Aptamers as therapeutic and diagnostic reagents: problems and prospects

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Aptamers are nucleic acid molecules that bind specific ligands. Barriers to the application of aptamers as therapeutic and diagnostic reagents have been overcome in the past several years. In particular, aptamers that bind biomedically relevant targets have proven to be efficacious at modifying cellular metabolism. Such aptamers can be stabilized by chemical modifications and potentially used *in vivo*. Researchers have begun to devise aptamer-based diagnostic assays that may rival more conventional immunoassays.

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Abbreviations

AMV	avian myeloblastosis virus
AZT	3′-azido-3′deoxythymidine
bFGF	basic fibroblast growth factor
ED	effective dose
FIV	feline immunodeficiency virus
HNE	human neutrophil elastase
IC	inhibitory concentration
IL	interleukin
KDF	keratinocyte growth factor
PKC	protein kinase C
RT	reverse transcriptase
SELEX	systematic evolution of ligands by exponential enrichment
VEGF	vascular endothelial growth factor

Introduction

Even though the *in vitro* selection (also known as SELEX) of nucleic acid binding species (aptamers) was only popularized in 1990 [1,2], this technology is already being used for the development of pharmaceutical reagents and diagnostic assays. Originally, SELEX involved the selection of RNA or DNA molecules from random sequence pools that could bind to small or large ligands. Numerous modifications of this technology have greatly expanded its potential [3••,4,5•]. Partially randomized pools have been used as starting points for defining functional sequences and structures in natural nucleic acids [6]. Modified nucleotides have been introduced into selection experiments, resulting in the isolation of aptamers that are surprisingly stable *in vivo*. The increased stability provides the potential for the five natural (and chemically mundane) nucleotide monomers to be outfitted with new structures and functions [7,8]. Enzyme substrates have been appended to random sequence pools, and chimeric molecules have been selected in which substrate binding is augmented by enzyme : aptamer interactions [9••]. Some appended ligands have been designed to participate in reactions of their own, such as photochemical cross-linking, blurring distinctions between selections for nucleic acid binding activity and selections for nucleic acid catalytic activity [10••].

The chief advantages of SELEX for biotechnology applications are its technical versatility and applicability to a wide array of target molecules, while the chief stumbling block is the widely held bias that nucleic acids are too large, too expensive, and in the end too synthetically cumbersome to serve as drugs or assay components. Nonetheless, recent results serve to validate the speculations of Tuerk and Gold [1] that "SELEX could...provide unpredictable and unimaginable molecular configurations of nucleic acids...with any number of targeted functions." and the visionary claim of Leslie Orgel [11] that "selective experiments with ethidium bromide...strongly suggest that specific positive interactions could evolve relatively quickly....It should be trivial to select for molecules that stick (or fail to stick) to a chosen surface." This review focuses on the solutions to problems that must be overcome before aptamers are utilized as therapeutic or diagnostic reagents.

Drug development

Advantages of aptamers as drugs

The therapeutic potential of aptamers hinges on many of the same issues that apply to more conventional pharmaceuticals. First, aptamers must interact tightly and specifically with their targets. The large size and surface area of nucleic acids is a decided advantage, in that they can potentially form many more interactions with targets than can smaller molecules. Some of the best aptamers are reported to form complexes that have dissociation constants in the 100 pM range [12–14,15••], while many have dissociation constants that are similar to the antigen-binding fragments (Fabs) of antibodies [5•]. Similarly, the large size of aptamers gives them multiple opportunities to discriminate between epitopes on related proteins, and aptamers have been shown to distinguish between even closely related targets, such as protein kinase C (PKC) isozymes that are 96% identical [16]. Second, aptamers must specifically disrupt the function of their targets. Anti-HIV reverse transcriptase (HIV-RT) aptamers selected from a single-stranded DNA pool bound the enzyme with K_i values as low as 1 nm [17•]. Both RNA and modified RNA aptamers selected to bind basic fibroblast growth factor (bFGF) have been shown to successfully inhibit bFGF binding to cell surface receptors at concentrations as low as 1 nM [12,14].

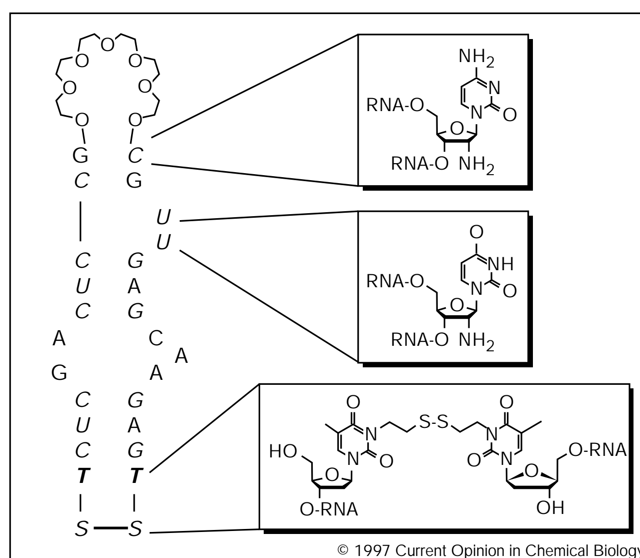
Similarly, anti-vascular endothelial growth factor (VEGF) aptamers selected from modified RNA pools inhibited receptor binding with an ED_{50} of 20–40 nM [13]. Finally, aptamers must be able to not only disrupt the function of a particular target, but also inhibit or modify the metabolism associated with that target. For example, anti-thrombin aptamers have been shown to block blood clotting [18,19]. Anti-human neutrophil elastase (HNE) aptamers have been shown to inhibit interleukin-1 (IL-1) induced, neutrophil-mediated damage in rat lung [9••]. Aptamers selected to bind to whole Rous sarcoma virus can inhibit infection when pre-incubated with the virus [20•]. Aptamers selected to bind HIV-1 Rev can functionally substitute for the wild-type *Rev-binding element* [21•] and can block viral replication when expressed in cells [22]. Anti-IgE aptamers can inhibit IgE-mediated serotonin release from cells in tissue-culture [23]. Modified RNA aptamers specific for keratinocyte growth factor (KDF) can inhibit the mitogenic activity of that ligand in cell culture with IC_{50} values as low as 92 pM [24]. Taken together, these examples emphasize the features of aptamers that warrant their consideration as drugs.

Potential disadvantages of aptamers as drugs

The question thus becomes whether these advantages are unique enough to prompt researchers to overcome some daunting disadvantages. Some of the most obvious problems have already begun to be solved. For example, while natural RNA and DNA molecules are relatively unstable in sera or within cells, chemically modified nucleic acids have been shown to be nuclease-resistant 'rocks'. Pyrimidines containing 2′-amino moieties are recognized by T7 RNA polymerase and AMV RT, and can be incorporated into the selection process. The stability in serum of the modified anti-bFGF aptamer was increased at least 1000–fold relative to unsubstituted RNAs [14], while the modified anti-VEGF aptamer could survive for up to 17 hours in urine [15••]. The development of stable nucleic acid aptamers now allows researchers to proceed to the more difficult problems of delivery and bioavailability. It is unlikely that aptamers will be orally available, and thus will have to be injected either naked, conjugated to biopolymer carriers such as polyethylene glycol, or packaged within liposomes.

Similarly, researchers have begun exploring the pharmacokinetic properties of aptamers, but these are still largely unknown. Even if the aptamers can be made bioavailable, their size — and hence cost of production — remains problematic. Fortunately, many aptamers can form tight and specific interactions with their targets via domains of only 30 to 40 nucleotides. While these 10 000 to 15 000 Dalton molecules are still orders of magnitude larger than a conventional drug (≈200 to 800 Daltons), advances in synthetic methods may soon allow their economical synthesis. Finally, while the specificity of aptamers for their targets may ward off the systemic side effects often associated with pharmaceuticals, this same specificity may encourage the evolution of metabolic or viral resistance. A theoretical analysis by Eaton and co-workers [25] has suggested that high-affinity binding species fit so snugly to binding sites on their protein targets that they would be perturbed by even minor changes in amino acid sequence or conformation. At least some data suggests that this conclusion may be flawed. Gold and co-workers [26•] have subsequently shown that aptamers selected to bind the RT from feline immunodeficiency virus (FIV), a virus similar to HIV-1, can act as inhibitors of the polymerase activity from both wild-type FIV and an AZT-resistant mutant of FIV.

Figure 1



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Designing a chemically modified, minimal anti-HIV-1 aptamer based on the results of Nelson *et al.* [34•]. One end of the aptamer has been replaced with a (hexa)ethylene glycol loop (top) [29] while the other end is stabilized by a disulfide cross-link (bottom) [27•]. Internally, pyrimidine residues have been replaced with 2′-amino ribonucleotides (italicized letters). Such modifications have been shown to make aptamers more nuclease resistant, and to increase their conformational stability.

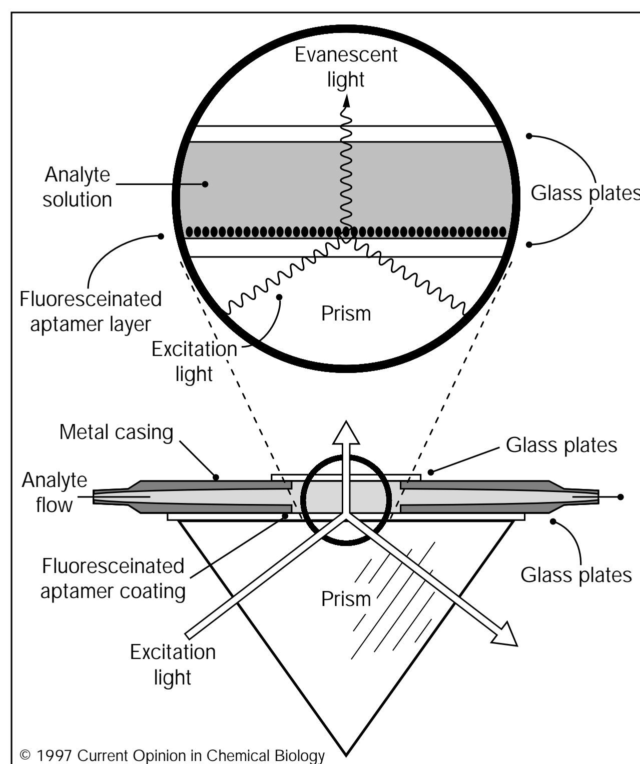
Potential solutions and applications

In fact, many of these potential problems serve to highlight unique advantages of aptamers relative to conventional drugs or even other biopolymers. The fact that nucleic acid shapes are largely determined by relatively simple secondary structural motifs implies that aptamers can be readily engineered (Fig. 1). For example, Glick and co-workers [27•] have shown that disulfide cross-links can be introduced into nucleic acid secondary structures and thus provide a large increase in thermal stability without compromising structural integrity. Structure-forming helices in aptamers have now been successfully replaced with disulfides and other compact chemical struts [28,29]. Similarly, aptamers have been stabilized against exonuclease degradation by simply adding nucleotides bridged by phosphorothioate linkages

to their 5′ and 3′-termini [15••]. The selection process itself can potentially be geared to anticipate and overcome problems associated with delivery and bioavailability. Aptamers selected for their ability to bind to complex targets, such as cell lines or organs, could potentially be used for tissue-specific delivery. Aptamers selected to interact with coated pits or for the ability to localize to specific organelles might provide another layer of sophistication for drug targeting.

Even if nucleic acids selected from combinatorial biopolymer libraries cannot compete with the numerous small organic leads flowing from combinatorial chemical libraries, *in vitro* selection can further the development of drug leads in several ways. The selection process can help identify targets for drugs, either by better defining natural nucleic acid targets or by validating protein targets. For example, aptamers can help locate effector sites on protein targets that are essential for viral, cellular, or organismal function. The aptamers can then be introduced into complex assay systems to determine what metabolic effects a drug with similar binding properties might have; the aptamers could in effect serve as functional mimics of drugs that have yet to be developed. Alternatively, the selection process can be used as an assay to define and refine the selectivities of anti-nucleic acid drugs, including those derived from combinatorial chemical libraries.

Figure 2



An aptamer-based evanescent wave fluorescence detector. A low intensity beam of coherent light reflects off a glass plate coated with a layer of fluoresceinated aptamer. If target molecules in the analyte solution bind to the aptamer, the conjugated fluoresceins emit light that can be detected by an attached photomultiplier tube.

Diagnostic assays and research reagents

While the prospects for aptamer therapeutics remain uncertain, it is likely that selected RNA and DNA molecules will find use in diagnostic assays. Like antibodies, aptamers that react with a variety of targets can be selected. Like antibodies, aptamers have high affinities and specificities for these targets. Aptamers are smaller and less complex than antibodies, however, and consequently may be easier to manufacture and modify. Thus, it is not unreasonable to speculate that the large markets associated with immunodiagnostics will see an increasing challenge from kits based on nucleic acid shape recognition.

Current methods for detecting nucleic acid : protein complexes generally require the partitioning of complexes from their component macromolecules. For instance, radiolabeled aptamers bound to a protein target can be separated from unbound aptamers with nitrocellulose filters, which generally bind protein but not RNA or DNA. Anti-PKC aptamers have been shown to function quantitatively and specifically in such an assay system [30]. In a clinical setting, however, it would be preferable to avoid radioactive labels and extensive enzymatic manipulations. One way to simplify the detection of aptamer : protein complexes is to link the aptamer to an enzyme that has an activity that is easily assayed. Drolet *et al.* [31] synthesized an aptamer specific for VEGF with a fluorescein group attached via a phosphodiester bond at the 5′ end. They coated microtiter plates with a monoclonal antibody specific for VEGF, then reacted samples containing known amounts of VEGF to the bound antibodies. The fluoresceinated aptamers were bound to the VEGF, and these were in turn bound to anti-fluorescein antibodies conjugated to alkaline phosphatase. The relative quantities of VEGF in each well were measured by the addition of a chemiluminescent alkaline phosphatase substrate. This multistep procedure is relatively easy to perform because each step requires only the addition and removal of a premade reagent.

Aptamers may also be useful in more direct methods that do not require immobilization. For example, fluoresceinated aptamers can be detected in a flow cytometer. Davis *et al.* [32•] modified a high affinity anti-HNE aptamer (Lin *et al.* [33]) by attaching fluorescein groups to different residues via a variety of linkers. The fluoresceinated aptamers were incubated with polystyrene beads coated with HNE and the resulting complexes were passed through a flow cytometer; some of the modified aptamers proved to be more sensitive for detection than fluoresceinated antibodies. This approach could be applied to detect cells that express particular proteins on their surfaces; for example, aptamers specific for tumor antigens might be used to detect the growth or spread of tumors.

These experiments may herald the use of aptamers in more novel assay configurations. If aptamers containing fluorescent labels undergo conformational changes on ligand binding, they might be able to directly detect analytes in solution. The recent determination of a number of aptamer structures may provide insights into where fluorescent labels should be placed to generate ligand-dependent signals. If not, fluorescent labels could be included in the selection reactions themselves and aptamers that yielded ligand-dependent changes in fluorescence could be identified by screening. These demonstrations should pave the way to sensitive but specific solution-phase diagnostic assays that would require very little sample preparation. For example, we are currently using fluoresceinated aptamers immobilized on glass surfaces to generate ligand-dependent signals that can be detected by evanescent wave fluorimetry (Fig. 2).

There are several reasons why aptamers may see increasing use as diagnostic and research reagents. Aptamers can be raised against toxic, small or otherwise poorly immunogenic antigens. They may circumvent problems with biopolymer denaturation during storage, and are ultimately cheaper than antibodies. The passage of aptamers from experimental novelties to research reagents should be accelerated by the identification of large numbers of aptamers recognizing large numbers of targets, and this radiation of aptamers may turn on the automation of aptamer selections. Just as monoclonal antibody facilities exist in many major corporate and academic research settings, it is possible that aptamer facilities will now begin to be set up. These facilities may reasonably be expected to include a robotic workstation that starts with purified targets or even target mixtures, and returns populations of binding species that can be quickly characterized by automated sequence acquisition and analysis. The selected aptamers can then be used to quantitate, localize, or inhibit proteins, even proteins whose function is unknown. As research reagents, aptamers may eventually contribute to nascent efforts in functional genomics, providing ready-made inhibitors of the multitude of new genes identified by genome projects.

Conclusions

The high affinities and specificities of aptamers for their targets originally suggested that aptamers might be good drug leads. Proofs that aptamers can specifically inhibit biomedically relevant proteins and modify cellular metabolism augur well for future drug development. Recent advances in the chemical modification of nucleic acids suggest that one of the major barriers to use, stability, can be overcome. If, however, aptamers are to be used as pharmaceuticals, methods of mass producing and delivering modified oligonucleotides will have to be developed; these methods will in turn be dependent on better defining the pharmacokinetic properties of oligonucleotides. The introduction of aptamer-based diagnostic reagents is more likely in the near future; this would require the development of simple methods for the detection of aptamer : target complexes.

Acknowledgements

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