Functional Influence of VASE Insertion on Neural Cell Adhesion Molecule Homophilic Binding

By

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A thesis submitted to Imperial College London in candidature for the Diploma of Imperial College and for the degree of Doctor of Philosophy.

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Declaration

I declare that, unless stated, the work described in this thesis was solely carried out by the author.
None of the work of the author has been submitted for any other qualification at this or any other university.

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Abstract

Neural cell adhesion molecule (NCAM) is a transmembrane, homophilic binding protein which has important roles in cellular migration, nervous system development, learning and memory. NCAM is estimated to have at least 27 different isoforms and the functional significance of one alternatively spliced isoform, called the variable alternatively spliced exon (NCAM-VASE) is the subject of this research.

NCAM-VASE differs from NCAM by the insertion of an extra ten amino acids into the fourth immunoglobulin domain. This converts NCAM function from a promoter of axonal outgrowth to an inhibitor. NCAM-VASE is expressed in nervous system areas of low synaptic plasticity in contrast to NCAM, which is generally found in areas of high plasticity. It has been postulated that NCAM-VASE functions differently by altering the homophilic binding strength of NCAM.

To test this hypothesis, single molecule force spectroscopy (SMFS) was carried out using an atomic force microscope (AFM) to measure the adhesion between single molecule NCAM±VASE proteins. A novel method for AFM surface functionalisation was developed to affinity capture Fc-tagged NCAM±VASE proteins via an adsorbed α Fc antibody. Adhesion measurements revealed that the homophilic binding strength of NCAM and NCAM-VASE proteins was similar but this data was only preliminary. Due to the potential problems associated with the use of a dimerising Fc tag and non-specific tip-sample interactions, another model was developed for measuring the adhesion between covalently bound PEGylated surfaces coordinating His-tagged NCAM±VASE proteins. Due to time constraints AFM measurements were not taken using this system.

A novel form of AFM, termed single cell force spectroscopy (SCFS), was used and an experiment was developed to measure the adhesion between parental cells and those expressing the NCAM or NCAM-VASE transgene. At long contact times (10 min), not short (5 s) NCAM-VASE cell adhesion was increased three-fold over two NCAM cells.
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<th>Description</th>
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<tbody>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic Force Microscope/Microscopy</td>
</tr>
<tr>
<td>APTES</td>
<td>Aminopropyltriethoxy silane</td>
</tr>
<tr>
<td>AvBBSA</td>
<td>Avidin incubated with biotinylated bovine serum albumin</td>
</tr>
<tr>
<td>BBSA</td>
<td>Biotinylated bovine serum albumin</td>
</tr>
<tr>
<td>Boc</td>
<td>t-butyloxycarbonyl</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CAM</td>
<td>Cell adhesion molecule</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CMC</td>
<td>Critical micelle concentration</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CTB</td>
<td>Cholera toxin B subunit</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
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<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DEAE</td>
<td>Diethylaminoethyl</td>
</tr>
<tr>
<td>DFS</td>
<td>Dynamic force spectroscopy</td>
</tr>
<tr>
<td>dH$_2$O</td>
<td>Distilled water</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dsDNA</td>
<td>Double stranded DNA</td>
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<tr>
<td>EBNA</td>
<td>Epstein-Barr virus nuclear antigen</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>EDT</td>
<td>1,2-Ethanediol</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>EFM</td>
<td>Electrostatic force microscopy/microscope</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>EndoN</td>
<td>Neuraminidase</td>
</tr>
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<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>FA</td>
<td>Formamide</td>
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<tr>
<td>F-actin</td>
<td>Filamentous actin</td>
</tr>
<tr>
<td>FAK</td>
<td>p125 focal adhesion kinase</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FGFR</td>
<td>Fibroblast growth factor receptor</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FMM</td>
<td>Force modulation atomic force microscopy/microscope</td>
</tr>
<tr>
<td>Fn_{III}</td>
<td>Fibronectin Type III domain</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast protein liquid chromatography</td>
</tr>
<tr>
<td>Fyn</td>
<td>p59fyn</td>
</tr>
<tr>
<td>GDNF</td>
<td>Glial cell line derived neurotrophic factor</td>
</tr>
<tr>
<td>GFRα</td>
<td>GPI-linked GDNF family receptor α</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycophosphatidylinositol</td>
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<tr>
<td>HABA</td>
<td>4’-Hydroxyazobenzene-2-carboxylic acid</td>
</tr>
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<td>HBD</td>
<td>Heparin binding domain</td>
</tr>
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<td>HCl</td>
<td>Hydrochloric acid</td>
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<td>HEK 293</td>
<td>Human embryonic kidney 293 cell line</td>
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<td>HEPES</td>
<td>4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid</td>
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<tr>
<td>HNK-1</td>
<td>Human natural killer cell glycan-1</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidise</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular cell adhesion molecule-1</td>
</tr>
<tr>
<td>ICC</td>
<td>Immunocytochemistry</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>IL-2</td>
<td>Interleukin-2</td>
</tr>
<tr>
<td>IP₃</td>
<td>Inositol 1, 4, 5-trisphosphate</td>
</tr>
<tr>
<td>I-set</td>
<td>Intermediate set</td>
</tr>
<tr>
<td>KPFM</td>
<td>Kelvin probe force microscopy/microscope</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny broth</td>
</tr>
<tr>
<td>LBD</td>
<td>Lectin binding domain</td>
</tr>
<tr>
<td>LFM</td>
<td>Lateral force microscopy/microscope</td>
</tr>
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<td>MAP 1A</td>
<td>Microtubule associated protein 1A</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>MFM</td>
<td>Magnetic force microscopy/microscope</td>
</tr>
<tr>
<td>Mono</td>
<td>Monomeric</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MTS</td>
<td>3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular weight cut off</td>
</tr>
<tr>
<td>N</td>
<td>Newton</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NCAM</td>
<td>Neural Cell Adhesion Molecule</td>
</tr>
<tr>
<td>NHS</td>
<td>N-Hydroxysuccinimide</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>nN</td>
<td>NanoNewton</td>
</tr>
<tr>
<td>NTA</td>
<td>Nitrilotriacetic Acid</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>O-Su</td>
<td>O-Succinimide</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDP</td>
<td>2-Pyridyldithiopropionyl</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PIP₂</td>
<td>Phosphatidylinositol 4, 5-bisphosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PIPES</td>
<td>Piperazine-1, 4-bis(2-ethanesulfonic acid)</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLL</td>
<td>Poly-L-Lysine</td>
</tr>
<tr>
<td>pN</td>
<td>Piconewton</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>PSA</td>
<td>Polysialic acid</td>
</tr>
<tr>
<td>PSPD</td>
<td>Photosensitive photodector</td>
</tr>
<tr>
<td>RP</td>
<td>Reverse phase</td>
</tr>
<tr>
<td>RPTPα</td>
<td>Receptor tyrosine phosphatase α</td>
</tr>
<tr>
<td>Rₜ</td>
<td>Retention time</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SAM</td>
<td>Self-assembled monolayers</td>
</tr>
<tr>
<td>SCFS</td>
<td>Single cell force spectroscopy</td>
</tr>
<tr>
<td>SCM</td>
<td>Scanning capacitance microscopy/microscope</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SECM</td>
<td>Scanning electrochemical microscopy/microscope</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology 2</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SMFS</td>
<td>Single molecule force spectroscopy</td>
</tr>
<tr>
<td>SNOM</td>
<td>Scanning near-field optical microscopy/microscope</td>
</tr>
<tr>
<td>SPPS</td>
<td>Solid phase peptide synthesis</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface Plasmon Resonance</td>
</tr>
<tr>
<td>SThM</td>
<td>Scanning thermal microscopy/microscope</td>
</tr>
<tr>
<td>STM</td>
<td>Scanning tunnelling microscopy/microscope</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris acetate EDTA buffer</td>
</tr>
<tr>
<td>TBME</td>
<td>Tert-butyl methyl ester</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>tBu</td>
<td>tert-butyl</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------------------------------------</td>
</tr>
<tr>
<td>TCEP</td>
<td>Tris (2-carboxyethyl) phospine</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TIS</td>
<td>Triisopropylsilane</td>
</tr>
<tr>
<td>$T_m$</td>
<td>Melting temperature</td>
</tr>
<tr>
<td>TM-AFM</td>
<td>Tapping mode atomic force microscopy/microscope</td>
</tr>
<tr>
<td>TPA</td>
<td>12-O-tetradecanoylphorbol-13-acetate</td>
</tr>
<tr>
<td>TRITC</td>
<td>Tetramethylrhodamine B isothiocyanate</td>
</tr>
<tr>
<td>TR mode AFM</td>
<td>Torsional mode atomic force microscopy/microscope</td>
</tr>
<tr>
<td>Trt</td>
<td>Trityl</td>
</tr>
<tr>
<td>V</td>
<td>Volt</td>
</tr>
<tr>
<td>VASE</td>
<td>Variable alternatively spliced exon</td>
</tr>
</tbody>
</table>
Chapter 1 Introduction

Neural cell adhesion molecule (NCAM) was first discovered in 1977 following immunoprecipitation with a cell adhesion blocking antibody raised against neural retinal chick cells (Thiery, Brackenbury et al. 1977). It was therefore named from the cells in which it was discovered, although it has since been found in non-neuronal tissue including the heart and skeletal muscle (Small and Akeson 1990). Phylogenetic experiments have revealed that NCAM homologues are expressed in all vertebrates tested and in some invertebrates including Caenorhabditis elegans (C. elegans) (Hall and Rutishauser 1985).

Initially NCAM was classified with the calcium independent immunoglobulin (Ig) family of cell adhesion molecules and was found to function as a homophilic binding protein on neuronal cell surfaces. It is comprised of five immunoglobulin-like domains (hereon referred to as Ig domains) and two fibronectin type 3 (Fn3) domains, which form the extracellular portion of NCAM, and a transmembrane and variable cytoplasmic domain. There are a number of size and alternatively spliced isoforms, which create diversity in NCAM structure and function, two of which form the basis of this study (Reyes, Small et al. 1991).

As a mediator of cell–cell interactions, NCAM is a key component in neural development, with diverse functions in synaptic plasticity, neurite outgrowth, cellular migration and learning and memory (Cremer, Lange et al. 1994; Maness and Schachner 2007). In recent years NCAM has been found to function as a membrane receptor to proteins including glial derived neurotrophic factor α (GDNF) and fibroblast growth factor receptor-1 (FGFR-1) (Saffell, Williams et al. 1997; Paratcha, Ledda et al. 2003).

1.1 NCAM isoforms

1.1.1 Genetics

Cloning and sequencing studies of the human NCAM gene revealed that it is encoded at a single locus on chromosome 11q23 (Nguyen, Mattei et al. 1986; Walsh, Putt et al. 1986). Although there is a large degree of conservation of the NCAM gene between species, the mouse NCAM sequence is found in a syntenic region on mouse chromosome 9 (D'Eustachio, Owens et al. 1985). In particular the position and size of the exons in NCAM are similar across the species studied. Exon 0 encodes the signal sequence and initiator methionine and is followed by 10 exons which encode 5 Ig-like domains; Ig I is encoded by exons 1 and 2, Ig II by exons 3 and 4, Ig III by exons 5 and 6, Ig IV by exons 7 and 8 and Ig V by exons 9 and 10. Between exons 7 and 8 is the site for alternative splicing of a 30 base pair sequence termed the variable alternatively spliced exon (VASE).
Exons 11 to 14 encode two fibronectin type III domains (FnIII). Within these exons a putative hinge may be alternatively spliced between FnIII 1 and FnIII 2 (Santoni, Barthels et al. 1989). In the same region a set of six exons termed the muscle specific domain (MSD) due to its discovery in human skeletal muscle, may be alternatively spliced (Dickson, Gower et al. 1987). Following the FnIII domain sequences, there are a number of stop codons to create alternative size isoforms of NCAM with varying methods of attachment to the cell membrane. NCAM can be attached to the cell membrane via a glycosylphosphatidylinositol (GPI) anchor due to the presence of a stop codon immediately after the FnIII domains or via a transmembrane domain (Cunningham, Hemperly et al. 1987).

1.1.2 Alternative splicing

Alternative splicing within NCAM alters its structure, function and properties dramatically (Fig. 1.1). Theoretically the 26 exons of NCAM can give rise to over 100 distinct messenger RNA (mRNA) transcripts (Reyes, Schulte et al. 1993). There are three major size isoforms of NCAM which are NCAM-120, NCAM-140 and NCAM-180, so-called due to their apparent molecular weights (MW) by SDS-PAGE and which differ in their method of attachment to the cell membrane (Hoffman, Sorkin et al. 1982). All three isoforms contain five Ig-like domains and two FnIII domains. NCAM-120 is attached to the cell membrane via a GPI anchor with no cytoplasmic domain; NCAM-140 inserts into the membrane with a transmembrane domain and has a short cytoplasmic domain; NCAM-180 also has a transmembrane domain and a longer cytoplasmic domain (Nybroe, Linnemann et al. 1988). All three size isoforms of NCAM have been found to be secreted by shedding from rat brain membranes (Olsen, Krog et al. 1993). Another secreted isoform of NCAM exists, called NCAMSec, which lacks FnIII 2 and is 115 kDa (Gower, Barton et al. 1988). Although the function of NCAMSec is mainly unknown, it is expressed during myoblast fusion in muscle development (Gaardsvoll, Krog et al. 1993). The role of secreted NCAM has been postulated to provide a concentration gradient of diffusing NCAM to encourage cell motility and growth in under-developed muscle areas (Gower, Barton et al. 1988).

Alternative splicing of the 30 base pair VASE sequence into NCAM Ig IV yields an important isoform named NCAM-VASE. The splicing of this sequence into NCAM is more prevalent in the adult brain and is developmentally and temporally regulated (Doherty, Moolenaar et al. 1992). NCAM-VASE expression on adjacent cells has been found to cause neurons to lose their ability to extend pre-axonal processes called neurites.

The MSD is encoded for by exons MSDa, MSDb, MSDc and a codon AAG. NCAM containing the MSD sequence is not only found in muscle, but also in the developing mouse cerebellum where the corresponding mRNA was found. The MSD isoform of NCAM plays a role in muscle development where NCAM MSD expression temporally correlates with myoblast fusion and the resultant
myotubes (Suzuki, Angata et al. 2003). The MSD has also been found to be expressed in all three size isoforms of NCAM in developed muscle, resulting in NCAM 125 kDa, NCAM 145 kDa and NCAM 185 kDa. Although the MSD has no direct effect on adhesion or signalling, conserved O-glycosylation sites in MSDb and MSDc may improve the stability of NCAM at the cell membrane, by raising those domains above the surface (Pizzey, Rowett et al. 1989; Suzuki, Angata et al. 2003). Interestingly insertion of MSDa, a proline-rich sequence ‘HSPPP’ between human FnIII 1 and FnIII 2, has been found to inhibit neurite outgrowth of cerebellar cells (Kasper, Stahlhut et al. 1996). It is thought that this proline-rich hinge mediated FGF receptor binding for neurite outgrowth.

![NCAM exon structure and sites for alternative splicing and polysialylation.](image)

The NCAM gene encodes 26 exons which confer a range of functions to NCAM. The basic structure of NCAM consists of five immunoglobulin (Ig-like) domains, two fibronectin type III (FnIII) domains and a C-terminal sequence of varying length. Alternative splicing of the VASE sequence ‘ASWTRPEKQE’ and muscle specific domain (MSD) exons may occur.

The AAG sequence introduces residues Gln and Gly into NCAM, although the significance of this switch is unknown. Overall the multitude of NCAM size and alternatively spliced isoforms yields a vast array of functionally significant proteins. This is demonstrated by the varying structures, expression patterns and functions of NCAM isoforms.

### 1.2 NCAM structure

#### 1.2.1 General structure

As a member of the immunoglobulin family of cell adhesion molecules (CAMs) NCAM contains five Ig-like domains and two FnIII domains (Fig. 1.2). The 5 Ig-like domains have been found to allow NCAM to engage in homophilic binding, while the FnIII domains have roles in NCAM receptor signalling (Ranheim, Edelman et al. 1996; Walsh and Doherty 1997). Interestingly the Ig-like domains of NCAM were found to be conserved across a number of species, including chicken mouse and frog, using a heterogeneous aggregation assay (Hoffman, Chuong et al. 1984). The assay tested for the binding of one species of NCAM in brain membrane vesicles for their ability to bind to another containing a difference species of NCAM. Cross-species NCAM-mediated adhesion indicated that homophilic binding patches in the Ig-like domains were conserved during evolution.
Figure 1.2: NCAM protein structure and insertion into the cell membrane.
NCAM protein consists of five Ig-like domains followed by two FnIII domains. NCAM alternative splicing can result in three common size isoforms, NCAM-120, NCAM-140 and NCAM-180. These isoforms differ in their method of attachment to the cell membrane and by the length or presence of a cytoplasmic domain. NCAM-120 attaches to the cell membrane via a glycosylphosphatidylinositol (GPI) anchor and has no cytoplasmic domain. NCAM-140 and NCAM 180 insert into the membrane using a transmembrane domain and have a short and long cytoplasmic domain respectively.

Initially, structural studies were carried out using a series of monoclonal antibodies raised against NCAM from chicken optic nerve (Frelinger and Rutishauser 1986; Watanabe, Frelinger et al. 1986). Antibodies were selected and tested for reaction against various purified isoforms of NCAM. In this way antibody epitopes were mapped to create a topographical model for NCAM. Electron microscopy analysis of deglycosylated chick NCAM-180 and NCAM-140 (corresponding to NCAM-160 and NCAM-130) revealed that they were uniformly thick, linear rods with a distinct bend near the centre (Hall and Rutishauser 1987). This hinge may be due to the proline-rich MSDa sequence, although the authors did not comment on this (Kasper, Stahlhut et al. 1996).

Predictions based on the sequence of NCAM, revealed that Ig domains followed an ‘intermediate set’ (I-set) structure of domain folding (Harpaz and Chothia 1994). Ig structure is tightly conserved and domains consist of approximately 100 amino acids although there is only 10-30% residue homology between proteins of the Ig CAM family (Lesk and Chothia 1982). The Ig fold is composed of two β sheets packed face to face with a cysteine disulfide bridge. Each cysteine residue is encoded for by a different exon within the Ig domain (Walsh and Doherty 1997). The residues which are not involved in the β sheets give the Ig fold individuality and determine its exact structure. NCAM Ig
domain structure has since been confirmed to be of the I-set, from assignment of crystal structures of Ig I, II and III to a 2 Å resolution (Soroka, Kolkova et al. 2003).

Great variability in NCAM structure and function arises not only from alternative splicing of sequences into NCAM but also due to post-translational modifications of its nascent and folded form.

1.2.2 Post-translational modification

Post-translational modifications to a protein allow alteration of its chemical properties and its function. NCAM can undergo a number of regulated post-translational modifications to confer functional diversity to the molecule.

HNK-1 Glycosylation

NCAM has six conserved sequences for N-glycosylation located at N-203, 297, 329, 415, 441 and 470 (numbering for human NCAM-VASE) (Albach, Damoc et al. 2004). These N-glycosylation sites are termed N-1 to N-6 respectively (Fig. 1.3). In murine NCAM, N-1 has been found to not always be glycosylated, whereas N-2, N-3 and N-4 have been found to be modified with a carbohydrate epitope human natural killer cell glycan (HNK-1). HNK-1 is a sulphated glycoside molecule and can modify a number of other CAMs and cell membrane proteins, including L1 and myelin associated proteins (Dodd and Jessell 1986). It has been implicated in synaptic plasticity and has been found to inhibit NCAM homophilic binding (Marzban, Sillitoe et al. 2004). Importantly mice with targeted knockouts of the glucuronlytransferase enzyme GlcAT-P exhibited defects in spatial memory formation due to their inability to synthesise HNK-1. Sulfated glycosides has also been incorporated into NCAM but their function is unknown (Sorkin, Hoffman et al. 1984; Suzuki, Hiraoka et al. 2001).

![Figure 1.3: Sites for N-glycosylation within the NCAM protein](image)

NCAM has six sites of N-glycosylation all of which fall within the extracellular domains of NACM. This figure is based on information from a paper by Albach, Damoc et al. (Albach, Damoc et al. 2004).

Palmitoylation

NCAM-140 and NCAM-180 have been shown to be palmitoylated on cysteine residues in the cytoplasmic domain (723, 729, 734 and 740, on human NCAM 1 lacking VASE) (Little, Edelman et al. 1990).
1998). NCAM palmitoylation was found to cause association with the membrane, even when NCAM was lacking a transmembrane domain, and recruitment to lipid rafts, which was necessary for activation of the fyn-FAK signalling pathway and neurite outgrowth (Niethammer, Delling et al. 2002).

**Phosphorylation**

NCAM can contain up to 49 total serine and threonine residues depending on its isoform. It has been shown to be phosphorylated on tyrosine 734 (in human NCAM1 lacking VASE) by receptor tyrosine kinase B (TrkB) to modulate NCAM’s neurite outgrowth promoting abilities (Cassens, Kleene et al.). Other serine and threonine sites are phosphorylated by casein kinase I and glycogen synthase kinase 3 (GSK-3) and this phosphorylation was found to be cell adhesion-dependent (Mackie, Sorkin et al. 1989; Matthias and Horstkorte 2006).

**Polysialylation**

The modification of proteins with polysialic acid (PSA), termed polysialylation, is another form of post-translational modification. NCAM is thought to be the only protein which is polysialylated in vivo although in vitro polysialylation has proved to be a popular method of modulating antibody pharmacokinetics (Finne, Finne et al. 1983; Constantinou, Epenetos et al. 2008). NCAM-deficient mice were found to show a complete absence of PSA, indicating that NCAM is the only protein to be polysialylated (Tomasiewicz, Ono et al. 1993). The function and developmental regulation of polysialylation of NCAM is well studied and understood.

Polysialic acid is a linear monomonomer comprised of α 2,8-linked N-acetyleneuraminic acid (NeuNAc). The addition of PSA to NCAM is regulated by two Golgi-associated polysialyltransferases: ST8SiaIV (PST) and ST8Sial (STX) (Eckhardt, Muhlenhoff et al. 1995; Nakayama, Fukuda et al. 1995). The attachment of sialic acid units to NCAM follows synthesis from UDP-N-acetylglucosamine and activation in the cytosol (Alcaraz and Goridis 1991). This leads to the addition of between 2 or more than 100 PSA units onto two asparagines residues in Ig V (See Fig. 1.3, N-5 and N-6) (Rutishauser, Acheson et al. 1988). For polysialylation of NCAM to occur, NCAM Fn_{III} 1 and Ig V only are required. The role of Fn_{III} 1 was proved by a triple alanine substitution of asparagine 511, glutamate 512 and glutamate 514 which resulted in complete abrogation of PSA addition to Ig V (Mendiratta, Sekulic et al. 2005).

The PSA polymer adopts a helical structure and creates a large, negative hydration sphere around NCAM (Fig. 1.4). This creates an electrorepulsive cloud around NCAM and local steric inhibition, which has been found to abrogate NCAM-mediated adhesion (Yang, Major et al. 1994; Bonfanti
2006). Aggregation assays have revealed that vesicles with NCAM lacking PSA aggregate four times faster than those with NCAM-PSA (Hoffman and Edelman 1983). Addition of the enzyme neuraminidase (EndoN), to remove PSA from the NCAM, caused the rate of vesicle aggregation to return to an NCAM level. Not only does PSA block NCAM-mediated homophilic binding, it can also block NCAM heterophilic interaction, specifically the interaction between the heparin-binding domain of NCAM to heparin sulphate proteoglycans on apposing cells (Storms and Rutishauser 1998).

![NCAM-PSA structure](image)

Figure 1.4: The fifth Ig domain of NCAM can be polysialylated on two Asn residues. NCAM-PSA is formed by the coupling of N-acetylglucosamine to one of the Asn residues in Ig V, followed by addition of branched mannose and galactose residues. Polysialic acid links to N-acetylglucosamine via an α 2, 3 link. PSA units are then linked to each other via α 2, 8 linkages. This figure is based on published data presented in schematic form in a paper by Bonfanti (Bonfanti 2006).

Although addition of PSA onto NCAM reduces its ability to bind homophilically, it increases its neurite outgrowth promoting ability. This enhancement due to the presence of PSA was proven by treatment of retinal ganglion cells, expressing NCAM-PSA, with EndoN which resulted in a return to basal levels of neurite outgrowth due to NCAM alone (Doherty, Cohen et al. 1990). It has been
postulated that by abrogating NCAM homophilic binding, interactions with NCAM Fn\textsubscript{III} domains are more likely, therefore NCAM-mediated neurite outgrowth is more prevalent (Kiselyov, Soroka et al. 2005).

The expression of PSA is developmentally regulated and highly present in the nervous system where it was first discovered. PSA can account for up to 30\% of the molecular mass of NCAM (Finne 1982). In the developing embryo, NCAM is found in an ‘embryonic’ form with high percentage content of PSA. During development polysialylation is down-regulated resulting in a so-called ‘adult’ form of NCAM (Hoffman and Edelman 1983). Many studies have been carried out to monitor the expression of NCAM-PSA and the two polysialyltransferases enzymes, which are found to correlate. In rodent, between E8 and E9, NCAM-PSA levels are high as are PST and STX (Brocco, Pollevick et al. 2003). The mRNA level of STX declines dramatically after this point but PST continues to be expressed into adulthood in areas of high synaptic plasticity, such as the glomerular layer of the olfactory bulb and the granule cell layer of the dentate gyrus (Hildebrandt, Becker et al. 1998). The presence of NCAM-PSA in areas of synaptic plasticity and development within the central nervous system (CNS) points to its function as a regulator of these processes.

The expression of NCAM-PSA in the nervous system has been mapped using various monoclonal antibodies against PSA (Chuong, McClain et al. 1982). Generally, NCAM-PSA is found to be distributed in a typical membrane staining pattern with punctate localisation across the whole cell (Bonfanti 2006). In the developing nervous system, there is a lack of NCAM-PSA until the later embryonic stages of rodent nervous system development, where it is found in discrete developing regions such as the olfactory placode and bundles of growing axons (Seki and Arai 1993; Aoki, Nakahara et al. 1999).

Experiments to remove PSA have revealed the extent to which PSA is involved in development, learning and memory. Contrasting reports of the effect of NCAM knockout mice have been found. One finding described the impaired long term potentiation following creation of knockout mice where the phenotype can be rescued by the injection of NCAM-PSA or PSA alone into the hippocampus of NCAM (Senkov, Sun et al. 2006). Another finding has indicated that NCAM lacking PSA only can rescue this phenotype (Stoenica, Senkov et al. 2006). PST and STX knockout mice reveal a postnatal mouse with severe brain abnormalities and with a lethal phenotype, where half did not survive before postnatal day 21 (Weinhold, Seidenfaden et al. 2005).

During development, NCAM-PSA is lacking on the fasiculated axons from chick embryo motor neurons until defasciculation occurs for rearrangement of nerve bundles (Bates, Becker et al. 1999).
During this process, NCAM-PSA is upregulated, but if these cells are treated with endoN to remove PSA from NCAM axons remain defasciculated (Tang, Rutishauser et al. 1994).

1.3 NCAM function
The multitude of NCAM isoforms created by alternative splicing and post-translational modification yield a series of proteins which are functionally opposing and expressed transiently and in differing areas. Historically NCAM has been thought of as a cell adhesion molecule, but in recent years its traditional view has been challenged as novel roles have been defined.

1.3.1 Axon growth and guidance
As has been previously described, NCAM is a modulator of the developing nervous system and is expressed in regions where neuronal guidance and targeting is required. One particular process, in which NCAM is involved, is neurite outgrowth. Neurites are pre-axonal processes extending from neural cells along a concentration gradient which develop into axons and dendrites (Doherty, Barton et al. 1989). The effect of NCAM on neurite outgrowth was first discovered using an in vitro model where primary cultured neurons were grown over fibroblast monolayer cells (Fig. 1.5) (Rutishauser, Gall et al. 1978). The transfection of NCAM into the NIH-3T3 mouse fibroblast cell line, which expresses extremely low levels of NCAM, allowed a model cellular system for use in determining the effect of NCAM in a cellular substratum on neurite outgrowth of cultured primary neurons. NCAM-140 transfected into NIH-3T3 cells yielded increased neurite lengths from cerebellar granule neurons grown over them (Doherty, Barton et al. 1989). An increase in NCAM concentration in the cell lines increased the neurite outgrowth length only above a certain threshold concentration. Before the threshold concentration, there was no effect on neurite outgrowth of the neurons.

![Figure 1.5: In vitro assay to measure the effect of NCAM transfected cells on primary cell neurite outgrowth.](image)

The role of NCAM on neurite outgrowth was first discovered in a cellular in vitro assay to measure the length of cerebellar granule neurons grown over a fibroblast monolayer of cells expressing NCAM. a) Triturated and rounded fibroblast cells were injected into a plastic dish and left to adhere for 16 hours. b) Primary rat cerebellar granule neurons were injected onto the fibroblast monolayer and incubated for 16 hours. c) The lengths of neurites (pre-axonal processes) extending from cerebellar granule cell bodies were calculated and compared to determine the effect of the fibroblast cell layer.

Interestingly NIH-3T3 cells transfected with NCAM-180 did not cause neurons to extend longer neurites. This was postulated to be due to increased cytoskeletal contacts between the longer
cytoplasmic domain of NCAM (Doherty, Rimon et al. 1992). This again indicates that different isoforms of NCAM exist for different functions and temporospatial expression.

NCAM-mediated neurite outgrowth occurs via fibroblast growth factor (FGF) signalling (Saffell, Williams et al. 1997; Kiselyov, Skladchikova et al. 2003). Interestingly, peptides from both NCAM Fn\textsubscript{III}-2 and from FGF have to been found to mimic NCAM and FGF mediated neurite outgrowth (Anderson, Kendal et al. 2005; Li, Christensen et al. 2009). These peptides have potential therapeutic relevancy for regenerating the neurite outgrowth promoting capabilities of the developed but injured nervous system.

1.3.2 Signalling

FGF signalling pathway

Alongside the function of NCAM as a typical CAM and modulator of neurite outgrowth, NCAM has an important role in cell signalling. NCAM mediated neurite outgrowth occurs via the FGF signalling pathway. NCAM has been found to bind directly via its Fn\textsubscript{III} domains to the two Ig-like domains of FGFR1 using expressed proteins on a surface plasmon resonance (SPR) instrument (Kiselyov, Skladchikova et al. 2003). At the cell surface, the Fn\textsubscript{III} domains of NCAM align with the Ig-like domains of FGFR as they extend from the membrane. CAMs are found to mediate neurite outgrowth via FGF-like signalling of the FGFR and its downstream receptors (Williams, Walsh et al. 1994).

A peptide from Fn\textsubscript{III} 1, DRIVEYSSTA (FRM peptide) has been found to inhibit NCAM-mediated neurite outgrowth (Anderson, Kendal et al. 2005). It is thought to do this by binding to FGFR and preventing NCAM Fn\textsubscript{III} 1 from binding and initiating FGFR signalling. Another peptide, EVYVVAENQQGKSQA (FGL peptide) from Fn\textsubscript{III} 2 binds with high affinity to FGFR and inhibits NCAM-mediated neurite outgrowth.

The FGF family ligands bind to FGFR with high-affinity and cause activation via receptor dimerisation and autophosphorylation (Eswarakumar, Lax et al. 2005). This leads to activation of the tyrosine kinase and tyrosine phosphorylation for docking of accessory proteins such as Src homology 2 (SH2) domains. Downstream signalling leads to action on phospholipase Cy (PLCy) and the cleavage of phosphatidylinositol 4, 5-bisphosphate (PIP\textsubscript{2}) into its precursors: diacylglycerol (DAG) and inositol 1, 4, 5-trisphosphate (IP\textsubscript{3}) (Fig. 1.6) (Schlessinger 2000). Breakdown of DAG into arachidonic acid (AA) may lead to the release of intracellular Ca\textsuperscript{2+} from intracellular calcium stores. The level of intracellular Ca\textsuperscript{2+} and inositol phosphates has been found to increase in cerebellar granule neurons in vitro when purified CAMs were added (Frei, von Bohlen und Halbach et al. 1992). DAG leads to
activation of protein kinase C (PKC), which activated GAP-43 and leads to neurite outgrowth.

Figure 1.6: NCAM mediated neurite outgrowth is achieved via intracellular signaling of FGFR and Fyn.
NCAM homophilic binding at the cell membrane and a heterophilic interaction with FGFR causes activation of the FGFR signalling pathway. FGFR dimerisation and autophosphorylation. PLCy is recruited to the cytoplasmic domains of FGFR and the cleavage of PIP2 into DAG and IP3. DAG signals via PKC and causes upregulation of GAP-43 to promote neurite outgrowth. DAG may also be broken down into arachidonic acid which leads to Ca2+ influx into the cytoplasm. NCAM can also signal via fyn and FAK recruitment to its cytoplasmic domain, which leads to neurite outgrowth. This figure has been modified from one presented in a paper by Williams, Walsh et al. (Williams, Walsh et al. 2003).

Fyn signalling pathway

NCAM also signals via an interaction with a protein p59fyn (fyn) (Fig. 1.6). Fyn is a member of the Src family of non-receptor tyrosine kinases and is highly expressed in neural tissue and has been found to be a positive regulator of neurite outgrowth (Maness 1992). The molecule has been found at the plasma membrane of growth cones and axons (Bare, Lauder et al. 1993). Activation of fyn is caused by dephosphorylation by receptor tyrosine phosphatase α (RPTPα). p125fak (FAK) is recruited to the complex and neurite outgrowth is induced. NCAM is involved in this neurite outgrowth process by interaction with RPTPα and fyn on the lipid raft membrane (Bodrikov, Leshchyns'ka et al. 2005).

NCAM-140 has been found to associate with fyn following immunoprecipitation of the proteins from postnatal day four (PND4) mouse cerebellar granule neurons (Beggs, Baragona et al. 1997). Immunoprecipitation with NCAM suggested that fyn associated preferentially with the GPI-anchored
isoform of NCAM. Fyn knockout mouse cerebellar neurons showed total inhibition of NCAM-mediated neurite outgrowth, when grown over fibroblast monolayers expressing NCAM-140, indicating the importance of fyn in NCAM-mediated neurite outgrowth (Beggs, Soriano et al. 1994).

MAP kinase signalling

NCAM-140 has also been found to play a role in the MAP kinase pathway (Schmid, Graff et al. 1999). This was elucidated using the B35 cell line, which expresses only the NCAM-140 isoform (excluding VASE). Analysis of cell lysates revealed the phosphorylation of MAP kinase (MAPK) and the activation of ERK1 and ERK2. Addition of a MAPK inhibitor PD98059 to rat cerebellar neurons, grown on NCAM-140 (excluding VASE) expressing mouse L-cells, caused inhibition of neurite outgrowth, indicating a role for MAPK in NCAM-mediated neurite outgrowth.

GDNF Signalling

NCAM-140 has been found to interact with glial cell line derived neurotrophic factor (GDNF) and this is promoted by association with the GPI-linked GDNF family receptor α (GFRα) (Paratcha, Ledda et al. 2003). GDNF ligands have been found to have protective effects on neuronal cell populations by interaction with GDNF receptors at the cell membrane. Following interaction, the Ret receptor tyrosine kinase is recruited to the complex to elicit downstream signalling effects (Airaksinen and Saarma 2002). However it has been noted in primary neurons and Schwann cells that in the absence of Ret, NCAM-140 may bind instead and induces the activation of Fyn. In tandem, treatment of these cells with GDNF has been found to increase migration and neurite outgrowth in an NCAM and Fyn dependent manner (Paratcha, Ledda et al. 2003). Interestingly FGFR and Ret were not involved in this response. The authors have postulated that NCAM may bind to GFRα in a higher affinity interaction than NCAM homophilic binding. This may prevent NCAM-mediated neurite outgrowth via FGFR binding and dimerisation and cause binding to GDNF instead. Recently, the GND binding site has been mapped to four acidic residues in Ig III of NCAM and may encompass NCAM homophilic binding patches (Sjostrand, Carlsson et al. 2007; Nielsen, Gotfryd et al. 2009). Therefore it is plausible that interactions between GFRα and GDNF mediate NCAM homophilic binding whilst inducing neurite outgrowth independent of NCAM homophilic binding.

Glucocorticoid receptor signalling

NCAM has been found to play a role in the inhibition of astrocyte proliferation (Sporns, Edelman et al. 1995). It is suggested by the authors that NCAM homophilic binding may be responsible for decreasing glial cell proliferation so that neurite outgrowth may be increased in the local region. This
is of great importance in CNS regions where injury may have occurred, as an increase in NCAM may decrease glial scar formation and aid regeneration (Krushel, Tai et al. 1998).

A link between NCAM and the glucocorticoid receptor signalling pathway was discovered by the addition of a glucocorticoid agonist RU-486, which was found to partially rescue the inhibition of astrocyte proliferation. This suggested that NCAM might have activated glucocorticoid signalling to prevent astrocyte proliferation (Crossin, Tai et al. 1997). Interestingly, addition of RU-486 to the cells correlated with activation of the MAP kinase signalling pathway. In the presence of NCAM alone, the MAP kinase activity was inhibited. This indicated that NCAM and the glucocorticoid pathways are linked, although their exact correlations are still unknown.

1.3.3 Homophilic adhesion

Functioning as a typical CAM, NCAM is able to bind homophilically to itself via its extracellular Ig domains. Cell-cell interactions in the developing nervous system rely on the presence of CAMs extending into the extracellular space, allowing them to interact with complementary proteins on apposing cell surfaces (Sperry 1963). NCAM fulfils this hypothesis, as retinal cells treated with αNCAM Fab' fragments against Ig I-V were inhibited from aggregating (Rutishauser, Gall et al. 1978). This indicated that the NCAM present on the cell surface was unable to function in NCAM-mediated adhesion due to antibody binding. Immunoprecipitation of detergent-solubilised chicken and frog brain membrane NCAM also revealed that NCAM-140 and NCAM-180 were found to bind to NCAM.

Initial electron microscopy images of chicken NCAM-130 and NCAM-160 revealed that 30% was present as single monomeric rods, whilst 50% was represented as dimers. Up to 10% of the NCAM population was formed from higher order oligomers (Hall and Rutishauser 1987). All experiments were carried out in detergent, which allowed the oligomerisation of NCAM.

Much work has been done to understand the mechanism and kinetics of NCAM homophilic binding. Affinity constants for the adhesion between homophilically interacting NCAM proteins purified from postnatal day 0 (PND 0) or adult rats has been carried out using an enzyme-linked immunosorbent assay (ELISA) (Moran and Bock 1988). The binding affinity constants (K_a) for the adhesion between newborn NCAM and adult NCAM were $1.23 \times 10^{-6}$ M and $6.9 \times 10^{-8}$ M indicating that they were in the low-affinity micromolar range. The addition of PSA to Ig V of NCAM was found to be sufficient for decreasing the affinity of NCAM homophilic binding.

Further work has been carried out to determine the amino acid sequences involved in NCAM homophilic binding. A starting point was the mapping of four monoclonal antibodies to the surface residues of Ig III (Frelinger and Rutishauser 1986). Following this information the effect of a series of
overlapping hexapeptides, from Ig III, on mouse L cell aggregation (transfected with chick NCAM-140) was investigated (Rao, Wu et al. 1992). The sequence KYSFNY was found to reduce cell adhesion and the optimal adhesion-abrogating sequence was found to be $^{243\text{KYSFNYDGS}}^{252}$ (numbering for chick NCAM-140 without VASE), with a 60% reduction in NCAM-140 transfected L cell adhesion. This sequence is from chick NCAM and shares only 50% homology with the human sequence (KYIFSDIDSSQ). Later experiments to measure the effect of deletion or alanine substitution of the 10 amino acid sequence $^{243\text{K-E}}^{252}$ from chick NCAM, resulted in abrogation of adhesion between NCAM covaspheres. This information indicated that this sequence in NCAM Ig III was required for NCAM homophilic binding.

![Diagram of putative homophilic interactions between NCAM proteins involving NCAM Ig III.](image)

Figure 1.7: Putative homophilic interactions between NCAM proteins involving NCAM Ig III. Data has indicated that NCAM Ig III is involved in an isologous interaction. At the cell membrane Ig III interactions may arise by cis interactions between two NCAM molecules on the same cell membrane or between two molecules on apposing cell membranes via a trans anti-parallel interaction.

To determine the substrate for the 10 amino acid Ig III $^{243\text{K-E}}^{252}$ and Ig III, NCAM Ig III binding to NCAM and its deletion mutants in transfected cells was assessed (Rao, Zhao et al. 1994). The full domain Ig III, which had been covalently coupled to covaspheres (fluorescently labelled microbeads), bound to adsorbed NCAM Ig III on a glass coverslip. This indicated that Ig III can bind homophilically to Ig III without any other portion of NCAM (Fig. 1.7). There was no indication as to whether the isologous interaction between Ig III would result in cis or trans interactions in the full length protein.
The authors postulated that $^{243}$K-$^{252}$ from Ig III itself was involved in isologous interaction with the same sequence on another Ig III domain.

The role of NCAM homophilic adhesion in NCAM-mediated neurite outgrowth has been studied by transfecting a mutant NCAM-140 lacking $^{243}$K-$^{252}$ into substratum L cells and assessing retinal cell neurite outgrowth promoting abilities (Sandig, Rao et al. 1994). NCAM lacking $^{243}$K-$^{252}$, which was found to reduce NCAM homophilic binding, did not increase neurite outgrowth to an NCAM level. Interestingly, addition of the $^{243}$K-$^{252}$ peptide to retinal cells grown over NCAM expressing cells induced neurite outgrowth, indicating perhaps that the peptide acted preferentially in outgrowth related function, as opposed to homophilic binding.

Although the role of Ig III was extensively investigated and was thought to account for NCAM homophilic binding alone, other data has been found which indicates Ig I-IV was involved (Cunningham, Hoffman et al. 1983; Cunningham, Hemperly et al. 1987). Studies of mouse NCAM protein revealed Ig I and II as well as FnIII 1 and 2 were essential for binding to neuronal cells (Frei, von Bohlen und Halbach et al. 1992). In experiments such as these, where cells were bound to protein substratum or vice versa, it is important to note that heterophilic interactions between NCAM proteins or domains could have occurred with cells. Consequently there was no guarantee that the interactions which occurred were due to NCAM homophilic binding. Therefore an explanation for the conflicting data regarding NCAM homophilic binding sequences may be due to this and to the species variation of the proteins studied.

To clarify the conflicting NCAM homophilic binding data, the adhesion of chicken NCAM coated microspheres to deletion constructs of NCAM Ig I-V was quantified (Ranheim, Edelman et al. 1996). Ig I was found to bind to Ig V, Ig II to Ig IV and Ig III isologously with Ig III. Although these experiments were studied in solution, it is plausible that NCAM undergoes trans binding where an NCAM molecule on one cell bind to NCAM molecules on the apposing cell with full anti-parallel overlap of all Ig-like domains (Fig 1.7).

Further experiments have revealed information contradicting homophilic binding data from the 1980’s and 1990’s. A crystal structure, at 1.85 Å resolution, of Ig I-II revealed a ‘crossed dimer’ of two Ig I-II fragments which interacted in trans via two salt bridges between $^{16}$Glu from Ig I of both fragments and $^{98}$Lys from Ig II of both fragments (Kasper, Rasmussen et al. 2000). A number of polar residues on the surfaces of Ig I and II aided to stabilise this interaction. The authors postulate the flexibility of NCAM allows this bent and crossed interaction of only the most N-terminal Ig domains which can be adopted when NCAM is polysialylated on Ig V.
Following on from this work, a novel mechanism of NCAM-mediated cell adhesion was proposed. An X-ray structure of NCAM Ig I-III at 2.0 Å resolution revealed structural similarities with the previously determined Ig I-II structure (Soroka, Kolkova et al. 2003). The two salt bridge interactions between two Ig I and II domains were visualised, which further corroborated the earlier structural analysis (Kasper, Rasmussen et al. 2000). Dimers of Ig I-III were revealed to interact in cis as well as trans interactions between Ig II and Ig III from apposing NCAM molecules in trans. These three structures yielded a proposal for NCAM-mediated cell adhesion via ‘double zipper’ adhesion complexes on apposing cell surfaces (Fig 1.8). In this proposal initially NCAM molecules interact in a ‘crossed dimer’ in trans between Ig I and Ig II. This conformation is stabilised by a trans interaction between Ig I from one NCAM and Ig III from another NCAM. Interestingly the amino acids from Ig III involved in Ig I binding were numbers 248-253 corresponding to part of the KYS sequence described previously. This interaction may be further stabilised by the interactions between two Ig III domains of apposing NCAM proteins. After a period of further cell-cell contact, cis interactions are postulated to occur between Ig II and III of two NCAM molecules on the same cell. A combination of these cis and trans interactions may yield a ‘double zipper’ adhesion complex.

Figure 1.8: Putative model for NCAM homophilic binding at the cell membrane.
a) There is supporting evidence to suggest that NCAM may bind homophilically via a partial overlap of Ig I and II. This interaction is thought to be stabilised via an Ig I to Ig III interaction. b) After further contact time, it is postulated that Ig III domains from trans interacting NCAM proteins bind isologously. c) After further contact time, NCAM clusters at the cell surface in a zipper like movement, due to the cis interaction between NCAM Ig II and III. This results in a progressive strengthening of the cell-cell interaction. This figure has been taken from Soroka, Kolkova et al (Soroka, Kolkova et al. 2003).

Overall this hypothesis seems to corroborate data from previous studies suggesting that NCAM Ig I-III domains are involved in homophilic binding. It does not take into account the interactions or structural differences due to Ig IV and V or insertion and effect of the VASE sequence.

Particular isoforms of NCAM have been found to have an effect on the adhesion of cells or vesicles expressing them. One example of this is the addition of PSA to NCAM Ig V (See section 1.2.2) which abrogates NCAM-mediated adhesion due to the large, negative hydration sphere surrounding NCAM caused by the glycosylation (Hoffman and Edelman 1983). Surface force measurements between
low-density NCAM-PSA functionalised surfaces revealed an earlier onset of repulsion which was larger than NCAM lacking PSA functionalised surfaces (Johnson, Fujimoto et al. 2005).

Figure 1.9: Schematic to show two putative NCAM homophilic binding interactions from surface force apparatus measurements.
The full extracellular portion of NCAM was affinity captured (via a C-terminal His tag) onto two NTA terminated self-associating lipid surfaces. Using surface force apparatus, functionalised surfaces were brought into apposition and the repulsions and bond failures were measured. Adhesive failure of the NCAM proteins was found to occur at 31 and 39 nm which correlated to a partial anti-parallel interaction between two Ig I and II domains and a full overlap of all domains (Ig I binds Ig V, Ig II binds Ig IV, Ig III bind Ig III) respectively. This figure is based on work carried out by Johnson and Fujimoto (Johnson, Fujimoto et al. 2004).

Single molecule surface force measurements using surface force apparatus (SFA) were carried out on chicken NCAM proteins (Johnson, Fujimoto et al. 2004). His-tagged extracellular domains were captured onto nitriioacetic acid (NTA) terminated lipids on two surfaces which were brought into apposition (Fig. 1.9). For full length ectodomains of NCAM, the onset of steric repulsion began when the monolayers were approximately 37 nm apart. The length of NCAM Ig I-V has been found to be 18 nm (Hall and Rutishauser 1987). After interaction, an unbinding event (or point of rupture between the interacting proteins) occurred after 18±0.5 nm of surface retraction, which corresponded to a full anti-parallel overlap of NCAM Ig I-V with Ig I interacting with Ig V, Ig II with Ig IV and an isologous
interaction of Ig III. A less frequent adhesive event occurred after a distance of 29±0.5 nm due to a partial overlapping interaction between Ig I-II of both NCAM molecules.

Addition of 1 mM of a 10 amino acid peptide sequence $^{243}$K-$^{252}$, used in previous adhesion blocking experiments, abrogated adhesion between full anti-parallel overlapping domains of NCAM in SFA experiments. The outer de-adhesion event, corresponding to a partial overlap of Ig I and II, became more prevalent. This indicated that $^{243}$K-$^{252}$ may have bound to NCAM Ig III and blocked Ig III isologous interactions. Addition of 1 mM of a peptide from Ig II whose sequence was ‘GRILARGEINFK’ was found to prevent the partial overlapping bond of Ig I and Ig II but permitted the adhesion between full overlapping Ig I-V domains (Soroka, Kiryushko et al. 2002).

1.3.4 Heterophilic adhesion

As a homophilic binding protein, NCAM-NCAM interactions at the cell-cell interface are able to maintain and modulate cell adhesion and stability. However homophilic binding is not the only means by which NCAM can interact; a number of heterophilic interactions have also been characterised (Fig. 1.10).

**Heparin**

NCAM has been found to interact heterophilically with heparin sulfate, a polysaccharide component of the extracellular matrix (ECM) which is found on collagen and agrin. NMR assignment has confirmed that a sequence in Ig II forms a heparin and chondroitin sulfate binding site which overlaps with an NCAM homophilic binding site (Kulahin, Rudenko et al. 2005). This provided an explanation for the inhibitory effect of heparin and chondroitin sulfate addition on the aggregation of NCAM Ig I and II (Pizsey, Rowett et al. 1989). The dissociation constant ($K_d$) of the interaction between heparin sulfate and NCAM was found to be $6.9 \times 10^{-8} \text{ M}$, which was similar to the binding constant for the interaction between homophilically interacting NCAM molecules (Moran and Bock 1988; Nybroe, Moran et al. 1989).

**L1**

L1, like NCAM, is $\text{Ca}^{2+}$-independent and belongs to the immunoglobulin superfamily of cell adhesion molecules (Fushiki and Schachner 1986). L1 is able to bind homophilically via its 6 Ig-like domains and can promote neurite outgrowth via its 5 Fn domains (Maness and Schachner 2007). Interestingly the signalling pathway which is responsible for L1 mediated neurite outgrowth is via pp60$c^\text{src}$. An interaction between L1 and NCAM was initially discovered when beads coated in L1 and NCAM formed larger and more stable aggregates than L1 coated beads alone (Kadmon, Kowitz et al. 1990). L1 was found to undergo L1 homophilic adhesion in trans between L1 coupled beads but,
upon addition of NCAM onto the beads, could participate in a cis interaction, which mediated and strengthened L1 adhesion.

The cis interaction between L1 and NCAM is carbohydrate-dependent so that the molecules bind to each other via oligomannosidic carbohydrates on L1 (Horstkorte, Schachner et al. 1993). The binding domain in NCAM determined to bind to L1 carbohydrates was a C-type lectin consensus sequence in Ig IV. This was determined by the addition of this peptide sequence which was found to inhibit L1 mediated neurite outgrowth and the interaction between L1 and NCAM (Kristiansen, Marques et al. 1999). The interaction of NCAM with L1 therefore introduces another facet of NCAM mediated signalling not only via p59fyn and FGFR, but also via the L1 mediated pp60c-src pathway.

Interestingly the oligomannosidic binding sequence in NCAM Ig IV is positioned closely to the alternatively spliced VASE sequence in NCAM Ig IV (Fig. 1.10). It is plausible therefore that L1 binding and insertion of the VASE sequence may confer alternate properties to NCAM regarding neurite outgrowth to inhibit neurite outgrowth.

**Cellular prion protein**

There is much mystery surrounding the function and structural changes in the cellular prion protein (PrP\(^c\)) which is thought to be converted into a disease-causing isoform scrapie prion protein (PrP\(^Sc\)) (Naslavsky, Stein et al. 1997). PrP\(^c\) is thought to be involved in cell adhesion and migration due to its expression patterns in vitro, and recently it has been found to interact with NCAM (Schmitt-Ulms, Legname et al. 2001). Formaldehyde crosslinking of mouse neuroblastoma cells yielded PrP\(^c\) protein in complexation with NCAM via a binding patch involving amino acids from both NCAM Fn\(^III\) domains.

As a GPI-anchored protein, PrP\(^c\) is localised in lipid rafts where it is thought to be involved in NCAM localisation and stabilisation to these domains (Santuccione, Sytnyk et al. 2005). Interestingly PrP\(^c\) knockout mice exhibit lower levels of lipid raft associated NCAM, but NCAM knockout mice can still become infected with PrP\(^c\). This indicates that NCAM may not be involved in PrP\(^c\) infectivity.

**Robo3**

NCAM has been found to associate with Robo3, a member of the roundabout family of proteins which function in cell migration and axon guidance in the developing CNS (Kidd, Russell et al. 1998). Robo3 is also a member of the immunoglobulin superfamily of cell adhesion molecules and contains 5 Ig-like domains responsible for homophilic binding and three Fn\(^III\) domains for neurite outgrowth and signalling.
The interaction between Robo-3 and NCAM was discovered using a bead aggregation assay but further investigation has not been carried out (Camurri, Mambetisaeva et al. 2005). It is unknown what the significance of this interaction is, but it is plausible that it may serve to strengthen cell-cell interactions in the CNS and to connect signalling pathways between NCAM and Robo-3.

Figure 1.10: Heterophilic interactions of NCAM.
Although NCAM is a well-known homophilic binding protein, it has been found to be involved in a number of heterophilic interactions. HBD= heparin binding domain, LBD= lectin binding domain, PSA= polysialic acid, HNK-1= human natural killer cell glycan-1. This figure is based on a diagram found in a paper by Walmod et al (Walmod, Kolkova et al. 2004).
**ADAM metalloproteases**

NCAM has been found to function as a secreted isoform which is translated and secreted from the cell without the need for processing from the cell membrane. However, NCAM-140 and NCAM-180 can be shed from expressing cell membranes by the action of metalloproteases of the ‘**A Disintegrin And Metalloprotease**’ (ADAM) family. TNFα converting enzyme, from the ADAM family, was found to cause NCAM-140 and NCAM-180 shedding of extracellular domains from NCAM transfected L cells (Hinkle, Diestel et al. 2006).

The exact function of NCAM ectodomain shedding is unknown; inhibition of shedding from primary hippocampal neurons has been found to decrease NCAM-mediated neurite outgrowth but had the opposite effect in primary cortical neurons (Hubschmann, Skladchikova et al. 2005; Hinkle, Diestel et al. 2006). Both methods inhibited the shedding of NCAM by inhibiting metalloprotease action so the reason for this disparity is not known although it may depend on different expression levels of NCAM on the tissue.

**Rabies virus protein**

The rabies virus causes encephalitis following transmission and transport to the brain via the peripheral nerves. Binding of the virus to host cells has been shown to involve receptors on the cell membrane including NCAM. *In vitro*, L cells were unable to be infected with rabies virus, until the NCAM-140 or NCAM-180 were transfected into the cell line. *In vivo*, NCAM knockout mice decreased the susceptibility of a cell for infection with rabies virus and delayed mortality (Thoulouze, Lafage et al. 1998).

**Cytoskeletal interactions**

Although the extracellular domains of NCAM play a key role in the homophilic and heterophilic binding of NCAM, the intracellular components of NCAM are also important. NCAM-140 has a short cytoplasmic domain and is found to be involved in neurite outgrowth, whereas NCAM-180 is a less favourable promoter of neurite outgrowth, has a longer cytoplasmic domain, and is thought of as a stabiliser of cell-cell contacts (Büttner, Reutter et al. 2004). The longer cytoplasmic domain of NCAM-180 has been found to interact with a number of cytoskeletal components, which is thought to strengthen cell mechanics by increasing the force transduction between the inside of the cell and the extracellular components interacting with NCAM (Pollerberg, Burridge et al. 1987).

A series of experiments using affinity chromatography against the cytoplasmic domains of NCAM-140 and NCAM-180 were carried out to determine its binding partners from rat brain protein
fractions (Buttner, Kannicht et al. 2003). The cytoplasmic domain of NCAM-140 was found to bind only α-tubulin, β-tubulin and α-actinin I whereas NCAM-180 was found to bind those proteins and microtubule associated protein 1A (MAP 1A), β-actin, tropomyosin and RhoA-binding kinase-α. α- and β-tubulin form microtubules. Therefore the NCAM short cytoskeletal domain is sufficient to confer direct links with microtubules. It is interesting that MAP 1A is only associated with the longer cytoplasmic domain on NCAM, which may indicate that an increased cytoskeletal link from NCAM-180 is achieved via association of this accessory protein. Tropomyosin and α-actinin I are accessory proteins for the actin cytoskeleton and are found to bind to the cytoplasmic domains of other adhesion proteins such as the integrins and adherins to form a cytoplasmic plaque. This plaque anchors the extracellular membrane contacts to the inside of the cell to relay external forces and control cell migration (Hirata, Tatsumi et al. 2008). The immunoprecipitation of RhoA-binding kinase-α with NCAM-180 was interesting as it indicated the potential for phosphorylation of this segment of the cytoplasmic domain. In fact, the longer portion of NCAM-180 contains a number of Ser and Thr sites for phosphorylation. This Rho kinase in particular is involved with growth cone collapse and formation which is involved in the process of neurite outgrowth, with which NCAM is so implicitly linked (Luo, Jan et al. 1997).

NCAM-180 has also been found to interact with ankyrin and TOAD-64 via its cytoplasmic domain (Pollerberg, Burridge et al. 1987; Buttner, Kannicht et al. 2005). Ankyrin is an actin accessory protein whilst TOAD-64 is involved in axon growth and neuronal differentiation (Minturn, Fryer et al. 1995). This information indicates that NCAM-180 is a stabiliser of cell-cell contacts due to its increased association with modulators of actin and microtubule accessory proteins and protein effectors of the axon outgrowth machinery.

The cytoplasmic domain of NCAM-140 and NCAM-180 have also recently been found to bind calmodulin via their calmodulin binding motif (Kleene, Mzoughi et al.). This interaction is essential for NCAM-mediated neurite outgrowth via activation of Fak.

1.4 NCAM expression and localisation

NCAM was first discovered in chick neural retinal cells and has been found to be expressed in tissues throughout the nervous system in embryonic and adult organisms. The localisation and temporal-spatial regulation of NCAM has been well-studied since its discovery. In general, NCAM expression is high and extensive in the developing embryonic nervous system, where novel connections and neuronal pathways are being formed (Chuong and Edelman 1984).
As development continues NCAM levels tend to decrease, although the exact time frame over which this occurs depends on the specific tissue. It is interesting to note that the expression of ‘embryonic’ NCAM-PSA is highest pre-natally in the nervous system (Edelman and Chuong 1982). As development continues, NCAM-PSA is replaced with NCAM lacking PSA. Since the adhesion between NCAM-PSA single proteins is far lower than between NCAM lacking PSA, NCAM-PSA is expressed in the developing nervous system to confer plasticity to synaptic regions (Johnson, Fujimoto et al. 2005). As NCAM-PSA is down-regulated in the developed nervous system, an increase in the insertion of the VASE sequence into NCAM is witnessed (Small and Akeson 1990). The functional effect of this insertion is the inhibition of neurite outgrowth from apposing neuronal cell bodies (Saffell, Walsh et al. 1994). NCAM-VASE is excluded from the areas of the brain with high synaptic plasticity and which have been subjected to this analysis; these areas included the olfactory bulb, adrenal gland and dorsal root ganglia (Small and Akeson 1990).

NCAM-120, NCAM-140 and NCAM-180 are also differentially expressed. NCAM-120 is localised in non-neuronal tissue including muscle, oligodendrocytes and astrocytes (Dickson, Gower et al. 1987). NCAM-140 is expressed in neuronal cells but can also be found in astrocytes, oligodendrocytes and muscle tissue (Walsh and Doherty 1997). NCAM-180 however is found at the postsynapse and in neuronal tissue (Persohn, Pollerberg et al. 1989). This information indicates that different isoforms of NCAM perform varying functions in separate tissues and cells within the nervous system.

1.4.1 Nervous system
The expression of NCAM isoforms in the developing nervous system is tightly controlled to confer the appropriate functional properties to specific regions. The expression of NCAM has been carefully mapped in the cerebellum, spinal cord, hippocampus and sciatic nerve. In the adult chicken cerebellum NCAM has been found in all four layers, the outermost molecular layer, the Purkinje cell layer, granule cell layer and the white matter fibre tracts (Daniloff, Chuong et al. 1986). However the molecular layer and white matter show the highest immunoreactivity against NCAM, which are the areas with axons from granule neurons and myelinated axons from Purkinje neurons. Other reports have indicated that the Purkinje cells weakly stain for NCAM in rat, and in mouse this is strongly expressed using in situ hybridisation (Goldowitz, Barthels et al. 1990; Filiz, Dalcik et al. 2002).

In the adult hippocampus, neurons are continually generated in the subgranular layer of the dentate gyrus and NCAM-PSA is expressed here in high levels (Seki and Arai 1993). In the adult spinal cord, NCAM is found in the grey matter in abundance, although low levels of NCAM are also found in the white matter (Joosten 1994; Filiz, Dalcik et al. 2002). The sciatic nerve in the peripheral nervous system (PNS) expresses NCAM in non-myelinating Schwann cells (Mirsy, Jessen et al. 1986). In fact,
NCAM is thought to be excluded from all myelinating cells due to inhibitory signals from the axon, which when removed cause upregulation of NCAM (Jessen, Mirsky et al. 1987).

1.4.2 The heart
Although NCAM is well studied and its expression is extensively mapped in the nervous system, in recent years it has been discovered in non-neuronal tissue. NCAM is thought to play an important role in the development of the heart. It was first found to be expressed using α NCAM antibodies during chick embryonic heart development in precardiac mesenchymal cells (Thiery, Duband et al. 1982). NCAM expression in preformed heart tissue is conserved across a number of species including human, rat and frog (al-Mahdawi, Shallal et al. 1990; Gaardsvoll, Krog et al. 1993; Probstmeier, Bilz et al. 1994). Expression persists after heart formation and into a number of cellular components including cardiomyocytes, epicardial and endocardial cells (Lackie, Zuber et al. 1991; Gerety and Watanabe 1997).

Interestingly, NCAM is found to be upregulated modestly in the ageing rat heart and in a rat myocardium model for myocardial hypertrophy (Gordon, Wharton et al. 1990; Linnemann 1994). A study of the specific isoforms of NCAM expression in the heart was found to be highly spliced with the MSD (Angata, Nakayama et al. 1997). NCAM is thought to have multiple functions in the heart; it can function homophilically to adhere neural and cardiac cells in vitro, but this has not been shown in vivo. NCAM is also found to be upregulated during innervation of the heart muscle. Therefore its signalling properties may be utilised in this function (Gordon, Wharton et al. 1990). Recently NCAM has been found to be a protective factor in cardiac stress as it was upregulated during remodelling of the rat heart and after induced myocardial infarction (Nagao, Ono et al.).

1.4.3 Skeletal muscle
The discovery of the alternatively spliced MSD sequence into NCAM was in mouse skeletal muscle (Dickson, Gower et al. 1987). NCAM containing the MSD was found to be of the GPI linked form. During muscle development NCAM is expressed in growing myofibers, which form the contractile units of skeletal muscle (Moore, Thompson et al. 1987). Following muscle formation in the adult, expression of NCAM is downregulated, which indicated that NCAM expression correlates with areas of regeneration (Covault and Sanes 1985).

1.4.4 Other cells
NCAM has been found to be expressed on a number of other non-neuronal tissues including cells within the kidney. In the early development of the kidney NCAM is expressed highly in the metanephrogenic mesenchyme (Klein, Langegger et al. 1988). Following kidney development, NCAM
expression in the kidney is downregulated but in the adult tissue it can still be found on the surface of a small subset of renal interstitial cells with the morphology (Markovic-Lipkovski, Muller et al. 2007). After the onset of interstitial fibrosis, NCAM has been found to be upregulated in interstitial cells, although the reason for this has not been ascertained.

NCAM is also expressed in cells within the pancreas, intestine, ovaries and testes. Although characterisation within these tissues has been minimal, the vast array of isoforms and functions of NCAM appear to be useful even in non-neuronal cells. NCAM has also been found to be expressed in human natural killer cells by PCR screening of cell RNA (Lanier, Chang et al. 1991). Investigations into the role of NCAM on NK cells revealed that NCAM was not required for resting or interleukin-2 (IL-2) cell-mediated toxicity. An aggregation assay revealed that NCAM expression on NK cells did not confer homophilic or heterophilic binding to the cells. Therefore NCAM was not thought to play a role in adhesion in this cell type.

1.5 Role in disease
1.5.1 Neuropsychiatric
As a modulator of neuronal organisation and synapse formation, NCAM has been postulated to be involved in neuropsychiatric disorders. Although there is much redundancy in CAM function, soluble NCAM was chosen as a marker for the prognosis of Alzheimer’s disease (Todaro, Puricelli et al. 2004). As the primary cause of dementia, Alzheimer’s disease is characterised by neurofibrillary degeneration, tangles and plaques. Therefore, with this devastating effect on regions of the nervous system, alterations in the expression and secretion of NCAM were to be expected. Serum from 50% of patients with Alzheimer’s disease was found to contain increased levels of all secreted NCAM isoforms. This finding was interesting but further research must be carried out to determine the exact role of the increase of secreted NCAM in patients.

1.5.2 Cancer
The role of the cadherin family of adhesion molecules in the epithelial-mesenchymal transaction (EMT) of cells has been documented in a number of tissue-specific cancers (Auersperg, Pan et al. 1999; Nakajima, Doi et al. 2004; Camara and Jarai 2010). EMT is characterised by the dysregulation of E-cadherin, a loss of cell adhesion via the adherens junction and increased cell motility, which may result in metastasis. Due to the similarities in cadherin and NCAM function, the role of NCAM in cancer has also been investigated.

NCAM has been found to be expressed on the surface of all of the following malignant tissues tested, including neuroblastoma, small cell lung cancer, rhabdomyosarcoma, glioma and
astrocytoma. This has lead to the conclusion that NCAM, as a cell surface receptor, functions as a tumour associated antigen and may be useful in the search for a number of antibody based cancer therapies. Investigators have been undeterred by data indicating that NCAM is present on healthy tissue in the CNS, PNS and non-neuronal tissue. It is noteworthy that in vivo experiments where NCAM-140 was transfected into rat glioma cells, cells exhibited a reduced tumour invasiveness (Edvardsen, Pedersen et al. 1994).

The addition of PSA to NCAM Ig V has been found to be involved directly in cancer progression. As PSA promotes plasticity by abrogating NCAM-mediated adhesion, it was unsurprising to find cancerous expressing NCAM-PSA prior to metastasis formation (Suzuki, Nakayama et al. 2005). Removal of PSA by EndoN, in an in vitro tumour cell model, inhibited tumour growth and differentiation indicating that PSA is directly involved in cancer progression (Seidenfaden, Krauter et al. 2003).

1.5.3 NCAM deficient mouse models
The creation of mouse models, with ablation of a protein of interest, have long been used to determine the function of proteins and whether there is any redundancy in their function (Gosselin, Stevenson et al. 1998; Plum, Wunderlich et al. 2005). NCAM knockout mice do not exhibit lethal phenotypes; they show only subtle morphological changes and impaired spatial learning and memory (Stork, Welzl et al. 1997).

NCAM-180<sup>−/−</sup> knockout mice exhibit a reduction in NCAM-PSA levels and neuronal migration abnormalities in the subventrical zone (Tomasiewicz, Ono et al. 1993). Their olfactory bulb, retina, hippocampus and cerebellum architecture were also altered. The NCAM180-/- knockout mice were subjected to further investigation to test the role of NCAM in schizophrenia. These mice were found to exhibit lateral ventricular enlargement, which is also seen in schizophrenia (Wood, Tomasiewicz et al. 1998).

In general, NCAM knockout mice exhibit a deficit in odour discrimination learning and this is thought to be due to the reduced size of the olfactory bulb (Schellinck, Arnold et al. 2004; Gheusi and Lledo 2007). A genetic knockdown mouse model with ST8SiaIV<sup>−/−</sup> revealed that NCAM was not polysialylated in the adult hippocampus and long term potentiation was impaired (Eckhardt, Bukalo et al. 2000).

1.6 NCAM-VASE
The literature describing the role of NCAM has revealed that it has diverse functions as a result of alternative splicing, post-translational modification and heterophilic interactions at the cell
membrane. Although many of the isoforms of NCAM are well-studied, one is not, even though it is of biological interest. The alternative splicing of a ten amino acid sequence, ASWTRPEKQE, into Ig IV of NCAM greatly alters the function of NCAM and yet there is almost no literature pertaining to it. The subject of this work will involve elucidation of the mechanism by which NCAM-VASE functions, therefore a thorough examination of the related literature has been carried out.

1.6.1 Introduction and discovery

The Variable Alternatively Spliced Exon (VASE) was first discovered in 1988 and was described as a 30 base pair sequence which was inserted between exons 7 and 8 in NCAM Ig IV (Small, Haines et al. 1988). VASE encodes a 10 amino acid sequence, ASWTRPEKQE, which has been postulated to convert the Ig IV domain from an Ig intermediate domain to an Ig variable domain. This is due to the hydrophilic sequence insertion into the hypervariable region of the Ig fold. In the Ig-like domain, as is the case in true immunoglobulin, the hypervariable region is used for antigen recognition (Small and Akeson 1990). Therefore insertion of VASE into this region may disrupt NCAM binding at this position or introduce a new site for an alternative interaction.

1.6.2 Phylogeny

Table 1.1: A protein sequence search to determine which species express the 10 amino acid VASE sequence, which is inserted into Ig IV.

<table>
<thead>
<tr>
<th>Species</th>
<th>VASE sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human, <em>Homo sapiens</em></td>
<td>ASWTRPEKQE</td>
<td>(Strausberg, Feingold et al. 2002)</td>
</tr>
<tr>
<td>Rat, <em>Rattus norvegicus</em></td>
<td>ASWTRPEKQE</td>
<td>(Small, Shull et al. 1987)</td>
</tr>
<tr>
<td>Mouse, <em>Mus muscularis</em></td>
<td>ASWTRPEKQE</td>
<td>(Santoni, Barthels et al. 1989)</td>
</tr>
<tr>
<td>Rhesus monkey, <em>Macaca mulatta</em></td>
<td>ASWTRPEKQE</td>
<td></td>
</tr>
<tr>
<td>Frog, <em>Xenopus tropicalis</em></td>
<td>ASWTRPLKQE</td>
<td>(Zorn and Krieg 1992)</td>
</tr>
<tr>
<td>Zebrafish, <em>Danio rerio</em></td>
<td>ASWTRPEKHK</td>
<td>(Mizuno, Kawasaki et al. 2001)</td>
</tr>
</tbody>
</table>

To further understand the role of the insertion of the VASE sequence into NCAM, a number of phylogenetic studies have been carried out to determine sequence conservation across NCAM genes (Table 1.1). The VASE sequence is alternatively spliced in NCAM in the mammals and birds investigated. There is high sequence conservation across these species and therefore protein structural similarities would be expected. It is interesting to note that the VASE sequence was not found in *Drosophila melanogaster* or *Caenorhabditis elegans*, although both of these organisms express NCAM homologues (Hall and Rutishauser 1985).
Table 1.2: A protein sequence search to determine whether the 10 amino acid sequence VASE is inserted into proteins excluding NCAM across a number of species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Protein</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human, Homo sapiens</td>
<td>NCAM</td>
<td>ASWTRPEKQE</td>
</tr>
<tr>
<td>Gram-positive bacterium, Kocuria rhizophila</td>
<td>Pfpl family peptidase</td>
<td>ASWTRPE</td>
</tr>
<tr>
<td>Funghal plant pathogen, Cochliobolus heterostrophus</td>
<td>Histidine kinase HHK8p</td>
<td>ASWTRPPK</td>
</tr>
<tr>
<td>Bacterium, Chryseobacterium gleum</td>
<td>Peptidyl-dipeptidase Dcp2</td>
<td>ASWTRAEK</td>
</tr>
<tr>
<td>Zebrafish, Danio rerio</td>
<td>Neogenin-like</td>
<td>SWTPEKNE</td>
</tr>
<tr>
<td>Butyrate-producing bacterium SS3/4</td>
<td>Superfamily II helicase</td>
<td>SWTPEKRE</td>
</tr>
<tr>
<td>Gram-negative bacterium, Erwinia amylovora</td>
<td>Dipeptidyl carboxypeptidase II</td>
<td>ASWTRAEK</td>
</tr>
<tr>
<td>Bacterial pathogen, Serratia odorifera</td>
<td>Peptidyl-dipeptidase Dcp</td>
<td>ASWTRAEK</td>
</tr>
<tr>
<td>Proteobacterium, Gallionella capsiferriformans</td>
<td>Sec-independent protein translocase, Tatc subunit</td>
<td>ASWTRPVEK</td>
</tr>
<tr>
<td>Gram-negative bacterium, Salmonella enterica</td>
<td>Peptidase family M3</td>
<td>ASWTRAEK</td>
</tr>
<tr>
<td>Bacterium, Paenibacillus curdlanolyticus</td>
<td>Sec-C motif domain protein</td>
<td>SWKRPEDEQ</td>
</tr>
<tr>
<td>Gram-positive bacterium, Streptomyces sp. SPB78</td>
<td>DeoR family transcriptional regulator</td>
<td>SWTRPEHQ</td>
</tr>
<tr>
<td>Gram-positive bacterium, Streptomyces sp. SPB78</td>
<td>Ribose/arabinose operon repressor, LacI-family transcriptional regulator</td>
<td>SWTRPEHQ</td>
</tr>
<tr>
<td>Gram-positive bacterium, Paenibacillus sp. JDR-2</td>
<td>Glycoside hydrolase family 3 domain protein</td>
<td>WTRPEKQ</td>
</tr>
<tr>
<td>Gram-positive bacterium, Mycobacterium avium 104</td>
<td>MbtD protein</td>
<td>SWTRPEIQ</td>
</tr>
<tr>
<td>Jumping ant, Harpagnathos saltator</td>
<td>28S ribosomal protein S15</td>
<td>WTRPEK</td>
</tr>
<tr>
<td>Gram-negative bacterium, Zunongwangia profunda SM-A87</td>
<td>DNA double-strand break repair rad50 ATPase</td>
<td>SWTRPDK</td>
</tr>
<tr>
<td>Parasite, Schistosoma japonicum</td>
<td>Glutamyl aminopeptidase</td>
<td>WTRPEK</td>
</tr>
</tbody>
</table>

A BLAST search for amino acid sequence homology of VASE (ASWTRPEKQE) revealed that a number of proteins from bacteria contained similar sequences (Table 1.2). Recurring proteins of interest included a series of peptidase enzymes and transcriptional regulators. The role of the VASE-like sequence in this protein has not been investigated, therefore its function is unknown. A neogenin-like protein from zebrafish contained a protein sequence with 70% homology to the VASE sequence. Neogenin is an Ig superfamily member and transmembrane receptor which is involved in axon outgrowth and guidance (Wilson and Key 2007). It contains 4 Ig-like domains and 6 FnIII domains. The sequence similarity to this protein was interesting as NCAM-VASE also modulates these functions, but the role of this sequence within neogenin has not been studied.
1.6.3 Function

Neurite outgrowth

NCAM lacking VASE neurons have been found to extend long neurites, which function to aid communication and plasticity within the developing nervous system (Doherty, Barton et al. 1989). However it was determined that neurons from rat extended shorter neurites with increasing age even though the expression levels of NCAM were similar throughout (Doherty, Cohen et al. 1990). Although a decrease in NCAM concentration was not responsible for this loss of neurite outgrowth promotion, the insertion of the VASE sequence into Ig IV was. The insertion of the VASE sequence into NCAM prevented the promotion of neurite outgrowth over basal levels (Doherty, Moolenaar et al. 1992). This inhibitory effect was confirmed in an in vitro neuronal cell model, where rat cerebellar granule neurons grown over a monolayer of NIH 3T3 fibroblast expressing transfected NCAM-VASE-140 did not extend long neurites.

The inhibitory effect on neurite outgrowth could simply be due to functional changes in Ig IV which abolish a protein binding site. However addition of a peptide corresponding to the VASE sequence specifically inhibits NCAM-mediated neurite outgrowth, indicating that the primary sequence alone is sufficient (Saffell, Walsh et al. 1994). However the VASE sequence has also been found to inhibit L1 mediated neurite outgrowth (Lahrtz, Horstkorte et al. 1997). This was determined by measuring the extension of neurites from cerebellar granule neurons grown on Ig IV±VASE protein in the presence of L1 or NCAM antibodies. The antibodies bound to the specific cellular receptors to block their interaction in NCAM-VASE mediated neurite outgrowth. The NCAM antibody had no effect on neurite outgrowth, but the L1 antibody inhibited all neurite outgrowth on cells grown on Ig IV-VASE protein. This indicated that a portion of VASE-mediated neurite outgrowth involved a trans heterophilic interaction with L1. It is also worth noting that there is a C-type lectin consensus sequence in Ig IV upstream of the VASE sequence. The insertion of VASE into this region may therefore affect the binding affinity for an interaction with L1, which may explain why the VASE-mediated neurite outgrowth effects via L1 are more marked than those with NCAM alone.

The mechanism of NCAM, L1 and N-cadherin mediated neurite outgrowth is thought to be the same as for FGF, via FGFR binding and downstream signalling (Williams, Walsh et al. 1994). All three proteins have been found to have sequence homology with FGFR within a portion of its Ig-like D2 domain, which has been named the CAM homology domain. For NCAM, the homology comes from the VASE sequence (Table 1.3) (Doherty and Walsh 1996). In N-cadherin, the ‘HAV’ sequence is involved in homophilic binding but the function of the L1 sequence is not known (Halbleib and Nelson 2006). Antibodies against the CHD and the acid box (directly upstream from the CHD)
abrogate FGFR mediated neurite outgrowth therefore this region of FGFR may be involved in L1, N-cadherin, NCAM and L1 mediated neurite outgrowth (Williams, Walsh et al. 1994; Sanchez-Heras, Howell et al. 2006). A FnIII domain within each of these proteins has been postulated to bind to the CHD and acid box of FGFR to elicit neurite outgrowth (Doherty and Walsh 1996). Therefore it has been postulated that the CAM homology sequence VASE can bind to the FGFR at its CHD domain to block NCAM FnIII 1 from binding and promoting neurite outgrowth (Saffell, Walsh et al. 1994).

**Table 1.3: The CAM homology domain in FGFR shares sequence homology with NCAM-VASE, L1 and N-cadherin.**

20 amino acids within FGFR share sequence homology with the VASE sequence, which may be inserted into NCAM, the ‘HAV’ motif in N-cadherin and the ‘APYW’ sequence in L1. Table modified from a table in a paper by Doherty and Walsh (Doherty and Walsh 1996).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGFR-1</td>
<td>VAPYWTSPEKMKLLHAVPA</td>
</tr>
<tr>
<td>L1</td>
<td><strong>AAPYW</strong></td>
</tr>
<tr>
<td>N-cadherin</td>
<td>RAHAVDI</td>
</tr>
<tr>
<td>NCAM</td>
<td>ASWTRPEKQE</td>
</tr>
</tbody>
</table>

**Adhesion**

There is an inextricable link between the adhesive properties of NCAM and its role in neurite outgrowth. Trans NCAM homophilic binding is required in order for the promotion of NCAM-mediated neurite outgrowth via FGFR (Lahrz, Horstkorte et al. 1997). As the insertion of the VASE sequence inhibits NCAM’s ability to promote neurite outgrowth, it is plausible that VASE affects the homophilic binding strength of NCAM. This hypothesis has been indirectly tested in a number of ways. L cells transfected with either NCAM±VASE-140, and subjected to a low-shear aggregation assay, were found to segregate in NCAM or NCAM-VASE L cell islands (Chen, Haines et al. 1994). The VASE sequence alone was responsible for the segregation of NCAM and VASE cell islands, therefore VASE altered the binding properties of NCAM. The segregation of the cells became more pronounced with increasing contact time. An explanation for this could be that the homophilic binding affinities of NCAM-NCAM and VASE-VASE are quantifiably different and therefore the cells were segregated. If the affinity between two VASE cells was larger than between an NCAM and a VASE cell, then the VASE cell would bind preferentially to another VASE cell. It is worth noting that both subsets of cells aggregated at the same rate, which would indicate that the protein adhesion binding constants may be similar.

An experiment to determine the effect of NCAM Ig I on NCAM homophilic binding also revealed some interesting data about the effect of the VASE sequence (Kiselyov, Berezin et al. 1997). Ig IV containing the VASE sequence was added to cerebellar granule neurons in a low shear, rapid aggregation assay. Ig IV-VASE was found to inhibit the aggregation of these cells, but not to the same
extent as NCAM Ig I. NCAM Ig IV-VASE was not found to bind to Ig II by SPR, even though NCAM Ig IV lacking VASE binds to NCAM Ig II, although the affinity of the individual domain interaction may be too low to measure (Ranheim, Edelman et al. 1996).

The beginning of the VASE sequence ‘ASWT’ is similar to ‘MSWT’, a sequence from NCAM Ig III, which has been found to be involved in NCAM homophilic binding (Small, Haines et al. 1988). The VASE sequence may therefore mimic this site in Ig III and bind to apposing NCAM molecules to prevent alternative binding.

1.6.4 Expression pattern

Initial experiments carried out following the discovery of the VASE exon found NCAM-VASE mRNA to be expressed in the adult rat brain (Small, Haines et al. 1988). The authors noted that up-regulation of NCAM-VASE mRNA occurred with increasing rat development. At embryonic day 15 (E15) NCAM-VASE mRNA accounted for only 3% of total NCAM transcripts. In the adult rat 43% of NCAM transcripts were found to contain the VASE sequence. This has been corroborated by data from the adult rat cerebellum, where 50% of NCAM mRNA transcripts contained the VASE sequence. This also fits with data which suggests that only 20-30% of NCAM transcripts require the VASE sequence in order to inhibit neurite outgrowth (Saffell, Walsh et al. 1994). Therefore only a minority of NCAM must contain VASE in order to have an inhibitory effect.

The expression pattern of NCAM-VASE is not well-studied but a few experiments have mapped mRNA expression using PCR. In the adult rat the VASE sequence in NCAM is highly expressed in the cerebellum, cortex, thalamus, hippocampus and the midbrain (Small and Akeson 1990). The cerebellum is generally an area of low plasticity which cannot undergo regeneration. Interestingly VASE mRNA is almost completely lacking in the dorsal root ganglia and olfactory epithelium and completely lacking in skeletal muscle and the adrenal gland.

Unpublished work carried out in the Saffell laboratory by Dr. M. Delves has attempted to map the subcellular localisation of NCAM-VASE in the developing and adult rat brain. Using an antibody against the VASE sequence in Ig IV, NCAM-VASE was found to be upregulated during myelination. It was found to be lacking from all sites of major neurogenesis in the adult CNS which were tested, including the dentate gyrus subgranular layer and the ependymal cell lining all ventricles of the brain (except the lateral ventricle).

The lack of NCAM-VASE in areas of plasticity and regeneration is supported by the function of VASE as an inhibitor of neurite outgrowth. If NCAM-VASE were introduced into areas of regeneration it would be interesting to see whether regeneration would be abrogated. Alternatively it would be
interesting to see if the knockdown of the VASE sequence in developed and low plasticity areas would allow regeneration.

NCAM-VASE is expressed in the adult rat heart but is completely absent from skeletal muscle, although NCAM is present (Fazeli, Wells et al. 1996). To further understand why NCAM-VASE is excluded from skeletal muscle, NCAM-VASE-125 was transfected into the skeletal muscle of mice. NCAM-VASE had no visible effect on the myoblast fusion process regulated by NCAM lacking VASE.

1.6.5 Role in disease
As a modulator of cell-cell adhesion, NCAM has been found to be expressed and secreted in patients with various neuropsychiatric disorders including Alzheimer’s disease. The expression level of NCAM-VASE in patients with a number of neuropsychiatric diseases has also been studied. NCAM containing the VASE sequence is upregulated in the membrane extracts of the hippocampus from patients with bipolar disorder (Vawter, Hemperly et al. 1998). NCAM-VASE was not upregulated compared with control individuals following schizophrenia or suicide. Interestingly the cerebrospinal fluid of schizophrenia patients was found to contain higher levels of NCAM-VASE than control individuals, although this was not the case in bipolar disorder patients or those who had committed suicide (Vawter, Frye et al. 2000).

The insertion of the VASE sequence into NCAM has also been found to play a role in age-related memory impairment of mice (Qin, Zheng et al. 2005). Senescence-accelerated mice were found to express higher levels of NCAM-VASE in their hippocampus than senescence-resistant mice and so performed worse in spatial learning Morris Water Maze tests. As NCAM-VASE inhibits neurite outgrowth, its presence in the hippocampus may have decreased the reorganisation and associated memory formation required for spatial learning. Interestingly aged rat hippocampi are found to contain a high proportion of astrocytes which, when cultured and differentiated, express increased levels of NCAM-VASE (Gegelashvili, Andersson et al. 1993). Astrocytes are known to provide structural support for CNS cells and are found in areas of scarring, post-repair (Niquet, me et al. 1993).

1.6.6 VASE vs. PSA
As has been previously mentioned, NCAM-VASE has not been found in any of the CNS areas with high plasticity tested so far. In contrast NCAM-PSA appears to be solely expressed in areas of regeneration and high synaptic plasticity, such as the olfactory bulb and the dorsal root ganglion. When comparing the temporal pattern of NCAM-VASE and NCAM-PSA, it is clear that they are diametrically opposed; whilst NCAM-PSA expression is high in the embryonic rat and is
downregulated as development continues, NCAM-VASE is upregulated and remains in the adult (Small, Haines et al. 1988; Seki and Arai 1993). NCAM-VASE and NCAM-PSA also have opposing effects on neurite outgrowth; NCAM-VASE inhibits neurite outgrowth whereas NCAM-PSA promotes it. These opposing functions fit with their opposing expression patterns and temporal distributions.

Although it would appear that the downregulation of NCAM-PSA may be linked to the upregulation of NCAM-VASE, there was found to be no increase in alternative splicing of VASE to force the downregulation of PSA (Soares, von Boxberg et al. 2000). There is no evidence as yet to suggest that NCAM-VASE cannot be polysialylated. NCAM-VASE from the membrane fractions of bipolar disorder patients was not polysialylated whereas the NCAM protein present was (Vawter, Hemperly et al. 1998).

The inverse correlation between VASE and PSA expression patterns, temporal distribution and function may indicate that while PSA plays a role in plasticity and regeneration, VASE promotes stability. NCAM-PSA has been found to abrogate NCAM-mediated adhesion, which reduces NCAM homophilic binding and allows cell-cell plasticity. Therefore it is plausible that the insertion of VASE may alter the homophilic binding strength of NCAM to promote adhesion and stability.

1.7 Atomic Force Microscopy

To test whether the insertion of the VASE sequence alters the homophilic binding strength of NCAM, a technique was chosen which was suitable for this investigation. The atomic force microscope has been used for a number of years to image surfaces and biological material. However in recent years applications have been modified to measure the forces between AFM surfaces. This has been optimised to measure the forces, and therefore adhesion, between single protein molecules and single cells. This technique therefore provides an exciting and powerful means of protein and cell characterisation.

1.7.1 Introduction and discovery

The first scanning probe microscope (SPM) was invented in tandem with the scanning tunnelling microscope (STM) in 1981 by Binnig and Rohrer (Binnig, Rohrer et al. 1982). SPM encompasses a large number of microscopy instruments which have many common features. The use of a scanning probe is paramount to this set of microscopies and requires a microfabricated flexible cantilever with a micrometer scale tip of known dimensions. Depending on the function of the tip, it may take the shape of a thin rectangle, triangle or pyramidal tip. Typically, silicon nitride tips are used, which are more flexible than their silicon counterparts. The properties of these tips vary but they behave as deformable springs which follow Hooke’s Law where ‘F’ is the force on the tip in Newtons (N), ‘k’ is
the cantilever spring constant (N/m) and ‘x’ is the vertical displacement of the cantilever tip (m) from its starting position:

\[ F = -kx \]

Figure 1.11: General components of an AFM instrument.
A laser is positioned onto the tip of a deformable spring or cantilever, which is under the control of a piezoelectric device, which controls the X-, Y- and Z-positioning of the cantilever. The cantilever approaches a surface and the deflection of the cantilever is measured by the change in position of the reflected laser onto a photodiode. Deflection may be due to attractive or repulsive forces between the tip and sample or due to changes in the local topology of the surface.

The force on the cantilever tip is therefore linearly correlated to the cantilever deflection and the force required to deflect the cantilever by an arbitrary distance. To image an immobilised surface or measure the interactions between a tip and sample, the cantilever is mounted into a specific tip holder. It is brought into contact with the immobilised sample of interest under the control of a piezoelectric device, over a distance of a few μm to a hundred μm depending on the properties of the piezoelectric material. Piezoelectric material expands and contracts depending on the voltage applied to it, which controls tip-sample separation. The deflection of the cantilever is monitored during an AFM experiment by different methods depending on the required function. This is monitored by reflection of a laser off the tip of the cantilever, which is reflected via a moveable mirror onto a photosensitive diode (PSD) (Fig. 1.11). A quad photodetector is common, which allows the user to detect horizontal and vertical cantilever deflection, as well as twisting or dithering of the tip.

The deflection of the cantilever can be converted from units of distance to force, knowing the cantilever spring constant, to determine the mechanical interactions between the tip and sample. In contact-mode between a tip and sample, the 3D topology of an immobilised surface may also be mapped by measuring the cantilever position and deflection. This function requires great precision and depends on an electronic feedback loop which can maintain a user-defined setpoint cantilever deflection or position. Cantilever movement is controlled by X-, Y-, Z-piezoelectric actuators, which
in some cases, due to their accurate positioning, give atomic resolution imaging of surfaces. This resolution can be achieved because it is not limited by diffraction, only by the tip-sample area. There are over 25 common SPM techniques, but the subject of this work involves the use of contact atomic force microscopy (AFM) (Table 1.4).

### 1.7.2 Evolution of SPM

There are many variations on the SPM, one of which is the STM, which is an instrument for imaging surfaces at nanometer resolution either in an ultra high vacuum, air or liquid (Table 1.4) (Binnig, Rohrer et al. 1982). A sharp tip is brought near to a conducting surface and the difference in voltage between the two allows electron tunnelling and measurement of the overall tunnelling current. The resultant data includes information regarding the tip position, sample properties and applied voltage.

Contact mode AFM was invented in 1986 by Binnig, Quate and Gerber and was first used as a non-destructive method to measure atomic resolution interaction forces as small as $10^{-18}$ N between a cantilever tip and sample (Binnig, Quate et al. 1986). It has since been used to image surfaces in air, liquid, or in a vacuum (Meyer and Amer 1988; Drake, Prater et al. 1989; Gould, Drake et al. 1990). High-resolution 3D AFM images have been obtained of lyophilised DNA and protein as well as cells in a liquid cell (Vadillo-Rodriguez, Busscher et al. 2004; Hamon, Pastre et al. 2007). The AFM topographs of a number of proteins have been compared with those from X-ray and electron crystallography and revealed that structure was not perturbed with contact imaging (Moller, Allen et al. 1999). This has allowed a fast method for the study of protein aggregation such as with amyloid fibres in Alzheimer’s disease (Adamcik, Jung et al. 2005). In recent years a ‘force mode’ application has been used to measure the mechanics between a tip and sample at high picoNewton resolution (Weisenhorn, Hansma et al. 1989). The authors measured the interactions between a Si$_3$N$_4$ cantilever and a cleaved mica substrate. By measuring the cantilever deflection in units of force, along the Z-axis only, they mapped this against the cantilever position to yield a series of force-distance curves and the first force spectroscopy experiment was carried out.

Molecular recognition and adhesion between receptor-ligand pairs plays a role in a large proportion of biological processes. Therefore a number of AFM instruments have been designed and optimised for biological application, including the Veeco Nanoscope IV with PicoForce controller and JPK Instruments CellHesion® 200 module. Using the force spectroscopy mode, the forces of interaction between proteins can be measured. It is also possible to measure the adhesive force between single cells and to map protein adhesion to patches of single cells using force-volume imaging (Gaboriaud,
Parcha et al. 2008; Helenius, Heisenberg et al. 2008). These two techniques will be discussed in further detail.

Table 1.4: The invention of scanning probe microscopy has lead to the invention of a series of imaging and spectroscopy techniques for characterising surfaces and their interactions.

This data has been taken from an Agilent Technologies publication detailing ‘A brief History of Scanning probe microscopy’ by Agilent Technologies.

<table>
<thead>
<tr>
<th>Scanning Probe Microscopy</th>
<th>Function</th>
<th>First used and Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scanning tunnelling microscope (STM)</td>
<td>Atomic resolution of conducting surfaces</td>
<td>1981, (Binnig, Rohrer et al. 1982)</td>
</tr>
<tr>
<td>Scanning capacitance microscope (SCM)</td>
<td>Measures tip-sample electrostatic capacitance</td>
<td>1981, (Matey and Blanc 1985)</td>
</tr>
<tr>
<td>Scanning near-field optical microscope (SNOM)</td>
<td>25 nm imaging resolution at visible wavelengths</td>
<td>1982, (Pohl, Denk et al. 1984)</td>
</tr>
<tr>
<td>Contact mode atomic force microscopy (Contact AFM)</td>
<td>Atomic resolution on surfaces in contact</td>
<td>1985, (Binnig, Quate et al. 1986)</td>
</tr>
<tr>
<td>Scanning thermal microscope (SThM)</td>
<td>Measures thermal conductivity and local temperature</td>
<td>1986, (Williams and Wickramasinghe 1986)</td>
</tr>
<tr>
<td>Magnetic force microscope (MFM)</td>
<td>Measures the magnetic structure of a surface to 100 nm lateral resolution</td>
<td>1986, (Martin and Wickramasinghe 1987; Saenz, Garcia et al. 1987)</td>
</tr>
<tr>
<td>Non-contact mode atomic force microscope (Non-contact AFM)</td>
<td>Topographic image of surface using non-contacting cantilever oscillating above surface</td>
<td>1986, (Martin, Williams et al. 1987)</td>
</tr>
<tr>
<td>Lateral force microscope (LFM)</td>
<td>Atomic scale imaging of tip-surface lateral fraction forces</td>
<td>1987, (Mate, McClelland et al. 1987)</td>
</tr>
<tr>
<td>Electrostatic force microscope (EFM)</td>
<td>Non-contact measurements of tip-sample electrostatic forces</td>
<td>1987, (Martin, Abraham et al. 1988)</td>
</tr>
<tr>
<td>Scanning electrochemical microscope (SECM)</td>
<td>Probing surface reactivity, deposition and etching</td>
<td>1989, (Husser, Craston et al. 1989)</td>
</tr>
<tr>
<td>Kelvin probe force microscope (KPFM)</td>
<td>Measures the potential of the surface in contact mode</td>
<td>1991, (Nonnenmacher, O'Boyle et al. 1991)</td>
</tr>
<tr>
<td>Tapping mode atomic force microscope (TM-AFM)</td>
<td>An oscillating cantilever in contact/non-contact mode</td>
<td>1992, US patent 5412980</td>
</tr>
<tr>
<td>Piezoresponse force microscopy using AFM</td>
<td>Measures forces between surfaces with AFM</td>
<td>1993, (Franke, Besold et al. 1994)</td>
</tr>
<tr>
<td>Atomic resolution non-contact AFM</td>
<td>Imaging surfaces at atomic resolution without contact</td>
<td>1995, (Giessibl 1995)</td>
</tr>
<tr>
<td>Magnetically actuated cantilever (MAC mode®) AFM</td>
<td>Measures the induced magnetic field between a magnetic filmed tip and sample</td>
<td>1996, (Han, Lindsay et al. 1996)</td>
</tr>
<tr>
<td>Dynamic friction force microscopy</td>
<td>Cantilever ‘dithering’ to measure changes in friction</td>
<td>1996, (Colchero, Luna et al. 1996)</td>
</tr>
<tr>
<td>Torsional resonance mode AFM (TR mode AFM)</td>
<td>Lateral force measurement between surfaces in non-contact mode</td>
<td>2001, (Pfeiffer, Bennewitz et al. 2002)</td>
</tr>
</tbody>
</table>
1.7.3 Single molecule force spectroscopy

Single molecule force spectroscopy (SMFS) is a method to measure the intermolecular interaction between two molecules using an AFM. It can also be used to measure the intramolecular forces and interactions of a molecule by measuring the unfolding of its domains.

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**Figure 1.12: Force spectroscopy can be carried out using atomic force microscopy.**
The interaction between a tip and sample can be measured during the approach and retraction of a cantilever to and from a surface of interest. The deflection of the cantilever is measured against the tip-sample separation. A typical force spectroscopy experiment can be described as follows: a) The cantilever tip is withdrawn from the surface and zero force is exerted on the cantilever. b) During tip approach, attractive force with the sample may occur to cause the tip to ‘jump into contact’. Cantilever deflection is negative. c) After contact, the piezoelectric device continues to lower the cantilever until a setpoint positive contact force is reached. d) The cantilever is raised from the surface, but the tip remains in contact, causing further negative deflection until a rupture event occurs. e) The tip jumps out of contact with the surface and tip-sample separation increases.
The intramolecular strength of titin has been investigated through forced unfolding of its Ig-like domains using an AFM (Schots, Van der Leede et al. 1988). SMFS has been used to determine protein secondary structure and to map the exact interacting portions of proteins (Wieland, Gewirth et al. 2005; Janovjak, Kedrov et al. 2006). The forces required to separate two single proteins engaging in intermolecular interactions can also be measured using SMFS. The adhesive force of protein separation increases with the logarithm of the binding affinity of the interaction; therefore a protein-protein interaction, which has a higher affinity, requires more force to separate it than an interaction which is non-specific or weak affinity (Kuo and Lauffenburger 1993).

To measure the adhesion between proteins by force spectroscopy, an AFM setup involving a cantilever and an immobilised sample is required (Fig. 1.12) (See Section 1.7.2). Protein attached to a tip and substrate is brought into apposition and the force required to separate the tip from the sample is recorded. The cantilever and substrate must be functionalised with the proteins to be studied using a suitable method of functionalisation. This method must maintain the quality of the biological sample and be reproducible to ensure successful data acquisition. Also, the method of protein functionalisation must result in a stronger adhesive link to the tip or surface than between the protein adhesion in question. This ensures that the unbinding events measured are due to the de-adhesion events between the protein and not due to removal of the protein from the surface.

Adsorption

Adsorption of a protein to a surface involves a variety of complex interactions and interactions between the solvent, solute, surface and properties of the protein, which results in a decrease in the Gibbs free energy (da Silva 2002). Interaction between the protein and glass or mica surface arises from hydrogen bonding, dipole and induced dipole moments and electrostatic potentials. If a protein is added to a surface in a buffer, it will adsorb over time and the rate and form in which it does this will also depend on the isoelectric point of the protein and pH of the solution (Schon, Gorlich et al. 2007). Although certain adsorbed protein conformations may be energetically favourable, proteins are orientated randomly on a surface as a result of adsorption.

Affinity capture

The production and purification of recombinant proteins often involves the use of a C- or N-terminal affinity tag for protein enrichment against an affinity capturing column. Affinity tags are also useful for capturing and orientating proteins in an AFM experiment. Many AFM surfaces have been functionalised with His-tag containing proteins via affinity capture by nitriloacetic acid (NTA) in the presence of Ni$^{2+}$ (Lata and Piehler 2005; du Roure, Buguin et al. 2006; Tang, Ebner et al. 2009). Tang
and Ebner described an elegant method using a bifunctionalised polyethylene conjugated linker (PEGylated linker), which was covalently coupled to a silanised glass coverslip at one end and to an NTA moiety at the other. His-tagged S-layer proteins were then affinity captured to the PEGylated surface in preparation for AFM measurements.

The interaction between one NTA moiety and two His residues has been found to be a suitable for capturing proteins whose adhesion will be measured by SMFS (Kienberger, Kada et al. 2000; Verbelen, Gruber et al. 2007). Two independent experiments revealed that the force required to separate NTA from a 6-His tag was 150-194 pN for loading rates of 4500-7000 pN/s.

**Covalent attachment**

Covalent attachment of proteins to AFM surfaces is the preferred method of functionalisation, due to their increased bond strengths (between 1 and 2 nN) (Grandbois, Beyer et al. 1999). Direct covalent links, formed between protein Lys side chains and amine reactive groups from surface silanisation, have been extensively used but may not be ideal (Chtcheglova, Shubeita et al. 2004; Denisov, Chtcheglova et al. 2008). This is not preferable because Lys groups involved in covalent linkages to the surface may be from protein active sites, which may abrogate protein function. Direct covalent linkages may also decrease protein mobility and abrogate function by this means.

Alternatively, chemical linkers of known length can be fashioned to covalently bind at one end to the AFM surface, and to capture a tagged protein by the other (Tang, Ebner et al. 2009). The flexible linker allows protein mobility and also raises the protein above the AFM surface to minimise non-specific interactions which may occur between the tip and sample. Instead of silanising a surface with aminopropyltriethoxy silane (APTES) and linking to amine groups in proteins, thiol chemistry can be employed. Cantilevers must firstly be coated with a chromium or titanium layer followed by a gold layer. Engineered Cys residues or 2-pyridyldithiopropionyl (PDP) tagged linkers may then be covalently coupled to the surface before AFM measurements. This method has been used to measure the adhesion between a number of proteins including ferritin and a ferritin antibody (Harada, Kuroda et al. 1999).

Another method for AFM surface functionalisation is to use self-assembled monolayers (SAM) on gold layered surfaces. This addition is a simple process, achieved by addition of bifunctionalised alkanethiols in EtOH, and allows the attachment of proteins via amine-reactive groups. This functionalisation method has been used for measuring the interaction between cell surface proteoglycans (Dammer, Popescu et al. 1995).
Data Acquisition

Following functionalisation and characterisation of AFM surfaces with the protein to be studied, force-distance curves exhibiting the unbinding between the proteins must be acquired. Prior to acquisition, careful instrument calibration must be carried out. Force spectroscopy requires picoNewton sensitivity since the putative forces required to separate single molecules may be in the sub piconewton range (Table 1.5) (Zlatanova, Lindsay et al. 2000).

Table 1.5: Single molecule force spectroscopy has previously been used to determine the adhesive forces of interaction between receptor-ligand pairs.
This data is taken from a review paper by Zlatanova et al. and contains some extra information (Zlatanova, Lindsay et al. 2000).

<table>
<thead>
<tr>
<th>Molecular Interaction</th>
<th>Proof of specificity</th>
<th>Most probable rupture force (pN)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG-Protein G</td>
<td>Free protein G</td>
<td>3000-4000</td>
<td>(Moy, Florin et al. 1994)</td>
</tr>
<tr>
<td>Fluorescein-α fluorescein</td>
<td>Non-specific antibody</td>
<td>200</td>
<td>(Stuart and Hlady 1995)</td>
</tr>
<tr>
<td>Fluorescein-α fluorescein (single chain)</td>
<td>Free fluorescein</td>
<td>50</td>
<td>(Ros, Schwesinger et al. 1998)</td>
</tr>
<tr>
<td>α human serum albumin-human serum albumin</td>
<td>Free serum albumin</td>
<td>250</td>
<td>(Hinterdorfer, Baumgartner et al. 1996)</td>
</tr>
<tr>
<td>α Ferritin-ferritin</td>
<td>Non-specific antibody</td>
<td>50</td>
<td>(Allen, Chen et al. 1997)</td>
</tr>
<tr>
<td>Intercellular adhesion molecule-1 (ICAM)-α ICAM</td>
<td>-</td>
<td>100</td>
<td>(Willemsen, Snel et al. 1998)</td>
</tr>
<tr>
<td>Proteoglycans</td>
<td>Antibody and non-specific antibody</td>
<td>Up to 400</td>
<td>(Dammer, Popescu et al. 1995)</td>
</tr>
<tr>
<td>Insulin-insulin</td>
<td>Free insulin, α insulin</td>
<td>1300</td>
<td>(Yip, Yip et al. 1998)</td>
</tr>
<tr>
<td>Citrate synthase-GroEL</td>
<td>Denatured substrate, ATP control, bare tips</td>
<td>420</td>
<td>(Vincikier, Dumortier et al. 1996)</td>
</tr>
<tr>
<td>B-lactamase-GroEL</td>
<td>Denatured substrate, ATP control, bare tips</td>
<td>240</td>
<td>(Vincikier, Dumortier et al. 1996)</td>
</tr>
<tr>
<td>P-selectin-P-selectin glycoprotein ligand-1</td>
<td>EDTA, non-glycosylated protein</td>
<td>160</td>
<td>(Fritz, Katopodis et al. 1998)</td>
</tr>
<tr>
<td>Myelin basic protein-lipid bilayers</td>
<td>Bare mica, low vs. high ionic strength</td>
<td>140</td>
<td>(Mueller, Butt et al. 1999)</td>
</tr>
<tr>
<td>Ganglioside GM1-cholera toxin B subunit (CTB)</td>
<td>Free CTB</td>
<td>90</td>
<td>(Luckham and Smith 1998)</td>
</tr>
</tbody>
</table>

AFM force-distance curves relate the position of the cantilever with its deflection during cantilever approach, contact and retraction from a surface. For force spectroscopy, deflection is measured in Newtons and position is optimised at nanometer resolution in keeping with the scale of the proteins.
of interest. Cantilever spring constants are normally determined using a method called ‘thermal
tune’ where the frequency of thermal fluctuations of the cantilever are recorded and Fourier
transformed to determine its resonant frequency (Christopher and et al. 2005). This allows
determination of the cantilever stiffness to relate deflection of the cantilever in distance and force
units. Alternative methods of spring constant determination have been employed, including:

1. estimation from cantilever geometry
2. comparison with and without added mass
3. hydrodynamic model

Estimation of cantilever geometry is usually carried out on a representative cantilever, but cantilever
properties vary due to intrinsic quality control issues with microfabrication processing (Charles and
Martin 2005). This method has therefore been found to be unreliable. Addition of a known mass to
the cantilever has been found to be highly accurate but time-consuming (Maeda and Senden 2000;
Jericho and Jericho 2002).

Following calibration of the AFM instrument, force-distance curves can be acquired to measure the
interaction between two surfaces (Fig 1.12). When the tip is brought into close apposition with the
surface, a number of repulsive forces act on the cantilever to deflect it away from the surface. These
may include steric, electrostatic and hydration forces. As the cantilever tip approaches the surface,
the tip may jump into contact with the surface or ‘snap in’, shown by negative deflection on the
force-distance curve, when the attractive forces exceed the spring constant of the cantilever. After a
period of contact, the cantilever is retracted from the surface, but the tip remains in contact until
the deflection reaches the total adhesive force of the interaction between the tip and sample. When
this point is reached, the tip snaps out of contact with the surface and this corresponds to the ‘pull-
off’ or unbinding force. Before the unbinding of proteins, there may be a period of ‘elongation’
corresponding to the peeling or lifting of the protein from the surface. This distance extension may
be longer if the protein is attached to a flexible linker.

Specificity of the interaction measured must be proved to ensure the unbinding forces are due to
the proteins of interest and not due to non-specific interactions. This may be one by the addition of
free protein to block binding sites, or by comparison to control proteins captured by the same
means.

Although it is possible to measure the affinity between interacting proteins using a number of
methods such as an ELISA, this is not ideal, as it only allows an average of thousands of interaction
and is devoid of spatial knowledge (Schots, Van der Leede et al. 1988). Single molecule AFM
techniques allow the measurement of the force required to separate a single protein pair using statistical analysis; by decreasing the concentration of protein on the AFM surfaces, the frequency of unbinding events will also decrease, therefore the probability that the unbinding events were between single protein pairs is increased (Brogan, Shin et al. 2004).

**Dynamic Force Spectroscopy**

Measuring the force required to separate single proteins using AFM alone provides evidence for the adhesive strengths of the interactions. Further information can be gained by carrying out dynamic force spectroscopy (DFS), which explores the relationship between the unbinding of interactions and the rate at which it is separated (the loading rate). It has been proven therefore that as the loading rate applied to an interaction varies, so too does the force required for separation of the interaction (Merkel, Nassoy et al. 1999). By varying the loading rate applied to the interaction, kinetic information and parameters can be ascertained which relate to the nature of the interaction.

**1.7.4 Single cell force spectroscopy**

A relatively new AFM application is single cell force spectroscopy (SCFS), which measures the adhesion between a single pair of living cells (Evans, Ritchie et al. 1995). Although it may be advantageous to study the isolated interaction between two proteins, the benefit of measuring adhesion in a cellular *in vitro* system is that heterophilic and cytoskeletal interactions are present which may be similar to those which occur in vivo. Many SCFS experiments have been carried out where cell lines were transfected with the protein of interest and specificity was proven with the use of antibodies against it (Alsteens, Dupres et al. 2009; Bajpai, Feng et al. 2009). When studying an adhesive protein interaction, it is ideal to characterise adhesion on a single molecule and single cell level to elucidate its mechanism of action.

For single cell adhesion measurements on an AFM instrument, the setup is similar to that of a single molecule AFM experiment. Two AFM surfaces, the cantilever and a tissue culture plastic dish must be ‘functionalised’ with living cells. The surfaces must then be brought into apposition, allowed to interact for a period of time and separated. One marked difference between SCFS and SMFS is that the piezoelectric device controlled cantilever movement must exhibit increased vertical range, as the separation of cells has been found to occur over up to 100 μm.

Another difference in an SCFS experiment is that the cantilever, to which the cell is attached, is tipless to prevent damage and to increase the contact area between the tip and cell. The functionalisation of a cantilever with a cell is possible due to optical imaging, which may not be present using SMFS. Live imaging of cells is useful because positioning onto the tip is easier as this is
done in situ (Vesenka, Mosher et al. 1995). Probe cell attachment can be visualised due to the use of silicon cantilevers which are transparent.

The interaction between the cell and tip must be more adhesive than cell-cell adhesion, otherwise the point of rupture during the experiment will be the peeling of the cell from the cantilever. A method of cantilever-cell functionalisation must therefore be optimised for each cell line used to determine a strong adhesion. A number of methods have been carried out including coating of the cantilever with concavalin A, CellTak, poly-l-lysine and fibronectin. In tandem a plastic tissue culture dish is used to grow adherent cells for AFM measurements. The adhesion between a ‘probe’ cell and an adherent cell can therefore be measured in this way.

Following attachment of the cell to the cantilever, force-distance curves can be taken between the ‘probe’ cell and cells on the plastic substrate. A number of SCFS AFM instruments have heated chambers which allow measurements to be taken in physiological conditions (i.e. the JPK Instruments CellHesion® 200). Contact is maintained between cells for set time periods and in a constant-height mode. This involves a feedback mechanism which ensures that cantilever height is maintained by altering the deflection of the tip. During the contact period, the loading force on the adherent cell is not constant but is varied around a setpoint force. A number of setpoint forces have been investigated but this must be optimised for each cell line to ensure cell viability is maintained.

The resulting force-distance curves from cell-cell interactions are quantifiably different from those between individual proteins on an SFMS AFM instrument. This is due to the difference in the interactions which are occurring. Cell-cell interactions are far more complex than the interaction between single proteins, where only one discrete rupture event may occur between them. Cell-cell contact times may vary from a few seconds to 30 min, which is of great use to study an in vitro model using biologically relevant contact times. However longer cell-cell contact times allow increased opportunity for protein-protein binding at the interface, therefore the unbinding of these interfaces becomes more complex.

SCFS has been used to study the avidity of integrin to collagen under varying, physiologically relevant conditions (Tulla, Helenius et al. 2008). Addition of 12-O-tetradecanoylphorbol-13-acetate (TPA) to α2β1 expressing Chinese hamster ovary (CHO) cells increased their adhesion as measured by SCFS. The individual unbinding events occurring during the separation of the cells were also found to be higher when treated with TPA. The authors were able to provide direct proof that TPA caused stimulation of integrin clustering and strengthens adhesion.
1.8 Force measurements involving NCAM

1.8.1 Quantifying NCAM±VASE protein adhesion

As a cell adhesion molecule, NCAM functions as a homophilic binding protein. A number of experiments have been carried out to determine the adhesion between single NCAM proteins. However, to date, there is no published data investigating the effect of VASE insertion on homophilic binding strength.

![Figure 1.13: Schematic to show the single molecule force measurements taken between NCAM proteins which had been affinity captured (via their C-terminal His tags) onto nitroloacetic acid terminated PEG linkers on the cantilever and AFM surface.](image)

This figure is a modified version of one taken from the paper of Wieland et al (Wieland, Gewirth et al. 2005).

The adhesion between NCAM extracellular domains has been measured by determining the force required to separate proteins after they have been allowed to interact using atomic force microscopy (AFM) (Wieland, Gewirth et al. 2005). Briefly a micro-cantilever, which behaves like a flexible spring, was covalently functionalised with a PEGylated linker, which was terminated with an amine-reactive group to couple to primary amine groups within NCAM (Fig. 1.13). A glass microscope was functionalised with NCAM in an analogous fashion.

The functionalised NCAM cantilever was then brought into apposition with the functionalised NCAM coverslip under a controlled velocity and after a period of interaction the cantilever was retracted. Unbinding events, visualised as negative cantilever deflections, were pooled, binned and their frequencies were analysed. The frequency of force-distance curves which contained an unbinding event was less than 25%, which indicates that there was an 80% probability that unbinding events were due to the rupture of two single proteins (Brogan, Shin et al. 2004). Histograms of the rupture force following the interaction of full length NCAM molecules revealed a bimodal distribution which supports the occurrence of two independent protein unbinding events. With increasing loading rates
over an order of magnitude, peak 1 increased in size from 41±6 to 70±6 pN whereas peak 2 increased from 55±11 to 106±15 pN (Table 1.6).

Table 1.6: The adhesion between full length NCAM proteins was determined by single molecule force spectroscopy. The loading rate (calculated from the slope before the rupture event and the velocity of cantilever retraction) was compared with the frequency of force curves containing an unbinding event and the most probable unbinding force. Data were taken from Wieland et al. figure 7 (Wieland, Gewirth et al. 2005).

<table>
<thead>
<tr>
<th>Loading rate (pN/s)</th>
<th>Binding Frequency (%)</th>
<th>Modal Unbinding Force (pN)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peak 1</td>
<td>Peak 2</td>
</tr>
<tr>
<td>781</td>
<td>19.4</td>
<td>41±6</td>
</tr>
<tr>
<td>1152</td>
<td>24.2</td>
<td>49±8</td>
</tr>
<tr>
<td>7423</td>
<td>6.9</td>
<td>70±6</td>
</tr>
</tbody>
</table>

To further understand what interactions were occurring during the ruptures corresponding to peaks 1 and 2, the authors measured the force of unbinding between deletion NCAM products lacking Ig III (NCAM ΔIg III). The binding frequency of rupture events between NCAM ΔIg III proteins was less than 15% for loading rates used over an order of magnitude; therefore the probability of the unbinding events being between single interaction proteins was high (Table 1.7). Frequency distribution plots for the binned unbinding rupture forces revealed a unimodal normal data distribution, indicating that only one specific unbinding event occurred during NCAM ΔIg III rupture events. Interestingly the sizes of modal unbinding forces, for mutant NCAM ΔIg III, were similar to those between the non-mutant NCAM proteins (any discrepancy was accounted for by slight differences in the loading rate). This information revealed that Ig III was required for one particular interaction during NCAM homophilic binding. The interaction involving Ig III yields was found to be more adhesive than the interaction with Ig III. Since the adhesion between NCAM ΔIg III proteins was not significantly lower than between NCAM proteins, the NCAM-NCAM interaction resulting in rupture ‘peak 1’ was independent of Ig III.

Table 1.7: The adhesion between NCAM Δ Ig III proteins was determined by single molecule force spectroscopy. The loading rate (calculated from the slope before the rupture event and the velocity of cantilever retraction) was compared with the frequency of force curves containing an unbinding event and the most probable unbinding force. Data were taken from Wieland et al. figure 7 (Wieland, Gewirth et al. 2005).

<table>
<thead>
<tr>
<th>Loading rate (pN/s)</th>
<th>Binding Frequency (%)</th>
<th>Modal Unbinding Force (pN)</th>
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<tbody>
<tr>
<td></td>
<td>Peak 1</td>
<td>Peak 2</td>
</tr>
<tr>
<td>414</td>
<td>12.4</td>
<td>26.5±7.5</td>
</tr>
<tr>
<td>1042</td>
<td>14.5</td>
<td>50±19</td>
</tr>
<tr>
<td>9810</td>
<td>3.3</td>
<td>92±18</td>
</tr>
</tbody>
</table>
It is interesting to note that as the loading rates increased during the NCAM Δlg III measurements, there were increased standard deviations in the most probable binding force. To circumvent this problem and to further test the data, an AFM experiment was carried out to measure the adhesive force between full length NCAM proteins and NCAM Δlg III only (Table 1.8). Due to the presence of the full length NCAM on one surface, error was significantly reduced, and data was in agreement with the most probable binding forces for NCAM Δlg III adhesion measurements at the corresponding loading rates (there is a linear relationship between the logarithm of the loading rate and the unbinding force size).

Table 1.8: The adhesion between NCAM Δ lgIII and full length NCAM was determined by single molecule force spectroscopy.
The loading rate (calculated from the slope before the rupture event and the velocity of cantilever retraction) was compared with the frequency of force curves containing an unbinding event and the most probable unbinding force. Data were taken from Wieland et al. figure 7 (Wieland, Gewirth et al. 2005).

<table>
<thead>
<tr>
<th>Loading rate (pN/s)</th>
<th>Binding Frequency (%)</th>
<th>Modal Unbinding Force (pN)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Peak 1</td>
</tr>
<tr>
<td>450</td>
<td>6.5</td>
<td>37±13</td>
</tr>
<tr>
<td>1015</td>
<td>3.2</td>
<td>42±10</td>
</tr>
<tr>
<td>2815</td>
<td>3.1</td>
<td>57±12</td>
</tr>
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</table>

The higher unbinding force, ‘peak 2’, during the interaction between full length NCAM molecules was only seen in the presence of NCAM lg III; therefore the weaker ‘peak 1’ rupture event was thought to correspond to the rupture of a trans interaction between lg I+II domains. To test this, the authors measured the adhesion between full length NCAM and NCAMΔ lg I+II (Table 1.9). If one of the interacting NCAM lacked lg I+II there was a single interaction between the proteins which at all loading rates was similar to ‘peak 2’ (Table 1.6). This indicated that lg III seemed to be responsible for the higher unbinding force peak.

Table 1.9: The adhesion between NCAM Δ lgI+II and full length NCAM was determined by single molecule force spectroscopy.
The loading rate (calculated from the slope before the rupture event and the velocity of cantilever retraction) was compared with the frequency of force curves containing an unbinding event and the most probable unbinding force. Data were taken from Wieland et al. figure 7 (Wieland, Gewirth et al. 2005).

<table>
<thead>
<tr>
<th>Loading rate (pN/s)</th>
<th>Binding Frequency (%)</th>
<th>Modal Unbinding Force (pN)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Peak 1</td>
</tr>
<tr>
<td>252</td>
<td>11.5</td>
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</tr>
<tr>
<td>1547</td>
<td>8.5</td>
<td>-</td>
</tr>
<tr>
<td>7285</td>
<td>7.2</td>
<td>-</td>
</tr>
</tbody>
</table>
The data from this paper had indicated that NCAM homophilic binding on a single molecule level can involve two independent interactions, one of which is independent of NCAM Ig III and required less force to separate. These data also corroborate findings that NCAM proteins bind and overlap in two independent interactions, corresponding to a full anti-parallel overlap of all 5 Ig-like domains and a partial overlap between Ig I+II (Johnson, Fujimoto et al. 2004). The higher adhesion interaction between NCAM proteins seems to involve an isologous interaction between Ig III which may cause a full partial overlap of Ig domains. The weaker adhesion interaction between NCAM proteins seems to involve NCAM Ig I+II only. This seems to contrast data which suggested that NCAM homophilic binding occurs via a ‘zipper’ mechanism involving Ig I-Ig III and Ig II-Ig III (Soroka, Kolkova et al. 2003). However it is unknown whether interactions between cis proteins occurred due to the method of surface functionalisation.

The interaction and force required to separate interacting Ig I+II domains has been calculated using MD simulations using structural data from the NCAM Ig I-III crystal structure (Maruthamuthu, Schulten et al. 2009). At a loading rate of approximately 700 pN/s, the force required to separate two tethered Ig I+II fragments was estimated to be 40 pN. This compares with the data measuring the force of NCAM ΔIg III proteins using AFM where, between approximately 400 and 1000 pN/s, the most probable unbinding force increased from 26.5±7.5 to 50±19 pN (Wieland, Gewirth et al. 2005). Therefore it is plausible that the NCAM interaction measured by Wieland et al. in the absence of Ig III was between two NCAM Ig I-II domains.

1.8.2 Quantifying NCAM±VASE cellular adhesion

A number of experiments have been carried out to measure the average adhesion between cells expressing NCAM (See section 1.3.3). However relatively little work has been done to quantify the adhesion between NCAM expressing cells on a single molecule level. An experiment has been carried out to determine the force between interacting NCAM expressing cells using a centrifugation aggregation assay (Woo and Murray 1993). Briefly Lmtk’ fibroblast cells were transfected with NCAM-140 and selected for high expression. A monolayer of cells was grown on a centrifugal surface plate and freshly triturated cells were allowed to associate before centrifugation began. The centrifugal force required to remove NCAM expressing vs. Non-expressing cells was then calculated. The insertion of NCAM into the cells had no effect on their adhesion, both were dislodged from monolayers with a removal force of 5x10⁻⁶ dyn/cell, which is 50 pN. Cells were left to adhere for only 15 min before centrifugation, which may explain why the force per cell separated equates only to a low adhesion interaction between a single pair of proteins (Zlatanova, Lindsay et al. 2000).
In the same way, the force required to separate NCAM transfected and untransfected L cells from a retinal ganglion cell monolayer was indistinguishable at 500 pN per cell. Abolition of L cell binding to retinal ganglion cells was not prevented by addition of NCAM antibodies. The authors conclude that NCAM homophilic binding is weak, and after low contact times has no effect on cell adhesion.

Further aggregation assays have been carried out on NIH 3T3 cells transfected with NCAM (Pizzey, Rowett et al. 1989). Using a shear force aggregation assay, no difference in cell aggregation could be seen between parental NIH 3T3 and NCAM expressing cells. The authors measured the aggregation of cells following 30 min of incubation with no shear force, which revealed that NCAM expressing cells exhibited increased aggregation compared with parental NIH 3T3 cells. It is possible that the forces exerted during shear force aggregation assays are not conducive to NCAM-mediated adhesion or are representative of physiological conditions in any way.

Following these findings there is a need for an in vitro model to measure the adhesion between stationary cells expressing NCAM isoforms.
1.9 Project Aims

The aim of this project was to investigate and characterise cell adhesion mediated by NCAM and NCAM-VASE. To do this, a number of in vitro models were developed to measure the effect of two isoforms of NCAM on their ability to mediate adhesion. This was carried out as follows:

- Validation of the use of atomic force microscopy to measure cellular and molecular adhesion
- Development of an in vitro model to measure the adhesion between NCAM±VASE proteins using single molecule force spectroscopy
- Proving the specificity of NCAM homophilic binding interactions using single molecule force spectroscopy
- Validation of the use of AFM for single cell adhesion measurements
- Development of an in vitro model to measure the adhesion between cells expressing NCAM±VASE using single cell force spectroscopy
- Proving the specificity of NCAM homophilic binding interactions using single molecule force spectroscopy

This project will aim to elucidate the mechanism of action of the insertion of the VASE sequence into NCAM using techniques from the fields of Biochemistry, Biological Chemistry and Biophysics.
Chapter 2 Quantification of the adhesion between single molecule NCAM±VASE proteins using Atomic Force Microscopy

Atomic force microscopy (AFM) has been used extensively to measure the adhesion between single molecule proteins. The ‘force spectroscopy’ application on an AFM was therefore chosen to determine the adhesive strength between single molecule NCAM±VASE proteins. The work described in this chapter includes:

- A description of the use of force spectroscopy on an AFM to measure adhesion
- The calibration of an AFM
- Proof of principle AFM measurements between biotin and avidin
- Elimination of large, non-specific interactions during AFM
- Development of an AFM surface functionalisation method for NCAM±VASE deposition
- Measurements of adhesion between adsorbed NCAM±VASE by AFM
- Single molecule measurements of adhesion between adsorbed NCAM±VASE by AFM
- Dynamic force spectroscopy analysis of NCAM±VASE homophilic and heterophilic adhesion
- Development of a covalent AFM surface functionalisation method for NCAM±VASE
2.1 Single molecule adhesion measurements using AFM

The function of the neural cell adhesion molecule (NCAM) as a homophilic binding protein is of great biological importance, but little research has been carried out to quantify the adhesion between its isoforms. One isoform of NCAM, the variable alternatively spliced exon NCAM-VASE, converts NCAM from a promoter to an inhibitor of neurite outgrowth and is expressed in areas of low synaptic plasticity in the nervous system. It has been postulated that insertion of the VASE sequence into NCAM alters its homophilic binding strength. Plausibly, the insertion of VASE into NCAM may increase the homophilic binding strength of NCAM and may decrease growth cone and neurite migration because they are bound too tightly to their substratum. Alternatively a decrease in NCAM homophilic binding due to the insertion of VASE might inhibit neurite outgrowth due to a loss of the neurite outgrowth promoting ability of NCAM. Therefore the aim of the work presented in this chapter was to quantify and compare the adhesion of NCAM±VASE proteins.

Protein adhesion strength is normally measured by determining affinity and rate constants using techniques such as surface plasmon resonance (SPR), ELISA, or aggregation assays using cells or protein-containing lipid vesicles. However, these methodologies provide only an average value from a population of molecular interactions and no information regarding their spatial positioning or the nature of their interactions. Techniques measuring multiple single molecule interactions such as surface force apparatus (SFA) and AFM collect information regarding nanoscale contacts and picoNewton adhesion forces from thousands of individual interactions, which can be averaged by the investigator. Combining this information for NCAM±VASE adhesion will be of great use as it may indicate whether different domains are involved in NCAM or NCAM-VASE homophilic binding.

Single molecule AFM also provides greater control over the functionalisation of experimental surfaces and therefore over the type of interaction which occurs. This is of great importance with NCAM proteins as they are homophilic binding proteins and may cluster in solution. In SPR, covalent coupling of NCAM to the sensor chip would result in unknown interacting complexes with potentially varying oligomeric states.

Single molecule techniques require nanomolar quantities of proteins unlike those with averaging techniques, which depend on using protein above the expected binding affinity. For the low affinity NCAM homophilic interaction, this would require producing millimolar quantities, which is prohibitively time consuming and expensive.

AFM has been used since the 1980s to image surfaces and measure interactions, but in recent years a force spectroscopy application has been developed to measure adhesion between interacting
surfaces. This application has proved useful for measuring the adhesion forces of many protein-protein interactions (Zlatanova, Lindsay et al.). In protein force spectroscopy the cantilever and either a glass coverslip or mica surface are functionalised, brought into apposition and then separated. The force required to separate the surfaces is measured and depends on the adhesion between the interacting proteins. The measurements are taken from the deflection of a cantilever, determined by the deflection of a laser beam positioned on its tip, which is reflected onto a photosensitive diode (Fig. 1.11). By careful calibration and knowledge of the cantilever spring constant, cantilever deflection is measured in force units and is used to describe the resulting adhesion between the proteins.

2.2 AFM calibration
Before adhesion measurements between NCAM±VASE functionalised surfaces were taken using AFM, calibration of the machine and cantilever was required. This involved a number of steps to convert cantilever deflection from units of distance to force. The following steps were taken prior to AFM measurements:

2. Determination of the cantilever deflection sensitivity
3. Determination of the cantilever spring constant
4. Assessment of the reliability of the thermal tune method of spring constant assignment

2.2.1 Cantilever deflection sensitivity was determined from contact between the cantilever and substrate in force spectroscopy mode
Force spectroscopy can be used to measure picoNewton forces of adhesion between proteins with high resolution. A number of specialised commercially available AFM controllers are available for this process including the Veeco Nanoscope IV picoforce controller, which was used exclusively for protein adhesion measurement work. High resolution AFM force spectroscopy is only achieved following careful calibration before each experiment. Cantilever movement should only occur in the vertical or Z-plane (although both horizontal and vertical deflection are measured) and is controlled by a voltage applied to the piezoelectric scanner. Force spectroscopy requires knowledge of the cantilever deflection and cantilever displacement via the voltage applied to the piezo (Fig. 1.12). These factors are calibrated before each experiment as they are likely to vary between experiments. Therefore to calibrate the instrument, a series of experimental curves constituting the extension and retraction of the cantilever (in the Z-axis) to and from the AFM surface were acquired.
Figure 2.1: Calibration of an atomic force microscope (AFM) for force spectroscopy was carried out using force curves before the experiment.

The cantilever was brought into contact with the glass coverslip and its deflection, in nm, was compared to its position, in μm, and voltage applied to its piezo control. Cantilever extend and retract data were acquired at 22°C in fluid, with velocities at 498 nm/s and a voltage setpoint of 2V. a) A force plot shows one force cycle (the cantilever extension and retraction) relating the position of the cantilever to the surface as driven by a piezo applied voltage (Z-piezo sensor, µm) against time (s). Between 0–2 s, the cantilever approaches the surface, at 2 s, it makes contact and is raised from 2–4s. b) A force plot relating the position of the Z-piezo and the time (s). The cantilever is moved towards the AFM surface between 0 and 2 s then retracted between 2 and 4 s. c) Overlaid force plots showing cantilever extension and retraction of the deflection of the cantilever (nm, y-axis) against the Z-position (µm, x-axis). The slope of the curve in the contact region of the force curve gives the sensitivity determinant which relates cantilever deflection. Determination of the sensitivity determinant value converted Voltage applied to the piezo to drive the cantilever into a nm deflection distance.
During force spectroscopy calibration, the cantilever position was recorded on the force curves and calibrated as a function of time, resulting in a time-deflection curve (Fig. 2. 1 a). The cantilever tip position in relation to the voltage applied to the piezo was ascertained from a Volt-distance force curve and compared with cantilever deflection in nanometers which was measured from the piezo extension curve (Fig. 2. 1 b).

These curves were taken between a clean cantilever and glass coverslip with a contact time of 0 s (there was no delay between extension and retraction of the cantilever), a scan rate of 0.249 Hz, 1024 data points resulting in cantilever extend and retract velocities of 498 nm/s. The setpoint contact voltage was 2 V therefore the voltage to the cantilever and hence cantilever deflection desisted when 2V was reached, to maintain contact with the coverslip.

The resulting spectroscopic data were supplied in three channels; a cantilever deflection vs. time curve (Fig. 2. 1a); a cantilever Z-position sensor vs. time curve (Fig. 2. 1b) and cantilever deflection vs. Z-position sensor (Fig. 2. 1c). This information revealed that between 0 and 1.9 s, the cantilever moved towards the surface and made contact before maximal deflection (2 V, 145 nm) was reached at 2 s. The cantilever was immediately retracted and zero deflection was reached at 2.1 s followed by an unbinding event (negative deflection), complete retraction from the surface.

To determine the cantilever deflection, these curves were used to convert the reading from volts to nanometers. To do this, a deflection sensitivity value was calculated from the gradient of the slope during contact between the cantilever and coverslip (Fig. 2. 1c). Typically, on this instrument, when using a Si₃N₄ cantilever and glass coverslip, the sensitivity determinant was routinely between 70 and 100 nm/V. This converted the deflection displayed on the photosensitive diode from units of voltage to a nanometre distance. Determining this sensitivity parameter was the first step in converting cantilever deflection from volts to Newtons.

These data were the first example of force spectroscopy carried out on this AFM instrument.

2.2.2 The cantilever spring constant was determined by the thermal tune method and converted cantilever deflection into force units

Before adhesion measurements were taken, and once cantilever deflection had been converted from Volts to distance, it was necessary to convert the deflection into a force (N). The force required to deflect a cantilever depends on its spring constant (N/m). The spring constant details the force required to move the cantilever by a certain distance; stiffer cantilevers have larger spring constants than more flexible ones because they require a greater force to deflect them by an arbitrary distance. For picoNewton force sensitivity, which is required to measure protein-protein
interactions, cantilevers with spring constants in the order of 0.01-0.1 N/m are generally used (Liu
and Parpura 2009). The DNP-10 Si$_3$N$_4$ pyramidal tipped cantilever (Veeco) was used for all AFM
adhesion measurements using the tip with a nominal spring constant of 0.06 N/m.

![Graph](image)

**Figure 2.2: Cantilever spring constant determination allowed calculation of the force required to deflect a cantilever by
an arbitrary distance.**

To determine the force required to separate interacting surfaces during AFM, the force required to deflect the cantilever
by an arbitrary distance is calculated by determining the spring constant of the cantilever. Spring constant assignment is
calculated using the inbuilt thermal tune method in Veeco Nanoscope software prior to an AFM experiment. The spring
constant of the cantilever is determined when held 100 μm above the substrate surface, in liquid, following thermal
equilibrium at 23 °C, under no driving oscillation. The thermal fluctuations of the cantilever are measured and these
frequencies are recorded (x-axis, kHz) against their frequency (y-axis) to determine the most common resonant frequency.
The distribution of frequencies is fitted with a Lorentzian fit to yield its spring constant. Spring constants are taken from the
mean of three readings. The spring constant of the cantilever is reassigned whenever the cantilever is realigned. The
thermal tune method allowed conversion of cantilever deflection from units of distance to units of force. This allowed
an unbinding force (pN) to be assigned to unbinding events between the cantilever and substrate.

The spring constant was determined before each set of experimental data was collected, using the
inbuilt thermal tune method in Veeco Nanoscope software. Cantilever deflection data were
measured over a time interval at thermal equilibrium under no driving oscillation in fluid at 22°C
above the surface of the coverslip. The resulting information was Fourier transformed to obtain a
power spectral density (PSD, y-axis) vs. frequency of fluctuation (x-axis) (Fig. 2. 2). A Lorentzian fit
was applied and the area under the curve was integrated to measure the cantilever thermal
fluctuations. The resulting spring constant was determined three times and the mean was applied to
the following data set. In this case it was 0.0539 N/m and within 10% of the manufacturer’s
estimates (0.06 N/m). This indicated that 539 pN was required to deflect the cantilever by 10 nm and
allows future experimental data to be measured in force-distance curves.
2.2.3 The thermal tune method of spring constant determination was reproducible

It is important that the thermal tune method of spring constant assignment is reliable. A small error in measuring the spring constant would result in a large error in the adhesion forces measured between proteins. It was therefore necessary to test the reproducibility of this method. To do this the four cantilever tips on the DNP-10 cantilever were assigned with spring constants and resonant frequencies using this method and a mean was calculated from three values taken in succession and compared with those in three independent experiments. These mean values were compared to the manufacturer’s estimates (Fig. 2.3).

<table>
<thead>
<tr>
<th>MANUFACTURER’S</th>
<th>CALCULATED</th>
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<tbody>
<tr>
<td>Length (µm)</td>
<td>Width (µm)</td>
</tr>
<tr>
<td>A</td>
<td>200-210</td>
</tr>
<tr>
<td>B</td>
<td>115-120</td>
</tr>
<tr>
<td>C</td>
<td>115-125</td>
</tr>
<tr>
<td>D</td>
<td>200-210</td>
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Figure 2.3: The reproducibility of the thermal tune method for spring constant determination was tested by assigning all DNP-10 cantilevers tips in three independent experiments.

The reliability of the thermal tune method was assessed by calculating the spring constant values of all four cantilevers, encompassing an order of magnitude of values. Cantilever spring constants were assigned using the inbuilt thermal tune method in Veeco Nanoscope software. Spring constants and the cantilever resonant frequencies were calculated in three independent experiments using the same unfunctionalised cantilevers each time. The readings were taken in fluid at 22°C above the surface of the AFM chamber and under no driving oscillation. Values were compared to the manufacturer’s estimates. The thermal tune method of spring constant determination was reliable.

For biological force spectroscopy measurements cantilevers have spring constants in the region of 0.01–0.1 N/m. The longer cantilever tips ‘A’ and ‘D’ were assigned with spring constants of 0.0546±0.007 and 0.0911± 0.001 N/m respectively and were therefore suitable for this application. These spring constants were in good agreement with the manufacturer’s estimates.

The resonant frequencies for all cantilever tips were lower than the manufacturer’s estimates which were carried out in air. This may be due to the fact that this data acquisition was carried out in fluid and therefore thermal fluctuations differed.

For all tip assignments, the standard error between independent experiments was within approximately 10%. The thermal tune method was therefore suitable for determining spring constants.
2.3 Following AFM calibration, force curves revealed quantifiable data points relating to the interaction which has occurred

Once the AFM had been successfully calibrated and the sensitivity determinant and spring constant were assigned, force-distance curves were collected to measure the interaction occurring between the cantilever and coverslip. To highlight the information gained from force-distance curves, a number were collected for the interaction between a Piranha-cleaned (3:1 H₂SO₄: 30% H₂O₂, 10 min at RT) cantilever and glass coverslip in a closed AFM chamber containing DMEM HEPES at 22°C. It was important that the AFM surfaces were cleaned to remove any residue before data acquisition or surface functionalisation. The data were collected following a closed loop contact setpoint of 0.6 nN, 10 ms of contact time and with extend and retract cantilever velocities of 498 nm/s (Fig. 2.5).

The example force curves shown display the retraction of the cantilever away from the surface only, as these will show the de-adhesion events following contact. The deflection of the cantilever was measured in pN (y-axis) against its Z-axis position (x-axis) following 10 ms of contact time and 498 nm/s extension and retraction cantilever velocities. The first force curve shows that no adhesive interaction has occurred between the cantilever and coverslip as no de-adhesion event was seen (Fig. 2.5a) This was due to the cantilever deflection returning from 0.6 nN to 0 deflection, without any negative deflection (which would indicate a de-adhesion event between the tip and sample). It is worth noting that the noise in this experiment appears to be approximately 15 pN.

The second force curve (Fig.2.5b) revealed that an interaction occurred between a protein functionalised tip and sample (BSA-BSA) following 0.6 nN of contact force (Fig. 2.5b point 1). The cantilever was raised from the surface but the tip remained in contact as the deflection returns from 0.6 nN to 0 (Fig. 2.5b point 2). A de-adhesion event occurred, as shown by the negative cantilever deflection between 20 and 90 nm Z-distance height (Fig. 2.5b point 4). The length of the negative deflection is equal to the distance over which unpeeling or stretching of a protein from the surface occurs and the cantilever tip is being pulled down to maintain interaction between the tip and sample, while the back of the cantilever was being raised by the piezo.

Once a critical force was reached, the maximal deflection force was reached (Fig. 2.5b point 3), and an unbinding event occurs between the tip and sample (Fig. 2.5b between points 3 and 5). In this force curve the unbinding of the interaction occurs at 90 nm and corresponds to a de-adhesion event of 200 pN which returns the cantilever to zero deflection (Fig. 2.5b point 6). An adhesive interaction was also measured between a bare cantilever and bare coverslip (Fig. 2.5c).
Figure 2.4: Force spectroscopy curves between a bare cantilever and glass coverslip highlighted measureable parameters by AFM.

AFM force spectroscopy was used to measure the interaction between a bare or protein functionalised cantilever and coverslip in DMEM HEPES at 22°C. The data were collected following a contact setpoint of 0.6 nN and 10 ms of contact time, with 498 nm/s extend and retract cantilever velocities. a) An example retract curve measuring the interaction between a bare cantilever and coverslip, with no interaction. b) An example retract curve where an interaction has been recorded between a protein functionalised cantilever tip and coverslip. This reveals the following quantifiable points: 1. Setpoint contact force (pN). 2. Sensitivity slope (nm/pN). 3. Unbinding event position (nm). 4. Separation distance over pulling regime (nm). 5. Unbinding Force (pN). 6. Non-contact region. c) A retract force curve revealing an adhesive interaction between a bare cantilever and coverslip.
The gradient of the slope from the raising of the cantilever to the point of loss of contact between the tip and sample (0–35 nm) was constant indicating that once a threshold cantilever deflection, and hence a force, was reached, the cantilever snaps out of contact with the sample and zero deflection is reached.

These force curves have revealed that interactions between a cantilever and coverslip require picoNewton forces to break and that such forces can be measured by force spectroscopy. A minority of measurements between these surfaces did not yield an unbinding event (See Fig. 2.4a), indicating that a negative deflection, following a return to zero deflection, is not the default response following contact between a cantilever and coverslip. For protein-protein interactions, the distance over the pulling regime, Z-position of the unbinding event, and its size, will provide valuable information concerning the nature of the interaction.

2.4 Proof of principle adhesion measurements between biotin and avidin by AFM
2.4.1 The adhesion between adsorbed biotin and avidin was measured by AFM as a proof of principle experiment

Following calibration of the AFM instrument and acquisition of example force curves, the AFM instrument was ready to use for protein adhesion measurements. Before quantifying an unknown interaction such as NCAM±VASE, a well-studied AFM interaction was chosen for preliminary measurements and validation. The resultant adhesion force could be compared to those in the literature to assess reliability of the instrument and to optimise the experimental technique. The interaction between biotin and avidin fulfils this remit and is well studied using force spectroscopy on the AFM and the reagents are readily available. The adhesion between biotin and avidin has one of the highest affinities measured and therefore large forces (167±20–442±17 pN for unloading rates of 39000–628000 pN/s) are required to separate them (Moy, Florin et al. 1994; Lo, Huefner et al. 1999; Lo, Zhu et al. 2001).

To determine the force of adhesion between biotin and avidin by AFM, the method of measurement was taken directly from the literature (Lo, Huefner et al. 1999). In this paper, the authors described the adsorption of biotinylated bovine serum albumin (biotinBSA) to provide a biotin surface for AFM measurements. Biotinylation was carried out by the reaction of BSA with biotin O-succinimide (biotin O-Su). Avidin was affinity captured on the apposing AFM surface, which had been pre-functionalised with biotinBSA. In this experiment the interaction between a biotinBSA surface and an avidin (captured by biotin BSA) was measured.
Figure 2.5: Schematic to show the proposed AFM experiment to measure the interaction between adsorbed biotin and avidin by AFM.

The interaction between biotin and avidin is measured by adsorbing biotinylated BSA (BiotinBSA) onto a cantilever or glass coverslip and capturing avidin on one side only. BSA can be biotinylated by reaction with biotin O-succinimide (biotin O-Su). The following measurements were taken to understand all possible interactions which might occur during the experiment: a) between biotinylated BSA (biotinBSA) and glass, b) biotinBSA and BSA, c) biotinBSA and biotinBSA, d) biotinBSA and avidin incubated via biotinBSA, e) avidin flooded biotinBSA: biotinBSA.

The work described by Lox et al did not mention any control AFM experiments. Here the following control experiments were conducted to measure all plausible interactions by AFM. These included those between avidin, biotinBSA, BSA and the glass coverslip (Fig. 2.5):

- biotinBSA and glass to determine if biotin BSA adsorbs and desorbs to and from the coverslip under the experimental conditions
- biotinBSA and BSA to see if biotin interacts with unreacted BSA
- biotinBSA and biotinBSA to determine whether biotin interacts homophilically
- biotin-avidin (via biotinBSA and biotinBSA captured avidin) to compare to the literature adhesion values
- avidin-avidin (avidin flooded biotin BSA on both the cantilever and coverslip) to see if interactions between biotin and avidin are blocked

These controls would enable us to determine whether the interactions measured were specifically between biotin and avidin or non-specific interactions.
2.4.2. BSA was biotinylated with biotin O-succinimide in preparation for measuring the biotin-avidin interaction by AFM

To provide a biotinylated surface for the measurement of biotin and avidin adhesion, BSA was biotinylated by attachment of biotin to primary amine groups using a 1:100 molar ratio of BSA: biotin O-Su at RT for 1 hr in PBS/10% DMSO. Following the reaction, unreacted biotin O-Su and DMSO were removed by centrifugation with a molecular weight cut off (MWCO) of 5 kDa and dialysed against PBS.

Biotinylation of BSA was confirmed by SDS-PAGE and Western blotting using a high affinity Neutravidin HRP conjugated antibody (Fig. 2.6). 20 μl of 1 mg/ml BSA, or BSA which had been biotinylated, was loaded in duplicate onto two non-reducing SDS gels along with biotinylated standards and a protein ladder for size comparison. Coomassie dye staining was carried out to highlight the total protein and revealed that equal amounts of BSA and biotinylated BSA product were loaded. The second gel was transferred onto nitrocellulose, blocked in PBS/10% milk and blotted with Neutravidin HRP (1:1000 in PBS). The antibody specifically bound to the biotinylated protein standards (Fig. 2.6 lane 2) and biotinBSA (Fig. 2.6. lane 4) and showed that BSA has been successfully biotinylated by the addition of biotin O-Su.

BSA was present under non-reducing conditions in various oligomeric states including a 60 kDa monomer (the majority), dimeric BSA at 120 kDa as well as other higher order oligomers. This indicated that BSA adheres homophilically, which may introduce problems in the AFM experiment when measuring the interaction between biotin and avidin.

To elucidate the extent of biotinylation of BSA, the number of moles of biotin per mole of BSA was estimated using a ‘biotin quantitation kit’ (Piercenet). The assay involved the addition of the sample to be tested to an aliquot of avidin prebound with 4’-hydroxyazobenzene-2-carboxylic acid (HABA). HABA binds in the same avidin binding pockets as biotin. The HABA-avidin complex absorbs at 500 nm and upon addition of biotin was outcompeted from the avidin binding sites due to biotin’s higher affinity for avidin. When this occurred, absorbance at 500 nm decreased, which correlated linearly with the amount of biotin in the sample. The decrease in absorbance of biotin BSA was compared to that upon addition of a biotinylated positive control sample and a negative control (Fig. 2.7).
Figure 2.6: Biotinylation of BSA was confirmed by Western blotting with a Neutravidin HRP.
The biotinylation of BSA was carried out by the addition of biotin O-Su in 10% DMSO/PBS to yield biotinBSA for later
measurement of biotin-avidin interaction. Biotinylation of BSA was assessed using Western blotting with a Neutravidin™
HRP antibody, which binds specifically to biotin. a) BSA, biotinBSA and standards were loaded in equal concentration onto
an SDS-PAGE non-reducing 12.5% stacking/5% resolving gel. The gel was run for 15 min at 80 V, and for 60 min at 120 V*. A
colloidal coomassie stain revealed BSA was present in equal amounts whether biotinylated or not and as a series of
oligomers. b) BSA, biotin BSA and standards were loaded in equal concentration onto nitrocellulose membrane for
Western blotting, run at 100 V for 90 min and blotted with Neutravidin™ HRP (1:1000)*. Lane 1 unbiotinylated standards
are visible due to overlay of the chemiluminescent image with one taken under white light. *Lane 1. Prestained protein
BSA. 4. Biotinylated BSA. Neutravidin HRP reacts specifically with biotinBSA and biotinylated standards only.

Addition of an unbiotinylated protein sample to the HABAg-avidin complex showed no decrease in
absorbance of the complex at 500 nm. Therefore no biotin was present to outcompete HABA from
the avidin binding sites as expected. Upon addition of the positive biotinylated control sample
absorbance decreased by 15%, which equated to 1.84 moles of biotin per mole of control protein.
Addition of biotinBSA to HABAg-avidin decreased the absorbance at 500 nm by 85% which equated
to 108 moles of biotin per mole of BSA and confirmed that BSA was extensively biotinylated in
preparation for the biotin-avidin adhesion measurements by AFM. BSA contains 60 lysine residues,
which can be biotinylated via their side chain primary amine. Therefore for every BSA molcule, 60
biotin molecules could be attached at pH 7.2. The discrepancy between the calculated amount
of biotinylation and the potential amount (108 vs 60 respectively) indicates that the assay provided
only an approximatex value.

As there were many molecules of biotin per molecule of BSA, biotin-avidin interactions by AFM may
be frequent. The likelihood of measuring single molecule unbinding events was therefore limited
unless low concentrations of biotinBSA were used. The method of analysis used in the literature
involved poisson statistics, which extract single molecule adhesion forces from those with multiple
unbinding events (Lo, Hufner et al. 1999). Biotinylation of BSA was confirmed, therefore the AFM experiment measuring the interaction between biotin and avidin was carried out (Fig. 2.5).

![Figure 2.7: The amount of biotinylation of BSA was quantified using the Biotin Quantitation kit (Piercenet). Biotinylation of BSA was compared to a negative control of unbiotinylated protein and a positive control of biotinylated protein, to determine the number of moles of biotin per mole of BSA. The biotinylation assay is competitive and compares the amount of absorbance at 500 nm due to a HABA-avidin₄ complex before and after the addition of protein sample. Addition of biotinylated protein sample to the HABA-avidin₄ complex displaces HABA from the complex and decreases absorbance at 500 nm because biotin has a greater affinity for avidin than HABA. The percentage inhibition of absorbance at 500 nm of the HABA-avidin₄ complex is therefore measured for each protein sample. This data consists of three independent experiments. Statistical analysis compares the significance of the decrease of absorbance at 500 nm after biotin BSA and control biotinylated protein addition to the unbiotinylated standard. * P<0.05 (statistically significant), *** P<0.0005 (extremely statistically significant). BSA was successfully biotinylated as confirmed by quantification using the competitive displacement assay with HABA-avidin₄.]

2.4.3 Avidin-biotin control AFM measurements reveal large non-specific interactions

Following the successful biotinylation of BSA, the adhesion between biotin and avidin was measured using AFM as described previously (Fig. 2.5). This included measuring the adhesion between an adsorbed layer of biotin BSA and avidin captured via biotin BSA as well as all control interactions. To achieve this the cantilevers and coverslips were Piranha cleaned, as before, and rinsed thoroughly. If required, 60 μl of 1 mg/ml of biotin BSA in DMEM HEPES was incubated on a coverslip or a cantilever was inserted into a 60 μl droplet for 1 hour at RT, in a humid chamber to prevent evaporation. The solution was aspirated and the surfaces were rinsed with DMEM HEPES. For surfaces that were treated with avidin, 100 μg/ml avidin in DMEM HEPES was incubated on pre-adsorbed biotinBSA surfaces and rinsed away after 1 hr. The glass coverslip and cantilever were loaded into the AFM chamber and contact was made between the tip and coverslip surface. Force curves with extend and retract velocities of 498 nm/s were acquired using a setpoint force of 3 nN to maintain contact and with interaction times varying between 0.05 and 5 s.
To assess the adhesive strength between biotin and avidin, the forces required to separate the interactions occurring between the tip and sample were assessed using the size (Fig. 2.4b point 5) and frequency of unbinding events on the retract force curves. The mean size of the unbinding events from all force curves was compared against the contact time for each interaction between biotinBSA, BSA, glass and avidin (Fig. 2.8a, see appendix for example force-distance curves). Specifically, only attractive interactions on retract force curves were pooled. If a force curve did not contain such an event, it was excluded from the data set. If an interaction was less than 20 pN, it was also excluded. Approximately 50 force-distance curves were collected for each interaction. Strikingly, the size of unbinding events at each contact time for all interactions studied were similar. This unexpected finding indicated that the specific interaction between biotin and avidin was difficult to differentiate from interactions with glass or biotinBSA.

![Graph](image)

**Figure 2.8: AFM experiment to measure the interaction between adsorbed biotin-avidin and a comparison of all potential interactions.**

The interaction between biotin-avidin was measured by AFM following the schematic presented in Fig. 2.5. The setpoint contact force was 3 nN and the cantilever forward and retract velocities were 498 μm/s. a) The mean unbinding forces for each interaction type were compared with increasing cantilever-substrate contact times between 0.05–5 s. b) Table to show the percentage of force curves with at least one unbinding event following a contact time of 0.05 s, during the interactions between all possible interactions. All experiments were carried out in DMEM/20 mM HEPES pH 7.4 at 22°C. Each data point is the mean of approximately 50 interactions from force curves. Large (>200 pN) unbinding events occur between biotinBSA and glass as well as all other interactions including biotin-avidin. Therefore unbinding events which occur following biotin-avidin interaction may not be specific.

After 0.05 s contact time between the tip and sample, there was no difference between the mean force required to separate biotinBSA from glass and biotin from avidin; both were approximately 180 pN. All other interactions at 0.05 s of contact time resulted in high unbinding forces (200-280 pN). Interestingly the unbinding events between biotinBSA and glass increased the least over increasing contact time (180–470 pN) of all of the interactions. This interaction was therefore qualitatively
different from the others and may be due to the de-adsorption of biotinBSA from the glass or cantilever.

As contact time increased, the size of the mean unbinding events increased dramatically for all other interactions. When biotinBSA protein was present on both the cantilever and the glass, the large interactions seen were non-specific between either the protein molecules or glass. Since the majority of all force curves contained an interaction, it is probable that they contain multiple unbinding events, which are more frequent with increasing contact time (Fig. 2.8b) (Chesla, Selvaraj et al. 1998), (Lim, Vedula et al. 2007). This would explain why the mean unbinding forces increase dramatically with time.

When both biotinBSA surfaces were incubated with avidin, to prevent any possible biotin and avidin interactions, the frequency of force curves with an unbinding event decreased slightly. It is possible that some of the biotin-avidin interactions were blocked.

Overall, it was impossible to differentiate between specific interactions of biotin and avidin due to the large non-specific interactions occurring. Therefore the method proposed by Lo et al was not suitable to measure the interactions between biotin and avidin. A new experiment was therefore designed to minimise the non-specific interactions which occurred before the true biotin and avidin adhesion force were measured.

2.5 Removing non-specific interactions from AFM experiments

The aim of a proof of principle AFM experiment was to quantify a known interaction, compare the adhesion values to the literature and to optimise experimental parameters in preparation for NCAM±VASE adhesion AFM measurements. During the biotin and avidin AFM experiment (Fig. 2.8), it became apparent that there was a high frequency of large unbinding events occurring between the tip and sample when avidin was not present. It was therefore necessary to minimise the probability of these interactions by investigating the experimental conditions.

2.5.1 A comparison of the effect of buffer (DMEH HEPES vs. PBS) on the adsorption of protein to glass

During an AFM experiment, the buffer which is used can have an effect on the size of unbinding events (Weisenhorn, Maivald et al. 1992). In the previous AFM experiment, unbinding events between biotinBSA on the cantilever and glass were measured indicating that it had adsorbed during contact and desorbed during cantilever retraction (Fig. 2.8). To test the role of buffer, NCAM Fc was adsorbed onto glass in either DMEH HEPES (used in previous AFM experiment) or PBS. A simple ELISA method was used to detect the amount of adsorbed NCAM. NCAM Fc was chosen due to its
easy detection using a well optimised NCAM antibody, α N16, which binds to a surface epitope in Ig III of NCAM.

Briefly, NCAM Fc was allowed to adsorb onto glass at RT with or without pre-adsorbed α human Fc to capture the Fc tag of NCAM, in either PBS, or DMEM HEPES. Following blocking and the addition of α N16, a secondary HRP conjugated antibody and BM blue POD substrate, the absorbance was read at 370 nm and revealed the amount of adsorbed NCAM protein on the glass (Fig. 2.9).

**Fig 2.9: The effect of buffer (DMEM HEPES vs. PBS) on the adsorption of NCAM Fc protein and detection antibodies by ELISA.**

The amount of adsorbed NCAM Fc to glass 96 well plates was compared in two buffers; PBS and DMEM HEPES, and with either NCAM Fc being orientated by pre-adsorption of an Fc capturing antibody (α human Fc) or adsorbed directly. The amount of adsorption was measured by addition of an NCAM antibody (α N16) and HRP conjugated secondary, which reacted with BM blue substrate and the absorbance was measured at 370 nm. **a** The relative absorbance of affinity adsorbed NCAM Fc in DMEM HEPES vs. PBS was compared. **b** The relative absorbance of non-specifically adsorbed NCAM Fc (no α human Fc) in DMEM HEPES vs. PBS was compared. This data presents the mean of three independent experiments. The relative level of affinity adsorbed NCAM Fc in PBS was decreased compared with when in DMEM HEPES.

When NCAM Fc was captured to glass via the α human Fc antibody (affinity adsorption), the relative absorbance at 370 nm was greater in DMEM HEPES than PBS (Fig. 2.9). Also when α N16 and the secondary antibody were added to α human Fc (no NCAM Fc added), the relative absorbance was greater in DMEM HEPES than PBS. This evidence indicated that the antibodies adsorbed non-specifically onto the glass to a greater extent in DMEM HEPES than in PBS. Therefore it is possible that the use of DMEM HEPES coupled with a high contact force between tip and sample during the AFM experiment increased the adsorption of proteins to the AFM surfaces. By changing the buffer to PBS, it is plausible that less protein was adsorbed from tip to sample and vice versa, therefore fewer large desorption events were seen.

When NCAM Fc was directly adsorbed (without α human Fc capture) onto glass, the levels were only measurable when in DMEM HEPES (Fig. 2.9b). NCAM Fc was not adsorbed directly to glass in PBS. This information will be an important factor in the assay development for measurement of
NCAM±VASE Fc adhesion by AFM. PBS was therefore chosen as the preferred buffer for AFM experiments to decrease the probability of adsorption and hence desorption events measured during the force measurements. PBS is also a preferable buffer for AFM as it consists of four salts, whereas DMEM HEPES contains a number of amino acids and vitamins, whose adsorption and desorption may have been interfering during the previous AFM experiment (Fig. 2.8).

2.5.2 High force unbinding events occurred between the bare cantilever and glass coverslip at low contact time and contact force

Having realised the importance of choice of buffer and its role in the AFM experiment, it was necessary to understand the underlying interactions that might have occurred during the biotin and avidin AFM experiment (Fig. 2.8). Following the functionalisation of the AFM surfaces, it was possible that areas of the tip and coverslip were bare and so interactions could occur between them. Therefore the adhesion between a bare Piranha-cleaned cantilever and glass coverslip was determined using AFM in PBS at 22°C with extend and retract velocities of 498 nm/s (Fig. 2.10). The effect of increasing the contact force between the tip and sample on the size of the unbinding events occurring between them was also investigated. A range of setpoint forces between 0.5 and 3 nN were chosen and the contact times were varied between 0–0.5 s. Data were acquired in 3 different positions for each contact parameter to assess adhesion between different areas and typically more than 100 force curves were included for each data point. In total, at last 50 force-distance curves were collected and analysed for each interaction.

![Figure 2.10: AFM experiment to measure the interaction between a Piranha cleaned cantilever and glass coverslip.](image)

The effect of increasing contact time (0–500 ms) and contact force (0.5–3 nN) on the frequency and mean unbinding events between cantilever and coverslip was investigated to determine the best parameters for future single molecule force spectroscopy experiments. The cantilever extend and retract velocities were 498 nm/s and the experiment was carried out at 22°C in PBS. a) The mean unbinding force between glass and cantilever was compared with increasing contact force and contact time. b) Table to show the frequency of retract force curves with one or more unbinding events. Each data point contains at least 50 unbinding events. **Frequent and large forces (> 200 pN) were recorded from the interactions between a bare cantilever and glass coverslip.** For future protein AFM experiments these interactions must be blocked and contact forces and times must be lowered.

<table>
<thead>
<tr>
<th>AFM parameter</th>
<th>Force curves with an attractive interaction (%)</th>
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<tbody>
<tr>
<td>0.5 nN</td>
<td>0 ms</td>
</tr>
<tr>
<td>10 ms</td>
<td>96</td>
</tr>
<tr>
<td>1.5 nN</td>
<td>0 ms</td>
</tr>
<tr>
<td>10 ms</td>
<td>94</td>
</tr>
<tr>
<td>3 nN</td>
<td>0 ms</td>
</tr>
<tr>
<td>10 ms</td>
<td>98</td>
</tr>
</tbody>
</table>
When comparing the mean unbinding force, it was clear to see that at all contact times and forces, the event sizes were larger (150 – 1900 pN) than typical protein unbinding events (50 – 200 pN) (Zlatanova, Lindsay et al.). The range of unbinding force sizes seen was similar to those in the previous AFM experiment (Fig. 2.8). Therefore it was impossible to differentiate between the true biotin and avidin interactions and those between the bare cantilever and glass, which could have occurred in that experiment.

In general, increasing the contact time between the glass and cantilever increased the mean unbinding force and also increasing the contact force dramatically increased this. Almost all force curves contained an unbinding event (Fig. 2.10b). Since these interactions are not between proteins, but between the tip and sample, it is important that they are prevented prior to protein adhesion measurements. The probability of an interaction was high due to an increased contact force, which was maintained by increasing the contact area of the cantilever and glass. The standard errors presented in this data set were in general larger than in the previous AFM experiment (Fig. 2.8) and reveal that there was larger variation in the adhesion of different areas of the coverslip and cantilever.

It was therefore necessary to prevent the large non-specific interactions between glass and cantilever by blocking both surfaces before addition of protein. In the following AFM experiments, the contact force and contact times between the tip and sample were therefore minimised to prevent sample damage and to decrease the probability of measuring an unbinding event.

2.5.3 Addition of surfactant P105 onto both the cantilever and glass coverslip lowered the mean unbinding events seen by AFM measurements

To block the non-specific interactions between the bare cantilever and bare coverslip during AFM experiments the effect of deposition of a non-ionic surfactant Pluronic 105 (P105, average MW = 6500 Da) was investigated. Non-ionic surfactants have been used in AFM experiments to block non-specific interactions whilst measuring the adhesive forces between proteins (Brogan, Shin et al. 2004). The interaction between P105 coated layers has also been studied by AFM (McGurk, Green et al. 1999).

A number of concentrations of P105 were used to determine which blocked the large non-specific interactions. Briefly, the cantilever and coverslip were Piranha-cleaned and to them was added a solution of P105 in PBS for 1 hr at RT. After brief rinsing, AFM measurements were taken between the cantilever and glass in PBS at 22°C. Concentrations below 3 mg/ml were ineffective as large interactions similar to those seen between a bare cantilever and coverslip (Fig. 2.10) were seen.
The critical micelle concentration of P105 is 3 mg/ml, therefore deposition of this concentration was investigated (Alexandridis, Holzwarth et al. 1994). 3 mg/ml P105 was adsorbed onto either the glass coverslip only or the coverslip and cantilever for 1 hr at RT to see what effect this had on the size of unbinding events between the tip and sample during AFM measurements. In this experiment lower contact forces of 0.5 and 1.5 nN were used as well as short contact times of 0 and 10 ms. Data were acquired in 3 different positions for each condition to assess different areas and at least 35 force-distance curves were collected for each interaction.

If P105 was adsorbed onto the glass coverslip only (Fig. 2.11e), large unbinding events (300–400 pN, approximately the same as between a bare cantilever and coverslip) occurred between the tip and P105 coated coverslip in most force curves at 0 and 10 ms of contact time and 0.5 nN contact forces (Fig. 2.11a-b). When increasing the contact force to 1.5 nN, the unbinding events seen between the P105 coated cantilever and bare coverslip were lower than those after 0.5 nN of force. During any AFM experiment there is likely to be cantilever drift, which alters the contact area. These areas had potentially different coverage of P105. The decrease in mean unbinding force at 1.5 nN, compared with 0.5 nN, indicated that the P105 coverage is patchy.

Experimentally this point was proven as when P105 was adsorbed onto the cantilever and coverslip (Fig. 2.11f), unbinding forces were consistently less than 50 pN after 0 s contact and 0.5 nN contact force. There was a dramatic decrease in the unbinding forces between P105 coated tip and sample (50 pN) from that of the bare surfaces (380 pN).

It appears that addition of 3 mg/ml P105 onto both cantilever and coverslip prevented the large non-specific interactions seen between the cantilever and coverslip during an AFM experiment. The interactions that occurred between P105 layers were lower than most protein-protein interactions measured by AFM.

Interestingly, although the size of unbinding events between P105 coated cantilever and coverslip was reduced compared with 1 side coated, the frequency of force curves containing an interaction is not significantly different. This indicates that although the nature of the interactions was different, there were still interactions occurring. This was investigated further.
Figure 2.11: AFM experiment to compare the effectiveness of blocking the interacting cantilever and glass coverslip surfaces with a non-ionic surfactant Pluronic 105 (P105).

The unbinding events during the interaction between a bare cantilever and a P105 coated coverslip and P105 covered cantilever and P105 coverslip were measured in PBS at 22°C using AFM. Cantilever extend and retract velocities were 498 nm/s. P105 was adsorbed to surfaces for 1 hr at RT in PBS. The experiment was carried out in a fluid cell in PBS. The effect of increasing contact times (0–10 ms) and set point contact forces (0.5–1.5 nN) was investigated with cantilever extend and retract velocities at 498 nm/s. 

a) The mean unbinding force following contact between a cantilever and coverslip with P105 on one side or both sides was compared following a set point contact force of 0.5 nN and contact time of 0 ms.

b) The mean unbinding force following contact between a cantilever and coverslip with P105 on one side or both sides was compared following a set point contact force of 0.5 nN and contact time of 10 ms.

c) The mean unbinding force following contact between a cantilever and coverslip with P105 on one side or both sides was compared following a setpoint contact force of 1.5 nN and contact time of 0 ms.

d) The mean unbinding force following contact between a cantilever and coverslip with P105 on one side or both sides was compared following a setpoint contact force of 1.5 nN and contact time of 10 ms.

e) Schematic to show the AFM experiment with P105 on one side.

f) Schematic to show the AFM experiment with P105 on both sides.

g) Table to show the percentage of force curves with an attractive interaction during the separation of P105 coated and uncoated AFM surfaces. Statistical analysis compares the significance of mean unbinding forces with P105 on 1 side vs. both. N.S. Not significant, ** P<0.001 (very statistically significant), *** P<0.0005 (extremely statistically significant). Each data point contains at least 35 unbinding events.

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<table>
<thead>
<tr>
<th>Force curves with an attractive interaction (%)</th>
<th>P105 1 side (Coverslip only)</th>
<th>P105 2 sides</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 nN 0 ms</td>
<td>46</td>
<td>62</td>
</tr>
<tr>
<td>10 ms</td>
<td>37</td>
<td>71</td>
</tr>
<tr>
<td>1.5 nN 0 ms</td>
<td>81</td>
<td>68</td>
</tr>
<tr>
<td>10 ms</td>
<td>77</td>
<td>78</td>
</tr>
</tbody>
</table>
2.5.4 Addition of surfactant P105 to both cantilever and coverslip decreased the frequency of large non-specific unbinding events

In previous AFM experiments, designed to optimise the experimental parameters, individual unbinding forces were averaged from all force curves to give an approximate force of the interaction occurring. In force spectroscopy, the distribution rather than the mean of different forces seen provides a more accurate picture of interactions and reveals whether a number of quantitatively varied interactions are occurring. To determine the nature of the small interactions occurring between P105 coated cantilevers and coverslips, all unbinding event forces (from the data in Fig. 2.11a-d) were pooled and binned into 20 pN intervals and plotted against the frequency in the total data set. These were compared with all unbinding events between bare cantilevers and coverslips (from the data in Fig. 2.10a) (Fig. 2.12).

![Graphs showing the frequency of binned unbinding events following the interaction of bare cantilever and glass coverslip with surfactant P105 on both sides or neither.](image)

Figure 2.12: AFM experiment to compare the frequency of binned unbinding events following the interaction of bare cantilever and glass coverslip with surfactant P105 on both sides or neither. These data were taken from the same experiment as Fig. 2.5.3. a) The frequency of binned unbinding events following contact between a cantilever and coverslip with P105 on both sides or neither was compared following a setpoint contact force of 0.5 nN and contact time of 0 ms. b) The frequency of binned unbinding events following contact between a cantilever and coverslip with P105 on one side or both sides was compared following a setpoint contact force of 0.5 nN and contact time of 10 ms. c) The frequency of binned unbinding events following contact between a cantilever and coverslip with P105 on one side or both sides was compared following a setpoint contact force of 1.5 nN and contact time of 0 ms. d) The frequency of binned unbinding events following contact between a cantilever and coverslip with P105 on one side or both sides was compared following a setpoint contact force of 1.5 nN and contact time of 10 ms. Data here was taken from the same experiment as Fig. 2.11 and follows the same experimental parameters. n > 35 per data point. Adsorption of P105 on both cantilever and coverslip decreases the frequency of large unbinding events, whilst increasing the frequency of smaller events.
At each contact time and contact force the data show that the frequency of large unbinding events was decreased between cantilever and glass upon addition of P105. For 0.5 nN force and 0 ms of contact time, 95% are less than 100 pN whereas for P105 it is 70%. Visually the shift to lower unbinding forces was clear and the trend was followed for all contact times and forces (% below 100 pN ratio of Bare: P105; 0.5 nN, 10 ms = 80%: 70%; 1.5 nN, 0 ms = 75%:35%; 1.5 nN, 10 ms = 60%:40%). Interestingly there appeared to be only one population of similarly sized unbinding forces (the mode was roughly the same as the mean unbinding force) for the measurements between P105 coated cantilevers and coverslips. An assay was therefore developed to measure NCAM±VASE adhesion in the presence of P105 to block the large, non-specific interactions between the cantilever and glass. Once the AFM adhesion measurements between NCAM±VASE were taken, a comparison with its unbinding force frequency distributions with those of P105 only revealed whether the interactions were quantitatively different.

2.5.5 AFM experiment optimisation between biotin and avidin removed large non-specific unbinding events

Following optimisation of the AFM experiment to remove the large non-specific interactions occurring between the tip and sample, the adhesion between biotin and avidin was measured. In previous experiments the addition of P105 on the cantilever and coverslip reduced the size and frequency of unbinding events and the use of PBS as the AFM buffer was preferred. High contact forces and contact times were also excluded to reduce the size and frequency of unbinding events.

This information was used to improve the AFM assay to measure the interaction between biotin and avidin.

To do this, 1 mg/ml biotinBSA (in PBS) was deposited onto a clean cantilever or coverslip in the presence of 3 mg/ml P105. If required, avidin was captured onto pre-adsorbed biotinBSA surfaces, also in the presence of 3 mg/ml P105. The interaction between biotinBSA captured avidin or biotinBSA alone and P105 was measured to determine whether they interacted non-specifically. Adhesion was assessed by comparing the mean unbinding force at low contact times (0–10 ms) and contact forces (0.5–1.5 nN) (Fig. 2.13).

The mean unbinding forces for all interactions (Fig.2.13a) were greatly reduced compared with those measured in the experiment where AFM surfaces were not blocked with P105 (Fig. 2.8). When P105 was present, the interactions between avidin and P105 did not increase with increasing force or contact time. This indicated that avidin was not interacting specifically with the Pluronic layer. The mean unbinding force between P105 layers (measured in Fig. 2.11) was similar to that between
avidin and biotin indicating that avidin may not interact with P105 at all; it may be due to the interaction between the Pluronic layers only.

The interaction between biotin and P105 was as adhesive as the interaction between avidin and P105, which indicated that biotin was not specifically interacting. The mean unbinding forces between avidin and biotin, in the presence of P105, were between 55 and 150 pN over the contact parameters compared with 200 and 1500 pN for the interactions without P105 coverage. There was no significant difference between the avidin and biotin mean unbinding force and those between of the control interactions until 0.5 nN of contact force and 10 ms of contact time. After this threshold, the interaction between avidin and biotin resulted in significantly higher unbinding forces. This indicates that an interaction was occurring specifically between avidin and biotin because when they were not present in the AFM experiment, the interactions were smaller.

When avidin was flooded into the AFM chamber to block all of the biotin sites on the cantilever and coverslip, the mean unbinding force decreased and the mean sizes were approximately the same as the control interactions. This indicates that the larger interactions seen during the interaction between biotin and avidin were reduced and that only non-specific, smaller interaction occurred.

On comparison of the frequencies of binned unbinding events between the control and biotin-avidin interactions, the control interactions between avidin-avidin, avidin-P105 and biotin-P105 displayed fewer large unbinding events (frequency >100 pN = 0%, 12%, 10 % respectively) (Fig. 2.13b); whilst 55% on interactions between biotin and avidin adsorbed surfaces in the presence of P105 were above 100 pN.

Following blocking of the AFM surfaces with P105 and measurement of the adhesion between biotin and avidin, the frequency of force curves containing an unbinding event was greatly reduced in comparison with those where surfaces were unblocked (Fig.2.8b vs. 2.13c). For example the percentage of force curves containing an unbinding event, following 3 nN and 0.05 s of contact, between avidin and biotin (no P105) was 98% and 90% between avidin coated surfaces; whilst the percentage of force curves with an unbinding event after 1.5 nN and 0 ms of contact were 44% and 45 % respectively. This decrease in frequency indicates that the interactions were more likely to be specific biotin-avidin single molecule interactions.
Fig 2.13: AFM experiment measuring the interaction between biotin and avidin following optimisation.

The interaction between biotin and avidin functionalized AFM surfaces was investigated by force spectroscopy following coating with P105 to remove non-specific interactions between the cantilever and coverslip. a) The mean unbinding force was measured between all plausible interactions between a cantilever and coverslip functionalised with biotinBSA and avidin (captured by biotinBSA) in the presence of P105. The effect of contact time and force was investigated. b) The frequency of binned unbinding events was compared for the interactions between P105 blocked avidin and biotinBSA functionalized AFM surfaces at 1.5 nN contact force and 10 ms contact time. Table C shows the frequency of force curves containing an attractive interaction. c) Table to show the frequency of retract force curves which contained an unbinding event following tip-sample interaction for interactions between avidin and biotin, avidin and avidin, biotin and P105 and avidin and P105. Addition of a P105 layer onto AFM surfaces decreased the percentage of force curves with large interactions.
Overall it may be concluded that the addition of P105 to the AFM assay prevented large non-specific interactions in the presence of adsorbed biotin and avidin. The retraction velocity used in this experiment was 498 nm/s and for the unbinding events between avidin and biotin following 1.5 nN of contact time, equated to an unloading rate of between 20000 and 25000 pN/s corresponding to a mean unbinding event of approximately 150 pN. The unloading rate used in this experiment was lower than those seen in the literature where a mean unbinding event of 167±20 pN was found following an unloading rate of approximately 39000 pN/s. The fact that these unloading rates were in the same order of magnitude and unbinding forces were similar indicated that the AFM experiment to measure the adhesion between NCAM±VASE proteins was carried out.

2.6 Measurement of the interaction between affinity captured NCAM±VASE Fc

The large and non-specific interactions between the cantilever and coverslip were reduced in AFM experiments by:

- Changing the experimental buffer from DMEM HEPES to PBS
- Depositing a non-ionic surfactant, P105, on the AFM surface
- Minimising the contact force and time during tip-sample interaction by AFM

Using this information, a methodology was developed to measure the interaction between NCAM±VASE proteins. A starting point for this experiment was the use of an α human Fc antibody to capture NCAM±VASE by its C-terminal Fc tag onto AFM surfaces (see Fig. 2.9). In this experiment NCAM Fc was affinity captured via adsorbed α human Fc and detected by the addition of a α N16 (which binds to NCAM Ig III), illustrating successful capture and the availability of the homophilic binding domains of NCAM. It is probable that NCAM was orientated in a way which allowed potential homophilic interaction in an AFM experiment.

To block non-specific interactions, P105 was incorporated into this capture method by adding it to the AFM surfaces with the α human Fc. Overall the proposed method of NCAM±VASE functionalisation of AFM surfaces involved deposition of P105 and α human Fc, followed by incubation and affinity capture of NCAM±VASE via its C-terminal Fc tag (Fig. 2.14). In reality adsorption of P105 and α human Fc would result in randomly oriented NCAM±VASE Fc proteins but only those with accessible Fc binding sites would bind the protein thus increasing the chance that NCAM±VASE Ig domains will be extending from the solid surface into the liquid chamber. This therefore provided a means of testing the protein adhesion by AFM.
2.6.1 NCAM±VASE Fc was produced in Cos-7 cells and purified by protein A purification

The NCAM±VASE Fc required for the AFM experiment was engineered in the laboratory of Prof. Doherty (Guy’s Hospital, UK) and has been expressed in the Saffell lab for many years. It consists of all extracellular domains of NCAM (Ig I, II, III, IV, V and Fn1 1-2). The transmembrane and cytoplasmic domains were excluded to circumvent difficulties in purification. Instead, an N-terminal signal sequence peptide, for targeting for cell secretion, was engineered in. The vector DNA contains tetracycline and ampicillin resistance for colony selection.

NCAM±VASE Fc protein was produced for the AFM experiment by transient transfection of Cos-7 cells. Briefly cells were plated at 75% confluence and the following day were serum starved for 30 min before addition of 2 μg/ml NCAM±VASE Fc DNA, 51.6 μg/ml chloroquine diphosphate and 0.4 mg/ml DEAE dextran. After 3.5 hours, cells were osmotically shocked with PBS/10% DMSO for 1.5 min and replaced with full serum media (low IgG). After 6 days of incubation, the media was removed and Fc containing proteins were purified by the addition of Protein A sepharose beads. Proteins were eluted from the beads by the addition of low pH buffer.

The success of the NCAM±VASE Fc protein pulldown was assessed in duplicate by non-reducing SDS-PAGE and Western blotting with an antibody against NCAM, α N16 (Fig. 2.15). 20 μl of protein samples were loaded into each lane. Samples loaded included the cell supernatant before removal of Fc containing proteins, cell media following the removal of Fc proteins and the eluted NCAM±VASE Fc fractions.
**Figure 2.15: NCAM±VASE Fc protein production in Cos-7 cells and pulldown with Protein A.**

NCAM±VASE Fc was produced in Cos-7 cells following transient transfection by the DEAE method. After 6 days of incubation, Fc proteins were purified from cell supernatant using Protein A sepharose beads and eluted with a low pH buffer. a) 7.5%/5% resolving/stacking non-denaturing gel stained with coomassie to show the purification of NCAM±VASE Fc produced transiently in Cos-7 cells. b) Western blot with an α NCAM antibody (α N16) to show the purification of NCAM±VASE Fc produced transiently in Cos-7 cells. Lane 1 = Cell supernatant, lane 2 = cell supernatant following purification, lanes 3 – 7 = 1st – 5th eluted fractions. **NCAM±VASE Fc was successfully produced by Cos-7 transient transfection and purified by Protein A to yield a monomer at 110 kDa and larger order oligomers.**

The coomassie stained gel revealed that the eluted fractions contain one or two enriched proteins which were more concentrated than in the media before pulldown (Fig. 2.15a Lanes 1, 3, 4). This is also the case with the NCAM-VASE Fc pulldown (Fig. 2.15a Lanes 1, 3, 4). Western blotting with the α N16 antibody in the NCAM Fc pulldown showed that NCAM was hardly visible in the media before and after pulldown but was successfully enriched following elution from the protein A beads (Fig. 2.15b). The same is true of the NCAM-VASE Fc pulldown, except that not all NCAM-VASE Fc was purified from the cell supernatant. Protein yields were verified by ELISA and BCA assay and determined to be approximately 500 μg from 20 plates of Cos 7 cells.

In both eluted proteins, a series of oligomers were seen. The monomer was present as a 115 kDa protein as well as another population of protein greater than 250 kDa in size, which corresponded to the dimer or trimer. The clustering of NCAM±VASE Fc as a higher order oligomer may have been due to the homophilic binding property of the protein but it is likely that it may have been due to the self-association of two Fc tags from different protein constructs. One α human Fc antibody captures two NCAM±VASE Fc proteins (see schematic Fig. 2.14). In this way, the NCAM±VASE Fc used in the AFM experiments can be thought of as ‘dimeric’.

It is worth noting that size exclusion chromatography was not used to purify NCAM±VASE Fc further, due to low yields (less than 1 mg). However, the affinity capture of the proteins onto the AFM surfaces itself could serve as a further purification.
2.6.2 NCAM Fc was orientated and captured on glass via its C terminal Fc tag

After production of NCAM±VASE Fc was successful, the method for AFM surface functionalisation was investigated using ELISA. The objective was for the NCAM±VASE Fc to be orientated onto glass in PBS via its affinity tag, Fc, binding to pre-adsorbed α human Fc on glass (Fig. 2.16b). The usefulness of this method was assessed by ELISA using increasing concentrations of α human Fc to capture NCAM Fc. This was monitored by the addition of α N16 and a secondary HRP conjugated antibody reacting with BM blue substrate.

![Graph showing relative NCAM Fc adsorption](image)

**C**

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**Figure 2.16:** Orientated NCAM Fc was captured on glass and detected by ELISA for use in a later AFM experiment. An ELISA was used to detect whether NCAM Fc could be captured on glass by its Fc tag via an antibody α human Fc in PBS. NCAM Fc was adsorbed via its Fc tag on glass at 37°C for 1 hr. After blocking, α N16 was added and detected with a secondary antibody and BM blue substrate. The absorbance was read at 370 nm. a) A representative experiment of the ELISA to show the detection of increasing concentrations of NCAM Fc captured by α human Fc with and without α NCAM antibody detection. b) Schematic to show proposed ELISA experiment with the capture of orientated NCAM Fc and detection with an α NCAM antibody. c) Table to show the mean and normalised data of three ELISA experiments comparing the concentration of captured NCAM Fc against its detection. **NCAM Fc was captured by α human Fc on glass in PBS and detected by an antibody against Ig III of NCAM.**

As the concentration of α human Fc was increased, more NCAM Fc was captured and this lead to an increased absorbance value at 370 nm. There was low background adsorption of secondary antibody onto the glass in PBS, so α N16 bound specifically to NCAM present on the coverslip. The
representative data presented (Fig. 2.16 a) follows the same pattern as the mean data taken from four independent experiments (Fig. 2.16c).

Functionalisation of the Si$_3$N$_4$ cantilever was carried out using this non-specific chemical adsorption method (Fischer, Heyn et al. 1993). Capturing NCAM±VASE Fc in this way in PBS on glass was successful and provided a good basis for measuring the interactions in an AFM experiment.

2.6.3 α human Fc captured NCAM Fc in the presence of 1:1 ratio of P105 was detected by an α NCAM antibody

P105 was required on the cantilever and coverslip to prevent large non-specific interactions during AFM experiments; therefore it was necessary to incorporate this layer into the ELISA to measure adsorbed NCAM. To do this 3 mg/ml P105 was adsorbed onto the coverslip with α human Fc (Fig. 2.17).

Two different ratios of 3 mg/ml P105: a 1:200 dilution of 200 µg/ml α human Fc in PBS were used to see which would allow the adsorption of both molecules. The relative NCAM adsorption was assayed following the same method as the previous ELISA, with α N16 and HRP conjugated secondary (Fig. 2.17). By using α N16 to detect adsorbed NCAM, it also tested the accessibility of its surface epitope of NCAM Ig III and therefore revealed whether this Ig domain is available for homophilic binding in the AFM experiment.

![Graph](image)

**Figure 2.17:** Orientated NCAM Fc was captured in the presence of P105 on glass and detected by ELISA for use in a later AFM experiment. α human Fc (1:200) was adsorbed onto glass in the presence of 1 or 10 molar equivalents of P105 in PBS. 4 µg/ml NCAM Fc was captured via the antibody and detected with an α NCAM antibody (α N16). a) ELISA to show the detection of α human Fc captured NCAM in the presence of a 1:1 or 10:1 ratio of surfactant P105: NCAM Fc. b) Schematic to show the proposed capture and orientation of NCAM Fc in the ELISA experiment in the presence of P105. The data presented is the mean and normalised of three independent experiments. Capture of NCAM Fc with a 1:1 ratio of α human Fc: P105 revealed specific detection and hence the presence of NCAM Fc on glass in PBS.
With a 10:1 ratio of P105: α human Fc, there was no significant difference between the absorbance whether NCAM was present or not. This indicated that NCAM Fc was not present in the P105 adsorbed layer and that the antibodies involved in the detection of NCAM were adsorbing onto the glass and gave high absorbance values of 0.4.

When the ratio of P105: α human Fc was 1:1, there was a significant increase in the absorbance seen when NCAM was present compared to when it was not. Interestingly, whether the P105: α human Fc ratio was 1:1 or 10:1 the absorbance with no NCAM present was the same, indicating again that this was due to non-specific adsorption of the antibodies onto the glass. This experiment concluded that P105 and α human Fc co-adsorb onto glass in PBS, and facilitated the affinity binding of NCAM Fc. Since the presence of NCAM Fc was proved on the coverslip, the adsorption method was used to measure the adhesion between NCAM±VASE by AFM.

It was not known whether the presence of the surfactant had any effect on the structure and therefore function of NCAM Fc. To check this it would be necessary to assess structural changes of NCAM Fc in the presence of P105 or in PBS alone using a technique such as circular dichroism. However in the ELISA, the surface epitope of NCAM Ig III, which binds to the α N16 antibody was still present, which indicated that no major structural changes had occurred in that domain. Under the same ELISA conditions, the relative absorbance of NCAM Fc by α human Fc capture was similar whether in the presence of P105 or not which may indicate that the affinity between the antibody and NCAM was similar in both cases.

2.7 Measuring the adhesion between orientated and adsorbed NCAM±VASE Fc proteins by AFM

2.7.1 NCAM and VASE homophilic adhesion was measured by single molecule AFM and was found to be similar

To measure the interaction between NCAM±VASE proteins using single molecule AFM, the cantilever and coverslip were first Piranha cleaned before the addition of 3 mg/ml P105 with a 1:200 dilution of α human Fc for 1 hr at RT. Non-adsorbed molecules were rinsed away with PBS before 4 μg/ml NCAM Fc was added to allow affinity binding of the protein via its Fc tag. After rinsing, the cantilever and coverslip were mounted into the AFM instrument. At least 50 force-distance curves were obtained per interaction and the effect of the contact force and contact time was investigated (Fig. 2.18a–d).
The size and frequency of unbinding events following the interaction of adsorbed (at 4 µg/ml) and orientated NCAM±VASE Fc proteins (on the cantilever and glass coverslip) was compared with the interaction between NCAM±VASE, α human Fc and α human Fc captured human IgG. Measurements were carried out in PBS at 22°C. The effect of increasing contact times (0–10 ms) and setpoint contact forces (0.5–1.5 nN) was investigated with cantilever extend and retract velocities set at 498 nm/s. a) Comparison of mean unbinding force of NCAM-NCAM, NCAM-α Fc, NCAM-h IgG and the corresponding VASE interactions following a setpoint contact force of 0.5 nN and contact time of 0 ms. b) Comparison of mean unbinding force of NCAM-NCAM, NCAM-α Fc, NCAM-h IgG and the corresponding VASE interactions following a setpoint contact force of 0.5 nN and contact time of 10 ms. c) Comparison of mean unbinding force of NCAM-NCAM, NCAM-α Fc, NCAM-h IgG and the corresponding VASE interactions following a setpoint contact force of 1.5 nN and contact time of 0 ms. d) Comparison of mean unbinding force of NCAM-NCAM, NCAM-α Fc, NCAM-h IgG and the corresponding VASE interactions following a setpoint contact force of 1.5 nN and contact time of 10 ms. e-g) Schematic to show the capture of NCAM±VASE Fc, α human Fc, human IgG and their corresponding AFM experiment. N.S. not significant, * P<0.05 (statistically significant), ** P<0.001 (very statistically significant), *** P<0.0005 (extremely statistically significant). At least 50 force distance curves were collected and analysed per interaction. The mean unbinding forces between NCAM±VASE Fc proteins were significantly higher than between control interactions.
The cantilever extend and retract velocities were kept constant at 498 nm/s. The mean unbinding forces of interactions from the retract force curves were compared with other control interactions. To test whether NCAM±VASE-NCAM±VASE was a specific interaction, the size of unbinding events was compared with the interaction of NCAM±VASE with α human Fc. In theory, no interactions should occur between NCAM±VASE Fc and α human Fc because the only interacting portion of the NCAM±VASE protein (the Fc tag) is already in complexation with its capturing antibody on the cantilever. The interactions between NCAM±VASE Fc and another Fc containing protein human IgG, which was captured by α human Fc was measured as a true negative control to test the specificity of the interactions occurring between NCAM±VASE Fc pairs with a protein with which it should not interact specifically.

A statistical analysis shows that following 0.5 nN of contact force and 0 ms of contact time, the interaction between NCAM-NCAM (104±9 pN) was statistically much higher than NCAM-α human (65±7 pN) and extremely higher than NCAM-human IgG (56±6 pN). The mean force of unbinding for VASE-VASE was statistically similar to that of NCAM-NCAM which provided the first evidence that there may not be a difference in the homophilic binding strength of the isoforms. Due to the variation in the unbinding forces seen between VASE-VASE interactions, there was no statistical difference in size with VASE-α h Fc. This indicates that it was important to ensure all α human Fc sites were blocked with an NCAM±VASE Fc protein to prevent non-specific interactions.

At other contact times and forces, the control interactions with IgG and α human Fc were significantly lower than NCAM-NCAM and VASE-VASE indicating that the interactions between these proteins was quantitatively different and more likely to be specific. Interestingly, at the higher contact force (1.5 nN) the interaction between NCAM-NCAM was larger than VASE-VASE.

2.7.2 The distribution of frequencies of binned unbinding events were similar for NCAM±VASE and all control interactions

Following the analysis of the size of the mean unbinding events of the interaction between NCAM±VASE and control interactions, the frequency of binned unbinding force distributions was compared. All unbinding force events were binned into forces of 20 pN wide and were compared against the percentage from the total data pool of interactions, after 0.5 nN and 0 ms of contact time (Fig. 2.19). For NCAM-NCAM, 60% of unbinding events were less than 100 pN but the mean unbinding size was 104±9 pN (Fig. 2.18). This indicated that the 40% of events over 100 pN increased the mean unbinding force. The unbinding data did not follow a normal distribution as it appeared that there were a number of different interactions present.
There was almost no difference in the binned unbinding force distribution pattern for the interaction between NCAM-NCAM and NCAM-α human Fc; for NCAM-α human Fc, 85% of unbinding events were less than 100 pN, which was larger than for NCAM-NCAM. However it was difficult to distinguish between an interaction due to NCAM homophilic binding or between NCAM Fc and the α human Fc capture antibody. These results indicated again the importance of blocking α human Fc binding sites with NCAM to prevent measuring the affinity capture of the Fc tag.

![Graph](image.png)

**Fig.2.19:** AFM experiment to compare the frequency of binned unbinding events following the homophilic interaction of NCAM±VASE Fc proteins and heterophilic control interactions with α human Fc and IgG.

This data was taken from the same experiment as Fig. 2.18. All attractive unbinding events for each interaction were pooled and sorted into force bins with a width of 20 pN. The frequency compared to the total number of unbinding events was presented as frequency distribution curve. **a)** The frequency of binned unbinding events following contact between a cantilever and coverslip with P105 and NCAM Fc and itself, or α human Fc or IgG was compared following a setpoint contact force of 0.5 nN and contact time of 0 ms. **b)** The frequency of binned unbinding events following contact between a cantilever and coverslip with P105 and NCAM-VASE Fc and itself, or α human Fc or IgG was compared following a setpoint contact force of 0.5 nN and contact time of 0 ms. At least 50 force distance curves were collected and analysed for each interaction.
Interestingly, the unbinding events between NCAM and human IgG functionalised surfaces was quantifiably different from NCAM-NCAM due to 95% of interactions being less than 100 pN and a mean unbinding force of 56±6 pN. These values for the interaction between NCAM and human IgG were statistically similar to those between two P105 functionalised surfaces (Fig. 2.12).

Using the same bin width for all unbinding events in the data set, the interactions between VASE and controls was compared in the same way as with NCAM. The frequency of unbinding events under 100 pN involving VASE were as follows VASE-VASE = 45%, VASE-α human Fc = 75% and VASE-human IgG = 70%. There was therefore a quantifiable difference between the frequency distribution of unbinding events between VASE functionalised surfaces and the interactions between VASE and the α human Fc or human IgG. This indicated that it may be possible to distinguish non-specific interactions of VASE with those involved in VASE homophilic binding.

2.7.3 The homophilic interactions between NCAM±VASE may not be single molecule as the frequency of force curves with an unbinding event was high

The first measurements of the homophilic interactions of NCAM±VASE by AFM revealed a similarity in their mean size at 0.5 nN of contact force. However this information alone was not enough to conclude the nature of these interactions. The percentage of retract force curves containing an unbinding event was therefore calculated to estimate the probability that the interactions were between single molecule pairs (Table 2.1). According to the literature, if two proteins have been allowed to interact, if 30% of the unbinding data (i.e. in this case the retract force-distance curves) contained a single unbinding event, there is an 80% probability that these interactions are between single molecule proteins (Brogan, Shin et al. 2004).

Table 2.1: Figure to show the percentage of retract force curves with an unbinding event, following the interaction between NCAM-NCAM. Table 2.1 shows the percentage of retract force curves with an unbinding event, following the interaction between NCAM-NCAM (Table a) and VASE-VASE (Table b). Frequencies of unbinding events following each interaction were compared for all contact times and forces from the data set in Fig. 2.18.

<table>
<thead>
<tr>
<th>Table a NCAM-NCAM</th>
<th>Force curves with an interaction (%)</th>
<th>Table b VASE-VASE</th>
<th>Force curves with an interaction (%)</th>
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<td>0.5 nN 0 ms</td>
<td>67</td>
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<tr>
<td>0.5 nN 10 ms</td>
<td>79</td>
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</table>

In the AFM experiment measuring the interaction between NCAM±VASE, the percentage of force curves with an unbinding event revealed that most contained an unbinding event (between 63 and 93%). Therefore it was improbable that the unbinding events corresponded to those between single NCAM-NCAM and NCAM-VASE-NCAM-VASE protein pairs.
2.7.4 The force of adhesion between the Fc tag and \( \alpha \) human Fc was stronger than the NCAM-NCAM interaction

During the measurement of the adhesive force between NCAM±VASE Fc proteins, it was possible that the unbinding events were due to unbinding of the Fc tag from the capturing antibody \( \alpha \) human Fc. To test this, the adhesion between human IgG, which contains an Fc domain, and \( \alpha \) human Fc was quantified using single molecule AFM. Briefly, \( \alpha \) human Fc was adsorbed onto a cleaned glass coverslip in the presence of 3 mg/ml P105 to minimise non-specific binding. In parallel human IgG in the presence of P105 was adsorbed onto a bare cantilever. Approximately 100 force-distance curves were collected to measure this interaction and the mean unbinding force was quantified from the retract force-distance curves. This was compared against the mean unbinding forces for NCAM±VASE Fc from the previous experiment (Fig. 2.18) for varying contact times and parameters (Fig. 2.20).

![Graph showing mean unbinding force for different conditions](image)

**Figure 2.20:** Single molecule AFM experiment to measure the interaction between human IgG (Fc containing) and \( \alpha \) human Fc to assess the adhesive strength of the NCAM±VASE Fc capture method.

The mean unbinding force was calculated for the interaction between an \( \alpha \) human Fc adsorbed surface and a human IgG adsorbed surface. The forces were compared for increasing contact times and forces, which were used in previous NCAM±VASE Fc AFM experiments. The mean force required to separate an \( \alpha \) human Fc functionalised surface and a human IgG surface was significantly higher than NCAM-NCAM and VASE-VASE for each contact parameter.

On first inspection, the mean unbinding forces for unbinding between an \( \alpha \) human Fc surface and a human IgG surface was significantly higher than those measured for NCAM-NCAM and VASE-VASE at each contact parameter. The mean unbinding force for \( \alpha \) human Fc- human IgG increased with increasing contact time and force from 175±8-335±32 pN. These events were unlikely to be due to the desorption of \( \alpha \) human Fc or human IgG from the AFM surfaces, as these forces have been found
to be generally larger. Neither protein was orientated specifically on the AFM surfaces for optimal α Fc-Fc binding, therefore it was likely that fewer interactions would be measured. The frequency of force curves with an attractive unbinding event was approximately 30%, which indicated that these interactions were likely to be due to a single molecule unbinding event.

Although the mean unbinding forces between α human Fc and IgG were higher than those between NCAM±VASE Fc, there was some overlap in the individual unbinding forces. Therefore a larger number of force-distance curves will be collected in future experiments to increase the number of NCAM or VASE homophilic unbinding events. As the adhesion between the Fc tag and capture antibody is likely to be greater than between NCAM or VASE proteins, it is likely that this method of antibody capture is reliable.

2.7.5 Addition of an NCAM homophilic binding peptide KYI did not reduce the size and frequency of unbinding events during an AFM experiment

In a previous AFM experiment measuring the interaction between NCAM±VASE, the mean unbinding forces following interaction were higher than the control interactions, but the unbinding event size distribution charts were similar for the two isoforms, indicating that there was nothing qualitatively different about the interactions. To test the specificity of the NCAM±VASE interactions, a peptide KYI, which has been shown to block full extracellular domain overlap in NCAM homophilic binding using SFA, was added to the AFM chamber (Fig. 2.21) (Johnson, Fujimoto et al. 2004).

1 mM KYI was incubated in the AFM chamber for 30 min following calibration, after which measurements were taken between the NCAM±VASE Fc proteins functionalised onto the cantilever and coverslip. The mean unbinding force of all interactions, which occurred between the cantilever and coverslip, was taken at different contact times (0–10 ms) and contact forces (0.5–1.5 nN). At least 100 force-distance curves were collected and analysed for each interaction.

The effect of the peptide on the mean unbinding event size for NCAM-NCAM and VASE-VASE was revealed by calculating it as a percentage of the control ‘no peptide’ measurements taken prior to peptide addition. For NCAM-NCAM, addition of KYI peptide decreased the size of unbinding events following 0.5 nN and 0 ms of contact by 20%. There was no effect at other contact parameters. For the VASE-VASE interactions, the peptide increased the mean unbinding forces by 50–85 % at three of the contact parameters (0.5 nN 0 ms, 0.5 nN 10 ms and 1.5 nN 0 ms).

Interestingly the frequency of force curves containing an unbinding event was the same for control ‘no peptide’ measurements and following peptide addition for both NCAM isoforms interactions. Typically in AFM protein adhesion blocking experiments, addition of a blocking peptide would result
in a decrease in the frequency of force curves with an interaction. As this was not the case in this experiment, the effect of the VASE interaction was deemed not to be specific and the effect of a scrambled version of the KYI peptide was not investigated. If the effect of the KYI peptide on the VASE unbinding force sizes was specific, the value would have decreased, indicating that VASE-VASE interactions were prevented and that the smaller sized P105-P105 unbinding events were the majority.

![Graph showing effect of 1 mM KYI peptide on unbinding force](image)

**Figure 2.21:** AFM experiment to measure the interaction between NCAM±VASE Fc in the presence of a homophilic binding peptide KYI from Ig III of NCAM.

To test the effect of a potential homophilic blocking peptide of NCAM (KYI), the interaction between NCAM±VASE Fc was measured by AFM and the mean unbinding events were compared to those in the presence of 1 mM KYI peptide. The peptide was incubated in PBS for 30 min prior to data acquisition. The effect of contact time and force was quantified and the experiment was carried out in PBS at 22°C with extend and retract velocities of 498 nm/s. **a)** Comparison of the effect of 1 mM KYI peptide addition on NCAM-NCAM and VASE-VASE adhesion measurements, as a percentage decrease from the control protein measurements taken directly before. **b)** Table to show the effect of 1 mM KYI peptide on the frequency of force-distance curves with an attractive unbinding event following the interaction between NCAM and VASE functionalised AFM surfaces. At least 100 force-distance curves were collected and analysed for unbinding events for each interaction. NCAM and NCAM-VASE homophilic adhesion was not blocked in AFM by the addition of 1 mM KYI peptide from NCAM Ig III.

Overall, the addition of the KYI peptide did not prove the specificity of the NCAM-NCAM and VASE-VASE protein interactions measured by AFM. It is plausible that the Ig III peptide did not block NCAM or VASE adhesion because this domain was not involved in adhesion and Ig I and Ig II were.
2.7.6 Lowering total protein concentration reduced the number of unbinding events following an NCAM±VASE interaction measured by AFM

Following the measurement of NCAM±VASE Fc protein interactions and the ineffectiveness of the KYI peptide in preventing specific adhesion, it was necessary to prove the specificity of the interactions by some other means. By decreasing the concentration of the adsorbed and captured proteins, but by maintaining the concentration of adsorbed P105 on the AFM surfaces, it was hoped that a decrease in the number of interactions would be seen. This would indicate that the interactions occurring were between the proteins present. The concentration chosen was decreased by an order of magnitude from 4 to 0.4 µg/ml. The Fc capturing α human Fc capturing antibody was used at a 1:2000 dilution (previously 1:200) and NCAM±VASE Fc was incubated at 0.4 µg/ml but the concentration of adsorbed P105 was maintained (3 mg/ml).

Table 2.2: Effect of lowering the concentration of captured NCAM±VASE Fc protein on the size and frequency of unbinding events following interaction in an AFM experiment.

<table>
<thead>
<tr>
<th>Interaction</th>
<th>Parameters</th>
<th>Mean Unbinding Force (pN)</th>
<th>Force curves with an interaction (%)</th>
<th>Mean Unbinding Force (pN)</th>
<th>Force curves with an interaction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCAM-NCAM</td>
<td>0.5 nN 0 ms</td>
<td>103.6±8.7</td>
<td>63</td>
<td>95.2±10.2</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>0.5 nN 10 ms</td>
<td>136.2±11.6</td>
<td>79</td>
<td>118.8±11.0</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>1.5 nN 0 ms</td>
<td>195.0±14.3</td>
<td>81</td>
<td>147.4±4.2</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>1.5 nN 10 ms</td>
<td>252.0±16.8</td>
<td>93</td>
<td>173.8±14.1</td>
<td>35</td>
</tr>
<tr>
<td>VASE-VASE</td>
<td>0.5 nN 0 ms</td>
<td>130.2±13.9</td>
<td>67</td>
<td>96.8±11.4</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>0.5 nN 10 ms</td>
<td>125.2±12.2</td>
<td>64</td>
<td>106.8±14.7</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>1.5 nN 0 ms</td>
<td>123.2±8.1</td>
<td>87</td>
<td>117.3±3.3</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>1.5 nN 10 ms</td>
<td>188.9±25.0</td>
<td>89</td>
<td>109.4±6.4</td>
<td>38</td>
</tr>
</tbody>
</table>

The mean unbinding forces were compared for the interaction between the two captured concentrations of NCAM±VASE Fc for a range of contact parameters (4µg/ml values taken from Fig. 2.18a). Upon decreasing the concentration of NCAM and VASE proteins, the frequency of force curves containing an unbinding event decreased (Table 2.2). For NCAM-NCAM and VASE-VASE interactions, the percentage frequency drop from the higher concentration to the lower was between 30 and 45%. This frequency drop indicated that the probability of single molecule unbinding events has increased and that the NCAM and VASE proteins present were responsible for the unbinding events (as opposed to P105-P105 interactions). For most contact parameters, the mean unbinding forces were lower and indicate that there were weaker unbinding events occurring, probably due to the weaker non-specific interactions between NCAM±VASE and P105 or P105-P105.
To test for specific adhesion between protein pairs, 1 mM KYI peptide was added into the AFM chamber and the mean unbinding force and frequency of force curves containing an interaction was quantified. For 0.4 µg/ml NCAM-NCAM control measurements, after 1.5 nN of contact force and 0 ms of contact time, the percentage of curves with an interaction was 32% and the mean unbinding force was 147±4 pN. After addition of 1 mM KYI peptide the mean unbinding force was statistically similar (155±7 pN) and the percentage of force curves with an interaction was 34%. For 0.4µg/ml VASE-VASE control measurements, in the same conditions, the percentage of curves with an interaction was 29% and the mean unbinding force was 117.3±3 pN. After addition of 1 mM KYI peptide the mean unbinding force was 180.7±7.4 pN and the percentage of force curves with an interaction was 27%. This increase in unbinding force between VASE proteins only in the presence of KYI peptide was interesting and was also in agreement with the higher concentration AFM measurements (Fig. 2.21a). This effect could be due to a non-specific peptide effect, which caused blocking of smaller interactions.

As was the case with the high concentration NCAM±VASE Fc interactions, there was no decrease in unbinding event size or frequency. Therefore adhesion between the proteins in AFM was not prevented by addition of the ‘blocking’ peptide KYI.

2.8 Probing the kinetics of the NCAM±VASE Fc single molecule interaction by dynamic force spectroscopy

2.8.1 Homophilic and heterophilic NCAM±VASE Fc single molecule interactions were identical in size

Following optimisation of the AFM method for measuring the mechanical force required to separate interacting NCAM±VASE Fc proteins, the adhesion between heterophilic interactions was quantified. This experiment was the first attempt to measure the interaction between NCAM and VASE proteins. In the previous experiments, measuring the adhesion between homophilic interactions of NCAM-NCAM and VASE-VASE, the AFM surfaces were functionalised separately and different cantilevers were used, with different spring constants. There was therefore the possibility of error since a direct comparison between the NCAM-NCAM and VASE-VASE interacting surfaces using the same cantilever was not possible.
Fig 2.22: Dynamic force spectroscopy experiments measuring the interaction between NCAM-NCAM, NCAM-VASE, VASE-VASE and VASE-NCAM at various retract velocities.

The homophilic and heterophilic interactions, between affinity captured NCAM and VASE Fc, were compared for a number of retraction velocities to probe its effect on unbinding force. The setpoint contact force was 0.5 nN, the cantilever forward velocity was 498 nm/s and the contact time was 0 s. a) A comparison of the mean unbinding force between NCAM and VASE homophilic and heterophilic protein interactions under varying retract velocities of the cantilever. b) A comparison of the mean unbinding event position between NCAM and VASE homophilic and heterophilic protein interactions under varying retract velocities of the cantilever. c) A comparison of the frequency of individual unbinding events between NCAM and VASE homophilic and heterophilic protein interactions under varying retract velocities of the cantilever. d) Table to show the percentage of retract force curves containing an attractive interaction. For interactions measured at each retraction velocity there were between 50 and 1000 force-distance curves that were collected and analysed. The large variation in the number of force-distance curves was due to a fixed pulling distance following interaction and a set experiment time for each set of force-distance curves at a particular velocity.

An AFM experiment was therefore designed to quantify all interactions between NCAM and VASE using 2 cantilevers and 2 coverslips, each functionalised with low concentration NCAM or VASE (0.4 µg/ml incubated) by the previous adsorption method in the presence of P105. Cantilever ‘1’ was functionalised with NCAM Fc, cantilever ‘2’ with VASE Fc, coverslip ‘A’ with NCAM and coverslip ‘B’ with VASE. AFM interactions were quantified in succession as follows:
• Cantilever ‘1’ (NCAM): Coverslip ‘A’ (NCAM) = Homophilic NCAM-NCAM
• Cantilever ‘1’ (NCAM): Coverslip ‘B’ (VASE) = Heterophilic NCAM-VASE
• Cantilever ‘2’ (VASE): Coverslip ‘B’ (VASE) = Homophilic VASE-VASE
• Cantilever ‘2’ (VASE): Coverslip ‘A’ (NCAM) = Heterophilic VASE-NCAM

Spring constants were determined following each laser alignment. Mean unbinding forces were compared following 0.5 nN of contact force and 0 ms of contact time at 22°C in PBS. At a cantilever retraction velocity of 498 nm/s there was no significant difference between the mean unbinding forces following interaction of NCAM-NCAM, VASE-VASE and both orientations of NCAM-VASE (Fig. 2.22a, see appendix for example force-distance curves).

All forces required to separate the homophilic and heterophilic interactions of proteins were approximately 120 pN for a loading rate of approximately 1200 pN/s. In the previous AFM experiment measuring the adhesion between NCAM-NCAM and VASE-VASE at this concentration (Table 2.2) the mean unbinding forces were approximately 100 pN. This discrepancy in size indicates that there is variation between experiments probably due to error in the thermal tune method of spring constant assignment. As noted in previous frequency distribution curves, there were still interactions occurring between the P105 layers during the AFM experiment. Therefore these smaller forces may have skewed the true mean unbinding force corresponding only to NCAM-NCAM or VASE-VASE interactions.

The distance between the tip and sample, following interaction and cantilever retraction, where an unbinding event occurred, was calculated from each retract force-distance curve. The mean unbinding event position was calculated from the retract force curves following the interactions between NCAM and VASE proteins (Fig. 2.22b). For NCAM-NCAM and VASE-VASE these distances were 30 nm. Interestingly during SFA measurements of NCAM ectodomain homophilic binding, the onset of repulsion as the protein layers were brought into apposition occurred at approximately 37 nm and de-adhesion events occurred following 31 and 39 nm of surface retraction (Johnson, Fujimoto et al. 2004). Not only does this agreement in distance indicate that the unbinding event is plausibly occurring around the length of the expected protein size, but it also corroborates these previous findings by the SFA. For the heterophilic interactions between NCAM-VASE and VASE-NCAM, the unbinding events occurred following 25 and 35 nm respectively of cantilever raising. It is unlikely that interchanging NCAM or VASE functionalisation between the cantilever and coverslip would affect the tip-sample distance of the heterophilic unbinding event position; therefore this difference may not be of significance.
Important thermodynamic information may be extracted from dynamic force spectroscopy data from AFM measurements. Dynamic force spectroscopy involved the measurement of unbinding forces between NCAM±VASE Fc functionalised AFM surfaces at varying cantilever retraction velocities. By varying the velocity of retraction, the unloading rate applied to the interaction was varied to affect the length scales and relative heights of the energy barriers to be overcome before the unbinding of the interaction. Cantilever retraction velocities were varied between 49.8 and 498 nm/s, over an order of magnitude. Higher velocities were not used, to limit hydrodynamic effects on the cantilever interactions.

The mean unbinding force was taken for each cantilever retraction velocity for all interactions following 0.5 nN and 0 ms of contact (Fig. 2.22c). Approximately 50 force-distance curves were collected and analysed for attractive interactions for slower retraction velocities including 50 and 100 nm/s. For retraction velocities of 205 and 388 nm/s, the number of force curves collected was approximately 200. The discrepancy between the numbers of acquired force-distance curves was due to a fixed cantilever pulling distance following interaction, regardless of the cantilever retraction velocity. Therefore one tenth of the force-distance curves were collected at 50 nm/s compared to 500 nm/s. In general there was a slight increase in unbinding forces as the retraction speeds increase. However to gain a more conclusive insight into the relationship between retraction velocity and the size of unbinding events, further data acquisition is required at lower velocities.

It is interesting to note that the frequency of force curves containing an unbinding event for all interactions and retraction velocities were compared and revealed that most had less than 30%. This indicates that there is approximately an 80% probability that the unbinding events were between single molecules (Fig.2.22d) (Brogan, Shin et al. 2004).

2.8.2 Increasing the unloading rate during the interaction of NCAM±VASE Fc single molecules increased the unbinding force of adhesion

The single molecule AFM measurements between NCAM and VASE Fc proteins revealed that their homophilic and heterophilic binding strengths were similar and that there may be a positive linear correlation between the speed of cantilever retraction and the force required to unbind homophilically and heterophilically interacting NCAM±VASE proteins (Fig. 2.22a). To test this, further dynamic force spectroscopy analysis was carried out to probe the energetic properties of the homophilic interactions between NCAM and NCAM-VASE protein pairs.
Fig 2.23: Dynamic force spectroscopy analysis to determine the relationship between the unloading rate of the cantilever retraction and the unbinding event force for homophilic NCAM and VASE interactions. Data from the AFM experiment in Fig. 2.22 was collated for each cantilever retraction velocity and individual unbinding event unloading rates were assigned and compared against the unbinding event force. a) NCAM-NCAM homophilic binding dynamic force spectroscopy plot to show the natural logarithm (ln) of the individual unbinding event unloading rate vs. the unbinding event force. b) VASE-VASE NCAM homophilic binding dynamic force spectroscopy plot to show the natural logarithm (ln) of individual unbinding event unloading rate vs. the unbinding event force. c) Comparison of the mean natural logarithm of the unloading rate for each cantilever retraction velocity vs. the corresponding mean unbinding force.
The unbinding forces previously measured allowed a comparison of the relative strengths of adhesion but it is worth noting that these values are due to the dependence of the unbinding force on the unloading rate applied to the complex. The intricacies of this complex relationship are lost when comparing the velocity with the mean unbinding force. There was only a slight positive linear correlation between the mean unbinding forces as the retraction velocity increased following interactions between NCAM±VASE proteins, (Fig. 2.22c).

In this experiment, using the data from Fig. 2.22, the proteins are thought of as compressible polymers with a specific elasticity dependent upon their properties. The system therefore consists of two springs in series, the cantilever and the interacting protein. Therefore, the actual spring constant, which determines deflection and hence the protein adhesive forces, is a combination of the protein and cantilever spring constant, or \( K_{\text{eff}} \). \( K_{\text{eff}} \) was calculated as follows, where \( K_c \) is the effective spring constant, \( K_s \) is the slope of the pulling off region before the unbinding event (Fig. 2.3 point 3) and \( K_c \) is the assigned spring constant:

\[
(K_{\text{eff}})^{-1} = (K_s)^{-1} + (K_c)^{-1}
\]

The effective spring constant (\( K_{\text{eff}} \) in units of pN/nm) is the actual spring constant for the interaction and relates the force of cantilever deflection vs. the distance over which it occurs. Once this was known, \( K_{\text{eff}} \) was multiplied by the cantilever retraction velocity to yield the unloading rate of the interaction. The natural logarithm (\( \ln \)) of the unloading rate was plotted against the individual unbinding event force to determine the relationship between the factors (Fig. 2.23a, b).

On comparison of these curves with the retraction velocity plot vs. mean unbinding force (Fig. 2.22c) it was clear that plotting vs. individual unloading rates revealed more marked differences in the retraction velocity effects.

There appeared to be a positive linear correlation between the loading on the interaction and the size of the unbinding force for both the NCAM-NCAM and VASE-VASE interactions. The data scatter seen in both plots was typical of those seen in dynamic force spectroscopy experiments. The linear equation of the line for the unbinding of NCAM-NCAM was \( y=39.8x–158.2 \) whereas the line for VASE-VASE was \( y=45.0x–190.8 \). The x-intercept of the line was 4.2, which corresponded to an unloading rate of 67 pN/s and indicated that below this threshold there would not be an unbinding event between NCAM-NCAM or VASE-VASE following interaction (the rupture would fail elsewhere).

To determine whether there were multiple energy barriers during the unbinding of NCAM-NCAM and VASE-VASE proteins, the mean unbinding forces and unloading rates were pooled and compared for each retraction velocity (Fig. 2.23c). The unbinding profile for NCAM-NCAM and VASE-VASE was
similar on first glance. Between the natural logarithm of the unloading rates of 2.5–5.5, the mean unbinding force increased linearly but increased dramatically between y=5.5–7. This indicated that there may be another energy barrier at high unloading rates which leads to higher unbinding forces. To determine whether this was correct, increased unloading rates would need to be applied to the protein interactions. This was not attempted due to the resulting hydrodynamic effects which would have occurred if velocities above 1 μm/s were used.

2.9 Design for measuring the adhesion between NCAM±VASE monomers

2.9.1 Improved SMFS experimental design using PEGylated conjugates to minimise non-specific binding

The work carried out in this section measured the adhesion between NCAM proteins and the effect on the insertion of the VASE sequence. To do this single molecule AFM was carried out to measure the force required to separate interacting proteins. Proteins were functionalised onto the AFM surfaces, the cantilever and coverslip, by adsorbing an antibody, α human Fc, which captures Fc tags. NCAM±VASE Fc were then incubated on these surfaces and bound via their C-terminal Fc tags to the antibodies. The concentration of NCAM±VASE Fc on the surfaces was sufficiently lowered to give approximately an 80% probability that the unbinding events occurring, following the interaction between the proteins, were between single molecules.

During these measurements, the surfaces were blocked by the adsorption of a non-ionic surfactant, P105, to prevent large non-specific interactions between cantilever and coverslip. Due to the method of surface functionalisation, using capture of the Fc tag on NCAM±VASE, there is no way of knowing whether the interactions measured by AFM are between two sets of dimers or single molecules. A more elegant method of measuring the adhesion would involve AFM surface blocking by the covalent coupling of polyethylene glycol (PEG) conjugates and capture of single molecules of NCAM±VASE. PEGylated conjugates have been used in single molecule AFM experiments previously and successfully block non-specific interactions due to their highly branched structure and covering of functionalised surfaces (Kawakami, Byrne et al. 2006).

Typically, a bifunctional PEGylated conjugate is used to covalently link glass or cantilever and the other end to the C-terminal of the protein. This would allow specific protein orientation and in the case of NCAM, would provide a good model for measuring NCAM adhesion in a ‘model membrane’ environment. The PEGylated conjugate flexibility may perhaps allow NCAM motility to bind in more physiological conditions. To measure the adhesion between NCAM±VASE proteins in this way, a bifunctionalised PEGylated conjugate was chosen with a terminal 3-(2-pyridyldithio) propionyl (PDP) group and terminal N-hydroxysuccinimide (NHS ester) (PDP-PEG-NHS) (Molecular weight = 1635 Da).
The length of the extended PEGylated conjugate was 6nm, which is approximately a fifth of the length of NCAM (Johnson, Fujimoto et al. 2004).

To attach the PEGylated conjugate to the cantilever or glass coverslip, the surface was firstly silanised for the addition of primary amine groups, and then reacted (pH > 7) with the PEGylated conjugate to allow the hydrolysis of its NHS ester and attachment via amine coupling (Fig. 2.24). Mono NTA was covalently attached to the PEGylated conjugate following removal of the PDP group and formation of a disulfide bond. NCAM±VASE can be attached via affinity to a C-terminal His tag to a nitriloacetic acid group (NTA) in the presence of Ni²⁺. This method has been optimised previously for measuring the adhesion between His-tagged S-layer proteins (Tang, Ebner et al. 2009). The affinity between NTA and the His tag has been found to be suitable for capturing His tagged proteins and measuring their adhesion by AFM (Kienberger, Kada et al. 2000), (Verbelen, Gruber et al. 2007).

![Diagram](image)

**Figure 2.24: Schematic to show the proposed capture of Histidine tagged NCAM±VASE proteins by a covalently linked bifunctionalised PEGylated conjugate (PDP-PEG-NHS) in a single molecule AFM experiment.**

The capture of NCAM±VASE to AFM surfaces can also be carried out using a bifunctionalised PEGylated conjugate which covalently couples to an amine functionalised cantilever. The ‘N-terminus’ of the PEGylated conjugate provides a protected thiol functionality which can be reacted with a single nitriloacetic acid (NTA) molecule. NTA coordinated Ni²⁺ and affinity binds to two histidine residues in the C-terminal 6-His tag of the NCAM or VASE protein. This functionalisation technique provides an alternative to the adsorption technique used previously.

By using this new method of surface functionalisation there is an opportunity to add in ‘capped’ PEGylated conjugates, which are ‘blocked’ by the addition of a short peptide sequence instead of mono NTA. These capped PEGylated conjugates could therefore be mixed in at different ratios with reactable mono NTA coupled PEGylated conjugates to decrease the overall amount of captured NCAM without decreasing the area of blocked surface. This would enable an easy method for decreasing the concentration of surface NCAM and therefore the percentage of force curves with an
unbinding event. This would give a greater probability that the interactions were between single molecules.

2.9.2 Synthesis and purification of CLL peptide by SPPS and HPLC
To measure the adhesion between NCAM±VASE His proteins captured by affinity to NTA functionalised PEGylated conjugates, test tube reactions were carried out to optimise the reaction of the conjugated PEG linker with mono NTA and CLL. Firstly, a short three amino acid peptide, CLL, was synthesised using solid phase peptide synthesis (SPPS) to later react with the PEGylated conjugates to form a capped non-reactive PEGylated conjugate. The CLL peptide was synthesised with a C-terminal rink amide and the N-terminus was acetylated to create an uncharged version. This peptide could be used to react with the PEGylated conjugate to give a capped version for surface functionalisation and blocking.

2.9.3 The bifunctional PEGylated conjugate reacted with thiol and amine group containing entities
To test the chemistry reactions of the PEGylated conjugate which would be used in the surface functionalisation prior to AFM measurements of NCAM±VASE His, test tube reactions were carried out. Prior to this, the NHS-PEG-PDP conjugate, CLL peptide and mono NTA compounds were characterized by liquid chromatography-mass spectrometry (LC-MS) to determine their retention time and molecular masses (Fig.2.25).

Briefly a 20 μl aliquot of each reaction sample was loaded into the LC-MS (mobile phase 0.1% FA, MeOH/dH2O). The LC-MS trace for NHS-PEG-PDP, which had been dissolved in water prior to the run, was separated into a number of peaks (between 9 and 9.5 min of retention time) which were assigned as follows: reduced and hydrolysed PEGylated conjugate, without the PDP and NHS groups, the hydrolysed PDP-PEG, as the NHS group is not present, a reduced NHS-PEG with no PDP group and most importantly, the NHS-PEG-PDP conjugate. The most prevalent compounds following separation were the full-length PEGylated conjugate and the hydrolysed PEG without the NHS group. It is worth bearing in mind that a driving force for attachment of the PEG to the amine functionalised surfaces will be required because half of the PEGylated conjugates may already be hydrolysed following dissolving in water. Following LC-MS, CLL was found as a single peak at approximately 8 min of retention time and mono NTA at about 1.3 min.
Figure 2.25: Proof of principle chemical reactions between bifunctionalised PEGylated conjugate (PDP-PEG-NHS) and truncation peptide CLL, monoNTA and controls for use in AFM NCAM1-VASE His capture experiment.

LC-MS traces to show the retention time of the starting materials (NHS-PEG-PDP, CLL peptide, monoNTA) and reactions A, B and C against their UV absorbance. All reactions were carried out at 2, 4 and 16 hr at RT. Reactions were complete after 2 hrs and so this data were chosen as representative for the curves above. Reaction A contains 1 mM MonoNTA, 1 mg/ml PDP-PEG-NHS and TCEP, reaction B contains CLL peptide, 1 mg/ml PDP-PEG-NHS and TCEP) and reaction C contains CLL peptide and 1 mg/ml PDP-PEG-NHS. Reactions were carried out in buffer 'A' (300 mM NaH₂PO₄, 2mM EDTA, pH 7.5). Table to show the assigned LC-MS traces with information regarding the molecular mass and peak compound assignment.
The next step was to react these starting materials together to test the reaction conditions for later use in AFM functionalisation and to prove the functionality of the PEGylated conjugate. Mono NTA and CLL were attached to the PDP end of the PEGylated conjugate under reducing conditions (TCEP) to remove the PDP group for 2 hours at RT (Fig 2.25, reaction A, reaction B respectively).

The success of these reactions was tested by LC-MS. The ‘Reaction A’ trace shows two peaks which corresponded to the formation of a mono NTA clipped PEGylated conjugate (with no NHS) and the full length unreacted PEGylated conjugate. There was no unreacted mono NTA therefore this was the limiting factor in the production of NTA clipped PEGylated conjugate. The ‘Reaction B’ trace showed two peaks, which correspond to some unreacted peptide and the CLL clipped PEGylated conjugate (with NHS). ‘Reaction C’ showed the reaction between mono NTA and the PEGylated conjugate under non-reducing conditions. Overall these test tube reactions indicated that the synthesis of the PEGylated conjugate would be possible following the methodology used. Before AFM experiments, it was necessary to attempt to silanise the AFM surfaces and attach the PEG linker before addition of the NTA moiety and His tagged NCAM±VASE proteins or CLL peptide to confirm surface functionalisation. Unfortunately due to time constraints this was not optimised for inclusion in this thesis.

2.10 Conclusion
The aim of this chapter was to measure the adhesion between single NCAM±VASE proteins using a single molecule force spectroscopy application on an AFM. To do this the validity of the AFM approach was investigated by carrying out careful AFM calibration. PicoNewton force sensitivity was achieved following elucidation of calibration constants and determination of cantilever spring constants prior to measurements between a cantilever tip and sample (Fig. 2.4). By collecting a series of extension and retraction force-distance curves measuring the interaction between a tip and sample, it was determined that the thermal noise was approximately 15 pN.

Prior to measurements between the proteins of interest, the interaction between biotin and avidin was investigated using AFM to optimise parameters and compare findings with those in the literature. This was investigated using adsorption of biotinBSA and avidin capture onto a biotinBSA surface. However, during this experiment it became apparent that large adhesive interactions were occurring during the experiments, which were not prevented by addition of free avidin to block all biotin binding sites (Fig. 2.8). Therefore the underlying interactions between the tip and sample were investigated and it was found that ‘unblocked’ surfaces were able to interact to yield large unbinding events, which may have been visualised during the biotin-avidin experiment. The effect of coating the surfaces with a non-ionic surfactant, P105, was investigated and addition above its
critical micelle concentration prevented large interactions between the coated tip and sample (Fig. 2.11)

The interaction between biotin and avidin was measured again using AFM, in the presence of P105 coated surfaces. At a 1.5 nN contact force, the mean unbinding force was statistically higher than for the interaction where avidin was flooded into the chamber to block biotin (Fig 2.13). The loading rates of the interactions between biotin and avidin were found to be between 20000 and 25000 pN/s, which resulting in a mean unbinding force of approximately 150 pN. These values corroborate findings in the literature.

Since the proof of principle AFM experiment was successful, an in vitro model was developed to measure the adhesion between NCAM±VASE Fc proteins. This design involved the adsorption of antibodies with affinity to the Fc tag (from a human immunoglobulin) onto a glass coverslip and Si3N4 cantilever in the presence of P105 to coat surfaces and prevent non-specific tip-sample interaction. NCAM±VASE proteins which had C-terminal Fc tags would then be captured onto the antibody coated surfaces in preparation for the AFM experiment (Fig 2.14). Proteins were produced from the transient transfection of Cos-7 cells and purified via protein A affinity (Fig 2.15). An ELISA determined that α Fc capture of NCAM Fc proteins in the presence of P105 was plausible due to binding of an antibody, α N16, against a surface epitope of native NCAM Ig III (Fig 2.17). It is possible that presence of the surfactant had an adverse effect on NCAM and NCAM-VASE structure. Although the ELISA under these conditions suggested that NCAM Ig III was accessible for binding, there may have been structural differences in other domains. To test this, a spectroscopic technique such as circular dichroism or differential scanning calorimetry could be used to assess any differences in NCAM±VASE structure in the presence of surfactant.

Following the design and preliminary testing of this in vitro adhesion model, AFM surfaces were functionalised and the adhesion between NCAM±VASE proteins were measured and compared with interactions between NCAM±VASE and control proteins (Fig. 2.18). The mean homophilic interactions between NCAM and NCAM-VASE proteins were generally higher than for the heterophilic interactions with IgG and α Fc. It was also noted that the frequency of retraction force curves with an unbinding event was between 60 and 90% which indicated that it was statistically improbable for the interactions to be between single protein pairs (Table 2.1). The adhesive strength of α Fc to an Fc domain (from IgG) was found to be higher at all forces and contact times tested indicating that it was unlikely that the interactions measured between functionalised NCAM±VASE surfaces were due to rupture of the Fc tag from α Fc (Fig 2.20).
Addition of a peptide from NCAM Ig III, KYI, did not reduce the percentage of retraction force curves containing an unbinding event. Therefore specificity of the interaction between NCAM and NCAM-VASE functionalised surfaces could not yet be proven (Fig. 2.21). The concentration of α Fc and NCAM±VASE Fc captured to the AFM surfaces was reduced by an order of magnitude (to 0.4 μg/ml) and this decreased the percentage of force curves with an unbinding event to between 20 and 40% (Table 2.2). In most instances less than 30% of retraction force curves contained an unbinding event. Therefore there was an 80% probability that these interactions were between single protein pairs (Brogan, Shin et al. 2004).

Following optimisation of the experiment, further force-distance curves were attained to measure the interaction between homophilic and heterophilic NCAM and NCAM-VASE interactions (Fig. 2.22). It was found that the mean unbinding forces for the separation of all of these interactions were statistically similar at 120 pN, after a contact force of 0.5 nN and a cantilever retraction velocity of 498 nm/s. The modal unbinding forces for each interaction were also similar. For these interactions the mean event position, that is the distance by which the cantilever was raised prior to an unbinding event, was between 25-35 nm, which corresponded approximately with the length of a full extracellular domain overlap of NCAM (31 nm) and partial overlap via Ig I and Ig II (39 nm) (Wieland, Gewirth et al. 2005).

This is the first evidence which suggests that on a single molecule level there is no difference in adhesive strength between NCAM±VASE Fc captured proteins. Therefore the role of insertion of the VASE sequence into NCAM may not be adhesive. The 120 pN unbinding force between NCAM±VASE occurred with a loading rate of approximately 1200 pN/s. In comparison Wieland et al found that for a loading rate of 1152 pN/s, the unbinding force between full length NCAM proteins was either 49±8 or 76±15.5 pN. By comparing the distribution frequency of binned unbinding events, it was possible that there were two independent interactions occurring during NCAM-NCAM and VASE-VASE homophilic binding, which became more apparent with increasing retraction velocities (and hence loading rates) (Fig. 2.26). These modal unbinding forces were approximately 100 and 150 pN for NCAM-NCAM and VASE-VASE interactions with 498 nm/s retraction velocity (1200 pN/s loading rate). These values were double the size of those found in the experiments carried out by Wieland et al (Wieland, Gewirth et al. 2005).

It is statistically probable that these interactions were between single molecule NCAM and NCAM-VASE proteins, but this does not take into account the method of capture. The Fc tag from IgG is dimeric in IgG and therefore an Fc tag from one protein can bind homophilically to another. Therefore NCAM±VASE Fc proteins may have dimerised via the Fc tag in solution and been captured
onto the α Fc antibody as dimers, since the antibodies have two antigen binding sites. Therefore although the SMFS interactions may have been between single molecules, statistically the NCAM±VASE proteins may already be dimeric before surfaces are brought into apposition. This could account for why NCAM±VASE Fc proteins exhibit double the adhesive force of NCAM His proteins when measured by AFM.

![Graph](image.png)

**Figure 2.26**: AFM experiment to compare the frequency of binned unbinding events following the homophilic interaction of NCAM±VASE Fc proteins after 0.5 nN and 0 s of contact.

This data was taken from the same experiment as Fig. 2.22. All attractive unbinding events for each interaction were pooled and sorted into force bins with a width of 50 pN. The frequency compared to the total number of unbinding events was presented as frequency distribution curve. a) The frequency of binned unbinding events following contact between a cantilever and coverslip coated with affinity captured NCAM Fc, following a setpoint contact force of 0.5 nN and contact time of 0 ms. b) The frequency of binned unbinding events following contact between a cantilever and coverslip with NCAM-VASE Fc following a setpoint contact force of 0.5 nN and contact time of 0 ms.

To test whether Fc tags on the NCAM±VASE Fc proteins accounted for the larger adhesive strengths, an alternative *in vitro* model was suggested, which involved His-tagged proteins and capture to covalently bound PEGylated linkers terminated with NTA. Preliminary experiments suggested that the coupling chemistry proposed was suitable but experiments were not carried out due to time constraints (Fig 2.25) (Tang, Ebner et al. 2009).
Chapter 3 Quantification of the adhesion between single
NCAM±VASE expressing cells using Atomic Force Microscopy

Following single molecule force measurements between NCAM±VASE proteins a new type of AFM, single cell force spectroscopy, was used to quantify the adhesion between single cells expressing NCAM and NCAM-VASE. The work described in this chapter involves:

- The use of force spectroscopy in measuring single cell adhesion
- Development of an assay to measure adhesion between cells using AFM
- Characterisation of NCAM±VASE expressing cells for use in the AFM experiment
- Calibration of the AFM
- Measurement of the adhesion between single control and NCAM expressing cells
- The effect of VASE insertion on the adhesion of NCAM cells
- The effect of the actin depolymerising agent latrunculin B on cell adhesion
- The effect of putative blocking peptides on NCAM±VASE cell adhesion
3.1 Single cell adhesion measurements using AFM

The aim of this research was to elucidate the effect of the insertion of the VASE sequence on the homophilic binding strength of NCAM. In the previous chapter this was assessed *in vitro* by measuring the adhesion between NCAM and NCAM-VASE functionalised surfaces using single molecule force spectroscopy using an AFM. Preliminary experiments using affinity capture and adsorption of Fc-tagged proteins revealed that large, non-specific interactions were occurring between the cantilever tip and coverslip surface, unless a surfactant layer was present. Although non-specific unbinding events were minimised in the presence of pluronic 105 (P105), small interactions (less than 50 pN) accounted for a number of unbinding events during protein measurements by AFM. Preliminary data indicated that the measurements between NCAM and NCAM-VASE proteins were on a single molecule (80% probability) and the forces required to separate them were roughly equal.

To gain more confidence in this result, a new surface functionalisation method was suggested to covalently couple bifunctional PEGylated conjugates and affinity capture His tagged NCAM±VASE proteins to AFM surfaces. Preliminary data confirmed the capability of the conjugate for this purpose, but experiments were not continued due to time constraints.

Adhesion measurements between cells expressing NCAM±VASE have indicated that VASE cells segregated from those expressing NCAM (Chen, Haines et al. 1994). The authors inferred that insertion of the VASE sequence into NCAM was sufficient for this response, but did not postulate on its effect on affinity. An explanation for this segregation could be that the affinity between VASE proteins expressed on the surface of the cells is greater than the affinity between NCAM and NCAM-VASE. If a VASE cell were to encounter an NCAM cell it could bind, but if a VASE cell came in contact with the VASE cell it would bind preferentially. The benefit of measuring protein adhesion in cells is that heterophilic interactions are supported and NCAM or VASE can form native oligomeric complexes, which is not possible in single molecule experiments (as seen in Chapter 2). The problem with quantifying adhesion by cell aggregation assays is that adhesion between cells is averaged by the size and number of aggregates.

For this reason, the role of the VASE sequence on NCAM adhesion in *vivo* was investigated using single cell AFM. Single cell force spectroscopy is a relatively new AFM application which has been used since 2000 and measures the adhesion between two cells; one cell is bound to the cantilever and is termed the ‘probe cell’ and another is grown in the AFM chamber or Petri dish and is termed the ‘adherent cell’ (Benoit, Gabriel et al. 2000). Adhesion between the two cells is measured using the method of cantilever deflection, which was described in the previous chapter. Briefly a laser is
pointed at the tip of the cantilever and reflected onto a photosensitive diode, which records the changes in cantilever deflection during the extension and retraction of the cantilever (Fig 3.1a).

All single cell adhesion measurements were carried out on a JPK CellHesion® 200 module fitted on an Axiovert 200M inverted microscope for optical imaging. This instrument was on a short-term loan from JPK Instruments for 6 months. The instrument was designed specifically to determine the details of cell separation over long-range distances (up to 100 μm) controlled by a piezoelectric scanner.

The cantilevers used in single cell AFM are tipless to prevent damage to the probe cell and to allow a greater surface area for cell attachment.

Figure 3.1: Schematic to show a single cell force spectroscopy experiment using an AFM to measure the adhesion between single cells.

Two cells are brought into apposition (one on the cantilever termed the probe cell, the other adhered to a Petri dish). The force required to separate the cells is taken from the deflection of the cantilever when it is raised following contact between the cells. a) A cantilever is functionalised with a probe cell and a laser is aligned to its tip. This reflects onto a photodiode which records the deflection position of the cantilever. b) Voltage applied to a piezo controls the Z-position of the cantilever to lower the probe cell towards the adherent cell. c) The cantilever is lowered until contact is made between the probe cell and adherent cell. This continues until a setpoint contact force is reached. d) After a set contact time the cantilever is raised and the deflection of the cantilever is measured and plotted against the tip-sample position. The cantilever is raised to the starting position approximately 100 μm above the surface to fully separate the cells. Single cell AFM can be used to measure the adhesion between two interacting cells.
During single cell force spectroscopy a probe cell is attached onto the cantilever tip in situ (See section 3.4.1) and lowered onto an adherent cell for adhesion measurements (Fig. 3.1b). The cantilever Z-axis movement is piezo controlled and can encompass long-range distances of up to 100 μm. When the probe cell comes in contact with the adherent cell, the cantilever tip begins to positively deflect until an arbitrary contact force is reached (Fig. 3.1c). The cells then remain in contact for a set time before the cantilever is raised (Fig. 3.1d). During the initial 10 μm of cantilever retraction, the cantilever deflects negatively, indicating that the cells remain in contact. This continues until the maximal cantilever deflection is reached. A series of major rupture events occur between the cells and the cantilever begins to deflect positively before returning to its starting position, by which time the cells have been separated.

The deflection of the cantilever during the separation of two cells can therefore be used to quantify the strength of their adhesion. To measure the adhesion between cells expressing NCAM±VASE, and to determine whether there was a difference in their in vitro adhesion, an AFM assay was designed following extensive cell characterisation.

3.2 Characterisation of cells for use in single cell adhesion experiments by AFM

To test whether insertion of the VASE sequence increased NCAM cell adhesion, a series of model NIH 3T3 mouse embryonic fibroblast cells were used. NIH 3T3 cells express very low levels of NCAM-140, are adherent and synthesise and secrete their own extracellular matrix (Ramanathan, Wilkemeyer et al. 1996). NIH 3T3 were previously stably transfected with NCAM-140 in the laboratory of Prof. F. Walsh (see Material and Methods) and were termed ‘N24’ cells. The NCAM-140 construct contained a signal sequence for direction of the protein to the cell membrane, and the transmembrane domain for membrane localisation. The N24 cell line has been used routinely in the Saffell lab for a number of years (Saffell, Walsh et al. 1994). Recently Saffell group member Dr M. Delves created another NIH 3T3 cell line by stably transfecting it with NCAM-VASE-140 to yield a monoclonal V140G cell line, which expressed statistically similar levels of NCAM-VASE as NCAM in N24 cells (see section 3.2).

These three cell lines, NIH 3T3 cells, expressing low NCAM levels, N24 cells and V140G cells, expressing similar levels of NCAM and NCAM-VASE protein, formed the basis of a single cell AFM assay to determine differences in cell adhesion caused by:

- Transfection of NCAM into control cells
- Insertion of the VASE sequence into NCAM

It was necessary to characterise the model cells before AFM measurements were carried out.
3.2.1 Transfected NIH 3T3 cells expressed similar levels of surface NCAM and NCAM-VASE by immunostaining

Before using any cells for adhesion measurements by AFM it was important to ascertain their homology. The NCAM±VASE-140 protein is inserted into the cell membrane and extends its homophilic binding Ig-like domains into the extracellular space. These domains can be targeted using surface immunostaining with the α N16 antibody, described in the previous chapter, which binds to Ig III of NCAM. It was used to test the expression levels and distribution pattern of surface NCAM±VASE on all three cell types (Fig. 3.2).

![DAPI, α N16, TritC Phalloidin images for 3T3, NZ4, V140G cells](image)

**Figure 3.2:** NCAM±VASE surface expression on transfected and control NIH 3T3 cells by immunostaining.
Fluorescent microscope images to show the expression and localisation of NCAM on 3T3, NZ4 (expressing NCAM) and V140G (expressing NCAM-VASE) NIH fibroblast cell lines, as well as fibroblast morphology. The DAPI images show nuclear staining; α N16 stains both NCAM and NCAM-VASE and TritC phalloidin binds to polymerised actin. NCAM and VASE transfected NIH 3T3 cells showed similar expression levels, distribution patterns and morphology.

Briefly cells were plated at low density and incubated for 16 hours at 37°C before fixation with 4% paraformaldehyde and gentle but extensive rinsing with PBS. Cells were blocked and stained with α N16 then incubated with a secondary FITC antibody. Cell shape was determined by co-staining with TRITC phalloidin, which binds to filamentous actin (F-actin), following membrane permeabilisation.
with MeOH. Staining with TRITC phalloidin revealed bundles of F-actin, which extended throughout all three cell lines in a similar distribution. Variation in morphology was typical of the NIH 3T3 cell line but there seemed to be no effect of NCAM−VASE transfection. Staining with α N16 revealed extremely low levels of NCAM present on the surface of NIH 3T3 cells. Expression of NCAM and NCAM-VASE on the surface of N24 and V140G cells respectively was similar. The level of fluorescence and its distribution appeared to be similar, with NCAM and NCAM-VASE expressed across the surface of N24 and V104G cells in a homologous fashion.

Overall the expression level of NCAM and NCAM-VASE in transfected NIH 3T3 cells was similar and had no qualitative effect on the cell lines. In tandem, in three independent experiments, the clonality of the cells was assessed to determine what percentage of N24 and V140G cells expressed NCAM−VASE. To do this all three cell lines were co-stained with α N16 and DAPI and the percentage of expressing cells was calculated from the 200 taken. This revealed that 97.3±1.2% of N24 cells expressed NCAM and 94±2% of V140G cells expressed NCAM-VASE. Using statistical analysis these expression levels were found to be similar, and indicated that there was less than a 5% chance of using a non-expressing N24 or V140G cell in the cell adhesion measurements.

3.2.2 Transfected NIH 3T3 cell lines expressed similar levels of surface NCAM and NCAM-VASE by ELISA

Although the percentage of N24 and V140G cells expressing NCAM and NCAM-VASE respectively was high, the amount of surface protein had to be compared. Prior to measuring the adhesion of NCAM and NCAM-VASE expressing single cells, it was necessary to quantify precisely the level of surface NCAM−VASE protein on the cells. The precision was necessary as it has been found that a slight difference in the surface concentration of NCAM was sufficient to dramatically increase their neurite outgrowth promoting capability (Doherty, Fruns et al. 1990). Therefore it is plausible that a similar threshold effect may be seen on the adhesion of cells expressing NCAM. The V140G cell line was created from a single transfected cell which was chosen from a polyclonal cell pool to match the surface levels of NCAM on the N24 cell line. Following a number of years of storage, it was essential to quantify the amount of surface NCAM and NCAM-VASE on N24 and V140G cells using an ELISA (Fig. 3.3).

50000 NIH 3T3, N24 and V140G cells were plated on pre-treated PLL and fibronectin 96 well plates so that 16 hours after incubation they would form a confluent monolayer. Cells were then fixed, as previous, blocked and stained with α N16 before incubation with a secondary HRP conjugated antibody. After reaction with BM blue POD substrate, the resulting coloured product was quantified by reading the absorbance at 370 nm, which corresponded to the amount of bound antibody and
hence surface NCAM±VASE. Before comparison the cell number was determined from an MTS assay of a duplicate 96 well plate of cells. Raw optical density values were normalised against these cell number controls (and were found to be within 5% of each cell type in each experiment). The normalised expression values were compared with negative controls for each cell type, which consisted of a ‘no primary’ where no α N16 was present. This value was termed the ‘blank’ and was deducted from the normalised data values to give the relative NCAM expression on each cell type.

![Bar chart showing relative NCAM expression for 3T3, N24, and V140G cell types.](image)

**Figure 3.3: Cell ELISA to show the levels of surface NCAM±VASE on transfected and untransfected NIH 3T3 cells.** The amount of surface NCAM on N24 and NCAM-VASE on V140G cells was compared with control NIH 3T3 cells by ELISA, using an NCAM antibody (α N16). Briefly monolayers of fibroblast cells were plated 16 hours before fixing, then fixed with PFA and blocked before addition of antibodies. In parallel a duplicate plate was set up and MTS was added to each cell type to measure the number of living cells. Cell NCAM expression was then corrected using the differences in cell number revealed by the MTS assay. The ELISA is the result of four independent experiments. Relative surface NCAM expression is shown for each cell type where 3T3 cells express very low levels of NCAM and N24 and V140G cells express similar high levels. NCAM and NCAM-VASE transfected NIH 3T3 cells showed statistically similar surface expression levels.

The ELISA revealed that there was no significant difference between the NCAM and NCAM-VASE expression levels on N24 and V140G cells. This is in agreement with data from the initial ELISA experiment carried out when the V140G cell line was first selected. The ELISA also showed that NIH 3T3 cells express low levels of NCAM, apparently less than a quarter of the NCAM expressed on N24 cells. However by comparing this information with the α N16 staining data, it revealed that the expression was far less than that revealed by ELISA.

### 3.2.3 NCAM and NCAM-VASE are expressed in fibroblast cells in equal amounts, as shown by Western blot

To further characterise the levels of NCAM in the transfected N24 and V140G cell lines, a Western blot was carried out. The three cells lines were grown to confluency then lysed in concentrated SDS-loading buffer. The whole cell lysate was then centrifuged to remove cell debris before the
concentration of protein was determined by BCA assay. Equal amounts of supernatant were then loaded onto SDS-PAGE gels for electrophoresis. A coomassie gel and Western blot was carried out in tandem to compare the whole cell lysate staining with NCAM and tubulin bands (Fig. 3.4).

Figure 3.4: Western blot to show the expression levels of NCAM on the surface of NIH 3T3 transfected fibroblasts. To qualitatively compare the levels of NCAM and NCAM-VASE on NIH 3T3, N24 and V140G cells, a Western blot was carried out using an NCAM antibody, α N16 which binds to Ig III and a tubulin antibody loading control. Cell lysates were obtained by addition of 5x SDS-PAGE buffer to confluent monolayers for 15 min at RT. Cells were then scraped, centrifuged and their concentration was determined by BCA assay. Equal amounts of the supernatant were loaded onto a non-reducing 12.5% stacking/5% resolving gel. The gel was run for 15 min at 80 V, and for 60 min at 120 V. a) A coomassie stained gel to show the total protein present in NIH 3T3, N24 and V140G cell lysate. b) Western blots were transferred at 100 V for 1.5 hours, blocked in PBS/10% milk and probed with 1:1000 α tubulin or 1:10000 α N16. NCAM was found in the cell lysate of N24 and V140G cells. A tubulin loading control revealed that equal amounts of cell lysate were loaded onto the gels.

Coomassie staining of whole cell lysate revealed that there was no quantifiable difference in protein levels within the cell, although proteins of different molecular weights varied in their prevalence across all three cells lines. A Western blot using an antibody against alpha tubulin revealed that relatively equal amounts of NIH 3T3, N24 and V140G cell lysate were loaded onto the SDS-PAGE gels (Fig 3.4b). Alpha tubulin was used as a loading control in this experiment due to its prevalence in this fibroblast cell line.

NCAM staining revealed that there was a small amount of NCAM in NIH 3T3 cells. However the amount in N24 cells was much greater than this basal level. The amount of NCAM-VASE in V140G cells was relatively similar to the amount in V140G cell lysate. Although this Western blot is not conclusive, it provides another piece of evidence which suggests that the level of NCAM-VASE on V140G cells is similar to the level of NCAM on N24 cells.
3.3 Calibration of the AFM gives picoNewton force sensitivity, ideal for single cell adhesion measurements

3.3.1 AFM calibration and determination of a sensitivity factor related z-piezo voltage to cantilever deflection

Before AFM measurement could be taken between interacting cells and prior to cantilever probe cell attachment, calibration was carried out. The cantilever was mounted onto the cantilever glass block holder at a 10° downward angle and inserted into the instrument over a Petri dish containing the adherent cells to be probed. The system was left to equilibrate for 15 min in DMEM HEPES at 37°C before any calibration was carried out. Following this, the cantilever sensitivity and measurement of the cantilever spring constant had to be determined. The methods used for both of these assignments were similar to those carried out on the Veeco Nanoscope IV described in Chapter 2.

![Extension Curve, Retraction Curve](image)

**Figure 3.5: Calibration of the sensitivity determinant of the cantilever converts cantilever deflection from units of Voltage to distance in an AFM experiment.**

Prior to the attachment of a probe cell to the cantilever, force curves are collected showing the interaction between the bare AFM chamber and fibronectin-functionalised cantilever. The slope of the area of the force curve where tip and sample were in contact was quantified and averaged (from 3 Volt-distance curves) to determine the relationship between the Voltage deflection of the cantilever and the distance of deflection. The experimental parameters were as follows: 37° in DMEM HEPES, 2 V contact setpoint, 10 μm pulling length, 5 μm/s cantilever extend and retract speed, contact time = 0, delay mode = constant height, closed loop mode, pixel rate 410 Hz or 4096 data points.
The cantilever sensitivity factor was determined by acquiring three Volt-distance curves between the fibronectin-functionalised cantilever and a plastic dish in DMEM HEPES at 37°C (Fig. 3.5). DMEM HEPES was the medium of choice for cell adhesion measurements because of its ability to buffer around neutral pH. FCS was removed from the buffer due to reported problems in attaching probe cells to the cantilever and blocking adhesion measurements (unpublished observation, JPK Instruments, Germany). Contact was maintained between the cantilever and dish using a 2V cantilever deflection setpoint. During this calibration, the contact time between the surfaces following contact was zero, but contact was controlled by a closed loop feedback mechanism in ‘constant height mode’. This indicated that a constant height, as opposed to a constant force, was maintained during contact.

The cantilever sensitivity was determined by measuring the slope (X-axis/Y-axis yielded units of nm/V) of the Volt-distance curve in the period of contact (in the linear portion) between the cantilever and plastic dish. Typically the sensitivity factor was between 70 and 100 nm/V which was the same as in the single molecule experiments (See chapter 2) and converted the cantilever deflection from units of voltage to distance.

3.3.2 PicoNewton force resolution was achieved following determination of the spring constant using the thermal tune method

Following the determination of the cantilever deflection factor and its conversion from units of Volts to distance, the cantilever spring constant was measured using the inbuilt thermal tune method in JPK Image Processing software (Fig. 3.6). This method was the same as that used in the single molecule AFM chapter to determine spring constants. Cantilever spring constants were determined in DMEM HEPES at 37°C, 100 μm above the surface and under no driving oscillation (See Chapter 2 for scientific background).

In the single molecule AFM experiments the cantilever spring constant was determined using the first frequency peak. However on the CellHesion® 200 instrument the spring constant was assigned from the first overtone peak, using a correction factor of 0.251. ‘Arrow TL1’ spring constants were typically between 0.01 and 0.03 N/m. Spring constant assignment allowed the conversion of cantilever deflection from units of distance to force. Following this calibration the adhesion between cells could therefore be measured by knowledge of the force required to separate cells following interaction under single cell AFM conditions.
Figure 3.6: Cantilever spring constant determination was carried out using the thermal tune method on a CellHesion® 200 AFM instrument.
To determine the force required to deflect the cantilever by a certain distance, the spring constant of the cantilever was measured. The frequencies of thermal fluctuations of the ‘Arrow TL1’ cantilever, in DMEM HEPES at 37°C, 100 μm above the surface under no driving oscillation, were collected and the resonant frequency was calculated. The first overtone peak was assigned using a correction factor of 0.251 for greater reliability. Spring constants for the ‘Arrow TL1’ cantilever were typically between 0.01 and 0.03 N/m.

3.4 Development of an AFM assay to measure the adhesion between NCAM±VASE expressing cells

3.4.1 Probe cell in situ cantilever functionalisation in an AFM assay
Characterisation of the N24 and V140G cells for use in single cell AFM experiments revealed that both cell lines expressed statistically equal amounts of NCAM and NCAM-VASE. This was the main requirement of cell lines for use in a single cell measurement assay; cells expressing equal levels of surface adhesion molecule could therefore be directly compared for differences in their adhesion as a property of the proteins transfected. An AFM assay therefore had to be developed to measure the adhesion between single N24 and V140G cells.

After calibration of the AFM instrument and cantilever, attachment of a ‘probe’ cell was optimised for cantilever functionalisation. The ‘Arrow TL1’ tipless cantilever was used in all measurements and had a nominal spring constant of 0.03 N/m. This spring constant value is similar to those used in single molecule AFM experiments because picoNewton sensitivity is required to measure individual...
rupture events between the cells. The method used for supporting fibroblast monolayers on plastic was adopted to attach a probe cell to the cantilever (Fig. 3.7). Following Piranha-cleaning of the cantilever, it was rinsed and inserted into a droplet of 16 mg/ml poly-l-lysine in DMEM HEPES to create a positively charged cantilever surface. After rinsing, the cantilever was incubated in 10 μg/ml fibronectin in DMEM HEPES and incubated at 37°C until probe cell attachment was carried out. The fibronectin coating on the cantilever was used to bind integrin proteins, which are readily expressed on the surface of fibroblast cells, for attachment of a probe cell.

![Image](image.jpg)

**Figure 3.7: in situ cell functionalisation of a cantilever for a single cell adhesion AFM experiment.** Images taken at x40 magnification to show the ‘Arrow TL1’ cantilever, probe cell and adherent cells used during a single cell force spectroscopy experiment. 

a) The ‘Arrow TL1’ silicon nitride cantilever was functionalised with poly L-lysine, followed by fibronectin to provide a ‘sticky’ surface for binding of the probe cell. Fibroblast cells were scraped from confluent monolayers (plated 16 hours previously), and triturated through a 19G needle to de-adhere cells. These cells were then injected into the AFM chamber and the cantilever was brought into contact (contact force < 1 nN) with one settled cell as close to the tip as possible. Once the cell was attached, it was allowed to equilibrate for 5 min before an experiment was started. 

b) The cantilever with a functionalised probe cell was then brought into contact with an adherent cell on the Petri dish directly over the nucleus. Adhesion measurements were then taken. **Successful probe cell functionalisation allowed a cantilever probe cell to be brought into contact with an adherent cell during a single cell AFM experiment.**

Probe cells were prepared by plating in 24 well plates so that confluency was reached after 16 hr. In tandem, individual 35 mm plastic dishes were plated with islands of NIH 3T3, N24 and V140G cells at low density (200 cells in a 200 μl droplet). Cell types were plated in different dishes because NCAM has been found to be secreted from the surface of N24 cells (unpublished observation, Dr. M. Delves) and this could affect the adhesion results between other cell types in the same plate.

Before AFM experiments, all cells were incubated in DMEM HEPES for 30 min at 37°C to remove excess FBS, which has been found to block cell adhesion using AFM (unpublished observation, JPK Instruments). Confluent cells were then scraped and triturated through a 19G needle before injection of a few cells into the adherent cell chamber (away from the cell islands). The rounded probe cells were allowed to settle, then contact was made between the fibronectin-functionalised
cantilever tip and the cell in force contact mode (Fig 3.7a). This contact was maintained by a contact force of less than 1 nN, to reduce force-associated damage to the cell, and during a contact time of 5 s. The cantilever was raised and in the majority of attempts, the cell had attached first time. If, within 5 min of cell injection, a cell had not attached, more cells were injected into another area of the dish and the process was repeated. If attachment was still unsuccessful, a new fibronectin-functionalised cantilever was used and the process was repeated.

Once a probe cell had been attached, the cantilever was raised to 100 µm above the Petri dish and left to equilibrate for 5 min to form more stable contacts before AFM measurements were taken (Fig 3.7b).

3.5 Single cell AFM force-distance curves revealed quantifiable data points relating to the separation of interacting cells

Following determination of the cantilever sensitivity and spring constant, a NIH 3T3 cell was used as a probe cell and force-distance curves were collected to measure the interaction with an adherent NIH 3T3 cell in the AFM chamber. This experiment was carried out to illustrate the quantifiable points during a force-distance cycle. A force-distance curve tracing the interaction between separating cells contains more information than that in a single molecule AFM experiment. This is due to the increased complexity of the interactions between cells.

In this force-distance curve measuring the interaction between two NIH-3T3 cells, the cantilever probe cell was lowered at a velocity of 5 µm/s from a distance of approximately 80 µm until it made contact with the adherent cell above the nucleus (Fig. 3.8 extend curve, orange). This contact position was chosen for all cell-cell interactions due to the ease with which it was located and to maintain consistency in adhesion measurements. Once contact had been made between the cells the cantilever continued to lower in the Z-direction, until a feedback deflection force of 0.5 nN had been reached. Contact was maintained in a closed-loop mode which maintained the cantilever at a constant height. This allowed the deflection of the cantilever to change to maintain the height of the cantilever. Contact was visualised using phase microscopy to confirm the probe cell was in contact over the nucleus of the adherent cell (Fig. 3.7b).

Following 5 s of contact time, the cantilever was raised at a velocity of 5 µm/s but the cells remained in contact initially due to their adhesion. This resulted in a negative cantilever deflection and so the cantilever tip was pulled downward (Fig. 3.8 retraction curve, maroon). The maximal unbinding force, which was the greatest negative deflection seen on the cantilever, represented the maximal force required to maintain cell contact. This occurred just before the start of de-adhesion events.
between the cells and typically occurred within the first few microns of cantilever retraction. This maximal force was used to assess the adhesion between the cells.

![Graph showing force-distance curves](image)

**Figure 3.8:** Single cell force-distance curves exhibit the separation of two cells following interaction during an AFM experiment. A force-distance curve shows the extension (orange) and retraction (maroon) curves resulting from the approach, contact and separation of two cells during a single cell AFM experiment. The probe cell on the cantilever is brought into contact with an adherent cell above the nucleus, until a setpoint deflection is reached on the cantilever. This corresponds to the maximal value on the Y-axis. The cantilever is lifted from the adherent cell after a set contact time and the resulting negative deflection is due to the strength of adhesion between the interacting cells. The total adhesion is taken from the maximal unbinding force unbinding (i.e. the maximal deflection on the cantilever). At this point interactions between the cells begin to break which leads to a series of jumps on the force curve. While the probe cell is being lifted, interactions remain between the probe and adherent cell, which are represented on the force-distance curve by plateaus (i.e. distances where there is no change in deflection), which are due to the pulling of membrane tethers from the cells. The end of a plateau is followed by an unbinding event corresponding to a rupture between the two cells. The experimental parameters were as follows: 37°C in DMEM HEPES, 0.5 nN contact setpoint, 98 μm pulling length, 5 μm/s cantilever extend and retract speed, contact time = 5 s, delay mode = constant height, closed loop mode, pixel rate 410 Hz or 4096 data points.

Following the maximal unbinding deflection, a number of positively and negatively deflecting jump events occurred, which indicated that small contacts between the two cells were broken, whilst others were re-adhering. The ‘jump’ period usually occurred within the first 10 μm of cantilever retraction when the contact area between the interacting cells is still large. Over the next 80-90 μm of retraction, vertical unbinding events occurred which corresponded to discrete unbinding events between the interacting cells. The unbinding events occurred rapidly (within a few nm of cantilever retraction) and, unlike the jump regions, decreased cantilever deflection. After each unbinding event
there were periods of no change in cantilever deflection, which were termed plateaus and correspond to maintenance of interactions between the cells, while the probe cell was moving further away. This is possible due to the extrusion of membrane nanotubes, or tethers, from one or both of the cells.

Following 98 µm of cantilever retraction, it was expected that the cells would be separated as shown on the force-distance curve by a return to zero deflection of the cantilever. However in most force-distance curves, there was a slight offset between the start deflection on the extension curve position and the end deflection of the retraction curve. This has been previously noted and was due to laser drift or thermal fluctuations during the experiment (Franz, Taubenberger et al. 2007).

Another parameter, which can be quantified from each force-distance curve, was the total work (in units of energy, joules (J)) required to separate the two cells. This value was calculated by integrating the area underneath the extension curve, which encompassed the entire retraction trace area. The work takes into account the number of unbinding events, their size and the maximal cantilever deflection.

By quantifying the maximal unbinding force between cells, the individual unbinding events, which occur during cell separation and the number of unbinding events, form the basis of an extensive comparison between the adhesion of NCAM±VASE expressing cells.

3.6 Measuring the homophilic adhesion between single cells expressing NCAM±VASE by AFM

3.6.1 Expression of NCAM in NIH 3T3 cells increased their adhesion after 5 s contact time

Before the effect of the insertion of the VASE sequence into NCAM was tested, it was necessary to optimise the parameters for measuring the adhesion between two cells. It was also essential to see the effect of NCAM transfection on the adhesion of the control NIH 3T3 cells, which express extremely low levels of NCAM.

The method for probe cell functionalisation on the cantilever (Section 3.4.1) was successful and allowed sturdy attachment of a series of individual NIH 3T3 cells. It was decided that an individual probe cell would used for up to 2 hours or in 30 force-distance curves, whichever came first. This time limit was chosen to minimise stress on the cell and to minimise the probability of using a cell in apoptosis. An MTS assay was carried out on NIH 3T3 cells at 25% confluency in DMEM HEPES at 37°C, which were set up under the conditions used for preparing adherent cells for AFM. The assay revealed that cell viability did not change between 30 min and 2 hour incubation in DMEM HEPES, and it was statistically similar for all three cell types. Although this assay did not take into account
the role of force on cell viability, it was a good indicator for these parameter choices. A low contact force of 0.5 nN was chosen to maintain contact between the cells in the AFM experiment, whilst minimising destruction due to force. 0.5 nN was also the contact force used in the single molecule experiments between NCAM and NCAM-VASE proteins.

![Figure 3.9: Single cell AFM experiment to measure the interaction between NIH 3T3 cells and NCAM expressing N24 cells.](image)

The adhesion between control NIH 3T3 cells (expressing extremely low levels of NCAM) and N24 cells expressing NCAM was compared using single cell AFM. Cantilever deflection was measured in units of force (N) and allowed quantification of adhesion from the maximal deflection force required to separate the two cells (Y-axis). The mean maximal unbinding force was calculated from at least 30 force-distance curves in three independent experiments in DMEM HEPES at 37°C. At least 10 different cell pairs were used with each contact time repeated in triplicate. The mean maximal unbinding force (nN, Y-axis) was plotted against the contact time (s, X-axis) between cells. The setpoint contact force between the cells was 0.5 nN, the sensitivity determinant was between 70-100 nm/V and the spring constant was approximately 0.03 N/m. *** P<0.0005 (extremely statistically significant). **After 5 s of contact time, N24 cells expressing NCAM adhered more tightly than control NIH 3T3 cells.**

The adhesion between NIH 3T3 and N24 cell pairs was measured after 5 and 60 s of contact time and 0.5 nN of contact force using single cell AFM between probe and adherent cells(Fig. 3.9). This contact force was chosen to provide a direct comparison with the single molecule NCAM Fc protein adhesion measurements (See Chapter 2). To assess the variability and adhesion between NIH 3T3 and N24 cells, at least 10 probe cells were quantified in triplicate for each contact time and in three independent experiments. Cells were probed one after the other with increasing contact time. The extension and retraction cantilever velocities used were 5 μm/s, which were 20 times more than those in the single molecule experiments.

The mean maximal unbinding forces measured between single NIH 3T3 and N24 cells were on a nanoNewton scale, whereas the single molecule measurements were on picoNewton scale (See Chapter 2). The maximal unbinding force between 3T3 cells increased slightly with increasing contact time, from 0.21±0.02 to 0.53±0.12 nN between 5 and 60 s. However the mean maximal unbinding force for N24 cells, after 5 s of contact time, was 0.40±0.04 nN which was extremely statistically
significantly higher than that for NIH 3T3 cells. After 60 s of contact, the mean maximal unbinding force between N24 cells increased dramatically to 1.52±0.24 nN which was a highly significant increase over 3T3 cell adhesion after 60 s. This evidence indicates that after short contact times, N24 cells display increased adhesion over control cells lacking NCAM. The only difference in the cell lines is that N24 cells contain NCAM; therefore this increase in adhesion may be due to NCAM binding homophilically to itself at the cell-cell interface or an indirect effect. The increase in adhesion over time indicated that either more adhesive bonds were formed over time or their strength increased. Cells therefore required more force to separate them.

This data revealed that the transfection of NCAM into NIH 3T3 cells increased their adhesion on a single cell level significantly following short contact times between cells and provided the first evidence of in vivo NCAM single cell adhesion measurements.

3.6.2 Addition of a peptide KYI from Ig III of NCAM reduced the adhesion between N24 cells

Although the presence of transfected NCAM-140 in NIH 3T3 cells increased the adhesion between N24 cells, it was not known whether this was due to direct adhesion between NCAM proteins at the cell-cell interface or due to upregulation of another protein. To test this, it was necessary to specifically block NCAM protein adhesion to knock down N24 cell adhesion. This would prove that the increased N24 cell adhesion was due to the presence of NCAM-140. To do this, the KYI peptide, which corresponds to a 10 amino acid NCAM homophilic binding sequence in Ig III, and which was used in previous single molecule AFM experiments (Section 2.7.5), was used. Following control N24 measurements using single cell AFM, 1 mM KYI was added to the AFM chamber and the adhesion between cells was measured. This experiment was repeated with a new set of cells but a scrambled version of the peptide, ‘KYIScr’, was added to test for any non-specific, charge-related peptide effects on N24 adhesion. It is worth noting that neither of the peptides reduced the efficacy of in situ probe cell functionalisation or maintenance.

The mean maximal unbinding force was compared for contact times of 5 and 60 s using the parameters previously described (Section 3.6.1). Firstly, the control ‘no peptide’ measurements taken between the N24 cells were statistically similar to those presented previously (Fig. 3.9). After addition of 1 mM KYI peptide, the mean maximal unbinding force of N24 cells was statistically similar to the control force. The scrambled peptide control appeared to increase the mean unbinding force, but overall there appeared to be no adhesion blocking effect of the peptide at this contact time.
Figure 3.10: Effect of an NCAM blocking peptide KYI (from NCAM Ig III) on the adhesion of N24 (NCAM expressing) cells in single cell AFM.

The effect of increasing contact time on adhesion between N24 cells, which express NCAM, was measured using SCFS. The maximal unbinding force is determined from the retract force curve and is the maximal deflection required before interacting cells are separated. The setpoint contact force between the cells was 0.5 nN, the sensitivity determinant was between 70-100 nm/V and the spring constant was approximately 0.03 N/m. a) Comparison of the mean maximal unbinding force (nN, y-axis) against contact time for cells treated with either no peptide, 1 mM KYI or 1 mM scrambled sequence KYI. b) Comparison of the mean number of unbinding events (y-axis) against contact time for cells treated with either no peptide, 1 mM KYI or 1 mM scrambled sequence KYI. c) Comparison of the mean individual unbinding event size (pN, y-axis) against contact time for cells treated with either no peptide, 1 mM KYI or 1 mM scrambled sequence KYI. Data was taken for each contact time from at least 10 different cell pairs and at least 30 individual measurements taken in three independent experiments. N.S. not significant, ** P<0.001 (very statistically significant), *** P<0.0005 (extremely statistically significant). After 60 s of contact time in single cell AFM, 1 mM KYI specifically reduced the adhesion between N24 (NCAM) cells.
On the same cell population, addition of 1 mM KYI peptide after 60 s of contact time decreased the mean maximal unbinding force to 19±1.5% of the ‘no peptide’ control force. This decrease in adhesion was extremely significant. Addition of the scrambled version of the peptide decreased the mean maximal unbinding force to 58±3.4% of the ‘no peptide’ control. The difference between the structurally specific peptide reduction and the non-specific charge reduction in adhesion was extremely statistically significant. The structurally specific sequence component of 1 mM KYI peptide therefore accounted for an approximate 40% knockdown in adhesion from the ‘no peptide’ control. Therefore addition of a peptide from Ig III reduced N24 cell binding significantly. This peptide has been found to block NCAM binding on a single molecule level (Wieland, Gewirth et al. 2005), therefore it can be postulated that the extra adhesion seen between N24 cells is due to the presence and homophilic binding of NCAM. N24 adhesion may only be knocked down at 60 s after peptide addition because short-term adhesion may be NCAM independent.

To understand the mechanism behind the decrease in adhesion between N24 cells in the presence of KYI peptide, the mean number of individual unbinding events seen on each retract force curve during cell separation was compared (Fig. 3.10b). For the control N24-N24 separations, there were on average 5 and 16 unbinding events following 5 and 60 s of contact time respectively. For 5 s contact time, there was no significant difference of the number of unbinding events during cell separation upon addition of KYI or KYILscr. This indicated that specific protein interactions at the cell-cell interface were not prevented by the addition of this blocking peptide. Since KYI is known to bind to NCAM Ig III it was inferred that the adhesion at 5s of contact time was largely independent of NCAM, therefore the peptide did not block individual NCAM binding units.

After 60 s of contact time in the presence of KYI peptide, the mean number of unbinding events during N24 cell separation was decreased to only 5. This corresponded to the mean number of unbinding events seen following 5 s of contact between the control N24 cells. This indicates that the individual NCAM containing binding units did not form during cell interaction, thus decreasing the overall number during separation. The scrambled peptide version also non-specifically reduced the mean number of unbinding events but was very significantly higher than the effect of the KYI peptide.

The mean size of individual unbinding events during N24 cell separation was also investigated. Before the addition of peptide, the mean unbinding event size was 30 pN at both 5 and 60 s of contact times. Upon addition of 1 mM KYI, there was no effect on their size but interestingly the scrambled peptide increased the mean unbinding force to approximately 50 pN at both contact times.
Overall it is plausible that the decrease in adhesion between N24 cells, which occurred following addition of KYI peptide, was accounted for by a decrease in the number of unbinding events during the separation of the cells. The peptide specifically prevented a majority of NCAM-NCAM interactions from occurring between N24 cells and therefore decreased the maximal unbinding force by this means. The mean size of unbinding events was similar between ‘no peptide’ and KYI treated cells. After 5 s of contact time, there may be no effect on the number of interactions occurring between the cells in the presence of KYI because early adhesion may not be due to NCAM-NCAM interactions.

AFM measurements using a higher concentration of KYI peptide were not attempted due to the high non-specific effect of the scrambled peptide and the already high concentration which had been used. However this experiment using 1 mM KYI has shown that the increased adhesion measured by AFM between N24 cells is in part due to the presence of NCAM on the surface of the cells.

3.6.3 Insertion of the VASE sequence into NCAM increased the adhesion of NIH 3T3 cells after 120s contact time using single cell AFM

Single cell AFM measurements revealed that the transfection of NCAM into NIH 3T3 cells increased their adhesion. Now that this adhesion difference had been quantified, it was necessary to determine the effect of insertion of the VASE sequence into NCAM. To do this the adhesion between V140G cells was determined by single cell AFM. In keeping with the previous cell adhesion experiments the same experimental parameters were used (Section 3.6.1). The mean maximal unbinding force was compared against three contact times, ranging from 5 to 120 s (Fig. 3.11).

Briefly, a contact force of 0.5 nN and cantilever velocities of 5 μm/s were used. At least 30 force-distance curves were collected between 10 cell pairs. V140G cells were found to attach to the cantilever as easily as N24 or NIH 3T3 cells. After 5 s of contact the mean maximal unbinding force between V140G cells was extremely statistically greater than that between 3T3 cells, but statistically similar to N24 cells. This indicated that at the shortest contact time measured, insertion of the VASE sequence into NCAM did not disrupt the increase in adhesion which NCAM exhibited over control cells. It was interesting to note that the adhesion between V140G cells was not increased above N24 cells after immediate contact.

It was not until the contact time between the cells reached 120 s that there was a very statistically significant increase in the mean force required to separate V140G cells compared with N24 cells. The mean force required to separate V140G cells after 120 s of contact and 0.5 nN was 5.0±0.59 nN. It is worth noting that the only apparent difference between the V140G and N24 cell is that they have an
extra 10 amino acids in Ig IV of NCAM. It is plausible however that the transfection of NCAM-VASE into NIH 3T3 cells may have caused the upregulation of another protein or disruption of the cell in some way, so as to cause an increase in cell adhesion. It is possible that NCAM-VASE is directly responsible for this increase in adhesion as it is present on the cell surface and has been known to function as an adhesion molecule (Fig. 3.2) (Chen, Haines et al. 1994). This finding was the first piece of data to indicate that the function of NCAM-VASE may be to increase adhesion. At this stage, the data did not reveal whether NCAM-VASE increased the homophilic binding strength of NCAM or whether it bound heterophilically to achieve this.

![Figure 3.11: Effect of VASE sequence insertion on NCAM cell adhesion using single cell AFM.](image)

The effect of increasing contact time on the homophilic adhesion between control (NIH 3T3), N24 (NCAM expressing) and V140G (NCAM-VASE expressing) cells, was measured using single cell AFM. The maximal unbinding force was determined from the retract force curve and corresponded to the maximal deflection of the cantilever required to separate the single cells, one on the cantilever and another on a Petri dish. The setpoint contact force between the cells was 0.5 nN, the sensitivity determinant was between 70-100 nm/V and the spring constant was approximately 0.03 N/m. The mean of the maximal unbinding force at each contact time was calculated from at least 10 different cell pairs and at least 30 individual measurements in three independent experiments. ** P<0.001 (very statistically significant). After 120 s of contact, VASE expressing cells adhere more tightly than NCAM expressing cells.

This experiment also highlighted that the adhesion between NIH 3T3 cells did not increase dramatically over time and that the presence of NCAM±VASE in cells was required to increase their contact dramatically with increasing contact time.

By comparing the adhesion of the N24 and V140G cells measured by AFM and the expression of NCAM±VASE on the cell surface (Fig. 3.3), it was probable that the extra adhesion of the VASE cells was not due to there being more VASE protein than NCAM on the cell surfaces.
3.6.4 A peptide from NCAM Ig III, KYI, did not affect the adhesion, work, or unbinding events between V140G cells

To prove that the insertion of the VASE sequence alone was responsible for the extra adhesion seen between V140G cells, an experiment was attempted to specifically prevent cells from adhering. To do this, the effect of 1 mM KYI peptide on adhesion was tested by AFM using a previously employed experiment (Section 3.6.2). This peptide specifically blocked the adhesion between N24 cells. The effect of KYI and KYIscr on the mean maximal unbinding force, work, mean individual unbinding event size and their number was compared for contact times of 120 and 300 s (Table 1). These contact times were chosen to see if the extra adhesion due to the VASE sequence could be knocked down to an N24 or control NIH 3T3 level.

The control ‘no peptide’ values of adhesion between V140G cells at 120 s of contact time were in good agreement with those previously recorded (See Fig. 3.11). Following addition of 1 mM KYI peptide, the mean maximal unbinding force between cells was 35±5.0% of the no peptide’ reading. Addition of the scrambled version to another population of V140G cells indicated that this adhesion had dropped to 30±5.0% of the original value. There was no significant difference between the non-specific effect of the scrambled peptide and the KYI peptide. Therefore V140G cell adhesion was not blocked between V140G cells by 1 mM KYI peptide, even though it decreased adhesion between N24 cells at short contact times.

**Table 3.1: Comparison of the effect of a NCAM blocking peptide mimic KYI (from NCAM Ig III) on V140G cell adhesion by single cell AFM.**

The effect of contact time on the mean maximal unbinding force, work, size and number of unbinding events was investigated. The results were compared to measurements carried out directly before the addition of peptide. The peptides were added for 30 min (whilst the probe cell was attached to the cantilever in situ) before measurements were taken. Each data point consists of at least 20 data points and 7 unique cell combinations taken in three independent experiments. The experiment was carried out at 37°C. SEM = standard error of the mean. 1 mM KYI peptide had no effect on the extra adhesion seen between V140G (NCAM-VASE expressing) cells after 120 and 300 s of contact time.

<table>
<thead>
<tr>
<th>Contact Time (s)</th>
<th>Maximal Unbinding Force (nN)</th>
<th>Work (E-14 J)</th>
<th>Unbinding Event Force (pN)</th>
<th>Number of Unbinding Events</th>
</tr>
</thead>
<tbody>
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<td>SDYIDFSSQ</td>
<td>300</td>
<td>5.17</td>
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</tr>
</tbody>
</table>

To test whether the peptides had a more subtle effect on V140G cell adhesion, the work required to separate the cells was quantified by measuring the area of the retraction force-distance curve, under y=0 (See Fig. 3.8). This also revealed that although the work decreased by approximately 50%, it also
occurred when the scrambled version of the peptide was added. Although the mean size of unbinding events during V140G cell separation was decreased, the scrambled peptide also decreased the size of the unbinding events. This indicates that the peptide had a non-specific effect on these quantifiable points but did not block adhesion specifically.

Interestingly the number of unbinding events, after 120 s of contact, was increased by the addition of 1mM KYI and the scrambled sequence version indicating again that the peptide had no specific blocking effect. After 300 s of contact time the effect of the peptides were more complicated; the KYI scrambled peptide increased the mean number of unbinding events by 67±30% but the KYI peptide had no effect. It could be that the non-specific effect of the charge of the peptide masked the true effect of the KYI peptide on the mean number of unbinding events. To further investigate this it would be necessary to use a number of scrambled sequence versions of KYI and to see their effect. However this was not attempted due to time constraints.

Overall a peptide from NCAM Ig III, which reduced adhesion between N24 cells, did not affect V140G cell adhesion after 120 and 300 s of contact. It is possible that longer term adhesion (over 1 min) was independent of NCAM±VASE. 1 mM KYI peptide may have decreased V140G cell adhesion had its effect been tested at 5 and 60 s (as was the case in the N24 blocking experiment, see Fig. 3.10). However the extra VASE adhesion may not have been affected by binding of KYI to its receptor (either NCAM or another protein on the cell surface) as this binding patch in Ig III may not have been involved in NCAM-VASE adhesion. It is possible that NCAM-VASE is not responsible for the extra adhesion seen in V140G cells.

3.6.5 The effect of putative NCAM blocking antibodies and peptides on the homophilic adhesion between N24 and V140G cells using AFM

To prove that NCAM-VASE was directly responsible for the extra adhesion seen between V140G cells, it was necessary to carry out further experiments to specifically block cell adhesion using AFM. Since NCAM is a well studied protein, there are a number of other peptides and proteins which have either been shown to block NCAM homophilic binding or were postulated to. In the previous sections a peptide from NCAM Ig III blocked N24 cell adhesion after 60 s of contact, but had no effect on V140G cell adhesion after longer contact times. Three other peptides were synthesised using solid phase peptide synthesis (SPPS) for the AFM experiments. The first was termed the C3 dendrimer (C3d) peptide and corresponded to the dendrimer of an 11 amino acid sequence from a peptide combinatorial library, which was found to bind tightly to NCAM Ig I (Kd = 10⁻⁶ M) and blocked NCAM homophilic protein adhesion (Ronn, Olsen et al. 1999). In this experiment, a C3 monomeric peptide was synthesised and used at a higher concentration for adhesion blocking experiments. The
second peptide was termed the GRIL peptide and corresponded to 12 amino acids from Ig II of
NCAM which have been found to prevent NCAM homophilic binding on a protein level (Wieland,
Gewirth et al. 2005). The final peptide corresponded to the 10 amino acid VASE peptide sequence,
with four flanking residues at the N- and C-terminus. This peptide alone has been found to inhibit
neurite outgrowth of cerebellar granule neurons grown on a NIH 3T3 monolayer (Saffell, Walsh et al.
1994). However the role of the peptide in adhesion is unknown.

A number of antibodies were tested for their role in adhesion of N24 and V140G cells. These
included α N16, used in previous experiments, which bound to Ig III and α VASE which was raised
against the VASE peptide with flanking amino acids, described previously.

Figure 3.12: Schematic to show the NCAM extracellular domain and the positions of putative adhesion blocking
antibodies and peptide binding sites.
The five Ig-like domains of NCAM function as homophilic binding domains. A number of peptide and antibodies were
chosen to investigate their effect on blocking the adhesion between NCAM and VASE expressing cells. The C3m peptide
binds to a site in NCAM Ig I and -as been found to block NCAM protein homophilic adhesion. The GRIL peptide mimics a
putative homophilic binding sequence in Ig II and has been found to block NCAM homophilic binding. α N16 is an antibody
which binds to NCAM Ig III. The KYI peptide mimics a putative homophilic binding sequence in Ig III. α VASE is an antibody
which binds to the VASE sequence in NCAM-VASE Ig IV only. The VASE peptide mimics the VASE sequence, which is present
only in NCAM-VASE Ig IV.

3.6.6 Putative adhesion blocking peptides and antibodies had no specific effect on the adhesion
between N24 and V140G cells using AFM
Table 3.2: The effect of a number of putative NCAM adhesion blocking peptides and antibodies on N24 cell adhesion using single cell AFM.

The effect of a series of peptides and antibodies on the adhesion of NCAM expressing cells (N24) was investigated using single cell AFM. The peptides or antibodies were added to the cells for 30 min prior to the AFM experiment and measurements were carried out in situ with the putative blocking factors present. Control measurements between N24 cells (which were within 10% of the pooled data and were not presented in this table) were taken prior to addition of the putative adhesion blockers. The mean maximal unbinding force, work required to separate the cells, mean unbinding event and number of unbinding events within each force curve were analysed. Each data point is the mean of at least 30 cell combinations using at least 10 unique cells taken in three independent experiments. Setpoint contact force=0.5 nN, extend and retract cantilever velocities were 5 μm/s. A series of putative NCAM adhesion blocking peptides and antibodies had no effect at the concentrations used on the adhesion between NCAM expressing cells.

<table>
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<th>Contact Time (s)</th>
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</table>

To further understand the mechanism of N24 and V140G cell adhesion, the effect of a number of putative adhesion blocking peptides was tested using single cell AFM. Briefly, control, ‘no peptide’, adhesion measurements were taken between a series of probe cells on the cantilever and adherent cells on the Petri dish immediately prior to cell treatment with a peptide or antibody, to see whether the values were within 10% of previously pooled adhesion data using identical parameters (See Fig. 3.2).
3.11). The experiment was only continued if this were the case. The peptide, or antibody, to be studied was then added into the AFM chamber in DMEM HEPES and incubated at 37°C for 30 min.

Adhesion between the single cells was then quantified at different contact times using at least 10 cell pairs and in three different experiments. The mean maximal unbinding force, work and the size and number of unbinding events were calculated from retract force-distance curves (Table 3.2).

A contact time of 60s was chosen to measure the effect of C3m on N24 cell adhesion. After this contact time, 0.5 mM C3 m peptide had no effect on the mean maximal unbinding force required to separate N24 cell pairs. It also had no effect on any of the other parameters chosen for study. 20 mM C3m peptide also had no effect (data not shown). The scrambled version of the peptide at 0.5 mM also had no significant effect on N24 cell adhesion. Therefore at this concentration and at this contact time, C3m did not block the adhesive complexes forming between N24 cells. Since C3m binds with high affinity to Ig I of NCAM (far higher than the putative NCAM-NCAM affinity) it may be that this binding patch is not involved in N24 cell adhesion following only 60 s of contact.

Interestingly, a 200 nM dilution of α N16 (the NCAM antibody which binds to NCAM Ig III, see Fig. 3.2), significantly increased the mean maximal unbinding force and work required to separate N24 cell pairs. Control measurements taken in 200 nM foetal bovine serum (FBS) had no effect on N24 cell adhesion which may indicate that the effect of α N16 was specific. Although the mean size of the individual unbinding events did not increase, the number did in the presence of α N16 but it also did in control 200 nM serum. The increase in N24 cell adhesion in α N16 was unusual and may be accounted for by increased NCAM clustering at the cell-cell interface.

The effect of 250 µg/ml VASE peptide was investigated on N24 cell adhesion. This concentration was chosen because it was found to have the maximal inhibition effect on neurite outgrowth of PC12 cells grown over an N24 cell monolayer (Saffell, Walsh et al. 1994). A contact time of 300 s was chosen between N24 cells for this experiment. Although the mean maximal unbinding force and unbinding events were lower than for control N24 cells, this effect also occurred following addition of the scrambled peptide sequence. This indicated that at this long contact time and concentration the VASE peptide had no specific effect on N24 cell adhesion.

Finally, the effect of 1 mg/ml of the GRIL peptide, from Ig II, on N24 adhesion was tested at contact times of 5 and 60 s to see if the extra N24 adhesion, seen over control NIH 3T3 cells, could be knocked down. On all four parameters studied there was no significant difference between the adhesion of N24 cells in the presence of 1 mM GRIL peptide, the scrambled version or the ‘no peptide’ control. This indicated that either at the concentration of peptide, or with the experimental
parameters used, it did not bind to an NCAM Ig II binding partner to block NCAM binding on the N24 cells. Another explanation could be that at these contact times NCAM Ig II is not involved in N24 cell adhesion. NCAM Ig III however is involved in N24 cell adhesion after this contact time as shown by the knock down in N24 cell adhesion after addition of a peptide from Ig III (Fig. 3.10).

Table 3.3: The effect of a number of putative NCAM-VASE adhesion blocking peptides and antibodies on V140G cell adhesion using single cell AFM.

The effect of a series of peptides and antibodies on the adhesion of NCAM-VASE expressing cells was investigated using single cell AFM. The peptides or antibodies were added to the cells for 30 min prior to the AFM experiment and measurements were carried out *in situ* with the putative blocking factors present. Control measurements between N24 cells (which were within 10% of the pooled data and were not presented in this table) were taken prior to addition of the putative adhesion blockers. The mean maximal unbinding force, work required to separate the cells, mean unbinding event and number of unbinding events within each force curve were analysed. Each data point is the mean of at least 30 cell combinations using at least 10 unique cells taken in three independent experiments. Setpoint contact force was 0.5 nN, extend and retract cantilever velocities were 5 μm/s. In general these peptides and antibodies had no effect at the concentrations used on the adhesion between VASE expressing cells.

<table>
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<tr>
<th></th>
<th>Contact Time (s)</th>
<th>Maximal Unbinding Force (nN)</th>
<th>Work (×10⁻¹⁴ J)</th>
<th>Unbinding Event (pN)</th>
<th>Number of Unbinding Events</th>
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<td>8.30</td>
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<td>20.1</td>
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</table>
To test whether the adhesion between V140G cells was specifically due to the presence of NCAM-VASE at the cell surface, a number of peptides and antibodies were added into the AFM chamber prior to adhesion measurements. As with the N24 experiments described previously, control ‘no peptide’ adhesion measurements between V140G cells were taken and compared with previous pooled data. The effect of a peptide or antibody was only tested if the control measurements were within 10% agreement with pooled data (Table 3.3).

To test whether the Ig III domain of NCAM was involved in V140G cell adhesion, the α N16 antibody was added into the cell media prior to AFM measurements. Contact times between 5 and 300 s were chosen to determine whether the antibody had an effect on short or long term cell adhesion. There was no significant difference between the mean maximal unbinding force and number of unbinding events between ‘no peptide’ V140G cell measurements and 2% α N16 treated cells. However the work required to separate the cells, in the presence of the antibody, decreased. On comparison, the adhesion of V140G cells in the presence of 200 nM FCS also decreased the work required to separate the cells. Therefore this may have been a non-specific adhesion blocking effect.

The effect of the VASE antibody, which binds to the VASE sequence of NCAM-VASE, was also investigated at contact times between 5 and 300 s. 200 nM α VASE had no significant effect on the mean maximal unbinding force, work and other parameters used to investigate V140G cell adhesion using an AFM. Therefore this concentration of α VASE did not block adhesion using these parameters and this technique. This was a surprising finding because it has been postulated that insertion of the VASE sequence into NCAM creates a novel binding site or alters its structure to increase the adhesion between VASE expressing cells. It is plausible that either a higher concentration of antibody must be used to increase the probability of binding to VASE or that the VASE epitope was involved in an interaction which was of a higher affinity than α VASE to VASE.

Addition of 250 μg/ml of VASE peptide also did not decrease the adhesion between V140 G cells. It was postulated that the VASE peptide may bind to the unknown VASE binding partner and therefore prevent NCAM-VASE protein from forming a high affinity interaction. It may be possible that even though the VASE peptide inhibits neurite outgrowth at this concentration, this is unrelated to the increase in adhesion between V140G cells. Insertion of VASE into NCAM may simply alter its structure and increase adhesion by this means. The GRIL peptide from Ig II of NCAM also had no effect on the adhesion between V140G cells, indicating that this epitope of Ig II may not be involved in V140G cell binding or that it was inaccessible for peptide binding.
To fully investigate the effects of these peptides and antibodies it would be necessary to use a wider range of concentrations and contact times during the AFM measurements. Since the adhesion between V140G cells is so high, the individual protein adhesive units may be due to large affinity interactions. Therefore by increasing the concentrations of the peptides and antibodies a knock-down in adhesion between the cells may be seen.

3.6.7 The increased adhesion seen between V140G cells was not due to an increased probe cell contact area

Since the addition of putative adhesion blocking peptides had failed to decrease the adhesion between V140G cells, a series of experiments were carried out to disprove alternative hypotheses on the increased V140G cell adhesion. One reason for an increase in adhesion between V140G cells may be that rounded V140G cells were larger than N24 and NIH 3T3 cells. This would mean that cantilever probe cells were larger and the contact area between the adherent cell and probe cells during the AFM measurements would be increased. An increased contact area corresponds to a larger cell-cell interface, therefore there would be a greater area for protein-protein interactions and clustering which could increase the adhesion between the cells.

![Bar chart showing mean cell area (µm²) for 3T3, N24, and V140G cells.](image)

**Figure 3.13:** Measurement of control, NCAM and VASE cell areas under single cell AFM experimental parameters. Bar chart to show the mean cell areas (µm², y-axis) of probe 3T3, N24 and V140G cells and adherent cells. The probe cells were grown for 16 hours so that a confluent monolayer had formed. They were then scraped, triturated and injected into a Petri dish where their areas were calculated using Axiovision microscope software under 20x phase magnification. Adherent cells were plated at low density for 16 hours before being measured in the same way. Each data point is the mean of three independent experiments where 200 cells were measured in each. The area of rounded probe and adherent control, NCAM and NCAM-VASE expressing NIH 3T3 cells was statistically similar.

The area of 200 adherent and probe cells was measured in three independent experiments following the same plating and preparation used in the AFM experiments. Areas were calculated using a
macro produced for Axiovision KS300 software. After plating and overnight incubation, cells were transferred into DMEM HEPES for 30 min before measurements were taken or cells were scraped and injected into the adherent cell Petri dish. The mean cell areas were compared for all three cell types used (Fig. 3.13). Adherent cell areas, grown on a 35 mm Petri dish at low density, were consistently between 1500 and 1700 μm² for all three cell types. There was no statistically significant difference between 3T3, N24 and V140G cell area. This meant that V140G probe cells were not larger than N24 cells so the extra V140G cell adhesion was not due to a larger contact area between cells.

3.6.8 VASE expressing cells adhered three times more rapidly than NCAM expressing cells

In previous experiments the introduction of NCAM into NIH 3T3 cells was found to increase the adhesion between single cells, and could be blocked by addition of a peptide from NCAM Ig III at short contact times. Insertion of the VASE sequence into NCAM was sufficient to increase the adhesion of transfected cells, following at least 120 s of contact. However this extra adhesion was not blocked by peptide addition and it had not been proved that VASE was directly responsible.

![Graph showing the effect of time on the adhesion between control, NCAM and NCAM-VASE expressing cells by single cell AFM.](image)

Figure 3.14: The effect of time on the adhesion between control, NCAM and NCAM-VASE expressing cells by single cell AFM.

Line graph to show the relationship between contact time and the mean maximal unbinding force between homologues cell interactions of control 3T3 cells expressing extremely low amounts of NCAM, N24 cells expressing NCAM and V140G cells expressing NCAM as determined by single molecule force spectroscopy using an AFM. The setpoint contact force between the cells was 0.5 nN, the sensitivity determinant was between 70-100 nm/V and the spring constant was approximately 0.03 N/m. Each data point is the mean of at least 30 cell combinations using at least 10 unique cells and contains data from 3 independent experiments. The equation of the fitted lines are \( y = 0x + 0.357 \), \( y = 0.011x + 0.860 \) and \( y = 0.0033x + 0.461 \) for 3T3, N24 and V140G cell adhesion respectively. The rate of increase in adhesion of cell expressing NCAM-VASE was three times that of NCAM expressing cells.
To fully understand the interaction occurring between NIH 3T3, N24 and V140G cells, a larger range of contact times was chosen to determine its effect on the adhesion between the cell. To assess cell adhesion, the mean maximal unbinding force during the separation of cells was investigated as a function of contact time (Fig. 3.14). Contact times were investigated up to 10 min to see the effect of long-term adhesion. For all three homologous interactions between NIH 3T3, N24 and V140G cells, there was a linear correlation between contact time and mean maximal unbinding force. However there was no positive correlation between these for homologous NIH 3T3 cell adhesion. The mean maximal unbinding force did not increase over time. The increase in adhesion between 5 and 600 s was only less than three times. In comparison the increase in N24 cell adhesion during this time frame was nearly twenty times and between V140G it was approximately forty-five.

The linear correlation between N24 and V140G cell adhesion and contact time was in agreement with the early time points between 5 and 120 s which revealed the same pattern. Above 120 s of contact time, the adhesion between V140G cells was extremely significantly increased over N24 cell adhesion. It is interesting to note that the standard error of the mean for the maximal unbinding forces at higher contact times revealed there was little variation in the cell population. These results were the first indication that the presence of the VASE sequence in NCAM expressing cells increased the adhesion at both short and long contact times.

Comparing the gradient (m) from the equation of the line (y=mx+c), and hence the rate of increase in adhesion, revealed that m = 0, 0.011 and 0.033 for NIH 3T3, N24 and V140G cells respectively. The gradient of zero for NIH 3T3 cell adhesion indicates that there is no significant increase in adhesion as contact time increases. However the rate of V140G cell adhesion increase was three times greater than that of N24 cells. This indicates that interactions between V140G cells either form three times as quickly or are three times more adherent, or a combination of the two.

Contact time between the cells was limited to 10 min due to the lack of a perfusion system. However evaporation was minimised during measurements using a polyethylene ‘skirt’ around the AFM holder, which covered the Petri dish. Interestingly a few adhesion measurements were taken between V140G cells following 30 min of contact time, which revealed that the cells could not be separated. The point of the failure during their attempted separation was the binding of the extracellular matrix to the plastic dish. This resulted in removal of the adherent cell from the Petri dish. If a lack of a perfusion system were not an issue then cell adhesion after longer contact times would have been carried out using a cantilever with a larger spring constant than 0.03 N/m.
3.6.9 Higher maximal unbinding forces between NCAM-VASE expressing cells were more frequent than NCAM expressing cells

Following the data indicating that insertion of the VASE sequence increased NCAM cell adhesion, in depth analysis was carried out on the maximal unbinding forces during cell separation. Data following 120 s of contact time for each cell type was pooled into bins of 2.5 nN and their frequency was compared (Fig. 3.15). 120 s was chosen as this was the first contact time where an increase in VASE cell adhesion over NCAM was seen. For each cell type the mode values of maximal unbinding force were between 0 and 2.5 nN. However, it is clear that during the separation of V140G cells there was an increased frequency of higher unbinding forces than with N24 cells. For example the frequency of maximal unbinding forces below 2.5 nN was 100: 60: 25% for 3T3: N24: V140G cell separation respectively.

![Figure 3.15: A comparison of the frequency of binned maximal unbinding forces after 120 s of contact between NIH 3T3 cells expressing no NCAM, NCAM and NCAM-VASE by single cell AFM.](image)

Maximal Unbinding Force (nN)

The frequency of binned higher maximal unbinding forces of separating VASE expressing was greater than for N24 cells.

Although the mean maximal unbinding force for V140G cells was 5.0±0.59 nN, 25% of the data still required less than 2.5 nN of force to initially de-adhere them. This data indicated that not all cells adhered as tightly as the mean force. This may be due to variation in the VASE expression in the cell population or it may indicate a number of quantitatively different binding mechanisms occurred during cell adhesion.

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3.7 Measuring the heterophilic adhesion between single cells expressing NCAM±VASE by AFM

Following the measurements of homologous cell adhesion between NIH 3T3, N24 and V140G cells and the finding that VASE expressing cells bound tighter than NCAM cells, the adhesion between heterophilically interacting cells was investigated. NCAM has been found to bind heterophilically with proteins such as L1 as well as others. These heterophilic interactions have been found to contribute towards the adhesive strength of NCAM expressing cells (Woo and Murray 1993; Schmid and Maness 2008). Therefore it was important to determine what role heterophilic interactions with NIH 3T3 proteins played in N24 and V140G cell adhesion. To do this the mean maximal unbinding force was compared with increasing contact time for the following interactions:

3T3-N24 to determine whether N24 surface NCAM binds to NIH 3T3 surface proteins
3T3-V140G to determine whether V140G surface NCAM binds to NIH 3T3 surface proteins
N24-V140G to determine whether V140G VASE protein binds to NCAM on N24 cells

It is important to highlight that during these measurements cis interactions between NCAM±VASE, in the same cell, can still occur. Therefore the heterophilic interactions described in this section probe the effect of heterophilic trans interactions.

3.7.1 After long contact times, the adhesion between single N24 cells was similar to the adhesion between N24 and NIH 3T3 cells

To measure the heterophilic interaction between NCAM expressing cells and control NIH 3T3 cells, single cell AFM was used. The method has been described previously (Section 3.6) and allowed the quantification of maximal unbinding force required to separate cells attached on a cantilever and adherent cells in the AFM chamber. This force was compared at different contact times and revealed an interesting pattern for the adhesion between N24 and NIH 3T3 cells (Fig. 3.16). At short contact times (5, 60 s) the adhesion between NIH 3T3 and N24 cells was low and statistically similar to the interaction between control 3T3 cells. This indicated that the presence of NCAM in one cell only had no effect on the adhesion of NIH 3T3 cells. However after 120 s of contact the cells began to adhere more tightly, and by 300 s of contact their adhesion was statistically similar to homophilic N24 cell adhesion at that contact time. After 300 s the mean maximal unbinding forces were 3.19±0.81 and 4.08±0.56 nN for 3T3-N24 and N24-N24 cells respectively. This information indicates that for the heterophilic interaction of an NIH 3T3 cell to a N24 cell, longer contact times were required before increased adhesion was seen. NCAM on the N24 cells was able to bind to surface receptors on the NIH 3T3 cell to increase adhesion. Therefore the adhesion measured between N24 cells cannot be
accounted for by NCAM homophilic binding in \textit{trans} at the cell-cell interface; it may be accounted for solely by heterophilic interactions.

![Graph showing force versus contact time for different cell interactions]

\textbf{Figure 3.16: Comparison of heterologous adhesion by AFM with homologous interactions on the maximal unbinding force required to separate single cells.}

The mean maximal unbinding forces for all possible homologous and heterologous cell interactions were compared by single cell AFM with increasing contact times. The setpoint contact force between the cells was 0.5 nN, the sensitivity determinant was between 70-100 nm/V and the spring constant was approximately 0.03 N/m. The cantilever extend and retract velocities were 5 μm/s. Each data point is the mean of at least 30 cell combinations using at least 10 unique cells taken in at least three different experiments.

3.7.2 The homophilic adhesion between V140G cells was dramatically increased over heterophilically interacting V140G and NIH 3T3 cells

Measuring the heterophilic interactions between NIH 3T3 cells and V140G cells revealed differences with N24 and NIH 3T3 cell adhesion. In contrast, the adhesion between a population of V140G and NIH 3T3 cells is statistically similar to 3T3-3T3 cell adhesion between contact times of 5 and 120 s (Fig. 3.16). Therefore the larger adhesion seen between homophilically interacting V140G cells cannot be accounted for by heterophilic interactions between NCAM-VASE on V140G cell and NIH 3T3 proteins on the apposing cell.

After 300 s of contact time there was an increase in the adhesion between NIH 3T3 and V140G cells, which was statistically similar to 3T3-N24 and N24-N24 cell adhesion at that contact time. After 600 s of contact time, 3T3-V140G cell adhesion was not quite statistically different (p<0.1) from 3T3-N24 cell adhesion, but was extremely statistically lower than V140G homophilic adhesion. This indicated
that although heterophilic interactions occurred and appeared to be quantitatively similar to those between 3T3 and N24 cells, they did not account for the large adhesion seen between V140G cells.

3.7.3 NCAM-VASE was required on both cells in single cell AFM for increased adhesion

The mean maximal unbinding force between N24 and V140G cell pairs was statistically similar to the mean between N24 cell pairs for all contact times (Fig. 3.16). This data revealed that a heterophilic interaction between an NCAM and a VASE expressing cell was not sufficient to increase the adhesion to a V140G-V140G level. Therefore it appears that NCAM-VASE protein was required on both cells, and that a homophilic trans interaction was necessary for the extra adhesion seen. This was the first piece of evidence to suggest that an NCAM-VASE complex interacting in trans formed between the cells to cause an increase in cell adhesion. An explanation for why N24-V140G adhesion is quantifiably similar to N24-N24 would be that there was less NCAM on N24 cells than VASE on V140G cells. Therefore the amount of NCAM protein could have been the limiting factor in N24-V140G cell adhesion and would explain why it was similar to N24-N24 cell adhesion. However this argument has proved to be invalid as an ELISA revealed there are statistically similar amounts of NCAM and NCAM-VASE on N24 and V140G cells (Fig. 3.3).

3.7.4 The role of cell elasticity in cell adhesion

Although probe cell size has already been investigated to determine if a difference in contact area between cell pairs in AFM was responsible for increased V140G cell adhesion (Fig. 3.13), cell elasticity may play a role in this. When contact is made between the probe and adherent cell during AFM, the cantilever continues to lower until a contact force of 0.5 nN is reached. If V140G cells were more elastic than N24 cells, the contact area between them would be larger than between two N24 cells. This would account for the increased adhesion measured and would increase the number of interactions possible. Therefore the separation of a larger number of interactions, rather than a property of the transfected VASE protein, would be responsible for larger maximal unbinding forces between V140G cells.

Even though the contact area between V140G cells was not three times larger than that between N24 cells (to account for the three times increase in V140G adhesion over N24), it has been found that past a threshold concentration of NCAM, adhesion increases exponentially (Doherty, Fruns et al. 1990). Therefore a small difference in contact area may increase the number of interacting NCAM-VASE proteins and cause an increase in V140G cell adhesion.

To test whether the elasticity of cells was the reason for difference in NCAM and NCAM-VASE cell adhesion, the heterophilic interaction between N24 and V140G was investigated further. It was
found that the adhesion between these cells was only as adhesive as the homophilic interaction between N24 cells (Fig. 3.16). Within this data set, the mean maximal unbinding force was compared for a series of N24 probe cells and V140G adherent cells and V140G probe cells probing N24 adherent cells (Fig. 3.17). If V140G cells were more elastic than N24 cells, the contact area between the V140G probe cell and N24 adherent cell would be larger than that between an N24 probe cell and an adherent V140G cell. This would mean that there would be more interactions between a V140G probe cell and adherent N24 cell and therefore the adhesion would be larger than that between a stiffer N24 probe cell and an adherent cell. However if the elasticity of the V140G cell was similar to an N24 cell, then the number of interactions would be the same as those between an N24 probe cell and a V140G adherent cell and adhesion would be similar.

![Image](image.png)

Figure 3.17: AFM experiment to compare the heterophilic adhesion of N24 and V140G cells using both N24 and V140G probe cells.

A single cell AFM experiment was carried out to measure the mean maximal unbinding force between an N24 probe and V140G adherent cell and a V140G probe and N24 adherent cell. By comparing heterophilic adhesion using N24 and V140G probe cells, an idea of the probe cell elasticity could be estimated to see if V140G cells were more elastic than N24 cells to account for increased V140G cell adhesion. The mean maximal unbinding forces for contact times between 5 and 600 s were compared. The setpoint contact force between the cells was 0.5 nN, the sensitivity determinant was between 70-100 nm/V and the spring constant was approximately 0.03 N/m. The cantilever extend and retract velocities were 5 μm/s. Each data point is the mean of at least 30 cell combinations using at least 10 unique cells taken in at least three different experiments.
This experiment only tested for the relative contact areas during cell-cell interaction as a result of potential elasticity differences and did not look at other effects of elasticity. It also assumed that the adherent cells, attached to the polystyrene dish, were stiffer than the probe cells which had weaker connections to the cantilever. This assumption was made as when rounded and triturated NIH 3T3 cells attach to a surface and spread due to cytoskeletal rearrangements, their elasticity decreased corresponding to an increase in the Young’s modulus values (Yeung, Georges et al. 2005).

The mean maximal unbinding forces between N24 probe cells and adherent V140G cells compared with V140G probe cells and N24 adherent cells revealed they were statistically similar at all contact times except 120 s. This similarity indicates that adhesion with an NCAM-VASE expressing cell is not simply increased due to an increased contact area, because when V140G was present as an adherent cell, adhesion was not less than when it was a probe cell. Therefore the increased adhesion measured between V140G cells is due to the transfection of the NCAM-VASE protein and potentially due to a property of that protein. Specific blocking of the adhesion between the VASE cells was not previously successful; therefore NCAM-VASE cannot be directly linked to the increase in adhesion. From the measurement of the heterophilic interaction between N24 and V140G, it has been proved that cells transfected with NCAM-VASE interaction in trans to elicit an increased adhesion.

To provide further evidence for the similarity in elasticity of the N24 and V140G cells, the Young’s modulus should be calculated. To do this an incompressible glass bead would be glued to a tipless cantilever and a series of force-distance curves, over the nucleus of the adherent cell and the centre of a probe cell, would be collected by AFM. Information regarding the indentation distances and forces could then be used to calculate the Young’s modulus. This would provide direct information about the elasticity of the cells and hence whether the contact areas between the cells were similar.

3.7.5 The work required to separate homologous and heterologous cell combinations follows the same pattern as for maximal unbinding force

Following the measurement of heterophilic cell interactions by AFM, further information was deduced from the force –distance curves. The work required to separate cells was analysed by calculating the area under the extension curve. The work required to separate the cells was plotted as a function of contact time for all homophilic and heterophilic interactions (Fig. 3.18). Most remarkably, the work required to separate V14G cells increased dramatically as a function of time. After 600 s of contact time, the work required to separate two V140G cells was four times that required to separate homophilically interacting N24 or heterophilically interacting N24 and V140G cells. This increase was more marked than with the mean maximal unbinding force presented previously where there was an increase of three times (Fig. 3.16). This indicates that there was an
extra component involved in the work required to separate cells, which contributed to the large increase in adhesion between the cells. The work of separation involved the number and size of individual unbinding events which occurred following the maximal unbinding force between the cells.

![Graph showing mean work (J) vs. contact time (s) for different cell combinations: 3T3-3T3, N24-N24, V140G-V140G, N24-V140G, T3-N24, and 3T3-V140G.](image)

**Figure 3.18: Comparison of homologous and heterologous cell adhesion by AFM on the work required to separate single cells.** The mean maximal unbinding forces for all possible homologous and heterologous cell interactions were compared by single cell AFM with increasing contact times. The setpoint contact force between the cells was 0.5 nN, the sensitivity determinant was between 70-100 nm/V and the spring constant was approximately 0.03 N/m. The cantilever extend and retract velocities were 5 μm/s. Each data point is the mean of at least 30 cell combinations using at least 10 unique cells taken in at least three different experiments. The work required to separate all single cell combinations followed the same pattern as for maximal unbinding force.

Interestingly, the pattern of work required to separate interacting cells was the same as for the mean maximal unbinding force measured. This indicates that the work required and maximal unbinding forces were intrinsically linked.

3.7.6 At most contact times the mean unbinding event size during the separation of V140G cells was higher than for all other cell combinations

To further understand the role of factors involved in the unbinding of V140G cells, the individual unbinding events during cell separation of homologous and heterologous cell combinations were calculated and compared against increasing contact times (Fig. 3.19). These individual unbinding events occurred following a plateau or a period of extrusion of a membrane nanotube from either
one cell, or both, where there is a protein interaction at the cell-cell interface. JPK image processing software selected unbinding events and these were collated and averaged from at least 30 force-distance curves.

![Graph showing mean unbinding event (pN) vs. contact time (s)](image)

*Figure 3.19: Comparison of the mean unbinding events during the separation of all combinations of control, NCAM and VASE expressing cells.*

Heterologous and homologous interactions between 3T3, N24 and V140G cells were measured by single cell AFM. The unbinding events, which are the vertical jumps (positive changes in cantilever deflection after the rupture of an interaction between cells) after a plateau (area of increasing contact time where there is no change in cantilever deflection), were collated and averaged to see the effect of increasing the contact time on them. Each data point contains all of the unbinding events from at least 30 curves, which were taken from at least 10 different cell pairs in three independent experiments. Setpoint contact force=0.5 nN, the sensitivity determinant was between 70-100 nm/V and the spring constant was approximately 0.03 N/m. The cantilever extension and retraction velocities were 5 μm/s. N.S. Not statistically significant, *P<0.05 (statistically significant), ** P<0.001 (very statistically significant), *** P<0.0005 (extremely statistically significant). **At most contact times the individual unbinding events between V140G cells are larger than those between N24 cells.**

The most striking part of the mean unbinding event size data was that the pattern seen on the maximal unbinding force and work bar charts was similar. At almost all contact times, except 300 s, the mean individual unbinding events between V140G cells were higher than for all other interactions. At these contact times the V140G unbinding events were statistically significantly higher than those occurring during N24 cell separation. This indicated that the interactions occurring between V140G cells were more adhesive or that the linkage between the cytoplasmic portion of the cell membrane proteins and the cytoskeleton was stronger. Transfection of NCAM-VASE into NIH
3T3 cells therefore affected the adhesive properties of the cell type by increasing the adhesive strength of the cell.

At a contact time of 5 s, which was the closest to the parameters used in the single molecule force spectroscopy experiment, statistical analysis revealed that the mean unbinding event size between V140G cells was significantly increased over N24 cells. After 5 s of contact time, the mean individual unbinding event between NIH 3T3 cells was 21 pN, 38 pN for N24 and 45 pN for V140G cells. Single molecule measurements between NCAM and NCAM-VASE proteins (See Chapter 2) revealed that the force required to separate single proteins was approximately 120 pN for a contact force of 0.5 nN and a retraction speed of 0.5 μm/s. The cell unbinding events were approximately a third of the apparent single molecule protein unbinding events. This indicates that either the unbinding events occurring during cell separation were not due to the unbinding of NCAM±VASE protein at the cell-cell interface, or the ‘single molecule’ AFM measurements were due to the unbinding of multimeric NCAM±VASE proteins. Single molecule measurements were carried out following Fc tag capture; therefore it is plausible that the unbinding events were due to the separation of two pairs of protein (Fc can dimerise).

Although the individual unbinding events during cell separation may not be due to direct unbinding of NCAM±VASE, the introduction of NCAM or NCAM-VASE into the cells increased the strength of these individual units. The extra adhesion seen between N24 cells, compared with control cells, could be due to the size of unbinding events and therefore the stronger individual interactions between the cells.

During the separation of heterophilic cell interactions, N24-V140G and 3T3-N24, the mean individual unbinding events were similar to those during N24 cell separation. This pattern was seen during the measurement of the work and maximal unbinding force. The unbinding events between 3T3 and V140G cells were lower than those between 3T3 and N24 cells at shorter contact times, but increased with increasing contact time and by 600 s contact time were statistically similar. This data indicates that the maximal unbinding force and work directly correlates with the size of the individual unbinding events occurring during cell separation by AFM.

Interestingly the mean individual unbinding event between 3T3 cells increased from approximately 20 to 45 pN between 5 and 600 s of contact time. The mean for N24 cells increased less dramatically, from approximately 40 to 55 pN during the same contact time period. The mean unbinding events during V140G cell separation were between 45 and 70 pN. It is plausible that these unbinding events were due to the breakage of a membrane nanotube, either rupturing at its anchor points or along its
axis, or due to the rupture of protein complexes at the nanotube interface between the cells (Schmitz, Benoit et al. 2008). The data suggests that these interactions between the three cell lines were quantitatively different due to the transfection of either NCAM or NCAM-VASE into the cells.

### 3.7.7 Increasing contact time between cells increased the frequency of larger unbinding events particularly between V140G cells

There are multiple methods of force spectroscopy analysis which can be employed to gain further insight into interactions measured by AFM. Comparisons of mean forces have been used extensively in this chapter but it is also necessary to compare the frequencies of the interactions which have occurred. This can indicate if there was any size periodicity in the interactions and whether one or more quantitatively different interactions were occurring.

![Graphs showing the effect of contact time on the size of unbinding events](image)

**Figure 3.20:** The effect of contact time on the size of unbinding events during the separation of homologous interactions of NIH 3T3, N24 and V140G cells were investigated by SCFS on the AFM.

All unbinding events during 30 force curves taken from at least 10 cell combinations were binned into 10 pN intervals and plotted against their frequency. a) The unbinding event frequency between 3T3 cells during their separation at varying contact times. b) The unbinding event frequency between N24 cells during their separation at varying contact times. c) The unbinding event frequency between V140G cells during their separation at varying contact times. d) A comparison of the frequency of unbinding events after 120 s of contact time between homologous combinations of 3T3, N24 and V140G cells. Individual unbinding events between V140G cells at all contact times were frequently higher than all other cell combinations.

To determine the unbinding force profile of separating cells, the individual unbinding event forces were pooled for each contact time and cell parameter and separated into forces of 10 pN bin widths. The frequency of each bin was calculated and compared (Fig. 3.20). Even at low contact times, there
was an increased frequency of large unbinding events during V140G cell separation compared with other cell types. For example, the frequency distribution of unbinding events following NIH 3T3 cell separation after 5 s of contact followed a single normal distribution (where the mean was approximately the same as the mode). However the equivalent V140G cell profile appeared to show a bimodal or possibly trimodal distribution, where a percentage of the unbinding events have deviated from the modal distribution at 20 pN resulting in distribution ‘shoulders’ at 40 and 60 pN. This may indicate a periodicity in the unbinding forces with unbinding event multimers of 20 pN.

3.7.8 At all contact times, the percentage of unbinding events, during cell separation, over 50 pN was higher for V140G cells than for all other cell combination

To further understand the distribution of high force unbinding events during V140G cell separation, the percentage of individual unbinding events which were over 50 pN was compared for all contact times and for all homophilic and heterophilic interactions (Table 3.4).

In general, the percentage of unbinding events over 50 pN calculated from the separation of all cell types increased with increasing contact time. For NIH 3T3 cell separation, the vast majority of events (between 75 and 99%) were less than 50 pN, whereas for V140G cell separation this was between 45 and 80%. There was no significant difference between the frequency of unbinding events over 50 pN for homophilic and heterophilic NCAM interactions, which provides further evidence that these interactions were qualitatively similar.

Table 3.4: Comparison of the percentage of unbinding events during the separation of all cell combinations using single cell AFM, which are over 50 pN.
The percentage of unbinding events over 50 pN from heterologous and homologous interactions of 3T3, N24 and V140G cells was measured by single cell AFM. Each data point contains all of the unbinding events from at least 30 curves, which were taken from at least 10 different cell pairs in three independent experiments. Setpoint contact force=0.5 nN, extend and retract cantilever velocities are 5 μm/s. The percentage of unbinding events between V140G cells over 50 pN is larger than all other cell combinations for all contact times.

<table>
<thead>
<tr>
<th>Contact Time (s)</th>
<th>3T3-3T3</th>
<th>N24-N24</th>
<th>V140G-V140G</th>
<th>N24-V140G</th>
<th>3T3-N24</th>
<th>N24-V140G</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>1.2</td>
<td>8.9</td>
<td>20.8</td>
<td>7.6</td>
<td>13.3</td>
<td>23.0</td>
</tr>
<tr>
<td>60</td>
<td>11.4</td>
<td>20.0</td>
<td>35.1</td>
<td>23.8</td>
<td>16.4</td>
<td>17.5</td>
</tr>
<tr>
<td>120</td>
<td>8.0</td>
<td>19.2</td>
<td>40.9</td>
<td>23.5</td>
<td>22.2</td>
<td>12.8</td>
</tr>
<tr>
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<td>34.6</td>
<td>25.8</td>
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<td>31.5</td>
<td>54.4</td>
<td>34.0</td>
<td>27.1</td>
<td>28.3</td>
</tr>
</tbody>
</table>

3.7.9 There were more unbinding events during V140G homologous cell separation than for any other cell combination

Although the individual unbinding events during V140G cell separation were generally larger than for other cell interactions, this alone did not account for why V140G cell adhesion was dramatically
higher. To provide further insight, the number of individual unbinding events was calculated from each force curve for each cell combination and contact time and was averaged (Fig. 3.21). Following a short term contact time of 5 s, there were approximately 5 unbinding events during cell separation for each cell-cell interaction. The mean maximal unbinding force for this contact time was between approximately 0.3 and 0.5 nN for each. Excluding the jump region which accounted for approximately one fifth of the deflection return to the baseline, five unbinding events or interactions between 20 and 45 pN each accounted for 0.2-0.4 nN of adhesion. Therefore a combination of the number of unbinding events and their size indicated the maximal unbinding force required to separate all interactions at the cell-cell interface.

Figure 3.21: Comparison of the mean number of unbinding events during single cell AFM measurements between all cell combinations. The mean number of unbinding events was calculated from the separation of all homologous and heterologous cell interaction between control NIH 3T3, NCAM expressing and NCAM-VASE expressing cells using single cell AFM. Each data point consists of the total number of unbinding events from at least 30 curves, which were taken from at least 10 different cell pairs in three independent experiments. Setpoint contact force=0.5 nN, extend and retract cantilever velocities are 5 μm/s. At longer contact times, there are more individual unbinding events during the separation of V140G cells than other cell combinations.

After 120 s of contact time, which was the first instance where VASE cell adhesion was increased over NCAM, there was a statistically significant (p <0.05) increase in the number of unbinding events between V140G cells. Beyond this contact time, there was a dramatic increase in the number of unbinding events between V140G cells, which was not the case for other cell combinations. From this data it appeared that the increase in the number of jumps between V140G separating cells accounted for their dramatic increase in cell adhesion. For all cell types the relationship between the
number of unbinding events was the same as in the work, mean maximal unbinding force and mean size of individual unbinding events.

3.8 Effect of latrunculin on the adhesion of NCAM±VASE cells

3.8.1 200 nM latrunculin B depolymerises actin in NIH 3T3, N24 and V140G cells but does not affect the expression of surface NCAM

Single AFM measurements between cells expressing NCAM-VASE revealed that there was an increase in the force required to separate cells over those expressing NCAM. To further understand the mechanism of increased V140G cell adhesion, the role of the actin cytoskeleton on adhesion was investigated.

The actin cytoskeleton has been found to be important in increasing the strength of cell adhesion due to cytoskeletal accessory proteins, which bind to the cytoplasmic tails of CAMs (Murray and Jensen 1992). Although the insertion of VASE is in Ig IV of NCAM and may not be involved directly in the recruitment of strengthening cytoskeletal accessory proteins via its cytoplasmic tail, NCAM-VASE may form a larger clustering complex on the surface, which recruits more accessory proteins to increases transmembrane mechanical strength. To test whether actin plays a role in this hypothesis the effect of an actin depolymerising agent, latrunculin B, on cell-cell adhesion was investigated by AFM. Addition of cytoskeletal altering drugs has previously been employed in single cell AFM experiments to see whether filamentous actin (F-actin) played a role in E-cadherin mediated cell-cell interactions (Bajpai, Correia et al. 2008).

The effect of 200 nM latrunculin B on control NIH 3T3, N24 and V140G cells was tested using a TRITC phalloidin and NCAM antibody (α N16). Briefly, cells were grown at low density so that after 16 hours their confluence was 25%. Prior to cell fixation, cells were incubated as they would be if AFM measurements were to be taken. If latrunculin B treatment was required, cell media was removed and replaced with DMEM HEPES for 30 min at 37°C. Cells were then incubated in DMEM HEPES with 200 nM latrunculin B for 30 min. Cells were observed by live phase microscopy and revealed that cells had lost contact area with the Petri dish. Cells were fixed immediately by adding paraformaldehyde up to a v/v concentration of 4%. If the latrunculin B media was removed and replaced with full serum media before fixation, cells would revert back to their original spread phenotype. Cells were blocked before addition of α N16 and a FITC conjugated secondary antibody. Cells were permeabilised with MeOH and blocked before TRITC phalloidin was added to the cells to bind to an epitope in F-actin.
Finally cells were mounted onto glass slides using PBS/10% glycerol/1 μg/ml DAPI and visualised by fluorescence microscopy. Addition of 200 nM latrunculin B had a dramatic effect on the morphology of all three cell lines (Fig. 3.22). Their area appeared to be dramatically decreased in comparison with cells which had not been treated (Fig. 3.2). TRITC phalloidin staining revealed that less F-actin was present in the cell cytoplasm than in untreated cells. Actin was not present in long filamentous bundles following treatment. This indicated that the depolymerisation of actin had occurred due to the addition of latrunculin B. Staining with α N16 revealed that addition of latrunculin B to NIH 3T3 cells had no effect on the expression level of surface NCAM; they still did not express quantifiable levels of NCAM. However addition of latrunculin B seemed to have no effect on the distribution and levels of NCAM and NCAM-VASE on N24 and V140G cells respectively.

![Fluorescence images](image)

**Figure 3.22**: Fluorescence images to show NCAM surface expression and the effect of 200 nM latrunculin B. Fluorescent microscope images to show the expression and localisation of NCAM on NIH 3T3, N24 (expressing NCAM) and V140G (expressing NCAM-VASE) cell lines, and on morphology after 30 min treatment with 200 nM latrunculin B, which depolymerises actin. The DAPI images show nuclear staining; α N16 stains both NCAM and NCAM-VASE and TRITC phalloidin binds to polymerised actin. Latrunculin B depolymerised actin and destabilises cells by decreasing the contact area between cell and surface.
Overall treatment of NIH 3T3, N24 and V140G cells with 200 nM latrunculin did not appear to affect the amount and distribution of NCAM on the cell surfaces but it did alter the size of cells and the organisation of the actin cytoskeleton.

3.8.2 Addition of 200 nM latrunculin B to 3T3, N24 and V140G did not alter their expression of surface NCAM and NCAM-VASE by ELISA

Latrunculin B had a dramatic effect on the morphology of fibroblast cell lines used in the AFM experiments. By fluorescence microscopy it was determined that there had been no visible effect on NCAM and NCAM-VASE surface expression after addition of latrunculin B. To quantify this, an ELISA was carried out using surface binding of α N16 to judge NCAM±VASE surface expression levels. The method used for this ELISA has been previously presented (Section 3.2.2).

![Bar chart showing relative NCAM expression](image)

**Figure 3.23**: ELISA to show NCAM surface expression on 200 nM latrunculin B treated control, NCAM and NCAM-VASE expressing cells.

The amount of surface NCAM on N24 and NCAM-VASE on V140G cells was compared to control 3T3 cells by ELISA, using an NCAM antibody (α N16). Briefly monolayers of fibroblast cells were plated 16 hours before fixing, then fixed with PFA and blocked before addition of antibodies. In parallel a duplicate plate was set up and MTS was added to each cell type to measure the number of living cells. Cell NCAM expression was then corrected using the differences in cell number revealed by the MTS assay. The ELISA is the result of four independent experiments. Relative surface NCAM expression is shown for each cell type where 3T3 cells express very low levels of NCAM and N24 and V140G cells express similar high levels. NCAM and VASE transfected NIH 3T3 still express surface NCAM and NCAM-VASE following latrunculin B treatment.

200 nM latrunculin B was added to cells for 30 min in DMEM HEPES before fixation and staining. The relative NCAM expression was compared for all three cell lines used in AFM experiments (Fig. 3.23). There was no statistical difference between the amounts of surface NCAM on control and latrunculin B treated NIH 3T3 cells. Therefore the depolymerisation of F-actin had no effect on the amount of surface NCAM on the basic NIH 3T3 cell line. Interestingly, after addition of latrunculin B there was significantly more NCAM-VASE present on V140G cells than NCAM on N24 cells. However the
relative amount of surface NCAM on N24 was statistically similar to that on untreated N24 cells. Addition of latrunculin B to V140G cells slightly increased the amount of NCAM-VASE present on the surface. This may be due to difference in cell area, which was apparent after cell treatment (Fig. 3.22).

Overall the addition of 200 nM latrunculin B did not reduce the amount of NCAM and NCAM-VASE on the surface of N24 and V140G cells.

3.8.3 Addition of 200 nm latrunculin B to adherent 3T3, N24 and V140G cells reduces their area significantly

Although treatment of fibroblast cell lines with 200 nM latrunculin B had no dramatic effect on the expression levels of NCAM and NCAM-VASE, fluorescence microscopy revealed that cellular morphology had changed. It was apparent that cell area had been reduced. To quantify the effect of latrunculin B treatment on NIH 3T3, N24 and V140G cells, their area was compared using a method employed previously (Section 3.6.7). Areas were calculated following 30 min incubation in 200 nM latrunculin B (Fig 3.24).

![Figure 3.24: Measurement of control, NCAM and VASE cell areas following latrunculin B treatment using single cell AFM experimental parameters.](image)

Bar chart to show the mean cell areas (µm, y-axis) of probe 3T3, N24 and V140G cells and adherent cells. The probe cells were grown for 16 hours so that they were low density. 200 nM latrunculin B was then added to the cells for 30 min at 37°C. Cell areas were calculated in the presence of latrunculin B using Axiovision microscope software under 20 x phase magnification. Each data point is the mean of three independent experiments where 200 cells were measured in each. The area of adherent control, NCAM and NCAM-VASE expressing NIH 3T3 cells is statistically similar but reduced upon treatment with latrunculin B.

Before latrunculin B treatment cell areas were between 1500 and 1600 µm². After addition, the area of N24 and V140G cells was reduced by 50% to approximately half of the untreated area. It is
interesting to note that NIH 3T3 cell area was least affected by latrunculin B and was only reduced by approximately 35%. Probe cell areas were not significantly different after treatment with latrunculin B (data not shown). This was because these untreated cell areas were only 200 µm² and due to their rounded nature had very different cytoskeletal organisation.

3.8.4 Addition of 200 nM latrunculin B reduced the adhesion of N24 and V140G to 3T3 levels measured by single cell AFM

![Mean Maximal Unbinding Force vs Contact Time](image)

Figure 3.25: Single cell AFM experiment to measure the interaction between NIH 3T3 cells and those expressing NCAM and NCAM-VASE following treatment with 200 nM latrunculin B. The effect of 200 nM latrunculin B on adhesion between N24 cells (expressing NCAM) and V140G cells (NCAM-VASE) were compared using single cell AFM. Cells were treated with 200 nM latrunculin B in DMEM/HEPES for 30 min prior to measurements in the latrunculin B. A probe cell was picked up in situ. Cantilever deflection was translated into force (N) which allowed quantification of adhesion from the maximal force required to separate the two cells (µN) and this was plotted against the contact time (s, x-axis). The setpoint contact force between the cells was 0.5 nN, the sensitivity determinant was between 70-100 mm/V and the spring constant was approximately 0.03 N/m. The mean of the maximal unbinding force at each contact time was calculated from at least 10 different cell pairs and at least 30 individual measurements during three independent experiments. Addition of latrunculin B to N24 and V140G cells significantly decreases the mean maximal unbinding force by single cell AFM.

To test the role of the actin cytoskeleton in NCAM±VASE mediated cell adhesion, adhesion between single cells was measured using single cell AFM. The method of cantilever probe cell functionalisation and all other experimental parameters were the same as those employed previously (Section 3.6). 200 nM latrunculin B was added to the cells, following calibration and probe cell attachment to the cantilever, for 30 min at 37°C before measurement were taken in situ.
The cells were left in latrunculin B during adhesion measurements otherwise they would revert to their untreated phenotype.

The mean maximal unbinding force was compared for a range of contact times between 5 and 300 s following a cantilever velocity of 5 μm/s and contact force of 0.5 nN (Fig. 3.25). The effect of latrunculin B on longer term adhesion was not investigated to minimise the experiment length. The mean maximal unbinding forces of untreated, control N24 and V140G cells were in good agreement with pooled data presented previously (Fig. 3.16). After addition of 200 nM latrunculin B, adhesion between N24 and V140G cells was dramatically decreased after each contact time. After 300 s of contact time, V140G cell adhesion was reduced by 75%, whereas for N24 this was 90%. After 120s this reduction was 35% for V140G cells and 20% for N24 cells. It seemed that latrunculin B treatment had a greater effect on cell adhesion in N24 cells than V140G cells. This extra decrease in N24 adhesion was not due to a difference in cell area, as probe cells were statistically similar for N24 and V140G cells. After treatment of N24 cells with latrunculin B, the adhesion of the cells was similar to control NIH 3T3 adhesion readings. This may indicate that the NCAM component of cell adhesion has been completely removed following actin depolymerisation.

It is plausible that adhesion of VASE expressing cells is less dependent on the actin cytoskeleton after longer contact times than NCAM expressing cells.

3.8.5 200 nM latrunculin B significantly reduced the size of mean unbinding events during the separation of N24 and V140G cells

To understand how treatment of NCAM and NCAM-VASE expressing cells with latrunculin B reduced their adhesion, the mean size of individual unbinding events, occurring during cell separation, was calculated. Mean unbinding events sizes were compared after cell contact times between 5 and 300 s and were compared those with untreated cells, taken immediately before latrunculin B treatment, and NIH 3T3 events (Fig 3.26).

Untreated cell unbinding events were in agreement with those presented from pooled data previously (Fig. 3.19), although the difference between the means of N24 and V140G was not as marked. Treatment of V140G cells with 200 nM latrunculin B decreased the mean unbinding event size by approximately 60%, from 45-60 pN to 15-20 pN for contact times between 5 and 120 s. This dramatic difference was interesting and indicated that the rupturing complexes were weaker than those between untreated cells. A similar decrease in the mean size of unbinding events between N24 occurred following treatment with latrunculin B. It is interesting to note that at longer contact
times, the mean unbinding event size for N24 and V140G cells were markedly smaller than those between NIH 3T3 control cells.

These unbinding events may be due to the rupturing of NCAM-VASE complexes at the cell-cell interface or due to the rupturing of membrane nanotube or cytoskeletal filaments. It is likely that these unbinding events were decreased due to disruption of the links to the actin cytoskeleton due to depolymerisation.

![Graph showing mean unbinding event size (pN) vs contact time (s) for different cell types.]

**Figure 3.26:** Single cell AFM experiment to measure the size of unbinding events during the de-adhesion of NIH 3T3 cells and those expressing NCAM and NCAM-VASE following treatment with 200 nM latrunculin B. The effect of 200 nM latrunculin B on the size of individual unbinding events between N24 cells (expressing NCAM) and V140G cells (NCAM-VASE) were compared using single cell AFM. Cells were treated with 200 nM latrunculin B in DMEM/HEPES for 30 min prior to measurements in the latrunculin B. A probe cell was picked up in situ. Cantilever deflection was translated into force (N) which allowed quantification of adhesion from the maximal force required to separate the two cells (y-axis) and this was plotted against the contact time (s, x-axis). The setpoint contact force between the cells was 0.5 nN, the sensitivity determinant was between 70-100 nm/V and the spring constant was approximately 0.03 N/m. The mean of the maximal unbinding force at each contact time was calculated from at least 10 different cell pairs and at least 30 individual measurements during three independent experiments. **Addition of latrunculin B to N24 and V140G cells significantly decreases the size of unbinding events by single cell AFM.**

### 3.9 Conclusion

The aim of this chapter was to determine whether insertion of the VASE sequence into cells expressing NCAM, altered cell-cell adhesion binding strength. To do this, a single cell force spectroscopy method was chosen using an AFM instrument. Prior to AFM measurements between
cells, it was necessary to develop an in vitro cellular assay to measure the adhesion between adherent cells and a probe cell on the cantilever. The NIH 3T3 cell line was chosen for experimentation due to the availability of NCAM-140 and NCAM-VASE-140 transfected NIH 3T3 cells. Cells were characterised by ELISA, immunocytochemistry and Western blotting to reveal that they expressed statistically similar levels of NCAM and NCAM-VASE.

After this characterisation, a cantilever functionalisation method was optimised using poly-l-lysine and fibronectin coating to attach scraped and trituated probe cells in situ (Fig 3.7). The adhesion between single probe and adherent cells was measured by SCFS. Transfection of NCAM-140 into NIH 3T3 cells (N24 cells) resulted in a threefold increase in adhesion after 60 s of contact time (Fig 3.9). Addition of a peptide KYI, which corresponded to a sequence in NCAM Ig III, reduced N24 cell adhesive strength to the parental NIH 3T3 level after 60 s of contact (Fig. 3.10). This indicated that the KYI site in Ig III was essential for N24 cell adhesion.

Transfection of NCAM-VASE-140 into NIH 3T3 cells (V140G cells) increased the adhesion between cells after 120 s of contact, which was a two-fold increase over N24 cells (Fig. 3.11). After 10 min of contact, V140G cells required, on average, 21 nN to separate them, which was a three-fold increase over N24 cells (Fig. 3.16). The area of rounded probe cells was measured using light microscopy and was determined to be similar for all three cell types. Therefore an increased V140G probe cell area did not account for the increased V140G adhesion measured (Fig. 3.13).

Addition of the KYI peptide to V140G cells did not decrease their adhesion (Table 3.1). This indicated that although NCAM expressing cells required the KYI site to undergo NCAM homophilic binding, V140G cells did not. Therefore a series of other peptides were tested to determine what NCAM-VASE binding sites were involved in V140G cell adhesion. Two peptides which either bound to Ig I or mimicked the action of NCAM Ig II did not abrogate V140G cell adhesion nor did the VASE peptide (Table 3.3). This was most surprising since the VASE peptide has been found to be functionally active and inhibits neurite outgrowth of cerebellar granule neurons grown over fibroblast monolayers (Doherty, Moolenaar et al. 1992). Insertion of the VASE sequence may therefore increase NCAM adhesion by altering the structure of NCAM or introducing a new binding site to NCAM so addition of a linear VASE peptide will have no effect. Due to time constraints a range of peptide concentrations were not tested although this may be necessary to prove that the increased adhesion measured between V140G cells was due to the presence of NCAM-VASE.

A comparison of the force required to separate homophilically and heterophilically binding single cells revealed further insights into NCAM and NCAM-VASE adhesion (Fig. 3.16). N24 cells were able
to bind heterophilically to parental cells and between 5 and 10 min of contact time, were found to bind with similar adhesion to two N24 cells. This indicated that N24 cell adhesion at longer contact times involved trans heterophilic interactions which were only possible if NCAM was present on at least one cell. In contrast, the interaction between V140G and parental cells was extremely significantly lower than between two V140G cells indicating that a trans heterophilic interaction did not account for the increased adhesion measured between V140G cells. Finally it was determined that NCAM-VASE was required on both cells to increase their adhesion and therefore a trans interaction between NCAM-VASE proteins may be involved in a more adhesive complex to increase adhesion.

All parameters quantified from the force-distance curves, for the separation of all types of interacting cells, followed the same pattern of adhesion where V140G-V140G > N24-N24 = N24-V140G. During the separation of V140G cells, the number of unbinding events increased from a mean of 5 to 30 between 5 s and 10 min of contact and the mean size increased from 45-75 pN (Fig. 3.19, Fig. 3.21). In comparison during the separation of N24 cells over this contact period the mean numbers of unbinding events were between 5 and 15 and their size increased from 40-50 pN only. Therefore V140G cells required more force to separate them because there were more adhesive contacts between the cells and individually they required more force to separate than those between N24 cells.

The individual unbinding events, which were quantified as the vertical jumps immediately following tethers on the force-distance curve, were similar in size to the NCAM extracellular domain rupture forces described by Wieland et al., which were between 40 and 100 pN for loading rates of 780-7400 pN/s (Wieland, Gewirth et al. 2005). It would be useful to compare the single molecule and single cell individual unbinding forces and loading rates to determine whether they were similar. However it is difficult to determine the loading rate during the separation of cells as the interactions are far more complex than those in SMFS. It would be too simplistic to determine the loading rate of the unbinding forces from the cantilever velocity and spring constant since this does not take into account the cellular component of the effective spring constant. Therefore it is not appropriate to conclude whether the individual unbinding event forces during N24 and V140G cell separation are similar to those presented in single molecule experiments.

An argument has been presented by Marcus et al., which describes that rupture events during membrane extrusion in cell-cell separation are due to the tether breakages and not due to the presence of a protein-protein interaction at the cell-cell interface (Marcus, McEver et al. 2004). It is unknown whether this would be the case for the NIH 3T3 cell line.
Addition of an actin depolymerising agent, latrunculin B, to N24 and V140G cells reduced the surface area of cells but did not dramatically alter the level of NCAM±VASE protein present on the cell surface (Fig 3.23). SCFS measurements between latrunculin treated cells revealed that they were unable to adhere as strongly as control cells, indicating that adhesion of cells within the pulling region was not possible without polymerised actin and the associated cytoskeletal linkages (Fig 3.25). It was interesting to note that the mean unbinding event sizes were reduced after addition of latrunculin B, to parental NIH 3T3 unbinding event sizes (Fig. 3.26). This may indicate that NCAM-NCAM or VASE-VASE protein interactions did not occur, were weaker or that the rupture events were due to the breakage of membrane nanotubes which were extruded from the cell during separation.
Chapter 4 Discussion and future work

As a key component in neuronal development, NCAM has been the object of many studies to elucidate its function in plasticity, neurite outgrowth and learning and memory (Cremer, Lange et al. 1994). NCAM functions as a homophilic binding protein via its Ig-like domains and is able to migrate towards the site of cell-cell contact to strengthen adhesion (Diestel, Schaefer et al. 2007). During development different isoforms of NCAM are upregulated in regions where their properties are required. For example, NCAM-PSA is expressed in the early rat embryo in areas of high synaptic plasticity whereas NCAM-VASE is expressed later in development and in areas with low synaptic plasticity (Finne 1982). NCAM-VASE monolayers have also been found to inhibit neurite outgrowth from cells grown on them (Doherty, Moolenaar et al. 1992). Therefore it has been hypothesised that insertion of the VASE sequence into NCAM alters its adhesive strength to prevent plasticity and neurite outgrowth of surrounding neurons.

The aim of this thesis was to determine the effect of insertion of the VASE sequence into NCAM. To do this, the adhesive forces of recombinant NCAM±VASE proteins were quantified using single molecule force spectroscopy using an AFM. The effect of insertion of the VASE sequence into NCAM expressing cells was also investigated using single cell force spectroscopy using an AFM. These two methods were chosen because they allowed the measurement of single interactions and provided spatial data as well as the separation forces of adhesion. In comparison, averaging techniques such as SPR and aggregation assays do not provide precise mechanical details regarding the unbinding of proteins and cells under force.

An in vitro model was developed to measure the force of NCAM±VASE Fc proteins attached to a glass coverslip and cantilever. The method of functionalisation chosen was adsorption due to the ease with which this could be carried out. However NCAM±VASE Fc was not directly coupled to the AFM surfaces as this may have decreased protein activity; instead it was affinity captured via its C-terminal Fc tag by adsorbed α human Fc. Therefore the extracellular domains of NCAM±VASE were more likely to be orientated outwards away from the solid surface than if the protein were adsorbed directly. Addition of a surfactant P105 was necessary to reduce large interactions between the cantilever tip and sample during AFM experiments. Although the presence of this surfactant layer was not ideal, as it may have altered the local pH properties of the solid-liquid interface and interfered with the native structure of NCAM±VASE, it was found to decrease the number of large, non-specific interactions measured. To test whether P105 had an effect on the secondary structure of NCAM it would be necessary to assess any structural changes using circular dichroism. NCAM has been found to bind homophilically in a detergent, NP-40, which may indicate that it could be
possible for NCAM to function in P105 (Hall and Rutishauser 1987). It was also possible that addition of a P105 surfactant layer to the AFM surfaces would not allow adsorption of α human Fc therefore an ELISA was used to test this. The ELISA showed that NCAM±VASE Fc was specifically captured on glass coverslips coated with α human Fc in the presence of P105.

AFM measurements carried out between NCAM±VASE Fc captured layers lead to the quantification of mean unbinding forces which were significantly higher than for interactions between NCAM±VASE Fc and proteins to which they should not interact specifically (IgG, α human Fc). However these control experiments were not ideal as they did not prove the specificity of the NCAM-NCAM or VASE-VASE interaction. Instead the effect of a peptide from NCAM Ig III was tested to determine whether protein adhesion could be abrogated. The Ig III KYI peptide has been found to inhibit NCAM homophilic binding on a single molecule level using SFA but it had no effect on NCAM or NCAM-VASE homophilic binding in this work using SMFS (Wieland, Gewirth et al. 2005). This may indicate that the Ig III domain of NCAM±VASE was not involved in homophilic binding measured by AFM.

To try to prove the specificity of the interactions between NCAM±VASE Fc proteins the concentration of capturing antibody and protein was reduced by an order of magnitude. This caused a decrease in the number of force-distance curves containing an adhesion event. As the concentration of P105 remained constant, this experiment provided proof that unbinding events measured were due to NCAM±VASE. These AFM measurements also revealed that the homophilic and heterophilic adhesive strengths between the proteins were statistically similar at 120 pN for loading rates of 1200 pN/s. The approximate modal unbinding force for NCAM-NCAM and VASE-VASE separations was 100 pN, which was a two-fold increase in strength over the separation of two NCAM proteins using SFA at the same loading rate (Wieland, Gewirth et al. 2005). Use of the α Fc protein to capture NCAM±VASE Fc may have resulted in the capture of NCAM dimers. It is plausible therefore that the unbinding forces measured between NCAM±VASE Fc proteins using AFM were due to the separation of two dimers (one dimer on the cantilever and one on the coverslip).

The adhesion measurements carried out gave a preliminary indication that the homophilic binding strength of NCAM and NCAM-VASE proteins may be similar. To prove this, it would be necessary to carry out further dynamic force spectroscopy AFM experiments to determine affinity constants for the interaction as well as other thermodynamic parameters describing the energetic barriers involved in each system. More data would need to be collected to produce reliable frequency distribution unbinding force curves to elucidate the true adhesive binding strengths for NCAM and NCAM-VASE.
Due to the discrepancy over whether NCAM±VASE Fc was present as a dimer during the experiment, a more elegant method was sought for SMFS measurements. The idea was to use a bifunctionalised PEGylated linker, which could covalently attach to a silanised tip or coverslip at one end, and to an NTA moiety at the other. Each PEGylated conjugate could then coordinate a C-terminal His-tagged NCAM±VASE protein in the presence of Ni$^{2+}$. The PEGylated conjugate would also serve to raise the bound proteins above the surface of the tip and sample, which would reduce non-specific interactions and remove the need for a surface-blocking surfactant in the experiment. \textit{In vitro} reactions were carried out to test the viability of this method and this was found to be successful. Due to time constraints AFM measurements using the PEGylated conjugate were not attempted.

A future comparison between the unbinding forces of NCAM±VASE proteins with either Fc or His-tags may allow determination of whether the Fc tag causes dimerisation. The values for NCAM±VASE His rupture forces would be more easily compared with those presented by Wieland et al. and would serve to determine whether VASE has no effect on NCAM homophilic binding on a single molecule level (Wieland, Gewirth et al. 2005).

Since cells expressing NCAM and NCAM-VASE were found to segregate in an \textit{in vitro} assay, the role of the VASE sequence on NCAM cell expression was tested (Chen, Haines et al. 1994). It was postulated that the VASE sequence may create a novel binding site in NCAM, or destroy one, to mediate NCAM homophilic and neurite outgrowth promoting properties. This interaction may be heterophilic in nature or require formation of a specific oligomer, which may explain why no difference in adhesion was seen in single molecule adhesion experiments.

To test this NIH 3T3 cells were transfected with NCAM-140, which lead to an increase in the adhesive strength between the cells, measured by AFM, after 60 s of contact time. It was interesting to note that there was no significant difference in N24 and NIH 3T3 cell adhesion after 5 s of contact. This indicated that early cell-cell adhesion may be independent of NCAM homophilic binding. A short term contact time of 5 s more than exceeds the on rate for a protein-protein interaction yet the effect of NCAM was not seen at this contact time. It is well-known that when cells are in contact, CAMs migrate to the site of contact at the cell-cell interface (Diestel, Schaefer et al. 2007). The rate of migration may differ depending on initial protein location in the cell membrane. The NCAM-mediated component of N24 cell adhesion may therefore not occur until longer contact times, due to migration, upregulation of the protein or until specific oligomers have formed.

This finding that NCAM-mediated adhesion was not seen until after 60 s of contact time corroborates a ‘zipper’ type mechanism of NCAM-mediated cell-cell adhesion where single proteins
bind in trans before strengthening cis contacts are made followed by ‘zipping’ of NCAM across the cell-cell interface to yield the strongest interaction (Fig. 1.8).

Abrogation of N24 adhesion occurred in the presence of the KYI peptide from Ig III of NCAM after 60 s of contact time but not after 5 s. This finding strengthens the argument that early cell-cell contact (on a scale of seconds) was independent of NCAM, but for longer contact times (on a scale of minutes) adhesion was NCAM-mediated. The effect of the Ig III peptide on longer contact times was not tested but it would be interesting to test this. It is interesting to note that the KYI maps to a portion of NCAM which was found to bind with Ig I in a trans interaction (Soroka, Kiryushko et al. 2002). Presumably the KYI peptide bound to Ig I of NCAM and prevented opposing NCAM Ig III domains from binding to it, which caused a decrease in adhesion between the cells.

Insertion of the VASE sequence into NCAM expressing cells increased their adhesion after 120 s of contact and by 600 s of contact cells required 21 nN to separate them (compared with 7 nN for N24 cells). This dramatic increase in adhesion was the first evidence to indicate that NCAM-VASE functions by increasing the adhesion between cells. A number of putative peptide blocking agents were added to the V140G cells prior to AFM measurements but these had no specific effect on adhesion. It was interesting that the Ig III peptide, KYI, abrogated adhesion of N24 cells but had no effect on V140G cell adhesion after 120 and 300 s of contact time. At shorter contact times, which were not investigated, it is possible that the Ig III- Ig I trans component of NCAM adhesion would have been prevented. The Ig III peptide however appeared to have no effect on the VASE component of NCAM binding. This data indicates that there is a conformational difference in the NCAM and NCAM-VASE adhesive units at the cell-cell interface and that they involve different domain binding epitopes.

The individual unbinding events which occurred during cell separation were only possible due to the extrusion of tethers or membrane nanotubes from the cell surface. For most cell-cell interactions, an interface was maintained between the cells for at least 90 μm of cantilever and probe cell retraction. It is unknown whether the individual unbinding events were due to the rupture of tethers at the cytoskeletal linkage or if they were due to protein unbinding events at the tether interface. It is plausible that they could be either due to their size (Marcus, McEver et al. 2004). These tethers were able to be extruded due to linkages from cell membrane proteins, such as NCAM, to the cytoplasm and the cytoskeleton. This was proven by the addition of latrunculin B to N24 and V140G cells, which reduced adhesion to a parental cell level indicating that the cells could be easily separated when actin was depolymerised.
Although the cells used in this experiment extruded tethers due to a force applied to the cells, tethers have also been found to occur in different biological systems. Leukocytes have been found to extend thin membrane tethers which are thought to stabilise cell rolling in a selectin-dependent manner (Ramachandran, Williams et al. 2004). As leukocyte rolling continues the network of membrane tethers become more complex, which is essential for the leukocyte adhesion cascade and subsequent immune response. Membrane tethers have also been found to be extended from natural killer cells to their target cells (Chauveau, Aucher et al. 2010). The tethers are thought to drive cell movement and may provide a direct link to cause lysis of the target cell. NCAM is also found to be expressed in natural killer cells and may mediate adhesion at the cell-cell membrane nanotube interface (Lanier, Chang et al. 1991).

Although NCAM-VASE may not have a direct role in the extrusion of membrane nanotubes, it is possible that insertion of VASE into NCAM strengthens cell-cell linkages by increasing the affinity of NCAM homophilic binding or the linkage to the cytoskeleton.

To validate the conclusions made from the SCFS experiments, it would be necessary to prove that increased adhesion between V140G cells was due to a property of the VASE protein and not due to abrogation or upregulation of another protein following transfection of the parental cell line. To test this, small interfering RNA sequences (siRNA) would be designed, synthesised and transfected into N24 and V140G cells to target total NCAM or NCAM-VASE mRNA for destruction and to reduce protein levels. Protein and mRNA knock down could be quantified by SCFS adhesion measurements between parental and siRNA transfected cells. If NCAM and NCAM-VASE homophilic adhesion were reduced, this would prove that adhesion was specifically due to the protein. If time permitted, addition of a range of concentrations of NCAM blocking peptides to N24 and V140G cell adhesion experiments would be tested using an AFM to ascertain which Ig domains were involved in adhesion.

The SCFS experiments used transfected cells to determine the effect of NCAM and NCAM-VASE on cell adhesion. However it would be more interesting to use primary cell lines to measure the adhesion between cells. After 2 days in vitro, oligodendrocytes express only NCAM but by 17 DIV NCAM-VASE is upregulated and accounts for a large proportion of total NCAM (Dr. M. Delves, unpublished observation). It would be interesting to develop an in vitro method for measuring the adhesion of these two oligodendrocyte cell types to compare with the adhesion levels seen between transfected NIH 3T3 cells and to determine how primary cell line expression levels compare. However it would be difficult to compare the relative adhesiveness of the cells since their morphology, structure and expression levels are not similar.
Although further experiments could be carried out using the AFM, the next step in this project would be to compare NCAM-VASE-140 and NCAM-140 binding partners. Since VASE expression in NIH 3T3 cells exhibited increased adhesion over those expressing NCAM, but not on a single molecule level, it may be due to alternative binding partners. NCAM-VASE has been found to be involved with L1-mediated neurite outgrowth and is postulated to bind L1 glycans upstream of the VASE sequence in Ig IV (Lahrtz, Horstkorte et al. 1997). Therefore it would be interesting to carry out a series of immunoprecipitations from parental NIH 3T3, N24 and V140G cells lines using covalently coupled NCAM±VASE protein columns. Determination of NCAM and NCAM-VASE binding partners would allow further elucidation of the mechanism of homophilic binding.

Alternatively it would be interesting to test which Ig domains of NCAM and NCAM-VASE bind homophilically with which domains, since there is much controversy surrounding this (Rao, Wu et al. 1992; Walmod, Kolkova et al. 2004). NCAM Ig IV (lacking VASE) has been found to bind to NCAM Ig II, therefore insertion of the VASE sequence into NCAM may alter this interaction to modulate cell adhesion therefore determining whether these domains bound to each other could be important. During SMFS experiments between NCAM±VASE Fc functionalised surfaces, the position at which unbinding events occurred was approximately 25-35 nm. These values bear a strong similarity to the length of the extracellular domain of NCAM and may contain information regarding whether the interacting Ig domains were in a partial overlapping homophilic conformation or a full overlap (Wieland, Gewirth et al. 2005). To test this further, careful calibration of the Z-piezo would be required using an AFM imaging standard with nanometer contours. Alternatively SFA measurements of the domain overlap between NCAM-VASE proteins would be a valid experiment since spatial resolution on this instrument is higher.

Overall the findings from this research have pointed towards a role for the VASE sequence in increasing the binding strength of NCAM in a cellular context. Two novel assays have been set up for the measurement of NCAM molecular and cellular adhesion using AFM, which provide a useful means to elucidate the exact adhesive mechanisms of NCAM and its isoforms.
Chapter 5 Materials and Methods

5.1 Tissue Culture

5.1.1 Basic cell culture

Cells were cultured on 10 cm tissue culture plates (Appleton Woods) in Dulbecco’s Modified Eagles Medium with Glutamax (DMEM, Invitrogen), containing 10% (v/v) foetal calf serum (FCS, Invitrogen) and 1x penicillin/streptomycin (Invitrogen) in a 37°C, 8% CO₂ humidified incubator. Cells were split at approximately 80% confluency by aspirating the growth medium and adding 0.25% trypsin/1 mM EDTA (Invitrogen) for 10 min at 37°C. Growth medium was then added to inhibit trypsinisation of the cells, followed by centrifugation at 2000 rpm for 5 min to yield a cell pellet, which was resuspended with trituration in full serum growth medium. If necessary, cell number was determined by counting in a haemocytometer (VWR) and plating the required number.

Long term storage of cell lines was achieved by freezing at 80 °C in cryotubes (VWR) in DMEM/10% FCS/10% DMSO (Sigma) and transfer to liquid nitrogen.

All cell manipulations were carried out aseptically in laminar flow tissue culture hoods.

5.1.2 Cell lines

Cell lines used during this investigation include:

- NIH 3T3 mouse fibroblasts (America Cell Type Collection, Rockville)
- N24 mouse fibroblasts, NIH 3T3 cells transfected with human NCAM-140 (a gift from Dr. F. Walsh, Wyeth Research, Princeton, USA (Saffell, Walsh et al. 1994)
- V140G mouse fibroblasts, NIH 3T3 cells transfected with human NCAM-VASE-140 by Dr. M. Delves (cDNA a gift from Dr. P. Doherty, Kings College London (Walsh, Furness et al. 1992)
- HEK 293 human embryonic kidney cells
- HEK EBNA human embryonic kidney cells transfected with Ebsstein-Barr virus nuclear antigen
- Cos-7 African green monkey cells (a gift from Prof Y. Ushkaryov, Imperial College London).

5.2 Light and fluorescence microscopy

5.2.1 Fixed cell immunostaining

Cells were plated onto sterile 13mm glass coverslips (VWR) and were fixed by the addition of an equal volume of 4% paraformaldehyde in PBS (PFA, VWR) to DMEM/10% FCS for 7 min. The solution was then aspirated and replaced with neat 4% paraformaldehyde for a further 7 min before removal. Cells were then rinsed with PBS for 5 min x3. If intracellular staining was required, cells were permeabilised with ice-cold MeOH for 10 min before being washed with PBS as before. Cells were blocked with PBS/10% FCS for 45 min before removal and washing with PBS. The cells were
incubated with the primary antibody of choice in PBS for 1 hour and then washed. A secondary antibody in PBS was then added to the cells for 1 hour before removal and washing. Coverslips were then mounted on glass slides (VWR) on a droplet of PBS/10% glycerol containing 1 μg/ml 4’,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) and an anti-fade reagent DABCO (Sigma-Aldrich).

5.2.2 Antibodies used in immunocytochemistry

Primary antibodies:

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<th>Antibody</th>
<th>Source</th>
<th>Species</th>
<th>Reactivity against</th>
<th>Working concentration</th>
</tr>
</thead>
<tbody>
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<td>α N16</td>
<td>Gift Dr. J. Hemperly (Ackley, Madison et al. 1997)</td>
<td>Mouse</td>
<td>Human NCAM Ig III</td>
<td>1:10000</td>
</tr>
<tr>
<td>Crude α VASE</td>
<td>Gift Dr. J. Hemperly (Vawter, Hemperly et al. 1998)</td>
<td>Rabbit</td>
<td>VASE sequence</td>
<td>1:500</td>
</tr>
<tr>
<td>α VASE</td>
<td>Purified crude</td>
<td>Rabbit</td>
<td>VASE sequence</td>
<td>1:20</td>
</tr>
<tr>
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<td>Filamentous actin</td>
<td>1:5000</td>
</tr>
</tbody>
</table>

Secondary antibodies:

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<th>Source</th>
<th>Reactivity against</th>
<th>Working concentration</th>
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<td>Mouse IgG</td>
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<td>Invitrogen</td>
<td>Mouse IgG</td>
<td>1:4000</td>
</tr>
<tr>
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</tr>
<tr>
<td>α rabbit IgG A555</td>
<td>Invitrogen</td>
<td>Rabbit IgG</td>
<td>1:4000</td>
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</table>

5.2.3 Microscopy

A Zeiss Axiocvert 200 inverted fluorescence microscope with a mercury light source was used to visualise virtually all images during this investigation. Images with this microscope were taken using x10, x20, x40 and x63 (phase and oil immersion) objectives.

5.3 Cellular assays

5.3.1 Cell area determination

Fibroblast cells were plated in DMEM/10% FCS onto 10 cm plates at 25% confluency and incubated for 16 hours before live measurements were taken. Adherent cell area was then assessed at 40 magnification using a cell area macro (written by Paul Whetten, Zeiss) in the program KS300 (Zeiss), which allows the user to draw an accurate outline of a multi-sided cell. The area of, on average, 200 cells was measured. For the area measurement of scraped (or probe) fibroblast cells, cells were

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plated in 24 well plates, so that 16 hours after seeding 90% confluency was achieved. Cells were scraped with a cell scraper (Fisher Scientific) and triturated through a 19 gauge needle (VWR) 40 times, then injected into a 10 cm plate containing DMEM/10% FCS. The area of the probe cells was then measured as previously stated.

5.3.2 NCAM cell surface expression determination by ELISA

The surface expression of NCAM and NCAM-VASE on fibroblast cell lines was determined by fixed cell ELISA. Cells were plated in triplicate, on duplicate 96 well plates (Sterilin), in DMEM/10% FCS, so that after 16 hours confluency had been reached. If the effect of a compound was being studied, the compound was incubated at 37 °C. Cells were then fixed by the addition of an equal volume of 4% formaldehyde in PBS, to DMEM/10% FCS in the wells for 7 min. The solution was then carefully aspirated and replaced with neat 4% paraformaldehyde for a further 7 min before removal. Cells were rinsed with PBS for 5 min x3. Cells were blocked with PBS/5% BSA for 45 min before removal and washing with PBS. The cells were then incubated with α N16 in PBS (1: 10000) for 1 hour and then washed. A secondary α mouse IgG HRP conjugated antibody in PBS (1:10000) was then added to the cells for 1 hour before removal and washing. All solution was removed and BM blue POD substrate (Roche) was added to the wells and the absorbance was read in a spectrophotometer at 370 nm after 5, 10 and 15 min of incubation. An equal volume of 1 M H₂SO₄ was then added to stop the colour development and the plate was read at 450 nm.

The duplicate cell plate was used for assessment of cell number by (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) assay (CellTiter96® Aqueous non-radioactive assay, Promega). An equal volume of MTS was added to DMEM/10% FCS in tandem with cell fixation for immunostaining, with incubation at 37 °C, for 3.5 hours. The plate was then measured in a spectrophotometer at 490 nm to determine living cell number. The cell counts for NIH-3T3, N24 and V140G was optimised so that cell densities were within 10% of each other, otherwise an experiment would not be continued. Surface NCAM expression on each cell line was then normalised using the minor differences in cell count.

5.3.3 Statistical analysis

Mean values are presented ± standard error of the mean (SEM) and if necessary are tested using a two-tailed Student’s t test. Differences in the data were considered to be statistically significant if p<0.05 (indicated by *), very statistically significant if p<0.01 (indicated by **) and extremely statistically significant if p<0.001 (indicated by ***)

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5.4 Molecular Biology

5.4.1 Competent cell preparation
The bacterial strain used in this study is:
- E. coli DH5α (Genotype: F' φ80dlacZ15, lacZ YA-argF)U169, deoR, recA1, endA1, hsdR17, (r eks +, m eks +) phoA, supE44 λ', thi-1, gyrA96, relA1)

A lysogeny broth (LB) agar plate (VWR) was streaked from a DH5α glycerol stock, which had been stored at -80 °C, and incubated at 37 °C for 16 hours. Competent DH5α cells were prepared by inoculating a single colony from an agar plate into sterile LB (VWR). This was grown at 37 °C with agitation (180 rpm) until an optical density (OD) of 0.5 at 600 nm was reached. The flask was then cooled on ice and centrifuged at 4 °C, 3000 rpm for 10 min. The media was removed and the cells were resuspended in 1/3 of the original volume of ice-cold transformation buffer (0.01 M PIPES, 0.015 M CaCl2, 2H2O, 0.25 M KCl, 0.5 mM MnCl2•4H2O in dH2O). Cells were incubated on ice for 10 min then re-centrifuged. The media was removed and cells were resuspended in 1/25 of the original culture volume with transformation buffer/7% DMSO. Cells were incubated on ice for 10 min then aliquoted into pre-chilled microtubes (VWR) before storage at -80 °C.

5.4.2 Glycerol stock preparation
Overnight bacterial cultures in LB were grown and 1 ml was removed and mixed with 400 µl of autoclaved and filter-sterilised 50% glycerol in a cryotube. Aliquots were flash-frozen and stored at -80°C.

5.4.3 Heat shock transformation
A 50 µl aliquot of competent DH5α cells was thawed on ice and to it was added 5µl of a ligated DNA with gentle mixing. The cells were incubated on ice for 30 min then heat shocked at 42 °C in a heat block for 1 min. Cells were immediately returned to ice for a further 2 min. Pre-warmed LB (950 µl) was added to the cells and this was incubated at 37 °C for 1 hour with agitation (180 rpm). The cells were centrifuged and resuspended in a smaller volume of LB before plating on agar plates with the appropriate antibiotics. The plates were incubated at 37°C for 16 hours before colonies were picked.

5.4.4 Plasmid DNA isolation
A bacterial colony, expressing the DNA of choice, was picked from a pre-spread agar plate containing necessary antibiotics and was inoculated into LB and grown at 37 °C with agitation (180 rpm). Once the mixture reached an OD of 0.5 at 600 nm, cells were centrifuged and the media was removed. Plasmid DNA was isolated from bacterial cells using a Mini or Maxi-prep kit (Qiagen). Briefly the cell pellet was lysed and the membrane and proteins were precipitated and removed by centrifugation.
Plasmid DNA was extracted from the supernatant after centrifugation by trapping on a spin column membrane. The column was rinsed and the plasmid DNA was eluted in sterile dH₂O.

5.4.5 DNA Restriction digestion
The plasmid DNA was confirmed by DNA digestion using restriction endonucleases (New England Biolabs). Typically 0.5 µg of isolated plasmid DNA was diluted in sterile dH₂O and to this 0.5 unit of restriction endonuclease was added, with appropriate buffers. The reactions were incubated at 37°C for 3 hours followed by incubation on ice. The results were assessed by agarose gel electrophoresis.

5.4.6 Agarose gel electrophoresis
The apparatus used for agarose gel electrophoresis was purchased from Biorad. Agarose (Invitrogen) was dissolved in TAE buffer (2M Tris-acetate, 0.05M EDTA, Sigma-Aldrich) by boiling in a microwave. After cooling, ethidium bromide (Sigma) was added (0.5 µg/ml) and the mixture was poured into a casting tray with appropriate well combs. The gel was left to polymerise at RT for 30 min. DNA samples to be analysed were diluted with 6x DNA loading buffer (30% glycerol in dH₂O, 50 mM EDTA, 0.25% bromophenol blue) and loaded onto the gel with DNA standards. Electrophoresis was carried out at 80 V until clear separation of samples was seen. The gel was visualised using the UV filter in a Fuji Image Analyser LS3000 machine.

5.4.7 Polymerase chain reaction (PCR)
For amplification of plasmid DNA for use in cloning, 33 ng of template dsDNA was amplified by the addition of 1.25 units of PfuUltra™ hotstart DNA polymerase (Promega) and 0.1 µM of each forward and reverse PCR primer. Alternatively a bacterial colony picked from an agar plate was waved into the PCR reaction tube. The reaction included final concentrations of 200 µM of each dNTP, 0.2 µM for each forward and reverse primer and 1x PCR buffer (20 mM Tris-HCl, 10 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1% triton X-100 and 0.1 mg/ml BSA) up to a final volume of 50 µl. The PCR amplification was carried out on a Techne TC 4000 using the following program:

Preheat lid 105 °C

1. Denaturation - 94 °C, 1 min
2. Annealing – (Average melting temperature (Tₘ) of the primers minus 5 °C), 1 min
3. Elongation - 72 °C, 1min per 1000 template nucleotides

Repeat steps 1-3 x25

End 4 °C

Amplified DNA mixtures were cleaned up using the PCR purification kit (QIAGen) and isolated DNA was analysed by agarose gel electrophoresis.
5.4.8 DNA ligation
Doubly digested insert dsDNA was added to doubly digested vector in molar ratios of 1:1, 1:3 and 3:1 insert:vector in total sample volumes of 10 μl diluted in nuclease free dH₂O. These DNA fragments were ligated by 0.5 units of T4 DNA ligase in the presence of DNA ligase buffer (30 mM Tris-HCl, 10 mM MgCl₂, 10 mM DTT and 1 mM ATP, Promega) for 15 min at RT. Samples were transformed into competent DH5α cells and spread on agar plates for colony screening.

5.4.9 DNA concentration determination
DNA concentration was quantified by UV spectrophotometry at an absorbance of 260 nm knowing that an absorbance of 1 corresponds to 50 μg/ml of dsDNA.

5.4.10 DNA sequence analysis
Plasmid DNA was isolated from an overnight culture using a QIagen Mini-prep kit and was sequenced by Beckman Coulter Genomics using appropriate oligonucleotides.

5.5 Protein expression, purification and modification
5.5.1 NCAM± VASE Fc protein expression
The expression of a chimeric fusion protein of the extracellular domains of NCAM± VASE with a C-terminal Fc tag (from human IgG) was carried out in the Cos-7 eukaryotic cell line. Cos-7 cells were plated in 10 cm plates in DMEM/10% FCS (no P/S) so that after 16 hours they were at 75% confluency (Meiri, Saffell et al. 1998). The cells were then transiently transfected following the DEAE dextran method with plasmid DNA which was isolated by Maxi-prep as described previously (See 5.4.4). Cells were washed with DMEM (no P/S) whilst 2 μg/ml DNA, 51.6 μg/ml chloroquine diphosphate (Sigma) and 0.4 mg/ml Diethylaminoethyl- (DEAE) dextran (Sigma-Aldrich) was allowed to equilibrate in DMEM (no P/S) for 15 min at RT. This solution was added to aspirated cells for 3 hours at 37 °C or before the cells became vacuolated. The solution was then aspirated off and cells were osmotically shocked in PBS/10% dimethylsulfoxide (DMSO) for 1.5 min before replacement of the solution with full serum media. Transfected cells were incubated at 37 °C for 24 hours and then the media was replaced with DMEM/1% IgG free FCS. Cell supernatant was then collected after 6 days for protein purification.

5.5.2 NCAM±VASE Fc protein purification
NCAM± VASE Fc transfected cell supernatant was centrifuged at 2000 rpm for 5 min and the supernatant was filtered through 0.5 μm syringe filters (VWR). The cell-free supernatant was then dialysed overnight in SnakeSkin dialysis tubing (Thermo Scientific) with a molecular weight cut off (MWCO) of 10 kDa against PBS with agitation on a stirring plate. 150 μl of Protein A-sepharose beads
(Sigma-Aldrich) was added to the dialysed mixture (to capture the Fc-tagged proteins) overnight at 4 °C on a roller. The mixture was then passed through a column with porous polyethylene disks (Thermo Scientific) to collect the beads and the filtrate. The beads were washed with PBS until no further protein was eluted (as determined by spectrophotometry at 280 nm). Protein bound to the beads was eluted by the addition of 100 mM glycine-HCl at pH 2.7 (Sigma). 500 µl fractions were collected into 50 µl 1M Tris-HCl at pH 9 samples for neutralisation. Samples from the protein purification were separated by SDS-PAGE electrophoresis and stained with coomassie blue G-250 (Sigma-Aldrich) or transferred for Western blotting. Fractions containing pure NCAM± VASE Fc protein were pooled and concentrated using 10 kDa MWCO spin concentrators (GE Healthcare) and centrifugation dialysis with PBS. Protein samples were concentrated to 1 ml and their concentration was determined by spectrophotometry at 280 nm and ELISA.

5.5.3 NCAM± VASE His protein expression
The expression of a chimeric fusion protein of the extracellular domains of NCAM± VASE with a C-terminal polyhistidine tag (His tag) was carried out in the HEK-293 cell line. HEK-293 cells were plated in 10 cm plates in DMEM/10% FCS (no P/S) so that after 16 hours they were at 75% confluency. The cells were then transiently transfected following the DEAE dextran method as described previously (See 5.5.1). Transfected cells were incubated at 37 °C for 7 days until protein purification.

5.5.4 NCAM± VASE His protein purification
NCAM±VASE His transfected cell supernatant was centrifuged at 2000 rpm for 5 min and the supernatant was filtered through 0.5 µm syringe filters. The cell-free supernatant was then dialysed overnight in SnakeSkin dialysis tubing with a MWCO of 10 kDa against PBS with agitation on a stirring plate. 0.5 ml of pre-hydrated Ni-NTA agarose beads (Qiagen) were added to the dialysed mixture and with 300 mM NaCl and 10 mM imidazole. The beads were incubated on a roller for 1 hour at RT. The mixture was then passed through a column (with resin) to collect the beads and to collect the filtrate. The beads were washed with aliquots of 20 mM imidazole in PBS until no further protein was eluted (as determined by spectrophotometry at 280 nm). Protein bound to the beads was eluted by the addition of 200 mM imidazole in 0.5 ml fractions. Samples from the protein purification were separated by SDS-PAGE electrophoresis and stained with coomassie blue G-250 or transferred for Western blotting. Fractions containing pure NCAM± VASE His protein were pooled and concentrated as previously described (See 5.5.2).
5.5.5 NCAM±VASE Fc protein concentration determination by ELISA
A 96 well plate was coated in a human IgG-Fc specific (Sigma) in DMEM/HEPES for 1 hour at 37 °C and then washed in DMEM/HEPES. The plate was then blocked in DMEM/HEPES/5% BSA for 1 hour at 37° before washing with DMEM/HEPES. The wells were then coated with varying concentrations of pre-sonicated NCAM± VASE Fc in DMEM/HEPES and left for 16 hours at 4 °C. Plates were then aspirated and washed with PBS, then blocked with PBS/5% BSA for 45 min at RT. The plate was washed again then coated with α N16 antibody (1:10000) in PBS for 1 hour at RT. The plate was washed and coated with a secondary α mouse-HRP (1:15000) (Amersham Biosciences) antibody in PBS for 1 hour at RT and then washed. BM blue POD substrate was added to the wells and the absorbance was read in a spectrophotometer at 370 nm after 5, 10 and 15 min of incubation. An equal volume of 1 M HCl was then added to stop the colour development and the plate was read at 450 nm. Each data point was assayed in triplicate to calculate the mean and its standard error. The results were used to compare and normalise NCAM± VASE Fc concentrations.

5.5.6 Protein concentration determination
Protein concentrations were determined by spectrophotometry at 280 nm and calculated by knowledge of each protein’s extinction coefficient. Protein purity was determined by coomassie G-250 staining followed by a BCA assay to determine total protein concentration.

5.5.7 Size exclusion chromatography
Gel filtration was conducted on a Superose 6 10/300 GL column (GE healthcare) connected to an ACTA+PFC system (Amersham). Running and sample buffers were syringe filtered and degassed before use. The column was stored in 70% ethanol (EtOH) at 4 °C and once connected to the system it was rinsed with two column volumes (48 ml) of dH2O and 1.5 column volumes (36 ml) of PBS. A 20 µl protein sample in PBS was then loaded into a 1ml injection loop and injected into the column. A column flow rate of 0.4 ml/min was used in all experiments. Fractions from the column were eluted in 0.5 ml volumes and collected using a Frac950 fraction collector (Amersham). Following elution, the column was rinsed with two column volumes of dH2O and stored as described above.

The retention time of eluted fractions was compared to a series of molecular weight standards using a calibration curve, which was calculated from the following equation:

A calibration curve was calculated linking an elution volume parameter, \( K_{sv} \), with Log MW.

\[
K_{sv} = V_o \cdot V_o/V_{T} - V_o
\]

Where \( V_o = \) Elution volume, \( V_c = \) Void Volume and \( V_T = \) Total bed volume.

The equation of the line of the calibration curve was used to estimate the molecular weight of the eluted protein species.
5.5.8 Concentration of protein samples

100% (v/v) trichloroacetic acid solution (TCA), at a quarter of the volume of the protein samples to be concentrated, was added to the protein samples and incubated at 4 °C for 10 min. The samples were centrifuged at 13000 rpm for 10 min at 4 °C and the pellet was resuspended in 1 ml of ice-cold acetone. Samples were incubated at -80 °C for 15 minutes and were re-centrifuged. The supernatant was removed and the remainder was allowed to evaporate off. The protein pellet was resuspended in the desired volume of PBS.

5.5.9 Protein Biotinylation

1 mg of BSA (Sigma-Aldrich) in 1 ml of PBS/10% DMSO was incubated with 0.49 mg Biotin O-Su at RT for 30 min. The biotin mixture was then centrifuged in a 5000 MWCO spin concentrator and dialysed against PBS. Biotinylated BSA (BBSA) was aliquoted and stored at -20°C.

5.5.10 Protein Biotinylation quantitation assay

Biotinylation of BSA was confirmed and quantified using the ‘Biotinylation Quantitation Kit’ (Piercenet). The 4’-hydroxyazobenzene-2-carboxylic acid HABA₄: avidin reagent was added to MilliQ dH₂O and allowed to equilibrate at RT. A blank, cuvette containing PBS was used as the reference for absorbance measurements at 500 nm. 100 µl of the (HABA)₄: avidin reaction was added into the PBS cuvette and the absorbance was measured at 500 nm. 100 µl of protein sample was then added into the cuvette and the absorbance at 500 nm was measured immediately, and every minute until the value remained constant. The number of moles of biotin was then calculated from the drop in absorbance upon addition of biotinylated protein sample.

5.6 SDS-PAGE and Western blotting

5.6.1 Protein sample preparation

Concentrated protein was diluted to the required concentration in PBS and mixed with 5x SDS sample buffer (10% (w/v) SDS, 10 mM β-mercaptoethanol, 20% (v/v) glycerol, 0.2 M Tris HCl and 0.05 % bromophenol blue). Samples were then heated at 95 °C for 10 min, mixed, then centrifuged before loading.

5.6.2 Cell lysate preparation for SDS-PAGE

Cells were seeded in DMEM/10% FCS in 10 cm plates to achieve 95% confluency 16 hours after incubation at 37°C. Fibroblast cell lysates were prepared by treating cells with 300 µl of 5xSDS sample buffer and using a cell scraper (Triple Red). Following scraping, plates were placed on ice for 15 min. Cell lysate was centrifuged at 13000 rpm for 10 min at 4°C. The supernatant was then aliquoted and total protein concentrations were determined by BCA protein assay (Piercenet) and
adjusted accordingly by dilution in sample buffer. Samples were assayed by SDS-PAGE electrophoresis.

5.6.3 SDS-PAGE gel casting

5:7.5% stacking: running SDS-PAGE gels were cast in Mini-PROTEAN 3 electrophoresis equipment (Biorad). Samples were loaded onto the gel and run in SDS running buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine (Biorad) and 0.1% SDS) against prestained protein markers of known molecular weight. Electrophoresis was carried out at 80 V until the protein samples had reached the stacking: running gel interface, then at 120 V for the duration.

Following electrophoresis, if coomassie staining was required, gels were rinsed in dH2O then fixed in fixing solution (0.1% (w/v) coomassie blue G-250, 2% (v/v) phosphoric acid and 10% (w/v) ammonium sulphate) for 3 hours. The gel was then rinsed again before being equilibrated in pre-stain buffer (50% EtOH, 2% (v/v) phosphoric acid) for 1 hour. 0.02% coomassie G250 (Sigma-Aldrich) was then added into the solution with agitation. The gel was then covered and left for up to 3 days at RT for colour development. Gels were scanned on a flatbed scanner.

5.6.4 Western Blotting

If Western blotting was required the electrophoresed gel was placed into a Mini Trans-Blot cartridge (Biorad), on a piece of PVDF membrane (Amersham) between sponges and blotting paper (Whatman). The gel was transferred for 1.5 hours at 100 V in ice-cold transfer buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine and 20% (v/v) MeOH). The membrane was then blocked in PBS/10% milk for 1 hour and rinsed in PBS, then incubated with primary antibody in PBS and blocking reagents, followed by washing and secondary HRP conjugated antibody incubation (See tables 5.3). The membrane was then washed and visualised by the addition of ECL Western chemiluminescent reagents (GE Healthcare) in a Fuji Image Analyser LS3000 machine.
5.6.5 Antibodies used in Western blotting

Table 5.3: Protocol for Western blotting antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Titre</th>
<th>Block</th>
<th>Washes</th>
<th>Secondary</th>
</tr>
</thead>
<tbody>
<tr>
<td>α N16 (Ackley, Madison et al. 1997)</td>
<td>1:10000 in PBS + 5% milk (1 hour RT or overnight 4°C)</td>
<td>PBS + 10% milk (1 hour RT or overnight 4°C)</td>
<td>PBS 3x10 min</td>
<td>α mouse - HRP (Biorad) 1:3000 in PBS + 5% milk (1 hour RT)</td>
</tr>
<tr>
<td>NCAM H-300 (Santa-Cruz)</td>
<td>1:1000 in PBS + 0.05% Tween (Sigma) + 1% BSA (Sigma) (overnight RT)</td>
<td>PBS + 0.05% Tween (Sigma) + 10% milk (2 hours RT)</td>
<td>PBS + 0.05% Tween 3x5 min</td>
<td>α rabbit - HRP (Biorad) 1:10000 in PBS + 1% BSA (1 hour RT)</td>
</tr>
<tr>
<td>Crude α VASE antiserum (Vawter, Hemperly et al. 1998)</td>
<td>1:1000 in PBS + 0.05% Tween + 1% BSA (overnight RT)</td>
<td>PBS + 0.05% Tween (Sigma) + 10% milk (2 hours RT)</td>
<td>PBS + 0.05% Tween 3x5 min</td>
<td>α rabbit - HRP (Biorad) 1:10000 in PBS + 1% BSA (1 hour RT)</td>
</tr>
<tr>
<td>Affinity purified α VASE</td>
<td>1:200 in PBS + 0.05% Tween + 1% BSA (overnight RT)</td>
<td>PBS + 0.05% Tween (Sigma) + 10% milk (2 hours RT)</td>
<td>PBS + 0.05% Tween 3x5 min</td>
<td>α rabbit - HRP (Biorad) 1:10000 in PBS + 1% BSA (1 hour RT)</td>
</tr>
<tr>
<td>α human Fc – HRP (Amersham)</td>
<td>1:10000 in PBS + 5% milk (1 hour RT)</td>
<td>PBS + 10% milk (1 hour RT)</td>
<td>PBS + 0.05% Tween 3x5 min</td>
<td>N/A</td>
</tr>
<tr>
<td>α tubulin (Sigma-Aldrich)</td>
<td>1:1000 in PBS + 0.05% Tween + 5% milk</td>
<td>PBS + 10% milk (1 hour RT)</td>
<td>PBS + 0.05% Tween 3x5 min</td>
<td>α rabbit - HRP (Biorad) 1:10000 in PBS + 1% BSA (1 hour RT)</td>
</tr>
</tbody>
</table>

5.7 Peptide production and purification

5.7.1 Solid Phase Peptide synthesis

Automated peptide synthesis was carried out on an Advanced ChemTech Apex 396 multiple peptide synthesiser. Peptides (AGTC Bioproducts) were synthesised using the Fmoc/tBu method of solid phase peptide synthesis (SPPS). The N-α-amino acids were protected with Fmoc, whilst the side chain protecting groups were tBu (Asp), Trt (Cys) and Boc (Lys). Peptide sequences were elongated from the C- to N-terminus.

25 µmol of amino acid-loaded Wang resin (Novabiochem) or unloaded rink amide resin (200-400 mesh, Novabiochem) were swelled in dimethylformamide (DMF) (peptide grade, Sigma-Aldrich) for 1 hour prior to the synthesis. Elongation of the peptide chain began with N-α-amino acid Fmoc deprotection in 20% (v/v) piperidine (Sigma-Aldrich) in DMF. 125 µmol of the next amino acid was
coupled by 125 µmol HBTU ‘in situ’ activation with 250 µmol DIPEA. Repeated cycles of deprotection and amino acid coupling were then carried out until the entire peptide chain was synthesised. If necessary, difficult-to-couple amino acids entered two repeat coupling reactions.

5.7.2 Peptide deprotection and acetylation

Following coupling of the final amino acid, terminal amino acid deprotection was carried out, then the resin was removed and washed in DMF, dichloromethane (DCM), MeOH and diethyl ether before drying *in vacuo*. If N-terminal acetylation was required, the resin was swelled in DMF for 20 min, then reacted in 10% N, N-diisopropylethylamine (DIPEA, Sigma-Aldrich), 20% acetic anhydride (Sigma-Aldrich) in DMF for 1 hour at RT with agitation (300 rpm). Resin was then dried by gravity flow and rinsed as previously then dried *in vacuo*.

One step side chain deprotection and cleavage from the resin was carried out in 94% trifluoroacetic acid (TFA), 2.5% H₂O, 2.5% 1,2-ethanedithiol (EDT) and 1% triisopropylsilane (TIS) for 2 hours at RT. The solution was filtered and the resin was washed with more TFA mixture and added to the filtrate. This was then precipitated and washed (repeated 3 times) in ice-cold tert-butyl methyl ester (TBME) and centrifuged at -4 °C, 4000 rpm for 10 min before the supernatant was discarded. The crude peptide was dried *in vacuo* overnight and purified using RP-HPLC.

5.7.3 Peptide purification and characterisation

Analytical HPLC was carried out on a Gilson system with a 322 Pump, a 234 Autoinjector and 151 UV/vis detector set at 223 nm. Peptide purification by HPLC was carried out on a Gilson semi-preparative reverse phase (RP) HPLC system equipped with 306 pumps and a Gilson 151 UV/vis detector. Both systems were fitted with an ACE 5 C18 column (250x4.6 mm) and a SecurityGuard cartridge system (C18, 4x3.0 mm) and carried out in a gradient of degassed acetonitrile (ACN) and ultrapure dH₂O (MilliQ) with 0.1% TFA. Masses were determined by MALDI-TOF using the Micromass MALDI-TOF.

Alternatively peptides were analysed and purified on a Waters LCMS system with a 2545 quaternary gradient module, 2767 sample manager, 3100 mass detector and SFO system fluidics organiser. The system was fitted with an analytical XBridge™ C18 (5 µm, 4.6x100 mm column) column and a preparative XBridge™ C18 (5 µm, OBD™, 19x100 mm) column and carried out in a gradient of MeOH and ultrapure dH₂O (MilliQ) with 0.1% formamide (FA).
5.7.4 Peptides synthesised

Table 5.4: Table to show peptides synthesised during this study by solid phase peptide synthesis (SPPS) and modifications to their C- and N-terminals. See appendix for mass chromatograms.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>m/z (M)+</th>
<th>tR (min)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KYI</td>
<td><em>KYIFSDDSSQ</em></td>
<td>1190</td>
<td>16.1</td>
<td>13.1</td>
</tr>
<tr>
<td>KYI scr</td>
<td><em>SDYIDFSKKQ</em></td>
<td>1190</td>
<td>16.1</td>
<td>5.8</td>
</tr>
<tr>
<td>CLL</td>
<td><em>CCL</em></td>
<td>389</td>
<td>8.3</td>
<td>11.6</td>
</tr>
<tr>
<td>GRIL</td>
<td><em>GRILARGEINFK</em></td>
<td>1375</td>
<td>18.9</td>
<td>26.3</td>
</tr>
<tr>
<td>GRIL scr</td>
<td><em>ILAEIGNFGRK</em></td>
<td>1375</td>
<td>18.9</td>
<td>11.5</td>
</tr>
<tr>
<td>VASE</td>
<td>^CSEEKASWTPKEQETLDG^N</td>
<td>2237</td>
<td>9.4</td>
<td>63.2</td>
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<tr>
<td>VASE scr</td>
<td>^CKKQSDWTEPSAILREEGT^N</td>
<td>2237</td>
<td>9.4</td>
<td>74.1</td>
</tr>
<tr>
<td>C3m</td>
<td>^ASKKPKRNKIN^N</td>
<td>1282</td>
<td>11.7</td>
<td>40.6</td>
</tr>
<tr>
<td>C3m scr</td>
<td>^KKAKRKRINASP^N</td>
<td>1282</td>
<td>11.7</td>
<td>19.2</td>
</tr>
</tbody>
</table>

*=charged, ^=acetylated, ^=amide

5.8 NHS-PEG-PDP functionalisation time course

The functionalisation of the bifunctional polyethylene glycol linker was investigated by LCMS. 20 µl of 1mg/ml PEG-NHS-PDP was reacted with maleimido 1 mM C3 NTA or 1 mM CLL peptide in 40 µl of ‘Buffer A’ (300 mM NaH2PO4, 2 mM EDTA, pH 7.5) with 5 µl of 20 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl). The reaction was carried out at RT and samples were taken after 2 hours, 4 hours and 16 hours and analysed by LCMS.

5.9 Single cell force spectroscopy

5.9.1 CellHesion® 200 Microscope

Single cell force spectroscopy was carried out on a CellHesion® 200 AFM (JPK Instruments, Germany) mounted on an Axiovert 200M inverted microscope (Carl Zeiss, Germany). The AFM head is equipped with a 100 µm Z direction piezoelectric scanner with electric laser. Tipless silicon nitride (Si3N4) Arrow™ TL1 cantilevers (500±5x100±5x12±0.5 µm, Nanoworld) or CSC12 E cantilevers (350±5x35±3x1±0.3 µM, MikroMasch) were used with a nominal spring constant of 0.03 N/m. The AFM experiment was temperature-controlled at 37°C on a PetriDishHeater (JPK Instruments). Cantilever control and data acquisition was carried out by JPK Image Processing software.

5.9.2 Cell preparation

The day before the AFM experiment, 200 fibroblast cells were seeded in 35 mm plates in 100 µl droplets of DMEM/10% FCS for 16 hours at 37°C. Plates were stored in humid chambers at 37 °C to prevent droplet evaporation. In parallel 24 well plates were seeded with fibroblast cells so that 16 hours later cells had reached 95% confluency. On the day of the experiment, 2 ml of serum free DMEM/20 mM HEPES, pH 7.4 was added to the cell dishes and stored at 37 °C for 30 min. If the effect of a drug or peptide was being studied, it was added in situ in the experiment and remained during it.
5.9.3 Cantilever functionalisation
On the day of the experiment cantilevers were cleaned with piranha solution (VERY DANGEROUS- add H₂SO₄ to H₂O₂, 30% H₂O₂/ 70% H₂SO₄) for 15 min at RT then rinsed with dH₂O x5. Cantilevers were then incubated in poly-L-lysine ((PLL) 16 mg/ml in dH₂O) for 30 min at RT. The cantilevers were rinsed with dH₂O and incubated in fibronectin (10 µg/ml in DMEM/10% FCS) for 3.5 hours at 37°C until the start of the AFM experiment.

5.9.4 AFM calibration
The functionalised cantilever was removed from the fibronectin, without washing, and quickly mounted onto a glass cantilever holder to maintain a 10° downward angle. The glass block was loaded onto the AFM above the cell dish. The laser was aligned on the tip of the cantilever, with a sum signal of 6V and left for 15 min to allow equilibration.
Sensitivity was determined by calibration of the tipless cantilever against the cell dish in an area without cells. Volt-distance measurements were taken using the following parameters:

• 2 V applied contact setpoint
• 10 µm pulling retraction distance
• 5 µm/s approach and retract velocities
• Contact time = 0
• 4096 data points
• Closed loop mode-constant height during contact mode

The average slope of three force curves measuring this interaction were used to determine the distance (nm) of cantilever deflection as a function of piezo applied voltage. The experiment was continued only if this value was between 70 and 100 nm/V.

The spring constant of the cantilever was calculated 100 µm above the surface of the dish, in fluid, using the JPK integrated thermal tune method by Fourier transform and fitting the second resonance peak using a correction factor of 0.251. Spring constants were determined to be within 10% of manufacturers’ estimates or the experiment was not continued. Calibration was carried out following every major laser realignment or cantilever repositioning.

5.9.5 in situ probe cell attachment to cantilever
Following calibration, cells were scraped from confluent 24 well plates, tritivated 40x through a 19G needle and injected into the edge of the experiment dish and allowed to settle (30 s). One probe cell was attached to the cantilever using the user controlled ‘Cell Capture’ mode, where the cantilever is brought into contact with the cell with a contact force of 0.5 nN for 10 s and raised. If a cell was not
attached within 5 min, a new well of probe cells was injected into another region of the experiment
dish. Once a cell was attached to the cantilever, the cantilever was raised 100 μm above the surface
of the dish and the cell was allowed to spread for 5 min before the experiment began.

5.9.6 Cell adhesion measurements
The cantilever probe cell was brought into contact with the adherent cell, at a velocity of 5 μm/s,
from 100 μm above the cell to the area above the nucleus until the cantilever deflection reached 0.5
nN. The measurements were taken in a closed-loop mode, where constant height was maintained
during cell-cell contact. The contact time between cells was varied from 5 to 600 s. After this the
cantilever was retracted at a velocity of 5 μm/s until the cantilever was raised 100 μm above the
adherent cell. Each contact time was repeated 3 times per cell, followed by a break of 20 s for cell
recovery before a new contact time was tested. A probe cell was removed and replaced after 30
force curves had been collected. At least 30 force curves were collected per contact time with at
least 10 different cell combinations. Each force curve contains 4096 data points.

5.9.7 SCFS Data Analysis
Data analysis was carried out in JPK Image Processing software version 3.3.4. Force curves were
batch analysed, first by applying spring constant and sensitivity parameters, to determine the force
of unbinding from cantilever deflection. Force curves retract baselines were then offset on the z and
y axis. Force curves were analysed to determine the maximal unbinding force between two cells
(nN), the work required to separate the cells (J) and the size and number of unbinding events.

5.10 Single molecule force spectroscopy
5.10.1 Nanoscope IV Picoforce microscope
Single molecule force spectroscopy was carried out on a Veeco Nanoscope IV Multimode Scanning
Probe Microscope with Picoforce controller (Veeco Metrology group, USA) mounted on an upright
microscope (Veeco Metrology Group). All experiments were carried out in fluid at 22°C. Pyramidal-
tipped silicon nitride cantilevers (Si₃N₄) (Veeco) were used with a nominal spring constant of 0.06
N/m. Cantilever control and data acquisition was carried out by Nanoscope Software version 6.0.

5.10.2 Cantilever and substrate functionalisation
Cantilevers and 13 mm glass coverslips were cleaned with piranha solution (VERY DANGEROUS-add
H₂SO₄ to H₂O₂, 30% H₂O₂/ 70% H₂SO₄) for 15 min at RT then rinsed with MilliQ ultrapure ddH₂O x5.
Protein was either adsorbed onto glass coverslips and cantilevers or covalently coupled.
5.10.3 NCAM± VASE Fc surface adsorption

The method of protein adsorption was adapted from the NCAM Fc ELISA (Section 5.5.5). 100 μl of 1 μg/ml α human Fc in PBS was added to the coverslip and cantilever for 1 hour at 37°C before rinsing with PBS. If required a surfactant, P105, was added to the antibody mixture at 3 mg/ml (0.46 mM) and incubated. An optional blocking step was carried out with 100 μl of 1% BSA in PBS for 45 min. After washing, 0.4-4 μg/ml NCAM Fc (3-30 nM) in PBS was incubated overnight at 4°C. After washing with PBS, an optional blocking step with 1% BSA was carried out. The cantilever and coverslips were then rinsed ready for the AFM experiment.

5.10.4 Biotinylated BSA surface adsorption

Following biotinylation of BSA, piranha cleaned cantilevers and glass coverslips were coated in 250 μg/ml of BSA or BBSA, as required, in PBS or DMEM/20 mM HEPES for 1 hour at RT. Surfaces were then rinsed and blocked with DMEM/20 mM HEPES/0.5-1% BSA for 1 hour at RT. If required, surfaces were then rinsed and incubated in 1 mg/ml avidin (Sigma-Aldrich) for 1 hour at RT. After washing, the AFM measurements were carried out. For flooding of avidin in the AFM experiment, 1 mg/ml avidin was injected into the AFM chamber and incubated for 30 min at 22°C then measurements were carried out in situ.

5.10.5 AFM calibration

The functionalised cantilever was mounted into a fluid cell, containing the buffer of choice, to maintain a 10° downward angle of the cantilever and loaded onto the AFM above the functionalised cantilever. The laser was aligned on the tip of the cantilever and reflected onto the photodiode, resulting in a sum signal of 7V. The rig was then left for 15 min to allow equilibration at 22°C. Sensitivity was determined by calibration of the cantilever against the functionalised coverslip. Volt-distance measurements were taken using the following parameters:

- 1 V applied contact setpoint
- 1 μm pulling retraction distance
- 0.5 μm/s approach and retract velocities
- Contact time = 0
- Scan rate = 0.249 Hz
- Number of data points = 1024
- Integral gain = 0.5
- Proportional gain =2
- Closed loop mode, constant height during contact
- Retraction delay = 50 ms

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• Trigger threshold = 50 nm

The average slope of three force curves measuring this interaction were used to determine the distance (nm) of cantilever deflection as a function of piezo applied voltage. The experiment was continued only if this value was between 70 and 100 nm/V.

The spring constant of the cantilever was calculated above the surface of the coverslip, in fluid, using the Nanoscope integrated thermal tune method. This method involved the collection of cantilever frequency fluctuations followed by a Fourier transform and fitting of the first resonance peak. Spring constants were determined to be within 10% of manufacturers’ estimates and of each other or the experiment was not continued. Calibration was carried out following every major laser realignment or cantilever repositioning.

5.10.6 Protein adhesion measurements
The functionalised cantilever was brought into contact with the functionalised coverslip in fluid at 22°C, at a velocity of 0.5 μm/s, with a ramp size of up to 1 μm until varying cantilever deflections between 0.5 and 3 nN were reached. The measurements were taken in a closed-loop mode, where constant height was maintained during contact by feedback from proportional and integral gains. The contact time between cells was varied from 0 to 0.5 s. Following this the cantilever was retracted at varying velocities between 0.05 and 0.5 μm/s. Between force curves acquisitions, the cantilever was rested above the surface for 50 ms before beginning another. Force curves were captured in an automatic continuous capture mode at varying scan rates and with 1024 data points per curve. If required, the cantilever position was manually moved following collection of one data set, to probe another area.

5.10.7 SMFS Data Analysis
Data acquisition was limited only by cantilever retract velocity and scan rate. Retract force curves were analysed by hand to remove all curves with no visible interaction within the first micron of cantilever retraction. Retract curve baselines were then offset on the z and y axis. Unbinding events, where 70% of the unbinding force had occurred over greater than a 5 nm separation distance, were excluded. Unbinding events smaller than 20 pN were also excluded. Curves which had viable unbinding events were then collated to determine the size and position of unbinding events between the interacting partners in question.

5.10.8 Dynamic Force Spectroscopy
To probe the energy landscape of single molecule protein interactions during force measurement acquisition, the approach velocity was kept constant and the retract velocity was varied between
0.05 and 0.5 μm/s. Protein unbinding events were then analysed as before, but individual unloading rates (pN/s) for each unbinding event were calculated from knowledge of the retraction velocity and the slope of the retract curve prior to the rupture event. The natural logarithm of each unbinding event was then plotted against the individual unbinding event and the plot was analysed for kinetic information.
Appendix

Enclosed within the appendix:

- Example force-distance curves
- NCAM sequence
- Mass chromatograms for peptides synthesised
Example force-distance curves

Supporting data for Figure 2.8

Supporting data for Figure 2.8: AFM experiment to measure the interaction between adsorbed biotin-avidin and a comparison of all potential interactions.

The interaction between biotin-avidin was measured by AFM following the schematic presented in Fig. 2.5. The setpoint contact force was 3 nN and the cantilever forward and retract velocities were 498 μm/s. a) An example force-distance curve representing the interaction between biotinBSA and glass after 5 s of contact time. b) An example force-distance curve representing the interaction between biotinBSA and BSA after 5 s of contact time. c) An example force-distance curve representing the interaction between biotinBSA and biotinBSA after 5 s of contact time. d) An example force-distance curve representing the interaction between biotinBSA and avidin after 5 s of contact time. e) An example force-distance curve representing the interaction between avidin and avidin after 5 s of contact time. All experiments were carried out in DMEM/20 mM HEPES pH 7.4 at 22°C.
Supporting data for Figure 2.22

Supporting data for Figure 2.22: Dynamic force spectroscopy experiments measuring the interaction between NCAM-NCAM, NCAM-VASE, VASE-VASE and VASE-NCAM at various retract velocities.

The homophilic and heterophilic interactions, between affinity captured NCAM and VASE Fc, were compared for a retraction velocity of 498 nm/s to probe its effect on unbinding force. The setpoint contact force was 0.5 nN, the cantilever forward velocity was 498 nm/s and the contact time was 0 s. **a)** An example force-distance curve representing the interaction between NCAM and NCAM. **b)** An example force-distance curve representing the interaction between VASE and VASE. **c)** An example force-distance curve representing the interaction between NCAM and VASE. **d)** An example force-distance curve representing the interaction between VASE and NCAM.
NCAM extracellular domain sequence

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atgctgcaaaactaaggatctc
MLKTDL
atctgacattggttttctcttgggactctgccaggttgatttgtttcttcagctgc
IIWTLFPFLGTAVSLQVDIVPS
aggggaggatcagcttggaagatctcacaattctttatgcgaagttgaggtgtgc
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The 30 base pair, 10 amino acid VASE sequence is underlined.
VASEscr

CKKQSDWTEPSAELREEGT
References


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