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Aptamers in oncology: a diagnostic perspective

Review Article

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Abbreviations: Aptamer beacons, (AB); fluorescence resonance energy transfer, (FRET); horseradish peroxidase, (HRP); human epidermal growth factor 2, (HER2); Molecular beacons, (MB); platelet derived growth factor, (PDGF); ribonuclease H, (RNase H); RNA interference, (RNAi); short hairpin RNA, (shRNA); surface plasmon resonance imaging, (SPRI); Systematic Evolution of Ligands by EXponential enrichment, (SELEX); tymidine, (T); vascular endothelial growth factor, (VEGF)

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Summary

Nucleic acid sequences can produce a wide variety of three-dimensional conformations. Some of these structural forms are able to interact with proteins and small molecules with high affinity and specificity. These sequences, comprising either double or single stranded oligonucleotides, are called "aptamers" based on the Greek word aptus, which means "to fit". Using an efficient selection process, randomised oligonucleotide libraries can be rapidly screened for aptamers with the appropriate binding characteristics. This technology has spawned the development of a new class of oligonucleotide therapeutic products. However, while interest among pharmaceutical companies continues to grow with some candidates already in clinical trials and one in the market, there appears to be some reluctance to fully explore the diagnostic potential of this technology. This article will review aptamer developments in diagnostics, compare them with other oligonucleotide therapeutics and highlight both potentials and pitfalls of technological development in this area.

I. Introduction

Advances in genomic and proteomic technology have allowed the identification of genes and/or gene products involved in the development and progression of cancer. This, in turn, has facilitated the development of novel drug compounds, leading to a variety of molecules showing promising therapeutic and diagnostic potential as anti-cancer agents. Such molecules include small compounds, peptides, antibodies and more recently oligonucleotides.

Apart from their role in the evolutionary archives of life, the use of DNA and RNA as natural ligands in evading cellular defence mechanisms was first discovered in the late 1970's with their application in antisense therapy. In this approach, the synthesis of disease-related proteins is disrupted by blocking the expression of their encoding mRNA. This is achieved by using complementary single stranded nucleic acids to hybridise in a sequence-dependent manner to the target mRNA. In this way, inhibition at the genetic level (and therefore protein production) occurs by the steric blockage of ribosomal scanning of the mRNA or by the activation of endogenous ribonuclease H (RNase H) (Younes et al, 2002; Famulok and Mayer, 2005). This simple approach to gene regulation, although promising, has vital limitations which hinder its wide scale application, as has been the case with other genetic manipulation strategies (for example, gene knockout and short interfering RNAs). For example, the target mRNA sequence may be inaccessible to the inhibiting oligonucleotide as other proteins may pre-occupy the binding sequence (Famulok and Mayer, 2005). Most proteins are found to operate as part of a multi-protein complex. Hence, completely abolishing the production of a protein may incur secondary effects on the complexes function (Thompson et al, 2002; Blank and Blind, 2005). In addition, for *in vivo* applications, the intracellular target is often difficult to access due to the low permeability of the oligonucleotide through the cell membrane (Pestourie et al, 2005). Despite these drawbacks, the use of oligonuclotides as therapeutic and diagnostic agents has however been explored further, where they are applied to function at the proteomic rather than the genetic level. Discovered around 15 years ago (Ellington and Szostak, 1990; Tuerk and Gold, 1990), aptamers offer an exciting prospect in their application as a novel class of nucleic acid inhibitors/ligands. Aptamers are short, single stranded oligonucleotides which inherently adopt stable three dimensional sequence-dependent structures. This intrinsic property makes them efficient binding molecules, such that they are capable of binding to an array of molecular targets ranging from small ions and organic molecules to large glycoproteins and mucins. Aptamers are typically isolated from

combinatorial libraries by a process of *in vitro* evolution, termed SELEX. These novel oligonucleotides exhibit high specificity and affinity for their target, in addition to often having high inhibitory potential. Additionally, aptamers are easily modified, making them valuable molecules for the delivery of therapeutic agents and/or for imaging and clinical diagnosis. Consequently, this technology, now approaching its second decade of development, shows considerable promise with two therapeutic aptamers in phase III trials and one already in the market. Relevant oncology targets are also attracting some interest, not least due to the potential benefits that aptamers have over antibody-based therapeutics. However, despite the advances and the huge body of literature documenting the success of the technology, the commercial application of aptamers in the field of diagnostics remains relatively undeveloped, not least due to the exclusive IP portfolio that prohibits potentially interested companies to invest in this area of research and development. Furthermore, there is already a significant antibody diagnostic market developed, and aptamers would need to offer significant improvements on current technologies to warrant the substitution of antibodies in current assay formats.

A. Selection of aptamers by the traditional method of SELEX

The generation of aptamers towards a specific target is typically achieved through a process called SELEX (Systematic Evolution of Ligands by EXponential enrichment). This procedure can be considered as an *in vitro* evolutionary selection process which allows the isolation of aptamer(s), with unique binding properties, from a large library of oligonucleotides. The core of the SELEX procedure consists of iterative cycles of two steps (i) selection of aptamers having an affinity to the target and (ii) amplification of the binding aptamers (**Figure 1**).

The starting point of SELEX begins with the synthesis of a combinatorial library of single stranded nucleic acids. The oligonucleotides synthesised for the library usually consist of a central region of variable base sequence (20-40 nucleotides) flanked by regions of known sequences on either end. The flanking sequences act as primers for amplification during the SELEX procedure. Typically, libraries contain up to 10^{15} different sequences which, combined with the innate ability of oligonucleotides to form stable sequence-dependent

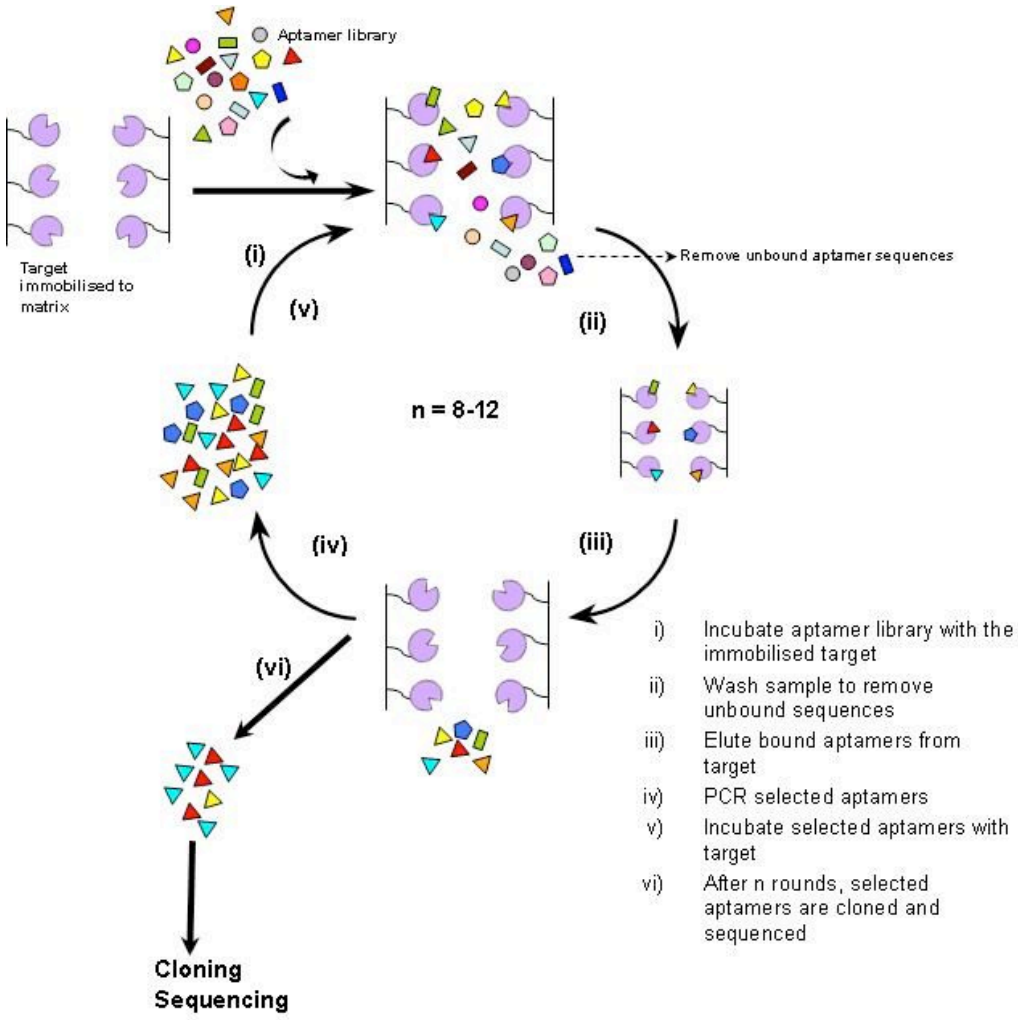

Figure 1. Schematic representation of a typical SELEX process

structures, provide an array of molecular shapes available for the selection process. In the selection steps, the library is initially incubated with the immobilised target under conditions which would mimic the *in vivo* application of the binding aptamers (e.g. physiological conditions). Subsequently, non- or weak-binding sequences are removed and those nucleic acids which adopt conformations that favour their binding to the target are eluted. Effective partitioning of the binding aptamers from the non-binding species can be achieved by methods such as affinity chromatography, nitrocellulose filtration, and magnetic functionalised beads. The binding nucleic acids are subsequently amplified by PCR (DNA) or reverse-transcription PCR (RNA) using primers complementary to the flanking sequences in the nucleic acid library. The enriched pool of binding oligonucleotides then acts as the library for the next round of selection. By repeating the selection and amplification steps iteratively, the library of oligonucleotides becomes less complex owing to competitive binding. However, the amplification process is susceptible to producing mutations, which can further add to the variability in the number of sequences available for binding. The selection and amplification step is referred to as one round or cycle and a typical SELEX procedure requires 8-12 cycles for the isolation of nucleic acids which have the highest affinity to the target, though as little as 1 cycle have been performed. To enhance the specificity of aptamers, some researchers incorporate additional "negative selection" steps at any stage in the SELEX process. In such steps, the aptamers recovered (and amplified) from any given round are incubated with variants/isoforms of the target of interest, and the nucleic acids which do not bind are now selected and amplified. Using this additional step ensures that the aptamers selected at the end of the SELEX experiment are able to discriminate (and therefore unlikely to cross react *in vivo*) between proteins which have similar conformations or properties to the target of interest.

Selected aptamers from the last round of SELEX are subsequently cloned, sequenced and characterised for their ability to bind to the target, possibly inhibiting its function, and for their application *in vivo*. Following sequence identification, the selected aptamer can be easily produced by chemical or enzymatic synthesis. Furthermore, additional chemical modifications can be easily made, at desired positions of the aptamer, to improve its therapeutic and/or diagnostic application. For example, given their susceptibility to nucleases, aptamers have often been found to be unstable in biological fluids, thus limiting their therapeutic applicability. However, modifications made to the backbone of these oligonucleotides (mainly at the 2' ribonucleotide position) have shown to significantly increase their plasma stability (Dougan et al, 2000; Darfeuille et al, 2004; Yan, 2004). Alternatively, to overcome degradation by nucleases, mirror-image (L-DNA/RNA) aptamers, termed spiegelmers, have recently been employed (Eulberg and Klussmann, 2003). Spiegelmers are isolated using typical SELEX methods, where a standard aptamer library is used to bind to the enantiomer form of the desired target. The isolated aptamer(s) is then synthesised as L-oligonucleotide which, in principle and largely in practice, binds to the physiological form of the target. While spiegelmers lose their susceptibility towards endogenous nucleases, they still bind to their target with the same affinity and specificity as the aptamer isolated for the enantiomer target (Williams et al, 1997; Leva et al, 2002). Spiegelmers have been isolated to bind to D-adenosine (Klussmann et al, 1996), vasopressin (Floege et al, 1999) and GnRH I (gonadotropin releasing hormone). Antagonists to GnRH I may have important implications into the treatment of hormone related benign and malignant tumours (Leva et al, 2002).

Other modifications made to aptamers include attachments of large hydrophilic and hydrophobic moieties such as PEG (Floege et al, 1999) or liposomes, respectively. Attachments of such molecules serve to decrease the renal clearance time of aptamers, as the pharmacokinetic properties of these molecules are limited by their small size (5-25 KDa). Signalling molecules such as fluorophores can also be incorporated into the aptamer for imaging/signalling purposes, while the attachment of drugs can facilitate the therapeutic effect of the aptamer, if required (Floege et al, 1999). Although chemical modifications can be completed relatively easily, it is important that such alterations to the backbone structure of the aptamer do not affect its binding to the target, particularly given that binding is largely governed by shape-shape interactions between the two molecules. Consequently, once modified, the aptamers need to be re-tested for their specificity/affinity towards the target. Modifications to the aptamer can sometimes be a daunting task if it leads to a decrease in the aptamers' affinity or even completely hindering their binding to the target. Taking this into account, many researchers employ modifications to the aptamer library at the start of the SELEX process so that any alteration to the aptamer(s) selected, will be those that favour binding initially. However, this too poses problems in the amplification stages of the SELEX procedure, as the modified nucleotides must still be recognised by the enzyme (e.g. Taq polymerase) used in the PCR step and they increase the cost of the production of the molecules. This, in turn, initiated research to focus on engineering prospective modified oligonucleotides which can act as substrates for amplification enzymes or, alternatively, finding amplification enzymes with low substrate specificity, which has been done with some success. T7 RNA polymerase, for example, is able to recognise 2'-fluoropyrimidine modified RNA, which has allowed the successful isolation of RNA aptamers for VEGF (Ruckman et al, 1998) and oncostatin M (Rhodes et al, 2000) from modified libraries. Such aptamers have shown increased stability towards nucleases without significantly compromising their binding affinity and specificity towards their target. The utility of aptamer libraries with other modifications, such as the attachment of PEG, in standard SELEX methods, however, has yet still to be achieved. Equally, the evolution of non-SELEX methods has potentially overcome the limitations associated with the use of modified aptamer libraries and may open the market in diagnostic applications by offering alternatives

to the SELEX patented technology that has limited their development in the past years.

B. Non-SELEX methods for the selection of aptamers

SELEX has undoubtedly proved to be a very robust and powerful method in allowing the isolation of many aptamers directed towards desired targets. However, modern science has improved on this technique by developing methodologies which overcome some of the drawbacks associated with traditional SELEX procedures. Apart from making automated robotic systems to improve the efficiency of traditional SELEX, other "non-SELEX" based methods for the selection of aptamers have recently been put into practice. The utilisation of capillary electrophoresis has been shown to be a highly efficient approach for the partitioning of aptamers with desired properties from a randomised pool (Drabovich et al, 2005; Berezovski et al, 2005) (see also Krylov, 2006 for a review into capillary electrophoresis). Using this technique, aptamers to h-Ras, a protein involved in the development and progression of cancer, were isolated with predetermined kinetic parameters (Berezovski et al, 2005). The isolation of aptamers with predefined kinetic and thermodynamic properties of their interaction with the target has so far been obstructed by standard SELEX technology. Furthermore, this method of aptamer selection only employed the partitioning steps of SELEX without the need for amplification between them. Hence, one of the most significant advantages of this non-SELEX method is its application to libraries which are difficult or cannot be amplified, thus overcoming the problems associated with using modified oligonucleotide libraries, as mentioned above. As well as the relative simplicity and easy-to-use nature of this procedure, aptamers are selected within only a few hours, which contrasts the several days or weeks needed for standard SELEX systems.

Exploitation of computational methods has also led the way into the development of non-SELEX methods for aptamer selection. More importantly, computational methods have been powerful in selecting aptamers with inhibitory activities or sequences that undergo ligand dependent conformational changes, a property useful for the design of molecular and aptamer beacons (see below). One of the major drawbacks associated with SELEX is the selection of aptamers that may not have any inhibitory activity towards its target, since the selection of aptamers is based on affinity. Consequently, researchers have used this drawback to drive the engineering of alternative selection methods based on inhibitory activity of aptamers.

Algorithm methods have shown to be sufficiently effective in selecting aptamers with such properties (Ikebukuro et al, 2005). This computational method has been used to predict the secondary structure of nucleic acids under different conditions e.g. in the presence and absence of a ligand (Hall et al, 2006). In general, algorithmic methods use aptamers with known structures and/or features (such as aptamers that undergo ligand-induced conformation changes) to rapidly select oligonucleotides from virtual pools which may present similar properties or adopt similar structures. Hence, sequences are selected that match a defined profile. One of the most valuable structures applied to computational selection is that of the G-quartet. Such structures are suggested to have important implications in the biology of cancer and thus, aptamers which adopt such configurations are of intense interest. An aptamer selected for thrombin has been thoroughly investigated and is known to adopt such a configuration. Consequently, this aptamer has been used as a model to investigate the potential of new selection methods, based on inhibitory activity. By randomising the sequence of the duplex region of the anti-thrombin aptamer, Ikebukuro and colleagues selected in 2006 inhibitory aptamers using genetic algorithm on a library of limited sequences. In another report, evolution mimicking algorithms were used to select aptamers with potent inhibitory activity from a pool that was designed to form G-quartet structures and contain a limited number of sequences (Noma and Ikebukuro, 2006). Other reports have used the structure of aptamers that undergo ligand-induced conformational changes as a basis for the selection of new aptamers that will effectively function as biosensors (Hall et al, 2006). Computational selection methods of aptamers require detailed information on the pre-requisites of the basis of selection e.g. the structure of the aptamer that new aptamers are to be modelled on or the structural changes that the profile aptamer undergoes upon ligand binding. Given that this is not always feasible and that gaining this information can sometimes be a lengthy process, computational selection of aptamers may be somewhat delayed. SELEX itself, however, is still widely used for aptamer selection and has advanced (and still is) rapidly to overcome many of its challenges.

II. Aptamer like reagents

The versatility of aptamers has greatly facilitated modern drug development. Based on the principal properties of aptamers, research into other novel compounds for diagnostic and therapeutic purposes has been progressively initiated. Consequently, many other aptamer-like reagents have evolved and are currently at the forefront of medical research with aptamers themselves. Such biomolecules are briefly described below in terms of their diagnostic and/or therapeutic potential.

A. Aptamer like reagents for therapeutics

1. Peptide aptamers

Peptide aptamers can be described as molecules consisting of a peptide domain, with a high affinity for a target, integrated as part of a stable scaffold protein. The peptide domain of these novel aptamers are frequently selected from a peptide library, similar to aptamer libraries. A highly complex peptide library is conceived by synthesising random oligonucleotides encoding for 20mer peptides which can contain any of the 20 amino acids at any of the 20 positions. These diverse sequences are then cloned within the open reading frame region of a vector which encodes for a scaffold protein. This, consequently, allows for the peptide domain to be expressed directly as part of the scaffold protein. The most common scaffold protein used is the *E. coli* thioredoxin A protein (TrxA)

(Buerger et al, 2003; Borghouts et al, 2005). However, alternative scaffolds such as green fluorescent protein and the inactive derivative of staphylococcal nuclease (Klevenz et al, 2002; Martel et al, 2006) have also been successfully integrated into peptide aptamers. In contrast to other man-made proteins, where a random peptide sequence and a protein are fused via their terminal ends, the variable peptide sequence of peptide aptamers is inserted directly within the scaffold (Baines and Colas, 2006). This use of scaffolds constrains the conformational freedom of the peptide domain, therefore forcing it to adopt a distinct three-dimensional shape. This conformational constraint has many advantages: (i) it renders the peptide aptamer less susceptible to proteases (ii) it aids in exposing amino acids which would otherwise be buried in flexible peptides and (iii) increases the binding efficacy of the peptide towards its target (Klevenz et al, 2002; Crawford et al, 2003). Selection of peptide aptamers that bind to a pre-determined target is commonly performed using a modified yeast two-hybrid assay (see Colas, 2000 and Hoppe-Seyler and Butz, 2000 for an overview). However, other methods such as phage displays and bacterial selection have also been employed (Colas, 2000).

Peptide aptamers have shown to not only bind to their target protein, but also inhibit protein function directly (e.g. blocking the active site of an enzyme) or indirectly. For example, many proteins need to form dimers (homo- or hetero-dimers) to be active, or recruit other proteins (e.g. kinases, ras proteins) to mediate their function. Such interactions can be masked if the peptide aptamer binds to distinct domains within the protein target. Advantageously, peptide aptamers have a subtle inhibitory effect, since, by binding to a specific domain, only one property of the protein (in proteins with multifunctional domains) is inhibited, while others may remain intact (Borghouts et al, 2005).

Peptide aptamers have been selected against a plethora of targets, including proteins involved in the development and progression of cancer. A peptide aptamer (pep8) has been shown to block cell cycle progression by competitively inhibiting the enzymatic activity of protein kinase Cdk2 (Cohen et al, 1998). The peptide aptamer KDI1 was selected to bind to the intracellular domain of EGFR, a protein involved in signalling cascades which lead to cell growth and proliferation. By binding to EGFR, this peptide aptamer suppressed EGFR-mediated signalling, thereby reducing the growth and metastasis of tumor cells (Buerger and Groner, 2003). The high expression of protein kinase CK2 in human cancers, and its involvement in tumorigenesis, has made this enzyme an attractive therapeutic target in cancer. Using a two-hybrid system approach, a peptide aptamer was selected to bind to protein kinase CK2, which furthermore showed to induce apoptosis in mammalian cells (Martel et al, 2006).

The inhibition of cancer protein targets, and their downstream signalling events, highlights the therapeutic potential peptide aptamers hold. However, as with nucleic acid aptamers, these small molecules also have limitations that need to be addressed, such as their method of delivery, their low cell membrane permeability and their potential immunogenicity (Borghouts et al, 2005). Researchers within the peptide aptamer field are focusing on addressing these issues, but as yet these small drug molecules are still in their early years of development.

2. Decoys

Aptamers can also be used to function as decoys. Decoy molecules are short lengths of double stranded DNA or RNA. These oligonucleotides are synthesised so as to contain the same nucleotide sequence as part of the target gene. As a result, these molecules compete with the target gene for binding proteins which act as transcriptional activators. In this way, decoys attract away the transcription protein which would otherwise bind to the promoter of the target and activate transcriptional events (Opalinska and Gewirtz, 2002). While decoy oligonucleotides are designed so that they are made of the same nucleotide sequence as that of their target gene (or part of it), an alternative method used to inhibit translation or destabilise/destroy RNA employs the use of antisense technology: oligonucleotides with a sequence complementary to their target genes. The more recent approach in this field focuses on the use of catalytically active RNA and DNA for therapeutic (ribozymes, DNAzymes) and diagnostic use (aptazymes/riboreporters and molecular beacons).

3. Antisense agents

While aptamers are mainly generated to target proteins that are believed to be involved in causing diseases, the use of antisense agents is primarily to function at the genetic level of the disease and involves preventing mRNA transport, inhibition of mRNA splicing and inhibition of 5' cap formation of mRNA, all of which prevent maturation of mRNA (Chan et al, 2006). By targeting the genetic cause of cancer, antisense molecules can effectively block the expression of a protein, a process that offers higher specificity than many protein-targeting therapies. For example, many enzymes belong to large families which share high sequence and structural homology, consequently making the ability of small therapeutic molecules to discriminate between individual members a difficult task. This is particularly important when one wants to inhibit the function of an enzyme which undertakes a reaction that is used widely by many enzymes. For example kinases are implied to have an important role in signalling pathways that ultimately lead to the development of many diseases. The function of these enzymes is to primarily phosphorylate other proteins leading to their activation or inactivation, a reaction which is an attractive target for many drug-like molecules. However, given that a large number of kinases exist (each having different targets), the ability to develop small drug molecules to isolate a specific kinase can be challenging. Consequently, many researchers have put considerable efforts into directly targeting the mRNA encoding for such proteins. Thus, a number of antisense oligonucleotides have currently entered clinical trials for a number of targets, both in oncology (**Table 1**) and for other diseases.

Table 1. Antisense oligonucleotides in clinical trials in oncology, with details on their target, trial phase and the companies that have developed them.

Drug	Target	Phase	Company
OGX-011	Clusterin	II	ISIS and OncoGenex
LY2181308	Survivin	I	ISIS and Lilly
LY2275796	eIF-4E	I	ISIS and Lilly
OGX-427	Hsp27	I	ISIS and OncoGenex
Oblimersen (genasense/G-3139)	Bcl-2	III	Genta Inc.
GTI-2040	Ribonucleotide reductase	II	Lorus
GTI-2501	Ribonucleotide reductase	II	Lorus
LErafAON-ETU	c-raf	I	Neopharm
AEG35156	XIAP	II	Aegera therapeutics
AP12009	TGF-□2	II	Antisense Pharma
ISIS-2503	H-ras	II	NCI

The use of traditional antisense oligonucleotides has now mostly been superseded by other types of antisense oligonucleotides, such as siRNA. First discovered in the late 90's by Fire and Mello in 1998, RNA interference (RNAi) has since received considerable interest in its application as a powerful research tool and more importantly as a potent therapeutic. Their research in the nematode *Caenorhabditis elegans*, showed that naturally occurring dsRNA molecules effectively led to the down-regulation of gene expression, for which they coined the term RNAi. Since then, this natural process for gene silencing has been observed in many organisms ranging from plants to humans (Cullen, 2002; Hannon, 2002). Acting at the post-transcriptional level, RNAi is a process whereby dsRNA molecules trigger the degradation of target mRNA by utilising two proteins conserved among all multicellular organisms. Hence, whilst the traditional antisense mechanism exploits the use of RNase H, RNAi differs by utilising Dicer, and primarily RISC, as a means to regulate gene expression.

RNAi is a naturally occurring phenomenon; hence multicellular organisms contain innate dsRNA molecules to trigger RNAi. This highly conserved method of gene regulation is widely believed to be an evolutionary mechanism to protect the genome of organisms from invading pathogens such as virus' and transposans and defective mRNAs (Cullen, 2002; Hannon, 2002). Scientists, however, have exploited this natural defence mechanism as a powerful therapeutic tool, by rationally designing short dsRNA molecules i.e. siRNAs and introducing them into cells to silence targeted genes. Transfection of larger dsRNAs (analogous to the endogenous ones that trigger RNAi) in mammalian cells often leads to activation of the innate immune response (interferon response) and cell death. siRNAs are typically 21-25 nucleotides in length, with the antisense strand designed to be complementary to any target mRNA, hence these drugs can be used to treat a variety of diseases which involve alterations in gene expression patterns. Several papers have reported the successful transfection of chemically synthesised siRNAs into mammalian cells, which lead to the down regulation of target genes by RNAi. Although RNAi and siRNA drugs are still in their infancy, they are considered to be one of the strongest candidates, among antisense and other types of drug molecules, for the future treatment of a range of diseases including infection, neurodegenerative diseases and cancer.

A number of therapeutic approaches have been adopted with siRNAs. Using liposomes as their delivery vehicle, Alnylam and Protiva were the first group to demonstrate the effective targeted delivery of a siRNA, targeting the apoB gene in the liver of monkeys. Sirna, a leading pharmaceutical company in the design of antisense agents, also work on the design of lipid nanoparticles that change under certain biological conditions, such as a change in pH, which leads to disruption of the endosomal membrane setting the antisense molecule free (Arnaud, 2006). Nastech, on the other hand, used peptide carriers as a delivery method, whereby a siRNA is directly conjugated to a peptide. The siRNA is synthesised as a slightly longer version (around 25-30 nucleotides) so that it can act as substrate for Dicer. As a result, following delivery, the peptide moiety is cleaved by Dicer, which concomitantly frees the siRNA molecule allowing it to be incorporated into RISC for gene silencing (Arnaud, 2006). Various other types of targeting ligands are also being investigated, such as the use of fusion proteins (e.g. antibody-protamine fusion protein complexed to siRNA, for delivery and targeting of siRNAs; Song et al, 2005) and aptamers. A recent study showing promising results in xenograft models demonstrated the use of an aptamer conjugated to siRNA via a double stranded RNA linker, for the targeting of prostate cancer cells. The aptamer used was specific for PMSA, a receptor that is overexpressed and internalised in prostate cancer cells, while the siRNA was used to silence survival genes overexpressed in cancer cells. Incubation of the aptamer-siRNA complex in cells expressing PMSA, led to internalisation of the complex and subsequent incorporation of the siRNA into the RNAi pathway, ultimately leading to targeted gene silencing and cell death. The aptamer-siRNA complex, however, did not bind or function in control cells that didn't express PSMA. Furthermore, the antisense complex also inhibited prostate tumour growth in xenograft models (McNamara et al, 2006). In another, recent, application of aptamers coupled

to a short hairpin RNA (shRNA), these constructs have demonstrated dose-dependent inhibition of RNAi by theophylline. To achieve this, the aptamer was coupled to the loop region of shRNA designed to silence fluorescent reporter genes and, by changing its conformation upon binding to theophylline, conferred the ability to modulate gene expression in a theophylline dependent manner (An et al, 2006). In a similar approach to this, though using antisense oligonucleotides, has also been used to modulate gene expression based on aptamer recognition of a target molecule. In this approach, modular riboregulators or antiswitches were designed to regulate expression of target transcripts as a response to ligand binding. The riboregulators use an antisense domain to control gene expression and an aptamer domain to recognise the specific regulating ligand. Ligand binding to the aptamer induces conformational changes that allow the antisense domain to interact with the target mRNA to affect translation, thus controlling translation (Bayer and Smolke, 2005).

Although siRNAs have entered clinical trials against various other diseases, such as HIV, asthma and age-related macular degeneration, currently no clinical trials for cancer have been established.

4. Ribozymes and DNAzymes

Ribozymes (RNA enzymes) are RNA molecules capable of catalysing the cleavage of either their own RNA or other RNA substrates (Tang and Breaker, 1997). Many naturally occurring ribozymes exist, of these RNAse P and the hammerhead ribozyme have been extensively studied (with their structures being widely used as a foundation for engineering other ribozymes; see below). RNAse P catalyses the cleavage of precursor tRNAs leading to their maturation. Conversely, hammerhead ribozymes are mostly self-cleaving enzymes (Khan, 2006).

The principal structure of these enzymes consists of a catalytic motif flanked by substrate binding domains. The substrate-binding domain entails a sequence antisense to the target mRNA, allowing the enzyme to hybridise specifically to its substrate by Watson-Crick base pairing. This brings the RNA substrate close to the catalytic domain, which cleaves the substrate at a specific site recognised by the ribozyme (Famulok and Verma, 2002). The mechanism of catalysis undertaken by these RNA enzymes is a 2' oxygen nucleophile attack of the adjacent phosphate in the RNA backbone, leading to the formation of 2', 3'-cyclic phosphate and 5' hydroxyl terminus (Khan, 2006). The resultant products are subsequently degraded by ribonucleases, guaranteeing permanent inactivation of the target. Following cleavage, the ribozyme is able to dissociate itself from the RNA products and bind to another RNA molecule to be cleaved. The significant advantage of ribozymes over other pharmaceutical reagents stems from their enzymatic property of recycling.

Ribozymes can be potentially synthesised to target almost any RNA sequence. By incorporating the catalytic domain of ribozymes into short oligonucleotides, antisense to any gene of interest (i.e. changing the sequence of the substrate recognition domains), highly specific enzymes have been engineered. For example, Angiozyme targets the vascular epidermal growth factor receptor and is currently being used in clinical trials to treat metastic colorectal cancer. Another ribozyme in clinical trials to treat breast and ovarian cancer is Herzyme, used to target human epidermal growth factor 2 (HER2) (Zaffaroni and Folini, 2004). Therefore, the application of RNA cleaving enzymes in suppressing the expression of a range of therapeutically relevant genes has shown considerable promise. However, as with other oligonucleotide based therapeutics, limitations such as the mode of delivery, target site identification and accessibility, cleavage efficiency and *in vivo* stability of ribozymes need to be addressed. Chemical modification of ribozymes offers the potential of overcoming such problems. For example, the use of phosphorothioate nucleotides in ribozymes considerably improves their nuclease stability (Pan and Clawson, 2006). However, such modifications often dramatically reduce the catalytic activity of the enzymes. Alternatively, a more "natural" approach is to use DNA based enzymes, as DNA are less sensitive to nucleases than RNA. DNAzymes are DNA backbone based enzymes capable of specifically cleaving RNA targets. Their structure (catalytic domain and two substrate binding domains) and mechanism of catalysis is analogous to ribozymes (for examples of DNAzymes targeting cancer related genes see Dass, 2006). DNAzymes are developed by *in vitro* selection as they are not known to exist naturally (Dass, 2006). Hence, ultimately DNAzymes (and engineered ribozymes) can be considered as catalytic DNA aptamers. As with ribozymes, DNAzymes suffer from the same drawbacks which hinder their therapeutic application. Conversely, these small molecules are being successfully applied as signalling molecules in the diagnostic field of research and medicine (see below).

B. Aptamer-based molecules for diagnostics

The accurate detection of cancer markers in blood and tissue is a matter of increasing importance in the early diagnosis and thus, the treatment of cancer. The versatility of aptamers is exemplified in their diagnostic application as biosensors. Their exploitation in this field stems from the ability of some aptamers to undergo conformational changes upon ligand binding. Combined with their high affinity and specificity for their target, aptamers can be efficiently used to produce signals in response to the binding of a ligand in two ways: i) the direct use of aptamers conjugated to dyes, so that they themselves detect and produce the response signal (i.e. as molecular/aptamer beacons), or ii) the conjugation of aptamers to a secondary biomolecule, which produces the signal response (i.e. as aptazymes).

1. Molecular/aptamer beacons

Molecular beacons (MB) are single stranded oligonucleotide probes used to detect the presence of target oligonucleotides (Tyagi and Kramer, 1996). These molecules are designed to form hairpin structures, with the loop region designed to contain a complementary sequence to the pre-determined sequence of the target (**Figure 2**). The stem structure is composed so that ends of

the oligonucleotide interact with each other. At either end of the stem, a fluorophore and its complementary quencher is covalently attached. The hairpin structure of MB is designed so that the fluorophore and quencher stay in close proximity to each other, leading to the quenching of any fluorescence that may be emitted by the fluorophore by means of fluorescence resonance energy transfer (FRET). In the presence of its target, the loop region of MB hybridises in a sequence-dependent manner to the oligonucleotide target, which in turn causes conformational changes in the MB. Such ligand-induced conformational changes cause destabilisation of the hairpin and the stem region to separate. This results in the spatial separation of the fluorophore and quencher, resulting in a loss of FRET and a readily detectable signal (Vet et al, 2002; Rajendran and Ellington, 2003; Tan et al, 2004). Ultimately, MB are designed to act like switches, where in the absence of their target they are normally switched off (i.e. in the hairpin state), while in the presence of their target they are switched on (i.e. duplex structure forms between target and loop region of MB and destabilises the stem). These signalling molecules are thus ideally suited to function in diagnostic assays. Indeed, MB have found a variety of applications such as in real time PCR, *in vivo* gene expression analysis and RNA-DNA interactions (Stewart, 2005), with each MB being designed to meet the needs of its application. MB assays offer the advantage of having low background signal and high specificity, as these probes are able to discriminate between two targets that differ only by a single nucleotide (Potyrailo et al, 1998). However, this specificity is only directed towards oligonucleotides targets as their ability to detect other analytes is limited. Their inability to detect proteins in particular has largely prevented their universal use in diagnostic assays. The conceptual framework of MB has, however, been applied to function for more versatile targets and this, not surprisingly, exploits the use of aptamers. Aptamer beacons (AB) combine the exceptional signal transduction mechanism of MB and the high specificity and affinity of aptamers for proteins. While labelled aptamers can be used as substitutes for antibodies in diagnostic platforms such as ELISAs and western blots, one powerful application is as AB. Although many aptamers undergo ligand-dependent conformational changes, they can also be engineered to undertake such transformations so that they efficiently function as AB. Adding complementary nucleotides conjugated to a fluorophore and quencher onto the 5' and 3' end of the anti-thrombin aptamer, permitted the engineering of an AB designed to detect thrombin. In the absence of thrombin, the nucleotides at the 5' and 3' end form a duplex which forces the AB to adopt a stem loop structure and destabilises the native binding structure of the aptamer. This non-native state thus exists as a quenched hairpin in the absence of its target. In the presence of thrombin, the native aptamer structure is adopted and stabilised by ligand binding, which ultimately results in the separation of the fluorophore and quencher molecules leading to the concomitant increase in fluorescence (Hamaguchi et al, 2001). Other AB, most using the anti thrombin aptamer as a model, have also been developed by either modifying the conceptual framework of the beacon, i.e. quaternary structure, or the signalling method employed to transduce ligand binding into a signal (see Nutiu and Li, 2004; Wang et al, 2005 for overviews of different AB/MB designs). An AB for the detection of TAT (HIV protein) was designed, where the anti-TAT aptamer was split into two pieces, with one piece being converted into a MB. In the presence of TAT, reassembly of the anti-TAT aptamer pieces was promoted leading to the opening of the AB piece and the generation of a fluorescent signal (Yamamoto and Kumar, 2000). Potyrailo and colleagues immobilised in 1998 the anti-thrombin aptamer onto a glass support which allowed the sensitive transduction of thrombin concentrations into changes of fluorescence anisotropy. AB for the platelet derived growth factor (PDGF) have also been developed using various fluorophore-quencher pairs, and each one being successful in the sensitive detection of PDGF.

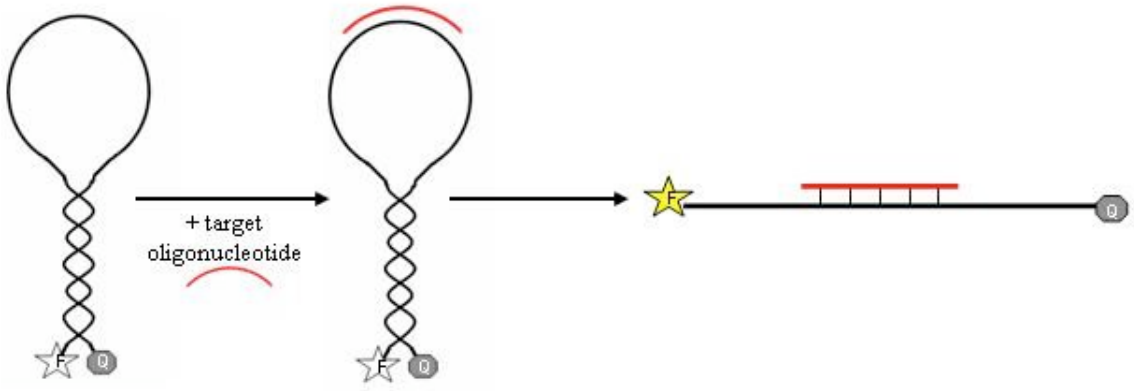

Figure 2. Structure and mechanistic function of molecular beacons. In the closed state, molecular beacons (MB) exist as hairpin structures with the fluorophore and quencher being in close proximity and thus fluorescence being quenched. In the presence of its target, binding to the loop region causes the destabilisation of the duplex region of the MB which in turn causes the spatial separation of the reporter molecules, ultimately leading to fluorescence emission.

Sensitivity of the AB was such that as little as 10 ng PDGF per µg of serum proteins from cell culture media could be detected (Vicens et al, 2005). The ability to use AB with different FRET pairs highlights the potential that multiple AB for selected targets can be engineered with different FRET pairs and consequently used in multiplex bioassays (Vicens et al, 2005). Quantum dots have also been engineered into AB as substitutes for the fluorescence-quencher pairs. Quantum dots hold many advantages over standard fluorophores, such as being more photostable, having longer fluorescence lifetimes, sharper emission bands and although they all respond to the same excitation wavelength, their emission wavelengths differ. In one study, the anti-thrombin aptamer was conjugated to a quantum dot and another oligonucleotide, designed to hybridise and destabilise the aptamer structure, was conjugated to a quencher. Binding of thrombin led to the stabilisation of the native quadruplex structure of the aptamer, which caused the displacement of the quencher-oligonucleotide and the concomitant increase in fluorescence. Quantum dot based AB also have the potential to be used in multiplex assays. The fact that different quantum dots have different emission wavelengths, even with the wavelength for excitation being the same, allows multiple targets to be screened by appending different quantum dots onto different aptamers (Levy et al, 2005). Although AB offer a sensitive and versatile method into target detection, their engineering is significantly challenged by the fact that a detailed knowledge of the aptamer sequence and structure is required. MB based assays are also liable of producing false positive results under certain unexpected conditions. For example, the duplex structure formed between the target and MB (or AB) loop could be forced to dissociate under conditions such as low metal ion concentrations, changes in temperature or denaturing reagents (Nutiu and Li, 2004). The susceptibility of these beacon molecules towards nucleases in biological samples also needs to be considered. The progressive research into aptamers themselves will not only help to overcome the challenges associated with MB/AB but also facilitate the development of other aptamer-based signalling molecules, such as aptazymes, which function using an alternative method of signal transduction-enzyme catalysis.

2. Aptazymes/Riboreporters

The ability of enzymes to modify the intrinsic property of a substrate to one representative of the product(s) makes them powerful molecules in producing signal changes/response. For example, if a substrate has an absorbance that differs from that of the cleaved products, then a simple assay for substrate detection can be devised (where either a decrease in substrate absorbance or increase in product absorbance is monitored). Furthermore, many enzymes can be allosterically regulated, where an effector (such as ions, nucleotides, co-factors, proteins) can either activate or inhibit the activity of an enzyme by binding to it. Effector binding usually induces conformational changes in the enzyme which in turn influences its activity. Hence, enzymes can be employed to either detect the presence of its substrate or the effector which regulates its activity, by simply measuring the signal from substrate cleavage. Ligand-induced conformational changes have been shown with aptamers and this property has conveniently been engineered into ribozymes, yielding sensitive biosensors called aptazymes (or RiboReporters™). Aptazymes are synthesised by appending an aptamer, specific for a ligand, to a ribozyme via a linker (or "communication module"; see **Figure 3**). In this way, interaction of the ligand (or effector) with the aptamer is coupled to the ribozyme, via the communication module, by either activating or inhibiting the enzyme activity (Kertsburg and Soukup, 2002; Silverman, 2003). Hence, the conformational adaptability of aptamers permits the allosteric regulation of ribozyme activity, as in nature, allosteric regulation of ribozymes has not yet been identified (Silverman, 2003).

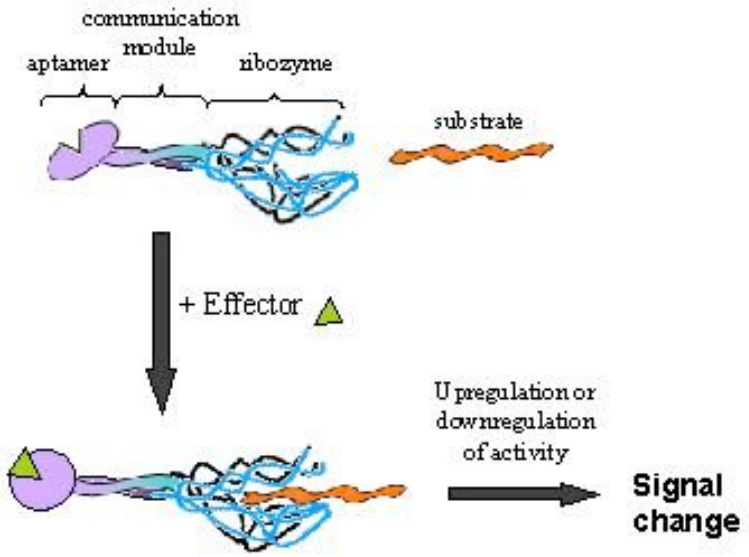

Figure 3. Schematic composition and function of an aptazyme. Aptazymes are typically made by conjugating an aptamer to a ribozyme via a communication module. Effector binding induces conformational changes in the aptamer, which is relayed to the ribozyme via the communication module. The change in ribozyme activity (by either upregulation or downregulation), as a result of effector binding, can then be monitored by suitable methods.

Although small peptides have been known to increase ribozyme activity, the extent of activation compared to basal activity is relatively small. Conversely, upon conjugation of an aptamer, ribozyme activity has shown to be greatly facilitated by an effector, typically between 10 to 10^3-fold (Silverman, 2003). Aptazymes also show exceptional specificity for their effectors, whereby they are able to discriminate between small differences in functional groups and protein isoforms, primarily owing to the aptamer domain. When conjoined, the specificity of an anti-theophylline aptamer has shown to be conferred to a theophylline aptazyme. Although a modest 18-fold activation in aptazyme activity was observed in the presence of theophylline, no activation was prevalent in the presence of caffeine, a molecule which differs by only one methyl group (Thompson et al, 2002). The theophylline-induced aptazyme has also been used in the design of an aptazyme-based riboswitch that can offer a detector-free and label-free detection of aptamer binding to its target, through the expression of the gene of a reporter protein (Ogawa and Maeda, 2007). In this work, the whole construct of aptazyme, ribosome-binding site, anti-ribosome-binding site, necessary stem loops and reporter gene are included in a DNA template that, during transcription results in a conformation that does not allow ribosome binding and expression of the reporter protein. However, binding of the cofactor theophylline induces conformation changes that results in ribosome binding and expression of the reporter protein. Subsequent detection of the reporter protein levels corresponds to cofactor binding to the aptazyme (Ogawa and Maeda, 2007).

Aptazymes modified with a fluorescence based signalling system have also been engineered and such molecules have been designated RiboReporters™. An ADP aptamer appended to the core unit of a hammerhead ribozyme with an integrated fluorescent-quencher system, showed ADP-dependent ribozyme self cleavage, which led to the release of the quencher and the generation of a fluorescent signal in the presence of ADP. This RiboReporter™ was able to specifically discriminate between ADP and over 100-fold molar excess of ATP, demonstrating the exceptional specificity of the aptamer domain (Srinivasan et al, 2004). Ferguson and colleagues also generated in 2004 fluorescence based RiboReporter™ sensors able to detect caffeine or aspartame. The aspartame RiboReporter™ demonstrated exquisite specificity whereby ribozyme activity was facilitated 50-fold by aspartame, while it remained unaffected in the presence of the closely related molecules, β-aspartame and phenylalanine. The function of aptazymes fundamentally entails conformational changes in the aptamer domain upon effector binding, which via the communication module, leads to changes in ribozyme activity. However, the detailed coupling mechanism of effector binding to ribozyme activity is yet unknown, although it is generally postulated that the aptamer-ligand complex stabilises the structurally active conformation of the ribozyme (Robertson and Ellington, 1999; Najafi-Shushtari and Famulok, 2005).

The remarkable utility of aptazymes as biosensors stems from their ability to act as molecular switches whereby enzyme activity can be turned on or off in response to the target being screened (i.e. effector/ligand for the aptamer domain). This has been demonstrated by the Ikebukuro group, who measure thrombin activity based on the ability of the construct to change conformation upon binding to the ligand for detection, whether a small molecule, such as adenosine (Yoshida et al, 2006a) or a complementary piece of DNA (Yoshida et al, 2006b). Furthermore, combining the multiple turnover activity of ribozymes with the high specificity and affinity of aptamers, allows the engineering of biosensors with unique sensitivity. That is to say, signal (i.e. the response to effector detection) amplification can be easily achieved even at low effector/target concentration, by serial addition of the substrate (RNA) which enhances catalytic turnover (Cho et al, 2005). Consequently, the combined advantages offered by both the aptamer and ribozyme domain, allows for aptazymes to be used as efficient substitutes for antibodies in assays used to detect specific disease related makers. Aptamer-based sensors overcome many of the drawbacks associated with the use of antibodies (see below) for the detection of proteins. In addition, the use of aptazyme-based assays omits the use of other secondary molecules typically used to produce the signal, such as secondary antibodies used in ELISAs.

The aptazyme approach to diagnosis can, in principle, be applied to any marker for any disease if aptamer selection towards the target is achieved. Furthermore, the unique ability to select aptamers binding to any molecule allows the application of aptazymes as sensors to be extended outside the field of medical diagnosis. Such an attribute is not available in many antibody-based assays, which are only employed in the biomedical field. For example, aptamer-based assays can be used as environmental sensors whereby they are applied to detect persistent pollutants (such as heavy metals or explosives) in soil and water. Although aptazymes show promising potential as biosensors, the problems associated with their engineering has hindered their success. One of the main problems associated with all aptamer-based modifications, is that it is often difficult to maintain the binding affinity of the aptamer towards the target. The catalytic activity of the ribozyme unit can also be affected by conjugation of the aptamer. Moreover, the innate self-cleaving activity of ribozymes sometimes causes a high background signal (Araki et al, 1998; Kertsburg and Soukup, 2002; Cho et al, 2005). However, this challenge can be overcome by the engineering of an aptazyme which can be allosterically regulated by an inhibitory molecule and an allosteric activator. The hairpin ribozyme consists of two domains which together form a catalytic complex when in the correct conformation. The introduction of an FMN aptamer domain into the ribozyme was shown to facilitate catalysis upon formation of the FMN-aptamer complex. Alternatively, in the presence of an oligonucleotide complementary to the aptamer domain, ribozyme activity was completely abolished. In the presence of FMN, however, a 150-fold increase in activity was observed (Najafi-Shoushtari and Famulok, 2005). Hence, hybridisation of the small inhibitory oligonucleotide can ultimately be employed to reduce

background signal. In addition, other complications associated with all aptamer-based technologies, such as nuclease sensitivity, cost-effectiveness and non specific binding of RNA binding proteins in biological fluids need to be considered before the practical application of such molecules is achieved.

3. Aptamers microarrays

Oligonucleotide microarrays have been used for genomic applications for some time with interesting results. Their widespread use for monitoring gene expression has generated valuable insight into various disease states, analysing gene clusters and revealing polymorphisms. Aptamers, usually oligonucleotides in nature, have also the ability to be used in microarrays and have recently found their way into the microarray scientific literature and market. Different aptamer microarrays with varying detection systems have been described. The basic principle lies on the spotting of oligonucleotide aptamers, RNA or DNA on a surface / chip, the interaction of the aptamers with the analyte containing the target molecule, and the detection of the bound oligonucleotides/aptamers by some physical method.

One such application of the traditional nucleic acid microarray technology to aptamers has been described by Ellington (Collett et al, 2005; Cho et al, 2006). In this, aptamers against specific target molecules, such as thrombin, lysozyme, ricin or IgE have been printed on streptavidin or neutravidin coated glass slides. The total protein content of a cell or a biological medium is that was to be analysed was fluorescently labelled and the analyte solution was incubated with the aptamers on the array. Following washes to eliminate non-binding and non-specific binding proteins from the array, the bound molecules were detected. The array was shown to be particularly sensitive, allowing detection of proteins in the low picomolar to nanomolar range (Collett et al, 2005; Cho et al, 2006). Furthermore, this indicated that multiple protein analytes can be identified simultaneously. This has been stipulated to have great potential in proteomic applications. However, this could equally have substantial applications in diagnostic arrays, where, for example, aptamers against multiple tumour markers could be printed on the slide, allowing simultaneous measurement of various tumour marker levels for better cancer prognosis/early diagnosis.

A similar approach on aptamer microarrays that differs on the detection method is based on enzymatically amplified surface plasmon resonance imaging (SPRI) detection of protein biomarkers in aptamer microarrays. SPRI is perhaps one of the primary optical methods for the detection of affinity adsorption onto biomolecular microarrays, including oligonucleotide (DNA and RNA) and protein arrays. However, enzymatic amplification of the signal can further enhance the sensitivity of the assay. This is a sandwich format, where the aptamers are dotted in the array, the protein is allowed to interact with the aptamer, followed by several washes and that is exposed to a protein-specific antibody-horseradish peroxidase (HRP) conjugate. An HRP substrate is then used to create a localised precipitation reaction that amplifies the SPR signal (Li et al, 2006, 2007). This enzymatically enhanced SPR sensor has demonstrated the ability to also detect multiple analytes, though still at an early stages and not tested in biological fluids as the fluorescent detection microarrays, working only with multiple purified protein samples. However, in these experiments it has demonstrated superior detection capabilities. The fluorescent detection microarray has been reported to detect thrombin in a solution at a 5nM concentration (Cho et al, 2006), whereas the enzymatically enhanced SRPI sensor has shown a detection limit for thrombin of 500fM (Li et al, 2007); a tenfold improved limit of detection. On the other hand, the enzymatically enhanced SPRI is based on the assumption that there is a second binding site on the molecule which is recognised by a HRP-conjugated antibody in a sandwich assay. This would not apply to small molecules or peptides, where the aptamer covers the entire, or the majority of the analyte, leaving little or nothing to be recognised by the antibody part, or where the aptamer would substantially change the structure of the target molecule upon binding, thus inhibiting secondary interactions with the antibody. Furthermore, this implies that such technology would not take advantage of the ability of aptamers to bind to non-immunogenic ligands where antibody development is a difficult, if not impossible task.

SPRI detection in microarrays has also been used with peptide aptamers. However, like most protein microarrays, this is a more complicated task than using DNA microarrays, which is now a standard technology. Yet, peptide aptamer microarrays have achieved a sensitivity of 1nM for their target protein (Davis et al, 2007) and, though still in its infancy, may yet prove to be an interesting alternative in the microarray detection technology.

4. Photoaptamers

One of the potential diagnostic applications of aptamers in the area of proteomics and linked with the development of the aptamer microarrays discussed above is the concept of photoaptamers, developed and championed by SomaLogic Ltd, one of the major players in the aptamer technology development. Though photoaptamers are also used in a microarray format, to allow for detection of multiple analytes, it does not use previously developed aptamers and varying the detection methodology. Instead, it is based on a different approach and a modified selection process, named PhotoSELEX.

In photoaptamer libraries, the tymidine (T) of normal DNA is substituted, usually, by 5-bromo-2'-deoxyuridine, which confers to the aptamers the capability of crosslinking to specific sites of the protein analyte, particularly on tyrosine residues, when exposed to UV light. This has allowed a modified selection process, in which oligonucleotides that do not crosslink with the protein are eliminated from the pool, whilst those that bind and crosslink are subsequently amplified in a fashion similar to the traditional SELEX methodology. Thus, like in the normal SELEX, photoaptamer libraries containing 5-bromo-2'-deoxyuridine instead of thymines are exposed

to the target of interest. Oligonucleotides that do not bind or bind only weakly to the target are washed away in the various wash steps. Here, in contrast with the normal SELEX, what remains bound is exposed to UV radiation which facilitates crosslinking of the aptamer to the target through the formation of a covalent bond between the modified base and an appropriate amino acid (usually tyrosine) that is in close proximity to the binding site. Oligonucleotides that do not crosslink are also washed away. To amplify the bound aptamers by PCR, crosslinked proteins are digested by proteases to reveal the aptamers (www.somalogic.com). The photoSELEX process is described in **Figure 4.**

Once selected, photoaptamer can be used in microarray formats, for proteomic applications, exhibiting a specificity ranging from 100 to $>10^6$ fold for the target proteins over non-specific proteins, with usual values in the 10^4 range (Smith et al, 2002). Examples of photoaptamers in proteomics multiplex photoaptamer-based arrays have been described successfully and have been presented for protein analytes such as interleukin 16 (IL-16), vascular endothelial growth factor (VEGF) and endostatin, with unparalleled sensitivity. The sensitivity reported for such aptamers reaches a detection limit of 10fM, the lowest detection limit reported in the current aptamer microarray technologies (Bock et al, 2004). Even so, photoaptamers have not yet achieved their potential, with only a handful of publications in the literature and no known significant developments. Various potentially limiting factors may include the fact that appropriate amino acids need be close to the binding site of the aptamers to affect crosslinking, or else no hits will be achieved given the nature of the photoSELEX methodology. Furthermore, this technology would not benefit from the multitude of selected aptamers from various groups around the world and photoaptamers for each target would need to be generated. Finally, the strong intellectual property control of SomaLogic prohibits other companies to show an interest in the diagnostic applications of aptamers in general and photoaptamers specifically.

5. Aptamers vs Antibodies

It is far from realistic to suggest that aptamers could replace the role of antibodies in diagnostics. However, aptamers do represent a valuable and viable complimentary technology that as yet appears to have gone unnoticed by all but a handful of research groups. Claims around aptamers have been that they offer marvellous advantages over their antibody counterparts. Yet, while not everything is the way the Gurus would have us imagine, aptamers have both advantages and

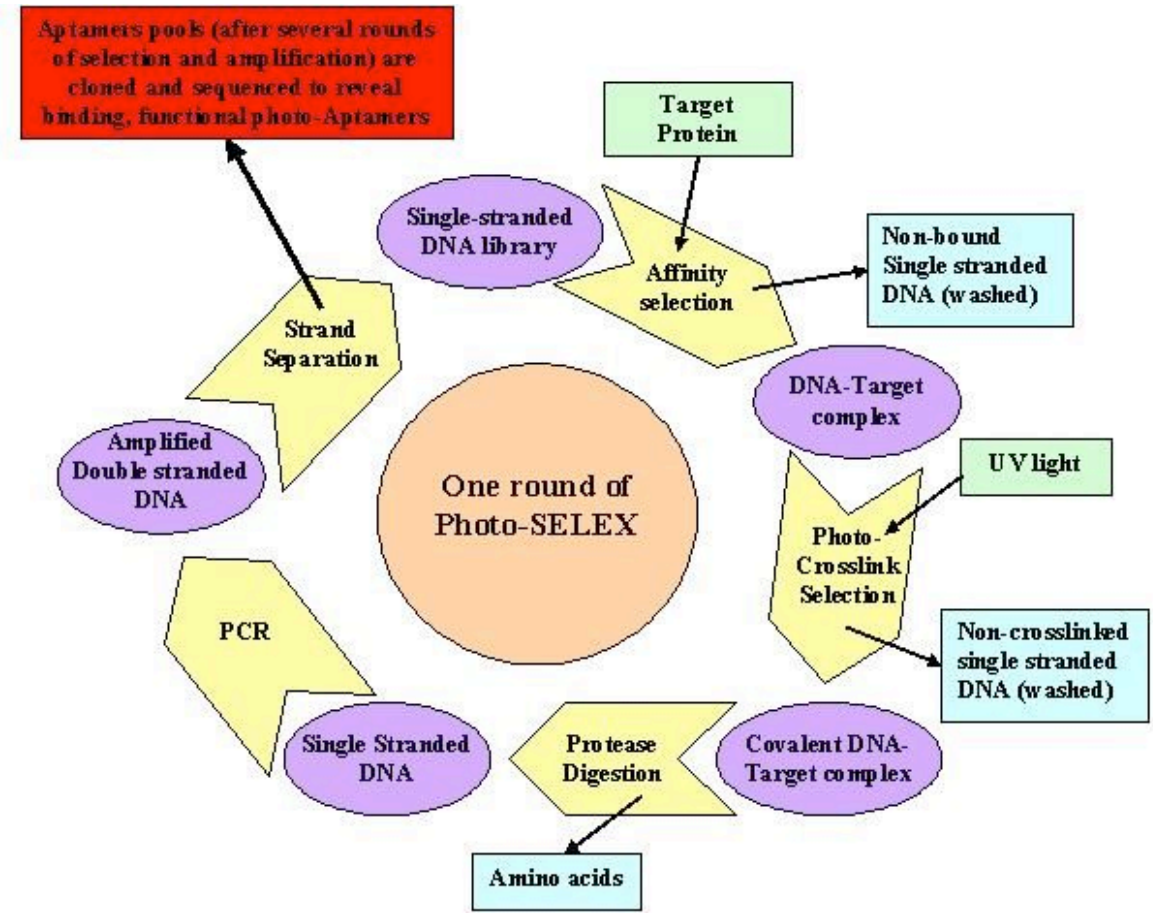

Figure 4. A schematic description of the photoSELEX process (www.somalogic.com).

disadvantages over their older and larger protein relatives. Aptamer recognition affinity and specificity is comparable to those of monoclonal antibodies, and sometimes higher, as aptamers are selected for high affinity, whilst nature often prefers more reversible interactions. Furthermore, aptamers can be selected to recognize and bind a wide range of targets, including toxic compounds, toxins and drug molecules, as well as inherently non-immunogenic molecules that antibodies cannot be raised against (Jayasena, 1999). Such examples include ricin and cocaine which has been a target in sensor development, as we saw above (Stojanovic et al, 2001; Stojanovic and Landry, 2002; Baker et al, 2006; Cho et al, 2006).

Aptamers can act as mimics of antibodies in that they can recognise molecular targets with high specificity and are able to carry therapeutic agents, fluorescent or MRI agents, radioisotopes, etc, directly to solid tumour masses or to individual cells that may have metastasised with greater efficiency (Brody and Gold, 2000; Cerchia et al, 2002; Ferreira et al, 2006; Borbas et al, 2007), thus offering great potential in diagnostic imaging. Furthermore, as aptamers are 10 to 100 times smaller than antibodies, they are expected to achieve higher tumour penetration than their counterparts.

When compared to antibodies, aptamers have advantages and disadvantages as therapeutic and biological reagents (Jayasena, 1999). Aptamers present faster tissue penetration and wider applicability and present the opportunity for simple base modifications to improve functionality by comparison. Furthermore their small size (molecular weights between 3000-20000) may reduce steric hindrance and increasing surface coverage during immobilization on chromatography matrices (Deng et al, 2001), sensors or immunoassays (Ferreira et al, 2008). The *in vitro* selection process (SELEX) can be more precisely monitored than can organismal immunization, and the affinities and specificities of aptamers can thus be better tailored than can those of antibodies (Jayasena, 1999). And yet, as antibody technology continues to evolve, one of the basic advantages often discussed with regards to aptamer, the one of their in vitro generation compared to antibodies' generation within an organism, is getting minimised. Antibody production technologies, such as the SLAM technology used by UCB (www.ucb-group.com), can also result in the raising of antibodies outside animals and the use of hybridomas. This technology, however, still exploits the use of living cells, which does not alter the fact that antibodies could not be raised to toxic chemicals.

One of the main advantages of aptamers is that, once identified, they can be reliably synthesized by automated methods, reducing batch to batch variations. Furthermore, during synthesis, aptamers can be easily modified to facilitate further modifications such as attachment points for enzymes or a variety of other reagents, and the introduction of modified bases for nuclease resistance (**Figure 5**) (Eaton et al, 1997; Gewirtz, 1999; Brody and Gold, 2000).

Aptamers can be stored for long term and transported in ambient temperatures, which gives them an advantage on thermal stability over their antibody counterparts, and are resistant to denaturation and degradation when lyophilised. Furthermore, in sensors and chips can be successfully regenerated, unlike most antibody arrays. However, aptamers are sensitive to nucleases, which presents a problem on diagnostic assays based on the assay of disease markers in biological fluids. The above mentioned modification of aptamers prolongs their lifetime on the sensor and their resistance to nucleases, but mostly delays rather than completely averts their degradation.

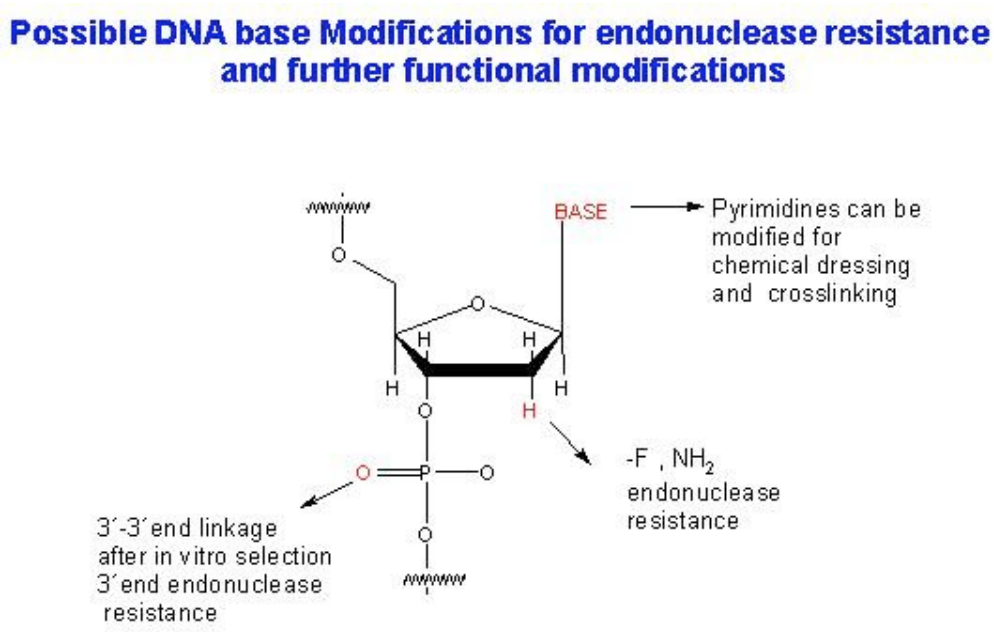

Figure 5. Possibilities for chemical modification of a nucleotide base

Finally, although aptamer production costs are lower than those of an antibody and aptamers can generally be produced faster than antibodies, the price of aptamer synthesis still remains much higher of their protein counterparts. This is largely due to the fact that antibody production technologies have been around for much longer and the need for large amounts of antibodies have pushed technological developments in the area. Instead, only a handful of aptamers have been produced at large enough scale for clinical trials and diagnostic applications to warrant production in gram scale. Thus, the technological focus of oligonucleotide synthesis is limited to small scale experiments for laboratory use. Companies like Pfizer that are now producing the only aptamer in the clinic and Antisoma that are currently conducting clinical trials on aptamers as cancer therapeutics, with the need of large quantities of the therapeutic preparation, are pushing the current technological limits for large scale aptamer synthesis. Yet, a number of pharmaceutical and biotech companies do not yet consider aptamers as valuable reagents due to prohibitive costs and a technology that is not yet robust enough. Finally, the complex Intellectual Property scene on aptamers and the proprietary technology for aptamer production, SELEX, spreads further scepticism over the use of aptamers in diagnostic applications. Yet, with the original patents on SELEX and aptamer production expiring in the next few years, this field remains open and ripe for further exploitation.

Overall, aptamers and their related biomolecules are still in an early stage of development for therapeutic and diagnostic purposes. They do however, clearly demonstrate their versatility in potentially binding a plethora of targets, hence they can find application to fields outside medicine, a benefit that antibodies have not yet overcome. Within the medical field itself, aptamers offer many significant advantages that cannot be dismissed and as such they can rival antibodies on some assays or work with them in others.

III. Concluding remarks

It is undeniable that aptamers offer a great potential for diagnostic and sensor applications and these bypass some of the problems nucleic acid based agents face as therapeutic and clinical agents. The SELEX procedure has offered the possibility to develop aptamers against any kind of target, whilst subsequent technologies for aptamer selection have offered alternatives and improved such selection processes in cost, time or project specific requirements. Photo-SELEX (www.somalogic.com) has promised potential developments in the field of proteomics, whereas SELEX NADIR has offered similar capabilities with photo-SELEX, but with the use of a nanopore reporting mechanism instead of the optical probe reporting one (Winters-Hilt, 2006). However, due to IP restrictions, the commercial applications of these agents are still limited. Thus, very few aptamer microarrays are currently in development from companies like Affymetrix, and one is currently in the market by LC Sciences, which contains 1,500 aptamers against various targets of interests from the published literature (www.lcsciences.com). On the other hand, photoaptamers

Apart from microarrays, a number of other sensor applications have been pursued by various academic and commercial groups. The Archemix group, which have developed fluorescent microarrays described above, have also used fluorescent aptamers in chips and microspheres (Potyrailo et al, 1998; Kirby et al, 2004). Other groups have also used fluorescent detection sensors based on aptamer technologies to recognise small molecules, such as L-arginamide (Ozaki et al, 2006) and ATP (Yamana et al, 2003). A novel application of fluorescent aptamer detection is with the use of quantum dots and a few applications have recently appeared in the literature. The ability of different quantum dots to emit at different wavelengths whilst excited at the same wavelength could offer a solution for multiple analyte detection in solutions, as demonstrated for adenosine and cocaine by Liu and colleagues in 2007. Quantum dot use with aptamers for detection of protein analytes has also been described (Levy et al, 2005; Choi et al, 2006), as well as detection of bacillus spores (Ikanovic et al, 2007), which further demonstrates the flexibility of these constructs.

Apart from fluorescence, a number of other sensor detection methodologies have been adopted with varying degrees of success. These include colorimetric changes (Liu and Lu, 2004, 2006; Cho et al, 2005), electrochemical detection (Xiao et al, 2005; Mir et al, 2006; Lai et al, 2007; Papamichael et al, 2007) and piezoelectric quartz crystal sensors (Bini et al, 2007).

The above methods, fluorescent, electrochemical and colorimetric detection, have also been used in a number of molecular switch approach sensors or modular sensor assemblies, where the aptamers usually change conformation upon binding to either emit a fluorescent signal based on an approach similar to an aptamer beacon on sensor, where the aptamer is labelled both by a fluorophore and a quencher, or through non-covalent interaction with the fluorescent label, triggering of an electrochemical sensor or change of colour (Frauendorf and Jaschke, 2001; Stojanovic et al, 2001; Stojanovic and Landry, 2002; Stojanovic and Kolpashchikov, 2004; Baker et al, 2006; Zuo et al, 2007). These sensors have been shown to be particularly sensitive in recognising small molecules like cocaine (Stojanovic et al, 2001; Baker et al, 2006; Zuo et al, 2007), ATP (Stojanovic and Kolpashchikov, 2004; Zuo et al, 2007) or theophylline (Frauendorf and Jaschke, 2001) with high selectivity.

However, aptamers have been used in enzymatic sensing, without the use of any label or signal related directly to the aptamer. These applications are again based on changes in aptamer conformation of bifunctional aptamers that recognise the target ligand and an enzyme or ribosome. The binding of the aptamer to the ligand results in conformational changes that affect enzymatic activity or protein expression, and it is the later that is subsequently measured (examples in Ogawa et al, 2007; Yoshida et al, 2006a,b). Finally, an enzymatic detection system that is however different in that the enzyme is used not for detection but to ligate proximally bound aptamers to large protein targets and allow their subsequent PCR amplification has been described. This has allowed detection of ligands at zeptomole ($40x10^{-21}$ mol) amounts,

significantly reducing previously described detection limits (Fredrikson et al, 2002).

The breadth of the above techniques available for analyte detection based on aptamer technologies has opened a variety of possibilities for aptamer-based sensors in different applications, ranging from early disease diagnosis and prognosis, to control of substances, such as anabolics and steroids in anti-doping control of athletes, or environmental sensors for the detection of pollutants, explosives or potential infectious agents, giving aptamers a breadth of application that has not been feasible with other agents to date.

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