

Aptamers for proteomics

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To my parents

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Abstract

Changes in post-translation modifications are very important in the regulation of biological processes. Many modifications occur at very low levels, resulting in a low-abundance of the modified proteins in cells, and therefore assessing those modifications is not an easy task. Modern proteomics needs improved methods for identifying such changes.

In this thesis, we focus on generating aptamers that can bind phosphoproteins with high affinities and therefore would be able to detect even low-abundance proteins. Aptamers are short sequences of nucleic acids that can be selected from libraries through a process called SELEX to bind targets of interest with high affinity and specificity. In this work, a phosphotyrosine (pY) peptide in a consensus sequence, commonly found in a class of phosphoproteins recognised by SH2 domains of signalling cascades in cells, was chosen as the target. By choosing this peptide target, we aim to create aptamers that can bind a class of proteins that carry this peptide sequence, mimicking the action of the intracellular SH2 domains.

An RNA library with 7×10^{14} molecules with 30 nucleotides in the random region was employed for the selection and aptamers that bind the pY peptide were selected. Using surface plasmon resonance (SPR), binding affinities of these

aptamers with their peptide target were determined (K_d values in high nanomolar (nM) range). In addition, aptamers that bind streptavidin tightly (K_d values in low nM range) were also isolated, as streptavidin was used as the matrix in partitioning step during the selection. Affinities of these aptamers were also determined by SPR. Moreover, fluorescence quenching suggested that the streptavidin binding aptamers bound in or near the biotin binding site. These aptamers can be used as affinity tags for RNA molecules. The secondary structures of both types of the aptamers were predicted based on their random-region sequences using the Mfold program.

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Abbreviations

A	Adenine
C	Cytosine
G	Guanine
T	Thymine
U	Uracil
Ala	Alanine
Arg	Arginine
Asp	Aspartic acid
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
His	Histidine
Leu	Leucine
Lys	Lysine
Met	Methionine
Phe	Phenylalanine
Pro	Proline
Ser	Serine
Trp	Tryptophan
(p)Tyr	(phospho)Tyrosine

Val	Valine
2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis
Ab(s)	Antibody(ies)
BB	Binding buffer
bp	Base pair
BSA	Bovine serum albumin
CE	Capillary electrophoresis
CPK	Corey-Pauling-Koltun
(k)Da	(kilo)Dalton
dNTPs	Deoxynucleoside triphosphates
<i>E. coli</i>	<i>Escherichia coli</i>
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
EDTA	Ethylenediaminetetraacetic acid
HABA	2-(4'-hydroxyazobenzene) benzoic acid
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
K _d (s)	Dissociation constant(s)
LB	Luria-Bertani
LBA	Luria-Bertani agar
MS	Mass spectrometry
NA(s)	Nucleic acid(s)
NECEEM	Non equilibrium capillary electrophoresis of equilibrium mixture
NHS	N-hydroxysuccinimide
NMR	Nuclear magnetic resonance
ORF(s)	Open reading frames
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PDB	Protein data bank

PDGF	Platelet-derived growth factor
pI(s)	Isoelectric point(s)
PTB	Phosphotyrosine-binding
PTM(s)	Post-translational modification(s)
RTKs	Receptor tyrosine kinases
pY	Phosphotyrosine
SA	Streptavidin
SELEX	Systematic evolution of ligands by exponential enrichment
SH2	Src homology-2
SPR	Surface plasmon resonance
TBE	Tris-Boric-EDTA
VEGF	Vascular endothelial growth factor

Chapter 1: Introduction

1.1. Proteomics

The complete sequence of many genomes has been solved (Perna *et al.* 2001; The International Human Genome Mapping Consortium 2001; Mouse Genome Sequencing Consortium 2002). As the results emerged, the number of genes comprising the genome ranges from a few hundreds for bacteria (Perna *et al.* 2001) to tens of thousands for mammalian species (The International Human Genome Mapping Consortium 2001; Mouse Genome Sequencing Consortium 2002). However, the number of expressed proteins is much higher as the same gene can generate multiple protein products that differ as a result of combinatorial splicing, processing and modification (Aebersold 2003). In the cellular context, apart from the amino-acid sequence defining a protein, its properties, such as the level expressed, the specific activity, the state of modification and association with other molecules, are crucial for the description of the biological system.

The concept of the proteome emerged to complement physical genomic research and the reason is clear: to really understand biological processes, we need to understand how proteins function in and around cells. The term “proteome” was first used by Wilkins a decade ago, to describe the set of proteins encoded by a genome

(Wilkins *et al.* 1996). The study of the proteome, called proteomics, now encompasses not just the proteins in a given cell, but also their modifications, activities, localization, and interactions of proteins in complexes to provide a comprehensive view of the structure, function and regulation of biological systems (Patterson and Aebersold 2003). In my opinion, proteomics can be viewed as protein characterisation in the cellular context as described through Figure 1-1.

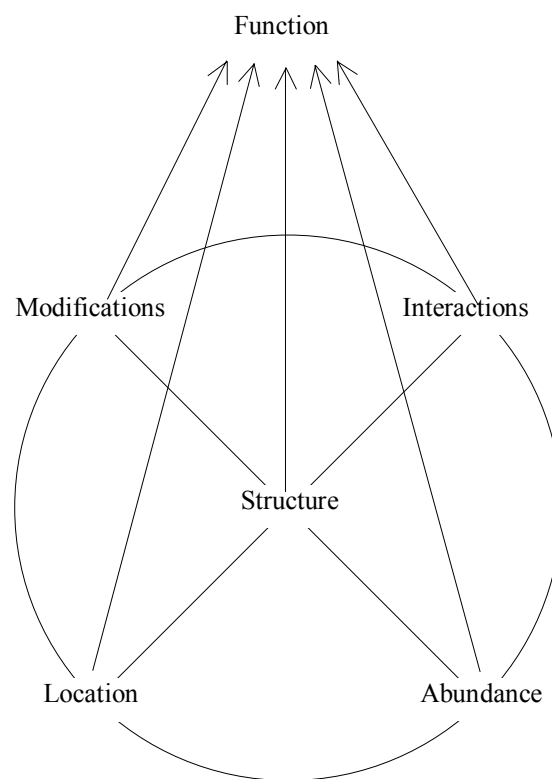


Figure 1-1: Characteristics of a given protein in proteomic context. Protein function in a cell is defined not only by its amino-acid sequence but also by the level expressed, the specific activity, state of modification and association with other molecules.

By studying global patterns of protein content and activity and how these change during development or in response to changing conditions such as disease, proteomic research is poised to boost our understanding of system-level cellular behaviour. Clinical research also hopes to benefit from proteomics by both the identification of new drug targets and the development of new diagnostic markers (Hanash 2003). In this project we focus our proteomic research on protein modifications, particularly for phosphorylation.

1.1.1. Protein modifications

Release of a completed polypeptide chain from a ribosome may not be the final chemical step in the formation of a protein. Various covalent modifications can occur either during or after assembly of the polypeptide chain and they are known as co- or post-translational modifications, respectively. Determination of these modifications is extremely important because these processes can lead to changes in physical and chemical properties: folding, conformation, stability, activity and therefore protein function. Often, post-translational modifications add functional groups to the modified protein. Examples of the biological effects of protein modifications include phosphorylation for signal transduction, ubiquitination for proteolysis, attachment of fatty acids for membrane anchoring and association, glycosylation for protein half-life, targeting or cell:cell and cell:matrix interaction. Therefore, proteomic analysis of modifications, particularly for post-translational modifications, paves the way to understanding how a cell works and that would particularly benefit the fields of diagnostics and drug discovery.

1.1.2. Protein phosphorylation

Protein phosphorylation is addition of phosphate group(s) to a protein. Reversible protein phosphorylation, principally on serine, threonine and tyrosine residues, is a very common post-translational modification and has been studied intensively for many years (Cohen 2000).

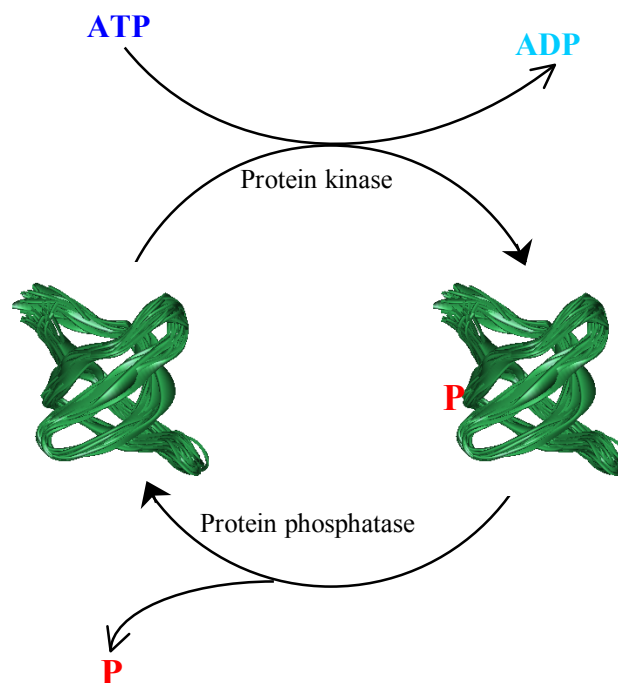


Figure 1-2: Protein phosphorylation/ dephosphorylation in cells. In phosphorylation processes, protein kinases catalyse the transfer of the terminal phosphate group of ATP to the acceptor protein. The reverse reaction, called dephosphorylation, is catalysed by protein phosphatases.

In phosphorylation processes, protein kinases catalyse the transfer of the terminal phosphate group of ATP to the acceptor protein. The reverse reaction, called dephosphorylation, is catalysed by protein phosphatases. A schematic view of phosphorylation/dephosphorylation is illustrated in Figure 1-2.

Genome sequencing suggests that about 2% of human genes encode protein kinases (Manning *et al.* 2002), and it is thought that hundreds of distinct types of protein kinases are present in a typical mammalian cell (Rubin *et al.* 2000). There are two main classes of protein kinases that operate as intracellular signalling proteins. The majority are serine/threonine kinases and the others are tyrosine kinases, which phosphorylate proteins on tyrosines. However, an occasional kinase can do both (Kyriakis *et al.* 1991). The added phosphate group forms an ester-linkage to the side-chain hydroxyl group of the residues. In addition to phosphate esters involving side chains of the hydroxyl residues, phosphoramidates of arginine, histidine, and lysine have also been observed (Duffy and Matthews 1995).

Addition of phosphate groups causes changes in protein charges and therefore alterations of protein structure. Protein phosphorylation generally exerts its regulatory function by altering the structure and thus the function of the targeted protein (Karin and Hunter 1995). Phosphorylation of a protein can therefore determine its activity states and interactions with other proteins. In signalling, for example, kinase cascades are turned on and off by the reversible addition and removal of phosphate groups (Pawson and Nash 2000). The mechanisms of activation and signalling have been studied intensively through analysis of modular interactions of receptor tyrosine kinases (Sadowski *et al.* 1986; Anderson *et al.* 1990;

Hooshmand-Rad *et al.* 2000; Hunter 2000; Hubbard and Miller 2007). The involvement of modular protein-protein interactions in signaling was originally recognised in the context of *src-homology 2* (SH2) domains, which were first discovered by researchers in Pawson lab in 1986 (Sadowski *et al.* 1986). SH2 domains are protein modules that recognise proteins through their phosphotyrosine (pY) peptide sequences (Eck *et al.* 1993; Waksman *et al.* 1993; Pascal *et al.* 1994). Most SH2 domains require phosphorylation of the peptide ligand for high affinity binding, but differ in their ability to recognise residues carboxyl-terminal to the pY, thereby imbuing each SH2 domain with an ability to bind preferentially to a pY peptide motif (Songyang *et al.* 1993; Payne *et al.* 1994; Bradshaw *et al.* 1999). In our project, one of these peptide motifs was chosen as our target and the choice of the peptide is discussed in more detail in Chapter 5.

1.1.3. Proteomic analysis of post-translational modifications

Post-translational modifications (PTMs) modulate the activity of most eukaryote proteins. Therefore, changes in post-translation modifications are very important in the regulation of biological processes. Many modifications occur at very low levels resulting in low-abundance of the modified proteins in cells, and therefore assessing those modifications is not an easy task. Identification of cellular proteins as well as their modification states has relied largely on mass spectrometry (Aebersold and Goodlett 2001) and this route is regarded as mass spectrometry-based proteomics.

1.1.3.1. Mass spectrometry-based proteomics

Mass spectrometry (MS) measures mass-to-charge ratio (m/z), yielding the molecular weight and the fragmentation pattern of peptides derived from proteins. For this reason, it is the most common method used to map the primary sequence of individual proteins.

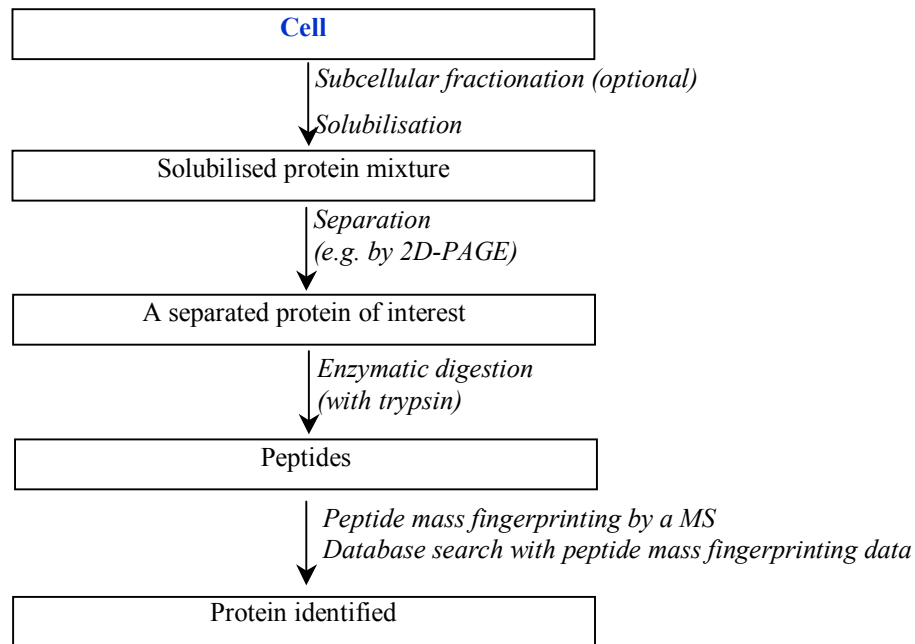


Figure 1-3: A general procedure for protein identification based on mass spectrometry. Proteins in a mixture are separated by 2D-PAGE. A protein of interest is then proteolytically cleaved into smaller peptides. The resultant peptides are analysed in a mass spectrophotometer to determine the sequence identity of the protein.

This method revolves around the following outline: Proteins in a mixture are separated (often by 2 dimensional polyacrylamide gel electrophoresis (2D-PAGE)). A separated protein is then proteolytically cleaved into smaller peptides. The resultant peptides are analysed in a mass spectrophotometer, the data processed through a series of computer algorithms that determine the sequence identity of the protein and to some extent its state of modification. Figure 1-3 describes a general protein based-MS identification procedure.

As shown in Figure 1-3, protein identification using mass spectrometry needs to be accompanied by protein separation methods. Initial proteomic efforts relied on 2D-PAGE for protein separation prior to mass spectrometric identification of protein spots (Wilkins *et al.* 1996). With 2D-PAGE, proteins in a mixture are separated according to differences in their masses and isoelectric points (pIs) and then the proteins are visualised by staining reagents (Görg *et al.* 2000). An inherent limitation of this approach is that it is constrained to the moderate to high-abundance proteins in the sample due to limitation in the visualisation sensitivity (Jenkins and Pennington 2001). This makes it particularly challenging to analyse post-translational modifications, including phosphorylation, where many changes occur at very low levels. In addition, co-migration of proteins can also contribute to the difficulties in separation of proteins of interest. Therefore, more functionally oriented strategy in proteomics is required, one that focuses on one type of modifications or on a group of proteins present in a sample. For example, separation of low-abundance proteins can be achieved by using high affinity reagents, such as using antibodies to capture proteins of interest in the samples (Wang and Hanash 2005).

1.1.3.2. Antibodies

Antibodies, the most popular class of molecules providing molecular recognition for a wide range of applications have been used in research for many decades. Proteomic analysis can rely on antibodies for fishing proteins of interest from a complex protein mixture for subsequent identification. For example, if high affinity antibodies have been raised against a particular protein of interest, the protein can be immuno-precipitated from the complex mixture for separation and enrichment prior to further analysis (Kalo and Pasquale 1999). Although effective in selectively isolating a particular modified protein, this procedure requires production of a specific antibody for each protein to be analysed. A more general useful tool would be antibodies that are able to recognise a group of proteins sharing a specific property. For example, antibodies directed against non-sequence-specific pY proteins have been developed. These pY-specific antibodies have been used to enrich pY-containing proteins (Daniel *et al.* 1985) as well as pY peptides (Kalo and Pasquale 1999). In addition, antibodies also act as affinity reagents for various functional protein assays and their employment in protein detection using microarray formats has been fast growing in the last few years (MacBeath 2002).

However, antibodies have their own limitations. Firstly, the antibody identification process starts within an animal and therefore antibody generation becomes difficult when raise for molecules that are not tolerated by animals, such as toxins, or less immunogenic molecules. Secondly, most antibodies are not stable as they are often quite sensitive to temperature and can undergo irreversible denaturation. In addition, as the number of antibodies is still small compared to the

number of proteins, there is a huge demand for finding new materials as alternatives that can fulfil the need.

1.1.3.3. Aptamers as alternatives to antibodies

To meet those shortcomings of antibodies, other types of affinity reagents have been considered. Particularly versatile probes with tremendous potential for use as affinity molecules are aptamers.

Aptamers are functional nucleic acids selected from combinational oligonucleotide libraries by *in vitro* selection. A number of aptamers selected against protein targets with affinities generally ranging from pM to nM have shown increasing utility as affinity reagents and can compete with antibodies in a number of applications, including in the analytical and clinical fields (James 2000; Luzi *et al.* 2003; Tombelli *et al.* 2005). In addition to having selectivities comparable to those of monoclonal antibodies (Bell *et al.* 1998; Cox and Ellington 2001; Daniels *et al.* 2002; Mori *et al.* 2004), some advantages of aptamers over traditional antibody-based reagents are that they can be developed against toxic targets, stabilised against degradation by the introduction of modified nucleotides and easily refolded following denaturation. In addition, labels for detection and linkers for conjugation can be introduced at specific sites. Furthermore, *in vitro* selection of aptamers can be automated. Its automation should allow a rapid, parallel production of multiple aptamers against complex target sets such as proteomes (Cox *et al.* 1998). For these advantages, it would be extremely useful to expand scale of aptamers for proteomic studies.

1.2. Aptamers

1.2.1. Introduction

Aptamers are DNA or RNA molecules of several tens of nucleotides in length that bind target molecules with both high affinity and specificity. Amongst the people who contributed most to triggering of the development of aptamers were Ellington and Szostak (Ellington and Szostak 1990), who first termed “aptamers”, and Gold and Tuerk, who first dubbed the *in vitro* selection procedure as “systematic evolution of ligands by exponential enrichment” (SELEX) (Tuerk and Gold 1990). Since then a number of aptamers have been generated against a various types of targets, including small molecules (Sassanfar and Szostak 1993; Geiger *et al.* 1996; Mannironi *et al.* 1997), peptides (Nieuwlandt *et al.* 1995; Gilbert *et al.* 1997) and proteins (Famulok 1999; Famulok and Mayer 1999; Brody and Gold 2000; Hesselberth *et al.* 2000a).

Nucleic acids are composed of sugar phosphate backbones and nucleobases. The differences between the two forms are DNA bears a hydrogen at the 2' carbon on the sugar moiety while RNA has a hydroxyl group in this position. RNA also utilises uracil instead of thymine, which contains a methyl group on the five-carbon of the base as shown in Figure 1-4. Adenine and guanine are purines, whilst the other bases are pyrimidines.

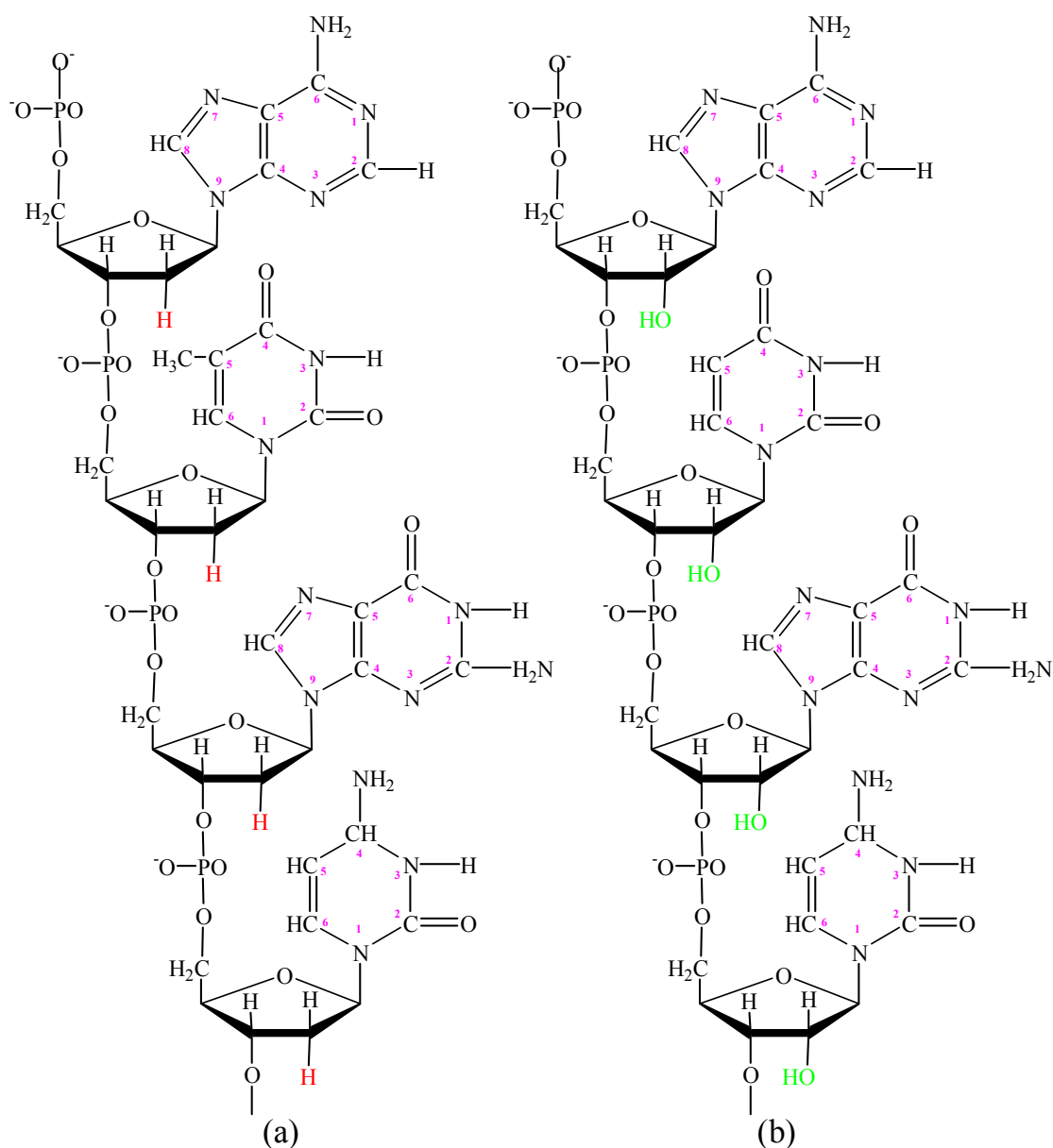


Figure 1-4: Structures of nucleic acids. Structures of a DNA chain ATGC (a) and an RNA chain AUGC (b).

In the double stranded form, the highly charged phosphate backbone is exposed on the surface of the molecule. However, in the single-stranded form, an array of polar and nonpolar surfaces is available to participate in the formation of secondary

and tertiary structures. This provides a host of possibilities for binding surfaces. Single stranded DNA and particularly RNA can fold into secondary structures that place specific functional groups at precise locations. This gives them optimal target binding properties, including the precise stacking of flat moieties, specific hydrogen bonding, and molecular shape complementation (Hermann and Patel 2000a).

1.2.2. Generation of aptamers

Aptamers are selected from oligonucleotide libraries *in vitro* through a cyclic process of binding, isolation followed by amplification known as SELEX (systematic evolution of ligands by exponential enrichment).

Selection of both DNA and RNA aptamers starts with a chemically synthesised DNA library. A library often consists of a random region of several tens of nucleotides, flanked by constant regions used for polymerase chain reaction amplification. A DNA library can be converted to an RNA library through transcription if a RNA polymerase promoter is included in one of the constant regions. A typical library contains from 10^{11} to 10^{15} different sequences. During selection process, following binding, isolation of unbound sequences (from the complexes of target molecules with bound sequences) is achieved by filtration, immobilisation/affinity chromatography or electrophoresis. After isolation, bound sequences are subjected to amplification to produce a library for the next round of the selection. RNA selection requires extra steps (conversion of RNA to cDNA and transcription of amplified DNA back to RNA) in amplification of the pool compared to that of DNA. Multiple selection rounds result in the preferential enrichment of the

sequences that bind the target with the highest affinities. A round of the aptamer selection is illustrated in Figure 1-5.

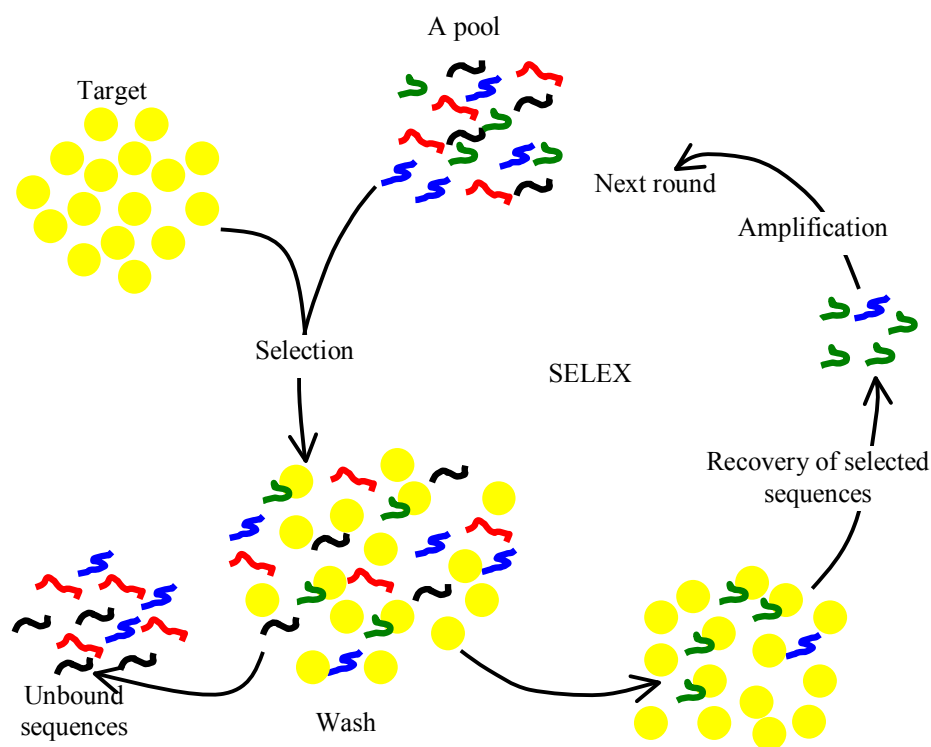


Figure 1-5: A general scheme for aptamer selection. A random nucleic acid library (beginning with 10^{11} to 10^{15} molecules) is incubated with the target (in yellow). Unbound sequences are washed away from the of bound-sequence/target complexes. The bound sequences are then eluted from the target molecules and amplified to make a library for a next round of the selection.

One aspect that greatly influences the efficacy of the selection is partitioning. A better degree of separation between bound and unbound sequences would lead to less

rounds of selection being performed. Unbound sequences can be separated from complexes by filtration through nitrocellulose membranes or washing following affinity capture of bound sequences/complexes on solid supports that (Hager and Szostak 1997; Marshall and Ellington 1999; Jhaveri and Ellington 2000; Cox and Ellington 2001; Robertson and Ellington 2001; Stoltenburg *et al.* 2005; Gopinath *et al.* 2006; Morse 2007; Sando *et al.* 2007). One of the problems countered when using these partitioning approaches is co-enrichment of matrix-bound sequences and, therefore, it may be necessary to add an extra matrix-screening step into selection protocols to exclude those undesired sequences from enriched pools. Furthermore, using solid supports in cases that targets are pre-immobilised on the solid supports for partitioning may have effects on accessibility of nucleic sequences to immobilised target molecules. Further discussion on different approaches for partitioning will be presented in Chapter 3.

It has recently been shown that capillary electrophoresis (CE) can lead to effective partitioning during *in vitro* selection. CE has for years been used for sensitive separation in the analytical field and researchers in the Bowser and Krylov labs have applied it to *in vitro* selection and the approach is called CE-SELEX or NECEEM-SELEX (NECEEM: Non-Equilibrium Capillary Electrophoresis of Equilibrium Mixtures) (Mendonsa and Bowser 2004b; Mendonsa and Bowser 2004a; Berezovski *et al.* 2005; Drabovich *et al.* 2005). A schematic representation of CE-SELEX is shown in Figure 1-6. Results published by these two labs demonstrated that the partitioning efficiency is higher than that of either membrane filtration or affinity chromatography (discussed earlier) and only a few rounds of selection are required instead of tens of rounds in the traditional selection,

exemplified by selection of aptamers against HIV-1 RT isolated by traditional SELEX and CE-SELEX (Schneider *et al.* 1995; Mosing *et al.* 2005).

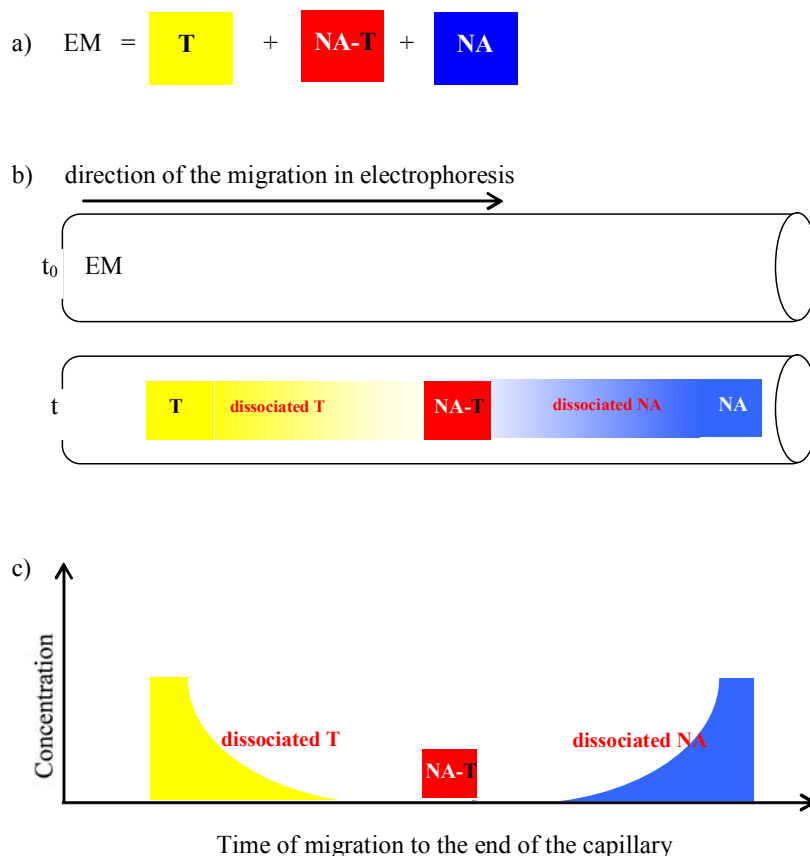


Figure 1-6: Schematic diagram of CE-SELEX. Where NA stands for nucleic acid (DNA or RNA); T is for target. (a) Incubated binding mixture (equilibrium mixture: EM), (b) Partitioning by CE, and (c) Collection of the bound sequences.

Libraries of modified nucleotides can also be used for selection of aptamers. For example, aptamers selected from libraries of species include a 5-iodo or 5-bromo

substituted on thymine (for DNA) or uracil (for RNA) has been demonstrated as shown in Figure 1-7.

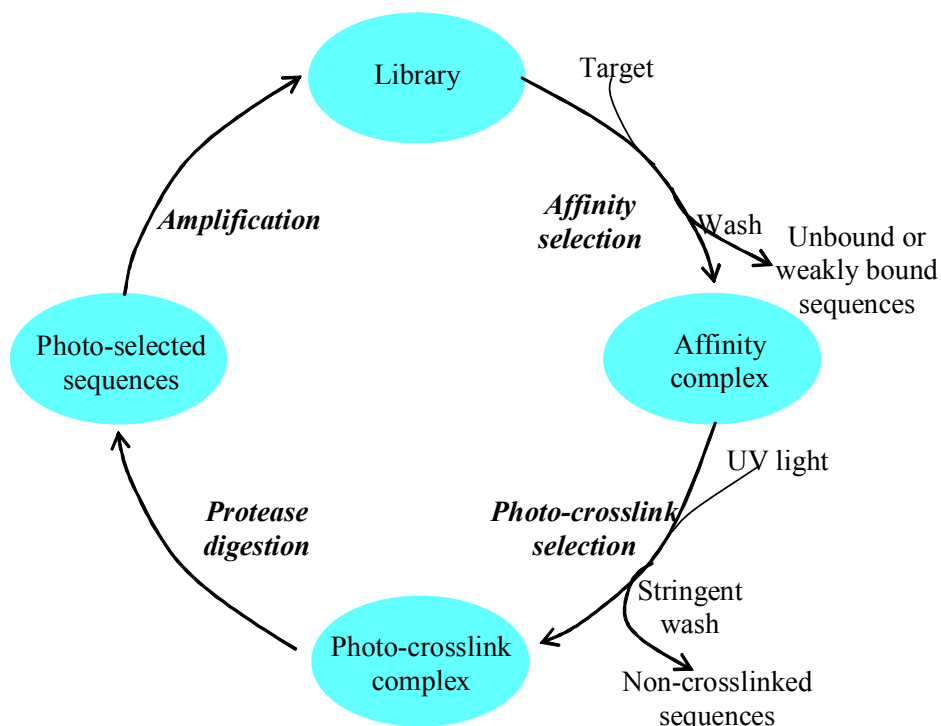


Figure 1-7: A general scheme of photo-SELEX. A library is incubated the target of interest. Unbound or weakly bound sequences are washed away. Those that remain bound to the target are exposed to UV radiation, allowing them to form covalent bond with the target if a photoactivable group is in close proximity to a suitable residue. A stringent wash regime is then applied to sweep sequences that do not form crosslink to the target whilst those were those that crosslink are amplified to make the library for the next round.

It has been reported that such substitutions can still lead to evolution of tight binding species that contain photo-active groups so-called photo-aptamers (Willis *et al.* 1994; Golden *et al.* 2000). Such SELEX experiments (known as photo-SELEX)

generate photo-aptamers that can specifically photo-crosslink to their cognate proteins through the substitute group by irradiating with UV light (at λ in 310 nm range) for photo-activation (Golden *et al.* 2000; Petach and Gold 2002; Bock *et al.* 2004).

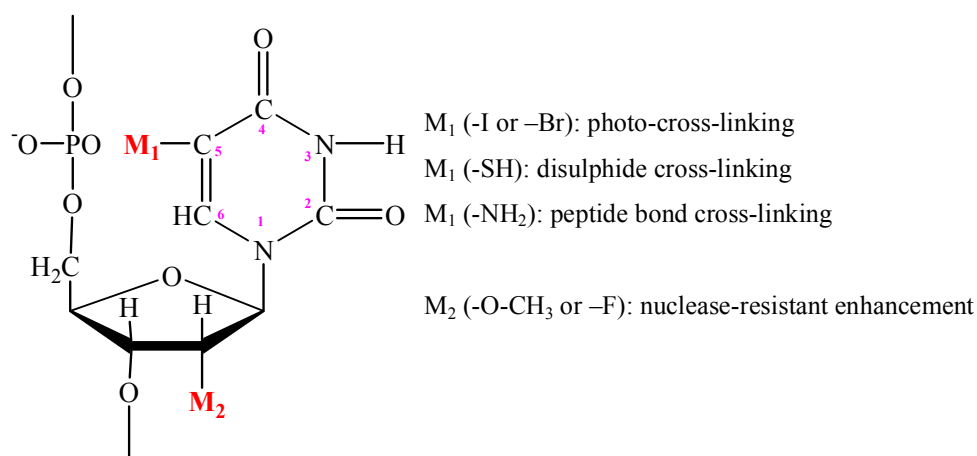


Figure 1-8: Possible modifications (M_i) on an oligonucleotide strand.

Modification at the 2' position of the sugar confers nuclease resistance whilst modification at the C5 of the base can be used for cross-linking.

For another purpose, modified libraries have also been employed to enhance nuclease-resistance for aptamers. For instance, anti-VEGF aptamers selected from a 2'-O-methyl modified library that composed entirely of 2'-O-methyl nucleotides showed resistance to degradation in plasma at 37°C for 96 hours or after it was autoclaved at 125°C (Burmeister *et al.* 2005). Possible modifications on aptamer are shown in Figure 1-8.

Since the first aptamer papers published 17 years ago (Ellington and Szostak 1990; Tuerk and Gold 1990), hundreds of aptamers have been successfully generated against a wide variety of targets including small molecules (Geiger *et al.* 1996; Berens *et al.* 2001), peptides (Nieuwlandt *et al.* 1995; Xu and Ellington 1996) and proteins (Macaya *et al.* 1993; Cox and Ellington 2001). Anti-protein aptamers have made up the largest group of the generated aptamers. This is partly due to the important biological roles of proteins and therefore the high demand for creating anti-protein aptamers to study their functions and, in fact, a great number of anti-protein aptamers have been reported to be able to recognise characteristic patches on protein surfaces such as binding pockets or active sites of enzymes (Tuerk *et al.* 1992; Conrad *et al.* 1994; Bell *et al.* 1998; Shi *et al.* 2007). These patches can act as scaffolds for aptamers in folding into their defined three-dimensional structures upon binding with their protein targets. In addition, proteins own variety of functional groups and rigid structures that could also make them ‘easy’ targets. Beside that, as proteins bind nitrocellulose membranes, simple filtration can be used for partitioning and therefore no conjugation needed.

Aptamers can be selected not only against purified targets, but also against heterogeneous mixtures of targets, such as whole trypanosome cells (Homann and Goring 1999), which were later found to bind a variant surface glycoproteins (Lorger *et al.* 2003), or red blood cell membranes (Morris *et al.* 1998). Selections against complex targets enable aptamers to be generated even when the targets are not known. This enables identification of new biomarkers and would particularly benefit proteomic research as well as the diagnostics field.

1.3. Methods for characterisation of aptamers

1.3.1. Methods for determination of affinity and specificity

Methods used for binding assays such as fluorescence, electrochemistry, electrophoresis, chromatography or surface plasmon resonance can be employed to investigate binding of aptamers to their targets. Some methods that tend to be used more than others depending on sensitivity, amount of sample required and availability. In the following section, the methods that are frequently employed for aptamer binding assays are described.

Radiolabeling has been amongst the most popular methods to determine binding strength of the selected aptamers to their targets. Radioactive aptamers can be generated by incorporation of an [α - ^{32}P]-nucleoside triphosphate during amplification and the aptamer-protein complexes can be separated from the solution by filter binding. Filter-binding assays have been used to determine dissociation constants of aptamers with their targets in a large number of publications (examples in Table 1-1). This method has advantages in term of high sensitivity and small sample amounts but it does not provide information on kinetics.

Table 1-1: Methods for characterising bindings of aptamers with their targets.

Method	Example targets	Basic for detection	Interaction information	References
Radiolabeling of aptamer and membrane capture	Lysozyme, a tyrosine phosphatase, basic fibroblast growth factor (bFGF)	Radioactivity	Binding strength	(Conrad <i>et al.</i> 1996) (Bell <i>et al.</i> 1998) (Hesselberth <i>et al.</i> 2000b) (Cox <i>et al.</i> 2002) (Levy and Ellington 2002)
Capillary Electrophoresis	Protein farnesyltransferase (PFTase), HIV-1 RT	Change in size, shape, or charge	Binding strength, kinetics	(Berezovski and Krylov 2002) (Mosing <i>et al.</i> 2005)
Surface Plasmon Resonance	HIV-1 glycoprotein, Liver X receptors, NF- κ B, Human influenza virus H3N2	Change in mass bound to surface (technically change in reflective index)	Binding strength, kinetics	(Sayer <i>et al.</i> 2002) (Rhie <i>et al.</i> 2003) (Surugiu-Warnmark <i>et al.</i> 2005) (Gopinath <i>et al.</i> 2006)
Quartz Crystal Microbalance	α -thrombin, Human IgE	Change in mass bound to surface	Binding strength, kinetics	(Hianik <i>et al.</i> 2005) (Liss <i>et al.</i> 2002)
Isothermal Titration Calorimetry	Neomycin B	Heat release or uptake	Binding strength	(Cowan <i>et al.</i> 2000)
Analytical size exclusion chromatography	G-protein-coupled receptor for neurotensin	Change in hydrodynamic size	Binding strength	(Daniels <i>et al.</i> 2002)
Electrochemistry	17 β -estradiol, Neuro inflammatory cytokine PDGF, IgE	Change in electrical properties	Conformational change, kinetics of slow reaction	(Kim <i>et al.</i> 2007) (Liao and Cui 2007) (Papamichael <i>et al.</i> 2007)
Fluorescence	IgG3, MS2 coat protein	Change in fluorescence	Conformational change	(Missailidis <i>et al.</i> 2005) (Parrott <i>et al.</i> 2000)

Capillary Electrophoresis (CE) was also employed to monitor binding of aptamers to their targets, particularly when information on kinetics of the binding is desired. CE uses the same separation mechanism used in conventional electrophoresis but not under denaturing conditions and with electro-osmotic flow having to taken into account. The technique offers advantages of speed, small sample

amounts, high sensitivity and possible automation as well for parallel selection processes. The Bowser and Krylov labs have employed CE during partitioning as well as kinetic measurements for the binding assays (Berezovski and Krylov 2002; Mendonsa and Bowser 2004a; Okhonin *et al.* 2004; Drabovich *et al.* 2005; Mosing *et al.* 2005).

Kinetic and affinity measurements of aptamer-target bindings can also be carried out using surface plasmon resonance (SPR). Commercially available SPR systems, such as BIAcore devices, have facilitated the evaluation of association and dissociation kinetics of aptamers and their targets. To determine affinity constants, targets or aptamers are immobilised on sensor chip surfaces using covalent attachments through coupling to carboxyl dextran chips or plain gold chips or affinity attachments of biotin-tagged molecules through streptavidin/avidin-coated chips or histidine-tagged molecules *via* Ni-NTA-coated chips (Sayer *et al.* 2002; Mori *et al.* 2004; Surugiu-Warnmark *et al.* 2005; Gopinath *et al.* 2006). A more detailed discussion on characterisation of aptamer-target binding by SPR is presented in the chapters 4 and 5 where SPR data on aptamers selected in this project are described.

In addition, fluorescence can also be employed to investigate bindings of aptamers to their targets as interactions between them often cause conformational changes, which may be observed through fluorescence intensity changes. Fluorescent reporter groups can be incorporated into either aptamers or targets and interactions can be monitored through changes in fluorescence behaviours of the reporter groups (Nutiu and Li 2005; Ozaki *et al.* 2006; Morse 2007).

1.3.2. Structural studies of aptamer-target complexes

The structure of several aptamers has been determined by enzymatic modification and chemical probes, nuclear magnetic resonance (NMR) spectroscopy and X-ray crystallography (Feigon *et al.* 1996; Patel 1997; Carothers *et al.* 2006). NMR has been a popular choice as aptamers are relatively amenable to the methodology due to small sizes and rigidities of aptamers when complexed with their targets.

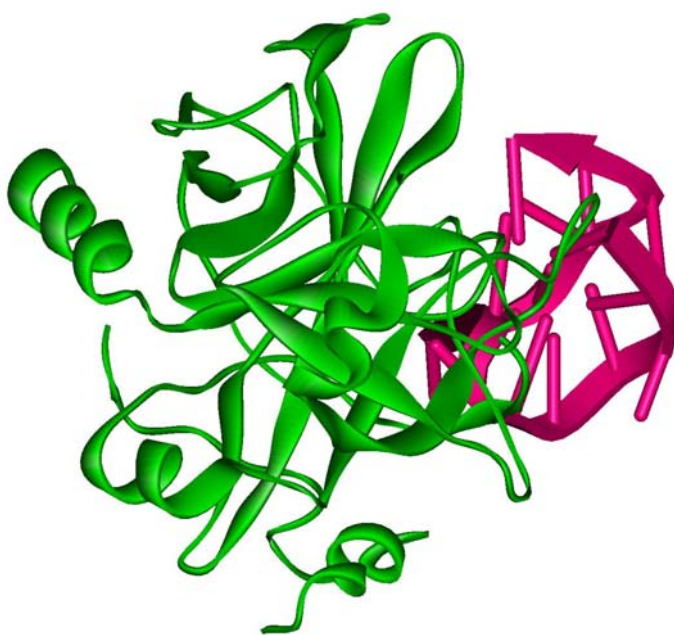


Figure 1-9: Structure of the anti-thrombin aptamer in complex with its target. The anti-thrombin aptamer, shown as the pink ladder model, bound its target, shown as the green ribbon model. The image created from pdb file 1HUT using WebLab ViewerLite (Padmanabhan *et al.* 1993).

One of the earliest aptamer structures studied was the 15-mer DNA aptamer against thrombin, d(GGTTGGTGTGGTTGG), a ‘high profile’ aptamer isolated by researchers at Gilead Sciences Inc. (Bock *et al.* 1992). Using two-dimensional NMR, it was shown that the aptamer folded into a four-stranded structure, stabilised by two stacked G tetramers, with each of the two pairs of the strands having a TT-loop (Macaya *et al.* 1993). Furthermore, Mao and Gmeiner used NMR to study the folding-unfolding mechanism of the aptamer upon its target (Mao and Gmeiner 2005). They proposed that when folding, the DNA first folded two TT-loops before forming the compact quadruplex and the unfolding was a reverse of the folding (Figure 1-9).

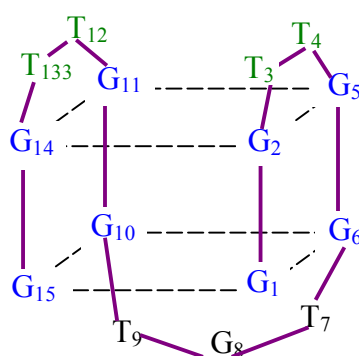


Figure 1-10: Quadruplex structure of the thrombin aptamer upon binding to its target (Macaya *et al.* 1993). The aptamer folds into four-stranded structure (purple) stabilised by two stacked G tetramers (blue), with each of the two pairs of the strands having a TT-loop (green).

The 15-mer aptamer-thrombin complex structure was also investigated using X-ray crystallography. Similarly to the NMR studies, it suggested that the aptamer in complex form folds into a structure of G-quartets. In addition, it reported that the DNA aptamer is sandwiched between two different positively charged regions of two symmetry-related thrombin molecule, making ionic and hydrophobic interactions (Padmanabhan *et al.* 1993).

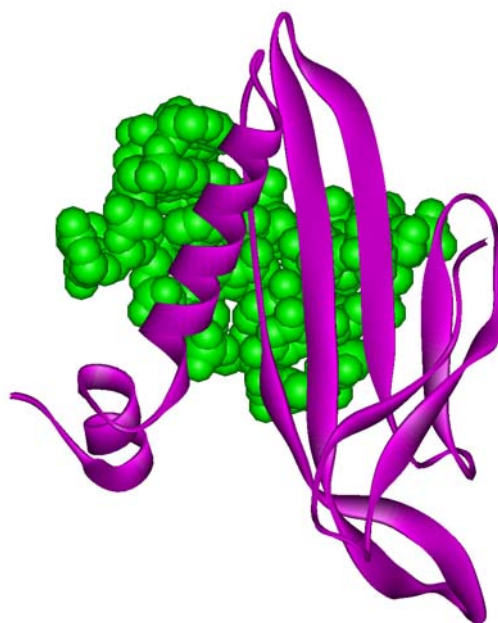


Figure 1-11: Structure of the anti-MS2 coat protein in complex with its target. The aptamer, shown as the green CPK model, bound its target, shown as the violet ribbon model. The image created from pdb file 6MSF using WebLab ViewerLite (Convery *et al.* 1998).

In another example, the crystal structure of an RNA aptamer bound to bacteriophage MS2 coat protein has been determined (Figure 1-10). Studies using X-

ray crystallography showed that the RNA aptamer “kisses” the antiparallel β sheet of the protein complemented by a number of loop-out bases, which are inserted into cavities and involved in stacking interactions (Convery *et al.* 1998; Horn *et al.* 2004).

A number of structural studies suggested when complexed with its target, an aptamer folds into a defined three-dimensional structure as a result of different forces: hydrogen bonding, electrostatic interactions, hydrophobic interactions, base stacking, and shape complementary (Fan *et al.* 1996; Patel *et al.* 1997; Jiang *et al.* 1999; Hermann and Patel 2000b). Structures of dozens of aptamer-ligand complexes can be found in protein data bank (www.pdb.org).

1.4. Applications of aptamers

As previously discussed in section 1.1.3.3, several properties of aptamers make them attractive analytical and therapeutic reagents that rival, and in some cases surpass, antibodies. When bound to their targets, they fold into defined three-dimensional structures and act in the same way as antibodies.

Similarly to antibodies, aptamers have been employed as affinity reagents for analytical technology as well as therapeutics. For instance, a anti-thrombin aptamer was employed as a recognition molecule of a sensor for thrombin detection (Schlecht *et al.* 2006) and potentially as a drug (currently in phase II trial), in which it replaced heparin for anti-coagulation during heart bypass surgery in canines (DeAnda *et al.* 1994). The structure of the anti-thrombin aptamer in complex with its target is shown in Figure 1-9.

Compared to antibodies, aptamers are less vulnerable to denaturation but they are more susceptible to degradation by nucleases. However, aptamers can be modified to become nuclease-resistant as described in page 38. In addition, it has been reported that aptamers usually retain their binding abilities and inhibitory behaviours when immobilised on solid supports (Farokhzad *et al.* 2004; Farokhzad *et al.* 2006), delivered into animals (Nimjee *et al.* 2005a; Nimjee *et al.* 2005b), labelled with fluorescent groups (Yamana *et al.* 2003; Ozaki *et al.* 2006) and expressed in cells (Blind *et al.* 1999). These give aptamers high potential in both analytical and clinical fields.

1.4.1. Analytical technology applications

The demand for analytical tools that allow rapid detection and quantitation of analytes is high in fields such as food and environmental issues or diagnostics in healthcare. Aptamers have been used extensively in biosensing application. The first sensor that used an aptamer as the recognition molecule was for measurements of L-adenosine (Kleijnung *et al.* 1998). The adenosine-binding RNA aptamer was labelled with fluorescein isothiocyanate (FITC) and immobilised on a streptavidin-coated fibre surface through biotin at its 5'-end and the sensor was used for direct quantitation of the ligand through fluorescence readings. Other examples include aptamer sensors for detection of cocaine (Stojanovic *et al.* 2001) or anthrax (Bruno and Kiel 1999). Examples of aptamer-based analytical applications are shown in Table 1-2.

Table 1-2: Examples of aptamers being developed as analytical reagents.

Target	Analytical application	Methods involved	Detection limit	Linear range	Reference
α -Thrombin	Affinity probe	Capillary electrophoresis and fluorophore-labelled aptamer	40 nM	40 nM – 4 mM	(German <i>et al.</i> 1998)
	Biosensor	Fluorophore-labelled aptamer (anisotropy)	5 nM	5 nM – 5 μ M	(Potyrailo <i>et al.</i> 1998)
Cocaine	Biosensor	Fluorophore-labelled aptamer (quenching)	10 μ M	10 μ M-4 mM	(Stojanovic <i>et al.</i> 2001)
Human IgE	Biosensor	Quartz crystal microbalance	0.5 nM (100 μ g/L)	0.5 nM-50 nM	(Liss <i>et al.</i> 2002)
	Affinity probe	Capillary electrophoresis and fluorophore-labelled aptamer	46 pM	46 pM-4.6 μ M	(German <i>et al.</i> 1998)
Platelet-derived growth factor	Biosensor	Fluorophore-labelled aptamer (anisotropy)	220 pM	0-100 nM	(Venter <i>et al.</i> 2001)
HIV-1 RT	Affinity probe	Capillary electrophoresis and fluorophore-labelled aptamer	10 nM	10 nM-50 nM	(Jhaveri <i>et al.</i> 2000)
Theophylline	Chemical sensor	Fluorescence	10 μ M	10 μ M – 2 mM	(Frauendorf and Jaschke 2001)

1.4.2. Therapeutic applications

In 2004, the US Food and Drug Association (US-FDA) approved the first aptamer, an anti-VEGF aptamer, as a drug under the commercial name Pagaptanib (Ng *et al.* 2006). There are a number of aptamers have been under trial or in pre-clinical studies including anti-thrombin aptamer, which just entered phase II trial and an anti-insulin receptor aptamer. Table 1-3 gathers information on some of the aptamers that have been developing for therapeutic uses.

Table 1-3: Examples aptamers developing for therapeutic applications.

Target	Aptamer type	K _d	Function	Clinical data	References
Vascular Endothelial Growth Factor (VEGF)	RNA	50 pM	Treatment of neovascular age-related macular degeneration	License by US FDA under name Pegaptanib	(Ruckman <i>et al.</i> 1998; Burmeister <i>et al.</i> 2005; Ng <i>et al.</i> 2006)
α -Thrombin	DNA	20 nM	Anticoagulation reagent	Phase II Trial	(DeAnda <i>et al.</i> 1994; Archemix 2007)
Hepatitis C	RNA	10 nM	Inhibiting NS3	Pre-clinical studies	(Kumar <i>et al.</i> 1997)
Insulin receptor MA20	RNA	30 nM	Inhibiting MA20	Pre-clinical studies	(Doudna <i>et al.</i> 1995)
HIV-1 Tat	RNA	120 pM	Preventing HIV-1 replication	Pre-clinical studies	(Ferguson <i>et al.</i> 2006)
Platelet-derived growth factor (PDGF)	DNA	100 pM	Inducing PDGF in mesangial proliferative nephritis	Pre-clinical studies	(Pietras <i>et al.</i> 2002)
Human nonpancreatic secretory phospholipase A ₂ (hnps-PLA ₂)	RNA	1.7 nM	Inhibiting hnps-PLA ₂	Pre-clinical studies	(Bridonneau <i>et al.</i> 1999)
Prostate specific membrane antigen	RNA	2.1 nM	Binding to a prostate cancer cell line	Pre-clinical studies	(Hicke <i>et al.</i> 2001)

1.5. Potential of “aptamers-based proteomics”

For proteomics, aptamers can be considered as a newly emerging class of affinity reagents compared to antibodies, which are still very ubiquitous in the field.

However, not long after their discovery, aptamers showed to have huge potential in rivalling antibodies as affinity reagents (Jayasena 1999).

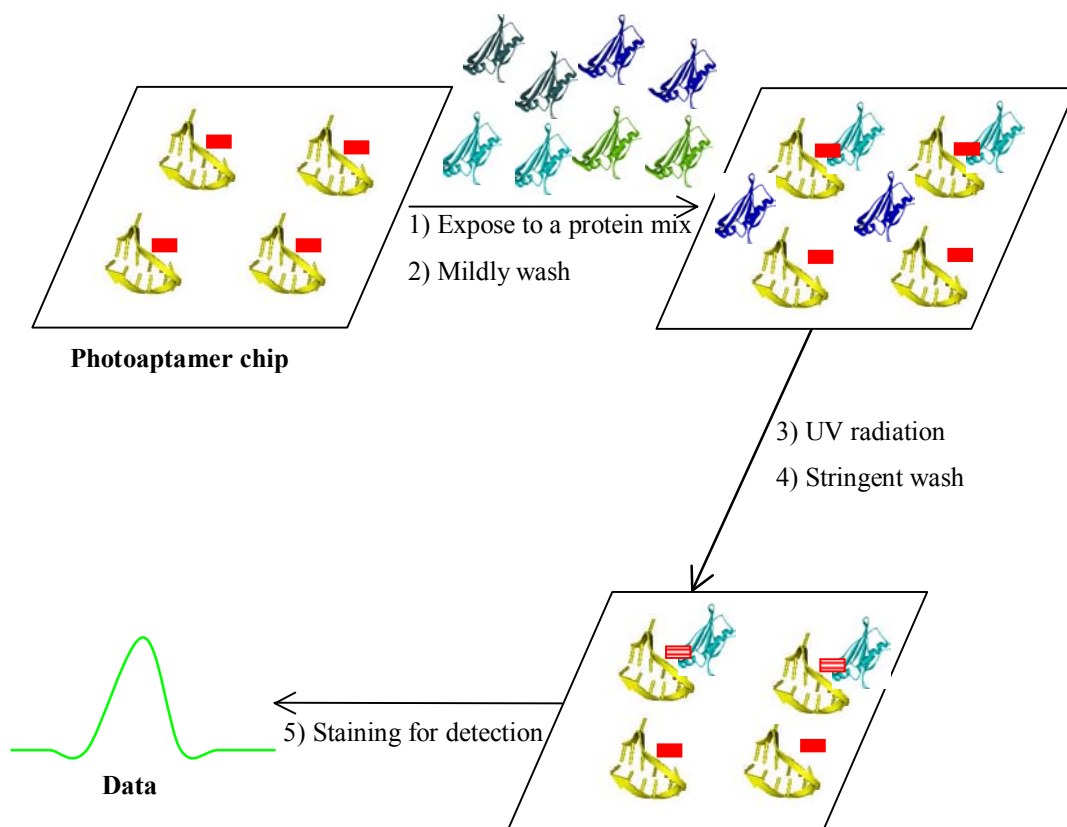


Figure 1-12: A general scheme of proteomic analysis using photoaptamer-based array chips. A photoaptamer chip is exposed to a protein sample and then gently washed to remove non-specifically bound analytes. The chip is photo-crosslinked to covalently attach specifically bound protein and then washed and stained for analysis.

Following this path, SomaLogic Inc. has been developing photo-aptamer chips (as illustrated in Figure 1-12). It was reported that the aptamer chips can be

quantitative means for multiple protein detection under a wide range of conditions, including stringent washes, high ion strength and extreme pHs that were not tolerated by antibodies (Petach and Gold 2002; Bock *et al.* 2004). Other examples using aptamer beacons, thrombin and platelet-derived growth factor (PDGF) can be detected at concentrations as low as nanomolar (Heyduk and Heyduk 2005; Vicens *et al.* 2005). These achievements proved that aptamers firmly have their stamps on proteomic analysis.

Aptamer-based microarrays for the quantitation of multiple protein analytes have also been developed by the Ellington group (Cho *et al.* 2006). A multiplex aptamer microarray was generated by printing two RNA aptamers (anti-lysozyme and anti-ricin) and two DNA aptamers (anti-IgE and anti-thrombin) onto either streptavidin or neutravidin coated glass slides. Target protein concentrations as low as 5 pM for lysozyme, 0.5 nM for ricin, 0.01 nM for IgE and 5 nM for thrombin could be detected. This result showed that aptamer arrays can potentially be used with numerous proteins in parallel, furthering the notion that aptamer arrays can be a powerful tool for proteomic studies.

1.6. Aims of the project

This work focuses on the selection of aptamers for phosphorylation analysis. A pY peptide was chosen as the target for our selection. The peptide sequence comprises a consensus sequence, which is found in a number of proteins involving in cell signalling and recognised by SH2 domains. By using this peptide as the selection target, we aim to isolate aptamers that can tightly bind phosphoproteins, mimicking

the action of SH2 domains in cells. Emergence of these aptamers would boost proteomic research of phosphorylation and potentially benefit the fields of diagnostics and drug discovery.

The work includes (1) isolation of aptamers that bind the pY peptide target and (2) characterisation of the isolated aptamers. In addition, a research plan for application the discovered aptamers to proteomic analysis of pY proteins in cells is also proposed.

Chapter 2: Materials and methods

2.1. Materials

2.1.1. Chemicals and biochemicals

10 mM dNTP mix was purchased from Invitrogen, 25 mM dNTP mix was from Promega and NTPs were from Epicentre. DNA ladders were from Promega. Antibiotics were from Sigma-Aldrich. Streptavidin magnetic beads were obtained from Promega. Other common chemicals used in the experiments were bought in analytical grade from Sigma-Aldrich and Fluka unless indicated. Water used in all experiments was purified by ion-exchange and carbon filtration and sterilised by filtration through a 0.2 µm cellulose membrane.

2.1.1.1. Oligonucleotides

Oligonucleotides as DNA templates and primers for PCR and DNA sequencing were custom synthesized by MWG-biotech. Oligonucleotides were dissolved in the water at desired concentrations upon the first use and stored at -20°C.

2.1.1.2. Peptides and proteins

The peptides (Biotin-Gly-Gly-Gly-Gly-Ser-Phe-Val-(p)Tyr-Ala-Lys-Leu) were ordered from Jerini Peptide Technologies, streptavidin (SA) was purchased from Invitrogen and bovine serum albumin (BSA) was bought from Sigma.

2.1.1.3. Enzymes

Taq DNA polymerase was purchased from Invitrogen. *Pfu* DNA polymerase was from Stratagene. Reverse transcriptase (SuperScriptTM III) was from Invitrogen. T7 DNA polymerase (AmpiScribeTM T7) was from Epicentre. T4 DNA ligase was from Invitrogen.

2.1.1.4. Bacterial strains

E. coli strain InV α F' chemically competent cells were obtained from Invitrogen.

2.1.1.5. Plasmids

Linearised pCR[@]2.1 was bought from Invitrogen.

2.1.2. Culture media, buffers and stock solutions

2.1.2.1. Culture media

Luria-Bertani (LB): 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl.

Luria-Bertani agar (LBA): LB solidified with 1.5% (w/v) agar.

SOC: 2% (w/v) tryptone, 0.5% (w/v) yeast extract; 10 mM NaCl; 2.5 mM KCl; 10 mM MgCl₂; 10 mM MgSO₄, 20 mM glucose.

(The media were sterilised by autoclaving).

2.1.2.2. Buffers

All buffers were adjusted to the desired pH at room temperature with HCl (or CH₃COOH) and NaOH.

2×transcription stop dye: 95% (v/v) formamide, 20 mM EDTA, 0.05 % (w/v) bromophenol blue.

10×TBE buffer: 0.89 M Tris, 0.89 M boric acid, 0.02 M EDTA, pH 8.0 adjusted by NaOH.

Acetate buffer: 10 mM acetate buffers (pHs 4.0, 4.5 and 5.0) adjusted by CH₃COOH and NaOH.

10×Hepes buffer or binding buffer: 200 mM Hepes, 1.5 M NaCl, 50 mM MgCl₂, pH 7.6 adjusted by HCl and NaOH.

EDTA solution: 5 mM EDTA.

(Buffers were sterilised by filtering through 0.2 µm cellulose membrane using sterilised Nalgene filter bottles after final dilution).

2.1.2.3. Stock solutions

Ampicillin (Amp) stock solution: 100 mg Amp per 1 mL water and sterilised by filtration through a 0.2 µm membrane. Stored at -20°C.

D-biotin stock solution: 2 mM biotin in 0.1 M phosphate buffer (pH 7.0). Stored at -20°C.

HABA (2-(4'-hydroxyazobenzene) benzoic acid) solution: 0.02 M HABA in 0.02 M NaOH. Stored at 4°C.

Streptavidin (SA) stock solution: 100 μ M in water. Stored at -20°C.

Bovine serum albumin (BSA) stock solution: 100 μ M in water. Stored at -20°C.

2.2. General molecular biology methods

2.2.1. DNA separation

DNA separation was carried out by agarose gel electrophoresis using a Pharmacia GNA-100 unit. In our experiments, as the desired DNA fragments are around 100 base pairs (bp), 3% agarose gels (Bio-rad Mini Ready Agarose Gel, TBE, 3.0% plus ethidium bromide, 12-well), were employed. The 25 bp DNA step ladder (Promega) was used as the marker. In each run, a gel was placed in the gel tank and submerged in 1×TBE buffer. The 5 μ L marker solution (made from 2 μ L of the DNA ladder solution from Promega and 3 μ L 1×TBE buffer) and the 5 μ L of the DNA amplification reaction mixtures were each added with 1 μ L of 6×loading buffer from Promega (containing 0.4% (w/v) orange G, 0.03% bromophenol blue (w/v), 0.03% xylene cyanol (w/v), 15% Ficoll 400 (w/v), 10mM Tris-HCl (pH 7.5) and 50mM EDTA (pH 8.0)). Upon mixing, 5 μ L sample volumes were loaded into wells of the gel. Gels were run for 45 min with a constant voltage of 200 V and a starting current of 400 mA.

2.2.2. DNA/RNA purification

2.2.2.1. Ethanol precipitation

An aliquot of each DNA/RNA sample had 2.5 volumes of absolute ethanol and 0.1 volumes of 3 M sodium acetate (pH 5.5) added, and was kept at -80°C for at least 40 min to facilitate precipitation of DNA/RNA. Precipitated DNA or RNA was obtained by centrifugation at 4°C with a centrifuging force of 15,500 ×g for 40 min. The obtained pellet was then washed with cold 90% ethanol and dried using a SpeedVac (Thermo Scientific).

2.2.2.2. Gel electrophoresis purification

RNA purification was carried out using an Invitrogen XCell SureLock™ Mini-Cell system. To purify RNA fragments with length of around 80 nucleotides, denaturing 10% polyacrylamide gels from Invitrogen (10% TBE-Urea Gels 1.0 mm, 10 well) were used with 1×TBE buffer. The RNA obtained from *in vitro* transcription reaction was mixed with an equal volume of the 2×transcription stop dye (2.1.2.2). A volume of 25 µL was loaded in each well of the gel. The gel was then run at a constant voltage of 200 V and a starting current of 35 mA for 40 min.

Separated RNA bands were then visualised by UV shadowing on a fluorescent TLC plate (VWR) covered with plastic wrap. A disposable scalpel blade was used to cut out the shadowed regions that contain the bulk of the RNA. The shadowed gel pieces were then divided into smaller ones and transferred into DNA-LoBind Eppendorf tube(s). A pipette tip was used to crush the gel into fine pieces and 5 mM EDTA solution was used to eluate RNA from the gel. Roughly, a volume of 500 µL

of the EDTA solution was applied for 1 cm² of the gel. The elution was carried out at 37°C overnight with mixing by a platform rocker (Stuart-Barloworld Scientific). RNA was then recovered by ethanol precipitation as described in Section 2.2.2.1.

2.2.3. DNA/RNA quantitation

Purified DNA/RNA was quantitated by measuring the absorbance at 260 nm. The extinction coefficient used for RNA was 0.025 mL cm⁻¹ µg⁻¹ (or 40 ng cm µL⁻¹), for ssDNA was 0.027 mL cm⁻¹ µg⁻¹ (or 37 ng cm µL⁻¹), and for dsDNA was 0.02 mL cm⁻¹ µg⁻¹ (or 50 ng cm µL⁻¹).

Absorbance measurements were performed with a UV-Vis spectrophotometer ND-1000 (NanoDrop) using 1 µL sample for each measurement.

2.2.3. Polymerase chain reaction

2.2.3.1. Reverse transcription

Conversion of the selected RNA into cDNA was carried out using SuperScriptTMIII Reverse Transcriptase (Invitrogen). A total of 40 µL of the reverse transcription reaction was run for each round of the selection as follows. Ethanol precipitation of the selected RNA was suspended in 24 µL water and 1 µL of 10 mM dNTP mix and 1 µL of 400 µM reverse primer, Arev, were added and incubated at 65°C for 5 min to facilitate denaturation of secondary structures present in the selected RNA that could inhibit reverse transcription. The mixture was briefly chilled on ice before adding 8 µL of the supplied 5×RT-PCR buffer (250 mM Tris-HCl (pH 8.3 at room

temperature), 375 mM KCl, 15 mM MgCl₂) and 2 µL of DTT (100 mM). This mixture was then preheated to 50°C and 4 µL of reverse transcriptase (200 U/µL) was added and the reaction was run for 1 hour. Finally, the temperature was lifted up and maintained at 70°C for 15 min to deactivate the enzyme and stop the reverse transcription reaction. The mixture was then used for DNA amplification.

2.2.3.2. DNA amplification

cDNA was then amplified using *Taq* DNA polymerase (Invitrogen). For each round, a total volume of 2 mL of the PCR reaction mix was employed as follows. The 40 µL of cDNA from the reverse transcription reaction (2.2.3.1) was transferred to a 2 mL DNA-LoBind Eppendorf tube containing 1670 µL of water and incubated on ice. It was then followed by addition of 200 µL of 10×PCR reaction buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl); 60µL of 50 mM MgCl₂; 10 µL of 25 mM dNTP mix; 1 µL of forward primer, Afor, and 1 µL of reverse primer, Arev, both at the same concentration of 400 µM and mixed well.

In order to obtain a desired yield as well as to avoid over-amplification, small-scale (100 µL) reaction was carried out prior to a large-scale amplification. 1 µL recombinant *Taq* DNA polymerase (5 U/µL) (Invitrogen) was added to 100 µL aliquot of the PCR mix. The amplification was then run with the following parameters: denaturing at 94°C for 30 sec; annealing at 45°C for 30 sec; and extension 72°C for 1 min. An extra 3 min of denaturation at the beginning and another 5 min of extension at the end of the amplification were applied. During the amplification process, 6 µL aliquots of the PCR mixture were taken out every two

cycles. The samples were analysed by agarose gel electrophoresis as described in Section 2.2.1 to determine if DNA amplification has occurred. This precaution guarded against under- or over-amplification of DNA and the subsequent selection of cantenated or deleted PCR products.

After the optimum cycles for the amplification had been found, 19 μL of the recombinant *Taq* DNA polymerase was added to the remaining 1.9 mL and mixed well. This PCR mix was then split into 100 μL volumes in 96-well plate format and the amplification was then performed at the previously optimised conditions.

DNA obtained from the amplification was ethanol precipitated as described in Section 2.2.2.1. The precipitated DNA was then suspended in 50 μL of the water.

2.2.3.3. Transcription

Two fifths of the obtained DNA (from Section 2.2.3.2) was transcribed into RNA and a total of 80 μL of the transcription reaction mix was employed as follows. 20 μL of the suspended DNA was taken into a 0.5 mL DNA-LoBind Eppendorf tube, where 8 μL of 10 $\times$ transcription buffer was added, followed by 6 μL of each ATP, UTP, CTP, and GTP (all the NTPs were at 100 mM) and mixed well. 8 μL of 100 mM DTT and 12 μL of the water were also added to make up 72 μL volume. Transcription was initiated by adding 8 μL of T7 RNA polymerase (AmpliScribeTM T7-FlashTM from Epicentre). The reaction was allowed to occur at 42°C for 2 hours. After transcription time, 5 μL of DNase (Epicentre) was added to the reaction mix and incubated at 37°C for 30 min to degrade the DNA template molecules.

The obtained RNA was then purified using polyacrylamide gel electrophoresis, as described in Section 2.2.2.2.

2.2.4. Cloning DNA

Taq DNA polymerase has a nontemplate-dependent activity that adds a single deoxyadenosine (dA) to the 3' ends of PCR products. The linearised vector pCR@2.1 has single deoxythymidine (dT) residues. This allows the PCR inserts to ligate efficiently with the vector. Figure 2-1 shows the concept behind the cloning method.

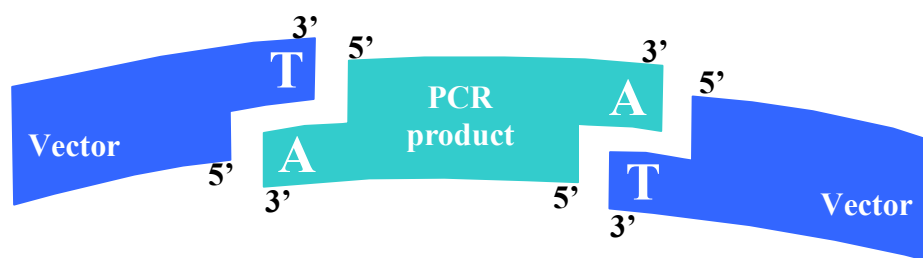


Figure 2-1: Cloning of PCR a product into the linearised pCR@2.1 plasmid. *Taq* DNA polymerase has a nontemplate-dependent activity that adds a single deoxyadenosine (A) to the 3' ends of PCR products. The linearized vector supplied in this kit has single 3' deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the vector.

The ligation reactions were carried out with 10 μ L of reaction volumes as follows. 1 μ L of the amplified DNA mix (from Section 2.2.3.2), 5 μ L sterilised

water, 1 μ L 10 $\times$ ligation buffer (100 mM Tris-HCl (pH 8.3), 500 mM KCl, 25 mM $MgCl_2$, and 0.01% gelatin), 2 μ L linearised pCR@2.1 vector solution (25 ng/ μ L) were added into a 0.2 mL Eppendorf tube and mixed well. The tube was incubated at 16°C for a 2 min and the ligation reaction was initiated by adding 1 μ L of T4 DNA ligase (4 U/ μ L) (Invitrogen). The reaction mix was incubated at 16°C for 9 hours and then kept at 4°C (within hours) for transformation.

2.2.5. Transformation of the plasmid DNA into competent cells

All transformation experiments were performed within hours after the ligation reactions. Transformation of the newly ligated pCR@2.1 plasmids was carried out as follows. Frozen competent cells (InVF' α) were thawed on ice and 50 μ L volumes of the aliquot cells was transferred to a 1.5 mL pre-chilled Eppendorf tube that sat on ice. 1 μ L of plasmid DNA sample from the previous ligation reaction was added and the tube was kept on ice for 30 min. The cells were then heat-shocked by placing the tube in a water bath at 42°C for 30 sec. Following the heat-shock, the tube was placed on ice for 2 min before being added with 500 μ L of SOC medium. The cell culture was then incubated at 37°C with shaking at 200 rpm for 40 minutes. The culture was finally spread on the LB-agar plate(s) (containing 100 μ g/mL ampicillin) using sterilised spreaders. The plate(s) were left in a 37°C incubator overnight.

2.2.6. Preparation of the plasmid DNA

A single colony was picked and inoculated in 10 mL of LB medium containing ampicillin (100 μ g/mL). The cell culture was grown overnight at 37°C with shaking

at 200 rpm. The culture was then pelleted and the plasmid DNA was prepared using Qiagen QIAprep[®] plasmid purification kit.

Purification of plasmid DNA using the kit was performed with an Eppendorf micro-centrifuge. The micro-centrifuge was set to run at maximum force (16,000×g) unless indicated. The overnight culture was pelleted by centrifuging for 1 min in 1.5 mL Eppendorf tubes. The cell pellet collected from 5 mL overnight culture was first suspended in 250 µL suspending buffer. Another 250 µL of alkaline buffer (containing NaOH and SDS for cell lysis) was added and the tube was gently inverted 4-6 times. It was followed by 300 µL neutralised buffer (containing acetic acid) and this was mixed gently before being centrifuged for 15 min. The supernatant was then applied to a QIAprep spin column and centrifuged for 1 min. The column was then washed with ethanol buffer (containing 80% (v/v) ethanol in water). The obtained plasmid DNA was eluted from the column by 50 µL sterilised water.

2.2.7. DNA sequencing

PCR products were cloned using the TOPO TA cloning kit (Invitrogen) as described in Section 2.2.4. Sequencing of the constructed plasmid DNA was carried out by the Dundee University sequencing services with primer set M13-forward and M13-reverse. The plasmid DNA samples were supplied with 50 µl (concentrations of around 5 ng/µl) and the sequencing primers were supplied by the Dundee sequencing service. DNA sequencing data were analysed using the Vector NTI Advance 10 program (Invitrogen).

2.3. Generation of RNA aptamers against the peptide target

2.3.1. The synthesised DNA pool

The synthesized N30 pool was designed and synthesized in the Ellington lab according to previous reported methods (Pollard *et al.* 2000) on an ABI 394 DNA synthesizer using standard phosphoramidite chemistry. The pool contained a core region of 30 random nucleotides flanked on both sides by constant regions (5'-GGGAATGGATCCACATCTACGAATTC-N30-TTCACTGCAGACTTGACGAAGCTT-3'). The phosphoramidite mixture contained a 3:3:2:2 molar ratio of dA:dC:dG:dT. The synthesized pool used in our selection was HPLC purified and contained around 7×10^{14} molecules.

The primers used for amplification were “Afor”, GAT AAT ACG ACT CAC TAT AGG GAA TGG ATC CAC ATC TAC GA (T7 RNA polymerase promoter underlined was included in this primer for subsequent transcription), and “Arev”, AAG CTT CGT CAA GTC TGC AGT GAA. The synthesized pool was amplified using polymerase chain reaction (PCR). 36 μ L of PCR reaction (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 125 μ M each of the dNTPs, 2 μ M of each of the primers, 0.5 U/ μ L of *Taq* DNA polymerase) was carried out. This reaction mix was divided into 100 μ L portions in 96-well plates (VWR). The plates were covered with a 96-well plate PCR sealing mat (BioRad). A 100 μ L sample was carried out prior to the amplification of the DNA pool to find the optimum conditions and the temperature regime for amplification was described in Section 2.1.1.1. The resulting double-stranded DNA pool was ethanol precipitated and then suspended in sterilised water. The pool was stored at -80°C.

2.3.2. RNA pool

The RNA pool was made by transcribing from the double-stranded DNA pool using T7 RNA polymerase from Epicentre. One third of the obtained doubled-stranded DNA pool was transcribed into RNA in a total volume of 2 mL. The transcription reaction contained 200 μ L double-stranded DNA pool; 200 μ L of 10 $\times$ buffer (provided along with the enzyme by Epicentre); 150 μ L each of the NTPs at 100 mM; 600 μ L water; 200 μ L DTT at 100 mM; and 200 μ L T7 RNA polymerase. The 2 mL mix was split into 100 μ L portions and transcription reaction was run as described in Section 2.3.3.3.

RNA obtained from the reaction was purified using denaturing polyacrylamide gels as described in Section 2.2.2.2. The RNA pool was suspended in sterilised water and quantitated using the NanoDrop-1000. The pool was then divided into 50 μ L portions and stored at -80°C.

2.3.3. *In vitro* selection

In the selection experiments, magnetic beads were used to capture the biotinylated complexes to separate target-bound RNA sequences from the unbound ones. The selection process is shown in Figure 2-2.

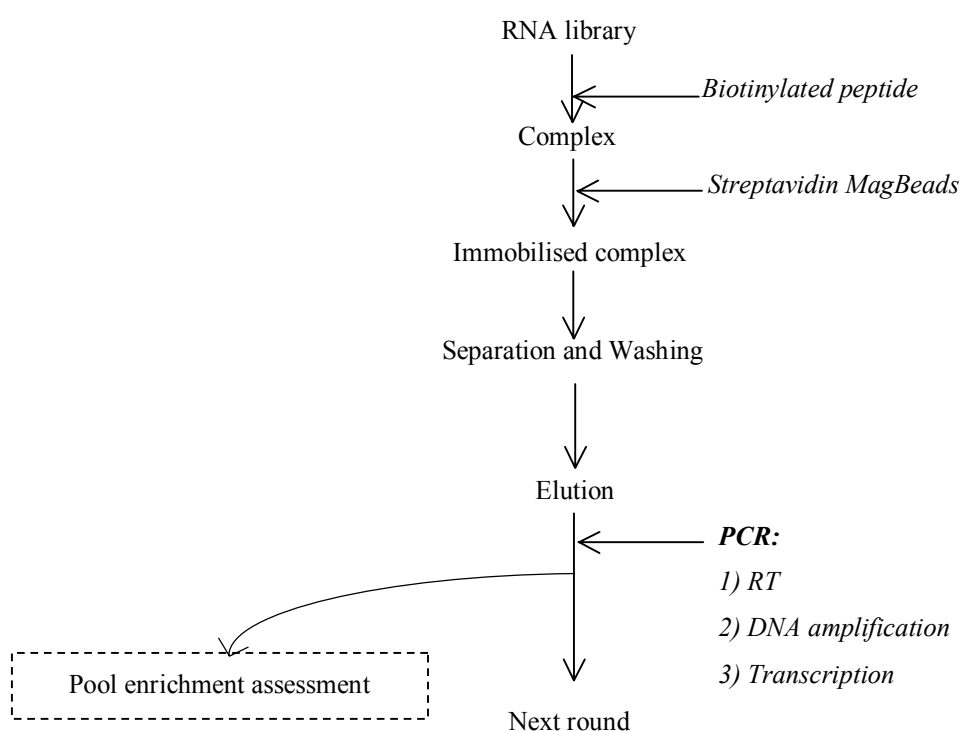


Figure 2-2: A round of selection of RNA aptamers against the pY peptide.

The RNA library is incubated with the pY peptide, which has a biotin tag at the N-terminus. The biotinylated peptide-RNA complex is then immobilised on streptavidin magnetic beads to allow unbound sequences to be washed away. The bound RNA is eluted and amplified for the next round of selection.

In the first round of the selection, 18 μL of the RNA pool (containing 38.5 μg or 1470 pmol RNA pool) was added to a 0.2 mL LoBind Eppendorf tube, which was preloaded with 50 μL of sterilised water and 10 μL of the 10 $\times$ binding buffer. The mix was incubated at 70°C for 3 min and was allowed to cool down to room temperature for 20 min. 22 μL of the phosphorylated peptide (containing 2.2 μg of the peptide) was added (making the binding mix of a total volume of 100 μL). The binding mix was then pipetted several times and incubated at 37°C with gentle shakes for 40 min. The binding mix was then added to a 0.5 mL LoBind Eppendorf tube containing 1.8 mg Streptavidin Mag Beads from Promega (the beads were pre-washed 6 times with 1 $\times$ binding buffer) and incubated at room temperature for 15 min with 3 periods of pipette mixing. The beads were then manipulated using a magnet and the supernatant was removed. 100 μL of the 1 $\times$ binding-buffer was added to the beads, mixed thoroughly and the beads were again captured and the supernatant removed. The washing process was repeated another 2 times. To elute bound RNA molecules from the beads, 100 μL of 5 mM EDTA solution was added and incubated at 95°C for 5 min. The supernatant was collected and this process was repeated with two further 150 μL aliquots of the EDTA solution. A total volume of 400 μL supernatant was collected and ethanol precipitated as described in Section 2.2.2.1. The RNA obtained was then suspended in 26 μL of sterilised water and reverse transcribed to cDNA with a total reaction volume of 40 μL as described in Section 2.2.3.1. The cDNA was then amplified in 2 mL reaction volume as described in Section 2.2.3.2. Two fifths of the DNA obtained from amplification was then used for transcription into an RNA pool for the next round. For the following rounds of selection, 500 pmol (13.1 μg) of the RNA pool was used, with an equal molar ratio of the target peptide for the selection. The selection strategy is described Figure 2-2.

2.3.4. Pool enrichment assessment

2.3.4.1. Sequence analysis

Following DNA amplification from Rounds 10, 21, 32, the products were cloned into pCR@2.1 vector as described in Section 2.2.4 and transformed into *E. coli* InV α F' cells as described in Section 2.2.5. DNA plasmids were isolated using the Plasmid Mini Kit (Qiagen) as described in Section 2.2.6. The sequencing process was described in Section 2.2.7, with 50 clones randomly picked and sequenced. The products were then analysed for the frequency of individual sequences in the population and the presence of consensus sequences using the Vector NTI Advance 10 program.

2.3.4.2. Affinity evaluation

Pool enrichment can be assessed through affinities of the pools towards the target. Binding analysis of the pools with the target was measured using SPR (surface plasmon resonance).

2.4. Aptamer-target binding assays

2.4.1. Surface plasmon resonance measurements

2.4.1.1. Introduction of surface plasmon resonance measurement

Surface plasmon resonance (SPR) is a phenomenon that occurs at thin metal films (typically gold) at an interface between media with different refraction indexes (such as water and glass) when an incident light beam strikes the film at a particular angle (SPR angle).

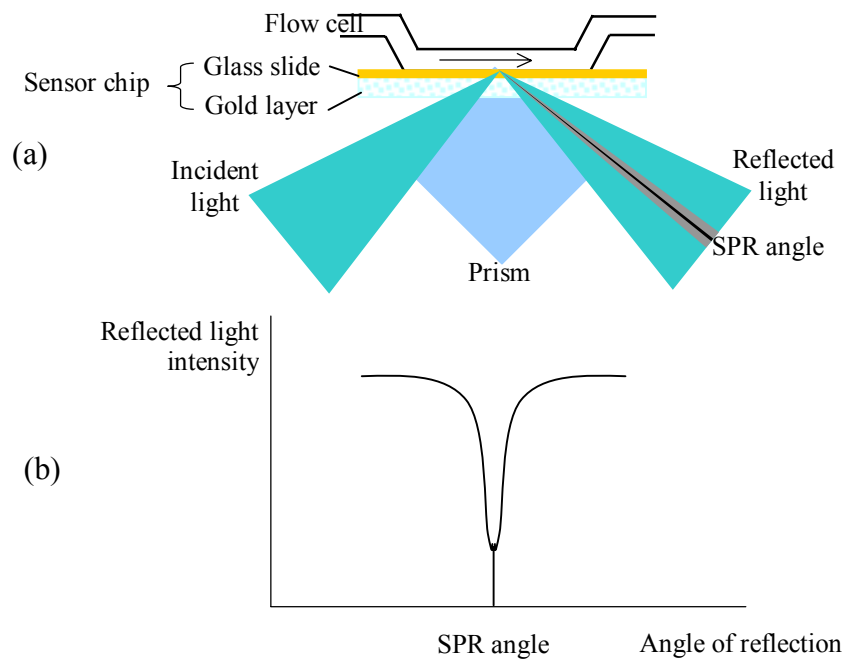


Figure 2-3: Principle of surface plasmon resonance measurement. At a certain combination of angle of incidence and energy (wavelength), the incident light excites plasmons (electron charged density waves) in the metal film (a). As a result, a characteristic absorption of energy *via* the evanescent wave field occurs and SPR is seen as drop in the intensity of the reflected light (b).

Changes of the environment on the metal film cause changes in the reflective index, which can be measured as changes in SPR angle. If binding occurs to the target immobilised at the metal film, the refractive index changes, leading to a change in SPR angle, which can be monitored in real-time by detecting changes in the intensity of the reflected light, producing a sensorgram. The rates of change of the SPR signal can be analysed to yield rate constants for the association and dissociation phases of the reaction.

All of the surface plasmon resonance (SPR) measurements were carried out on a BIAcore3000 (BIAcore) at 25°C on CM5 or gold chips.

2.4.1.2. Immobilisation on CM5 chips

Streptavidin (SA) and bovine serum albumin (BSA) (2 μ M solutions) were amine-coupled on carboxyl dextran chips (CM5 chips) in 10 mM acetate buffers at an optimal pH. The immobilisation was run at a flow rate of 5 μ L/min for all the steps as follows. A mixture of EDC/NHS (EDC 0.4 M and NHS 0.1 M in water) was injected onto the CM5 chip over a course of 7 min to activate of the carboxyl groups. A 2 μ M solution of the desired protein (SA or BSA) was then injected over a course of 30 min for coupling of the protein's amine groups with the activated carboxyl groups of the surface. Finally, an ethanolamine solution (1 M in water, pH 8.5) was applied over a course of 7 min to block any vacant activated carboxyl groups. A series of immobilisation experiments at different pHs ranging from pH 4.0 to pH 5.0 was carried out to determine optimal pH for immobilisation of the protein.

2.4.1.3. Attachment of TaL1 linker on gold chips

A thiol polynucleotide TaL1 acting as a linker for the aptamer immobilisation was covalently attached on a gold chip by thiolation. For each experiment, 5 consecutive injections were performed. With each injection, a 5 μM solution of TaL1 in water was applied on the gold chip at a flow rate of 2 $\mu\text{L}/\text{min}$ and over a course of 100 min.

2.4.1.4. Target binding

Aptamer stock solutions were heated at 70°C for 4 min and then cooled at room temperature for 20 min before being diluted to make up solutions of desired concentrations for the binding assays.

a) Streptavidin-binding aptamers

A series of Tai ($i = 15$ or 23) samples with concentrations ranging from 10 nM to 2 μM in binding buffer and a blank sample (of the binding buffer) were prepared for an experimental set. Each sample was run on a BIAcore3000 as follows. For association phase, a Tai sample of a desired concentration was injected onto the sensor chip surface with a flow rate of 2 $\mu\text{L}/\text{min}$ and over a course of 3000 s. For dissociation phase, initiated when the injection of the sample for association phase ended, a flow of binding buffer was applied to the sensor chip surface with the same flow rate of the association phase and over a course of up to 3000 s. Finally, for regeneration of the surface, a solution of 10 nM glycine, pH 2.5 was injected onto the surface with a flow rate of 30 $\mu\text{L}/\text{min}$ and over a course of 30 s. The process was repeated for other samples in the series.

b) pY peptide-binding aptamers

With streptavidin-coated chips (immobilised peptide)

A series of Tap_j (j = 1 or 2) samples with concentrations ranging from 20 nM to 2 µM in binding buffer and a blank sample (of the binding buffer) were prepared for an experimental set. Each sample was run on a BIAcore3000 as follows. For immobilisation of the pY peptide on the streptavidin-coated chip, a 2 µM solution of the biotinylated pY peptide was injected onto the chip with a flow rate of 10 µL/min and over a course of 300 s and followed by a buffer flushing at the same flow rate of the immobilisation over a course of 600 s. For an association phase, a Tap_j sample of a desired concentration was injected onto the sensor chip surface, in which the pY peptide was previously immobilised, at a flow rate of 2 µL/min and over a course of 3000 s. For a dissociation phase, initiated when the injection of the sample for the association phase ended, a flow of binding buffer was applied to the sensor chip surface with the same flow rate of the association phase and over a course of up to 3000 s. Finally, for regeneration of the surface, solutions of 10 nM glycine, pH 2.5 and 40 mM NaOH were injected one after the other onto the surface with a flow rate of 30 µL/min and over a course of 30 s. The process was repeated for other samples in the series.

With gold chips (immobilised aptamer)

A series of the pY peptide samples with concentrations ranging from 50 nM to 10 µM in binding buffer and a blank sample (of the binding buffer) were prepared for an experimental set. Each sample was run on a BIAcore3000 as follows. For

immobilisation of the aptamer Tap_j ($j = 1$ or 2) on the surface, in which the TaL1 linker was previously attached on by thiolation, a 5 μM solution of the Tap_j was injected onto the chip with a flow rate of 2 $\mu\text{L}/\text{min}$ and over a course of 1800 s and followed by a buffer flushing at the same flow rate of immobilisation over a course of 600 s. For an association phase, a sample of the pY peptide at a desired concentration was injected onto the sensor chip surface with a flow rate of 10 $\mu\text{L}/\text{min}$ and over a course of 900 s. For a dissociation phase, initiated when the injection of the sample for association phase ended, a flow of binding buffer was applied to the sensor chip surface with the same flow rate of the association phase and over a course of up to 900 s. Finally, for regeneration of the surface, solutions of 40 mM NaOH and 1 mM HCl were injected one after the other onto the surface with a flow rate of 30 $\mu\text{L}/\text{min}$ and over a course of 30 s. The process was repeated for other samples in the series.

2.4.1.5. Analysis of SPR data

A curve (total curve) shown in a BIAcore sensorgram consists of 2 components: binding of the analyte to the surface and a bulk reflective index effect. A bulk reflective index effect is excluded from the total curve when the data are analysed. Figure 2-4 shows the contribution of a bulk reflective index effect to the total curve. Therefore, to start analysing the data, the bulk reflective index has to be subtracted from the total curve to obtain the binding curve. Binding curves can be analysed to gain affinity constants.

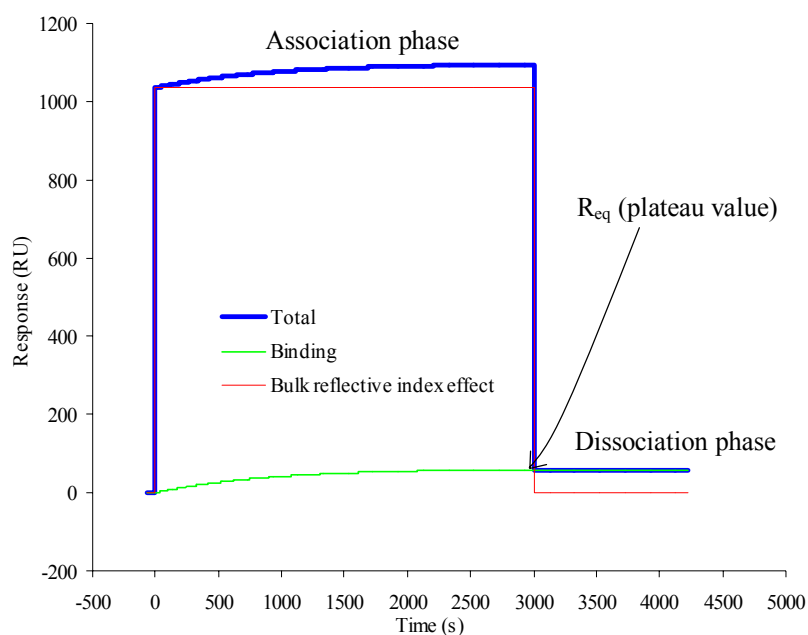


Figure 2-4: A total curve consists of a binding component and a bulk reflective index effect. A total curve is shown in blue. A binding curve (light green) is obtained from subtracting the total curve to bulk reflective index effect (red).

In order to obtain affinity constants, a data set that consists a number of binding curves corresponding to different concentrations, was fitted into binding models. In these binding assays, it was assumed that binding of the aptamers to their targets followed 1:1 stoichiometry and therefore the 1:1 Langmuir isotherm was chosen as the binding model. Two types of fitting regimes were used to analyse the SPR data: a global fit and a plateau value (steady state binding level) fit. The global fit used a huge number of data points (response was recorded in an interval of a second) for

each binding curve to fit into the binding model whilst the plateau value used one value at association phase end for each curve to fit into the binding model.

b) Global fitting

Affinity constants of the binding of the aptamers to their targets were determined by a global fit using the kinetic simultaneous k_a/k_d model, assumed 1:1 Langmuir isotherm through a series of the following equations.

$$K_d = \frac{k_d}{k_a}$$

$$R_{eq} = R_{max} \frac{k_a \times Conc}{k_a \times Conc + k_d} = R_{max} \frac{Conc}{Conc + K_d}$$

$$k_{obs} = k_a \times Conc + k_d = \ln \left(\frac{dR}{dt} \right)$$

$$R = R_{eq} \left(1 - e^{-(k_a \times Conc + k_d)t} \right)$$

Where: K_d is equilibrium dissociation constant (M); k_a is associate rate constant ($M^{-1}s^{-1}$); k_d is dissociate rate constant (s^{-1}); R_{eq} is steady state binding level (plateau value) ; R_{max} is maximum analyte binding capacity; Conc is analyte concentration (M); k_{obs} is observed rate constant (s^{-1}); R is response (binding) at time t.

The fitting minimizes sum squares of the differences between the calculated (model) and experimental curves. The fitting provided χ^2 as a criterion for assessing how well the real data fitted into the model.

b) Plateau value fitting

Dissociation constant (K_d) can also be obtained by fitting a set of plateau values corresponding to a series of analyte concentrations using 1:1 Langmuir isotherm as described in the equation presented below.

$$R_{eq} = R_{max} \frac{Conc}{Conc + K_d}$$

Where: R_{eq} is steady state response level (plateau value), R_{max} is maximum response (binding capacity), and Conc is analyte concentration.

2.4.2. Fluorescence measurements

Fluorescence measurements were performed on a Perkin-Elmer LS-50B spectrofluorimeter with quartz cuvettes at room temperature. The excitation and emission monochromator slit widths were both set at 10 nm and wavelengths were 280 nm and 340 nm, respectively. Appropriate aliquots of the aptamer stock solution were added into 300 μ L of sample containing 1 μ M streptavidin in binding buffer (pH 7.4). Fluorescence emission spectra were obtained as an average of three repetitive readings.

2.4.3. Absorbance measurements for effect of aptamers on streptavidin-HABA complex

The absorbance measurements were performed on a NanoDrop spectrophotometer, in which 1 μ L was needed for each measurement. A solution of streptavidin-HABA

complex (called Mo) was prepared by adding 10 μL of the HABA stock solution (0.02 M) and 10 μL of the streptavidin stock solution (100 μM) into 100 μL of the 0.2 M phosphate buffer (pH 7.0). 10 μL of desired solutions for absorbance measurements were prepared by mixing 6 μL of the Mo solution with appropriate aliquots of the aptamer stock solution (500 μM). The absorbance measurements were carried out at 500 nm and each data point was obtained as an average of three readings.

Chapter 3: Selection of RNA aptamers for the pY peptide target

3.1. Introduction

In cells, RNA was at first identified as a mediator between DNA and protein and it was considered only in the context of the flow of genetic information: the DNA sequence encodes genes that are transcribed into RNA sequences, which are subsequently translated into proteins, as illustrated in Figure 3-1.

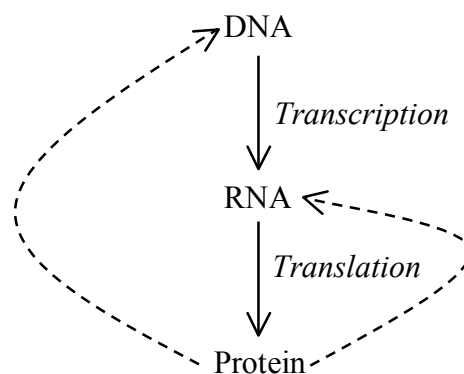


Figure 3-1: “Mediating RNA”. The role of RNA was first described as an intermediate state in the flow of genetic information, a theory which has since been proven to be too limited. (— indicates information flow and - - - shows action flow)

This initial description (regarded as “the central dogma of molecular biology”) has been proven to be too limited. In the early 1980’s, Cech and Altman independently published their work, in which they observed RNA’s role in facilitating the complex biochemical interplay of the cells for the first time (Kruger *et al.* 1982; Guerrier-Takada *et al.* 1983). Altman’s team showed that the RNA-cutting activity of the RNase P enzyme resided in the enzyme’s RNA cofactor; one RNA molecule catalyzed the cleavage of another. On the other hand, Cech and colleagues discovered a self-splicing RNA molecule, in which the RNA molecule was able to cut itself in a process known as splicing. The finding on catalytic properties of RNA earned them the 1989 chemistry Nobel Prize. RNA catalysts were then called ribozymes to distinguish from protein-based enzymes. The discovery led to a hypothesis for structure of primitive biological system. The name “RNA world” was then coined by Gilbert as he tried to describe a period where RNA served both as catalytic and genetic material (Gilbert 1986).

Since then, a number of RNA molecules have been characterised that have catalytic activity (Epstein and Gall 1987; Saville and Collins 1990; Tuschl *et al.* 1995; Boudvillain *et al.* 2000). RNA can catalyse the RNA-splicing reactions (Kruger *et al.* 1982) or be involved in directing assembly of proteins from amino acids (Moore and Steitz 2003). In addition to acting as a catalyst, RNA is also capable of serving as a molecular switch (dubbed “riboswitch”). For example, riboswitches specific for vitamin B12 (Nahvi *et al.* 2002) and for riboflavin (Winkler

et al. 2002) have been reported. These new findings are illustrated in Figure 3-2 for the roles of RNA within cells.

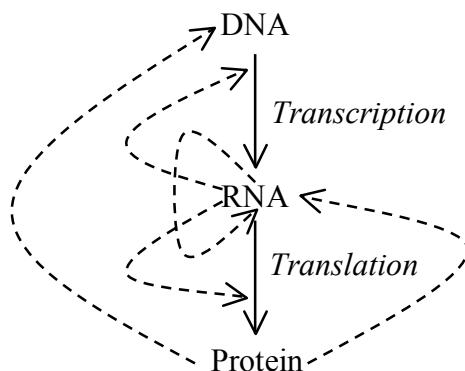


Figure 3-2: The new concept of the roles of RNA in cells. Several additional roles of RNA have been discovered, including catalytic RNA, genetic regulation coordinator (riboswitch) and ribosomal RNA used in directing protein synthesis. (— indicates information flow and - - - shows action flow)

Whilst a few naturally occurring catalytic RNA molecules have been discovered, it is the development of *in vitro* selection techniques (described in Chapter 1) that has convincingly demonstrated that RNA is not only capable of catalysis, but also a variety of other reactions (Wilson and Szostak 1999; Hermann and Patel 2000b).

Since then, by using *in vitro* selection, RNA molecules (so-called RNA aptamers) that can catalyse a variety of reactions have been isolated such as carbon-

carbon formation (Tarasow *et al.* 1997), amide bond formation (Wiegand *et al.* 1997; Sun *et al.* 2002; Nieuwlandt *et al.* 2003), alkylation (Wilson and Szostak 1995; Wecker *et al.* 1996), Michael addition (Sengle *et al.* 2001), Diels-Alder reaction (Seelig and Jaschke 1999; Amontov and Jaschke 2006), nucleotide synthesis (Unrau and Bartel 1998). RNA molecules are also capable of acting as ligases (Bartel and Szostak 1993; Hager and Szostak 1997; Robertson and Ellington 1999) and kinases (Lorsch and Szostak 1994). Furthermore, it has also been reported that *in vitro* selection led to successful isolation of RNA acting as molecular switches (Tang and Breaker 1997; Soukup and Breaker 1999; Robertson and Ellington 2001).

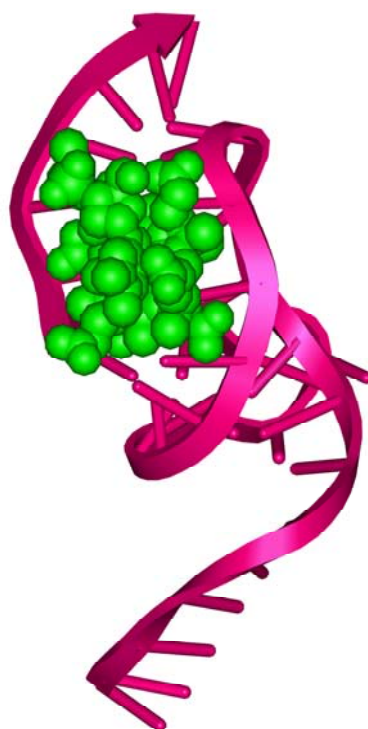


Figure 3-3: Molecular recognition in an RNA aptamer-target complex. The structure shows RNA aptamer (pink) in complex with the vitamin B12 (green). The image created from pdb file 1ET4 using WebLab ViewerLite (Sussman *et al.* 2000; Sussman and Wilson 2000).

Structure of the aptamer bound to vitamin B12 (an aptamer that has the highest affinity yet reported for a small molecular ligand) has been intensively studied (Sussman *et al.* 2000; Sussman and Wilson 2000). Figure 3-3 shows recognition of the target by the aptamer from studying the complex using X-ray crystallography.

While the examples listed above demonstrated that *in vitro* selection has been used to create RNA molecules that have enzyme-like activity or molecular-switch behaviour, the selection process has also been successfully used to isolate RNA sequences that are capable of binding to specific targets that do not naturally have an RNA binding partner. *In vitro* selection has also been used for targets that do have natural RNA binding partners, where the selection has been designed to alter affinity and/or specificity. For example proteins such as MS2 bacteriophage coat protein (Stockley *et al.* 1995) or TCF-1 protein (Lee *et al.* 2005b), human immunodeficiency virus type 1 Rev peptide (Symensma *et al.* 1996) or Alzheimer's disease amyloid peptide (Ylera *et al.* 2002), and also small molecules such as arginine (Geiger *et al.* 1996) or tetracycline (Berens *et al.* 2001).

Whilst the discovery of catalytic RNA was seen as the foundation for the emergence of aptamers and the first aptamers were RNA (Ellington and Szostak 1990; Tuerk and Gold 1990), the development of *in vitro* selection techniques is not limited to RNA. However, it is worth noting that the 2'-hydroxyl in RNA may make it more capable of forming complex tertiary structures (Cate *et al.* 1996), which expands its chemical and structural repertoire compared to DNA and therefore

provides it with additional binding functionalities and more possibilities to bind targets. Nevertheless, DNA aptamers have been isolated that also proved to be capable of catalysis (Bock *et al.* 1992; Emilsson and Breaker 2002; Levy and Ellington 2003). The very “high profile” aptamer for thrombin, which was mentioned in Chapter 1, is a DNA aptamer.

Since their discovery, researchers have isolated numerous aptamers against proteins that typically bind their targets with high affinity (K_d values in pM- μ M range), demonstrating that aptamers can have recognition properties equivalent to those of antibodies.

3.2. *In vitro* selection of RNA aptamers against a pY peptide target

As introduced in Chapter 1, selection of aptamers goes through a cyclic process involving two main steps: (1) separation of the bound sequences from unbound ones (partitioning); and (2) amplification of the partitioned sequences to produce the pool for the next round of selection. Selection of RNA aptamers requires extra steps in amplification of the pool compared to selection of DNA aptamers as RNA molecules are needed to be reverse-transcribed into DNA molecules for PCR amplification as shown in Figure 3-4.

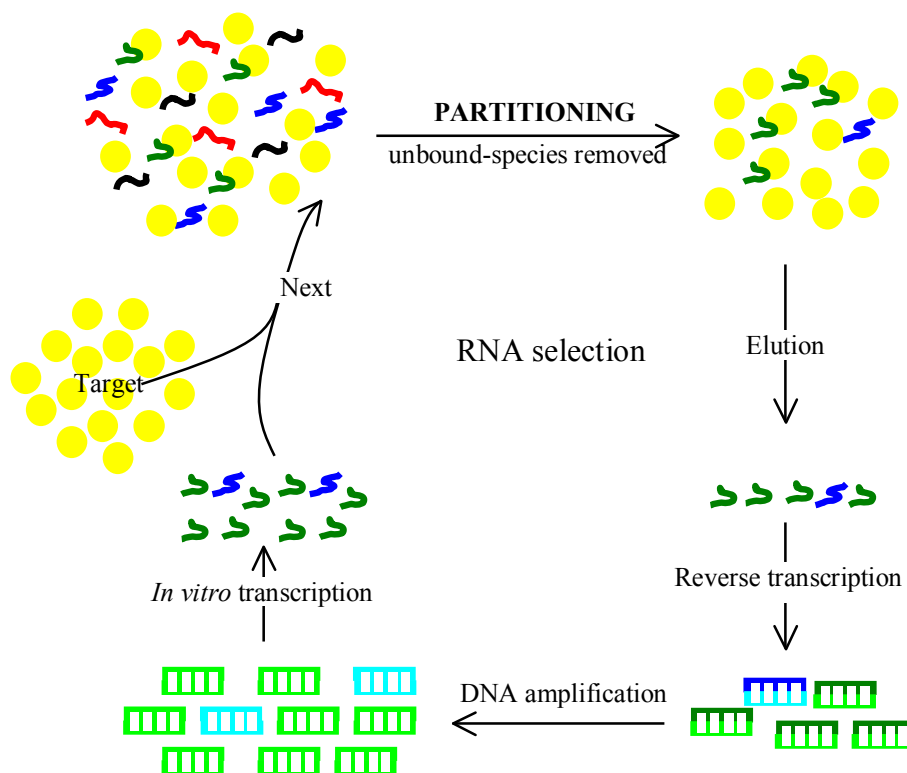


Figure 3-4: A general scheme for selection of RNA aptamers. A random RNA pool is incubated with a target (top left). The low-affinity nucleic acid species were removed from the RNA-target complexes. The bound sequences were then eluted from the target and reverse transcribed to cDNA, then amplified and transcribed to an RNA pool for the next round of selection.

For a selection scheme, the actual partitioning of the non-binding sequences of the random pool from those species that specifically bind the target of interest is the most important step in *in vitro* selection. There have been several methods published describing the physical partitioning (Conrad *et al.* 1996; Xu and Ellington 1996;

Jhaveri and Ellington 2000). Often, a protein target is non-specifically absorbed onto a nitrocellulose membrane. Sequences complexed to the protein are retained on the membrane whilst uncomplexed species are washed away. Complexed sequences are then eluted under denaturing conditions, for example by boiling (Jhaveri and Ellington 2000). An alternative partitioning method that has been used in selection against small targets that are not suitable for nitrocellulose membrane filtration involves immobilising the target of interest onto a solid support, and uncomplexed sequences are removed by centrifugation or washing through a column. Complexed sequences are then eluted by changing wash buffer to high salt (Xu and Ellington 1996).

The research presented here employed *in vitro* selection to isolate RNA aptamers for a peptide target from a library that contained 30 nucleotides in the random region. The target was a pY peptide of 11 amino acids in length. As it is a short peptide sequence, the method of partitioning was immobilisation of the target on solid support. Instead of using a resin with the target covalently attached, we used streptavidin-coated magnetic beads to capture the target, which has a biotin tag at its N-terminus. The manipulation of magnetic beads could be facilitated by using a magnet.

3.3. Results and discussion

The RNA pool used for our *in vitro* selection was produced from a synthetic DNA pool (N30 pool) estimated as having 7×10^{14} molecules, and was described in Section 2.3.2. The N30 pool consisted of 30 nucleotides, in the random region, flanked by

constant regions required for PCR amplification. The 3'-constant region also allowed cDNA to be synthesised from the selected RNA. The 5'-constant region also contained a T7-promoter sequence that allowed RNA molecules to be generated from the double-stranded DNA templates by *in vitro* transcription. The library structure is shown in Figure 3-5.

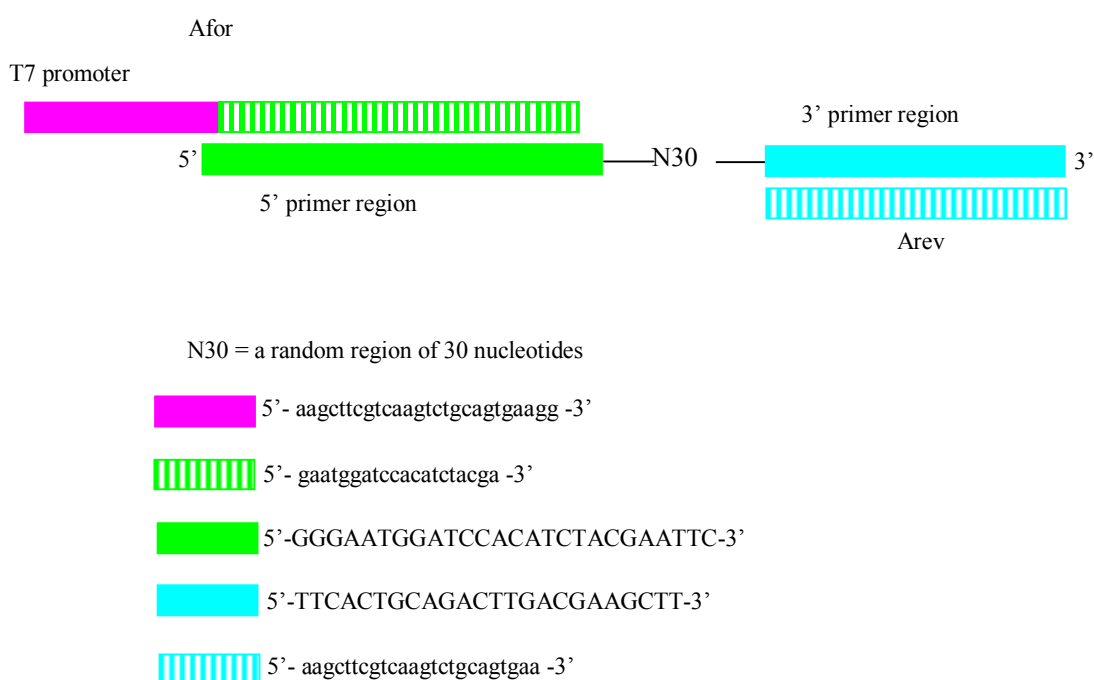


Figure 3-5: The N30 pool. N30 indicated that the random region consisted of 30 nucleotides. The capitalised residues represent bases transcribed into RNA pool; lower-case bases represent priming sequences. The Afor sequence incorporates a T7 RNA polymerase promoter sequence shown in pink.

A distinctive property of this pool is that it had been designed with a “weak” (W) 3’ end “... CTT”, contrary to traditional primer design. Usually, references designate the primer ends described in the literature have “strong” (S) bases, such as “...GGC”, to increase the efficiency of priming (Chen *et al.* 2002). This strategy of poorly hybridising 3’ clamps (“SWW” ends) has been previously shown to facilitate ‘infinite amplification’ of PCR products without generation of higher molecular mass amplicons that is usually seen with serially-cycled nucleic acids amplified with “WSS” clamps (Crameri and Stemmer 1993).

In our selection, partitioning was achieved by using streptavidin-coated paramagnetic beads rather than membrane filtration, as the target is a small peptide 11 amino acids in length and therefore unlikely to bind nitrocellulose membranes. There have been several papers published recently describing streptavidin- or Ni-NTA-coated magnetic beads for partitioning during *in vitro* selection (Cox and Ellington 2001; Murphy *et al.* 2003; Stoltenburg *et al.* 2005). In these papers, the targets were pre-immobilised onto magnetic beads prior to adding nucleic acid library.

Using affinity-capture with streptavidin-coated or Ni-NTA-coated magnetic beads to bind biotinylated or His-tagged molecules could have an additional benefit of allowing the target and aptamer to complex in solution followed by capture of the complex onto the solid support (the beads). This is closer to the situation with the membrane partitioning where the complex forms in solution and is then absorbed onto nitrocellulose membrane and can avoid difficulties in accessing the target

molecules as well as folding of the aptamers that may occur if the target is pre-immobilised on a solid support.

The first round of the selection was initiated with 38.5 µg of the pool of approximately 7×10^{14} unique RNA molecules, and with that amount it was expected to have all the possible sequences present in the pool. Approximately 20-40 µg of RNA was typically recovered for each round of selection. Given the initial complexity of the pool, while some high affinity binding species may have been lost following the first round of selection, no high affinity binding sequences should be lost during the subsequent rounds.

Following ten rounds of selection, carried out as described in Section 2.3.3, the double-stranded DNA pool of Round 10 was cloned and sequenced. The sequences from randomly selected clones were subjected to a global sequence analysis using Vector NTI Advance 10.0 (Invitrogen) to search common sequences for the whole population. As shown in Table 3-1, no such common sequences were found in a population of 31 clones.

Table 3-1: Global sequence analysis of the randomly-picked clones from Round 10. The blue highlights show an occasional common nucleotide occurs at particular positions but there are no regions of sequence of more than 2 nucleotides in length.

	(1)	1	10	20	39
_clipseq	(1)	--ACTGTCCACTCATCGC--	--AAGCCTTGGCAGGC----		
_clipseq#31	(1)	--GCTTTAGCATCGTTGCGGG	AAGGCCGGGCA-----		
_clipseq#27	(1)	-----CAAATTATGTCATGCCGATA	TGCAGCCGC--		
_clipseq#5	(1)	---CATTAAAA	TCTCTCGT--ACGTTCTAACGGT-----		
_clipseq#10	(1)	--GCCTTAAGTAGAATCTTTA	AAGCCATAGC-----		
_clipseq#26	(1)	--GCCCTCTGCTGGAAGAATGTGGC	TATGCT-----		
_clipseq#12	(1)	--AATTAGTAATAAGCAGGTC	AGCCGATAGTT-----		
_clipseq#6	(1)	----AGTGCGTAGAGCAGGTGAAGCTGT	CCTGGA----		
_clipseq#11	(1)	----GCTGTGCTCCTGCGTAC	AACGGTGTAAAC-----		
_clipseq#3	(1)	----GTGTCTTCATGCTTAT	AACATTCTGCGGCC----		
_clipseq#18	(1)	----TATGTGTTACTAACAGGGGCA	TGGCGCTT-----		
_clipseq#21	(1)	--CTGTTCTTTGTAAACCGCA	ACGCGTATGT-----		
_clipseq#22	(1)	----CACTTCTGCTAACTCCAACCAT	TGATG-----		
_clipseq#15	(1)	-----CTAGATATAGAGGACG	CCTCAGCGGAAC--		
_clipseq#30	(1)	AGCTTGAGTATAGCACTATTG	ACTCAGTCA-----		
_clipseq#7	(1)	--GTTTAGTATGGCTATAATATCGG	CCTCCT-----		
_clipseq#25	(1)	----GACGGCTCGCCACTATTGTTCT	TCACGAG----		
_clipseq#9	(1)	-----AACTTCGCTAACGTT	ATTCGTATACTCCTC---		
_clipseq#2	(1)	-----CGCTTCCCGCAGCAGGGAT	CCGAACCGCTCA--		
_clipseq#4	(1)	-ACTGCAGACTTGACGAAGCTTAAG	CCGAAT-----		
_clipseq#28	(1)	----ATCACTGGGGGACACGCTCG	CATCACTGTC----		
_clipseq#29	(1)	-----TCGCTTGCTGAC--	ATGCTGTAAACAGGTTCTCT		
_clipseq#14	(1)	--AGTCGCATTTACAGTAATCCGGCT	TCCAA-----		
_clipseq#20	(1)	---GTTTGCAGTTCCGGTTGGTGAGCA	TCCGTC-----		
_clipseq#23	(1)	-----CGCGTTTAACGCCGTTGGCC	CTCAGTTGAA---		
_clipseq#13	(1)	----GGCAGATGCGCGCCTT	ATCTCATTTGACCG----		
_clipseq#19	(1)	---AATAGACATACCCTCAGT	ACCTCCTCTGTC-----		
_clipseq#24	(1)	-----CGTCAACTCAAAGTCCT	TTTTTCGCACA		
_clipseq#16	(1)	----ATAGCTTTATCTA--	GATAGCATCCGTTTTG--		
_clipseq#8	(1)	-ACCAACAAGCGAAACGAGTG	ATATCTCAG-----		
_clipseq#17	(1)	-AGTAAGAGGATAGAGTATTTGGCC	CCGCGT-----		
Consensus	(1)		TT	A	C T C

However, the sequencing results showed the selection had been progressing with evidence of slight enrichment as shown by a “one-to-many” sequence comparison. For example, the sequence of clone-1 was analysed to find common regions with the remaining clones (Table 3-2) and regions of 5 nucleotides long homology in four other clones were seen. As common regions were shared only between pairs of clones rather than several, the degree of enrichment of the pool had not been significant enough to show preferred sequences for the target. Therefore, for the following rounds of selection, we increased the stringency of washing to speed up the selection.

Table 3-2: Sequence analysis of the clone 1 against the other clones from Round 10. In the table, _clipseq indicates sequence of clone 1 whilst _clipseq#i (i = 2 to 31) indicates sequence of clone i. The table shows five-conserved sequences of clone 1 with clones 10, 21, 23, 24 and 28.

Clone	Sequence comparison	Clone	Sequence analysis
(1) 1	10 20 38	(1) 1	10 20 32
_clipseq (1)	ACTGTCCACTCATCGCAAGCCTTGGCAGGC-----	_clipseq (1)	ACTGTCCACTCATCGCAAGCCTTGGCAGGC--
_clipseq#2 (1)	-----CGTTGCCAGCAGGATCCGAACCGCTCA	_clipseq#17 (1)	--AGTAAGAGGATAGATATTGGCCCGG
Consensus (1)	C C TC C AGC G G	Consensus (1)	GT AT G T G C GC
(1) 1	10 20 31	(1) 1	10 20 31
_clipseq (1)	ACTGTGCAC-TCATCGCAAGCCTTGGCAGGC	_clipseq (1)	ACTGTCC-ACTCATCGCAAGCCTTGGCAGGC
_clipseq#3 (1)	-GTGTGTCATGCTTATAACATTCTGGGCC	_clipseq#18 (1)	TATGTGTTACTAACAGGGGCACTGGGCGTT-
Consensus (1)	TGTC C T T AA T GC G C	Consensus (1)	TGT ACT A G CT GGC
(1) 1	10 20 31	(1) 1	10 20 31
_clipseq (1)	ACTGTCCACTCATCGCAAGCCTTGGCAGGC-	_clipseq (1)	-ACTGTCCACTCATCGCAAGCCTTGGCAGGC
_clipseq#4 (1)	ACTGCAGACTTGACG-AAGCTTAAGCCGAAT	_clipseq#19 (1)	AATAGACATACCTCAGTA-CCTCCTCTGTC
Consensus (1)	ACTG ACT CG AAGC T GC G	Consensus (1)	A G C C TC A CCT C G C
(1) 1	10 20 31	(1) 1	10 20 34
_clipseq (1)	ACTGTCCACTCATCGCAAGCCTTGGCAGGC-	_clipseq (1)	----ACTGTCCACTCATCGCAAGCCTTGGCAGGC
_clipseq#5 (1)	-CATTAAAACTCTCTGTACGTTCTAACG	_clipseq#20 (1)	GTTTGCACTTC-CGGTTGGTGACATCCGTC---
Consensus (1)	C T A TC TC C C TT A G	Consensus (1)	C GT C C T G AGC T G
(1) 1	10 20 30	(1) 1	10 20 35
_clipseq (1)	ACTGTCCACTCATCGCAAGCCTTGGCAGGC	_clipseq (1)	ACTGTCCACTCAT----CGCAA-GCCTTGGCAGGC
_clipseq#6 (1)	AGTGCCTAGAGCAGGTGAAGCTGTCCTGGA	_clipseq#21 (1)	-CTGTCTTTTGTAAACCGCAACGCCTATG----
Consensus (1)	A TG A CG A CT C GG	Consensus (1)	CTGT C T T CGCAA GC T G
(1) 1	10 20 34	(1) 1	10 20 32
_clipseq (1)	ACTGTCCACTCATCGCAAGCCTT--GGCAGGC--	_clipseq (1)	-ACTGTCCACTCATTCGCAAGCCTTGGCAGGC
_clipseq#7 (1)	---GTTTAGT-ATGGCTATAATTCGGCCTCCT	_clipseq#22 (1)	CACCTTCTGCTAACTTCCAACATTGATG--
Consensus (1)	GT A T AT GC A T GGC C	Consensus (1)	ACT TC CT A T CAA C TT G G
(1) 1	10 20 34	(1) 1	10 20 38
_clipseq (1)	ACTGTCCACTCATCGCAAGCCTTGGCAGGC----	_clipseq (1)	ACTGTCCACTCATCGCAAGCCTTGGCAGGC-----
_clipseq#8 (1)	---ACCAACAAGCGAAACGAGTGATCTCAG	_clipseq#23 (1)	-----CGCGTTTAACGCCGTGGGCCCTCAGTTGAA
Consensus (1)	CCA A CG AA TG A C	Consensus (1)	C C T A GCC TTGGC C
(1) 1	10 20 38	(1) 1	10 20 32
_clipseq (1)	ACTGTCCACTCATCGCAAGCCTTGGCAGGC-----	_clipseq (1)	ACTGTCCACTCATCGCAAGCCTTGGCAGGC--
_clipseq#9 (1)	-----AATTTCGCTAAGCTTATTCGTATACTCCTC	_clipseq#24 (1)	-CGTCACTCAAAAGTCCTTTTTCGCACA
Consensus (1)	C TCGC A C TT G	Consensus (1)	GTC ACTCA TT C G
(1) 1	10 20 34	(1) 1	10 20 31
_clipseq (1)	---ACTGTCCACTCATCGCAAGCCTTGGCAGGC	_clipseq (1)	-ACTGTCCACTCATCGCAAGCCTTGGCAGGC
_clipseq#10 (1)	GCCTTAAGTAGAATCTTTA-AAGCCATAGC----	_clipseq#25 (1)	GACGCTGGCACTATTGTTCCCTTCAGAG-
Consensus (1)	GT A TC T AAGCC T GC	Consensus (1)	AC G C C T CCTT C G
(1) 1	10 20 31	(1) 1	10 20 33
_clipseq (1)	ACTGTCCACTCATCGCAAGCCTTGGCAGGC	_clipseq (1)	ACTGTCCACTCATCGCAAGCCTTGGCAGGC--
_clipseq#11 (1)	GCTGTGTC-CTGCGTAAACGGTTGTAAAC	_clipseq#26 (1)	---GCCCTGCTGGAAGATGTGGCCTATGCT
Consensus (1)	CTGT C C C CG A A C T G A C	Consensus (1)	G CC CT T G A TGGC A GC
(1) 1	10 20 37	(1) 1	10 20 35
_clipseq (1)	ACTGTCCACTCATCGCAAGCCTTGGCAGGC-----	_clipseq (1)	ACTGTCCACTCATCGCAAGCCTTGGCAGGC--
_clipseq#12 (1)	-----AATTAGTAATAGCAG-GTCAGCCGATAGTT	_clipseq#27 (1)	-----CAATTATGTATGCCGATATTGCAGCCGC
Consensus (1)	A T T AAGC G CAG C	Consensus (1)	C A T AT CA GCC T GCAG C
(1) 1	10 20 35	(1) 1	10 20 36
_clipseq (1)	---ACTGTCCACTCATCGCAAGCCTTGGCAGGC	_clipseq (1)	---ACTGTCCACTCATCGCAAGCCTTGGCAGGC
_clipseq#13 (1)	GGCAGATGCGCGCCTTATCTCAATTGCACCG----	_clipseq#28 (1)	ATCAGTGGGGAACGC-TCGCATCACT-GTC----
Consensus (1)	A C C CT ATC CA C G	Consensus (1)	ACTG CAC C TCGCA CT G C
(1) 1	10 20 32	(1) 1	10 20 34
_clipseq (1)	ACTGTCCACTCATCGCAAGCCTTGGCAGGC--	_clipseq (1)	ACTGTCCACTCATCGCAAGCCTTGGCAGGC----
_clipseq#14 (1)	AGTCGATTTCAAGTAATCG--GGCTTCCAA	_clipseq#29 (1)	----TCGCTTGCTGACATGCTGTAACAGGTTCCCT
Consensus (1)	A T C TCA G AA CC GGC C	Consensus (1)	TC T T CA GC T CAGG
(1) 1	10 20 32	(1) 1	10 20 32
_clipseq (1)	ACTGTCCACTCATCGCAAGCCTTGGCAGGC--	_clipseq (1)	ACTGTCCACTCATCGCAAGCCTTGGCAGGC--
_clipseq#15 (1)	---CTAGATATAGAGGACGCCCTCACGCGGAAC	_clipseq#30 (1)	-AGCTTGAAT-ATAGCACTATTGACTCAGTCA
Consensus (1)	T A A G A GCCT GG	Consensus (1)	T A T AT GCA T CAG C
(1) 1	10 20 33	(1) 1	10 20 33
_clipseq (1)	---ACTGTCCACTCATCGCAAGCCTTGGCAGGC	_clipseq (1)	ACTGTCCACTCATCGC--AAGCCTTGGCAGGC
_clipseq#16 (1)	ATAGCTTATCTAGATAGCATCCGTTTGT---	_clipseq#31 (1)	GCTTAGCATGCTTGGGGGAAGCGCGGCA---
Consensus (1)	CT T AT GCA CC T	Consensus (1)	CT T TC T GC AAG C GGCA

In addition to the enrichment progress of the pool, the size of cloned DNA showed no evidence of expanding or shrinking in length in the random region of 30 nucleotides. This proved that the amplification had worked well and no over-amplification had occurred.



Figure 3-6: Agarose gel electrophoresis analysis of the amplification of the DNA pool from Round 10. The gel was 3% agarose in TBE buffer and the DNA was visualised by ethidium bromide and UV light. The numbers in red indicated the PCR cycles whilst the light green indicated the base pairs of the DNA marker.

From Round 11, the partitioning of the subsequent rounds was intensified by increasing the molar ratio RNA to peptide from equimolar to 3:1 as well as increasing the washing stringency from 3 washes to 5 washes. Increasing molar ratio of the input RNA library to the peptide leads to more competition for binding between RNA molecules and the target whilst increasing washing stringency results in dissociating less strongly bound sequences. Sequences of clones from Round 21 are analysed and shown below in Table 3-3.

Table 3-3: Sequence analysis of the emerged clones from Round 21.

Common regions between clones are marked by colours. The number of times a clone appeared is shown in parentheses.

Aptamer		Clone		Clone
Ta23	Tgtgggtcagctgctgctttatagcaccg	23 (2)	Atgtggctgcttcggccttaactgtgatc	8
	Ttaatagtgggtcagccgtcataatgtgat	35	Tgtggactacgcttcgcgcgtgtgtgtt	9
	Tgtgggtcactgtaatatgtctgcttg	7	Tgtattgcctgtggatatactgagtttgc	12
	Tgtgggtcatggtcttcgatttacactgct	24	Atgtggagatgcttaactgtcgcgtctata	26
	Tgggtccacatgtattaaatgcgcagtgcc	32	Tgtgggaagctcatcggtttttcgtgctg	27
	Aatatcattcaaatgcgcagtgatggtgat	37	Agtggatcacgcttttagctgcagttcgtgt	2
	Tatagttattaaaccttcactgtttgtgca	10	Gtggactttgcttcggcgcgcagctga	6
	Atcgtttattaaagtggttagctctgcc	30	Tacgaaatatattagctcggtgattagcta	5
Ta15	Tgtggaaactgctcattgtcgcaagtcgt	15 (2)	Actactcggtcagcatgtggactctgcacg	3
	Ttgtggaaagggtcgttacggctgcctccga	19	Ttgtggtaaccgacaatgtaatgctagtat	21
	Ttgtggacgagcggctgcgcagagatttgcc	18	Atgtggtacagtttcgactcttgccagtt	33
	Ttgtggacaactcgccgtctgtacaccata	28	Taaagaatctggatctaggcttacgtgtc	1
	Ttacagggatcctttgtgtctgcgcacac	4	Cacagggtccttgcttcttaagtcaa	14
	Tttaacggatccaagcatcgacggccgcac	11	Taagaatggctcgtaattcagcgcctctg	22
	Actgatggggatcataatgtagttctgct	17	Ttatgcttctgtctgaagatgattttgat	31
	Tatgtatttgctacttctgggtgtgttga	13	Aggatcggtttgtcatcagtaaattgtgc	36
	Caatgggtcctacgtcaaatacttgcata	16		
	Tgtacgtagtggcgatcttatgcgttaagt	25		
	Gtgggagctgcacgttaagttcattgcttgt	29		

This selection strategy appeared to work as by Round 21 of the selection, enriched species emerged and yielded a population in which clones 15 and 23 appeared twice (Table 3-2). A conserved octamer sequences (gtgggtca, red) was

found in clone 23 and clones 35, 7, and 25. This octamer was expanded to a decamer sequence (gtgggtcagc, red) between clones 23 and 35. The other clone that twice occurred, Ta15, also shared a common sequence (tgtggaa/c, turquoise) with 3 other clones. In addition, clones in the sequenced population could be paired together based on shared sequences such as a nonamer region for both clones 32 and 37. The sequences of the two clones that appeared twice in population, so-called aptamers Ta15 and Ta23, were assayed to investigate their interaction to the peptide target. Binding was assessed by surface plasmon resonance (SPR) using a BIAcore3000.

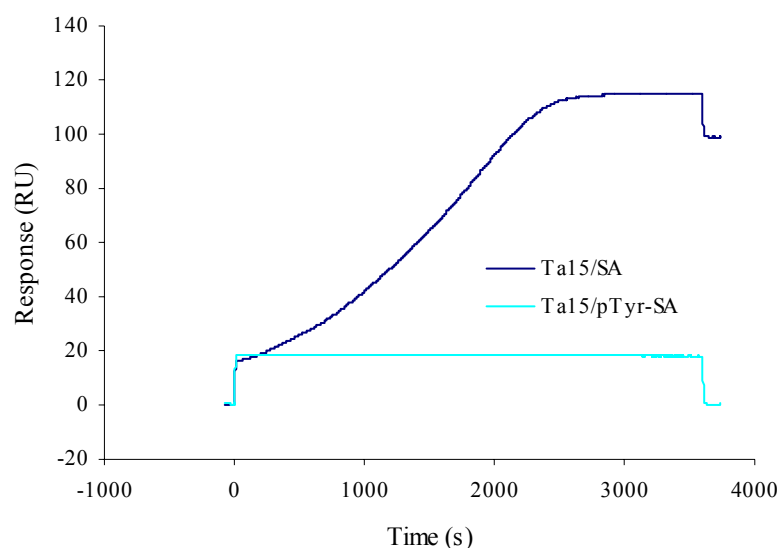


Figure 3-7: Interaction of Ta15 with streptavidin and the pY peptide determined using surface plasmon resonance (SPR). 2 μ M Ta15 solution was injected onto streptavidin- and pY peptide/streptavidin-coated surfaces at a flow rate of 2 μ L/min over a period of 60 min. Interaction was indicated by an increase in response units. Ta15 showed no response (turquoise) with the pY peptide target, which was immobilised through a streptavidin-coated surface, whilst a clear increase was seen with a streptavidin-coated surface (blue).

As shown in Figure 3-7, Ta15 did not bind the pY peptide target but did instead to streptavidin. A similar observation was made with Ta23. It is concluded from these SPR experiments that both aptamers were selected against streptavidin rather than the pY peptide. The emergence of anti-streptavidin aptamers was a consequence of using streptavidin-coated magnetic beads to capture the complex of aptamer and peptide target in the partitioning step. During partitioning aptamers bound to streptavidin on the beads and were subsequently enriched in the selection. Further characterisation and discussion of possible applications of these streptavidin-binding aptamers are presented in Chapter 4. It is however worth pointing out that the lack of binding to the peptide-streptavidin complexes seen in the SPR measurements suggests that the aptamers bind in or near to the biotin binding pocket. This is consistent with the general observation (described in Chapter 1) that aptamers are often bound to ‘pockets’ or ‘clefts’ in proteins such as active sites or ligand binding sites.

Thus the selection so far resulted in isolation of aptamers binding to the matrix (streptavidin) instead of the intended target. This possibility of selection outcomes has been discussed previously (Conrad *et al.* 1996). Isolation of undesired aptamers against the matrix could be avoided by introducing an extra step during selection (pre-absorption to the matrix) and this had been applied in a number of examples (Xu and Ellington 1996; Bell *et al.* 1998). However, the pre-absorption step was not always necessary as many successful selections were achieved without it (Gilbert *et al.* 1997; Cox and Ellington 2001; Cox *et al.* 2002; Murphy *et al.* 2003). In the work presented by researchers in the Ellington lab, the N30 library was successfully used to select aptamers against biotinylated proteins using streptavidin-coated magnetic

beads for partitioning (Cox and Ellington 2001; Cox *et al.* 2002). These selections were performed without the matrix pre-absorption step, however in these cases targets were pre-complexed to the beads and therefore the biotin binding sites were already occupied.

As far as can be ascertained from the literature the method described in this thesis of capturing the aptamer-target complex after solution complexation has not previously been used. As discussed earlier in this chapter such strategy should make the partitioning step more like that using nitrocellulose membranes.

The difference in the selection outcomes is probably due to the difference in immobilisation procedures of targets on the streptavidin-coated magnetic beads. In the experiments performed by the Ellington group, the biotinylated targets were pre-immobilised on the streptavidin-coated beads. As a result, the immobilised protein molecules probably occupied all the biotin-binding sites and covered the streptavidin surfaces and therefore prevented streptavidin-bound sequences from accessing to the streptavidin molecules. However, in our experiment, as described in Chapter 2 (see Figure 2-2), the target was incubated with the library before being immobilised on the streptavidin-coated beads. This probably allowed sequences in the library to get access to streptavidin, causing a subsequent enrichment of streptavidin-binding aptamers. In addition, the target properties can also contribute to the difference in the selection outcomes. In the Ellington group's experiment (Cox and Ellington 2001), lysozyme was used as the model target and in fact it was considered as an "easy target" and has often been used to test the selection procedures (Cox and Ellington 2001; Lee *et al.* 2005a; Hybarger *et al.* 2006).

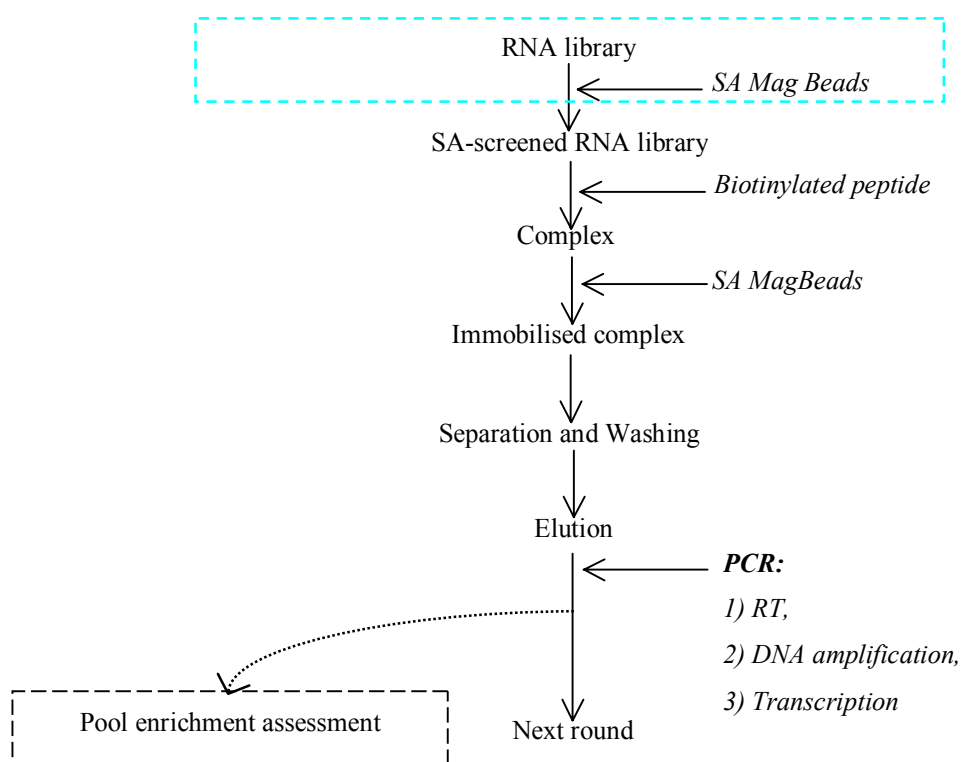


Figure 3-8: The modified protocol of RNA aptamer selection against the pY peptide. The extra pre-absorption step (in the turquoise frame) was added at the beginning of each round to eliminate streptavidin-binding sequences from the library.

Consequently, our original selection protocol had to be modified. The possibility was that aptamers bound to the pY peptide target were co-enriched with the streptavidin-binding aptamers, and therefore it was decided to pre-absorb the streptavidin-binding sequences from the pool and hence enrich our described target-binding aptamers. This was achieved by adding an extra screening step to the selection protocol and continuing the revised selection from Round 20. To exclude

streptavidin-binding sequences from the population, the pool was first incubated with the streptavidin-coated beads. The modified protocol is illustrated in Figure 3-8.

After another 11 rounds of selection, the enriched double-stranded DNA pool of Round 32 was cloned and sequenced. Sequence analysis in Table 3-4 showed that the selection pool had been significantly winnowed to just a few variants with 2 sequences comprising the majority of the population. Both clones appeared 10 times each out of 30 randomly picked clones in Round 32. This implies an enrichment of better than 10^{14} -fold, given that the initial random pool had a diversity of approximately 7×10^{14} unique members.

Table 3-4: Sequence analysis of the emerged sequences from Round 32.

The frequency a clone appeared in the sequenced population is shown in parentheses.

Aptamer		Clone
Tap1	Atgtggaaagctccgaacagcctctatgaa	1 (10)
Tap2	Cgtgtgggtgccatattcaattgattggaa	4 (10)
	Aatgtggaattgtcaatctcttga	17 (2)
	Atgtgggaagctcatcgttttctgactg	22 (2)
	Tggacaagcttcagtcacagtcataccg	2
	Atcatgtggaagcttttaactcctgctca	6
	Aagggggaattgcctcgctcttgcca	9
	Ttgtgggggttcgatcacgtgctgctggg	10
	Atgtggaatgcttaactgctgctgata	13
	Tgcagtaccagtggtccttagataaggg	23

The two dominant clones, Tap1 and Tap2, were assessed using SPR to find their binding affinities with the peptide target. Figure 3-9 shows the results of this measurement for addition of Tap2 to the phosphorylated target. The increase in response upon adding the aptamer is as expected for a binding reaction between it and the peptide. In contrast there was no response to streptavidin other than bulk refractive index change. The selection, therefore, appeared to successfully isolate aptamers for the pY peptide, our intended target. Binding of the aptamers to the pY peptide is further described in Chapter 5.

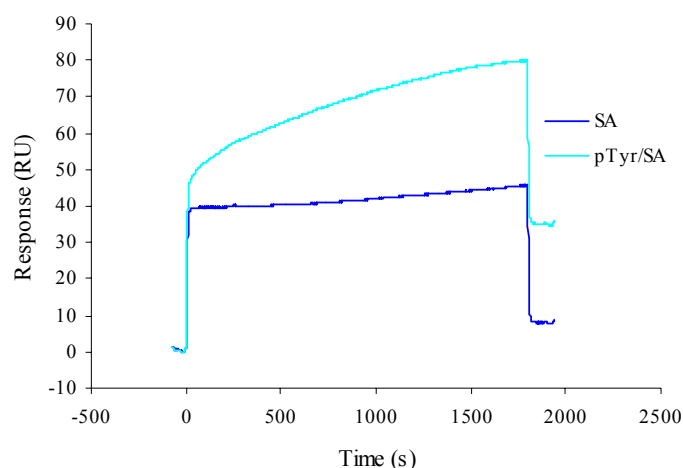


Figure 3-9: Interaction of Tap2 with streptavidin and with the pY peptide and to streptavidin using surface plasmon resonance. A 2 μ M solution of Tap2 was injected onto streptavidin- and onto the pY peptide/streptavidin-coated surfaces with a flow rate of 2 μ L/min over a course of 30 min. Interaction was indicated by changes in response. Tap2 showed little response to streptavidin (blue) whilst it bound to the pY peptide target, which was immobilised on top of streptavidin-coated surface (turquoise).

3.4. Conclusions

Two classes of RNA aptamers were isolated by *in vitro* selection. The first appeared to bind in the biotin binding pocket of streptavidin and were a consequence of using streptavidin-coated beads to capture the target peptide during the partitioning step. These aptamers were selected because the protocol relied on adding the streptavidin-coated beads after complexation of the aptamer library to the target peptide and hence empty binding pockets on streptavidin were available for binding to the aptamers. Subsequently modifying the selection procedure to incorporate a pre-absorption step at the start of each round remove streptavidin binding sequences and allowed enrichment in aptamers that could bind to the pY peptide. In this case the enrichment was more than 10^{14} fold and the properties of the both classes of aptamers are described in the following chapters.

Chapter 4: Streptavidin binding aptamers

4.1. Introduction

As presented in the previous chapter, the streptavidin binding aptamers were not intentionally selected but they were isolated as the selection was carried out without pre-adsorption of matrix-binding sequences. Although not our primary target, high affinity streptavidin binding aptamers have potential application in immobilisation of nucleic acids as an alternative to biotinylation. This chapter describes these aptamers and their binding to streptavidin.

4.1.1. Streptavidin

Streptavidin (SA) is a homotetrameric protein isolated from the actinobacterium *Streptomyces avidinii* (Chalet and Wolf 1964). Each native monomer of the protein contains 159 residues with molecular mass of 16.5 kDa (Bayer *et al.* 1989), but purified protein from different sources can vary in length (Argarana *et al.* 1986). Full length streptavidin is produced by *Streptomyces avidinii* but the bacterium also contains endogenous proteases that can cleave at residues 14 and 134 and therefore each subunit of streptavidin that is fully cleaved by these enzymes consists of residues 15-133 and is referred to as the core streptavidin subunit (with molecular

mass of 13.5 kDa) (Weber *et al.* 1989). Figure 4-1 shows a streptavidin molecule that consists of the 4 core subunits. The molecule has S2 symmetry and can be seen as a dimer of dimers. As there are no cysteine residues; a pair of monomers that form a dimer are not held together by disulfide bonds but through hydrogen bonds and salt bridges (Hendrickson *et al.* 1989; Weber *et al.* 1989; Hyre *et al.* 2006). The dimer-dimer interface is held together more weakly by hydrophobic interactions (Coussaert *et al.* 2001).

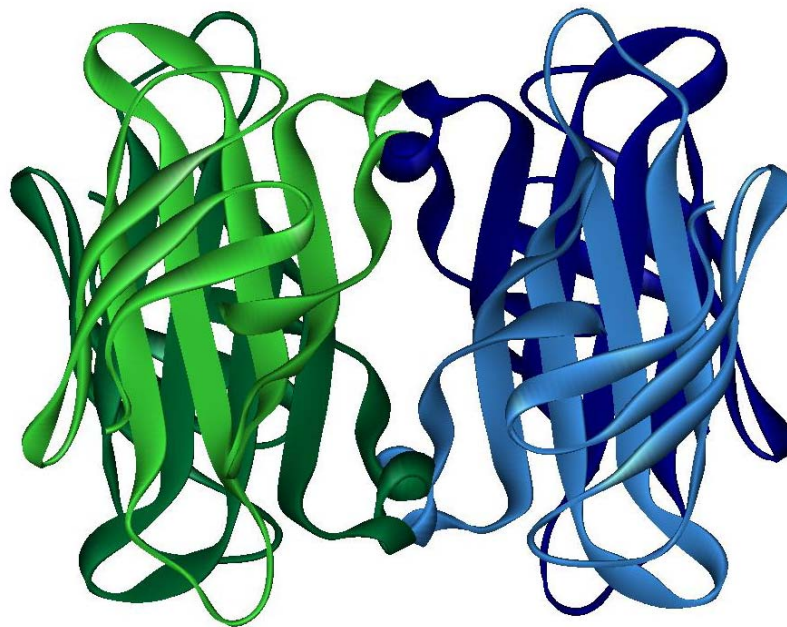


Figure 4-1: Crystal structure of a streptavidin molecule (Hyre *et al.* 2006).

A molecule of the protein consists of 4 identical units (marked as light green, green, light blue and blue) shown in ribbon model. Two monomers of dimer are marked by the same colour band (green or blue). The image created from pdb file 1MK5 using WebLab ViewerLite.

Streptavidin is well-known for its ability to bind D-biotin with very high affinity, the dissociation constant K_d value was estimated to be around 10^{-15} M (Chalet and Wolf 1964), and in this aspect it is very similar to a protein found in egg white called avidin (Green 1963). The strong specific binding of streptavidin with biotin is utilized in numerous biological applications (Schetters 1999) since streptavidin can be used as a molecular “hook” for biotinylated biomolecules such as proteins and nucleic acids (Ding *et al.* 2001; Crucifix *et al.* 2004). In addition to binding biotin, streptavidin is also known to bind other ligands including 2-(4'-hydroxyazobenzene) benzoic acid (HABA) (Weber *et al.* 1992), peptides (Schmidt and Skerra 2007) and nucleic acid aptamers (Srisawat and Engelke 2002; Tahiri-Alaoui *et al.* 2002; Stoltenburg *et al.* 2005).

4.1.2. Ligands for streptavidin

4.1.2.1. Biotin

D-biotin or vitamin H (molecular formula $C_{10}H_{16}N_2O_3S$) is required for synthesis of functional biotin carboxylases and decarboxylases. It has a molecular mass of 244 Da and, as mentioned above, is known to bind streptavidin with an exceptionally high affinity (K_d of 10^{-15} M). Apart from streptavidin, biotin also binds avidin (Green 1963; Livnah *et al.* 1993) and a number of other proteins (Mandella *et al.* 1978) and these proteins are sometimes referred to as biotin-binding proteins.

Binding of biotin to streptavidin has been well-studied (Weber *et al.* 1989; Kurzban *et al.* 1990; Gonzalez *et al.* 1997). Figure 4-2 shows the structure of biotin bound to a streptavidin monomer. The structure was established based on residues 15-133 (*i.e.* the core subunit) and reported that this core protein binds biotin with a similar affinity to the native form (Weber *et al.* 1989). Each streptavidin subunit has a biotin-binding pocket formed from 4 tryptophan residues (marked by the blue and green ball and stick model in Figure 4-2) (Argarana *et al.* 1986). These tryptophan residues are clustered in the hydrophobic binding site in close contact with biotin (Hendrickson *et al.* 1989; Weber *et al.* 1989). The biotin binding pocket is located in a shared interface; one of the tryptophan residues (marked in green) of the binding pocket is known to connect to an adjacent subunit (Sano and Cantor 1995) and replacing this tryptophan residue causes weakening of the tetrameric structure of streptavidin. It was also reported that in the presence of biotin, this tryptophan residue makes contact with the bound biotin and may help to seal biotin site (Kurzban *et al.* 1991). As tryptophan residues form the biotin binding pocket, the presence of biotin can cause changes in their fluorescence properties. Fluorescence measurements showed that when biotin binds streptavidin, there is a blue-shift of 9 nm (Kurzban *et al.* 1990) or 5 nm (Gonzalez *et al.* 1997) in the emission wavelength of the tryptophan with a decrease of 39% (Kurzban *et al.* 1990) or 25% (Gonzalez *et al.* 1997) in the fluorescence intensity.

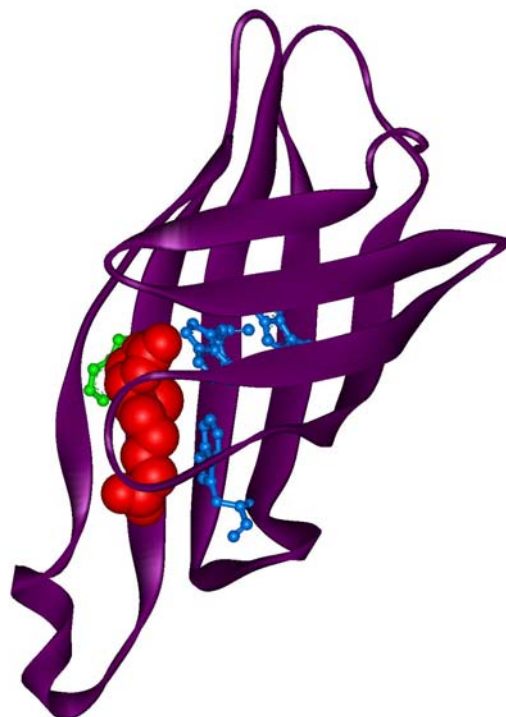


Figure 4-2: Crystal structure the streptavidin-biotin complex (Weber *et al.* 1989). The streptavidin subunit is shown as the violet ribbon model; the biotin molecule is shown as the red Corey-Pauling-Koltun (CPK) model; the tryptophan residues of the biotin binding pocket are marked by the blue or green ball and stick model. The image created from pdb file 1STP using WebLab ViewerLite.

Biotin can be chemically (Larsson *et al.* 2003; Lue *et al.* 2004) or genetically (Beckett *et al.* 1999; Polyak *et al.* 1999) incorporated into biomolecules. Biotinylated peptides, proteins and nucleic acids have been used widely for immobilisation in analytical applications (Edgar *et al.* 2006) .

4.1.2.2. HABA

HABA (2-(4'-hydroxyazobenzene) benzoic acid) is an organic dye with absorbance at 348 nm in aqueous solution. Although this molecule is structurally different from biotin, it was found to bind streptavidin in the biotin binding pocket (Weber *et al.* 1992). However, HABA forms a far less tight complex with streptavidin (K_d value of 10^{-4} M) compared to biotin (K_d value of 10^{-15} M) (Weber *et al.* 1992). Figure 4-3 shows the structure of streptavidin-HABA complex.

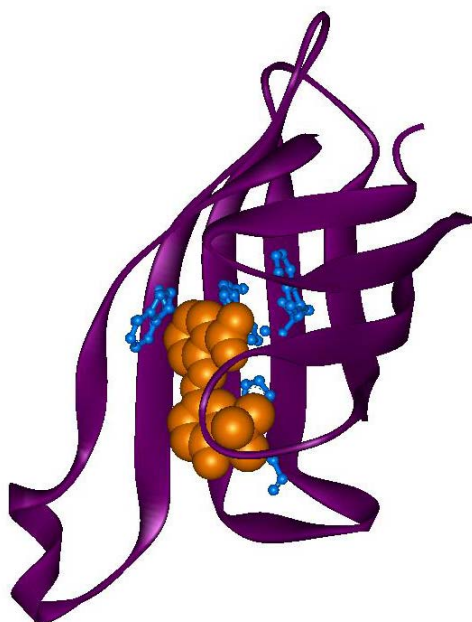


Figure 4-3: Crystal structure of the streptavidin-HABA complex (Weber *et al.* 1994). The streptavidin subunit is shown as the violet ribbon model; the HABA molecule is shown in orange CPK model; the tryptophan residues of the biotin binding pocket are marked by blue ball and stick model. The image created from pdb file 1SRF using WebLab ViewerLite.

This dye shows a shift in its absorbance maximum from 348 nm to 500 nm when bound to streptavidin. Addition of biotin to streptavidin-HABA complex results in the absorbance at 500 nm decreasing while the absorbance at 348 nm increases due to displacement of HABA by biotin in the binding sites and release of free HABA. This change in absorbance has been used to determine the degree of biotinylation of biomolecules and this method was originally known as Green method (Green 1970).

4.1.2.3. Streptavidin binding peptides

The Strep-tag is a nine-amino acid peptide sequence (Ala-Trp-Arg-His-Pro-Gln-Phe-Gly-Gly) with high specificity and affinity towards streptavidin. Its sequence was derived by selection from a phage library (Schmidt and Skerra 1993). The peptide was reported to bind streptavidin at the biotin binding pocket with a K_d value of 37 μ M (Schmidt *et al.* 1996). Subsequently a second streptavidin binding peptide (Strep-tag II) was discovered and this peptide binds a mutated streptavidin with a slightly higher affinity (K_d of 13 μ M) (Schmidt *et al.* 1996; Voss and Skerra 1997).

4.1.2.4. Streptavidin binding aptamers

A number of DNA and RNA aptamers have been isolated from nucleic acid libraries that bind streptavidin tightly, such as DNA aptamers for streptavidin with K_d values in sub-micromolar range (Stoltenburg *et al.* 2005) and 2'-Fluoro-substituted RNA aptamers that bind streptavidin with K_d values in low nanomolar range (Tahiri-Alaoui *et al.* 2002).

4.2. Characterisation of the streptavidin-binding aptamers

As briefly mentioned in Chapter 3, the sequences that emerged from Round 21 were found to bind streptavidin through SPR responses when added to streptavidin-coated sensor chips. Further characterisation of the streptavidin-binding aptamers using SPR, fluorescence spectroscopy, and spectrophotometry is presented in this section.

4.2.1. Determination of affinity constants of the streptavidin-binding aptamers using surface plasmon resonance

Surface plasmon resonance (SPR) has been widely used as a powerful tool for label-free monitoring in real-time of biomolecular interactions (Parsons *et al.* 1995; McDonnell 2001). Label-free measurement is an advantage for biomolecular binding assays since the labelling groups (except for biosynthetically incorporated isotopes) may lead to misfolding or surface steric hindrance and hence changes in binding constants. A commercial SPR device, the BIAcore3000, was employed in this work to investigate binding of the aptamers. Binding is monitored in real-time and indicated by changes in response (measured by response unit (RU)). However, when a sample is injected onto a sensor surface, changes in response often include a bulk refractive index effect. The bulk effect can be subtracted and the difference in response between the values recorded at the beginning and at the end of an injection indicates the response caused by binding of the analyte to the surface over a period of the injection time.

In order to determine binding affinities of the streptavidin-binding aptamers, streptavidin was covalently attached to carboxyl dextran CM5 chips through side-chain amino group of lysine residues using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) as coupling reagents. With this type of coupling, the carboxyl groups of dextran matrix on the CM5 sensor chip surface were first activated with a mixture of EDC/NHS to give reactive succinimide esters. Streptavidin was then passed over the surface and the esters react with side-chain amino groups (of lysine residues) to link streptavidin covalently to the dextran. After the binding of streptavidin, ethanolamine was passed over the sensor surface to deactivate any remaining active esters. Details of an immobilisation time course were described in Chapter 2.

A series of immobilisation experiments at different pHs (ranging from 4.0 to 5.0) showed that pH 4.1 was suitable for streptavidin immobilisation in 10 mM acetate buffer. The loading of streptavidin was aimed to reach around 15,000 RU and this is equal to 1.7×10^{11} molecules per square millimetre, assumed that 1 RU is equal to an immobilised amount of 1 pg/mm² on BIAcore CM5 chips (Biacore handbook). Figure 4-4 shows a sensorgram of an immobilisation time course.

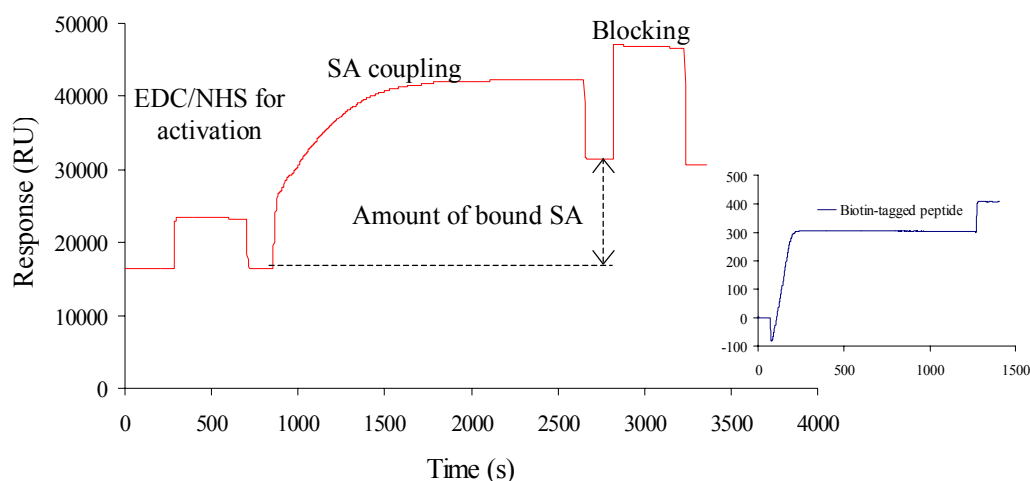


Figure 4-4: Immobilisation of streptavidin on a CM5 chip. The coupling of streptavidin was performed in 10 mM acetate buffer, pH 4.1 using EDC/NHS activation. A 2 μ M solution of streptavidin was injected onto the activated surface over a period of 30 min at a flow rate of 5 μ L/min. The amount of immobilised streptavidin reached 14,300 RU. The inset shows binding of a biotin-tagged peptide to a CM5 chip that was coated with SA using this attachment chemistry.

Affinity constants of the streptavidin-binding aptamers were obtained from fitting data that were recorded through association and dissociation phases, as described in Chapter 2. Figures 4-5 and 4-6 show association and dissociation phases of 4 different concentrations of aptamers Ta15 and Ta23, respectively. The curves in the figure included bulk refractive index effects. In the fitting regime, the bulk responses were excluded from the curves. Parameters of the fitting are presented in Appendix 1 and Appendix 4.

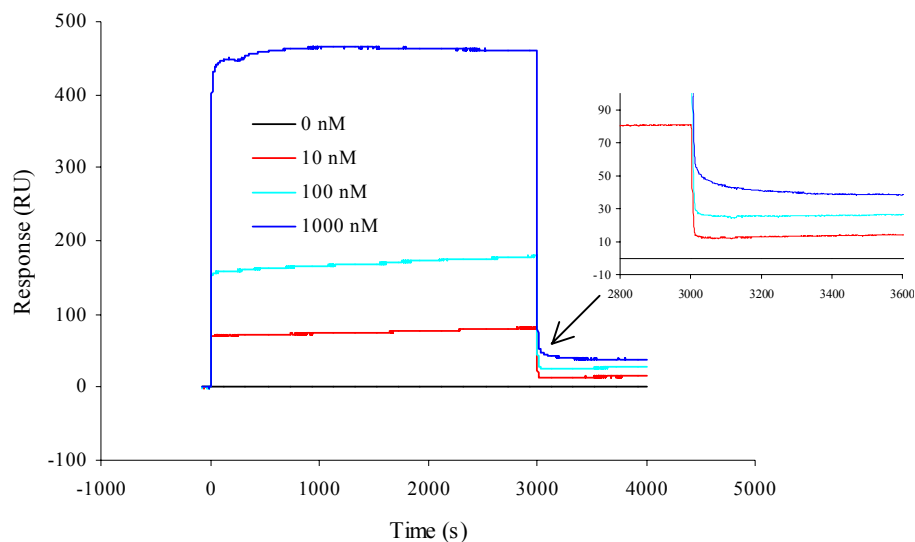


Figure 4-5: A BIAcore3000 sensorgram demonstrating binding of the Ta15 to streptavidin. Streptavidin was immobilised on a CM5 chip. Interaction was monitored through changes in response (RU). Interaction between Ta15 and streptavidin was assessed through association and dissociation phases. The diagram shows 4 curves corresponding to association and dissociation phases of 4 different concentrations of Ta15, as representatives. In the fitting regime, a total of 11 curves corresponding to samples of Ta15 at 9 different concentrations ranging from 0 nM to 2000 nM (with the 1000 nM sample run in triplicate) were used as shown in Appendix 1. Global fitting, assuming a 1:1 Langmuir binding model, resulted in a K_d of 12.6 nM and the model was fitted with χ^2 of 23.9. The inset shows a zoom-in of the dissociation phases.

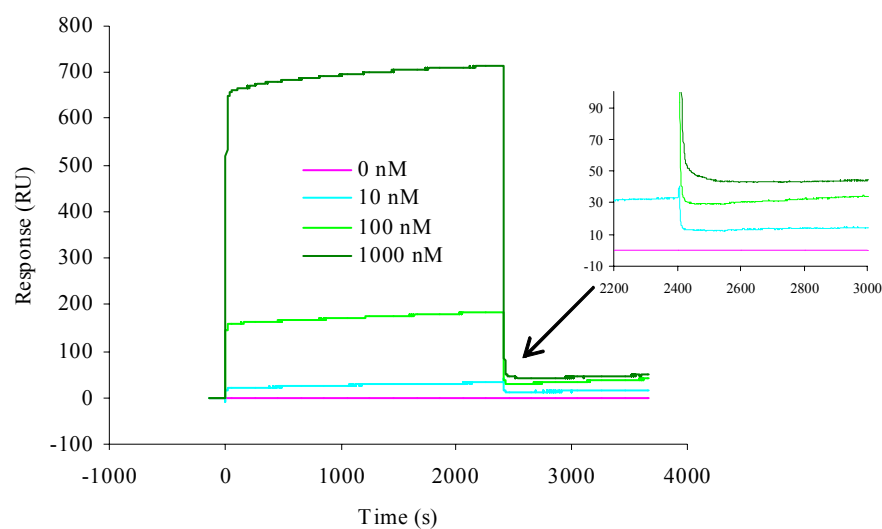


Figure 4-6: A BIAcore3000 sensorgram demonstrating binding of the Ta23 to streptavidin. Streptavidin was immobilised on a CM5 chip. Interaction was monitored through changes in response (RU). Interaction between Ta23 and streptavidin was assessed through association and dissociation phases. The diagram shows 4 curves corresponding to association and dissociation phases of 4 different concentrations of Ta23, as representatives. In the fitting regime, a total of 10 curves corresponding to samples of Ta23 at different concentrations ranging from 0 nM to 2000 nM (the 1000 nM sample run in duplicate) were used as shown in Appendix 4. Global fitting, assuming a 1:1 Langmuir binding model, resulted in a K_d of 2.6 nM and the model was fitted with χ^2 of 62.3. The inset shows a zoom-in of the dissociation phases.

The affinity constants of the streptavidin-binding aptamers obtained from an experimental plateau value (R_{eq}) fit were also calculated to compare with the ones obtained from the global fit. Figure 4-7 shows fitting a set of the plateau values of different concentrations of Ta15 of Ta23 using the Grafit 5.0 program (Erithacus).

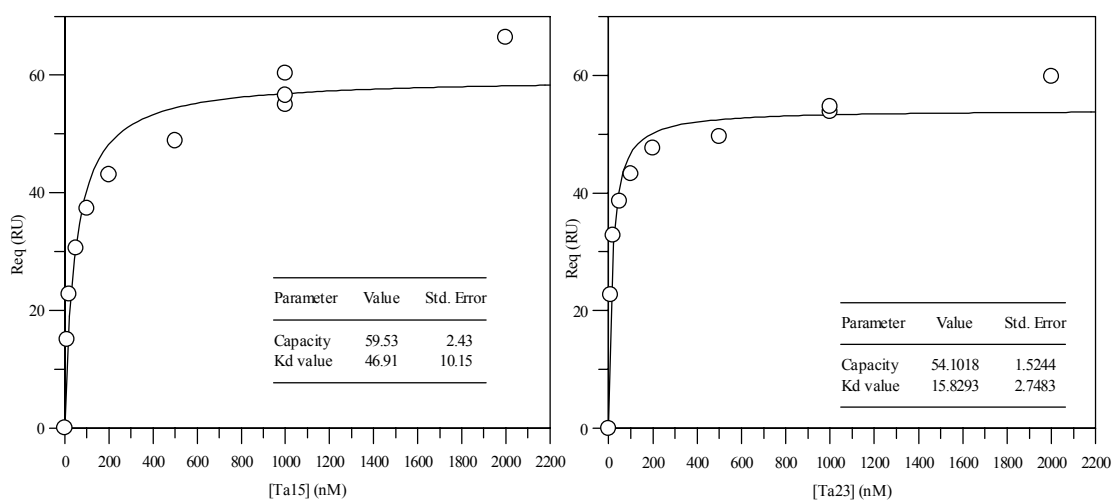


Figure 4-7: Ta15 and Ta23 binding curves. Data were obtained from SPR measurements. Streptavidin was immobilised on CM5 chips. Experimental plateau values (R_{eq} s) from different concentrations of Ta15 or Ta23 were used to calculate K_d . Data were fitted to single binding site equation using Grafit 5.0. The K_d values obtained from the fits were 46.9 ± 10.2 nM and 15.8 ± 2.7 nM, respectively.

For a comparison, the values of dissociation constants obtained from the two fitting regimes from an SPR experiment set for both Ta15 and Ta23 are presented in Table 4-1 below.

Table 4-1: Dissociation constants of the streptavidin-binding aptamers, Ta15 and Ta23, obtained from SPR measurements

Aptamer	K_d (global fit) (nM)	K_d (plateau value fit) (nM)
Ta15	$12.6 \pm 1.58^*$	46.9 ± 10.1
Ta23	$2.6 \pm 0.42^*$	15.8 ± 2.7

* was calculated from replicate run of a sample of the experimental set.

As shown in Table 4-1, affinity constant values of the streptavidin-binding aptamers Ta15 and Ta23, which were obtained from the global fit and the plateau value fit, were different. For Ta15, the values calculated from the two fitting regimes were within 4-fold of one another whilst the difference was 6-fold with Ta23. Statistically, as the global fitting regime used thousands of data points instead of a single plateau value for each curve, data obtained from its model should be more reliable. Therefore, the dissociation constant values obtained from global fits are preferred. For consistency of data presentation though out the thesis, the dissociation constants gained from SPR experiments will from now on be reported using only the values from global fitting. Table 4-2 below shows the dissociation constant values that obtained from 3 independent experimental sets for each of the aptamers, Ta15

and Ta23. This represents a total 33 separate Biacore experiments taking over several weeks. As there was no randomisation of concentration order, the differences could represent systematic errors. The full data of the SPR measurements and data analysis using global fits are presented in the appendices.

Table 4-2: Dissociation constants of the streptavidin-binding aptamers, Ta15 and Ta23, obtained from SPR measurements

Aptamer	SPR data from global fits**					
	Experimental set 1		Experimental set 2		Experimental set 3	
	K _d (nM)	χ^2	K _d (nM)	χ^2	K _d (nM)	χ^2
Ta15	16.7	3.38	12.6	23.9	50.5	3.5
Ta23	5.8	22.4	2.6	62.3	17.9	151

** Data analysis using global fits is fully presented in the appendices.

In addition, binding specificity of the streptavidin-binding aptamers was also investigated using SPR measurements through binding of the aptamers with bovine serum albumin (a protein that has similar molecular mass and pI as streptavidin).

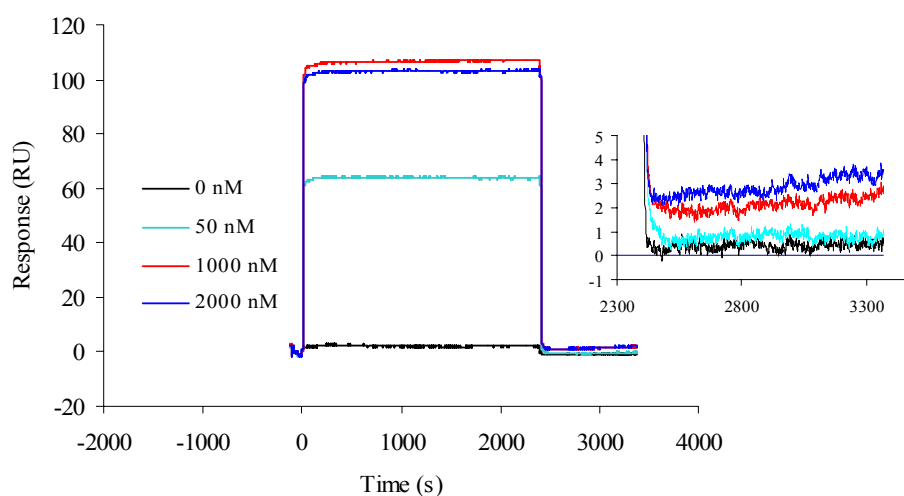


Figure 4-8: A BIAcore3000 sensorgram of Ta15 with bovine serum albumin. Bovine serum albumin (BSA) was immobilised on a CM5 chip at around 6,000 RU. Interaction was monitored through changes in response (RU). The diagram shows 4 curves corresponding to 4 different concentrations of Ta15. Injections of the Ta15 samples of different concentrations onto the BSA-coated chip resulted in insignificant changes in response (BIAcore3000 signal noise was 2 RU) as seen in the inset. This indicated that the aptamer did not bind the BSA-coated chip.

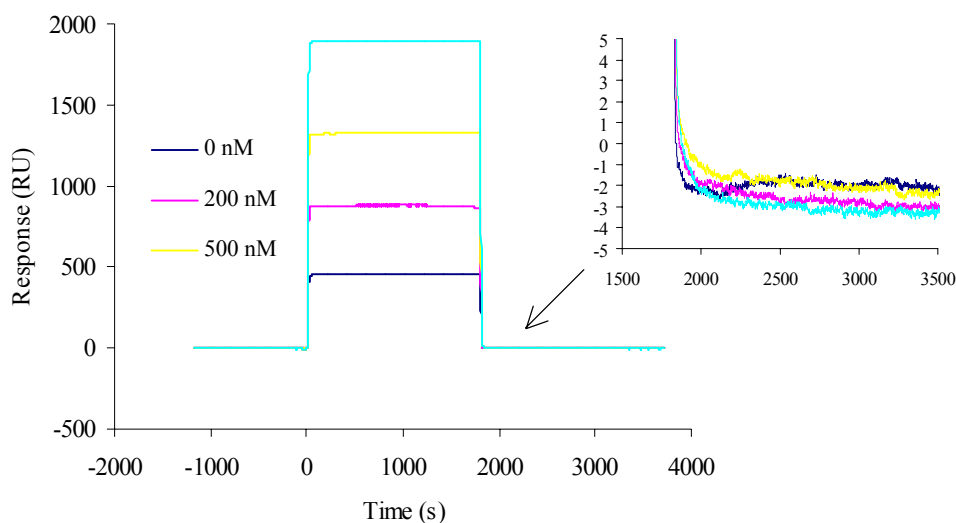


Figure 4-9: A BIAcore3000 sensorgram of Ta23 and bovine serum albumin. Bovine serum albumin (BSA) was immobilised on a CM5 chip. Interaction was monitored through changes in response (RU). The diagram shows 4 curves corresponding to 4 different concentrations of Ta23. Injections of the Ta23 samples of different concentrations onto the BSA-coated chip resulted in insignificant changes in response (signal noise: 2 RU) as seen in the inset. This indicated that the aptamer did not bind the BSA-coated chip.

The measurements showed no significant response when a series of samples with different concentrations of the aptamers, Ta15 or Ta23, were injected over bovine serum albumin, which was also immobilised on CM5 sensor chips. The changes in the responses before and after injections of the samples in the range of the instrument's signal noise range (2 RU) for both aptamer Ta15 and Ta23 as shown in figure 4-8 and 4-9 and therefore indicate no binding between the aptamers and BSA.

4.2.2. Investigation of the aptamer-target binding using fluorescence spectroscopy

As previously demonstrated, the affinity constants of the aptamers to streptavidin were obtained from SPR measurements. However, this method does not provide any structural information about the interaction. Therefore, in addition to using SPR, fluorescence was also employed to investigate the interactions. Fluorescence is a spectroscopic method of analysis that results from a process which occurs when a fluorophore absorbs light at a particular wavelength and subsequently re-emits the absorbed light at a longer wavelength (Lakowickz 2006). The emission spectrum can provide information for both qualitative and quantitative analysis. Binding of aptamers to their targets often lead to conformational changes that can be monitored through fluorescence changes of fluorophores located in proximity to the binding site.

Aptamers form defined secondary structures upon binding their targets. Therefore, any methods that can yield information on conformational changes of molecules are useful for studying interaction of aptamers with their targets. Fluorescence has been widely used to study conformational changes of biomolecules. By incorporating environmentally sensitive fluorescent reporter groups, such as dansyl (5-dimethylaminonaphthalene) or NBD (7-nitrobenz-2-oxa-1,3-diazole), into either aptamers or targets, interaction can be monitored through changes in the fluorescence of the incorporated fluorophore. In particular, the intrinsic fluorescence of tryptophan residues, which is sensitive to its surroundings, can be employed to

study interaction of the tryptophan-containing proteins themselves with their interactants without being labelled with extrinsic fluorescence reporters. As the biotin-binding pocket of streptavidin formed from 4 tryptophan residues, interaction of streptavidin and the aptamers could be monitored through the intrinsic tryptophan fluorescence. Information gained from the fluorescence studies can provide some clues on whether the aptamers bind streptavidin in proximity to the biotin binding pocket that could lead to changes in fluorescence behaviour of the tryptophan residues of the biotin binding pocket.

Fluorescence measurements showed that the interaction of the aptamers to streptavidin caused changes in fluorescence of the intrinsic tryptophan residues. Figures 4-10 and 4-11 show fluorescence emission scans of different concentrations of Ta15 and Ta23 with 1 μ M streptavidin.

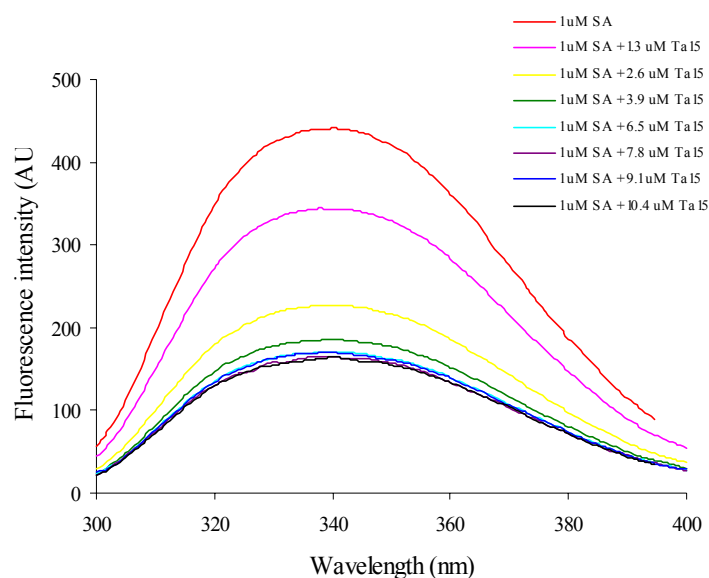


Figure 4-10: Fluorescence emission spectra of streptavidin in the absence and with increasing amounts of Ta15. The solution (300 μ L) contained 1 μ M streptavidin in binding buffer was excited at 280 nm. Aliquots of Ta15 stock solution (130 μ M) were added to make the final desired concentrations.

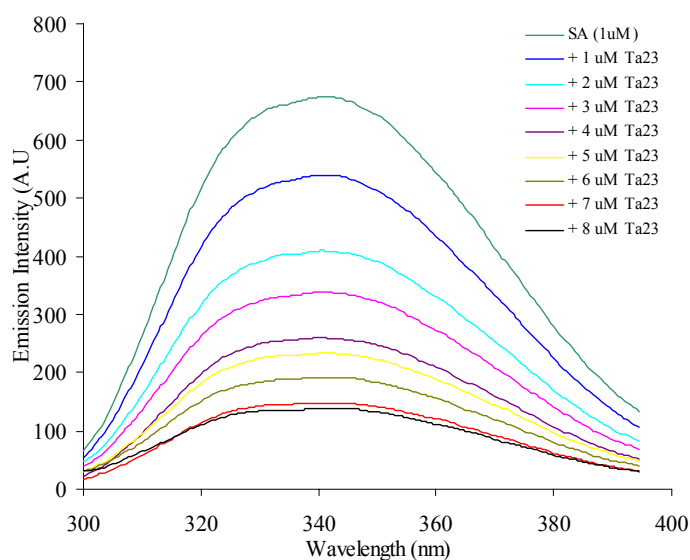


Figure 4-11: Fluorescence emission spectra of streptavidin in the absence and with increasing amounts of the Ta23. The solution (300 μ L) contained 1 μ M streptavidin in binding buffer was excited at 280 nm. Aliquots of Ta23 stock solution (100 μ M) were added to make the final desired concentrations.

The streptavidin (SA) solution was titrated with increasing amounts of Ta15 and changes in maxima of fluorescence emission intensities were plotted against ratio of the $[Ta15]/[streptavidin]$. As the concentration needed to get a good fluorescence signal was high ($1\text{ }\mu\text{M}$ is higher than the K_d value that was previously obtained from the SPR measurements by several orders of magnitude), the experiment yielded a stoichiometric titration curve.

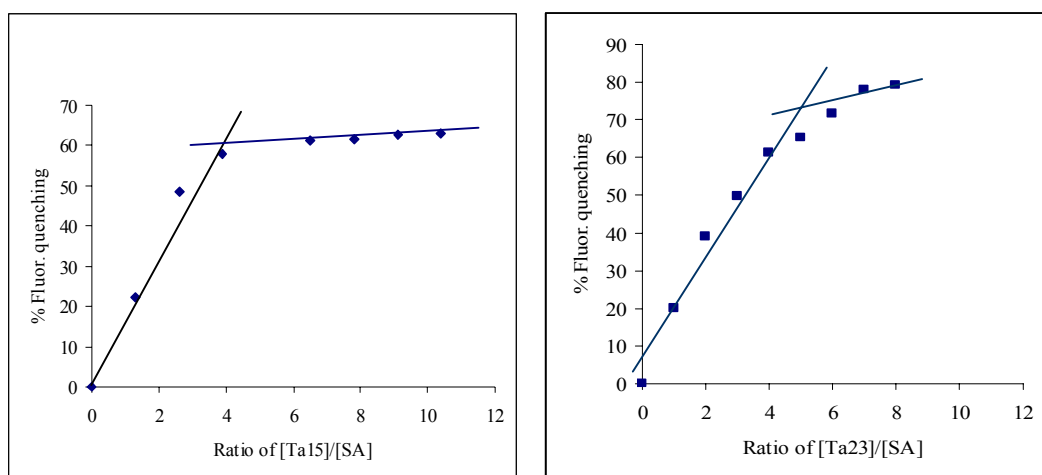


Figure 4-12: Binding curves of the streptavidin binding aptamers Ta15 and Ta23. The percentages of quenching fluorescence were calculated by dividing the differences between the fluorescence intensity at a given concentration of Ta15 or Ta23 to the fluorescence intensity of the streptavidin in the absence of the aptamer (zero concentration). The percentages of quenching fluorescence are plotted against the ratio of $[aptamer]/[SA]$.

The intersection of the linear parts, which represented concentrations of the aptamer below and above the stoichiometric ratio, was 4. This is consistent with a stoichiometry of a single aptamer molecule binding to each streptavidin subunit.

4.2.3. Effects of the aptamers on the streptavidin-HABA complex

HABA (2-(4'-hydroxyazobenzene) benzoic acid) is a synthetic organic dye that is known to bind streptavidin in the biotin-binding site and is displaced by biotin. The HABA assay has been used to calculate the degree of biotinylation of biomolecules. The purpose of HABA assay for the streptavidin-binding aptamers here is to determine if the aptamers mimic biotin and are also able to displace HABA from the complex, causing a decrease in absorbance of the streptavidin-HABA complex at 500 nm.

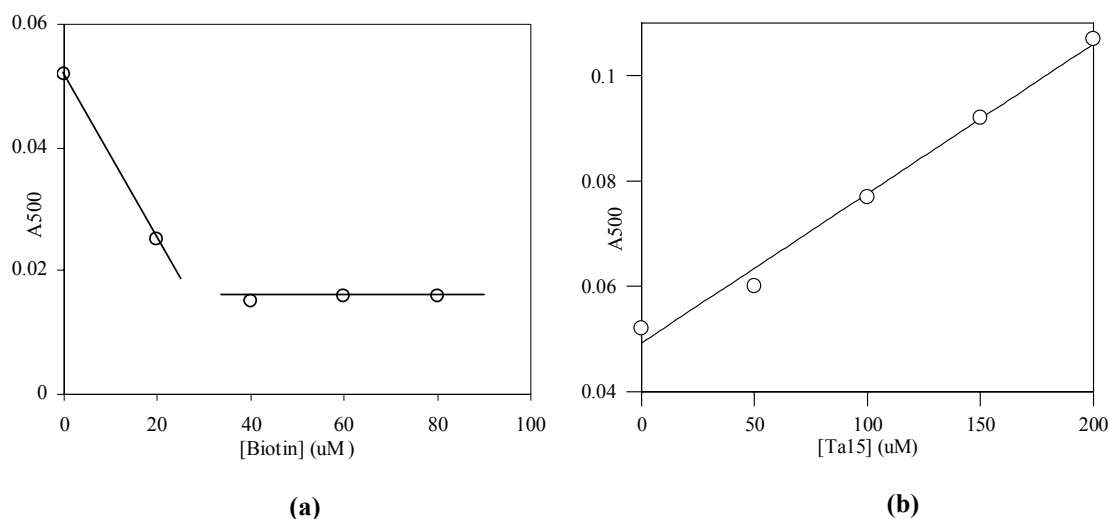


Figure 4-13: Effects of biotin (a) and Ta15 (b) on the absorbance of streptavidin-HABA complexes. The graph shows changes in A_{500} with increasing amounts of Ta15 or biotin at constant concentrations of streptavidin (5 μM) and HABA (1000 μM) in 0.1 M phosphate buffer, pH 7.0.

As shown in Figure 4-13 (a), titration of the streptavidin-HABA complex with biotin led to the expected decrease in A_{500} , as the dye was displaced from the binding pocket. However when Ta15 was added to the complex, the absorbance increased linearly with increasing aptamer concentrations (Figure 4-13 (b)). In addition, the absorbance was increasing even at an aptamer concentration of 200 μM , which was 40 times higher than the streptavidin concentration and 2×10^4 times the K_d values as determined by SPR. The increase was not due to Ta15 binding to HABA as control experiments where the HABA was titrated with increasing aptamer concentrations showed no absorbance at 500 nm nor any change in absorbance at 348 nm. Whilst the reasons for the effect of Ta15 on the streptavidin-HABA complex can only be speculative, one possibility is that the molecules form a tertiary complex such that the absorbance is larger in this than in the binary complex. The high concentration of Ta15 necessary to observe such the absorbance change is consistent with a weak binding compared to that to streptavidin alone. This may be due to the HABA and aptamer occupying adjacent locations within the biotin binding pocket or due to the aptamer binding elsewhere on the streptavidin monomer and affecting the environment of HABA through a conformational change. Results described earlier in this chapter on the changes in intrinsic fluorescence of streptavidin monomer and the SPR results on Ta15 binding to streptavidin in presence and absence of biotin suggest that the former is more likely explanation. Ta23 showed quantitatively same behaviour with the streptavidin-HABA complex and therefore no further experiments were carried out on this method.

4.3. Discussion

Affinity constants of the aptamers for streptavidin (SA) were determined by SPR using a BIAcore3000. The affinity constants were obtained from a global fit using a single binding site model, assuming a Langmuir isotherm. As a streptavidin molecule has 4 identical subunits, it was assumed that each subunit acts as a “single molecule”. The SPR data showed aptamers bind streptavidin with high affinity (K_d values were 16.7 nM and 5.8 nM for Ta15 and Ta23, respectively). The dissociation constants of these aptamers were comparable with the values of the streptavidin-binding aptamers that reported by James and colleagues (Tahiri-Alaoui *et al.* 2002) whilst another group isolated less tight binding sequences with dissociation constants in high nanomolar range (Srisawat and Engelke 2002). In addition, SPR data also showed no significant responses of the aptamers to bovine serum albumin, a protein that has similar molecular mass and pI as streptavidin. This indicated that the aptamers bind streptavidin specifically.

However, the BIAcore binding assays using streptavidin-coated chips were performed in a similar fashion to the selection process, in which streptavidin was immobilised on a solid support (magnetic beads). The binding affinities determined by SPR reflect the binding of the aptamers to the immobilised streptavidin rather than soluble streptavidin. Therefore, other types of binding assays were needed to see if the aptamers also bind soluble streptavidin. In addition, SPR experiments cannot provide any structural information on the binding. Taking these requirements into account, fluorescence measurements were carried out in solution to search for

information on binding of the aptamers with soluble streptavidin as well as possible conformational changes caused by the interaction. The fluorescence data could be obtained by measuring changes in the intrinsic fluorescence of tryptophan residues within streptavidin upon binding with the aptamers. Since it is known that the biotin-binding pocket are formed from 4 tryptophan residues, changes in the fluorescence behaviour can indicate if the aptamers bind streptavidin in proximity to the biotin binding pocket. Whilst it is known that biotin binding quenches streptavidin fluorescence by up to 40% (Kurzban *et al.* 1990), we found binding of Ta15 quenches up to 60% (shown in Figure 4-10) and binding of Ta23 quenches up to 80% (shown in Figure 4-11). The results implied that the aptamers may have bound soluble streptavidin in proximity to the biotin-binding pocket. To try and confirm this, competitive binding between Ta15 and HABA for streptavidin was performed. Biotin is known to displace HABA from its complex with streptavidin. The results showed an unexpected behaviour; instead of decreasing in A_{500} as the complex dissociated, it showed an increase. This could be instead of displacing HABA from streptavidin the aptamer co-occupied the biotin binding pocket. A control showed no effect of the aptamer on HABA absorbance in the absence of streptavidin. However, the competitive binding was performed in buffer condition that may have favoured HABA-streptavidin interaction and not aptamer binding. In particular, the measurements were carried out in the absence of Mg^{2+} , which is known to play an important role in stabilising the tertiary structure of RNA (Grilley *et al.* 2006). This also showed in the data, the formation of Ta15 to the complex continued even at the concentration of the aptamer 10 times over the stoichiometry of $[Ta15]/[streptavidin]$ (Figure 4-13(b)) whilst the previous fluorescence experiment had shown insignificant

further changes when the concentration of the aptamers exceeded the stoichiometric ratio (Figure 4-12).

As no dissociation constant values for the aptamers were obtained from the solution binding experiments (the fluorescence measurements), conclusions cannot be drawn whether immobilising streptavidin on the surfaces would have any restrictions on binding of the aptamers to streptavidin. For the binding assays, it is important that once streptavidin immobilised on the sensor chip, it is still active, accessible to its ligands and ideally in a specific orientation. In term of binding between biotin to streptavidin, binding of a biotin-tagged peptide on the streptavidin-coated surface (as shown on the inset of Figure 4-4) indicated the immobilisation of streptavidin on the surface met the first two criteria. As the streptavidin-binding aptamers showed they bind streptavidin in proximity to the biotin binding pocket, these criteria may be met as well with binding of the aptamers to streptavidin. However, as streptavidin was covalently attached on the surface through amide bond between an amino group of lysine's side chain with a carboxyl group of the dextran layer of the CM5 chip, there are several possible different orientations of the immobilised streptavidin on the surface. For this reason, access of aptamer molecules from solution to immobilised streptavidin molecules may not be uniform. This could affect the consistency of the signal as well as association and dissociation rates that obtained from different immobilisation batches.

Secondary structural models of the Ta15 and Ta23 aptamers were generated using the Mfold program (Jaeger *et al.* 1990; Zuker 2003) and showed similarity in shape despite having quite different sequences. As shown in Figure 4-14, in both

structures, a stem loop was formed in the middle region of the sequence that apparently aligned the surrounding regions. The aligned structure was formed from almost complementary base pairs, except for one mismatched pair G-U, in both models. In addition, both structures have a bulged loop in between the aligned regions. This is consistent with their comparable affinities for streptavidin.

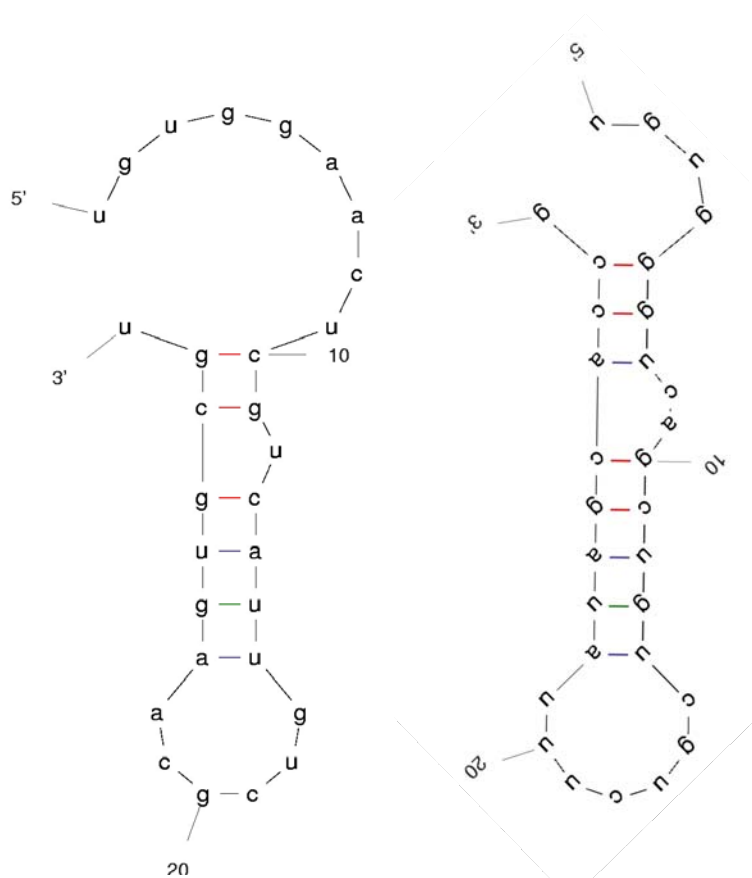


Figure 4-14: Predicted structures of the aptamers Ta15 (left) and Ta23 (right). The secondary structures of the aptamers were predicted using the Mfold program (Jaeger *et al.* 1990). Double hydrogen bonds (A:U pairs) are shown by blue lines while triple hydrogen bonds (G:C pairs) are indicated with red lines.

As the aptamers both bind streptavidin tightly and specifically, they can be employed as affinity tags for the immobilisation of RNA. Aptamer tags for streptavidin binding have advantages over biotinylation for a couple of reasons. Firstly, they can be incorporated into RNA molecules both *in vitro* and *in vivo*. Secondly, they can be included in the desired RNA at specific locations (at 5'- or 3'-ends) whilst biotin is incorporated through *in vitro* transcription at random locations.

Anti-protein aptamers that recognise functional regions such as binding pockets or active sites of the targets proteins have often been observed (Conrad *et al.* 1994; Bell *et al.* 1998). This is probably due to these regions can act as “frames”, in which specific aptamers can fold into defined secondary structures to fit into. The streptavidin-binding aptamers emerged from our selection showed evidence of their binding to streptavidin in the proximity of the biotin binding pocket and they may use the pocket as their recognition site. However, in order to be able to locate the precise binding site of the aptamers to streptavidin, further structural studies using NMR or X-ray crystallography are needed.

4.4. Applications of the streptavidin binding aptamers

Affinity tags have been applied widely to facilitate purification as well as immobilisation of biomolecules (Wada *et al.* 2003; Ashraf *et al.* 2004; Schmidt and Skerra 2007). His-tag and biotin tag have been popularly used as they both have high affinities towards their capturing systems, *i.e.*, Ni-NTA and streptavidin/avidin systems. Even though His-tag binds Ni-NTA less tight compared to biotin binding to

streptavidin/avidin, it has been hugely popular due to its reversible binding (Schmid *et al.* 1997) and easy fusion with proteins (Paramban *et al.* 2004). Efforts have been made to develop peptide tags for streptavidin/avidin system that can be easily fused into the expressed proteins as well as reversibly bind its captors as His-tag (Skerra and Schmidt 1999; Schmidt and Skerra 2007).

For DNA molecules, incorporation of biotin tag(s) has mostly been done through synthesis using either biotin-modified nucleosides or at the 5'- or 3'-ends. For RNA, incorporation of biotin is usually through transcription and *in vitro* occurs randomly. To avoid random incorporation of biotin, streptavidin-binding aptamers with high affinity can be used as affinity tags for RNA molecules as a mean for rapid detection, immobilisation, and purification through the streptavidin capture system. Those tags can be included in the desired polynucleotide sequences and it is possible to be produced both *in vitro* and *in vivo* at 5'- or 3'-end analogues to the peptide-tags that are fused at N- or C-terminal in proteins. A number of RNA affinity tags have already been suggested (Bachler *et al.* 1999; Srisawat and Engelke 2002; Tahiri-Alaoui *et al.* 2002). As our isolated aptamers have high affinity towards streptavidin, they can also be used as affinity tags for nucleic acids.

4.5. Conclusions

Aptamers that emerged from Round 21 of the selection were found to bind streptavidin specifically. The dissociation constants of these aptamers were determined by SPR, in which the K_d values for Ta15 and Ta23 were 16.7 nM and 5.8 nM, respectively. Fluorescence measurements indicated that the aptamers bind

soluble streptavidin in proximity to the biotin binding pocket. The aptamers can potentially be used as affinity tags for RNA molecules.

Chapter 5: Phosphotyrosine peptide-binding aptamers

5.1. Introduction

5.1.1. Choice of the pY peptide sequence for the aptamer selection

Over the last two decades, a new and unifying concept of cellular organisation has emerged, in which modular protein-protein interactions provide an underlying framework through which signalling pathways are assembled and controlled. From proteomic points of view, post-translational modifications such as phosphorylation commonly exert their biological effects by regulating molecular interactions, exemplified by the ability of *Src* homology-2 (SH2) domains to bind selectively to pY sites and regulate protein-protein interactions by inducing conformational changes through phosphorylation /dephosphorylation of interactants upon binding (Sadowski *et al.* 1986; Koch *et al.* 1991; Songyang *et al.* 1993; Pawson 2004).

SH2 domains were discovered and named in 1986 by Pawson and colleagues when they studied a group of cytoplasmic protein tyrosine kinases and discovered these proteins possess an additional region to the expected kinase region (known as the SH1 domain). The then newly discovered region (named the SH2 domain) was

not required for catalytic activity but modified kinase activity by substrate recognition (Sadowski *et al.* 1986).

Since then, a huge amount of research has been focused on this type of protein domain. It is now known that the SH2 domains are found within a wide variety of cytoplasmic signaling molecules that bind proteins having specific pY-containing motifs with high affinity (K_d values ranging from nM to μ M) (DeClue *et al.* 1987; Cantley *et al.* 1991; Koch *et al.* 1991; Fantl *et al.* 1992; Payne *et al.* 1993; Ladbury *et al.* 1995). The SH2 domains are thought to mediate specific protein-protein interactions (Kuriyan and Cowburn 1997). Through binding to substrates with specific pY-containing motifs, SH2 domains regulate phosphorylation. On the other hand, the phosphorylation/dephosphorylation of a specific tyrosine residue acts as an on/off switch for protein interactions. SH2 domains themselves are found in two groups of proteins. One group has enzymatic activity that is necessary for signal transduction, such as the tyrosine protein kinases of the Src-related family (Sadowski *et al.* 1986; Payne *et al.* 1994). The other group has no enzymatic activity and act as so-called adaptor molecules in bridging protein-protein interactions, such as EGF (epidermal growth factor) and Grb2 (growth factor receptor-bound protein 2) (Sierke *et al.* 1993; Nioche *et al.* 2002). In 2001, when the first draft of human genome was published, it reported that the human genome codes for 87 proteins that contain a total of 95 SH2 domains (Venter *et al.* 2001).

SH2 domains are independently folding modules of around 100 amino acid residues that have ability to specifically bind pY containing proteins (Anderson *et al.* 1990; Moran *et al.* 1990). More specifically, SH2 domains bind directly (Waksman

et al. 1992) to the phosphorylated state only (Lemmon and Ladbury 1994) and different SH2 domains have distinct binding preferences for specific pY-containing sequences (Songyang *et al.* 1993; Long *et al.* 1999b; Beebe *et al.* 2000). In 1992, Kuriyan and colleagues published the first crystal structures of complexes of a SH2 domain with two pY peptides and revealed the molecular basis of the recognition of the pY moiety by the SH2 domain (Waksman *et al.* 1992). However, the preferential binding of an SH2 domain to a specific pY-containing sequence only came clear later when researchers in the Cantley lab used a phosphopeptide library to determine sequence specificity (Songyang *et al.* 1993). In this experiment, a pY-peptide library (Gly-Asp-Gly-pTyr-X-X-X-Ser-Pro-Leu-Leu-Leu, where X is any of the amino acids) was constructed to explore the optimal sequence for binding various SH2 domains of tyrosine kinases. The results indicated that different SH2 domains have different “tastes” in pY peptides: one group of SH2 domains preferred sequence with the general motif pY-hydrophilic-hydrophilic-Ile/Pro while another group selected the general motif pY-hydrophobic-X-hydrophobic. More specifically, individual members of these groups preferred unique sequences, except the Src family, in which all selected the sequence pTyr-Glu-Glu-Ile (Songyang *et al.* 1993). Thus, in addition to the discovery of binding preferences for SH2 domains to pY peptide sequences, results gained from this experiment also hinted that apart from having a pY-binding pocket as shown by an earlier crystal structural study (Waksman *et al.* 1992), SH2 domains also possess other elements that interact with residues to support the overall binding. This implication was subsequently confirmed by a number of structural studies using X-ray crystallography and NMR, which revealed SH2 domains have a second binding surface that engages with the specific residues in addition to the pY-binding pocket (Eck *et al.* 1993; Waksman *et al.* 1993; Lee *et al.* 1994; Mikol *et al.*

1995; Narula *et al.* 1995; Pletneva *et al.* 2006). Figure 5-1 shows an NMR structure of a SH2/peptide complex.

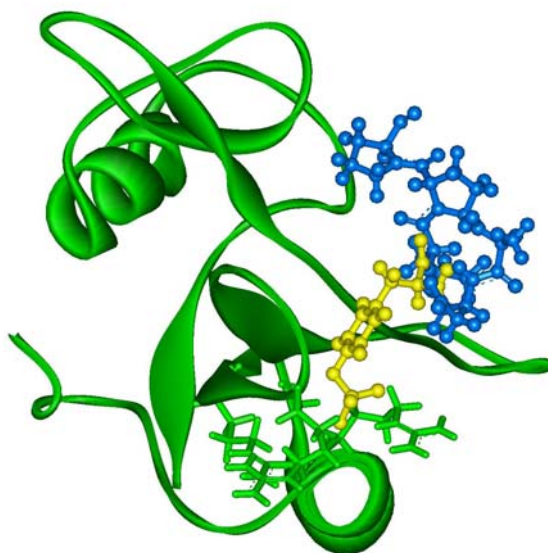


Figure 5-1: NMR structure of a SH2/pY peptide complex (Pletneva *et al.* 2006). The SH2 domain was of the Itk SH2 and is shown as the green solid ribbon model. The peptide sequence was pTyr-Val-Pro-Met-Leu shown as the stick and ball model with pY shown in gold and the other residue of the peptide shown in blue. The pY binding pocket of the Itk SH2 domain consists of three arginines (positively charged residues) shown as the green stick model. In addition to the binding of pY, other residues of the peptides also make contact with the SH2 domain. The image created from pdb file 2ETZ using WebLab ViewerLite.

In addition to the pioneering work by Cantley and colleagues that established three residues immediately C-terminal to the pY are key determinants of specificity, and that sequence preferences in targets could be correlated with particular residues in the SH2 domains (Songyang *et al.* 1993), a number of studies followed to determine the effects of specific residues flanking on both sides of the pY (Long *et al.* 1999a; Beebe *et al.* 2000; Staub *et al.* 2004) and also with more distant residues to be examined (Larose *et al.* 1995). In the 2000 publication of Pei and colleagues, a synthetic random pY peptide library (X-X-pTyr-X-X-X, where X is any amino acid) was used to screen against the N- and C-terminal SH2 domains of protein tyrosine phosphatase-1 (SHP-1) to determine the optimal binding sequences. The results revealed consensus sequence domains that have high affinity (K_d values in low micromolar range) for both SH2 domains in the protein. The N-terminal SH2 domain can recognise two classes of peptides with consensus sequences Leu-X-pTyr-(Met/Phe)-X-(Phe/Met) and Leu-X-pTyr-Ala-X-Leu. The C-terminal SH2 domain selects consensus sequence X-X-pTyr-Ala-X-(Leu/Val). These findings also fit to the model pTyr-hydrophobic-X-hydrophobic consensus sequence that was determined by Cantley and colleagues.

Taking these data into accounts, we chose a peptide target for the aptamer selection with a sequence Biotin-Gly-Gly-Gly-Gly-Ser-Phe-Val-pTyr-Ala-Lys-Leu-NH₂, where the region Gly-Gly-Gly-Gly-Ser acts as a linker. Biotin at the N-terminus was incorporated to facilitate the capture of the peptide on solid support using streptavidin-coated magnetic beads.

5.1.2. Rationale for the selection of pY peptide-binding aptamers

As presented in the chapters 1 and 3, aptamers have been generated that bind various targets with specificity and high affinity and in this respect they are conceptually equivalent to antibodies. Our intention is to generate aptamers for use in proteomic analysis of protein phosphorylation. A given mammalian cell has thousands of phosphoproteins (Garcia *et al.* 2005; Mumby and Brekken 2005). Even though there have been a number of aptamers isolated for individual proteins such as Erk2 (extracellular signal-regulated kinase 2) (Seiwert *et al.* 2000; Vaish *et al.* 2002), generation of specific aptamers for all the phosphoproteins in cells not only requires an enormous amount of effort but also would take many years to fulfil. In addition, many phosphorylated proteins, especially the ones with low abundances in cells, have not been mapped since not all of proteins expressed can be predicted from the genome sequences due to gene splicing as well as other modifications. Therefore, we are testing a possibility of generating aptamers that can recognise not individual phosphorylated proteins but a class of those that share common features. So pY proteins, which are recognised by SH2 domains, were chosen as the representative class. There are a couple of reasons for the pursuit of this idea. Firstly, SH2 domains serve as the prototype of a growing family of protein-interaction modules involved in transmitting signals from external and internal cues by their ability to specifically bind a large number of proteins through recognition of specific pY peptide sequences. Therefore, by choosing a pY peptide sequence that is recognised by SH2 domains, we are trying to generate aptamers mimicking the SH2 domains, and can subsequently recognise a class of proteins that contain the target sequence. Secondly, as aptamers are considered as antibody analogues and antibodies against pY proteins have been successfully developed that bind the pY proteins, therefore it should be

possible to develop equivalent aptamers. The antibodies have been employed in purification of phosphoproteins (Daniel *et al.* 1985) or enrichment of pY peptides gained from proteolysed pY proteins prior to mass spectrometry identification (Jensen 2004). One of examples of using aptamers to probe protein phosphorylation is the research at Ribozyme Pharmaceutical Inc. that discovered ribozymes, which were selected with the purpose of being inhibitors for Erk2, that bind only unphosphorylated Erk2 with high affinity ($K_d = 500$ pM) (Vaish *et al.* 2002). In combination with the aptamers isolated that bind doubly phosphorylated Erk2 (Seiwert *et al.* 2000), those aptamers have been used to monitor phosphorylation of Erk2 (Vaish *et al.* 2004).

In addition, aptamers not only exhibit highly specific molecular recognition properties, but also are able to modulate the function of their cognate targets in a highly specific manner (Famulok *et al.* 2001). A number of papers demonstrates controlled expression of aptamers inside cells (dubbed intramers) demonstrated their potential as intracellular inhibitors of biomolecules (Morinelli *et al.* 1997; Blind *et al.* 1999; Konopka *et al.* 2000; Theis *et al.* 2004; Choi *et al.* 2006). This suggested that functional proteomics can also benefit if this type of aptamers is successful developed.

5.2. Characterisation of the pY peptide binding aptamers

The two most abundant clones from the final round of the selection (Round 32), Tap1 and Tap2, were analysed to determine their binding affinities to the pY peptide. Surface plasmon resonance (SPR) measurements were conducted using a

BIAcore3000, in which streptavidin-coated chips were used for immobilising the biotinylated pY peptide whilst gold chips were employed for immobilising the aptamers on sensor chip surfaces.

5.2.1. SPR measurements using immobilised peptide

Streptavidin-coated chips were employed as matrix sensor surfaces for immobilisation of the pY peptide. Preparation of the streptavidin-coated chips is described in Chapter 2. The pY peptide had a biotin tag at N-terminus, which was used to immobilise it on the streptavidin-coated chips. This approach had been applied in partitioning during the selection process but in the SPR measurements, the biotinylated peptide was pre-immobilised on the surfaces and interaction was then monitored through changes in responses corresponding to different concentrations of the aptamers. Figure 5-2 shows association and dissociation phases of interaction of Tap1 with the pY peptide, in which the pY peptide was immobilised on the streptavidin-coated surface.

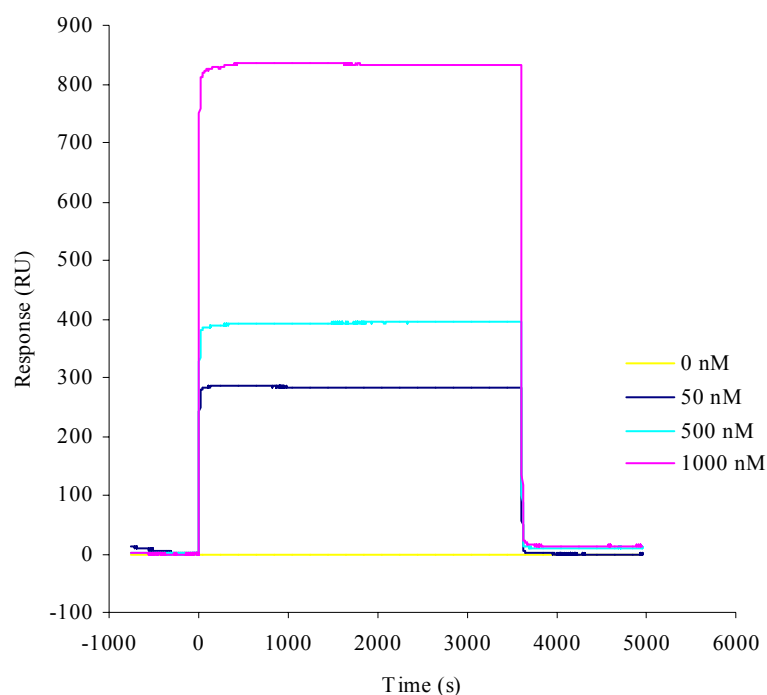


Figure 5-2: BIAcore3000 sensorgrams demonstrating binding of Tap1 with the pY peptide. The pY peptide, which had a biotin tag at its N-terminus, was immobilised on streptavidin-coated chip. Interaction was monitored by changes in response (RU). Interaction between the aptamer and the pY peptide was assessed through association and dissociation phases. The diagram shows 4 curves corresponding to association and dissociation phases of 4 different concentrations of Tap1, as representatives. In the fitting regime, a total of 10 curves corresponding to samples of Tap1 at different concentrations ranging from 0 nM to 5000 nM were used as shown in Appendix 7. A global fit, assuming a 1:1 Langmuir binding model, resulted in a K_d of 422 nM and the model was fitted with χ^2 of 6.67

Figure 5-3 shows association and dissociation phases of interaction of Tap2 with the pY peptide, in which the pY peptide was immobilised on the streptavidin-coated surface.

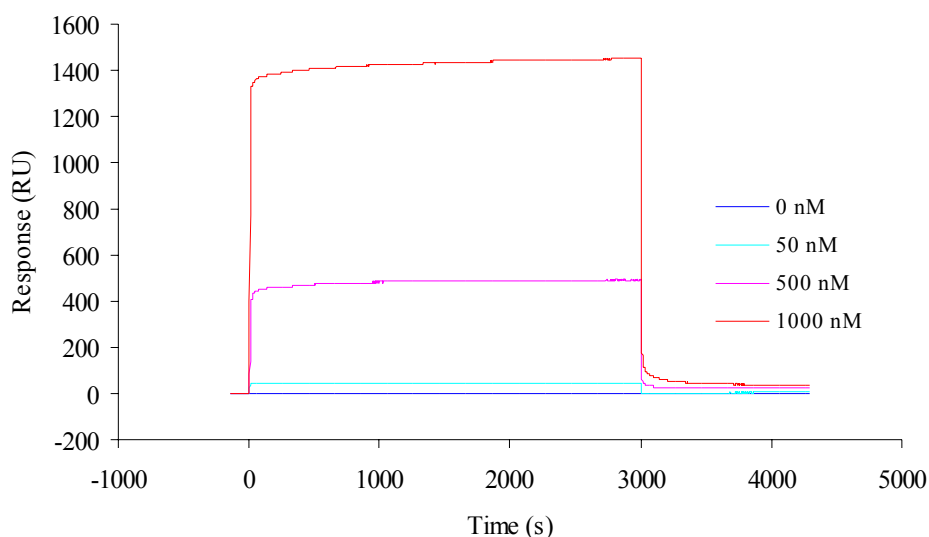


Figure 5-3: BIAcore3000 sensorgrams demonstrating binding of Tap2 with the pY peptide. The pY peptide, which had a biotin tag at its N-terminus, was immobilised on streptavidin-coated chip. Interaction was monitored by changes in response (RU). Interaction between the aptamer and the pY peptide was assessed through association and dissociation phases. The diagram shows 4 curves corresponding to association and dissociation phases of 4 different concentrations of Tap2, as representatives. In the fitting regime, a total of 11 curves corresponding to samples of Tap2 at different concentrations ranging from 0 nM to 1000 nM were used as shown in Appendix 8. A global fit, assuming a 1:1 Langmuir binding model, resulted in a K_d of 128 nM and the model was fitted with χ^2 of 12.2.

Dissociation constant values obtained from independent SPR experiment sets using streptavidin-coated chips are shown in Table 5-1.

Table 5-1: Dissociation constants of the pY peptide-binding aptamers, Tap1 and Tap2. The values were obtained from a global fit of independent sets of data of SPR measurements using streptavidin-coated surfaces.

Aptamer	SPR data from global fits**			
	Experimental set 1		Experimental set 1	
	K_d (nM)	χ^2	K_d (nM)	χ^2
Tap1	422	6.67	fitting failed	
Tap2	128	12.2	567	61

** Data analysis using global fits is fully presented in the appendices.

Three independent SPR-based determination of K_d were made each over a range of concentrations. These experiments were carried out over a period of several weeks and the results of global fitting to each independent data set are given in Table 5-1. The estimated K_d values show experiment to experiment differences that are larger than the within experiment variation. This probably reflects systematic variations in the experimental circumstances, such as changes in the chip surface. This can be seen by the substantial difference in RU for the same concentration of aptamer in different experiments. It is for this reason that a global fitting of the entire

data of all three independent experimental sets was not carried out. Therefore, deriving an “average” K_d value from independent experiments is not appropriate.

In addition, the specificity of the phosphopeptide-binding aptamers to their target was also investigated using SPR. Nonphosphorylated peptide was immobilised on the SA-coated chip (also through biotin-streptavidin binding of a biotin-tag at N-terminus of the peptide). However, the binding assay was only carried out with Tap2 and the preliminary result is shown in Figure 5-4. The responses recorded on SPR indicated the nonphosphorylated peptide did not bind with a typical saturation response expected from the Langmuir binding model. The absence of a saturating response to increasing aptamer suggests only a weak and non-specific interaction in the former. Although this represents only a single experiment and must therefore be treated with caution the SPR response is entirely consistent with no specific interaction between the aptamer and the nonphosphorylated peptide. Control experiments with a streptavidin-coated chip described in Chapter 3 (section 3.3) revealed that the aptamer isolated at Round 32 did not bind to the streptavidin and the results obtained with the nonphosphorylated tyrosine peptide are also consistent with this.

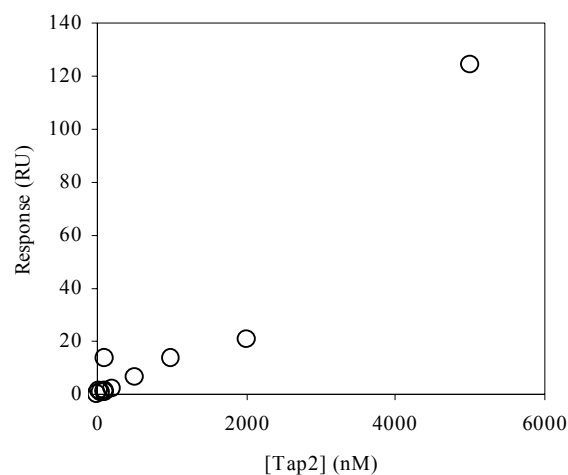


Figure 5-4: Binding to Tap2 to nonphosphorylated state of the peptide. The measurements were carried out on a streptavidin-coated chip. The nonphosphorylated tyrosine peptide was attached on the chip through its biotin tag at the N-terminus.

As previously discussed in Chapter 4, the affinity constants obtained by these SPR measurements were for the binding of the aptamers to the pY peptide target immobilised on the streptavidin-coated surface and this was similar with the binding format used for partitioning during the selection. Therefore, we designed a reverse binding format, in which aptamers were immobilised on the surface of gold sensor chips.

5.2.2. SPR measurements using immobilised aptamers

In addition to using immobilised peptide for the binding assays, which monitored interaction through changes in response with variation in aptamer concentration, we also performed a reverse binding format, in which the aptamers were immobilised on the gold sensor chips and pY peptide samples of different concentrations were injected onto the surfaces to monitor the binding.

Typically, attachment of synthetic DNA on gold is through a 5'- or 3'-thiohexyl group that self-assembles on the surface (Herne and Tarlov 1997; Bamdad 1998). In the case of RNA, although thiol modified synthetic RNA could be made the chemical synthesis of RNA is much more expensive than DNA. Moreover we wanted a versatile RNA immobilisation method that could be used with any RNA aptamer. To achieve this we took advantage of the constant regions flanking the aptamer which were used for PCR amplification during selection. Figure 5-5 shows the approach that we adopted; a synthetic DNA linker (TaL1) was prepared that carried a 3'-thiol sequence complementary to the 5'- constant region of the aptamer. Attachment of TaL1 to gold was through 3'-thiol group and the aptamer subsequently immobilised *via* DNA-RNA hybridization in the constant region. This approach has the advantage that the TaL1 linker can be used to immobilise any aptamer carrying the constant sequence.

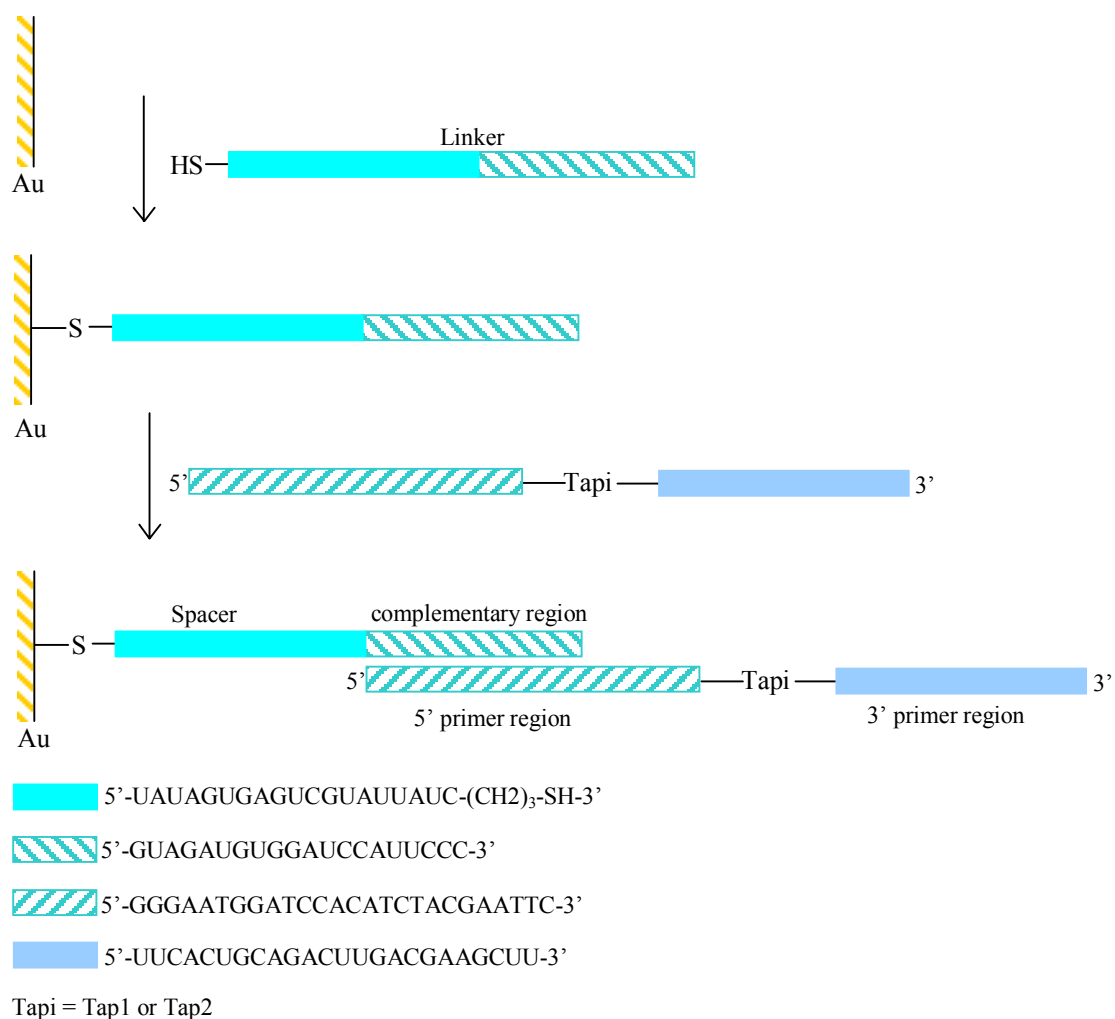


Figure 5-5: The concept of using a DNA linker. A thiol DNA linker was attached on the gold chips through formation of -S-Au bond. The aptamer was then immobilised on the surface through hybridizing with its complementary region of the linker.

For the formation of a dense thiol TaL1 layer on gold substrate, it is important that the gold surface is well-cleaned. It is known that although gold is a relatively inert metal, it easily gets contaminated with organic substances that form a thin layer

on the surface and prevent thiol attachment. Highly oxidative solutions such as Piranha or warm and concentrated nitric acid solutions can help to clean gold substrate effectively. The clean gold can be stored in sealed bags contained nitrogen gas. In addition, the BIAcore system must be subjected to an intensive clean before each immobilisation. We applied a combination of acidic, basic, detergent, urea and ethanol solutions for washing the system. The attachment of the TaL1 linker on a gold chip is shown in Figure 5-6.

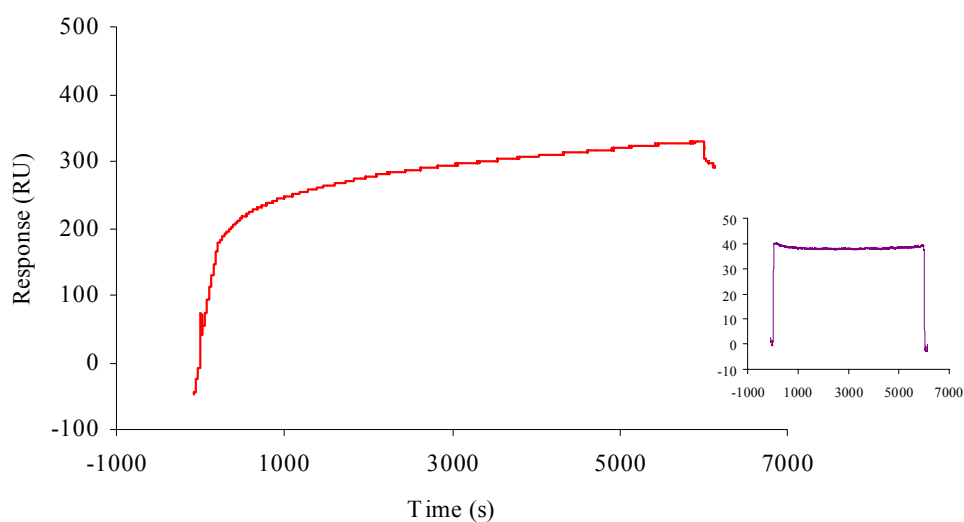


Figure 5-6: Attachment of a 3'-thiol linker TaL1 on a gold chip. The reaction of the 3'-thiol DNA linker with the gold substrate was performed in water. A 5 μ M thiol solution was used for the immobilisation. The thiol solution was injected at a flow rate of 2 μ L/min and over the course of 6000 s. After each injection, the baseline was reset to 0 RU. The injection was repeated 5 times consecutively to ensure a saturated level was reached. For the fifth injection (inset), no further binding was observed as the surface was saturated.

The aptamers were then immobilised on to the TaL1-modified surfaces. Figure 5-7 shows association and dissociation phases of interaction of the pY peptide with Tap1, in which Tap1 was immobilised on the surface through the TaL1 linker.

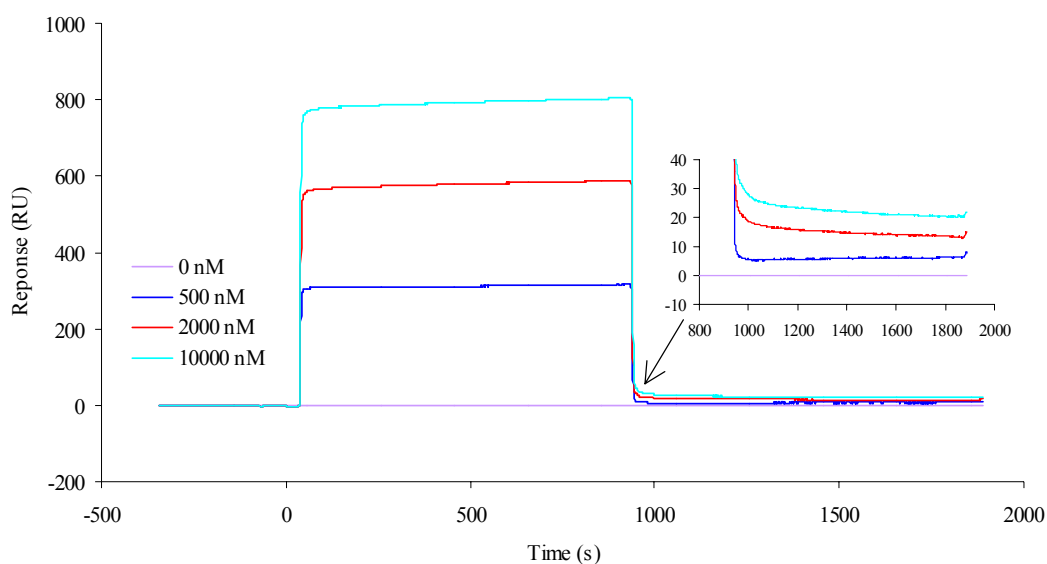


Figure 5-7: BIAcore3000 sensorgram demonstrating binding of the pY peptide to Tap1. Tap1 was immobilised on a gold sensor chip through hybridizing with TaL1 that was previously attached on the chip. Interaction was monitored by changes in response (RU). Interaction between the pY peptide and the aptamer was assessed through association and dissociation phases. The diagram shows 4 curves corresponding to association and dissociation phases of 4 different concentrations of the pY peptide, as representatives. In the fitting regime, a total of 10 curves corresponding to samples of the pY peptide at different concentrations ranging from 0 nM to 10000 nM were used (with the 1000 nM sample run in duplicate) as shown in Appendix 10. A global fit, assuming a 1:1 Langmuir binding model, resulted in a K_d of 6.63 nM and the model was fitted with χ^2 of 5.63. The inset shows a zoom-in of the dissociation phases.

Figure 5-8 shows association and dissociation phases of interaction of the pY peptide with Tap2, in which Tap2 was immobilised on the surface through the TaL1 linker.

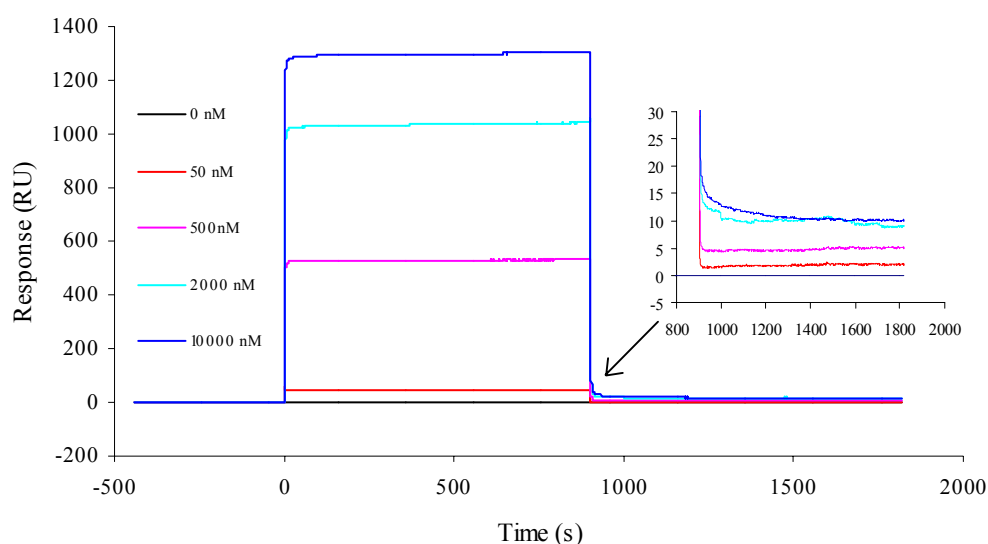


Figure 5-8: BIAcore3000 sensorgram demonstrating binding of the pY peptide to Tap2. Tap2 was immobilised on a gold sensor chip through hybridizing with TaL1 that was previously attached on the surface by thiolation. Interaction was monitored by changes in response (RU). Interaction between the pY peptide and the aptamer was assessed through association and dissociation phases. The diagram shows 5 curves corresponding to association and dissociation phases of 5 different concentrations of the pY peptide, as representatives. In the fitting regime, a total of 11 curves corresponding to samples of the pY peptide at different concentrations ranging from 0 nM to 10000 nM were used (with the 1000 nM sample run in duplicate) as shown in Appendix 12. A global fit, which assumed a 1:1 Langmuir binding model, resulted in a K_d of 219 nM and the model was fitted with χ^2 of 5.31. The inset shows a zoom-in of the dissociation phases.

Affinity constants of binding of the aptamer Tap1 to the peptide target were obtained in the same way. The results presented in Table 5-2 show that the two aptamers have comparable affinities for their target and this is consistent with the sequencing result that found both sequences have the same abundance in the population (~30%).

Table 5-2: Dissociation constants of the pY peptide-binding aptamers, Tap1 and Tap2, obtained from SPR measurements using immobilised aptamer on gold chips. The table shows values obtained through global fits for the pY peptide-binding aptamers, Tap1 and Tap2.

Aptamer	SPR data from global fits**					
	Experimental set 1		Experimental set 2		Experimental set 3	
	K _d (nM)	χ^2	K _d (nM)	χ^2	K _d (nM)	χ^2
Tap1	663	5.63	525	0.71	Fitting failed	
Tap2	889	1.38	219	5.31	475	1.36

**Data analysis using a global fit is fully presented in the appendices.

5.2.3 Comparison of the binding affinities obtained with immobilised peptide and immobilised aptamer

Dissociation constants of the pY peptide-binding aptamers Tap1 and Tap2 determined by SPR using immobilised peptide showed slightly lower values than those obtained from measurements using immobilised aptamer but in the same order of magnitude. The values in tables 5-1 and 5-2 are collected in Table 5-3 for ease of comparison.

Table 5-3: Dissociation constants of the pY peptide-binding aptamers, Tap1 and Tap2, obtained from SPR measurements. The values, which obtained from global fit, are presented for a comparison.

Aptamer	Modified surface type	SPR data from global fits **					
		Experimental set 1		Experimental set 2		Experimental set 3	
		K _d (nM)	χ^2	K _d (nM)	χ^2	K _d (nM)	χ^2
Tap1	Immobilised peptide	422	6.67	Fitting failed		No experiment	
	Immobilised aptamer	663	5.38	525	2.71	Fitting failed	
Tap2	Immobilised peptide	128	12.2	567	61	No experiment	
	Immobilised aptamer	889	1.38	219	5.31	475	1.36

** Data analysis using global fits is fully presented in the appendices.

From these data we can draw two main conclusions. Firstly, binding of the peptide to streptavidin does not affect its interaction with the aptamer. This suggests that the streptavidin does not cause a steric interference between peptide and aptamer, nor does the aptamer bind to the peptide-streptavidin interface. Secondly, the use of the TaL1 linker to immobilise the aptamer has not affected its affinity for the peptide. The second point confirms that the analytical utility of the Tap1 and Tap2 aptamers is not compromised by their immobilisation. Further discussion of this is presented in Chapter 6.

Comparing the dissociation constant for both aptamers in both formats revealed no significant differences in affinity. In fact the variation between aptamers and/or formats was comparable to that between replicate independent experiments with the same format (Table 5-3). This is a significant finding as it is well known that when protein-hapten conjugates are used to raise antibodies the antibodies often recognise the linker as part of the protein as well as the hapten (Hoffman *et al.* 1972; Sykulev *et al.* 1992; Siuzdak *et al.* 1994). As a consequence the antibody often shows lower affinity for the hapten than anticipated.

5.3. Discussion

The aptamers that emerged from the final round (32) of the selection were found to bind the pY peptide target and the affinity constants were determined using SPR using immobilised peptide and immobilised aptamer.

Affinity constants of the aptamers were initially determined using a similar format to that used in partitioning during the selection process, in which streptavidin-coated surfaces were used to immobilise the biotinylated pY peptide. This raised the question whether the affinity constants were of aptamers bound solely to the peptide or whether the streptavidin was also involved in the interaction. To address this, we designed a reverse binding format with the aptamer immobilised on the SPR chip.

As described for the SA binding aptamers, predicted secondary structures were obtained from the Mfold program and these are shown in Figure 5-9. Between the two aptamers, affinity data showed Tap1 had a slightly lower affinity to the pY peptide compared to Tap2. However, the difference was in the same order of magnitude and therefore was probably not significant. This is consistent with the sequence analysis results, which showed both sequences had the same frequency in the population. Although Tap1 and Tap2 showed similar affinities, predicted secondary structures obtained from the Mfold program were different. Tap1 had a cylinder-like shape with two loops separated by stem regions whilst Tap2 had a V-shape with two stems terminating in loops.

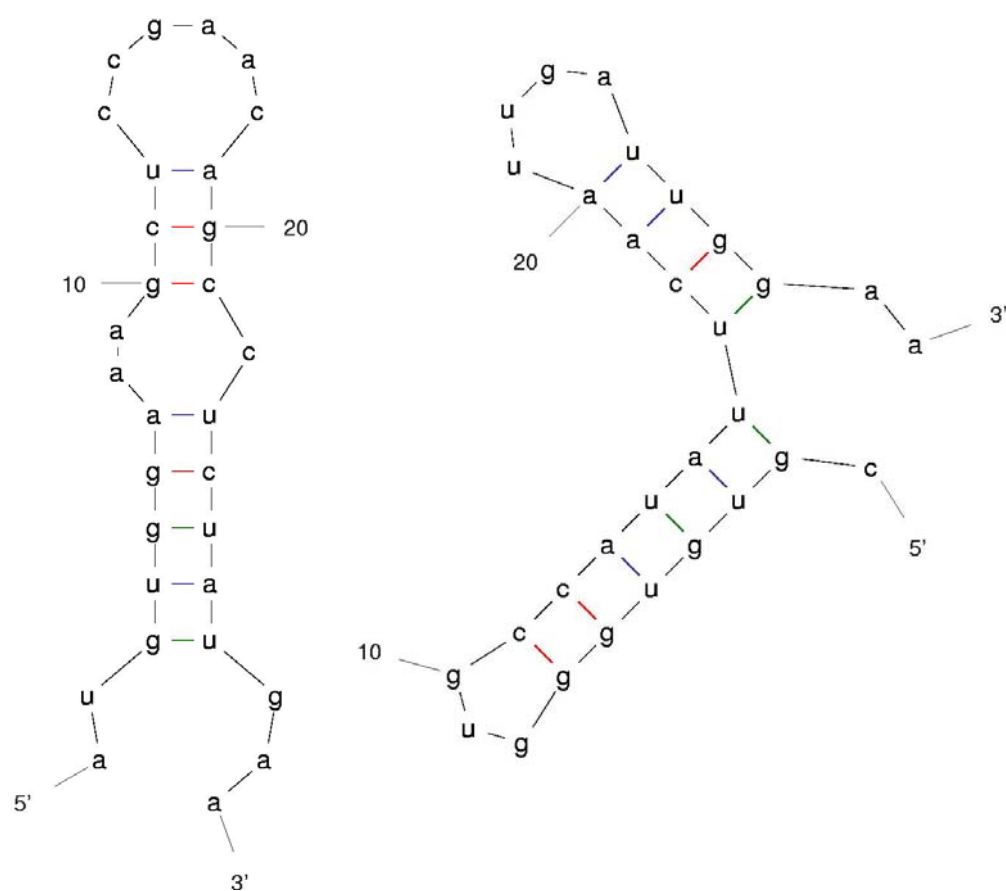


Figure 5-9: Predicted secondary structures of the aptamers Tap1 (left) and Tap2 (right). The secondary structures of the aptamers were predicted using the Mfold program (Jaeger *et al.* 1990; Zuker 2003). Double hydrogen bonds (A:U pairs) are shown by blue lines while triple hydrogen bonds (G:C pairs) are indicated with red lines.

When a comparison of the dissociation constant obtained from the immobilised peptide was made with that of immobilised aptamer, the difference was in the same order of magnitude and was probably insignificant given the inter-experimental variation. This suggests that there are no significant surface steric independent effects on the binding. It is worth noting that SPR measurements that used immobilised peptide were carried out under binding conditions very similar to those used during the selection. It is not therefore surprising that tight binding was observed and has been reported for other ligands (Tahiri-Alaoui *et al.* 2002). Aptamers are often used to bind their targets in complex mixtures and therefore it is important that they also show tight binding when they are immobilised on solid supports and their targets are solution. This applies to aptamers for example in biosensors or affinity chromatography. Immobilised aptamers that retain their binding abilities had been observed in previous work (Kirby *et al.* 2004; Collett *et al.* 2005). Our binding assays using immobilised aptamer demonstrated the pY peptide-binding occurred with very similar affinity to those for immobilised peptide. We can therefore conclude that they retained their binding abilities when immobilised on the solid support.

Immobilisation of RNA aptamers presents more of a challenge than DNA aptamers. The latter are typically synthetic and can be prepared with a 5'-thiohexyl group that self assembles on gold. Synthetic RNA is much more expensive than DNA and the aptamers are usually produced through *in vitro* transcription, giving less opportunity to introduce additional chemistries. We therefore took advantage of DNA-RNA hybridisation to build-up a multi-layer structure on the chip surface. The thiol layer was self assembled thiohexyl-DNA with a sequence complementary to the

primer region of the aptamers and the RNA aptamers then bound to this linker sequence. This strategy offers a completely generic method of RNA aptamer immobilisation as the primer sequence will be common to all aptamers isolated from this library. Results from this work also suggests that non of the primer sequence is involved in ligand binding as if it were then ligand binding would be competitive with the hybridisation and lead to RNA strand displacement.

As the selected aptamers were aimed to bind proteins that contain the pY peptide sequence, it is important that the aptamers are also able to bind pY-containing proteins tightly and specifically. Therefore, further binding assays are needed to investigate binding of the aptamers with the protein analogues that share the pY peptide sequence. Another approach to test this is using aptamers to capture proteins from cells, which are known to contain pY proteins, rather than binding assays with individual proteins. Description of this approach is presented in the future work section in Chapter 6.

Furthermore, if these aptamers are able to bind the protein analogues, expression of the aptamers in cells (dubbed as intramers) should also be considered. As the selected aptamers are expected to mimic SH2 domains, a type of protein domains that involve in cell signalling by modulating the proteins, the aptamers are possibly able to act as modulators as well. Evidence of intramers acting as modulators or inhibitors when expressed in cells have also been reported (Blind *et al.* 1999; Famulok *et al.* 2001). This would make the aptamers have high potential in drug discovery.

5.4. Conclusions

RNA aptamers have been isolated that bind the pY peptide target with dissociation constants in the high nanomolar range. Affinities of the pY peptide binding aptamers and their target were determined by SPR. Results obtained from using two formats of binding assays showed no significant difference (values in the same order of magnitude). This indicated that the contribution of surface steric hindrances was not significant in the bindings of the aptamers to their target. In addition, we also demonstrated that the pY peptide aptamers were able to bind their target when they were immobilised on the solid support and this is an important criterion for their applications in analytical and clinical fields.

Chapter 6: Conclusions and future work

6.1. Summary

The overall aim of this project was to select aptamers that bind pY containing sequences in a particular context (those are that bound by a SH2 domain). The reason for attempting this was to generate a new class of reagents for proteomic applications.

The selection strategy involved a reaction between the aptamer library and the target peptide in solution, followed by isolation of the complex using streptavidin coated magnetic beads. The complexes were bound to the beads through an N-terminal biotin group on the target peptide.

The first aptamers emerged (Round 21) were found to bind streptavidin (SA) instead of the peptide target. After modifying the selection protocol to pre-absorb SA-binding sequences from the library, the second class of aptamer emerged (Round 32) was found to bind the pY peptide. Both these classes were further characterised.

6.1.1. Streptavidin binding aptamers

Two sequences appeared more than once in the population of Round 21 (Ta15 and Ta23) and were found to bind streptavidin. These were characterised using surface plasmon resonance (SPR), fluorescence spectroscopy and spectrophotometry. The SPR measurements were conducted using a BIAcore3000 and provided information on affinities of the aptamers for streptavidin. The dissociation constants of Ta15 and Ta23 were 16.7 nM and 5.8 nM, respectively. In addition, SPR measurements for specificity showed the aptamers had no significant affinity for bovine serum albumin (BSA), a protein that has similar molecular mass and pI to streptavidin. This implied that the streptavidin-binding aptamers specifically interacted with their target. However, the dissociation constants obtained from SPR measurements were for the interactions of the aptamers to immobilised streptavidin. Therefore, other types of binding assays were needed to determine if the aptamers also bind soluble streptavidin. In addition, SPR data yielded no structural information on the binding between the aptamers and their target. Fluorescence measurements in solution were designed to meet these shortcomings of the SPR experiments. Fluorescence studies showed that the aptamers bound streptavidin in solution and quenched the intrinsic tryptophan fluorescence by around 60% and 80% compared with quenching of up to 40% caused by biotin. This suggests that binding of the aptamers to their target was not in proximity of the biotin binding site, which has 4 tryptophan residues in it. Competitive binding assays through spectrophotometric measurements subsequently showed that the aptamers did not displace 2-(4'-hydroxyazobenzene) benzoic acid (HABA) from biotin binding pocket. It would be expected that if the HABA was displaced then its absorbance at 500 nm would decrease. In contrast the absorbance

actually increases probably due to the aptamer and HABA co-occupying the binding pocket.

Secondary structure models were generated using the Mfold program (Jaeger *et al.* 1990; Zuker 2003) and showed a similarity in shape for both aptamers. As shown in Figure 4-14, both structures had a stem loop that was formed in the middle region of the sequence and apparently aligned the surrounding regions. The aligned structure was formed from almost complementary sequences, except for one mismatched pair G-U, in both models. In addition, both structures have a bulged loop in between the aligned regions.

Interestingly, both experimental data and the models suggested that these two streptavidin-binding aptamers were similar. As they both bind streptavidin tightly and specifically, they can be employed as affinity tags for immobilisation of RNA. RNA aptamer tags for streptavidin have advantages over the biotin tag for a couple of reasons. Firstly, they can be incorporated into RNA molecules both *in vitro* and *in vivo*. Secondly, they can be included in the desired RNA at specific locations (at 5'- or 3'-ends) whilst biotin is incorporated through *in vitro* transcription at random locations.

6.1.2. Phosphorylated peptide binding aptamers

The selected aptamers were found to bind a chosen pY peptide. Two of the most abundant clones, Tap1 and Tap2, which appeared 10 times each out of 30 clones in the sequenced population, were assayed to determine the binding affinities to the pY

peptide. Affinity measurements were performed with a BIAcore3000 using streptavidin-coated chip for immobilised peptide and gold chip for immobilised aptamer. With the former, the biotinylated peptide was immobilised on the surfaces through high affinity interaction of biotin-streptavidin. Dissociation constants for Tap1 and Tap2 measured on this surface type were 422 nM and 128 nM, respectively. For the latter type, a reverse binding format was used. A 3'-thiol polynucleotide linker, TaL1, was used to facilitate immobilisation of the aptamers on the surface. The thiol linker was covalently attached to the gold surface of chips by thiolation, and the aptamers are then immobilised through hybridisation of the complementary regions between the aptamers and TaL1. Affinity measurements using immobilised aptamer resulted in dissociation constants of 663 nM and 475 nM for Tap1 and Tap2, respectively and were comparable with the result obtained from measurements using immobilised peptide (in the same order of magnitude). The consistency of the affinity constants obtained from the two types of binding assays indicated that there were no significant surface steric hindrance effects that influenced the binding of the aptamers to their pY peptide target. In addition, the binding assays using immobilised aptamer also demonstrated that the aptamers were able to bind their target when they were immobilised on solid supports. This retention of ability of the aptamers is important in their application to bioanalysis as well as drug delivery.

Between the two aptamers, affinity data showed Tap1 had a slightly lower affinity to the pY peptide compared to Tap2. However, the difference was in the same order of magnitude and therefore was probably not significant. This is consistent with the sequence analysis results, which showed both sequences with the

same frequency in the population. However, predicted secondary structures (Figure 5-9) obtained from the Mfold program showed different folding shapes. Tap1 had a cylinder-like shape with two loops separated by stem regions whilst Tap2 had a V-shape with two stem terminated in loops.

6.2. Future work

6.2.1. Further characterisation of Tap1 and Tap2

Since the aptamers were selected against a pY peptide sequence that is present in a class of pY proteins, further research is recommended to investigate if these aptamers can bind different proteins in phosphorylated states in this class.

The desired experiments include:

- Repeat binding assays for non-phosphorylated peptide;
- Screen pY containing proteins for binding to Tapi.

Screening pY-containing proteins can be done by dot-blot assays. Different proteins are spotted on a nitrocellulose membrane. After probing with Tapi, the membrane is washed and exposed to Biotin-TaL, where TaL is reverse and complementary to a primer region of Tapj ($j = 1,2$), for hybridisation. The protein-aptamer complexes are then identified with Horseradish Peroxidase (HRPO). A general scheme is described and Figure 6.1.

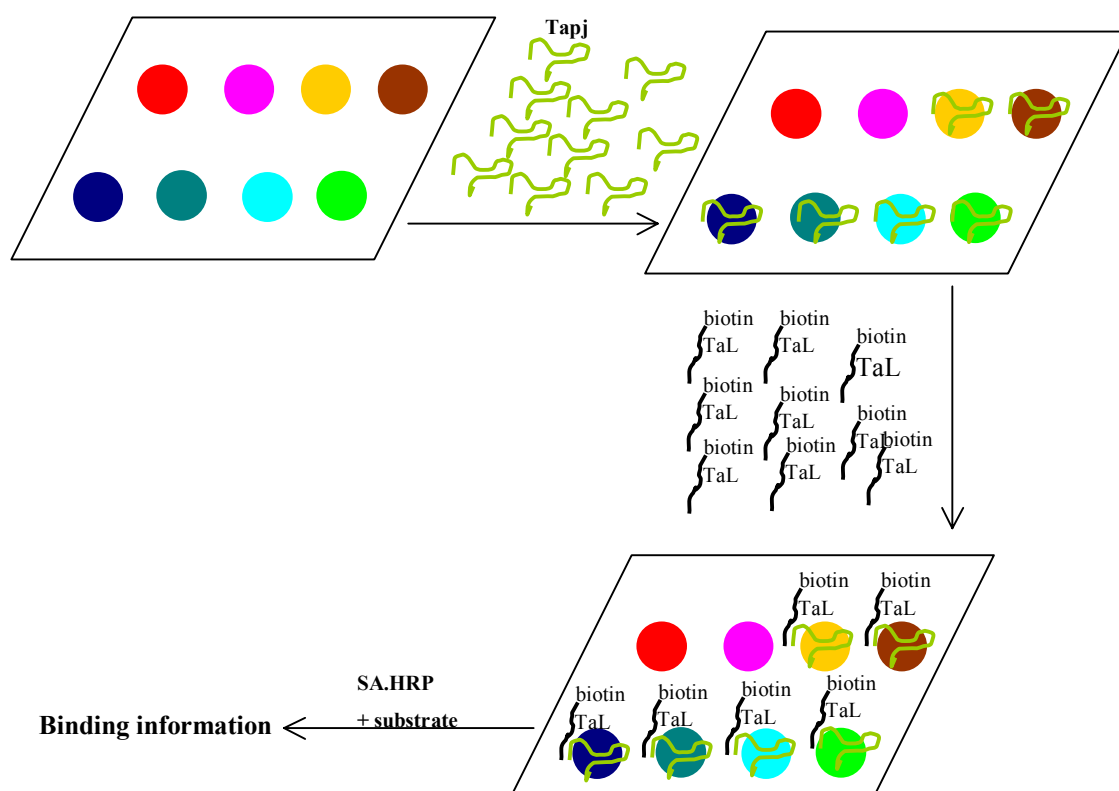


Figure 6-1: A general scheme of a dot-blot experiment to screen pY-containing proteins for binding to Tapj. Different pY proteins are illustrated dots in different colours whilst Tapj indicates either Tap1 or Tap2.

6.2.2. Analysis of cell lysates: Compare Tapi with anti pY antibodies

Along with the screening experiments, the selected aptamers can also be tested directly for their abilities to bind pY proteins in cell lysates. As anti-pY antibodies have been raised and used in probing pY containing proteins (Daniel *et al.* 1985; Kalo and Pasquale 1999), ability of Tapj for probing this protein group can be compared using simple experiments such as western blot. A western-blot experiment is similar to the dot-blot experiment that is described in the previous section but an

extra step needed to separate proteins. Proteins in a cell lysate sample can be separated by PAGE before being transferred into the cellulose membrane.

However, as it is known that RNA is subjected to degradation in biological systems due to presence of ribonucleases, it is necessary to enhance nuclease-resistance for the aptamers before they are able to work with cells or lysates. To enhance resistance to nuclease degradation, these aptamers can be modified with functional groups to decrease nuclease sensitivity. For instance, 2'-O-methyl nucleotide derivatives are resistant to many nucleases, and this modification was found to substantially increase the half-life of RNA in blood (Uhlmann *et al.* 2000). In another example, anti-VEGF aptamers composed entirely of 2'-O-methyl nucleotides showed resistance to degradation in plasma at 37°C for 96 hours or after it was autoclaved at 125°C (Burmeister *et al.* 2005). Total methylation can be achieved by using a T7 RNA polymerase double-mutant Y639F/H784A that is known to show better incorporation of modified nucleotides (Padilla and Sousa 2002). The introduction of 2'-O-methyl and other substitutions into RNA may be further enhanced by evolution of RNA polymerases that can incorporate modified nucleotides (Chelliserrykattil and Ellington 2004).

6.2.3 Using the aptamer-NP conjugation for analysis of pY containing proteins in cells

One possible approach for analysis of the pY proteins in cell lysates is to immobilise the aptamers on nanoparticles (NPs). The aptamer-coated NPs can then be added to cell lysates to capture pY proteins. The bound proteins are then eluted and subjected

to identification and/or quantitation using mass spectroscopy. This proposed analysis scheme is shown in Figure 6-2.

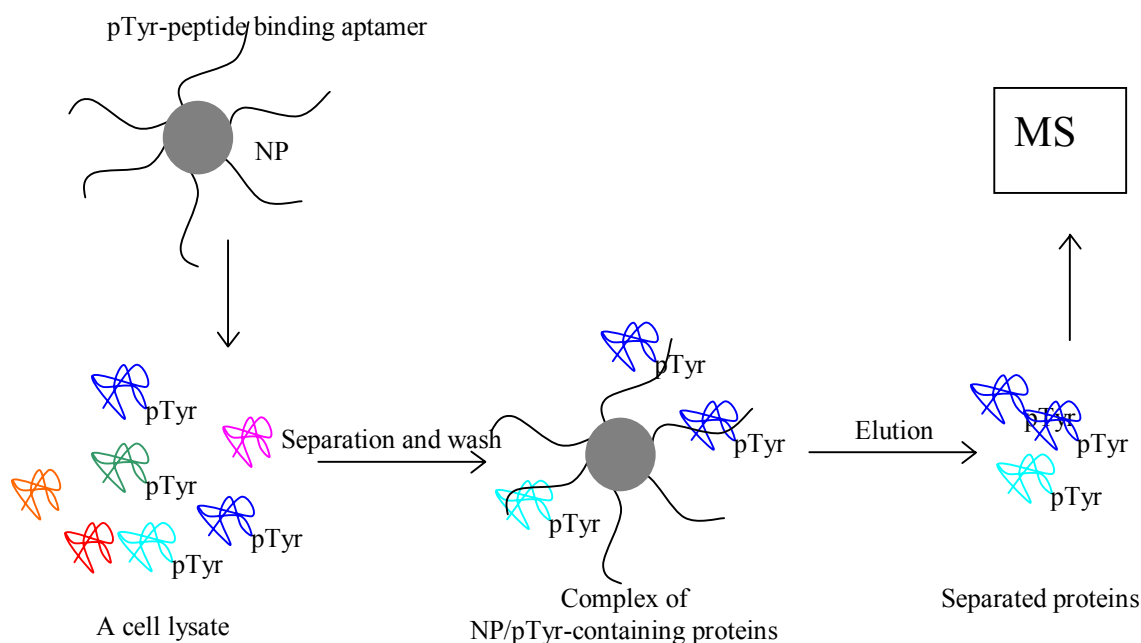


Figure 6-2: A possible scheme of pY protein analysis in cell lysates. The pY peptide binding aptamers are immobilised on nanoparticles (NPs). The aptamer-coated NPs are added to a cell lysate and incubated to allow pY proteins bind the aptamers. The NPs are then separated from the cell lysate by centrifugation or magnetic separation, depending on the nature of the nanoparticles. The bound pY proteins are then eluted from the nanoparticles and subjected to further analysis for identification and/or quantitation using mass spectrometry.

6.2.4. Incorporation with photoactivable groups

When working with complex matrix such as cell lysates, it often required strong binders to work in harsh conditions such as stringent washes or dramatic change physical or chemical environments to get rid of variety of non-specific binding components in cells. In order to achieve this, photoactivable groups can be incorporated into the aptamers to a possible covalent bond between aptamer and bound protein. For example, incorporation of 5-bromo-uridine into the aptamers provides a means to covalently cross-link the photoaptamers to complexed proteins by irradiating at 308 nm light (Golden *et al.* 2000; Meisenheimer *et al.* 2000; Petach and Gold 2002; Bock *et al.* 2004). However, as the photo-crosslinking is an irreversible process, in order to elute bound proteins from the nanoparticles, the aptamers must be able to be eluted from the nanoparticles if alkaline hydrolysis is not used. Therefore, considerations have to be made with the methods used to attach the aptamers. Feasible immobilisation approaches include using a polynucleotide as a linker so the aptamers can be immobilised on the nanoparticles through hybridizing with the linker, or using an affinity tag that binds reversibly the nanoparticle matrix, such as tagging the streptavidin-binding aptamers, Ta15 or Ta23, for streptavidin-coated nanoparticles. The elution for both immobilisation approaches can be achieved by applying heat or denaturing conditions.

6.2.5. Kinase assays

Phosphorylation using a kinase can be investigated by using the pY peptide binding aptamers (Tapi) for detection of the phosphorylated form. A general scheme of the experiment is described in Figure 6-3.

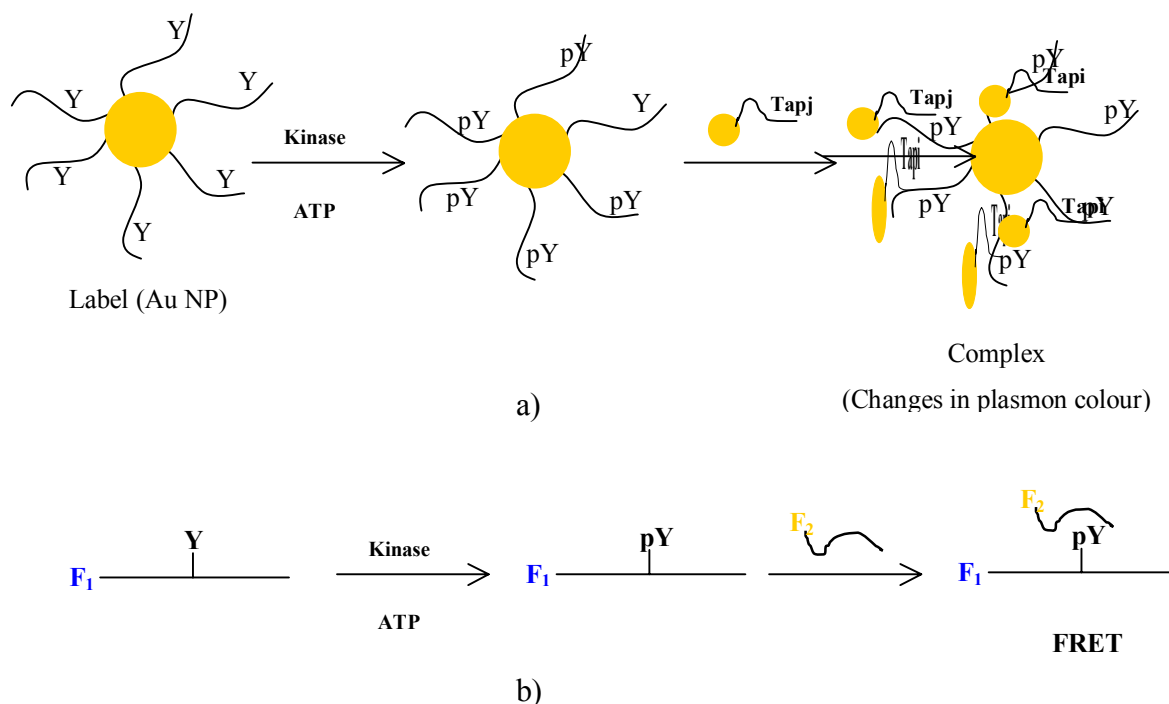


Figure 6-3: Kinase assays using Tapi for detection of the phosphorylated form. (a) Non-phosphorylated tyrosine (Y) protein is immobilised on Au nanoparticles (NPs). ATP and a specific kinase for the Y-containing protein were added to convert the protein to phosphorylated state. Tapi immobilised on NPs binds pY protein causing aggregation of NPs. NP aggregation can be observed by changes in plasmon colour. (b) Non-phosphorylated tyrosine (Y) protein is labelled with a fluorophore F_1 . ATP and a specific kinase for Y were added to convert the protein to phosphorylated state. Tapi labelled with a fluorophore F_2 , which is donor/receptor of F_1 for FRET, binds pY protein bringing the two fluorophore close that can have FRET.

6.3. Improvement of selection technologies

Over the last seventeen years, a great number of aptamers have been selected that bind their targets with high affinity and specificity. Aptamers have demonstrated that they can rival antibodies in many fields, including but not limited to analytical technology, and their abilities to take part in proteomic research have been since approved. However, despite the many useful attributes of aptamers, they have a notable drawback, which is the large amount of tedious time required to select them. To obviate this difficulty, the Ellington lab has demonstrated an automation system that can perform selection faster, in days instead of months (Cox *et al.* 1998; Cox and Ellington 2001; Cox *et al.* 2002; Goertz *et al.* 2004). In another effort, a small system called microfluidic SELEX prototype was also introduced to cut short the selection time (Hybarger *et al.* 2006).

Along with developing automated systems for *in vitro* selection, continuous improvements in selection technologies should allow the generation of aptamers with greater efficiency. One aspect that greatly influences the efficacy of the selection is the partitioning step. A better degree of separation between bound and unbound sequences would lead to less rounds of selection being performed. It has recently been shown that capillary electrophoresis (CE) can be used for the partitioning during *in vitro* selection. CE has for years been used for sensitive separation in the analytical field and researchers in the Bowser and the Krylov labs have applied it to *in vitro* selection (CE-SELEX) (Mendonsa and Bowser 2004b; Mendonsa and Bowser 2004a; Berezovski *et al.* 2005; Drabovich *et al.* 2005). They have been

demonstrated that the partitioning efficiency is higher than for filtration or affinity chromatography and only a few rounds of selection are required instead of tens of rounds in the traditional selection. They exemplified this by selection of aptamers against HIV-1 RT isolated by traditional SELEX and CE-SELEX (Schneider *et al.* 1995; Mosing *et al.* 2005). These developments suggest that the next selection experiments should take advantage of CE in partitioning. In addition, apart from CE, other highly effective separation methods, such as high performance liquid chromatography (HPLC), also have the potential to improve partitioning in *in vitro* selection and are also worth pointing out for applying for partitioning in the selection as well. However, up to now, no papers have been published on this method suggesting that HPLC may not practically have sufficient resolving power for aptamer selection.

Whilst aptamers have been demonstrated on many occasions to have binding characteristics (affinity and specificity) comparable to antibodies they are still relatively little used compared to the latter. It is anticipated that improvements in selection efficiency as well as more examples of their use in different areas of biomedical research and application will lead to their more widespread use over the next decade.

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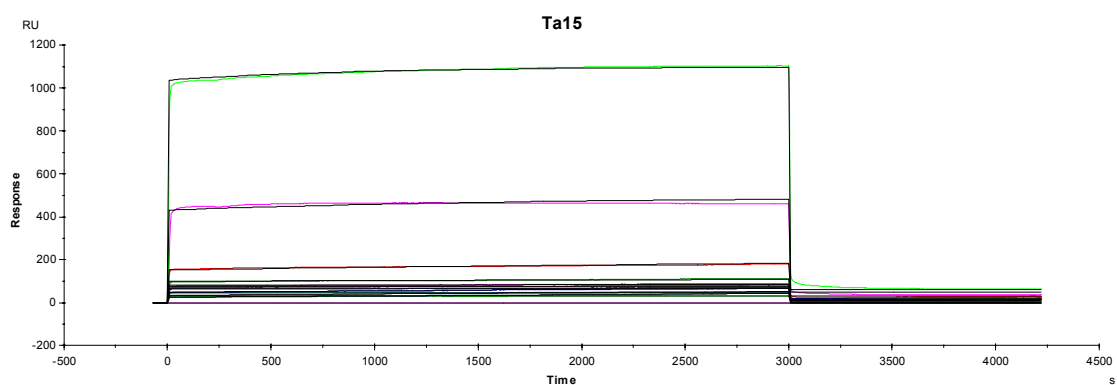
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Appendices

Appendix 1: Binding of Ta15 to immobilised streptavidin



ka (1/Ms)	kd (1/s)	Rmax (RU)	Conc of analyte	KA (1/M)	KD (M)	Req (RU)	kobs (1/s)	Chi2
114	1.43E-06	61.7	0	7.94E+07	1.26E-08	0	1.43E-06	23.9
			1.00E-08			27.3	2.57E-06	
			2.00E-08			37.9	3.71E-06	
			5.00E-08			49.3	7.12E-06	
			1.00E-07			54.8	1.28E-05	
			2.00E-07			58	2.42E-05	
			5.00E-07			60.2	5.83E-05	
			1.00E-06			60.9	1.15E-04	
			1.00E-06			60.9	1.15E-04	
			1.00E-06			60.9	1.15E-04	
			2.00E-06			61.3	2.29E-04	

Figure A1: BIAcore3000 sensorgrams demonstrating binding of the Ta15 to SA. SA was immobilised on a CM5 chip. Interaction between the aptamer and SA was indicated by changes in RU over the course of 3000 s injection interval. After 3000 s of the association phase, binding buffer took over for the dissociation phase. The K_d obtained from the Langmuir binding model was 12.6 nM and the model was fitted with χ^2 of 23.9.

Appendix 2: Binding of Ta15 to immobilised streptavidin

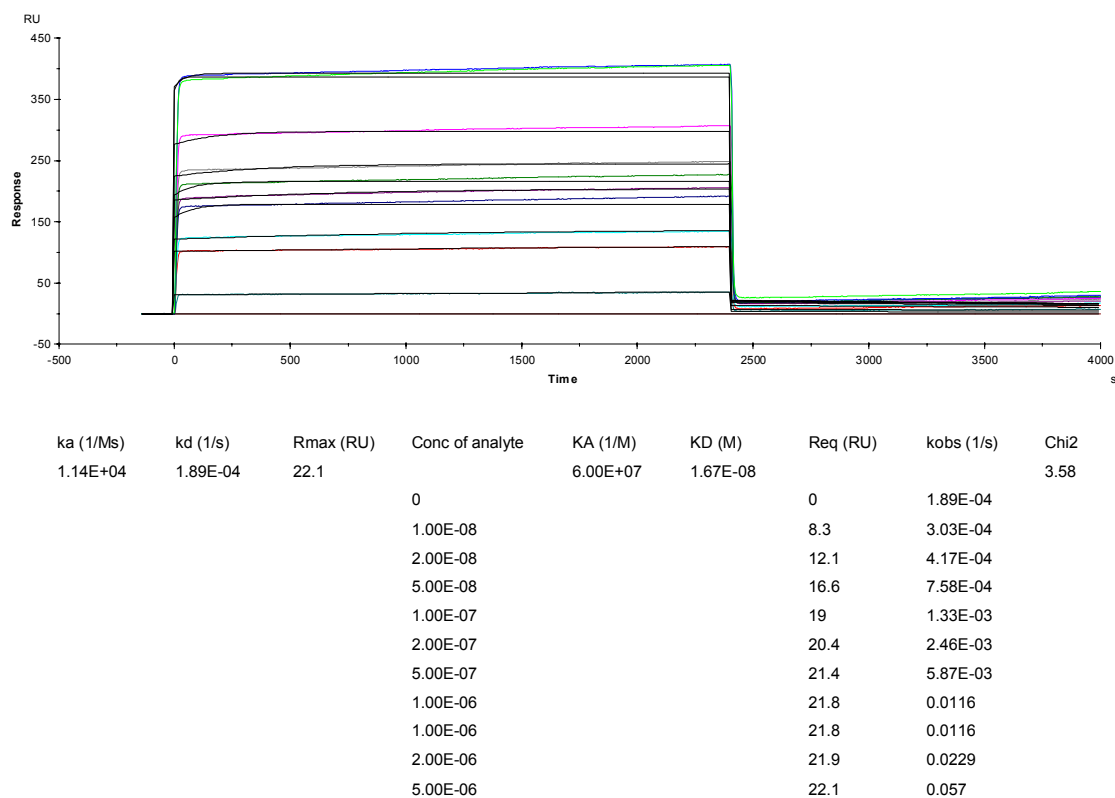


Figure A2: BIAcore3000 sensorgrams demonstrating binding of the Ta15 to SA. SA was immobilised on a CM5 chip. Interaction between the aptamer and SA was indicated by changes in RU over the course of 2400 s injection interval. After 2400 s of the association phase, binding buffer took over for the dissociation phase. The K_d obtained from the Langmuir binding model was 16.7 nM and the model was fitted with χ^2 of 3.58.

Appendix 3: Binding of Ta15 to immobilised streptavidin

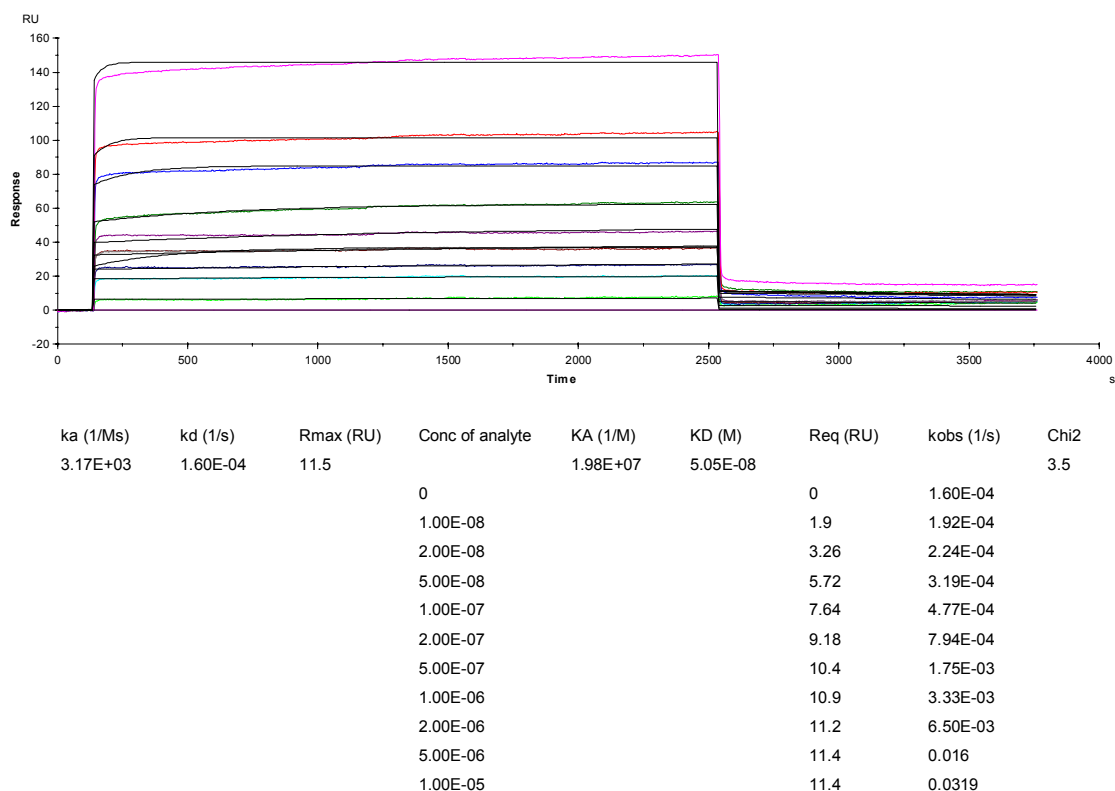
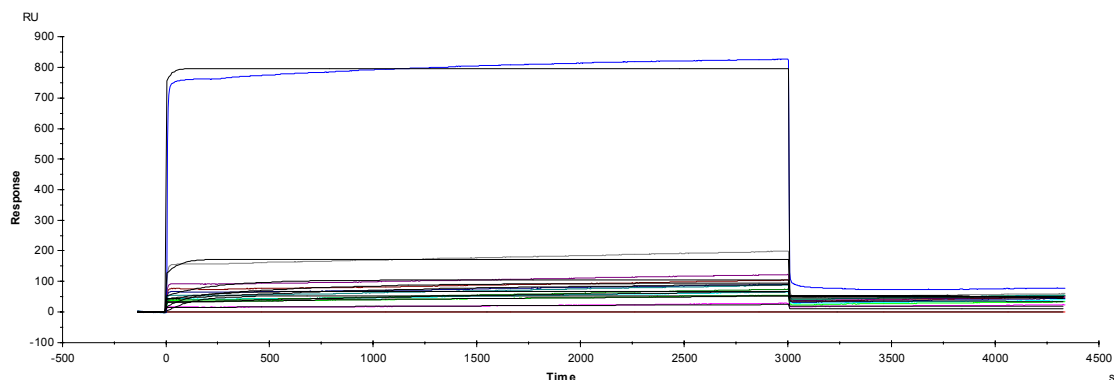


Figure A3: BIAcore3000 sensorgrams demonstrating binding of the Ta15 to SA. SA was immobilised on a CM5 chip. Interaction between the aptamer and SA was indicated by changes in RU over the course of 2400 s injection interval. After 2400 s of the association phase, binding buffer took over for the dissociation phase. The K_d obtained from the Langmuir binding model was 50.5 nM and the model was fitted with χ^2 of 3.5.

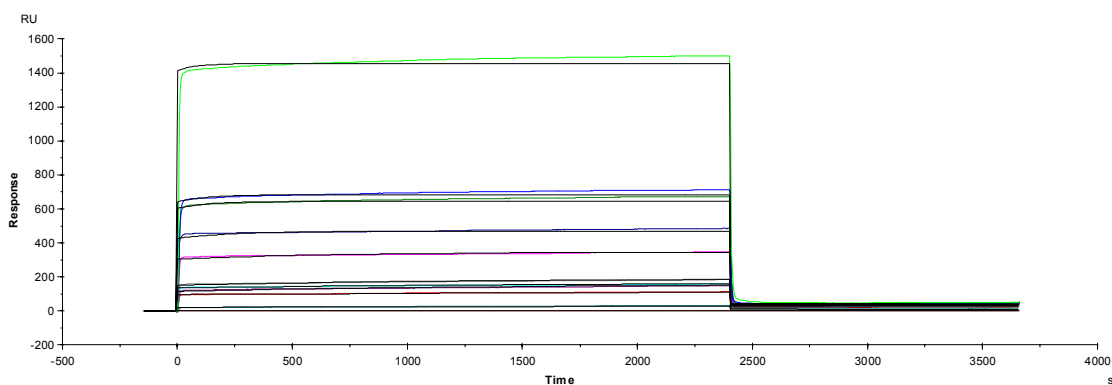
Appendix 4: Binding of Ta23 to immobilised streptavidin



ka (1/Ms)	kd (1/s)	Rmax (RU)	Conc of analyte	KA (1/M)	KD (M)	Req (RU)	kobs (1/s)	Chi2
8.16E+03	2.14E-05	52		3.80E+08	2.63E-09			62.3
			0			0	2.14E-05	
			1.00E-08			41.2	1.03E-04	
			2.00E-08			46	1.85E-04	
			5.00E-08			49.4	4.29E-04	
			1.00E-07			50.7	8.37E-04	
			2.00E-07			51.3	1.65E-03	
			5.00E-07			51.8	4.10E-03	
			1.00E-06			51.9	8.18E-03	
			1.00E-06			51.9	8.18E-03	
			2.00E-06			52	0.0163	

Figure A4: BIAcore3000 sensorgrams demonstrating binding of the Ta23 to SA. SA was immobilised on a CM5 chip. Interaction between the aptamer and SA was indicated by changes in RU over the course of 3000 s injection interval. After 3000 s of the association phase, binding buffer took over for the dissociation phase. The K_d obtained from the Langmuir binding model was 2.6 nM and the model was fitted with χ^2 of 62.3.

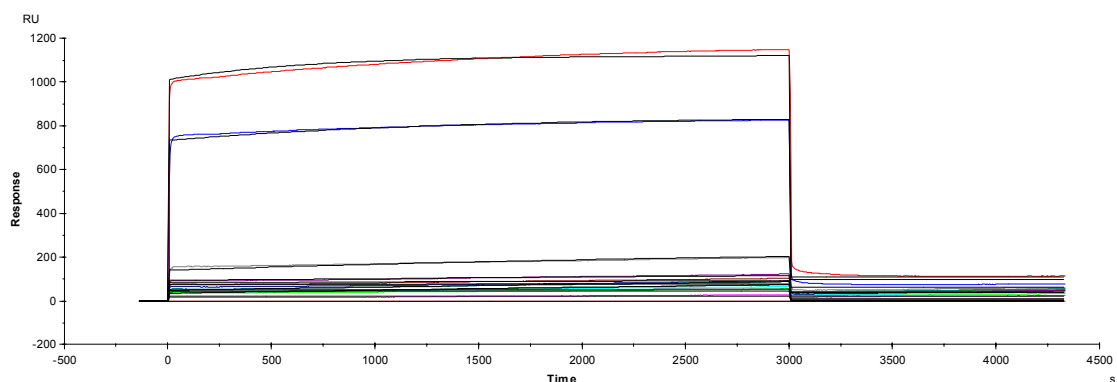
Appendix 5: Binding of Ta23 to immobilised streptavidin



ka (1/Ms)	kd (1/s)	Rmax (RU)	Conc of analyte	KA (1/M)	KD (M)	Req (RU)	kobs (1/s)	Chi2
7.51E+03	4.38E-05	42.6		1.72E+08	5.83E-09			22.4
			0			0	4.38E-05	
			1.00E-08			26.9	1.19E-04	
			2.00E-08			33	1.94E-04	
			5.00E-08			38.1	4.19E-04	
			1.00E-07			40.2	7.95E-04	
			1.00E-07			40.2	7.95E-04	
			2.00E-07			41.4	1.55E-03	
			5.00E-07			42.1	3.80E-03	
			1.00E-06			42.3	7.56E-03	
			1.00E-06			42.3	7.56E-03	
			2.00E-06			42.5	0.0151	

Figure A5: BIAcore3000 sensorgrams demonstrating binding of the Ta23 to SA. SA was immobilised on a CM5 chip. Interaction between the aptamer and SA was indicated by changes in RU over the course of 2400 s injection interval. After 2400 s of the association phase, binding buffer took over for the dissociation phase. The K_d obtained from the Langmuir binding model was 58.3 nM and the model was fitted with χ^2 of 22.4.

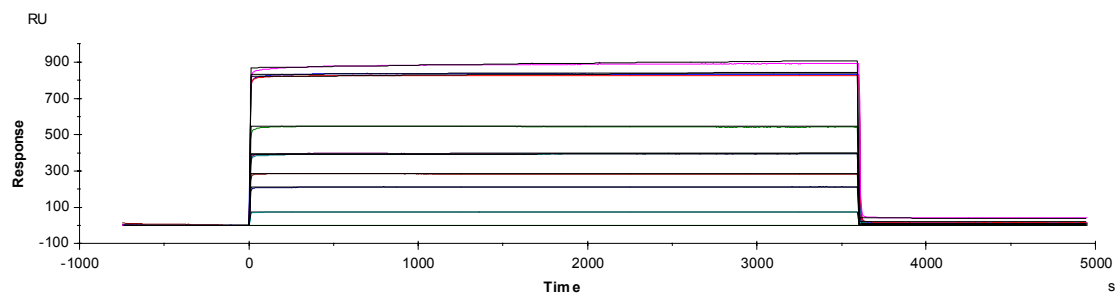
Appendix 6: Binding of Ta23 to immobilised streptavidin



ka (1/Ms)	kd (1/s)	Rmax (RU)	Conc of analyte	KA (1/M)	KD (M)	Req (RU)	kobs (1/s)	Chi2
146	2.61E-06	110		5.58E+07	1.79E-08			151
			0			0	2.61E-06	
			1.00E-08			39.5	4.07E-06	
			2.00E-08			58.1	5.53E-06	
			5.00E-08			81.2	9.90E-06	
			1.00E-07			93.5	1.72E-05	
			2.00E-07			101	3.18E-05	
			5.00E-07			106	7.55E-05	
			1.00E-06			108	1.48E-04	
			1.00E-06			108	1.48E-04	
			2.00E-06			109	2.94E-04	
			5.00E-06			110	7.32E-04	
			1.00E-05			110	1.46E-03	

Figure A6: BIAcore3000 sensorgrams demonstrating binding of the Ta23 to SA. SA was immobilised on a CM5 chip. Interaction between the aptamer and SA was indicated by changes in RU over the course of 3000 s injection interval. After 3000 s of the association phase, binding buffer took over for the dissociation phase. The K_d obtained from the Langmuir binding model was 17.9 nM and the model was fitted with χ^2 of 151.

Appendix 7: Binding of Tap1 to immobilised pY peptide



ka (1/Ms)	kd (1/s)	Rmax (RU)	Conc of analyte	KA (1/M)	KD (M)	Req (RU)	kobs (1/s)	Chi2
50.3	2.12E-05	70.1	0	2.37E+06	4.22E-07	0	2.12E-05	6.67
			1.00E-08			1.62	2.17E-05	
			2.00E-08			3.18	2.22E-05	
			5.00E-08			7.43	2.37E-05	
			1.00E-07			13.4	2.62E-05	
			2.00E-07			22.6	3.13E-05	
			5.00E-07			38	4.64E-05	
			1.00E-06			49.3	7.15E-05	
			2.00E-06			57.9	1.22E-04	
			5.00E-06			64.7	2.73E-04	

Figure A7: BIAcore3000 sensorgrams demonstrating binding of the Tap1 with the phosphotyrosine peptide. Biotinylated phosphotyrosine peptide was immobilised on a streptavidin-coated chip. Interaction between the aptamer and the phosphotyrosine peptide was indicated by changes in RU over the course of 3600 s injection interval. The surface was generated by 10 mM glycine at pH 2.5 and then 40mM NaOH. Following a series of analysis, the K_d obtained from the Langmuir binding model was 422 nM and the model was fitted with χ^2 of 6.67.

Appendix 8: Binding of Tap2 to immobilised pY peptide

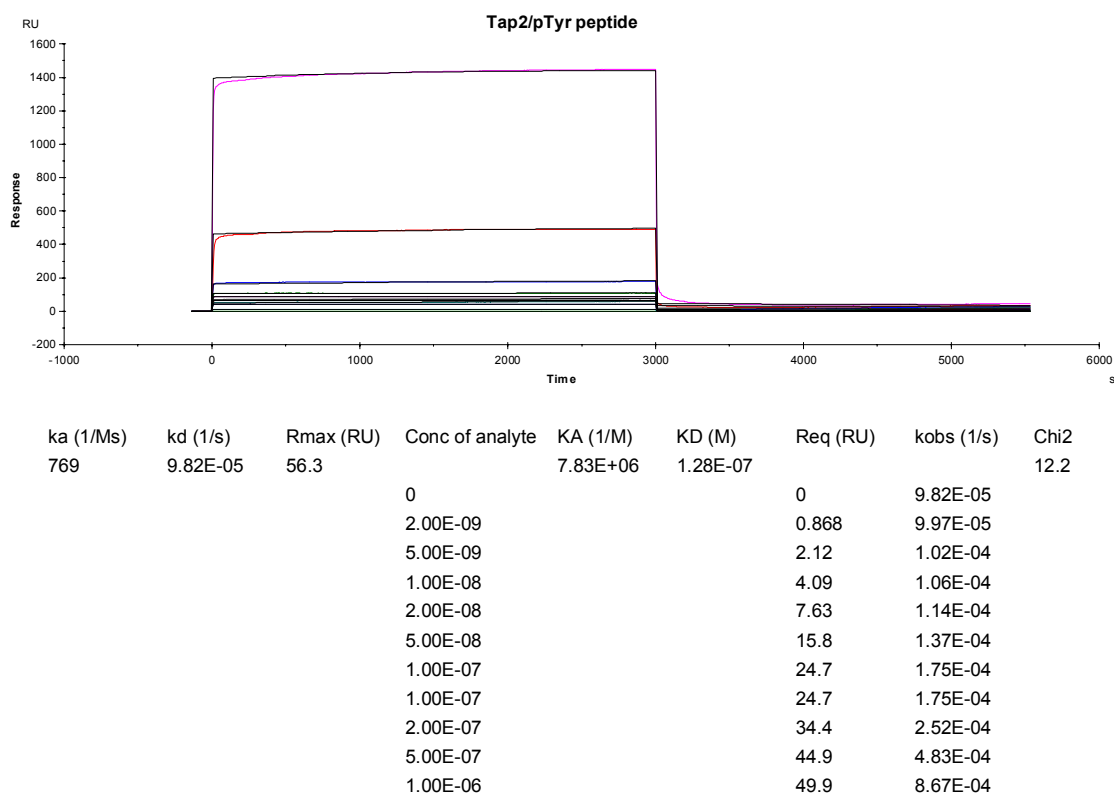
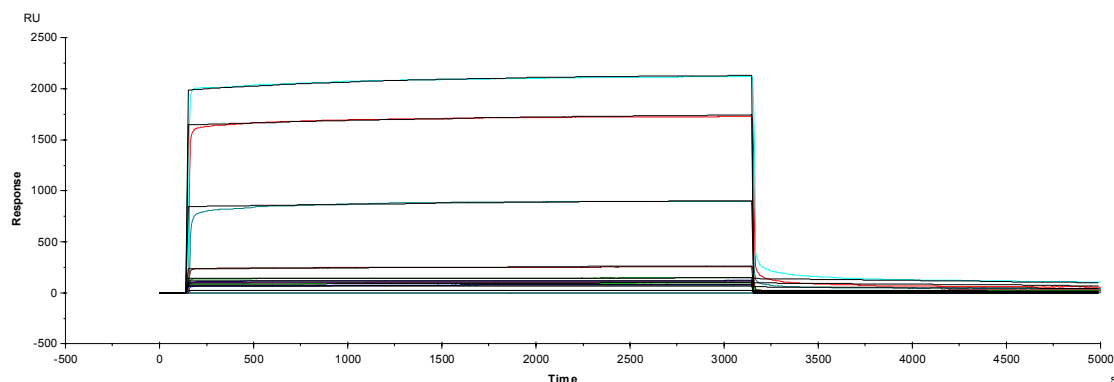


Figure A8: BIAcore3000 sensorgrams demonstrating binding of the Tap2 with the phosphotyrosine peptide. Biotinylated phosphotyrosine peptide was immobilised on a streptavidin-coated chip. Interaction between the aptamer and the phosphotyrosine peptide was indicated by changes in RU over the course of 3000 s injection interval. The surface was generated by 10 mM glycine at pH 2.5 and then 40mM NaOH. Following a series of analysis, the K_d obtained from the Langmuir binding model was 128 nM and the model was fitted with χ^2 of 12.2.

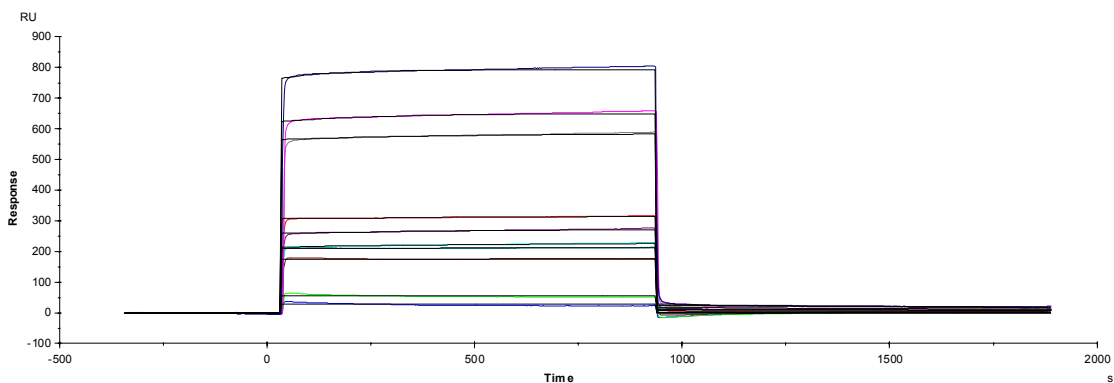
Appendix 9: Binding of Tap2 to immobilised pY peptide



ka (1/Ms)	kd (1/s)	Rmax (RU)	Conc of analyte	KA (1/M)	KD (M)	Req (RU)	kobs (1/s)	Chi2
323	1.83E-04	200		1.76E+06	5.67E-07			61
			0			0	1.83E-04	
			2.00E-09			0.702	1.84E-04	
			5.00E-09			1.75	1.85E-04	
			1.00E-08			3.46	1.86E-04	
			2.00E-08			6.81	1.89E-04	
			5.00E-08			16.2	1.99E-04	
			1.00E-07			30	2.15E-04	
			1.00E-07			30	2.15E-04	
			2.00E-07			52.1	2.47E-04	
			5.00E-07			93.6	3.44E-04	
			1.00E-06			128	5.06E-04	
			2.00E-06			156	8.28E-04	

Figure A9: BIAcore3000 sensorgrams demonstrating binding of the Tap2 with the phosphotyrosine peptide. Biotinylated phosphotyrosine peptide was immobilised on a streptavidin-coated chip. Interaction between the aptamer and the phosphotyrosine peptide was indicated by changes in RU over the course of 3000 s injection interval. The surface was generated by 10 mM glycine at pH 2.5 and then 40mM NaOH. Following a series of analysis, the K_d obtained from the Langmuir binding model was 567 nM and the model was fitted with χ^2 of 61.

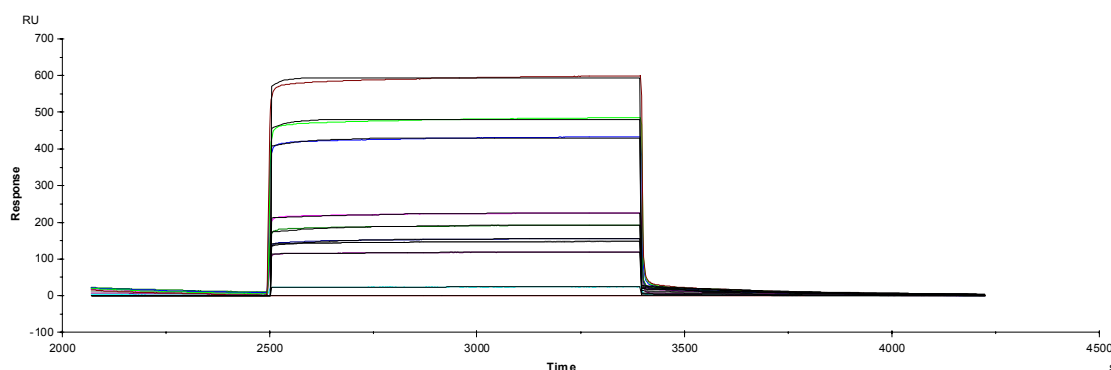
Appendix 10: Binding of the pY peptide to immobilised Tap1



ka (1/Ms)	kd (1/s)	Rmax (RU)	Conc of analyte KA (1/M)	KD (M)	Req (RU)	kobs (1/s)	Chi2
709	4.70E-04	29.4	1.51E+06	6.63E-07			5.63
			0		0	4.70E-04	
			5.00E-08		2.06	5.05E-04	
			1.00E-07		3.85	5.41E-04	
			2.00E-07		6.81	6.12E-04	
			5.00E-07		12.6	8.24E-04	
			1.00E-06		17.7	1.18E-03	
			1.00E-06		17.7	1.18E-03	
			2.00E-06		22.1	1.89E-03	
			5.00E-06		25.9	4.02E-03	
			1.00E-05		27.6	7.56E-03	

Figure A10: BIAcore3000 sensorgrams demonstrating binding of the phosphotyrosine peptide with Tap1. Tap1 was attached on the chip through hybridization with the linker TaL1, which was covalently attached on a gold chip by thiolation. Interaction between the aptamer and the phosphotyrosine peptide was indicated by changes in RU over the course of 900 s injection interval. The surface was generated by 40mM NaOH and then 1 mM HCl. Following a series of analysis, the K_d obtained from the Langmuir binding model was 663 nM and the model was fitted with χ^2 of 5.63.

Appendix 11: Binding of the pY peptide to immobilised Tap1



ka (1/Ms)	kd (1/s)	Rmax (RU)	Conc of analyte	KA (1/M)	KD (M)	Req (RU)	kobs (1/s)	Chi2
4.54E+03	2.39E-03	28.7		1.90E+06	5.25E-07			2.71
			0			0	2.39E-03	
			5.00E-08			2.49	2.62E-03	
			1.00E-07			4.59	2.84E-03	
			2.00E-07			7.91	3.30E-03	
			5.00E-07			14	4.66E-03	
			1.00E-06			18.8	6.93E-03	
			1.00E-06			18.8	6.93E-03	
			2.00E-06			22.7	0.0115	
			5.00E-06			26	0.0251	
			1.00E-05			27.3	0.0478	

Figure A11: BIAcore3000 sensorgrams demonstrating binding of the phosphotyrosine peptide with Tap1. Tap1 was attached on the chip through hybridization with the linker TaL1, which was covalently attached on a gold chip by thiolation. Interaction between the aptamer and the phosphotyrosine peptide was indicated by changes in RU over the course of 900 s injection interval. The surface was generated by 40mM NaOH and then 1 mM HCl. Following a series of analysis, the K_d obtained from the Langmuir binding model was 525 nM and the model was fitted with χ^2 of 2.71.

Appendix 12: Binding of the pY peptide to immobilised Tap2

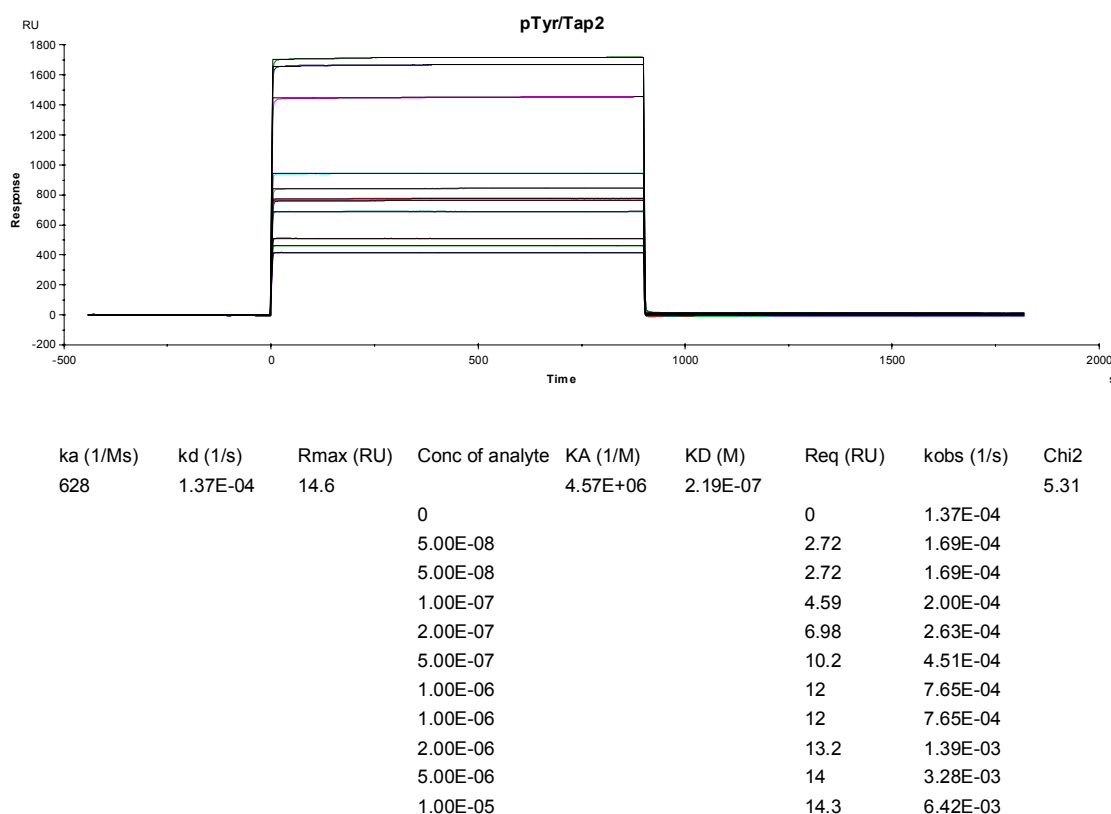


Figure A12: BIAcore3000 sensorgrams demonstrating binding of the pTyr peptide to Tap2. Tap2 was immobilised on the sensorchip through hybridizing with TaL1, which was thiolated on gold surface. Association between the aptamer and the phosphotyrosine peptide was obtained by injecting a phosphotyrosine peptide solution over the course of 900 s and a buffer chase was followed for the dissociation analysis. The surface was generated by two injections of 40 mM NaOH and 1 mM HCl, respectively. Following a concentration series analysed, the K_d obtained from the Langmuir binding model was 219 nM and the model was fitted with χ^2 of 5.31.

Appendix 13: Binding of the pY peptide to immobilised Tap2

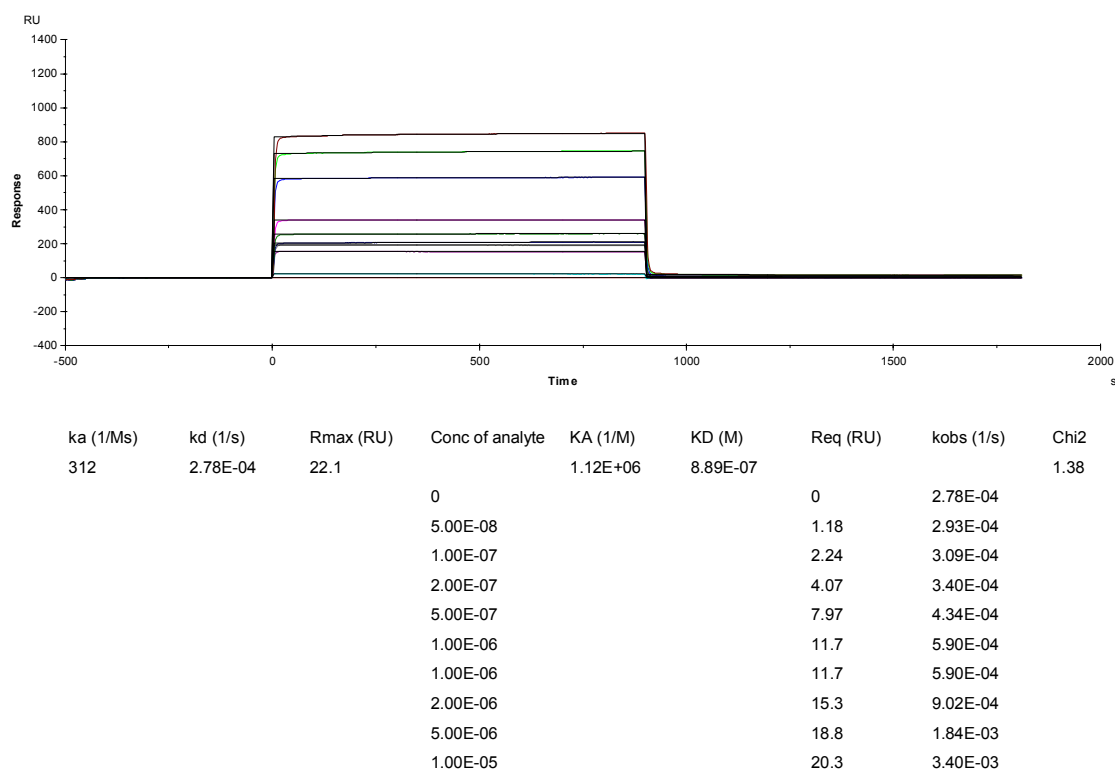


Figure A13: BIAcore3000 sensorgrams demonstrating binding of the pTyr peptide to Tap2. Tap2 was immobilised on the sensorchip through hybridizing with TaL1, which was thiolated on gold surface. Association between the aptamer and the phosphotyrosine peptide was obtained by injecting a phosphotyrosine peptide solution over the course of 900 s and a buffer chase was followed for the dissociation analysis. The surface was generated by two injections of 40 mM NaOH and 1 mM HCl, respectively. Following a concentration series analysed, the K_d obtained from the Langmuir binding model was 889 nM and the model was fitted with χ^2 of 1.38.

Appendix 14: Binding of the pY peptide to immobilised Tap2

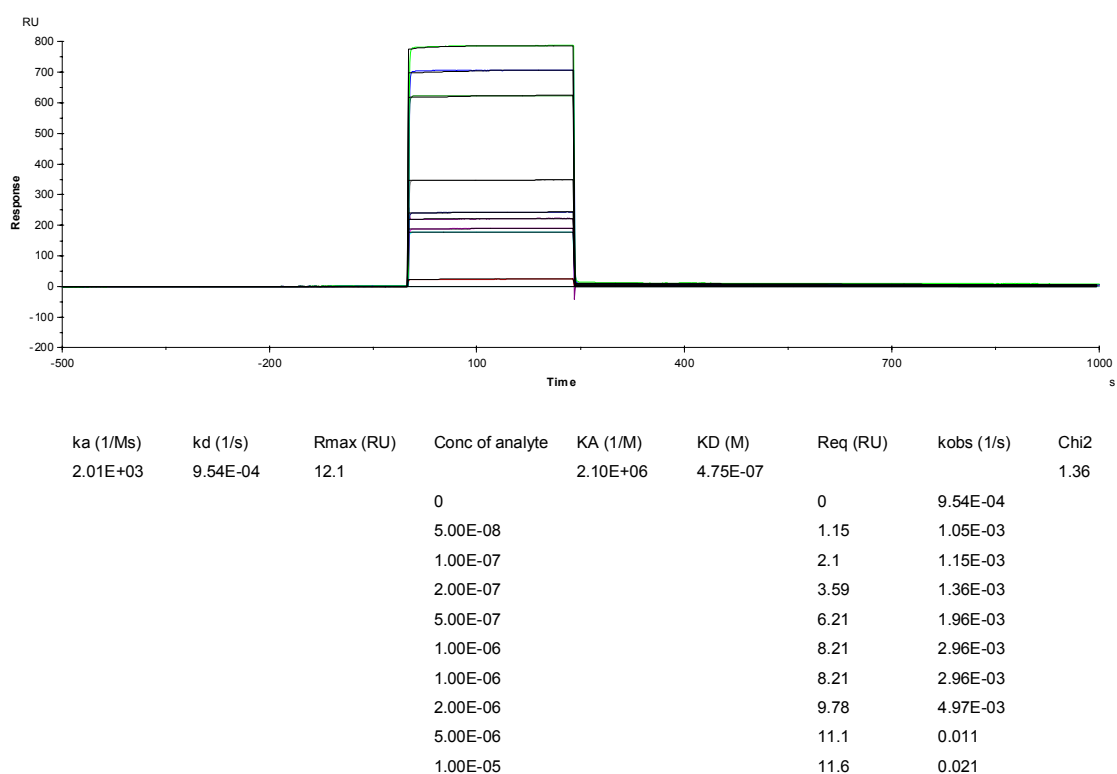


Figure A14: BIAcore3000 sensorgrams demonstrating binding of the pTyr peptide to Tap2. Tap2 was immobilised on the sensorchip through hybridizing with TaL1, which was thiolated on gold surface. Association between the aptamer and the phosphotyrosine peptide was obtained by injecting a phosphotyrosine peptide solution over the course of 180 s and a buffer chase was followed for the dissociation analysis. The surface was generated by two injections of 40 mM NaOH and 1 mM HCl, respectively. Following a concentration series analysed, the K_d obtained from the Langmuir binding model was 475 nM and the model was fitted with χ^2 of 1.36.