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# Aptamers: An Emerging Class of Molecules That Rival Antibodies in Diagnostics

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**Antibodies, the most popular class of molecules providing molecular recognition needs for a wide range of applications, have been around for more than three decades. As a result, antibodies have made substantial contributions toward the advancement of diagnostic assays and have become indispensable in most diagnostic tests that are used routinely in clinics today. The development of the systematic evolution of ligands by exponential enrichment (SELEX) process, however, made possible the isolation of oligonucleotide sequences with the capacity to recognize virtually any class of target molecules with high affinity and specificity. These oligonucleotide sequences, referred to as "aptamers", are beginning to emerge as a class of molecules that rival antibodies in both therapeutic and diagnostic applications. Aptamers are different from antibodies, yet they mimic properties of antibodies in a variety of diagnostic formats. The demand for diagnostic assays to assist in the management of existing and emerging diseases is increasing, and aptamers could potentially fulfill molecular recognition needs in those assays. Compared with the bellwether antibody technology, aptamer research is still in its infancy, but it is progressing at a fast pace. The potential of aptamers may be realized in the near future in the form of aptamer-based diagnostic products in the market. In such products, aptamers may play a key role either in conjunction with, or in place of, antibodies. It is also likely that existing diagnostic formats may change according to the need to better harness the unique properties of aptamers.**


The detection and quantification of molecules play an essential role in basic discovery research as well as in clinical practice. Technologies that allow specific detection and precise quantification of molecules are evolving to cater to new analytes as well as to improve existing techniques. As a result, novel approaches that challenge traditional methods are being discovered. Today, technological advancements have enabled researchers to change the properties of molecules on demand, thereby generating variants that suit various applications. This is quite contrary to what Nature has designed these molecules to be. For example, the antibody molecule that evolved to recognize antigens with high affinity and specificity is now being intentionally modified to catalyze different reactions *(1)*. This is the task that enzymes have been designed to undertake. In a different area of research, the development of the in vitro selection and amplification technique *(2, 3)* has allowed the discovery of specific nucleic acid sequences that bind to a wide array of target molecules with high affinity and specificity. The technique by which these oligonucleotide ligands are obtained is called the systematic evolution of ligands by exponential enrichment (SELEX)[1] process *(2)*. The resulting oligonucleotide ligands are referred to as "aptamers", derived from the Latin word "aptus", meaning "to fit" *(3)*. As the name implies, aptamers are suitable for applications based on molecular recognition needs that include diagnostics and therapeutics. On the therapeutic front, aptamers have made tremendous progress and are already in clinical trials only 8 years after the inception of the technology. Another area of research that is being actively pursued is the application of aptamers for in vivo diagnostics or in vivo imaging *(4)*. In this report, I have limited the discussion of aptamers to in vitro diagnostics and summarized some promising developments in the field.

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[1] Nonstandard abbreviations: SELEX, systematic evolution of ligands by exponential enrichment; $K_d$, equilibrium dissociation constant; VEGF, vesicular endothelial growth factor; HNE, human neutrophil elastase; FP, fluorescence polarization; CE, capillary electrophoresis; APCE, affinity probe capillary electrophoresis; hTSH, human thyroid-stimulating hormone; hLH, human luteinizing hormone; and hCG, human chorionic gonadotropin.

## Antibodies

A discussion on diagnostic applications would not be complete if one omits antibodies from the subject. Antibodies have made a tremendous contribution to a wide range of applications that are based on molecular recognition. The use of antibodies in detecting analytes predates the 1950s *(5)* and became widespread by the 1970s when polyclonal sera from immunized animals was the popular choice. The demand for large quantities of polyclonal antibody preparations was too high to meet the requirements for immunological assays. During that time, Kohler and Milstein *(6)* reported the discovery of the monoclonal antibody technology that allowed the production of a unique antibody in large quantities. Because of the enormous practical value of antibodies, monoclonal antibody technology became very popular and was embraced by researchers in all corners of the world. Monoclonal antibodies were produced in cultured cells in sufficient quantities to allow optimization and further refinement of antibody-based assays and development of the nonradioactive detection technologies that are popular today. Today, selected clones that produce a monoclonal antibody can be cultured continuously to produce the antibody of choice. In theory, the life span of an antibody-producing cell culture and the amount of antibody it can produce have no limits. Furthermore, the immunogen used in the identification of monoclonal antibodies does not have to be pure. These are some of the benefits of the monoclonal antibody technology that has revolutionized immunology research and further broadened the applications of antibodies. There are certain limitations, however, associated with antibodies:

- The antibody identification process starts within an animal; therefore, antibody generation becomes difficult with molecules that are not well tolerated by animals, such as toxins. Furthermore, antibodies against molecules that are inherently less immunogenic are difficult to raise.
- By and large, the generation of hybridomas are restricted to rat and mouse, limiting the use of antibodies in therapeutic applications. Antibodies of non-human origin have implications in diagnostic applications as well *(7)*. Heterophilic antibodies (human antibodies that recognize antibodies of non-human origin) that exist in humans could potentially link a capture antibody with a detector antibody of non-human origin in the absence of the specific analyte, leading to false-positive results. Rheumatoid factors and autoantibodies also interfere in immunoassays *(8)*.
- The identification and production of monoclonal antibodies are laborious and could become very expensive in searches for rare antibodies that require screening of a large number of colonies.
- The production of antibodies has challenges of its own. Frozen stocks of antibody-producing cells should be stored at multiple sites to overcome accidental losses or the death of cell lines. Typically, high yields of monoclonal antibodies are obtained by growing the hybridomas in the peritoneal cavities of animals and purifying the antibody from ascites fluid. Some hybridomas are difficult to grow in vivo, thus restricting this route of antibody production.
- The performance of the same antibody tends to vary from batch to batch, requiring immunoassays to be reoptimized with each new batch of antibodies.
- Although the production of antibodies is subject to in vivo variations, the identification of antibodies is restricted by in vivo parameters. In other words, identification of antibodies that could recognize targets under conditions other than physiological conditions is not feasible.
- The kinetic parameters of antibody-target interactions cannot be changed on demand.
- Antibodies are sensitive to temperature and undergo irreversible denaturation. They also have a limited shelf life.

It is important to note, however, that various approaches are being developed to circumvent limitations associated with the current hybridoma-based antibody technology. These approaches, which include humanization of antibodies *(9, 10)*, displaying peptide libraries on phages *(11, 12)* and ribosomes *(13–15)*, antibody engineering *(16)*, and in vitro immunization *(17)*, are at various stages of development.

Alternatively, it is possible to consider an entirely different class of molecules, aptamers, to meet the shortcomings of antibodies. In this regard, aptamers have the following advantages:

- Aptamers are identified through an in vitro process that does not depend on animals, cells, or even in vivo conditions. As a result, the properties of aptamers can be changed on demand.
- Selection conditions can be manipulated to obtain aptamers with properties desirable for in vitro diagnostics. For example, aptamers that bind to a target in a nonphysiological buffer and at nonphysiological temperatures could be identified. Similarly, kinetic parameters, such as the on- and off-rates of aptamers, could be changed on demand.
- Because animals or cells are not involved in aptamer identification, toxins as well as molecules that do not elicit a good immune responses can be used to generate high-affinity aptamers.
- Aptamers are produced by chemical synthesis with extreme accuracy and reproducibility. They are purified under denaturing conditions to a very high degree of purity. Therefore, little or no batch-to-batch variation is expected in aptamer production.
- Reporter molecules such as fluorescein and biotin can be attached to aptamers at precise locations identified by the user. Functional groups that allow subsequent derivatization of aptamers with other molecules can

also be attached during the chemical synthesis of aptamers.

- Aptamers undergo denaturation, but the process is reversible. Once denatured, functional aptamers could be regenerated easily within minutes. They are stable to long-term storage and can be transported at ambient temperature.

## Aptamers

### APTAMER IDENTIFICATION: THE SELEX PROCESS

The identification of rare nucleic acid sequences with unique properties from very large random sequence oligonucleotide libraries was described in 1990 *(2, 3)*. This method, called the SELEX process *(2)*, is a technique for screening very large combinatorial libraries of oligonucleotides by an iterative process of in vitro selection and amplification. Combinatorial libraries based on replicable biopolymers such as nucleic acids (and peptides) offer the convenience of iterative amplification of their members, making the screening process fast and easy.

The SELEX process begins with a random sequence library obtained from combinatorial chemical synthesis of DNA. Each member in a library is a linear oligomer of a unique sequence. The complexity, or the molecular diversity, of a library is dependent on the number of randomized nucleotide positions. Theoretically, a library containing a 40-nucleotide random region is represented by $1.2 \times 10^{24}$ individual sequences ($4^{20} = 1.2 \times 10^{24}$). However, in practice, the complexity of a typical combinatorial oligonucleotide library obtained from 1-$\mu$mol scale solid-phase DNA synthesis is limited to $10^{14}$ to $10^{15}$ individual sequences. The success of finding unique and rare molecules that interact with a target parallels the diversity of the libraries used. The degree of molecular diversity present in random sequence oligonucleotide libraries supersedes that of other combinatorial libraries used for screening. These include peptide libraries used for phage display as well as the libraries made up of small organic molecules.

In the screening process, a random sequence oligonucleotide library is incubated with a target of interest in a buffer of choice at a given temperature (Fig. 1). During this step, a very small fraction of individual sequences tends to interact with the target, and these sequences are separated from the rest of the library by means of any one of the physical separation techniques. Typically, nitrocellulose filter partitioning is used with protein targets that are retained on nitrocellulose. Small molecular targets are generally immobilized on a solid support to generate an affinity matrix, in which case sequences that do not interact with the target on the solid support can be removed easily by a simple washing step. The population of sequences bound to the target is isolated and amplified to obtain an enriched library to be used for the next selection/amplification cycle. The enrichment efficiency of high-affinity binders is governed by the stringency of selection at each round. The progress of the enrichment of high-affinity binders can be determined by carrying out binding analysis of enriching populations against the target. Once affinity saturation is achieved after several rounds of selection/amplification, the enriched library is cloned and sequenced to obtain the sequence information of each member. Individual sequences are further charac-

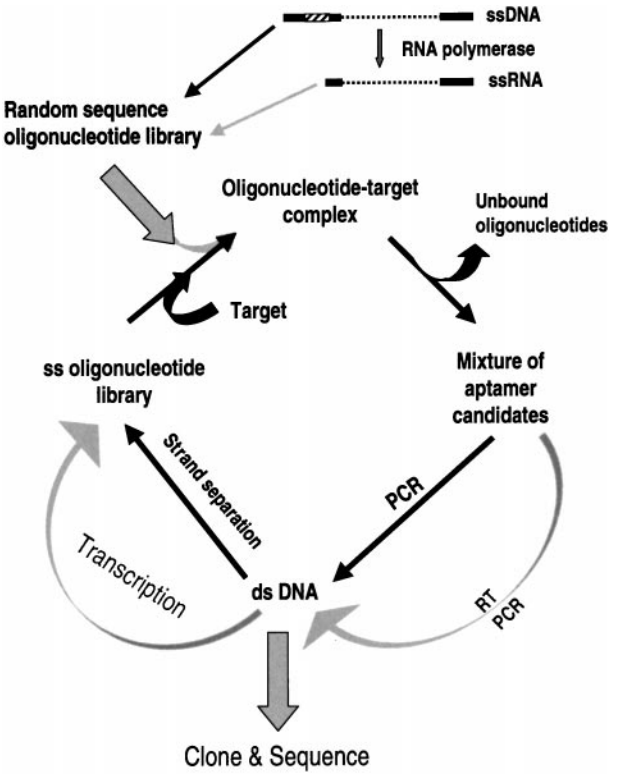



Fig. 1. Generalized scheme indicating the key steps in the SELEX process.

The process starts with a chemically synthesized random sequence DNA library containing approximately $10^{14}$ to $10^{15}$ individual molecules. A library is designed to contain a contiguous randomized region (*dashed lines*) flanked by two fixed sequence regions (▬). Each nucleotide position in the contiguous random region is synthesized upon delivery of a mixture of phosphoramidites containing all four building blocks: A, G, C, and T. To obtain an unbiased library with equal representation of all four nucleotides, the ratios of the four phosphoramidites in the mixture are adjusted based on the coupling efficiencies of individual monomers. Fixed sequences are used for primer binding sites in enzymatic amplification of individual sequences. Because aptamers are identified from DNA as well as from RNA libraries, chemically synthesized DNA libraries are enzymatically converted to RNA libraries. A promoter for an RNA polymerase (for example, T7 RNA polymerase) is engineered within a fixed sequence (▨) of the library to accommodate in vitro RNA synthesis *(177)*. A random sequence oligonucleotide (RNA or DNA) library is exposed to the target of interest under defined buffer conditions. Sequences that bind to the target are separated from the unbound species by a suitable partitioning technique. This population of sequences recovered from the target (mixture of aptamer candidates) represents a mixture containing both high- and low-affinity binding molecules to the target. Because further screening is required to eliminate the sequences with low-affinity binding, individual sequences are amplified for the next round of selection. The aptamer candidate mixture is amplified directly by PCR, provided it is DNA. *Black arrows* indicate steps in a DNA-based aptamer selection; *gray arrows* indicate the steps in an RNA-based selection. RNA sequences are amplified by PCR after converting to copy DNA by reverse transcription (*RT*). The single-stranded DNA population obtained by strand separation of PCR products is incubated with a fresh sample of the target for the next round of selection. The RNA population is obtained by in vitro transcription. The enrichment of the high-affinity sequences at the expense of the low-affinity binders requires several iterations of the selection process carried out under increasingly stringent conditions. Once affinity saturation is observed in an enriched library, the corresponding PCR products are used for cloning and sequencing.

terized on the basis of their ability to bind to the target. Usually, the majority of individual sequences, >90%, in an enriched library are "winners", aptamers that bind to the target used for selection.

Aptamers that come out of a SELEX experiment are full-length sequences containing the fixed sequences that were included to aid the amplification process. These full-length aptamers are generally 70–80 nucleotides long and could be truncated to eliminate nucleotide stretches that are not important for direct interaction with the target or for folding into the structure that facilitates target binding. The identification of truncated aptamers restricted to the minimal target-binding domain requires some effort, but it has been successfully carried out to obtain functional aptamers less than 40 nucleotides long *(18–23)*. In the majority of cases, the fixed sequence regions used for primer binding are unimportant for aptamer function and can be eliminated. Technological advances have already been made to eliminate the requirement for the fixed regions in random sequence libraries used for the SELEX process (Pagratis et al., manuscript in preparation), thereby producing short aptamer sequences.

The number of cycles required for aptamer identification is usually dependent on the degree of stringency imposed at each round as well as on the nature of the target. For most targets, affinity enrichment is reached within 8–15 cycles. In general, a researcher could accomplish one cycle of SELEX every 2 days. Including cloning and sequencing, a typical SELEX experiment may take approximately 2–3 months. Once the sequence is identified, an aptamer is produced by chemical synthesis. Small-scale synthesis and purification of an aptamer do not take more than 3 days and provide ample quantity of aptamer (several nanomoles) for the design and optimization of a diagnostic assay. This whole process is faster than the amount of time typically spent to generate a cell-line to produce a specific monoclonal antibody and purification of the antibody. The SELEX process recently was automated to make aptamer discovery even faster and more economical [Jenison et al., manuscript in preparation and Ref. *(24)*]. The automated platform carries out the iterative SELEX process around the clock with little or no human intervention and intuition (Jenison et al., manuscript in preparation). It has parallel processing capabilities to handle multiple SELEX experiments run on microtiter plates, allowing fast and high-throughput discovery of aptamers.

Aptamers are known for their remarkable specificity. Aptamers can discriminate targets on the basis of subtle structural differences such as the presence or absence of a methyl *(25, 26)* or a hydroxyl group *(27, 28)* and the D- vs L-enantiomer of the target *(27, 29)*. The high degree of specificity often seen in aptamers, sometimes even better than antibodies *(25)*, is a result of the selective demand in the SELEX process that eliminates sequences that bind to closely related analogs of the target. Practically, this is achieved by the process called "counter-SELEX" that effectively discards ligands that have ability to bind the target as well as closely related structural analogs of the target *(25)*. During selection, the population of aptamers bound to the target is subjected to affinity elution with structural analogs and the sequences eluted are discarded. In some diagnostic applications of small molecule targets, it may be important to measure the analyte as well certain structural variants of the analyte. In that case, aptamers retained on the target could be specifically eluted with the structural analogs to select the species that do not discriminate them.

The counter-SELEX strategy could be a valuable tool in identifying aptamers aimed at a unique target in a complex mixture, probably even without knowing what the target is, for example, in the search for aptamers that bind to an "epitope" present exclusively on the surface of cancer cells but not in healthy cells, or to find aptamers that interact with molecules present in the serum of patients infected with a pathogen but not present in uninfected individuals. In these applications, it would be possible to use the cells from healthy tissue (or serum from a healthy individual) to remove sequences that bind to the background that does not contain the epitope of interest before the library is challenged with cancer cells (or serum from the patient).

### MODIFIED LIBRARIES FOR APTAMER DISCOVERY

Chemically modified oligonucleotide libraries have been introduced to the SELEX process for several reasons. Unmodified oligonucleotides, especially RNA, are degraded by nucleases commonly present in biological fluids. Researchers in the antisense field have made impressive advances toward making oligonucleotides resistant to nucleases by introducing various modifications, predominantly to the oligonucleotide backbone *(30, 31)*. Most of these backbone modifications are introduced during the chemical synthesis and are not compatible with the enzymes used in the SELEX process. An exception to this is phosphorothioate modification. Oligonucleotides modified with phosphorothioate linkages exert nonspecific interactions with proteins *(32, 33)*; hence, they may not be suitable for identifying ligands that confer specificity. Certain modifications at the 2′ position of the sugar (Fig. 2) make RNA nuclease resistant *(34, 35)*. Because the nucleases that are most abundant in biological fluids appear to be pyrimidine-specific endonucleases, substitutions at the 2′ positions of pyrimidine nucleotides alone is sufficient to protect an RNA sequence from degradation in biological fluids *(35, 36)*. Most importantly, pyrimidine nucleotides substituted with amino ($NH_2$) and fluoro (F) functional groups at the 2′ position of the sugar are substrates for the enzymes used in the SELEX process. As a result, aptamers with enhanced survival times in biological fluids have been selected successfully from libraries containing pyrimidines modified with 2′-$NH_2$ and 2′-F functional groups *(19, 21, 37–*

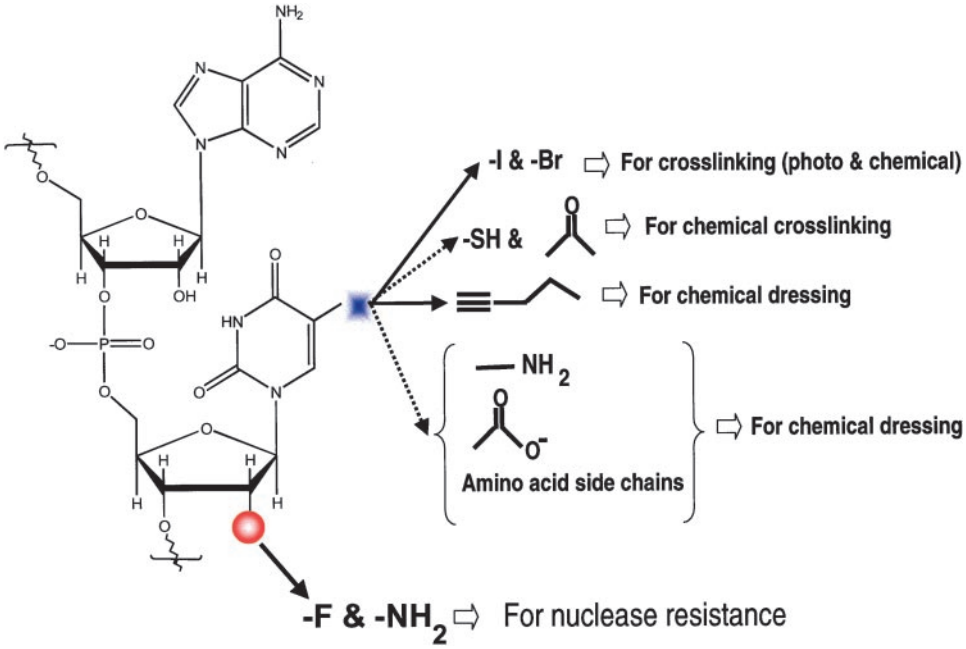



Fig. 2. Possible modifications on an oligonucleotide strand to generate modified oligonucleotide libraries for the SELEX process.

Modification at the 2′ position of the sugar confers nuclease stability, whereas various modifications at the C-5 position of the pyrimidines could be used either to attract certain classes of targets or to generate covalent cross-links with targets. Modifications indicated by *solid arrows* have been used in aptamer identification; those shown indicated by *broken arrows* represent some examples of the potential substitutions that could be used for the SELEX process.

*40)*. These aptamers, resistant to nucleases, are well suited for both diagnostic and therapeutic applications.

Only four building block monomers are available for making replicable oligonucleotide libraries. Attachment of different substituents to nucleotide bases of these four monomers effectively increases the molecular diversity of oligonucleotide libraries. Such modified libraries will be useful for aptamer technology provided they can be substrates for the enzymes used in the SELEX process. There are several elegant examples in which novel unnatural base pairs have been specifically incorporated into DNA and RNA (Fig. 3). Piccirilli et al. *(41)* and Bain et al. *(42)* demonstrated the incorporation of the IsoG/IsoC pair and the 2,6-diaminopyrimidine/xanthine base pair into DNA and RNA in experiments aimed at expanding the genetic code. Tor and Dervan *(43)* used the methylisoC and (6-aminohexyl)isoG base pair to introduce aminohexyl modification into unique positions in an RNA sequence by in vitro transcription. Other appendages, such as biotin and EDTA, were then introduced to unique alkyl amine functional groups of RNA posttranscriptionally. Libraries can be tailor-made for certain classes of targets by decorating nucleotide bases with different functional groups. For example, modified libraries equipped with hydrophobic groups on nucleotides may be suitable for finding winning aptamers to hydrophobic targets. Recently, Schweitzer et al. *(44)* and Guckian et al. *(45)* described a novel base-pair analog that does not require hydrogen bonding for recognition. Nonpolar, non-hydrogen-bonding shape mimics (6-methyl purine and 2,4-difluorotoluene) for an AT base pair had been enzymatically incorporated into DNA. These base-pair analogs have the ability to become building blocks of replicable oligonucleotide libraries, and yet they represent a radical departure from the classical Watson-Crick base pairing. These modifications will increase the molecular diversity of oligonucleotide libraries and further enhance the probability of finding aptamers with unique properties.

Similar to the nuclease-resistant aptamers isolated from the libraries containing 2′-modified pyrimidines, aptamers modified at the C-5 position of pyrimidines have also been identified using the corresponding modified libraries. Aptamers consisting of 5-(1-pentynyl)-2-deoxyuridines have been selected to recognize human thrombin using a library containing the modified thymidine nucleotide analog *(46)*. Aptamers isolated from the modified library were strikingly different from those derived from an all-natural DNA library *(47)* at the level of the primary and the secondary structure. Thus, it is possible that the substitution at the C-5 position of thymidine may have affected the shape repertoire of the library. The attachment of fairly bulky groups, such as a benzoyl group, at the C-5 position of pyrimidines appears to be tolerated by the enzyme used in the SELEX process *(48, 49)*, which expands the possibilities for chemically decorating oligonucleotide libraries. Interestingly, amino acid side chains could also be introduced to create pseudo peptide/nucleic acid libraries.

Oligonucleotides containing certain modifications can be activated by light to generate reactive groups that can form covalent linkages with another molecule in close proximity *(50–54)*. A special class of aptamers called photo-cross-linkable aptamers can undergo cross-linking between the aptamers and their cognate targets upon brief exposure to ultraviolet light. Modified libraries containing 5-bromouracil and 5-iodouracil residues have been used

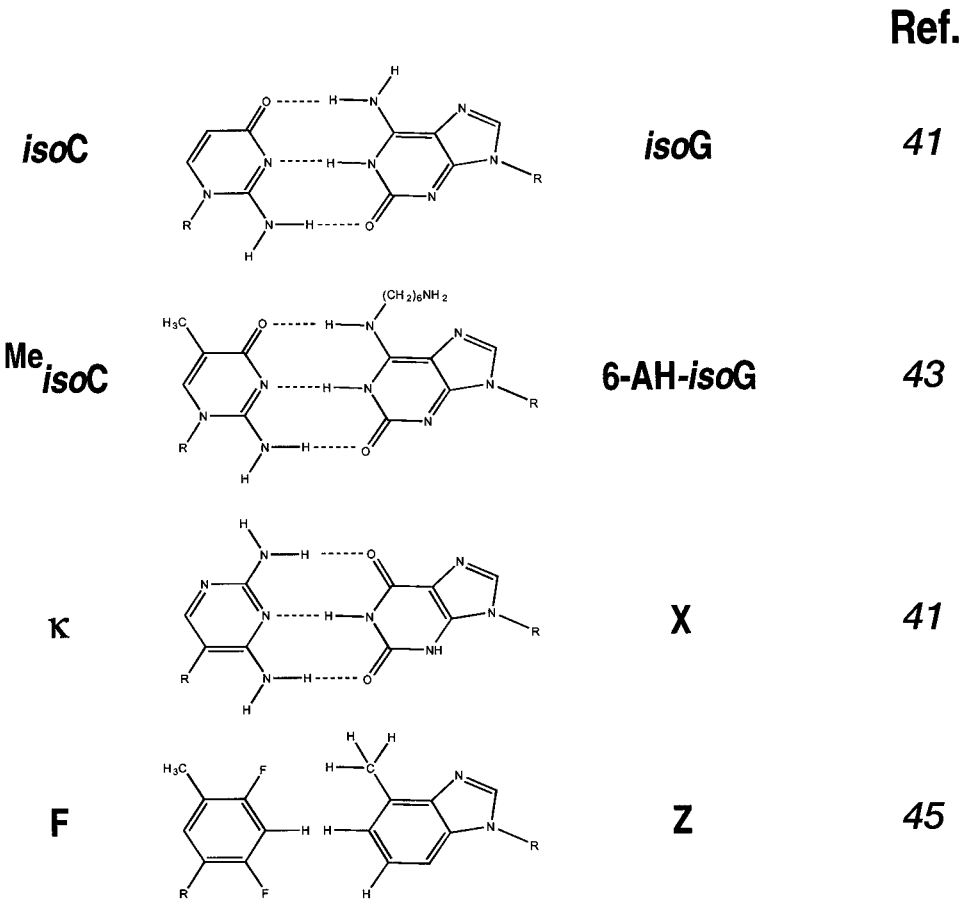



Fig. 3. Structures of unnatural base pairs that have been incorporated by either DNA polymerases or RNA polymerases, or both.

These unnatural base pairs are potential candidates for creating modified oligonucleotide libraries for the SELEX process. κ represents 2,6-diaminopyrimidine and *X* represents xanthine *(41)*. *F* represents 2,4-difluorotoluene and *Z* represents 6-methyl purine, which mimic the shape of an A-T base pair *(45)*.

to generate photo-cross-linkable aptamers [Golden et al., manuscript in preparation, and Ref. *(55)*]. The photo-cross-linking mediated by a 5-halo-substituted thymidine analog takes place between the C-5 position of the nucleobase and an electron-rich amino acid at close proximity. The potential amino acid candidates for cross-linking are tryptophan, tyrosine, histidine, phenylalanine, and cysteine. A priori, the requirement for a photo-cross-linkable amino acid side chain to reside at close proximity with the correct geometry to a single 5-halo-uracil residue of an aptamer is very stringent. Such a high stringent requirement for photo-cross-linking is expected to bring added specificity to aptamer-protein interactions. Long wavelength monochromatic light sources, 308 nm for 5-bromouracil and 325 nm for 5-iodouracil, used in the photo-cross-linking of aptamer-protein complexes substantially reduce the photodegradation of proteins and aptamers *(53, 55, 56)*. Although identification of photo-cross-linking aptamers directly from 5-halo-substituted libraries is preferred, post-SELEX substitution experiments could also lead to the identification of positions to be substituted by 5-halouracil residues within an aptamer to afford specific photo-cross-linking *(20, 57)*.

The serendipitous discovery of aptamers that undergo covalent cross-linking in the absence of light has been reported *(55)*. Although these aptamers undergo light-independent cross-linking to their cognate protein target, they require 5-iodouracil to do so. The chemistry of light-independent cross-linking has been attributed to the formation of a Michael adduct between the C-6 position on 5-iodouracil and a suitable nucleophile on the protein. These aptamers that undergo light-independent target cross-linking grant all the benefits provided by photo-cross-linking aptamers for diagnostic applications and do not require a light source for cross-linking.

The characteristics that are most important to the success of a diagnostic assay are the affinity and the specificity of the ligand that provides molecular recognition. In heterogeneous assays, nonspecifically retained molecules are washed away under conditions that do not affect the binding equilibrium of the specific target. Ideally, all molecules bound nonspecifically must be eliminated without losing any of the specific target. Chemistries that trigger the formation of a covalent linkage between an aptamer and the specific target will permit the use of extremely harsh conditions to remove molecules that contribute to the background in heterogeneous assays. In homogeneous assays, the generation of such a covalent linkage overcomes any possible dissociation of targets, especially ones with fast off-rates.

PROPERTIES

Single-stranded oligonucleotide sequences have an exceptional propensity to assume an array of secondary (and tertiary) structural motifs with different shapes. The number of possible thermodynamically stable structural variants of an oligonucleotide sequence is much higher than the number of variants available for a peptide sequence of the same length. This is simply based on the ability of nucleotide bases to interact with each other through canonical Watson-Crick as well as unusual base pairing. The existence of oligonucleotide sequences that could assume a myriad of shapes within a random sequence library is the basis for the remarkable success of generating aptamers to a wide variety of target molecules *(58, 59)*.

To date, there has not been a restriction on the type of target for which high-affinity aptamers could be identified. Aptamers have been identified that bind to small molecular targets, including metal ions *(60, 61)*, organic dyes *(3, 62)*, drugs *(25, 28)*, amino acids *(29, 63–65)*, cofactors *(66–70)*, aminoglycosides *(71–74)*, antibiotics *(75, 76)*, nucleotide base analogs *(77)*, nucleotides *(26, 27, 78)*, and peptides *(79, 80)*.

There are a growing number of protein targets to which selected aptamers bind. These include enzymes *(2, 20, 22, 23, 37, 47, 81–85)*, growth factors *(19, 39, 86, 87)*, antibodies *(38)*, gene regulatory factors *(88–92)*, cell adhesion molecules *(21, 40, 93, 94)*, and lectins *(95)*. Intact viral particles *(96)* and pathogenic bacteria have also been used to obtain high-affinity aptamers against complex targets whose binding epitopes were not well understood. The equilibrium dissociation constants, $K_d$, of aptamers range between a few picomoles per liter and a few nanomoles per liter. Therefore, the affinities and specificities of aptamers are comparable to, if not better than, those of antibodies that are used in diagnostic applications. The reader will find several interesting articles reviewing the properties of aptamers, including their affinities, specificities, and molecular structures, that have been identified to recognize a broad spectrum of target molecules *(58, 59, 97–100)*.

As discussed above, RNA libraries bearing suitable modifications at the 2′ position of the sugar have yielded aptamers that are nuclease resistant, thus circumventing the potential limitation of RNA aptamers to measure analytes in biological fluids that contain nucleases. On the other hand, compared with the unmodified RNA sequences, unmodified DNA sequences are generally more nuclease resistant. In fact, DNA aptamers with and without terminal modifications have been effective in biological fluids [Lin and Jayasena, submitted for publication, and Refs. *(20, 47, 101, 102)*]. Therefore, it is reasonable to assume that DNA aptamers without further modifications could perform in diagnostic assays in which aptamers may come into contact with biological fluids for a brief period of time. If necessary, additional protection from exonucleases can be provided through terminal capping with small molecules such as an amine linker, a phosphate group, or an inverted thymidine residue. For most diagnostic formats, terminal modification of aptamers is expected to be common and provides a route to conjugate aptamers either to reporter molecules or to solid supports.

In the case of the monoclonal antibody technology, a desirable antibody is selected upon screening of a large number of hybridomas. When the antibody in hand is not optimal for use, another antibody with improved characteristics is sought either by the screening of more clones or by starting the entire process from the beginning. In other words, improvement of the properties of an existing antibody is not generally feasible. On the other hand, aptamer technology is gifted by its capacity to further optimize the characteristics of aptamers obtained from a SELEX experiment (Fig. 4). The binding affinity and specificity of an aptamer could be further improved by subjecting the sequence to a second selection in which case a biased library is created based on the primary sequence of an existing aptamer. The biased library increases the complexity of molecules, mostly represented by the variants of the primary aptamer, that were not present in the completely randomized library used in the initial SELEX experiment. As discussed above, the complexity of a working library is not completely represented by all of the theoretically possible individual sequences. Thus, the biased library may present another opportunity

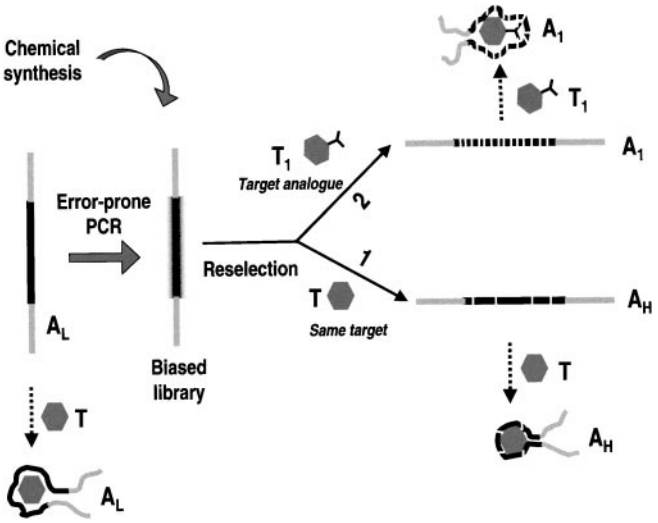



Fig. 4. Aptamer optimization through biased library generation.

An aptamer ($A_L$) with low-affinity interaction with the target ($T$) could either improve its affinity for the same target or change its affinity and specificity to recognize a closely related analog of the target ($T_1$). This is accomplished by creating a biased library based on the sequence of $A_L$ by either chemical synthesis or error-prone PCR. Based on the primary sequence, a biased library can be synthesized chemically *(55, 64, 106)*, in which process the ratios of the four nucleotides at each position are controlled by adjusting the ratio of phosphoramidite mixture. Alternatively, conditions can be chosen in PCR to introduce random mutagenesis *(178–180)*. Under error-prone PCR conditions, variants of the primary sequence are generated by the misincorporation of nucleotides by the polymerase with an error rate of 0.6% per position *(103, 178)*. The resulting library is used for reselection with the same target to identify an aptamer variant(s), $A_H$, with high affinity for the target T (*route 1*) *(79)*. Alternatively, the biased library could be used to identify aptamers that recognize an analog ($T_1$) of the target (*route 2*) *(64)*.

to select the best aptamer, which could have been missed during the initial selection. Conceptually, this approach is analogous to the "lead optimization" strategy used by medicinal chemists to identify a potent drug candidate based on the structure of a lead compound. Biased libraries have been used to optimize characteristics of aptamers and ribozymes successfully *(64, 68, 79, 103–106)*. This approach has also been used to change the specificity of an existing aptamer. A biased library, based on an aptamer sequence that recognizes L-citrulline, was subjected to reselection for binding to L-arginine. This second selection yielded aptamers that bound to L-arginine and completely lost their ability to bind to L-citrulline *(67)*. This is an interesting example that demonstrates the power of in vitro selection that allows for changing and further modifying the properties of aptamers on demand.

## Aptamers in Different Diagnostic Assay Formats

### TWO-SITE BINDING ASSAYS

The two-site binding assay, also referred to as the sandwich assay, is one of the most commonly used diagnostic formats today. In this approach, the analyte is sandwiched between two ligands, one used as the capture, and the other used as the detector. Aptamers have also been tested in two-site binding assays. In one example, an RNA-based aptamer selected to recognize vesicular endothelial growth factor (VEGF) with high affinity and specificity was used as the detector ligand in the sandwich assay format *(107)*. The RNA-based aptamer labeled with fluorescein at the 5′ end was detected with an anti-fluorescein Fab fragment conjugated to alkaline phosphatase. The reason for the use of Fab-enzyme conjugate was its availability as a commercial source. It is also conceivable to replace the antibody-enzyme conjugate with an aptamer-enzyme conjugate. This assay was able to detect VEGF in serum samples. In another example, the simultaneous binding of an aptamer and an antibody to human CD4 expressed on cell surfaces was demonstrated *(40)*. Because the binding sites of the two ligands were confined to the termini of the rod-shaped CD4 molecule, the interaction of one ligand did not affect the other. In both of these examples, aptamers were not immobilized on a solid support. Aptamers immobilized on beads have also been used successfully in a sandwich assay format to capture the target protein (Heil et al., manuscript in preparation), indicating that aptamers can function as the capturing reagent as well as the detector reagent in two-site binding assays.

The two-site binding assays based on aptamers reported to date involved an antibody as the second ligand. This has been mainly because of the lack of two aptamers that do not share identical or overlapping binding sites on a target of interest. One of the reasons for the lack of aptamers that bind a target at nonoverlapping binding sites could be related to the way in which the selections are generally performed. In a typical SELEX experiment directed to identify the highest affinity aptamers, the affinity enrichment of evolving libraries is pushed to the limit. These efforts generally lead to aptamers with the tightest binding directed to a unique site on the target. It would be reasonable to expect to find aptamers that bind to different binding sites of a target, perhaps with somewhat lower affinity, in the intermediate cycles of the selection. In addition, alternative strategies could also be used to identify aptamers with nonoverlapping target-binding sites. An aptamer already identified for a target could be used in a subsequent SELEX experiment to direct aptamers elsewhere on the same target, or the target could be presented in a different manner in two different SELEX experiments to expose different epitopes of the same target. Aptamers that share nonoverlapping binding sites on a target do exist without the use of any of these techniques. Lochrie et al. *(91)* had selected aptamers that bound either to nucleocapsid protein or to the matrix protein in the HIV type-1 gag polyprotein target. These two classes of aptamers did not compete for binding to the HIV-1 gag polyprotein. Tasset et al. *(20)* described the identification of DNA aptamers that bound human thrombin at a site different from the site bound by aptamers isolated by Bock et al. *(47)*. There is a striking similarity between the DNA aptamers identified by the two groups aimed at thrombin binding. Both classes of aptamers have the ability to adapt intramolecular G-quartet structures. However, one class of aptamers bound thrombin at the fibrinogen-recognition exosite *(47)*, whereas the other class bound at the heparin-binding exosite *(20)*. As described previously *(20)*, the identification of two classes of aptamers with nonoverlapping binding sites could have been the result of the two different partitioning methods used in the selections. In one case, thrombin free in solution had been challenged with the single-stranded DNA library, whereas in the other, thrombin immobilized through a glycosylated site was used as the target. Immobilization of the target may have hindered an otherwise preferred binding site, directing aptamers to a different site. Such oligonucleotide ligands with nonoverlapping binding sites on a target protein were used to design a sandwich assay exclusively based on aptamers (Y. Lin and S.D. Jayasena, unpublished results) to detect human thrombin.

Understanding of how aptamers interact with their cognate targets is an intense area of research *(108–112)*. Molecular details of how aptamers interact with small molecular targets have been obtained by nuclear magnetic resonance spectroscopy *(110–116)*. These experiments revealed several interesting insights on the subject: (*a*) Aptamers are folded into unique overall shapes to form intricate binding pockets to accommodate their targets. (*b*) Functional groups scattered on an aptamer are brought to close proximity to form a cluster of molecular forces that specify target interaction. (*c*) Aptamers discriminate molecules that are closely related to cognate targets at the atomic level. Nuclear magnetic resonance studies of several aptamer-target complexes indicated that small molec-

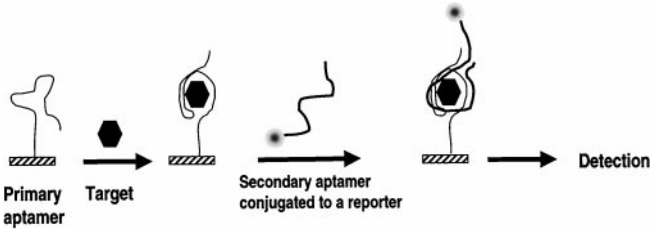



Fig. 5. Aptamer-based assay using a secondary aptamer that specifically recognizes primary aptamer-target complex.

A format of a heterogeneous assay is shown. The target is captured by the primary aptamer immobilized on a solid support to form a target-aptamer complex, which is then specifically recognized by the secondary aptamer conjugated to a suitable reporter molecule. The same concept could also be used in homogeneous assay format, such as FP, in which the change in fluorescence characteristics of a fluorophore conjugated to the secondary aptamer is measured upon binding to the primary aptamer-target complex.

ular targets are buried within the binding pockets of aptamers, leaving very little surface to interact with a second molecule. This may limit the possibilities of finding a second aptamer that could interact with a small molecular target that is already bound to the first aptamer.

One experiment that has great value in diagnostics, especially in detecting small molecular targets, and is still waiting to be carried out with aptamers is the identification of aptamers that bind to aptamer-target complexes. One could be optimistic on the success of isolating aptamers to aptamer-target complexes, based simply on the fact that nucleic acids interact well with one another. Such aptamers would be useful in designing two-site binding assays on small molecular targets and would eliminate the need for antibodies on large protein targets (Fig. 5). A diagnostic assay that utilizes an antibody that recognizes an antibody-target complex is referred to as an "anti-immune complex immunoassay". An example of this format has been described for measuring digoxin in serum samples *(117)*. In this assay, a secondary antibody that specifically recognizes digoxin only when it is bound to the primary antibody was used. Although the basic idea of this assay format warrants rapid expansion, the difficulty in generating secondary antibodies that bind small analyte-primary antibody complexes may have hampered these efforts. The identification of secondary aptamers that recognize targets bound to their primary aptamers should expand this assay format.

The exquisite specificity of aptamers should be considered carefully in diagnostic applications. Aptamers have been shown to discriminate closely related molecules from their targets on the basis of small structural changes, such as a methyl group *(25, 26)*, a hydroxyl group *(27, 28)*, and a urea vs a guanidino group *(64)*. They also exhibit a high degree of chiral discrimination of target molecules. This level of specificity in aptamers rivals that of monoclonal antibodies *(118–120)*. Monoclonal antibodies have been used to develop assays that distinguish minute differences between targets; for example, immunoassays that specifically detect glycated proteins *(121)* or isoenzymes of different tissue origin *(122)* have been described. Similar assays that require extreme specificity should be possible to design with aptamers. Assays based on monoclonal antibodies aimed at detecting certain types of targets with molecular heterogeneity, such as pituitary glycoprotein hormones, have been troublesome *(123–126)* because of the high specificity of antibodies. These challenges may also exist in aptamer-based assays for detecting protein heterogeneity. However, a cocktail of aptamers, each designed to bind specific variants of the target, could be generated with speed—especially using the automated SELEX process—for assays that are specific for target analytes with molecular heterogeneity.

The catalytic power of enzymes is often harnessed to enhance sensitivity of diagnostic assays. In ELISAs, the secondary antibody is conjugated to an enzyme such as

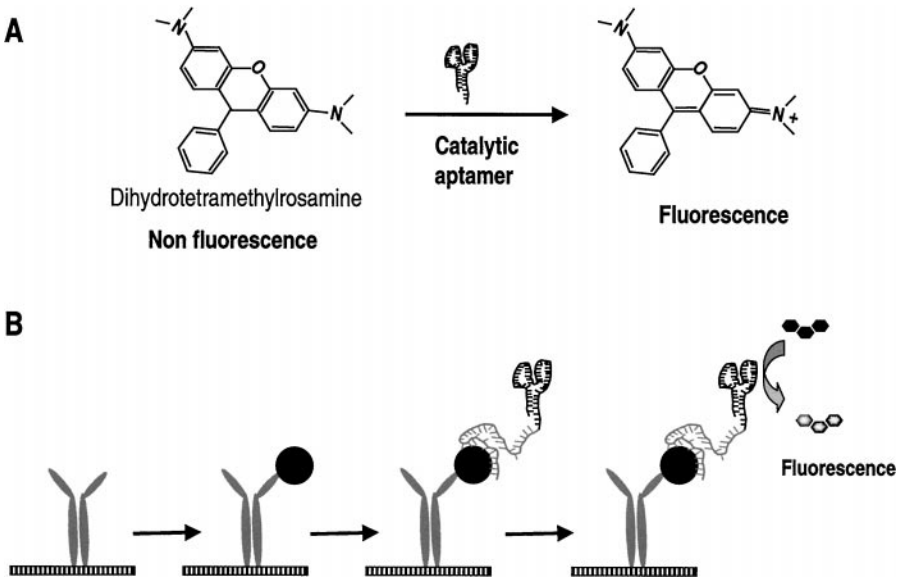



Fig. 6. Potential application of catalytic oligonucleotides (ribozymes and deoxyribozymes) to in vitro diagnostics.

Aptamers with the ability to catalyze the transformation of a fluorogenic substrate into a product that emits fluorescence have been described *(127)*. (*A*), nonfluorescent dihydrotetramethylrosamine is oxidized by catalytic DNA aptamers to generate tetramethylrosamine containing an extended $\pi$-electron system capable of fluorescing. (*B*), a possible extension of such catalytic aptamers to in vitro diagnostic applications. This example shows an ELISA format in which an aptamer is the secondary ligand in the two-site binding assay. The binding aptamer is conjugated to a catalytic aptamer that catalyzes the fluorogenic transformation to generate fluorescence signal.

alkaline phosphatase or horseradish peroxidase to catalyze a reaction that generates the signal. Although it is not discussed here, in vitro selection, the same technology that is used to discover aptamers, is being used to discover oligonucleotides that catalyze novel reactions. In the future, it should be possible to use catalytic oligonucleotides (ribozymes or deoxyribozymes) to carry out signal generation in diagnostic assays (Fig. 6), pushing proteins completely out of the picture. Recently, Wilson and Szostak *(127)* described the isolation of aptamers that bind to a fluorophore, a sulfonated rhodamine derivative. Upon screening of several aptamers, the authors found aptamers (or deoxyribozymes) that catalyze oxidation of a related molecule, dihydrotetramethylrosamine, to its oxidized form that emits fluorescence. Although the activity of the described oligonucleotides is weak, these deoxyribozymes could be further optimized to enhance their catalytic power, making them valuable for diagnostic applications.

### FLOW CYTOMETRY

Flow cytometry is a powerful analytical tool that allows multiparameter analysis of cells and microsphere particles. Today, the technique is being used in basic research as well as in clinical diagnostics. Diagnostic assays can be constructed on microsphere particles bearing ligands or analytes. Because particles can be distinguished on the basis of their color and size, flow cytometry is an attractive platform for multiplex analysis. Flow cytometers equipped with state-of-the-art signal processing software programs can measure these properties on thousands of particles in samples containing very small amounts of material in a matter of a few seconds. Microspheres dyed with a spectrum of colors coated with different ligands such as antibodies and oligonucleotides provide a way to multiplex diagnostic assays in flow cytometry *(128)*.

Flow cytometry has been used to detect the binding of aptamers to their cognate proteins presented on either microspheres or cell surfaces. A DNA aptamer selected to recognize human neutrophil elastase (HNE) *(23)* was modified to attach fluorescein at different positions, away from the target binding site, using different linkers, and was used to stain HNE-coated beads for flow cytometry *(129)*. Although every single derivative of the aptamer tested bound the target-coated beads, the signal intensity was dependent on the way in which fluorescein was attached. Fluorescein conjugated through an ethylene glycol linker gave the highest signal intensity, which was even better than that of the fluorescein-conjugated antibody. For cellular staining experiments, aptamers have been selected using recombinant proteins immobilized on beads either by chemical biotinylation *(40)* or by an expression tag *(21, 102, 130)*. Full-length aptamers containing 2′-F-pyrimidines obtained by in vitro transcription have been conjugated to either fluorescein or phycoerythrin to analyze their binding to human CD4 expressed on cell surfaces *(40)*. Both monovalent fluorescein-conjugated RNA aptamers and multivalent aptamer-phycoerythrin conjugates stained CD4-expressing cells. The results clearly indicated that the aptamers specifically stained subsets of cells, namely T-helper cells and monocytes, expressing CD4 in a heterogeneous cell mixture of human peripheral blood mononuclear cells. Aptamers labeled with one color probe performed well in combination with antibodies labeled with a second color probe in experiments aimed at two-color analysis of two different targets on cell surface.

Truncated DNA aptamers labeled with fluorescein have been chemically synthesized for staining cells that express L-selectin *(102, 130)*. Anti-L-selectin aptamers stained both leukocytes and neutrophils that express L-selectin in human peripheral blood mononuclear cell preparations. This result was very similar to what was obtained with an anti-L-selectin antibody. The addition of the unlabeled aptamer completely inhibited the staining of neutrophils by the labeled aptamer but only partially blocked the binding of the anti-L-selectin antibody. The inability to completely block the antibody binding has been attributed to the undesirable binding of the antibody to the Fc receptors on neutrophils. The staining of P-selectin on human platelets was demonstrated with a 2′-F-pyrimidine-containing RNA aptamer *(21)*. The truncated RNA aptamer specific for P-selectin was chemically synthesized and subsequently derivatized with fluorescein. As expected, this aptamer showed specific binding to activated human platelets that express P-selectin but not to resting platelets with no P-selectin on the cell surface.

Antibodies of the IgG class are bivalent, whereas aptamers that come out of selections are typically monovalent. To understand their behavior in their bivalent forms, aptamers have been dimerized and studied in flow cytometry *(129, 130)*. Dimeric, therefore bivalent, aptamers had been obtained by solid-phase chemical synthesis starting with a commercially available solid support (symmetric branching CPG) that allows symmetric divergent synthesis of two aptamer sequences linked at 3′ ends. The divalent anti-HNE aptamer showed a 10-fold higher affinity than the monovalent form in binding to the target either in solution or immobilized on beads *(129)*. The effects of aptamer dimerization on cellular staining were elegantly studied by Ringquist and Parma *(130)*, who used a DNA aptamer isolated to recognize L-selectin. The aptamer binding to L-selectin on cells showed a 20-fold affinity improvement upon dimerization. The rate of dissociation of the divalent aptamer on cells was 10-fold lower than the monomeric form. The half-life of the divalent aptamer on cell surface was superior to that of the divalent antibody (14 min vs 5.5 min) *(130)*, suggesting that aptamer engineering may lead to the discovery of aptamers with further improved characteristics.

Research aimed at testing the performance of aptamers in flow cytometry revealed some interesting features. Aptamers conjugated to small fluorophores, such as flu-

orescein, as well as to structurally large proteins such as phycoerythrin (molecular mass, 240 kDa) retain their binding characteristics, indicating their ability to accommodate a wide variety of the reporter molecules generally used in diagnostic applications. However, the signal intensity may be a function of the chemistry of fluorophore attachment and can easily be optimized. Aptamers can perform equally well in lieu of, or in combination with, antibodies. The generation of divalent, or even multivalent, aptamers with enhanced performance characteristics is relatively easy. The presence of the Fc region on antibodies that may interact with the Fc receptors on cells could complicate the outcome of certain cellular staining experiments *(130, 131)*, making aptamers more attractive for such applications.

### SENSORS

Certain applications demand analyte detection within a very short period of time. The need for techniques that allow rapid detection and quantification of analytes cannot be underestimated, especially when dealing with emergency medical situations or in a battlefield. Portable equipment that could be used as small hand-held devices would be ideal for such applications. Sensors based on molecular recognition coupled to a transducer have been developed to meet the needs of rapid detection. Ideally, at least three basic criteria should be met in an affinity sensor: (*a*) the ability to transduce the binding event without an extra reagent added; (*b*) the ability to detect and quantify the target within the desired concentration range and the time period; and (*c*) the ability to turn over the sensing capacity, i.e., to make repeated measurements on the same transducer multiple times.

Antibody-based immunosensors have been developed as an alternative to immunoassay techniques. In immunosensors, antibodies have been immobilized on sensor surfaces that come into contact with analytes *(132)*. One of the obvious limitations of immunosensors is their poor capacity to regenerate the antibody surface. Mild conditions are required to preserve the integrity of the antibody function. Although immunosensors have been demonstrated to be reusable *(133)*, the loss of activity of surface-immobilized antibodies is inevitable. On the other hand, several advantages are apparent in aptamer-based sensors. The ability to regenerate the function of immobilized aptamers would be the most attractive feature of aptamers. Being nucleic acids, aptamers could be subjected to repeated cycles of denaturation and renaturation. Several methods are available for aptamer regeneration: heat, salt concentration, pH of the medium, and chelating agents. Except for pH, the other conditions can be varied to any extreme without damaging the aptamer. Extremes of pH should be avoided because they could potentially damage aptamers. As discussed earlier, the ease with which aptamers could be modified for immobilization purposes bestows the second benefit. The attachment of oligonucleotides on different surfaces in a controlled and reproducible manner has been demonstrated *(134–139)* and could easily be adapted to aptamers. Homogeneous preparations of chemically synthesized aptamers with appropriate linkers could be deposited precisely on solid surfaces at a desired density. The third benefit of aptamers is the ease of labeling with a wide range of reporters, enabling the design of a variety of detection methods. As discussed below, a fluorophore could be attached to an aptamer such that its characteristics would change upon target binding. One of the attractions of the SELEX technology is that the selections could be carried out under any condition defined by the user to obtain aptamers with the desired characteristics. For example, modified random sequence oligonucleotide libraries containing fluorophores could be subjected to selections to identify aptamers that have the desired binding and fluorescence characteristics for applications in affinity sensors.

Recently, aptamers have been tested in affinity sensors. In one application, a biotinylated RNA aptamer selected to recognize L-adenosine was immobilized on an optical fiber surface derivatized with streptavidin *(140)*. The RNA aptamer on the sensor surface detected L-adenosine conjugated to fluorescein with an affinity similar to that measured in solution. The sensor showed selective binding to L-adenosine with a chiral discrimination of at least 1700-fold. In another application, a DNA aptamer specific for human thrombin was used to detect binding of the target protein by evanescent wave-induced fluorescence anisotropy *(141)*. Here, the 5′ end of the aptamer was labeled with fluorescein and its 3′ end was modified with an alkyl amine attached to a glass surface. The advantage of this sensor is that it does not require its target to be labeled and, therefore, could be extended to in vivo measurements. The aptamer-sensor was specific for thrombin because there was no change in fluorescence anisotropy when it was challenged with elastase, another basic protein. There was no response when a scrambled DNA sequence labeled with fluorescein was attached, indicating that the sensor was dependent on the specific interaction between the aptamer and target. Repeated measurements of thrombin have been made on the sensor, with the aptamer surface regenerated by the removal of bound thrombin with a denaturant. Although research on aptamer-based sensors has been limited, the promising results obtained thus far indicate their potential utility in developing sensors designed for specific molecules.

An unexplored area of aptamers is in sensors based on electrochemical detection. Aptamers, being polyanionic, may be attractive for sensing the changes in conductance in the presence and absence of target binding. An interesting area of nucleic acid research is the understanding of the principles behind the charge transfer within the DNA helix *(142, 143)*. Although this research is still in infancy, it has great potential in the area of molecular sensing. To date, electron transfer studies have focused purely on DNA strands, but soon they will expand to understand the changes mediated by interacting DNA

with other molecules. Applications of aptamers in various diagnostic formats may benefit from these novel discoveries.

### FLUORESCENCE POLARIZATION

Homogeneous assay formats are attractive for their simplicity, ease of use, speed, and applicability in high-throughput screening. One homogeneous assay format based on fluorescence polarization (FP) is being used to measure small target analytes such as steroids in clinics *(144)*. These are competitive assays based on the interaction between unlabeled antibodies and their small analytes conjugated to a fluorophore. The change in FP is a direct reflection of the change in the tumbling rate of the molecule conjugated to the fluorophore upon binding to a structurally large antibody (Fig. 7). The competitive binding of the unlabeled analyte present in the test sample to the antibody decreases the FP signal in the assay. Competitive assays are rapid, easy-to-use, and amenable for high-throughput screening. There are, however, several shortcomings associated with competitive assays. They are less sensitive, have a narrow dynamic range, and require precise control of the reagents used. The inherent nature of antibodies dictates that antibody-based FP assays be competitive. Structurally bulky antibody molecules tumble slowly in solution. Therefore, the change in the tumbling rate of an antibody molecule upon its association with a small analyte is insignificant. As a result, the change in FP that occurs when a fluorescently labeled antibody binds to its target could be very small or even undetectable. In addition, homogeneous preparations of antibodies labeled with a fluorophore are difficult to obtain. The scenario with aptamers is, however, expected to be different. In aptamer-based FP assays, the fluorescence tag could be on the aptamer itself rather than on the analyte. This arrangement allows these assays to become noncompetitive. The potential use of fluorescently labeled aptamers in FP assays is justified in two ways: (*a*) Aptamers are relatively small compared with antibodies (one-tenth of the size of an antibody), and therefore tumble faster than antibodies. The change in tumbling rate, and thus the change in FP, is expected to be large when the aptamer interacts with the target. (*b*) Results obtained from nuclear magnetic resonance experiments indicate that aptamers selected to bind small targets undergo target-induced conformational changes *(111, 113)*. These conformational transitions induced by target recognition on aptamers are likely to affect the tumbling rates of aptamers as well.

A DNA aptamer identified to recognize HNE was labeled with fluorescein at the 5′ end and used in a FP assay to detect HNE (Fig. 8). A significant change in FP was observed when the labeled aptamer interacted with HNE. The addition of a nonspecific target, human serum albumin, produced a very small change in FP only at high concentrations. This demonstration suggests that fluorescein-labeled aptamers could be used to design noncompetitive FP assays for target detection. Aptamers selected to recognize aminoglycoside antibiotics have been used in competitive FP assays to understand their binding characteristics to a family of aminoglycosides *(74, 145)*. The change in FP, or anisotropy, of a fluorescein molecule attached to an antithrombin aptamer has been monitored during its interaction with thrombin in an aptamer-based biosensor *(141)*. The fluorophore on the aptamer was excited by an evanescent field to measure the change in polarization as a function of thrombin concentration at the interface. The thrombin biosensor detected between 0.7 and 700 amoles of thrombin in a 140-pL volume. Contrary to the thrombin assay, the fluorescein-labeled aptamer was free in solution in the HNE assay described in Fig. 8.

Aptamers that change the fluorescence characteristics

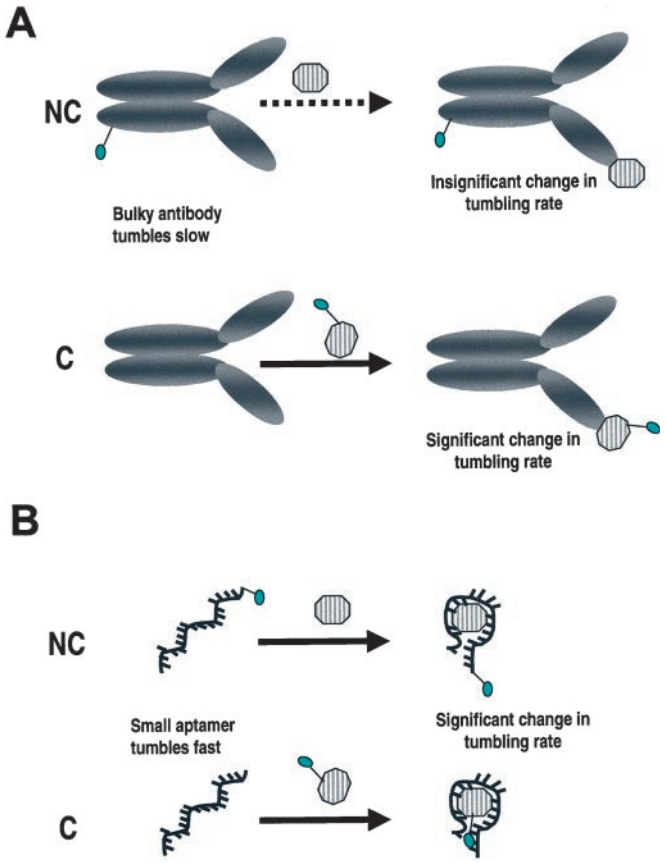



Fig. 7. Different formats of FP assays based on either an antibody (*A*) or an aptamer (*B*) for detecting small molecule targets in a homogeneous manner.

The change in FP relates directly to the change in tumbling rate of the molecule conjugated to a fluorophore upon association with another molecule. The change in FP brought about by the association of a target with its ligand is measured in FP-based assays. (*A*), noncompetitive (*NC*) homogeneous detection is generally not feasible (*dashed arrow*) with bulky antibodies labeled with fluorophores. This is because of the insignificant (or very small) change in the tumbling rate of fluorescein-labeled antibody upon binding to a small molecule target. Therefore, unlabeled antibodies are used in competitive (*C*) formats in which the target is conjugated to a fluorophore (*solid arrow*). (*B*), in contrast to antibodies, aptamers could be used in both noncompetitive and competitive formats in FP assays. The noncompetitive format is possible because the small size of an aptamer (approximately one-tenth the size of an antibody) should allow a significant change in tumbling rate upon interaction with the target.

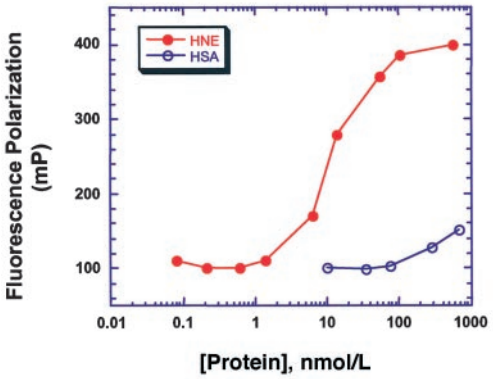




Fig. 8. Aptamer-based FP assay to detect HNE.

The structure of the single-stranded aptamer labeled with fluorescein at the 5′ end used in the assay is shown (*right*). As illustrated, this aptamer exists as a G-quartet structure with duplexed ends *(23)*. The DNA aptamer (100 pmol/L) labeled with fluorescein was incubated with various concentrations of either HNE (●) or human serum albumin (○) in a buffer consisting of 150 mmol/L NaCl, 100 mmol/L Tris-HCl (pH 7.0), 6 mmol/L KCl, and 2 mmol/L $MgCl_2$ at 37 °C for 10 min. FP was measured at ambient temperature using a Beacon Fluorescence Polarization System, model P2000 (Pan Vera).

of a fluorophore attached either to the aptamers themselves (for noncompetitive assays) or to the analyte (for competitive assays) would be useful. In fact, aptamers directed to bind flavins have been shown to quench the fluorescence of the target when it is bound by the aptamer *(66)*. Thus, it is possible that targets that inherently fluoresce could be detected by aptamers by measuring the changes in fluorescence.

### COMMUNICATING WITH MOLECULAR BEACONS

Recently, a novel class of fluorogenic probes called molecular beacons was introduced for homogeneous detection of nucleic acid sequences *(146, 147)*. Molecular beacons are simple hairpin-loop probes in which a fluorophore is attached to one terminus and a quencher is attached to the other (Fig. 9). This mode of attachment brings the fluorophore close to the quencher when the molecular beacons are folded into hairpins; the fluorescence is then quenched by the formation of a nonfluorescent complex between the fluorophore and the quencher *(147)*. The nucleic acid sequence in the loop of the molecular beacon is designed to be complementary to the target of interest. The loop of the molecular beacon interacts with its target sequence to form an intermolecular hybrid, during which the stem of the beacon unfolds to move the fluorophore away from the quencher. The end result is the emission of fluorescence from the previously nonfluorescent beacon. Homogeneous detection of nucleic acid sequences specific to pathogens has been demonstrated using molecular beacons *(148)*. Until recently, molecular beacons were limited to detecting nucleic acid targets because of their lack of productive interaction with other classes of targets such as proteins.

Aptamers with the ability to interact with nucleic acids as well as other classes of targets have been used to close the communication gap between molecular beacons and targets other than nucleic acids (Lin and Jayasena, submitted for publication). Molecular beacons used in this approach are designed to interact with aptamers and are called ligand beacons. The loop of the ligand beacon is complementary to a nucleotide region, preferably containing unpaired nucleotides, in the aptamer. The nucleotide region of the aptamer targeted by the ligand beacon should allow efficient intermolecular hybridization only in the absence of the target protein. However, when the aptamer is bound to its target, the ligand beacon should be unable to interact productively with the aptamer. This strategy allowed the design of homogeneous and competitive assays to detect proteins for which high-affinity aptamers have been isolated (Lin and Jayasena, submitted for publication). Target proteins have been detected in buffer as well as in plasma, demonstrating the potential application of this approach to clinical practice.

Simultaneous analysis of more than one analyte (multiplexing) provides another dimension into the advantages in an assay. Several molecular beacons, each conjugated to a unique fluorophore that emits at a wavelength different from the others, have been used to analyze the presence of multiple nucleic acid targets *(147)*. The same multiplex approach could be adapted to the ligand beacon assay to measure more than one target in a single tube. A variation of the theme is the use of beads that are easily distinguishable in flow cytometry. The ligand beacon assay has been extended to multiplex analysis to detect more than one protein in a single tube using a flow cytometry platform (Heil et al., manuscript in preparation). This assay, called the solid-phase ligand beacon assay, has aptamers immobilized on beads and ligand beacons in solution. Because the fluorescent measurements take place on beads and not in solution, this format eliminates the need for actual beacons containing a quencher and does away with fluorescein-labeled DNA probes complementary to aptamers.

In this format, ligand beacons are expected to displace molecules that nonspecifically interact with aptamers, further improving the specificity of assays. In addition, a

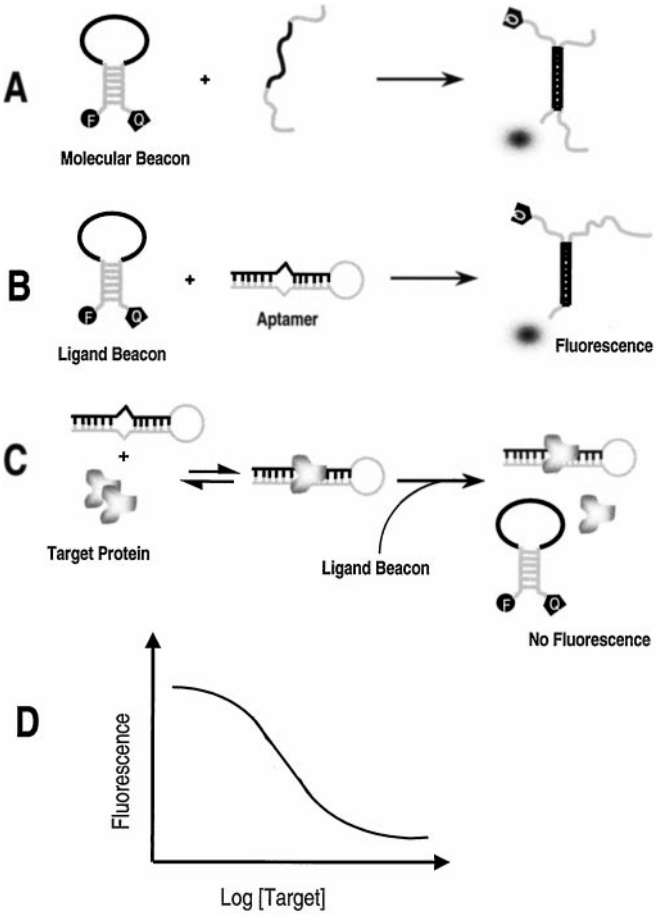



Fig. 9. Schematic representation of the principle behind the ligand beacon assay designed to detect target molecules in a competitive and homogeneous manner.

(*A*), use of a molecular beacon in homogeneous detection of a specific nucleic acid sequence *(146)*. (*B*), a ligand beacon is a molecular beacon that interacts with an aptamer to generate fluorescence. The black loop sequence (*black loop*) hybridizes to the target nucleotide region (shown in *black*) within an aptamer to form an intermolecular complex that emits fluorescence. (*C*), the nucleotide region within an aptamer that hybridizes with a ligand beacon becomes unavailable upon target binding. Thus, when the target protein is present in excess, the ligand beacon remains dark because it cannot interact with the aptamer. (*D*), expected outcome of the ligand beacon assay. When the target concentration is very low, all of the aptamer molecules form intermolecular hybrids with the ligand beacon, generating fluorescence. At the other extreme, when the target protein concentration becomes very high, the ligand beacons remain free in solution, generating virtually no signal because the aptamers are sequestered by the target protein. When the target concentration is not at the two extremes, the fluorescence signal is expected to be inversely proportional to the concentration of the target.

ligand beacon could also interfere with the specific aptamer-target interaction. This is expected to be especially prominent in cases in which the aptamer-target interaction becomes weak. Thus, aptamers that form tight complexes with slow off-rates are the ideal candidates for the ligand beacon assay. Photo-cross-linkable aptamers that form covalent linkages with their cognate targets are expected to further improve the performance of the ligand beacon assay. Clearly, the ligand beacon assay represents a unique application of aptamers that cannot be replaced by antibodies.

### CAPILLARY ELECTROPHORESIS

Capillary electrophoresis (CE) uses the same separation mechanism used in conventional electrophoresis in a capillary format. The technique offers the advantages of speed, the use of small sample volumes, suitability for automation, sensitivity, and possible multiplex analysis. In CE, component separation occurs on the fly, and the separated components are identified by online detection. Whereas conventional gel electrophoresis requires additional detection steps after electrophoresis, CE combines these two steps. CE is now being adapted to immunoassays in which antibody-antigen complexes are separated on a fluidic stream under an applied electric field. Both competitive *(149)* and noncompetitive immunoassays *(150)* have been performed with CE. In competitive assays, the unlabeled target in a test sample competes with the same target labeled with a fluorophore for binding to a limited amount of antibody. In contrast, in noncompetitive assays, a large excess of fluorescently labeled antibody interacts with unlabeled target. As described above, competitive assays have certain limitations. Therefore, noncompetitive assays are preferred whenever possible. Practical challenges associated with immunoassays run on a CE format make it difficult to develop noncompetitive assays. Poor analytical separation is expected between labeled antibody-target complexes and free labeled-antibodies when the targets are small and uncharged. Antibodies could exert heterogeneous electrophoretic patterns, possibly resulting from different degrees of glycosylation. Moreover, homogeneous preparations of antibodies labeled with small molecules are difficult to obtain *(151)*.

These limitations of antibodies have prompted German et al. *(152)* to use an aptamer in CE based on affinity probes, the method referred to as affinity probe capillary electrophoresis (APCE). The authors used a fluorescently labeled DNA aptamer previously isolated to recognize human IgE *(38)* to detect and quantify human IgE in the presence and absence of serum in APCE. APCE yielded well-separated peaks corresponding to the free aptamer and aptamer-IgE complex as detected by laser-induced fluorescence. The addition of serum-containing IgE samples had little or no effect on the separation and the characteristics of the two peaks, indicating that the binding of the aptamer to the target IgE was not affected in the context of a complex mixture. The apparent $K_d$ value of the fluorescein-conjugated aptamer measured by APCE was approximately sixfold higher than the value obtained for the unlabeled aptamer using the nitrocellulose filter binding technique *(38)*. Although the reduced affinity of the labeled aptamer in APCE could be attributable to the attachment of fluorescein *(152)*, the most likely cause could be the electric field applied to separate the free and bound aptamer. Successful results in APCE have been obtained with an aptamer that interacts weakly ($K_d \sim 200$ nmol/L) with human thrombin as well.

Aptamers have uniform charge/mass ratios, and there-

fore have predictable behavior in electrophoresis. They also undergo structural transitions when they interact with small targets. Such structural transitions, accompanied with the change in mass upon binding to the target, could lead to changes in electrophoretic patterns. On the other hand, aptamer-target interactions may be sensitive to an electric potential because of the charged nature of aptamers. The advantage of the aptamer technology is the ability to demand desirable characteristics from aptamers. For example, selection conditions and separation methods could be defined to obtain aptamers that not only form stable complexes with targets but also give a resolvable mobility shift in an electric field when they bind to targets. In other words, CE itself could be used as a separation technique during the SELEX process to isolate aptamers aimed at APCE applications. Interestingly, the aptamers that have been successfully used in CE were not identified in this manner.

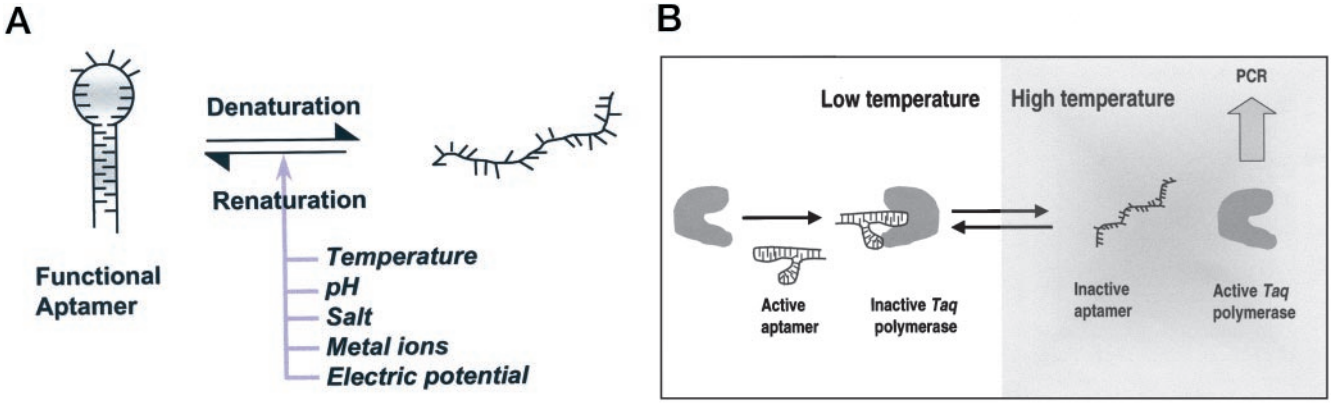
### APTAMERS AS MOLECULAR SWITCHES

One of the inherent characteristics of oligonucleotide aptamers is their robustness under repeated cycles of denaturation and renaturation conditions mediated by a variety of environmental factors, including heat (Fig. 10). This characteristic is uncommon among proteins, except in proteins derived from thermostable organisms. This feature of aptamers, coupled with the ability to select them under conditions defined by the user, makes aptamers a unique class of reagents that can be used as molecular switches. What differentiates an aptamer from other nucleic acid sequences is its ability to fold into a tertiary structure to create a binding pocket to precisely and specifically interact with the target. The folding of nucleic acids is sensitive to a variety of environmental factors, including salt, pH, divalent ions, dehydrating agents, and temperature. As a result, aptamers that bind to their targets could be selected to be responsive to any one or a combination of such factors. In fact, aptamers that are sensitive to pH, chelating agents, and temperature have been selected by demand *(84, 94, 95, 102, 153)*. Romig et al. have used one such aptamer in aptamer-affinity chromatography (manuscript submitted for publication). A DNA aptamer selected to bind L-selectin in a metal ion-dependent manner *(94)* was immobilized on a solid support to create an aptamer-affinity matrix. This matrix was used to purify an L-selectin-Ig fusion protein from cell-conditioned medium with an 83% single-step yield by eluting aptamer-retained protein with EDTA. This application is very similar to immunoaffinity chromatography, in which antibodies are immobilized on a solid support *(154)*. Repeated regeneration of the affinity matrix is a clear advantage associated with aptamer-based affinity matrices.

Diagnostic tests that are vulnerable to contamination pose a major challenge in clinical applications. Carryover contamination in diagnostic PCR tests represents a good example. Once the reagents are added to PCR, it is not advisable to open sample tubes for further manipulations because of the possibility of amplicon contamination. Therefore, reagents that could be turned "on and off" by an external stimulus, such as heat, are attractive in these settings. PCR amplification of low copy number target sequences in biological samples that contain a large excess of nonspecific nucleic acids often leads to amplification of undesirable sequences. Coamplification of undesirable sequences decreases the sensitivity of PCR, complicates the interpretation of final results, and demands the use of alternative techniques to confirm the presence of the desired specific amplicons in PCR *(155, 156)*. These undesirable spurious amplifications have been attributed to the ambient temperature activity of *Taq* DNA polymerase on primers annealed nonspecifically to one another (primer-dimers) and/or to nonspecific sequences *(157–161)*. Such nonspecific primer annealing takes place at temperatures below the optimum for specific annealing.

"Hot start" PCR is an approach designed to eliminate undesirable amplification of products by withholding a reagent essential for amplification until the reaction temperature is sufficient for stringent primer annealing. Although hot start PCR performed manually improves sensitivity and specificity, the technique is contamination prone and becomes tedious, especially when a large number of samples are to be handled. Therefore, a variation of the hot start method, called "in situ hot start", has been introduced. Under in situ hot start conditions, *Taq* polymerase is kept inactive at a low temperature and is activated at a high temperature. A monoclonal antibody that neutralizes the activity of *Taq* polymerase *(162)* or a modified form of *Taq* polymerase (AmpliTaq Gold) *(163)* has been used to accomplish in situ hot start PCR. In both cases, the polymerase that remains inactive at low temperature becomes active in situ during the permanent heat denaturation of the antibody or the permanent inactivation of the modification in AmpliTaq Gold enzyme. Although these reagents were quite effective in improving the specificity and sensitivity of PCR mediated by *Taq* polymerase, neither reagent is suitable for amplifying RNA targets. This is because of the temperatures required to initiate the polymerase activity in these two methods, i.e., >75 °C for the antibody and >90 °C for the AmpliTaq Gold enzyme. These high temperatures can lead to the degradation of RNA targets. Amplification of RNA targets is essential for detecting certain viral pathogens and has substantial commercial value. Amplification of RNA targets by reverse transcription-PCR requires an initial reverse transcription step to convert an RNA sequence to the DNA copy, before being amplified by PCR. The initial reverse transcription step typically carried out at relatively low temperatures requires a reagent that could activate the polymerase between 40 and 50 °C.

Aptamers selected to bind *Taq* polymerase with high affinity ($K_d$ values between 30 and 50 pmol/L) have been described *(84)*. When they bound to *Taq* polymerase, these DNA aptamers inhibited the activity of *Taq* poly-



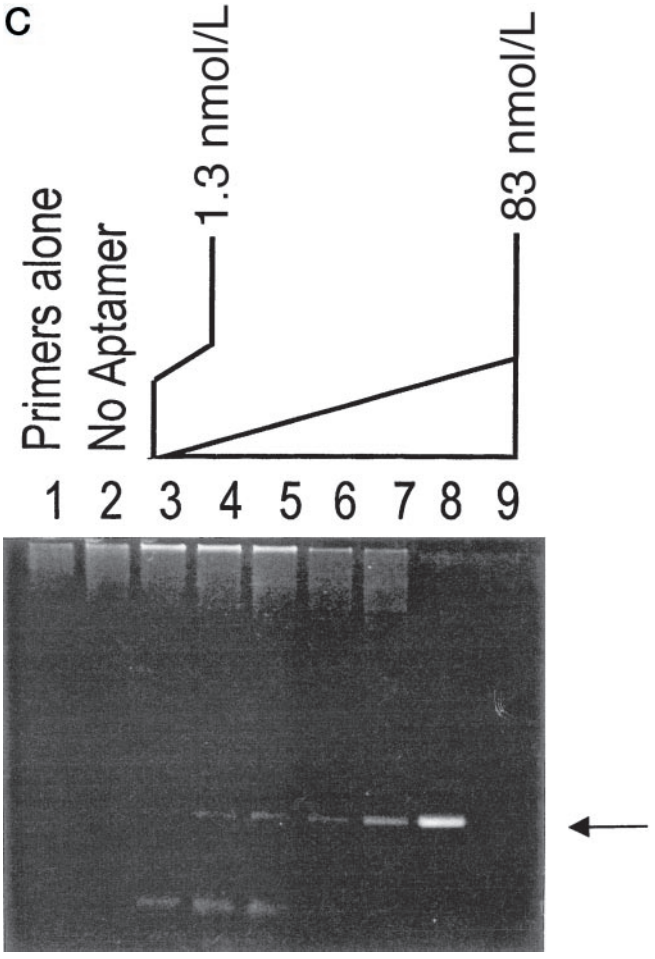




Fig. 10. Schematics of the reversible nature of aptamer denaturation (*A*) and a temperature-sensitive aptamer for in situ hot start PCR (*B*), and an example of improved amplification under in situ hot start conditions (*C*).

(*A*), reversible transformation could take place in response to several different environmental factors, including temperature. This feature allows aptamers to be used as sensor molecules that respond to various external stimuli. (*B*), a temperature-sensitive aptamer that has been developed to improve the performance, specificity, and sensitivity of PCR-based diagnostic assays by generating in situ hot start conditions. An aptamer that has been isolated to bind *Taq* DNA polymerase with high affinity and specificity only at low temperature (<40 °C) is added to the PCR, along with the other ingredients. At low temperature, the aptamer binds to *Taq* DNA polymerase with high affinity and inhibits the polymerase activity, preventing the extension of primers annealed nonspecifically at low stringent conditions. When the temperature is increased to initiate thermocycling, the aptamer denatures and dissociates from the polymerase, leaving the active polymerase to carry out PCR. (*C*), example depicting the improvement of low copy number PCR target amplification under in situ hot start conditions generated by a single-stranded DNA aptamer that inhibits *Tth* DNA polymerase in a temperature-dependent manner. A PCR assay designed to amplify a 104-bp amplicon in the *K-ras* gene from human genomic DNA *(181)* was used. The reaction contained 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 2.5 mmol/L $MgCl_2$, 1 $\mu$mol/L each primer, 200 $\mu$mol/L each dNTP, and 2.5 U of *Tth* DNA polymerase in a 30-$\mu$L volume. The reaction in *lane 1* did not contain the template, whereas those in *lanes 2–9* contained 25 ng of human placental DNA as the template. The aptamer concentration was doubled from one reaction to the next, starting at 1.3 nmol/L in *lane 3* and ending at 83 nmol/L in *lane 9*. The *arrow* indicates the mobility of the specific amplicon. Specific amplification of the intended amplicon requires an appropriate concentration of the aptamer. A high concentration of the aptamer completely inhibited PCR (*lane 9*), whereas the low concentrations generated nonspecifically amplified products that appear as a smear in the gel (*lanes 3–7*). All PCRs were performed in a GeneAmp PCR System 9600 (Perkin-Elmer) under the following thermocycling conditions: initial denaturation at 94 °C for 2 min, followed by 34 cycles at 94 °C for 30 s, 55 °C for 30 s, and 75 °C for 45 s. Amplified products were resolved on 3% agarose (NuSieve GTG; FMC BioProducts) gels in Tris-borate-EDTA buffer and visualized under ultraviolet light after staining with ethidium bromide.

merase at temperatures below 40 °C and were effective in generating in situ hot start conditions in PCR. As expected, the polymerase inhibition mediated by an aptamer was reversible. Moreover, the aptamers selected on *Taq* polymerase inhibited polymerases isolated from other species of the *Thermus* genus but not polymerases isolated from other thermostable microbes. As a result, these aptamers inhibit *Tth* DNA polymerase, which has the ability to reverse transcribe RNA targets and, therefore, are expected to be a valuable reagent in diagnostic PCR assays aimed at amplifying viral targets.

*Taq* DNA polymerase, the Stoffel fragment derived from *Taq* polymerase, and *Tth* polymerase are three enzymes useful for different PCR applications. *Tth* polymerase is used to amplify RNA targets *(164)*. Taq polymerase is the most popular enzyme for PCR. The Stoffel fragment of *Taq* is more thermostable than the parent enzyme and exhibits less processivity with improved discrimination against mismatch extensions. These qualities attract the Stoffel fragment for specific applications, such as multiplex PCR, arbitrarily primed PCR, and rare sequence-specific PCR *(165, 166)*. Two truncated aptam-

ers derived from two distinct sequence families identified in the affinity-enriched library for *Taq* polymerase demonstrated different specificities in inhibiting the above three thermostable DNA polymerases. A heterodimer of the two truncated aptamers synthesized as a single DNA strand effectively inhibited all three DNA polymerases *(153)*. This result shows that, like bispecific antibodies *(167)*, two aptamers with different functions could be combined into a single bispecific aptamer. The heterodimer, or the bispecific aptamer, served as a single reagent that generated in situ hot start conditions with all three DNA polymerases. Aptamers that can be switched "off" at temperatures >40 °C have been isolated by using high temperature selection conditions (Lin and Jayasena, manuscript in preparation), demonstrating the versatility of in vitro-derived oligonucleotide ligands. The above are examples of aptamers that function as thermal sensors or temperature-sensitive molecular switches.

Chemical modifications present in a starting library would be useful in isolating aptamers that respond to different environmental stimuli. For example, inclusion of ionizable groups in a library may provide aptamers that are sensitive to salt, and the presence of metal chelating groups may lead to aptamers that are sensitive to specific metal ions. This hypothesis is supported by the binding characteristics of aptamers isolated from two different oligonucleotide libraries to recognize the same target protein, HNE. The two selections had been carried out in the same buffer, using the same partitioning method but using either an RNA library consisting of 2′-$NH_2$-modified pyrimidines *(37)* or a single-stranded DNA library *(23)*. The binding of the RNA aptamer containing 2′-$NH_2$-pyrimidines to HNE is very sensitive to the pH of the binding buffer (Fig. 11), especially near the p$K_a$ of the 2′-$NH_2$ group, which has been reported to be 6.2 *(168)*. Such a drastic pH-dependent change in affinity was not observed with the DNA aptamer. These results indicate that the aptamer containing ionizable 2′-$NH_2$ functional groups responds to protons in the medium and exhibits pH-dependent interaction with the target. Consequently, such aptamers could be used as molecular switches sensitive to pH.

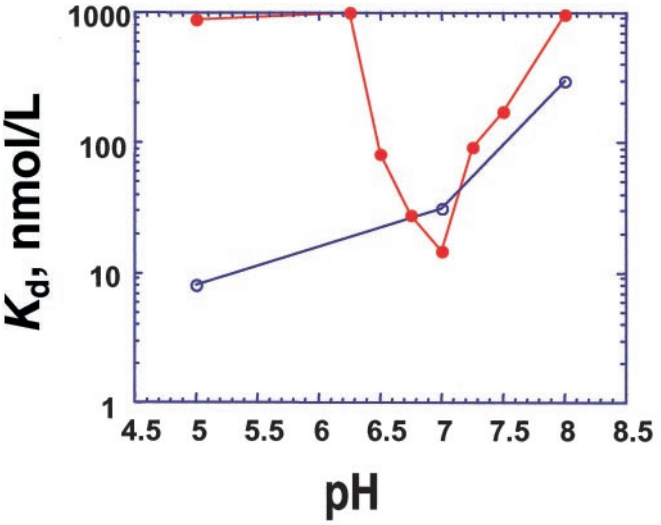



Fig. 11. Characteristics of a pH-sensitive aptamer.

The binding of two types of aptamers, a single-stranded DNA aptamer (○) and an RNA aptamer consisting of 2′-$NH_2$ pyrimidines (●), to HNE as a function of pH was studied using the nitrocellulose filter binding technique. Although these two aptamers were selected under identical conditions to HNE *(23, 37)*, the binding of the RNA aptamer containing 2′-$NH_2$ pyrimidines to the target protein is extremely sensitive to pH.

### DETECTION OF PROTEINS IMMOBILIZED ON MEMBRANES

One of the simplest formats of target detection involves immobilization of a target on a membrane utilizing hydrophobic (or hydrophilic) interactions. Once immobilized on a solid matrix, the target is detected by a specific ligand conjugated to a suitable reporter. Stringent washing conditions are required to eliminate the background originating from nonspecific binding of the ligand. In Southern and Northern blotting applications, oligonucleotide probes labeled with a reporter are commonly used to detect DNA or RNA targets, respectively, immobilized on a membrane. However, in Western blotting, proteins immobilized on a membrane are detected by antibodies bearing reporter molecules. The presence of aptamers that recognize proteins with high affinity and specificity allows the detection of proteins immobilized on a membrane by an oligonucleotide and can be referred to as "Eastern blotting", thus completing the nomenclature of blotting techniques.

Several examples exist for this Eastern blotting approach. In one example, an aptamer isolated to bind human thyroid-stimulating hormone (hTSH) was used to detect the hormone immobilized on a nitrocellulose filter membrane *(169)*, akin to the dot-blot technique. hTSH belongs to a closely related family of glycohormones whose other members include human follicle-stimulating hormone, human luteinizing hormone (hLH) and human chorionic gonadotropin (hCG). All four hormones share a high degree of structural similarity. Each member hormone is made up of noncovalently associated $\alpha$ and $\beta$ subunits. The $\alpha$ subunit is identical in all four hormones, and there is considerable structural similarity among the $\beta$ subunits of the four hormones as well. The structural similarities among members of this glycohormone family have posed a challenge in developing immunological assays that are specific to each member hormone *(126)*. Interestingly, aptamers that interact with hTSH with high affinity ($K_d$ = 3 nmol/L) and discriminate the other members, exhibiting high specificity, have been isolated from a modified RNA library containing 2′-$NH_2$-pyrimidines. This observed specificity of an anti-hTSH aptamer is quite impressive because it was isolated without a counter-SELEX step *(169)*. Various amounts of hTSH immobilized on a nitrocellulose membrane in a dot-blot format were detected with a radiolabeled aptamer in a dose-dependent manner.

Another SELEX experiment using hCG as the target led

to the isolation of aptamers that recognize the target hormone with high affinity [Lin et al., manuscript in preparation, and Ref. *(170)*]. One of the aptamers that binds hCG with a $K_d$ of 4 nmol/L discriminates its binding to hTSH and human follicle-stimulating hormone by more than 400-fold. However, the aptamer bound hLH with almost equal affinity, with a twofold higher $K_d$. This presumably is attributable to the high degree of structural similarity between the two hormones. hCG and hLH not only share identical $\alpha$ subunits but also have 85% sequence similarity within the first 114 amino acids in the two $\beta$ chains *(171)*. hCG has an additional 24 residues at the C terminus of the $\beta$ chain that hLH lacks, and this feature has been exploited to generate monoclonal antibodies that do not cross-react with hLH. Such antibodies directed to the $\beta$ subunit of hCG have been generated using purified $\beta$ subunit as the immunogen *(172)*. Some of the highly specific antibodies have been directed to the unique carboxyl tail of hCG *(173)*. However, the aptamers have been isolated by using the intact hCG but without using a counter-SELEX strategy to specifically remove sequences that interacted with hLH. It is reasonable to expect to identify aptamers with extreme specificity to hCG, using the counter-SELEX strategy. The radiolabeled aptamer detected hCG immobilized on a nitrocellulose membrane in a concentration-dependent manner [Lin et al., manuscript in preparation, and Ref. *(170)*]. The aptamer gave an intense signal for urine samples from pregnant women, which are known to contain hCG. This was in contrast to the signal obtained with urine samples from nonpregnant females.

In Western blotting applications, proteins resolved on polyacrylamide gels are transferred to a membrane and detected with a labeled antibody. Drolet et al. *(107)* demonstrated an elegant example of the use of an aptamer to specifically detect an isoform of an angiogenic factor, VEGF. Four isoforms of VEGF, expressed as a result of alternative splicing of mRNA, have been described *(174)*. An enzyme-linked oligonucleotide assay described by Drolet et al. utilized a monoclonal antibody to VEGF as the capture and an RNA aptamer as the detector *(107)*. The assay was intended to detect the predominant isoform of VEGF ($VEGF_{165}$), but it showed some degree of interference from $VEGF_{121}$. Protein blots prepared from the two isoforms resolved on sodium dodecyl sulfate-polyacrylamide electrophoresis gels were probed with the monoclonal antibody and the aptamer. The results indicated that the aptamer stained the $VEGF_{165}$ form but not $VEGF_{121}$. The antibody, on the other hand, stained both isoforms, which explained the origin of the interference observed in the assay.

### APTAMER ARRAYS TO UNDERSTAND PROTEOMICS

As the human genome project advances at a rapid pace, more and more genes will be discovered, gene banks will be expanded, and a wealth of information at the DNA level will be available. The real value of this wealth of information at the genetic level lies in its ability to predict the outcome, or the phenotype, with accuracy. It is becoming clear, however, that the function of gene products is difficult to assess at the DNA level or even at the RNA level. This is simply because of the regulation during translation, which leads to differential protein expression. Moreover, posttranslational modifications that determine functional forms of translated proteins further complicate the picture. Because of this discrepancy between the genotype (at DNA and RNA level) and the phenotype (at protein level), it would be meaningful to study proteins to better understand the true cellular function of the genome. This would be invaluable for early detection of diseases and for monitoring the progression of existing diseases and responses to therapeutic agents.

Although it is easy to rationalize and comprehend why proteins must be studied, proteomics represents a daunting task waiting to be undertaken. At the very base of the proteomics pyramid lies the tools that are required to analyze almost all of the proteins expressed in a tissue or, for diagnostics, the majority of proteins secreted into a biological fluid. Microarray technology provides a means of analyzing a large number of molecules simultaneously. For example, the entire yeast genome has been analyzed on a single array confined to a microscope slide *(175)*. Such analyses carried out at the DNA level are facilitated by the high affinity and specificity of interactions driven by complementary base pairing. For proteomics, ligands that could capture proteins with high affinity and specificity provide the basis for an array technology aimed at protein capture and detection. Although antibody-based microarrays are being developed for the analysis of a large number of proteins *(176)*, microarrays based on high-affinity aptamers would be very attractive for the following reasons:

- Aptamer identification is a rapid process that can be performed on an automated platform.
- Aptamers can be immobilized at a defined density at precise locations on a solid surface with existing technologies used to generate DNA microarrays.
- Homogeneous preparations of aptamers are readily available from chemical synthesis.
- Microarrays based on aptamers are robust and are expected to have a long shelf life.
- Aptamers containing 5-halo-uracil analogs could be used to lock the bound proteins irreversibly.
- The formation of irreversible cross-linking between aptamers and proteins is highly specific and provides another dimension of specificity in addition to the specificity provided by affinity. This dual specificity provided by cross-linkable aptamers eliminates the need for secondary ligands specific for each protein for detection. Thus, it is conceivable that proteins captured on a cross-linkable aptamer microarray could be de-

tected by a reagent that is specific to proteins and does not react with nucleic acid.

In the near future, aptamer microarrays are expected to play a dominant role in the arena of proteomics that not only will facilitate better disease management by analyzing the expression of proteins by patients but will also help discover new therapeutics by target validation.

## Conclusion

Over the past three decades, antibodies have been the reagent of choice for the development of diagnostic assays. Consequently, diagnostic platforms that are commonly used today were evolved to better suit antibodies. The discovery of aptamers whose affinity and specificity parallel those of antibodies is expected to have a future impact on diagnostics. As discussed here, aptamers that have been tested in different diagnostic platforms show very encouraging results. Aptamers have an unleashed potential to circumvent limitations associated with antibodies and are waiting to be utilized in practical settings where their performance could be compared directly to that of antibodies. Aptamer research is in its pre-puberty age and will require time before examples of aptamer products are available. The aptamer research already moving in a fast pace is expected to get even better with a completely automated SELEX process that allows high-throughput aptamer identification. It is important to point out that aptamers may not offer solutions for all molecular recognition needs. The value of aptamers in most cases will be in applications or formats where the performance of antibodies is inadequate rather than in replacement of assays that work well with antibodies. The ability of aptamers to function well in combination with antibodies in diagnostic formats will have considerable impact in the designing of assays that have not been feasible before. Covalent capture of targets is unheard of in antibody research but feasible with aptamers. It is reasonable to expect that microarrays generated by aptamers with the ability to covalently capture proteins will make inroads to proteomics in the near future.

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