Electrochemical aptamer-based assays coupled to isothermal nucleic acid amplification techniques: new tools for cancer diagnosis

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Abstract

Highly sensitive detection of cancer biomarkers in blood is key not only to find cancer at an early stage but also to help clinicians to decide the best treatment plan and to find how well treatment is working. To quantify the small changes in clinically validated biomarkers associated with carcinogenesis both selective receptors and signal amplification strategies of the recognition event between the receptor and the biomarker are highly in demand. This report covers the most recent developments in the integration of aptamer-based recognition of blood-circulating cancer biomarkers and isothermal nucleic acid amplification platforms with electrochemical readout, highlighting the potential of these novel tools, and the challenges to translate these assays to the clinical practice.

Keywords: aptamer, biosensor, cancer biomarker, electrochemical detection, isothermal amplification

Introduction

Early detection of cancer is crucial to increase the survival rate of patients. Nowadays, the efforts are focused on liquid biopsy, a method that relies on the presence of specific biomarkers in the serum of cancer patients. These tumor markers are mainly proteins present in low concentration levels in a high protein content medium, which hinders their detection. Enzyme-linked immunosorbent assays (ELISA) are the gold standard method for protein biomarker detection. These approaches rely on the use of antibodies, which are among the most selective, naturally occurring molecular recognition elements. However, they suffer from some limitations such as the batch-to-batch variations in their production, and the cumbersome and challenging process needed for generating specific monoclonal antibodies, especially against non-immunogenic molecules [1]. For this reason, new receptors able to overcome these drawbacks are needed.

First reported in 1990 [2,3], aptamers are short single-stranded DNA or RNA oligonucleotides able to adopt special three-dimensional conformations that allow them to bind with great affinity and specificity almost any type of targets, from ions to cells. These affinity receptors are raised through an in vitro process called SELEX (Systematic Evolution of Ligand by EXponential enrichment) that mimics the natural selection. Besides holding advantages such as thermal and chemical stability, production by chemical synthesis without batch-to-batch variation and easy labeling with any marker molecule, aptamers have a dual behavior, both as nucleic acids and affinity receptor. This unique characteristic for an affinity ligand allows to transform the detection of any type of target into a nucleic acid quantification event, making possible the use of different nucleic acid amplification reactions to develop ultrasensitive analytical assays [4,5••]. Within this context, isothermal amplification methods, a set of

increasingly popular alternatives to PCR, are of outstanding interest in the developing of sensitive aptamer-based analytical systems, as they do not require the cycling of temperature that is required for PCR, which is the most well stablished method for nucleic acid amplification.

The combination of the selective biochemical recognition of cancer biomarkers by aptamers with isothermal nucleic acid amplification and the inherently simplicity, rapidity, cost-effectiveness and easy miniaturization of electrochemical readout, provides new opportunities for clinical diagnosis, with great potential for creating integrated platforms useful in decentralized point-of-care analysis [6•,7].

This short review focuses on the latest advances made in the field of aptamer-based electrochemical bioassays and biosensors coupled to isothermal nucleic acid amplification techniques to achieve enhanced sensitivity in the detection of cancer-related targets. We classify these approaches into three general groups, according to the isothermal nucleic acid amplification method employed. (1) Strategies implying DNA/RNA synthesis mediated by polymerases, including rolling circle amplification (RCA) and amplification by terminal deoxynucleotidyl transferase (TdT), (2) methods based on recycling schemes mediated by nucleases, such as exonuclease-assisted and nicking endonuclease-aided amplifications, and, (3) systems based on toehold-mediated DNA strand displacement, such as hybridization chain reaction (HCR) and catalytic hairpin assembly (CHA). Assays for thrombin have been included in this work because, though it is not a cancer biomarker, this protein has been widely used as a model target for the development of aptamer-based analytical systems.

Polymerase-mediated amplified assays

The most representative isothermal polymerase-mediated amplification method is rolling circle amplification (RCA). First reported in the mid-1990s [8,9], this *in vitro*

amplification technique consists in the replication of a circular single-stranded DNA template, performed by certain DNA and RNA polymerases (φ29, Bst, and Vent exo- DNA polymerases for DNA, and T7 RNA polymerase for RNA), which extend a short DNA or RNA primer. It results in a long single-stranded DNA or RNA containing multiple tandem repeats of a sequence complementary to the original circular template [5••,10•].

RCA has already been successfully integrated in aptamer-based assays for different targets of interest in biomedical analysis [10•] including proteins used as cancer biomarkers. Many of these assays rely on a sandwich format that implies the immobilization of antibodies or aptamers on the surface of an electrode or microplate well to capture target molecules from samples. Afterwards, the addition of a reporter aptamer extended with a primer region triggers RCA in the presence of a circular template and proper enzymes (Figure 1A). The linear extension of the aptamer around the circular template allows the sensitive detection of the target through the measurement of the thousands of oligonucleotide repeats generated as it will be explained below [11••–14].

RCA has also been coupled to target-induced strand displacement assays. For this purpose, aptamers are immobilized as a duplex through hybridization with a partially complementary single-stranded DNA sequence (cDNA). Several formats are possible as indicated previously [15], depending on which strand is anchored on the assay platform. The target-aptamer complex formation induces the release of cDNA [16] or the aptamer [17•,18]. In any case, the cDNAs are designed with a primer region to anneal to the RCA template. Depending on which element is immobilized, target binding may lead to increasing signals (signal-on) or a signal decrease (signal-off). When the aptamer is immobilized, the more the target binds to the aptamer, the more cDNA is displaced

(Figure 1C), thus the RCA will not occur extensively resulting in a signal-off architecture [16]. On the contrary, when cDNA is immobilized higher target concentrations displace a higher number of hybridized aptamer molecules, leading to an increase in RCA events (signal-on assays) [17•,18]. In the latter case, cDNA is designed to anneal the RCA template only when it is not hybridized with aptamer (Figure 1B). These strategies circumvent the requirement of two different binding sites per target protein, although they need two different oligonucleotide sequences as reagents. The implementation of competitive assays would allow the use of a single aptamer, however, to the best of our knowledge, competitive assays coupled to RCA have not been developed for the detection of cancer biomarkers until now.

Preferred place for Figure 1

Although classic RCA has many attractive features such as being isothermal and having a simple reaction mechanism, amplification occurs only linearly over time. To overcome this drawback and achieve exponential amplification, hyperbranched RCA (HRCA) [19], also known as cascade RCA [20] or ramification amplification (RAM) [21], has been developed (Figure 1B). In this method, the repeats in the linearly extended strand around the circular RCA simultaneously serve as binding site for an extra reverse primer, which is also extended. Since strand displacement also takes place HRCA result is an exponential growth of large ramified DNA duplexes [21]. Electrochemical aptamer-based systems that make use of HRCA have been reported for the detection of thrombin and platelet-derived growth factor BB (PDGF-BB) with limits of detection (LOD) as low as 1.2 aM [18] and 1.6 fM [17•], respectively, whilst reported RCA-based electrochemical systems for these targets led to an LOD of 35 fM (thrombin) [14] and 10 fM (PDGF-BB, Table 1) [11••].

In order to electrochemically detect the RCA extended strands, either linear or hyperbranched, some well stablished DNA detection schemes have been employed, usually involving additional reagents. The detection methods can be categorized into two classes. The first is based on the use of tagged DNA probes, typically biotin-probes, which hybridize with the multiple repetitions obtained after RCA [11••]. The second one relies on the interaction of DNA with electroactive reagents or precursors of electroactive compounds [13,16–18,22]. These include the use of intercalating agents, compounds that interact with the bases or the reaction of phosphate groups on the DNA backbone with molybdate to give the electroactive reporter molecule. The assembly of hemin/G-quadruplex complexes [23–25] and the formation of metal nanoparticles using the newly synthesized DNA as a template [12••] have also been described. RCA has proven to be a powerful strategy for the sensitive detection of clinically relevant protein biomarkers, producing high amplification efficiency and extremely low LOD, for example 0.07 aM (0.020 fg/mL) for prostate specific antigen (PSA) [12••] and 0.25 fM (0.05 pg/mL) for carcinoembryonic antigen (CEA) [13] with a dynamic range across four orders of magnitude (Table 1).

Another amplification strategy that implies nucleic acid synthesis is the amplification mediated by terminal deoxynucleotidyl transferase (TdT), also termed as surface initiated enzymatic polymerization (SIEP) [26•]. TdT is an unusual DNA polymerase that catalyzes the extension of DNA at its 3’-terminal without using a template [27]. TdT is also able to incorporate functionalized deoxynucleoside triphosphates (dNTPs), thus leading to an extended and labeled aptamer. Redox-active reporter molecules, such as aminophenol and nitrophenol [28] or tags as biotin and digoxigenin, for subsequent labelling with an enzyme conjugate, can be directly incorporated to facilitate the detection. Of note, the length of the extension depends on

the incorporation rate of each dNTP, which varies with the experimental conditions (dNTP:primer ratio, dNTP concentration and divalent cation present), the formation of G-quadruplex or hairpin structures during the extension and the dNTP nature, being more efficient when adding natural ones [28–30].

This TdT-mediated template-independent DNA amplification method has been applied for the detection of CEA using a sandwich format with two different aptamers as capture and signaling receptors [26•]. In this sensor, the capture aptamer was immobilized on a gold electrode via thiol on its 3'-end to avoid its extension during the TdT amplification and the reporter aptamer was attached to gold nanoparticles (AuNPs) via thiol on its 5'-end to form nanoprobes. Upon sandwich assembly, only the 3'-terminals of the oligos on the nanoprobe were elongated using biotin-modified dATP as a substrate for TdT, followed by enzymatic labeling with avidin-peroxidase conjugate and amperometric detection of enzyme substrates. This strategy led to a LOD as low as 5 fM for CEA. This approach provides two levels of amplification: the TdT extension with only biotin-dATP and the high load of short reporter oligonucleotides on the AuNPs. Since the length of TdT extension with labeled dNTPs is expected to be rather limited it is unclear whether the observed signal amplification could mainly come from the multidecorated gold nanoparticles. A comparison with a similar detection scheme using naanoprobes directly modified with biotin-labeled oligonucleotides, without TdT, would allow to estimate the contribution of both amplification strategies separately. Another concern is the lack of a template for the TdT extension, which could compromise the selectivity of the assay. This means that successful application of this amplification for clinical diagnosis will demand for a strict control of background signal, which could lead to false positive results.

Nuclease-assisted amplified assays

Nucleases are a group of hydrolases catalyzing the cleavage of nucleic acids. Two types of these enzymes, exonucleases and endonucleases, have been used as molecular biology tools to develop aptamer-based assays with signal amplification schemes based on target recycling. On the one hand, exonucleases (Exos) catalyze the stepwise removal of nucleotides from single stranded or double stranded DNA at either 3' or 5'-ends. Three Exos with different activities have mainly been used in exonuclease-assisted amplification assays: Exo III, $RecJ_f$ and Exo I. On the other hand, endonucleases catalyze the removal of nucleotides from the middle of a polynucleotide chain, which properly designed allows to recycle the target and provide the amplified signal [5••,31,32•].

Exo III removes nucleotides from duplex DNA in a 3' to 5' direction only when the 3'-terminus is a blunt or recessed end. Therefore, single stranded DNA and double stranded DNA with 3' overhangs are resistant to Exo III digestion [5••,32•]. Taking advantage of this property, hairpin structure-switching aptamers can be employed in Exo III amplification methods. In such assays, the absence of target prompts the aptamer to fold into a structure with a 3' overhang, avoiding hydrolytic digestion by Exo III. Upon target binding, the aptamer suffers a conformation change that leads to the pairing of its 3'-end, thus enabling Exo III to hydrolyze the aptamer with the consequent target liberation [33]. An electroactive tag, such as ferrocene, at 3' end is responsible for the electrochemical signal that increases after Exos-mediated release due to the higher diffusivity and lower repulsion of mononucleotides toward an electrode surface negatively charged.

Preferred place for Figure 2

Exo III can be used in other assay formats, some of them very complicated and somewhat unpractical. For instance, Huang et al. developed a signal off sensing strategy for the detection of PDGF-BB using three ssDNA sequences: the aptamer, a complementary DNA (cDNA) and a biotin-labeled signal probe (bDNA) [34]. In the absence of target, the aptamer hybridizes with cDNA, forming a duplex resistant to Exo III digestion, and the bDNA is captured on the transducer for signal generation. On the contrary, in the presence of target, the aptamer preferably binds it and the bDNA hybridizes with the cDNA to form a duplex with a 3'-overhang, prone to be hydrolyzed by Exo III, with the consequent signal decrease (Figure 2A). The assay achieves a LOD of 20 fM. However, this approach suffers from several shortcomings. Although the LOD is very low, the scheme does not achieve target recycling. It is not clear whether the increased signal is actually related to the claimed exonuclease-based signal amplification or to the particular nature of the transducer, a layered molybdenum selenide–graphene ($MoSe_2$–Gr) composite onto glassy carbon surfaces, which shows excellent electronic conductivity and large specific surface area. The need for three different ssDNA, specifically designed for each aptamer-target system, represents another limitation.

Among single-stranded DNA specific exonuclease, $RecJ_f$ is the only known 5′ exonuclease, which catalyzes the removal of mononucleotides from single-stranded DNA in the 5'→3' direction, with similar processivity on 5′ phosphorylated and unphosphorylated ends. The use of complementary DNAs (cDNAs) is widespread in $RecJ_f$ exonuclease-assisted amplification methods. The cDNA can be immobilized through several strategies on an electrode and it hybridizes with the aptamer sequence. In the presence of target, the formation of the aptamer-target complex must break the aptamer-cDNA duplex. In this way, $RecJ_f$ can digest the aptamer from the complex, and

the released target can sequentially trigger another binding-digestion round resulting in a recycling amplification scheme [35,36]. This general scheme has been recently applied to the detection of the breast cancer biomarker HER2 (human epidermal growth factor receptor-2) [36]. The authors implemented a homogeneous assay, which requires four different auxiliary oligonucleotide sequences immobilized on two types of Au-nanospheres (Figure 2B). One type of Au-nanospheres are modified with ferrocene-labeled DNA sequences (Fc-DNA) and a complementary DNA to aptamer anti-HER2 (cDNA1), and the other Au-nanospheres are linked to peroxidase-labeled DNA sequences (POD-DNA) and a partially complementary DNA to cDNA1 (cDNA2). Only when the target is recognized by the aptamer, cDNA1 hybridizes with cDNA2, leading to the assembly of the two types of Au-nanospheres. The subsequent addition of $RecJ_f$ gives target recycling and the claimed signal amplification. The above reactions take place in solution (homogeneous assay) and after that, the ferrocene-labeled nanobeads are entrapped onto cyclodextrin-modified transducers. The signal is related to the peroxidase activity, linked to the nanospheres hybridized with entrapped Fc-cDNA. This approach exploits the amplification of the signal by the multiple enzyme molecules loaded onto the sDNA-nanospheres, but the efficiency of the exonuclease-mediated recycling process is limited (an increase of less than twice compared to the assay in the absence of exonuclease is observed in a single point experiment).

A simpler approach for monitoring aptamer-based homogeneous assay implies the use of electrodes placed within the reaction medium to detect the presence of a diffusing electroactive reporter associated with cDNA. Reporter molecules are most usually anchored to the 5´-end of the oligonucleotide, so single-stranded DNA specific exonucleases that perform their catalytic activity in the 3’→5’ direction are needed. Exo I, belonging to this class of exonucleases, has been recently used in the design of a

homogeneous aptamer-based assay for mucin 1 detection [37•]. To improve the binding efficiency between the aptamer and the target a methylene blue-labeled cDNA, complementary to a mucin-1 aptamer, is designed to form a bulge-loop structure after the hybridization reaction. In the presence of target, the aptamer was displaced from the duplex aptamer-cDNA to form the aptamer-target complex, and Exo I digested both single-stranded DNA sequences, thus achieving target recycling and signal generation (Figure 2C). The detection exploits the improved diffusivity that occurs when the reporter molecule is bound to the short mononucleotide fragments obtained after exonuclease digestion, resulting in an increased voltammetric signal [37•]. This approach, which does not work in the absence of exonuclease, has a reported LOD of 0.40 pg of mucin mL^{-1} and a dynamic range of four orders of magnitude (Table 1).

Taking a different approach, Miao et al. developed an electrochemical aptasensor for PSA based on Exo T-aided target recycling amplification [38]. Exo T is another single-stranded DNA specific exonuclease that removes nucleotides in the 3’→5’ direction. Specifically, their approach uses an anti-PSA aptamer, which hybridized to a complementary sequence (cDNA) placed on the vertex of a DNA tetrahedron, previously immobilized on a gold electrode (Figure 2D). In the presence of PSA, the target displaces the aptamer from the duplex to bind it, thus being digested by Exo T, with the consequent target recycling. In this case, the aptamer is labeled with an amine group at the 5’-end and silver nanoparticles are used as electrochemical reporters. The nanoparticles interact with the aptamer via silver-amine reaction and are detected by linear sweep voltammetry. Using this system, a signal-off response curve is obtained, with a LOD of only 0.11 pg mL^{-1} (Table 1). However, it is not possible to estimate the signal amplification efficiency, as the authors do not give information about the performance of the assay without exonuclease-based target amplification, which would

have allowed the comparison with other strategies. A second concern is that the reporter they used for the transduction of the aptamer-target interaction may interact unspecifically with amine groups in the nucleobases.

Nicking endonucleases (NEases) are a special type of endonucleases that can recognize specific sequences on double stranded DNA and cleave only one strand at that particular site, leaving the other strand intact [5••,32•]. For this reason, structure-switching aptamer and complementary DNAs (cDNAs) are generally employed in endonuclease-assisted assays. The cDNAs are usually hairpin probes which contain the recognition site for the NEase, and become labile for enzymatic hydrolysis when they hybridize with the aptamer [23]. This amplification scheme has scarcely been used in the electrochemical detection of cancer biomarkers, and in general, it requires a careful design of the multiple oligonucleotides needed, which precludes its generalization. Both endonuclease and exonuclease-assisted target recycling amplification strategies have been combined with other nucleic acid isothermal amplification methods, such as RCA [23,25] or HCR [39,40], to further improve sensitivity, which may be indicative of the low efficiency of these amplification strategies.

Preferred place for Figure 3

Exponential amplification reaction (EXPAR) has recently gained attention as it holds high amplification efficiency ($10^6 - 10^8$) in a very short time (< 30 min). First used for the detection of nucleic acids, it has already been applied to protein targets and ions [41••]. The main feature of EXPAR is that it is based on the performance of two enzymes, a DNA polymerase with strand displacement activity and a nicking endonuclease (NEase). A DNA template, a trigger sequence (the target itself in the case of RNA) and deoxynucleotides (dNTPs) are also required. The EXPAR template is designed to contain two repeat regions that are complementary to the trigger sequence

and separated by a short NEase recognition sequence. The trigger DNA hybridizes to the template and is extended by a DNA polymerase. After extension, the formed duplex is cleaved by a NEase generating a nick, and DNA polymerase can extend the 3′-end of the nicked site simultaneously displacing the newly synthesized strand. Since the template contains two equal sequences, the displaced DNA strand has the same sequence as the trigger DNA, and it can hybridize with another template molecule, initiating a new amplification, while amplification of the target DNA from the initial trigger-template complex continues. Thus, two copies of the trigger DNA are produced in each reaction cycle from a single target molecule, achieving exponential amplification (2^n) [41••].

This isothermal amplification method has been integrated in an electrochemical aptasensor for PDGF-BB, based on target-induced proximity hybridization [42•]. This system takes advantage of the two identical aptamer-binding sites in the target protein. Both of them were lengthened with short and complementary sequences and only one of them is previously hybridized with the EXPAR trigger sequence. The transducer surface is modified with the EXPAR template hybridized to a partially complementary sequence. The binding event in solution place aptamers closely enough on the protein to induce the partial hybridization of the extended aptamers, leading to the release of the EXPAR trigger, which sequentially displaces the template from the surface to initiate the amplification reaction (Figure 3). The electrochemical detection is achieved through hybridization of the cDNA remaining on the electrode with DNA/AuNPs/G-quadruplex/hemin probes. This strategy led to a LOD of 52 fM [42•], slightly over the values obtained using other amplifications such as RCA [11••] or HRCA [17•] (Table 1). This fact can be attributed to high background amplification in EXPAR, the main

shortcoming of this technique that limits its practical applications [41••] and the limited catalytic activity of artificial DNAzymes.

Toehold-mediated DNA strand displacement amplified assays

This name encompasses various enzyme-free approaches initially devised to quantify nucleic acid targets that have been extended to other analytes coupled to aptamer recognition. They are rooted in a unique characteristic of DNA as analytical reagent: its programmed and predictable self-assembly through Watson-Crick base pairing. Toehold-mediated strand-displacement reactions consist on the displacement of one or more prehybridized strands from a DNA duplex containing a toehold overhang. Toehold designates a single-stranded region of usually 5-8 nucleotides where strand displacement is initiated [5••,43]. The most representative toehold-mediated strand-displacement method is hybridization chain reaction (HCR). First reported by Dirks and Pierce [44], HCR consists in a cascade of hybridization events which gives rise to long DNA duplexes. It requires two species of DNA hairpins that are stable in solution until an initiator strand is unveiled by the presence of the target and triggers the first hybridization reaction (Figure 4A).

Preferred place for Figure 4

HCR has been widely used to develop biosensors for the assessment of small molecules, nucleic acids, proteins and cells, due to its attractive features of being isothermal and enzyme-free [45,46•]. The initiator DNA sequence is usually part of the aptamer [47,48] but a complementary DNA strand that can be immobilized on the transducer surface has been proposed for other targets different from cancer biomarkers [39,40]. Peroxidase-mediated detection is one of the most exploited strategies for achieving electrochemical transduction in HCR-based methods. For this purpose, biotin-tagged hairpin probes are commonly used, so that the resulting long DNA duplexes

contain multiple biotin molecules that can be labeled with an enzymatic avidin-peroxidase conjugate [39,47,49••,50]. An alternative is using hemin/G-quadruplex peroxidase-mimicking DNAzymes. These structures can be generated by designing the hairpin probes with guanine-rich sequences at their ends which can bind hemin to acquire its peroxidase-like catalytic activity [40,48,51•]. In-solution pre-cast supersandwiches comprising a QD-labeled strand and an aptamer overhang can bind cancer cells tethered on the electrode surface. The limit of detection, 50 cells mL^{-1}, matches the sensitivity of the assay with fluorescent detection and is 120-fold lower than the one reported by impedance without HCR amplification [52••]. Similar sensitivity is achieved using QDs as ECL probe. The aptamer anchored to the electrode is hybridized with a cDNA-labeled AuNPs where hyperbranched HCR is initiated with auxiliary QD-DNA strands. Upon target binding, the AuNPs containing the superstructure are released decreasing the analytical signal [53].

Similar to HCR, catalytic hairpin assembly (CHA) make use of two hairpin probes (H1 and H2) that are stable in solution until the initiator DNA strand hybridizes with one of the probes, to be consequently displaced by the other probe to form a duplex H1-H2 [54,55]. Thus, the released initiator strand can trigger another toehold strand displacement, and recycling circuits like those of nuclease-assisted amplification can be designed. It has been envisioned as a sensitive method of real-time detection of isothermal amplifications [52••] but also for aptasensing. Zao & Ma have implemented this enzyme-free amplification strategy in an aptasensor for PSA [56•]. They used magnetic particles modified with a duplex of aptamer and a cDNA strand. The aptamer-PSA binding induced the release of the cDNA, which was designed to trigger the CHA reaction between two hairpins probes, one of them previously immobilized on the transducer surface (Figure 3B). The electrochemical signal was achieved by

hybridization of the formed hairpin duplexes with Au/Pt-polymethylene blue/DNA probes. This strategy led to a limit of detection as low as 2.3 fg mL^{-1}. CHA has also been combined with HCR in a immobilization-free fully non-enzymatic aptasensor for the detection of carcinoembryonic antigen [51•], or TdT-mediated amplification to synthesize hemin/G-quadruplex DNAzymes, using a dNTP pool with a molar ratio of dGTP:dATP = 6:4 [57]. Only the combination of two enzyme-free strategies successed in pushing down the LOD, which is a warming about random arrangements of amplification schemes.

Preferred place for Table 1

Concluding remarks

Aptamers hold a great potential in early cancer diagnosis, as they can theoretically be raised towards many different targets, including some potential biomarkers that are not easily accessible to antibodies such as post-translational modifications in proteins. The SELEX process can be tuned to direct the selection of aptamers toward specific parts of molecules suffering specific changes during carcinogenesis, with potential, for example, to detect aberrant glycosylation of proteins, which has recently gained attention as a better predictive biomarker for early cancer diagnosis than total protein levels [58]. Moreover, their dual nature of affinity receptors and nucleic acids can be exploited to transform the detection of non-nucleic acid targets into a wide variety of nucleic acid-based isothermal amplification reactions, thus making aptamers a powerful class of synthetic affinity reagents, suitable for ultrasensitive detection of cancer biomarkers, which may favorably compete with antibodies.

Even though aptamers are being raised to bind promising cancer biomarkers such as neutrophil gelatinase-associated lipocalin (NGAL) [59] and α-fetoprotein (AFP) [60,61], most current aptamer-based assays are concentrated on a few well-known

targets, including PDGF-BB, PSA and CEA. For this reason, greater efforts should be made in the selection and thorough characterization of new aptamers for clinically validated cancer biomarkers or new molecules identified for its potential in early detection of cancer. Moreover, since sandwich approaches present obvious advantages it would be also wise to screen aptamers for two separate binding sites on large molecules.

For highly sensitive detection of those cancer related-targets, a great variety of isothermal nucleic acid amplification methods have been coupled to aptamers. Unfortunately, these new platforms have not yet fulfilled their potential for the improved detection of cancer biomarkers in the clinical practice. The most significant barriers to overcome are the following. Many of these strategies are hard to generalize as they have complex mechanisms and reaction schemes that sometimes include more than one enzyme, increasing the cost per assay. In addition, numerous amplification schemes described in the literature are poorly characterized in terms of their efficiency, robustness and practical utility in biological samples. Finding easier, more effective solutions are still urgently needed to meet the growing demand for sensitivity and selectivity in biomedical and clinical analysis.

Finally, the discovery of new cancer biomarkers should be accompanied by the development of analytical methods for their detection, which makes essential both multidisciplinary work and fluent communication among clinicians, biologists and chemists.

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CAPTIONS FOR THE FIGURES

Figure 1. Schematic illustration of aptamer-based assays incorporating rolling circle amplification (RCA). (A) Sandwich format. (B) Target-induced strand displacement assays where the aptamer or (C) a partially complementary DNA sequence (cDNA) are immobilized on the transducer surface (inset: hyperbranched RCA).

Figure 2. Electrochemical aptasensors based on exonuclease-assisted target recycling amplifications for different cancer biomarkers: (A) platelet-derived growth factor BB, (B) human epidermal growth factor receptor-2, (C) mucin-1, and (D) prostate specific antigen. Reprinted and adapted from [34] (A), [36] (B), [37•] (C) and [38] (D) with permission from Elsevier.

Figure 3. Electrochemical aptasensor based on proximity hybridization-mediated exponential amplification reaction (EXPAR) for PDGF-BB.

Figure 4. Schematic illustration of aptamer-based assays incorporating toehold-mediated strand displacement amplifications. (A) Hybridization chain reaction (HCR) coupled to a sandwich format. (B) Target-induced catalytic hairpin assembly (CHA).

Figure 1

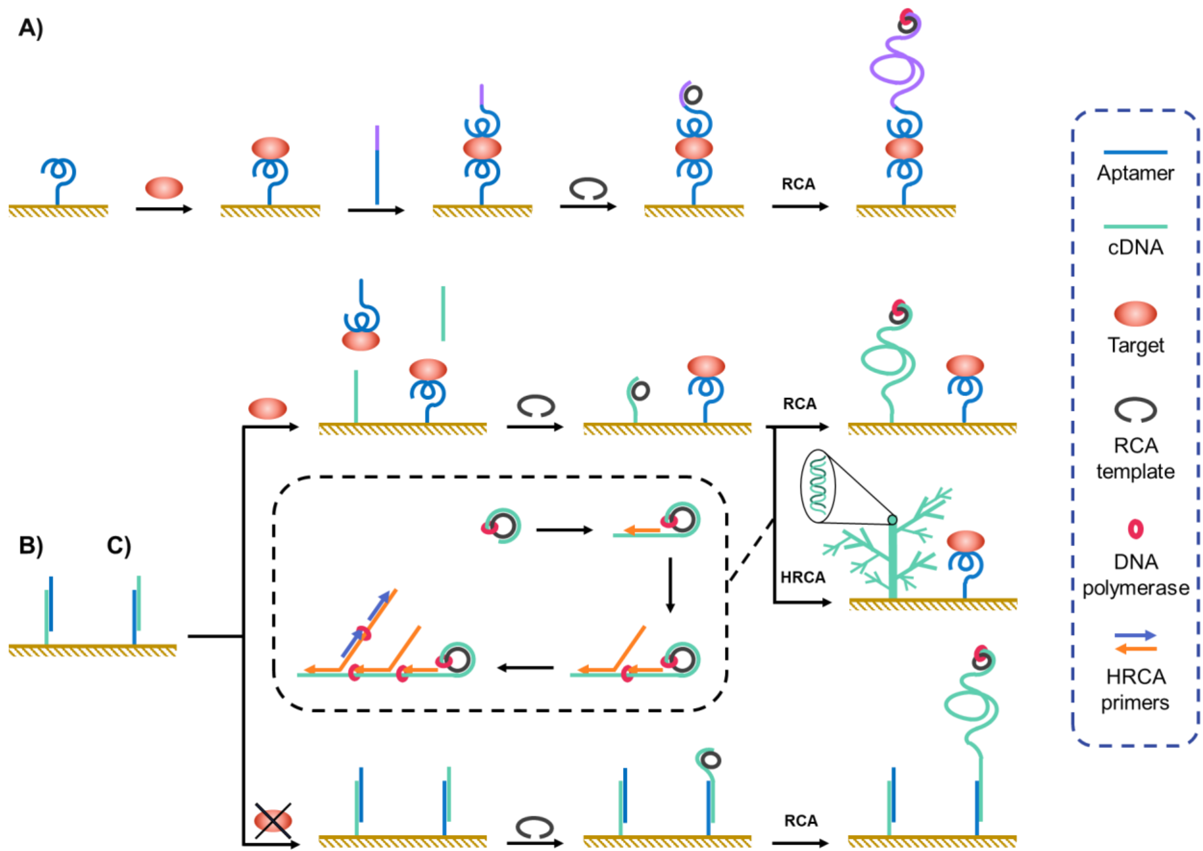

Figure 2

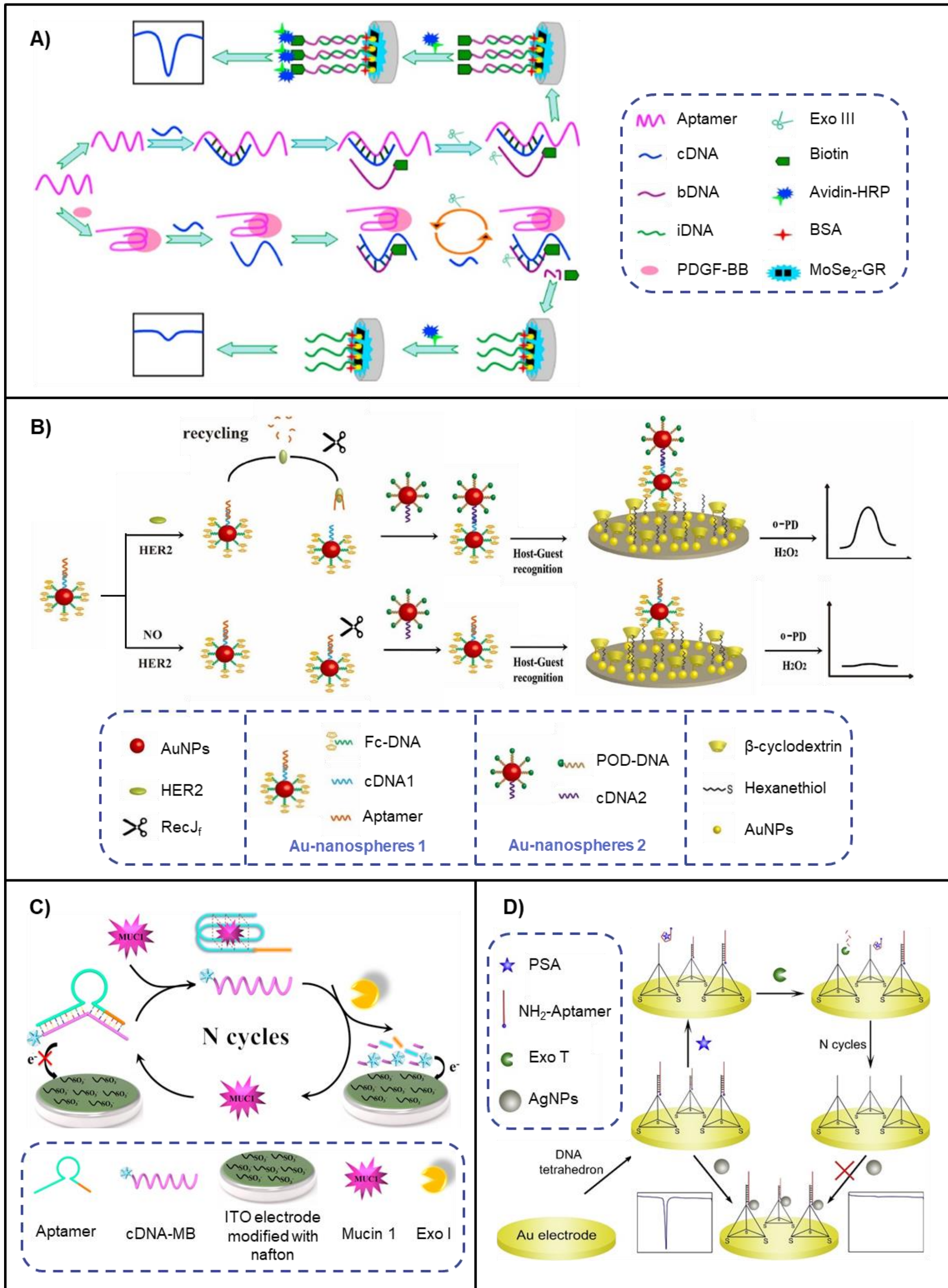

Figure 3

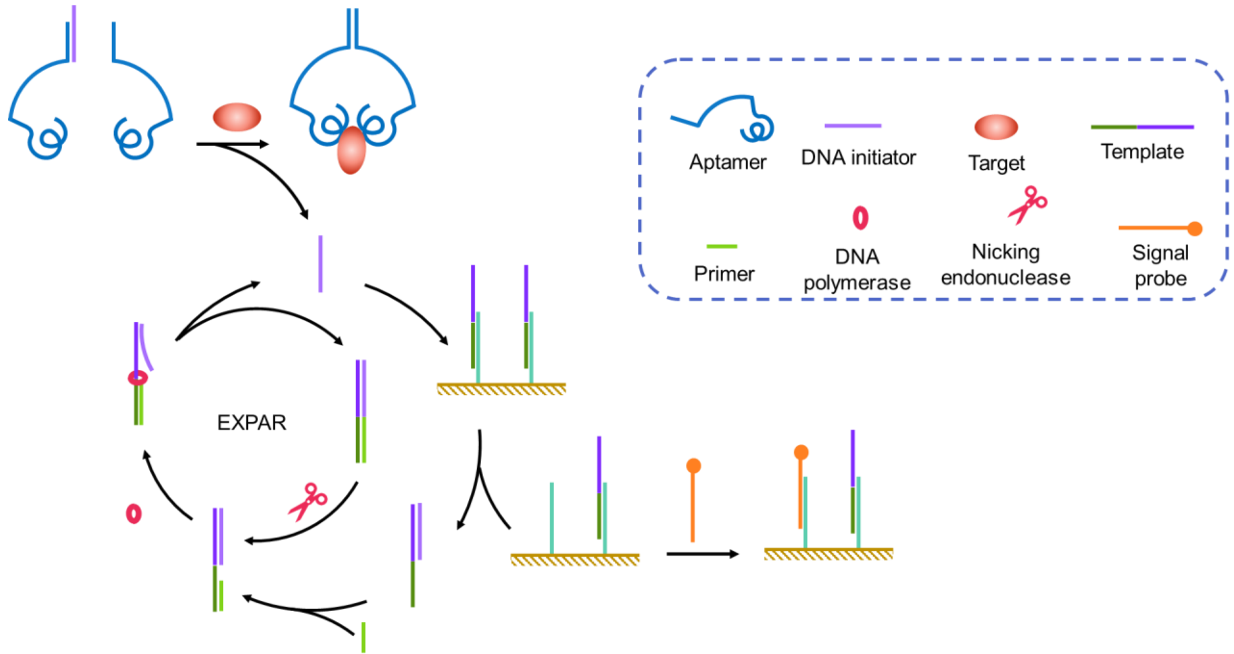

Figure 4

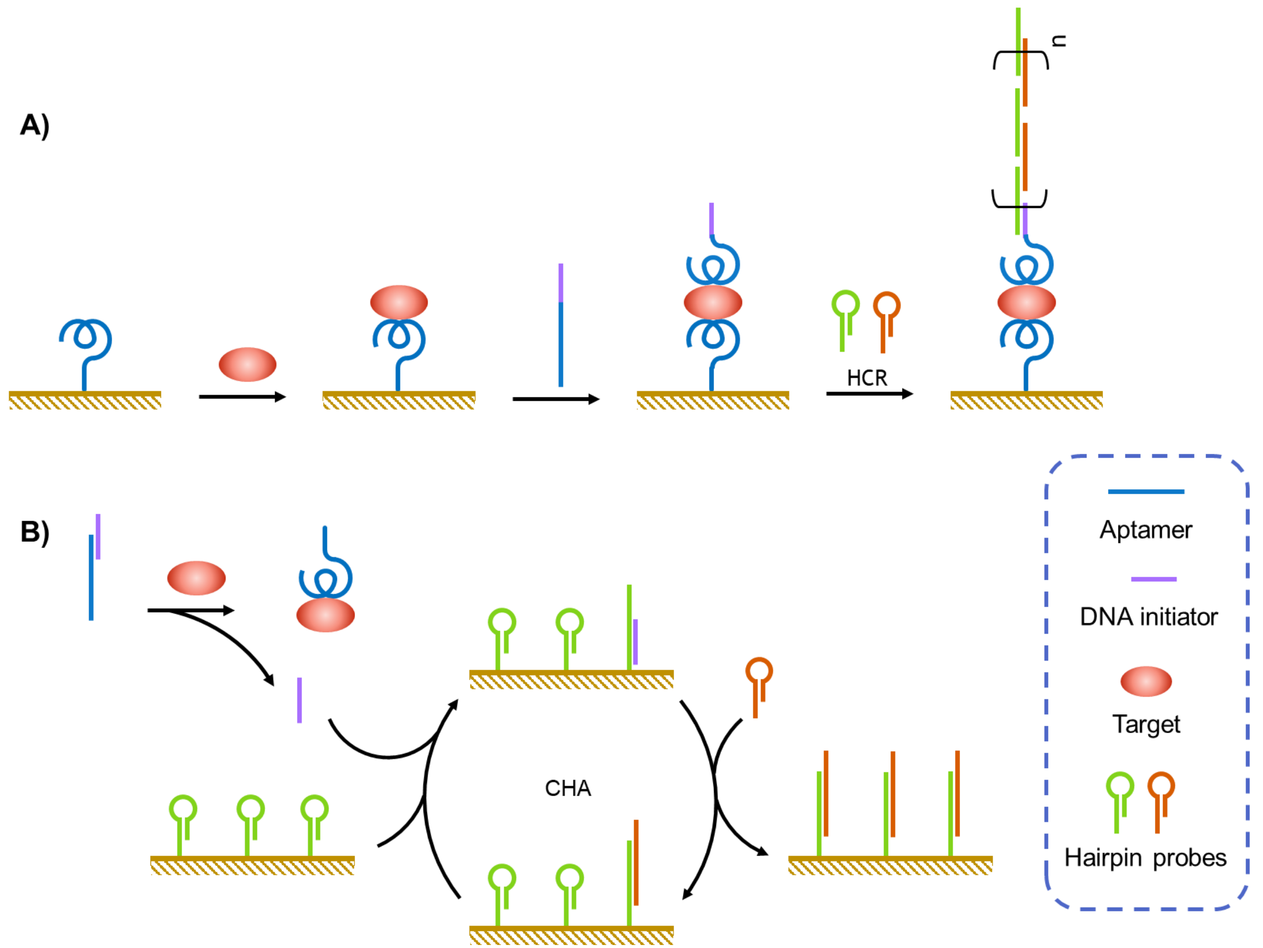

Table 1. Overview of current electrochemical aptamer-based assays and sensors coupled to isothermal nucleic acid amplification methods for cancer-related targets.

Target	Amplification system	Transduction method	Detection limit	Linear range	Reference
CCRF-CEM cells	HCR	ASV	50 cells mL^{-1}	10^2 to 10^6 cells mL^{-1}	[52••]
CEA	RCA	SWV	0.05 pg mL^{-1} (0.25 fM*)	0.1 pg mL^{-1} to 1 ng mL^{-1}	[13]
	TdT	CA	5 fM	50 fM to 500 nM	[26•]
	CHA-HCR	PEC	70 ag mL^{-1}	70 ag mL^{-1} to 500 fg mL^{-1}	[51•]
HepG2 cells	RCA	DPV	3 cells mL^{-1}	10 to 10^6 cells mL^{-1}	[24]
	HCR	DPV	5 cells mL^{-1}	10^2 to 10^7 cells mL^{-1}	[48]
HER2	Exonuclease ($RecJ_f$)	DPV	4.9 ng mL^{-1}	10 to 150 ng mL^{-1}	[36]
MCF-7 cells	HCR	CA	4 cells	4 to 1000 cells	[49••]
Mucin 1	NEase-RCA	ECL	0.71 fg mL^{-1}	10^{-2} to 10^4 pg mL^{-1}	[23]
	Exonuclease (Exo I)	DPV	0.40 pg mL^{-1}	1 pg mL^{-1} to 50 ng mL^{-1}	[37•]
PDGF-BB	RCA	LSV	10 fM	10 fM to 100 pM	[11••]
	HRCA	LSV	1.6 fM	5 fM to 80 pM	[17•]
	Exonuclease (Exo III)	DPV	20 fM	0.1 pM to 1 nM	[34]
	EXPAR	DPV	52 fM	0.1 pM to 1 nM	[42•]
PSA	RCA	DPSV	0.020 fg mL^{-1} (0.07 aM*)	0.05 to 500 fg mL^{-1}	[12••]
	RCA	SWV	22.3 fM	100 fM to 10 nM	[16]
	RCA-Exonuclease (Exo-III)	PEC	16.3 pg mL^{-1}	0.05 to 40 ng mL^{-1}	[25]
	Exonuclease (Exo T)	LSV	0.11 pg mL^{-1}	1 pg mL^{-1} to 160 ng mL^{-1}	[38]
	CHA	SWV	2,3 fg mL^{-1}	10 fg mL^{-1} to 100 ng mL^{-1}	[56•]
Ramos cells	HCR	ECL	230 cells mL^{-1}	500 to 100000 cells mL^{-1}	[53]

Abbreviations: ASV ,anodic stripping voltammetry; CA ,chronoamperometry; CEA ,carcinoembryonic antigen; CHA ,catalytic hairpin assembly; DPSV ,differential pulse stripping voltammetry; DPV ,differential pulse voltammetry; ECL ,electrochemiluminescence; EXPAR ,exponential amplification reaction; HCR ,hybridization chain reaction; HER2 ,human epidermal growth factor receptor-2; HRCA ,hyperbranched rolling circle amplification; LSV ,linear sweep voltammetry; NEase ,nicking endonuclease; PDGF-BB ,platelet-derived growth factor BB; PEC ,photoelectrochemical; PSA ,prostate specific antigen; RCA ,rolling circle amplification; SWV ,square wave voltammetry; TdT ,terminal deoxynucleotidyl transferase).

(*) In order to compare the different methodologies, LOD values were expressed as molarity using 200 and 28 kDa as molecular weights for CEA and PSA, respectively.