTG CAG CTG TTG CAG TCT GGG</td>
</tr>
<tr>
<td>C6.5 NotI</td>
<td>Reverse</td>
<td>CTT TAC CTT TGC GGC CGC ACC TAG GAC GGT CAG CTT</td>
</tr>
<tr>
<td>LS N-terminal</td>
<td>Forward</td>
<td>ACT AAG GAT CTC ATC TAC TGG</td>
</tr>
</tbody>
</table>

### 2.1.7 Mammalian Cell Lines and Culture

**Mammalian cell lines:**

Various cell lines were cultured for expression or genetically engineering purposes. Cell types, origins and resources were listed in Table 2.3.
### Table 2.3. Mammalian cell lines utilized during this project and their origin.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>Gifted or Purchased from</th>
</tr>
</thead>
<tbody>
<tr>
<td>NB2a</td>
<td>Murine neuroblastoma cancer cell</td>
<td>Professor Yuri Ushkaryov (Imperial College London)</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary cell</td>
<td>Cancer Research UK (CR-UK)</td>
</tr>
<tr>
<td>COS-1</td>
<td>African green monkey kidney cell. SV40 transformed</td>
<td>CR-UK</td>
</tr>
<tr>
<td>3T3</td>
<td>Primary mouse embryonic fibroblast cell</td>
<td>Dr. David Mann (Imperial College London)</td>
</tr>
<tr>
<td>HeLa</td>
<td>Human cervical cancer cell</td>
<td>Dr. David Mann</td>
</tr>
<tr>
<td>HEK-293</td>
<td>Human embryonic kidney cell</td>
<td>Dr. David Mann</td>
</tr>
<tr>
<td>LS174T</td>
<td>Human colonic adenocarcinoma cell</td>
<td>CR-UK</td>
</tr>
<tr>
<td>LoVo</td>
<td>Human colon-carcinoma cell</td>
<td>Professor Kerry Chester (University College London)</td>
</tr>
<tr>
<td>KB</td>
<td>Human epidermal carcinoma cell</td>
<td>CR-UK</td>
</tr>
<tr>
<td>SKOV-3</td>
<td>Human ovarian cancer cell</td>
<td>CR-UK</td>
</tr>
<tr>
<td>SKBr-3</td>
<td>Human breast carcinoma cell</td>
<td>CR-UK</td>
</tr>
</tbody>
</table>

### Culture media:

#### Standard tissue culturing medium (TCM): Dulbecco’s modified Eagle’s medium (DMEM) (PAA Laboratories) with 10% heat-inactivated fetal bovine serum (FBS) and 2mM L-glutamine (from 200mM stock, Invitrogen) plus 1% of penicillin-streptomycin (from 5000 units/ml penicillin + 5000µg/ml streptomycin stock, Invitrogen).

To harvest the cells for recombinant protein secretion from stably selected cell lines, FreeStyle™ CHO Expression Medium or FreeStyle™ 293 Expression Medium (both from Invitrogen) were used with extra 4mM L-glutamine.

#### Dulbecco’s PBS (1x): Cell culture specialised, without Ca & Mg ions was purchased from PAA Laboratories.

#### Zeocin solution: Zeocin powder was purchased from Malford, and made into 100mg/ml solution with tissue culture specialised water, and 0.2µm PES filter sterilized.

#### Cell lysis buffer: 10mM Tris-HCl, 10mM NaH₂PO₄, 130mM NaCl and 1% TritonX-100 in dH₂O, at pH7.5. Each EDTA-free protease inhibitor cocktail tablet (Roche) was added in every 100ml of the lysis buffer.
**Immunoprecipitation buffer:** 50mM Tris-HCl, 150mM NaCl, 5mM EDTA, 0.5% NP-40, 0.1% SDS, and EDTA-free protease inhibitor, pH 7.5.

**Cell freezing buffer:** 1% dimethyl sulfoxide (DMSO) in FBS.

Tissue culture flasks (150, 75cm²), plates (3 and 10 cm²), and multi-well plates (96-, 24-, and 6-well) were purchase with brand name BD Falcon™, Nunc™ or Corning.

### 2.1.8 Protein Purification Buffers

**Imidazole elution buffer:** 200mM imidazole dissolved in 1xPBS or 1xTBS, pH 7.4, and filter sterilized.

**Anion exchange running buffer:** 100mM Tris-HCl in dH₂O, pH 7.4. Degassed and sterilized through 0.2µm PES filter.

**Anion exchange high salt buffer:** 100mM Tris-HCl, and 1M NaCl in dH₂O, pH 7.4. Degassed and sterilized through 0.2µm PES filter. The high salt buffer was also used to make other NaCl concentration buffers for differential protein elution condition.

### 2.1.9 Antibodies, Antigens and Affinity Proteins

**Antigens:**

**N-A1:** CEA domains with interaction with MFE23, expressed from *Pichia pastoris*. Purified native N-A1 has a molecular weight ranging from 38 to 188 kDa, due to glycosylative post-modification, and was prepared in-house (Sainz-Pastor et al. 2006) and provided by Professor Kerry Chester, University College London.

**hErbB2/Fc:** Chimeric Her2 antigen fused with human IgG Fc domain. Provided by R&D system. Recommended 100µg/ml stock was prepared in sterile 1x PBS, and stored in -20°C.
**Commercial Antibodies:** Details of commercial antibodies used in the project for various purposes were listed in Table 2.4.

Table 2.4. Commercial antibodies and their descriptions. HRP – horseradish peroxidase. FITC- fluorescein isothiocyanate. AP- alkaline phosphatase.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Description and Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-His HRP (Sigma)</td>
<td>Mouse monoclonal IgG recognizes poly-histidine conjugated to HRP. Dilution 1:4000.</td>
</tr>
<tr>
<td>Anti-NCAM or CD56 (Abcam)</td>
<td>Mouse monoclonal (123C3) IgG1 to NCAM/CD56 recognises NCAM epitope between exons 11-13 (first fibronectin type-III, FN1). Dilution 1:1000 (for Western blot and ELISA), and 1:500 (for live cell experiments). Reactivity: Human and rat.</td>
</tr>
<tr>
<td>Anti-PSA (BD Pharmingen)</td>
<td>Rat monoclonal (12F8) IgM recognizes the polysialic acid on NCAM, with sialic acid residue number &gt; 7. Dilution 1:1000 (for Western blot and ELISA), and 1:200 (for live cell experiments). Reactivity: all species.</td>
</tr>
<tr>
<td>Anti-c-Myc (CR-UK)</td>
<td>Mouse monoclonal IgG1 recognizes c-Myc tag. Dilution 1:1000 (for Western blot and ELISA), and 1:500 (for live cell experiments)</td>
</tr>
<tr>
<td>Anti-V5 (AbD serotec)</td>
<td>Mouse IgG2a against V5 tag. Dilution 1:1000 (for Western blot and ELISA), and 1:500 (for live cell experiments)</td>
</tr>
<tr>
<td>Anti-Mouse IgG-HRP (Fc specific) (Sigma)</td>
<td>Goat whole IgG recognising the Fc portion of mouse IgG. Polyclonal conjugated to HRP. Dilution 1:10,000.</td>
</tr>
<tr>
<td>Anti-Rat IgM-HRP (Pierce)</td>
<td>Goat whole IgG polyclonal specific for recognising the μ-Chain of rat IgM. Dilution 1: 10,000.</td>
</tr>
<tr>
<td>Anti-His (C-term)-FITC (Invitrogen)</td>
<td>Mouse monoclonal IgG recognizes poly-histidine conjugate to FITC. Dilution 1:500</td>
</tr>
<tr>
<td>Anti-Mouse IgG-FITC (Fc specific) (Sigma)</td>
<td>Dilution 1:200.</td>
</tr>
<tr>
<td>Anti-Rat IgM-FITC (Abcam)</td>
<td>Dilution 1:1000.</td>
</tr>
<tr>
<td>Anti-Galactose-α(1,3) Galactose (Millipore)</td>
<td>Baboon polyclonal antibody against porcine Gal-α(1,3) galβ1-4GlcNAc-BSA. Dilution 1:200.</td>
</tr>
<tr>
<td>Anti-human IgG-HRP (Fc specific) (Sigma)</td>
<td>Goat monoclonal IgG binds to human IgG only. Conjugated with HRP. Dilution: 1: 60,000.</td>
</tr>
<tr>
<td>Alexa Fluor® 488 goat anti-mouse IgG (Invitrogen)</td>
<td>Purified whole goat antibody recognizing mouse IgG and conjugated to the bright and photostable Alexa Fluor 488 dye. Dilution 1:2000.</td>
</tr>
</tbody>
</table>
**Lectins:** Apart from isolectin GS-IB₄ (biotinylated) was purchased from Invitrogen, all the other lectins were pre-labelled with digoxigenin and obtained within DIG glycan differentiation kit from Roche (Table 2.5).

Table 2.5. Indications of sugar linkages through positive reaction with specific lectins, and corresponding glycoprotein controls.

<table>
<thead>
<tr>
<th>Lectins</th>
<th>Sugar linkage indication</th>
<th>Glycoprotein controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNA (Sambucus nigra agglutinin)</td>
<td>Indicates sialic acid, terminally linked (2–6) to galactose or N-acetylgalactosamine.</td>
<td>Transferrin</td>
</tr>
<tr>
<td>MAA (Maackia amurensis agglutinin)</td>
<td>Indicates sialic acid terminally linked (2–3) to galactose.</td>
<td>Fetuin</td>
</tr>
<tr>
<td>DSA (Datura stramonium agglutinin)</td>
<td>Indicates galactose-β(1–4)-N-acetylgalactosamine.</td>
<td>Fetuin</td>
</tr>
<tr>
<td>PNA (Peanut agglutinin)</td>
<td>Indicates galactose-β(1–3)-N-acetylglactosamine.</td>
<td>Asialo-fetuin</td>
</tr>
<tr>
<td>GS-I B₄ (Griffonia simplicifolia)</td>
<td>Indicates Galactose-α(1–3)-Galactose.</td>
<td>Transferrin (Bovine)</td>
</tr>
</tbody>
</table>

**Buffers required for DIG glycan differentiation kit:**
- **Buffer 1:** 1mM MgCl₂, 1mM MnCl₂, 1mM CaCl₂, in 1xTBS, pH 7.5.
- **Buffer 2:** 0.1M Tris-HCl, 0.05M MgCl₂, 0.1M NaCl in dH₂O, pH 9.5.
- **DIG blocking buffer:** supplied with the kit.

**Others:**

**MFE23 scFv:** produced in *E. coli*, and provided by Professor Kerry Chester, University College London.

**Avidin, streptavidin, protein A, and protein G** were supplied by Sigma-Aldrich, and dissolved in dH₂O with recommended stock concentration. Stored in -20°C for long term use.

**Marvel® milk power** (Premier Products): dried skimmed milk powder (99.5%). Diluted within 1xPBS or 1xTBS for blocking nitrocellulose membrane used in Western blots.
**BM-Blue POD** (Roche): 3, 3’-5’, 5-Tetramethylbenzidine (TMB) ready to use soluble peroxidise substrate solution.

**ECL Kit** (GE Healthcare): enhanced chemiluminescence detection kits.


**EZ-Run protein gel staining solution** (Fisher Scientific): ready-to-use Coomassie blue G-250 based reagent for mini-gel staining.


2.1.10 Glycosidases

Different glycosidases removing specific glycol-linkages were used in this project for basic glycol-structure illustration of interested glycoproteins. Details of each glycosidase are listed in Table 2.6, and their specific disaccharide linkage recognitions are illustrated in Figure 2.2.

**Table 2.6. Different glycosidases and their specific functions.** All required reaction buffers were supplied with the enzymes when purchasing.

<table>
<thead>
<tr>
<th>Glycosidase</th>
<th>Source</th>
<th>Unit/µl</th>
<th>Functional specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endo-Neuraminidase (Endo-N) (Abcys)</td>
<td>Phage K1</td>
<td>0.7</td>
<td>Recognises α2-8 linked sialyl residues, and requires minimum 5 sialyl residues for activity.</td>
</tr>
<tr>
<td>Acetyl-neuraminyl hydrolase (Exo-N) (New England Biolabs)</td>
<td><em>Clostridium perfringens</em></td>
<td>50</td>
<td>Also known as sialidase, mainly catalyzes the hydrolysis of α2-3, α2-6 linked N-acetyl-neuraminic acid residues from glycoproteins and oligosaccharides. It also hydrolysis the α2-8 linked, but very less efficient.</td>
</tr>
<tr>
<td>N-Glycosidase F (PNGase F) (New England Biolabs)</td>
<td><em>Flavobacterium meningosepticum</em></td>
<td>500</td>
<td>An amidase that cleaves between the innermost GlcNAc and asparagine residues of high mannose, hybrid, and complex oligosaccharides from N-linked glycoproteins.</td>
</tr>
</tbody>
</table>
2.1.11. Murine Models

Female healthy BALB/c nude mice (6 – 8 weeks old) were used for the purpose of all in vivo work (Home Office Project Licence: 70/6982 under Dr. Deonarain, Imperial College London). Mice were quarantined for 6 weeks in Central Biomedical Service (CBS) unit of Imperial College London before any experiment and maintained in individually vented cages with sterile bedding and supplied with autoclaved food and water by fully trained animal technicians from CBS Unit.

Figure 2.2. Schematic illustration of glycosidase enzymatic specificities. (a) A typical complex N-glycan structure model showing the breaking sites of each glycosidase. (b) Glycosidase specificities indicated from an O-glycan. Asn: asparagine; Ser: serine.
2.2 Methodologies

2.2.1 Bacteria Culture

2.2.1.1 Preparation of Chemically Competent *Escherichia coli*

Both competent *E. coli* strains XL-1 Blue and HB2151 were prepared according to the following protocol. *E.coli* single colonies grown on 2TY-agar plate (containing 15µg/ml tetracycline for the XL-1 Blue strain) were inoculated into a 5ml 2TY broth and cultured overnight at 37°C shaking (280rpm) in an orbital shaking incubator. This culture was then sub-cultured (1:100) into 100ml of fresh 2TY broth (containing 15µg/ml tetracycline for the XL-Blue strain). The culture was grown at 37°C with shaking (250rpm) until the culture optical density at 600nm (OD$_{600}$) reached between 0.6 and 0.8. The culture was chilled on ice for 30 minutes, and split in 50ml volumes into two centrifuge tubes. Subsequent centrifuge was carried out at 3000rpm for 10 minutes at 4°C. Each pellet was responded in 50ml pre-cooled 100mM CaCl$_2$ (0.2µm filter sterilized) and left on ice for another 30 minutes. The bacteria solution were span down once again at 3000rpm for 10 minutes at 4°C and each pellet was dissolved in 1.5ml of 100mM CaCl$_2$ with 15% glycerol (0.2µm filter sterilized). The competent bacterial cells were aliquoted in 50µl volumes, snap-frozen by liquid nitrogen and stored at -80°C.

2.2.1.2 Transformation of Chemically Competent *E.coli*

The bacteria transformation was carried out by heat shock method. An aliquot of competent *E. Coli* was mixed with 1µg of plasmid DNA with gentle agitation with the pipette, and incubated on ice for 30 minutes. Heat-shock of the bacteria was applied in 42°C water bath for 60 seconds, followed by incubation on ice for at least 2 minutes. The 37°C pre-warmed SOC medium (1ml) was added for recovering the bacteria, which was then incubated at 37°C shaking incubator (280rpm) for an hour. Depends on the size of the agar plates, transformed bacteria culture (between 25µl to 100µl) was plated onto the agar plates with 1% glucose and 100µg/ml of carbenicillin (analogous antibiotics like ampicillin, but more stable) , as most of the vectors used in this project containing ampicillin resistant gene. For pEGFP-C1 vector, 100µg/ml kanamycin is used instead of carbenicillin for plasmid selection. Additional
15µg/ml tetracycline was added for XL-1 Blue strain only. The plates were incubated in 37°C for 12 to 16 hours for the colonies to form.

2.2.2 Molecular Biology Techniques

2.2.2.1 Isolation and Purification of DNA
DNA required to be isolated in this project were mainly from bacterial plasmid, PCR mixtures and agarose gels. QIAquick™ Spin range of DNA isolation and purification kits manufactured by QIAGEN were used for this purpose. This establish technology utilises a spin column containing a silica based membrane which selectively binds DNA molecules in the presence of high concentration of chaotropic salts and lower pH conditions (pH 5.0 optimum). Contaminants pass through the column, and following ethanol-based washing steps. DNA is eluted in nuclease free water or a Tris based buffer, pH 7.4. The protocol for isolating DNA varies depending on the type and source of acquired DNA. Detailed information and protocol can be obtained from appropriate manufacturer handbooks.

Plasmid DNA
QIAGEN Mini and Midiprep kits were used to produce and purify bacteria plasmid DNA from transformed E.coli XL-Blue strain. Cultures of 10ml (for Miniprep) or 200ml (for Midiprep) were grown in 2TY broth with 100µg/ml of carbenicillin or kanamycin, 1% glucose at 37°C for 12-16 hours and 15µg/ml tetracycline. The bacteria were pelleted by centrifugation at 4000rpm for 10 minutes at room temperature. The pellets were lysed and DNA were purified throughout the manufacturer protocols and using the buffers and reagents supplied in the kits. Purified DNA was stored at -20°C for long term usage.

DNA Fragment Isolation by Gel Extraction
In this project, DNA fragments were generated mostly from PCR and enzymatic digestions. A high standard purity was required in applications such as ligation to the DNA fragments. Isolation of the fragment can be done through agarose gel, and consequent clean-up method using QIAquick Gel Extraction Kit. Samples containing interested DNA fragments underwent electrophoresis in a 1% agarose gel with ethidium bromide staining. The DNA fragment of interest was visualised under low intensity UV light using a UV transilluminator, and separated from the gel by excision with a scalpel blade. Briefly, the extracted gel slice was
weighted and dissolved in buffer QG which contains guanidine thiocyanate. The DNA was then isolated and cleaned by 100% isopropanol through a silica-based spin column, according to the manufacturer protocol. Following the wash step with buffer PE, the DNA was eluted in 30µl of nuclease free water, and stored in -20ºC.

2.2.2.2 Agarose Gel Electrophoresis
DNA samples were typically visualized and analysed by agarose gel electrophoresis. For the gel size of the electrophoresis system (Anachem/Scotlab) used requires 30ml of agarose gel solution (1%) together with ethidium bromide (0.5µg/ml final concentration). DNA samples were prepared by mixing with 5 x DNA loading buffer in a ratio of 1:4. The voltage applied as 100V for electrophoresis. Results were detected by BioDoc-It™ UV Transilluminator (Jencons-PLS) or FujiFilm LAS-3000 CCD imaging system (UV setting) after gel running. Gel images were formatted and edited using CorelDRAW Graphic Suite X3 software.

2.2.2.3 DNA Concentration Determination
Plasmid DNA was quantified using a Thermo Scientific NanoDrop™ 1000 Spectrophotometer. The device allowed accurate determination of concentration and purity using only 2.5µl of neat sample. The sample absorption at 260nm and 280nm was measured, and the 260nm value was converted to DNA concentration based on the calibration that 1.0 OD reading equates to plasmid DNA concentration of 50µg/ml. The 260nm/280nm ratio was used as a measurement for sample purity determination, and standard pure sample should have this value higher than 1.6.

For concentration determination of PCR products, laser scanning densitometry was employed by using FujiFilm LAS-3000 instrument. The density degree of ethidium bromide stained sample bands were compared with the molecular weight marker Hyperladder I or IV, which has standard indications between DNA concentration and density degree.

2.2.2.4 Polymerase Chain Reaction (PCR)
PCR was used to amplify specific DNA fragment for this research. The exact details for individual experiments are outlined in the relevant results chapters. Briefly for this project,
PCR was involved in amplifying inserts for ligation, and conducting colony PCR to check insert sizes post-ligation before sending to sequence. Generally TAQ polymerase (of *Thermus aquaticus*) was applied mainly in all PCRs, and the reactions were taken place by using Mastercycler® gradient (Eppendorf).

Table 2.7. Common PCR reaction constituents and cycling conditions. For 25µl final reaction volume.

<table>
<thead>
<tr>
<th>Reaction Constituents</th>
<th>Standard PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer 1 (10 pmol/µl)</td>
<td>2.5µl</td>
</tr>
<tr>
<td>Primer 2 (10 pmol/µl)</td>
<td>2.5µl</td>
</tr>
<tr>
<td>10x Buffer*</td>
<td>2.5µl</td>
</tr>
<tr>
<td>MgCl₂ (25mM)</td>
<td>2.5µl</td>
</tr>
<tr>
<td>dNTP Mix (25mM)</td>
<td>2.5µl</td>
</tr>
<tr>
<td>TAQ DNA polymerase (5U/µl)</td>
<td>0.25µl</td>
</tr>
<tr>
<td>Template (culture or 50ng/µl)</td>
<td>Trace culture or 0.5µl</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>10.5µl</td>
</tr>
</tbody>
</table>

* 10x Buffer: 750mM Tris-HCl (pH 8.8), 200mM (NH₄)₂SO₄ and 0.1% (v/v) Tween-20.

**General PCR cycling condition:**

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 0</td>
<td>Hot lid (100°C)</td>
</tr>
<tr>
<td>Step 1</td>
<td>Initial denaturation at 96°C for 5 minutes</td>
</tr>
<tr>
<td>Step 2</td>
<td>Denaturation at 94°C for 1 minute</td>
</tr>
<tr>
<td>Step 3</td>
<td>Annealing for 1 minute at a determined temperature for both primers or a serial of temperature gradient (if the annealing temperature is unknown initially)</td>
</tr>
<tr>
<td>Step 4</td>
<td>Extension for 1 minute at 72°C</td>
</tr>
<tr>
<td>Step 5</td>
<td>Repeat of Step 2-4 for 30 cycles</td>
</tr>
<tr>
<td>Step 6</td>
<td>Final extension for 10 minutes</td>
</tr>
<tr>
<td>Step 7</td>
<td>Hold at 4°C until use</td>
</tr>
</tbody>
</table>

**Colony PCR (cPCR)**

Rather than using DNA template, trace amount of bacterial cells selected from single colonies were used to donate their chromosome as PCR template after initial 100°C boiling step. Same PCR cycling conditions were applied to the cPCR.

**2.2.2.5 Enzymatic Restriction Digestion**

Typical restriction enzymes digestions were set up according to the suppliers’ protocols, and used mostly for DNA double digestions. In order to achieve maximum digestion all reaction incubations were carried out for overnight. A typical reaction would contain 10 units of each
enzyme used, genomic DNA (up to 5µg), reaction buffer and any other supplements such as bovine serum albumin (BSA), and the reaction conditions is shown in Table 2.7 below. Nuclease-free water was used to dilute the reaction buffer and enzyme associated glycerol concentration.

Table 2.8. Conditions for restriction enzyme DNA double digestions.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Enzyme Ratios</th>
<th>Reaction Temperature (ºC)</th>
<th>Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>PciI (or PstI)* and NotI*</td>
<td>1:1</td>
<td>37</td>
<td>Buffer O*</td>
</tr>
<tr>
<td>NcoI* and NotI*</td>
<td>4:1</td>
<td>37</td>
<td>Buffer O*</td>
</tr>
<tr>
<td>HindIII* and NotI*</td>
<td>1:4</td>
<td>37</td>
<td>Buffer R*</td>
</tr>
<tr>
<td>EcoRV† and XbaI†</td>
<td>2:1</td>
<td>37</td>
<td>Buffer 2† + BSA</td>
</tr>
</tbody>
</table>

*: Purchased from Fermentas
†: Purchased from New England Biolabs.

Buffer Composition:

10x Buffer O:
50mM Tris-HCl, 10mM MgCl₂, 100mM NaCl, and 0.1mg/ml BSA, pH 7.5.

10x Buffer R:
10mM Tris-HCl, 10mM MgCl₂, 100mM KCl, and 0.1mg/ml BSA, pH 8.5.

1x Buffer 2:
50mM NaCl, 10mM Tris-HCl, 10mM MgCl₂, 1mM Dithiothreitol, pH 7.9.

2.2.2.6 DNA Ligation

Following double digestion with relevant restriction endonuclease enzymes, both vector and insert were purified by gel extraction and quantified by laser scanning densitometry. Two insert to vector molar ratios, 1:1 and 3:1 were applied for ligation. In practice, normally 100ng of vector was used, and following the molar ratios, appropriate insert amount can be worked out. T4 DNA ligase (0.1units) was added for catalysing the ligation process with associated T4 DNA ligase reaction buffer (for 1x buffer: 50mM Tris-HCl, 10mM MgCl₂, 1mM ATP, and 10mM dithiothreitol, pH7.5) The reaction was then made up to 20µl by nuclease-free water, and incubated at 16ºC overnight in the PCR thermo-cycler.
2.2.2.7 DNA Sequencing

Various DNA sequencing services were used during this PhD research. Initially, the service was done by Dr Wonda Stow at the Advanced Biotechnology Centre, Imperial College London, using ABI377 Automated DNA Sequencers following in-house standard protocols. External DNA sequencing was taken place in Cogenics (http://www.cogenics.com/sequencing/service/primerwalking.cfm), and the sequencing protocol is available from the website as well. The DNA sequencing results were viewed and analysed by ChromasPro 1.41 (Technelysium Pty Ltd). Alignment and comparison of DNA sequences were achieved through National Center for Biotechnology Information (NCBI) website specialised BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.2.3 Tissue Culture Techniques

2.2.3.1 Cell Growth and Maintenance

All cell lines were grown in standard DMEM tissue culture medium (TCM) with 10% FBS, plus 2mM L-glutamine and 1% of penicillin-streptomycin (see section 2.1.7), and sustained in a humidified incubator at 37°C and constantly supplying 5% CO₂. All cell lines listed in Table 2.3 were adherent and sub-cultured when they reached to 70%-80% confluent (culturing days varies to different cell type). To sub-culture the cells, the old culturing media was removed and the cells were washed once with 1xPBS. Trpsin/EDTA (1x) from Invitrogen was added to cover the culturing surface (approximately 1ml/75cm²), and left for 5 minutes or more at 37°C in the cell culture incubator until cells were dislodged from the surface. The cells were then diluted in TCM and split in an appropriate ratio depending on the cell growth speed and usage of other experimental purpose. The more dilute, the less cell numbers will be split out in each aliquot for seeding.

2.2.3.2 Cell Lysis

To lyse the cells grown in 60mm culture dishes, 1ml ice cold cell lysis buffer was added and incubated on ice for 30 minutes, with repeated pipetting every 5 minute. Soluble material was separated from the cell debris by centrifugation at 13,000rpm for 10 minutes. The soluble material was decanted and stored in -20°C until ready for analysis.
2.2.3.3 Tissue and Cell Homogenation
Embryonic (E11.5) mouse brain (dissected and gifted by Dr. Anita Hall, Imperial College London) was frozen at -80ºC initially. After thawing, 1ml of ice-cold immunoprecipitation buffer was added to each brain. Homogenation of the brain was carried out by syringing the brain through a needle (26-gauge) 7 times, followed by top speed centrifugation for 5 minutes. The supernatant was then collected, aliquoted, and frozen stored before use.

2.2.3.4 Cell Storage Techniques
Cells reached 80% confluent in a 75cm² tissue culture flask were normally enough to be put into a 2ml Cryo™Tube vial (Nunc™) as one stock. Generally, the cells was washed once with 1x PBS, trpsinized, and collected by TCM in 10ml total volume. After centrifuging at 1500rpm for 5 minutes, TCM was aspirated and the cell pellet was resuspended in cell freezing buffer (1ml for one vial), before transferring into the vial. The vials were immediately moved in -80ºC and can be store there for a short time period (normally no more than 6 months) and later transferred to liquid nitrogen tank for long term storage.

2.2.3.5 Cell Counting
Relatively accurate cell numbers were required occasionally depending on the experiment. To do this, cells were firstly washed with 1x PBS and trypsinized as described in section 2.2.3.1. Dislodged cells were then collected in an appropriate volume of TCM, and 50µl of the cell suspension was removed to mix with 50µl of 0.4% trypan blue solution (1:1 ratio). Approximately 15µl of the mixture was added through the edge into the counting area of Neubauer hemocytometer affixed with a clean cover-slip. Viable cells appeared white under microscope while the dead cells were dyed into blue. The number of viable cells (N) in the middle big square of the grooved/counting area on the hemocytometer was counted, and the concentration of the cell suspension collected in TCM equals to N x 10⁴ (viable cells/ml).

2.2.3.6 Cell Culture Transfection
Cell transfection protocols involved in this research were based on cationic lipids. The lipids mix with plasmid DNA to produce liposomes, which fuse with the cell membrane and deposit the cargo inside. This brings the minimum damage to live cells. A few existing transfection
reagents utilizing this protocol were selected at the beginning of the research, and an optimization test was carried out in order to find the best reagent and condition to induce the highest transfection efficiency. The reagents tested were Fugene® 6, Fugene® HD (both from Roche), and Lipofectamine™ 2000 (Invitrogen). The methods of using each transfection reagent and corresponding condition settings will be described in the results section.

After this optimization process, Fugene® HD was chosen and used with optimized condition for cell transfection throughout the PhD research. The transfection protocol was modified from Fugene® HD handbook. Normally, Cells (~ 3 x 10⁶) were plated on 10cm tissue-culture dishes (achieving ~80% confluent for an overnight incubation) prior to transfection. On the following day, all media, reagents, and plasmid DNA solutions were pre-warmed in room temperature before use. For a single transfection on a 10cm cell culturing surface area, 10µg of plasmid DNA was required to cooperate with 40µl of Fugene® HD according to the optimized DNA (w) to transfection reagent (v) ratio for best transfection efficiency, and both the plasmid DNA and Fugene® HD were mixed with serum free DMEM in a total volume of 500µl. The transfection complex was then vortex mixed, and incubated at room temperature for 15 minutes. Cells ready for transfection were washed with 1x PBS once, and replaced with 4.5ml serum free DMEM in the 10cm tissue culture dishes, together with all the transfection complex. Incubation of the cells was taken place in the 37ºC cell culture incubator overnight. A double transfection can be achieved by sampling mixing both plasmid DNA (10µg each) and 80µl of Fugene® HD with serum free DMEM in a total volume of 500µl to form the transfection complex.

2.2.3.7 Selection of Stable Cell Line

For stable cell selection, cells after transfection were gently wash once with 1x PBS, then trypsinized and collected with TCM for a total volume of 5ml. Five different cell dilution plates were set up by loading 0.1, 0.25, 0.5, 1, and 2ml dilutions of the collected cell suspension respectively into 5 different 10cm tissue culture dishes and supplemented with TCM to 6ml in total. Zeocin (100mg/ml stock) or G418 sulphate solution (50mg/ml, PAA) was used for selecting plasmid DNAs containing zeocin or neomycin resistant genes, and they were added into each plate to make a final concentration of 1mg/ml. A transfection-free plate was also applied the same way to observe antibiotic killing. Cell culturing medium was changed every 5 days with serum medium containing 1mg/ml zeocin. Normally after two
rounds of 1mg/ml antibiotic incubation, large amount of dead cells was observed, and the zeocin concentration was reduced to 0.5mg/ml. However, this selection phenomenon varies to different cell types. Another few days, untransfected cells were all killed according to the negative control plate, and survived transfected cells formed into monoclonal cell colonies, which were beginning to be visualized without microscopes. The antibiotic level was dropped down to 0.25mg/ml for minimum selection pressure to maintain the stable cell line.

**Polyclonal Selection**
After antibiotic elimination of un-transfected cells, the survived transfectants from the same DNA transfection were trypsinised and pooled together. Such collections were also made for colonies left over after monoclonal selections. Sub-culturing these cells in the same way described in section 2.2.3.1 in TCM with 0.25mg/ml corresponding mammalian selection antibiotics, and stocked in liquid nitrogen for long term use.

**Monoclonal Selection**
The initial antibiotic treatment after transfection trigged apoptosis to cells without incorporation of induced resistance expressing DNA. Visualized individual monoclonal cell colonies were then selected from the cell dilution plates. The plates were gently washed with 1x PBS first. Selected colonies on the plate were picked by trypsin soaked 5mm autoclave sterilized paper discs (cut from 3MM Whatman paper by hole punches), and transferred into a 24-well plate (surface area of each well = 1.9 cm²) pre-loaded with 100µl trypsin in each well. TCM (900µl) was then applied to each well with gentle suspension in order to shear off the cells sticking to the paper discs, and finally the plate was incubated in normal cell culture condition. The paper discs were removed after cells were stably growing on the plate surface. Sub-culturing of the cells from each well of the 24-well plate was carried out the same way described in section 2.2.3.1 in 1ml TCM with 0.25mg/ml corresponding mammalian selection antibiotics, in order to control the cells in a similar growth rate for expression selection purpose. Selected monoclones were increasingly cultured step-by-step from transferring into 6-well plates to 75cm² culture flasks.

**2.2.3.8 Transient Protein Expression after Cell Transfection**
After cell transfection (see section 2.2.3.5), the cells were cultured immediately in 10ml TCM without any antibiotic selection followed by removal of the transfection complex. The
medium was collected after 3 or 4 days normal cell incubation, and centrifuged at 2000rpm for 5 minutes to remove the cells involved in the medium before further applications.

2.2.3.9 Protein Expression from Stable Cell Lines
Selected stable cell lines were increasingly cultured in TCM with normal cell culturing condition until they reached ~80% confluence. For cells with polysialylatable functionality, additional 1mM valproic acid (VPA) was added in TCM to enhance polysialyltransferases activities. Cells were then washed twice with 1x PBS, and replaced with FreeStyle™ CHO Expression Medium (for stable NB2a cell lines) or FreeStyle™ 293 Expression Medium (for stable HEK293 cell lines), supplemented with 4mM L-glutamine. With incubation in 37°C cell culture incubator, supernatant was collected after 4 days, and the cells was repeatedly cultured in the same serum-free medium for another 1 or 2 rounds depending on the healthy status of the cells. All collected medium were pooled together, and dialysed through Spectra/Por® Dialysis Membrane (MWCO: 12-14,000; vol/length: 18ml/cm) (Fisher Scientific) against 1 x PBS before purification.

2.2.4 Protein Expression and Purification

2.2.4.1 Selection of Stable Cell Lines with Optimal Protein Expression
Medium of each selected stable cell lines cultured in 24-well plates was collected when the cells have grown approximately above 70% confluent (normally after 4 days culturing), and centrifuged at 2000rpm for 5 minutes to remove the cell debris. Two main selection methods were used throughout the project: (1) Dot-blot; and (2) ELISA. Both methods were applied for checking interested proteins expression from other expression formats and scales.

Dot-Blot
Bio-Dot® microfiltration apparatus (Bio-Rad) was used for detecting protein expression level of each monoclonal. It is quick and feasible, and only tells the expression level from a single protein characteristic through each immunodetection. By following the manufactory protocol of the dot-blot apparatus, 500µl of cell supernatant was loaded on top of pre-wetted nitrocellulose membrane embedded in dot-blot apparatus outlined by a 96-well plate format lid. Samples were transferred in the membrane by vacuum through the apparatus. The membrane
was then blocked in 5% milk in 1x TBS for 40 minutes. Primary antibody was applied onto the membrane consequently. Additional secondary antibody conjugated with HRP was added when none HRP-conjugate primary antibody was used, with three times of vigorous 1x TTBS wash step in between. After another 3 washes in TTBS and then 3 times in TBS, the blot was developed by ECL™ (GE Healthcare), and the image was taken by Fujifilm LAS-3000.

**Enzyme-Linked Immunosorbent Assay (ELISA) for Selection**

For selecting proteins having two important characteristics from an un-purified buffer containing contaminants carrying one or the other characteristics, dot-blot is not accessible in such application, but an ELISA principle derived method was designed for the dual-selection. Specifically for the selection purpose, two characteristics from the fusion protein needed to be determined simultaneously were scFv antigen binding epitope, and polysialic acid. To carry out the method, scFv antigens were firstly immobilized on the surface of 96-well plates overnight at 4°C. For selection of monoclones with the best yield of polysialylated MFE23-Ig5-FN1, 100µl of N-A1 (1µg/ml) was directly coated in each well, while for the polysialylated C6.5-Ig5-FN1 selection, 100ng of hErbB2/Fc was immobilized in each well with Fc domain interacted with pre-coated protein A (1µg per well). Followed by twice washing twice with 1x PBS, the antigen coated plates were blocked with 3% milk in 1x PBS (300µl per well), and left at 37°C for 2 hours. After washing the plates 3 times with 1x PBS, 250µl of collected medium from the stable cell lines were loaded into each well, and a duplicate of each sample was applied. The following steps were continued by standard ELISA protocol described in later context (see section 2.2.5.6), and the final read-out was measured at 450nm wavelength from 96-well plate reader Spectramax 340pc (Molecular Devices).

**2.2.4.2 Immobilised Metal Affinity Chromatography (IMAC)**

Most proteins generated from both bacteria and mammalian expression systems within this project were tagged with a hexa-histidine (His<sub>6</sub>) epitope which coordinates strongly with certain transition metals such as cobalt. Cobalt metal affinity resin (TALON™, Clontech) was used to purify the proteins. For every litre of protein expressing bacterial culture, 2ml of TALON™ resin slurry was added to either the bug lysate or concentrated, dialysed supernatant. For mammalian expression system, 2ml TALON™ slurry was used for 500ml dialysed cell culture medium. Typically the resin was incubated with the bacterial lysate, supernatant or cell culture medium overnight at 4°C with gentle rolling, and then applied to a
10ml gravity flow column containing a scinter for collection of the resin. The flow-through was collected, and re-purified if the purification step is not efficient enough. Subsequent washing steps were conducted with 1x PBS or 1x TBS, and 4 column volumes of wash buffer were normally used. Protein was eluted in 1ml fractions of ice-cold 200mM imidazole buffer (pH 7.4). During IMAC purification, elution total protein content was quantitatively traced with Coomassie protein assay reagent using 100µl of the reagent and 10µl of sample. Fractions were pooled and dialysed exhaustively into the buffers that were required for consequent experiments or storage.

2.2.4.3 Immunoprecipitation
TALON™ anti-6xHis cobalt resin and anti-Myc antibody coated sepharose resin (gift from Dr. John Silva) were used. Both types of anti-protein tag resin beads were supplied as slurries, and incubated with the unpurified medium solution (approximately 100µl in 12ml medium) overnight at 4ºC. The medium was then run through a column containing a scinter for collection of the resin, and the resins were washed twice with 1xPBS followed by collection in 1x PBS as well. Centrifugation to the resin collection was carried out at 4000rpm for 5 minutes, and the supernatant was carefully removed before applying the resin for further experiment. Typically, the resin was diluted by 1x Laemmli (SDS-sample) buffer (Sigma-Aldrich) and directly used for SDS-PAGE. Other immunoprecipitations were achieved by firstly incubating the unpurified medium with specific precipitant recognizing monoclonal antibodies, followed by incubation with protein A or G immobilized on the sepharose resins. Further steps were processed as standard immunoprecipitation protocol described above.

2.2.4.4 Ion Exchange Chromatography
Further purification of separating proteins with different degrees of polymerization (DP) after IMAC purification was achieved by utilizing anion exchange chromatography based on the negative charge nature of sialic acid. An AcroSep™ Chromatography Column (1ml bed volume) with strong anion exchanger Q Ceramic HyperD® F (Pall Life Sciences) was used and optimised for separating proteins with different polysialylation degree. The column is suitable for connecting with both automated and pumped chromatography systems. In terms of automatically programmed purification, the anion exchange column was connected with the BIO-RAD BioLogic DuoFlow fast protein liquid chromatography (FPLC) with gradient
valve was served as the automatic system paired up with BioLogic QuadTec UV-Vis Detector for simultaneous wavelengths monitoring. Recommended by the column handbook, Tris buffer (100mM) with neural pH was selected as basic running and mixing buffer. The anion exchanger was firstly activated by washing with 5ml (equally to 5 column volumes) of loading buffer and 5ml of high salt elution buffer, and equilibrated with 5-10ml of loading buffer. Prior to loading the sample into the FPLC, samples were buffer-exchanged to the running buffer by exhaustive dialysis, and injected into the column with a speed of 1ml/minute based on the pre-designed wizard system. Protein samples were dialysed against the ion exchange running buffer and filter sterilized prior to be injected into the column. A few optimisation steps were processed and a final optimal programme was determined as routine protocol for automated anion exchange chromatography to classify fusion proteins with 3 major types of glycosylation or polysialylation feature. Basically, the FPLC instantly mixes the 1M high salt elution buffer with running buffer to prepare buffer conditions for featured elutions. The salt in the solution competes for binding to the immobilized matrix and releases the protein from its bound state at a given concentration. Proteins separate due to the amount of salt needed to compete varies with the external charge of the protein. Extra washing volumes were applied for better purity of the glycoproteins. During each elution steps, proteins were stripped off and collected with 20ml of corresponding elution buffer and finalised by 1M NaCl high salt elution buffer. Table 2.9 below outlines the optimised process of anion exchange chromatography

Table 2.9. Optimised anion exchange chromatography for sia/polysialylated protein purification. The glycoprotein was classified into 3 glyco-forms throughout the purification steps. Buffer A: running buffer; Buffer B: 1M NaCl high salt elution buffer.

<table>
<thead>
<tr>
<th>Steps</th>
<th>Buffers</th>
<th>Buffer Content (%)</th>
<th>NaCl concentration (mM)</th>
<th>Volume (ml)</th>
<th>Flow Rate (ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isocratic flow</td>
<td>Buffer A</td>
<td>100</td>
<td>0</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>Injection*</td>
<td>Buffer A</td>
<td>100</td>
<td>0</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>Isocratic flow*</td>
<td>Buffer A</td>
<td>100</td>
<td>0</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>Isocratic flow</td>
<td>Buffer A</td>
<td>95</td>
<td>50</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>Isocratic flow</td>
<td>Buffer A</td>
<td>90</td>
<td>100</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>Isocratic flow (Wash 1)</td>
<td>Buffer A</td>
<td>90</td>
<td>100</td>
<td>200</td>
<td>2</td>
</tr>
<tr>
<td>Isocratic flow</td>
<td>Buffer A</td>
<td>80</td>
<td>200</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>Isocratic flow (wash 2)</td>
<td>Buffer B</td>
<td>80</td>
<td>200</td>
<td>300</td>
<td>1</td>
</tr>
<tr>
<td>Isocratic flow</td>
<td>Buffer B</td>
<td>20</td>
<td>200</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>Isocratic flow</td>
<td>Buffer B</td>
<td>100</td>
<td>1000</td>
<td>20</td>
<td>2</td>
</tr>
</tbody>
</table>

* Both injection and the following isocratic flow steps can be multiplied to increase the sample loading into the anion exchange column. The column has an ion exchange capacity > 85mg/ml.
The chromatographic results were illustrated by BioLogic DuoFlow system (BIO-RAD), and indicated NaCl concentration dependent protein elutions were collected and pooled together. Consequent dialysis steps removed the associated high salt content. To be noted, proteins eluted from 1M salt solution were confirmed to be polysialylated (see results section) and dialyzed against 1x TBS instead of 1x PBS for improved preservation of PSA.

2.2.5 Protein Characterisation

2.2.5.1 Protein Concentration Determination

The concentration of protein was determined using either (1) the BCA™ Protein Assay Kit (Pierce, Thermo Scientific) or (2) NanoDrop™ 1000 Spectrophotometer by measuring protein absorbance at 280nm.

(1) Most commonly, the total protein concentration in samples was measured by the BCA assay. This method consists of Cu$^{2+}$ to Cu$^{+}$ by protein in an alkaline medium (biuret reaction) and subsequent colorimetric detection of Cu$^{+}$ with a reagent containing bicinchoninic acid. Protein standards of BSA at 0, 31.25, 62.5, 125, 250, 500, 1000, and 2000µg/ml were prepared, and were dissolved in the same buffer as the tested sample proteins. Using a 96-well microtitre plate, 25µl of each sample was transferred to each well, and analysed in triplicate, together with the BSA standards. The BCA working reagent was a clear green solution prepared by mixing 50 parts of BCA reagent A (sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartarate in 0.1 sodium hydroxide) with 1 part of reagent B (4% cupric sulphate). To each well, 200µl of BCA working reagent was added (1:8 sample to WR ratio) and mixed thoroughly for 30 seconds. The plate was allowed to stand for 30 minutes at 37ºC with a purple colour change. After cooling down to room temperature, the colorimetric detection was then taken place, and the absorbance of the samples was measured at 562nm by the 96-well plate reader Spectramax 340pc. A calibration curve using the protein standards was constructed and the concentration of the unknown samples was determined by extrapolation. The method was often used for protein samples without fully genetic information, and provided a detection range between 20 – 2000µg/ml.

(2) The protocol for NanoDrop™ 1000 Spectrophotometer was mentioned in section 2.2.2.3 for DNA concentration determination. For protein (working range: 0.1 to 100mg/ml), the
standard protein concentration detection at 280nm option was chosen, and a triplicate reading was tested for each sample. Following the Beer-Lambert law \( A = \varepsilon \cdot C \cdot l \), where \( A \) is absorbance (no units), \( \varepsilon \) is the molar absorption (extinction) coefficient (in \( \text{L mol}^{-1} \text{cm}^{-1} \)), \( C \) is the molar concentration (in \( \text{M}, \text{i.e.} \text{mol L}^{-1} \)) and \( l \) is the path length of the cuvette (in cm; almost always 1 cm), the equations below illustrate conversion between the absorbance and protein concentration.

\[
\frac{\text{Read-out of } A_{280}}{\text{Extinction coefficient}} = \text{Concentration (M)}
\]

\[
\text{Concentration (M)} \times \text{Molecular Weight (Da)} \times 1000 = \text{Concentration (µg/ml)}
\]

Both the protocols were very accurate for protein concentration detection, however the NanoDrop™ provides a quicker way but requires known extinction coefficient and calculated molecular weight of the protein sample, which can be obtained from the protein amino acid sequence by using ProtParam on the ExPASy Server (http://www.expasy.ch/tools/protparam.html) (Wilkins et al. 1999).

### 2.2.5.2 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Coomassie Staining

SDS-PAGE was executed using the BIO-RAD Mini-PROTEAN® 3 gel system in accordance with the manufacturers’ instructions. The gel components have been mentioned in section 2.1.3, and all gels used for this project were reducing gels. Typically, samples were mixed with laemmli loading buffer (6x) in a 2:1 ratio, and boiled for 5 minutes. The polysialylated samples were mixed with semi-reducing loading buffer, and heated at 65ºC for 10 minutes. Samples were then span down and chilled on ice before electrophoresis. The SDS-PAGE was normally running under a constant current at 20mA per gel, and the operation time was determined by the migration level of sample associated loading dye and standard pre-stained protein molecular weight ladders (Fermentas). For Coomassie staining, gels were carefully moved out from the glass plate cassettes, and soaked in PAG fix buffer for more than 2 hours at room temperature on a gentle rocking platform after electrophoresis. Followed by 3 times dH\(_2\)O wash, the gels were then stained by EZ-Run protein gel staining solution (approximately 20ml per gel), and left rocking until clear protein bands appeared. Remove the
staining solution and wash the gel another three times with dH₂O before taking an image from FujiFilm LAS-3000 laser scanning densitometer (White light EPI setting).

2.2.5.3 Western Blotting
Polyacrylamide gels (above) were transblotted onto Amersham™ Hybond™-ECL nitrocellulose membrane (GE Healthcare) using a BIO-RAD Trans-Blot SD semi-dry transblotter. The gel was laid over pre-wet nitrocellulose and sandwiched between two think slices of Whatman filter paper. All of these components were pre-soaked in transfer buffer for 5-10 minutes. The transfer was conducted at 15 volts for approximately 40-60 minutes, and subsequently the nitrocellulose membrane was blocked with 5% Marvel milk powder in 1x PBS (mPBS) for one hour. Antibody detection stages were for 1 hour per layer using dilutions recommended by the antibody manufacturers (see Table 2.4). All antibodies were diluted in 3% mPBS, and the blots were washed vigorously 3 times by 1x PBS-Tween between each antibody detection stage. Extra 3 times of 1x PBS wash were performed at the very last round of antibody-HRP incubation. Detection of chemiluminescent signal was done using the luminance based HRP substrate and imaged using the FujiFilm LAS-3000 densitometer with the chemiluminescence detection setting. The pre-stained protein marker image of the same blot was taken at white light (DIA) setting, and fused back to the western blot image by CorelDRAW Graphics Suite X4 software.

2.2.5.4 Glycosidase Treatment
Typically, excessive amount of each glycosidases listed in Table 2.6 was used to catalyse the hydrolysis of the oligosaccharides from the interested proteins (less than 10µg) during an overnight incubation at 37°C. For Endo-Neuraminidase, 2 Units of the enzyme were added directly into the protein samples maintained in PBS buffer. Acetyl-neuraminyl hydrolase or sialidase (Exo-N) (25 Units) catalysed with 1x G1 Reaction Buffer (50mM sodium citrate, pH 6.0) in a total reaction volume of 10 µl. Before PNGase F hydrolysis, proteins were denatured with 1x Glycoprotein Denaturing Buffer in a total volume of 10µl at 100°C for 10 minutes. Together with the additional addition of 1% NP-40 and 1x G7 Reaction Buffer (50mM sodium phosphate, pH 7.5), 20 Units of PNGase F are added to form another 10µl total reaction volume (20µl in total) and the reaction mix is incubated overnight at 37°C.
2.2.5.5 Digoxigenin (DIG) Glycan Differentiation

The DIG Glycan Differentiation Kit (Roche) that employs lectins to detect sugar linkages was used to give a general identification of certain oligosaccharide linkages. Lectins (listed in Table 2.5) extracted from different plant species and labelled with DIG, were found to bind to specific glycosidic linkages. The specificity of lectin affinities enables positive identification of the presence of certain oligosaccharides. Proteins with or without glycosidase treatments were tested. Samples were loaded on SDS-PAGE, and electrically transferred to nitrocellulose membrane as described in section 2.2.5.3. Following the manufacturer’s protocol, the nitrocellulose membranes were immersed in 1x blocking solution supplied with the kit for at least 30 minutes. After 3 times washing with 1x TBS buffer, the blots were incubated with lectins labelled with digoxifenin (10µl of SNA and DSA; 50µl of MAA; 100µl of PNA to 10ml Buffer 1) for 1 hour. Similar to Western blot, the secondary antibody, anti-digoxigenin-AP (dissolved in 1x TBS) was induced consequently, and incubated for another hour. With 3 times TBS washing, blots were stained by immersing without shaking in the staining solution (200µl NBT/BCIP solution supplied by the kit dissolved in 10ml Buffer 2), until grey to almost black bands developed. Staining reaction was quenched by rinsing with distilled water several times. The filters were dried on paper towels and photographed by the FujiFilm LAS-3000 densitometer with white light (DIA) setting.

2.2.5.6 Antigen Biotinylation

The EZ-Link® Sulfo-NHS-LC-LC-Biotin reagent (sulfosuccinimidyl-6-[biotinamido]-6-hexanamidohexanoate) is an N-Hydroxysuccinimide ester of biotin which reacts with primary amino groups available in lysine residues. According to the manufacturer’s protocol, a 20:1, reagent to protein coupling ratio should be used for an actual 4 or 5 biotin molecule to incorporate with 1 molecule of target protein. However, exceeded amount of biotin incorporation can mask the antigen binding site, and fatally damage the antibody recognition. A 5:1 reagent to protein couple ratio was also prepared to the target protein (for an actual 1:1 biotin to protein binding) to minimize the hazard of biotin blockage to the antigen binding site. Briefly, 10mM biotinylation reagent solution was firs prepared freshly in dH₂O. To biotinylate 1ml of 1mg/ml N-A1 (~26kDa MW) with 20:1 biotinylation ratio, the amount of 10mM biotinylation reagent solution was calculated from the following equations:
1ml N-A1 * 1mg N-A1/1ml N-A1 * 1mmol N-A1/26,000mg N-A1 * 20mmol Biotin/1mmol N-A1 = 0.000769 mmol Biotin
0.000769mmol Biotin * 1,000,000µl/L * 1L/10mmol = 76.9µl Biotin reagent solution

Following the same equations, the amount of 10mM biotinylation reagent solution required for 5:1 ratio was 19.23µl. The biotinylated reagent solution was mixed with 1ml N-A1 stored in 1x PBS, and the reaction was incubated on ice overnight or within 1 hour at room temperature. The free biotins were removed through a PD-10 desalting column (GE Healthcare). Before using the PD-10 column, the column was pre-conditioned by 25ml of the appropriate buffer that aimed to exchange to. Protein sample was prepared to 2.5ml in volume and added directly into the column, which was eluted by 3ml appropriate exchanging buffer.

2.2.5.7 ELISA for Affinity Binding Measurement
Enzyme-Linked Immunosorbent Assay was mentioned in the above paragraphs for monoclonal mammalian cell selection (see section 2.2.4.1). Both N-A1 and hErkB2/Fc antigens were coated in the same way for the affinity binding measurement. For biotinylated antigen, 100µl per well of 20µg/ml avidin (in 1x PBS) was pre-coated, followed by one hour incubation with 100µl biotinylated antigen (10µg/ml) in each well after two washes with 1x PBS. The antigen plate was then blocked with 300µl/well of 3% mPBS for 2 hours at 37ºC consequently followed by another two washes with 1x PBS. Unlike the ELISA for selection procedures, a serial concentration dilution of samples (especially scFvs or scFv fusion proteins) was prepared by 3% mPBS with a typical range between 5000nM to 0.05nM. Each dilution was tested in a triplicate, and 100µl of each was added for each well followed by 1 hour incubation. Unless stated otherwise, all subsequent wash steps consisted of three washes with 1x PBS-Tween and three times with 1x PBS. Like Western Blotting, antibody detection stages were then processed (100µl per well) with manufacturers’ recommended dilution (in 3% mPBS) until incubation with HRP conjugated antibodies. BM Blue POD soluble substrate (100µl per well) was used for colour development under dark condition, and quenched by 1M HCl (100µl per well) until distinctive blue colour change appeared. Absorbance at 450nm was taken afterwards by Spectramax 340pc, the 96-well plate reader. The data points in ELISA dissociation equilibrium constant ($K_D$) determinations were fitted, using Sigmaplot® 10 software, to a standard 3-parameter sigmoid curve with maximum, minimum, hill-slope,
standard errors and EC50 \( (K_D) \) which corresponds to the concentration of antibody yielding 50% of the saturating signal.

### 2.2.5.8 Surface Plasmon Resonance Binding Analysis: BIACore™

Real time binding analysis of MFE23 to its antigen targeting site N-A1 was performed by surface plasmon resonance (SPR) on a BIACore 3000 instrument. The running buffer used was 100mM Tris buffer pH7.4 containing 300mM NaCl, 0.005% P20 surfactant and 0.005% sodium azide. This buffer was thoroughly degassed and sterilized through a 0.2µm PES vacuum filtration unit. All experiments were carried out using two flow-cells of each BIACore™ chip using one for test (with immobilized ligand) and the other as reference.

#### Ligand Immobilisation

The new BIACore® streptavidin (SA) sensor chips were allowed to adapt room temperature for 30 minutes before use. After docking and priming the chip with the running buffer, the flow cells were then conditioned with three 1-minute pulses of 50mM NaOH + 1M NaCl at a flow rate of 30µl/min. Biotinylated N-A1 (1:5, NA-1: biotin ratio) was diluted with the running buffer to 2µg/ml, and injected onto the designated “test” flow-cell only from the SA chip with initially short 5µl pulse at 20µl/min until approximately 700 to 900 resonance units (RU) the immobilized antigen signals were reached.

#### Kinetic Experiments:

A serial dilution containing a concentration range of 185nM to 0.4nM of each type of MFE23 recombinant fusion protein sample was prepared using the running buffer. A kinetic wizard was set up using a flow rate of 20µg/min, implementing a 2 minute association phase, 10 minute dissociation phase and a regeneration phase of 1 minute at a flow rate of 30µg/min with 10mM HCl (Sainz-Pastor et al. 2006), followed by a 15 minutes stabilization period before the next cycle. All concentration were run in duplicate, preceded with 1 buffer cycle before and after of each sample cycle, in order to provide double references. The sample solutions were run following the order from the most concentrated (185nM) to the least concentrated (0.4nM). Same amount of blank running buffer solution was injected into the instrument and run under the same wizard programme for subtraction from the sample run to minimize the noise signals.

#### Evaluation of Kinetic Experimental Data

The scFv and scFv fusion protein kinetic binding experiments were evaluated by the BIAevalutation software (version 4.1). Values from the kinetic binding experiments were
firstly displayed as the subtractions between the test (with immobilized ligand) and reference flow-cells. Next, the response curves were plotted as the subtraction of the responding blank buffer run, and the injection and regeneration spikes were removed as well as the pre-injection areas from the curves. Then, the curves were “Y-transformed” all together by averaging the injection starting area to the zero baseline level. The simultaneous $k_{on}/k_{off}$ approach was used to all experiments to fit the data into a suitable kinetic model in order to measure the rate constants. Injection start and stop points were set and the association and dissociation phases were outlined. Prior to initiate the kinetic fit, the reflection index (RI) values were set to zero as constant to avoid software inappropriate default setting, and the maximum resonance values ($R_{max}$) were selected as locally fittings. The 1:1 (langmuir) binding model was then conducted with visual inspection of fit lines overlaid to the plotted curves and the assessment of statistically analysed rate constant values.

2.2.5.9 Fluorescence Activated Cell Sorting (FACS)

FACS provides a powerful method for sorting a heterogeneous mixture of cells based upon the specific light scattering and fluorescent characteristics carried by each cell. It was served for two major purposes in this project, which are cell surface binding detection and intracellular protein detection.

FACS for Cell Surface Antigen Binding Detection

This detection was focused on antibody and modified antibody derivatives in their antigen binding features to live antigen expressing cells. Antibodies tagged with FITC amplify the fluorescent signal and indicate the binding activities to the live cell antigen. Typically, adherent antigen expressing cells were cultured for 3 days and dissociated from the culture flasks by accutase (PAA), a much gentle proteolytic and collagenolytic enzymes for preservation of cell surface antigen. Cells ($4 \times 10^5$) were transferred to autoclaved Eppendorf tubes, and centrifuged for 30 seconds at 10,000rpm. Cell pellets were resuspended by 10µg antibody samples in a volume of 50µl, and incubated at 4ºC for 1 hour. Re-pelletize the cells followed by washing twice with 500µl of FACS buffer (2% FBS and 1mM EDTA in 1x PBS). The pellet was mixed with 100µl of serial immunodetection antibodies until incubation with FITC conjugated antibodies. All antibodies used were diluted with manufacturers’ instruction in FACS buffer for 1 hour incubation interval. Incubation with FITC conjugated antibody was carried out in dark. Repeated centrifugation and FACS buffer washing were carried out twice.
after each antibody incubation time. Finally, cells were resuspended in 500µl of FACS buffer and transferred to round bottom FACS tubes kept at 4°C with foil covered before analysed by dual laser 4 colour Becton Dickinson FACS Calibur analyser linked with Cellquest software (FITC setting).

**FACS for Endogenous Protein Detection**

This method was used to confirm PolyST transfected cells expressing functional endogenous polysialytransferases. Followed by the same protocol for detecting cell surface antigen binding, pelletized cells were resuspended in 100µl of 2% formaldehyde (16% methanol-free stock, Thermo Scientific) diluted in 1x PBS. The cells were fixed in this buffer for 10 minutes at room temperature and washed and re-pelletized by 500µl of FACS buffer. The pellet was then resuspended and detected by serial antibodies (100µl each round) until incubation with FITC conjugated antibodies. All antibodies used were diluted with manufacturers’ recommendation in FACS buffer for 1 hour incubation interval. Cells were lastly washed in 500µl of FACS buffer and transferred to round bottom FACS tubes kept at 4°C in dark. The analyses were carried out by Becton Dickinson FACSCalibur analyser and Cellquest software as described above.

**2.2.5.10 Confocal Laser Scanning Microscopy**

Confocal laser scanning microscopy (CLSM) was used for obtaining high-resolution optical images with selected depth. It has featured ability, also known as optical sectioning to acquire in-focus images from selected depths. Point-by-point images can be acquired and reconstructed with a computer, allowing three-dimensional reconstructions of topologically-complex objects. For this project, CLSM was used for tracking live cell surface antigen binding and internalization process, especially comparing these processes between normal scFv and the scFv fusion after internalization. Cells were cultured on 13mm cover slips for specimen preparation on the glass slides. Basically, individual well of a 24-well plate was placed with a piece of 13mm cover slip. The cover slip was disinfected with 0.5ml 70% ethanol per well for 1 minute. Followed by washing with 1ml tissue-culture specialized water (TC H2O), 0.5ml 0.05mg/ml poly-D-lysine (diluted with TC H2O) was applied per well, and the incubation at 37°C was carried out for 2 hours or at room temperature (sterile condition) overnight. Two 1ml TC H2O washing steps were processed after poly-D-lysine attachment achieved after the incubation. Typically, 20,000 cells were coated onto each cover slip, and
incubated for 2 to 3 days for antigen expression with approximately 50% confluence. Prior to antibody binding, cells were washed twice by TCM, and 300µl of antibody constructs (5µg/ml) diluted by TCM were added onto each cover slip. One hour antibody-antigen binding incubation was allowed prior to the further incubation conditions. Two temperature conditions (4°C and 37°C) paired up with two incubation period options (5 minutes and 1.5 hours) were set up specifically for surface antigen binding and internalization detections. After the live cell binding and extra conditioning incubation processes, the cells were gently washed once with TCM, followed by 1x PBS, and fixed by 250µl of 4% paraformaldehyde (in 1x PBS) for each cover slip on ice for 10 minutes, followed by 5 minutes permeabilization treatment by 250µl of 0.1% Triton X-100 on ice. Secondary antibodies, such as anti-Myc, anti-6xHis, anti-FN1 or anti-PSA antibodies (see Table 2.4 for dilution factors) were prepared in TCM, and applied to the cells (250µl/cover slip) for 1 hour incubation. Tertiary immunofluorescent antibodies against the secondary were consequently introduced to the cells after three times TCM washes. Finally, the tertiary antibodies were removed and the cells were washed three times with TCM and twice with 1x PBS, followed by an additional fixing step by 4% paraformaldehyde. For cells applied with anti-PSA as secondary antibodies, no fixing steps were used to avoid chemical oxidation of PSA. The cover slip was mounted (cell-face down) on the specimen slide with one drop of aqueous mounting gel (Sigma) in between. The slides were stocked in dark at 4°C.

Images of prepared specimen were taken by CLSM 510 on the Axioplan 2 microscope stand (Carl Zeiss) through control software LSM 510. For both fluorescent dyes, Alexa Fluor® 488 and 594 were exited at wavelength 488nm with emission filter setting between 505 to 550nm. Images of planes at various depths (z-stack) with 1µm increments were taken throughout the cells. LSM Image Brower was used as the software for image analysis.

2.2.5.11 Gel Filtration and Size Exclusion Chromatography (SEC)
SEC was used for the identification of protein apparent hydrodynamic volume. HiLoad™ Superdex 200 prep grade column (MW selectivity ranging from 10 to 600 kDa) filled with gel filtration media composing dextran covalently bound to highly cross-linked porous agarose beads (GE Healthcare) was used for filtrating molecules according to their apparent sizes. The BIO-RAD BioLogic DuoFlow fast protein liquid chromatography (FPLC) serving as the automatic system was connected with the gel filtration column, which was paired up with the
BioLogic QuadTec UV-Vis Detector for simultaneous wavelengths monitoring. Protein samples were dialysed to the column running buffer (1xPBS with 0.005% sodium azide, degassed and filter sterilized), and concentrated to a high concentration (>0.5mg/ml). Samples (0.5ml) were separately injected into the system with buffer flow-rate at 1ml/minute, and 160ml of running buffer was applied for the gel filtration programme.

**Table 2.10. Standard molecular weight markers used for size determination.** Approximate MW values are displayed in kDa. Information from (http://www.sigmaaldrich.com/life-science/proteomics/protein-chromatography/mw-markers.html)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Approximate Molecular Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbonic Anhydrase, Bovine Erythrocytes</td>
<td>29,000</td>
</tr>
<tr>
<td>Albumin, Bovine Serum</td>
<td>66,000</td>
</tr>
<tr>
<td>β-Amylase, Sweet Potato</td>
<td>200,000</td>
</tr>
<tr>
<td>Blue Dextran</td>
<td>2,000,000</td>
</tr>
</tbody>
</table>

MW standards (Sigma) listed in Table 2.10 were used for gel filtration column calibration prior to applying the protein samples. One ml of each MW marker (1mg/ml) were injected into the column and running at standard 1ml/minute flow-rate.

### 2.2.6 Mass Spectrometry (MS) for Polysialylated Glycoprotein Structure Determination

Initial MS studies (Chapter 3) were carried out by M-Scan Ltd (Wokingham, UK). The MS analysis for the polysialylated fusion protein (Chapter 4 and 5) was performed in the Glycobiology Centre, Imperial College London with the MS technical support from Dr Kevin Canis, and investigatory consultations from Dr Stuart Haslem and Professor Anne Dell.

#### 2.2.6.1 Reduction and Carboxymethylation

Approximately 100µg glycoprotein samples were lyophilised, and re-dissolved in 300µl of 2mg/ml dithiothreitol (DTT) in degassed 0.6 M Tris buffer (pH 8.5). Reduction was carried out by incubation for 60 minutes at 37°C. The carboxymethylation was then achieved by addition of 300µl of 60mg/ml iodoacetic acid in degassed 0.6M Tris buffer (pH 8.5) and incubation for one hour at room temperature. The reaction was stopped by dialysing samples

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against a 50mM ammonium hydrogen carbonate buffer (pH 7.4) for 36 hours at 4°C, and samples were lyophilised afterwards.

2.2.6.2 Trypsin Digestion
Reduced-carboxymethylated and lyophilised samples were digested using 300µl of a 100µg/ml L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPKC) treated bovine pancreas trypsin in 50mM ammonium hydrogen carbonate buffer (pH 8.4). Samples were incubated for 14 hours at 37°C and reaction was terminated by addition of 2 drops of glacial acetic acid. Digested samples were then immediately purified by passing through a Sep-Pak® C₁₈ cartridge. The reverse-phase Sep-Pak® C₁₈ cartridge (Waters corporation) was attached to a glass syringe and conditioned successively with methanol (5ml), 5% (v:v) acetic acid (5ml), propan-1-ol (5ml), and 5% (v:v) acetic acid (3 times with 5ml each time). Samples were directly loaded onto the cartridge and washed by 20ml of 5% (v:v) acetic acid (elution of hydrophilic contaminants). Peptides and glycopeptides were then eluted sequentially with 3ml of each 20%, 40% and 100% (v:v) propan-1-ol in 5% (v:v) acetic acid. The volume of each fraction was reduced on a Savant Speed-Vac and samples were lyophilised

2.2.6.3 PNGase F Digestion (N-glycan Release)
Release of the N-glycans was achieved by using the PNGase F (Roche) from Flavibacterium meningosepticum. Lyophilised 20% and 40% propan-1-ol fractions resulting from the Sep-Pak® C₁₈ purification were mixed and dissolved in 200µl of 50mM ammonium hydrogen carbonate buffer (pH 8.4). Samples were digested by addition of 5 Units of PNGase F and incubation for 36 hours at 37°C. Released N-glycans were separated from digested samples using a Sep-pak® C₁₈. A reverse-phase Sep-pak® C₁₈ cartridge was attached to a glass syringe and conditioned as before. The samples were directly loaded onto the cartridge. N-glycans were eluted by 5ml of 5% (v:v) acetic acid, and peptides and glycopeptides were then eluted sequentially with 3ml of each 20%, 40% and 100% (v:v) propan-1-ol in 5% (v:v) acetic acid. The volume of each fraction was reduced on a Savant Speed-Vac and samples were lyophilised.
2.2.6.4 Reductive Elimination (O-glycan Release)
The lyophilised 20% propan-1-ol fractions resulting from the post-PNGase F digestion were dissolved in 400µl of 55mg/ml potassium borohydride (KBH₄) in a 0.1M potassium hydroxide (KOH) solution and incubated for 24 hour at 45°C. The reaction was stopped by addition of 5 drops of glacial acetic acid. An ion-exchange column (2ml bed volume) with Dowex® 50W-X8 H cation exchanger resin (DOW) was assembled and washed with 15ml of 5% (v:v) acetic acid. Sample was loaded and eluted with 2.5ml of 5% (v:v) acetic acid. Samples were collected and dried on a Savant Speed-Vac. Excess borates resulting from the reductive elimination were removed by a co-evaporation with 4 times 500µl of a 10% (v:v) acetic acid in methanol solution and samples were finally dried under a steam of nitrogen.

2.2.6.5 Polysialylic Acid Digestion by Endo-N
Purified and dried native oligosaccharide samples (from section 2.2.6.4) were dissolved in a 50mM ammonium acetate pH 5.5 buffer. Enzyme (25mUnit) was added, and samples were incubated for 18 hours at 37°C. Another aliquot of the enzyme was added afterwards for completion PSA digestion. Samples were again incubated for 18 hours at 37°C, and finally freeze-dried.

2.2.6.6 Polysialylic Acid Lactonization on MALDI Plate
Purified native oligosaccharides (from section 2.2.6.4) were spotted onto a MALDI TOF-MS target and allowed to dry. The dried samples were dissolved in 1µl of a 0.1% orthophosphoric acid, incubated for 30 minutes at room temperature and dried under vacuum prior to MS analyses.

2.2.6.7 Oligosaccharide Cores Permethylation
Permethylation was carried out on purified and lyophilised glycans. Five pellets of sodium hydroxide (NaOH) were placed in a glass mortar and crushed in presence of 3ml of dry DMSO. The resulting slurry (1ml) was added to the lyophilised samples and 0.5ml of methyl iodide (ICH₃) was added. The mixture was vigorously mixed on an automatic shaker for 15 minutes at room temperature. The reaction was stopped by dropwise addition of water, while constantly shaking the tube. Permethylated samples were extracted in 1ml of chloroform and
washed several times with 3ml of dH₂O. Chloroform was finally evaporated under a stream of nitrogen, prior to the C₁₈ purification of derivatised glycans. A reverse-phase C₁₈ cartridge was attached to a glass syringe and conditioned successively with methanol (5ml), dH₂O (5ml), acetonitrile (5ml), and dH₂O (3 times of 5ml). The samples were dissolved in 1:1 (v:v) methanol:water and loaded onto the cartridge. Samples were washed with 5ml of dH₂O, and then eluted stepwise with 3ml of each 15%, 35%, 50% and 75% (v:v) aqueous acetonitrile solution. Organic solvent from each fraction was removed on a Savant Speed-Vac and samples were lyophilised prior to MS analyses.

2.2.6.8 Matrix Assisted Laser Desorption Ionisation / Time of Flight (MALDI-TOF) MS Analyses

Lactonized samples were dissolved in 1µl of the ATT matrix (20mg/ml 6-aza-2-thiothymine in 50:50 (v:v) acetonitrile/50mM ammonium citrate) and allowed to dry under vacuum. MALDI-MS analysis was performed using a Voyager DE-STR™ (Applied Biosystems) mass spectrometer in the linear negative mode with delayed extraction. The 4700 calibration standard, calmix, was used as the external calibrant for the MS mode. Data were acquired using Voyager 5 Instrument Control Software, and were processed using Data Explorer MS processing software.

Permethylated samples were dissolved in 10µl of 1:1 (v:v) methanol:water. One µl was mixed with 1µl of the DHB matrix (10mg/ml 2,5-dihydroxybenzoic acid in 50:50 (v:v) methanol:water) and was spotted onto a MALDI plate and allowed to dry under vacuum. MALDI-MS analysis was performed using the same mass spectrometer in the reflectron positive mode with delayed extraction. The 4700 calibration standard, calmix, was used again as the external calibrant for the MS mode. Data were acquired by Voyager 5 Instrument Control Software, and processed by Data Explorer MS processing software as described above.

2.2.6.9 Matrix Assisted Laser Desorption Ionisation (MALDI) MS/MS

Permethylated samples were dissolved in 10µl of 1:1 (v:v) methanol:water. Samples (1µl) were mixed with 1µl of the DHB matrix and was spotted onto a sample plate and dried under vacuum. MS/MS data were acquired using a 4800 MALDI TOF/TOF™ (Applied Biosytems).
mass spectrometer. The collision energy was set to 1 kV and air was used as collision gas. The instrument was calibrated using [Glu\(^1\)]-fibrinopeptide G human (Sigma-Aldrich) as external calibrant for the MS/MS mode and the 4700 calibration standard, calmix, was used as the external calibrant for the MS mode. Data were acquired using 4000 Series Explorer Instrument Control Software and were processed using Data Explorer MS processing software.

2.2.7 *In vivo* Studies of Radio-labelled Fusion Proteins

All mouse maintenance, injections and culling were performed by fully trained staff at the CBS unit of Imperial College London.

2.2.7.1 Preparation of Radio-labelled Samples

Protein samples were dialysed extensively into 1x PBS buffer prior to iodination to remove any glycerol or preservatives, and concentrated to 100μg/ml. Pierce® Pre-Coated Iodination Tubes (Thermo Scientific) were used to iodinate the protein samples. The tubes were pre-wet with 1 ml Tris iodination buffer (25 mM Tris-HCl, pH 7.5, 0.4 M NaCl) and decanted. Another 100μl of the Tris buffer was added directly to the bottom of the tube, followed by mixing with 10μl (1.0 mCi) Na\(^{125}\)I (Amersham). The buffer was allowed to stand for 6 minutes at room temperature with swirling every 30 seconds for iodide activation. Consequently, the buffer was transferred to the protein solution (1ml). For “over-oxidation” iodination, the protein solution (1ml) was added directly to the activated iodide tube for iodination. The reaction was incubated at room temperature for 9 minutes with swirling every 30 seconds. To end the reaction, 50μl of scavenging buffer (10mg/ml tyrosine in 1x PBS) was added and mixed for 5 minutes at room temperature. In order to remove the free radio-labelling materials and tyrosine residuals, the radio-labelled protein samples were buffer-exchanged to 1x PBS through pre-conditioned PD-10 columns (see section 2.2.5.6), and 3ml elutions of each radio-labelled protein sample were collected. All samples (with final concentration around 33μg/ml individually) were sterilized through 0.2μm PES syringe filters before injecting to the mice.
2.2.7.2 Sample Injections and Mouse Maintenance

Up to 300μl (~10μg) of each radio-labelled fusion protein were injected intravenously into the mouse tail vein. Due to radioactive nature of the samples, the experiment mice were caged and maintained behind purpose made lead-shielding during the time course after sample injection.

2.2.7.3 *In vivo* Blood Clearance Studies

Group of 4 tagged mice was carried out for each protein sample test. At the appropriate time points (0.5, 1, 2, 4, and 6 hours) after injection, a cut was made from the mouse tail tip, and approximately 20μl blood samples were taken from each mouse by using microhematocrit capillary tubes (VWR). Blood crur inside the capillary tube was stored in the sample tube. At 24 hours, all mice were culled under terminal anaesthesia and blood was immediately collected by cardiac puncture and transferred to sample tubes.

For the purpose of studying *in vivo* blood clearance pharmacokinetics data values were fitted to curves that conform to the two-compartmental intravenous model of clearance, which takes into account the biexponential clearance phases; distribution phase and elimination phase, of single intravenous doses. This is described by the exponential decay, double, 4-parameter equation: \( y = ae^{bx} + ce^{dx} \); where the distribution phase clearance rate \( t_{1/2\alpha} \) can be determined by \((\ln2)/b\), and the elimination clearance rate \( t_{1/2\beta} \) can be determined by \((\ln2)/d\).

2.2.7.4 *In vivo* Biodistribution Analysis

Group of 6 tagged mice was carried out for each protein sample test. Within each group, three mice were culled under terminal anaesthesia at 6 hours after sample injection, and the other 3 were culled under the same way at 24 hours after injection. Immediately after culling, blood was collected by cardiac puncture and transferred to a sample tube. The mouse was then dissected and kidney, liver, spleen, intestines, lung and heart were collected for biodistribution analysis.
2.2.7.5 Radiation Activity Measurement

The gamma radiation activities of each tissue sample collection were measured by Wallac-1470 gamma counter with settings for $^{125}$I isotope. For the PK studies, increased measuring time limit was set (1200 seconds) with maximal count limit of 200000; whilst the biodistribution analysis was measured with time limit of 60 seconds. The radiation values (counts per minute, cpm) were recorded and converted as a percentage of the initial injected dose per gram of tissue (% ID/Gram). The weights of each collected tissue samples were calculated after the deduction of the pre-weighted corresponding empty containers (i.e. sample tube and/or capillary tube).
CHAPTER 3

Initial Attempts to Develop a Recombinantly Polysialylated Antibody in Neuroblastoma (NB2a) Cells
3.1 Introduction

With the understanding that polysialylation is a potentially superior drug delivery technique, this project focused on producing polysialylated scFvs using a recombinant method conjugating PSA chains through a natural orientation, rather than chemical polysialylation. As described in section 1.5, the concept of a recombinant polysialylation system was derived from naturally occurring NCAM polysialylation, and a fusion construct containing the scFv together with the minimal polysialylatable NCAM domains (Ig5 and FN1) was generated (Constantinou 2005).

As described in sections 1.4.1 and 1.4.2, NCAM in mammals is predominantly expressed in brain glial and neuronal cells. Normally polysialylated NCAM disappears during brain development; but certain types of neuroblastoma cells can consistently express PSA-NCAM, in order to enhance cancer cell mobility and metastasis (Soroka et al. 2010). Human neuroblastoma CHP-134 cells were the first cell line reported to produce NCAM with PSA moieties with 55 or more sialyl residues (Livingston et al. 1988). During several later studies, human SK-N-SH and IMR-32 neuroblastoma cells, rat PC-12 pheochromocytoma, and mouse neuro-2A (NB2a) neuroblastoma cells were confirmed as PSA-NCAM positive cell lines (Hildebrandt et al. 1998; Gallagher et al. 2000; Poongodi et al. 2002; Beecken et al. 2005). The expression of polysialyltransferases (i.e. PST and STX) were also correspondingly reported within these neuroblastoma cell lines, which closely correlated to the degree of polysialylation and the cancer differentiation (Stoykova and Glick 1995; Seidenfaden and Hildebrandt 2001; Poongodi et al. 2002). Based on this evidence, these neuroblastoma cell lines were examined as they express essential enzymes for polysialylating endogenous NCAM and therefore can be considered candidate mammalian cell hosts for producing polysialylated versions of the scFv and NCAM polysialylatable domains fusion protein. The mouse NB2a neuroblastoma cell line (kind gift of Professor Yuri Ushkaryov, Imperial College London), one of the previously confirmed endogenous PSA-NCAM positive cell lines, was selected as the cell host for initial attempts to express and produce recombinantly polysialylated scFv fusion protein in this section of the project.

Constantinou, A. et al. initiated the genetic fusion work to construct recombinantly polysialylatable MFE23 scFv, in comparison with the chemical conjugation of PSA to the same scFv (Constantinou 2005; Constantinou et al. 2009). DNA constructs including MFE23-
Ig5-FN1, MFE23-FN1, FL-NCAM, and LS-MFE23, were inherited from this previous study (Constantinou 2005). As shown in Figure 2.1(b), a mammalian cell leader sequence (LS) – MLQTKDLIWTLLFLGTAVSLQVD, previously used for extracellular expression of NCAM protein, was added at the +1 position for all four constructs. This leader sequence is the signal peptide and was artificially created as a synthetic construct for the purpose of human NCAM expression and post-modification, but latterly was used as a powerful directory peptide for generating other human or humanised recombinant proteins and peptides following the mammalian cell secretion pathway through the Golgi apparatus to the cell surface, and eventually extracellular matrix (Wiemann et al. 2001). The full length (FL) NCAM gene was originally supplied as a recombinant fusion with an Fc ligand constructed in the pIg vector (kind gift of Dr. Jane Saffell, Imperial College London) (Appendix 2). The NCAM originated from the NCAM-140 isoform, and covered all five Ig-like domains and two fibronectin type-III domains. Truncation of the NCAM sequence to obtain the fragments encompassing the minimal NCAM polysialylation domains (Close et al. 2003) was achieved through DNA mutagenesis, digestion and ligation, which has been described in detail previously (Constantinou 2005). pcDNA4\textsuperscript{TM}/TO/myc-His (Appendix 1) was used as the vector system for the genetic incorporation of all the fusion elements, and expression of recombinant proteins in the mammalian cell hosts. This vector was chosen based on the following (1) It provides a strong expression promoter, also known as human cytomegalovirus (CMV) immediate-early promoter, which allows high-level protein production in a wide range of mammalian cells; (2) Double C-terminal peptide epitopes encoding both Myc and polyhistidine (His\textsubscript{6}) metal-binding tags are favourable for purification and detection of expressed protein; (3) Most importantly, the vector contains ampicillin and zeocin resistance genes for both bacterial and mammalian cell selections. Selection of highly expressing stable mammalian cells was one the main issues involved in this project, in order to provide enough research material for structural characterisation, and \textit{in vitro} and \textit{in vivo} investigations. The Zeocin\textsuperscript{TM} resistance gene enables selection of mammalian cells stably expressing the protein of interest incorporated within the pcDNA4\textsuperscript{TM}/TO/myc-His vector after transfection (Mulsant et al. 1988).

The antibody fragment, MFE23 scFv was investigated as the scFv portion of the fusion protein in the recombinantly polysialylated construct. It has been characterised by its carcinoembryonic antigen targeting specificity and affinity, good extravasation and tumour penetration and localization, and rapid blood clearance rate as a drawback (Casey et al. 1995;
Verhaar et al. 1995; Chester et al. 2000; Mayer et al. 2000). Chemically conjugating PSA to MFE23 in order to increase the pharmacokinetics of the single chain antibody was previously studied (Constantinou et al. 2009), which offered a direct contrast to the recombinant fusion approach. Furthermore, the scFv has also been repeatedly reported in many recombinant fusion formats for other investigations, such as antibody-directed enzyme prodrug therapy (ADPET) (Michael et al. 1996; Chester et al. 2000; Kousparou et al. 2002; Francis et al. 2004; Mayer et al. 2004), gene therapy (Whittington et al. 1998; Chowdhury et al. 2004), and other targeted therapies (Cooke et al. 2002). With all this previous fusion engineering experience, MFE23 scFv was selected as the first model to be genetically fused with the polysialylatable domains from NCAM.

3.2 Aims and Objectives

The aim of this section is to continue the previous research methodology (Constantinou 2005) in order to develop a recombinant polysialylation system for antibody fusion proteins as an alternative approach to chemical polysialylation, using NB2a as a naturally polysialylating cell line to fulfill this purpose.

The objectives are:

■ Optimizing reagents and conditions for mammalian cell line transfection.
■ Selection of stable NB2a cell lines expressing the MFE23 fusion proteins (i.e. the polysialylated format and other fusion controls).
■ To further characterize the fusion proteins to confirm true recombinant polysialylation.
■ To characterize the glycan nature of the MFE23-Ig5-FN1 to see if it can potentially be polysialylated.
■ To examine the antigen-binding potential of the recombinant scFv fusion protein in comparison with the scFv alone.
3.3 Results

3.3.1 Preparation and Examination of MFE23 scFv fusion Plasmid DNAs

The plasmid DNA constructs including LS-MFE23, MFE23-FN1, MFE23-Ig5-FN1 and FL-NCAM were inherited from previous research (Constantinou 2005) and prepared as described in section 2.1.5. These were verified by DNA double digestion before DNA was introduced into the mammalian cell hosts for protein production (Figure 3.1).

Figure 3.1. Schematic diagrams showing genetic information of FL-NCAM and all MFE23 fusion constructs fused in pcDNA4a vector. Localizations of both Hind III and XhoI restriction sites are addressed, and detailed fusion insert conformations are also illustrated corresponding to each fusion construct. The base pair (bp) numbers of each insert are calculated from the referred DNA sequences.
Corresponding restriction endonucleases were used in the double digestion for each construct. As illustrated in Figure 3.2, the double-digested DNA fragments showed the expected sizes for each fusion insert matching their DNA sequence calculations (indicated in Figure 3.1).

**Figure 3.2. XhoI/HindIII double digestions of DNA vectors containing MFE23 fusion constructs.** fragments ~2 kbp, ~1.5 kbp, ~1.2 kbp and ~0.9 kbp referred to the insert sizes from FL-NCAM, scFv-Ig5-FN1, scFv-FN1 and LS-scFv (respectively from lane 2, 3, 4, and 5). Lane 6 was loaded with empty pcDNA4a vector alone. Hyperladder I was used as base pair markers in lane 1.

### 3.3.2 Optimizing Reagents and Conditions for NB2a Cell Transfection

Among all the transfection methods, using liposomes as a carrier to deliver DNA is considered to be one of the mildest and efficient methods to treat cells. Many liposome-mediated transfection products are commercially available nowadays. Here, to identify the optimal transfection conditions, the three most popular and highly rated liposomal transfection reagents - Fugene® 6, Fugene® HD (Roche), and Lipofectamine™ 2000, were purchased and compared for the expression level of green fluorescent protein (GFP) after pEGFP-C1 DNA vector transfection.

In addition to the transfection procedure described in section 2.2.3.6, specific optimization protocols were tested for each transfection reagent. For Fugene® 6, the recommended reagent (μl) to DNA (μg) ratios – 3:1, 3:2 and 6:1 were prepared, whilst for Fugene® HD, a serial of ratios were prepared including 3:2, 4:2, 5:2, 6:2, 7:2, and 8:2 (as the protocol recommended). Fresh medium was added after overnight transfection complex incubation for all three transfection reagents. After transfection and two days recovery, the cells were lysed and the GFP fluorescent intensity from each lysate was consequently analysed. The same number (2x10^5) of NB2a cells that have been previously transfected with the same DNA vector (gift from Dr. John Silva, Imperial College London) with identified transfection efficiency (8.5%) were treated and analysed in the same way for its fluorescent reading.
In comparison, remarkable results were obtained with GFP extracted from NB2a cells transfected with Fugene® HD transfection reagent (Figure 3.3). Particularly, more than 90% transfection efficiency was obtained (reagent to DNA ratios at 7:2 and 8:2). Low cytotoxicity of Fugene® HD was also observed after long-term transfection incubation (overnight). Hence, Fugene® HD was selected as the optimal transfection reagent for transfecting DNA constructs into the NB2a cell line under the reagent to DNA ratio of 8:2.

![Transfection efficiency determination](image)

**Figure 3.3. Transfection efficiency determination.** Comparisons include Fugene® 6 with three different reagent to DNA ratios – 6:1, 3:1 and 3:2; Fugene® HD with six recommended transfection mix ratios, and Lipofectamin™ 2000 transfection system, together with positive control with known transfection efficiency as 8.5%.

### 3.3.3 Selection of Stable Monoclonal NB2a Cell Line Expressing MFE23 Fusion Proteins

With the optimised transfection conditions, pcDNA4 vectors containing the genes for the fusion proteins were transfected into the NB2a cells respectively. Stable cell lines were selected by zeocin resistance as described in section 2.2.3.7. Selection of isolated cell colonies were cultured in 24-well plates and the culture media from surviving healthy and growing monoclonal cell lines were collected and used for dot-blot, a screening tool for initial detection of the expression level of the induced fusion proteins. Monoclonal cell lines stably secreting relatively high levels of His<sub>6</sub>-tagged protein were further selected.
Dot-blot results indicated the monoclones which had the best protein expression, as shown by the intensity of the anti-\text{His}_6 dot blot signals. With distinct visual intensity differences of the dots shown in Figure 3.4 for each MFE23 fusion constructs, clone 3 of LS-MFE23, clone 6 of MFE23-FN1, and clone 3 of MFE23-Ig5-FN1 were considered to be the monoclones that had the highest fusion protein expression levels. Monoclonal media collection and dot-blot tests were processed again under the same conditions, and the same result was obtained. For cells transfected with the FL-NCAM construct DNA, no positive signal was obtained in the dot-blot. Further FL-NCAM monoclonal cell lines were also tested, and none produced a positive result (results not shown). Comparatively, monoclone 1 (shown on Figure 3.4) of FL-NCAM was chosen to be the highest expressor among all FL-NCAM monoclone tested in the dot blot. All 4 monoclonal cell lines including monoclone 3 and 7 for LS-MFE23, monoclone 2 for MFE23-FN1, and monoclone 1 for MFE23-Ig5-FN1 were also selected and cultured as backup lines.

Figure 3.4. Anti-\text{His}_6 dot-blot results for selecting monoclones for each fusion construct with best protein expression levels. In total, six FL-NCAM, ten LS-MFE23, six MFE23-FN1, and eight MFE23-Ig5-FN1 monoclonal cell lines were screened by dot-blot. Three negative controls, which were TCM (a), PBS (b) and untransfected NB2a cell culturing medium (c) were used for background reading. Approximately 10μg of a control \text{His}_6-tagged tetanus toxin protein was employed as a positive control.

Additionally, all of the selected monoclones from the four recombinant fusion constructs were dot-blot screened in the same manner with immuno-detection of anti-PSA (12F8) antibodies.
However no positive signal was obtained from any monoclonal supernatant sample (results not shown).

3.3.4 Characterisation of the MFE23 Fusion Protein Constructs Purified from Selected NB2a Monoclones

The selected NB2a monoclines with best expression levels for all four fusion constructs were cultured in 75cm² flasks under normal tissue culture conditions with serum supplemented TCM (section 2.2.3.1). In order to eliminate serum albumin contamination (which will hinder protein purification quality), the serum-free medium, FreeStyle™ CHO Expression Medium was applied to the cells once they reached approximately 80% confluence. Two rounds of the culture medium were collected following the procedure described in section 2.2.3.9. A third round of serum-free incubation of the cells was tested as well, and massive cell death was observed, and this media was not used in case it contaminated the fusion proteins.

PBS Dialysed serum-free media was used to purify His₆-tag fusion protein immobilised by metal (TALON™) affinity chromatography (section 2.2.4.2). Purified samples of each construct were loaded onto SDS-PAGE with Coomassie staining in order to visualize the purity, integrity, and molecular weight of the produced protein. Western-blotting followed by immuno-detection was also utilised to identify the recombinant proteins. Results of Coomassie stained and Western Blotted purified MFE23 fusion proteins are shown in Figure 3.5. All three MFE23 fusion proteins (LS-MFE, MFE23-FN1, and MFE23-Ig5-FN1) were successfully purified (Figure 3.5a). Each of the MFE23 recombinants also showed the correct molecular weight based on their amino acid compositions (Table 3.1). Bands shown in the sample flow-through and wash flow-through were considered as major contaminants (i.e. serum or growth factor related proteins), which were significantly eliminated after the IMAC purification. Unfortunately, the FL-NCAM was not purified from His₆ affinity column, as measured by Bradford assay of protein elution fractions during the purification process (results not shown). The cause of the problem was considered to be His₆-tag detachment or mis-folding, and this predication was further confirmed by the Western blot results.

Both anti-His₆ and anti-FN1 immuno-detection are shown in Figure 3.5b and c. All three MFE23 fusion proteins were detected by anti-His₆ antibody with the correct MW, but no band was seen with FL-NCAM, which may indicate a malfunctioning or missing His₆-tag. Concentrated supernatant from FL-NCAM monoclones was subsequently subjected to an anti-FN1 Western blot immuno-detection, and a positive result was obtained together with
Figure 3.5. Recombinant fusion proteins purified from selected stable NB2a monoclonal cell lines. (a) Coomassie staining of His₆ tag affinity column purified MFE23-Ig5-FN1, LS-MFE23 and MFE23-FN1 recombinant constructs. Red arrows indicate the purified fusion proteins shown in lane 4 and 5. Lane 2 and 3 represents sample flow-through and first wash flow-through from the affinity column in purification steps. (b) Anti-His₆ and (c) Anti-FN1 Western blot analysis. Lane 2, 3, 4, and 5 were loaded with FL-NCAM (concentrated but unpurified), MFE23-Ig5-FN1, MFE23-FN1, and LS-MFE23 in both Western blots. The recombinant Hc fragment of tetanus neurotoxin (TT-Hc) (Scott et al. 2008) and NCAM-Fc (gift from Dr. Jane Saffell) proteins were used respectively as positive controls for both anti-His₆ and anti-FN1 immuno-detections, and shown in lane 6 of each blot. Untransfected NB2a cell supernatant was prepared as negative control in lane 7.

MFE23-Ig5-FN1 and MFE23-FN1 constructs, which also encode a FN1 domain. LS-MFE23 had no FN1 domain DNA in its fusion construct, and was not detected by anti-FN1 antibody as expected. A Myc tag-specific immuno-affinity column was also used to purify FL-NCAM, but also failed (Results not shown). The anti-FN1 Western blot confirmed the expression of FL-NCAM from the cell medium. However, from all the evidence, it is more likely that the expressed FL-NCAM protein did not maintain functioning C-terminal peptide tags. However,
results from SDS-PAGE and Western blot showed that the selected monoclones from dot-blot were expressing intact MFE23 fusion proteins with correct molecular weights. Anti-PSA Western blot detection was also carried on all recombinant fusion proteins including concentrated unpurified FL-NCAM monoclonal supernatant. Unfortunately, nothing was detected by Western blot.

Another important finding was that the apparent molecule weight values of all three recombinant proteins (LS-MFE23, MFE23-FN1, and MFE23-Ig5-FN1) indicated by the protein gels and immuno-detections are appeared to be bigger than the computed calculated MW values (Table 3.1) according to the proteins corresponding DNA sequences.

Table 3.1. Calculated amino acid molecular weight and extinction coefficient of all three MFE23 fusion proteins by ExPASy ProtParam programme.

<table>
<thead>
<tr>
<th>MFE23 Fusion Proteins</th>
<th>Molecular Weight (Da)</th>
<th>Extinction Coefficient (M$^1$ cm$^{-1}$) at 280nm in water</th>
</tr>
</thead>
<tbody>
<tr>
<td>LS-MFE23</td>
<td>29251</td>
<td>53860</td>
</tr>
<tr>
<td>MFE23-FN1</td>
<td>43706</td>
<td>76320</td>
</tr>
<tr>
<td>MFE32-Ig5-FN1</td>
<td>53615</td>
<td>97750</td>
</tr>
</tbody>
</table>

Amino acid MW was derived from corresponding protein gene sequences. All MWs include both Myc and His$_6$ tags molecular weights.

More precisely, the apparent MW increases of both LS-MFE23 and MFE23-FN1 were within ~6 kDa, while a significant MW increase (~20 kDa) was demonstrated by MFE23-Ig5-FN1 fusion protein, and this indirectly indicated that additional post-translational modification was involved especially when a polysialylatable protein domain was carried alongside.

Following the same method as above, large scale production of MFE23-Ig5-FN1, MFE23-FN1, and LS-MFE23 was carried out in multiple 125cm$^2$ culture flasks. The purity and integrity of the fusion proteins were checked again (shown in Figure 3.6). All three large-scale purifications produced recombinant proteins with the correct molecular weights, according to their calculated MW outlined in Table 3.1. Both Coomassie staining and Western blot also proved the purity and integrity of the proteins. Therefore, the serum-free production system is very likely to be an adequate routine system for obtaining fusion proteins expressed in mammalian cells, despite some degree of degradation appearing in the MFE23-Ig5-FN1 fusion protein, as three bands are observed for the protein in Figure 3.7 with one major band indicating the glycosylated fusion protein (~70 kDa), and two minor bands with reduced MWs.
Figure 3.6. Purity and integrity detection of MFE23-Ig5-FN1, MFE23-FN1, and LS-MFE23 recombinant fusion proteins produced in large scale. (a) Coomassie staining and (b) anti-His<sub>6</sub> tag Western blot. All three recombinant proteins were produced from the corresponding NB2a stable monoclones.

### 3.3.5 Affinity Binding Assay – ELISA

*In vitro* antigen binding profiles were subsequently investigated for further characterisation of recombinant MFE23 fusion proteins selected from stable monoclonal NB2a cell lines. The ELISA tests were carried out to determine the $K_D$ value of all three MFE23-recombinant fusions: LS-MFE23, MFE23-FN1 and MFE-23-Ig5-FN1, in order to show that the fusion proteins produced from the stable mammalian cell lines have retained antigen binding affinity compared to the bacteria-expressed MFE23 scFv and the fusion domains together with the potential polysialylatable post-modifications have no significant effect on the binding affinity compared to the original scFv. As described in section 2.2.5.7, an ELISA method was used with N-A1, the recombinant binding domains of CEA as the actual antigen target in the assays (Figure 3.7).

Previously, *in vitro* studies confirmed that the $K_D$ of scFv MFE23 against N-A1 is approximately 4.5nM (Sainz-Pastor et al. 2006). As shown in Figure 3.7, results from the antigen binding test revealed a similar $K_D$ value of MFE23 scFv expressed by both *E. Coli* bacterial and NB2a mammalian cell expression systems, which is almost the same as the published $K_D$ value. More importantly, NB2a cell expressed MFE23-Ig5-FN1 fusion protein also showed a consistent $K_D$ value, indicating the recombinant fusion had no effect on antigen binding.
3.6 Glycosidase Treatment of MFE23-Ig5-FN1 Fusion Protein Expressed from Stable NB2a Cell Line

Due to the lack of anti-PSA immuno-detection of the MFE23-Ig5-FN1, further glycoprotein investigation was carried out involving different glycosidases. Glycosidases have unique specificities recognising and removing different oligosaccharide structures. The defined functions and targets of various glycosidases are mentioned previously in the Material and Method section (see Table 2.6 and Figure 2.2). MFE23-Ig5-FN1 was the fusion protein that was hypothesised to be polysialylated during the post-translational modification of mammalian glyco-protein expression process; therefore the glycosidase treatment was focused on this protein (Figure 3.8). Many positive controls were also utilized in order to define the specific activities of each glycosidase.

Glycosylation increases the molecular weight of a protein simply through the addition of multiple monosaccharides. Glycosidases can hydrolyse specific oligosaccharides. This glyco-structure removal directly induces a MW loss, which can be easily revealed by SDS-PAGE. As shown in Figure 3.8(a) above, Coomassie staining suggested no band shift after either Endo-
Figure 3.8. Glycosidase treatment analysis. (a) SDS-PAGE analysis (Coomassie staining) of MFE23-Ig5-FN1 fusion constructs before and after different glycosidase treatments. Coomassie staining of both (b) Exo-N treatment for transferrin, and (c) fetuin with Exo-neuraminidase and PNGase F treatments. (d) Anti-PSA/NCAM (12F8) Western blotting of homogenized embryonic mouse brain (HEMB) with/without Endo/Exo-N treatments, together with intact MFE23-Ig5-FN1 fusion protein. Transferrin, fetuin, and HEMB were used as positive controls carrying the corresponding glycosylation features to test the activities of Exo-Neuraminidase, PNGase F, and Endo-Neuraminidase.

Neuraminidase or O-Glycosidase treatments, indicating no corresponding sugar linkages (α2-8 linked sialyl residues and GalNAc-Ser/Thr) were present in the MFE23-Ig5-FN1 fusion construct. In other words, it is likely that the fusion was not polysialylated. Exo-N (NeuNAcα2-3/6Gal specific) treatment indicated a molecular weight shift of approximately 5 kDa as compared to the intact fusion protein, and a ~10 kDa difference was also detected when all N-linked glycans were removed by PNGase F. The activity tests of the glycosidases were confirmed simultaneously. Blood proteins, transferrin and fetuin are well acknowledged to be heavily sialylated, and specifically, transferrin has been reported to have NeuNAcα(2-6)Gal terminal sialylation (Finne and Krusius 1979), while NeuNAcα(2-3)Gal linkages appeared more often in fetuin (Nilsson et al. 1979; Edge and Spiro 1987). This made these blood proteins ideal positive controls for monitoring Exo-N catalytic activity (Figure 3.8). Fetuin is also an N-glycan rich protein, so its band shift after PNGase F treatment confirmed
the enzyme function (Figure 3.8c). Homogenated embryonic mouse brain (HEMB) containing polysialylated NCAM was used as positive control for the anti-PSA Western blot (Figure 3.8d). This also confirmed the intriguing activity of Endo-N, in terms of PSA hydrolysis, and the specific polysialic acid recognition of the anti-PSA (12F8) antibody. A smear between 250 kDa and above was revealed with the immuno-detection of HEMB, suggesting variable polysialylation level of the NCAM (the extracellular domain of non-glycosylated NCAM is only ~80 kDa) in the fetal mouse brain. The same smear was also detected in Exo-N treated HEMB, indicating the inefficiency of Exo-N to cleave the protruding PSA chains that also inhibited the glycosidase ability to reach its preferred cleavage sites: Neu5Acα(2-6)Gal or Neu5Acα(2-3)Gal, inside the N-glycan structures. Again, no anti-PSA detection was detected with the intact MFE23-Ig5-FN1 fusion protein produced by NB2a cells.

### 3.3.7 Oligosaccharide Differentiation by Digoxigenin Linked Lectins

Lectins have specific abilities to recognise unique glyco-structures. The lectin binding exposes the specific sugar linkage existence within glycoproteins. The specificities of each lectin are shown in Table 2.5. After failing to detect PSA by Western blot, the MFE23-Ig5-FN1 fusion construct was then examined in various lectin binding tests, in order to reveal the presence of essential oligosaccharides that are responsible for sialylation, and discover the epitopes required for further sialic acid elongation – polysialylation.

Bands shown in Figure 3.9 indicated linkages including Neu5Acα(2-6)Gal, Neu5Acα(2-3)Gal, and Galβ(1-4)GlcNAc were present in carbohydrate compositions of MFE23-Ig5-FN1 fusion protein made from NB2a cells. Galβ(1-3)GlcNAc linkage was not detected by PNA detection, suggesting such linkage may not be involved in the fusion protein surface carbohydrate presentation. After the glycosidase treatments, no band shift was found with the fusion protein after Endo-N treatment (Figure 3.9, SNA and MAA detection) comparing with the non-glycosidase treated form. This implied that no Endo-N hydrolysis occurred with the MFE23-Ig5-FN1 fusion protein, which indicated no PSA was attached to on the fusion protein. After Exo-N treatment, no band was detected in the SNA lectin binding test, but bands were seen with both MAA and DSA detection, and the band in the MAA detection appeared to be weaker in intensity. Both bands found in MAA and DSA after Exo-N treatment also showed minor migration differences compared to the non-treated ones. No bands were seen in any lectin detection after PNGase F treatment that removes the N-linked glycans.
Figure 3.9. Lectin detections of sugar linkages from MFE23-Ig5-FN1, MFE23-FN1, and LS-MFE23 stably expressed from NB2a monoclonal. Four different lectins SNA, MAA, DSA, and PNA were used to detect Neu5Acα(2-6)Gal, Neu5Acα(2-3)Gal, Galβ(1-4)GlcNAc, and Galβ(1-3)GlcNAc respectively. Glycosidase treatments were also applied for the oligosaccharide removal.

Neither MFE23-FN1 nor LS-MFE23 had any of the four glyco-linkages being detected by any lectins (Figure 3.9). All the positive control glycoproteins showed positive results after binding with the corresponding lectins, but no bands were seen after glycan removal by glycosidases, proving the validity of the DIG glycan differentiation tests, and good catalytic abilities of all glycosidases involved. Evidence of MFE23-Ig5-FN1 protein degradation was
also revealed by lectin detection similar to that seen in the protein immuno-staining (Figure 3.6b).

### 3.3.8 Structural Analysis of the Carbohydrate Presentation on NB2a Expressed MFE23-Ig5-FN1

The aim of this part of the work was to provide as much information as possible concerning the potential N-glycans on the MFE23-Ig5-FN1 peptide backbone using a combination of protein and carbohydrate chemistry plus mass spectrometry. The structural analysis services were provided by M-Scan Ltd (Wokingham, UK) and approximately 500µg of purified NB2a-expressed MFE23-Ig5-FN1 dissolved in 1 x PBS (see section 3.2.4 for purification procedure) was used for the analysis.

**N-linked oligosaccharide population screening by MALDI-MS**

The samples were reduced and carboxymethylated. A small amount of precipitation was observed in the reaction products. The supernatant was removed and purified using a Microcon spin cartridge. Trypsin digestion was then performed. The lyophilised products were digested using PNGase F and then purified using a C18 Sep Pak. The 5% aq. acetic acid (N-linked oligosaccharide containing) fraction was permethylated and DE-MALDI-TOF mass spectra were obtained using a portion of the derivatised oligosaccharides in a high mass range for molecular ions. MALDI-TOF mass spectrometry was performed using a Voyager-DE STR Biospectrometry Research Station laser-desorption mass spectrometer using Delayed Extraction (DE) technology. Table 3.2 lists the predominant molecular ions present in the MALDI spectra (Appendix 12).

**N-linked oligosaccharide population screening by ES-MS**

Following MALDI-MS analysis, a fraction of the permethylated N-glycans were analysed by Electrospray-Mass Spectrometry (ES-MS). ES-MS was performed using a quadrupole-orthogonal acceleration time of flight (Q-TOF) instrument using Argon as the collision gas. Glu-Fibrinopeptide fragment ions in MS/MS mode were used to calibrate the instrument. Dried permethylated glycans were redissolved in methanol:0.1% TFA (80:20) before analysis. Table 3.3 lists the predominant fragment ions present in the electrospray spectrum (Appendix 13).
Table 3.2. Masses observed in the MALDI spectra of permethylated N-glycans derived from MFE-Ig5-FN1. Possible carbohydrate structures according to the corresponding masses were also assigned.

<table>
<thead>
<tr>
<th>Signals observed (m/z, peak top masses)</th>
<th>Possible Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1089.7</td>
<td>Hex$_x$ +Na$^+$ (contaminant)</td>
</tr>
<tr>
<td>1293.9</td>
<td>Hex$_y$ +Na$^+$ (contaminant)</td>
</tr>
<tr>
<td>1362.7</td>
<td>Hex$_z$HexNAc$_a$ A-type fragment ion</td>
</tr>
<tr>
<td>1416.9</td>
<td>Hex$_b$HexNAc$_c$ +Na$^+$</td>
</tr>
<tr>
<td>1526.1</td>
<td>Hex$_d$HexNAc$_e$ A-type fragment ion</td>
</tr>
<tr>
<td>1580.3</td>
<td>Hex$_f$HexNAc$_g$ +Na$^+$</td>
</tr>
<tr>
<td>1730.3</td>
<td>Hex$_h$HexNAc$_i$ A-type fragment ion</td>
</tr>
<tr>
<td>1784.5</td>
<td>Hex$_j$HexNAc$_k$ +Na$^+$</td>
</tr>
<tr>
<td>2111.8</td>
<td>Not assigned</td>
</tr>
<tr>
<td>2432.4</td>
<td>NeuAcHex$_l$HexNAc$_m$ +Na$^+$</td>
</tr>
<tr>
<td>2881.6</td>
<td>NeuAcHex$_n$HexNAc$_o$ +Na$^+$</td>
</tr>
<tr>
<td>2922.7</td>
<td>Not assigned</td>
</tr>
<tr>
<td>3127.0</td>
<td>Not assigned</td>
</tr>
<tr>
<td>3243.1</td>
<td>NeuAc$_p$Hex$_q$HexNAc$_r$ +Na$^+$</td>
</tr>
<tr>
<td>3604.3</td>
<td>NeuAc$_s$Hex$_t$HexNAc$_u$ +Na$^+$</td>
</tr>
<tr>
<td>3692.2</td>
<td>NeuAc$_v$Hex$_w$HexNAc$_x$ +Na$^+$</td>
</tr>
<tr>
<td>4053.8</td>
<td>NeuAc$_y$Hex$_z$HexNAc$_a$ +Na$^+$</td>
</tr>
<tr>
<td>4415.1</td>
<td>NeuAc$_b$Hex$_c$HexNAc$_d$ +Na$^+$</td>
</tr>
<tr>
<td>4777.0</td>
<td>NeuAc$_e$Hex$_f$HexNAc$_f$ +Na$^+$</td>
</tr>
</tbody>
</table>

Table 3.3. Low mass fragment ions observed in the Electrospray spectrum of permethylated N-glycans derived from MFE-Ig5-FN1. The data obtained show fragment ions which are consistent with antennal structures expected to be present in the complex glycans detected by MALDI-MS.

<table>
<thead>
<tr>
<th>Signals observed (m/z)</th>
<th>Possible Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>260.1 (minor)</td>
<td>HexNAc$^+$ A-type fragment ion</td>
</tr>
<tr>
<td>344.2</td>
<td>HexNAc$^+$ A-type fragment ion (-methanol)</td>
</tr>
<tr>
<td>376.2 (minor)</td>
<td>NeuAc$^+$ A-type fragment ion</td>
</tr>
<tr>
<td>432.2 (minor)</td>
<td>HexHexNAc$^+$ A-type fragment ion (-methanol)</td>
</tr>
<tr>
<td>464.2</td>
<td>HexHexNAc$^+$ A-type fragment ion</td>
</tr>
<tr>
<td>477.2</td>
<td>Hex2 +Na$^+$ (contaminant)</td>
</tr>
<tr>
<td>793.4</td>
<td>NeuAcHexHexNAc$^+$ A-type fragment ion (-methanol)</td>
</tr>
<tr>
<td>825.4</td>
<td>NeuAcHexHexNAc$^+$ A-type fragment ion</td>
</tr>
<tr>
<td>1186.5 (weak)</td>
<td>NeuAc2HexHexNAc$^+$ A-type fragment ion</td>
</tr>
<tr>
<td>1293.6</td>
<td>Hex6 +Na$^+$ (contaminant)</td>
</tr>
</tbody>
</table>
The structural analysis data conclusively confirm the presence of high mannose and complex N-glycan structures on the glycoprotein. The major structures present are high mannose representing early structures in N-glycan biosynthesis. Complex structures were detected with masses consistent with bi-, tri- and tetra-antennary structures with varying levels of sialylation. Evidence of polysialylated structures was found but these were detected at minor levels on the tetra-antennary glycans. A weak signal (1186.5m/z) consistent with an antennal fragment ion containing two sialic acid residues was observed in the electrospray mass spectrum.

3.4 Discussion

By predominantly following the protocols and indications from a previous study (Constantinou 2005), the recombinant polysialylation of MFE-23 scFv research initiated by Dr. A. Constantinou was further investigated and characterised as the first part of this PhD project. All the DNA constructs including polysialylatable MFE-23 scFv and other control constructs MFE23-FN1, LS-MFE-23, and FL-NCAM were inherited from previous research (Constantinou 2005) and successfully reproduced as confirmed by DNA double digestion (Figure 3.2). The original project design was to transfect the polysialylatable fusion construct into a neuroblastoma-2a (NB2a) cell line, a well known PSA-NCAM positive cell line, that makes its own polysialylated NCAM, and use this cell line to secrete polysialylated fusion proteins. If the NB2a cells express the essential enzymes to polysialylate their own NCAM protein, theoretically the fusion protein containing NCAM polysialylatable domains should be glycosylated and polysialylated through the cell Golgi apparatus by the responsible enzymes.

Transfection optimization tests using a GFP containing pEGFP-C1 plasmid vector were processed initially to optimize the transfection conditions, including plasmid DNA uptake and cell viability after transfection, to determine the optimal conditions for following stable cell line selections. Moreover, the NB2a cell line is not a standard expressing cell-line and as a type of neuronal cell line, is often difficult to transfect, so it was important to verify the transfection efficiency prior to the experiments with the MFE23 fusion constructs. Based on the high transfection efficiency (Figure 3.3), as well as gentle and low post-transfection
toxicity, Fugene® HD was selected as the transfection reagent with an 8:2, reagent (µl) to DNA (µg) ratio and overnight transfection incubation time for optimal transfection efficiency. These conditions were consequently applied to MFE23 fusion construct transfection and stable cell selection.

As shown in Figure 3.5, stable monoclonal cell lines were successfully selected from LS-MFE23, MFE23-FN1, and MFE23-Ig5-FN1 plasmid DNA transfectants, and fusion proteins were characterised with the correct information based on the MW and fusion domains. Furthermore, fusion proteins were produced in serum-free medium in order to diminish the FBS contamination in serum supplemented culture. With relatively good expression yield (~700µg/ml), the stable cell line can be used to constantly produce protein under increased production scale within 8 days (every four-day incubation interval) in serum-free medium. A 72 kDa protein band was predominantly observed for the MFE23-Ig5-FN1 fusion protein (Figure 3.5 and 3.6), and two additional smaller bands (~60 and 54 kDa) of the fusion protein were also observed (Figure 3.6), which were potentially caused by incomplete post-translational modifications or protein degradation. To overcome this problem, more rigorous cell culturing conditions may need to be applied, such as a suitable serum-free culture medium specialized for the NB2a cell line, applying sub-culturing protocols for cells cultured in serum-free conditions, elimination of detrimental cell products (e.g. lactic acid and glutamine), and many other aspects in routine and scaled-up mammalian cell-culturing conditions. Further investigations on essential enzymes and factors influencing the fusion protein glycosylation should also be considered for reducing the expression level of poorly glycosylated fusion species.

In this initial attempt at making recombinantly polysialylated antibody fragment, no PSA could be detected in all recombinant fusion proteins, neither the proteins that theoretically could be polysialylated, nor the negative control constructs. It seemed that polysialylation was not occurring, or only occurred at an undetectably low level in the fusion constructs (MFE-Ig5-FN1 and FL-NCAM) that were designated to be polysialylated, as neither fusion proteins was detected by any anti-PSA antibodies. Supportive data at the monoclonal selection stage also showed no reaction in dot-blots by anti-PSA antibody, which might already indicate negative polysialylation outcome would be seen in the protein characterization stage as some of the same samples on the dot-blot was strongly detected by anti-His6 immunoblot (Figure 3.5), primarily showing the presence of the fusion proteins. Moreover, the increase in molecular
weight of MFE23-Ig5-FN1 produced by NB2a cell system was discussed in the context, however this MW augment was not dramatic when compared to the previous NCAM polysialylation studies investigated by other groups (Georgopoulou and Breen 1999; Franceschini et al. 2001; Close et al. 2003; Angata et al. 2004), as they have suggested around 50 kDa – 100 kDa increases in MW through protein polysialylation.

By comparing the apparent MWs (Figure 3.6a) and calculated MWs (Table 3.1) of MFE-23-Ig5-FN1, MFE-23-FN1, and LS-MFE-23 the later two were found to be more or less the same as their calculated MW according to their amino-acid sequences (~45 kDa and 33 kDa). The polysialylatable fusion was found to have a higher molecular weight (~70 kDa) compared to the calculated weight (~54 kDa). The most plausible reason was due to glycosylation of the protein, and sialylation may be also involved according to the innate property of NCAM Ig5-FN1 domain. However, it was not clear whether the 16 kDa MW increment is specifically due to the multiple sialic acid repeats (not long enough for anti-PSA detection), or other more complicated glycosylated structures. Further studies on fusion protein associated glycosylation features were initiated to clarify the glycosylation status of the fusion proteins (i.e. lectin binding, glycosidase treatment, and mass spectrometry investigations).

FL-NCAM that contains the entire extracellular domains (Ig1 to 5 domains and fibronectin 1 & 2 domains) of NCAM was constructed as a positive control to show polysialylation. Unfortunately, this FL-NCAM protein could not be purified from anti-His6 and anti-Myc affinity purifications, but it could be detected by anti-NCAM antibody from concentrated cell supernatant prior to the purification (Figure 3.5c). This might imply that the C-terminal peptide tags of the FL-NCAM were cleaved during protein expression by proteases or were mis-folded leading to malfunction of protein tag specific affinity bindings. Also, as pointed out by previous studies (Constantinou 2005), the protein tag malfunction could also be a potential problem encountered in the dot-blots for selecting FL-NCAM monoclones (Figure 3.4). However, the anti-NCAM (FN1 specific) Western blot applied on concentrated cell culture supernatant from FL-NCAM revealed a positive result with a band having reasonably higher MW (~120 kDa) than predicted (~100 kDa) according to its primary sequence. The successful FN1 domain detection indicated expression of FL-NCAM from the chosen monoclonal cell line, and the increase of molecular weight was highly likely to be caused by glycosylation of FL-NCAM protein produced by NB2a cells. However, due to the problems
in purifying and identifying the mammalian expressed FL-NCAM fusion protein, it was very difficult to obtain this protein to serve as a positive control to show successful polysialylation.

The low yield and difficulties in generating purified FL-NCAM protein, and the failure of having it to show polysialylation as a positive control in this initial research, led to the key requirement for positive materials to illustrate anti-PSA immuno-detection in order to confirm our finding of having no polysialylated recombinant fusion protein from the present NB2a system. Mouse embryonic brain that contains naturally occurred polysialylated NCAM has been repeated reported previously (Eckhardt et al. 2000; Weinhold et al. 2005; Angata et al. 2007). The homogenized mouse embryonic brain (HMEB) was consequently tested as a positive control for anti-PSA (12F8) immunoblot. As seen in a short smear-like pattern in Figure 3.8(d), the HMEB successfully demonstrated the efficacy of using IgM antibody specifically targeted to NCAM associated polysialic acid. The smear-like immunoblot result is a common indication of protein polysialylated to differing degrees resulting in a dispersed molecular weight depiction. Compared with the positive result of PSA detection on HMEB, it was more certain that the NB2a cell was not producing polysialylated MFE23 fusion proteins.

The following glycosidase treatments, and lectin directed glycan differentiation experiments were applied to understand the basic sugar components involved in the glycosylated MFE23-Ig5-FN1 fusion protein produced from transfected NB2a stable cell line. Clearly shown in Figure 3.8a, both sialidase and PNGase F produced a band shift towards lower molecular weight, and the distances of band migration were proportionally corresponding to the sugar residues being removed by each glycosidase (Figure 2.2 shows the orientation of glycosidase treatment to N-glycan). This evidence indicates the existence of NeuNacα(2-3), and/or α(2-6)-Gal, and linkage between the innermost GlcNAc and asparagine residues from N-linked glycoproteins.

Plant lectins have been widely used for the detection, isolation, and characterization of glycoconjugates using their characteristic carbohydrate binding properties (Shibuya et al. 1987). The DIG glycan differentiation experiment mediated by specific lectin bindings (Figure 3.9) suggested the existence of the polysialylation epitopes, NeuNacα(2-3), and α(2-6)-Gal, alongside the potential subterminal Galβ1-4GlcNAc linkage on the N-glycan structure from the MFE23-Ig5-FN1 fusion protein. Although the precise structural composition of these linkages can not be determined by the lectin binding assays, the presence of these
disaccharides provides strong evidence for a typical N-linked glycan core with terminal polysialylation epitopes based on the current understanding of mammalian glycosylation patterns (Oltmann-Norden et al. 2008). No disaccharide linkages were revealed by any lectin binding assay with either MFE-FN1 or LS-MFE. This finding indicated the glycosylation is probably taking place entirely on the Ig5 domain, which corresponds to the concept that Ig5 is the main polysialylation site of NCAM.

From both band shift and glycan differentiation tests, Endo-N, an enzyme catalysing PSA hydrolysis, did not have any effect on fusion proteins produced in the NB2a MFE23-Ig5-FN1 stable cell line. This was probably due to insufficient α2-8 linked sialic acids being present in the glycoprotein, as Endo-N requires a minimum of 5 sialyl residues to be active (Hallenbeck et al. 1987). Anti-PSA immuno-detection on homogenate embryonic mouse brain before and after Endo-N treatment confirmed Endo-N activity on highly polysialylated proteins (Figure 3.9d). This result provided further evidence that no polysialylation occurred on the MFE23-Ig5-FN1 fusion protein.

All the data presented (anti-PSA/NCAM immuno-detection, band shift after glycosidase treatments, and mass spectrometry) strongly suggests that the fusion protein expressed in the NB2a MFE23-Ig5-FN1 stable cell line is sialylated (possibly di-sialylated), but not polysialylated. However, the determination of N-linked glycosylation with estimated polysialylation epitopes presence on the Ig5 domain of the MFE23-Ig5-FN1 fusion protein suggested promising evidence for the elongation of the terminal sialic acid into PSA.

So, why are NB2a cells unable to produce polysialylated fusion protein, even though they are fully equipped to mediate endogenous NCAM polysialylation? The problem could be the NB2a cell batch itself. The cell line is a cancer cell line, and genetically unstable. Although polysialylated NCAM was found to re-express in some metastatic cancers including neuroblastoma (Livingston et al. 1988), under laboratory conditions, after excessive passage numbers and modifications (i.e. transfection), the cells could acquire mutations, which may cause downregulation or deletion of polysialyltransferases genes. Also, the PolyST genes or the actual enzyme may become inactivated. Negative detection in anti-PSA immunoblots using lysed and homogenized NB2a cells (results not shown) may support this assumption, as no essential PolySTs were available in the transfected NB2a cells to polysialylate endogenous...
protein. As mentioned previously, in the adult and late brain development stage, PolySTs become absent within the neuronal cells (Edelman and Chuong 1982; Rothbard et al. 1982). As a key function, studies investigating the affinity binding of the fusion protein are another significant part of the project. It is clear that without maintaining the antibody-antigen binding affinity and specificity, there will be no point performing PK manipulations. In the case of this project, the scFv molecule was recombinantly fused with additional protein domains that were also glycosylated. Polymeric chain structures were also desired to be recombinantly conjugated to the fusion as well. Therefore, it is very important to determine whether the antigen binding affinity was perturbed after all these structural modifications. ELISA results of all MFE23 recombinant fusion proteins expressed by NB2a cells (Figure 3.7) illustrated unchanged in vitro antigen binding affinity as compared with the original bacterial produced MFE23 scFv, which indicated the fusion protein retains normal targeting ability. Moreover, as we have confirmed that the MFE23-Ig5-FN1 fusion protein was only sialylated, it would be interesting to know the $K_D$ of the polysialylated fusion version.

In conclusion, during this initial part of the proposed project, we confirmed that the NB2a cells utilised were not capable of producing any polysialylated protein. However, we do show recombinant MFE23 fusion proteins can be expressed in mammalian cells and high-quality purification of the fusion proteins can be obtained. FL-NCAM was not purified due to C-terminal tag problems. Homogenized embryonic mouse brain was proved to be an alternative positive control for PSA-specific immuno-detection. In vitro antigen binding experiments also validated the preservation of antigen affinity of mammalian expressed MFE23 fusion proteins. However, concerns still remain that changes in the $K_D$ value for antigen binding could be seen with polysialylated MFE23-Ig5-FN1. Lastly, results from anti-PSA immunoblot, lectin binding, glycosidase treatment, and mass spectrometry suggested that the MFE23 fusion protein carrying theoretically polysialylatable NCAM domains can only be modified with sialylated N-glycosylation by NB2a cells. However, the glycan core structure containing terminal polysialylation epitopes provided promising indications that the fusion protein is available for polysialylation. According to the PSA biosynthesis process, it was consequently deduced that polysialyltransferases are likely to be the stumbling block in accomplishing MFE23 fusion protein polysialylation in the mammalian expression system. Investigations focused on introducing PolySTs to the mammalian cell lines for recombinant fusion protein polysialylation are described in the following ch

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CHAPTER 4

Generation of Recombinant Polysialylated MFE23 scFv Fusion Protein from NB2a Expression System
4.1 Introduction

The initial attempt at making recombinantly polysialylated MFE23 scFv fusion protein from mammalian neuroblastoma cell line - NB2a alone was not successfully achieved, and the problems reported in Chapter 3 pointed to problems in the intracellular biosynthesis of polysialic acid on the specific protein substrate, such as NCAM Ig5-FN1 domain (section 1.4.2 and section 1.4.3). With the successful detection of essential polysialylation epitopes - NeuNAcα(2-3 or 6)Gal disaccharide composed in the N-glycan core structure associated with the MFE23-Ig5-FN1 fusion protein, the missing factor to accomplish the elongation of α2-8 linked sialic acid alongside the existing polysialylation epitopes during the entire mammalian PSA biosynthesis pathway was considered to be the lack of essential polysialyltransferases. As illustrated in Figure 1.17, PolySTs recognise a specific protein epitope, and specifically transfer CMP-Neu5Ac to form α2-8 linked PSA as N-terminal in the Golgi apparatus.

Natural PolySTs specifically polysialylating NCAM during brain development are known as ST8SiaII (STX) and ST8SiaIV (PST), which have been studied genetically and successfully cloned. In order to introduce these two PolySTs into mammalian cells to compensate for missing factors required for mammalian PSA biosynthesis, full length human PST and STX cDNAs (including the Golgi transmembrane domain) were kindly provided by Professor Karen Colley (University of Illinois, USA), whose group has been investigating the two enzymes catalysing NCAM polysialylation (Close and Colley 1998; Close et al. 2003; Mendiratta et al. 2005; Mendiratta et al. 2006; Colley 2008). The PolyST genes (for PST and STX DNA sequences see Appendix 9 and 10) were inserted into the mammalian expression vector pcDNA3.1 V5/His B vector using EcoRV and XbaI restriction sites (Close et al. 2003), and the two translated PolySTs have a similar MW of approximately 56 kDa (Close et al. 2000; Foley et al. 2009). V5 and His\textsubscript{6}-tags were encoded as the C-terminal peptides fused with the PolySTs. The vector also contains ampicillin and neomycin antibiotic-resistant genes for bacterial and mammalian selection respectively (Appendix 3).

Several different cell lines have been experimentally transfected with the PolyST cDNAs in previous studies of mammalian protein polysialylation research. Endogenous NCAM and PolyST negative cell lines including COS-1 (Kudo et al. 1998; Close et al. 2000), CHO (Close and Colley 1998), 3T3 (Franceschini et al. 2001) and HeLa (Mahal et al. 2001) have been co-transfected with PolyST and NCAM to produce polysialylated NCAM. In particular,
the PST and STX cDNAs provided by Professor Colley were previously co-transfected into COS-1 cells with truncated NCAM domains revealing the minimal structural domains (Ig5-FN1) for NCAM polysialylation (Close et al. 2003). In addition, tumour function involving PSA has also been studied with a number of neuroblastoma cell lines after transfection of the PolySTs (Mahal et al. 2001; Seidenfaden et al. 2003). Positive polysialylation has been shown by all of these previous investigations after introducing PolyST DNAs into the cells, which supported our theory that PolySTs are the key factors required to achieve recombinant polysialylation in mammalian expression systems. As shown in Chapter 3, the NB2a MFE23-Ig5-FN1 monoclonal cell line stably expressing sialylated fusion protein with a good yield was considered an ideal model for investigating the systematic polysialylation of the fusion protein after the introduction of the PolyST genes.

In order to accurately analyse the glyco-profile of recombinant proteins, mass spectrometry (MS) is currently the most sensitive and accepted tool for analysing and characterizing large biomolecules of varying complexity (Lennon and Glish 1997). Already utilized in Chapter 3, the matrix assisted laser desorption/ionization (MALDI) technique was chosen as the main analytical tool to measure PSA and other associated glycosylation structures on the recombinant fusion protein. With the addition of time-of-flight (TOF) MS instrument, MALDI TOF-MS operates on the principle that when a temporally and spatially well defined group of ions of differing mass/charge (m/z) ratios are subjected to the same applied electric field and allowed to drift in a region of constant electric field, they will traverse this region in a time which depends upon their m/z ratios (Lennon 1997). In order to improve the mass resolution in MALDI TOF-MS, a single-stage or a dual-stage reflectron has been utilized (illustrated in Figure 4.1).

In terms of the reflectron mode of MS, the reflectron, located at the end of the flight tube, is used to compensate for the difference in flight times of the same m/z ions of slightly different kinetic energies by means of an ion reflector (Figure 4.1), and eventually focuses the ion packets in space and time at the detector. Based on the concept of reflectron mass spectrum, better resolved isotopic multiplets can generate greatly enhanced mass resolution which also increases the mass accuracy when determining the ion's mass (Lennon 1997).
4.2 Aims and Objectives

The aim of this chapter’s work is to express Golgi targeted full length, human PST and STX cDNAs in conjunction with the MFE23-Ig5-FN1 construct (containing the polysialylatable NCAM motif) in mammalian cell hosts that have been shown to produce polysialylation outcomes in previous studies. Successful recombinant polysialylation was anticipated, which would lead to characterization and consequential optimization and establishment of a manufacturing system to produce the polysialylated fusion protein. Preliminarily, the carbohydrate composition of the fusion proteins and their antigen binding affinities will also be investigated in this part of the study.
The objectives are:

- Introduce the polysialyltransferases into mammalian cells for recombinant MFE23-Ig5-FN1 fusion protein polysialylation, and verify the catalytic capacity of the PolySTs in mammalian cells.
- Make stable transfections of a previously selected NB2a MFE23-Ig5-FN1 stable cell line with PolyST plasmid DNAs and select polyclonal cell lines for production of polysialylated MFE23-Ig5-FN1 fusion protein.
- Characterize and purify recombinantly polysialylated MFE23-Ig5-FN1 fusion protein from genetically-modified NB2a cells.
- Determine the carbohydrate composition and degree of polymerization from the expected polysialylated MFE23-Ig5-FN1 fusion protein.
4.3 Results

4.3.1 Molecular Cloning of Recombinant Human Polysialyltransferases

Both STX and PST cDNA vectors were produced in *E. Coli* XL-1 blue with ampicillin selection and purified with a DNA Midi-prep kit (QIAGEN). DNA double digestion at both *EcoRV* and *XbaI* restriction sites was carried out to verify the presence of the two polysialyltransferase genes.

![Figure 4.2. DNA double digestion of PST and STX gene containing pcDNA3.1-V5 vectors.](image)

As clearly depicted in the agarose gel presented in Figure 4.2, double digestion of propagated DNA produced bands at 1077 and 1128 bp which correspond to the expected sizes of PST and STX respectively (Close et al. 2003) and a band at 5.6 kbp corresponding to the “empty” pcDNA3.1-V5 vector. This result verified the propagated PST and STX plasmid DNAs before transfection into mammalian cells. The undigested PolyST plasmid DNAs were running slightly further than the double digestion linearized plasmid DNA, due to the supercoiled compactness of bacterial plasmid DNA.

4.3.2 Transient Transfections of Polysialyltransferases

4.3.2.1 With the Appearance of MFE23-Ig5-FN1

Transient co-transfection of the two PolySTs was carried out on NB2a cells that stably expressing MFE23-Ig5-FN1 fusion protein, which were initially selected (Chapter 3). In
addition, PST, STX and MFE23-Ig5-FN1 plasmid DNAs were transiently triple-transfected into 3T3 and HeLa cell lines, which are known as NCAM-negative cell lines, and have been previously used as host cells for truncated or mutated NCAM transfection for polysialylation research (Nakayama and Fukuda 1996; Angata et al. 2000; Franceschini et al. 2001). Using NCAM-negative cell lines avoids the unnecessary problem of polysialylation and contamination from endogenous NCAM proteins.

Sixty-mm cell-culture dishes were coated with \(10^6\) cells from each cell line and were incubated overnight with 5ml of tissue culture medium (TCM). Using the optimized transfection protocol described in section 3.3.2, both 3T3 and HeLa cells were triple-transfected with 5µg each of PST, STX and MFE23-Ig5-FN1 plasmid DNAs, while the NB2a MFE23-Ig5-FN1 stable cell line was only co-transfected with 5µg of PST or STX. After overnight incubation with the transfection complex, cells were further cultured for 3 days (5ml TCM) before the collection of cell supernatants. Both anti-NCAM (FN1-specific) and anti-PSA antibody were subsequently used for immunoblotting after separating the collected cell-culture samples on reducing SDS-PAGE. In order to avoid physiochemical breakage of the PSA chain during protein sample denaturation, reduced heating temperature and semi-reducing laemmli sample loading buffer were carefully applied (section 2.1.3 and 2.2.5.2).

Compared to non-transfected cells, results from anti-PSA immunoblotting demonstrated the existence of PSA secreted into the cell culture medium after MFE23-Ig5-FN1 co-transfection with PST and STX plasmid DNAs in NB2a cells, as well as 3T3 and HeLa cells triple-transfection with the two PolyST and polysialylatable MFE23-Ig5-FN1 plasmid DNAs (Figure 4.3).

As shown in Figure 4.3, smears revealed by anti-PSA immuno-detection ranging approximately between 100 kDa to over 250 kDa (indicated by HEMB as anti-PSA positive control) depicted a possible polydispersive degree of polymerization caused by PSA, which were only seen in samples where PolySTs were introduced. The anti-NCAM (FN1 specific) antibody only detected single protein band from both NB2a MFE23-Ig5-FN1 cell secretions with or without PolyST co-transfection, and the bands were showed to have the same molecular weight which (~70 kDa) means both bands were more likely to be the sialylated MFE23-Ig5-FN1 as a constant product secreted by the stable NB2a cell line (Chapter 3). Neither 3T3 nor HeLa cells triple-transfected samples showed any detection by anti-FN1
antibody from cell medium collections, which raises the question – whether the polysialylation signals detected with anti-PSA immunoblot were induced by fusion protein specific polysialylation?

We suspected the results of anti-FN1 detection in Figure 4.3 was caused by the PSA chains masking the FN1 domain of the fusion protein, which consequently hindered the anti-FN1 antibody binding to its target. To test this, glycosidase treatments by Endo-N (removing PSA) and PNGase F (removing N-glycan) were applied to the triple-transfected HeLa sample used in Figure 4.3.

As shown in Figure 4.4, both glycosidases removed the polysialic acid content that was seen in the non-glycosidase-treated cell medium from triple-transfected (TT) HeLa cells. Endo-N efficiently hydrolysed the α2,8-linked sialic acid residues leaving no detection at all for anti-PSA immunoblotting, while PNGase F cleaves the last GlcNAc connecting the whole N-glycan to the peptide and left a faint smeary signal by anti-PSA detection, suggesting insufficient PNGase F treatment to the polysialylated species, which was possibly hindered by the PSA masking. Samples were also processed for anti-FN1 immunoblotting, but the bands detected were weak (see Figure 4.4). However, the blurry results of anti-FN1 immunoblotting did show a trend of de-polysialylation band shift after the two glycosidase treatments.
Figure 4.4. Anti-PSA and anti-FN1 immunoblots of glycosidase treated culture medium collected from transient triple-transfected HeLa cells. Glysosidases (Endo-N and PNGase F) were used to remove PSA and N-glycan respectively. TT: triple-transfection of the two PolySTs and MFE23-Ig5-FN1 DNAs.

At the same time, two common mammalian expression cell lines, COS-1 and CHO cells were also tested by transient triple-transfection with the two PolyST and MFE23-Ig5-FN1 plasmid DNAs, following the protocols described above for 3T3 and HeLa cells, in order to confirm the reliability of the PolyST for fusion protein polysialylation. The soluble cell products from the culture medium were collected 3-days post-transfection, and detected by anti-FN1 and anti-PSA Western blot after separation via reducing SDS-PAGE (Figure 4.5).

Figure 4.5. Immunoblots of cell supernatant collected from transiently triple-transfected COS-1 and CHO cells. Both anti-FN1 and anti-PSA immunoblots were processed to COS-1 and CHO samples with (+) or without (-) transient triple-transfections with the two PolyST and MFE23-Ig5-FN1 DNAs. Samples (35μl each) were separated through 8% SDS-PAGE. Red arrows indicate two protein bands visualized after anti-NCAM (FN1 specific) immuno-detection.
Corresponding to the findings shown in Figure 4.3, positive polysialylation was also obtained from COS-1 and CHO cells after introduction of the PolySTs and MFE23-Ig5-FN1 genes into the cells (Figure 4.5). Both COS-1 and CHO smear ranges (~100 to >250 kDa) were identical to the anti-PSA findings from the previous three cell lines, and more promisingly the anti-FN1 detection revealed a major band at ~70 kDa and a weaker band signal with higher MW (~200 kDa) from the triple-transfected COS-1 cells. The 70 kDa band has a strong correlation with the sialylated MFE23-Ig5-FN1, while the ~200 kDa band could be considered to be the polysialylated MFE23-Ig5-FN1 fusion protein, due to the immuno-detection specificity of the FN1 domain. However, no bands were obtained in CHO cells upon anti-FN1 immunoblotting. No bands were seen with all untransfected cells by both immunoblots as expected.

Additional immunoblotting against protein-tags (His<sub>6</sub> or c-Myc) genetically fused with the MFE23-Ig5-FN1 protein were also processed, and either the same results as anti-FN1 immunoblotting were obtained (results not shown).

4.3.2.2 Cells without MFE23-Ig5-FN1 Fusion Protein Expression
The immunoblot results from the transient co- or triple-transfections of the two PolySTs and MFE23-Ig5-FN1 plasmid DNAs into several different cell types indicated the existence of polysialic acid in the cell culture which suggested the correct polysialylation process with the additional incorporation of PolyST genes compared to previous findings in Chapter 3. However, this does not conclusively prove that the MFE23-Ig5-FN1 fusion protein was produced and recognized as the protein substrate for polysialylation under the catalysis of the PolySTs. In the case of triple-transfection of 3T3, HeLa, and CHO cells, polysialylation was detected despite any expression of the MFE23 fusion protein. Even in the more promising cases of the NB2a MFE23-Ig5-FN1 stable cell and COS-1 cells which showed positive anti-FN1 detection from the collected cell culture, no strong immunoblotting results correlated with the smear found in the corresponding anti-PSA immuno-detections. Further proof was needed to determine that the MFE23-Ig5-FN1 fusion protein was undergoing specific polysialylation. One experiment to test the protein substrate specificity of the two PolySTs was to examine the cell secretions after PolyST transfection into cells not expressing NCAM or the Ig5-FN1 domains (responsible for polysialylation). According to the findings above, COS-1 cells, having the most potential to study the potencies of PolySTs in MFE23-Ig5-FN1 fusion protein polysialylation, were chosen for the PolyST alone transfection tests.
As shown in Figure 4.6, the result interestingly suggests that the PolyST-catalysed polysialylation was not NCAM Ig5-FN1 domain exclusive, as the culture medium from COS-1 cells doubly or individually transfected with PST and STX plasmid DNAs showed strongly positive anti-PSA immuno-detection, which also showed as a smear with almost identical MW ranging (~100 to more than 250 kDa) as the MFE23-Ig5-FN1 incorporated polysialylation immuno-detection (Figure 4.4).

One possible explanation for the smears in Figure 4.6 is that it is caused by the PolySTs themselves (by cell lysis or alternative splicing), as both PST and STX have the capacity to autopolsialylate (Close and Colley 1998). However, both anti-FN1 and anti-V5 showed no detection from the same COS-1 samples (results not shown), suggesting the polysialylation seen in Figure 4.4 was very likely to take place through neither NCAM polysialylatable domains nor autopolsialylating PolySTs. In addition, individual catalytic activity of both PST and STX was also demonstrated after individual transfections.

4.3.2.3 Immunoprecipitation (IP) of Culturing Medium from PolyST Transfectants
Immunoprecipitating the MFE23-Ig5-FN1 fusion protein through the genetically fused protein-tags was a direct and feasible way to investigate the production level of the fusion protein after transient triple-transfections of various cell lines, and to check the glycosylation pattern in the presence of the PolySTs. TALON™ anti-His₆ cobalt resin was used for precipitating MFE23-Ig5-FN1 fusion protein from the cell culture medium after mammalian
cell transient transfections, which was consequently analysed by SDS-PAGE and Western blottings (Figure 4.7).

As shown in Figure 4.3(a), immunodetections of triply transfected COS-1 cell culture mediated by three different antibodies were inconclusive. Both anti-His\textsubscript{6} and anti-Myc antibodies did not detect any bands before the His\textsubscript{6}-tag specific IP, and the IP flow-through, which also reflects similar findings by anti-FN1 antibody (see in Figure 4.4), while the \( \frac{1}{4} \) of IP concentrated His\textsubscript{6}-tag containing protein was detected by the two antibodies with major bands slightly below 70 kDa and weak signals visualized from high MW range (> 130 kDa)

(a) COS-1 cell with triple-transfections of PST, STX and MFE23-Ig5-FN1 cDNAs

(b) COS-1 cell transfected with PST and/or STX cDNAs

Figure 4.7. Immunoblots of TALONTM anti-His\textsubscript{6} cobalt resin immunoprecipitated culturing medium collected after different transient transfections. (a) The cell medium (12ml) from triple-transfected COS-1 cells was immunoprecipitated and specifically detected by anti-His\textsubscript{6}, anti-Myc and anti-PSA antibodies. (b) Anti-PSA immunoblotting for immunoprecipitated cell culture medium from COS-1 cells transfected with PST and STX jointly or individually. Wide type COS-1 cell culture medium and HEMB were used as negative and positive control respectively. For all IP samples, only one quarter of each immunoprecipitated samples were loaded onto the SDS-PAGE.
by anti-Myc detection. However, the other immunoblot using anti-PSA antibody illustrated positive polysialylation detection of all three samples with MW ranging approximately between 100 to > 250 kDa, indicating not only the protein pulled down by IP was polysialylated, but also other substances in the cell supernatant. On the other hand, immunoprecipitated samples from COS-1 cells transfected with the PolySTs (Figure 4.7b) were specifically detected by anti-PSA antibody before IP (same results as Figure 4.6), but no detection by the antibody to the His<sub>6</sub>-tag specifically immunoprecipitated culturing media, despite the joint transfection of the two PolySTs or the individual incorporations.

**Immunoprecipitation of MFE23-Ig5-FN1 from NB2a PolyST Cells**

The His<sub>6</sub>-tag specific IP was also carried out on the NB2a MFE23-Ig5-FN1 cells transiently co-transfected with the two PolySTs or transfected individually. Results illustrated in Figure 4.8 strongly support MFE23-Ig5-FN1 fusion protein polysialylation. Sample pulled down by His<sub>6</sub>-tag specific IP indicated several features that are uniquely and genetically possessed by the MFE23-Ig5-FN1 fusion protein. Such features including protein-tags (His<sub>6</sub>- and Myc-tags) and domains (FN1) which were specifically detected by the corresponding antibodies producing similar smear-like immunoblotting results ranging approximately from 70 to 250 kDa. The anti-PSA antibody detection also intensively depicted a smear but ranging from ~100 to 250 kDa. The shortened smear range of anti-PSA immuno-detection indicated that protein detected between 70 to 100 kDa by other three antibodies was the MFE23-Ig5-FN1 fusion protein, but not the polysialylated format. Potential protein degradations was also suggested by two bands with smaller MWs (~60 and 50 kDa respectively) from the

![Image of immunoblot results](image_url)

**Figure 4.8.** Immunoblot and biochemical analysis of immunoprecipitated media from NB2a MFE23-Ig5-FN1 cell line transiently co-transfected with PST and STX. Four antibodies, including anti-His, anti-Myc, anti-FN1, and anti-PSA antibodies were used to identify the corresponding properties of samples after running through 7.5% SDS-PAGE. Lane 1: PolyST co-transfected before IP (35µl loaded); Lane 2: IP flow-through (35µl loaded); Lane 3: immunoprecipitated samples (1/4 of total IP).
immunoblots, which were also found in previously purified sialylated MFE23-Ig5-FN1 fusion protein (Chapter 3). A weak single band (~ 70 kDa) was found in the pre-IP lane from both protein-tag and domain specific immunoblots, while no bands were detected by these antibodies from the IP flow-through samples. However, smear-like detection was demonstrated in both pre-IP and IP flow-through samples from the anti-PSA blot, and both smears showed a similar high MW, but less intensively visualized than the immunoprecipitated sample.

Additional IP experiments were also carried out with NB2a MFE23-Ig5-FN1 and original unmodified NB2a culture medium with or without single PolyST transfections (Figure 4.9). Further confirmation of MFE23-Ig5-FN1 fusion protein polysialylation is shown in Figure 4.9, as all PolyST (single or co-transfected) samples of NB2a MFE23-Ig5-FN1 cells confirmed similar findings to those illustrated in Figure 4.8. Other than the ~70 kDa sialylated MFE23-Ig5-FN1 protein band and its degraded smaller MW bands, no smear (100 – 250 kDa) detection was detected with either anti-Myc or anti-FN1 antibodies to the non-PolyST

![Immunoblots](image)

**Figure 4.9. Immunoblots of immunoprecipitated culturing medium from NB2a MFE23-Ig5-FN1 and original NB2a cells with/without PolyST transfections.** Anti-Myc, anti-FN1 and anti-PSA antibodies were used for specific Western blots. One quarter of the total IP samples were investigated. Samples from left to right for each cell line were non-PolyST transfected (lane 1), PST transfected (lane 2), STX transfected (lane 3) and PST + STX co-transfected (lane 4) from NB2a MFE23-Ig5-FN1 cells and original NB2a cells.
transfected MFE23-Ig5-FN1 expressing cells, which also had no anti-PSA detection. None of the immunoblots revealed any bands with the original un-modified NB2a cells, even with additional PolyST incorporations.

**Immunoprecipitation of Other MFE23 Fusion Proteins from NB2a PolyST Cells**

The capacity of PolyST to polysialylate other MFE23 fusion protein expressing NB2a cell lines was also tested by transiently transfecting PolyST DNAs into NB2a MFE23-FN1 and LS-MFE23 monoclonal cell lines selected previously (Chapter 3). In order to detect the glycosylation status of the MFE23-FN1 and MFE23 scFv proteins with the expression of PolyST intracellularly, the fusion proteins after transfections were specifically immunoprecipitated and characterized by Western blots (Figure 4.10).

Produced from MFE23-FN1 and LS-MFE23 expressing NB2a cell lines, the cell culture samples under the co-expression of PolyST were concentrated by TALON™ anti- His6 cobalt resin and demonstrated to have the same MW (~55 kDa for MFE23-FN1 and ~35 kDa for MFE23 scFv) by anti-Myc immunoblotting, as compared to the immunoprecipitated culture medium from two cell lines un-transfected with the PolyST (Figure 4.10). In addition, anti-PSA antibodies did not detect any signal in the same samples used in Figure 4.10.

![Figure 4.10. Anti-Myc immunoblot of immunoprecipitated (His6-tag specific) culturing medium collected from NB2a MFE23-FN1 and LS-MFE23 cell lines with/without transient PolyST transfections. Single and co-transfections of PST and STX DNAs were introduced to the two cell lines. “-” denotes cells without transfection with the PolyST. Previously purified MFE23-FN1 (Chapter 3) was used as positive control (+) for anti-Myc antibody detection.](image)

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4.3.3 Stable Transfection of PolyST in NB2a MFE23-Ig5-FN1 Cell Line

Following the protocols described in section 2.2.3.7, NB2a MFE23-Ig5-FN1 cells were stably co-transfected with PST and STX, and the polyclonal cell line was selected by the neomycin resistance gene co-encoded within the PolyST cDNAs. This polyclonal cell line was then used to largely express recombinantly polysialylated MFE23-Ig5-FN1 fusion protein on a large scale and the protein was subsequently purified for further functional and structural investigations.

Based on the information obtained from the immunoprecipitation experiments (Figure 4.8 and 4.9), the MFE23-Ig5-FN1 fusion protein was specifically purified by the His6-tag binding TALON™ resins. As shown in Figure 4.11, immunoblots of the IMAC eluate using anti-FN1, anti-Myc and anti-PSA antibodies confirmed the presence of the fusion protein as well as the associated polysialylation, which were in correspondence with previous results with transiently transfected cells (Figure 4.8 and 4.9). Thorough washing steps using 1xTBS were

![Coomassie staining](image)

**Figure 4.11. IMAC purification of MFE23-Ig5-FN1 fusion protein from NB2a MFE23-Ig5-FN1/PolyST polyclonal cell expression.** TALON™ (anti-His6) cobalt metal affinity resin was used in the purification. Coomassie protein staining, and three different immunoblottings were applied to identify the IMAC purified protein. Number denotations: 1. column flow-through; 2. first 1xTBS wash of the resin in purification; 3. second wash of the resin; 4. purification eluate.
applied in the IMAC until no protein remained in the eluate as measured by Coomassie blue staining. Fusion protein specific characteristics were weakly shown in anti-FN1 and anti-Myc immunoblotting (bands ~70 kDa) from the sample flow-through, which were used to repeat the IMAC purifications for more efficient fusion protein collection. These results demonstrate that the selected polyclonal cell line is capable of producing polysialylated MFE23-Ig5-FN1 stably.

4.3.4 Ion Exchange Purification of Recombinantly Polysialylated Proteins

4.3.4.1 Manual Optimization Tests

As already suggested from the IP results (Figure 4.8 and 4.9) and the IMAC purification (Figure 4.11), proteins eluted from the TALON™ cobalt resin were a mixture of differentially glycosylated MFE23-Ig5-FN1 fusion proteins, and even the polysialylated forms showed a range of DP. In order to further separate the polysialylated fusion proteins from the non-polysialylated isoforms, and to refine the desorption of the polysialylated protein, anion exchange chromatography was used for further purification based on the negative charge of the sialic acid residues within the fusion protein’s carbohydrate composition. AcroSep™ Chromatography Column (1ml bed volume) with the strong anion exchanger Q Ceramic HyperD® F was selected for this purification purpose. As described in section 2.2.4.4, the anion exchanger resin was firstly activated and equilibrated with the anion exchange running buffer, and manually tested prior to utilisation with automatic chromatography systems. Protein collected from the IMAC purification was dialysed to the anion exchange running buffer before being manually injected into the column at a flow rate of 1ml/min. After washing the column with 10ml running buffer, protein elution was optimised using 5 different NaCl concentrations (50mM, 100mM, 200mM, 400mM, and 1M), which were injected (10ml each) from low to high salt concentration. Eluates were collected every 1ml.

In order to determine the elution conditions required to separate fusion protein with different polysialylation levels, five NaCl concentrations were investigated for anion exchange protein elution. As shown in Figure 4.12(a), the anti-Myc antibody was used to identify all glycosylation types of MFE23-Ig5-FN1 fusion protein produced by PolyST stably transfected fusion protein expressing NB2a polyclonal cells. Initially, 50mM NaCl buffer was unable to elute any negatively charged proteins from the anion exchange column. The fusion protein was eluted with the second NaCl concentration, as all 10 fractions eluted by 100mM NaCl buffer were levelled up and strongly revealed a band at approximately 70 kDa in anti-Myc
immunoblots. However, the anti-PSA antibody did not detect a band in the 100mM NaCl elutes (results not shown). The polysialylated fusion proteins began to be eluted with the 200mM NaCl buffer, as short smears (around 70 to 100 kDa) were predominately detected by anti-Myc antibody from the first five 200mM NaCl elution fractions, and corresponding anti-PSA detection of these fractions also showed positive signals, which were weakly indicated and restricted to bands with MW around 90 or 100 kDa, rather than smears (Figure 4.12b). Fusion proteins with higher polysialylation levels were found to be eluted at 400mM NaCl.

Figure 4.12. Immunoblots of manually eluted MFE23-Ig5-FN1 fusion protein from anion exchange column. Both anti-Myc and anti-PSA antibodies were utilized for specific immunoblots. All five different concentrations of NaCl buffers were tested for eluted fusion proteins, and every 1ml fraction of each elution buffer was collected and applied to the Western blots. From left to right, the sample sequence of each set of NaCl concentration followed the elution order, from fraction 1 to 10. Samples (40μl each) were loaded into SDS-PAGE (10% acrylamide) initially.
concentrations, as both anti-Myc and anti-PSA immuno-detected smears were revealed up to 250 kDa. The final 1M NaCl elution did not have any fusion protein immuno-detection at all (results not shown). Immunoblots also revealed that smears detected by the anti-Myc antibody in 200mM and 400mM elution fractions began from ~70 kDa which did not match the MW ranges of the forms detected with anti-PSA antibodies (starting from ~ 90 kDa or higher MW).

4.3.4.2 Programmed Anion Exchange

The anion exchange column was connected to a BIO-RAD BioLogic DuoFlow system which was subsequently used to achieve an automated ion exchange purification protocol for polysialylated fusion protein. With the understanding gained from the manual optimisation tests of the anion exchange column, different purification programmes were designed, and analysed to establish an ideal automatic purification protocol for separating proteins with different polysialylation level.

Elution under Increasing NaCl Concentration Gradient

Fusion protein elution from the anion exchange column was closely correlated to the NaCl concentration. In order to further investigate this correlation, a steadily increasing NaCl concentration gradient was programmed with 10mM increase per 1ml elution buffer with the concentration ranging from 0 to 1M.

The chromatography profile is showed in Figure 4.13(a) with the elution gradient initiated at fraction number 50. An elution peak was clearly seen in the chromatogram encompassing fractions 61 to 80, which corresponded to NaCl concentrations of 100 - 300mM. The peak of the elution was found at fraction 63 or 64 eluted by 130mM NaCl buffer. Immunoblotting analysis of these fractions was performed with anti-Myc antibody (shown in Figure 4.13b) to detect, and anti-PSA was utilised to identify the polysialylated fusion proteins. The anti-Myc immunoblotting suggested that all investigated fractions shared very similar detection as a protein smear with MW between approximately 70 to 250 kDa was detected in all fraction samples. This implied that different fusion protein glyco-forms were not distinctly separated by the gradually-increasing NaCl concentration. Degraded fusion protein fragments with Myc-tag were also found at lower MWs. Anti-PSA immunoo-detection showed a weakly polysialylated protein smear from fraction 61 to 70, and much stronger smear signals from fraction 71 to 80. Despite the intensity of the anti-PSA immunoblotting, polysialylated protein smears implied a trend that started with a wider MW range (~ 90 to 250 kDa) from fraction 62,
leading to nearly no detection (fraction 69), which then gradually widened out again and finally returned to the MW range covering between 90 to 250 kDa at fraction 79 and 80 (with probably more eluates up to fraction 90).

Figure 4.13. Automatic anion exchange purification with a steadily increasing NaCl concentration gradient. (a) Chromatography profile of protein purification process. UV absorbance at 280nm was used for monitoring the protein elution, alongside NaCl concentration gradient curve; (b) Western blotting analysis of peak fractions. Both anti-Myc and anti-PSA antibodies were used for specific protein and carbohydrate characteristic detections. Peak fractions from eluate 61 to 80 were analysed, and 40μl of each sample was applied on SDS-PAGE (8% acrylamide) directly after the ion exchange purification.
Step-wise NaCl Elution

Based on the unsuccessful separation scheme under the gradually increasing NaCl concentration, step-wise NaCl concentrations were then applied automatically according to the same protocol used in the manual anion exchange purification with approximately four times more materials injected through the column. Five stepped phases (10ml/phase) were tested, including elution buffers with NaCl concentrations of 50mM, 100mM, 200mM, 400mM, and 1M. Peaks of protein eluates under each NaCl concentrations were clearly demonstrated by the chromatogram shown in Figure 4.14.

![Chromatography of automatic anion exchange purification with different five phases of NaCl concentration for protein elution](image)

Figure 4.14. Chromatography of automatic anion exchange purification with different five phases of NaCl concentration for protein elution. Plots of protein absorbance at UV wavelength 280nm and 214nm Quad-Tec laser, alongside NaCl concentration changes respectively against eluate fraction number were depicted with different colours.

After sample injections into the purification system, the elution procedure commenced from fraction 60 with the 50mM NaCl phase, although no absorbance peak was detected with this concentration. Subsequently, the remaining four NaCl concentrations were applied, and absorbance peaks were detected with each concentration. Generally, the higher the eluting salt concentration, the higher peak absorbance values were obtained. Although the 214nm wavelength Quad-tec detector depicted more sensitive signal than the UV for absorbance detections, the same chromatographic trend was shown. Moreover, the 400mM NaCl elution had a sharper peak with its maximum at fraction 92, while the other two peaks seen in 100mM and 200mM elutions were relatively broader. In order to characterize the purification
product, eluates from each NaCl concentration were separated by SDS-PAGE and analysed by anti-Myc and anti-PSA immunoblots (Figure 4.15).

**Figure 4.15. Immunoblotting of peak fractions eluted from each NaCl concentration phase.** Both anti-His$_6$ and anti-PSA antibodies were used and 40μl of each sample was applied on SDS-PAGE (8% acrylamide) directly after the ion exchange purification.

The two Western blots shown in Figure 4.15 indicate some similarities to the manual anion exchange purification also using the same five NaCl concentrations for elution purpose (Figure 4.12). For fusion protein detection, anti-Myc antibody outlined three different smear ranges of protein eluted by 100mM, 200mM and 400mM salt concentrations, which all began at a MW of ~70 kDa. The peak fractions of 1M NaCl elutions were also detected by anti-Myc
antibodies but only found at high MW ranges. Anti-PSA immunoblotting showed no
detection of all fractions collected by 100mM salt elution, and limited smear range (~90 to
110 kDa) for 200mM NaCl eluates. Most of the fractions from 400mM and 1M NaCl elutions
were intensively detected by anti-PSA antibody, which predominantly covered a MW range
between 90 to 250 kDa, although the very last two fractions of the 400mM NaCl elution and
very beginning two 1M NaCl eluates did not contain a robust anti-PSA signal. More precisely,
the 1M NaCl fractions seemed to have anti-PSA detection with a larger MW range, however
most of these fractions were not correspondingly detected by the anti-Myc antibodies. No
bands were revealed with the 50mM NaCl elutions by either immunoblots.

**Optimized Automatic Anion Exchange Purification Programme**

With all the findings from the data above, an optimized anion exchange purification of the
polysialylated fusion protein was programmed for the automatic system. In general, the
phase-wise elution scheme with step-wise increase in NaCl concentrations was modified with
prolonged additional inter-phase washing steps.

Compared to the chromatogram shown in Figure 4.14, the final optimized protocol for the
anion exchange purification employed two additional washing steps with 100mM and 200mM
NaCl elution buffers after the adjacent sample elution phases, which were indicated in the
absorbance chromatography in Figure 4.16(a). Moreover, the 400mM NaCl elution phase was
substituted by the highest salt molarity (1M) to directly remove all highly negatively charged
molecules. For each 100mM, 200mM and 1M NaCl elution phase, extra 10ml collections
were made, and the washing steps in between were added with the intention of removing
weakly negatively charged molecules from the highly polysialylated fusion protein.

Maximal fractions of each chromatographic peak under 100mM, 200mM and 1M NaCl
eluting concentrations were taken for anti-Myc and anti-PSA immunoblotting analysis.
Results are shown in Figure 4.16(b), which particularly shows the smears detected by anti-
Myc antibody were not initiated from ~70 kDa for all eluates anymore (outlined by a red
dotted line), leaving only the 100mM NaCl eluates (fraction 63 & 64) with a strong anti-Myc
detection around 70 kDa, whereas the 200mM NaCl peak eluates revealed bands between a
MW of ~80 to 110 kDa, and the 1M NaCl peak fractions showed smears started around 100
kDa and migrated even further towards the higher MW. The anti-PSA blot showed roughly
the same results as the anti-Myc detection with much stronger sensitivities, while no bands
were detected with the 100mM NaCl eluates. Overall, the optimized anion exchange purification method successfully separated the polydispersively polysialylated MFE23-Ig5-FN1 fusion proteins into three major isoforms which were eluted under 100mM, 200mM and 1M NaCl buffers (Figure 4.16a), and based on the indications from Figure 4.16(b), they were identified as sialylated, weakly polysialylated and highly polysialylated glycol-forms of the fusion protein.

Figure 4.16. Optimized automatic anion exchange purification process for separating different glycoforms of polysialylated MFE23-Ig5-FN1 fusion protein. (a) Chromatography. Two additional washing steps were applied by using 100mM and 200mM NaCl buffers (200ml and 300ml respectively) after the corresponding elution phases. (b) Immunoblotting of peak fractions from 100mM, 200mM and 1M NaCl buffer elutions. Both anti-Myc and anti-PSA antibodies were used for immuno-detections. The red dotted line outlines the MW at 70 kDa cross both blots.
### 4.3.5 Glycosidase Treatment of Anion Exchange Purified Fusion Protein

Further confirmation of carbohydrate compositions from each MFE23-Ig5-FN1 fusion isoform were carried out by specific glycosidase treatments and protein band-shift observation. As mentioned in Chapter 3, treatments by glycosidases including Endo- and Exo-Neuraminidases and PNGase F were investigated followed by other specific analysis.

As shown in Figure 4.17, three glyco-forms of MFE23-Ig5-FN1 were clearly displayed by

![Coomassie Stain](image)

![Anti-FN1](image)

![Anti-PSA](image)

**Figure 4.17. Glycosidase treatment of MFE23-Ig5-FN1 fusion protein after anion exchange purification.**

Three glyco-forms (sialylated, weakly, and highly polysialylated) of the fusion protein were treated by specific glycosidases including Endo-N, Exo-N, and PNGase F. The results were revealed by Coomassie staining, and immunoblots mediated by anti-FN1 and anti-PSA antibodies. S, W and H are abbreviations of sialylated, weakly and highly polysialylated MFE23-Ig5-FN1 fusion proteins. Samples were separated by SDS-PAGE (7% acrylamide)
Coomassie staining with the majority of sialylated isoform at a MW of 70 kDa, weakly polysialylated isoform at ~80 to 110 kDa, and highly polysialylated isoform at approximately 110 to 200 kDa. Similar results were also obtained with the anti-FN1 immunoblot. As indicated by previous glycosidase treatment (section 3.3.6), no significant protein band-shift was observed with the sialylated fusion protein after the glycosidase treatments. For the two polysialylated fusions some degree of band-shifts towards the lower MW was detected after Endo-N, and combinational Endo-N plus Exo-N treatment in Coomassie stained gels, which were more clearly revealed by the anti-FN1 antibody detection. The most detectable band-shift of the two polysialylated isoforms was the Endo-N plus PNGase F treatment that sequentially removes PSA and N-glycans from the fusion protein and approximate 40 and 160 kDa band-shifts respectively could be estimated for the weakly and highly polysialylated fusion isoforms.

More importantly, the total N-glycan (PSA inclusive) removed fusion isoforms aligned at the same MW of ~65 kDa. The anti-PSA immunoblot depicted the expected results with no PSA detection from sialylated and Endo-N treated polysialylated isoforms. The weakly polysialylated MFE23-Ig5-FN1 was detected with confined MW, while the highly polysialylated form showed a wider MW range covering between 100 to 250 kDa.

4.3.6 Initial PSA Speculation by Mass Spectrometry

With the confirmation of polysialylation and successful separation of MFE23-Ig5-FN1 from the non-polysialylated glyco-forms, we were now in a position to carry out mass spectrometry analysis (Biopolymer Mass Spectrometry Group, Imperial College London) to understand the fusion protein carbohydrate composition and polysialylation profile. The highly polysialylated MFE23-Ig5-FN1 fusion protein samples were used for the MALDI-MS analysis according to the procedure outlined in Scheme I below, and the corresponding methodological preparations are described in section 2.2.6.

Based on the understanding of NCAM polysialylation (section 1.4.2), and the previous MS investigation of the sialylated fusion protein, the initial attempt to analyse the polysialylation profile from the MFE23-Ig5-FN1 fusion protein was processed by PNGase F removal of the entire N-glycans from the trypsin digested fusion protein glycopeptides (Scheme I-a).
Indicated by the previous experiments (Powell and Harvey 1996), standard permethylation using ICH₃ in DMSO (section 2.2.6.7) was carried out to block the carboxyl groups in order to get stabilized volatile sialic acid derivatives from the N-linked polysaccharides. However, this method failed to initiate either positive or negative ion MALDI-MS, suggesting the traditional permethylation method was not able to stabilize the highly negatively charged PSA, which led to the potential spectral signal split due to the formation of both positive and, to a greater extent, negative ions (Powell and Harvey 1996). Subsequently, the polysialylated N-glycan was hydrolysed with Endo-N, and the mixture of oligosialic acid fragments and N-glycan core compositions were permethylated for MALDI-MS analysis (Figure 4.18).

Analysis by MALDI TOF-MS revealed the general carbohydrate composition of the polysialylated MFE23-Ig5-FN1 fusion protein which is illustrated in Figure 4.18. As indicated in Scheme I-a, the Endo-N hydrolysis of the N-terminal PSA from the PNGase F cleaved N-glycans enabled the permethylation process to create volatile derivatives in both oligosialic acid and N-glycan cores. Signals of short oligosialic acid fragments were more sensitively demonstrated than the relatively longer oligosialic acid fragment, and signals for
fragments containing more than 12 NANA residues were considered as insignificant. Both complex and oligomannosidic N-glycan core structures were deduced from the MALDI-MS analysis with potential polysialylation along one antenna on their N-termini. Di-, tri- and tetra-sialylated glyco-structures also possibly occurred based on the peaks from this MALDI-MS spectrum with potentially multiple sialic acid elongations of each antenna.

Figure 4.18. MALDI (reflectron positive) MS analysis of Endo-N hydrolysed N-glycan from highly polysialylated MFE23-Ig5-FN1 fusion protein. Peaks on the spectrum were predominately produced by [M+Na]⁺ species. Based on the biosynthesis of mammalian glycosylation, possible oligosaccharide structures were indicated according to the mass to charge ratio (m/z). Information of each monosaccharide (symbol denotation, structure, permethylated monoisotopic mass) was provided in Appendix 11.

In an alternative process, the polysialylated fusion protein glycopeptide was repeatedly treated with Endo-N initially for complete PSA hydrolysis and removal, and subsequently samples of both the PSA portion (Scheme I-b) and the de-polysialylated N-glycan portion (Scheme I-c) were prepared for MALDI-MS analysis under the standard permethylation process.

As shown in Figure 4.19, several fragmented sialic acid oligosaccharides containing different NANA residues aligned on the MALDI-MS spectra resulted from the PSA portion after Endo-N hydrolysis of the polysialylated glycopeptide from MFE23-Ig5-FN1 fusion protein. Shorter sialic acid fragments (≤7 NANA residues) possibly existed compared to the sialic acid oligosaccharides produced by the regular Endo-N treatment found in Figure 4.18. In Figure 4.19(b), further MALDI-MS/MS analysis by re-ionization of the longest fragment (2597.2 Da) causing cleavages of glycosidic linkages confirmed that Neu5Ac was the only sugar component within this oligosaccharide fragment with unique α2-8 ketosidic linkage to each
other. This was based on the average mass of permethylated Neu5Ac molecule, which has five –OH groups methylated when the monosaccharide is involved in the chain structure, while the monosaccharide at one end of the oligosaccharide fragment can be additionally methylated with one extra –OH group and one oxygen molecule can be contained or not depending on the breakage of the α2-8 ketosidic linkage (showed in Figure 4.19b). As shown in Appendix 11, the MW of permethylated Neu5Ac is 361 Da. For one single ionized Neu5Ac molecule in the MALDI TOF-MS analyses, one dominant isoform contains an extra methylated –OH group, one oxygen element and was produced as [M+Na]+ species, which gives an overall MW of 415 Da (detected as 416.3 Da in Figure 4.19b). The other single Neu5Ac isoform released by MALDI-MS/MS without the oxygen molecule from the α2-8

![Figure 4.19. MALDI (reflectron positive) MS analysis of Endo-N digested PSA removed from highly polysialylated MFE23-Ig5-FN1 fusion protein. (a) MS spectrum of the Endo-N hydrolysed oligosialic acid fragments. (b) Oligosaccharide with MW of 2597.2 Da was further ionized and analysed by MALDI- MS/MS. Oligosialic acid fragment structures were speculated based on the peaks produced by [M+ Na]+ species. Two isotypes of each fragments were detected, which were differentiated by one oxygen element content due to the breakage of α2-8 ketosidic linkage of Neu5Ac schematically illustrated in the inset diagram.](image)
ketosidic link breakage did not show a significant signal, however other fragmented sialic acid oligosaccharides appeared as two isoforms with the difference of one oxygen element content.

The de-polysialylated N-glycan properties of the fusion protein was analysed simultaneously by MALDI-MS, and all possible N-glycan core structures are presented based on the m/z signals (Figure 4.20 and 4.21). Further analysis of the N-glycan structures of the polysialylated MFE23-Ig5-FN1 fusion protein after complete removal of the PSA content (Figure 4.20 and 4.21) also revealed the same core structure compositions that was found in Figure 4.18. Potential cleavages were observed to occur at each glycosidic linkage of the N-glycan alongside its MALDI-MS spectrum, and additional non-primate mammalian glycosylation featuring the glycosidic linkage, Gal(α1-3)Gal was also determined according to the spectral signals.

![Figure 4.20. MALDI (reflectron positive) MS analysis of de-polysialylated N-glycan cores of the highly polysialylated MFE23-Ig5-FN1 fusion protein. Peaks on the spectrum were predominately produced by [M+Na]+ species. “C” indicates contaminating polymer signals and the red arrow points out the xenogenic galactose(α1,3)galactose or Gal(α1-3)Gal disaccharide expressed in one of the fusion protein N-glycan variations. Information of each monosaccharide (symbol denotation, structure, permethylated monoisotopic mass) was provided in Appendix 11.](image-url)
In order to determine whether polysialic acid was elongated as a mono-antennary structure of the N-glycan from the fusion protein, one oligosialylated N-glycan indicated in Figure 4.21(c), containing three NANA residues, was re-ionized and analysed by MALDI-MS/MS for further dissection of this molecule (Figure 4.22).

Figure 4.21. Enlarged MALDI-MS spectra analysis of de-polysialylated N-glycan cores of the highly polysialylated MFE23-Ig5-FN1 fusion protein. Spectra mass ranges from (a) 1499 to 2500 m/z, (b) 2500 to 3500 m/z, and (c) 3500-5000 m/z were interpreted respectively. Oligoman standards for oligomannose structure with two GlcNAc residues and up to 6 Mannose residues. Red arrows point out the xenogenic Gal(α1-3)Gal disaccharide expressing in the fusion protein N-glycan structures. “MS-MS” highlighted N-glycan structure was re-ionized for MALDI-MS/MS analysis consequently. Information of each monosaccharide (symbol denotation, structure, permethylated monoisotopic mass) was provided in Appendix 11.
Figure 4.22. MALDI-MS spectrum of an oligosialylated N-glycan from polysialylated MFE23-Ig5-FN1 fusion protein. Fragment ions are indicated by the arrows. Information of each monosaccharide (symbol denotation, structure, permethylated monoisotopic mass) was provided in Appendix 11.

As indicated in Figure 4.22, the fragmentation of the selected oligosialylated N-glycan demonstrated that the three Neu5Ac monosaccharide were continuously linked to each other on the N-terminal of the N-glycan core, which suggested polysialylation of the fusion protein was highly likely to occur as a single antennary structure.

4.3.7 Antigen Binding Affinity Determination of the Fusion Proteins
Recombinant polysialylated of MFE23 scFv fusion protein was successfully achieved after introducing PolySTs to the NB2a MFE23-Ig5-FN1 stable cell line, but the retention of antigen binding affinity from the glycosylated isoforms of the scFv fusion protein required study. This information is very important before investigating whether there is any pharmacokinetic improvement, as reduction of target binding affinity is considered to be the most common drawback of polymer or any conjugation to proteins. ELISA was initially carried out to identify the $K_D$ values of the fusion proteins comparing to the original MFE23 scFv. As described in previous chapters, the recombinant protein N-A1 containing the binding sequences of MFE23 scFv, was used to serve as the antigen. Fusion proteins including LS-
MFE23, MFE23-FN1, and the three glyco-forms of MFE23-Ig5-FN1 were prepared for the ELISA experiment (Figure 4.23).

**Figure 4.23. ELISA analysis of antigen binding affinity of all MFE23 fusion proteins produced by NB2a PST cells.** In triplicate, a serially diluted protein concentrations (from 0.5nM to 1μM) was prepared for each fusion protein including bacteria made MFE23 scFv, and NB2a cell expressed MFE23 scFv (LS-MFE23), MFE23-FN1, and three types of glycosylated MFE23-Ig5-FN1 isoforms (depicted in different colours). The ELISA was mediated by Myc-tag specific antibodies. N-A1 antigen-binding was confirmed for the polysialylated MFE23-Ig5-FN1, and the $K_D$ values of each type of fusion protein were also indicated ± standard errors.

Determined by the ELISA analysis mediated by anti-Myc antibody (Figure 4.23), all transfected NB2a cell expressed fusion proteins and MFE23-Ig5-FN1 glyco-forms shared very similar $K_D$ values of approximately 7nM, similar to that obtained with bacterially expressed MFE23 scFv. The MFE23-FN1 fusion protein showed a slight decrease in N-A1 binding affinity with the $K_D$ increased to 10.32nM. However, the polysialylated MFE23-Ig5-FN1 isoforms showed excellent antigen binding affinities which were similar to the $K_D$ of the scFv alone.
4.4 Discussion

As clearly shown by the experimental results described in Chapter 3, the NB2a neuroblastoma cells used in this investigation were not able to polysialylate the MFE23-Ig5-FN1 fusion protein. Additionally negative results were obtained from NB2a endogenous PSA and NCAM immuno-detections further confirming the existing NB2a cells used for fusion protein transfection were different to NB2a cells previously reported where the expression of PSA-NCAM was obtained (Gallagher et al. 2000; Poongodi et al. 2002; Beecken et al. 2005), and they no longer contained the machinery to achieve NCAM polysialylation.

From the studies described in Chapter 3, the MFE23-Ig5-FN1 fusion protein expressed by NB2a cells was found to be N-glycosylated with terminal sialic acid residues by mass spectrometry analysis, and the essential polysialylation disaccharide epitope - Neu5Aca(2-6)Gal and Neu5Aca(2-3)Gal were also indicated by the corresponding lectin-detections suggesting sialic acid has been successfully biosynthesized and sialyltransferases catalysing Neu5Aca(2-6)Gal and Neu5Aca(2-3)Gal disaccharides existed within the cell. Therefore, according to the polysialic acid biosynthesis pathway (Figure 1.17), the only missing factors were postulated to be the PST and STX polysialyltransferases. Kindly provided by Prof. Karen Colley, both Golgi resident PST and STX cDNAs were subsequently introduced into the NB2a MFE23-Ig5-FN1 stably transfected cell. In order to monitor the performance of transfected PolySTs, transient co-transfections of the fusion protein and PolyST cDNAs were also carried out in a few endogenous NCAM- and PolyST-free cell lines including 3T3, HeLa, COS-1 and CHO cells, which successfully produced PSA confirming previous studies (Close and Colley 1998; Kudo et al. 1998; Close et al. 2000; Franceschini et al. 2001; Mahal et al. 2001; Close et al. 2003). As illustrated by Figure 4.3 and 4.5, culture media from all transfected cells showed strong positive immuno-detection by anti-PSA antibodies. This result encouragingly suggested successful polysialylation after the cells incorporated the PolyST cDNAs. However, apart from the NB2a MFE23-Ig5-FN1 transfectants that stably expressing the fusion protein, other cell lines co-transfected with the fusion cDNA did not show noticeable MFE23-Ig5-FN1 fusion expression according to the FN1 domain specific immunoblotting (Figure 4.3, 4.4 and 4.5). More interestingly, culture medium from COS-1 cell transfected with the PolyST cDNA alone also demonstrated polysialylation as evidenced by anti-PSA immunoblotting (Figure 4.5). Further negative results from anti-FN1 and anti-V5 immunoblots on the same sample failed to reveal endogenous NCAM or the soluble form of
autopolysialylated PolySTs. According to this experimental outcome, it was speculated that unexpected polysialylation was likely to have occurred on unidentified cellular secretions, whilst the soluble format of autopolysialylated PolySTs missing the V5-tags could be another possible explanation.

With the contamination of the unidentified polysialylated protein from the culture media of PolyST transfectants, the specific MFE23-Ig5-FN1 fusion protein polysialylation induced by PolySTs was consequently determined by His$_6$-tag specific immunoprecipitation. As recombinantly engineered to contain a His$_6$-tag, the MFE23-Ig5-FN1 fusion protein was pooled and isolated from the rest of the culture medium by this method. The following immunoblot detection for the immunprecipitated samples (Figure 4.8 and 4.9) specifically determined that MFE23-Ig5-FN1 fusion protein was polysialylated and was distinguished from the unexpected polysialylation contaminants. The credibility of this conclusion was proved by COS-1 and the original NB2a cells transfected by PolyST alone after their culture media was processed through the same His$_6$-tag specific IP and immunoblotting. As expected, none of these IP samples showed any positive results (Figure 4.7b and 4.9). Further more, immunoprecipitation from PolyST and MFE23-Ig5-FN1 co-transfected COS-1 cells also indicated fusion protein specific polysialylation (Figure 4.7a).

Further data on the PolyST transient transfections of different cell lines (under the same cell culturing and transfection protocol and conditions) implied different polysialylation capabilities based on the variations of the PSA specific immuno-detections from 5 different types of cells investigated in Figure 4.3 and 4.5. It is already known that the two polysialyltransferases displaying different catalytic efficiencies under different circumstances (section 1.4.4), but this study also suggested that polysialylation was a cell type specific glycosylation feature too.

Both polysialylation negative fusion proteins, MFE23-FN1 and LS-MFE23 were also co-expressed with PolySTs, and as expected, neither polysialylation nor MW increase were seen with direct Western blot analysis (Figure 4.10). All the demonstrable results confirmed the successful polysialylation of MFE23-Ig5-FN1 fusion protein achieved by the PolyST catalysis, and consequently both PST and STX cDNAs were stably co-transfected into the previously selected NB2a MFE23-Ig5-FN1 expressing monoclonal cell line, followed by polyclonal neomycin selection. This cell line was then considered to be the first mammalian host capable
of systematic polysialylation of MFE23-Ig5-FN1 fusion protein, and the system stability, fusion protein production, purification and characterizations were subsequently analyzed.

His6-tag specific IMAC purification shares the same principle as the IP, and results presented in Figure 4.11 suggested the polysialylated fusion protein was isolated from the culture medium and concentrated. However, a mixture of polysialylated and non-polysialylated (mostly sialylated with MW ~70 kDa) was also indicated by Figure 4.11. The polysialylated material illustrated by anti-PSA immunoblotting only covered the MW range ~100 kDa to 250 kDa, while the protein domain specific immunoblots (anti-FN1 and anti-Myc) suggested a wider MW range (~65 kDa to 250 kDa) which contained the non-polysialylated fusion protein isoforms as well. Furthermore, the MW range demonstrated by the anti-PSA immunoblotting implied a polydisperse degree of polysialylation of the fusion protein, which was similarly suggested by previously reported NCAM polysialylation studies (Troy 1995; Rutishauser 1998; Georgopoulou and Breen 1999). In order to separate the polysialylated fusion protein from its non-polysialylated isoforms, anion exchange purification was utilized as described in previous studies (Angata et al. 1998; Jain et al. 2003). This system is based on sialic acid being a negatively charged molecule (Figure 1.16). However, it is very important to purify the fusion protein by IMAC first, as contaminating polysialylated substances were also detected in the cell culture (discussed above). Several optimisations based on the ion exchange elution profile were examined through chromatography and Western blot analysis (Figure 4.12 to 4.16). Initial manual ion exchange using five NaCl concentration (50, 100, 200, 400, and 1000mM) increments illustrated a rudimentary profile capable of separating different glyco-forms of the MFE23-Ig5-FN1 fusion protein. As shown in Figure 4.12, eluates with the 100mM NaCl buffer only demonstrated a single band around 70 kDa by anti-Myc Western blot, but no bands when subjected to anti-PSA immunoblotting. However protein eluted by 400mM NaCl buffer showed a smear signal covering the correct MW ranges in response to both anti-Myc and anti-PSA immunoblots. Western blot analysis of the eluates collected according to the automatic anion exchange chromatogram (Figure 4.14 and 4.15) showed similar results to that observed from the manual purification. In addition, polysialylated fusion proteins eluted by 1M NaCl buffer after 10ml 400mM NaCl elution were showed by the automatic anion exchange chromatography as three-times more purification material was loaded than the manual purification, of which revealed that all fusion proteins were eluted by 400mM NaCl buffer. Experimental attempts to elute the anion exchanged MFE23-Ig5-FN1 fusion proteins under a gradually increased NaCl
concentration gradient failed to separate the polysialylated and non-polysialylated fusion proteins (Figure 4.13), which was predominately contaminated by the sialylated isoform and degraded fragments (≤70 kDa) indicated by anti-Myc Western blot. However, as also seen in Figure 4.13, fractions from 70 (correlates to 200mM) onwards showed an expected elution profile according to the anti-PSA immuno-detections, indicating a gradual polysialylation degree increase alongside the NaCl concentration gradient.

Taking these findings together, an optimized anion exchange purification programme was obtained (Table 2.9) and its elution profile is reported in Figure 4.16. Three NaCl concentrations (100mM, 200mM and 1M) was used for eluting the MFE23-Ig5-FN1 fusion proteins within three MW categories (~70 kDa, 70-110 kDa, and 100-250 kDa) based on the anti-Myc Western blot. Two separate and prolonged washing steps with 100mM and 200mM NaCl buffer were added in order to remove the corresponding fusion protein glyco-forms which contaminated the following purification processes (Figure 4.16). By doing this, the non-polysialylated fusion protein was efficiently eliminated from the polysialylated population. Eluates were subjected to two UV detections, 214nm and 280nm wavelengths. As reported previously, sialic acid had a strong absorption at UV-215nm (Spyridaki and Siskos 1999). Due to different levels of sialic acid present on the sialylated or polysialylated fusion proteins, more sensitive detections were made by UV-214nm than UV-280nm, which detects the aromatic amino acids and protein absorptions.

Specific glycosidase treatment and band shift experiments (Figure 4.17) indirectly illustrated α2-8 linked PSA and other glycosidic linkages within MFE23-Ig5-FN1 fusion protein carbohydrate compositions. Efficient oligosaccharide/glycan release by the glycosidases was ascertained before, mass spectrometry as one of the most accurate and sensitive methods to analyse the glyco-composition of glycoprotein/peptide was consequently utilized for further investigation of the polysialylated fusion protein. Following the three investigatory routes shown in Scheme I, the released sugar samples were methylated to volatile derivatives prior to application on the MALDI TOF-MS instrument. Based on the glyco-biosynthesis of mammalian glycoprotein, glycan and oligosaccharide structures were analysed according to the m/z peaks from MS or MS/MS spectra (Figure 4.18 to 4.22). The overall MS information suggested the polysialylated fusion protein was a complex and/or oligomannosidic N-glycan core structure, and the α2-8 linked Neu5Ac existed in an oligo-chain structure (limited by the
detection power), which could possibly be elongated as a mono-antenna on the \(N\)-terminal of the \(N\)-glycan.

On the other hand, due to the highly negative charge of PSA, the polymer was very labile and resistant to becoming volatile after the standard permethylation process. Endo-\(N\) hydrolysis fragmented the polymer into oligomers with different NANA residues, which were successfully methylated and ionized for MS investigation. Unfortunately, these hydrolysed PSA polymer fragments were not usable to determine the entire degree of polymerization and to structurally understand the polydisperse profile of the PSA obtained from the MFE23-Ig5-FN1 fusion proteins. In addition, non-primate mammalian glycoprotein specific glycosidic linkage, Gal(\(\alpha 1\)-3)Gal was also detected by MALDI-MS as expected. After fusion protein polysialylation on introduction of the PolySTs to the mouse NB2a MFE23-Ig5-FN1 expressing monoclonal cell line, the next phase of establishing a polysialylation system in a human cell line was consequently required. Similar sialylated \(N\)-glycan structure detections by MALDI TOF-MS were also reported previously in adult rat brain tissue suggesting mono-, di- and trisialylated components, together with components substituted with four (or more) Neu5Ac residues (Zamze et al. 1998). Other important glycosidic linkages including NeuAc(\(\alpha 2\)-3 or -6)Gal and Gal(\(\alpha 1\)-3)Gal were also indicated from their studies (Zamze et al. 1998).

Furthermore, \textit{in vitro} antigen binding affinity was determined especially for the polysialylated MFE23-Ig5-FN1 fusion proteins. As shown in Figure 4.23, no antigen binding hindrance was found with the polysialylated fusion protein comparing to the MFE23 scFv, and the \(K_D\) values were consistently retained with all MFE23 fusion proteins.
CHAPTER 5

Generation and Characterization of Recombinant Polysialylated MFE23 scFv Fusion Protein from HEK-293 Cells
5.1 Introduction

Generation of polysialylated MFE23-Ig5-FN1 fusion protein was successfully achieved after transfection of PST and STX cDNAs into the mouse NB2a fusion protein-expressing monoclonal cell line (Chapter 4). Having the optimized purification procedures, the only issue arising from the fusion protein produced from the mouse cell line was the expression of xenogenic glycosidic linkage, Gal(α1-3)Gal within the fusion protein glyco-composition. As a product of the enzyme α1,3galactosyltransferase (αGalT), galactose-α1,3galactose-β1,4N-acetylglucosamine-R (αGal) epitopes are synthesised in all mammals except Old World primates (Galili et al. 1988). During the course of evolution, αGalT has been mutationally inactivated from Old World primates, and the α-gal epitope is therefore absent in humans but largely expressed on glycolipids and glycoproteins in non-primate mammals, prosimians and New World monkeys (Galili et al. 1988; Galili 2005). More significantly, natural anti-Gal antibody is abundantly present in all humans. As a result, transplantation of tissue xenografts from animals to humans is rejected by the natural anti-Gal anti-sera binding to the α-gal epitopes, and injection of substances exposing the α-gal epitopes can cause serious immunogenic problems to humans (Galili 2005). This fact raised the seriousness and requirement for producing the fusion proteins without the α-gal epitopes. The human embryonic kidney (HEK)-293 cell line has been widely used by the biotechnology industry to produce therapeutic proteins (Thomas and Smart 2005; Pham et al. 2006; Backliwal et al. 2008). I was therefore considered an appropriate human cell host for the systematic expression of polysialylated fusion proteins without the biosynthesis of the xenogenic α-gal epitopes within the protein carbohydrate composition.

Successful polysialylation from the mouse NB2a cell line confirmed the importance of active PolySTs for elongating α2-8 linked Neu5Ac from N-glycosylated proteins within mammalian cells. In order to establish a proper polysialylation system, human cell lines (or cells which do not produce α-gal epitopes) stably expressing active intracellular PolyST are therefore needed to be used as a “universal cell host” for producing recombinantly polysialylated proteins with available polysialylatable domain structures.

For analyzing oligo or polysialic acids, methods include nuclear magnetic resonance (NMR) (Michon et al. 1987), high-performance capillary electrophoresis (Cheng et al. 1998), thin-
layer and high performance anion-exchange chromatography (HPAEC) combined with mild acid hydrolysis (Kitazume et al. 1992; Zhang and Lee 1999), high performance liquid chromatography (HPLC) combined with fluorometric detection (FD) (Sato et al. 1999; Galuska et al. 2006), fluorometric C7/C9 analysis (Sato et al. 1998), and Western blotting using specific antibodies/lectins combined with different glycosidase treatment (Sato 2004). However, limitations and inaccuracies are associated with these detections. Methylation modified sialic acid derivatives were analysed through mass spectrometry (Finne et al. 1977), however, understanding the integrity and degree of polymerization of the PSA molecule was problematic. Such limitation with analysis was also observed from the MALDI TOF-MS results described in Chapter 4 for the polysialylated MFE23-Ig5-FN1 fusion protein. As a very rapid, and probably one of the most sensitive structural analysis approaches, MALDI TOF-MS was continuously used for characterizing oligo- and polysialic acids. In addition, recent research demonstrated unambiguous identification of polysialylation based on the exact mass determinations after direct lactonization on the MALDI target (Galuska et al. 2007). According to the research, lactonized oligo/polysialic acid also provided specific information including the type of sialic acid, potential side-group substitutes, and the number of NANA residues in the polymeric chain through MALDI TOF-MS analysis (Galuska et al. 2007).

The concept of oligo/polysialic acid lactonization was suggested many years ago, firstly highlighted by the existence of lactones in oligo/polysialic acid (McGuire and Binkley 1964). Reported by different investigators, natural sialic acid lactones were found on glycolipids extracted from cell lines and brain specimens (Gross et al. 1980; Riboni et al. 1986; Kawashima et al. 1994; Kielczynski et al. 1994). These studies suggested that the lactone derivation significantly preserves labile and reducible Neu5Ac in an alkaline condition, in order to ensure the recognition and signalling through the sialic acid presented on the glycolipids. However, occurrences of sialic acid lactonization are still poorly understood whether acid-catalysed through chemical reactions or actively controlled by specific enzymes. In terms of chemical reactions, previous laboratory examinations of α2,8- and α2,9-linked oligo/poly-Neu5Acs under acidic conditions detected rapid formation of lactonized polymer derivatives. This modification was then suspected to have physicochemical and biological alterations to the properties of sialic acid polymers, such as charge density and conformation (Lifely et al. 1981; Lifely et al. 1984). Mild and slow lactonization of PSA was also induced simply by dialysing the polymer dissolved in a sodium salt solution against water (Zhang and Lee 1999). Loss of negative charge for PSA after lactonization was determined by several
experiments. Insufficient de-lactonization of oligo- and polysialic acid mixture severely limited their separation by capillary electrophoresis and anion exchange column (Cheng et al. 1998; Zhang and Lee 1999).

As shown in Figure 5.1, two adjacent α2,8-linked Neu5Ac residues within PSA chain can be lactonized. The COOH-group of one Neu5Ac residue esterifies the OH-group linked to the C9 and forms a 6-membered lactone ring. The same ring structure was also observed by NMR between naturally occurring α2,8-linked sialic acid residues in GD3 and GD1b ganglioside lactones (Ando et al. 1989; Acquotti et al. 1991).

![Figure 5.1. Acid-catalysed α2,8 linked PSA lactonization. Lactone rings are marked by asterisks. Carbon atoms are numbered in the reducing end monosaccharide. Figure is take from (Galuska et al. 2007).](image)

This acid-catalysed lactonization method (chemically removing the negative charge from PSA) was firstly described by Zhang et al. (protocol mentioned in section 2.2.6.6), and then expanded to MALDI TOF-MS for studying PSA polymerization (Zhang and Lee 1999; Galuska et al. 2007). As mentioned above, apart from the precise MALDI MS results Galuska et al. obtained from their studies on PSA, the successful MS investigations and DP indications on highly polysialylated colominic acid and PSA bound to NCAM after lactonization treatment suggested a promising way for understanding of the scFv-Ig5-FN1 fusion protein polysialylation (Galuska et al. 2007).
5.2 Aims and Objectives

In order to eliminate the α-gal xenogenic glycosidic linkage featured by mammalian protein glycosylation, the human HEK-293 cell line was positioned to replace the mouse cell line to produce MFE23-Ig5-FN1 fusion protein with human-type glycosylation. A polysialylating HEK-293 cell host was planned to be engineered with stable expression of PolyST that could actively polysialylate any suitable protein format (i.e. scFv-Ig5-FN1) in the future. As already proved to be polysialylated in Chapter 4, MFE23-Ig5-FN1 fusion protein was then scheduled for HEK-293 cell host transfection and monoclonal selection for best fusion protein expression and polysialylation levels. Following the successfully optimized purification process, this part of the study focused on more detailed glyco-structure investigation on PSA by MALDI-MS by using the lactonization preparation method, and exploring the polysialylated fusion protein antigen binding profiles by several sensitive measurement methods and instruments.

The objectives are:
- Stable transfection and polyclonal selection of human cell line, HEK-293 for PolySTs expression.
- Transfection of MFE23-Ig5-FN1 and other MFE23-bond fusion protein cDNAs to the HEK-293 PolyST cells and selection of individual monoclonal cell lines with best protein expression and polysialylation level.
- Generation and purification of recombinantly polysialylated MFE23-Ig5-FN1 fusion protein.
- Determination of degree of polymerization of lactonized PSA from the polysialylated MFE23 fusion proteins by Mass Spectrometry.
- *In vitro* antigen binding affinity investigation of polysialylated MFE23 fusion proteins and other recombinant MFE23 proteins – ELISA and BIAcore™.
- *In vitro* live cell binding studies of polysialylated MFE23 fusion proteins and other recombinant MFE23 proteins – FACS and confocal microscopy.
- Molecular size property studies of the polysialylated MFE23 fusion proteins and other recombinant MFE23 proteins – Size exclusion chromatography.
5.3 Results

5.3.1 Polyclonal Selection of HEK-293 Cells Transfected with PolyST DNA

The human cell line, HEK-293, was used to substitute the NB2a (mammalian cell) protein production system, in order to avoid the expression of xenogenic oligosaccharide such as Gal(α1-3)Gal sugar linkage in the carbohydrate composition of the glycosylated fusion proteins. With the intention of creating an in situ recombinant protein polysialylation system from a human cell line, HEK-293 cells were stably transfected with the PolyST plasmid DNA, and the polyclonal PolyST transfected HEK-293 cell lines were collected under the neomycin antibiotic selection.

In order to confirm the endogenous expression of PST and STX after stable transfections, FACS analysis was used to investigate the polyclonally selected cell lines (section 2.2.5.9). As shown in Figure 5.2(a), the PolyST encoded V5 protein-tag was specifically targeted. The peak-shifts were clearly noticed from both HEK-293 PST and STX transfectants by comparing the ones without the involvement of anti-V5 antibody and the untransfected HEK-293 cells. The corresponding values of average fluorescent intensity of each tested samples were statistically analysed, and the two PolyST transfectants had approximately a threefold increase of the mean fluorescent intensity compared to the non-transfected HEK-293 cells.

As mentioned in Chapter 1, autopolysialylation was found in both PST and STX (Close and Colley 1998), and as is also suggested in Chapter 4, the active enzymes were potent enough to polysialylate other endogenous proteins. Therefore, positive polysialylating enzymatic activities of the two PolyST transfectants were confirmed specifically by anti-PSA antibody detections. The FACS results showed in Figure 5.2(b) clearly indicated a peak-shift of PST transfected HEK-293 cells with or without the mediation of anti-PSA antibody, while only a slight shift was observed from the STX transfectants. A doubled geometric mean fluorescence value (GMean) mediated by anti-PSA antibody was indicated for the PST transfectants in comparison with the wild type HEK-293, whereas the STX transfectants had almost the same value as the untransfected.
The lysed cell samples from original HEK-293 and the two PolyST transfected cells were also immunoblotted with an anti-PSA antibody (Figure 5.2c). Smears ranging from ~120 to 250 kDa showed strongly from the PST transfectant lysate, whilst only a weak signal was obtained from the lysate of STX transfected HEK-293 cells covering the similar MW range. No anti-V5 immunoblotting results were found for all lysates.
5.3.2 Monoclonal Selection of MFE23 Fusion Protein Expressing HEK293 PolyST Cell Lines

The creation of sustainable human cell lines with expression of active polysialyltransferase provided the cellular mechanism for engineered protein recombinant polysialylation under a human glycosylation pattern. With the successful polysialylation of MFE23-Ig5-FN1 fusion protein produced by the mammalian NB2a PolyST cells, the polysialylatable fusion construct was stably transfected into the polyclonal HEK-293 PST or STX cells under zeocin antibiotic selection pressure, and monoclonal cell lines performing the best fusion protein expression as well as the polysialylation level was selected by an ELISA-derived selection method (section 2.2.3.7 and 2.2.4.1).

Two separate bar-charts shown in Figure 5.3 demonstrate the monoclonal selection status of polysialylated MFE23-Ig5-FN1 expressing cell lines from both HEK-293 PST and STX cells. Generally speaking, the majority of monoclonal cell lines picked from HEK-293 PST MFE23-Ig5-FN1 transfectants expressed polysialylated fusion protein in their culturing medium, whilst the STX MFE23-Ig5-FN1 monoclonal cells only showed an equally good fusion protein production yield, but none of them was found to express the polysialylated version of MFE23-Ig5-FN1 (almost the same level as the un-transfected cells). The idea of the monoclonal selection was to find a cell line that had a high fusion protein expression levels and high polysialylation efficiency of the fusion proteins. Clone 2D2 from HEK-293 PST MFE23-Ig5-FN1 transfectants indicated the best anti-PSA ELISA result among all isolated monoclonal cell lines, and also had a relatively good fusion protein yield implied by the anti-Myc ELISA result. Clone HEK-293 PST 2D2 was chosen as the optimal cell line for expressing polysialylated MFE23-Ig5-FN1 fusion proteins with human glycosylation features. Other monoclonal cell lines including HEK-293 PST 1D1, 2A1, 2A5, 2B1, 2B3, 2C4, and 2C6, alongside the HEK-293 STX MFE23-Ig5-FN1 polyclonal cell line were selectively retained as well.

In addition, MFE23-FN1 plasmid DNA was also transfected into the HEK-293 PST cells (as it appeared to be more effective for MFE23-Ig5-FN1 protein polysialylation) as a negative polysialylation control, and the polyclonal cell line was selectively collected for MFE23-FN1 protein expression, in order to avoid unnecessary xenogenic glycosylation.
**Figure 5.3. Monoclonal selection of MFE23-Ig5-FN1 transfected HEK-293 PolyST cell line.** Culturing medium samples of each MFE23-Ig5-FN1 transfected HEK-293 PST or STX monoclonal cell line were immuno-detected by anti-Myc and anti-PSA antibodies respectively, and correspondingly, their consequent ELISA results were analysed for determining fusion protein yield and polysialylation status. “+” denotes 10μg purified highly polysialylated MFE23-Ig5-FN1 proteins made by NB2a cells; “-” indicates cell culture medium from HEK-293 PolyST polyclonal cells without fusion protein transfection. Clone 2D2 is indicated with red arrow.

**5.3.3 Expression and Purification of MFE23 Fusion Proteins from HEK-293 PST Cells**

Standard serum-free medium culturing and purification procedures (TALON™ cobalt anti-His₆ IMAC and ion exchange purifications) described for the NB2a cell expression system in
Chapter 4 were accordingly applied to the selected HEK-293 PST MFE23 fusion protein transfectants. Subjected to the polysialylated MFE23-Ig5-FN1 purification, the automatic anion-exchange chromatography optimized in section 4.3.4.2 with additional washing steps successfully separated the HEK-293 cell line-expressed polysialylated fusion protein into sialylated, weakly polysialylated and highly polysialylated isoforms. Results from Coomassie staining, various immunoblottings and glycosidase treatments indicated the same findings that illustrated previously by the NB2a cell products (Figure 4.16 and 4.17 in Chapter 4).

In addition, confirmation of the abolishment of Gal(α1-3)Gal disaccharide glyco-composition from HEK-293 expressed MFE23 fusion protein was determined by immunoblotting using an antibody that recognises the Gal(α1-3)Gal linkage (Figure 5.4).

![Figure 5.4](image)

**Figure 5.4. Anti-Gal(α1-3)Gal disaccharide immunoblotting for MFE23 fusion proteins produced from both NB2a and HEK-293 cell expression systems.** Sia, Weak, and High indicate three different glycosylation isoforms of polysialylated MFE23-Ig5-FN1 fusion protein. Bovine and human transferrins were used to show positive and negative signals from Gal(α1-3)Gal glycosidic linkage. Arrows indicate detected disaccharide containing protein bands.

Illustrated by the sugar linkage specific immunoblotting in Figure 5.4, xenogenic Gal(α1-3)Gal disaccharide was weakly displayed according to protein MWs by murine NB2a cell-expressed sia- or polysialylated MFE23-Ig5-FN1 fusion proteins. However, the NB2a-made MFE23-FN1 and LS-MFE23 fusion proteins did not show any signals from Gal(α1-3)Gal disaccharide specific immuno-detection. All human HEK-293 cell-produced MFE23 fusion proteins showed the expected negative results, indicating the abolishment of the mammalian cell specific glycosylation feature. Transferrins from both bovine and human origins depicted positive and negative outcomes from the sugar linkage specific antibody.
5.3.4 MALDI TOF-MS for Polysialylation Speculation

5.3.4.1 Colominic Acid (DP≥100)

Different concentrations of commercially purified colominic acid were initially analysed as controls for exploring MALDI TOF-MS detected PSA profiles. As suggested by Galuska et al., on-target lactonized colominic acid was combined with ATT matrix and detected in the negative-ion mode MALDI TOF-MS (protocol described in section 2.2.6.8) for satisfactory signal-to-noise ratios and better resolution signals particularly when high mass range (i.e. m/z > 10,000) involved in the MS detection (Galuska et al. 2007). Because of the PSA lactonization, no additional permethylation process was required for masking the negative charge from sialic acid, leading to each Neu5Ac residue appearing on their monoisotopic mass, 291 Da (Appendix 11). However, the formation of the lactone ring removed one OH-group and H molecule from each sialic acid residue. Therefore, receiving the addition of a proton from ionization, the MW of lactonized α2,8-linked Neu5Ac monomer should be 274 Da. The exact mass determination of each peak signal is indicated in Figure 5.5 and 5.6, implying different multiplications of lactonized Neu5Ac MW.

Also showed in Figure 5.5, an average 274 Da difference of two adjacent peak values was clearly stated indicating the homogenous constitution of the polymer by α2,8-linked Neu5Ac monomers. The maximally detectable colominic acid chain length was found to be more than 100 NANA residues (Figure 5.5a inset) when a relatively large sample amount (6.25μg) was MALDI-MS analysed by negative linear mode. Improving peak resolution for more accurate identification of molecule masses was achieved by setting the MALDI TOF-MS instrument into a negative reflection mode (Figure 5.5b). Although sharper peak signals were observed, the detectable mass range was reduced compared to the linear mode.

Furthermore, the increased peak resolution enabled the observation of satellite peaks differing by mass increment of 18 Da, of which was caused by a water molecule under the detection condition. Showed in Figure 5.6, the sensitivity (i.e. peak signal-to-noise ratios) and detectable mass range was considerably reduced when analysing a much smaller amounts of colominic acid (3ng). However, peak signals were still clearly revealed from the MS spectra with unambiguous MW information identifying unique α2,8-linked Neu5Ac polymerization as well as the determination of the chain length. The detected mass range indicated up to approximately 70 residues maximum from the sialic acid polymer by linear negative
Figure 5.5. Analysis of colominic acid (6.25μg) by MALDI TOF-MS. Colominic acid was lactonized on-target and analysed in: (a) negative-ion linear mode with enlarged spectrum inset of mass range m/z 25573-34477; (b) negative-ion reflectron mode with enlarged spectrum (mass range m/z 3707-5744) inset of DP14-20 species and their satellite peaks caused by additional water molecule detected under the condition used for analysis. Average masses [M – H] and corresponding DP values are given for selected signals.
Figure 5.6. Analysis of colominic acid (3ng) by MALDI TOF-MS. Colominic acid was lactonized on-target and analysed in: (a) negative-ion linear mode with enlarged spectrum inset of mass range m/z 14442-23432; (b) negative-ion reflectron. Average masses [M – H]⁻ and corresponding DP values are given for selected signals.
mode (Figure 5.6a inset), while the reflection mode could only detect m/z signals showing DP of 30 NANA residues in the polymer (Figure 5.6b).

5.3.4.2 Polysialylated MFE23-Ig5-FN1 Fusion Protein
Successfully indicated by colominic acid MALDI TOF-MS studies, polysialic acid species and the degree of polymerization were clearly determined. Lacking this type of information from polysialylated MFE23-Ig5-FN1 fusion protein analysed in previous MS studies described in Chapter 4, glyco-structural studies of the polysialylated fusion protein newly expressed from the human monoclonal cell line were consequently performed by the mass spectrometry analysis. Outlined in Scheme II below, three investigatory pathways were explored.

Scheme II. Preparation procedures for MALDI-MS and MS/MS analysis of HEK-293 cell made polysialylated MFE23-Ig5-FN1 fusion protein with additional lactonization treatment. Three analytical MS preparations were made for (a) PSA, (b) N-glycan core and (c) O-glycosylation detections. Glycosidases and chemical β-elimination for sugar residue removal were marked in blue within the preparation process. O-phosphoric acid was used for PSA lactonization on target-plate.

Based on the o-phosphoric acid catalysed lactonization method (section 2.2.6.6) initially used by Galuska et al (Galuska et al. 2007), this lactonization treatment was also performed on the sialic acid derivatives of all three MFE23-Ig5-FN1 fusion protein glyco-forms (sialylated,
weakly and highly polysialylated isoforms) and analysed by MALDI TOF-MS (preparation outlined in Scheme II-a). High signal intensities from PSA NANA residues were subsequently detected.

Without any further pre-treatment, direct MALDI TOF-MS analysis of lactonized MFE23-Ig5-FN1 fusion proteins revealed PSA chains with up to ~40 α2,8-linked Neu5Ac residues from the linear negative-ion mode and halved residue numbers detected from the reflectron mode (Figure 5.7). On the other hand, higher resolution of peak signals was received from the reflectron mode with more exact mass determination (Figure 5.7b). An additional water molecule co-ionized with the PSA fragment was also revealed by the reflectron mode (Figure 5.7b inset). Furthermore, according to the masses suggested by each MS peak signal, PSA chain structures were the only species detected on the MALDI TOF-MS spectra, which facilitated the determination of the polysialylation degree occurring on the fusion protein. However, the homogenous PSA chain signals also suggested a release of lactonized PSA from the glycopeptide which did not hamper the MS measurement. This release of lactonized PSA from glycopeptide also occurred in the polysialylated NCAM analysed by MALDI TOF-MS, and the reason of this occurrence is still under investigation (Galuska et al. 2007).

Weakly polysialylated and sialylated MFE23-Ig5-FN1 fusion proteins (100μg of each) were also treated for lactonization preparation, followed by direct detection by MALDI TOF-MS. Unlike the highly polysialylated glycol-form, weaker signal resolution and sensitivity were produced. Shown in Figure 5.8(a), available signals suggested approximately ≤15 NANA residues of the weakly polysialylated fusion protein after lactonization. Although DPs extended to 20 and 28 were speculated according to the masses indicated by the spectral signals, these mass values were ambiguous and not accurate enough to confidently declare them as sialic acid repeats. Only one strong MS spectral signal of lactonized sialylated fusion protein suggested possible oligosialic acid structure (DP = 4), while other detectable signals seen from the spectrum did not represent any clear and decisive masses for Neu5Ac chain structure (Figure 5.8b).
Figure 5.7. Analysis of polysialylated N-glycan from highly polysialylated MFE23-Ig5-FN1 fusion protein (100μg) by MALDI TOF-MS. Polysialylated N-glycan was lactonized on-target and analysed in: (a) negative-ion linear mode with enlarged spectrum inset showing mass range m/z 5933-14443; (b) negative-ion reflectron mode with enlarged spectrum (mass range m/z 2624-4204) inset of DP10-15 species and their satellite peaks caused by additional water molecule detected under the condition used for analysis. Average masses [M – H] and corresponding DP values are given for selected signals.
Figure 5.8. Analysis of N-glycans of other MFE23-Ig5-FN1 fusion protein glyco-forms by negative-ion linear mode MALDI TOF-MS. N-glycans from (a) weakly polysialylated fusion protein (100μg) and (b) sialylated fusion protein (100μg) were lactonized on-target. Average masses [M – H] and corresponding DP values are given for selected signals. “?” marks indicate unconfidently speculated DP values due to the ambiguous mass correspondences.
Another investigation was focused on the N-glycan core structures of MFE23-Ig5-FN1 fusion protein (Scheme II-b). All three glyco-forms of the fusion protein were Endo-N treated for PSA removal followed by N-glycan isolation by PNGase F treatment. The permethylation process was carried out prior to sending the samples to the MALDI TOF-MS instrument. As expected, with the same fusion protein backbone, all three fusion glyco-forms share the same N-glycan core carbohydrate profile that is illustrated in Figure 5.9.

Figure 5.9. Analysis of N-glycan core structures of MFE23-Ig5-FN1 fusion proteins by positive-ion reflectron mode MALDI TOF-MS. Biosynthetically logical glycan structures were speculated according to the average mass [M – Na]+. Information of each monosaccharide (symbol denotation, structure, Permethylated monoisotopic mass) was provided in Appendix 11.

In comparison with the N-glycan core structures detected from mouse NB2a cell expressed polysialylated MFE23-Ig5-FN1 fusion protein (Figure 4.18), both complex and oligomannosidic core N-glycan constitutions were also detected from the HEK-293 made fusion proteins with di-, tri- and tetra antenmary structures involved, alongside bisecting N-acetylglucosamine which was occasionally detected. Moreover, fucoses were typically found within the human cell made fusion protein N-glycan cores, which were not indicated from the MS spectrum of the fusion protein with the mouse glycosylation format.

Additionally, the O-glycosylation profile of the fusion protein was also measured by mass spectrometry. Introduced in Scheme II-c, O-glycans from all three fusion glyco-forms (100μg
each) were removed by reductive elimination (or β-elimination) process (standard protocol described in section 2.2.6.4), followed by permethylation treatment. According to the m/z signals received from MALDI TOF-MS spectra (positive reflectron), no clear O-glycosidic linkage/structure as well as polysialylation could be postulated (results showed in Appendix 14).

5.3.5 In vitro Antigen Binding Affinity Investigation

It is very important to understand the effect of polysialylation and recombinant protein domain fusion to the MFE23 scFv in terms of the antibody-antigen binding affinity. The exploration of the binding affinities of the fusion protein antigen target was achieved by two important in vitro antigen binding methods, of which were ELISA and BIAcore™. In both cases, N-A1, the recombinant protein representing CEA binding-site to the MFE23 antibody was used as the antigen target.

5.3.4.1 ELISA

In order to compare the $K_D$ data received from NB2a cell expressed MFE23 fusion proteins (section 4.3.6), the same ELISA protocol was employed to identify the antigen targeting profiles of the HEK-293 cell made fusion proteins.

Confirmed from the previous study (Figure 4.18), the NB2a cell expressed LS-MFE23 scFv molecule had an excellent N-A1 antigen binding affinity, and the scFv was used as a positive control to identify the in vitro antigen targeting motifs of other MFE23 fusion proteins produced by HEK-293 PST cells. Almost the same results of all MFE23 fusion constructs were shown in Figure 5.10 by comparing with the ELISA data from the NB2a products showed in Figure 4.23. Most N-A1 binding molecules tested here depicted their $K_D$ values between 4-6nM, including the positive control, whilst the MFE23-FN1 and sialylated MFE23-Ig5-FN1 fusion protein showed approximately tripled and doubled $K_D$ values in this binding test.
Figure 5.10. ELISA analysis for determining antigen binding affinities of all MFE23 fusion proteins produced by HEK-293 cells. A serially diluted protein concentrations (from 0.5nM to 1μM) was prepared in triplicate for each fusion protein including NB2a cell expressed MFE23 scFv (LS-MFE23), HEK-293 PST cells produced MFE23-FN1 and three types of glycosylated MFE23-Ig5-FN1 isoforms (depicted in different colours). The ELISA was mediated by Myc-tag specific antibodies and the $K_D$ values (± standard errors) of each type of fusion protein were also signified.

5.3.4.2 BIAcore™ Affinity Kinetics Studies

Behind the complex physical principles of surface plasmon resonance (SPR), BIAcore, the SPR-based biosensor system, has been recognised as one of the most widely used technologies by far for obtaining accurate quantitative data particularly in the investigations of molecular bindings and interactions. Particularly, BIAcore offers advantages such as sensitive analysis of weak molecular interactions, and specific on-rate and off-rate measurements under the real-time binding models. Subjected to the kinetic studies of the MFE23 scFv, and its different fusion and polysialylation formats, the BIAcore 3000 instrument was used and the corresponding operation/analysis protocols have been described in section 2.2.5.8.

Again, the recombinant N-A1 proteins were immobilized on the BIAcore chip to offer antigen binding. Previous BIAcore experiments using *E.Coli* expressed MFE23 scFv against the recombinant N-A1 antigen showed the $K_D$ of 5.33nM (Sainz-Pastor et al. 2006), and their
suggested protocol was followed in part. However, other than coupling the N-A1 antigen to a BIAcore® CM5 sensor chip via amine coupling, the antigen proteins were initially biotinylated and consequently immobilized to a BIAcore® SA sensor chip which was pre-coated with streptavidin. Both 5:1 and 20:1 biotinylated N-A1 antigens were prepared (section 2.2.5.6) to improve antigen immobilization on the SA sensor chip. Standard ELISA assays were carried out using LS-MFE23 scFv against the two differently biotinylated N-A1 antigens. Affinity constants were preserved from both ELISA tests (results not shown). Both biotinylated N-A1 showed good antigen immobilizations to the SA sensor chip with relatively low response unit signals (700-900 RU) reached. It is expected to have low enough antigens immobilized on the BIAcore chip surface in order to minimize the kinetic artifacts such as rate-limited mass transport (effecting on-rate measurement) and re-binding (effecting off-rate measurement). In order to minimize structural changes of the antigen, the 5:1 biotinylated N-A1 was used in all BIAcore experiments.

The results of BIAcore kinetics experiments with LS-MFE23 scFv, MFE23-FN1 and three different MFE23-Ig5-FN1 glyco-forms were shown in Figure 5.11. Exactly the same experimental process (i.e. association/dissociation period and regeneration condition) and evaluation method were carried out on all five MFE23 fusion samples (method described in section 2.2.5.8). Twenty cycles of each fusion protein BIAcore experiment covered sample concentrations from 0.4nM to 185nM. In terms of the data evaluation, the 7 highest concentrations (from 2.89nM to 185nM) of each fusion protein were used to fit best to the 1:1 langmuir binding model, which evaluated the $K_D$ values and other kinetics parameters including $k_{on}$, $k_{off}$ and their standard errors listed in Table 5.1. The $\chi^2$ values were also indicated after the fitting of each fusion proteins BIAcore data, suggesting the closeness and statistical significance of each fit.

Showed in Figure 5.11, well fitting models were evaluated and plotted as overlays of kinetic bindin of each sample concentration. Statistically approved in Table 5.1, the $\chi^2$ values of each fusion protein were all less than 5% of their corresponding $R_{max}$ values indicating the adequacy the corresponding fitting models. In terms of the real-time kinetic binding, almost the same $k_{on}$ and $k_{off}$ values, consequently the $K_D$ values were obtained from the LS-MFE23 scFv and MFE23-FN1 fusion protein. Much slower on-rates (~20-fold less than the scFv $k_{on}$) were found for the two polysialylated fusion glyco-forms, however their off-rates were kept consistent, and approximately an 8-fold increase of the $K_D$ value of weakly polysialylated MFE23-Ig5-FN1 fusion protein, and a 10-fold increase to the highly polysialylated were
observed. The standard errors of both $k_{\text{on}}$ and $k_{\text{off}}$ values were at least 10-fold less than the corresponding kinetic parameters suggesting the reliability of the real-time binding kinetics.

![Figure 5.11. Kinetics of binding analysis of MFE23 recombinant fusion proteins.](image)

Dilution series ranging from 0.4nM to 185nM were prepared respectively for fusion proteins including LS-MFE23 scFv, MFE23-FN1, sialylated MFE23-Ig5-FN1, weakly and highly polysialylated MFE23-Ig5-FN1, and their kinetic binding to NA1 recombinant antigen peptide were measured by SPR using Biacore 3000 instrument. Each concentration was tested in duplicate. Due to low binding signals at the low concentrations, only traces for the highest seven concentrations (2.89nM to 185nM) are shown. The best fit (Langmuir 1:1 binding) lines (black) were also indicated as overlays of each corresponding kinetic bindings. Injection start (I-start) and stop (I-stop) are indicated, alongside association (2 minutes) and dissociation (only less than 4 minutes out of total 10 minutes were showed) phases marked in grey.
Figure 5.11 - Continued
The $K_D$ increases found in the polysialylated MFE23-Ig5-FN1 fusion proteins were not suggested by the ELISA results (Figure 5.10). As mentioned previously, the N-A1 antigen peptides were biotinylated for coating on the BIAcore sensor chip, which has a carboxymethylated dextran matrix for pre-immobilizing with streptavidins, and a highly negative surface on the SA sensor chip due to the free carboxyl group in the dextran matrix (http://www.biacore.com/lifesciences). In this case the negative charges from the PSA were speculated as the reason that hindrance of the on-rate of the antibody-antigen binding occurred, and the electronic repulsion generated opposite force against the affinity orientation. In order to support this idea, a de-polysialylated MFE23-Ig5-FN1 fusion proteins kinetic analysis (Figure 5.12) was processed consequently by the same BIAcore method (section 2.2.5.8) after treating the polysialylated fusion proteins with Endo-N for PSA removal (section 2.2.5.4).

![Figure 5.12. Kinetics of binding analysis of de-polysialylated MFE23-Ig5-FN1 fusion protein](image)

Figure 5.12. Kinetics of binding analysis of de-polysialylated MFE23-Ig5-FN1 fusion protein. A dilution series of the fusion protein was prepared ranging from 0.4nM to 185nM and kinetic binding to N-A1 recombinant antigen peptide was measured by SPR using BIAcore 3000 instrument. Each concentration was tested in duplicate. Due to low binding signals at the low concentrations, only traces for the highest seven concentrations (2.89nM to 185nM) are shown. The best fit (Langmuir 1:1 binding) lines (black) were also indicated as overlays of each corresponding kinetic bindings. Injection start (I-start) and stop (I-stop) are indicated, alongside association (2 minutes) and dissociation (only less than 4 minutes out of total 10 minutes were showed) phases marked in grey.

With all the kinetic parameters and adequate statistic values summarized in Table 5.1, the kinetic binding and corresponding fitting model of the de-polysialylated MFE23-Ig5-FN1 fusion protein (Figure 5.12) indicated a restored on-rate, and consequently a decreased $K_D$ value, which was only $\sim 2.5$-fold higher than the scFv $K_D$. 
Table 5.1. Summary of SPR Biacore kinetics analysis. The kinetic and statistical values were generated according to the best fit models showed in Figure 5.11 and 5.12.

<table>
<thead>
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<th>Fusion proteins</th>
<th>$k_{on}$ (M$^{-1}$s$^{-1}$)</th>
<th>SE* - $k_{on}$ (M$^{-1}$s$^{-1}$)</th>
<th>$k_{off}$ (s$^{-1}$)</th>
<th>SE* - $k_{off}$ (s$^{-1}$)</th>
<th>$K_D$ (M)</th>
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<tr>
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<td>4.75x10$^{-5}$</td>
<td>3.74x10$^{-8}$</td>
<td>8.13</td>
</tr>
</tbody>
</table>

*: Standard error (SE) – For excellent statistical reliability, SE values are needed to be at least 10-fold less than the corresponding parameter;

Chi$^2$: A score of less than 10% of the corresponding $R_{max}$ value is needed to determine the model used for fitting adequately describes the data.

5.3.6 In vitro Live Cell Binding Studies

The effect of recombinant polysialylation of antibody targeting on antigens expressed on live cells under culturing conditions was consequently investigated. The findings from the live cell models would give more appropriate answers of how suitable polysialylation could be in terms of serving as a drug delivery system for antibody fragments. For this part of the investigation, FACS and confocal microscopy were employed to understand the targeting profiles under different live cell conditions.

5.3.6.1 FACS for Cell Surface Antigen Binding Detection

Two colonic cancer cell lines, LS174T and LoVo, were cultured to supply CEA antigen expression, while KB, the epidermal carcinoma cell line was used for negative antigen expression control. Following the protocol described in section 2.2.5.9, all MFE23 containing fusion proteins were analysed by FACS for comparing their binding profiles and efficiencies to the live cell surface expressed CEA antigen.

The CEA antigens expressed on the surface of colonic cancer cells, LS174T and LoVo, were specifically targeted by MFE23 containing recombinant fusion proteins, and this live cell CEA-MFE23 binding detected by FACS was bridged up by the antibodies recognising the characteristics brought by the fusion protein. Shown in Figure 5.13(a), anti-Myc antibody directed FACS indicated all MFE23 containing fusion proteins recognised the antigen
Figure 5.13. In vitro live cell binding investigation by FACS. Antibodies specifically recognising Myc-tag (a), and PSA (b) were used, and all mammalian expressed MFE23 fusion proteins (0.2μg/μl each) were analysed in replicate upon LS174T, LoVo, and KB cells. Protein constructs were differentiated by colours (denotations on the left) with the average GMean values indicated respectively.
expressing cells by comparing with the cells absent of the mammalian cell made MFE23 proteins. Generally, according to the associated GMean values from cells bound with the MFE23 protein, the LS-MFE23 scFv and MFE23-FN1 constructs depicted better binding efficiencies than the three glycosylated MFE23-Ig5-FN1 isoforms. However, in the case of anti-PSA directed FACS analysis, only the highly polysialylated MFE23-Ig5-FN1 showed a significant shift, where approximately a 12-fold increase in average fluorescent value was evaluated by comparing to the other constructs which had almost the same fluorescent intensity as the non-MFE23 protein incubated colonic cancer cells (Figure 5.13b). CEA-negative KB cells were prepared and analysed the same way as the other two cell lines, and no live cell binding evidence could be detected.

5.3.6.2 Confocal Microscopy for Cell Surface Antigen Binding Detection

In addition to FACS live-cell binding analysis, more in-depth detection was carried out by using confocal laser scanning microscopy (CLSM) (section 2.2.5.10), which allowed focused observation of antigen-binding profiles by all MFE23 fusion proteins on live cells with high-resolution optical images, and topologically investigation of the binding from multiple depths of a cell. Again, LS174T cells were used as the CEA expressing cell line, and KB cells were prepared as the CEA-negative cell line. Antibodies recognising different features of recombinant MFE23 fusion proteins were exploited for antigen-binding development.

Fluorescent CLSM images mediated by Myc-tag specific identifications shown in Figure 5.14, 5.15 and 5.16(a), directly illustrated the mammalian cell produced MFE23 scFv and fusion proteins had generally equivalent CEA antigen binding ability under live cell binding conditions. Images received from different post-binding conditions including temperature (4°C and 37°C) and incubation time (10 minutes and 2 hours) showed the same results revealed in the figures above according to each corresponding fusion protein construct (results not shown). The in-depth z-stack images of individual cell models demonstrated an identical surface antigen binding profile to all MFE23 involving recombinant proteins, especially the highly polysialylated MFE23-Ig5-FN1 fusion protein. This proved to have a live cell antigen-binding profile as the non-glycosylated MFE23 scFv as well, and the overall antibody-antigen binding only happened around the cell surface with hardly any internalization interpretations indicated by images of planes at various depths. Phase-contrast CLSM images outlining the cell shapes further identified the cell surface binding observation after
Figure 5.14. Binding of cell surface CEA antigen expressed on LS174T cells by LS-MFE23 scFv and MFE23-FN1 fusion proteins. Fluorescein (Alexa Fluor® 488) associated anti-Myc specific detection (I) and phase-contrast (II), alongside their merged CLSM images (III) are shown. Images of planes at various depths (from bottom to top with 1µm depth interval between each plane) within individual cell samples are illustrated. The middle plane images of cell clusters are displayed for the overall CEA binding profiles of both recombinant MFE23 proteins.
Figure 5.15. Binding of cell surface CEA antigen expressed on LS174T cells by sialylated and weakly polysialylated MFE23-Ig5-FN1 fusion proteins. Fluorescein (Alexa Fluor® 488) associated anti-Myc specific detection (I) and phase-contrast (II), alongside their merged CLSM images (III) are shown. Images of planes at various depths (from bottom to top with 1µm depth interval between each plane) within individual cell samples are illustrated. The middle plane images of cell clusters are displayed for the overall CEA binding profiles of both MFE23 fusion proteins.
Figure 5.16. Binding of cell surface CEA antigen expressed on LS174T cells by highly polysialylated MFE23-Ig5-FN1 fusion protein. Alexa Fluor® 488 associated anti-Myc (a) and Alexa Fluor® 594 associated anti-PSA (b) specific CLSM images (I) are shown alongside their corresponding phase-contrast (II) and fluorescent merged images (III). Images of planes at various depths (from bottom to top with 1µm depth interval between each plane) within individual cell samples are illustrated. The middle plane images of cell clusters are displayed to depict the overall CEA binding profile.

overlapping with the fluorescent images. The fluorescent imaging signals mediated by the anti-PSA antibody (Figure 5.16b) were only discovered from the highly polysialylated MFE23-Ig5-FN1 protein as expected, which also depicted the cell surface targeting orientation of the fusion protein. Negative control experiments using KB cell line repeated the CLSM preparation protocols using all MFE23 recombinant protein and the positive control IgG under the same post-binding incubation conditions. No positive fluorescent signal was
revealed under the same CLSM detection settings (described in section 2.2.5.10) (Figure 5.17). Positive control experiments using a commercial bivalent IgG antibody against the CEA antigen expressed on the LS174T cells also revealed live cell binding (Figure 5.17), suggesting the MFE23 motif containing fusion proteins followed the same antigen targeting principle.

**Figure 5.17. CLSM control experiments.** (a) Negative control experiment using KB cells were carried out by all MFE23 fusion proteins with the same fluorescent negative results. (b) Binding of cell surface CEA antigen expressed on LS174T cells by a commercial bivalent anti-CEA IgG antibody. Alexa Fluor® 488 conjugated anti-Fc specific CLSM images (I) and phase-contrast (II), alongside their merged CLSM images (III) are shown. Images of planes at various depths (from bottom to top with 1µm depth interval between each plane) within individual cell samples are illustrated. The middle plane images of cell clusters are displayed to depict the overall CEA binding profile. CLSM setting of fluorescent detector-gain reduced 200 degrees comparing to recombinant MFE23 proteins.
5.3.7 Gel Filtration and Size Exclusion Chromatography

SEC is a widely used technique for the purification and analysis of synthetic and biological polymers. The gel filtration column (HiLoad™ Superdex 200 prep grade) was used to compare and define the hydrodynamic radius of each protein construct under aqueous environment. Based on the concept of SEC, the larger the size of the water-soluble molecule the faster it is filtrated through the column, while a longer time and higher running buffer volume will be spent by smaller size molecules trapped in the column gel pores. To identify the size improvement after differential sia- and polysialylation post-translational modifications, the fusion protein samples were separately injected into the gel filtration column (protocol described in section 2.2.5.11). Samples were compared to standard MW marker molecules (Table 2.10) for plotting the standard size exclusion curve, of which was important to calculate the sample proteins’ apparent molecular size and hydrodynamic radius increment.

Showed in Figure 5.18(a), the gel filtration column was calibrated by the four MW markers indicating standard analytical SEC profile. The peak fraction of dextran polymer (2000 kDa) defined the void point (52ml fraction) suggesting the largest molecule that the column can analytically separate. Under the same physiological buffer conditions, the SEC profiles of all MFE23 fusion proteins and glyco-forms are illustrated in Figure 5.18(b). Following the MW difference of each fusion protein, fusion proteins containing higher MW were eluted quicker than the lower MW ones. Having the same amino acid backbone, the highly polysialylated MFE23-Ig5-FN1 fusion protein was eluted more rapidly than the other two glyco-forms, which showed its peak fraction just within the maximum column capacity (void point). This finding may suggest that under physiological buffer conditions, the increase of sialic acid chain length (causing small MW increment) amplified the hydrodynamic volume possession of the fusion protein and eventually increased its apparent molecular size. Secondary peaks (peak-2s in Figure 5.18b) of each fusion protein and glyco-form were also found from the SEC, which were eluted earlier than the predominant peaks (Peak-1s in Figure 5.18b). On the other hand, highly polysialylated MFE23-Ig5-FN1 fusion protein showed an opposite eluting profile with a major peak (~55ml fraction) appearing more rapidly than a minor eluting drag (covering approximately from 60 – 70ml fractions). In addition, protein collected from the peak fractions indicated in the SEC (Figure 5.18b) were revealed by SDS-PAGE, and specifically identified by various immunoblottings (Figure 5.19).
Figure 5.18. Analytical SEC on HiLoadTM Superdex 200 prep grade gel filtration column under physiological buffer condition. Size exclusion chromatograms of (a) Standard MW markers and (b) MFE23 motif containing recombinant proteins were plotted according to the normalized absorbance data (recorded at laser wavelength 214nm) against running buffer consumptions for protein elution. Predominantly, two elution peaks were found for each MFE23 construct, and were labelled by 1 (higher peak) and 2 (lower peak) with corresponding colour indications of the protein samples. Void limit of SEC were signified in both plots based on the peak elute value of dextran polymer (2000 kDa). PBS (pH7.4) was used as the running buffer for the SEC analysis.
Figure 5.19. Western blotting and Coomassie staining analysis of the SEC peak fractions eluted from each MFE23 fusion protein or glyco-form. Both anti-Myc and anti-PSA antibodies were used respectively for fusion protein and PSA detections. The SEC peak numbers are indicated in correspondence to the labelling showed in Figure 5.18(b). Reducing SDS-PAGE was used for separating the proteins and four times more sample materials were loaded for Coomassie staining in comparison with the immunoblottings.
Dimerization of most of the fusion proteins was speculated to cause the appearances of the secondary peaks that eluted more quickly, while the minor SEC elution fractions found in the highly polysialylated fusion protein were thought to be caused by the polydisperse PSA chain lengths associated with the fusion protein. Based on the SEC, protein fractions were collected and concentrated before loading into the reducing SDS-PAGE. Western blot and Coomassie staining analysis were followed to identify the protein content. Shown in Figure 5.19, almost identical results were found for both SEC peaks of each fusion protein, particularly demonstrated by anti-Myc immunoblotting and protein staining. The anti-PSA antibodies only picked up weakly and highly polysialylated MFE23-Ig5-FN1 fusion protein as expected and the smear indicating polysialylation of peak-1 fraction from highly polysialylated fusion protein reached higher MW level than its peak-2 elution fraction. In addition, weak anti-Myc antibody detections were shown for the LS-MFE23 scFv and MFE23-FN1 fusion protein suggesting potential hydrolysis to these proteins without the “watery shield” masking established by the additional glycosylation components.

To further analyse the SEC results, the standard elution curve (Figure 5.20) was plotted based on the MWs of the four standard markers against their peak elution volumes showed in Figure 5.18(a). According to the standard curve equation, the apparent MWs (or MW\text{app}) of each MFE23 recombinant protein formats were determined in correspondence with their higher peaks showed in Figure 5.18(b), as well as the lower peaks apparent MW correspondences. Presented in Table 5.2, the apparent MW of the MFE23 scFv indicated by SEC peak 1 was almost the same as its calculated MW, while the other fusion proteins’ apparent MWs determined by their SEC peak 1 were much higher than their calculated MWs. Particularly, the hydrodynamic volume recruited by the highly polysialylated MFE23-Ig5-FN1 fusion protein was worth a protein of 746 kDa alone, however, its calculated MW was only 54 kDa despite additional MW from glycan cores and polysialylation (70 kDa maximally). SEC peak 2 found as an earlier eluate of all protein formats apart from the highly polysialylated MFE23-Ig5-FN1, determined approximately doubled or tripled apparent MWs of the recombinant proteins than the SEC peak 1 indications, while the highly polysialylated MFE23-Ig5-FN1’s second peak showed an reduced apparent MW. Overall, based on the SEC measurement the highly polysialylated MFE23-Ig5-FN1 fusion protein demonstrated approximately a 25-fold increase of the apparent MW than the MFE23 scFv, and a 6-fold increase over the sialylated MFE23-Ig5-FN1. Comparing with the calculated MWs, the apparent MWs determined by reducing SDS-PAGE (Table 5.2), on the other hand, suggested relatively consistent LS-
MFE23 scFv and MFE23-FN1 fusion protein MWs, whilst the three MFE23-Ig5-FN1 glycoforms showed increased MWs but not in proportion with their glyco-possession increments.

![Figure 5.20. SEC standard curve on HiLoadTM Superdex 200 prep grade gel filtration column. The standard curve was plotted according to the MW logarithm values of standard markers: (a). Beta-amylase (200 kDa); (b). BSA (66 kDa); (c). Carbonic anhydrase (29 kDa), against their peak elution volumes. Aligned on the standard curve, the apparent MW logarithm values of each MFE23 recombinant fusion protein were calculated according to the standard curve equation from their maximum (peak 1) elution volumes.](image)

<table>
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<tr>
<th>Calculated Monomer MW (kDa)</th>
<th>Apparent MW (kDa)</th>
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<tr>
<td></td>
<td>SDS-PAGE</td>
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<tr>
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<tr>
<td>Highly PolySia MFE23-Ig5-FN1</td>
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Table 5.2. The SDS-PAGE and SEC measured MWs against the calculated MWs of all MFE23 recombinant fusion formats. The table contains the statistical conversion of SEC information display in Figure 5.18(b) and Figure 5.20, including the two protein chromatogram peaks of each MFE23 fusion protein species indicated in Figure 5.18(b), and the direct MW readings from the Coomassie staining showed in Figure 5.19. The calculated MWs of each protein were displayed previously in Chapter 3, Table 3. Additional MW that MFE23-Ig5-FN1 fusion protein gained from different degree (n ≤20 in average) of polysialylation (291 Da per Neu5Ac residue) was also indicated.
5.4 Discussion

Following on from the success of recombinantly polysialylating MFE23-Ig5-FN1 fusion protein in the mouse NB2a cell line (described in Chapter 4), this chapter develops the technology via a human cell line. The proof-of-concept investigation in the mouse cell line distinctively proved the indispensable involvement of PolyST for PSA elongation along the minimal polysialylatable NCAM structure. However, the production of rodent specialized Gal(1,3)Gal disaccharide linkage associated in the fusion protein glyco-composition made it xenogenic to humans. Consequently, the human cell host (HEK-293 cell line) was transfected for the production of recombinantly polysialylated fusion protein.

Prior to switching the fusion protein expression cell host from a mouse to a human cell line, a universal biological polysialylation system was established initially through selection of HEK-293 cells stably expressing polysialyltransferases - PST or STX. The same transfection and selection procedures were performed for both of the PolyST cDNAs on the HEK-293 cells, and the polyclonal cell lines of both transfectants were collected. The polysialylation enzymatic activities of the two polyclonal cell lines were preliminarily determined from the autopolysialylation exhibited by the two PolySTs, which was reported previously (Close and Colley 1998; Close et al. 2000). From the experimental results, the HEK-293 PST transfectants appeared to have a better PolyST endogenous expression level (indicated by anti-V5 FACS, Figure 5.2b) and autopolysialylation activity (indicated by anti-PSA Western blot and FACS, Figure 5.2a & c).

Rather than selecting a monoclonal cell line with optimal PolyST performance, only a broad polyclonal selection was made to generate HEK-293 PST or STX expressing cell lines. This was because a secondary monoclonal selection was consequently performed for picking the cells with comparatively the best MFE23-Ig5-FN1 fusion protein expression and polysialylation levels. An ELISA selection platform was established for this monoclonal selection with double parameters after transfection of MFE23-Ig5-FN1 fusion cDNA to the HEK-293 PST or STX polyclonal cells. Shown in Figure 5.3, culture media from each of the selected monoclonal cell lines were immuno-screened by anti-Myc (for protein expression level) and anti-PSA (polysialylation level) antibodies respectively. Monoclonal cell line 2D2 was eventually selected for producing the polysialylated MFE23-Ig5-FN1 fusion protein with a better expression yield and consistency. In addition, poor polysialylation levels were
detected from all monoclones selected from STX transfectants (Figure 5.3), which conflicted with the positive STX polysialylation activity indicated from the results shown in Figure 5.2. It was speculated that the polyclonal HEK-293 STX cell population might contain a number of cells that were unable to polysialylate but survived during antibiotic selection alongside active STX expressing HEK-293 transfectants. Through a number of cell sub-culturing steps, the population of unpolyssialylating cells might have overwhelmed the total cell population of HEK-293 STX cells, which led to a very low polysialylation level from the MFE23-Ig5-FN1 fusion construct transfected STX cells. Re-selection of HEK-293 STX cells with stable and active STX expression might be required for further study of STX induced polysialylation as well as the dual expression of PST and STX to the polysialylatable scFv-Ig5-FN1 fusion proteins.

The generation and purification of the polysialylated fusion protein from the selected monoclonal cell line 2D2 were followed using the procedures optimized in Chapter 4. Consistent purification steps (His$_6$ specific IMAC followed by anion exchange chromatography) separated the polysialylated MFE23-Ig5-FN1 fusion protein into sialylated, weakly and highly polysialylated glyco-forms again under 100mM, 200mM and 1M NaCl eluting conditions. Confirmed by anti-Myc, FN1 and PSA immunoblottings and different glycosidase treatment (same results were showed in Figure 4.16 and 4.17 from Chapter 4), the glyco-complexity of the fusion protein was analysed by mass spectrometry again. As previously encountered, the highly negatively charged PSA chain associated with the fusion protein N-glycans was too labile to be measured by MALDI TOF-MS. In order to mask the negative charge from the sialic acid carboxyl group, the fusion protein was treated with o-phosphoric acid, which induced lactonization between the two adjacent Neu5Ac residues within the PSA chain. Several different acids and matrix substances for lactonization were examined in previous studies, and the current method using o-phosphoric acid and ATT (section 2.2.6.8) was suggested to offer the best mass spectra quality (Galuska et al. 2007).

**MALDI TOF-MS Analysis**

Purified colominic acid (commercially available) was initially on-target lactonized and measured by MALDI TOF-MS. In correspondence to the MALDI TOF-MS results initially showed by Galuska, et al (Galuska et al. 2007), sialic acid residues of colominic acid were measured with high resolution signal and accurate mass (Figure 5.5 and 5.6). The sensitivity of the MS signals was strongly increased when more material was used for the analysis.
Signals indicating DP more than 100 NANA residues were able to be detected with reasonably high mass accuracy when 6.25μg of colominic acid was measured under the linear mode of MALDI TOF-MS (Figure 5.5a), while the reduced amount (3ng) only supported a detection range up to approximately 70 NANA residues under the same MS settings (Figure 5.6a). In comparison with the linear mode for MALDI TOF-MS detection, previously described in Figure 4.1, reflectron mode was added to reflect ions, which focuses detection of each m/z molecule with same mass but different kinetic energies at the detector. By doing this, greatly enhanced mass resolution is achieved compared to the linear mode spectrum with consequent increased mass accuracy and reduced noise signals. Particularly shown in Figure 5.5(b), more sharp and distinguishable mass signals were illustrated in the MS spectrum in comparison with the linear mode (Figure 5.5a), and the zoom-in analytical windows clearly identified differently ionized small molecules. Shown in the inset of Figure 5.5(b), an H$_2$O molecule often associated with the sialic acid fragment was co-ionized and detected with exactly 18 Da difference to the singly ionized sialic acid fragment. Signals indicating two or more co-ionized water molecules were also clearly detected in the zoom-in analytical windows (m/z values not pointed out in Figure 5.5b inset). This detailed information cannot be distinguished from the linear mode MALDI TOF-MS detection, and only average mass information can be offered from the ion peaks. In addition, shown in Figure 5.6, the detection sensitivity, mass resolution and range were greatly reduced when considerably less (3ng) sample amount was used for the MS analysis, whilst the reflectron mode remained as producing better mass resolution and mass indications than the linear mode, and also efficiently demonstrated an average of mass increment (273 Da) reflecting a Neu5Ac (291 Da).

Polydispersive chain lengths of the commercial colominic acid were also suggested by the MS data (Figure 5.5 and 5.6). As suggested by both of the linear and reflectron modes of MALDI TOF-MS analysis, different DPs of sialic acid chains were detected. Implied by the spectral intensities of both linear and reflectron modes (Figure 5.5), normal distributions of colominic acid species were shown based on the amount of each colominic acid species (varied by sialic acid chain length). Apparently, species with DP 10 to 25 sialic acid residues existed as the majority population in the commercial colominic acid sample, and the amount was gradually reduced when the DP increased. The maximal detectable DP reached more than 100 sialic acid residues when 6.25μg colominic acid was analysed through a linear mode MALDI TOF-MS (Figure 5.5a). Linear mode offers a relatively mild condition to measure
ionized molecules flying from one side to the other within the MALDI instrument, which considerably preserves the integrity of the PSA chain structure. However, despite the enhanced detection resolution, sialylated glycans have been reported with substantial loss of sialic acid when their spectra were recorded with reflectron-TOF instrument (Papac et al. 1996; Harvey et al. 2000; Mechref and Novotny 2002). This evidence explained the reduction of PSA DP detections on the reflectron mode MALDI MS spectrum (Figure 5.5b), as the loss of sialic acid residues or physical breakage of the lactonized PSA chains could easily occur in the reflectron mode, resulting differentially fragmented PSA chain structures being measured by the MS detector.

Other factors, including acid hydrolysis of PSA during mild acid treatment for lactonization, and incompletely lactonized PSA species could also affect the sensitivity, mass accuracy and reduce the DP detections for the polysialylated glycans, even under the linear mode. MALDI TOF-MS studies on lactonized colominic acid in low temperatures (below 10°C temperature for reducing PSA hydrolysis) (Galuska et al. 2007), or through linear positive ion mode analysis (Hsu et al. 2007) suggested similar findings shown in Figure 5.5 and 5.6. To conclude, unambiguous identification of PSA species from the polydispersive colominic acid sample was determined alongside the polymer chain lengths. Maximum DP value (~100 NANA residues) was determined by the linear negative ion mode MALDI TOF-MS under the average mass indications. The sensitive reflectron mode MALDI TOF-MS analysis confirmed the poly-Neu5Ac structure indicated by the linear mode through the exact mass determinations. In general, the dispersion of the PSA species was determined with majority populations having DP between 5 to 25 NANA residues.

Successful reproduction of colominic acid MALDI TOF-MS analysis confirmed the capability of the lactonization method for analysing the PSA composition associated with the fusion protein. Previous studies suggested that the maximally detectable PSA chain length depends strongly on the amount of material used (Galuska et al. 2007). MS spectra comparison between 6.25µg and 30ng colominic acid (Figure 5.5 and 5.6) also supported this suggestion, as a much more sensitive and milder detection range was obtained from the abundant polysaccharide sample. Also documented in previous studies, purified PSA-NCAM (500ng) from postnatal mouse brain was directly on-target lactonized and analysed by MALDI TOF-MS with up to ~40 sialic acid residues contained in the associated PSA chains (Galuska et al. 2007). The same group also recorded ~55 NANA residues from the same PSA-
NCAM with roughly 100-fold of the sample amount by HPLC/FD detection (Galuska et al. 2006). Therefore, in order to minimise the material amount attributed analysis discrepancy, 100μg of MFE23-Ig5-FN1 fusion protein was used for on-target lactonization treatment prior to MALDI TOF-MS measurement. With the colominic acid detections, sensitive and unambiguous MS spectra were demonstrated by both linear and reflectron modes for the PSA chains associated with the fusion protein (Figure 5.7). Increased mass resolution with accurate m/z signal details was again revealed by the reflectron mode (Figure 5.7b inset). Indicated by the average mass signals, maximally ~40 sialic acid residues were revealed by the linear mode MALDI TOF-MS for the highly polysialylated MFE23-Ig5-FN1 fusion glyco-form (Figure 5.7a), while the reflectron mode interpreted reduced maximal DP detections (~20 sialic acid residues) again due to the instrumental interruptions (Figure 5.7b). These data also showed that the highly polysialylated MFE23-fusion protein had a comparable DP and dispersion profile with the MOLDI TOF-MS detected the PSA-NCAM extracted from mouse brain (Galuska et al. 2007).

Based on the MS detection intensities, the polydisperse profile of highly polysialylated MFE23-Ig5-FN1 fusion protein showed most of the fusion proteins had PSA DP between 5 to 20 NANA residues (Figure 5.7a). In order to avoid the physical breakage conceived by the reflectron mode MALDI TOF-MS measurement, only the linear mode detection was used for analysing the oligosialic acid chain lengths from the weakly polysialylated and sialylated fusion protein glyco-forms. Due to the limited mass accuracy, approximately less than 15 NANA residues could be detected maximally in the weakly polysialylated glycans (Figure 5.8a), and their PSA polydispersity was much reduced with most of the chain lengths containing around 5 sialic acid residues. The spectrum of sialylated fusion protein confidently revealed mass signal for four Neu5Ac chains (Figure 5.8b), while other mass signals were not able to be interpreted for sialic acid chain structures. Furthermore, the MS data also supported the SDS-PAGE data (Figure 4.16 and 4.17) that was used to define the three fusion glyco-form with different polysialylation levels.

As an experimental phenomenon, the direct release of the on-target lactonized poly-/oligo-sialic acid chains from the fusion protein glyco-peptide was encountered during MALDI TOF-MS analysis. The question remained unresolved as to whether this release was caused by acidic lactonization treatment or partially cleaved by the MALDI instrumental process, however, the same PSA release was also reported when analysing lactonized PSA-NCAM by
MALDI TOF-MS (Galuska et al. 2007; Hsu et al. 2007). Therefore, the same preparation used in Chapter 4 was performed again for understanding the N-glycan core structures from the human cell line made fusion proteins. Spectra shown in Figure 5.9 clearly indicated all available N-glycan core structures present from the fusion protein, which were similarly found from the mouse NB2a cell made fusion protein (Chapter 4, Figure 4.20 and 4.21). In contrast, human glycosylation features were expressed from the fusion proteins produced by HEK-293 cells with no Gal(α1-3)Gal glycosidic linkage detected but additional fucose linkage to the first N-acetylgalactosamine was detected in most N-glycan core structures.

A specific mutation of the NCAM FN1 domain has been engineered in previous studies, which claimed additional polysialylation based on the O-glycans from the FN1 mutate (Mendiratta et al. 2006). In order to understand the polysialylation profile of the MFE23-Ig5-FN1 fusion protein, additional MS experiments were carried out for determining the polysialylated O-glycans from the fusion protein. Chemical O-glycan release and lactonization steps were processed prior to the reflectron mode MALDI TOF-MS analysis. Spectral results showed in Appendix 14 suggested no mass signals indicating NANA repeats from the O-glycans of all three fusion protein glyco-forms.

**In vitro Antigen Binding Experiment**

The antigen binding properties of all MFE23 fusion protein were detected by: (a) Kinetic affinity constant determination using N-A1 as antigen target, and (b) live-cell binding tests on CEA antigen expressing cells.

Two major sets of experiment - ELISA and BIAcore™ were used for determine the kinetic constants of each MFE23 fusion protein. According to the ELISA results (Figure 5.10), the \( K_D \) values (~3nM) were consistent for all MFE23 fusion proteins and remained unchanged by comparing with mouse NB2a made MFE23 fusion proteins (Chapter 4, Figure 4.23). The surface plasmon resonance based BIAcore™ system was used to measure the real-time kinetic binding profiles of the MFE23 fusion proteins with separate analysis of association \( (k_{on}) \) and dissociation \( (k_{off}) \) rates, which consequently determined the affinity constant \( (K_D) \) under the equation: \( K_D = k_{off} / k_{on} \). In comparison with the ELISA determinates, the BIAcore kinetic binding models (1:1 langmuir binding) shown in Figure 5.11 suggested slightly higher \( K_D \) values of MFE23 scFv and MFE23-FN1 (~3-5-fold of the ELISA \( K_D \)), which were more close to the MFE23 scFv \( K_D \) value (~6nM) determined by BIAcore against N-A1 (Sainz-Pastor et al.)
Moreover, a course of 20 cycles were experimented for each fusion protein sample on the same N-A1 immobilized BIAcore® sensor chip. Although the 10mM HCl regeneration buffer efficiently cleared the sensor chip for the next cycle sample-run after each dissociation phase, a slight drifting baseline perhaps due to the unnecessary bindings to the antigen chip could be observed, which led to an artificially increased off-rate, and increased $K_D$ value as a consequence. Treatments such as subtracting the sample-run with an immediate blank buffer-run during the evaluation analysis (“double-reference” treatment) was used to reduce the drifting baseline effect on the kinetic parameters. Strict BIAcore instrument maintenance and cleaning procedures were also carried out to avoid additional contaminations. Furthermore, during data evaluation, 1:1 langmuir binding with drifting baseline option was also used, however the conducted the fitting models were inadequate as the resulted Chi² values were higher than 10% of the $R_{max}$ value.

In contrast to the ELISA findings which indicated consistent $K_D$ values of all MFE23 fusion proteins (Figure 5.10), the BIAcore system presented slower on-rates and eventually higher $K_D$ values of the polysialylated MFE23-Ig5-FN1 fusion proteins. The high negative charge on the BIAcore® sensor chip surface (due to the free carboxyl groups within its carboxymethylated dextran matrix) was speculated to cause the hindrance to the association of the polysialylated MFE23 fusion proteins which also possess negatively charged PSA. As illustrated in Figure 5.21A, the electric repulsion between the two negative charge clusters – PSA associated with the MFE23 fusion protein and the SA sensor chip surface, could cause the slowing down of the interaction rate between MFE23 and N-A1. The off-rates listed in Table 5.1 of all MFE23 fusion proteins behaved consistently comparing to each other, which suggested the alterations of affinity constant were focused on the association phases, and additional potential reasons are hypothesized in Figure 5.21 to explain the decrease in apparent on-rates of the polysialylated fusion proteins.

The potential on-rate reducing factors such as Figure 5.21B, C and D were also suggested by the binding kinetics of PEGylated proteins (Kubetzko et al. 2005). The BIAcore kinetic analysis of site-specific PEGylated (20 PEG residues) scFv fragment indicated ~6-fold slower on-rate in comparison with the scFv alone (Kubetzko et al. 2005). In our case, the highly polysialylated MFE23-Ig5-FN1 had ~16-fold reduced on-rate compared to the MFE23 scFv alone. This was potentially due to the addition of extra PSA chains recombinantly
Figure 5.21. Potential reasons for the decrease in the apparent on-rates of the polysialylated scFv-fusion proteins from SPR based BIAcore system. In principle, there are several factors that could reduce the on-rates of polysialylated scFv fusion proteins. A. the presence of electric repulsion force ($F$) that generated by the negatively charged PSA possessions of the polysialylated scFv fusion proteins and the BIAcore sensor chip surface. This electric repulsion force could also happen between the adjacent polysialylated fusion proteins affecting the kinetic binding rates and the antigen sites (1+ n) occupied on the chip surface. B. The PSA chains if there were long enough could intramolecularly block the binding region temporarily with rate constants ($k_1$ and $k_{-1}$) for switching polymer conformation between binding-block and binding-unblock status. C. Translational ($k_t$) and rotational ($k_r$) diffusion limitations would slow the molecule association rate, especially after polysialylation, which potentially increases the apparent molecular size and slightly slows down the $k_t$ and $k_r$ rates in real-time binding. D. Polysialylated fusion protein in antigen bound state could temporarily mask other antigen sites (n) through its PSA tails, which may intermolecularly hinder the antigen binding of other scFv fusion proteins to these sites. Figure was modified from (Kubetzko et al. 2005).
conjugated to the fusion protein with electric repulsions mentioned above and illustrated in Figure 5.21A. The off-rate is concentration independent, and not affected by the factors shown in Figure 5.21 during BIAcore analysis. Referring to the BIAcore kinetic results shown in Table 5.1, decreased $k_{on}$ values were found from the scFv-Ig5-FN1 fusion proteins, but their $k_{off}$ values were kept relatively consistent in comparison with the MFE23 scFv. Shown in Figure 5.12, further BIAcore studies where PSA was removed from the fusion protein using Endo-N treatment restored the antibody-antigen binding on-rate and the $K_D$ value by comparing with the scFv kinetic results (Table 5.1), which further confirmed the effect of the polymeric glycosylation on reducing the scFv-fusion protein binding on-rates. In order to determine the PSA negative charge effect to the antibody-antigen binding kinetic parameters, a low or non-charged BIAcore sensor chip with alternative antigen immobilization method should be tested. Overall, with the kinetic binding results so far, the on- and off-rates and $K_D$ values of all MFE23-fusion proteins were significantly evaluated and less than 10-fold affinity reduction was determined to the highly polysialylated MFE23-Ig5-FN1 fusion protein. Although the highly negatively charged BIAcore sensor chip may exaggerate the electric repulsion hindering the association of the polysialylated scFv-fusion proteins, this gives us an insight into the biophysical affects of the charged PSA and highlights the need to carry out physiological binding studies on live cells.

The understanding of the MFE23-fusion proteins antigen-binding profiles under live cell conditions was determined by FACS and CLSM mediated by specific detecting antibodies. Based on the nature of CEA, a cell-surface expressing antigen, colonic cancer cell lines, such as LS174T and LoVo cells were used to express native CEA antigen, which can be targeted by the MFE23 fusion proteins. Shown in Figure 5.13(a), the anti-Myc mediated FACS detections indicated live cell antigen targeting by all MFE23 fusion proteins at the same saturating concentration (0.2μg/μl or ~3mM). However, slightly reduced curve shifts from the un-targeted cell population, which were also indicated by the mean fluorescent intensity values of the three N-glycosylated MFE23-Ig5-FN1 fusion isoforms might suggest some degree of inefficiency of antigen targeting to live cells. The potential reasons for decreased binding efficiencies can also be explained by Figure 5.21, which cause the polysialylated fusions slower on-rates in the BIAcore system. In Figure 5.13(b), only the anti-PSA antibody mediated FACS revealed significant positive shift from the highly polysialylated fusion as expected. However, rather than a distinguishable narrow cell population, the targeted cell populations detected by anti-PSA antibody were very broad. This could possibly be caused by
the polydispersity of the PSA chain lengths, which may alter the targeting ratio between the anti-PSA antibody and the PSA chains; and conformational variations and water molecule masking of the PSA chains in physiological buffer conditions, which may make the secondary antibody binding more difficult.

The anti-Myc antibody mediated fluorescent confocal microscopic images showed the same cell surface antigen binding profile for all MFE23 fusion proteins (Figure 5.14-5.16a), suggesting the polysialylated fusion proteins have comparable antigen localization capacity to the scFv. Particularly revealed by the z-stack images, after different post-targeting incubation periods (10 minutes and 2 hours) and temperatures conditions (4°C and 37°C), similar binding and cell surface localization profiles were demonstrated by all MFE23 fusion proteins. Potentially having the same issues seen in the FACS experiment, the anti-PSA mediated fluorescent CLSM images appeared to be weakly detected, but indicated typical CEA cell surface binding throughout the entire cell z-stack imaging by the polysialylated MFE23-Ig5-FN1 fusion proteins (Figure 5.16b).

**Molecular Size**

The concept of using water-attracting polymers to increase the apparent size has already been introduced with PEGylated proteins (Figure 1.13). Using PSA, an organic polysaccharide, polysialylation followed this concept and successfully proved a prolonged blood circulation period and improved pharmacokinetics through chemical conjugations to various proteins (section 1.3.1.3). As an alternative polysialylation approach, the apparent molecular size of recombinantly polysialylated MFE23-Ig5-FN1 fusion protein was studied by running it through a size exclusion column under physiological (1xPBS, pH 7.4) buffer conditions.

Calibrated by MW markers (Figure 5.18a), the size differences of different MFE23-fusion proteins and glyco-forms were clearly demonstrated from the SEC (Figure 5.18b). Only differing in PSA chain length, the three MFE23-Ig5-FN1 glyco-forms showed significantly distinctive elution profiles, and supported the theory of the highly polysialylated glyco-form drawing the largest hydrodynamic volume, followed by the weakly polysialylated and lastly the sialylated MFE23-Ig5-FN1 fusion protein. Rather than one predominate elution peak from the SEC shown in Figure 5.18(b), a secondary elution peak (labelled as 2) suggesting a larger molecular size was found for all MFE23 fusion proteins except the highly polysialylated MFE23-Ig5-FN1 glyco-form. Under physiological buffer conditions, these secondary peaks
were speculated as being dimers (or multimers) of each MFE23 fusion protein species, which were presumably caused by the cross-linking between two MFE23 scFvs, as the MFE23 scFv has been reported previously to form homogenous dimers (Verhaar et al. 1996; Boehm et al. 2000; Lee et al. 2002; Constantinou et al. 2009). This hypothesis was further developed by running all the peak fractions through reducing SDS-PAGE, which resolves the dimers into monomers. Shown in Figure 5.19, anti-Myc and anti-PSA immunoblots and Coomassie protein staining revealed identical results between the two peak eluates collected according to the SEC shown in Figure 5.18(b), which confirmed that peak 2 (earlier SEC elution peak) was a homogenous multimer formation of the MFE23 moiety. Compared to the other fusion species, the highly polysialylated fusion protein, however, showed a different gel filtration profile with a wider eluting volume range (Figure 5.18) indicating the polydispersive distribution of its apparent size caused by the differential PSA chain lengths associated. The SEC peaks suggested that most of the highly polysialylated fusion proteins drew the largest apparent size (peak 1 red), while the rest showed gradually smaller sizes until the smallest indicated as the peak 2 (red). The anti-PSA immunoblot (Figure 5.19) also demonstrated that higher MW was reached by the peak 1 eluate than the peak 2 eluate of the highly polysialylated fusion protein. Worth noticing was that the smallest apparent size lay out by the highly polysialylated fusion protein was still found to be larger in size than the monomeric weakly polysialylated fusion glyco-form (peak 1 pink, Figure 5.18b), which additionally confirmed efficient anion exchange separation of the polysialylated fusion protein glyco-species from the previous purification stages.

Another way to interpret the SEC measurement is to convert the proteins elution peaks corresponding in size to apparent molecular masses. Based on the standard conversion curve (y = -0.0343x + 4.7245, where y = logMW app and x = elution volume) plotted by the SEC standard MW markers (Figure 5.20), the average MW of a molecule could be calculated corresponding to its SEC elution volume (within the analytical gel filtration column measuring limit). Listed in Table 5.2, the SEC apparent MW conversions of each MFE23 fusion proteins were calculated according to their elution profiles and shown in Figure 5.18(b). Apart from the highly polysialylated fusion protein, the MW app s of other proteins’ minor peaks (peak 2) showed their ratios to the corresponding peak 1 indicated MW app s resulted in approximately a doubled multiple, which again suggests the possible dimerization formation of these fusion proteins. Based on the average MW app s of each monomeric fusion protein, the corresponding molecular sizes of each molecule can be directly compared following the
mathematical equation: \( V = \frac{m}{\rho} \), where \( V \) is the volume (or molecular size), \( m \) is the mass (or MW\text{app}), and \( \rho \) is the average density. Assuming the molecules being compared were all proteins as material, so they have the same average density, and their size comparison will be equally the comparison between their MW\text{app}s. In order to compare the hydrodynamic radius, a sphere shape assumption for each protein molecule under physiological buffer conditions was made, so that the hydrodynamic radius \( r \) of each sphere can be calculated based on the formula:

\[
V = \frac{4}{3} \pi r^3 \quad \Rightarrow \quad r = \sqrt[3]{\frac{3}{4\pi} V} = \sqrt[3]{\frac{3}{4\pi} m \rho}
\]

Therefore, the comparison of the hydrodynamic radius of the fusion protein \( (r_f) \) against the hydrodynamic radius of LS-MFE23 scFv \( (\Theta) \) can be calculated as:

\[
\frac{r_f}{\Theta} = \sqrt[3]{\frac{\frac{3}{4\pi} m_f \rho}{\frac{3}{4\pi} m_\Theta \rho}} = \sqrt[3]{\frac{m_f}{m_\Theta}}
\]

where \( m_f \) stands for the MW\text{app} of the MFE23 fusion proteins, and \( m_\Theta \) stands for MW\text{app} of the LS-MFE23 scFv.

With the MW\text{app} values availably presented in Table 5.2, the hydrodynamic radii of each fusion protein can then be interpreted as ratios of the hydrodynamic radius of LS-MFE23 scFv \( (\Theta) \), and the great circles implying each fusion protein spherical size are therefore shown in Figure 5.22(a) below.

Based on the prediction (Figure 5.22a), the maximal 2.94-fold hydrodynamic radius improvement was made by the polysialylated MFE23-Ig5-FN1 fusion protein in comparison with the MFE23 scFv. Although the apparent shapes of each fusion protein might not appear to be a complete sphere, the analytical postulations shown in Figure 5.22 illustrate the average proportional comparisons against the scFv molecules in terms of the apparent size differences under physiological aqueous conditions. Schematically explained in Figure 5.22(b), the effectiveness of molecular size enhancements after recombinant polysialylation of the MFE23 fusion proteins induced by the increased hydrodynamic volume from the water attracting PSA molecules has been clearly demonstrated by the SEC experiments. Based on these results, size-related improvement of antibody fragment pharmacokinetics including blood circulation
longevity and kidney elimination rate after intravenous injection (i.v.) were expected from the consequent in vivo studies.

Figure 5.22. Postulated schematic illustrations of MFE23 fusion protein apparent hydrodynamic radii. Molecules were postulated as sphere shape in aqueous condition. (a) Hydrodynamic radii indicated in great circles of each protein spheres. MFE23-FN1 (blue), sialylated (green), weakly polysialylated (pink) and highly polysialylated (red) MFE23-Ig5-FN1 fusion protein hydrodynamic radii were calculated and indicated as multiplications of MFE23 scFv (black) hydrodynamic radius (Ø). Due to the polydispersive degree of polysialylation, the dotted and solid red circles outlined the apparent size range the highly polysialylated MFE23-Ig5-FN1 induced. (b) Schematic comparison of apparent size differences in proportion to the hydrodynamic radius increments. Hydrodynamic volumes are interpreted by light blue circles masking the embedded proteins.
CHAPTER 6

*In vivo* Pharmacokinetic Studies of Recombinantly Polysialylated MFE23 scFv Fusion Proteins
6.1 Introduction

A series of SEC experiments described in Chapter 5 suggested an incremental relationship between polysialylation and hydrodynamic radius. Caused by the hydrophilicity and water attraction of the associated PSA molecules, different hydrodynamic volume sizes were also suggested by SEC based on variations from the PSA chain length (sialylated, weakly and highly polysialylated scFv-fusion proteins). After estimating the increase of the hydrodynamic radii of the polysialylated scFv-fusion proteins in comparison with the scFv, further understanding of how size affects the renal ultrafiltration, and protein blood clearance in live animal models was the next logical step.

As already mentioned in section 1.2.2.3, renal ultrafiltration is one of the main routes that small molecules, such as scFvs, is eliminated from the blood circulation (within hours after iv injection) (Tang et al. 2004; Mahmood and Green 2005). In the kidneys, glomerular permeability is selectively restricted by a double barrier constituting a basement membrane that primarily functions as a coarse filter, and an epithelial slit diaphragm that operates at a more distal site in the capillary wall as a fine screen (Deen et al. 1979; Caliceti and Veronese 2003). Despite other factors that kidneys utilize for permeaselectivity, such as charge and glycosylation, the glomerular capillary wall can simply be considered as a porous filter capable of size-selective discriminations. Having a MW cut-off of about 70 kDa for eliminating native globular proteins from kidney, which is close to the MW of serum albumin, macromolecules possessing effective sizes larger than the size of a 70 kDa protein or albumin, will avoid the ultrafiltration and return into the bloodstream (Deen et al. 1979; Rabkin and Dahl 1993).

Although renal clearance of macromolecules usually refers to their MW, it does not accurately define the effective size of macromolecules. Due to the compact and globular structure of proteins, the blood half-life of a protein, with its coiled and extended conformation, can be the same as a hydrophilic polymer with a much lower MW. For example, cytochrome c (13 kDa) displays the same hydrodynamic radius (2nm) as a 5 kDa PEG, and shares the same blood half-life of 18 minutes (Nakaoka et al. 1997; Sherman et al. 1997). Hydrophilic polymers, such as PEG and PSA have been effectively used to increase the antibody fragment hydrodynamic volume with minimum MW increase, and with improved pharmacokinetics, have eventually prolonged protein blood circulation time (Caliceti and
Veronese 2003; Constantinou et al. 2009). Other than knowing the MW cut-off for kidney elimination, previous studies also reported that the pore size of the glomerular basement membrane is in the range of 3-5 nm (Nakaoka et al. 1997), which more precisely defines the minimum hydrodynamic radius for macromolecules to avoid being sieved-off by the glomerular filtration. Furthermore, Yamaoka et al. studied the effect of MW on the time course of PEG elimination from blood. They concluded that PEGs smaller than 8 kDa cleared very quickly without any restriction via kidney ultrafiltration, whereas the elimination of PEGs in the range of 8-30 kDa was controlled by the apparent size difference. Lastly for PEG MW over 30 kDa, the blood clearance was slowed down and no significant difference could be observed (Yamaoka et al. 1994; Yamaoka et al. 1995). Nakaoka et al. consequently revealed that the 30 kDa PEG has a hydrodynamic diameter of about 8nm which indirectly outlines a size cut-off for kidney elimination as compared to its pore size (Nakaoka et al. 1997).

6.2 Aims and Objectives

In order to investigate on the in vivo blood clearance effect by the recombinantly polysialylated scFv-fusion protein, the laboratory-bred BALB/c mouse, the most widely used inbred strains used in animal experimentations was used for intravenous injections of radio-labelled protein samples through the tail vein. Blood samples at different post-injection time points were collected for analysing the clearance rates as well as tissue distribution studies.

The objectives are:

■ Determining the blood clearance rates of LS-MFE23 scFv, sialylated and highly polysialylated MFE23-Ig5-FN1 fusion proteins by measuring the radioactivity from of the collected mouse blood samples at different time points after intravenous injections.

■ Biodistribution analysis of the scFv and scFv-fusion proteins at 6 hours and 24 hours after in vivo intravenous injections, including the average radioactivity measurement of major tissues and the radioactivity ratio against the blood content for non-specific binding investigations.
6.3 Results

6.3.1 *In vivo* Blood Clearance Studies

In order to ascertain the effect of the recombinant MFE23 fusion protein and its polysialylated isoform on scFv blood clearance, laboratory-bred normal female BALB/c mice were injected with 300μl (~10μg) \(^{125}\)I-labelled LS-MFE23 scFv, sialylated MFE23-Ig5-FN1 and highly polysialylated MFE23-Ig5-FN1 fusion proteins respectively (section 2.2.7.1) intravenously into the tail veins (section 2.2.7.2). Collected according to different post-injection time points (0.5, 1, 2, 4, 6, 24 hours) by microhematocrit capillary tubes (section 2.2.7.3), blood sample radio-activity was measured by a gamma counter and analysed after correction for the same weight of blood (Figure 6.1).

![Figure 6.1. Blood clearance of different MFE23 fusion proteins.](image)

Comparing blood clearance curves between the three MFE23 recombinant fusions (Figure 6.1), the polysialylated MFE23-Ig5-FN1 fusion protein clearly showed the slowest blood
clearance profile and prolonged residence time within the body circulation. The sialylated fusion protein showed a comparatively steeper slope of the β phase to the polysialylated, suggesting quicker blood clearance rate, however it is clearly found to be much slower than the scFv within the course of 24 hours. A much slower redistributional α phase was also found for the highly polysialylated fusion protein than the sialylated and scFv. In addition, the protocol used for over-oxidizing the fusion proteins through radio-iodination by mixing with activated iodide in the Pierce® Pre-Coated Iodination Tubes (section 2.2.7.1) could potentially change the physiochemical property of PSA, hence its water-attracting capacity. This is because the iodination tubes are coated with the iodination reagent (1,3,4,6-tetrachloro-3α, 6α-diphenylglycouril; formerly called “IODO-GEN” Iodination Reagent), which is an effective oxidizer (Saha et al. 1989; Bailey 1996). As shown in Figure 6.1, a much less significant blood clearance effect was found for the highly polysialylated MFE23-Ig5-FN1 that was over-oxidized in comparison with its more gentle labelled form. Based on Figure 6.1, more precise statistical analysis of the fusion protein blood clearance profiles are listed in Table 6.1.

<table>
<thead>
<tr>
<th>Fusion proteins</th>
<th>t₁/₂α (hr)</th>
<th>t₁/₂β (hr)</th>
<th>Increase of t₁/₂β (fold)</th>
<th>AUC (% hour/gram)</th>
<th>Increase of AUC (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MFE23 scFv</td>
<td>0.301 ± 0.188</td>
<td>2.658 ± 0.438</td>
<td>1.0</td>
<td>19.791</td>
<td>1.0</td>
</tr>
<tr>
<td>Sialylated MFE23-Ig5-FN1</td>
<td>0.353 ± 0.067</td>
<td>4.784 ± 1.100</td>
<td>1.8</td>
<td>37.856</td>
<td>1.9</td>
</tr>
<tr>
<td>Highly PolySia MFE23-Ig5-FN1</td>
<td>1.474 ± 2.320</td>
<td>39.383 ± 7.265</td>
<td>14.8</td>
<td>519.140</td>
<td>26.2</td>
</tr>
<tr>
<td>Highly PolySia MFE23-Ig5-FN1 (over-oxidized)</td>
<td>0.441 ± 0.124</td>
<td>8.004 ± 2.189</td>
<td>3.0</td>
<td>54.501</td>
<td>2.8</td>
</tr>
</tbody>
</table>

**Table 6.1. Summary of Pharmacokinetic Parameters.** Based on the blood clearance profiles shown in Figure 6.1, the blood clearance profile was fitted to a biexponential decay model and t₁/₂α and t₁/₂β were determined. The bioavailability or blood exposure was determined by calculating the area under the blood clearance curve (AUC).

As listed in Table 6.1, short half lives of the α phase (t₁/₂α) were displayed by both MFE23 scFv (~18 minutes) and the sialylated MFE23-Ig5-FN1 fusion protein (~21 minutes) before venturing off into different tissues. Considerably improved, the highly polysialylated MFE23 fusion protein showed a much reduced clearance rate with longer t₁/₂α (~5-fold comparing with the scFv). In terms of blood circulation time, the highly polysialylated fusion protein
demonstrated a significantly increased half life of the elimination phase \((t_{1/2\beta})\), which was found to be \(~15\)-fold higher than the scFv that only appeared to be maintained in the body around 2 and half hours. The sialylated fusion protein also showed moderately improved blood circulation time, but only \(~1.8\)-fold enhancement was measured in comparison with the scFv. Also shown in Table 6.1, the AUCs indicating the bioavailability of the three MFE23 recombinant fusions were analysed according to the blood clearance profile shown in Figure 6.1. The AUC value of the highly polysialylated fusion protein was approximately 26-fold of that of the scFv and 14-fold of the sialylated fusion. This suggests that a high proportion of polysialylated fusion species were kept in the body without being eliminated within 24 hours due to the slowest blood clearance rate of the out of the three recombinant fusions. Already illustrated from Figure 6.1, the over-oxidized PolySia MFE23-Ig5-FN1 appeared to ineffective compared to the fusion protein under the normal radio-labelling process, and it had almost the same \(t_{1/2\alpha}\) as the sialylated MFE23 fusion protein. An inefficient blood circulation time was also found as only 3-fold increase of \(t_{1/2\beta}\) and 2.8-fold improvement of AUC were demonstrated by the over-iodinated PolySia MFE23-Ig5-FN1 compared to the scFv.

6.3.2 In vivo Biodistribution Analysis

In order to investigate the tissue distribution of the three MFE23 recombinant fusions after sample injection, mice culled at 6 hours and 24 hours were dissected, and the radioactivity of six different tissues including spleen, kidney, liver, heart, lung, and gastrointestines alongside blood collections were measured. The results (outlined in Table 6.2) are presented as the percentage of the radioactivity out of the injected dose (ID) against the weight (gram) of the measured tissue and compared in Figure 6.2.

Compared in Figure 6.2, the blood contents of the three MFE23 recombinant fusions follow the clearance profile shown in Figure 6.1, as more polysialylated fusions were kept in the circulation than the sialylated that was left more than the scFv at both 6 hours and 24 hours post-injection time points. Clear contrasts are demonstrated for each protein blood level between 6 hours and 24 hours, and more than 92% scFv was eliminated during the two time points, while \(~84\%\) and \(~82\%\) reductions were found for the sialylated and polysialylated fusion proteins (Table 6.2)
Table 6.2. Biodistribution of MFE23 scFv, sialylated and over-oxidized highly polysialylated MFE23-Ig5-FN1 fusion proteins in mice at 6 and 24 hour post-injection time points. Normal BALB/c mice were culled at 6 and 24 hour time point after i.v. The average radioactivities of each tissue were measured, and the results were displayed as mean values ± standard deviations (SD) of four mice.

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Blood</th>
<th>Spleen</th>
<th>kidney</th>
<th>Liver</th>
<th>Heart</th>
<th>Lung</th>
<th>GI</th>
</tr>
</thead>
<tbody>
<tr>
<td>MFE23 scFv</td>
<td>6</td>
<td>1.04 ±0.57</td>
<td>0.89 ±0.47</td>
<td>3.07 ±1.45</td>
<td>0.79 ±0.75</td>
<td>0.61 ±0.37</td>
<td>2.60 ±2.27</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.08 ±0.01</td>
<td>0.14 ±0.03</td>
<td>0.84 ±0.13</td>
<td>0.16 ±0.06</td>
<td>0.05 ±0.01</td>
<td>0.31 ±0.09</td>
</tr>
<tr>
<td>Sia MFE23-Ig5-FN1</td>
<td>6</td>
<td>1.65 ±0.40</td>
<td>0.67 ±0.15</td>
<td>1.87 ±0.77</td>
<td>0.83 ±0.24</td>
<td>0.68 ±0.18</td>
<td>1.49 ±0.50</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.27 ±0.06</td>
<td>0.12 ±0.05</td>
<td>0.78 ±0.39</td>
<td>0.13 ±0.08</td>
<td>0.12 ±0.05</td>
<td>0.32 ±0.16</td>
</tr>
<tr>
<td>PolySia MFE23-Ig5-FN1 (over-oxidized)</td>
<td>6</td>
<td>2.83 ±0.71</td>
<td>1.39 ±0.49</td>
<td>3.54 ±2.62</td>
<td>1.20 ±0.90</td>
<td>0.95 ±0.06</td>
<td>1.60 ±0.32</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.51 ±0.18</td>
<td>0.47 ±0.26</td>
<td>2.55 ±0.61</td>
<td>0.53 ±0.33</td>
<td>0.15 ±0.05</td>
<td>0.35 ±0.06</td>
</tr>
</tbody>
</table>

Figure 6.2. Biodistribution analysis of MFE23 scFv, sialylated and over-oxidized highly polysialylated MFE23-Ig5-FN1 fusion proteins. This bar-chart directly interprets the data listed in Table 6.3.

The biodistribution data shown in Table 6.2 can also be evaluated as the tissue to blood ratios. Based on the values shown in Table 6.3, if the ratio found is less than one, it means that a higher protein sample level remains in the blood than the specific tissue. Alternatively, if ratio is more than 1, an accumulation of the protein content in specific tissues can be explained, or
the blood clearance rate is faster than the protein transporting rate from tissues back to the circulation system. Displayed graphically in Figure 6.3, despite the kidney, in other tissues both sialylated and polysialylated fusion proteins showed tissue/blood ratios of less than one at both 6 hours and 24 hours after injection, suggesting a higher blood content due to the reduced clearance rate. The scFv showed spleen, liver and lung to blood ratios more than 1 particularly at the 24 hours time point, indicating a rapid blood elimination rate. For the kidney, all three recombinant fusion proteins showed kidney/blood ratios more than 1 at 24 hours. As the site where small molecules are being filtered out from the body, accumulations of the protein samples were indicated, especially the scFv which showed closely to a value of 10 for this ratio, while the polysialylated fusion protein only had a ratio value of 5, suggesting a prolonged period of the sample circulating in the blood. In addition, free iodines degraded from the conjugated protein are quickly eliminated from the circulation, leading to a relatively high radioactivity measurement in the kidney at early time points.

Overall, all these data suggested that the MFE23 fusion proteins did not produce non-specific tissue bindings. However, the biodistribution study for the polysialylated MFE23-Ig5-FN1 fusion protein after normal radio-labelling is still needed in order to understand the non-specific tissue binding profile of biosynthesized PSA, as the polysialylated MFE23 fusion proteins used in the biodistribution were over-oxidized during iodination.

Table 6.3. Mouse tissue/blood ratios of MFE23 scFv, sialylated and highly polysialylated MFE23-Ig5-FN1 fusion proteins at 6 and 24 hour post-injection time points. Results were displayed as mean values ± standard deviation (SD) of four mice.

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Spleen</th>
<th>Kidney</th>
<th>Liver</th>
<th>Heart</th>
<th>Lung</th>
<th>GI</th>
</tr>
</thead>
<tbody>
<tr>
<td>MFE23 scFv</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.86 ±0.45</td>
<td>2.95 ±1.39</td>
<td>0.76 ±0.72</td>
<td>0.58 ±0.36</td>
<td>2.50 ±2.19</td>
<td>0.87 ±0.43</td>
</tr>
<tr>
<td>24</td>
<td>1.75 ±0.33</td>
<td>9.97 ±1.58</td>
<td>1.94 ±0.70</td>
<td>0.61 ±0.16</td>
<td>3.69 ±1.04</td>
<td>0.35 ±0.05</td>
</tr>
<tr>
<td>Sia MFE23-Ig5-FN1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.41 ±0.09</td>
<td>1.14 ±0.47</td>
<td>0.51 ±0.14</td>
<td>0.41 ±0.11</td>
<td>0.91 ±0.30</td>
<td>0.35 ±0.15</td>
</tr>
<tr>
<td>24</td>
<td>0.45 ±0.20</td>
<td>2.83 ±1.44</td>
<td>0.46 ±0.31</td>
<td>0.45 ±0.17</td>
<td>1.17 ±0.57</td>
<td>0.24 ±0.10</td>
</tr>
<tr>
<td>PolySia MFE23-Ig5-FN1 (over-oxidized)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.49 ±0.17</td>
<td>1.25 ±0.92</td>
<td>0.42 ±0.31</td>
<td>0.34 ±0.02</td>
<td>0.55 ±0.11</td>
<td>0.52 ±0.31</td>
</tr>
<tr>
<td>24</td>
<td>0.92 ±0.51</td>
<td>5.01 ±1.20</td>
<td>1.05 ±0.64</td>
<td>0.30 ±0.10</td>
<td>0.68 ±0.13</td>
<td>0.16 ±0.07</td>
</tr>
</tbody>
</table>
Figure 6.3. Mouse tissue/blood ratios of MFE23 scFv, sialylated and highly polysialylated MFE23-Ig5-FN1 fusion proteins at 6 and 24 hour post-injection time points. This bar-chart directly interprets the data listed in Table 6.3. A red dashed line outlines the tissue/blood ratio equals to 1.

6.4 Discussion

Based on the hydrodynamic volume analysis using size exclusion chromatography (described in section 5.3.7), protein samples (LS-MFE23 scFv, sialylated MFE23-Ig5-FN1 and highly polysialylated MFE23-Ig5-FN1 fusion proteins) with in vitro apparent size discriminations were radio-labelled and intravenously injected into healthy laboratory-bred BALB/c mice for blood clearance and in vivo biodistribution studies.

Due to the size-selective function of animal kidney, rapid circulation elimination was found by the scFv molecule (eliminated within 1 day), which was also reported by previous investigations (Verhaar et al. 1995; Adams et al. 1998). As expected, having increased hydrodynamic volumes, the sialylated and polysialylated scFv-fusion protein indicated significantly delayed blood clearance rates, where the latter was found to be more slowly eliminated ($t_{1/2}\beta$) with longer bioavailability (AUC) within the mouse body (Figure 6.1 and Table 6.1). Mentioned in the introduction of this chapter, it is very important to understand
the actual size outlines of the MFE23 fusion proteins in comparison with the size cut-off for kidney elimination and how this correlated to the protein pharmacokinetic behaviours obtained from the in vivo studies. Previous study showed that human serum albumin (HSA) had only a minor difference between apparent MW from SEC and its calculated MW (66 kDa) (Stork et al. 2008). With the knowledge of the hydrodynamic radius of HSA (3.5nm) (Stork et al. 2007), the hydrodynamic radius of LS-MFE23 scFv (Ø) can be estimated following the equation introduced in the discussion section of Chapter 5:

\[
\frac{r_f}{\phi} = \sqrt[3]{\frac{m_f}{m_\phi}} \quad \Rightarrow \quad 3.5\text{nm} = \sqrt[3]{\frac{66\text{ kDa}}{29\text{ kDa}}} \quad \Rightarrow \quad \phi = 2.661\text{ nm}
\]

where the \(r_f\) and \(m_f\) values are the hydrodynamic radius and SEC apparent MW of HSA, and the MW\(_{\text{app}}\) of LS-MFE23 scFv (\(m_\phi\)) is obtained from Table 5.2.

Consequently, following the statistical comparison showed in Figure 5.22(a), the hydrodynamic radius can be calculated as \(~4.3\text{nm}\) for the sialylated MFE23-Ig5-FN1 fusion protein, and within the range of \(5.4 - 7.8\text{nm}\) for the highly polysialylated fusion. Clearly, both fusion glyco-forms exceeded the glomerular membrane pore size, whereas the polysialylated fusion also greatly surpassed the size cut-off limit (radius: \(~4\text{nm}\)) for kidney elimination. These findings correlate to the results from in vivo PK studies listed in Table 6.1, and explained the increases in \(t_{1/2\alpha}\) and overall AUC from the scFv to the polysialylated fusion protein.

Although studies on PEGs possessing hydrodynamic volumes exceeded the size cut-off for kidney elimination, they did not show a significant blood clearance difference between each other (Yamaoka et al. 1994; Yamaoka et al. 1995). Hydrolysis of biodegradable material (i.e. PSA) and the protein itself will lead to a gradual size reduction process until it becomes smaller than the size cut-off limit and is eliminated more rapidly through glomerular filtration. Therefore, for protein-polymer conjugates having an apparent size more than the kidney elimination cut-off, in general the longer of the associated polymer, the slower the blood elimination rate (\(t_{1/2\beta}\)) will become, alongside longer circulation exposure time. Also supported by PEGylated proteins (Knauf et al. 1988; Caliceti and Veronese 2003), and chemically polysialylated antibody fragments (Constantinou et al. 2008; Constantinou et al. 2009), proteins conjugated with higher MW of hydrophilic polymers resulted in significantly increased apparent sizes and prolonged residence in the blood. These polymers used for chemical conjugation were composed in linear forms, and their MW increases directly
correlated with the prolongations of polymeric chain length or DP. The *in vivo* experiments (Figure 6.1) using two fusion proteins with different degree of polymerization (DP) consequently indicated the importance of the polymer chain length in delaying the protein blood clearance rates. The DPs of sialylated and highly polysialylated MFE23-Ig5-FN1 fusion proteins were determined by MALDI TOF-MS previously, where average of approximately 4 and 20 sialic acid repeats were detected respectively (Figure 5.7 and 5.8). Therefore, statistically the highly polysialylated fusion protein had DP ~5-fold of the sialylated fusion protein, which led to its *in vitro* hydrodynamic radius ~3.8-fold of the MFE23 scFv (Figure 5.22) and ~26-fold of the scFv’s blood AUC. In comparison, the chemically conjugated MFE23 scFv with single 11 kDa (DP~37 NANA residues) has been previously reported to increase the blood AUC to ~10-fold of the scFv, as well as ~1.7-fold and 3.6-fold increase of *in vivo* $t_{1/2\alpha}$ and $t_{1/2\beta}$ respectively (Constantinou et al. 2009). It is then noticeable that the highly polysialylated MFE23 fusion protein gains additional Ig5 and FN1 NCAM-domains and one extra N-glycosylated PSA chain, leading to potentially a larger hydrodynamic volume and better AUC than the site-specific chemical-polysialylated MFE23 scFv.

Additionally, the negative charge masking from the PSA can also alter protein ultrafiltration through the kidney. Based on the structure of glomerular basement membrane, expression of very proximal sialoprotein-rich elements in the membrane epithelium and lamina rara interna predominately retard the filtration process of the circulating polyanions, whereas the more distal elements found in the lamina rara externa of the glomerular basement membrane retard the clearance of cationic macromolecules (Deen et al. 1979; Rabkin and Dahl 1993). Moreover, additional studies suggested that the anionic macromolecules appeared to be cleared by renal ultrafiltration more slowly than neural or positive ones (Takakura et al. 1990). Therefore, the negative property of the associated sialic acid can be more beneficial for prolonging protein stability in the bloodstream, and enhancing this negative charge possession (i.e. increased PSA DP) can be another potential factor to slow down the protein blood clearance rate. Other factors, such as polymer flexibility, conformation, and deformability, alongside the hydrolysis rate of polysialylated fusion protein, could also affect the protein kidney elimination profiles (Caliceti and Veronese 2003; Olafsen et al. 2004).

The biodistribution studies of the three MFE23 recombinant fusions showed rather similar results, suggesting the recombinant fusion of the polysialylated NCAM domains to the MFE23 scFv did not effect the antibody fragment localization within animal body tissues.
after injection. Especially suggested by Figure 6.3, the kidney/blood ratios of the polysialylated fusion protein were found to be lower than the scFv, indirectly indicating a slower clearance rate, as less polysialylated fusion content was found trapped in the kidney during the clearance process comparing too the scFv. In order to investigate the in vivo antigen targeting profile of the fusion proteins, further biodistribution studies can be made on mice bearing CEA expressing tumours. Properties including high tumour specific localization, prolonged tumour retention time and good tumour/blood ratio of the polysialylated MFE23-Ig5-FN1 fusion protein are expected in comparison with the MFE23 scFv.

The over-oxidization of the PSA from fusion proteins could possibly occur when radio-labelling the fusion protein with excessive iodination reagent (“IODO-GEN”), which is an effective oxidizer. This modification has been showed to reduce the PSA capacity for increasing the hydrodynamic radius (Figure 6.1 and Table 6.1). Shown in Figure 5.1, lactonized PSA as one major product can be easily formed from acid-catalysed oxidation process (Zhang and Lee 1999), and the hydrophilicity of lactonized PSA has not been reported so far. Although the normal iodination process avoided direct contact of the polysialylated fusion protein with the iodination tube (section 2.2.7.1), a lower degree of PSA oxidation can still occur during the radioactive iodine labelling process, which can affect the PK modulation performance from the polysialylated species. For improvements, alternative radio-labelling methods that avoid any potential physiochemical changes to the PSA and protein should be considered. In addition, it will be important to investigate the non-specific binding profile of the biosynthesized PSA from the polysialylated fusion protein, as only the potentially over-oxidized PSA fusion forms were used in the biodistribution studies.
CHAPTER 7

Generation of Recombinantly Polysialylated C6.5 scFv Fusion Protein
7.1 Introduction

Based on the successful development (in vitro and in vivo) of recombinant polysialylation using the MFE23-Ig5-FN1 fusion protein, another scFv model was consequently tried to further exemplify the concept of recombinant polysialylation through fusion with specific NCAM domains, and verify the adaptability of the recombinant polysialylation technology for different scFv models.

As determined from previous studies on chemically polysialylated MFE23 scFv (Constantinou 2005; Constantinou et al. 2009), as well as the recombinantly polysialylated MFE23-Ig5-FN1 fusion proteins (previous chapters), the antigen interaction between the polysialylated MFE23 scFv (or fusion) and the CEA were retained compared to the scFv alone. With a relatively good understanding of surface antigen binding by the polysialylated scFv, unanswered question was whether the association of the polyanionic PSA interrupts the process of internalization. In addition, studies on the surface charge of mammalian cells showed the negative charge of the inner leaflet of the plasma membrane, to be predominantly made up of two low-negatively charge of the high lipid content monovalent phospholipids, phosphatidylserine (PS) and phosphatidylinositol (PI) (McLaughlin and Murray 2005; Yeung and Grinstein 2007; Yeung et al. 2008; Goldenberg and Steinberg 2010). Differing to other bio-polymers, PSA possesses a highly negative charge which increases with the PSA DP. A potential electric repulsion between the cell membrane, particularly the inner-leaflet and the polysialylated scFv would be generated especially during transmembrane processes after scFv binding to internalizing cell receptors.

As an important oncogene product and therapeutic target particularly overexpressed in breast and prostate cancer, HER2 represents a type of cell surface tumour antigen that undergoes dimerization and internalization. Background on the HER2 antigen, especially in malignant conditions has been described previously in section 1.1.4.1 (trastuzumab) and its signal transduction was also showed in Figure 1.4. Based on the induction of HER2 internalization, C6.5 scFv was chosen as another antibody fragment model to be genetically engineered for recombinant polysialylation. Overcoming the limitations of whole IgG molecules (section 1.2.1), the human scFv molecule, C6.5 was selected by phage display of antibody gene repertoires, and has a high affinity ($K_D$ of 16nM) for the human c-erbB2, the extracellular domain of HER2 antigen, and a $K_D$ of 20nM to the HER2 expressing cell line – SKOV3
(human ovarian cancer cell) (Schier et al. 1995). Like trastuzumab, C6.5 scFv or its diabody format were used in previous studies for monitoring HER2 internalization process under live cell conditions (Nielsen and Marks 2000; Adams et al. 2004; Robinson et al. 2005; Robinson et al. 2008).

7.2 Aims and Objectives

The aim for this chapter was to genetically create another recombinant polysialylated scFv-Ig5-FN1 fusion protein, in order to expand the concept of antibody fragment polysialylation through a recombinant fusion approach. C6.5 scFv was chosen as the model scFv based on its transmembrane activity after HER2 ligand binding. Following the already established recombinant polysialylation protocols and materials (i.e. HEK-293 PolyST cell host), C6.5 recombinant fusion proteins could be produced. In addition to characterisation of the fusion protein, HER2-polysialylated C6.5 fusion protein internalization was also investigated.

**The objectives are:**
- Genetically clone C6.5 scFv cDNA into polysialylatable mammalian expression vector, alongside with other fusion motifs, and generate C6.5-Ig5-FN1, C6.5-FN1, and LS-C6.5 fusion construct cDNAs.
- Transfection C6.5-Ig5-FN1 and other C6.5 fusion protein cDNAs to the HEK-293 PolyST cells and selection of individual monoclonal cell lines with good protein expression and polysialylation level.
- Generation, purification and characterization of recombinantly polysialylated C6.5-Ig5-FN1 fusion protein, alongside other C6.5 fusion proteins.
- *In vitro* antigen binding affinity investigation of polysialylated C6.5 fusion proteins and other recombinant C6.5 proteins.
- *In vitro* live cell binding studies of polysialylated C6.5 fusion proteins and other recombinant C6.5 proteins.
- Preliminary *in vitro* investigations on C6.5 bound HER2 internalizations under different live cell incubation conditions
7.3 Results

7.3.1 Cloning of Recombinantly Polysialylatable C6.5 Fusion Protein
The construction of recombinantly polysialylatable C6.5 fusion protein cDNA was based on the successful genetic construction of MFE23 fusion proteins. Figure 7.1 illustrates the principle of genetically making recombinant fusion antibodies for polysialylation.

Previously generated by Dr Constantinou (Constantinou 2005), the intermediate constructs maintained in the mammalian expression vector pcDNA4a contained essential NCAM domain genes, and served as starting clones for incorporating the recombinantly
polysialylatable fusion constructs. Illustrated in Figure 7.1, double digestion of the intermediate constructs with PciI and NotI restriction endonucleases was shown to create ligation sites for incorporating other sub-genomes. Digested PciI and NcoI restriction sites can re-ligate to each other, however both restriction sites are lost after such ligation. The cloning scheme was firstly introduced to incorporate recombinantly polysialylatable MFE23 fusion proteins (Constantinou 2005), and was used here for generating the C6.5 scFv fusion constructs as the first succession of the MFE23 scFv fusions.

**C6.5 scFv Gene Extraction**

The C6.5 scFv gene was originally maintained in the bacterial expression vector pUC119 vector. Through PCR (protocols described in section 2.2.2.4), C6.5 scFv gene was isolated with additions of NcoI and NotI restriction sites on the N- and C-terminals respectively introduced by specifically designed primers (Chapter 2, Table 2.2). Temperature gradient PCR was performed for ensuring a successful reaction, and determining the optimal annealing condition. Shown in Figure 7.2, bands ~760bp appeared in lanes 1 to 12 indicated right base-pair size of C6.5 scFv sequence, and according to the band intensity, annealing temperatures around 56 ± 4ºC outlined the optimal conditions applied to this PCR.

![Figure 7.2. C6.5 gene extractions from bacterial expression vector.](image)

Temperature gradient PCR was used for determining the coordinative annealing temperatures of the specially designed C6.5 NcoI (forward) and C6.5 NotI (backward) primers. The annealing temperature gradient applied covered from 40ºC (lane 1) to 60.02ºC (lane 12) with 1.82ºC increment between each lane. Samples without DNA template were also prepared for PCR according to each temperature gradient, and their product were pooled together and revealed in the negative control (-) lane. Previous DNA template with corresponding primers determined to have successful PCR under annealing temperature at 60ºC was provided as positive control (+). Samples were loaded into 1% agarose gel, and Hyperladder I was used as base-pair marker.

**Selection of C6.5 scFv Recombinant Fusion Constructs**

Following the process outlined in Figure 7.1, the C6.5 scFv gene and intermediate constructs were ligated, and transformed into chemically competent *E. Coli* strain XL-1 Blue. Under
ampicillin antibiotic selection, fusion vector incorporated in bacteria formed colonies on the 2TY agar plates. As shown in Figure 7.3, colony PCR (cPCR) was then applied for selecting positive bacteria colonies correctly transformed with ligated DNA. The same primers were used as the ones in the C6.5 extraction PCR, and the cPCR was run under optimal annealing temperature at 56°C.

**Figure 7.3. Colony PCR for selecting positive bacterial colonies correctly transformed with ligated C6.5-Ig5-FN1 or C6.5-FN1 DNAs.** Each lane represents a candidate bacterial colony. Red arrows indicate colonies selected for DNA preparation and sequencing. PCR products were loaded into 1% agarose gel, and Hyperladder I was used as base-pair marker.

The ~760bp C6.5 scFv gene fragment from the plasmid DNAs were generated from the bacterial colonies correctly transformed with fully ligated C6.5 fusion DNAs after cPCR (Figure 7.3). The chosen colonies were then used for DNA preparation and analysed by DNA sequencing for final verification.

**Construction of LS-C6.5 scFv in pcDNA4a Vector**

Based on the successful incorporations of C6.5-Ig5-FN1 and C6.5-FN1 fusion constructs in mammalian expression pcDNA4a vector, the LS-C6.5 recombinant construct was consequently generated. Illustrated in Figure 7.4(a), the LS-C6.5 sequence was double digested and fused into the mammalian expression vector pcDNA4c. The selection of bacterial colonies transformed with LS-C6.5 sequence was processed by cPCR under the same conditions as C6.5-Ig5-FN1 and C6.5-FN1 cPCRs. As indicated in Figure 7.4(b), colonies presenting C6.5 fragment in the cPCR product were used for DNA preparation and sequence for further verification.
Figure 7.4. (a) Schematic genetic cloning process for LS-C6.5 construction in mammalian expression vector pcDNA4c. Important restriction sites are indicated, alongside different protein domain information. (b) Colony PCR for selecting positive bacterial colonies correctly transformed with ligated LS-C6.5 DNA in pcDNA4c vector. Red arrows indicate colonies selected for DNA preparation and sequencing. PCR products were loaded into 1% agarose gel, and Hyperladder I was used as base-pair marker.

Finally, all three C6.5 recombinant fusion DNA constructs were correctly cloned. As shown in Figure 7.1 and 7.4(a), double digestions by HindIII and XhoI release the fusion inserts LS-C6.5 (861bp), LS-C6.5-FN1 (1272bp), LS-C6.5-Ig5-FN1 (1535bp) from the corresponding DNA constructs (calculated base-pair number in brackets). Shown in Figure 7.5 are the correctly sized products of the restriction digestion for each C6.5 construct.

Figure 7.5. HindIII and XhoI double digestion of LS-C6.5, C6.5-FN1 and C6.5-Ig5-FN1 fusion constructs. All three samples were loaded into 1% agarose gel, alongside Hyperladder I as the base-pair marker.
7.3.2 Monoclonal Selection of C6.5 Fusion Protein Expressing Cell Lines

Based on the successful monoclonal selection of MFE23 fusion transfectants from HEK-293 PST cells (section 5.3.2), all three recombinant C6.5 fusion clones were transfected into HEK-293 PST polyclonal cells, followed by zeocin antibiotic treatment. As shown in Figure 7.6 and 7.7, selection of the ideal monoclonal cell lines of each fusion construct was achieved by dot-blot and ELISA method using collected cell-cultured medium as described previously (section 2.2.4.1).

Figure 7.6. Anti-Myc antibody detected dot-blot for LS-C6.5 and C6.5-FN1 monoclonal selections. HEK-293 PST cells were used in transfections. Only the monoclines (indicated with clone labels) showed positive signals by anti-His, antibody detection were displayed, and the best protein expression clones were outlined by red squares.

The Myc protein-tag specific dot-blot results (Figure 7.6), directly illustrate the monoclonal cell lines to exhibit the best fusion protein expression level for both LS-C6.5 and C6.5-FN1 HEK-293 PST transfectants. Clone 2-4A5 and 2-3B1 were selected for stable expression of LS-C6.5 and C6.5-FN1 fusion proteins respectively.

As described in section 5.3.2, the dual-characteristics of polysialylation and protein expression were required for selection of the scFv-Ig5-FN1 fusion protein from the PolyST expressing mammalian cell host. In the case of selecting the best monoclonal cell line expressing polysialylated C6.5-Ig5-FN1 fusion protein, the ELISA method was used, with hErbB2/Fc fusion protein coated as antigen trapping the C6.5-Ig5-FN1 fusion protein. Anti-PSA specific detection illustrated 10 out in total 40 collected monoclonal cell lines to exhibit high-levels of PSA, among which the clone numbered 4 showed the highest anti-PSA ELISA absorbance (Figure 7.7a). Protein expression yield was also detected by Myc protein-tag specific dot blotting (Figure 7.7b). In particular, the anti-PSA ELISA selected 10 clones,
clone 4 also demonstrated a relatively good fusion protein secretion level when compared to the others. Clone 8, 9 and 10 showed exceptional PSA expression results in Figure 7.7(a). C6.5-Ig5-FN1 HEK-293 PST clone 4 was chosen for consistent expression of polysialylated C6.5 fusion protein.

Figure 7.7. Monoclonal selection of polysialylated C6.5-Ig5-FN1 fusion protein expressing HEK-293 PST transfectants. (a) Anti-PSA specific ELISA monoclonal selection method for polysialylation screen of all 40 collected monoclonal cell lines after C6.5-Ig5-FN1 transfection. (b) Anti-Myc dot-blotting for screening the best 10 monoclonal clone samples selected from (a). For positive control (+), previously purified highly polysialylated MFE23-Ig5-FN1 (5μg) was used in both selection method (pink bar), and untransfected HEK-293 PST culturing mediam (green bar) was applied as the negative control (-). Culture media from LS-C6.5 (purple bar) and C6.5-FN1 (black bar) polyclonal cell lines were tested in the anti-PSA ELISA. Clone 4 was labelled in red.
7.3.3 Purification and Characterization of C6.5 Fusion Proteins

Both TALON™ IMAC and optimized anion exchange purification methods previously described for MFE23 fusion protein investigations (section 4.3.3 and 4.3.4) were routinely used for purifying the polysialylated C6.5-Ig5-FN1 and other C6.5 fusion proteins. The purified proteins are showed in Figure 7.8 and 7.9 below.

![Coomassie staining and anti-Myc Western blot](image1)

![Coomassie staining and anti-PSA immunoblot](image2)

Figure 7.8. Purified C6.5 recombinant fusion proteins. (a) TALON™ IMAC purified LS-C6.5 scFv and C6.5-FN1 fusion protein. Coomassie staining and anti-Myc Western blot were used for identifying the two proteins. Ion-exchange purified C6.5-Ig5-FN1 fusion protein isoforms were revealed by coomassie staining (b) and anti-PSA immunoblot (c). Sialylated (1), weakly polysialylated (2) and highly polysialylated (3) C6.5-Ig5-FN1 glyco-forms were analysed. Endo-N treated highly polysialylated fusion glyco-form (3') was also used in anti-PSA immunoblot.

As Shown in Figure 7.8(a), both LS-C6.5 and C6.5-FN1 fusion proteins were correctly generated and purified with corresponding MW from SDS-PAGE alongside recombinant protein-tag immunodetections. The two-step purification for separating the glycosylated C6.5-Ig5-FN1 fusion protein showed expected results after the first His$_6$-tag specific IMAC purification (results not shown), of which were similar to the findings of IMAC purified MFE23-Ig5-FN1 glyco-proteins as shown in Figure 4.11 previously. The following anion
exchange of the fusion protein produced the similar chromatographic image of the MFE23-Ig5-FN1 fusion protein (Figure 4.16a) and the DP defined C6.5-Ig5-FN1 fusion glyco-forms are shown in Figure 7.8(b), where the three fusion glyco-forms were separated with expected MW range. In comparison to the MFE23-Ig5-FN1 fusion proteins, C6.5 fusion proteins appeared to be easily degraded, as very weak anti-protein tag immunodetection signals were found (results not shown). A proteolysed fragment (~26 kDa) was also revealed from the fusion glyco-forms protein staining gel (Figure 7.8b). Without strong indications from the protein-tag immunodetections, the fusion protein content was defined by the anti-FN1 Western blot shown in Figure 7.9 below.

Anti-PSA immunoblot (Figure 7.8c) only revealed polysialylation signals from the highly polysialylated glyco-form (or 1M NaCl elution). No PSA detection was found from the weakly polysialylated glyco-form, suggesting the 200mM NaCl eluted C6.5-Ig5-FN1 fusion protein potentially composed higher DP than the sialylated isoform but not enough to be recognised by the anti-PSA antibodies. Endo-N treated highly polysialylated fusion isoform clearly removed the PSA immunodetection (Figure 7.8c), indirectly confirming the succession of recombinantly polysialylating C6.5 fusion protein. Additional glycosidase treatments are illustrated in Figure 7.9.

![Glycosidase treatments of polysialylated C6.5-Ig5-FN1](image)

**Figure 7.9. Glycosidase treatments of polysialylated C6.5-Ig5-FN1.** Three glycosylated fusion isoforms – sialylated, weakly polysialylated, and highly polysialylated C6.5-Ig5-FN1 were treated by glycosidases including Endo-N, Exo-N, and PNGase F, and consequently separated by SDS-PAGE and revealed by anti-FN1 Western blots.
Similar to the glycosidases treated MFE23-Ig5-FN1 fusion glyco-forms (Figure 4.17), different degrees of band-shift are also revealed by each C6.5-Ig5-FN1 fusion glyco-form in correspondence to each glycosidase specification (Figure 7.9), and an approximate 65 kDa band was produced for each C6.5 fusion glyco-form after total N-glycan removal by PNGase F. In addition, the anti-FN1 immunodetections of the three glyco-form before any glycosidase treatment showed no signal below ~70 kDa for the highly polysialylated C6.5 fusion suggesting an expected anion exchange separation of the glycosylated C6.5-Ig5-FN1.

7.3.4 Antigen Binding Affinity Determination of the C6.5 Fusion Proteins

The in vitro antigen binding profile of all C6.5 fusion proteins was determined by ELISA, and the human ErbB2/Fc fusion protein containing the extracellular domain of HER2 antigen was used to as antigen target. Following the ELISA protocol described in section 2.2.5.7, the EC$_{50}$ or dissociation constants ($K_D$) of each C6.5 fusion protein were determined (Figure 7.10).

![Figure 7.10](image)

**Figure 7.10.** ELISA analysis for determining antigen binding affinities of all C6.5 fusion proteins. A serially diluted protein concentrations (from 0.1nM to 1μM) was prepared in triplicate for each fusion protein (depicted in different colours), including LS-C6.5 scFv, C6.5-FN1, and the three glyco-forms of C6.5-Ig5-FN1 fusion proteins. The ELISA was mediated by Myc-tag specific antibodies and the $K_D$ values (± standard errors) of each type of fusion protein were also signified.

Figure 7.10 shows the $K_D$ values analysed by anti-Myc antibody mediated ELISA to be comparable for antigen binding capacities of all C6.5 fusion proteins. Although the
polysialylated C6.5-Ig5-FN1 fusion proteins displayed ~2-fold $K_D$ of the C6.5 scFv, the antigen binding affinity was retained again after the recombinant fusion and polysialylation modification of the scFv. Moreover, as suggested from the previous section (protein staining and immunodetections), the fusion protein glyco-forms were found to be easily degraded leading to protein-tag missing. This could also lead to an apparent increase in the $K_D$ values of the recombinant C6.5-Ig5-FN1 fusion proteins.

7.3.5 Antigen Binding Profile under Live Cell Conditions

In contrast to the recombinant MFE23 fusion proteins, which targets the CEA receptor expressed on the cell surface, the recombinant C6.5 fusion proteins interacts with the HER2 receptor, which dimerizes and internalizes. Conditions such as post-targeting incubation temperature and time were analysed during immuno-mediated FACS and CLSM analysis.

7.3.5.1 Detection of Antigen Expressing Live Cell Binding by FACS

After the $K_D$ determination by ELISA, SKOV-3, the HER2-expressing human ovarian cancer cell line was used for investigating live cell binding of the C6.5 recombinant fusion proteins using FACS analysis. In order to avoid protein-tag degradation problems, shown in Figure 7.11(a) anti-FN1 antibody mediated FACS was used, and C6.5-FN1 was considered as the positive control in comparison with the three C6.5-Ig5-FN1 fusion glyco-forms. Anti-PSA specific FACS detection was also carried out and demonstrated in Figure 7.11(b). The FACS analysis was only used for investigating the HER2 binding at the cell surface level, in order to prevent HER2 internalization, all incubation procedures were taken at 4°C (section 2.2.5.9).

Clearly shown in Figure 7.11, positive antigen binding was revealed by anti-FN1 antibody detected FACS for all three C6.5-Ig5-FN1 fusion glyco-forms after incubating with HER2 expressing SKOV-3 cells. In comparison with the non-glycosylated C6.5-FN1, the antigen targeting capacities of all three fusion glyco-forms under live cell condition remained consistent, particularly indicated by the geometric mean fluorescence values ($GMean$) from each sample detection. The anti-PSA mediated FACS results were also demonstrated in Figure 7.11 for the three C6.5 fusion glyco-forms, from which only the highly polysialylated C6.5-Ig5-FN1 fusion species was found to show a significantly positive curve shift. The PSA structure of the C6.5 fusion protein remained intact during targeting of the cell surface expressed HER2. The same sample preparations were also carried out on the HER2 negative
KB cell line, and as expected, no positive curve shift was detected by either antibody directed FACS (Figure 7.11).

7.3.5.2 CLSM for Cell Surface Antigen Internalization Detection

To investigate internalization between HER2 and the C6.5 scFv as well as C6.5 fusion proteins, live cell (SKBR-3) binding steps took place initially at 4°C for an hour, and confocal microscopy was used to capture the localization as well as potential cell uptake of HER2 bound C6.5 fusion proteins under different in vitro post-targeting incubation temperatures (described in section 2.2.5.10). In particular, the localization status of HER2 bound recombinantly polysialylated C6.5-Ig5-FN1 fusion proteins was studied, in order to understand the effect of negatively charged PSA on protein complex internalization.

Figure 7.11. C6.5 fusion protein in vitro live cell binding investigation by FACS. Antibodies specifically recognising NCAM FN1 domain and PSA were used for detecting the three C6.5-Ig5-FN1 isoforms (0.2μg/μl of each) live cell binding profile. Interactions with HER2 expressing SKOV-3 and negative control KB cell lines were analysed in replicate and the average GMean values of each FACS detection are indicated alongside. Colour denotations are provided on the left.
Figure 7.12. CLSM showing LS-C6.5 scFv and HER2 cell surface binding and internalization. After 1 hour antigen binding incubation at 4°C, CLSM images were taken under two post-targeting incubation conditions: (a) 4°C for 5 mins and (b) 37°C for 1.5 hours. Anti-Myc mediated fluorescein staining (I) and phase-contrast (III), alongside their merged CLSM images (II) are shown. Z-stack images of a representative cell were taken from a serial of plane levels (1 µm depth interval from bottom to top of the cell), and the z-stack middle plane images suggesting overall cell population fluorescent-staining profile were taken randomly within corresponding specimen areas.
Figure 7.13. CLSM showing C6.5-FN1 fusion protein and HER2 cell surface binding and internalization. After 1 hour antigen binding incubation at 4°C, CLSM images were taken under two post-targeting incubation conditions: (a) 4°C for 5 mins and (b) 37°C for 1.5 hours. Anti-FN1 mediated fluorescein staining (I), phase-contrast (III) and their merged CLSM images (II) are shown. Z-stack images of a representative cell were taken from a serial of plane levels (1µm depth interval from bottom to top of the cell), and the z-stack middle plane images suggesting overall cell population fluorescent-staining profile were taken randomly within corresponding specimen areas.
Figure 7.14. CLSM showing interactions between C6.5-Ig5-FN1 fusion glyco-forms and HER2 expressed by SKBR-3 cells. (a) With 5mins post-targeting incubation at 4°C. (b) Targeting status of sialylated or weakly polysialylated glyco-forms with 1.5 hours post-targeting incubation at 37°C. (c) Targeting status of highly polysialylated C6.5-Ig5-FN1 fusion protein with 1.5 hours post-targeting incubation at 37°C. Representative single cell confocal z-stack images were showed including anti-FN1 mediated fluorescein staining (I), phase-contrast (III) and their merged CLSM images (II) are shown. Z-stack images were taken with a serial of plane levels (1µm depth interval from bottom to top of the cell).
Figure 7.15. CLSM showing human anti-HER2 IgG and HER2 cell surface binding and internalization. After 1 hour antigen binding incubation at 4°C, CLSM images were taken under two post-targeting incubation conditions: (a) 4°C for 5mins and (b) 37°C for 1.5 hours. Anti-human Fc domain mediated fluorescein staining (I) and phase-contrast (III), alongside their merged CLSM images (II) are shown. Z-stack images of a representative cell were taken from a serial of plane levels (1µm depth interval from bottom to top of the cell), and the z-stack middle plane images suggesting overall cell population fluorescent-staining profile were taken randomly within corresponding specimen areas.
As shown in Figure 7.12 and 7.13, both C6.5 scFv and C6.5-FN1 fusion proteins bound to the SKBR-3 cell surface at 4°C, and internalized at 37°C after 1.5 hours post-targeting incubation. Same surface binding and more complete internalization profiles were demonstrated by the positive control anti-HER2 IgG (Figure 7.15) with the same post-targeting incubation conditions, of which outlines the standard HER2 activity from live cells. In terms of the C6.5-Ig5-FN1 fusion isoforms, the sialylated and weakly polysialylated showed comparable localization profiles as the C6.5 scFv and C6.5-FN1 fusion proteins (Figure 7.14a&b), which clearly indicated the differences between cell surface binding and internalization under corresponding preparation conditions. On contrast, the confocal images of the highly polysialylated C6.5-Ig5-FN1 fusion glyco-form showed minor difference between the 4°C and 1.5-hour 37°C post-targeting incubations (Figure 7.14a&c). However, a few fluorescent signals can be detected from the 37°C incubated cells, suggesting heterogeneous cellular activity of HER2 bound to the highly polysialylated C6.5-Ig5-FN1 fusion protein, which potentially due to the inconsistent chain length of the associated PSA. In addition, standard cell surface targeting profiles were detected by all C6.5 fusion protein as well as the anti-HER2 IgG at 4°C for 1.5 hours and 37°C for 5 minutes post-targeting incubations (results not shown).

7.4 Discussion

The investigation in this chapter expanded the application of recombinant polysialylation to another antibody fragment, C6.5 scFv. In comparison with the initial scFv example (MFE23 scFv), C6.5 scFv targets HER2 antigen expressed on the cell surface, and followed by dimerization and internalization (Adams et al. 2000; Nielsen and Marks 2000; Robinson et al. 2005; Robinson et al. 2008). With a high affinity binding C6.5 scFv and HER2, effects of the highly negatively charged polysialic acid associated with the C6.5-Ig5-FN1 fusion protein during HER2 internalization process was preliminarily investigated. Furthermore, charged molecule cannot freely pass across the lipid bi-layer and very negatively charged species could leave a weaker association with lipid membrane, therefore additional information could be provided from the study of recombinantly polysialylated C6.5 fusion protein.
As demonstrated in section 7.3.1, the recombinantly polysialylatable C6.5-Ig5-FN1 fusion construct as well as the control constructs, including LS-C6.5 scFv and C6.5-FN1 were constructed using the intermediate Ig5-FN1 and FN1 pcDNA4 vectors provided previously (Constantinou 2005). After DNA sequencing verifications, vectors containing fusion constructs were respectively transfected to the HEK-293 PST cell line (Chapter 5), and three monoclones were stably selected for LS-C6.5, C6.5-FN1 and C6.5-Ig5-FN1 expressions. The standard cell culture, expression, and purification procedures that utilized and verified for the MFE23 system (Chapter 4) were applied for the C6.5 system. In terms of the C6.5-Ig5-FN1 fusion protein, three glyco-forms (sialylated, weakly and highly polysialylated) were produced and similarly detected by SDS-PAGE and Western blots (Figure 7.8) in comparison with the MFE23-Ig5-FN1 fusion glyco-forms (Chapter 4). The polysialylation was also confirmed by Endo-N, Exo-N and PNGase F involved glycosidase treatments (Figure 7.9), and similar results were outlined by the MFE23-Ig5-FN1 fusion isoforms. In vitro antigen binding affinity test using ELISA determined comparable $K_D$ values of all recombinant proteins, whereas a small $K_D$ increase was found from the highly polysialylated C6.5-Ig5-FN1 fusion protein indicating ~2-fold higher than the LS-C6.5 scFv and ~3.5-fold higher than the originally determined C6.5 scFv $K_D$ value (16nM) (Schier et al. 1995). Consequently, live-cell binding tests using FACS further demonstrated the cell surface expressed HER2 targeting capacity of the recombinantly polysialylated C6.5-fusion protein. Having the FACS preparations at 4°C (avoiding HER2 internalization), slight reductions in terms of curve shifts and GMean values were showed by the three C6.5-Ig5-FN1 glyco-forms comparing with the C6.5-FN1 fusion protein through anti-FN1 antibody mediated detection, and only the highly polysialylated C6.5-fusion protein suggested considerable curve shift from anti-PSA antibody mediated FACS (Figure 7.11). Prepared with a saturating concentration (0.2μg/μl or ~3mM) for the FACS analysis, factors that deduced for MFE23 fusion proteins FACS analysis including PSA antigen masking and negative charge repulsion (Figure 5.21), as well as potential protein degradation issues could possibly affect the targeting efficiencies of the glycosylated C6.5 under live cell conditions. Further study of live cell binding affinities ($K_D$) can be determined with a serial dilution of concentrations of each C6.5 fusion protein by FACS verified GMean values, and $K_D$s can be thus measured.

CLSM was consequently performed for understanding the potential effect from PSA on HER2 internalization. As expected, standard cell surface localization was demonstrated by all C6.5 fusion proteins when incubated at 4°C, which slows down all receptor-mediated
endocytosis activity. Under normal culturing condition (37°C), HER2 internalization was revealed by most of the C6.5 fusion proteins after 1.5 hours post-targeting incubation time. However, the highly polysialylated C6.5-Ig5-FN1 fusion protein only showed a very low level of internalization, suggesting the inhibition of HER2 internalization potentially induced by the long PSA chain associated with the C6.5-Ig5-FN1 fusion protein. Compared with the sialylated and weakly polysialylated fusion glyco-forms, the highly polysialylated isoform possesses longer PSA chain leading to much larger hydrodynamic volume and higher negative charge of the fusion protein. The hydrodynamic radii of the polysialylated C6.5 fusion proteins were expected to be similar as the MFE23 fusion proteins. According to Table 5.2, the highly polysialylated MFE23-Ig5-FN1 fusion protein had a SEC determined apparent MW much bigger than the IgG molecule. It was not sure whether the excessive size increment restricts the internalization process of the HER2-fusion protein complex, as large protein molecule such as IgM has been found to be internalized (Della Torre et al. 1987; Chao et al. 1997). However, the increased negative charge from the highly polysialyalted C6.5 fusion protein could potentially enhance negative charge repulsion between the fusion protein and the cell membrane which counteract the internalizing process (Figure 7.16a).

Figure 7.16. Possible PSA involved mechanisms that inhibit HER2 mediated highly polysialylated C6.5-Ig5-FN1 internalization. (a) Negative charge repulsion (F); (b) HER receptor dimerization interference.

HER2 internalization is initiated by HER receptor dimerization (Maier et al. 1991; Gilboa et al. 1995; Spector and Blackwell 2009). In addition to the PSA associated negative repulsion, another possible internalization inhibition mechanism was explained in Figure 7.16(b). The close contact of highly polysialylated C6.5 fusion protein and HER2 after their high affinity interaction could potentially extend the long PSA chain to interfere the dimerization process.
of HER2 and other HER family receptors, which consequently hamper the receptor mediated internalization. Unaffected internalization outlined by the sialylated and weakly polysialylated C6.5-Ig5-FN1 glyco-forms further suggests an impact of PSA elongation to the HER2 internalization process. In addition, due to the dispersive hydrodynamic radius (2.05<Ø<2.94) predicted by SEC for the highly polysialylated MFE23-Ig5-FN1 fusion protein (Figure 5.22), individuals having shorter PSA chain lengths in the highly polysialylated category could potentially internalize as a low internalization level was spotted to the highly polysialylated C6.5-Ig5-FN1 fusion protein. Further CLSM studies as well as other analysis (i.e. FACS) should be carried out in order to prove the polysialylation affect on HER2 internalization. Presumably, a certain polysialylation level might also need to be decided for understanding the HER2-C6.5 fusion protein complex internalization

In addition, other studies undertaken in the investigation of polysialylated recombinant MFE23 – for example, measurement of antigen binding kinetics by BIAcore, MALDI-TF MS determination of the DP of PSA, measurement of hydrodynamic volume and in vivo pharmacokinetics – need to be applied to the polysialylated recombinant C6.5 fusion protein to develop a more detailed understanding of this molecule. Moreover, the effect of polysialylation on C6.5 activated HER2 internalization needs to be further studied in order to understand the applicability of the recombinant polysialylation in such circumstances. Internalization induced endocytosis can significantly accelerate the antibody fragment lost from the blood circulation, and should be considered during future in vivo PK study.
CHAPTER 8

General Discussion and Conclusions
Modern bioscience exploitation of the specificity and affinity of antibody targeting has been greatly advanced by various biotechnological developments. Described as a “magic bullet”, antibody drugs target specific disease areas significantly reduce the side-effect received from traditional radio- and chemo-therapies, particularly seen in cancer treatment. As shown in Table 1.1, at least 36 monoclonal antibody related products have already been approved and launched into the market worldwide with significant medical/therapeutic effects and commercial successes. Humanisation of recombinantly engineered antibodies enabled the development of antibodies into a more “human friendly” system, leading the new generation antibody products with much less side-effect problems seen from the pioneering murine-originated antibodies (Holliger and Hudson 2005; Wu and Senter 2005; Reichert 2008).

Also shown in Table 1.1, most available therapeutic antibodies are provided as an IgG format, which initiates the immunological function by recruiting the effectors cells (via the antibody Fc-domain) to take on the targeted cells. However, a few limitations of using full-length IgGs, including tissue cross-reactivity, poor tissue penetration, slow extravasation and capillary diffusion and heterogenous biodistribution significantly reduce the antibody therapeutic efficacy of a number of diseases, and therefore restrict their clinical applications (i.e. diagnostic imaging). Antibody directed therapies, using antibodies to deliver powerful therapeutic agents including drugs, toxins, radionuclides; or fusing antibodies with effective proteins (outlined in Figure 1.5), have also shown promised potencies in therapies, such as cancer and arthritis treatment (Wu and Senter 2005; Schrama et al. 2006). IgG antibody has a large molecular weight, exhibits slow blood clearance, and inappropriate Fc-domain cross-reactivity with Fc receptors (FcγR and FcRn), which often results in high exposure to normal tissues thus leading to high risk of cytotoxic effects inflicted on normal cells.

Antibody fragments (Fab₂, Fab and scFv) with reduced sizes in comparison with their parent immunoglobulins have been designed and produced to overcome the problems seen with the intact MAbs. Exceptionally amenable to various molecular library selection methods (e.g. phage-display), high affinity antibody fragments can be readily selected and produced in large-scale with high yields, economically. With the antigen targeting moiety, a number of antibody fragment multimers (e.g. diabodies, triabodies, and bis-scFv) and fusion variants have been developed to provide additional valency, specificity, function and stability for therapeutic efficacy improvements. Most importantly, the smaller size of the antibody fragment allows rapid tissue penetration, enhanced vascular extravasation and more uniform
biodistribution outcomes, which enables fast and specific delivery of powerful therapeutic agents to the disease areas and greatly lowers the unnecessary hazard to the healthy cells (Holliger and Hudson 2005; Jain et al. 2007; Weisser and Hall 2009). With a number of antibody fragments approved for medical diagnosis and imaging applications, at least 5 fragmented antibodies have been launched for direct disease treatment, and numerous pipeline products are under different development levels (Nelson and Reichert 2009).

Although there are significant advantages to the reduced molecular size of antibody fragments in comparison with intact antibodies in therapeutic applications, these fragments have been found to have very short circulation times in vivo, predominantly caused by rapid elimination through the kidneys. To overcome this drawback, various technologies (described in section 1.3) were developed principally following two PK modulation concept: (i) increasing hydrodynamic volume of antibody fragment via coupling with hydrophilic molecules (i.e. PEG and PSA); (ii) association with long-lived plasma proteins (i.e. albumin). Demonstrated by a number of commercially available drugs and protein products conjugated with polyethylene glycol (PEG) or PEG derivatives, and numerous PEGylated pipeline products during clinical and animal tests (introduced in section 1.3.1.2), hydrophilic polymer-linked PK modulation method has been effective for improving protein bioactivity after intravenous injection. Successfully introduced with antibody fragments, PEGylation showed significant improvement in blood circulation duration (Chapman 2002). Specific tumour localization and retention was also observed, of which was enhanced by the enhanced permeability and retention (EPR) effect associated with water soluble polymers (Maeda et al. 2000; Chapman 2002).

As a non-biodegradable material, repeated treatment with PEGylated therapeutics can lead to the accumulation of PEGs in the body (i.e. liver) with toxic effect to patients, and the long term clinical effect are not known. Immuno-response can also be generated over prolonged exposure of PEG (Gregoriadis et al. 2005; Jevsevar et al. 2010). Over the risk of using non-biodegradable synthetic polymers in vivo, an alternative natural polymer, polysialic acid (PSA) was consequently proposed by Gregoriadis et al. under the PK modulation principle that PEGylation indicates (Gregoriadis et al. 1993). The biodegradable PSA is hoped to extend the therapeutics half-life with minimized immunogenic and toxic side-effects that associated with PEG. A number of protein candidates were chemically polysialylated and showed in vitro and in vivo with increased solubility and stability, reduced immunogenicity,
preserved function, and improved pharmacokinetics (Gregoriadis et al. 2005). Focused on antibody fragments, both Fab and scFv were chemically polysialylated recently (Constantinou et al. 2008; Constantinou et al. 2009). Although random amine coupling detrimentally hampered antigen targeting of scFv, conjugating PSA via a C-terminal site-specific method thus avoiding interference with the antibody binding site further confirmed the polysialylation as a capable application for increasing the longevity of circulating antibody fragments and improve their in vivo PK. The latter work also opened up ideas for recombinant site-specific polysialylation.

It is well known that chemically conjugating polymers to drugs and proteins has been provided with decades of research experience and industrialized production scale. However, due to the process, major concerns including protein inactivation, downstream processing, high cost (production costs of both bio-therapeutics and polymers), and conjugation yield have been noticed from the development of PEGylation and chemical polysialylation (Constantinou et al. 2010). To overcome these problems, and setting up an alternative polysialylation approach, this thesis focuses on the establishment of a chemistry-free polysialylation method. As an extreme glycosylation feature, biosynthesized PSA on the NCAM protein with a significant level was found during mammalian early brain development. In order to preserve the advantages antibody fragment gained from size reduction, the polysialylation approach described in this thesis genetically fuses the minimal human NCAM polysialylatable domains (Ig5-FN1 domain, ~20 kDa) to the C-terminal of model scFvs, in order to produce recombinantly polysialylated scFv-Ig5-FN1 fusion proteins with enhanced PK properties.

8.1 Succession in Recombinantly Polysialylating scFv-Ig5-FN1 Fusion Protein

The recombinant polysialylation of scFv-Ig5-FN1 fusion protein investigated in this PhD project is principally following the process of intracellular PSA biosynthesis (Figure 1.17) and NCAM polysialylation pathways (section 1.4.2). Described in Chapter 3, the initial attempt of recombinant polysialylation by transfecting the MFE23-Ig5-FN1 fusion cDNA into
murine NB2a cells, a line that previously reported with PSA-NCAM expression (Hildebrandt et al. 1998; Gallagher et al. 2000; Poongodi et al. 2002; Beecken et al. 2005) produced the expected fusion protein composition. However, at best, two sialic acid monomers were found in this N-glycosylated fusion protein. Additional glycosidic linkage detection assays (using specific lectins) further indicated the presence of essential polysialylation epitopes - NeuNAcα(2-3/6)Gal, alongside the potential subterminal Galβ1-4GlcNAc linkage, suggesting potential polysialyltransferase deficiencies from the initially used NB2a cell line.

In order to accomplish the mammalian PSA biosynthesis alongside the polysialylation epitopes on the scFv-Ig5-FN1 fusion protein, the essential PolyST cDNAs were introduced to the fusion protein expressing NB2a cells (Chapter 4). This revolutionary modification for the first time enabled positive detection of PSA specifically associated with the scFv-Ig5-FN1 fusion protein. In correspondence with the previous NCAM and NCAM fragment polysialylation immunoblot results (Close et al. 2003; Mendiratta et al. 2005; Colley 2008), the polysialylated MFE23-Ig5-FN1 showed a polydispersive apparent MW covering from ~70 up to potentially over 250 kDa, whilst the previous sialylated version only had a defined MW about 70 kDa. Stable polyclonal selections of polysialylated MFE23-Ig5-FN1 NB2a cell line was consequently selected to provide with consistent fusion protein expression.

Alongside positive polysialylation detection from other PolyST transfected cell lines including COS-1, CHO, 3T3, HeLa cells (Chapter 4), a universal human recombinant protein expression cell line (HEK-293) that was capable of stably polysialylating scFv-Ig5-FN1 fusions was created for producing polysialylated fusion proteins with human glycosylation features (elimination of xenogenic antigens, such as Galα1-3Gal detected from murine NB2a expressed fusion proteins). Using the HEK-293 PolyST cell line, two polysialylated scFv-fusion proteins, MFE23-Ig5-FN1 (Chapter 5) and C6.5-Ig5-FN1 (Chapter 6) were produced respectively after transfecting their cDNAs to the cell host, and monoclonally selected, the polysialylated fusion proteins were stably expressed with a relatively good yield (~1mg/L). The success of producing more than one specificity of polysialylated scFv-Ig5-FN1 fusion proteins further suggested the systematical usability of the concept of recombinant polysialylation technology.
8.2 Polydispersity and the Degree of Polymerization of the Recombinantly Polysialylated scFv-Ig5-FN1 Fusion Proteins

One major concern of using polymer to modulate protein PK is the polydispersity of most water soluble polymers particularly natural ones. As a quality problem, protein conjugated with polydispersive polymer species can make proteins behave heterogeneously and potentially variable PK modulation efficacy as well as uncontrolled therapeutic effects. Either produced synthetically (i.e. PEG) or by living organisms (i.e. colominic acid), polymers are normally found as heterogeneous species, particularly differentiated in residual numbers of each individual. A high cost is normally associated with purifying or classifying polymer species with a relatively unified and low polydispersity before conjugating to the proteins (Gregoriadis et al. 2000; Veronese and Pasut 2005; Veronese and Mero 2008). In terms of natural polysialylation, both endogenous and recombinant PSA-NCAM has been reported with a broad MW range on SDS-PAGE due to the polydispersive PSA expression (Troy 1995; Fujimoto et al. 2001; Close et al. 2003; Colley 2008), of which was also confirmed by several structural studies such as using mass spectrometry (Galuska et al. 2007).

Following the NCAM polysialylation intracellular process, the polysialylated scFv-Ig5-FN1 also showed a polydispersive profile initially from the SDS-PAGE and Western blot covering a broad MW range. In order to narrow down the polydispersity of the scFv-fusion protein and the characterization of the glyco-species, an anion exchange purification protocol (Chapter 4) was used based on the negative charge composition of each sialic acid. Following the negative charge increase alongside the polysialylation DP, the anion exchange protocol was optimized to produce three glyco-forms of scFv-Ig5-FN1 fusion protein eluted by characterized NaCl concentrations. In turn, they were named as sialylated (eluted by 100mM NaCl), weakly polysialylated (eluted by 200mM NaCl) and highly polysialylated (eluted by 1M NaCl), and more restricted PAGE profiles were revealed showing the sialylated as one single band (~70 kDa), weakly polysialylated covering from ~70 kDa to 100 kDa, while the highly polysialylated occupying the rest above 100 kDa.

Delicate structural studies using mass spectrometry further investigated DP of PSA and the polydispersive distribution of each MFE23-fusion protein glyco-forms (section 5.3.4). Following the MOLDI TOF-MS protocol utilized for analyzing colominic acid and
polysialylated NCAM (Galuska et al. 2007). Based on the experimental results, both sialylated and weakly polysialylated MFE23-Ig5-FN1 fusion proteins showed much defined DP (4 and ~10 NANA residues respectively), as well as the highly polysialylated (~25 NANA residues on average), which appeared to be more dispersive than the former two. In comparison with the MOLDI TOF-MS analyzed PSA-NCAM, a rather similar mass spectrum profiles was received by the highly polysialylated MFE23-Ig5-FN1 fusion protein, and approximately 40 NANA residues were maximally indicated by both mass spectra (Galuska et al. 2007), suggesting a normal and natural recombinant polysialylation process was carried out on the highly polysialylated fusion species.

8.3 Recombinant Polysialylation Retains the Antigen Binding Affinity of Antibody Fragment

Reduction in antigen binding affinity was considered as one of the most severe drawbacks of many PK modulation approaches, including PEGylation and chemical polysialylation to small-size antibody fragment (Chapman 2002; Constantinou et al. 2009). Introduced previously in the PEGylation (section 1.3.1.2), chemically-modified active groups from hydrophilic polymers enable coupling reaction with the formation of carbamate, amine, amide and thioether bonds between protein and polymers. These chemical conjugations can be randomly mixed, leading to a high risk of coupled polymers at or near the antibody binding site and severely compromising antigen binding affinity (Pedley et al. 1994; Francis et al. 1998; Chapman et al. 1999; Constantinou et al. 2009). One way to effectively reduce this risk is to engineer specific binding sites from the antibody fragment, which avoid the unnecessary polymer intervention at the antigen binding site. However, other factors that may induce variations in targeting kinetics due to polymer involved conjugation methods (for example polymer masking of the binding domains from either antibody or antigen) were discussed in Chapter 5. Particularly relevant to PSA, the possession of a highly negative charge should also be considered during antigen binding and other bioactivities (Figure 5.21).

The recombinantly polysialylated scFv-Ig5-FN1 theoretically introduced an alternative site-specific approach for adding PSA to the antibody fragment, which ideally reduces the risk of
polymer interference at the antigen targeting site. In order to understand the actual antigen binding profile for the recombinantly modified fusion protein, both ELISA and BIAcore studies were carried out. Considerably, $K_D$ values received from the ELISA suggested that the polysialylated scFv-Ig5-FN1 fusion proteins (for both MFE23 and C6.5) had almost no effect to in vitro antigen binding in comparison with their corresponding scFvs. However, the kinetic binding studies using SPR (BIAcore) for the MFE23 fusion proteins revealed approximately 10-fold increase of overall $K_D$ to both weakly and highly polysialylated fusion formats, of which both of them was predominately caused by the decreased $k_{on}$ values (~20-fold decrease) (Table 5.1). This experimental phenomenon was speculated to be induced by the negatively charged surface (free –COOH group) of BIAcore® SA sensor chip used for immobilizing the antigen proteins. Receiving the polyanionic possessions from the associated PSA molecules, an electrical repulsion force can be generated against the interaction between the polysialylated MFE23-fusion proteins to their antigens coated on the negatively charged BIAcore chip surface, resulting decreased on-rates (Figure 5.21a). This hypothesis was approved by further BIAcore experiment using PSA removed (hydrolysed by Endo-N) MFE23-Ig5-FN1 fusion protein, which kinetically retrieved the fusion protein on-rate and overall $K_D$ back to the scFv level.

Generally speaking, the recombinant polysialylation approach genetically arranged the N-glycosylation sites on Ig5 domain for site-specific polysialylation of the scFv-Ig5-FN1 fusion protein, avoiding the polysialylation directly taking place on the scFv and interrupting the antigen binding site. The antigen binding affinity was retained comparing with the corresponding scFv under physiological buffer condition, but can be potentially reduced when targeting antigens located on a negatively charged surface. The cell plasma membrane is acknowledged as a weakly negative-charged surface with sialylated membrane protein covering from the outside, and low-negatively charged monovalent phospholipids on the inner leaflet (McLaughlin and Murray 2005; Yeung and Grinstein 2007; Yeung et al. 2008; Goldenberg and Steinberg 2010), leaving potential binding hindrance to cell membrane antigens by the polysialylated antibody fragments. Live cell binding experiments using CEA expressing cell lines suggested comparable results between MFE23 scFv and polysialylated MFE23-Ig5-FN1 fusion protein by both FACS and confocal laser scanning microscopy (CLSM). Surface staining of the cell membrane located CEA was also equally demonstrated by all MFE23 fusion proteins using CLSM. Furthermore, previous studies on chemically polysialylated insulin showed comparable insulin-receptor binding on the cell surface.
additionally suggesting the negatively charged PSA does not prevent the conjugated interaction with corresponding ligand expressed on the cell surface (Jain et al. 2003). Standard in vivo tumour uptake of chemically polysialylated Fab and scFv respectively targeting cell surface expressed placental alkaline phosphatase (PLAP) and CEA antigens also supporting the applicability of polysialylation (Constantinou et al. 2008; Constantinou et al. 2009). Standard HER2 receptor cell surface staining was also demonstrated by the recombinantly polysialylated C6.5-Ig5-FN1 fusion protein. However, the interference of HER2 internalization was initially found by the CLSM studies through interaction with the highly polysialylation C6.5 fusion species (Chapter 7). The highly negative-charge of PSA was speculated to generate an electric repulsion force against the cell membrane and hinder the HER2 internalization. Moreover, other possible reasons such as PSA interrupted HER2 dimerization were speculation (Figure 7.16).

8.4 Recombinant Polysialylation Increases the Molecule Hydrodynamic Volume and Prolongs the in vivo Bioactivity

This PK modulation can be classed as a method using hydrophilic polymer to increase molecule apparent size under physiological aqueous conditions. Experimentally the recombinant polysialylation approach successfully proved this effect. Using size exclusion chromatography (SEC) with physiological buffers, significant size increase was measured from the polysialylated MFE23-Ig5-FN1 fusion protein in comparison with the scFv molecule, and the hydrodynamic volume increased proportionally along with the degree of polysialylation (Figure 5.18). Discussed previously in Chapter 5 according to the SEC results, the three fusion glyco-forms were found to have excessive apparent MW than the calculated MW based on the MFE23-Ig5-FN1 amino acid sequence (Table 5.2). The increments of apparent MW were directly reflected by the hydrodynamic size improvements from each fusion molecule. The same concepts were also suggested by PEGylated proteins (Chapman 2002; Veronese and Harris 2002; Veronese and Mero 2008) and other hydrophilic polymers modified antibody fragments, such as dextran (Mikolajczyk et al. 1996) and homo-amino-acid polymer (Schlapschy et al. 2007).
A relationship between the protein’s hydrodynamic radius and its SEC-measured MW were postulated assuming the soluble fusion proteins displayed a globular shape within the aqueous condition (Figure 5.22). Furthermore, knowing that the human serum albumin (HSA) with the hydrodynamic radius of 3.5nm displays almost the same MW determined by SEC measurement comparing to its amino acid MW (Muller et al. 2007), the hydrodynamic radii of the MFE23-fusion proteins were estimated based on the equation indicating the ratio between hydrodynamic radius and SEC apparent MW (discussed in Chapter 6).

Approximately, the hydrodynamic radius of the sialylated MFE23-Ig5-FN1 was ~4.3nm, and ~5.4-7.8nm for the highly polysialylated fusion protein according to the SEC data, whereas the radius of scFv only appeared to be less than 2.7nm. Knowing the MW cut-off of kidney elimination is around 70 kDa, which can be more precisely interpreted as the pore size of glomerular basement membrane (3-5nm) (Nakaoka et al. 1997), the in vivo pharmacokinetic data (Chapter 6) outlined differential blood clearance profiles between MFE23 scFv, the sialylated and highly polysialylated MFE23-Ig5-FN1 fusion proteins regarding to their apparent size differences.

The results from in vivo PK studies on mice practically demonstrated the concept of the recombinant polysialylation to improve antibody fragment blood circulation longevity. Already mentioned from Chapter 6, longer distributional phase (t$_{1/2\alpha}$) and elimination phase (t$_{1/2\beta}$) alongside increased overall AUC were monitored by the MFE23-Ig5-FN1 fusion proteins in comparison with the MFE23 scFv, and the highly polysialylated glyco-form appeared to be significantly effective. Previously, approximate 10-fold blood AUC increase was achieved by the chemical-conjugated MFE23 scFv to the engineered cysteine site with one 11 kDa (DP~37) PSA chain (Constantinou et al. 2009). Gaining both PSA chains (with average DP of 20) from the two N-glycosylation sites on the Ig5 domain (Colley 2008), a much promised PK-improvement was observed from the highly polysialylated MFE23-Ig5-FN1 fusion glyco-form with ~26-fold increase of blood AUC than the scFv. Typically, IgG molecules have an average circulation time between 24-72 hours in mice, and 324 (% h/g) blood AUC was also reported previously (Constantinou et al. 2008). Found with similar circulation half-life, the highly polysialylated MFE23 fusion protein showed a blood AUC (514 % h/g), of which was ~1.6-fold of the IgG (Table 6.1), suggesting the significant capacity of the recombinantly polysialylated proteins with reduced blood clearance profile. Oxidation of PSA during protein sample radio-labelling was considered to reduce PSA hydrophilicity. The animal PK studies are needed to be repeated with more careful protein
sample preparation, alternative radio-labelling methods, and accurate time interval control as well as the blood sample measurement.

Results from the biodistribution study suggested minimal variations between the scFv and the polysialylated fusion protein. Other than kidney, most organs showed accumulation ratios of less than one suggesting a desired non-specificity of the fusion proteins. In terms of kidney accumulation for small size proteins due to its size-selective nature (Verhaar et al. 1995; Verhaar et al. 1996), progressively, deceased kidney/blood ratio was observed from the glycosylated scFv-Ig5-FN1 fusion proteins than the scFv alone, which indirectly indicated the improvement of prolonged circulation period for the polysialylated scFv-fusion protein, and this fact can also be beneficial in terms of reducing the non-specific kidney toxicity induced by the antibody fragment accumulation.

8.5 Future Studies, Refinements and Improvements

8.5.1 Future Studies

For establishing and developing the recombinant polysialylation technology, based on the research achievement so far, there are still many more investigatory aspects need to be determined, including (1) in vivo antigen localization and tissue penetration/uptake (e.g. for tumour treatment); (2) PSA immunogenicity; (3) polydispersity of polysialylated fusion protein. (4) New targets which could be benefit.

In vivo Antigen Localization and Tissue Penetration/Uptake

Previously, the site-specifically chemical-polysialylated MFE23 scFv demonstrated a high level of in vivo tumour uptake (tumour/blood ratio) and tumour retention (30-fold more than the scFv at 48 hours) (Constantinou et al. 2009). Based on these features, the recombinantly polysialylated MFE23-Ig5-FN1 is expected to show similar rational of results from CEA-tumour bearing mouse models, and the percentage of CEA-expressing tumour uptake, tissue/blood ratio as well as further investigations such as tumour penetration using immunohistochemistry on tumour sections.
**Immunogenicity**

In terms of PSA immunogenicity, indeed, as a naturally occurring polymer, PSA has been involved in a number of infectious microbial including the serogroup B capsular polysaccharide from Neisseria *meningitidis* B and *Escherichia coli* K1, the serogroup C capsular polysaccharide from *N. meningitidis* C, and the polysaccharide K92 from *E. coli* K92, as well as their shorter chain derivatives (Troy 1992; Troy 1995; Janas and Janas 2004). However, under the masking of bacteria capsular PSA, (also known as colominic acid with DP up to 200 NANA residues), these neuro-invasive bacteria can escape from immune surveillance to achieve virulence (Troy 1992; Troy 1995). Moreover, it is also found as a glycosylation feature for a number of mammalian proteins (e.g. polysialylated NCAM) and tumour cells, the naturally biosynthesized α2,8-linked polysialic acid (without a known receptor in human body) has important role in brain development and tumour progression (mentioned in section 1.4.2). Without provoking the immune response, the fact that PSA has been popularly used by bacteria or malignant cancer cells also suggests that PSA can be applied to masking the immunogenic determinant from therapeutic proteins that often raised with non-self properties eliciting anti-sera antibody productions. Furthermore, PSA masking may sterically hinder the approach of pre-formed antibodies and proteases to their corresponding sites on the therapeutic proteins (Gregoriadis et al. 1993). In terms of PSA conjugated proteins, the potential of enhancing immunogenicity from a PSA covalently coupled carrier protein has been previously suggested by converting them to thymus-dependent antigens (Devi et al. 1991). PSA was also reported as poor immunogen after conjugation with proteins, and expected potential alterations of the net surface charge as well as the three-dimensional structure of the conjugates with possibly enhanced immunogenicity (Jennings and Lugowski 1981; Jennings et al. 1986). An evaluation study was carried out in order determine the immunological properties of chemically polysialylated proteins using asparaginase as an example. To that end, they conjugated 10 kDa PSA chains to asparaginase via random amine-coupling, and found that without provoking additional immune response, polysialylation reduced the antigenicity of asparaginase and as a result prolonged its circulation in the blood even in the presence of anti-asparaginase antibodies (Fernandes and Gregoriadis 2001). Based on these findings, theoretically the recombinant polysialylation approach produces PSA via a human glycoprotein biosynthesis pathway is expected to have much less immunogenicity issues but equivalent antigenic/proteomic site masking purposes as the chemical-polysialylation. In terms of the immunogenicity of the entire fusion protein, *in vivo* experiments using immunized animal with MFE23-Ig5-FN1 (with or without
polysialylation) can be done to detect the generation of anti-fusion protein IgGs. In addition, although PSA is a biodegradable material, its toxicity at a high dose should also be studies as well as related side effects.

**Polydispersity**

Polydispersity can be considered as an inevitable feature of synthetic polymers. A measure of the distribution of molecular mass in a given polymer sample (also know as the polydispersity index or PDI) is normally used to define the polydispersity of the polymer. The PDI is calculated as a ratio between the weight average molecular weight ($M_w$) and the number average molecular weight ($M_n$), and gives indications to the distribution of individual MW in a batch of polymers. The value of PDI can be equal to or more than 1, although the closer to the value of 1, the uniform chain length the polymer batch displays (Rudin 1999). As a negative property, the polymer polydispersity is reflected in polydispersity of the conjugates, and significantly restricts the application of polymer derived PK modulation approaches to small protein therapeutics. Compare with other polymers, PEG has a relatively narrow polydispersity with PDI equals to 1.01 for PEGs less than 5 kDa, and 1.1 for the ones above 50 kDa (Roberts et al. 2002). However, it was still suggested that PEG-conjugate is composed of molecules with different number of PEG monomers, yielding a Gaussian distribution of MWs. Therefore, conjugating with a certain MW PEG in fact produces a diverse conjugate population, which might induce different functional impact, including blood circulation time and immunogenicity (Veronese and Pasut 2005; Veronese and Mero 2008). Based on the development of polymer synthesis and purification procedures, much less polydispersive PEGylated products have been introduced; however the polydispersity problem still needs to be considered for the low MW therapeutics, such as antibody fragments, as they can be more effective in relation to their molecular size changes (Veronese and Pasut 2005). In addition, high cost in producing more uniform chain length polymers for conjugation should also be considered. Consequently, the same polydispersity limitation also comes to the fore for the polysialylation technologies, and concerns of PSA polydispersive have been suggested by almost all the chemically polysialylated protein studies (Gregoriadis et al. 2005). Reported that natural bacterial colominic acid (DP~200) has a PDI value (<1.1) similar to high MW PEG (Troy 1992; Troy 1995), it is equally important to control the polydispersity of polysialylated antibody fragment. In terms of the recombinantly polysialylation, lower PDI could be found due to shorter PSA chain length. However, from the MOLDI TOF-MS and SEC results for the polysialylated MFE23-Ig5-FN1 fusion protein, a polydispersive profile
can be already detected even after ion-exchange purification (Chapter 5). Better purification methods, such as more specific ion exchange or SEC purifications should be involved in the future studies for further reducing the polydispersity of the polysialylated fusion protein species, and in terms of quality control, monitoring the PDI of recombinantly polysialylated product should be strictly considered.

In addition, questions on potential structure conformational change of scFv-Ig5-FN1 fusion proteins after recombinant polysialylation, as well as serum/in vivo degradation profile of recombinantly polysialylated antibody fragment may also need to be studied.

**New Targets**

New antibody fragment models (e.g. neutralizing antibody) as well as protein/peptide (i.e. TNF, VEGF, and toxins) examples should also be tested for recombinant polysialylation with pharmacokinetic investigations for understanding the universality of the modification approach. These proteins/peptides require prolonged circulation half-life for therapeutic purposes, making them perfect pipeline candidate to apply recombinant polysialylation as an alternative technology for PK enhancement.

### 8.5.2 Improvements for Current Recombinant Polysialylation Approach

As a novel PK modulation approach, the recombinant polysialylation technology described in this thesis requires a number of refinements, in order to keep the technology developing in a right direction with better application value. Based on the experimental data described from each result chapter, the predominant inefficiencies emerged from the control and the degree of polymerization, which again can easily lead to polydispersity involved quality problems.

Different from the chemical-conjugation method, recombinant polysialylation is an intracellular protein glycosylation process that involves the biosynthesis of polysialic acid from the specific polysialylation epitopes on specific protein substrate. A cascade of enzymatic reactions is involved in this process (Figure 1.17), and these enzyme activities are highly correlated to the culturing cell conditions. A strictly good and uniform cell culturing technique with a standard cell passage number control needs to be performed routinely for maintaining the enzyme activities with minimized polysialylation level changes.
From anion exchange purification results (Chapter 4 and Chapter 7), three different scFv-Ig5-FN1 fusion glyco-forms with increasing polysialylation levels were detected, and the sialylated glyco-form took a significant proportion of the total fusion protein production, which therefore reduced the yield of polysialylated fusions. Based on the same amino acid backbone, this phenomenon might suggest an inefficient polysialylation to the sialylated and weakly polysialylated fusion glyco-form, and potentially they can be pushed toward the highly polysialylated level. With correct polysialylation improvement strategies, it is achievable to produce more homogenous fusion proteins with probably a higher DP level.

Technically, more selective monoclonal selection methods (e.g. FACS) can be improved for identifying mammalian cell monoclonal clones with higher polysialylation and fusion protein production levels. Cell culture conditions, including incubation time, temperature, culture medium pH, and other cell metabolic product levels. More optimized purification protocols are also needed to purify and define the polysialylated species with more distinctive DP. A DP controllable polysialylation process will be beneficial for modifying protein with adequate half-lives to their corresponding applications, where a too long plasma circulation might not be an advantage to certain applications (e.g. radioisotope conjugate).

Moreover, several methods for increasing sialylation of recombinant glycoproteins have been introduced previously (Bork et al. 2009), and some of these methods are also very prospective for improving the recombinant polysialylation, such as (1) application of N-acetylmannosamine (Man-NAc) - the specific precursor for increasing intracellular sialic acid pool (Pels Rijcken et al. 1995), hence CMP-Neu5Ac level for polysialylation, (2) overexpression of enzymes involved in the biosynthesis of glycans (i.e. α2,3/2,6 and 2,8-sialyltransferases) (Jenkins et al. 1996), (3) over-expression of GNE (the rate-limiting step in the biosynthesis of sialic acid) (Bork et al. 2009) (see Figure 1.17), and (4) inhibition of sialidase (Munzert et al. 1996). In addition, a number of synthetic N-acyl-modified D-mannosamines have also been introduced as sialic acid precursors for sialic acid biosynthesis (Keppler et al. 1999), however, the ability of these unnatural sialic acid to form into PSA chain structure, and the consequent immunogenicity hazard have to be noticed. Combine with optimized purification process, the success of these cell or metabolite engineering methods can additionally increase the yield and decrease the PDI of polysialylated species.
Other than producing the recombinantly polysialylated from inside of the cell, it might be also possible to elongate the glycosylated scFv-Ig5-FN1 fusion protein in test tubes. Previously, Cho et al. successfully polysialylated several exogenous sialyl accepting glycosphingolipids (GSLs) with *E. coli* K1 polyST. They also reported that a structural feature common to the preferred sialyl acceptors was the disialylglycotope, Siaα2,8Sia, α2,3-linked to galactose (Cho and Troy 1994). Sharing the similar structural of the sialylated scFv-fusion protein, this “in test tube” method might be able to re-polysialylate the sialylated glyco-species using PST or STX, and also monitor the upper limit of recombinant polysialylation DP with sufficient CMP-Neu5Ac substrate.

Factors responsible for the yield of recombinantly polysialylated scFv-Ig5-FN1 from the mammalian cell expression system should also be improved for better application of the technology. Further more, based on the existing approaches and the development to the production of homogeneous human glycoproteins with human-type glycans from other biological expression systems, such as bacteria or yeast (Rich and Withers 2009), alternative ways for producing recombinantly polysialylated proteins could possibly be considered with increased production yield.

### 8.5.3 Future Development Concepts

Based on the successful application from recombinantly polysialylated scFv-Ig5-FN1 fusion protein, a number of pipeline fusion concepts (listed in Figure 8.1) can then be processed in order to further verify and accomplish the development of antibody fragment recombinant polysialylation technology and facilitate antibody fragments to apply their advantageous features after getting smaller.

As mentioned in section 1.4.4, the natural NCAM FN1 domain was suggested as a docking site for PolyST contacting with NCAM and consequently starting elongating the *N*-glycans on adjacent the Ig5 domain (Figure 1.18). In the case for scFv-Ig5-FN1 fusion protein, once the polysialylation completes, the FN1 domain becomes more redundant to the fusion protein. Minimizing the fusion protein moiety by the removal of FN1 domain post-polysialylation can be beneficial for antigen localization and tissue penetration. To achieve this, a recombinant protease-recognizing linker between Ig5 and FN1 domain can be introduced, and the specific enzymatic hydrolysis treatment to the collected fusion protein with new linker can specifically
detach the FN1 domain from the polysialylated scFv-Ig5 fusion moiety (Figure 8.1a, b, c). Based on the polysialylation evidence from an engineered O-glycosylation site of the recombinantly modified NCAM FN1 domain (also referred as Δhelix TT) (Mendiratta et al. 2006), linking the scFv moiety with this polysialylatable FN1 domain can lead to another fusion option (Figure 8.1d). Ultimately, the CDR grafts that responsible for the antigen binding specificity of an antibody can be genetically extracted and recombinantly fused with

![Figure 8.1. Schematic of future recombinantly polysialylatable scFv-fusion constructs.](image)

Based on the construct of scFv-Ig5-FN1 fusion protein (a), FN1 domain, the PolyST docking site can be removed by introducing a specific protease recognizing linker (pink) between Ig5 and FN1 domains (b), which leads to a polysialylated scFv-Ig5 fusion protein (c) that is smaller than the original. An engineered FN1 domain alone with additional O-glycosylation site was suggested to be polysialylated (Mendiratta et al. 2006). Fusing this polysialylatable FN1 domain with scFv (d) can be considered as another alternative construct. CDR grafts can be extracted from the scFv and fused with the original polysialylatable Ig5-FN1 domains (e), as well as the Ig5 and FN1 connected by the hydrolysable linker (f). Eventually the polysialylated CDR-Ig5 fusion construct can be produced with probably the smallest protein content. The amino acid MWs of each fusion construct are also indicated accordingly.
the immunoglublin-like Ig5 domain (Figure 8.1e). Such species with non-antibody format but effective antigen-binding affinity are also known as affibodies, and have been successfully produced through different protein manipulation studies (Nord et al. 1997; Gunneriusson et al. 1999). The first NCAM Ig-like domain has also been investigated for fusing with antibody CDR graft (Casey 2009). Consequently, by removing the redundant FN1 domain after polysialylation, the smallest polysialylated antigen binding motif (~10 kDa) among all polysialylatable fusion constructs can be produced (Figure 8.1f, g).

In addition to PK modulation, PSA has already been used as degradable scaffold material for making hydrogels for drug release (Haile et al. 2008). The recombinant polysialylation method also offers an alternative platform of using PSA as a potential scaffold for nanotechnology or drug conjugations. Based on the DP of PSA, a fine balance of the amount between short, medium and long PSA chains associated with the polysialylated therapeutic product can lead to a differential clearance portfolio, which serves in a sense of drug release concept with a continuous drug supply based on the circulation half-life.

### 8.6 Benefits of Recombinant Polysialylation

Overall, the recombinant polysialylation technique described in this thesis establishes a promising and alternative platform for modulating the pharmacokinetics of bio-therapeutics. Using antibody fragment (i.e. scFv) as example, small proteins which can lose their therapeutic potency significantly by rapid blood clearance through kidney filtration have been shown to re-gain in vivo circulation longevity after recombinant polysialylation modification. Fundamentally, this new technology follows the PK modulation principle demonstrated by PEGylation, chemical-polysialylation and other hydrophilic polymer involved hydrodynamic volume improving approaches. Suggested previously in the thesis (section 1.5.1), water-attracting polysialic acid was biosynthesized and specifically manipulated to increase the hydrodynamic size of the associated scFv molecule, and preventing fast kidney clearance as seen with scFv.

On contrast to all other polymer involved approaches using chemical-conjugation, the recombinant polysialylation offers a chemistry-free method that reduces the potential
immunogenic hazard induced from chemical engineered proteins and polymers, and particularly to antibody fragment, it retains the integrity of the antigen-binding moiety of the scFv without being chemically modified. The genetically arranged site-specific polysialylation also avoids the interruption of the antigen-binding site from polymer conjugation. Moreover, the polysialylated scFv through the recombinant approach requires only one production line, while the chemical-conjugation needs the production of scFv and the hydrophilic polymers as well as additional conjugation process of the two and the downstream purification of the conjugate. Significant material wasting from various purification processes greatly increases the production cost of the chemical-conjugation approaches. With further optimization, the recombinant polysialylation promises to offer an easy and cheaper platform technology to reduce the rapid kidney elimination of small biotherapeutics. Furthermore, already introduced by chemical-polysialylation (section 1.3.1.3), PSA is a polysaccharide, organically biodegradable, FDA approved polymer material, and has already been used as protein conjugate in clinical trial stages. PSA is expected to reduce the risk of accumulation within the body that leads to consequent toxicity and immune response stimulating by many synthetic polymers.
APPENDIX
Appendix 1:

Vector Map for pcDNA4™/TO/myc-His Vectors

The pcDNA4™ system was used for the construction of scFv MFE-23 and C6.5 alongside their fusion constructs. Transformed *E. coli* XL-1 blue cells were selected by ampicillin resistance conferred by this vector. Stable mammalian cell lines of NB2a and HEK-293 cells were maintained by selection against zeocin resistance conferred by the vector. All constructs were tagged by c-terminal Myc and His$_6$. The tetracycline controlled operator (TO) normally used for expression of potentially toxic proteins was not exploited in this study.
Appendix 2:

Vector Map for pIg Vector

The pIg vector system shown above originally contained a chimeric NCAM-140 fusion construct (incorporated via the Hind III/Bam HI sites of the multicloning region) with a C-terminal Fc tail (provided by Dr. Jane Saffell, Imperial College London). The NCAM domains including Ig5-FN1 and FN1 alone were extracted from the NCAM-140 sequence within this vector and fused with MFE23 scFv sequence consequently (Constantinou 2005).
Appendix 3:

Vector Map for pcDNA3.1/V5-His Vectors

Both PST and STX sequences were provided as insertions in pcDNA3.1/V5-His B vector between EcoRV and XbaI restriction sites (from Professor Karen Colley, University of Illinois, USA). Transformed *E. coli* XL-1 blue cells were selected by ampicillin resistance conferred by this vector. Stable HEK-293 cells were maintained by selection against neomycin resistance conferred by the vector. Both the inserted PolyST constructs were tagged by c-terminal V5 and His$_6$. 

* After the XhoI site, there is a unique BstE II site, but no XbaI or ApaI sites in version C.

** There is a unique SacII site between the ApaI site and the Sfu I site in version B only.
Appendix 4:

**pUC119 DNA Vector**

pUC119 vector was originally used for containing C6.5 scFv in its multi-cloning site. *E.coli* XL-1 blue cells were transformed with this vector and selected by ampicillin for DNA amplification.
Appendix 5:

pEGFP-C1 Vector

pEGFP-C1 encodes a red-shifted variant of wild-type GFP which has been optimized for brighter fluorescence and higher expression in mammalian cells. A bacterial promoter upstream of this cassette expresses kanamycin resistance in E. coli. For the research purpose of this project, pEGFP-C1 vector was used as positive control in transfection optimization experiments.
Appendix 6

Sequence of MFE23 scFv
Sequence Range: 1 to 732

CAGGTGAAACTGCAGCAGTCTGGGGCAGAACTTGTGAGGTCAGGGACCTCAGTCAAGTTG
GTCCACTTTTGACGTGTCAGACCCCGGTCTTGAAACACTCCAGTCCCTGGAGTCAGTTCAC
Q V K L Q Q S G A E L V R S G T S V K L

TCCTGCACAGCCTTCTGGCTTCAACATTAAGACTCCTATTATGCACTGGTGGGACTGGCAGGGG
AGGACGTGTACAGGAGCCAGGTTTTGTAATTTCTGAGGATATAGTGACAAACTCCGTC
S C T A S G F N I K D S Y M H W L R Q G

CCTGAAACAGGCGCTGGAGATTGAGATGATTGATCCTGAGAATGCTGAGTACTGAATAT
GGACTTGTACCCCGGACTTCACCTAACCTACTACCTACTAGGACTCTTACGACTATTATA
P E Q G L E W I G W I D P E N G D T E Y

GCCCGAAGTTCCAGGGCAAGGCCGACCTTTTACTACAGACATCCCTCACAAGCCTAC
CGGCGGTCTGGTCGTCAGCTGGACTGATGACTCCTGGGGACTGGGAGGGAGGTTGTCG
A P K F Q G K A T F T T D T S S N T A Y

CTGGCAGCTCAGCACTCTAGAGACACTGCCTGCTATTGTTGATAATGGAGGGACT
GAGCTCAGTGCAGCTGAGCTTACTGCTGACTCTGGGCGAGACTCCCTGGGCGAGGAGG
L Q L S S L T S E D T A V Y Y C N E G T

CCGACTGGCCGGCACTACTTCTGACTCTGGGGCAAGGACCAGCTCCGCAGTCCTGCTCTCA
GGCTAAGCCGATTGAGAACTGATGACCCCGTCTGGTGCAGTGCCAGGAGGAGGT
P T G P Y Y F D Y W G Q G T T V T V S S

GGTGAGGCGCTTGGAGCTCTGGGCTGGGCTGAGACAGTCAGAACAAATGCTGACC
CCACCTCCGCAAGTCCGCTCCACAGGGCAAGCCCCCGCAGCTGGTGTTGAGAGGT
G G G G S G G G G S G S G G S E N V L T
CAGTCTCCAGCAATCATGTCTGCATCTCCAGGGGAGAAGGTCACCATAACCTGCAG

TGCC

GTCAGAGGTCGTTAGTACAGACGTAGAGGTCCCCTCTTCCAGTGGTATTGGACGTCACGG

Q  S  P  A  I  M  S  A  S  P  G  E  K  V  T  I  T  C  S  A

AGCTCAAGTGTAAAGTTACATGCACTGGTCCAGCAAGCCACGGCAGTCTCTCTCCCAAACTC

TCGAGTTTCACATTCATAGTGACTCGACCAAGCTCTCTCCAGCTGGTATTGGACGTCACGG

S  S  V  S  Y  M  H  W  F  Q  Q  K  P  G  T  S  P  K  L

TGGATTTATAGCACATCCCAACCTGGCTCTCGAGTCCCTGCTCTCCGACTGGCAAGTGGA

ACCTAAATATCGTGTTAGTTGGAACCAAGACCTACGGAAGCGACGAGTAACCTTGCTACCT

W  I  Y  S  T  S  N  L  A  S  G  V  P  A  R  F  S  G  S  G

TCTGGGACCCTCTTTACTCTCTCAACATCACCGGAATGGAGCTGAAGATGCTGCCACTTAT

AGACCCTGGAGAATGGAGAGATGGTAGTGACAATTCCAGCTATCCGACTCTACTACGACGGTAAT

S  G  T  S  Y  S  L  T  I  S  R  M  E  A  E  D  A  A  T  Y

TACTGCCAGCAAAGGAGTAGTACCCACTCACTACGTTGCTGCTCCACCAAGCTGGAGCTG

ATGACGGTCTGTTTCTACTCAATGGGAGTGGACGACCCACAGCGGCTGCTGACCTCGAC

Y  C  Q  Q  R  S  S  Y  P  L  T  F  G  A  G  T  K  L  E  L

AAACGGGCGGCC

TTTGGCCCGCCGG

K  R  A  A
Appendix 7

Sequence of C6.5 scFv
Sequence Range: 1 to 768

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| 70| 80| 90| 100| 110| 120|
| TCTGTAGAGGTTCTGGATACAGCTTTACACAGCTACTGGATCCGCTTGAGGATCGTCCCAGATG|
| SCKGSGYTSWIAWVRQM|

| 130| 140| 150| 160| 170| 180|
| CCGGGAAAGGCCTGAGATACATGGGCTCATCTATCTCTGAGCTCTGAGCACAAATAC|
| PGKGLEMYMGLYPGDSDTSKY|

| 190| 200| 210| 220| 230| 240|
| AGCCGTCCCTTCCCAGGCCAGTCACCATCTCGACGACAAGCGTCACGCACCTGCTAC|
| SPSFQGQVTISVDSKSVSTAY|

| 250| 260| 270| 280| 290| 300|
| TTGCAAATGGAGCAGTCGTCAAGGCTCGAGCCGCTGTATTTTTTGTGGGAGACATGAC|
| LQWSSLKPSDSSAVYFCARHD|

| 310| 320| 330| 340| 350| 360|
| GGGGATATTGCAATATCGATAGTTCCAACCTGCGAAATGGGCTGATCTTTTTGTGGGAGACATGAC|
| VGYCSSSNCAKWPHEYFQHWG|

| 370| 380| 390| 400| 410| 420|
| CAGGGCACCCTCGGCTCACCGTGTCCTCCTCAGTTGGAGGCCGTTTCAGGCCAGGATGGTCCTGGC|
| QGTLVTVSSGGGGGSG|

| 430| 440| 450| 460| 470| 480|
| GGTGCGGGATACAGTCTGTGGAGCGCCAGCCCCTCAGTCTCGGCCGCCAGGACAG|
| GGGSQSSLTTQPPSSVSAAPGQ|

| 490| 500| 510| 520| 530| 540|
| AAGGTCAACCCTCCTCTGGAGGAGCAGCTCACAATGGGAAATTTATGTATCTGG|
| KVTSICSGBSNSNIGNNYVSW|
550 560 570 580 590 600
TACCAGCAGCTCCAGGAAACAGCCCCAAACTCCTCATCTATGGTCACACCCAATCGGCC
Y Q Q L P G T A P K L L I Y G H T N R P

610 620 630 640 650 660
GCAGGGGTCCCTGACCGATTCTCTGCTGCTCCAAGTGCTGGCACCTCAGGCTCCTCGCCATC
A G V P D R F S G S K S G T S A S L A I

670 680 690 700 710 720
AGTGGGTTCGCGTGCGGATGGCTGGATTATTACTGTGCAGCATGGGATGACAGCCTG
S G F R S E D E A D Y Y C A A W D D S L

730 740 750 760
AGTGGTTGGGTGTTCCGCGGAGGACCAGCTGACCGTCCTAGGT TAG
S G W V F G G G T K L T V L G
Appendix 8

Sequences of Recombinantly Polysialylatable Fusion Components

NCAM mammalian expression leader sequence:

ATGCTGCAAACCTAAAGGATCTCATTCTGGACCTTTTTTCTTTTCTTGGGACTGCAGTTTCTCTG
CAGGGGACATGG

NCAM Ig5 domain (264bp):

TATGCCCCAAAGCTACAGGGCCCTGTGGCTGTGTACAACCTGGGAGGGGAGCAGGGTGAAC
ATCACCTGCAGGCTATTTGCTATCCAGCTGACCAGTCATCTCCATTCTACAGCTACAACCCCTCTGCTCCAGCTAT
CTGGAGGTGACCCCAAGACTCTGAGAATGTATTGTGGAATCTACAACTGACTGTCACTGCACTG
CGCATTGGGCGGGAGTGCTTTGAA

NCAM FN1 domain (300bp):

TTCTATCTCTTTGTTTCAAGCAGACACCCCCCTCTTACCATCCATGAGGATGGGAGCCATAC
TCCACGACAGCCACAGGTGAGTTGTGTAACCAGAGCCACAGGTGGGCTGCTCCAAACCCCTGTTGAAGGCTGGCGGCGCTCAATGGCAAAGGGCTGGGTGAGATCAGCGGCCTCC
Appendix 9

ST8Sia IV (PST) DNA Sequence
ST8SIA4 ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 4 [ Homo sapiens ]

Sequence (1077bp):

ATGCGCTCCA TTAGGAAGAGTGCGACGATCTGCAAAATAGTAGTGCAACTCAGAGGAGAAATGCTGGCTCTATGCTCGTCAATAGCTCAATCGGAGATCCGAACACTTCTGCCATCTTTTACGCAGACAGCCCAGTACAAACAGAAGGACCTGTACACAGAGCTAACCTTCTG

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Appendix 10

**ST8Sia II (STX) DNA Sequence**


**Sequence (1128bp):**

```
ATGCAGCTGC AGTTCCGGA GCTGGATGCTG GCCGCGC TCA CGCTGCTCG
GGTCTTCCTC ATCTTCGCAG ACATCTCAG GATCGAAGAA GAAATCGGGA
ATTCGGGAGG CAGAGGTACA ATCAGATCAG CTGTGAACAG CTTACATAGC
AAATCTAATA AGAGCTGAAGT TGATTAATAAC GGCTTCTCAT CAGACCTGT
TGTTGACAGA AGTAATGAAA GCATCAAGCA CAACATCCAG CCAGCCTCGT
CCAAATGGA ACATAACCG AGCCTCTCCT TGAAGATCAG GAAGCAGATT
TTAAGGTCTT TGATGTCTGA AAAGGACATT TCTGTCCTAA AGGGAACCTT
GAAGCCTGGA GATATTATAC ATTACATCTTG CAGACAGGAC AGCACCATGA
ATGTCGGCCA GAGCCTCTAC GAGCTTCTCC CAGAGCTTCC GCCACTGAG
AATAAGCCAT TTGGGACTTG TGCCATCTGT GCCAACCTGGG GGGTCTTGCT
GAACAGCGGC TGTTGGGACC AGATTGACGC CCAGCCTTCTC GTCATCAGGT
GCAACCTGGG CCCAGTACAG GAGTATGCCA GGATGTGGG GCCAAAGACA
GACCTGGTAA CCATGAACTC CTGCTACATC CAGCCGGCCT TTGAGGACTT
GGTCAATGCC ACGTGGGCGG AGAAGCTGCT GCCACGGCTG CACAGCTTCA
ATGGCAGCAT CCTGTGGAAC CCTGGCTTCA TGGCCGCGGG GGGCAAGGAG
CGTGTTGAGT GGTGAAGCAG GCTTTATCTG AAGCACCAGC TCGACCCTGG
CAGTGATCAG CCGTCTCCTG GCCCTGTGCA GCGGACTTCC CAGACCTGAG
TGACCAACAA AGTCCAGACGT AAAGAAGCCA CCAGCAGCCT TTGATGATAT
ACCCTGGCCA CAGGTTCCTC CCAAACAAATCT CATCTCTCCG CCTTCTGCC
CTTTGTCCTG CGATCAGAAC AGAACCCAGT CAAGTACAC TATTTAGACA
GCCCTAACAC TGCTACACCC TCCAGCGCCA GCCGCATAC CAGGCCTTG
GAGTAAAGG CACCTGGAAG CCTAGATGAC CAGGGGCTTT TGAACAGTAC
TGTCGGCCAG TGCGATGGGG CCAGCTGAG
```
Appendix 11:

The principle monosaccharides found in mammalian glycoproteins

Information of symbols, structures, underivatised and permethylation derivatised monoisotopic mass (m/z) of each monosaccharides were listed and specifically applied for the mass spectrometry analysis in this project.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Name</th>
<th>Monosaccharide Structure</th>
<th>Underivatised Monoisotopic Mass (Average)</th>
<th>Permethylated Monoisotopic Mass (Average)</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Fucose" /></td>
<td>Fucose (Fuc)</td>
<td>α-D-Fucose</td>
<td>146.0579 (146.1430)</td>
<td>174.0892 (174.1968)</td>
</tr>
<tr>
<td><img src="image" alt="Mannose" /></td>
<td>Mannose (Man)</td>
<td>α-D-Mannose</td>
<td>162.0528 (162.1424)</td>
<td>204.0998 (204.2230)</td>
</tr>
<tr>
<td><img src="image" alt="Glucose" /></td>
<td>Glucose (Glu)</td>
<td>β-D-glucose</td>
<td>162.0528 (162.1424)</td>
<td>204.0998 (204.2230)</td>
</tr>
<tr>
<td><img src="image" alt="Galactose" /></td>
<td>Galactose (Gal)</td>
<td>β-D-Galactose</td>
<td>162.0528 (162.1424)</td>
<td>204.0998 (204.2230)</td>
</tr>
<tr>
<td><img src="image" alt="N-acetylglicosamine" /></td>
<td>N-acetylglicosamine (GlcNAc)</td>
<td>N-acetyl-α-D-glucosamine</td>
<td>203.0794 (203.1950)</td>
<td>245.1263 (245.2756)</td>
</tr>
<tr>
<td><img src="image" alt="N-acetylgalactosamine" /></td>
<td>N-acetylgalactosamine (GalNAc)</td>
<td>N-acetyl-α-D-galactosamine</td>
<td>203.0794 (203.1950)</td>
<td>245.1263 (245.2756)</td>
</tr>
<tr>
<td><img src="image" alt="N-acetyleneuraminic acid" /></td>
<td>N-acetyleneuraminic acid (NeuAc)</td>
<td>α-D-N-acetyleneuraminic acid (sialic acid)</td>
<td>291.0954 (291.2579)</td>
<td>361.1737 (361.3923)</td>
</tr>
<tr>
<td><img src="image" alt="N-glycolyneuraminic acid" /></td>
<td>N-glycolyneuraminic acid (NeuGc)</td>
<td>α-D-N-glycolyneuraminic acid (sialic acid)</td>
<td>307.0933 (307.2573)</td>
<td>391.16423 (391.4186)</td>
</tr>
</tbody>
</table>
Appendix 12:

The raw data obtained from MALDI-MS analysis of the permethylated N-glycans released from MFE-Ig5-FN1 using PNGaseF.

Signals consistent with some under and/or over methylation were observed, which was most noticeably in high mass complex structure.
Appendix 13:

The raw data obtained from ES-MS analysis of the permethylated N-glycans released from MFE-Ig5-FN1 using PNGase F.
Appendix 14

Analysis of $O$-glycan structures of MFE23-Ig5-FN1 fusion proteins by negative-ion reflectron mode MALDI-TOF MS. No typical $O$-glycosylation was indicated by the signals detected from the three glyco-forms, known as (a) highly polysialylated, (b) weakly polysialylated, and (c) sialylated MFE23-Ig5-FN1 fusion proteins.


Angata, K., M. Suzuki, et al. (2000). "Differential biosynthesis of polysialic acid on neural cell adhesion molecule (NCAM) and oligosaccharide acceptors by three distinct alpha 2,8-sialyltransferases, ST8Sia IV (PST), ST8Sia II (STX), and ST8Sia III." J Biol Chem 275(24): 18594-601.


formation also to a second antigen, if the latter is presented as a covalent adduct with the former." Adv Exp Med Biol 303: 199-206.


