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REVIEW

Surface immobilization methods for aptamer diagnostic applications

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Abstract In this review we examine various methods for the immobilization of aptamers onto different substrates that can be utilized in a diverse array of analytical formats. In most cases, covalent linking to surfaces is preferred over physisorption, which is reflected in the bulk of the reports covered within this review. Conjugation of aptamers with appropriate linkers directly to gold films or particles is discussed first, followed by methods for conjugating aptamers to functionally modified surfaces. In many aptamer-based applications, silicates and silicon oxide surfaces provide an advantage over metallic substrates, and generally require surface modification prior to covalent attachment of the aptamers. Chemical protocols for covalent attachment of aptamers to functionalized surfaces are summarized in the review, showing common pathways employed for aptamer immobilization on different surfaces. Biocoatings, such as avidin or one of its derivatives, have been shown to be highly successful for immobilizing biotin-tethered aptamers on various surfaces (e.g., gold, silicates, polymers). There are also a few examples reported of aptamer immobilization on other novel substrates, such as quantum dots, carbon nanotubes, and carbohydrates. This review covers the literature on aptamer immobilization up to March 2007, including comparison of different linkers of varying size and chemical structure, 3′ versus 5′ attachment, and regeneration methods of aptamers on surfaces.

Keywords Aptamer · Sensor · Immobilization · Linker · Optimization · Assay

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Introduction

The field of aptamer-based molecular recognition applications is a rapidly developing area that is anticipated to be competitive with biosensors, immunoassays, and other analytical formats currently in use [1]. Aptamers are artificial receptors, primarily DNA or RNA sequences, that are evolved in vitro (e.g., by SELEX methods [2]) toward binding target molecules of interest such as proteins, cells, and small molecules [3]. These biological-based artificial receptors can provide the molecular recognition unit that is required as the first step in any sensor or assay. Depending on how the aptamer sensor/assay is constructed, aptamers can also provide the transduction and signal-generation steps necessary to complete the sensor design [4–6]. Reflecting the growth in this field, there are a number of early reviews on the development of aptamers and aptasensors that can be consulted for aptamer syntheses, selection methods, and analytical techniques that incorporate aptamers [4, 7–11].

Because antibodies are routinely available for biological and chemical targets, these have been generally used for sensor/assay applications. However, aptamers have comparable affinities for target analytes [1, 9, 12, 13], and there are a number of advantages for using aptamers versus antibodies. In a comparison of aptamers versus antibodies that bind immunoglobulin E (IgE) immobilized on a quartz crystal microbalance (QCM), the aptamer could be regenerated multiple times without loss of sensitivity, while the antibody sensor suffered irreversible damage [14]. Furthermore, it has been shown that the thermal stability of the IgE aptamer is significantly better than that of the corresponding IgE antibody. Aptamers are prepared at lower cost via automated synthesis, which also allows directed modifications for further chemistry and engineering (e.g., immobili-

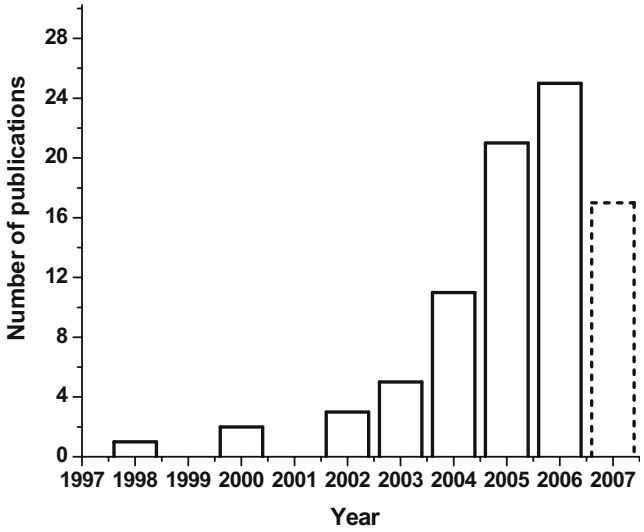


Fig. 1 Number of research articles on aptamer-based diagnostics up to March 2007 (search results from both SciFinder Scholar and Web of Science databases)

zation functionalities, fluorophores), or the aptamers can be incorporated into larger DNA sequences (e.g., DNA tiles) [15]. The small size of aptamers provides several advantages over antibodies; for example, a greater surface density of receptors can be achieved, and small size also facilitates multiple binding to analytes (avoiding steric problems) for "sandwich assays." Furthermore, under some conditions, certain analytical methods such as field effect transistor (FET) devices require receptors that bind within the Debye length (approximately 3 nm); antibodies are approximately 10 nm in size, providing binding outside the measurable range, whereas aptamers (1–2 nm) are within this Debye range [16, 17]. Finally, the production of aptamers is animal-free, allowing receptors toward analytes that are toxic or nonimmunogenic to organisms, and avoids batch variation exhibited by polyclonal antibodies. Among their drawbacks, aptamers can be sensitive to nuclease attack, although there are several strategies that can be employed to increase aptamer stability [18].

Because of the advantages of using aptamers as molecular recognition elements, there have been increasing reports on different analytical constructs incorporating aptamers for sensors and other applications (Fig. 1), reflecting the enormous potential and interest of aptamers for these applications. Analytical formats, such as those listed in Table 1, often require immobilization of the receptor to a surface for integration into a device. An important goal for aptamer immobilization is to maintain the binding affinity and selectivity that the aptamer displays in solution. This is usually achieved by covalently tethering the aptamer to a surface-bound linker, and in some cases noncovalently through physisorption. For example, surface plasmon resonance (SPR), acoustic-wave, and electrochemical analyses require the aptamer to be tethered within close proximity to a metallic surface to induce an analytical signal. Chromatographic methods such as affinity chromatography and capillary electrophoresis employ immobilization to keep aptamers stationary under solution flow conditions to effect the affinity separation. This is also the case for sandwich or immunosorbent-type assays. More generally, immobilization leads to easy recovery of the aptamer and repeated use of the sensor, chromatographic support, or spectroscopic device after washing and regeneration steps. Furthermore, immobilization allows the construction of multiarray devices, either exclusively with aptamers or in combination with antibodies or other receptors for highly parallel detection of many targets simultaneously. Overall, immobilization is important for the development and integration of aptamers into microanalytical, portable or solid-state devices.

Table 1 Different analytical methods used for the detection of aptamer-protein interaction along with the respective substrate used in each method

Surface and related modifications	Analytical method
Gold, self-assmebled monolayers on gold, biocoatings on gold	Surface plasmon resonance [19, 20], electrochemical impedance/resistance [21, 22], ellipsometry [20], surface acoustic wave [23, 24], quartz crystal microbalance [14, 25], cantilever [26], surface plasmon resonance imaging [27], fluorescence titration, fluorescence resonance energy transfer , atomic force microscopy [28], enzyme-linked aptamer assay [19, 29], UV [30], electrochemical indicator [31]
Silica, silicon, or TiO_2; Al_2O_3, biocoatings on silica; carbohydrates on silica	Fluorescence spectroscopy, whispering gallery mode optical spectroscopy [32], field-effect transistor [33], mass spectrometry [34], capillary electrophoresis [35], atomic force microscopy [36], affinity chromatography [37], fluorescence microscopy [38], fluorescence anisotropy [39]
Biocoatings on polymers	High-performance liquid chromatography [40]
Carbohydrates	Fluorescence microscopy [41]
Single-walled carbon nanotube	Conductance, single-walled carbon nanotube field-effect transistor [16, 17]
Quantum dots	Photoluminescence [42]
DNA tiles (on glass)	Fluorescence [15, 43]

Table 1 summarizes different analytical methods for aptasensor development, which often determines the choice of substrate for aptamer immobilization. Metallic substrates, almost exclusively gold, are used for SPR and many electrochemical measurements. In some cases such as QCM, Love-wave sensor, and atomic force microscopy, silicon can be used as a substrate, but because of the ease of forming gold–thiol monolayers, gold is frequently used. For optical measurements such as UV–vis and fluorescence, glass or polymer substrates are normally used because of their transparency for signal transmission and to avoid spectroscopic quenching that can occur with a gold surface. However, quenching for electrochemical measurements has been shown to be an advantage for aptasensors where changes in the conformation of the aptamer upon binding actually change the proximity of an electrochemical indicator with the gold surface with corresponding changes in signal intensity [4, 6]. Aptamers have also been used for chromatographic chiral separations; in these cases, aptamers are immobilized on base column materials, most frequently silica particles [37]. Polymer particles coated with streptavidin have also been used as a substrate for high-performance liquid chromatography [40]. Future developments are anticipated to further realize the potential of aptasensors using novel methods, such as recent reports employing quantum dots [42], DNA tiles [15, 43], and carbon nanotubes [16, 17].

Centi et al. [44] have stated, "The procedure to fix the aptamer to the surface is of paramount importance to obtain an ordered and oriented layer able to ensure, as much as possible, the flexibility of the bioreceptor without altering the affinity for the target molecule." This highlights the importance of properly designing immobilization strategies as a first consideration for the development of aptamer diagnostics. This aspect of analytical device development is important to optimize at an early stage, in order to maximize the performance of any aptamer-based analytical technique. Thus, the focus of this review is on immobilization chemistries and critical parameters for the optimization of immobilized-aptamer performance.

There are several different approaches for the immobilization of aptamers, which depend upon the chemical composition of the surface, the availability of suitable aptamer linkers, and the chemistries employed for attachment. While the number of literature contributions of analytical applications of aptamer-based devices is ever expanding, the purview of this review will follow reports up until March 2007.

Aptamer targets

A number of aptamers have been developed against proteins, oligopeptides, small molecules, cells, and microbes, and have been used for aptamer-based diagnostics. The majority of the targets are proteins, including enzymes (e.g., thrombin [6, 15, 16, 19, 20, 22–24, 28, 29, 33, 34, 37–39, 45–51], lysozyme [49, 50, 52–55], Taq DNA polymerase [26, 56]), biomarkers that are overexpressed on cancer cell membranes (e.g., prostate-specific membrane antigen, PSMA) [57–60], antibodies (e.g., IgE [14, 22, 36, 49, 61, 62], which plays an important role in allergic reactions), growth factors such as platelet-derived growth factor (PDGF) [30, 63] and vascular endothelial growth factor [27], Rev protein, a RNA-binding protein of HIV-1 [54], and biological toxins [49, 54] (e.g., ricin, a protein extracted from castor beans). Aptasensors for oligopeptides have been reported, such as arginine-vasopressin [40], and HIV-1 Tat (peptide), the trans-activator of transcription (Tat) protein, which is an oligopeptide controlling the early phase of the replication cycle of HIV-1 [25, 64]. Analytical formats have also been developed for aptamers elicited to small-molecule targets; first reported were capillary electrophoresis methods to evaluate retention of flavin nucleotides [35]. Subsequently, cocaine [65] and adenosine [33] were targeted for detection in the first small-molecule aptasensor studies; soon afterwards, novel detection strategies were further explored using adenosine [66] and ATP [41, 67]. A chromatographic application of an immobilized aptamer toward D-histidine was reported to be able to separate enantiomers of this amino acid [68]. Recently, aptamers against the bacterial spore *Bacillus thuringiensis* have been immobilized onto quantum dots to create a fluorescence-based assay [42]. In addition, increase of cell adhesion was demonstrated using an aptamer immobilized on a Ti-alloy surface [69].

Thrombin as a model system

In terms of aptamer covalent immobilization onto a sensor surface, the majority of cases reported have made use of the relatively short 15-base thrombin-binding aptamer 5′-GGTTGGTGTGGTTGG-3′, also known as HD-1, which binds exosite I of thrombin [70]. HD-1 was the first aptamer used to demonstrate the feasibility of aptasensor development using a number of substrates, including glass [39], silica [51], carbon nanotubes [16], gold nanoparticles [71], and quantum dots [50]. The thrombin model system was able to demonstrate the usefulness of several methods for the detection of aptamer–protein interactions for the first time, including SPR [19, 20], Love-wave sensor [23, 24], fluorescence methods [39], as well as a number of electrochemical methods [21, 22, 31]. Further, HD-1 is the first aptamer used to demonstrate the utility of different formats, such as reagentless detection [19], DNA tiles [15], sandwich-type assays [48], and structure-switching aptamers [6]. In addition, this aptamer was also generally used as a model for the optimization of different parameters

for aptasensor development [44, 72, 73]. The thrombin aptamer is also one of the most commonly used aptamers for demonstrating multianalyte arrays for sensor and detection formats [27, 50, 60].

The widespread use of the HD-1 aptamer as a model system for aptasensor development is due to a combination of several factors. First, the short sequence leads to higher yield and lower cost; furthermore, DNA is more stable than RNA, and the G-quartet structure of HD-1 is well characterized relative to other aptamer structures [74–77]. Historically, HD-1 was the first aptamer selected toward a protein target; hence, it was the first one used for aptasensor development. This, in combination with the fact that only a limited number of aptamers are available, led to its continued use for aptasensor development. Tasset et al. [78] in 1997 reported another aptamer for binding thrombin, a 29-oligonucleotide sequence, referred to as HD-22, which binds to exosite II of thrombin. The availability of two aptamers that bind at different sites has facilitated the development of aptamer sandwich assays for the detection of proteins [46, 48]. For detection of diseases such as hemophilia, thrombin aptasensors are good candidates in clinical settings, making thrombin an important target [79].

Chemistry of aptamer immobilization

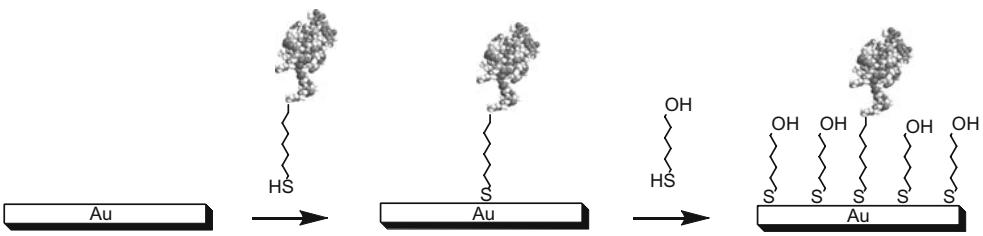
There are a number of chemical methods for the immobilization of aptamers, and all are based on methods previously developed for immobilization of DNA hybrids and other biomolecules [80, 81]. The following discussion begins with the most widely used methods, and expands into novel methods reported recently. Studies that employ covalent coupling chemistries generally use the same or similar reaction pathways (summarized in Scheme 1), and these are compared in cases where different immobilization methods are used for the same aptamer.

Direct attachment to gold

Thiols on planar gold

Aptasensors on gold were first reported in 2002 [14], while aptamers on other surfaces were reported for sensors as early as 1998 [39]. It should be noted that DNA tethered via thiol-terminated linkers had been reported much earlier for DNA-hybridization studies [80, 81]. To link aptamers to gold surfaces, methods generally follow those of other nonbiological monolayers formed through chemisorption of thiol onto gold films. Typically, a cleaned gold substrate is immersed in an aqueous buffer solution of thiol-terminated aptamer, which then forms a monolayer on the gold surface. The gold surface can be a planar substrate, such as those used in SPR [19, 20, 24] or QCM sensors [14, 25] and in ellipsometry [20]; and recently there have been several reports of monolayer formation on gold nanoparticles [30, 71]. Owing to the ease of formation of aptamer monolayers on gold surfaces, this method can also be used to functionalize probe tips such as atomic force microscope (AFM) tips [28] and cantilever tips [26] to study and monitor the interaction between the immobilized aptamer and its recognition partner.

Direct attachment of an aptamer to a gold surface is achieved by using a thiol–alkane linked to the aptamer sequence, utilizing methods similar to those found in the literature covering DNA hybridization on gold monolayer surfaces [82]. The use of gold as a substrate has many advantages, the most prevalent of which are the ease of immobilization of molecules to the surface of gold, its ability to form a monolayer, and the highly ordered nature of the monolayer. As stated earlier, aptamer-immobilization methods generally follow those of other monolayers, and operate through chemisorption of thiol onto gold. Thus, an aptamer with a thiol end group can be used to form a self-assembled monolayer (SAM) on the gold surface. With respect to metallic surfaces, gold is most commonly used for several reasons [83]. First, gold is air-stable and commercially available from several sources as films and particles. Second, gold binds thiols with a high affinity [83], and the films are stable to complex liquid media containing target biomolecules. Third, one of the most important features of planar gold surfaces is that they are not only easy for aptamer monolayer formation, but are also useful for physically characterizing the structure, composition, and binding properties of the aptamer-based films. Methods that have been used for surface analysis of structure and composition of the aptamer monolayers include X-ray photoelectron spectroscopy (XPS) [84, 85],



Scheme 1 Two-step procedure for direct formation of aptamer mixed monolayers on gold

optical ellipsometry [86, 87], scanning probe microscopy [88–90], reflectance absorption infrared spectroscopy [87, 91], and contact angle measurements [92].

Binding analyses and aptasensors have utilized a number of analytical methods for reading successful association events (Table 1), such as SPR spectroscopy [19, 20, 24], QCM [14, 25], and other mass-sensor methods; however, the most frequently used methods are fluorescence [15, 38, 39, 41, 43, 47, 49, 51, 64] and electrochemical methods [6, 22, 46, 52, 62, 65, 66, 71, 93]. Aptasensors have also been developed using a number of other readout methods of the association event, such as electrochemical impedance [21, 52], enzyme-linked aptamer assay [19, 29], UV [30, 71], and electrochemical indicators [31]. In addition to binding studies, aptamer-based monolayers can also be used to functionalize AFM tips and cantilever tips to study and measure force interactions between the immobilized aptamer and the protein [28].

Because monolayers on gold are an important avenue for immobilization of aptamers onto a surface, use of the appropriate thiol-terminated aptamers is equally important. The design of thiol-tethered aptamers consists of three segments: (1) a thiol (–SH) or disulfide (–SSR) terminus, (2) a linker or spacer, and (3) the aptamer sequence (Fig. 2). To date, thiol-tethered aptamers have only been reported for monolayers on planar gold; however, disulfide-terminated aptamers have been used for coating gold nanoparticles (vide infra). Most thiol-tethered aptamers are obtained commercially as a stable nonsymmetric disulfide, with one sulfur atom linked to the aptamer, and the other sulfur group linked to mercaptohexanol (MCH), i.e., aptamer–linker–S–S–$(CH_2)_6OH$.

To obtain the free thiol from the disulfide precursor, it is necessary to reduce the disulfide. Typically dithiothreitol (DTT) is used, which forms the free thiols for each side of the disulfide, i.e., aptamer–linker–SH and HS–$(CH_2)_6OH$. After reduction, the remaining DTT and MCH is removed by extraction [94], or by using a size-exclusion column [95]. This step is necessary because DTT and MCH are effectively chemisorbed onto the gold surface and will block attachment of the thiol-terminated aptamer. Alternatively, solid-phase DTT can be used, which can be easily separated by filtration [96]. Tris(2-carboxyethyl)phosphine (TCEP) has also been used to reduce the disulfide, and the cleaved MCH can be removed using a MicroSpin™ column [50]. TCEP has the advantage over DTT in that it does not adsorb onto the gold surface and, as such, can be left in the aptamer–thiol solution. Hence, addition of TCEP to the aptamer solution will maintain the aptamer in the thiol form (aptamer–linker–SH) until it is used. In the case of gold particles (discussed next), the disulfide has been used directly For gold nanoparticles (discussed in more detail in the next section), it has been reported that DNA-tethered disulfide compounds [aptamer–linker–S–S–$(CH_2)_6OH$] can be directly used for formation of monolayers on the particles. However, in the case of DNA hybridization studies on surfaces, this method has been reported to provide only half of the maximum density possible [97]. While this does not severely affect nanoparticle aggregation studies (vide infra), the lower density of aptamer may reduce the sensitivity of other analytical formats (e.g., planar formats) [20].

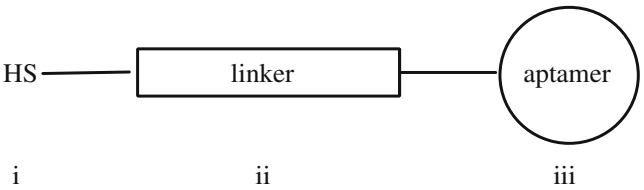


Fig. 2 General structural design of thiol-tethered aptamer

Aptamer monolayer formation is typically carried out by a two-step procedure, as first reported by Herne and Tarlov [82] for monolayer formation of thiol-terminated DNA hybrids. The procedure calls for first cleaning the gold surface by UV–ozone treatment, followed by rinsing with absolute ethanol and by distilled/deionized water, and finally drying in a nitrogen stream. Alternatively, brief immersion in piranha solution (1:2, 30% H_2O_2/H_2SO_4) has been employed in some cases, followed by rinsing the substrate with distilled/deionized water. The cleaned gold surface is then immersed in an aqueous buffer solution of aptamer–thiol for 2 h or more (Scheme 1). The aptamer is a negatively charged molecule with ionizable phosphate groups; thus, in low ionic strength buffer, the surface coverage of aptamers on the gold surface is low, owing to the repulsion between the negatively charged aptamer strands. Under high ionic strength conditions (greater than 400 mM), the intermolecular electrostatic repulsion between neighboring strands of DNA–aptamers is minimized because the charged strands are electrostatically shielded, thus allowing higher surface densities of aptamer–thiol [20, 82]. Thus, aptamer monolayers are generally prepared using a buffer concentration of 1 M. During the monolayer formation step, the aptamers become tethered to the gold surface via the terminal thiol group as intended; however, the aptamers can also nonspecifically adsorb to the gold surface via bonding of the nitrogen lone pairs of the nucleotide bases. To remove the nonspecifically adsorbed aptamers, the gold substrate can be immersed in a second solution of a different thiol not containing the aptamer, referred to as a "co-thiol," which forms a "mixed" monolayer (i.e., a monolayer comprising at least two components). For aptamer systems, the co-thiol must be water-soluble and is generally chosen with a low molecular weight, which affords fast kinetics for displacement of the

weaker bound nitrogen groups (relative to the gold–thiol bond) in the aptamer sequences. These small co-thiol molecules also fill the unoccupied sites on the gold surface, which eliminates nonspecific binding of substrates such as proteins to gold. This process must be controlled in order to avoid displacement of the thiol-terminated aptamer as well and can experimentally be followed by XPS (the free nitrogen in the DNA base shows a peak at approximately 400 eV and the chemisorbed nitrogen shows a peak at approximately 402 eV) [98]. Typically, MCH [$HS(CH_2)_6OH$] is used as the co-thiol [20], but other co-thiols include mercaptoethanol [$HS(CH_2)_2OH$] [19]. Balamurugan et al. [20] used EG_3 [$HS(CH_2)_{11}(OCH_2CH_2)_3OH$] in ethanol as a co-thiol, and found only a low level of aptamer-based thiols were displaced compared with MCH treatment, leaving the highest surface density of aptamer immobilization. Furthermore, the use of EG_3 as the co-thiol provided significantly better resistance to nonspecific protein binding

Thiols on gold particles

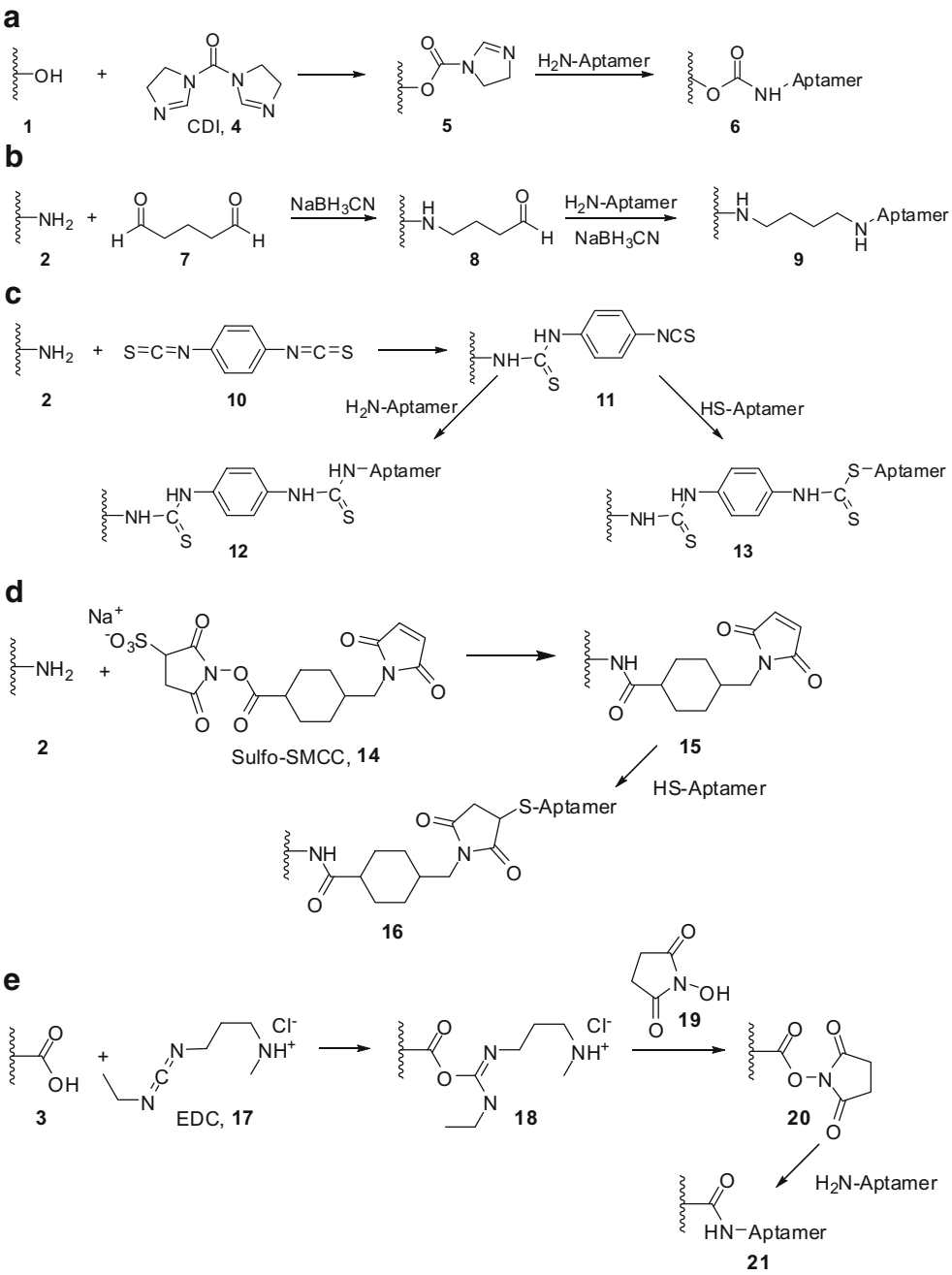
Aptamers have also been immobilized onto the surface of gold nanoparticles, which can be used for sensors through chromatic changes that result from directed aggregation of the nanoparticles following association of the target with its aptamer recognition element [30]. Chromatic sensors are advantageous because they often do not require sophisticated readout apparatus, and as such provide a convenient and simple sensor method. Sensing of molecular targets by receptor–target-directed aggregation of gold nanoparticles makes use of the phenomenon that gold nanoparticles change color from red to blue–purple upon aggregation [99]. This has been recently adapted to aptamer-directed aggregation systems as chromatic sensors [30, 100]. For example, Huang et al. [30] made use of the dimeric protein PDGF as a "cross-linker" for aggregating gold nanoparticles incorporating an aptamer that binds one site on each of the dimeric units of PDGF. The PDGF-binding aptamers were attached to the gold nanoparticles using standard techniques [99], which rely on a standard gold nanoparticle synthesis employing $HAuCl_4$ in a citrate solution [101].

In most cases, the immobilization of aptamers on gold nanoparticles for the experiments reported has been through direct attachment on gold using the asymmetric mixed disulfide [aptamer–S–S–$(CH_2)_6OH$] as received from commercial sources [100]. Immobilization is carried out as a sequential process where the mixed disulfide is first dissolved in a low ionic strength buffer (e.g., 50 mM phosphate buffer) with the ionic strength gradually increased by the addition of concentrated buffer followed by sonication. An incubation time of 20 min is allotted for each buffer addition until the solution reaches 1.0 M; afterward, the solution is left at room temperature overnight. Co-thiol adsorbents have not been reported for aptamer-based applications to these aptamer-coated gold nanoparticles as was the case for planar gold. It may be that non-specifically-bound proteins do not interfere with the aggregation behavior of the particles, or the MCH from the mixed disulfide may serve as a co-adsorbent. Other examples include a complex "dipstick" format reported by Liu et al. [102], who used two different gold nanoparticles: one conjugated to the aptamer and another one conjugated to its complementary DNA via terminal thiol groups using the procedures described above for direct attachment to planar gold surfaces. A last example, by Wu et al. [66], used similar chemistry to attach a thiol-terminated aptamer to a gold nanoparticle immobilized onto a gold surface. Other methods for immobilization of DNA to metal nanoparticles have been reported, and may provide more useful methods for immobilization of aptamers in the future [103].

Covalent attachment to functionally modified surfaces

General chemistry of modified surfaces

Many options for employing different conjugation chemistries are available once a particular surface (e.g., gold, silicate, polymers) has been functionalized with an "active" outer layer. The choice of surface functionalization strategy is dependent on what types of terminal functional groups linked to aptamers are available. Thus far, aptamers have been reported that are linked to amine, thiol, or biotin termini on aptamers, which limits the type of chemistry that can be used for conjugation to these functional groups. Biotin-terminated aptamers can be conjugated to surface-immobilized avidin or one of its derivatives. Covalent attachment of aptamers that have amine or thiol termini has been demonstrated using standard chemical methods previously developed for similar applications of other biomolecules. Most of these aptamer-immobilization methods use one or more of the reaction pathways summarized in Scheme 2, which only shows a subset of all possible conjugation chemistries available [104]. The three most common groups employed for surface attachment are hydroxyl, amine, and carboxylic acid surface functional groups. Hydroxylated surfaces are first modified with carbonyldiimidazole (CDI) to form a reactive intermediate, which forms a stable carbamate bond to an amino-terminated aptamer group (Scheme 2a) [14, 35, 39]. Amine-terminated surfaces provide several options for aptamer attachment. For example, a surface amine group can be modified with glutaraldehyde, a symmetric bifunctional dialdehyde linker, which forms an imine bond (sometimes referred to as a Schiff's base) with an aldehyde (**8**), leaving the other aldehyde free for repeating this chemistry with an amino-terminated aptamer group



Scheme 2 Common reaction sequences reported for covalent attachment of aptamers to surfaces. *CDI* carbonyldiimidazole, *SMCC* succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate, *EDC* 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide

(Scheme 2b) [51, 105]. Symmetrical diisothiocyanates have also been used as bifunctional linkers for attachment of amine-functionalized surfaces to either thiol-terminated or amine-terminated aptamers (Scheme 2c) [32].

Aptamer attachment onto self-assembled monolayers on gold

Gold surfaces can be easily functionalized with SAMs for subsequent conjugation to aptamers. A tremendous advantage of this method is that the underlying monolayer can be devised to prevent nonspecific adsorption of aptamer to the gold surface, which is a particular problem for long oligonucleotides with larger numbers of amine groups (in their base unit) that have a greater probability of nonspecific binding to the gold surface. Furthermore, this method is useful for creating lithographic patterns by exposure to UV light. For example, Gronewold et al. [23] and Schlensog et al. [24] used an 11-mercaptoundecanoic acid monolayer on gold to couple an amine-terminated aptamer using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)/*N*-hydroxysuccinimide (NHS) coupling (Scheme 2e). Li et al. [27] utilized the 11-amino-1-undecanethiol hydrochloride monolayer on gold to immobilize a thiol-tethered aptamer on the

surface using sulfosuccinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (Scheme 1d). This method can also be used to prepare a patterned surface via UV irradiation of the monolayer, which is potentially useful for preparing aptamer sensor arrays on a gold surface for SPR imaging [27]. In another example, Wu et al. [66] immobilized gold particles on a gold surface coated with electropolymerized tyramine using chemistry shown in Scheme 2b (**8**); subsequently, thiol-aptamers were directly attached to the gold nanoparticles as described above [66] Functionalized monolayers can also used to couple avidin (or its derivatives) on the gold surface for conjugation to biotin-terminated aptamers (vide supra).

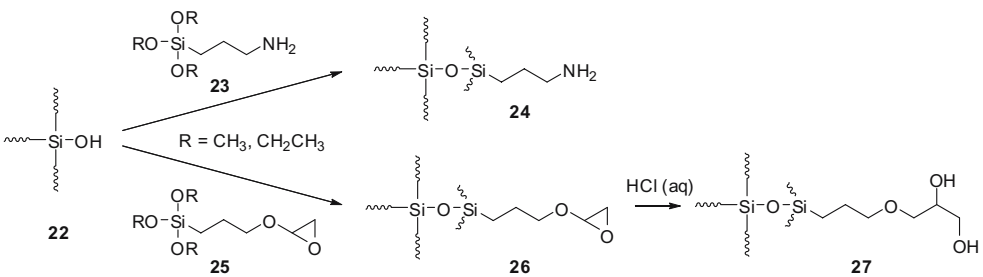
Silicates and silicon substrates

Silicates and silicon have been used as substrates for aptamer immobilization. Silicates are readily available, and are found in formats such as glass slides, capillary tubes, silica gel, or beads [32]. In addition to their low cost and widespread availability, silicate surfaces also offer the advantages of optical transparency for spectroscopic signal recovery and do not quench fluorescence signals as is the case for gold (or other metal) substrates. Covalent attachment to silicon surfaces, such as silicon wafers or AFM tips [36], make use of the silicon oxide outer layer to which silane reagents can be conjugated. Most applications of silicate and silicon substrates first require functionalization of the silanoxide surface using trialkoxysilane (e.g., trimethoxysilane or triethoxysilane) reagents incorporating aminopropyl or glycidoxypropyl moieties at the fourth valence site (e.g., Scheme 3, **23** and **25**). A general procedure begins with exposure of the silica surface for approximately 1–2 h to the desired silane reagent in the vapor phase or in a 2–25% (v/v) solution of the silane reagent in solvents such as acetone [51], xylene [35], or 90% methanol/water [49], followed by washing with pure solvent and drying in air or under nitrogen. The amino-functionalized silicates (**24**) can be used directly for subsequent aptamer conjugation steps; however, the glycidoxy group (**26**) is generally first hydrolyzed with dilute HCl solution to form a dihydroxyl group (**27**), which can be further modified in subsequent steps (Scheme 3). For example, one of the hydroxyl groups of the hydrolyzed glycidoxy group has been reacted with CDI to form a reactive intermediate, which forms a stable carbamate bond when introduced to an amino-terminated aptamer (Scheme 2a) [14, 35, 39].

The most frequently employed method uses aminopropyl silane functionalized surfaces (**24**) for conjugation to aptamers. For example, glutaraldehyde can be linked to the amine via Schiff's base formation to form the reversible imine bond, which can be reduced to form the nonreversible aminoalkyl bond (Scheme 2b) [51, 105]. This leaves a pendant aldehyde group for further conjugation to an amino-terminated aptamer sequence repeating the same chemistry. This method has been applied to aminopropyl silane modified silicon wafers as well [36]. Another method makes use of 1,4 phenylene diisothiocyanate, which is first reacted with the surface amino groups to form an isothiocarbamate intermediate (**11**), which forms a stable thiourea linkage in the presence of an amino-terminated aptamer sequence (Scheme 2c, **12**) [32]. Alternatively, the surface-tethered isothiocarbamate intermediate **11** can be reacted with a thiol-terminated aptamer sequence to link an aptamer via a thiocarbamate linkage (Scheme 2c, **13**). A third strategy makes use of the heterobifunctional linker sulfosuccinimidyl-4-(*N*-maleimidomethyl)-cylcohexane-1-carboxylate (**14**), which first reacts with surface-bound amino groups through displacement of the *N*-hydroxysuccinimidyl group (Scheme 2d, **15**). Subsequently, a thiol-tethered aptamer can be reacted with the pendant maleimide group to form the surface-conjugated aptamer **16** [34].

Biocoatings

The specific and strong interaction between avidin (or one of its derivatives) and biotin has been exploited for surface immobilization of a number of bioreceptors, including aptamers. This method is operationally easy, and mainly requires incubation of the biotin-tethered aptamer with the avidin-coated substrate at room temperature in a buffer solution to give efficient immobilization of biotin to avidin. For example, streptavidin can be either physisorbed or covalently immobilized onto the appropriate substrate. Biotin-tethered aptamers are available commercially, with



Scheme 3 General modification of silanoxide surfaces for subsequent aptamer conjugation

biotin conjugated to the aptamer using standard phosphoramidite chemistry. In addition, avidin (or a related derivative) coated substrates can be purchased, including avidin-, streptavidin-, or neutravidin-coated glass slides [14, 29, 49, 55], streptavidin-coated magnetic beads [53], and the perfusion chromatography column POROS BA, with streptavidin covalently linked to 20-μm flow-through polystyrene particles [40]. Rodriguez et al. [52] fabricated streptavidin-coated indium tin oxide electrodes for the immobilization of biotin-tethered aptamers. SAM surfaces have also been developed to couple avidin to gold substrates as well. For example, Hianik et al. [47, 72] used 3,3′-dithiopropionic acid di(*N*-succinimidylester) (DSP) to form a SAM on gold, which was subsequently used to covalently link random amine groups of avidin via the activated ester. Alternatively, Minunni et al. [25] developed a carboxylate modified 11-mercaptoundecanol SAM on gold for eventual coupling of avidin using EDC/NHS coupling chemistry (Scheme 2e).

Studies utilizing avidin (or derivatives thereof) on silicate have thus far used commercially available products. Similarly, streptavidin on polystyrene microtiter plates has been used as well. However, in general, avidin and its derivatives immobilized on gold are not commercially available; therefore, several covalent procedures have been developed. For example, Minunni et al. [25] immobilized streptavidin onto a gold-coated QCM crystal using a multistep process. First, they reacted the hydroxyl terminus of an 11-mercaptoundecanol SAM with epichlorohydrin to introduce epoxide groups, which are subsequently conjugated with dextran in basic solution. The hydroxyl groups of the dextran were carboxymethylated using bromoacetic acid, and finally the strepatavidin was covalently coupled to the carboxylic acid groups of the dextran using a combination of EDC and NHS (Scheme 2e). After washing with the immobilization buffer, the biotinylated aptamer for binding HIV-1 Tat protein was incubated for 20 min to achieve aptamer conjugation [25]. A more direct route to aptamer immobilization onto a gold-coated QCM crystal was reported by Liss et al. [14], who activated the gold surface with DSP to which streptavidin was directly coupled, then conjugated to biotin-tethered aptamers that bind IgE. Improved specificity and sensitivity was found utilizing this method versus direct attachment of amine-terminated IgE aptamers to a DSP monolayer. This method was also used by Hianik et al. [72], who found greater sensitivity in QCM measurements in comparison with other immobilization techniques such as secondary attachment to a DSP monolayer, or avidin-modified dendrimers on the gold surface. In the latter method, glutaraldehyde was used to conjugate avidin to a G1 polyamidoamine dendrimer (Scheme 2b) co-immobilized on the gold surface with hexadecanethiol.

Other substrates used to immobilize aptamers

Sensors have been developed incorporating aptamers into a single-walled carbon nanotube (SWNT) FET. The small size of the aptamer has a tremendous advantage over traditionally used antibodies in that the binding event can occur within the electrical double layer (i.e., approximately 3 nm) in millimolar salt concentrations. For this type of sensor, So et al. [16] followed the methods of Chen et al. [106], who first modified the sidewalls of the SWNT-FET using CDI–Tween, which has the reactive CDI moiety covalently attached to the surfactant Tween. Tween adsorbs to the SWNT through hydrophobic interactions through the surfactant tail, leaving the pendant CDI group available for reaction with the amine group on the 3′ terminus of the thrombin aptamer. Overall, the attachment chemistry is the same as that used earlier for covalent linking of aptamers to surfaces with hydroxyl groups (Scheme 2a). Greater sensitivity was obtained for a similar carbon-nanotube-based FET that incorporated an aptamer that binds IgE. Immobilization chemistry followed conceptually similar procedures; first, 1-pyrenebutanoic acid succinimidyl ester was hydrophobically adsorbed to the carbon nanotube via the pyrene moiety with subsequent amide formation from the activated ester, which was reacted with an amine group (Scheme 2e) and attached to the 5′ terminus of the IgE aptamer [17].

There has been interest in the development of "bioactive paper" which can detect, repel, or deactivate pathogens. In contrast to antibodies and many enzymes, DNA aptamers may survive dehydration on paper surfaces for paper-immobilized biosensing agents. Procedures reported by Su et al. [41] described immobilization of fluorescently labeled ATP aptamers on cellulose and cellulose powder through covalent linkages and physisorption, respectively. For the covalent linkage, the regenerated cellulose membrane was first oxidized with $NaIO_4$ to generate aldehyde groups on the surface of the membrane. Following Schiff's base formation with an amine-terminated aptamer, the mixture was reduced in $NaCNBH_3$ solution for 10 h in the dark and washed. Physisorption was carried out on microcrystalline cellulose (MCC) by mixing a fluorescently labeled aptamer solution in the buffer with the MCC suspension in water for 24 h at room temperature with continuous shaking. The suspensions were centrifuged and the supernatants were removed and the amount of aptamer physisorbed on the MCC was determined using fluorescence measurements. However, in rebinding assays it was determined that the physisorbed aptamer did not remain immobilized and therefore the covalent strategy was necessary.

Lin et al. [15, 43] have incorporated aptamer sequences into DNA tiles, which support the aptamer after the self-assembly of the two-dimensional DNA arrays. The DNA tile is formed by complementary annealed DNA oligonu-

cleotides, which self-assemble into DNA branched-junction blocks (or so-called DNA tiles), which are first constructed in solution and subsequently deposited onto glass slides. This method has been extended to the detection of multiple targets using a color-coded DNA-tile array that colorimetrically indicates which of multiple targets are bound.

A simple procedure for capping quantum dots with the thrombin-binding aptamer HD-1 has been reported by Choi et al. [107] that not only solubilizes the quantum dots, but also stabilizes them against aggregation for several months. The aptamer-capped PbS quantum dots were prepared by incorporating the unmodified (i.e., no linker or terminal functional groups) HD-1 aptamer with lead acetate in a sodium sulfide solution to obtain particles with diameters of 3–6 nm. Sensing by these particles occurs through a novel photoluminescent transduction mechanism that is specific for thrombin even in the presence of other proteins that adsorb, but apparently do not modulate photoluminescence. Yamamoto-Fujita and Kumar [64] have demonstrated an aptamer-immobilization strategy on the surface of an S-BIO®PrimeSurface®, a cyclic olefin copolymer surface with random copolymerization of 2-methacryloyloxyethyl phosphorylcholine, *n*-butyl methacrylate, and *p*-nitrophenyloxycarbonyl poly(ethylene glycol) methacrylate (MEONP). An amine-terminated HIV-1 Tat aptamer fragment was immobilized onto the surface of this polymer by substitution of the *p*-nitrophenyloxy group of MEONP by the amine-tethered aptamer to give a covalent amide bond between the polymer and the aptamer. Cheng et al. [108] covalently attached an aptamer elicited toward PSMA to polymer-based nanoparticles for targeted drug delivery. The nanoparticles were derived from a block copolymer comprising polylactic acid-*co*-glycolic acid and poly(ethylene glycol), and the amine-terminated aptamer was covalently bound using chemistry shown in Scheme 2e. In an example by Ho et al. [109, 110], the HD-1 thrombin aptamer was noncovalently complexed through charge–charge interactions to cationic imidazolium-modified poly(3-alkoxy-4-methylthiophene) in solution, which transduces an optical or fluorometric signal upon binding to thrombin.

Immobilization methods and critical parameters

Linkers

As shown in Fig. 3, the design of aptamers for surface immobilization includes a linker group connected to the terminal functional group (e.g., thiol, amine, or biotin). The function of the linker is to present the aptamer above the surface (e.g., gold, silicate) to promote accessibility of target analytes to the aptamer binding site. As shown in Fig. 3, there can be up to three different modular components in the linker that connect the aptamer to the terminal functional group. In all cases, the linker incorporates an alkyl group directly attached to the terminal functional group, followed in some cases by an oligoethylene glycol unit, and/or an oligonucleotide spacer. Each of the modular units is connected via the bonding unit "X," which is usually a phosphate group introduced as a result of phophoramidite chemistry used in automated DNA synthesis. The most commonly used alkyl group is a hexamethylene linker [–$(CH_2)_6$–] [16, 20, 22, 26, 28, 64]; however, there have been a few cases were trimethylene [–$(CH_2)_3$–] [23], nonamethylene [–$(CH_2)_9$–] [45] and dodecane [–$(CH_2)_{12}$–] [45] linkers were employed.

In all cases but one [22], an oligonucleotide spacer has been incorporated as part of the linker group before final attachment of the aptamer at the end. The choice of an oligonucleotide as the spacer group arises naturally from the ability to incorporate a number of nucleotides during automated DNA/aptamer synthesis, and most often comprises a variable number of thymidine (T) groups, which exhibit minimal nonspecific adsorption to various surfaces relative to the other DNA bases [111]. The number of T nucleotides in the spacer varies widely from T_1 to T_{20}, which is also the case for linker designs in the literature for DNA hybridization studies on surfaces [19, 20, 28, 71, 73]. Other oligonucleotides have been used as spacer groups; for example, Basner et al. [28] used a random 33-mer as a spacer to link the 15-mer thrombin HD-1 aptamer to gold-coated AFM tips. Shorter sequences were reported by Liss et al. [14], who used 5′-GCGC-3′ as a spacer for the IgE aptamer; and Mir et al. [45] utilized the sequence 5′-CCAAC-3′ to link the HD-1 thrombin aptamer to a gold surface.

Comparisons have been made in a few reports that illustrate how inclusion of a spacer group improves the overall dissociation constant as well as the amount of protein that specifically binds to the surface. For example, Liss et al. [14] reported an improvement in the dissociation constant of IgE to its aptamer of 3.6 nM (versus 8.4 nM without a spacer) by the addition of the 5′-GCGC-3′ spacer. Centi et al. [44] demonstrated that incorporation of a 20-base long oligothymidine spacer before the HD-1 thrombin aptamer increases thrombin binding twofold compared with binding for the aptamer without the oligothymidine spacer.

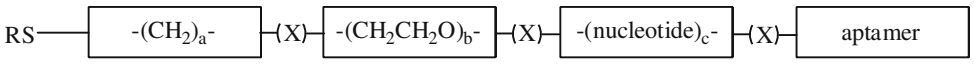


Fig. 3 Modular components in the overall design of an aptamer linked to a terminal functional group

Recently, Balamurugan et al. [20] reported the use of a hexaethylene glycol linker appended to a five-base oligothymidine spacer [HS–$(CH_2)_6$–OPO_3–$(CH_2CH_2O)_6$–TTTTT–aptamer], which increased the amount of thrombin bound to the sensor surface by a factor of 4 compared with using only the five-base oligothymidine spacer. The oligoethylene glycol spacer was selected owing to the fact that oligoethylene glycol modified surfaces are known to prevent nonspecific adsorption of proteins. Hurst et al. [112] have also reported that oligoethylene glycol linkers can be used to improve the hybridization efficiency of DNA immobilized on surfaces. Thus, the inclusion of the oligoethylene glycol modular component appears to be a general method for enhancing binding properties of surface-bound DNAs, including aptamers.

Comparison of 5′ versus 3′ attachment of aptamers

The linker and functional group termini can be attached to the solid support at either the 5′-end or the 3′-end of the aptamer. In the literature, both positions have been reported as having been used for aptasensor development. However, only a few reports have looked at differential effects of the two types of end functionalization. Cho et al. [49] examined this effect for the biotin-terminated aptamer for thrombin, lysozyme, IgE, and ricin. Their results showed that it depends on the particular aptamer. The anti-lysozyme and anti-IgE aptamers showed 14–30% higher sensitivity following 3′-end immobilization, whereas anti-ricin and anti-thrombin aptamers showed 26–28% higher sensitivity following 5′-end immobilization.

Sensor-surface regeneration

The regeneration of binding surfaces, i.e., the removal of the target analyte, is important for reusable biosensors, but can be difficult to achieve. Ideally the regeneration solution would only disturb the interactions of the aptamer with the target analyte, without degrading the aptamer. Many strategies have been reported for aptasensors and examples of reagents for aptamer regeneration have been identified. General strategies include the use of temperature, concentrated salt solutions, acidic or basic solutions, chaotropic reagents such as urea or guanidinium hydrochloride, surfactants such as sodium dodecyl sulfate, chelating agents such as EDTA, or a combination of two or more of the abovementioned regenerating agents [113]. As mentioned in the “Introduction,” aptamers are known to undergo denaturation/regeneration multiple times, but antibodies suffer from permanent degradation [14, 54].

In one report, simply using hot water was said to be successful for the removal of adenosine from its immobilized aptamer, which maintained 90% of its original response level after 40 regeneration cycles [66]. The use of concentrated salt concentrations for denaturing the analyte, aptamer, or both is a relatively mild method for disrupting binding interactions; for example, 2 M NaCl has been used to remove thrombin from its immobilized aptamer [19]. Another general strategy for disrupting noncovalent interactions of analytes bound to aptasensors is the use of acidic and basic solutions. For example, Schlensog et al. [24] used a 100 mM NaOH aqueous solution to remove thrombin from an HD-1 immobilized aptamer surface; and Kawde et al. [53] used 20 mM aqueous NaOH to regenerate a lysozyme-binding aptamer surface. Minunni et al. [25] reported that a 12 mM NaOH solution with 1.2% EtOH (v/v) allowed complete regeneration of HIV Tat 1 aptasensor using two sequential 30-s incubation steps when immersed in this solution, which was reproducible 15 times on the same sensor without noticing loss of sensitivity.

Alternatively, more specialized regeneration agents can be used. For example, surfactants can be used at levels below the critical micelle concentration to wash analyte away from the aptamer. Lai et al. [63] successfully recovered 95% of the original aptasensor signal for PDGF using a 4-min incubation period in an aqueous solution of 10% (v/v) sodium dodecyl sulfate, followed by rinsing with buffer. Another option was demonstrated by Liss et al. [14], who used 50 mM of the metal-chelating agent EDTA for removal of IgE from its aptasensor, presumably by removing metals responsible for secondary or tertiary structure important for molecular recognition by the aptamer. Chaotropic reagents, such as urea or guanidinium chloride, which disrupt noncovalent molecular interactions, can be used for the regeneration of aptasensor surfaces. A number of researchers have used aqueous solutions of 6 M guanidium chloride to regenerate HD-1 thrombin aptamer sensor surfaces [16, 22, 39, 51]. Savran et al. [26] used a 7 M aqueous urea solution to regenerate a Taq DNA polymerase aptamer sensor surface.

More specific reagent solutions can be used for aptasensor regeneration; for example, thrombin has been released from its immobilized aptamer using the competitive inhibitor hirudin, an oligopeptide which binds to the exosite I position of thrombin [114]. In a similar example, thrombin has actually been used to displace enzyme-labeled thrombin, which has been demonstrated to have 1 order of magnitude less affinity for the aptamer than native thrombin [29].

If a single regeneration agent is not sufficiently effective, a combination of regeneration reagents can be used. For example, Kirby et al. [54] used a regeneration solution comprising 100 mM sodium citrate, 10 mM EDTA, and 7 M urea at pH 5.0 in order to regenerate a lysozyme aptamer surface and an anti-ricin aptamer surface. Liss et al. [14] used the combination of acidic 0.2 M glycine–HCl buffer solution at pH 2.2 with 6 M urea as a regeneration solution

for an IgE aptamer surface. In all cases, the general procedure for regeneration of the aptasensor by removing the bound target species is to first wash with buffer, then treatment with the regeneration solution, followed by a final rinse with buffer solution.

Conclusion

The growing area of aptamer-based diagnostics can open new avenues toward the development of analytical tools requiring molecular recognition, and recently there has been an increasing interest to develop aptamer arrays for multi-analyte detection of different biomarkers, such as proteins and DNAs simultaneously. Perhaps the major limiting factor is the current limited number of aptamers directed for different targets, which may change in the future. Immobilization of aptamers on surfaces is an important first step for the development of these analytical tools, clearly pointing out the importance of understanding and optimizing the method for this step. Furthermore, different analytical methods require different substrates, including, but not limited to, gold, silica and silicon surfaces, carbon nanotubes, quantum dots, carbohydrates, and polymer substrates. These substrates can be used in a number of different formats as well, such as monolayers, DNA titles, chemisorption, covalent coupling, and biocoatings. The choice of substrate is predicated predominately by the readout format of the molecular association or application area, such as SPR, QCM, atomic force microscopy, cantilever-based methods, capillary electrophoresis, affinity chromatography, and electrochemical and fluorescence methods. This review summarized important chemical protocols for the immobilization of aptamers and compared different approaches for the immobilization. Particular emphasis was placed on presenting examples involving the optimization of the linker design and immobilization procedures. This included the identity of the molecular structure of the linker, the length of the linker, and its point of attachment, all of which are necessary to place the aptamer above the surface in a "solution-like" environment for the optimum binding of target molecules. In addition, methods used for the regeneration of aptasensors were reviewed as well. This was a subject often overlooked, but a necessary consideration for creating multiple-use analytical sensors for real-time monitoring. Overall, this review should be a valuable resource for determining the best method for initial development of aptamer-based diagnostics.

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