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Aptamer Sensors Combined with Enzymes for Highly Sensitive Detection

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1. Introduction

Diagnostics have taken on a larger role in patient care, with diagnostics for point-of-care testing (POCT) having greatly expanded during the last two decades. The expectation is that POCT will continue to grow because it can help to expand personalized therapy and theranostics, which will provide the right medicine to the right person, with the right dosage at the right time. For POCT, it will be necessary to construct miniaturized biosensors at a low cost that have high sensitivity with rapid sensing.

Affinity-based techniques that are used in clinical diagnostics rely on the high affinity and specificity of antibodies for highly sensitive detection of target molecules. Antibody-based diagnostic systems are well established, and enable the detection of various molecules. However, aptamers are expected to be the next-generation elements for molecular recognition.

Aptamers are short strands of nucleic acids that have been designed to specifically bind to various target molecules, and have comparable affinity and specificity to antibodies. They are selected using the SELEX (Systematic Evolution of Ligands by Exponential Enrichment) technique (Tuerk & Gold, 1990), also referred to as "in vitro selection" or "in vitro evolution." SELEX is a combinatorial chemistry technique that produces single-stranded oligonucleotides of DNA or RNA, which specifically bind to a target ligand(s).

It has been two decades since the first report on aptamers became public (Ellington & Szostak, 1990; Tuerk & Gold, 1990). During these two decades, many aptamers have been reported and an aptamer has been approved as a therapeutic medicine by the US Food and Drug Administration (FDA). Aptamers have many advantages as biosensors that antibodies do not have, such as:

1. The ability to be selected by SELEX.
2. The ability to be synthesized and modified.
3. The ability to renature.
4. More stability than antibodies.
5. The ability to change conformation upon binding to target molecules.

For theranostics, the first advantage is important when it is necessary to obtain affinity probes against various novel biomarkers. SELEX has many advantages, and is an excellent method to evolve aptamers so that they have an extremely high binding affinity to a variety of target ligands. We were able to successfully achieve the automatic in vitro screening of aptamers, which enabled us to obtain aptamers against more than 100 proteins per month (Cox & Ellington, 2001). Aptamers can therefore be developed more rapidly than antibodies.

In addition, since there is virtually no shortage of targets for aptamers, we can obtain aptamers against small molecules, peptides, proteins and even cells.
Aptamers selected by SELEX against purified membrane proteins often do not bind to native membrane proteins on the cell because the membrane protein will change its conformation after purification. Since Cell-SELEX enables us to treat native membrane proteins as target molecules, we can obtain more useful aptamers for cell identification (Pestourie et al., 2006). SELEX against unpurified protein, including Cell-SELEX, can be utilized to identify biomarkers as well (Berezovski et al., 2008; Noma et al., 2006a). Although in vitro selection is a powerful method to obtain aptamers, selected aptamers often require optimization for the best affinity and specificity. We can perform post-optimization of their sequences after SELEX through iterative cycles of mutated DNA synthesis and evaluation of their functions, which would be a cumbersome process for antibodies (Ikebukuro et al., 2005b; Ikebukuro et al., 2006; Knight et al., 2009; Noma & Ikebukuro, 2006; Noma et al., 2006b; Savory et al., 2010).
The fifth advantage is important so that aptamers can be perceived as unique reagents for analytical applications, not as alternatives to antibodies. Since aptamers are a kind of biopolymer consisting of nucleic acids that have many negative charges, their tertiary structures are destabilized by repulsion between phosphate backbones. In addition, since aptamers are formed by Watson-Crick base pairing, some aptamers have the ability to form different structures. Target molecule binding stabilizes a particular structure, even though the structure would have little chance of forming without the target molecules. Aptamers have the potential to change structures, and in some cases, drastically. Many aptameric sensors have been reported that rely on the transduction of structural changes into detectable signals (Han et al., 2010; Li et al., 2010).
In this chapter, we focus on enzymes that are combined with aptamer sensors. Since enzymes catalyze various kinds of reactions, they are used as biosensors for many biomarkers. In addition, since catalytic turnover of enzymes enables signal amplification at moderate temperatures, it enables affinity-based biosensors to measure target molecules with high sensitivity, and without the need for radioisotopes. Most antibody-based diagnostics systems use enzyme-labeled antibodies. For the combination of aptamers with enzymes, aptamers would work not only as a replacement molecule for antibodies, but also as a part of a new device that can exploit the unique properties of aptamers. First, we categorize aptamer sensors based on how to combine enzymes with aptamer sensors. Second, we describe the detail of the enzymes and discuss them based on biosensors for highly sensitive detection and POCT.

2. Strategy of enzyme combination with aptamer sensors

The key point of combining an aptamer sensor with an enzyme is the ability to distinguish aptamer–target molecular complexes from unbound aptamers before signal amplification. Figure 1 shows how aptamer sensors using enzymes are divided into two groups based on how to distinguish aptamer-protein complexes from unbound aptamer. Bound free (B/F) separation can easily purify aptamer-protein complexes by eliminating unbound aptamer. Ordinarily, to eliminate unbound aptamer and detect an aptamer-protein complex by its enzyme activity, a sandwich assay is applied. Most clinical diagnostic methods use an antibody-based sandwich assay such as the enzyme-linked immunosorbent assay (ELISA). However, we constructed a biosensor using an aptamer which had the unique properties of

aptamers, as well as their ability to bind to the target molecule. When we perform B/F separation using an antibody, we apply a competitive assay. However, a competitive assay requires purified target molecules, and the process is difficult when the purification of a target molecule is laborious. As mentioned above, aptamers have unique features: their structural change is accompanied by target binding. Therefore, we can construct an aptamer based B/F separation system that is not a competitive assay.

The second group does not require B/F separation, and regulates enzymatic activity by using target molecules. It can be divided into two subgroups. One group regulates enzymatic activity directly by using target molecules. The other group changes the input DNA structure into an output DNA structure that can serve as a substrate for polymerase. Since the polymerase can replicate the substrate DNA, we can control the polymerase reaction via regulation of the substrate DNA.

We will describe the details of each group separately.

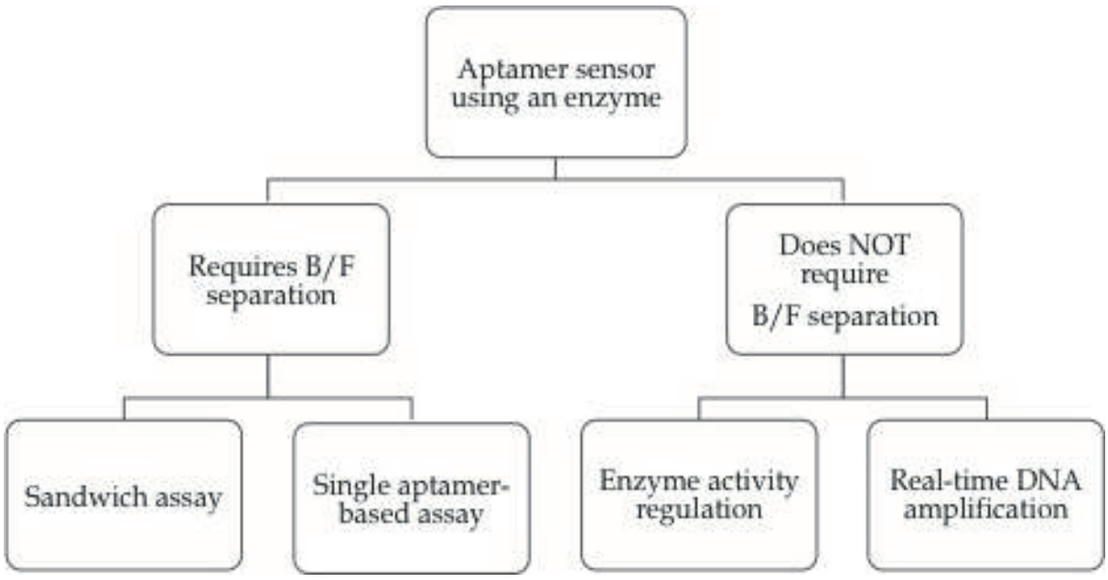

Fig. 1. A schematic of the two categories of aptamer sensors that use enzymes. Aptamer sensors that use enzymes are divided into two main groups. The first group requires B/F separation, and the second group does not require it. The first group is divided into two subgroups based on how the B/F separation is performed. The second group is divided into two subgroups based on how enzymatic activity is regulated. In one subgroup, enzymatic activity is regulated directly by the activity of the aptamer, and in the other, the structure of the aptamer is regulated by target binding for DNA amplification.

2.1 Sensing with bound free separation

2.1.1 Sandwich assay

B/F separation is an attractive process because it can eliminate not only unbound enzyme-modified molecular recognition elements, but also nonspecific binding and some molecules that affect noise. Since B/F separation reduces background signal and noise, it enables highly sensitive detection. Most clinical diagnostic methods using antibodies are based on the sandwich strategy. A sandwich assay requires two molecular recognition elements that bind to the target molecule simultaneously. One is immobilized on a solid support such as

beads or plates, and the other one is modified with enzymes. Using them, unbound molecular recognition elements modified with enzymes can be removed, and the target molecule can be detected without the need to label target molecules via enzyme activity measurements (Fig. 2). There have been many reports that describe the sandwich assay (Han et al., 2010) and many types of enzymes have been mentioned in these reports, as we will discuss later. Sometimes, the aptamers modified with enzymes are used as detection reagents, and antibodies are used for capturing the target reagent. A chemical crosslink is required when antibodies are modified with enzymes, and it often causes a loss of function. However, since aptamers can be easily modified with various molecules, they can simply be connected with any enzyme via interaction with modified molecules and their partner molecules, such as biotin and avidin.

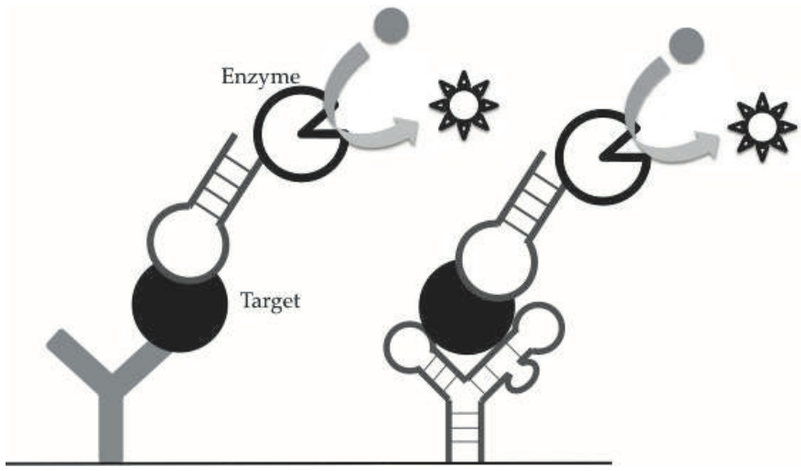

Fig. 2. Sandwich assay using an aptamer. On the left side, the antibody is used as a capture reagent and the enzyme-modified aptamer is used as a detection reagent. On the right side, different aptamers are used as capture reagents and detection reagents. For the sandwich assay, both reagents should recognize different regions of the target protein if the target protein is not a homomultimeric protein.

2.1.2 B/F separation based on a single aptamer

Since the sandwich assay requires two molecular recognition elements that can bind to the target molecule simultaneously, it is difficult to apply it to the sensing of small molecules and proteins that have only one superior molecular recognition element. Therefore, we constructed a simple B/F separation system based on single aptamers using a conformational change of aptamer. This system consists of two parts: an aptamer and its complementary DNA. We previously described two types of single aptamer-based B/F separation systems (Abe et al., 2011; Fukasawa et al., 2009; Ogasawara et al., 2009) (Fig. 3).

The first type of system makes use of the stability feature of aptamers by providing target molecules so that the aptamer will bind to them and stabilize its structure (Fig. 3(a)). Structures of aptamers are destabilized by repulsion between negative charges. Therefore, an aptamer can be easily hybridized with its complementary DNA without target molecule binding. On the other hand, since target binding stabilizes the whole structure of the aptamer, the aptamer-target complex inhibits hybridization with complementary DNA. We designed a complementary DNA named CaDNA that will bind to a part of an aptamer that

is amenable to hybridization inhibition upon binding to the aptamer target. We modified the aptamer with an avidin-conjugated enzyme and we succeeded in detecting thrombin, IgE (Fukasawa et al., 2009), and vascular endothelial growth factor (VEGF) (in preparation) via enzymatic activity measurement.

The second system makes use of the structural changes that aptamers undergo upon binding to their target molecules (Fig. 3(b)). We created a "capturable" aptamer by adding a sequence to it that gave it a new structure. Capturable aptamers cannot hybridize with CaDNA unless their target molecules are present. In this case, the structure of a capturable aptamer in the presence of its target molecule changes to a different structure from that which was present in the absence of the target molecule. We succeeded in the design of a capturable aptamer for thrombin (Abe et al., 2011) and a mouse prion protein (Ogasawara et al., 2009). In these studies, although fluorescent labeling was used for detection, enzyme labeling enabled a 10-fold lower detection of mouse prion protein than fluorescent labeling (unpublished data).

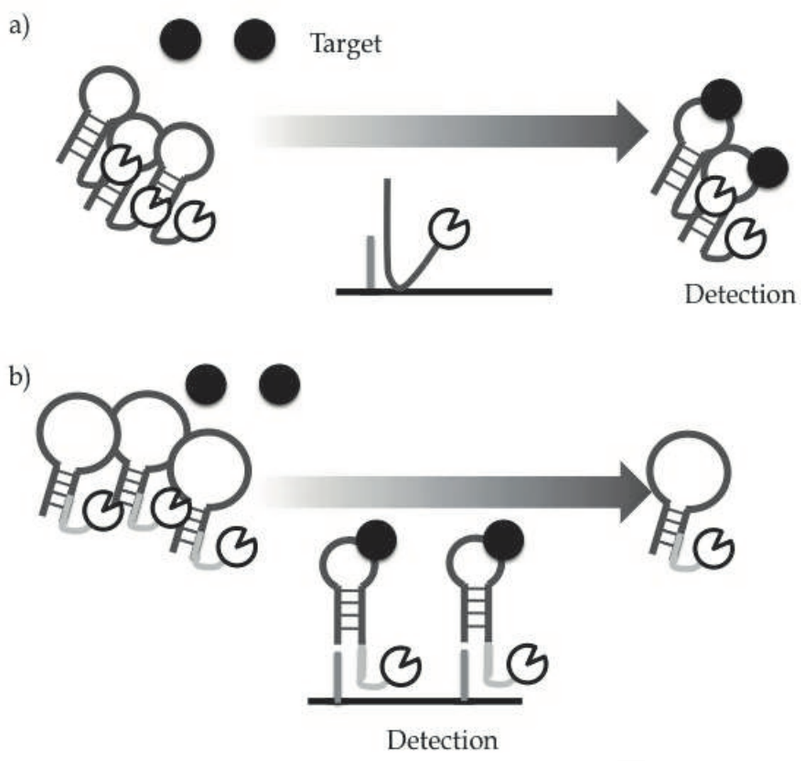

Fig. 3. The scheme of a single aptamer-based B/F separation system. (a) In the absence of a target molecule, the aptamers are trapped by the immobilized beads containing CaDNA, whereas in the presence of the target protein, aptamers that bind to the target are not trapped. The target protein can therefore be detected by means of simple B/F separations, and by measuring the fluorescence or enzymatic activity of the labeled aptamer in the supernatant. (b) The aptamer, which is able to be captured, undergoes a conformational change upon binding to the target molecule. This change induces the exposure of a partial single-strand that hybridizes with the CaDNA. Otherwise, any unbound capturable aptamer does not hybridize with the CaDNA and is removed by the bound/free separation.

Of these two types of single aptamer-based B/F separation systems, the first can be easily designed, because it does not require any additional sequences, whereas the second system requires careful design of the additional sequence of the aptamer with structural prediction. However, the benefit of the second system is that it can eliminate many interfering compounds. The first system can eliminate enzyme-modified aptamers that do not bind to the target molecule, but it is difficult to eliminate interfering compounds because aptamers that bind to the target molecule are present in the supernatant. It is therefore necessary to select a particular system to suit the needs of each particular target molecule.

Wei et al. reported a different type of single aptamer based B/F separation system without complementary DNA being present (Wei & Ho, 2009). They utilized steric hindrance between enzyme-modified antibodies and antigen-modified target-binding aptamers. They used fluorescein-modified aptamers and anti-fluorescein horseradish peroxidase (HRP)-conjugated antibody. The antibody cannot bind to the fluorescein-modified aptamer due to steric hindrance without its target molecule. The aptamers change conformation upon binding to the target molecule, and then the antibodies can bind to them. Since the aptamers were immobilized on the solid support, this sensing system enabled B/F separation to occur using an aptamer.

2.2 Homogeneous sensing

To measure the target molecules without B/F separation, regulation of signal output is required. Jhaveri et al. reported aptamers that changed their structure upon binding to the target molecule, which resulted in the regulation of fluorescent signals (Jhaveri et al., 2000). If we can introduce enzyme signal amplification into a signaling aptamer, a highly sensitive detection can be performed without the need for B/F separation. Reported homogeneous detection systems using enzymes are based on two strategies: enzyme activity regulation by the target molecule, and DNA amplification accompanied by the target molecule binding to aptamers.

2.2.1 Enzyme activity regulation by the target molecule

If we can find an enzyme that catalyzes a reaction with a target molecule, we can construct an effective sensing system such as the glucose sensor, which is already on the market and is being used daily. However, it is difficult to screen an enzyme that reacts with a given target molecule. Protein engineering allows us to improve the enzyme substrate specificity, and we have reported such examples (Igarashi et al., 2004), but it is still difficult to change the substrate specificity dramatically. Then we constructed an enzyme that has a novel subunit that can regulate enzymatic activity allosterically based on the aptamer. If the target molecule activates enzymatic activity, we can quantify the target molecule via an enzyme activity measurement. We named this sensing system the Aptameric Enzyme Subunit (AES) (Ikebukuro et al., 2008; Yoshida et al., 2009; Yoshida et al., 2006a, b, 2008).

An AES consists of two aptamers: an enzyme-inhibiting aptamer and a target molecule-binding aptamer. The enzyme does not generate signals because the AES inhibits enzymatic activity when it is not bound to the target molecules. However, upon binding of the target molecules to the AES, the AES changes its conformation, which results in a loss of enzyme inhibitory activity. Then we can measure the target molecule concentration via enzyme activity measurements without the need for B/F separation. Therefore, an AES acts as an enzyme subunit that can regulate its activity via the target molecule binding allosterically.

Figure 4 shows a design strategy for an AES. To act as an AES, the binding ability of an enzyme-inhibiting moiety against an enzyme should decrease upon binding of the target molecule to the target molecule-binding moiety. We used a 31-mer thrombin-binding aptamer (TBA) that we optimized as the enzyme-inhibiting aptamer (Fig. 4(a)) (Ikebukuro et al. 2005b). The TBA forms a G-quadruplex structure that plays an important role in its inhibitory activity. Then we inserted the target molecule-binding moiety into a loop region of the G-quadruplex that does not critically affect its binding ability against thrombin. This was done by inserting the DNA-binding domain into the TBA (Yoshida et al., 2006b) (Fig. 4(b)). DNA binding would disrupt the TBA's structure, resulting in an increase of thrombin activity. Next, we inserted an adenosine-binding aptamer into the TBA (Yoshida et al., 2006a) (Fig. 4(c)). We expected that adenosine binding would stabilize the TBA structure rather than disrupt it. As expected, we observed a decrease in thrombin activity that was dependent on the adenosine concentration. However, it was not obvious whether most aptamer stabilization occurred because of the aptamer's structure, or whether there was also influence from the TBA's structure upon binding to the target molecule. Then, we designed different types of AESs for the purpose of universal molecule sensing (Fig. 4(d)).

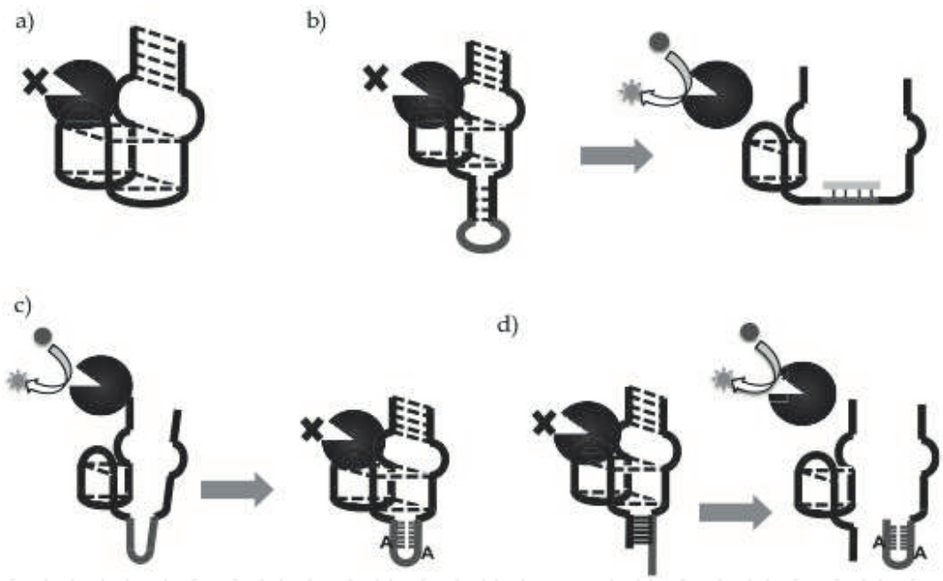

Fig. 4. Aptameric enzyme subunits using a thrombin-inhibiting aptamer. The target-binding aptamer was inserted into a loop of thrombin-inhibiting aptamer that was not a critical region for thrombin recognition. a) The structure of 31-mer thrombin-inhibiting aptamer b) The AES inhibits thrombin activity without a target DNA. Target DNA hybridization induces a destruction of the structure of thrombin-inhibiting aptamer, resulting in an increase of thrombin activity. c) There is more inhibition of thrombin activity when the AES binds to the target molecule as compared to when there is no target binding. d) The AES inhibits thrombin activity without a target molecule. Target molecule binding induces a break in hybridization between the target molecule binding aptamer and additional complementary DNA, resulting in an increase of thrombin activity.

We split the TBA into two parts in the same region where a target-binding aptamer was inserted. One strand is connected with the target-binding aptamer and another strand is

connected with its complementary strand (Fig. 4(d)). Without the target molecule, the target-binding aptamer moiety hybridizes with its complementary strand, which results in the stabilization of the TBA conformation. Then the TBA moiety inhibits thrombin enzymatic activity. Target molecule binding disrupts complementary base pairing and results in a single-stranded nucleic acid structure, which would destabilize the structure of TBA and increase thrombin enzymatic activity. Compared with former AESs, we would be able to design a type of AES that is easily split. We succeeded in designing a type of split AES for sensing adenosine (Yoshida et al., 2006a), IgE (Yoshida et al., 2008) and insulin (Yoshida et al., 2009).

Chelyapov and Fletcher et al. reported similar sensing systems for AESs (Chelyapov, 2006; Fletcher et al., 2010). Chelyapov used an aptamer that inhibited Russell's viper venom factor X activator (RVV-X), and Fletcher et al. used an aptamer that inhibited *Eco*RI.

AESs are advantageous because they sense rapidly and easily. Target molecule binding transduces enzymatic activity immediately. In addition, an AES does not require the modification of an enzyme with an aptamer. Therefore, enzymatic activity can be fully utilized. To design AESs for highly sensitive detection, it is most important that the aptamer has powerful enzyme inhibitory activity. When we used an aptamer with weak inhibitory activity, we had to add a large quantity of it in order to completely inhibit thrombin activity. Then most of the aptamer in solution will not bind to enzyme It is difficult to detect low concentrations of target molecules because target molecules bind to AESs that do not bind to enzyme. Therefore, we should use enzyme-inhibiting aptamers that have a high inhibitory activity.

2.2.2 Real-time PCR or RCA assay

Fredriksson et al. reported a proximity ligation assay (PLA) (Fredriksson et al., 2002). The PLA depends on the simultaneous and proximate recognition of target molecules by pairs of affinity probes modified with oligonucleotides. Each modified oligonucleotide can be hybridized with connector DNA, resulting in the formation of amplifiable DNA through ligation between modified oligonucleotides. Then we can detect target molecules through PCR amplification without B/F separation. Fredriksson et al. reported a PLA using an aptamer (Fig. 5(a)). Although PLA and immuno-PCR require oligonucleotide modification with affinity probes, oligonucleotide modification with an antibody is a cumbersome process. On the other hand, the aptamer can be easily connected to oligonucleotides by DNA synthesis. Therefore, the aptamer is more suitable for immuno-PCR and the PLA than the antibody.

Di Giusto et al. reported protein detection by rolling cycle amplification (RCA) based on proximity extension (Di Giusto, 2005) (Fig. 5(b)). This method used a circular aptamer and an aptamer that had a complimentary sequence with a part of a circular aptamer that could bind to the target molecule simultaneously. They reported circularization of the aptamer, enabling it to stabilize without loss of function. When both aptamers bind to the target molecule, complementary DNA hybridizes with a part of the circular DNA, and the rolling cycle amplification reaction starts. This method can detect protein, without the need for carrying out B/F separation or ligation.

Although proximity ligation or an extension assay will achieve highly sensitive detection of proteins without B/F separation, they require two aptamers that can bind to the target molecule simultaneously. There are some reports of protein detection by PCR or RCA that employs the conformational change of an aptamer. For PCR, binding to the target molecule

should induce a conformational change of the aptamer, and when the aptamer hybridizes to its complementary DNA, this will serve as a primer binding site (Yang & Ellington, 2008) (Fig. 5(c)). Then we can detect the target molecule by ligation of the aptamer to complementary DNA followed by PCR amplification. On the other hand, for RCA, Yang et al. designed an aptamer sequence for proximity ligation within the internal aptamer (Yang et al., 2007) (Fig. 5(d)). Upon binding of the target molecule, both the 5′ end and 3′ end form a stem and join with each other. Then an aptamer is formed by ligation of circular DNA, and it is amplified by RCA

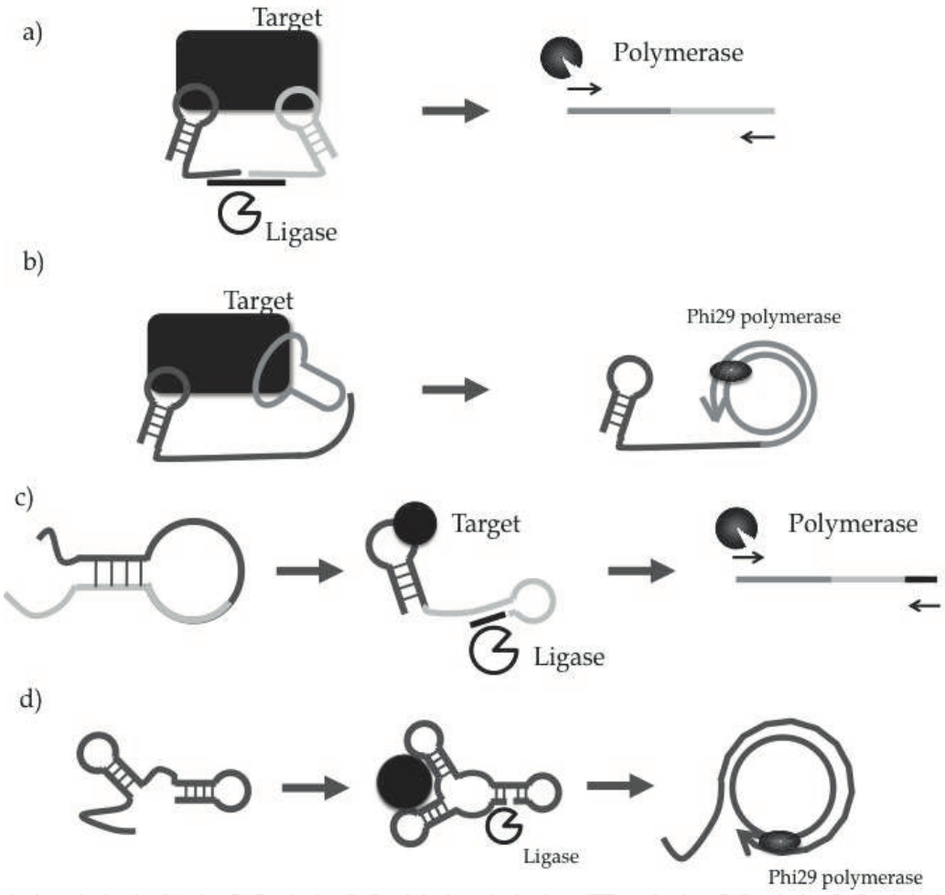

Fig. 5. Biosensing based on different methods of DNA amplification, accompanied by target molecule binding. a) Proximity ligation assay. Two aptamers are ligated after binding to the proximate site of target molecules, resulting in the detection of the target through PCR amplification. b) Proximity extension assay. An aptamer is circularized and a primer sequence that is complementary to a part of the circularized aptamer is added to the other aptamer. Proximate binding of both aptamers to the target molecules induce a RCA reaction. c) Target molecule binding induces a conformational change in the aptamers. Then, the aptamer hybridizes and ligates with probe DNA, resulting in the formation of amplifiable DNA, which enables detection of the target through PCR amplification. d) Target molecule binding induces a conformational change of the aptamer, resulting in the formation of circular DNA by intramolecular ligation. Circular DNA is amplified by RCA.

Conformational change of an aptamer is an attractive strategy for biosensing because only one aptamer is required. However, to design drastic conformational changes of the aptamer would be time-consuming. Although there are many reports of biosensing using conformational changes of aptamers, only a few target protein-binding aptamers are used because their conformational changes have been thoroughly studied. Wu et al. reported a universal aptamer sensing system using RCA (Wu et al., 2010). As previously mentioned, the structure of aptamers is stabilized upon binding to a target molecule, resulting in inhibition of hybridization with the captured DNA that is a part of the complimentary DNA of the aptamer. Wu et al. utilized free capture DNA that was not hybridized with an aptamer for formation of circular DNA by ligation, followed by RCA. This sensing system does not require careful design of the aptamer's desired conformational change. However, the addition of DNA to an aptamer or hybridization with an aptamer before target molecule binding results in decreasing binding affinity of the aptamer.

3. Transduction of binding events into measurable signals by enzymes

Enzymes can transduce binding events to various measurable signals and amplify them. As mentioned above, enzymes are combined with aptamer sensors using various sensing schemes. Table 1 shows a list of enzymes combined with aptamer sensors. There are many reports that aptamer sensors have been combined with ribozyme or deoxyribozyme (Breaker, 2002; Kuwabara et al., 2000). (Deoxy)ribozyme is attractive for use as a labelling tool of aptamer sensors because it can easily be connected to an aptamer by synthesis, whereas enzyme connections often require chemical crosslinking that sometimes causes a decrease in enzymatic activity. However, compared with enzymes, there is limited use for (deoxy)ribozyme combinations in detection schemes because their activities are much less than that of enzymes and they catalyze fewer types of reactions than enzymes. In the following subsection, we describe the features of enzymes and detection schemes. We have focused on electrochemical biosensors because they can be constructed with low cost and high sensitivity.

3.1 Oxidoreductase

Electrochemical sensing applications using aptamers are rapidly increasing (Cho et al., 2009). Electrochemical sensing systems enable highly sensitive detection of target molecules, and these systems can be readily miniaturized at a low cost. Therefore, an electrochemical sensing system is suitable for POCT. In fact, the most frequently used biosensor is a glucose biosensor, based on electrochemical sensing using glucose dehydrogenase. Since glucose-sensing systems are well-established and used commercially, they are attractive tools for sensing systems of various biomarkers that use aptamers.

We first reported thrombin sensing using an aptamer conjugated with pyrroloquinoline quinone-dependent glucose dehydrogenase (PQQGDH) (Ikebukuro et al., 2004; Ikebukuro et al., 2005a). PQQGDH has a high catalytic activity (about 5000 U/mg protein). We used glutaraldehyde to crosslink PQQGDH with avidin. Biotin-modified aptamers were labeled by PQQGDH through avidin-biotin interaction. Thiol-modified aptamers were immobilized on an Au electrode. A sandwich structure was formed on the Au electrode, and we observed a current that was dependent on the target molecule concentration via PQQGDH activity mediated by 1-methoxy-5-methylphenazinium methyl sulfate with a low detection limit of 10 nM. However, cross-linking between PQQGDH and avidin resulted in a decrease in enzymatic activity. Then we reported the accomplishment of PQQGDH labeling without

Name	Detection type
Polymerase	Fluorescence
Phi29 polymerase	Fluorescence or electrochemical
Dehydrogenase	Electrochemical
Peroxidase (HRP)	Electrochemical, Chemiluminescence or Fluorescence
Alkaliphosphatase	Electrochemical, Chemiluminescence or Fluorescence
Nuclease	Fluorescence
Protease	Fluorescence or others

Table 1. Enzyme list for signal amplification in aptamer sensors

crosslinking using a PQQGDH-binding aptamer (Abe et al., 2010; Osawa et al., 2009). The PQQGDH-binding aptamer that we screened was bound to PQQGDH with high affinity (K_d: c.a. 40 nM) and specificity, and it did not affect PQQGDH activity. Enzyme labeling of target-binding aptamer via noncovalent bonding with enzyme-binding aptamer would help us to make a construct for highly sensitive detection.

3.2 Polymerase

Since the development of Immuno-PCR in 1992 (Sano et al., 1992), polymerases have been used as biosensor signal amplification tools. As contrasted with the cumbersome step of antibody modification using oligonucleotides, aptamers are easily applicable to similar assays that use immuno-PCR. If the aptamer has sufficient length for primer binding, it can be amplified directly (Fischer et al., 2008). Since a PCR reaction can amplify DNA exponentially, signal amplification by polymerase enables more highly sensitive detection than by ELISA. The limit of detection of a given ELISA is, in general, enhanced 100 to 10000-fold by the use of PCR as a signal amplification system. The disadvantage of PCR is the requirement of a longer reaction time than for other enzyme reactions. Many researchers have attempted time reduction of PCR, and they succeeded in a PCR that took 20 minutes using Lab-on-a-chip technology (Kim et al., 2009; Kopp et al., 1998).

Phi29 polymerase has been used to catalyze RCA, and it is also used for signal amplification. As contrasted with a typical DNA polymerase, Phi29 polymerase can amplify hundreds of copies of a circular DNA template isothermally. This unique amplification was utilized for biosensing that could not be performed by a typical DNA polymerase. Isothermal amplification has a great advantage for use with biosensing because there is no requirement for specific devices.

The reaction products are ordinarily measured by fluorescence using Sybr® Green I or a related molecule that can generate a fluorescent signal upon specific recognition of double-stranded DNA. In addition, since RCA can isothermally produce a long strand of DNA that is connected to the aptamer, the aptamer can be labelled by fluorescence or enzymatic methods via DNA probe hybridization. A molecular beacon that can recognize DNA with more specificity than Sybr® Green I and can generate a fluorescent signal upon DNA binding will enable real-time detection with high specificity. Since RCA products have many probe binding

sites, multiple enzyme labelling in a RCA product will enable a 10 to 100-fold signal amplification compared with modification of an aptamer with an enzyme (Zhou et al., 2007).

3.3 Alkaline phosphatase and horseradish peroxidase

Alkaline phosphatase (ALP) and HRP are mainly used as biosensors when combined with an antibody and an aptamer. The most important advantage of these enzymes is that we can use commercial avidin conjugates, as well as commercial antibody conjugates. Then we can easily apply them to various sensing systems.

ALP catalyzes the dephosphorylation of various substrates, and is used in various sensing systems such as chemiluminescent detection, fluorescence detection and electrochemical detection. ALP allows a nonreductive substrate, ascorbic acid 2-phosphate, to be converted into reducing agent ascorbic acid at an electrode's surface. Finally, silver ions were reduced and deposited on the electrode surface as metallic silver, which was determined by linear sweep voltammetry. Zhou et al. combined RCA, to be used for the detection of Platelet-Derived Growth Factor (PDGF), with ALP by using an electrochemical assay based on silver deposition (Zhou et al., 2007). They succeeded in the detection of PDGF with a low detection limit of 10 fM. Xiang et al. combined diaphorase with ALP for further signal amplification (Xiang et al., 2010). They used *p*-aminophenylphosphate (*p*-APP) as a substrate for ALP. ALP catalyzes the dephosphorylation of *p*-APP to *p*-aminophenol (*p*-AP), and the *p*-AP was then subjected to an electrochemical oxidation process that caused it to change to *p*-quinonimine (*p*-QI) on the electrode. Diaphorase catalyzes the reduction of *p*-QI to *p*-AP, coupled with NADH oxidation. Successful thrombin detection occurred with a low detection limit of 8.3 fM. The dual amplified detection strategy substantially lowered the detection limit by four orders of magnitude compared to common single enzyme-based schemes.

HRP catalyzes reduction of various substrates that is accompanied by hydrogen peroxide oxidation. Using a specific mediator such as 3,3',5,5'-tetramethylbenzidine (TMB), HRP has been applied to electrochemical detection. TMB was also used for enhancement of surface plasmon resonance imaging (SPRI) (Li et al., 2007).

3.4 Nuclease

Specific nucleases are used for fluorescence signal amplification using a molecular beacon as the substrate. The molecular beacon is a stem-loop type of DNA that is labeled with a fluorescent molecule and has a quencher at each termini (Tyagi & Kramer, 1996). Although fluorescence is quenched with stem-loop structure formation, fluorescence is observed upon binding to the target DNA or the target molecule when structural disruption of the molecular beacon is induced. Although most molecular beacons bind to DNA, we can design the transduction of any molecule by controlling the binding event of the molecule to an aptamer so that specific DNA signals are transmitted, which are then detected by a molecular beacon. A simple example is the modification of complementary DNA of a molecular beacon with an aptamer in a sandwich assay. Xue et al. used Nb.*BbvC* I, which is one of the nick-end labeling nucleases used for fluorescence signal amplification (Xue et al., 2010). The molecular beacon recognizes the modified DNA of the aptamer, and then Nb.*BbvC* I cleaves the hybrid of the molecular beacon with the aptamer. Since Nb.*BbvC* I introduces a nick to the strands of the molecular beacon, the molecular beacon then dissociates from the aptamer. The released target strand could then hybridize to another

molecular beacon and initiate a second cycle of cleavage. Each DNA strand modified by an aptamer has the capability to go through many such cycles.
Fletcher et al. also used a molecular beacon inserted into the *Eco*RI recognition sequence (Fletcher et al., 2010). They used *Eco*RI to inhibit the aptamer and DNA, which consisted of target-binding of the DNA and the complementary DNA of *Eco*RI that would inhibit the aptamer. The binding of the target DNA induces hybridization of the complementary DNA to the *Eco*RI-inhibiting aptamer, resulting in an increase of fluorescence via cleaving of the molecular beacon by active *Eco*RI.

3.4 Protease

Since TBA is well characterized, some researchers, including ourselves, have used thrombin as a detection enzyme, utilizing ability of TBA inhibiting thrombin activity (Pavlov et al., 2005; Yoshida et al., 2006a). Protease activity was measured using a synthetic peptide labeled with a fluorescent molecule as the substrate. In the case of a protease such as thrombin and RVV-X factor X activator, we can measure protease activity via observation of the coagulation that results from enzymatic activity. Chelyapov constructed a biosensor that can evaluate RVV-X activity with the naked eye, using microspheres for signal amplification (Chelyapov, 2006). Chelyapov succeeded in the detection of VEGF with a low detection limit of 5 fmol. Despite semi-quantitative or qualitative assays, visible detection is suitable for POCT because it does not require specific devices.

4. Conclusion

Many aptamer sensors have been reported for the past two decades. However, antibodies are still commonly used for diagnostics because unlike aptamers, many kinds of antibodies can be utilized. Although different kinds of aptamers have been increasing every year, it is difficult to replace aptamer sensors with existing antibody-based devices. Therefore, we should not use aptamers as alternatives for antibodies, but instead, we should utilize their unique properties accompanied with their molecular structure for constructing sensors. There is a strong need for aptamer sensors to be developed for theranostics and POCT, since there is substantial growth in the demand for biomarkers that will be used in drug development and in vitro diagnosis.
As mentioned above, certain properties of aptamers enable us to construct biosensors that are suitable for POCT. They can easily measure target molecules with high sensitivity and rapidity. Aptamers enable us to construct homogeneous biosensors that can use any enzyme. Most homogeneous sensing systems that use antibodies require specific devices or are based on the aggregation of beads, resulting in a sandwich formation. However, we can construct various homogeneous biosensors, including those based on electrochemical systems, utilizing various enzyme activities. The AES is a most ideal sensing system because it can amplify signals without any cumbersome processes, although optimization would require rigorous control of the structural change of the aptamer in order to enable highly sensitive detection. If we can obtain the aptamer that inhibits glucose dehydrogenase, we would be able to construct attractive biosensors.
One of advantages of aptamers for theranostics is that they can measure target molecules by binding to them. Homogeneous detection with capturable aptamers enable the detection of a target molecule using a single aptamer. We can detect any molecules, from cells to small molecules, based on the same sensing strategies, and we do not have to select and optimize

two affinity probes. As a short-term goal, we should develop biosensors for novel biomarkers, since aptamers would be excellent candidates for affinity probes that facilitate the construction of a biosensing system for any biomarker.

5. Acknowledgment

This work was supported by the 2009 Industrial Technology Research Grant Program of the New Energy and Industrial Technology Development Organization (NEDO) of Japan.

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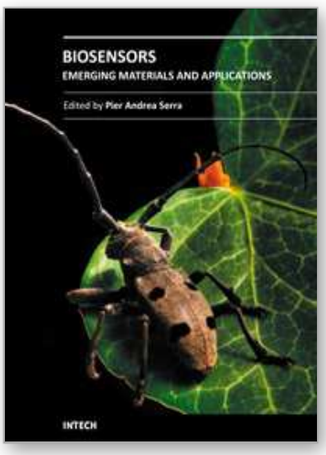

Biosensors - Emerging Materials and Applications

Edited by Prof. Pier Andrea Serra

ISBN 978-953-307-328-6
Hard cover, 630 pages
Publisher InTech
Published online 18, July, 2011
Published in print edition July, 2011

A biosensor is a detecting device that combines a transducer with a biologically sensitive and selective component. Biosensors can measure compounds present in the environment, chemical processes, food and human body at low cost if compared with traditional analytical techniques. This book covers a wide range of aspects and issues related to biosensor technology, bringing together researchers from 19 different countries. The book consists of 27 chapters written by 106 authors and divided in three sections: Biosensors Technology and Materials, Biosensors for Health and Biosensors for Environment and Biosecurity.

How to reference

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Koichi Abe and Kazunori Ikebukuro (2011). Aptamer Sensors Combined with Enzymes for Highly Sensitive Detection, Biosensors - Emerging Materials and Applications, Prof. Pier Andrea Serra (Ed.), ISBN: 978-953-307-328-6, InTech, Available from: http://www.intechopen.com/books/biosensors-emerging-materials-and-applications/aptamer-sensors-combined-with-enzymes-for-highly-sensitive-detection

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