



Aptamers as a replacement for antibodies in enzyme-linked immunosorbent assay



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ABSTRACT

The application of antibodies in enzyme-linked immunosorbent assay (ELISA) is the basis of this diagnostic technique which is designed to detect a potpourri of complex target molecules such as cell surface antigens, allergens, and food contaminants. However, development of the systematic evolution of Ligands by Exponential Enrichment (SELEX) method, which can generate a nucleic acid-based probe (aptamer) that possess numerous advantages compared to antibodies, offers the possibility of using aptamers as an alternative molecular recognition element in ELISA. Compared to antibodies, aptamers are smaller in size, can be easily modified, are cheaper to produce, and can be generated against a wide array of target molecules. The application of aptamers in ELISA gives rise to an ELISA-derived assay called enzyme-linked apta-sorbent assay (ELASA). As with the ELISA method, ELASA can be used in several different configurations, including direct, indirect, and sandwich assays. This review provides an overview of the strategies involved in aptamer-based ELASA.

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1. Introduction

The enzyme-linked immunosorbent assay (ELISA) technique was developed in 1971 to replace radioimmunoassay. ELISA consists of an antigen (target), an antigen-capturing antibody, and a detection antibody that produces a signal when the antigen is present. The robustness and simplicity associated with ELISA resulted in its widespread application as a measurement tool in parasitology (Ljungstrom et al., 1974; Voller et al., 1975), microbiology (Engvall, 1977), and oncology (Sipponen et al., 1976; Seppala et al., 1978; Uotila et al., 1981; Meenakshi et al., 2002; Lim et al., 2010). Indeed, ELISA became a sensation among researchers and commercial manufacturers such as Boehringer Mannheim (Germany), Abbott (United States), and Organon Teknika (The Netherlands) (Lequin, 2005). ELISA is highly specific, can detect antigens at ultralow concentrations (Simeonov, 2013), and is safer than radioimmunoassay for a wide variety of tests (Baker et al., 2007). Many enzyme detection methods involved in ELISA use standard spectrophotometric detection, which eliminates the need for other sophisticated and expensive equipment (Pierce Biotechnology Inc., 2005). However, ELISA assays that depend on antibodies have various downsides. In addition to batch-to-batch variations in the production of antibody, it is tedious and challenging to generate specific monoclonal antibodies, especially against non-immunogenic molecules.

These problems highlight the need for an alternative to antibodies in order to improve the ELISA method, and, among different options, modular replacement of the target-capturing agent with a more suitable probe is an ideal approach. The emergence of an alternative molecular recognition element (MRE), termed 'aptamer', has the potential to replace or complement the role of antibodies in ELISA, resulting in an improved ELISA-enzyme-linked apta-sorbent assay (ELASA). Different variations of the term 'ELASA' include enzyme-linked aptamer assay (ELAA), enzyme-linked oligonucleotide assay (ELONA), and aptamer-linked immobilised sorbent assay (ALISA). Aptamers have several appealing characteristics that make them superior to antibodies in these assays and have guaranteed the success of ELASA. This review focuses on aptamer as the new generation of MRE, its advantages over antibody and its application in ELASA. Following this, various configurations, applications and virtues of ELASA are reviewed.

2. Generation of aptamers

Aptamers (also known as chemical antibodies) are single-stranded DNA or RNA that binds to a wide range of molecules with high specificity and affinity. DNA aptamers and RNA aptamers do not differ from each other in terms of specificity or affinity, except that DNA aptamers have greater intrinsic chemical stability. RNA aptamers, on the other hand, have more flexibility and, hence, producing a greater variety of possible structural configurations (Rotherham et al., 2012). Aptamers are generated via two main methods: the Systemic Evolution of Ligands (SELEX) method or the non-SELEX method. SELEX involves three major steps:

incubation of the randomized oligonucleotide library with the target, separation of the bound from the unbound nucleic acid ligands, and amplification (Gopinath, 2011) (Fig. 1). These steps are usually repeated 8–15 times before an enriched pool of nucleic acids with high binding affinity and specificity is obtained, cloned, and sequenced (Stoltenburg et al., 2007). The sequences obtained are analysed and the classified sequence that is present at the highest percentage is the potential aptamer. Variations of the SELEX method include genomic SELEX, spiegelmer, *in vivo*-SELEX, photo-SELEX, monoLEX, whole cell-SELEX, and *in silico*-SELEX.

The main non-SELEX method involves non-equilibrium capillary electrophoresis of equilibrium mixture (NECEEM)-based partitioning, which consists of two major steps: incubation of the randomized oligonucleotide library with the target and separation of the bound from the unbound nucleic acid ligands without the need for amplification (Berezovski et al., 2006) (Fig. 2). These steps are usually repeated three times until a pool of nucleic acids containing high affinity binders are obtained (Berezovski et al., 2006). Other non-SELEX methods include HAPIScreen, a high-throughput method for identifying aptamers (Dausse et al., 2011).

3. Advantages of aptamers over antibodies

Despite having similar functions to antibodies, aptamers have huge advantages over antibodies. First, aptamers can be easily produced via chemical synthesis, which eliminates any batch-to-batch variations and reduces the cost and the time needed for production. Second, unlike antibodies, which undergo irreversible denaturation at room temperature or higher, aptamers can reform

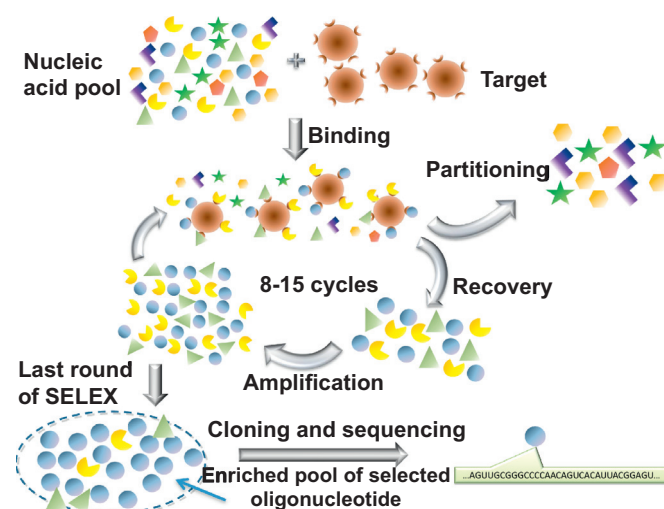


Fig. 1. Diagram of systematic evolution of ligands (SELEX). Starting with a DNA/RNA library, these oligonucleotides are incubated with the target in the binding stage. In the recovery stage, the unbound DNA/RNA molecules are partitioned from the target-bound molecules, and the bound molecules are eluted. PCR/RT-PCR is carried out in the amplification stage. The SELEX cycles, which consist of these three steps, are repeated for 8–15 rounds before an enriched pool of oligonucleotides is produced. Cloning and sequencing is performed, and the aptamer specific for the target is discovered.

to their original conformations when optimal temperature is restored (Jayasena, 1999). Furthermore, aptamers have dissociation constants that can reach as low as the picomolar–femtomolar range (Famulok, 2002; Cho et al., 2009; Gopinath and Kumar, 2013). Aptamers are also smaller in size compared to antibodies, can reach previously blocked or intracellular targets, and are less immunogenic than antibodies (Jayasena, 1999). Because they are nucleic acids, aptamers are easy to be labelled and modified with a variety of reporter molecules, linkers, and other functional groups (Luzi et al., 2003), therefore providing a simple means of detection (Jayasena, 1999). Aptamers can be used to detect a wide variety of targets ranging from small molecules to supramolecular complexes (Jayasena, 1999; Shamah et al., 2008; Nguyen et al., 2009). In addition, aptamers can discriminate between highly similar molecules, such as theophylline and caffeine, which differ by only a methyl group (Sassanfar and Szostak, 1993; Conrad et al., 1994; Jenison et al., 1994; Geiger et al., 1996; Haller and Sarnow, 1997; Mannironi et al., 1997; Wang et al., 2011) (Table 1).

Because of these features, aptamers have tremendous potential for use in therapeutic and diagnostic technologies. The first Food and Drug Administration (FDA)-approved therapeutic aptamer, Macugen, was designed to treat the wet form of age-related macular degeneration by binding to vascular endothelial growth factor and inhibiting its activity (Lee et al., 2005). Other examples of potential therapeutic aptamers include REG1, AS1411, APG7909, and IMO2055, which could act as drugs to treat cancer and various

other diseases (Krieg, 2006, 2008; Teng et al., 2007; Bates et al., 2009; Becker and Chan, 2009). In addition to aptamers being used as drugs, they can also be used in therapies, such as using aptamer-conjugated nanorods in targeted photothermal therapy to target and kill prostate cancer cells as shown by Wang et al. (2013a, 2013b). In fact, some aptamers have also been successfully used as a mode of delivering drugs to affected cells due to their cell-internalisation property. Li et al. (2014) have managed to use their ssDNA aptamer, L33 to deliver doxorubicin into human colorectal tumour cells (Cell line: HCT116). In the diagnostic field, aptamers have been widely used in different sensing tools, including microchips, microarrays, biosensors, and bio-imaging (Lee et al., 2005; Amaya-González et al., 2013; Han et al., 2013; Sachan, 2013; Sosic et al., 2013; Pilehvar et al., 2014a, 2014b). The applicability of the aptamer for aptasensing was also manifested for the detection of target molecules such as platelet-derived growth factor (Bi et al., 2014), prostate specific antigen (Cha et al., 2014), Microcystin-LR (Eissa et al., 2014), human activated protein C (Erdem and Congur, 2014), adenosine triphosphate/adenosine deaminase (Feng et al., 2014), mercury (II) (Gao et al., 2014), aflatoxin M1/aflatoxin B1 (Malhotra et al., 2014), chloramphenicol (Pilehvar et al., 2014a, 2014b), Pb^{2+} and whole cells (Gopinath et al., 2014a, 2014b). A range of aptamer-based assays were also developed such as electrochemical aptasensor (Deng et al., 2014; Zheng et al., 2014), surface plasmon resonance imaging aptasensor (Vance and Sandros, 2014), aptamer-based leaky surface acoustic wave biosensor array (Chang et al., 2014), amplified amperometric aptasensor (Zhang et al., 2014) surface-stress-based microcantilever aptasensor (Lim et al., 2014), chitosan-graphene oxide based aptasensor (Erdem et al., 2014) and amplified impedimetric aptasensor (Jiang et al., 2014). Because aptamers were being used in a variety of applications, scientists began to realise their potential for replacing antibodies in immunoassays.

4. Aptamers-alternative MREs for use in ELISA

ELISA consists of a target molecule (antigen) immobilised on the platform surface, which forms a complex with the specific antibody and subsequently with the detection antibody linked to an enzyme. The addition of a substrate that reacts with the enzyme produces colour, and the intensity of the colour is the signal readout that represents the quantity of the antibody. To assay or detect the presence of the antigen (for example in the sample), the capturing antibodies can be immobilised on the surface of the microtiter plate, followed by the addition of sample containing the target, detection antibody and substrate for signal production. An aptamer can be used to fill the role of the antibody as the capturing agent or detecting agent, or to work in concert with the antibody for target detection. The substitution of the antibody with aptamer engenders an ELISA-derived assay known as ELASA. Traditionally, ELISA has been marketed as a single-use disposable unit. Although the concept of reusable ELISA plates has

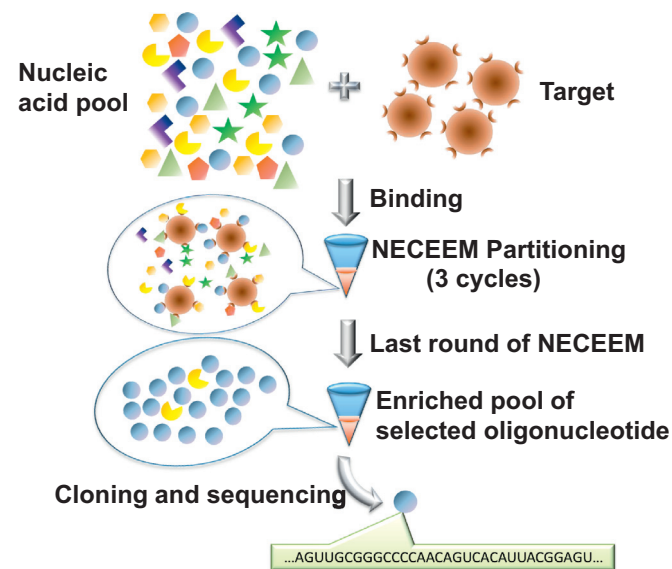


Fig. 2. Diagram of a non-SELEX method called Non-Equilibrium Capillary Electrophoresis of Equilibrium Mixtures (NECEEM)-partitioning. With an oligonucleotide library at hand, the oligonucleotides are incubated with the target. After the dissociation constant (K_D) of the mixture is determined, NECEEM partitioning is conducted. This step is repeated for a few times before an enriched pool of oligonucleotides is obtained. The oligonucleotides are then cloned and sequenced for aptamer discovery. The amplification stage is omitted in the non-SELEX method.

Table 1

Comparison of the properties of antibodies with aptamers.

	Antibodies	Aptamers
Cost of production	Reasonable at large scale	Less expensive
Size	Larger	Smaller
Types of targets	Only those with high immunogenicity	Almost all kinds
Transportation requirements	Dry ice	Ambient temperature
Chance of causing immunogenic response when used in vivo	Higher	Lower
Ability to conjugate to surfaces/resins	Less variety of surfaces/resins	Broader variety of surfaces/resins

been considered, but to the dismay of scientists worldwide, their concept has remained a concept ever since (Sibley et al., 1993). In contrast to antibodies, aptamers used in ELASA offer the advantage of reusability/regenerability.

5. The regenerability of aptamers as capturing agents in ELASA

Unlike antibodies, which suffer from permanent degradation, aptamers in ELASA can be easily regenerated for repeated use. An aptamer-immobilised ELASA system can be easily reused after the target has been unbound, which can be performed by a number of methods. For example, aptamers can be easily denatured by heat, which in turn releases the bound antigens. The aptamer structures can then be refolded into a functional configuration by lowering to ambient temperature. The process of heating and refolding can be repeated as many times as required. In one study, Wu et al. (2007) used hot water to successfully remove adenosine from the aptamers that captured it. Moreover, they regenerated the aptamers 40 times, after which ~90% of the aptamers were still able to refold into their original configuration (Wu et al., 2007).

In addition to heat, methods to remove the target from the aptamer include using concentrated salt solutions, acidic or basic solutions (Minunni et al., 2004; Schlensog et al., 2004; Kawde et al., 2005), chaotropic reagents (e.g., urea, guanidinium hydrochloride) (Potyailo et al., 1998; Lee and Walt, 2000; Savran et al., 2004; So et al., 2005; Xu et al., 2006), surfactants (e.g., sodium dodecyl sulphate) (Lai et al., 2007), chelating agents (e.g., EDTA) (Liss et al., 2002), competitive inhibitors (Porschewski et al., 2006), or various combinations of the aforementioned reagents (Andersson et al., 1999; Liss et al., 2002; Kirby et al., 2004). Furthermore, the steps required to regenerate the binding surfaces are very simple. After the bound targets are removed by using any type of the regenerating solution mentioned above, the immobilised aptamers are ready to be reused (Balamurugan et al., 2008b). Besides that, the aptamers can also be regenerated using proteinase K, which can specifically degrade the target protein. As the immobilised aptamer is a nucleic acid-based probe, it is unaffected by the enzyme. This is not the case with ELISA, as both the antibody and the antigen are proteins that will be degraded by proteinase K.

Because of their ability to be regenerated, effectiveness and specificity of using aptamers in ELISA, aptamers are widely used as the capturing agent in ELASA (Golden et al., 2000; Kiel and Holwitt, 2004; Guthrie et al., 2006; Ferreira et al., 2007; Feng et al., 2011; Kitamura et al., 2011; Zhu et al., 2012) (Table 2).

Table 2

Comparison of the features of ELISA with ELASA.

	ELISA	ELASA
Sensitivity	Similar	Similar
Can be automated	Yes	Yes
Temperature requirements	4 °C is required when antibodies are used	No requirements; should be able to tolerate temperatures up to ~60 °C
Shelf life	Short	Longer
Cost	Higher	Lower
Reusability	Single use	Repeated use

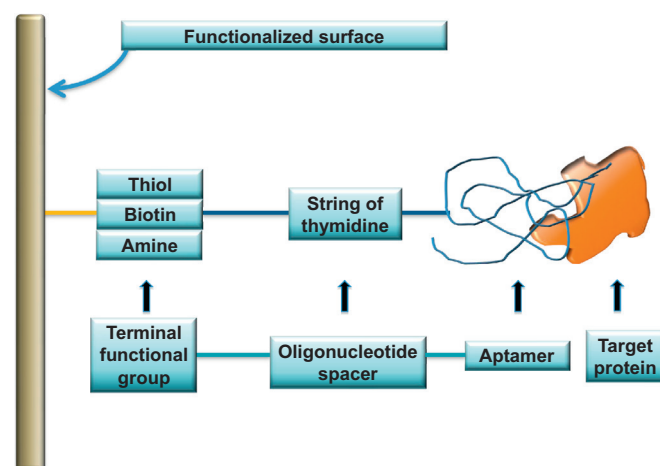


Fig. 3. This diagram shows the general structure and composition for the immobilisation of the aptamer. The aptamer immobilisation construct should have a terminal functional group for immobilisation to the surface via covalent linkage, an oligonucleotide spacer (optional but recommended; usually a string of thymidine), and the aptamer itself.

6. Immobilisation of the aptamer

The main criterion of ELAA is the immobilisation of the aptamer on the surface of the solid support, which is via controlled orientation. The immobilization orientation of the aptamer enables efficient capture of the target which engenders high binding efficiency. An appropriate chemical method must be chosen for the immobilization of the aptamer on the surface platform. Covalent linkage of the aptamers on different immobilisation platforms is the most preferred method (Balamurugan et al., 2008b). Generally, two major techniques are used: (i) direct attachment of aptamers with suitable linkers on the bio-coated sensing surface and (ii) conjugation of aptamers to functionally activated surfaces (Balamurugan et al., 2008b). To facilitate immobilisation, the aptamer must be functionalised with a terminal functional group such as biotin or amine (Balamurugan et al., 2008b) (Fig. 3). Sometimes an oligonucleotide spacer is added to the terminal functional groups to create flexibility in between the aptamer and functional group. The spacer usually consists of a string of thymidine nucleotides because thymidine has a lower chance of binding non-specifically to various immobilisation surfaces (Kimura-Suda et al., 2003). These spacers may increase target protein binding and improve the dissociation constant (Liss et al., 2002; Centi et al., 2007; Balamurugan et al., 2008a, 2008b). The immobilisation method can be applied to bio-coated surfaces, which include, but are not limited to, avidin-, streptavidin-, or neutravidin-coated surfaces. Then, for example, the biotinylated aptamers are incubated with the avidin-coated surface in an appropriate buffer to ensure maximum immobilisation.

7. Configurations of ELASA

One of the main differences between ELASA and other types of aptamer-based sensors are the availability of different types of configuration. Configuration of ELASA relies on the availability of aptamers or other complementary capturing agent that can bind to different region of the target. As such, ELASA can be classified into several different configurations such direct ELASA, indirect ELASA, and sandwich ELASA. Configurations of sandwich ELASA include aptamer–target–antibody, antibody–target–aptamer, aptamer–target–aptamer, aptamer–target–capture antibody–detection antibody, and aptamer–target–capture antibody–aptamer (Table 3).

Table 3

Summary of the aptamers used in ELASA, types of ELASA and the detection limit.

Target molecule	Aptamer type	Type/s of ELASA used	Limit of detection for ELASA	Limit of detection for ELISA	References
Aflatoxin B1	DNA aptamers	Direct	0.1 ng/ml	0.05 ng/g	Medibena (2014a), Shim et al. (2014)
Apple Stem Pitting Virus (ASPV) MT32 and PSA-H coat proteins	DNA aptamers	Sandwich	100 ng	Several groups of researchers have reported unsuccessful production of antibodies against ASPV coat proteins	Jelkmann and Keim-Konrad (1997), Komorowska and Malinowski (2009), Zsofia (2011)
Basic fibroblast growth factor (bFGF)	DNA aptamer	Sandwich	58 pg/ml	7 pg/mL (the kit detects both endogenous and recombinant bFGF)	Golden et al. (2000), Life Technologies (2014)
Cathepsin E	Peptide aptamer	Sandwich	~10 µg/ml	18.75 pg/ml	Kitamura et al. (2011), MyBioSource.com (2014)
<i>Francisella tularensis</i> antigen	DNA aptamer	Sandwich	~ 250 ng	–	Kiel and Holwitt (2004), Kiel and Vivekananda (2003), Kiel et al. (2003), Pierce Antibody (2014)
Hepatitis C Virus (HCV) Glycoprotein E2 and/or other HCV particles	DNA aptamer	Sandwich	16 ng/ml	5–8 nM	Chen et al. (2009), Park et al. (2013)
Anthrax 63 kDa–C-terminal fragment of protective antigen (PA63)	DNA aptamer	Direct	BH-2: 8.53 ± 4.3 nM BH-4: 1.75 ± 0.3 nM	Monoclonal antibody against anthrax is being developed	Choi et al. (2011), Elusys Therapeutics Inc. (2014)
Human vascular endothelial growth factor (hVEGF)	DNA aptamer	Sandwich	25 pg/ml (theoretical)	31.2 pg/ml (in human serum)	Drolet et al. (1996), Guthrie et al. (2006), ScienCell Research Laboratories (2014)
<i>Leishmania infantum</i> H2A antigen	DNA aptamer	Direct	16.5 pmol/4 pmol of aptamer	–	Martín et al. (2013), Ramos et al. (2007)
<i>Leishmania tropica</i> LRC590 and <i>Leishmania donovani</i> 2 unknown promastigote surface antigens (~60–100 kDa)		Direct	~20 µg per well	–	Bruno et al. (2007)
MPT64 antibody (tuberculosis)	DNA aptamer	Sandwich	2.5 mg/l	–	Zhu et al. (2012)
Mucin 1 (MUC1)	DNA aptamer	Sandwich and displacement	1 µg/ml	0.156–10 ng/mL	Ferreira et al. (2007), USCN Life Science Inc. (2014)
Chikungunya E1a peptide	DNA aptamer	Direct	–	–	Bruno et al. (2012)
Crimean-Congo haemorrhagic fever (CCHF) Altamura Gn 611	DNA aptamer	Direct	–	–	Bruno et al. (2012)
Crimean-Congo hemorrhagic fever (CCHF) 11E7a,b and c	DNA aptamer	Direct	–	–	Bruno et al. (2012)
Drosdov (Dros) strain of Crimean-Congo hemorrhagic fever (CCHF)	DNA aptamer	Direct	–	–	Bruno et al. (2012)
Tick-borne encephalitis virus (TBEV) CE/gE	DNA aptamer	Direct	–	–	Bruno et al. (2012)
West nile virus (WNV) E Protein	DNA aptamer	Direct	–	–	Bruno et al. (2012)
Recombinant dengue Types 1,2,3 and 4	DNA aptamer	Direct	–	–	Bruno et al. (2012)
Prion protein (PrP^{Sc})	DNA aptamer	Sandwich	–	–	Tayebi et al. (2011), Wang et al. (2011)
Tetracycline	DNA and RNA aptamers	Sandwich	DNA: 15.7 µg/ml RNA: 10.1 µg/ml	2.0 ppb	Jeong and Paeng (2012), Medibena (2014b)

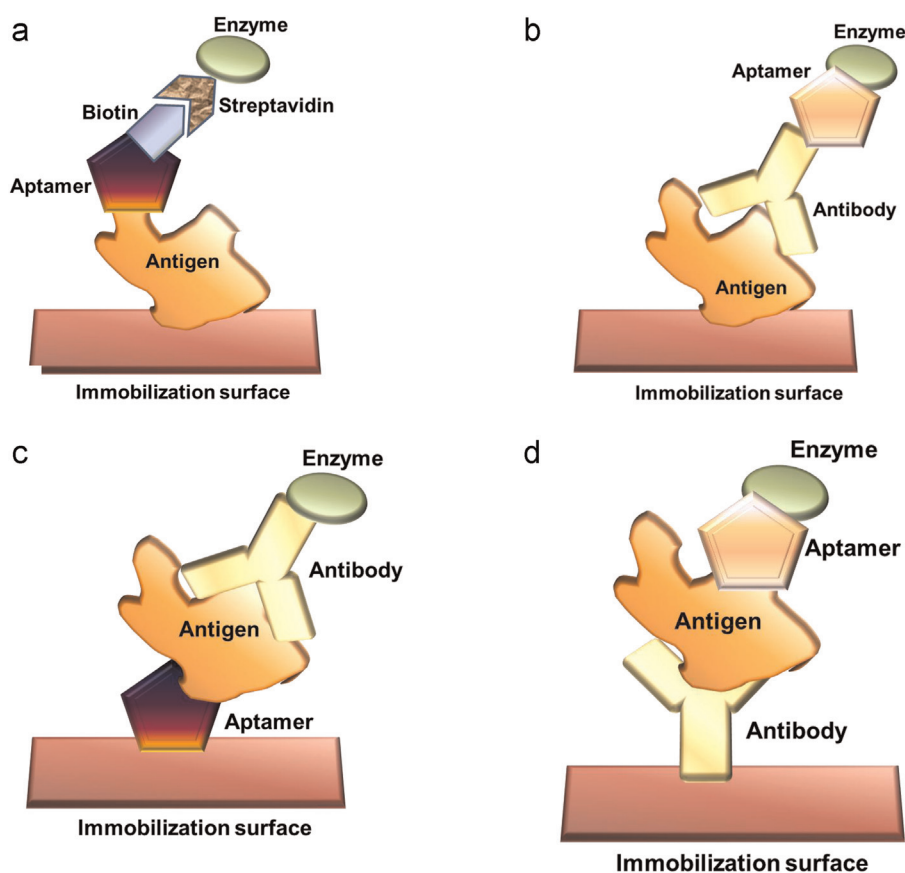


Fig. 4. Configuration of (a) direct ELISA: protein is immobilised onto the platform surface followed by the addition of the biotinylated aptamer and streptavidin–HRP conjugate. (b) Indirect ELISA: the aptamer acts as the detecting agent, whereas the antigen itself is immobilised onto the platform surface. An antibody can be used as a connector between the aptamer and the antigen. (c) Aptamer–target–antibody: in this structure, the aptamer is immobilised onto the immobilisation surface and acts as the capturing agent to capture the target, whereas the antibody acts as the detecting agent. (d) Antibody–target–aptamer: in an inverse arrangement of the aptamer–target–antibody configuration, the antibody acts as the capturing agent while the aptamer acts as the detecting agent.

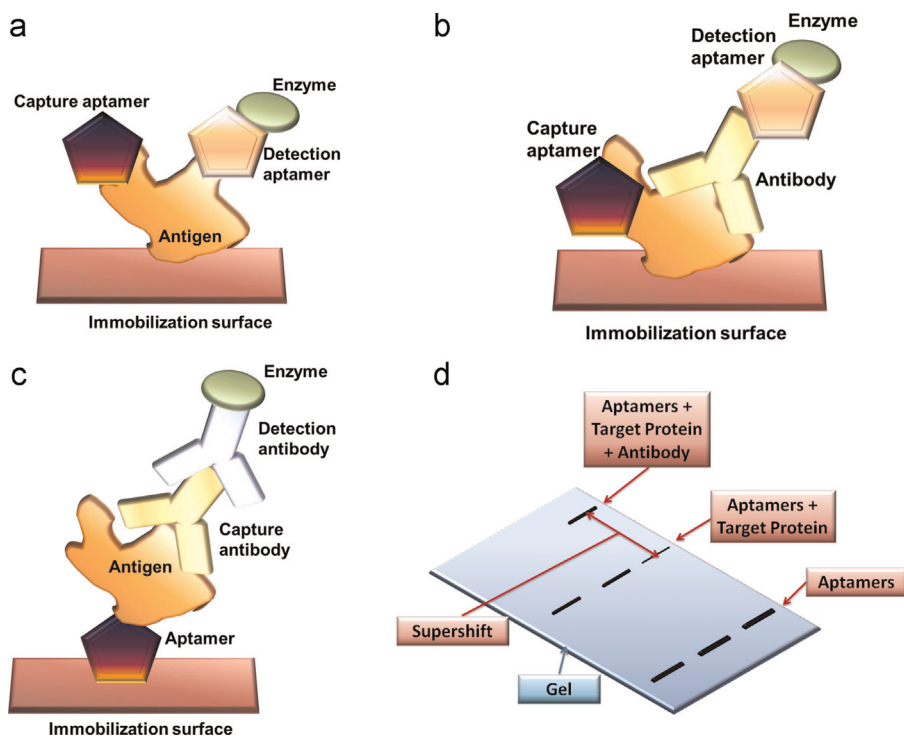


Fig. 5. Configurations of (a) aptamer–target–aptamer, (b) aptamer–target–antibody–aptamer and (c) aptamer–target–capture antibody–detection antibody. This configuration utilises only the reusability factor of aptamers without modifying the conventional method of ELISA. (d) Cartoon illustration of the gel shift assay, which is a useful way to check for the different binding sites of the antibody and the aptamer on the target protein.

7.1. Direct ELASA

In direct ELASA, the target is immobilised onto the platform surface followed by blocking with bovine serum albumin (BSA) and the addition of the biotinylated aptamer and streptavidin-conjugated horseradish peroxidase (HRP) (Fig. 4a). This is the simplest form of ELASA and can be performed easily. Direct ELASA is usually used to estimate the dissociation constant of the aptamers against the corresponding targets. Bruno et al. (2012) have obtained more than 10 candidate aptamer sequences for a single target arbovirus, and they used direct ELASA to determine the affinity of the aptamers against the target.

7.2. Indirect ELASA

In indirect ELASA, the target is immobilised on the platform surface and the capture antibodies are used to specifically bind the target (Fig. 4b). The enzyme-labelled aptamer (specific against the capturing antibodies) then binds to the antibodies, and with the addition of substrates signals are generated. Indirect ELASA is not very popular because the method of antigen immobilisation (passive absorption) is unspecific and therefore may recruit impurities alongside the target protein in the sample. Moreover, if the target is present in low concentration, it may not be able to compete with the other proteins in binding to the surface of the microtiter plate well, resulting in very little target immobilisation. Indirect ELASA also requires additional antibodies, which may translate into higher cost. Moreover, most of the aptamers generated are against the target (antigen) and less aptamers are isolated against the antibodies specific for the target. However, this configuration can avert the requirement for labelled antibodies for signal production as the enzyme-labelled aptamer can be a cost saving in comparison to the antibody. In addition, the availability of the aptamer that is highly specific against the target can minimise non-specific absorption associated with the labelled antibody.

7.3. Sandwich ELASA

Sandwich ELASA is perhaps a better version of ELASA compared to direct or indirect ELASA. Sandwich ELASA does not depend on the method of target immobilisation, purity, or concentration; instead, it depends on the effectiveness of the capturing antibody or aptamer in capturing the target. The capturing antibody or aptamer is immobilised onto the platform surface using the appropriate chemical technique, and the sample containing the target is added after blocking the remaining unbound sites with a suitable blocking agent (Narendran et al., 2012). Subsequently, an enzyme-labelled detection antibody/aptamer is introduced, followed by the addition of substrate that will result in signal emission. There are many variations of sandwich ELASA, several of which are described below.

7.3.1. Aptamer–target–antibody

In the aptamer–target–antibody configuration, the aptamer against the target must be immobilised onto a platform surface after functionalisation with functional groups that do not alter the structural configuration of the aptamer (Fig. 4c). As such, the immobilisation of the aptamer will not affect its interaction with the aptatope on the surface of the target. In addition, considering that some aptamers reportedly have higher binding affinity to the target compared to the antibody (Drolet et al., 1996; Zsofia, 2011), the sensitivity of the ELASA configuration is thought to be higher than that of ELISA. In addition, using an aptamer to capture the target offers the advantage of reusability, as the target can be recovered and the aptamer can be reused to capture a subsequent target.

In another sandwich arrangement, the antibody is immobilised prior to the addition of the target and the labelled aptamer (antibody–target–aptamer) (Fig. 4d). This configuration is the most commonly used among the various types of sandwich ELASA, as it is easier to label the aptamers than the antibodies. Moreover, labelling of the aptamer has a null effect on the conformation of the aptamer. As an example, Zsofia (2011) used ELONA in her study

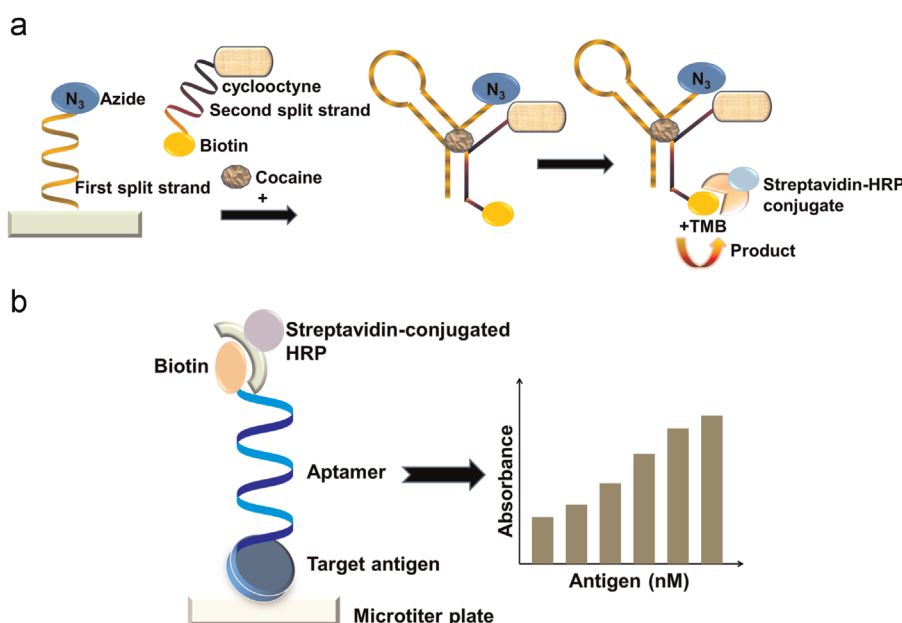


Fig. 6. (a) Split aptamer ligation: this conformation consists of two strands of aptamer: the first split strand and the second split strand. The presence of the target, cocaine, causes these two strands to assemble and facilitates ligation between the azide and cyclooctyne. Attachment of biotin to the surface of the microtiter plate and the subsequent addition of the streptavidin–HRP conjugate and the substrate TMB results in signal production that can be measured spectrophotometrically. (b) Application of ELASA to assess binding of the nucleic acid pool to the corresponding target in SELEX: coating of target proteins with different concentrations onto the microtiter plate followed by the addition of the biotinylated aptamer and streptavidin–HRP conjugate produces a signal that can be measured spectrophotometrically.

of the use of aptamers to detect apple stem pitting virus (ASPV) coat proteins.

7.3.2. Aptamer–target–aptamer

In the aptamer–target–aptamer configuration, two different aptamers against the target are used for capturing and detecting purposes. This assay has advantages, as the capture aptamers can be easily reused and may even have a higher affinity for the target than antibodies, and detection aptamers can be easily labelled. However, two high affinity aptamers must be available for the same target, and the binding sites of these aptamers must not overlap with each other (Fig. 5a). In this case, the application of the aptamer as both the capturing and detection agent can be much more cost-saving compared to antibody. A known example of the successful use of aptamer–target–aptamer sandwich ELISA was described by Park et al. (2013), who adopted this system to measure the amount of hepatitis C virus (HCV) envelope protein E2 present in their experimental system.

7.3.3. Aptamer–target–capture antibody–detection aptamer

This configuration is at the moment an untested concept (Fig. 5b). In this arrangement, an aptamer specific against the target is immobilised prior to the addition of the target and capture antibody specific against the target. Subsequently, an aptamer directed against the capture antibody is added; the aptamer has an enzyme, such as HRP, attached to it to produce a detectable signal. Immobilization of the functionalized aptamer as the capturing element on the surface of the platform is faster compared to the immobilization of antibody that is slower due to passive absorption. Aptamer as the capturing agent can also be subjected to reusability, by using regeneration buffer to allow the aptamer to be reused for subsequent application.

7.3.4. Aptamer–target–capture antibody–detection antibody

This configuration of sandwich ELISA assay can be applied when both the aptamer and antibody are specific against the target. However, the MREs must not overlap with each other, which means that the binding site of the aptamer and the antibody on the surface of the target must be different. This can be ensured by performing a gel shift assay of the aptamer, antibody, and corresponding target (Fig. 5c). The presence of the supershift, which is the band that is moving slower due to the formation of an aptamer–target–antibody complex as compared to the bands containing only aptamer–target or aptamer–antibody complex, gives an indication that the antibody binds to a different region of the target from that of the aptamer. Similar to the other configurations, the aptamer is first immobilised on the surface of the microtiter plate, followed by addition of the target, capture, and detection of the antibody (Narendran et al., 2012). As aptamer immobilisation is the very first step in most of the ELISA configurations, the following factors must be considered prior to immobilisation: type of immobilisation surface, type of aptamer linker used, and the method for aptamer immobilisation (Balamurugan et al., 2008b).

7.4. Split aptamer ligation

In this latest configuration of ELISA, an anti-cocaine DNA aptamer that consists of two strands was utilised as the proof-of-principle (Sharma et al., 2012). The two strands assemble upon binding to the target cocaine. In this assay, the first split strand (capture strand) is immobilised on the surface of the microtiter plate, and then the other split fragment (detection strand) is added to the sample that contains the target cocaine. The capture strand contains azide and amine at the respective terminus, whereas the detection strand contains biotin at one terminus and cyclooctyne

at the other end. The presence of the target cocaine causes both strands to assemble, which promotes ligation between the azide and cyclooctyne. This facilitates the attachment of the biotin to the surface of the microtiter plate. Addition of the streptavidin–HRP conjugate and the substrate 3,3',5,5'-tetramethylbenzidine (TMB) results in signal production that can be measured spectrophotometrically (Fig. 6a). The detection limits achieved were 100 nM–100 μ M in buffer and 1–100 μ M in human blood serum. This single cocaine aptamer can replace both the capture and detection antibody used in ELISA (Sharma et al., 2012). The split aptamer-based assay is also more advantageous than the corresponding antibody assay as the split fragments amalgamate only in the presence of the target to form a complete aptamer structure. Hence, the presence of any non-specific target gives less interference in the signal production owing to the high specificity of the target-split fragment aptamer complex formation.

7.5. Competitive ELISA

Competitive ELISA is designed in order to detect the presence of the target in the crude sample which does not require any prior purification step. In this format, the sample that contains the target is added for the competitive reaction with the immobilized pure target, which causes the decrease in the signal as the target concentration in the sample increases. Competitive enzyme-linked aptamer assay was developed for the detection of tetracycline residue in honey, which is a cheap antimicrobial agent. First, tetracycline–BSA conjugate was immobilized on the surface of the microtiter plate. This is followed by the addition of the biotinylated aptamer and various concentrations of the tetracycline–BSA conjugate. Finally, streptavidin–HRP conjugate and substrate TMB were added for signal production. The detection limit achieved was 9.6×10^{-3} ng/mL with a linear working range from 0.01 to 100 ng/mL (Wang et al., 2014). Competitive ELAA was also developed for the detection of oxytetracycline, an antibacterial agent used to prevent the bacterial growth in milk. Similar to the protocol exhibited by Wang and colleagues, after the immobilization of the target protein oxytetracycline, constant amount of the biotinylated aptamer and different concentrations of the unlabelled oxytetracycline were added. Signal production was aided with the addition of the streptavidin–HRP conjugate and substrate TMB. The detection limit achieved was 12.3 μ g/L and this assay corroborated the potential application of this simple assay for the detection of oxytetracycline in food product (Kim et al., 2014). In an inverse configuration of competitive ELAA, aptamer against dopamine was conjugated with biotin and was immobilized on the surface of the streptavidin coated plate. Following this, dopamine with a series of different concentrations were added for incubation with the immobilized aptamer. Then, a constant amount of dopamine conjugated to HRP was added, followed by substrate addition and signal measurement. Dopamine was not immobilized on the surface as it is very sensitive to oxygen and light which can lead to degradation after prolonged exposure. This simple assay achieved a detection limit of 1 pM and can be a potential assay for the detection of dopamine that is a clinical diagnostic marker of Parkinson's disease (Park and Paeng, 2011). Competitive ELISA can confer higher specificity against the target compared to the corresponding antibody-based assay due to the reaction specificity of the aptamer against the target.

7.6. ELISA–ELAA

ELISA–ELAA is a configuration that uses both ELISA and ELAA. As the proof-of-concept, Baldrich et al. (2004) used an anti-thrombin DNA aptamer. The HRP-labelled aptamer was mixed with different concentrations of thrombin and added to an anti-

thrombin antibody immobilised on the surface of the microtiter plate. Compared to ELAA alone, which had a detection limit of 1.8 nM, ELISA–ELAA had a detection limit of 1 nM. This result proved that using aptamer as the capturing agent can complement the role of an anti-thrombin capturing antibody (Baldrich et al., 2004).

8. Biolayer-interferometry-based ELASA

Biolayer-interferometry (BLI) is a label-free technology that relies on the interaction between the ligand immobilised on the sensing surface (which consists of a glass fibre) and the analyte. The interaction between the ligand and the analyte results in a shift of the interferometry profile, which can be monitored in real time. Gandhi et al. (2012) described direct ELISA using BLI as the platform. In their study, amine reactive second generation sensors (AR2G from ForteBio) were immobilised with the target protein at various concentrations using EDC (1-ethyl-3-(3-dimethylamino-propyl) carbodiimide) and NHS (*N*-hydroxysuccinimide) coupling. After quenching of the reactive surface using 1 M Tris buffer (pH 8), biotinylated aptamer was added followed by the addition of the streptavidin–HRP conjugate, substrate TMB, and spectrophotometric signal measurement, as in ELISA. Gandhi et al. (2012) are also currently developing a sandwich-based ELISA assay by functionalising the aptamer with 3'-amine for immobilisation onto the platform surface using identical EDC/NHS coupling. The addition of the various concentrations of the target and biotinylated secondary aptamer is carried out before the addition of substrate TMB and signal measurement (Gandhi et al., 2012). However, inverse configuration of immobilizing the aptamer on the platform surface is also possible by using amino group conjugated via a C12 linker at the 3'-end.

9. Colorimetric ELAA

In a format that is equivalent to the conventional sandwich ELAA, colorimetric ELAA was devised. Colorimetric ELAA has the advantage of allowing result interpretation by naked eyes without the need for any special equipment. It comprises of two different aptamers that bind to distinct regions on the surface of the target molecule. The first aptamer (capturing agent) was immobilized on the surface of the thiol-functionalized glass. This thiol group was conjugated to the amine-functionalized aptamer via sulfo-succinimidyl 4-(*N*-maleimido-methyl) cyclohexane-1-carboxylate (sulfo-SMCC) as a cross-linker. The detection aptamer was functionalized with HRP for detection. The limit of detection is 25 fM and the surface-functionalized aptamer can be reused by regeneration with 8 M urea due to the stability of the disulphide bond of the cross-linker (Park et al., 2014). The assay developed has a better linearity over a wide range than the study conducted by Nie et al. (2013), who have used enzyme-linked HRP and biotinylated aptamer rather than direct conjugation of the detecting aptamer with the HRP.

10. Exonuclease I assisted-ELAA

Selective degradation of exonuclease I (Exo I), which specifically cleaves ssDNA from the 3' to 5' was used as the underlying principle in a novel format of ELAA. This Exo I assisted-enzyme-linked aptamer assay was successfully developed using ATP and L-argininamide as the proof-of-concept. In this format, DNA aptamer integrated with fluorescein isothiocyanate (FITC) was hybridised with the complementary sequence before attachment onto the

streptavidin-coated microtiter plate. In the presence of the target, aptamer–target bound complex formation forbear the degrading action of Exo I. The FITC, which is conjugated at the 3'-end of the aptamer sequence, remains intact and produces signal as a result of interaction with the enzyme HRP-conjugated anti-FITC. In the absence of the target, Exo I degrades the exposed aptamer from the 3' to 5'. This action releases the FITC group and triggers no signal production. This assay can be potentially applied for detection of small molecules and macromolecules (Zhao et al., 2014). Exonuclease I assisted-ELAA is the first ELAA assay that harness enzyme to augment the assay specificity. Similar assay can be designed by using other enzymes such as S1 nuclease that degrades ssDNA/RNA (Vogt, 1973) or any RNA cleaving enzymes such as RNase T1 that cleaves RNA at the 3'-end of the G residues but does not cleave the RNA–protein complex (Sawadogo and Roeder, 1985).

11. Fluorescent enzyme-linked DNA aptamer-magnetic bead sandwich assay

Signal readout in a common ELAA method is based on the reaction between HRP and the substrate TMB. One alternative substrate that can offer better signal enhancement and a broader assay range than TMB is Amplex Ultra Red (AUR) reagent. Using AUR as the substrate, Fluorescent Enzyme-Linked DNA Aptamer-Magnetic Bead Sandwich Assay was developed for the detection of Leishmania in sandflies using two specific aptamers as the proof-of-principle. Magnetic beads are employed as the platform for the immobilization of the aptamers for the development of this enzyme-linked aptamer assay. Fluorescent peroxidase conjugated to biotinylated DNA aptamer was immobilized on the surface of the streptavidin-coated magnetic beads and incubated with *L. major* promastigote-infected sandfly homogenates (Bruno et al., 2014). After incubation, the magnetic beads were collected by magnetic racks and incubated with biotinylated reporter aptamer followed by subsequent addition of the streptavidin–HRP. AUR reagent was then added and a Handheld Fluorometer (FLASH Reader) was used to measure the fluorescence signal. The detection limit achieved is 100 ng of homogenate *L. major* promastigote. AUR can ensure consistency of the signal readout even with a low level of analyte as the fluorescent product of the AUR is readily detectable since this product is channelled into the bulk solution. In fact, AUR reagent can alleviate the shortcomings such as blue shifting and loss of fluorescence frequently associated with fluorophore quantum dot (Fitzpatrick et al., 2009).

12. Piezoelectric aptamer-linked immunosorbent assay

Piezoelectric aptamer-linked immunosorbent assay or PALISA is a novel method of frequency based ELASA that uses Acoustic Membrane MicroParticle (AMMP) as the platform for the sensitive detection of thrombin (Collins et al., 2013). AMMP is a platform that applies acoustic loading to track the surface capture on the surface of piezoelectric material termed ViBE platform. This platform enables the automatic introduction of the sample and the result is displayed in real-time by integrated software (ViBE, version 0.7.X). The aptamer-conjugated to the magnetic beads and fluorescein-labeled aptamer which bind to different regions of the target thrombin are incubated with the sample in 96-well microtiter plate. These beads are collected by magnetic field and the beads without the captured analyte are discarded. The analyte-captured beads are transferred to the sensor chip, which are conjugated with anti-fluorescein antibody. This antibody is able to bind to the beads that have captured the analytes. The

quantification is based on the vibrational frequency change of the magnetic beads bound to the surface of the sensor, which reflects the amount of target captured. The detection limit achieved is 386 pg/ml and this platform averts the usage of HRP/substrate TMB for signal production. The target-induced conformational change of the aptamer in the presence of the target ensures a large change in the frequency compared to the antibody-based target recognition that does not induce any conformational change of the antibody. Large alteration of the frequency translated from this conformational change can be a sensitive strategy of measuring the aptamer–target interaction.

13. Other non-enzymatic-based aptamer assays and applications

Also known as reporter-linked aptamer assay (RLAA), they are the new generation assays which does not depend on enzymes or even substrates. These types of assays are designed to go around the few disadvantages of using enzyme-labelling, such as multiple assay steps, some hazardous substrates and the possibility of interference from endogenous enzymes, at the cost of reagent stability and speed in obtaining visual results (Pierce Biotechnology Inc., 2005). Various types of reporters have been introduced, for example fluorophores which produce fluorescence. In fluorescence, instead of using a substrate, it uses blue light to activate the fluorophore in order to generate fluorescence. There are diverse types of fluorophores which generates different fluorescent colours. For example, GFP produces green colour, YFP produces yellow colour and BFP produces blue colour (Rizzo et al., 2009). Although phosphorescence has been around for quite some time, it has just recently been proven to be of use as a reporter in an assay by Yan and Wang (2011) in their effort to detect heparin. They have reported tuneable sensitivity and detection window in their experiment. This may mark a new branch in ELASA in which phosphorescence can be used.

14. Applications of ELASA

Because aptamers can be generated against virtually any target molecule, ELASA can be used to detect the corresponding targets. Potential targets include environmental hazards/pollutants (e.g., arsenic or cyanide levels in factories and mines), polychlorinated biphenyl, lead, and other contaminants in water (Fukata et al., 2006; Hayat and Marty, 2014). Besides being used as a virus genotyping tool (Gopinath et al., 2006), ELASA can also be used as a fast presumptive screen for illegal drug/stimulant usage in athletes and as a cheaper and more sensitive home pregnancy test than currently available ELISA-based tests. Another potential use of ELASA is for high-throughput screening for biomarker discovery, as aptamers are particularly useful in targeting proteins that have no known binding partners (Green et al., 2001). In summary, ELASA can perform all the tasks that an ELISA can perform, and probably has numerous advantages over ELISA.

Finally, ELASA can be a useful tool for assessment of binding between the nucleic acid pool from each SELEX cycle and the target antigen. For example, a constant amount of envelope peptide or protein is immobilized on the surface of the well of a microtiter plate. After washing to remove unbound antigen, a biotinylated aptamer was added for incubation, followed by washing and then the addition of streptavidin-peroxidase. Substrate was added for signal production and the absorbance was measured at 405 nm. The absorbance corresponds to the binding affinity of the nucleic acid pool till saturation point is reached.

This method facilitated the generation of an aptamer against *Shigella sonnei*, the causative agent of the enteric infection called Shigellosis. Among the five DNA sequences tested, one sequence was found to bind the target with the highest affinity (i.e., it had the highest absorbance reading) (Masoudipour et al., 2011). Nagarkatti et al. (2014) used ELASA to monitor binding of the nucleic acid pool from round 2 to 10 of SELEX against *Trypanosoma cruzi* excreted secreted antigens (TESA). A gradual increment of binding affinity was found for cycles 2, 4, 6, 8, and 10. Sequence analysis of the nucleic acid pool from round 10 of SELEX revealed seven different classes of sequences, with one particular sequence, Apt-L44, showing significant binding to the target (Nagarkatti et al., 2014). Bruno et al. (2007) reported that four aptamer candidates (LD-3F, LD-3R, LD-5F, and LD-5R) have high affinity against *Leishmania donovani* and *L. tropica* as determined by ELASA. This method does not require the use of radioisotope labelling to assess the binding of nucleic acid pool against the target in SELEX, which can be time-consuming and expensive.

15. Commercialisation

Years have passed, and while many aptamers have been discovered and reported in academic journals, the aptamer-based diagnostic revolution has not materialized. There have been many reasons and one of the major ones is antibody generation is a very well-developed method with a history of around 120 years (Llewelyn et al., 1992). There are standardised protocols in generating new antibodies that has become routine in nature. However, aptamers still undergoes several developmental stages compared to antibodies—aptamers only have around 20 years of history. This is because the process of discovering aptamer is very much dependent on the laboratory skills. In addition, complex target molecules such as glycosylated protein and small molecule can complicate the selection process. Selecting aptamer against complex target molecules such as glycosylated protein is plausible by using boronic acid modified randomized nucleic acid library (Li et al., 2008). Another issue with the delay of aptamer in drug discovery process is the specificity of aptamer against unknown proteins from the human system. However, aptamers are still holding advantages and it can be produced infinitely by using simple molecular biology techniques such as PCR and in vitro transcription, which can be economic compared to the antibody production. These aptamers can be modified prior to conjugation on the platform for ELASA. In fact, a recent ELASA-based dipstick test specific to detect aflatoxin B1 in grains and corn was developed by Shim et al. (2014) that shows the commercial potential of ELASA. Since aptamers can be very easily produced, the mass production of anything that is based on aptamers is definitely not a problem. Unlike ELISA-based kits or dipsticks, ELASA can be kept at room temperatures and has a longer shelf-life. The biotinylated aptamers can be pre-immobilized on the surface of the streptavidin-coated plate. The plate can be stored at RT and the wells can be used after the addition of the appropriate buffer, unlike that of antibody which requires storage at 4 °C for prolonged stability of the protein. Hence, it is very possible that ELASA-based kits and tests will be making their mark on the market soon enough. One rationale for the market potentiality of ELAA is that many aptamers are generated against the targets to which antibodies were previously generated. For example, aptamers were raised against erythropoietin (Citartan et al., 2014), human menopausal gonadotropin (Ohno et al., 2012), cocaine (Kawano et al., 2011) and insulin (Yoshida et al., 2009) though antibodies against these targets are already available.

16. Conclusion

From the many ELASA techniques discussed, the most optimal method would be the aptamer–target–aptamer configuration. Since most aptamers are directly selected against the target analyte, it is easier to use the aptamers that we already have, especially if they are more sensitive than their respective targets' antibodies. Another appealing character of aptamer over antibody is generation of aptamer against small molecules where antibody fails. In addition, aptamers can be more easily attached to a detecting agent (horseradish peroxidase, fluorophore molecule, or others) compared to antibodies without perturbing the binding sites. However, this configuration requires the availability of two different aptamers binding to different regions of the target. 'Tweaking' of the SELEX strategy is able to generate aptamers binding to the distinct regions of the target. This can be achieved by modulating the interaction of the nucleic acid pool with the target protein blocked at a specific binding site. Blocking of this site can actually skew the selectivity of the aptamer to be focused against the other 'unblocked' site (Hohmura et al., 2013). Another possible approach also involves the usage of complex target SELEX that results in the generation of several different classes of aptamers binding to different sites on the target. Target molecules such as whole cell surface protein of viruses, bacteria and cell fragments can be amenable candidates for complex target SELEX (Shamah et al., 2008). However, antibody can still complement the usage of aptamer in the form of sandwich assay, if only one aptamer binding to a single region of the target is available.

17. Future perspectives

"SciFinder" reports that between 2009 and 2010, more than 2000 publications on aptamers were published, which suggests that aptamer research is experiencing burgeoning growth (Iliuk et al., 2011). This increased interest has stimulated many studies of aptamer-based applications, such as the development of ELASA and the generation of somamers, which have much higher binding affinity than conventional aptamers. These advances suggest that the gradual replacement of antibodies with aptamers may begin to occur at a faster pace. The advent of locked nucleic acid (Kuwahara and Obika, 2013) and somamer (Park et al., 2013) techniques to enhance stability of aptamers and to heighten the binding affinity of aptamers, respectively, will increase the efficiency of ELASA. Another possible future development of ELASA will involve the use of an inert polymer as the platform to facilitate the application of multiple substrates in a single reaction, as was demonstrated by Dixit (2011).

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