CHAPTER 1

APTAMERS: LIGANDS FOR ALL REASONS

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1.1 INTRODUCTION

Several strategies were described over the last 25 years that make the use of synthetic oligonucleotides in different perspectives of interest for biology and medicine, thanks to the development of very powerful and relatively cheap methods for nucleic acid synthesis on solid support. These strategies generally do not take into account the genetic information borne by the oligonucleotide chain but rather, explore its wide potential of molecular interaction and recognition. The antisense approach was the first to be developed (Belikova et al., 1967). It rests on a simple hypothesis: the binding to a target mRNA of a complementary sequence (the antisense sequence), thus leading to the formation of a local double-stranded structure, might interfere with reading the message. This was demonstrated experimentally in the late 1970s by pioneering works of Zamecnik and Stephenson (1978), on the one hand, and Miller et al. (1974), on the other. The progress made in the sequencing of entire genomes offered multiple opportunities for validating this technique, which was used not only against messenger but also against pre-mRNA (Sazani and Kole, 2003) or viral RNA (Toulmé et al., 2001). Very quickly it turned out that regular DNA or RNA oligomers suffered from weaknesses for use in biological media. Numerous chemically modified oligonucleotide analogs were synthesized and evaluated (Wilson and Keefe, 2006). In particular, modifications were introduced for improving resistance to nucleases (Toulmé, 2001). A number of clinical trials have been and are still being carried out (Aboul-Fadl, 2005), but disappointingly, a single antisense
oligonucleotide was approved by the U.S. Food and Drug Administration for the treatment of cytomegalovirus–related retinitis (Orr, 2001). More recently, similar modifications were introduced in small interfering RNA (siRNA) that also bind their target RNA through Watson–Crick base pairing (Wilson and Keefe, 2006). siRNAs generally show a higher efficiency than the homologous antisense sequence, due to triggering of the enzymatic destruction of the target transcript by the interference machinery (Scanlon, 2004; Chakraborty, 2007). But both antisense and siRNA suffer from the same types of limitations: target access, specificity of interaction, and cell uptake (Shi and Hoekstra, 2004; Kurreck, 2006).

Antisense oligomers and siRNAs sequences are designed rationally on the basis of Watson–Crick complementarity with the target transcript. During the last 15 years, combinatorial approaches have been developed in both chemistry and biology (Fernandez-Gacio et al., 2003; Frankel et al., 2003; Li and Liu, 2004). In such methods a family of candidates is randomly synthesized. Molecules exhibiting the desired property are then extracted from this pool. There is no prerequisite to the use of such methods: The structure of the target does not need to be known nor does one need to postulate the interactions that will take place between the target and the successful candidate. The interest in a combinatorial approach is related directly to the size of the pool—the molecular diversity—that can be screened. From this point of view, oligonucleotide libraries surpass by several logs the complexity of any other type of library. In vitro selection of oligonucleotides can be undertaken in pools containing up to \(10^{15}\) different candidates (Gold et al., 1995). This is in large part related to the information encoded in the candidate. Consequently, in contrast to any other compound, oligonucleotide candidates from the pool can be amplified and analyzed very easily. Indeed, covalent fusion between an mRNA and the polypeptide it codes for allows the screening of very large libraries (\(10^{12}\) to \(10^{13}\) independent fusion candidates) and makes the in vitro selection of proteins by ribosome display a very powerful method (Roberts, 1999).

Pioneering work recognized the interest of either selection or in vitro evolution of nucleic acid mixtures for the identification of protein-binding sites or replication suitability (Mills et al., 1967; Blackwell and Weintraub, 1990). But in 1990, three laboratories independently described a procedure for the identification, within large pools of randomly synthesized molecules, of nucleic acid sequences exhibiting a predetermined property: affinity for a given target (Ellington and Szostak, 1990; Tuerk and Gold, 1990) or enzymatic activity (Robertson and Joyce, 1990). This was achieved through repeated rounds of selection and amplification, thus ensuring directed evolution of the starting pool in response to selection pressure on the population. This method, now known as SELEX (systematic evolution of ligands by exponential enrichment) leads to the selection of aptamers [i.e., oligomers able (apt) to carry out some function]. Since numerous papers described an entire range of applications for aptamers that take advantage of their wide potential due to both the high strength and the specificity of their interaction with their target (Osborne and Ellington, 1997; Famulok, 1999; Jayasena, 1999; Wilson and Szostak, 1999; Brody and Gold, 2000; Toulmé,
Examples are found in many different fields, and excellent reviews were recently published that cover the use of aptamers for the validation of targets (Toulmé et al., 2001; Blank and Blind, 2005; Bunka and Stockley, 2006), for the design of therapeutic agents (Nimjee et al., 2005), for cancer (Ireson and Kelland, 2006), for infectious diseases (Held et al., 2006; James, 2007), for gene therapy (Fichou and Ferec, 2006; Que-Gewirth and Sullenger, 2007), for the development of analytic tools (Tombelli et al., 2005; Mairal et al., 2007), or for the design of probes for imaging (Pestourie et al., 2005). We describe the SELEX process briefly and review a few points dealing with two wide areas of application for aptamers: regulation and sensing.

1.2 THE POWER OF SELECTION AND APTAMER REFINEMENT

Only a few general points are addressed here. The reader is referred to a recent review by Gopinath (2007) for details on procedures for the selection of aptamers. Like any other combinatorial method, the SELEX methodology first requires synthesis of the library. Compared to other libraries, it is easy to prepare an unbiased pool of DNA sequences, as the coupling efficiency of the A, T, G, or C phosphoramidite is very similar. One could compensate for the slightly different incorporation of nucleotides: a mixture of phosphoramidites in a ratio of 1.5 : 1.25 : 1.15 : 1.0 (A : C : G : U/T) is believed to produce a balanced mixture of sequences (Ho et al., 1996). The diversity of the library is fixed by the length of the random region. One generates \(4^n\) different sequences \(n\) nucleotides long. The experimental limit (about \(10^{15}\)) corresponds to the diversity obtained for a random region about 25 nucleotides long. The full theoretical diversity is not covered for pools of candidates with a wider randomized window. For instance, only 1/1000 of the theoretical population will be present in a library of candidates with 30 random nucleotides. However, as the fidelity of the polymerases used in the SELEX process is rather low, each amplification round will generate variants that were not present at the preceding selection step, hence increasing the size of the sequence space explored.

Aptamers are then isolated by an iterative process (typically, 7 to 15 rounds) of binding, partitioning, and amplifying nucleic acid variants (Figure 1.1). The evolution of the population is driven by the selection conditions; the stringency (concentration, incubation time, washes, etc.) is increased progressively from round to round for selecting the candidates exhibiting the highest possible affinity (Gopinath, 2007). Selection is a tedious and time-consuming process when carried out manually. Automated selection was reported about 10 years ago (Cox et al., 1998; Brody and Gold, 2000; Eulberg et al., 2005). Several biotech companies have developed specialized procedures for high-throughput production of aptamers that reduce the isolation time from several months to a few days (Blank and Blind, 2005). An automated microchannel-based platform was recently described (Hybarger et al., 2006). The aptamers generated by automated selection are equivalent to those derived from manual selection, and those isolated against proteins show dissociation constants in the nanomolar range.
Intramolecular base pairing defines higher-order structures. Therefore, a nucleic acid library of sequences is actually a library of three-dimensional shapes. Every candidate will display a unique combination of double-stranded helical segments, loops, and bulges. Each nucleotide may contribute hydrogen bonds and electrostatic and van der Waals interactions. The scaffold resulting from the intramolecular folding of oligonucleotides constituting the library offers a three-dimensional potential for interacting with any type of target. The selection process therefore corresponds to the capture of candidates that display a set of interacting groups complementary to that of the target. The association is even optimized through an induced fit mechanism: the aptamer acquires its final shape upon binding to its target (Patel et al., 1997; Hermann and Patel, 2000). This results in both a very strong affinity and a high specificity. For small molecules (e.g., amino acids, nucleosides, dyes, antibiotics), equilibrium dissociation constants in the micromolar range are frequent, whereas $K_d$ values from $10^{-9}$ to $10^{-12}$ M$^{-1}$ are typically obtained for proteins (Osborne and Ellington, 1997; Jayasena, 1999).

What makes aptamers ligands of great interest is their exquisite specificity. One of the clearest examples is the aptamer selected against theophylline, a purine derivative used for the treatment of asthma. This aptamer binds with a 10,000-fold-lower affinity to caffeine, another purine analog that differs from theophylline by a single methyl group on the N-7 position (Jenison et al., 1995). This exquisite selectivity was achieved through a careful selection procedure: oligonucleotides bound to the support functionalized with theophylline were first eluted with a solution of caffeine that made it possible to get rid of candidates that did not discriminate between the two purine compounds. A high degree of specificity could be reached even without such a counter-selection step. For example, the pseudoknot aptamer selected by Gold and co-workers against the reverse transcriptase (RT) of the human immunodeficiency virus does not bind to murine or feline RTs (Tuerk et al., 1992). But aptamers have been raised
against these enzymes that display similar affinity ($K_d = 5 \text{ to } 20 \text{ nM}$) and do not bind to the HIV enzyme (Chen and Gold, 1994; Chen et al., 1996). Strikingly, these aptamers are specific inhibitors of their cognate enzyme, indicating that they hinder and very likely bind to the catalytic site, as these enzyme are nucleic acid–binding proteins. Despite the identity of the function ensured by these polymerases, specific aptamers have been selected, suggesting that they do not interact with the conserved residues responsible for the catalytic activity. Similar results were obtained for targets that are not natural ligands of nucleic acids: aptamers raised against human immunoglobulin E (IgE) or against human matrix metalloproteinase 9 do not bind to their murine homolog (Mendonsa and Bowser, 2004; Da Rocha-Gomes et al., unpublished results). The same level of specificity can be reached with nucleic acid targets. It has been demonstrated that an aptamer raised against a hairpin structure and interacting with the loop through the formation of six base pairs was far more specific than the anti-sense sequence generating the same pattern of Watson–Crick pairing (Darfeuille et al., 2006). Compared to the antisense–sense duplex, the three-dimensional structure of the aptamer–hairpin kissing complex provides additional elements of recognition. Interestingly, in vitro selection was used to identify hybridization probes that discriminate strongly between variants of the human papilloma virus (Brukner et al., 2007). Optimizing the probes rather than the hybridization conditions generated oligonucleotides that show a “relaxed” binding potential (i.e., partial complementarity) but that minimize cross-reactivity. This method might be generalizable to the design of genotyping kits.

It has been possible, however, to select aptamers that cross-react with different molecular species. In toggle SELEX, the target is changed during alternating rounds of selection. This strategy yields aptamers that recognize both human and porcine thrombin and display similar properties toward the two proteins: plasma clot formation and platelet activation (White et al., 2001). This approach is useful when one wants to raise ligands recognizing a class of structurally and chemically related targets.

Due to these properties, aptamers are considered to be “chemical antibodies” and actually rival or sometimes surpass antibodies. Indeed, the ease and the reproducibility of the synthesis as well as several other properties make aptamers interesting alternatives: it is, for instance, possible to raise aptamers against toxic substances.

### 1.3 THE CHEMISTRY DRIVES THE SHAPE

The initial random library is always synthesized as DNA, but the starting pool used for selection can be made of RNA candidates. In this case the DNA library is transcribed prior to SELEX. Either RNA or DNA aptamers exhibiting similar properties can be selected against a given target. The sequences selected against the HIV-1 reverse transcriptase constitute a good example. DNA and RNA aptamers are competitive inhibitors of each other, indicating that there is
APTAMERS: LIGANDS FOR ALL REASONS

a major site for aptamer interaction on the surface of the enzyme that drives the selection (Tuerk et al., 1992; Schneider et al., 1995). Importantly, the anti-HIV-1 RT RNA and DNA aptamers have very different sequences and structures. Whereas the RNA aptamer is a pseudoknot, the DNA aptamer is an imperfect hairpin. The DNA version of the RNA pseudoknot does not bind to the HIV-1 RT. It should be remembered that the conformation of DNA and RNA double helices differs. As aptamers are shapes, not surprisingly, changing the chemistry changes—generally weakens or even abolishes—the binding properties. It is therefore of prime importance to define the chemistry of the library prior to performing the selection. The choice of the aptamer chemistry is partly guided by the intended use of the aptamer. DNA aptamers are far cheaper than RNA aptamers. But if post-SELEX modifications have to be introduced, there are more RNA-mimic oligonucleotide derivatives; in addition, RNA aptamers can be expressed inside cells from DNA expression vectors.

Chemically modified aptamers have been developed due to intrinsic limitations of regular RNA and DNA oligomers (Wilson and Keefe, 2006). In particular, it has long been recognized from studies on antisense sequences that unmodified nucleic acids are short-lived species in biological media. The presence of nucleases in serum leads to the rapid digestion of natural oligonucleotides. Numerous modifications have been described by chemists that render nucleic acid resistant to nucleases (Toulmé, 2001; Wilson and Keefe, 2006). These include substitution at the 2’ position (e.g., 2′-O-methyl, 2′-fluoro) (Kubik et al., 1997; Prakash and Bhat, 2007), phosphate modification (e.g., phosphorothioate, phosphoramidate, morpholino) (Koizumi, 2007), nucleoside modification (e.g., α anomer, bicyclic sugar) (Orum and Wengel, 2001), or even the use of a polyamide backbone (peptide nucleic acid) (Elayadi and Corey, 2001). However, most of these modifications cannot be introduced during the SELEX process, as the modified nucleotides are not substrates for polymerases and therefore cannot be used by the relevant enzymes either for generating the initial library or for amplifying the oligomers selected.

2′-Fluoro- or 2′-aminopyrimidine derivatives (Figure 1.2a) are widely used for the production of aptamers in which regular purine residues are incorporated (Aurup et al., 1992; Proske et al., 2002; Rhie et al., 2003). The resulting oligonucleotides show improved resistance to nucleases. Alternatively, the four phosphorothioate triphosphates in which a nonbridging oxygen of the internucleoside linker has been substituted by sulfur can be used in polymerase chain reaction (PCR) amplification (Andréola et al., 2000). Similarly, ribonucleoside boranophosphates have been demonstrated to be incorporated by T7 RNA polymerase (Shaw et al., 2003). This enzyme is also able to polymerize transcripts containing 4′-thiopyrimidines (Figure 1.2a), a modification that increases their stability by about 50-fold relative to unmodified RNA (Kato et al., 2005). It was recently reported that the combination of mutated T7 RNA polymerases, Y639F and Y639F/H784A, allows the efficient incorporation of all four 2′-O-methyl nucleotides (Chelliserrykattil and Ellington, 2004; Burmeister et al., 2005, 2006).
Figure 1.2  Modified nucleotides and nucleosides described in the text. (A) Nucleotides incorporated by polymerases yielding nuclease-resistant oligonucleotides. Top from left to right: 2'-fluoro, 2'-amino, 2'-O-methyl. Bottom from left to right: boranophosphate, phosphorothioate, 4'-thio. (B) Photosensitive residues. Left to right: 5-bromo-U, 2-(2-nitrophenyl)ethyl T, 2-(2-nitrophenyl)propyl T. (C) Amino–imino equilibrium used in 2D-SELEX (see the text).
The positions that remain unmodified at the end of the in vitro selection procedure (e.g., the purine residues in a selection carried out with 2′-fluoropyrimidine triphosphates) can be modified post-SELEX for further optimization of the aptamers. A systematic study of the 64 variants of the six-membered apical loop of an anti-TAR aptamer led to the identification of locked nucleic acid/2′-O-methyl chimeras fully resistant to nucleases that displayed anti-HIV-1 properties in a cell culture assay (Di Primo et al., 2007). Identification of the few residues that cannot be modified in an RNA aptamer can be carried out by chemical interference, a method used to identify chemical variants of the aptamer originally selected. Such an approach led to the synthesis of a modified anti-HIV-1 reverse transcriptase in which all but two of the positions of the RNA aptamer were substituted by 2′-O-methyl residues (Green et al., 1995). This was also the case for the aptamer used for age-related macula degeneration in human beings (Ruckman et al., 1998).

An original approach developed by Klussmann and co-workers relies on L-enantiomers of aptamers (called spiegelmers) (Vater and Klussmann, 2003). L-DNA (or L-RNA) is the mirror image of natural D-DNA (or D-RNA). L-nucleic acids are fully resistant to nucleases, but they cannot be processed by polymerases. Therefore, a natural D-aptamer will first be raised against the mirror image of the target of interest. Once identified, the L version of the sequence selected, the spiegelmer, will be synthesized chemically; it will give rise to a complex with the natural target characterized by properties identical to that formed between the D-aptamer and the mirror image of the target. This strategy is restricted to small molecules for which the enantiomer of the target can be synthesized. It has been applied successfully to amino acids, nucleosides, and peptides (Klussmann et al., 1996; Wlotzka et al., 2002). L-Aptamers targeted to calcitonin gene-related peptide binding and to the monocyte chemoattractant protein CCL2 were shown to be efficient in vivo in an animal model (Denekas et al., 2006; Kulkarni et al., 2007).

A new methodology has been described that aims at increasing the molecular diversity of aptamers by a process that rests on the simultaneous use of SELEX and dynamic combinatorial chemistry (Ganesan, 2002), called 2D-SELEX. The concept has been validated using oligonucleotides that contain unmodified purine nucleosides and 2′-aminopyrimidine nucleosides (Bugaut et al., 2004). Such oligonucleotides are amenable to standard in vitro selection (i.e., they can be amplified). The 2′-amino group can react reversibly with aldehydes, thus generating imines (Figure 1.2c). A random library of 2′-amino oligonucleotides was prepared as usual for selection. Upon mixing with a small library of aldehydes, this generates a dynamic pool of 2′-amino, 2′-imino oligonucleotides (Figure 1.2c). In the presence of the target, the pool will be enriched in such oligonucleotides. Therefore, both the scaffold (the oligonucleotide sequence) and the pendant groups (the 2′-imino substituents) will be selected at once. Following capture, the iminoooligomers selected are hydrolyzed. The regenerated amino oligonucleotides are then PCR-amplified and a new round of 2D-SELEX is carried out. At the end of the process the candidates selected are cloned.
and sequenced. The oligonucleotides identified are then reacted individually with the aldehydes in the presence of the target, and the mixture is reduced by cyanoborohydride. The 2′ pendant groups are then identified by mass spectrometry. 2D-SELEX has been applied successfully to the selection of aptamers to the HIV-1 TAR element, leading to sequences different from that obtained when the SELEX is carried out in the absence of aldehydes (Ducongé and Toulmé, 1999; Ducongé et al., 2000; Bugaut et al., 2006). The process could be applied to other reversible reactions, such as disulfide bond formation. It could be extended to other types of modified nucleotides that are incorporated by polymerases such as uracil modified in position 5 (Latham et al., 1994), thus allowing the selection of new fluorescent or electrochemical sensors.

One particular case of modified nucleobase is 5-bromouridin (BrU) (Figure 1.2b), a phoreactive derivative that is used in photo-SELEX (Jensen et al., 1995; Golden et al., 2000). The pool of BrU-containing candidates is mixed with the target protein. Following capture of the bound oligomers, the mixture is ultraviolet-irradiated to generate covalent cross-links between the oligonucleotides and the protein. Unbound oligomers are washed away and the photo-cross-linked material is subjected to protease digestion. The free oligonucleotides are then amplified for the next round of selection. Partitioning therefore relies on two features: affinity and accurate positioning of cross-linkable groups, resulting in very high specificity. Photo-SELEX allowed the selection of DNA aptamers against human basic fibroblast growth factor (Jensen et al., 1995).

In contrast to what was stated above about the difficulty of introducing an internal modification in an aptamer, it is fairly easy to modify the 3′ or the 5′ end of the oligomer during synthesis on the solid support, without altering its binding properties. For example, the optimization of selected sequences may include a terminal 3′, 3′ cap or disulfide cross-link that renders the oligonucleotide resistant to the abundant 3′, 5′ exonucleases. Any marker—biotin, fluorescent reporter, or groups that increase the bioavailability, such as polyethylene glycol or cholesterol (Boomer et al., 2005; Dougan et al., 2000)—can also be introduced. This constitutes a major advantage over antibodies and opens the way to the design of probes or sensors as well as to agents for in vivo applications, two highly challenging fields toward therapeutic or diagnostic applications.

### 1.4 APTAREGULATORS

The high affinity and specificity of recognition displayed by aptamers make them appropriate for the design of regulators of biological function. Indeed, very early on, experiments aiming at the identification of sequences recognized by RNA- or DNA-binding proteins were carried out (Henderson et al., 1994). Frequently and not unexpectedly, sequences that are stronger binders than the natural sequences were identified (Bartel et al., 1991). The biological function requires additional properties, in particular the reversibility of the target–regulator complex over a given physiological concentration range, to turn the process on and
off under control. Such aptamers are therefore able to trap the target protein and consequently, to act as efficient decoys. The competition between the aptamer and the natural binding site results in the control of the function ensured by the protein. Interestingly, aptamers selected in the test tube retain their properties inside cells. Therefore, the effect of these decoy aptamers can be investigated in vivo (Famulok et al., 2001). This was demonstrated by aptamers targeted to the Tat (Yamamoto et al., 2000) or Rev proteins of the HIV-1 (Bartel et al., 1991). These proteins are involved in the transcription of the HIV genome and in the nuclear export of incompletely spliced viral mRNA. In situ expression of anti-Rev and anti-TAR RNA aptamers inhibited the retroviral replication by more than 70% in cultured cells (Good et al., 1997).

Similar regulators of protein function can be developed against proteins that are not natural binders of nucleic acids. A very large number of proteins have been used for raising aptamers. One of the most popular examples is the G-tetrad-forming DNA aptamer selected against thrombin, a key regulator in the coagulation cascade (Bock et al., 1992). This aptamer prolonged clotting time in purified fibrinogen and in human plasma and displayed anticoagulant properties in vivo (Griffin et al., 1993).

Regulatory aptamers have also been developed for targeting RNA instead of proteins. Oligonucleotides are poorly adapted to the recognition of RNA structures (Toulmé et al., 2005). Folded RNA regions are not available for intermolecular pairing with the complementary sequence. Consequently, antisense or small interfering RNA targeted to structured regions shows a limited efficiency (Kurreck, 2006). In vitro selection has been carried out to identify aptamers that recognize the folded state of the target RNA region. The numerous interactions identified in tertiary RNA structures, besides Watson–Crick base pairing, suggest that it should be possible to take advantage of nonpaired nucleic acid bases in loops and bulges to engage intermolecular interactions with an aptamer. Additional interactions such as stacking would bring an additional contribution to the binding. Both DNA and RNA aptamers have been identified against the TAR RNA hairpin of HIV-1, an imperfect hairpin involved in the trans-activation of the transcription of the retroviral genome (Boiziau et al., 1999; Ducongé and Toulmé, 1999). The binding occurs through the formation of a loop–loop helix between the partially complementary apical loops of the two partners. For the RNA aptamer a six-base-pair helix is formed and a critical noncanonical GA pair closing the aptamer loop has been shown to play a crucial role in the kissing complex formation (Ducongé et al., 2000). A high affinity (a $K_d$ value of a few nanomolar) has been obtained. The in situ expression of this aptamer driven by a RNA Pol III promoter was shown specifically to reduce by 60% expression of a reporter gene under the control of the TAR element in cultured HeLa cells (Kolb et al., 2006). Chemically modified aptamers have been designed that show improved biological properties compared to the regular RNA molecule (Darfeuille et al., 2002a,b, 2004). Other RNA structures have been targeted successfully by aptamers, in particular in the internal ribosome entry site of the hepatitis
C virus RNA (Tallet-Lopez et al., 2003; Da Rocha-Gomes et al., 2004; Kikuchi et al., 2005).

It is of great interest to generate reversible regulators that can be activated or deactivated at will, in response to a signal. To this end, regulation can be achieved by aptamers raised against small molecules. When inserted in mRNAs, such aptamers mimic riboswitches that have been identified in prokaryotic mRNAs (Tucker and Breaker, 2005). For these RNAs, conformational changes in response to ligand binding may result in translational regulation, most frequently by switching the ribosome-binding site from a sequestered to a free status. The ligand-binding site on RNA that triggers the conformational rearrangement is functionally equivalent to an aptamer. It was therefore tempting to design artificial regulators of gene expression by inserting an aptamer sequence in the 5′ untranslated region (UTR) of a given gene. The interaction between the aptamer and its target molecule will alter or stabilize the RNA structure, which in turn might prevent the binding of the ribosome or the initiation of the translation. In a pioneering work, Werstuck and Green (1998) validated this approach by inserting an aptamer to Hoechst dye in the 5′ UTR of a reporter gene and demonstrated that they were able to specifically control the translation of the reporter gene by adding the dye either in vitro in wheat germ extract or in cultured CHO cells. Similar results allowing regulation in cis have been reported in Saccharomyces cerevisiae (Suess et al., 2003). Recently, the replication of a viral genome in which a crucial viral stem–loop was substituted by the theophylline aptamer could be controlled by this purine derivative (Wang and White, 2007).

It is also conceivable to design riboregulators acting in trans. For instance, the activity of an antisense RNA was controlled by an aptamer specific for theophylline fused to the antisense sequence (Bayer and Smolke, 2005). In the absence of theophylline, the antisense domain is sequestered in an intramolecular structure. Addition of theophylline induces a conformational change of the riboregulator that allows the antisense to interact with its target mRNA. The expression of a reporter gene (EGFP) in yeast cells expressing such a riboregulator (antiEGFP antisense) was shown to be inhibited in the presence of theophylline. The effect was specific: no inhibition was induced by caffeine that does not bind to the riboregulator.

Analogous strategies can be applied to the reversible control of protein function. The association between an aptamer and its target protein can be prevented by an oligonucleotide that base-pairs with part of the aptamer sequence, thus disrupting the active structure of the aptamer. A 2′-fluoropyrimidine-containing aptamer specific for the human coagulation factor IXa displays anticoagulant properties that can be reverted by the addition of a 2′-O-methyl oligonucleotide complementary to the 5′ end of the aptamer (Rusconi et al., 2002, 2004). The antisense oligonucleotide constitutes an antidote to the anticoagulant aptamer. This rational method is of wide interest for designing regulatable aptamers that are suitable for in vivo use, as both the aptamer and the antisense regulator are resistant to nucleases.
An alternative to the development of “aptamer antidotes” is to hide the aptamer’s active conformation by reversible chemical modification of some part of the sequence. The reversal of the protection will free the oligonucleotide, which will recover its binding properties as a consequence. The difficulty resides in the use of groups that could be unmasked at will. This was achieved elegantly by introducing caged nucleobases (i.e., bases that are protected by photo-labile groups). Caged analogs of thymidine T\(^{\text{NPP}}\) containing a photo-labile 2-(2-nitrophenyl) propyl (NPP) group (Figure 1.2b) have been introduced in the well-characterized antithrombine DNA aptamer (Heckel and Mayer, 2005). This G- and T-containing 15-mer folds into a G-tetrad structure that displays anticoagulant properties. The presence of a single residue T\(^{\text{NPP}}\) prevented the binding of the aptamer analog to thrombin, whereas the \(K_d\) value of the photo-reactivated derivative was similar to that of the wild-type aptamer, but a full anticoagulant activity was not restored following irradiation under physiological conditions.

Caged residues can be combined with the antidote approach to generating controllable aptamers, as described recently by Mayer and colleagues. The antithrombin DNA aptamer was extended on the 5\(^{\prime}\) side by a short sequence complementary to part of the G-tetrad-forming 15-mer. Initially, the association between the two complementary sequences is prevented by the presence of a C residue caged with an NPE [1-(2-nitrophenyl)ethyl] group (Heckel et al., 2006). The C\(^{\text{NPE}}\) nucleobase acts as a transient mismatch that can be converted into a match by irradiation at 366 nm, thus allowing intramolecular pairing between the aptamer region and the antidote complementary sequence. The caged oligomer displays reduced but significant anticoagulant properties, whereas it is totally inactive following uncaging. The use of caged residues therefore allows triggering by light of either activation or deactivation of aptamers.

Formation of an aptamer–protein complex can be controlled by a small molecule that upon binding to the aptamer triggers a conformational change of the aptamer, thus altering its binding properties (Buskirk and Liu, 2005). A conditional RNA aptamer to formamidopyrimidine glycosylase (Fpg), a DNA repair enzyme, was obtained through a clever selection procedure (Vuyisich and Beal, 2002). After a few selection rounds the RNA/Fpg retained on a filter was eluted by the addition of neomycin. Therefore, only Fpg-bound oligoribonucleotides that dissociated in the presence of the aminoglycoside were collected and used for further selection rounds. The aptamer isolated at the end of this process was an inhibitor of Fpg. This inhibition was reverted by the addition of neomycin. The structural analysis of this aptamer demonstrated that it contained two overlapping binding sites for the Fpg and for neomycin, respectively. Interestingly, the neomycin binding site shared similarities with previously identified neomycin aptamer (Wallis and Schroeder, 1997). The rescue of the Fpg activity by neomycin was specific; the addition of kanamycin, an antibiotic structurally related to neomycin that does not bind to the patamer, does not show any effect. Aptamers selected in parallel against the Fpg through a procedure that did not include the elution step by neomycin displayed similar
binding and inhibitory properties, except that the inhibition was not reverted by addition of the aminoglycoside (Vuyisich and Beal, 2002). Even though this is of wide potential interest, it remains to be demonstrated whether this approach can be generalized.

1.5 APTASENSORS

Aptamers, which show high affinity of binding and high specificity of target recognition are sometimes described as “chemical antibodies.” They actually display a number of advantages over antibodies, as they can in principle be raised against any type of target, including toxic compounds. Moreover, aptamers are smaller than antibodies (molecular mass 5 to 15 kDa) and can get access to regions that are poorly accessible to immunoglobulins. They are easily and reproducibly synthesized and are easy to store. They can be conveniently conjugated to various derivatives. Last, as SELEX is an iterative process (Figure 1.1), it has been possible to adapt in vitro selection to robotic procedures that considerably reduce the time needed to generate aptamers (Cox et al., 1998, 2002). It is therefore not surprising that aptamers became ideal tools for the development of analytical methods. Reports dealing with the purification of molecules, the design of optical, electrochemical, or acoustic sensors, the conception of signaling aptamers (beacons, quantum dots), and the development of aptamer arrays for high-throughput analysis appear on a weekly basis. Multiple applications will be described in detail in the following chapters, and several recent reviews are available (Tombelli et al., 2005; Mairal et al., 2007). We mention here only briefly the different areas in which aptamers have been used and outline a few recent examples that are of high potential interest.

Aptamers have been used for the purification by affinity chromatography of different peptides and proteins: thrombin, thyroid transcription factor 1, L-selectin, and so on (Ravelet et al., 2006). Affinity chromatography of proteins with aptamers is very attractive, as there is no need for a tag that might affect folding and also no need for the tag-cleavage step, thus ensuring quick purification procedures and high yield of recovery, as demonstrated recently for purification of the Taq polymerase (Oktem et al., 2007). Aptamers have been shown to discriminate efficiently between target enantiomers, allowing specific chiral separation. For instance, the D-enantiomer of the oligopeptide arginine–vasopressin was retained on a specific DNA aptamer column while the L-enantiomer was eluted in the void volume (Michaud et al., 2003). The biotinylated aptamer was easily immobilized on a streptavidin polystyrene–divinyl benzene support. Enantiomers of small biomolecules were also resolved efficiently (Michaud et al., 2004). To develop this strategy on a large scale, several problems have to be solved, notably the cost and the problem of the aptamer stability if samples contaminated with nucleases are used. Solutions discussed above (modified oligonucleotides) can be considered.
Aptamers could sense the presence of the target molecule they were selected against if they were combined with a transducer element that converts formation of the aptamer–target complex into a signal that can be measured or at least evaluated. Various formats have been described. Surface plasmon resonance (SPR) is of particular interest, as it is a label-free methodology that is used widely for the quantitative study of selected sequences and analysis of the binding sites. It allowed the characterization of protein–aptamer (Win et al., 2006) and RNA–aptamer complexes (Aldaz-Carroll et al., 2002). It was even used to select RNA aptamers against the human RNaseH1 and the hemaglutinin of the human influenza virus (Pileur et al., 2003; Misono and Kumar, 2005). Depending on the relative size of the two partners, either the aptamer or the target can be immobilized on the SPR chip. Careful analysis of the sensorgrams even allows monitoring the formation of ternary complexes (Di Primo, 2008). However, it is a low-throughput method that requires a sophisticated and rather expensive instrument. Electronic aptamer-based sensors have been developed (Willner and Zayats, 2007), but the most popular sensors are by far optical sensors, based mostly on fluorescence measurements (i.e., intensity, anisotropy, energy transfer, etc.). A very simple assay would make use of a fluorescently labeled analyte (Drolet et al., 1996). Conversely, the aptamer could be associated with a fluorophore whose emission properties will change upon binding to its ligand. The second possibility does not require the synthesis of a target analog that shows binding properties similar to that of the unmodified molecule and is therefore easier to use in real time. Moreover, conjugation of the aptamer to a fluorophore at either the 5′ or 3′ end is trivial. The binding to the analyte can be monitored by changes in the evanescent wave-induced fluorescence anisotropy as described in the pioneering work by Potyrailo et al. (1998). An immobilized FITC-conjugated DNA aptamer allowed the detection of as little as 0.7 amol of thrombin. Differences in the diffusion rates of fluorescently labeled aptamers free or bound to their target can also be measured in solution, as shown for the analysis of IgE (Gokulrangan et al., 2005) and angiogenin (W. Li et al., 2007). Fluorescent analogs of nucleic acid bases could also be incorporated into the aptamer sequence; the binding of the aptamer to its target might eventually induce a modification in the electronic environment of the fluorophore. Generally, multiple variants of fluorescent aptamers should be assayed before a responsive molecule is identified (Jhaveri et al., 2000; Katilius et al., 2006).

A light switch based on a ruthenium complex which shows a strong luminescence emission when it intercalates into double-stranded nucleic acids was used to convert an aptamer into a sensor. The binding of the aptamer to its ligand induces conformational changes that perturb intercalation of the ruthenium complex, leading subsequently to luminescence changes (Jiang et al., 2004). Interestingly, this requires no labeling of either the analyte or the aptamer. Angiogenin and IgE were detected in serum samples using such assays.

Molecular beacons are oligonucleotide probes designed originally for the detection of nucleic acid sequences (Tyagi and Kramer, 1996). They generally have a hairpin shape, which brings into close contact fluorescence reporters bound
at the 3′ and 5′ ends of the double-stranded stem. These reporters can be either a fluorophore and a quencher or a pair of dyes that constitute donor–acceptor molecules appropriate for fluorescence energy transfer (FRET). This stem–loop structure will unfold upon hybridization to a sequence fully complementary to the loop and stem regions, thus moving the reporters away from each other. This translates into a fluorescence change, either a dequenching of the fluorophore or a decrease in the FRET efficiency. Measurement of the fluorescence therefore allows quantitative detection of the target. Monitoring of fluorescence variation associated with conformational changes has been adapted to aptamers, as these molecules frequently undergo structural variation upon association with their cognate ligand (Hermann and Patel, 2000; Soukup et al., 2000). Beacons were engineered for the detection of thrombin by the G-quartet, forming the DNA aptamer identified previously (Bock et al., 1992). This oligonucleotide is in equilibrium between a random and a folded state; this equilibrium is shifted toward the four-stranded structure by binding to thrombin. This is associated with a significant fluorescent signal when the 3′ and 5′ ends of this 15-mer are conjugated to fluorescence reporters (Li et al., 2002). This is unlikely to be generalized, as not every aptamer will undergo a switch from an extended structure in the absence of its ligand to a folded structure in its presence. But a given aptamer might be truncated to give rise to a partly unfolded oligomer that will fold into a closed structure upon binding to the ligand. The three-way junction aptamer for cocaine was converted into a specific sensor by derivatizing a truncated DNA aptamer with fluorescein and dabcyl (Stojanovic et al., 2001). Such beacons signal the presence of their cognate analyte by decreased emission. An off-to-on transition in the presence of the ligand is more desirable, as the detection of the analyte is more sensitive. Many aptamers can be converted into hairpin-shaped beacons by extending one of their extremities by a few nucleotides so as to generate a short sequence complementary to the other end of the oligomer. The length of the resulting double-stranded stem should be adjusted to be opened in the presence of the ligand. Such a design has been used successfully for the antithrombin aptamer (Hamaguchi et al., 2001). The first aptamer beacon that was described was designed as a bipartite molecule from an RNA aptamer targeted to the Tat protein of HIV (Yamamoto et al., 2000). This aptamer was split in two parts, one part of which was reformulated to generate a hairpin-shaped beacon. In the absence of Tat, the two halves are independent. The addition of Tat induces the reannealing of the two RNA halves, which results in fluorescence emission (Yamamoto and Kumar, 2000). This design is appropriate for aptamers that show a rather long double-stranded stretch.

Tripartite beacons can be tailored as described previously for nucleic acid detection. In this case the hairpin structure is not labeled but displays single-stranded 3′ and 5′ extensions that are complementary to two oligomers bearing a fluorophore and a quencher, respectively. In the free folded hairpin state, the reporters are in close proximity, but they are moved apart in the extended unfolded state resulting from the binding of the loop region to its complementary sequence, thus generating a fluorescence signal (Nutiu and Li,
2002). This strategy has been adapted to aptamers: The aptamer is labeled with a fluorophore, whereas a short complementary oligonucleotide bears the quenching molecule. Binding the aptamer to its target induces release of the short oligonucleotide and results in fluorescence emission (Nutiu and Li, 2003, 2005a).

Modular biosensors assemble two aptamers into one molecule. The first is a recognition module corresponding to the binding site of the analyte; the second is a signaling domain that accommodates a reporter. The two modules communicate in such a way that binding of the analyte leads to increased affinity of the signaling domain for the reporter molecule, hence resulting in increased signal. Modular sensors for ATP, FMN, or theophylline were designed in combination with a malachite green sensing domain (Stojanovic and Kolpashchikov, 2004). This is highly reminiscent of small molecule–dependent switches tailored for the control of gene expression (Buskirk et al., 2004).

Very generally, engineered beacons from preidentified aptamers require some knowledge of the secondary or tertiary structure. In addition, design of the signaling aptamer needs critical adjustment of the modified version so that the addition of the ligand shifts the equilibrium efficiently between the two structures corresponding to the free and bound states. It would be much better to select not only on the binding but also on the signaling. A procedure was described for which the selection step involves a capture oligonucleotide. Some of the complexes formed by this oligomer and a complementary region in candidates of the random library are dissociated upon the addition of an analyte. This is assumed to be due to the conformational change of the candidate, which actually constitutes an aptamer for the added analyte. The released oligonucleotides are collected and amplified as usual in the SELEX procedure, thus allowing the identification of aptamers whose binding to their ligand induces a conformational change that in turn leads to disruption of the aptamer–capture oligonucleotide complex. If a quencher is introduced on the capture oligonucleotide and a fluorophore into its complementary region on the aptamer, binding of the analyte to the aptamer will result in increased fluorescence emission. This clever approach has been validated for the direct selection of molecular beacons against oligonucleotides (Rajendran and Ellington, 2003), purine nucleotides (Nutiu and Li, 2005b), and more recently, against the aminoglycoside antibiotic tobramycin (Morse, 2007).

1.6 PROSPECTS

The development of an automated selection process, the intrinsic properties of aptamers combined with their conversion into signaling elements, and the ease of conjugating oligonucleotides on different surfaces make them appropriate for their use in microarray formats. Interest in aptamer chips was outlined long ago (Brody and Gold, 2000). They could be used for the identification and quantification of multiple proteins or biomarkers. There are still few reports on the development of microarray-based aptamer assays. Detection of the analyte
can be carried out using fluorescent ligands (Collett et al., 2005), fluorescent aptamers (McCauley et al., 2003), or surface plasmon resonance imaging (Y. Li et al., 2006, 2007b). Photoaptamer-based arrays represent an interesting strategy (Golden et al., 2000). The use of aptamer chips for the simultaneous detection of multiple targets requires further optimization before it can be used for proteomics, but attempts along this line have been undertaken (Cho et al., 2006).

One major challenge is the simultaneous detection of a number of analytes in identical analytical conditions. Aptamers that can be selected at will under predetermined conditions are of particular interest to this end to the extent that they transduce different signals. The association of aptamers to quantum dots (QDs) offers an exciting perspective. QDs are fluorophores that can be excited at the same wavelength and show sharp emission profiles, a large Stokes shift, and a long fluorescence lifetime. They have been used in different beacon formats (Levy et al., 2005; Choi et al., 2006). Nanostructures combining quantum dots, quenching gold nanoparticles, and aptamers allowed the detection of adenosine and cocaine by monitoring the fluorescence emission at 525 and 585 nm, respectively (Liu et al., 2007). Recently, quantum dot–labeled aptamer was reported to allow the detection of *Bacillus thurigiensis* spores (Ikanovic et al., 2007).

Increased sensitivity of aptamer-based sensors might be achieved by the inclusion of an amplification step. To this end, modulation of enzymatic activity by the analyte is an interesting perspective. Allosteric ribozymes have been rationally designed by combining a hammerhead or a hairpin ribozyme to an aptamer module. Different formats have been described. Binding of the analyte to the aptamer might regulate the ribozyme activity directly (Tang and Breaker, 1998) or release its association with an inhibitory oligonucleotide (Famulok, 2005). A ribozyme conjugated to two aptamer modules—for the replicase and helicase of the hepatitis C virus—were shown to show high selectivity (Cho et al., 2005). Ribozyme–aptamer conjugates have been designed for screening small-molecule libraries in order to identify inhibitors of viral enzymes. The RNA pseudoknot aptamer inhibitor of the HIV-1 reverse transcriptase (Tuerk et al., 1992) prevented the activity of the conjugated ribozyme when bound to the retroviral polymerase. If a small molecule displaces an aptamer from its target, the ribozyme becomes active, which is visualized by a fluorescent signal, thus allowing identification of potential reverse transcriptase ligands (Yamazaki et al., 2007). The potential of an aptamer-based amplified signal was recently evidenced: a DNA sequence bearing the cocaine aptamer at its 3′ end was engineered to constitute a template for DNA polymerase in the presence—but not in the absence—of cocaine. The replication fragment was used to titrate out a fluorescent molecular beacon (Shlyahovsky et al., 2007). This opens the way to the design of highly sensitive sensors.

Whereas oligonucleotide libraries were traditionally partitioned by filtration or capture on beads or on affinity columns, capillary electrophoresis has been validated as a promising alternative. Aptamers displaying a high affinity were obtained in a very limited number of selection rounds. Nonequilibrium capillary electrophoresis of equilibrium mixtures led to the identification of DNA aptamers
with a $K_d$ value of about 1 nM against protein farnesyltransferase (Berezovski et al., 2005). Interestingly, this methodology allows the selection to be carried out according to kinetics criteria. Non-SELEX capillary electrophoresis (i.e., selection without amplification) has been described for the selection of DNA aptamers against the h-Ras protein (Berezovski et al., 2006a). This procedure is of particular interest for the identification of chemically modified oligonucleotides that cannot be synthesized by polymerases (Berezovski et al., 2006b). As the selection occurs in solution, there is no background of sequences targeted to the support (i.e., filter, beads, etc.). But this technique is restricted to targets of size larger than about 20 kDa, as complexes between oligonucleotide candidates and smaller molecules have a mobility close to that of oligomers.

Over the last 15 years, aptamers have emerged as compounds of wide potential for both therapeutic and diagnostic purposes. Many applications of interest have not been described here: for example, in the field of cell biology, molecular imaging, and delivery (Chu et al., 2006; Hicke et al., 2006). Last but not least, a targeted antivascular epithelium growth factor aptamer (pegaptanib) has been approved by the U.S. Food and Drug Administration for the treatment of neovascular age-related macular degeneration (Ng et al., 2006). This constitutes a milestone in the development of aptamers. It is anticipated that numerous aptamers will develop into drugs and diagnostic tools in the near future.

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REFERENCES


APTAMERS: LIGANDS FOR ALL REASONS


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