Applications of Aptamers as Sensors

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Abstract

Aptamers are ligand-binding nucleic acids whose affinities and selectivities can rival those of antibodies. They have been adapted to analytical applications not only as alternatives to antibodies, but as unique reagents in their own right. In particular, aptamers can be readily site-specifically modified during chemical or enzymatic synthesis to incorporate particular reporters, linkers, or other moieties. Also, aptamer secondary structures can be engineered to undergo analyte-dependent conformational changes, which, in concert with the ability to specifically place chemical agents, opens up a wealth of possible signal transduction schemas, irrespective of whether the detection modality is optical, electrochemical, or mass based. Finally, because aptamers are nucleic acids, they are readily adapted to sequence- (and hence signal-) amplification methods. However, application of aptamers without a basic knowledge of their biochemistry or technical requirements can cause serious analytical difficulties.

1. APTAMER SELECTION AND PROPERTIES

Aptamers are single-stranded nucleic acids (RNA, DNA, and modified RNA or DNA) possessing unique binding characteristics to their targets, unlike traditional nucleic acids. They are isolated from 10^{12}–10^{15} combinatorial oligonucleotide (DNA or RNA) libraries chemically synthesized by a process known as in vitro selection (1, 2). Over multiple rounds of selection (generally 6–18 rounds), quite large populations ($>10^{13}$ different sequences) can be sieved, and the few "fittest" nucleic acid species can be isolated. Although the traditional in vitro selection procedure usually requires several weeks to months, recent automation of the methods may provide a route from target to novel reagent within only a few days (3).

Numerous high-affinity and highly specific aptamers have been selected against a wide variety of target molecules including small organics, peptides, proteins, and even supramolecular complexes such as viruses or cells (4, 5). The binding affinities of aptamers are highly target dependent and range from the picomolar scale (1×10^{-12} M) to the high-nanomolar scale (1×10^{-7} M) for various protein targets. When small organics are targeted, the dissociation constants are higher (typically micromolar), as might be expected given the smaller number of interactions that form. In either case, interactions tend to be extremely specific, and aptamers can discriminate between even closely related compounds such as (*a*) the antitheophylline aptamer, which in turn can discriminate against caffeine on the basis of the presence of a single methyl group (6), or (*b*) antikinase aptamers that can distinguish between closely related isozymes (7). Beyond these features, aptamers have shown extraordinary promise in analytical applications because they can be readily produced by chemical synthesis and can thereby be easily modified with reporter molecules, linkers, and other functional groups (8). Aptamers can also be joined to nucleic acid enzymes (e.g., ribozymes and deoxyribozymes) to create allosteric enzymes or so-called aptazymes (9). Because aptazyme applications have recently been reviewed (10, 11), we only briefly describe some of their innovative analytical applications herein.

2. OPTICAL SENSORS

2.1. Incorporation of Single Reporters

Structural studies have shown that aptamers frequently undergo significant conformational changes upon binding to their cognate ligands (12, 13). By introducing organic fluorophores into conformationally labile regions of aptamers, one can transduce ligand binding into a change in the chemical environment of the fluorophore and hence to a change in fluorescence characteristics such as intensity and anisotropy. For example, by labeling antiadenosine RNA and DNA aptamers with a fluorophore adjacent to the adenosine-binding site during synthesis, so-called signaling aptamers can be created (14). The apparent K_d based on the dose-responsive fluorescence intensity change as a function of ATP concentration (~30 μM for the DNA-signaling aptamer and ~300 μM for the RNA-signaling aptamer) was much higher than that of the parental aptamers (~6 μM for the antiadenosine DNA aptamer and 6–8 μM for the antiadenosine RNA aptamer) (15, 16). A loss in binding affinity can be considered as a general theme of reagents (aptamers or otherwise) that rely upon conformational transduction for signaling.

There are numerous ways to introduce labels into aptamers, and the nature of the label and the site of introduction strongly influence the mechanism of signal transduction. In one study, for example, a BODIPY (boron-dipyrromethene) fluorophore was linked to the 2′ hydroxyl of ribose to convert three different aptamers (that bound AMP, tyrosinamide, and argininamide) into signaling aptamers (17). The sensitivity of the BODIPY fluorophore to its local environment resulted in a significant change in its fluorescence quantum yield (up to a 3.7-fold increase in

fluorescence was observed for the argininamide aptamer). Similarly, when a ATP-binding aptamer was internally modified with a bis-pyrene fluorophore whose excimer and monomer states were known to be highly sensitive to local structural changes (18), the modified aptamer exhibited a 2–3-fold fluorescence change upon binding ATP. Ribose labeling may probe structures in a less intrusive way than does labeling the backbone or nucleobases, and Kamekawa et al. (19) found that pyrene conjugation to a ribose adjacent to the ligand-binding pocket of the anti-ATP DNA aptamer allowed substantial retention of binding affinity compared with similarly labeled nucleobases (14, 20), which lowered the apparent K_d by 10-fold.

In addition to internal or external labeling with fluorescent dyes, replacing nonfluorescent nucleotides with fluorescent nucleotide analogs can be exploited for signal transduction. The quantum yield ($0.39 \sim 0.88$) of nucleotide analogs, such as 2AP (adenine analog, 2-aminopurine), 6MAP (adenine analog, 4-amino-6-methylpteridone), and 3MI (guanosine analog, 3-methylisoxanthopterin), is highly dependent upon the analogs' local environments, in particular the extent of base-stacking (21). This has made such modified nucleotides extremely useful for the study of nucleic acid structure and dynamics (22). In general, the fluorescence of these nucleoside analogs is quenched when they are base-paired in a stack. If interactions with an analyte induce the formation of a single-stranded region or otherwise disrupt base-stacking, a strong increase in fluorescence intensity can result. The sequences of several DNA aptamers [namely antithrombin, anti-IgE (immunoglobin E), and anti-PDGF (platelet-derived growth factor) aptamers] have been modified with 2AP, 6MAP, or 3MI (23). The positions for these modifications were chosen by modeling the known aptamer tertiary structure (for the antithrombin aptamer) or by simply screening a set of modified aptamers (for the anti-IgE and anti-PDGF aptamers) for which less tertiary structural information was available. The resulting signaling aptamers showed an analyte-specific increase in fluorescent signal of up to 30-fold. An advantage of using modified nucleotides is that because they are an integral part of the functional structure, little or no decrease in binding affinity is expected; also, they have high resistance to photobleaching.

A problem with these methods is that it may be difficult to identify where in an aptamer sequence or structure to put a label. However, in some selection experiments, labels were inserted into the original random sequence pool, and selection for binding (followed by screening for signaling) was carried out. An anti-ATP RNA aptamer bearing small numbers of fluorescein-UTP (uridine-5′-triphosphate) residues detected an amount of ATP as low as 25 μM, with an apparent K_d value of 175 ± 5 μM (24).

Other global conformational changes can also be monitored with fluorescence anisotropy. Proteins can be relatively large compared to aptamers; therefore, they can increase the overall molecular weight and size of the aptamer and can greatly alter the rotational diffusion rate and fluorescence anisotropy of fluorophores appended to aptamers. In an initial demonstration, the 5′ end of an antithrombin aptamer was labeled with FITC (fluorescein isothiocyanate), and the 3′ end was labeled with an alkyl amine that was used for surface immobilization (25). The immobilized aptamer showed a substantial change in molecular anisotropy in the presence of thrombin, detected as little as 5 nM of thrombin (in 140 pL of working volume), and had a dynamic detection range of three orders of magnitude. It also showed high selectivity for thrombin compared with elastase, another serine protease with an isoelectric point and a molecular weight similar to those of thrombin. Similar fluorescence anisotropy measurements were carried out using fluorescently labeled anti-PDGF aptamers (26). The twofold anisotropy change upon PDGF binding was completed within only a few seconds in homogeneous solution, in real time, with a limit of detection (LOD) of 0.22 nM. The sensitivity demonstrated by this method should be suitable for the detection of PGDF in serum samples ($0.4 \sim 0.7$ nM) and in biological fluids ($0.008 \sim 0.04$ nM) surrounding tumors. Fluorescence anisotropy is a relative (ratiometric) detection technique, and

thus the common problems associated with fluorescence intensity assays, such as bleaching and nonuniform emission of the fluorophore, are not major concerns.

2.2. Incorporation of Two Reporters

Strategies that rely on more than one reporter have also proven to be practical in that they allow one to attempt optical signal transduction based on fluorescence resonance energy transfer (FRET). As more and varied fluorophores and aptamer modifications have become commercially available, more and varied design strategies have been developed (**Figure 1**).

The simplest format is to label aptamers with a quencher and either one or two fluorophores (**Figure 1*a*,*b***), enabling both quenching and "light-up" strategies. An anticocaine DNA aptamer (27) and an anti-PDGF aptamer (28) were destabilized by truncating one of the stems of a three-way junction and labeling the termini with a 5′ fluorophore and a 3′ quencher (**Figure 1*a***). Ligand binding stabilized the engineered stem and brought the quencher and fluorophore in close proximity, resulting in a fluorescence decrease. However, a potential problem with quenching aptamer beacons is that there are a variety of ligands or solvents that may interfere with quenching, leading to a false positive signal. To obviate this problem, beacons can rely upon FRET to signal. For example, Tan's group (29) has noted that in the absence of the protein target, an equilibrium exists between the random coil state and the quadruplex states of an antithrombin aptamer. By incorporating two fluorophores, coumarin (donor) and fluorescein (acceptor), at the two termini of the aptamer, thrombin binding can be monitored. In this study, the K_d and limits of detection for

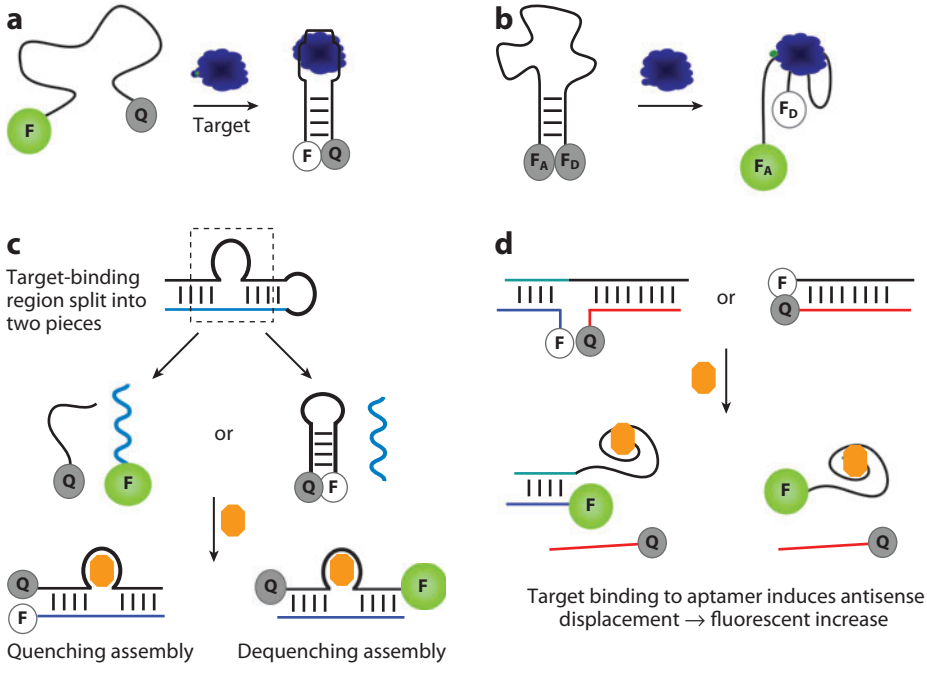

Figure 1

Schemas for optical sensors that incorporate two reporters. (*a*) Quenching aptamer beacon. (*b*) Fluorescence resonance energy transfer (FRET) aptamer beacon. (*c*) Assembly aptamer beacon. (*d*) Disassembly aptamer beacon. F, Q, F_A, and F_D stand for fluorophore, quencher, acceptor fluorophore, and donor fluorophore, respectively.

the two-fluorophore aptamer beacon were 4.87 ± 0.55 nM and 429 ± 63 pM, respectively. The FRET beacon gave a ~14-fold signal-enhancement factor (the ratio of acceptor to donor intensity before and after binding). FRET-type aptamer beacons may prove to be particularly useful for real-time analysis of proteins and may be used in living specimens in conjunction with ratiometric imaging. Such beacons have already been used for the detection of angiogenin in serum samples from healthy and lung cancer patients (30).

Aptamers can also be engineered to refold, rather than fold. In one experiment (31), sequence was added to an antithrombin DNA aptamer that fixed it in a molecular beacon–like hairpin structure. Upon the addition of thrombin, the conformational equilibrium was shifted from the quenched hairpin to the thrombin-bound quadruplex, resulting in dequenching of the fluorophore and the creation of an optical signal (**Figure 1*b***) (31).

The refolding process can be facilitated. Tang et al. (32) have developed a new light-up design similar to the in vivo antiswitch developed by Bayer & Smolke (33). The new aptamer beacon contained an antisense sequence attached to the 3′ end of the aptamer via 5~6 polyethylene glycol linkers. In the absence of target, the antisense sequence that terminated with a quencher preferentially hybridized with the aptamer, keeping fluorophore and quencher in close proximity. As the target bound to the aptamer, the conformational equilibrium shifted, displacing the quencher and turning on the fluorescence signal. The authors (32) demonstrated the feasibility of this technique with both anti-ATP and antithrombin aptamers. The anti-ATP aptamer beacon exhibited a 30-fold intensity enhancement immediately upon addition of 3.5 mM of ATP (>90% response within 5 s), but it did not respond to ATP analogs at 1 mM. The antithrombin aptamer beacon performed similarly, with a 17.6-fold signal enhancement in the presence of 300 nM thrombin, but it had no response to negative controls such as IgG, IgM, and bovine serum albumin (BSA) at 200 nM.

Quaternary structural rearrangements that result in the assembly or disassembly of aptamers can also be exploited for signaling. Aptamers can be split into two pieces, and fluorophores can then be added to each piece (**Figure 1*c***). In the absence of the target ligand, the two oligomers largely exist independently in solution, but target binding brings the oligomers together and leads to ternary complex stabilization, ultimately resulting in a quenched optical signal. Anticocaine and anti-ATP aptamers have been converted into aptamer beacons through this strategy (34). Within the concentration ranges of 10 μM to 1 mM ATP and 10 to 150 μM cocaine, it proved possible to simultaneously report the concentrations of the two analytes using the two aptamer beacons. In a similar set of experiments (35), an aptamer that binds to the Tat protein of human immunodeficiency virus (HIV) was converted into an aptamer beacon by causing one of the two half-aptamers to act as a molecular beacon. The Tat-detecting aptamer beacon had an extremely low K_d for Tat (~120 pM), and the engineered biosensor quantitated Tat samples as low as 100 nM (35). Of course, assembly strategies also extend to sandwich assay methods involving two aptamers that recognize two distinct epitopes of a protein (36). As a model system, two antithrombin aptamers [G15D and 60–18 (29)] were labeled with FRET fluorophore pairs, and cobinding events were monitored. Thrombin was detected with picomolar sensitivity in the presence of a complex biological background, and sample manipulation was minimal.

Disassembly strategies are also possible. Aptamer beacons have been generated in which a complementary, antisense DNA strand is used to intentionally denature the aptamer (**Figure 1*d***). Upon addition of ligand, the native aptamer structure is stabilized, and the equilibrium is concomitantly shifted away from the denatured, antisense duplex and toward the native structure. The aptamer can be labeled with a fluorophore and the antisense oligonucleotide with a quencher, leading to a quenched fluorescent signal in the absence of target, similar to the unimolecular aptamer beacons described above. Nutiu & Li (37) have pioneered this approach for aptamer beacon design by making antisense aptamer beacons that can detect ATP (K_d = 600 μM) and thrombin

(K_d = 400 nM), and such aptamer beacons have been further adapted to high-throughput drug screening (38). The antisense oligonucleotide can also contain fluorescent nucleobases, rather than dyes (39). Like many other aptamer beacon strategies, little or no foreknowledge of aptamer structure is required. In addition, because the aptamer and denaturing strand can be separately synthesized, a number of variables can be modularly optimized to improve performance.

These disassembly strategies are amenable to selection. The separable oligonucleotide can be immobilized on a column and used to trap a nucleic acid pool. In the presence of ligand, those aptamers that best transduce binding to conformational change should fall off the column, when they can then be collected, further amplified, and selected. This strategy has been used to select oligonucleotide-sensing beacons (40), ATP-sensing beacons (41), and zinc-sensing beacons (42). Although these selections yielded structure-switching aptamers, the sensitivities of these biosensors were for the most part no better than similarly designed aptamer beacons (14-fold for similarly designed aptamer beacons versus 4.5-fold for evolved beacons).

Covalent disassembly of fluorescently tagged oligomer can also be exploited. Aptamers can be adapted to serve as allosteric domains for nucleic acid enzymes, or analyte-dependent nucleic acid enzymes can be directly selected, as indicated above. An allosteric cleavage can therefore cut apart a fluorescently tagged oligomer on a hybridizing quencher–labeled oligonucleotide substrate. In recent work, a Pb^{2+}-activated deoxyribozyme was used as a model system for various optical sensing formats (43, 44). UO^{2+} was detected in <2 min at ambient temperature with 11 ppt (45 pM) sensitivity and >1 million–fold selectivity over other metal ions (45). Similar methods have been used to detect other analytes, such as small complementary oligonucleotides, organic molecules, and even proteins (46, 47).

2.3. Incorporation of Nanoparticle Reporters

The basic transduction strategies described above are generally applicable, and other reporters can be used to overcome problems typically associated with fluorescent dyes. In particular, quantum dots (QDs) and other nanoparticles offer a number of advantages over standard fluorescent dyes for monitoring biological systems in real time, including greater photostability, larger effective Stokes shifts, longer fluorescent lifetimes, and sharper emission bands than traditional organic fluorophores. In addition, QDs all respond to the same excitation wavelength but emit at different wavelengths, which assists with multiplexing. Aptamer:QD conjugates have been used for the detection of cancer cells (48), bacterial spores (*Bacillus thuringiensis*) (49), and proteins (thrombin, with a LOD of 1 nM) (50). QDs were first adapted to aptamer beacon strategies by Levy and coworkers (51), who coated the nanoparticle with multiple antithrombin aptamers hybridized to antisense strands bearing quenchers. In the presence of thrombin, the quenchers were displaced, and the QD beacon construct showed a 19-fold increase in fluorescence.

Gold nanoparticles (GNPs, or Au-NPs) have also proven to be interesting reagents because their absorption properties change depending upon the aggregation state. In particular, the color changes associated with aggregation (red) and disaggregation (purple) are easily visible to the naked eye without the need for sophisticated instruments. In addition, GNPs are more biocompatible, easy to bioconjugate, and less toxic than are QDs. Methods similar to those demonstrated with QDs have been developed. An aptamer beacon containing TAMRA (tetramethyl-6-carboxyrhodamine) was hybridized to an oligonucleotide on a GNP, effectively quenching the dye. Upon binding to the target, the fluorescence was recovered in a dose-dependent manner (52).

Analyte-dependent aptamer conformational changes have been coupled to GNP aggregation. An anti-ATP DNA aptamer was hybridized to a short complementary inhibitory oligonucleotide that in turn was attached to a GNP. The highly negatively charged phosphate groups in the

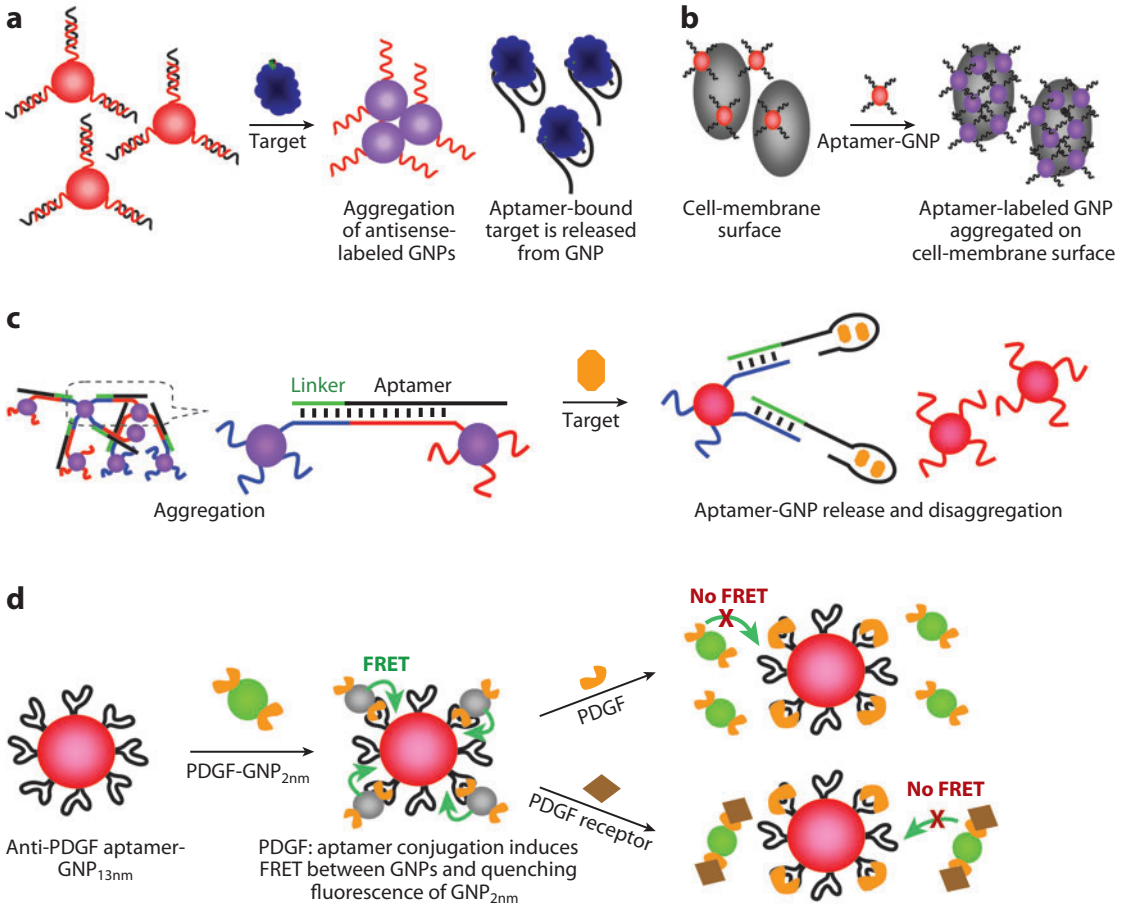

Figure 2

Schemas for optical sensors that incorporate gold nanoparticles (GNPs). (*a*) Aptamer release and GNP aggregation. (*b*) Avidity-mediated aggregation. (*c*) Aptamer release and disaggregation. (*d*) Competition and disruption of fluorescence resonant energy transfer (FRET). Throughout, black and red strands represent the aptamer and a complementary, inhibitory oligonucleotide, respectively. Abbreviation: PDGF, platelet-derived growth factor.

nucleic acids repelled one another and stabilized the GNP to aggregation (**Figure 2*a***) (53). As ATP molecules were bound, aptamers were displaced. The GNPs aggregated, leading to a color change from red to purple.

With larger targets, avidity-mediated aggregation may be possible. Recently, Medley et al. (54) coupled aptamers selected against cancer cell surfaces to GNPs (**Figure 2*b***). Assembly on the cell surface apparently places the aptamer-GNP conjugates in close enough proximity that a shift in optical properties can be detected. When 1×10^{10} particles of GNP were mixed with 1×10^{4} cells, as few as 1000 cancer cells per 1×10^{10} GNP in bulk solution were detected with the naked eye, and it was calculated that the LOD using a microplate reader was 90 cells. However, this method may not be generally applicable, given that the apposition of targets on the cell surface and the degree of aggregation necessary for signal change vary.

The use of GNPs in many assays is difficult because of the particles' strong tendency to aggregate over time. To deal with this limitation, disaggregation methods have been developed.

An aptamer hybridizes to and bridges two oligonucleotide-coupled GNPs, and in the presence of the appropriate analyte, it undergoes a conformational change and is released (**Figure 2*c***). This disaggregation method has been demonstrated for the detection of ATP, cocaine, Pb^{2+}, K^+, and their combinations (55–57). However, this method has also proved to have significant background due to aggregation; tens of target molecules are required to disassemble a single GNP aggregate. As an alternative approach to disassociation, Huang et al. (58) developed a variation on more standard ligand displacement assays. Two different-sized GNPs (2 nm and 13 nm) were conjugated to PDGF-AA and to an anti-PDGF aptamer, respectively, generating a particle couple that also yielded a FRET-based signal. Either PDGF or the PDGF-α-receptor competed with the particles for binding, disrupting the particle couple and yielding a fluorescent signal. This assay showed a LOD of 80 pM for PDGF-AA and 0.25 nM for PDGF-α-receptor, respectively (**Figure 2*d***). However, these results were somewhat surprising: The anti-PDGF aptamer had previously been shown to be specific for the BB isoform of PDGF and to not bind noticeably to PDGF-AA (59).

To move the red GNP-oligonucleotide monomers away from the aggregated purple background, the assay was coupled to a lateral-flow (or dipstick) technology. The released GNP-oligonucleotides were also biotin modified and were captured from the lateral flow on a streptavidin (SA)-modified membrane pad (60). Test kits were constructed for either ATP or cocaine, and the LODs were ∼20 μM (compared to 0.5 mM for equivalent bulk-solution assays) and ∼10 μM, respectively. Most significantly, this device was shown to detect cocaine down to 0.2 mM within 20 s in serum without any dilution or desalting.

In addition to their use as GNPs for optical signal transduction, these particles can also be used as platforms for generating high-avidity, highly fluorescent architectures. Huang et al. (61) displayed 80 fluorophore-labeled aptamers on 12 nm × 56 nm gold nanorods, which led to a 300-fold greater fluorescence signal than was seen with individual dye-labeled aptamer probes. The enhancement of signal was also due in part to the improvement in binding affinity on cancer cells displaying multiple copies of the target (26-fold higher than the intrinsic affinity of the original aptamer probe).

2.4. Dye Displacement Assays

Even given the various techniques described for the design, engineering, and evolution of aptamer beacons, it is not always possible to produce highly functional biosensors because the precise target-binding sites and the conformational changes of the aptamers are generally not specified in atomic detail. Moreover, the covalent incorporation of reporters into the aptamer can be time-consuming and expensive. These considerations argue for an even simpler method for adapting aptamers to function as optical biosensors, the noncovalent binding and subsequent analyte-dependent displacement of reporters. Such displacement strategies can be considered label-free methods, as none of the assay reagents (neither aptamer nor target) requires any further modification prior to assay and may prove to be especially useful for high-throughput screening in drug and environmental monitoring. However, the displacement of fluorescent dyes can lead to a decrease in signal, which is not desirable for assay development.

Displacement assays typically rely on competitive binding to an aptamer by a labeled reporter and an unlabeled target. The reporter molecules are highly fluorescent when they form complexes with the aptamer, but target binding induces displacement of the reporter and a decrease in fluorescence. Examples of reporters are traditional organic dyes (62, 63), metal complexes such as $Ru[(phen)_2\text{-}(dppz)]^{2+}$ [where phen = 1,10-phenanthroline; dppz = dipyrido(3,2-a:2′,3′-c)phenazine (also known as a molecular light-switching complex)] (64), and a water-soluble cationic polymer (65, 66). Experiments with this latter polymer were reported to detect femtomole

concentrations (2×10^{-15} mol) of human thrombin in only a few minutes. However, these experiments were carried out in water, whereas the aptamer was originally selected in a buffered solution; therefore, it is unclear whether interactions with the quadruplex aptamer structure were being detected. Similarly, cationic porphyrins (such as PMPyP4) are known to bind G-quadruplex structures, and this complexation event can be monitored via fluorescence changes (67).

A problem with dye displacement strategies is that aptamers may have multiple dye-binding sites, not all of which undergo displacement, thereby leading to background. To overcome this difficulty, modular sensors (also known as allosteric bifunctional aptamers) have been generated. These sensors are composed of (*a*) a reporting domain, which signals the binding event of an analyte through binding to a fluorophore; (*b*) a recognition domain, which binds the analyte; and (*c*) a communication module, which serves as a conduit between recognition and signaling domains (**Supplemental Figure 1*a***; follow the **Supplemental Material** link from the Annual Reviews home page at **http://www.annualreviews.org**) (68). In this system, ligand binding to the detector module stabilizes the communication and subsequently the reporter modules, aiding the binding of the fluorophore reporter. The first allosteric bifunctional aptamer was demonstrated using an antimalachite green aptamer as a reporting domain and either anti-ATP, -FMN, or -theophylline aptamers as signaling domains. In each instance, the authors obtained a sensor that lit up and responded to increasing analyte concentrations (68).

The modularity of displacement assays can be further increased by combining dye displacement and conformation-switching aptamer strategies. As a proof of principle, the canonical 15mer DNA antithrombin aptamer was mixed with a complementary oligonucleotide and ethidium bromide (**Supplemental Figure 1*b***) (69). The fluorescence intensity of ethidium bromide increases in the presence of duplex, but not quadruplex, DNA. The fluorescence intensity of the aptamer:competitor oligonucleotide duplex was therefore decreased (~2%) as thrombin shifted the equilibrium toward the quadruplex structure and released the competitor oligonucleotide. Although the authors claimed that they detected thrombin at concentrations as low as 2.8 nM, the statistical reliability for a 2% overall diminution in signal is doubtful. Displacement strategies, beacon strategies, and nanoparticle reagents can be conjoined as well. An anti-PDGF DNA aptamer was immobilized on a GNP, and an intercalating dye, DMDAP (N,N′-dumethyl-2,7-diazapyreneium dication), was added. The strong fluorescence of DMDAP at 424 nm was quenched due to the GNP (**Supplemental Figure 1*c***). However, DMDAP fluorescence was recovered when PDGF binding displaced the intercalated DMDAP (70). The LOD reported with this approach was 8 pM for PDGF-AA, and the relative order of responsivity was PDGF-AA>PDGF-AB>PDGF-BB, which was again at odds with the previously described specificity of the aptamer.

3. OTHER DETECTION MODALITIES

3.1. Electrochemical Detection

As shown in **Figure 3**, the analytical applications of aptamers are rapidly moving toward electrochemical strategies, in part because electrochemical techniques have high sensitivities and in part because the instrumentation is simple, can be readily miniaturized, and is low cost. As was the case with optical detection, various electrochemical detection methods have now been utilized with aptamers, including electrochemical impedance spectroscopy (EIS) (71), potentiometry with ion-selective electrodes (ISEs) (72), electrogenerated chemiluminescence (ECL) (73), cyclic voltammetry (74), and amperometry (75). Detection in the femtomolar range has been achieved with aptamer capture of a protein target followed by EIS and chemical amplification (10 fM) (76) as well as with protein-induced oligonucleotide displacement coupled with ECL (1 fM) (73).

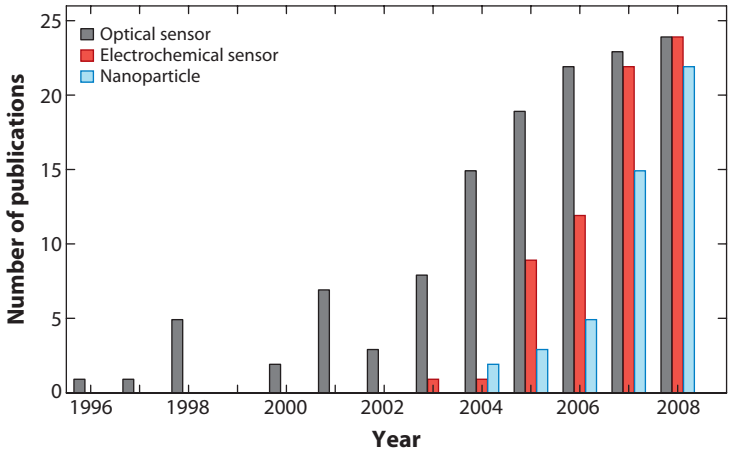

Figure 3

Number of publications relating to different types of aptamer-based sensors from 1996 through August, 2008. Data from Scifinder (**http://www.cas.org/SCIFINDER/SCHOLAR/**).

Interestingly, most of these detection methods have been limited to DNA aptamers. However, the first example of an RNA aptamer–based electrochemical sensor has recently been reported by Ferapontova et al. (77).

Many of the strategies previously examined for optical sensing are relevant to electrochemical sensing. However, electroactive reporters such as methylene blue (MB) (78), ferrocene (79), ferrocene-bearing polymers (80), ruthenium complexes (73), and $Fe(CN)_6^{4-/3-}$ (81) are used for signal transduction in electrochemical aptamer-based sensors. These reporters can be covalently conjugated to the aptamer itself (**Supplemental Figure 2*a*,*b*,*d***) (78, 79, 82), conjugated to a complementary oligonucleotide (**Supplemental Figure 2*c***) (83, 84), or indirectly complexed with aptamers (**Supplemental Figure 2*e***) (80). Following the incorporation of an electrochemical reporter, detection can be either "signal on" or "signal off," depending on how the redox reporter is shielded from the electrode [nicely reviewed by Song et al. (85)]. For example, the Plaxco group has pioneered the development of aptamer beacons to electrochemical sensors. Anti-PDGF (86) and anticocaine (78) DNA aptamers were labeled with MB. In the absence of targets, the partially unfolded aptamers led to MB being farther away from the electrode, resulting in reduced electron transfer (**Supplemental Figure 2*b***). Upon analyte binding, these aptamers became more structured, and the distance between MB and the electrode was reduced, leading to an increase in signal. Displacement (as opposed to folding) strategies that led to closer approach of MB to the electrode also proved possible (**Supplemental Figure 2*c***) (84). Aptazymes as well as aptamers can be adapted to electrochemical methods. The same Pb^{2+}-dependent aptazyme that was previously described as an optical biosensor has also been adapted to electrochemical methods, achieving parts-per-billion (nanomolar) sensitivity and excellent selectivity in a single, convenient measurement step (87). A displacement strategy was used in which aptazyme cleavage led to dissociation from an electrode-bound oligonucleotide and to closer approach of MB.

A major difference among optical methods is that electrochemical sensors are limited to heterogeneous assays because the aptamers, complementary oligonucleotides, or target molecules need to be attached to electrodes. In other words, the analytical methods may be more important than the molecular engineering. Because of the importance of methods, we focus on representative examples that utilize unique electrochemical techniques.

Numnuam et al. (72) have demonstrated the use of ISE for monitoring biomolecular interactions of an aptamer coupled to a nanocrystal label in an enzyme-linked immunosorbent assay (ELISA) microtiter-plate format (**Supplemental Figure 3*a***). Thrombin was captured by a thiolated aptamer anchored to the surface of a gold substrate. Then, a secondary aptamer with a CdS nanoparticle label was added, upon which the CdS labels were dissolved with H_2O_2. The released Cd^{2+} was detected on the basis of the potentiometric responses of a polymer membrane, the Cd^{2+}-selective microelectrode. Thrombin was measured in 200-μL samples with a low LOD of 0.14 nM, which corresponded to 28 fmol of analyte.

A similar sandwich assay format was used to measure the redox activity of adenine (A) nucleobases (88). The principle of this so-called bio barcode assay was to chemically or enzymatically release A nucleobases from the captured nanoparticle and its associated aptamers. The nucleobases were then detected on a pyrolytic graphite electrode (**Supplemental Figure 3*b***). The fact that the nanoparticle contained large numbers of aptamers led to substantial signal amplification, allowing thrombin detection down to 0.1 ng ml^{-1} (3 pM).

3.2. Mass-Sensitive Detection

Although conformational signal transduction is a major theme of this review, the fact that aptamers can be readily synthesized and conjugated to surfaces has led to their use in other analytical techniques, especially mass-sensitive detection methods where detection is performed on or at a surface. For example, aptamers have been adapted to surface plasmon resonance (SPR), surface acoustic wave (SAW), quartz crystal microbalance (QCM), and microchannel cantilever (measuring the degree of cantilever bending) sensors (85). These methods generally do not require that the target or other reagents contain labels, and they are therefore known as label-free techniques. Because these methods determine properties that are proportional to the differential changes in mass, they are generally most applicable to relatively large analytes (i.e., of the same size as the aptamer itself or larger), but not to small organic molecules or metabolites.

Recently, SPR has been utilized to monitor RBP4 (retinol-binding protein 4, a useful biomarker in the diagnosis of type 2 diabetes) in serum samples via an anti-RBP4 DNA aptamer immobilized on a gold chip. The sensitivity of the method was comparable to that obtained with a competitive ELISA kit or Western blot analysis with an anti-RBP4 antibody (89). In an effort to improve throughput, aptamers have been adapted to imaging SPR (SPRi), in which the fixed-angle reflectivity changes arising from analyte binding are interrogated in a microarray format. By using PDMS multichannels on a gold substrate, four different anti-IgE DNA aptamers were immobilized, and their affinities and dose responses were evaluated as IgE was introduced in real time (90). The authors demonstrated that aptamer binding to IgE showed a linear dependence on concentration, a LOD of 2 nM, and an apparent K_d of 2.7×10^{-7} M.

SPRi techniques have also been adapted to microarray formats by the Corn group (91). A 5×4 DNA oligonucleotide array was created on a gold surface, and RNA aptamers were ligated in place with T4 RNA ligase. Different sequences of aptamers against protein factor IXa were analyzed, and a LOD of 2 nM was observed (91). This method has also been adapted to a sandwich assay microarray format, in which the aptamer array captured target proteins that in turn were bound by antibodies conjugated to HRP (horseradish peroxidase). The immobilized HRP was treated with TMB (3,3′,5,5′-tetramethylbezidine), creating a localized dark blue precipitate that could be detected by SPRi with high spatial resolution. This sandwich SPRi methodology detected thrombin at concentrations as low as 500 fM and vascular endothelial growth factor at a biologically relevant concentration of 1 pM (92).

SAW devices work because mass changes on the surface lead to a change in the propagation velocity of acoustic waves, resulting in a reduction in resonance frequency or an alteration of the phase shift between the input and output signals. A special type of SAW sensor, the Love-wave sensor (which uses horizontal waves guided in a layer on the surface of the sensor to reduce energy dissipation of the acoustic wave, thereby increasing surface sensitivity), was combined with aptamers by Schlensog et al. (93) for the detection of thrombin and a HIV-1 Rev peptide. The detection limits for thrombin and HIV-1 Rev peptide were 72 pg cm^{-2} and 77 pg cm^{-2}, respectively. This method was somewhat cumbersome in that it required a separate determination of the total mass of protein loaded. However, these methods have been extended to sensing in a more complex milieu: the detection of binding of the anticoagulants heparin and antithrombin III to the blood-clotting cascade factor human thrombin. In this study (94), both RNA and DNA antiaptamers were immobilized on a SAW sensor without loss of affinity, which allowed thrombin to be loaded and its protein partners to be detected. The sensor was regenerated (with 0.1 N NaOH) and reused with additional samples. Recently, the same assay method was combined with mass spectrometry for target identification. Thrombin was captured on the chip, digested in situ with proteases, and directly identified by peptide mass fingerprint with MALDI-MS (matrix-assisted laser desorption/ionization–mass spectrometry) (95).

Like SAW, QCM employs piezoelectric quartz crystals, but the latter is a simpler and more cost-efficient technique. Aptamers on QCM have been used for the detection of thrombin (LOD < 1 nM) (96) and HIV-1 Tat protein (LOD of 0.25 ppm 100 μL^{-1} and a dynamic range of 0.25 to 2.5 ppm) (97) by measuring the gravimetric resonance frequency change. Aptazymes may be useful for changing the relative mass present on a QCM. Knudsen et al. (98) have adapted the catalytic activity of both ligation and cleavage aptazymes to the detection of a HIV-1 Rev peptide (ligation) or theophylline (cleavage). The catalytic activities of immobilized aptazymes on the sensor surface were observed in real time by monitoring changes in QCM frequency. These methods were particularly notable in that they overcame the problems associated with detection of a low-molecular-weight ligand such as theophylline by transducing ligand recognition into a change in nucleic acid mass.

A final example of the varied use of piezoelectric quartz crystals is the construction of micro-cantilevers that operate in a vibrational mode. Binding is measured by monitoring the differential bending between reference (random DNA) and sensor (aptamer) cantilevers. Taq DNA polymerase (99) and hepatitis C virus (100) were detected using DNA and RNA aptamers and metrics of 15 pM (K_d) and 2.1 pM (LOD), respectively.

4. ADAPTATION OF APTAMERS TO SEQUENCE AMPLIFICATION

One of the most obvious analytical formats for aptamers is simply to detect them as one detects other nucleic acids: via any of a variety of amplification formats such as the polymerase chain reaction (PCR), rolling-circle amplification (RCA), nucleic acid sequence–based amplification, loop-mediated isothermal amplification, or the proximity ligation assay (PLA). Moreover, by using detection reagents that can signal in real time such as molecular beacons, one can also follow amplification cascades in real time. Also, the sensitivity of detection can be improved by looking at the evolution of signals, as with real-time PCR.

4.1. Immuno–Polymerase Chain Reaction with Aptamers

Although antibody-based immunoPCR (IPCR) has proven to be extremely useful as a diagnostic method, there is still room for the development of similar aptamer-based methods. IPCR is

subject to the same limitations faced by other methods involving antibodies, including labor-intensive synthesis, low thermal stability, and potential batch-to-batch variation. Additionally, directly tagging proteins with nucleic acids can be somewhat cumbersome, although the use of protein moieties such as SA has to some extent reduced this shortcoming (101, 102). Direct conjugation to antibodies suffers from a lack of precision and often results in uneven numbers of oligonucleotides per antibody, thus introducing higher rates of error and potentially lowering sensitivity (103, 104). However, because aptamers are already made of nucleic acids, each aptamer can be readily extended to form a conjugation site for a hybridizing nucleic acid that in turn can be conjugated to virtually any molecule. Alternatively, DNA aptamers can be synthesized with a terminal biotin moiety for conjugation to SA, and RNA aptamers can be similarly transcribed with a terminal biotin (105, 106). Aptamers thus modified have already proven to be extremely beneficial in analytical assays such as fluorescent-activated cell sorting, as well as for the delivery of therapeutic molecules such as small interfering RNA to diseased cells (107–110).

Despite the obvious advantages of aptamer-based IPCR, it has only recently been implemented in a robust way by the Tok group (111). Using an aptamer-based IPCR, Tok et al. attained an antigen LOD of 100 fM. The protocol mirrored traditional IPCR: A 50-nt DNA sequence comprising a 15-nt antithrombin aptamer, a poly(A) linker sequence, and a short primer-binding site was captured by thrombin immobilized on antibody-coated magnetic beads (**Supplemental Figure 4*a***). The specifically bound aptamer-protein complexes were then heat-dissociated, and the DNA aptamers were amplified and detected via real-time PCR. At concentrations lower than 1 pM, amplification was nonspecific due to the nonspecific binding of DNA aptamers to the magnetic beads, indicating that aptamers are not immune from many of the same background issues that face antibodies. Nonetheless, aptamer-based amplification was possible in serum with only a 10% compromise in sensitivity.

Challenges with background can be mollified by prepurification of the target complex. For example, Le and coworkers (112) have described a sensitive aptamer-based affinity technique where tiny amounts of target were bound and captured by a cognate aptamer, and the complex was then isolated by capillary electrophoresis prior to IPCR. An anti-HIV reverse transcriptase (RT) DNA aptamer (RT-26) (113) was utilized to capture and purify as few as 180 molecules of the RT enzyme.

Although aptamers can probably perform as well as antibodies in IPCR, there are a number of unique applications of aptamers in this technique that cannot be readily mimicked by non–nucleic acid based reagents. For example, Wang et al. (114) adapted the well-known nuclease protection assay to IPCR. In this study, a 22-nt probe containing an antithrombin aptamer sequence was protected from nuclease degradation by thrombin, two adaptor oligonucleotides were ligated to generate an amplicon, and the amplicon was subsequently detected by real-time PCR. This unique aptamer-based reagent was sensitive enough to detect as few as several hundred molecules of thrombin (**Supplemental Figure 4*b***). The obvious advantage of this technique is that it does not require any purification of target or complexes and thus can be carried out with a single aptamer (as opposed to sandwich assays, which require at least two binding reagents).

The conformation-switching aptamers or aptamer beacons described above can also be adapted to amplification assays. In this instance, the change in conformation is again associated with the production of an amplicon. The secondary structures of antithrombin and anti-PDGF aptamers were engineered to hide a ligation junction in the absence of ligand and to form the ligation junction with an added oligonucleotide in the presence of ligand (**Supplemental Figure 4*c***) (115). These designs were aided by having the ligand-bound conformation fold to form a hairpin near the ligation junction that could readily accommodate the binding of the added oligonucleotide. As do other methods with conformation-switching aptamers, this technique benefits from being carried

out in homogeneous mixtures, although the ligation reaction (20 min) has to be separated from the real-time PCR step. As little as 0.8 nM of thrombin in BSA (a value well within normal thrombin concentrations in human blood) and 0.32 nM of purified PDGF-BB were detected by quantitative PCR. Interestingly, thrombin mixed with cell lysate prepared from human 293T fibroblast cells was detected not only with high specificity, but also at an even lower concentration (32 pM).

4.2. The Proximity Ligation Assay

Recently, Fredriksson, Landegren, and their coworkers (116–118) developed a brilliant innovation in which a sandwich assay was carried out without the need for reagent immobilization, which can be a significant source of background. PLA depends on nucleic acid probes that localize adjacent to one another on a target, and that when ligated via a templating oligonucleotide, form a unique amplicon that can be detected via real-time PCR, thereby in effect detecting the doubly bound target. The true advantage of PLA lies not only in its heterogeneous format, but in the fact that it all but eliminates background by requiring a quaternary recognition event for the production of signal: Two DNA-containing reagents must bind to a target, and then a hybridizing oligonucleotide must also bind and template the production of the amplicon. The odds of this happening in a configuration where the two probes are not already specifically localized adjacent to one another is vanishingly small; therefore, real-time PCR can be used to distinguish the few true molecular events from an even smaller number of false positive events.

PLA was originally employed for the detection of PDGF-BB, a notorious player in cancer (119, 120). DNA aptamers specific for PDGF were extended at their 3′ and 5′ ends to develop what was termed the proximity probe pair. Because PDGF is a dimer, two probes bound to the same target, and the extensions efficiently hybridized to a connector oligonucleotide (also known as a splint). Although background ligation can of course occur, target-specific signals proved to be strong, specific, and independent of probe length. Dose-response curves indicated that 24,000 molecules (~40 fM) of PDGF were detected. Given the sensitivity and the ease of the assay, PLA was soon adapted to nonaptamer reagents such as antibodies (116, 121).

Another advantage of PLA is that proximity recognition need not be limited to individual molecules; it can also involve the surfaces such as bacterial spores, vegetative cells, and cancer cell lines (122–124). RNA aptamers previously selected against the homodimer prostate-specific membrane antigen (PSMA) were used for the in situ detection of this protein on the cell surface. To adapt the RNA aptamers to DNA ligation, the aptamers were hybridized via sequence-specific tails to DNA oligomers, which in turn were ligated with a DNA splint. This use of reagents that can form a single, defined ligation junction may have led to less variance than would have been the case with comparable, multiply labeled antibodies (**Supplemental Figure 5*a***) (124). Upon cell binding and splint-initiated ligation, the anti-PSMA aptamer probe constructs detected as few as 10 LNCaP prostate tumor cells on their own or in the presence of 100,000 noncognate HeLa cells. The reagent concentrations required for this feat were in the picomolar range. The further use of reagents that can detect specific cells or tissue types, rather than particular molecular biomarkers, may greatly improve the diagnostic applications of PLA. In this regard, a number of aptamers have already been generated against whole cells (125–127).

4.3. Aptamers and Rolling-Circle Amplification

RCA assays are based on the ability of DNA or RNA polymerases to continuously replicate around a circular, single-stranded nucleic acid template under isothermal conditions (128, 129). The polymerase strand displaces the primer and product to produce a concatemeric linear sequence up to

0.5 Mb in length (130). Because no thermal cycling is required, this assay can often be implemented without expensive or cumbersome equipment (129, 131). RCA is often employed as a signal-amplification technique because the product contains hundreds to thousands of complements of the original template, and hybridization of probes to these sites can increase the original signal by several orders of magnitude with concomitantly lower detection limits (129, 132). The circular template utilized in RCA is resistant to nuclease degradation, enhancing stability in serum samples and making RCA attractive for clinical diagnostics (133). Finally, the long RCA products can be localized by tethering the primer to solid surfaces or immobilized molecules, which is beneficial to chip assays and microarrays (132, 134).

Just as aptamers were readily adapted to IPCR, they may be equally well adapted to RCA. Indeed, Fischer et al. (111) used the same antithrombin aptamer for RCA that they had previously used for IPCR. The aptamer was incubated with thrombin-conjugated magnetic beads. Bound aptamers were removed to serve as primers for real-time RCA. Real-time RCA detected the aptamers bound to 1 μM thrombin in less then 10 min and to a lower limit of 3 nM. In contrast, a picomolar detection range was observed with samples amplified by real-time PCR, as described above. Zhou et al. (135) used an antibody-aptamer sandwich to set up RCA detection of PDGF.

As was the case with IPCR, conformation-switching aptamers can be used to generate amplicons and amplified signals with RCA. Yang et al. (136) engineered an anti-PDGF DNA aptamer to switch between an inactive hairpin and a ligand-binding conformation that led to the formation of a ligation junction for circularization. Upon addition of DNA ligase and polymerase, circles formed and RCA was carried out. Amplification was quantitated in real time via the addition of complementary probes and SYBR Green. The aptamer-based assay showed a detection range of 0.3 to 80 nM PDGF-BB, specific detection of the B isoform, and the ability to detect a concentration of PDGF-BB as low as 1.6 nM in a complex sample of cell lysate (**Supplemental Figure 5*b***). The conformation-switching aptamer was be immobilized and used for PDGF detection. Because nucleic acid catalysts have been shown to carry out ligation reactions, a protein ligase is not necessary to transmogrify analytes to amplicons; the nucleic acid receptor can also be its own ligase. An anti-ATP DNA aptamer was coupled to a deoxyribozyme, yielding a DNA aptazyme (137). In the presence of ATP, the deoxyribozyme showed increased ligation activity. This aptazyme was engineered to circularize a linear, single-stranded DNA, which in turn became a template for RCA (132). A secondary function of the aptazyme was to serve as a primer for RCA. The ATP-dependent aptazyme was spotted to a chip via biotin SA linkage, and immobilized concatemers were detected in situ by hybridizing a fluorescently tagged oligonucleotide probe. RCA added 1500-fold signal amplification in solution and detected ATP from 10 to 100 μM on the chip surface.

Proximity formats are also possible for aptamer-mediated RCA. One experimental design involved binding two separate antithrombin aptamers, one a circularized aptamer that bound exosite 2 of thrombin and the other a linear aptamer that bound exosite 1. One advantage of the circular "captamers" is that they were more resistant to nucleases (133). When these two molecules were brought together on the surface of thrombin, one served as a primer and the other served as a template for RCA. Thrombin concentrations as low as 10 pM were easily detected and signaled via isothermal amplification, thus displaying an elegant yet simple alternate to IPCR (138). The marriage of proximity ligation with RCA is likely to be of increasing utility in the future. In a stunning example (121), antibody reagents were recently employed in a method similar to that described by Di Giusto et al. (138) to detect the transcription factors Myc and Max in situ at the single-molecule level.

In addition to fully exploiting the various capabilities of aptamers and aptazymes described above, RCA methods have been favored for adapting nucleic acid signal amplification to a variety

of detection modalities. Furukawa et al. (139) utilized rolling-circle transcription to construct tandem repeats of a RNA aptamer that binds malachite green. This fluorophore is known to be easily deexcited from vibration, and it therefore has a low quantum yield for fluorescence. The aptamer, in turn, stabilizes the vibrational state and gives a higher quantum yield of fluorescence (139). Thus, the nucleic acid products can be directly detected without the need for hybridization. The amplified nucleic acid can also serve as much more than a landing site for a hybridizing probe. Cheglakov et al. (140) used a molecular beacon to initiate RCA on a circular template encoding a deoxyribozyme that mimicked the activity of HRP. When the substrate $ABTS^{2-}$ [2,2′-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid] was added in the presence of H_2O_2, a colorimetric change from clear to green was observed. The assay was also adapted to chemiluminescence by the addition of luminal and H_2O_2. As little as 1 pM of phage DNA was detected. The RCA products were ultimately detected by capturing alkaline phosphatase that led to the production of ascorbic acid, which in turn led to silver deposition at the same site (**Supplemental Figure 5*c***). The deposited silver was sensitively detected via electrochemical methods. This assay reflected PDGF concentrations over four orders of magnitude with a detection limit of 10 fM. Although the assay also worked in the absence of RCA, the use of this molecular-amplification technique yielded a 90-fold increase in the amount of silver deposited (135). Unique optical detection modalities may also be used with RCA.

5. SUGGESTED STANDARDS FOR APTAMER PUBLICATIONS

The burgeoning field of aptamer research has resulted in a wide variety of publications, especially in the area of analytical applications of aptamers. Because these reagents are relatively new, standards for their use have not yet been promulgated or applied in the analytical community. For the information of both the community and journal editors, we suggest that the following standards may be of use in deciding whether an aptamer paper should be accepted.

5.1. Sequence Must Be Reported

A general standard of scientific publication is that experiments should be reported in sufficient detail that other skilled scientists can potentially reproduce the results themselves. If an aptamer sequence or reference (141) is not reported, then the experiment cannot be independently repeated.

5.2. Researchers Should Understand the Sequences They Are Using and Citing

Conversely, knowledge of the sequence of an aptamer is often taken as an indication that only this sequence itself is necessary for experimental repetition. In fact, it is not only the sequence, but many other variables—such as buffer conditions, temperature, purification method, and conformational state—that affect the function of an aptamer. If an analytical researcher does not know or heed these important variables, then their experiments may be meaningless. As a particularly embarrassing example, the Ellington lab published a manuscript detailing the use of aptamers on a sensor array (142). In Table 1 of that article, the authors gave the DNA template used for the production of an RNA aptamer rather than the sequence of the RNA aptamer itself (although the section on methods and its associated references were clear in this regard). As a result, other researchers took the table at face value and attempted to make antilysozyme aptamers out of DNA that should have been made out of RNA (141, 143). Worse, instead of the full aptamer sequences, only the random-sequence regions of these aptamers were reproduced, including primer-binding sites (despite having been fully described in the section on methods and the accompanying references).

The claims in a subsequent paper (144) suggest specific binding, but this cannot be true. Rather, the nonspecific binding properties of the basic protein lysozyme lead to interactions with many different nucleic acids. If this were an isolated incident, it would primarily be an indictment of the sloppiness of two labs, but it is not: There are several other papers in which DNA sequence was used instead of the proper RNA aptamer (145) and in which only the random region was used instead of the whole aptamer sequence (146).

It is possible that these mistakes were made not out of sloppiness, but rather because analytical chemists fundamentally misunderstand the reagents they are using. To help remedy this potential difficulty, researchers should note that DNA, RNA, and modified RNA are completely different chemical compounds. Although they may all form helices based on Watson-Crick base-pairing, the details of interactions with ligands completely differ among these different chemical compounds. In addition, because aptamers are selected in the presence of their constant regions (their primer-binding sites), these are an integral part of the reagent. Finally, although aptamers can sometimes be reproduced without their constant regions, the functionality of any foreshortened aptamer must be checked in advance.

5.3. Researchers Should Use Controls

This may seem to be such a bedrock of hypothesis-driven science that it not be necessary to repeat it. However, building on the same "sequence-centric" fallacy cited in Section 5.2 above, some researchers seem to think that if a sequence has been reported to bind to a protein, then it must bind to that protein and only that protein. In fact, nonspecific interactions between proteins and nucleic acids are abundant, and it is strongly suggested that for any given interaction that is demonstrated that two negative controls be carried out: one with a noncognate sequence (a random pool or scrambled version of the aptamer is best) and one with a noncognate protein (preferably a related protein, not BSA).

5.4. There Are More Analytes in the World than ATP, Thrombin, Platelet-Derived Growth Factor, and Immunoglobin E

The analytical community, including the Ellington lab, has frequently focused on the robust, extant DNA aptamers against ATP and these three proteins. Indeed, the popular use of these aptamers led Tombelli et al. (147) to summarize publications utilizing only these DNA aptamers and a few additional RNA aptamers. Although this approach suffices for proof of principle, most experiments at this juncture should strive to go well beyond proof of principle and to show potential utility in particular analytical applications. To this end, researchers should begin to couple the development of their analytical methods to certain aptamers for particular end uses. This recommendation goes hand-in-hand with the plea for controls, in that if two aptamers are used with two targets, then they can at least act as specificity controls for each other.

5.5. Buffer Conditions and Temperature Make a Difference

Aptamers are typically selected in a single buffer, and their performance may be very specific for that buffer. Nucleic acid structures are particularly sensitive to both the type and the amount of monovalent and divalent cations. By leaving out or changing the concentration of a given ion during the assay development, a researcher may greatly compromise aptamer function. Any deviation from the original selection buffer requires a reassay of function. Although a journal editor may not necessarily know when a deviation has occurred, reviewers should be on the lookout for this, and

researchers should dutifully report any deviations. A particularly good example of how aptamers can be adapted to operate under standard buffer conditions can be found in a paper by Cho et al. (148). A number of common buffer conditions were screened with four sets of aptamer:protein complexes selected under very different buffer conditions in order to find a universal buffer where the disparate aptamers could retain their affinities and selectivities for their corresponding protein targets. In a similar vein, aptamers have typically been evolved at one temperature, and their function at a different temperature is not assured. Moreover, because nucleic acids can notoriously fold into multiple conformations, an aptamer can become trapped in an inactive conformation, and without thermal equilibration, it cannot access its active conformation. It is for this reason that we typically denature and renature any aptamer we use for analytical applications (149).

6. SUMMARY

As described throughout this review, the sensitivities of aptamer sensors vary greatly. These sensitivities depend in part upon the affinities of the aptamers for their targets; greater affinities generally yield lower LODs. In this regard, the coming generation of aptamers with picomolar binding constants will likely greatly improve the utility of aptamer sensors. Although conformational transduction mechanisms can be uniquely engineered with aptamers and have proven to be adaptable to a wide range of formats (as evidenced by the optical, electrochemical, and amplification schemas described), the energy required to drive the conformational change comes from the binding of a ligand to the aptamer and must necessarily reduce the apparent affinity and sensitivity of the sensor (this requirement is known as the "no-free-lunch" principle). The exception to this rule is the assembly of quaternary structures via multiple binding sites on a target. In this case, the improbability of structure formation in the absence of the target greatly reduces background, allowing even very weak signals to be observed with confidence. Although aptamers can be readily adapted to widely used nucleic acid–amplification methods, these methods generally did not greatly improve sensitivity in homogeneous assay formats. In fact, signal-amplification techniques did not generally result in increases in sensitivity unless some immobilization or wash steps were included in the assay. The sensitivity of a given aptamer biosensor is most likely to be influenced by the sensitivity of the analytical method used for its detection. Overall, although aptamer biosensors will likely see increasing use because of their ease of synthesis and the possibilities for modular introduction of reporters, it will be newer and ever more sensitive methodologies adopted by analytical chemists that will ultimately drive their application as sensors. That is, of course, if analytical chemists continue to attend to the biochemical complexities inherent in these reagents.

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Contents

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