Chapter 2

Aptamer Arrays

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Abstract

In less than 40 years, aptamers have consolidated their role in biosensor development. Chemically related to nucleic acid probes, production of aptamers against targets of various sizes and compositions places them as ideal capture elements, alternative to more consolidated molecules such as antibodies. Thanks to their chemical simplicity and production, as well as their unique characteristics, aptamers have been successfully integrated in several innovative approaches. The incorporation of aptamers into the existing microarray technologies has lead to the reporting of various detection strategies, including direct fluorescence detection of fluorescent reporters, fluorescence anisotropy, FRET, SPR imaging, and electrochemical detection.

Key words: Aptamer, Aptamer array, Aptasensor, Reagentless detection

1. Introduction

1.1. Aptamers: Description, Advantages, and Drawbacks

Aptamers are artificial nucleic acid ligands, selected in vitro from DNA/RNA random pools against specific nonnucleic acid targets. The reported aptamers have shown equal or higher affinity and specificity for their targets than their equivalent antibodies. Furthermore, aptamers have been selected against a vast variety of targets, including small molecules, drugs, peptides, and hormones, and also complex objectives such as proteins, spores, and whole cells, showing surprising versatility compared to other biorecognition components (1–6). The fact that aptamers are selected and produced in vitro eludes the use of animals and related ethical concerns, ensures no batch-to-batch variation, and allows selection under nonphysiological conditions and toward small molecules and toxins. The whole procedure is potentially automated and easier, quicker, and cheaper than antibody production. From an integration point of view, aptamers are

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smaller and less complex than antibodies (5–25 kDa vs. 150 kDa), and are easier to modify during or after synthesis, favoring immobilization and labeling. In addition, the unique chemical and structural characteristics of nucleic acids permit aptamer reversible denaturation and thus the design of truly reusable devices. The main concerns regarding the real applicability of aptamers, related to inherent nucleic acids properties such as sensitivity to nuclease attack and chemical simplicity, are being circumvented in the shape of spiegelmers and chemically modified aptamers. The fact that each single DNA/RNA sequence can adopt multiple conformation, reducing assay efficiency and increasing cost, can be also minimized by careful folding characterization and assay optimization.

1.2. Aptamer Arrays

The first reports on aptamer arrays exploited either optical fiber arrays (7, 8), or agarose beads deposited in the wells of micromachined flow chips (9). Later works, however, used glass slides to produce aptamer arrays in a variety of assay formats that, taking advantage of detection strategies already proved for DNA, permitted detection of an assortment of targets (Fig. 1). Gold and

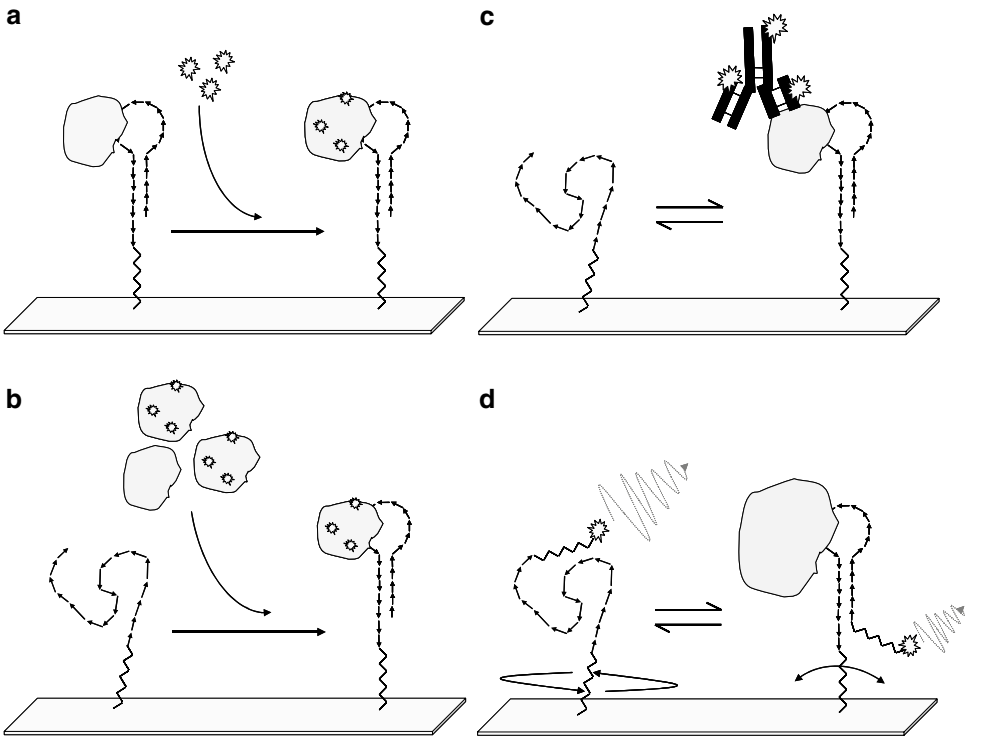

Fig. 1. Scheme of different fluorescence aptameric array assay formats. (**a**) Target capture, followed by labeling with a reactive fluorophore. (**b**) Sandwich assay using aptamer and labeled antibody as capture and detector biocomponents. (**c**) Competition between native and labeled target variants. (**d**) Molecular beacon format exploiting the use of a labeled aptamer immobilized on surface; target binding induces changes in fluorophore emission.

coworkers introduced the use of photoaptamers that, remaining covalently bound to their targets after photoactivated cross-linking, allowed of highly stringent washes and efficient removal of non-specific binders (10–12). In view of the enhanced signal-to-noise ratios and low detection limits registered, the SomaLogic team developed a 17-plex photoaptamer array on activated glass slides (10). Detection was based in a sandwich format, using either NH-reactive fluorophores or fluorophore-labeled antibodies, with detection limits below 10 fM for several analytes measured in 10% serum.

Ellington et al. reported on a number of arrays manufactured using the lysozyme, ricin, IgE, and thrombin RNA/DNA aptamers on streptavidin slides (13–15). As a novelty, they defined a "universal buffer" in which the four aptamers retained acceptable affinity for the fluorophore-labeled analytes at concentrations over seven orders of magnitude (10–10^7 pg/mL).

In a different approach, the team at the company Archemix immobilized fluorescein-labeled RNA/DNA aptamers on streptavidin slides (16). In this format, target binding is directly measured as changes in fluorescence polarization anisotropy in a completely reagentless format. The system detected and quantified four different proteins in the presence of serum and bacterial cell lysates. Alternatively, Lin self-assembled the aptamer into high-density nanoarrays, following modification with a fluorescent nucleotide analog near the target-binding site (17). Target binding generated increase in fluorescence, measurable by confocal fluorescence microscope imaging down to the low nanomolar range. Finally, Bera Aberem spotted onto silanized slides an aptamer, Cy3-labeled and hybridized with a chromic, cationic, water-soluble polythiophene (18). In the absence of target, the polymer quenched Cy3 emission; target binding induced polymer displacement and fluorescence increase.

Alternative approaches include the development of surface plasmon resonance imaging (SPRi) and electrochemical aptamer arrays (19–21).

2. Materials

2.1. Physical Support

1. Microscope slides, modified so as to incorporate on surface reactive groups (amino, aldehyde, maleimide, thiol, and epoxy) or components (streptavidin and biotin) are provided, among others, by Xenopore Corp. (Hawthorne, NJ), Pierce–Thermo Fisher Scientific Inc. (Rockford, IL), Corning (Corning, NY), Nanocs Inc. (New York, NY), Nalge Nunc International (Rochester, NY), Genetix (New Milton, UK),

and Erie Scientific (Portsmouth, NH). In some cases, customized coated glass slides can be produced.

2. Streptavidin-coated agarose and silica beads can be obtained from Sigma-Aldrich and Pierce-Thermo Fisher Scientific Inc. (Rockford, IL).
3. Gold-coated slides and substrates can be purchased from a number or companies, including Aldrich, Platypus Technologies (Madison, WI), Phasis (Geneva; Switzerland), Nanocs Inc. (New York, NY), and Asylum Research (Santa Barbara, CA); and Gentel Biosciences, Inc. (Madison, WI) produces gold-coated substrates especially optimized for SPRi.
4. Electrodes of different geometries, composition, and complexity can be obtained from providers such as BASi (West Lafayette, IN), DropSens (Oviedo, Spain), BVT Technologies, a.s. (Brno, Czech Republic), Palm Instruments BV (Houten; The Netherlands), Applied BioPhysics (Troy, NY), and Princeton Applied Research (Oak Ridge, TN).

2.2. Aptamer Production and Manipulation

1. Once its sequence is known, an aptamer is produced by classical DNA/RNA synthesis. In the lack for synthesis facilities, the aptamer can be ordered to any company commercially providing oligonucleotides. In this case, double check the sequence ordered and ensure that appropriate spacers/linkers are being added to the extreme chosen for aptamer immobilization/modification (see Note 1).
2. For novel aptamers, at least three companies produce customized aptamers: RNA-tec (Leuven, Belgium), AptaRes (Luckenwalde, Germany), and Nascacell (Munchen, Germany).
3. Reconstitute lyophilized aptamers to 100–500 μM using sterile water or binding buffer. Store frozen in small aliquots in order to avoid repeated thaw-freeze cycles (see Note 2).
4. The most widely used binding buffers, which often correspond to the solutions employed in aptamer SELEX, are the following ones:
   - PBS (10 mM phosphate buffer, 138 mM NaCl, 2.7 mM KCl, pH 7.4).
   - PBS, 1–5 mM $MgCl_2$.
   - 5 mM NaH_2PO_4, 5 mM KH_2PO_4, 2 mM $MgCl_2$.
   - 10–100 mM Tris–HCl, pH 7.5, 50–150 mM NaCl, 0–5 mM $MgCl_2$.
   - 20 mM Tris–acetate, pH 7.4, 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$.
   - 10–50 mM Hepes, pH 7.4, 0–150 mM NaCl, 0–5 mM KCl, 0–5 mM $MgCl_2$.

5. When possible, sterile plasticware, pipette tips with filter, and separate pipettes (specific for work with DNA/RNA) should be used. If working with RNA aptamers, additional measures should be considered such as using RNAse-free material, treating all solutions and surfaces with an RNAse inhibitor (such as DEPC), and working in a space physically separated and labeled for work with RNA.

2.3. Aptamer Immobilization and Labeling

1. Use an aptamer produced or modified with an amino/thiol/biotin group at one of the extremes, incorporating the appropriate spacer/linker (see Note 1).
2. Unless otherwise stated, use the following solutions. Binding buffer: sterile binding buffer of choice (see Subheading 2.2). Blocking buffer: binding buffer supplemented with 0.1% Tween (see Note 3). Washing buffer: binding buffer containing 0.05–0.1% Tween.
3. Amine-silane-based aptamer immobilization. Activation solution (1): 0.05 M dioxane solution of carbonyldiimidazole. Activation solution (2): 5% (v/v) glutaraldehyde in PBS, pH 7. Washing solution (1): dioxane and diethyl ether. Washing solution (2): PBS, pH 7. Chemical blocking: 1 M ethanolamine, pH 8.5, prepared in sterile water or binding buffer.
4. Thiol-silane-based aptamer immobilization. Chemical blocking: 0.1 M mercaptoethanol prepared in sterile water or binding buffer.
5. Self-assembly of thiolated aptamer on gold surfaces. Use 0.1 M in KH_2PO_4, pH 3.8.
6. Aptamer labeling with amine-reactive reagents. Reaction buffer: freshly prepared 0.1 M tetraborate buffer, pH 8.5 (see Note 4). Alternatively, 0.1 M sodium bicarbonate buffer pH 8–9 can be used (see Note 5). For labels/reagents insoluble in water, dissolve in dimethyl sulfoxide (DMSO) or in dimethyl formamide (DMF). *Caution*: DMF is a possible carcinogen and should be manipulated wearing protections.
7. Aptamer conjugation to COOH-bearing electroactive labels. Reaction buffer: 10 mM PBS or HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.4. For labels/reagents insoluble in water, dissolve in DMSO or in DMF. *Caution*: DMSO and DMF are possible carcinogens and should be manipulated wearing protections. Cross-linkers: 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and *N*-hydroxy succinimide (NHS).
8. Aptamer precipitation. 3 M NaCl, cold ethanol, and cold 70% (v/v) ethanol.

9. Aptamer modification by transcription (see Note 6). Dithiothreitol (DTT), each of the four unmodified ribonucleotides, modified ribonucleotide of choice, DNA-dependent RNA polymerase (e.g., SP6, T3, or T7), and RNAse-free DNAse. Transcription buffer (often provided 5×): 60 mM Tris–HCl, pH 8.0, 10 mM NaCl, 40 mM $MgCl_2$, and 8.0 mM spermidine.

2.4. Target Labeling

1. For labels/reagents insoluble in water, dissolve in DMSO or in DMF. *Caution*: DMSO and DMF are possible carcinogens and should be manipulated wearing protections.
2. Reaction buffer for succinimidyl and STP esters: 0.1–0.2 M sodium bicarbonate buffer, pH 8.3 (see Note 5).
3. Reaction buffer for TFP esters, isothiocyanates, and sulfonyl chlorides: 0.1–0.2 M sodium bicarbonate buffer, pH 9.0 (see Note 5).
4. Stop reaction: freshly prepared 1.5 M hydroxylamine hydrochloride, pH 8.5.
5. Gel filtration/desalting columns: G25, P-10, D-Salt, or similar (provided by Pharmacia; Supelco-Sigma Aldrich; GE Healthcare; and Thermo Scientific Pierce among others).
6. Centrifuge-filter devices and spin columns: Microcon, VectaSpin, Centri-Spin, Centrex, Quick-Spin, or similar (provided by Millipore; Whatman; Princeton Separations; Aldrich; Roche Applied Science; and GE Healthcare).

2.5. Regeneration Solutions

Table 1 summarizes the most widely used regeneration solutions.

3. Methods

Aptamer immobilization for microarray production can take advantage to certain extent of the fabrication and spotting techniques already developed for the more consolidated DNA microarrays. However, as target binding absolutely depends on aptamer folding into the appropriate conformation, aptamers performance can be impaired following immobilization. For this reason, using long spacers is essential for optimal aptamer performance, with length and chemical composition of the spacer strongly influencing detectability (see Note 1).

Even if aptamers, modified with the appropriate reactive groups, can be successfully conjugated on silanized glass/silica or self-assembled on gold-modified slides, most authors chose using streptavidin-coated surfaces that provide better blocking against nonspecific adsorption and a much faster and easier aptamer immobilization procedure. For this purpose, the aptamer can be

Table 1
Aptamer regeneration solutions reported by several authors

Reagent type	Regeneration solution	Target type
Buffer	Binding buffer	Small molecules
	Water	Small molecules
Temperature	Hot water, 80°C	Small molecules
Concentrated salt	2 M NaCl	Protein
pH	10–100 mM NaOH	Protein
	12 mM NaOH, 1.2% EtOH	Protein
	0.2 M Glycine–HCl, pH 2.2	Protein
	0.1 M HCl	Protein
Chaotropic	7–8 M Urea	Protein
	5–6 M Guanidinium hydrochloride	Protein
Chelating	50 mM EDTA	Protein
	10 mM EGTA	Small molecules
Detergent	0.03–10% SDS	Protein
Mixture	0.1 M Sodium citrate, 10 mM EDTA, 7 M Urea, pH 5.0	Protein and small molecules
	1 M NaCl in 0.02% Triton X-100	Protein
	7 M Urea in 25 mM Tris–HCl for 3 min at 70°C	Protein

obtained commercially biotinylated, or chemically or enzymatically biotinylated in-home. Although polylysine slides work well for the immobilization of oligonucleotide probes and nucleic acid detection, they have been reported to be unsuitable for aptamer immobilization. This is likely to be due to aptamer unfolding following electrostatic interaction between its negatively charged phosphodiester backbone and positively charged polylysines.

Assay performance, on the other hand, strongly depends on the binding buffer of choice. In this respect, the unique characteristics of aptamers make it difficult to define universal assay conditions. For this reason, most authors just use a buffer similar to that used for aptamer selection. Nevertheless, it has been demonstrated that buffer optimization can generate improved results or facilitate assay performance. Once an aptamer optimal binding buffer is defined, all the assay steps, from aptamer immobilization to target detection, should be carried in that buffer.

Fluorescence aptamer arrays mostly exploit competition, often using a suboptimal fluorophore-labeled target, or sandwich assay formats (Fig. 1). Alternatively, the molecular beacon format takes advantage of an immobilized aptamer whose free extreme has been modified with a fluorophore. In this case, aptamer rearrangement induced by target binding translates into a change

of either fluorescent emission or fluorescence polarization anisotropy. SPRi and impedance electrochemical arrays, on the other hand, are based in truly reagentless formats, in which target binding directly translates into changes in signal transduction (Fig. 2a, b).

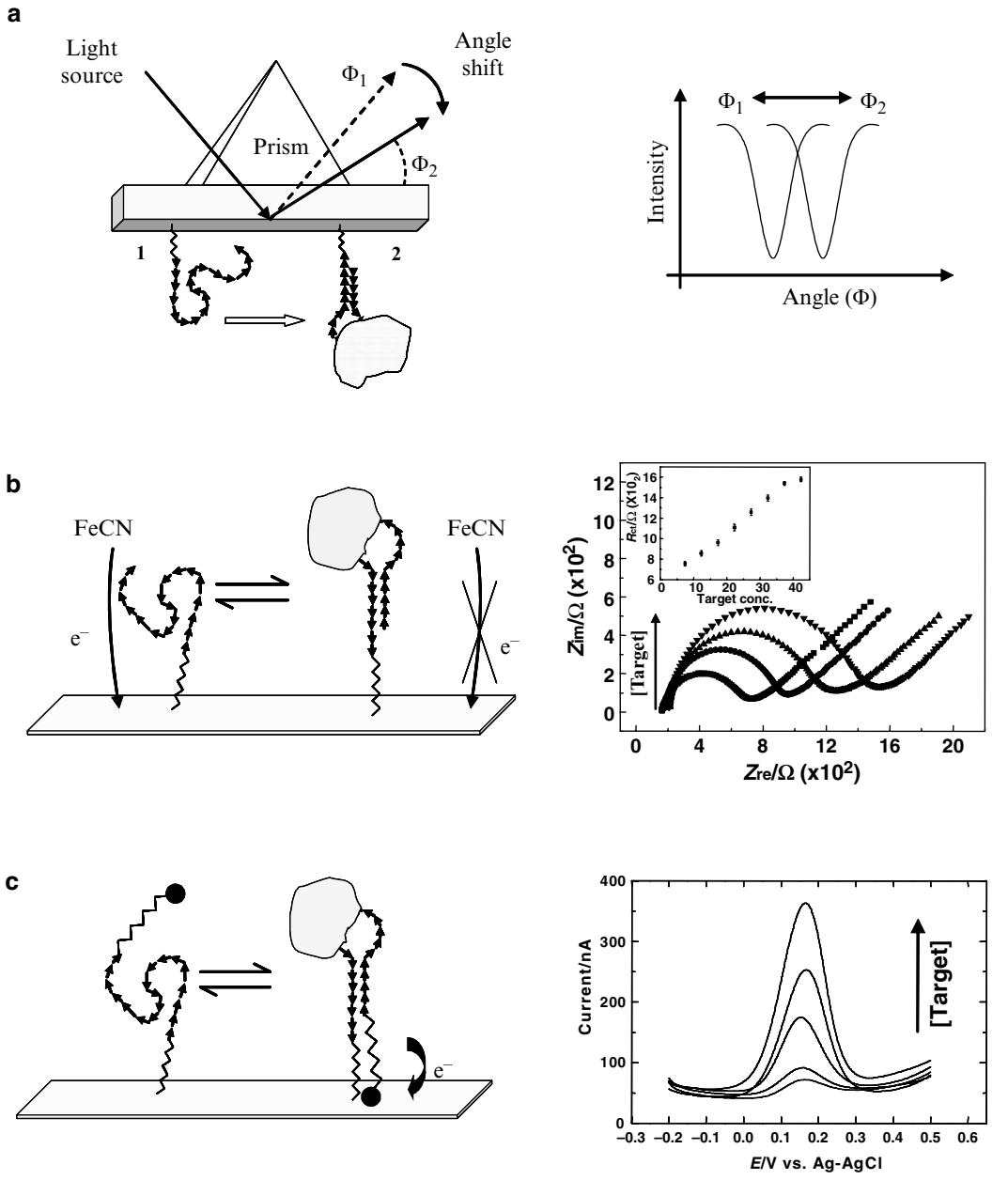

Fig. 2. Scheme of different SPRi and electrochemical aptameric array assay formats. In SPRi (**a**) and impedance sensing (**b**), target capture translates into direct label-less signal transduction. (**c**) Among the various electrochemical detection strategies, the molecular beacon format exploits the use of an aptamer immobilized on surface, which bears on its free extreme an electroactive label. Target binding correlates with a change in distance between surface and label, and thus change in electron transfer rate, in a reagentless assay format.

Other electrochemical sensing strategies, such as the amperometric or voltametric approaches, remind their fluorescent counterparts, with the exception that electroactive labels are used in place of classical fluorophores (Fig. 2c). In these specific cases, aptamer self-assembly onto gold electrodes is preferred to streptavidin capture. In the following sections, we summarize the most used protocols for aptamer immobilization and modification, and for the production of labeled targets. We will finish with a discussion on optimization of binding conditions and sensor regeneration.

3.1. Glass Slide Pretreatment and Silanization

1. Sequentially sonicate the glass/silica slide for 5 min in chloroform, 5 min in acetone, and five more minutes in isopropyl alcohol.
2. Extensively rinse with sterilized water and sonicate for 1 h in 3 M NaOH.
3. Thoroughly rinse with sterile water and dry under a nitrogen flow.
4. Dissolve the silane of choice in ethanol or methanol to final concentration 0.5% (v/v).
5. Silanization can be carried out by immersion of the glass surface into the silane solution for 15–60 min at room temperature. Alternatively, drop the solution on a compartment, place the slide on top, without touching the liquid with the aid of a physical support, and incubate overnight at room temperature for vapor deposition.
6. Rinse with ethanol or methanol, dry, and bake for 15 min at 110°C.

3.2. Pretreatment of Gold-Coated Surfaces and Gold Electrodes

3.2.1. Gold-Coated Slides

1. Gold-coated slides have to be handled carefully as to avoid scratching/damaging the gold cover. Wear gloves as not to leave fingerprints.
2. Although the best option is to seek for the provider's advice, high-quality slides are usually sufficiently cleaned by wiping the surface with an optical wipe, cotton-tipped swab, or a smooth brush moistened in high-purity alcohol.
3. If the facilities are available, the slides can be treated with UV/ozone for 10–25 min, or by oxygen or argon plasma etching (time to be experimentally determined), followed by ultrasonic washing for 1 min in isopropanol and/or extensive washing with water.
4. Reports exist on treatment of gold-coated surfaces with piranha (3/1 v/v H_2SO_4/H_2O_2. Caution!) or chromic acid for up to 30 min, but some substrates do not survive in good shape in these harsh conditions and can present gold detaching.

3.2.2. Electrodes

1. In addition to cleaning (as described in the previous section) to remove organic, inorganic, and ionic species not native to the surface, electrodes are often electrochemically activated as to promote more uniform metallic surfaces (e.g., to reduce the concentration/thickness of oxide on gold and/or remove adsorbed components).
2. Macroelectrodes (such as disk electrodes) are regularly cleaned by mechanical polishing with diamond, alumina, or silicon carbide of different particle sizes (BASi supplies 0.05 μm alumina polish and 1, 3, 6, and 15 μm diamond polishes), followed by ultrasonic washing with alcohol or water according to the provider's instructions.
3. Most electrodes can be electrochemically activated by cyclic voltammetry. For gold screen-printed electrodes, cycle between 0 and +1.4 V at scan rate 100 mV/s in 0.1 M H_2SO_4 until a sharp stable peak of surface gold oxide reduction is obtained (10–20 cycles in most cases). For microfabricated electrodes, cycle the potential from 0.8 to –1.5 V (vs. Ag/AgCl) in 0.1 M KCl until reproducible voltammograms are obtained. Hydrogen evolution at the cathode and oxygen generation at the anode accompany cleaning.
4. The electrode state can be traced by cyclic voltammetry in 1 mM ferrocyanide, 0.1 M KCl at the beginning, and at the end of each experiment.

3.3. Aptamer Immobilization (See Note 7)

3.3.1. Immobilization of Biotinylated Aptamer on Streptavidin-Coated Glass Slides

1. Wash the streptavidin-coated slide 2–3 times with binding buffer.
2. Dissolve the biotinylated aptamer in binding buffer and spot onto the surface. Aptamer concentration should be optimized in each case, but concentrations in the range of 100 nM to 1 μM will work in most cases.
3. Incubate for 30 min at room temperature (see Note 8).
4. Wash three times with washing buffer and store at 4°C in binding buffer (see Note 9).

3.3.2. Covalent Conjugation of NH-Aptamer to Amino-Silanized Surfaces

1. Activate the amine-modified slides by immersion for 1 h in either (1) a 0.05 M dioxane solution of carbonyldiimidazole or (2) 5% glutaraldehyde.
2. Wash with (1) dioxane and diethyl ether or (2) PBS, and dry under a nitrogen stream.
3. Spot the amino-modified aptamer, dissolved to 1–10 μM in binding buffer, and incubate for 30–60 min at 37°C or overnight at room temperature (see Note 8).
4. Rinse with binding buffer and block any remaining active groups by incubating in 1 M ethanolamine for 15 min (see Note 9).

5. Rinse with washing buffer and store at 4°C until used, either immersed in binding buffer, or dried under an argon or nitrogen stream.

3.3.3. Covalent Conjugation of SH-Aptamer to Thiol- or Maleimide-Silanized Surfaces

1. Spot the thiol-modified aptamer, dissolved in binding buffer to a concentration of 0.1–1 μM.
2. Incubate for 30–60 min at 37°C or overnight at room temperature (see Note 8).
3. Rinse with binding buffer and block any remaining active groups by incubating in 0.1 M mercaptoethanol for 15 min (see Note 9).
4. Rinse extensively with water and washing buffer, and incubate with binding buffer for at least 15 min before use.

3.3.4. Self-Assembly of SH-Aptamers onto Gold Surfaces

1. Spot the thiol-modified aptamer, containing a spacer of the appropriate length (to be optimized), dissolved in KH_2PO_4 buffer to a concentration of 0.1–1 μM.
2. Incubate for 60 min at room temperature (see Note 8).
3. Rinse with sterile water.
4. Spot a thiolated molecule of choice whose chain length has to be shorter than the aptamer spacer (e.g., mercaptohexanol has been widely used for this purpose), dissolved to 0.1 M in KH_2PO_4, or in ethanol depending on its solubility. This molecule will fill any pin holes existing on the aptamer cover, and will displace loosely bound molecules. Incubate for 60 min at room temperature.
5. Rinse extensively with water and washing buffer, and incubate with binding buffer for at least 15 min before use.

3.4. Aptamer Modification by Label/ Biotin Incorporation

3.4.1. Incorporation of Commercial Amine-Reactive Compounds to Amine-Modified Aptamer

1. This protocol is appropriate for the most commonly used amine-reactive reagents (e.g., fluorophore, biotin): sulfosuccinimidyl esters, isothiocyanates, sulfonyl chlorides, and tetrafluorophenyl esters (see Note 10). Label quantity has been calculated for an aptamer of 15–25 nucleotides. For optimal results, the most favorable aptamer:label molar ratio has to be experimentally determined by assaying 3–4 different ratios.
2. Dissolve 100 μg of the aptamer in 0.1 M tetraborate buffer (see Note 5). The final volume should be below 100 μl. If necessary, precipitate and redissolve the aptamer.
3. Immediately before use, dissolve the amine-reactive label in DMSO to a final concentration of 20 mg/mL, and vortex until completely dissolved.
4. While gently stirring, add drop-to-drop 10 μl of the fluorophore to each 100 μg of aptamer (the concentration of DMSO must not exceed 10% in the final reaction).

5. Incubate for 4–6 h at room temperature, in agitation, and protected from light.
6. Purify the modified aptamer by ethanol precipitation as follows. Add one-tenth volume of 3 M NaCl and mix. Add two and a half volumes of cold absolute ethanol. Mix again and incubate at –20°C for 30 min. Vortex for 1–2 min as to wash away any molecules attached to the tube walls. Centrifuge at ~12,000 × *g* for 30 min, using a refrigerated centrifuge, if possible. Carefully remove the supernatant, rinse the pellet twice with two volumes of cold 70% ethanol, and dry briefly avoiding that the labeled aptamer becomes completely dry. Dissolve in sterile water or binding buffer, aliquot, and freeze.
7. Alternatively, the labeled aptamer can be purified by reverse-phase HPLC or by gel electrophoresis.

3.4.2. Incorporation of COOH-Bearing Electroactive Labels to Amine-Modified Aptamer

1. This protocol is appropriate for most molecules exhibiting COOH-groups (e.g., ferrocene carboxylic acid). Label quantity has been calculated for an aptamer of 15–25 nucleotides. For optimal results, the most favorable aptamer:label molar ratio has to be experimentally determined by assaying 3–4 different ratios.
2. Dissolve 100 µg of the aptamer in 10 mM PBS or HEPES, pH 7.4, to a final volume of 100 µl. If necessary, precipitate and redissolve the aptamer.
3. Immediately before use, dissolve the label in DMSO (or DMF) to a final concentration of 200 mM and vortex until completely dissolved.
4. While gently stirring, add 1 µl of the label to each 100 µg of aptamer (the label will be at a final concentration of approximately 2 mM, equivalent to a tenfold molar excess vs. the aptamer. DMSO/DMF final concentration must not exceed 10%).
5. Add a 100-fold molar excess of EDC (e.g., 1 µl of a stock prepared by dissolving 3.82 mg in 200 µl PBS or HEPES) and NHS to a final concentration 5 mM (e.g., 2.5 µl of a 200 mM stock prepared by dissolving 2.3 mg in 100 µl PBS or HEPES).
6. Incubate for 2 h at room temperature, in agitation, and protected from light.
7. Purify the modified aptamer by ethanol precipitation as follows. Add one-tenth volume of 3 M NaCl and mix. Add two and a half volumes of cold absolute ethanol. Mix again and incubate at –20°C for 30 min. Vortex for 1–2 min as to wash away any molecules attached to the tube walls. Centrifuge at ~12,000 × *g* for 30 min, using a refrigerated centrifuge, if possible.

Carefully remove the supernatant, rinse the pellet twice with two volumes of cold 70% ethanol, and dry briefly avoiding that the labeled aptamer becomes completely dry. Dissolve in sterile water or binding buffer, aliquot, and freeze.

8. Alternatively, the labeled aptamer can be purified by reverse-phase HPLC or by gel electrophoresis.

3.4.3. RNA Aptamer Modification by Incorporation During Transcription

1. Use a commercially modified nucleotide (e.g., biotinylated, fluorophore-labeled) and, if required, deprotect following the provider's instructions.
2. Unless otherwise advised by the provider, prepare 20 µl reactions containing 800 ng of the template DNA (see Note 6), 30 mM DTT, 5 mM of each of the three unmodified nucleotides, 2 and 3 mM of native and biotinylated species for the modified nucleotide, and 2 U of T7 RNA polymerase, in transcription buffer.
3. Incubate for 4 h at 37°C.
4. Treat with 1 µl of RNAse-free DNAse for 15–30 min at 37°C, or according to the provider's instructions.
5. Purify by chromatography, gel electrophoresis, or ethanol precipitation as described above. Dissolve in sterile binding buffer containing RNAse inhibitor, aliquot, and freeze.

3.5. Target Labeling

3.5.1. Target Labeling with an Amine-Reactive Fluorophore

1. This protocol is appropriate to label amine-containing proteins using the most common amine-reactive reagents: sulfosuccinimidyl esters, isothiocyanates, sulfonyl chlorides, and tetrafluorophenyl esters (see Note 10). For smaller targets, specific protocols will have to be searched.
2. Dissolve the protein target in 0.1 M sodium bicarbonate buffer, pH 8–9, to a final concentration 2–20 mg/mL (see Notes 5 and 11).
3. Immediately before use, dissolve the amine-reactive fluorophore in DMSO to 10 mg/mL and vortex until complete dissolution.
4. While vortexing the protein solution, add drop-by-drop 5–10 µl (0.05–0.1 mg) of reactive fluorophore per mg of reacted target protein (the concentration of DMSO must not exceed 10% in the final reaction). In general, it is recommended to assay in parallel at least three different protein:fluorophore molar rates.
5. Incubate for 1 h at room temperature with continuous stirring and protected from light. For sulfonyl chlorides, incubate at 4°C instead.
6. The reaction can be optionally stopped by adding 0.1 mL of freshly prepared 1.5 M hydroxylamine per mL of reaction, and incubating for 1 h at room temperature.

7. Depending on the target size, the conjugate can be recovered through a gel filtration column using the buffer of choice, and concentrated using a centrifuge filter/spin device. Alternatively, the conjugate can be dialyzed or purified by column chromatography.
8. Determine the degree of fluorescence labeling according to the provider's protocol.
9. Add bovine serum albumin (BSA) to a final concentration of 1–10 mg/mL, or any other stabilizer of choice, and store at 4°C. For extended storage, either add glycerol up to 50% or distribute in small aliquots and freeze at –20°C.

3.6. Optimal Conditions for Target Capture

1. Every single aptamer molecule can fold into different structures, but only one or few of them exhibit on surface the binding pockets or clefts for the specific recognition of the target (see Note 7). Aptamer folding will thus be affected by a variety of external factors, such as incubation temperature and buffer composition, and will often depend on the presence of certain ions. For example, aptamer folding into hairpin and stem-and-loop structures usually requires the presence of magnesium ions, and quadruplex formation is favored and stabilized by the binding of cations such as sodium or potassium.
2. This implies that changes in the assay binding conditions (buffer ionic composition or strength, assay temperature, etc.) can affect aptamer folding, affinity for the target, and assay performance. This is of extreme importance when, for example, an aptamer evaluated in vitro is to be used in real samples of different ion concentrations.
3. For these reasons, aptamers are regularly assayed under similar conditions as those employed in their selection procedure (normally available in the literature and summarized in the methods section). Nevertheless, some works indicate that aptamer optimal working conditions are not necessarily the SELEX ones, especially if a multiplexed assay is to be optimized. In these cases, series of optimization experiments should be performed for each aptamer or structure type. In any case, and contrary to what happens with immuno and nucleic acids arrays, it is difficult to define universal conditions in the case of the aptamer arrays.
4. In the same way, optimal binding time has to be experimentally optimized for each aptamer-target pair and experimental setup, with authors having reported incubations from just few minutes in flow systems to up to 2 h in bulk experiments.
5. Although most aptamers perform well at room temperature in the appropriate binding buffer, in some few cases higher temperatures (i.e., 37°C) are required. Nonetheless,

higher temperatures usually contribute to increase the level of nontarget nonspecific adsorption, and complicate assay setup.

6. When required, nonspecific adsorption can be decreased by adding 0.05–0.1% of Tween to the binding buffer.
7. In all cases, incubations have to be followed by extensive washing (e.g., 10 min flowing), or at least three serial washes with detergent-containing washing buffer.

3.7. Array Regeneration and Reutilization

1. Compared to antibody-based immunosensors, which are difficult to regenerate preserving their properties and performance, aptasensors are potentially regenerated. Being nucleic acids, aptamers can be submitted to repeated cycles of denaturation and renaturation without seriously damaging their structure, and thus performance.
2. Wash the array with washing buffer, followed by a wash with binding buffer.
3. The binding of small targets to an aptamer, for example drugs and small molecules, is highly reversible. So, it is possible to regenerate these aptasensors by just washing extensively with water, washing buffer, or binding buffer, until the target has been completely removed.
4. In the case of more complex targets, the various strategies reported for aptamer regeneration are mainly directed toward disruption of the aptamer folding, disturbance of the aptamer–target interaction, and/or target denaturation (see Table 1). They range from the use of high temperature (to denature aptamer folding), to the use of concentrated salt solutions (to disrupt binding interactions and denature aptamer folding), surfactants (to wash away the analyte), chaotropic components (to disrupt noncovalent molecular interactions), or chelating agents (to remove metals responsible for aptamer secondary or tertiary structure). Even if extreme pHs should be avoided, because they can damage nucleic acids, successful results have been reported for the use of diluted NaOH, glycine, and HCl, which are believed to disrupt noncovalent interactions of analytes bound to aptasensors. Nevertheless, the choice of the regeneration procedure will be conditioned by the aptamer immobilization strategy (i.e., it is meaningless to regenerate the aptamer with a component that damages the streptavidin–BSA cover used for its immobilization) and by target nature.
5. Regeneration should always be followed by a final rinse with washing and binding buffer as to completely eliminate the regeneration agent.

3.8. Detection Strategy

3.8.1. Fluorescence Aptamer Microarrays

1. Fluorescence aptamer arrays exploit the use of fluorescent labels such as fluorophores and, more recently, quantum dots.
2. In the simplest approach, captured target is treated with a reactive fluorescent component, and thus labeled (Fig. 1a). For this system to work, the target has to present the reactive groups targeted by the label, and the aptasensing surface has to be 100% specific (as any nontarget components nonspecifically trapped onto the surface will be also labeled).
3. In most cases, competition assay formats are developed, in which a fluorescently labeled target competes in solution with the native target (i.e., present in the samples) for the immobilized aptamer (Fig. 1b). As an alternative, labeled aptamer can be used if competition occurs between native target in solution and modified target immobilized on the chip for the aptamer in solution. For competition optimization, several concentrations of the labeled component are assayed. The concentration generating 70–80% of the maximal signal registered is considered optimal for competition performance. In this respect, changing the concentrations of immobilized and labeled biocomponents allows the assay linear range to be shifted if required.
4. The sandwich assay formats usually provide extremely high sensitivity (Fig. 1c). However, few aptamers present two of more binding sites per target unit, and two or more aptamers have been seldom reported against different epitopes in the same target. Consistently, sandwich microarrays often take advantage of mixed aptamer/antibody formats. Using aptamer and antibody as capture and detection biocomponent, respectively, offers the additional advantage that the latter, being much bigger, can incorporate a higher number of label units, and thus generate "amplified" signals.
5. The unique characteristics of aptamers allow optimization of an alternative assay format using molecular beacons. This format takes advantage of an immobilized aptamer whose free extreme has been modified with a fluorophore (Fig. 1d). In this case, aptamer rearrangement induced by target binding translates into a change of either fluorescent emission or fluorescence polarization anisotropy.

3.8.2. Surface Plasmon Resonance Imaging

1. SPR is an optical label-free detection strategy based on the fact that, under certain conditions, part of a polarized light beam hitting a gold-coated prism is absorbed by the free electrons at the metal surface. The photons absorbed are in this way converted into surface plasmon waves, a phenomenon traceable in real time in the shape of a dip in light reflectivity.

Adjustments that occur at the biochip gold surface, such as those generated by target capture, will directly induce a change of resonance, and thus, a modification in light reflectivity.

2. In the case of SPRi, the whole biochip surface is visualized using a video camera. This enables the researcher to functionalize the biochip in an array format (e.g., with different aptamers) and to obtain data separately and simultaneously from the different spots. SPRi provides data in real time and does not require more biocomponent modification/labeling than aptamer immobilization (Fig. 2a).

3.8.3. Electrochemical Aptamer Microarrays

1. Target capture onto the aptasensor surface induces its partial blocking and thus increase of the interfacial electron-transfer resistance. This effect can be monitored in real time by impedance spectroscopy, without the need to use labels or labeled biocomponents (Fig. 2b). Nevertheless, several authors have described sandwich formats that, making use of nanoparticle or enzyme-labeled biocomponents, contribute to the increase in impedance signals registered in an important way.
2. Other electrochemical sensing strategies, such as the amperometric or voltametric approaches, remind their fluorescent counterparts, with the exception that fluorophores are substituted by electroactive labels such as redox mediators or metal nanoparticles. In this case, target capture correlates with changes in electron transfer rate or efficiency (Fig. 2c).
3. Electrode apta functionalization is mostly based on aptamer self-assembly onto gold instead of using streptavidin capture. In these cases, the length of the spacers used during aptamer immobilization has to be carefully optimized so as to provide surface specificity against nonspecific adsorption and efficient electron transfer.

4. Notes

1. Longer spacers generate better aptamer performance, in terms of signal amplitude, reproducibility, and selectivity, except for electrochemical assay formats in which the use of long spacers interferes with electron transfer. Spacers of DNA, hydrocarbon-based, and oligo(ethylene oxide) composition have been successfully used. With few exceptions, aptamer immobilization through its two extremes generates very similar results. It is thus recommended to order 3′ modifications, significantly cheaper than 5′ changes.

2. TE buffer (10–50 mM Tris–HCl, pH 8.0, 1 mM EDTA) can also be used, where EDTA contributes to inhibit nuclease activity. However, ion quelation interferes in aptamer folding, and buffer exchange might be necessary previous to any experiment.
3. Although some authors describe the use of BSA as a blocking agent, according to our experience, this molecule is too big for most aptamers. It is appropriate, on the other hand, for blocking the surface previous to aptamer capture (e.g., streptavidin-coated surfaces).
4. Tetraborate buffer can be aliquoted and frozen, but each aliquot should be thawed and used only once. If the frozen buffer precipitates, vortex until completely resuspended.
5. Avoid buffers containing primary amines, such as Tris, that might compete for conjugation with the amine-reactive compound.
6. RNA aptamer production and/or modification by transcription is carried out from an appropriate double stranded DNA template that incorporates a promoter for the RNA polymerase used. The reaction should be carried out under conditions that exclude contamination with RNases. All plasticware and solutions should thus be nuclease free.
7. The requirement for a denaturation step previous to aptamer immobilization or assay performance, in order to improve aptamer proper folding, is unclear. Among the few existing studies, some indicate that denaturing may be unnecessary or deleterious for certain aptamers, as it is the case of the quadruplex-folding 15-nucleotide thrombin-binding aptamer, while others demonstrate that aptamers submitted to denaturation show slightly better limits of detection and reproducibility, as happens for the hairpin-forming aptamer against HIV Tat protein. Denaturation can be carried out by incubating for 3–5 min at 70 and 90°C for single-strain RNA and DNA aptamers, respectively, followed by gradual cooling to 4°C over 1–2 min. Alternatively, aptamers can be denatured by washing in 7–8 M urea, followed by refolding in the appropriate binding buffer.
8. In order to avoid the spots drying during incubation, place the surface into a humid chamber (a closed box or tube with a humid towel inside will serve). In some cases, especially when using highly saline buffers, addition of glycerol up to a final 1–10% concentration can help to prevent desiccation.
9. Optionally, the slides can be additionally blocked to reduce nonspecific adsorption by incubating for 1 h at RT in blocking buffer. In this case, add 0.05–0.1% Tween to all buffers subsequently used.

10. Tetrafluorophenyl and succinimidyl esters are preferred because they form very stable amide bonds (TFPs are less susceptible to hydrolysis and provide better conjugation ratios in aqueous media). Isothiocyanates are also commonly used, but the resulting thiourea product has been reported to deteriorate over time. Sulfonyl chlorides, on the other hand, are more reactive and may conjugate also to aromatic amines, and are more difficult to manipulate.
11. Amine-reactive fluorophores react with nonprotonated aliphatic amine groups, including protein terminal amine and ε-amino groups in lysines. While the pK_a value of the latter is around 10.5, the terminal amine is lower. Using a slightly basic buffer will thus target the reaction toward the two groups that are kept protonated under these conditions. Using a buffer closer to neutral instead will favor labeling of the amine terminal.

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