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Sensitive and Homogeneous Detection System with Aptamer-Based Biosensor

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Rapid and highly sensitive methods for detecting target molecules are required to develop biomarker-based diagnoses that help medication or therapy. Thus, the development of homogeneous sensing methods with high sensitivity can provide the basis for detecting a biomarker in routine diagnostics and on site. Oligonucleotide aptamers, ligands that consist of nucleic acids, have been selected for numerous targets such as proteins and small compounds, and are applicable to biosensing platforms. In particular, attractive features of aptamers enable us to construct valuable sensing methods for clinical uses without the need to use nanomaterials. Recent discoveries of novel signal transducing elements derived from an enzymatic and a fluorescent oligonucleotide also allow for the development of homogeneous aptasensors. In this review, we describe recent progress in the development of aptamer-based biosensors with high sensitivity.

1. Introduction

Aptamers are oligonucleotide or peptide ligands that bind to various molecular targets. In 1990, two groups reported systematic evolution of ligands by exponential enrichment (SELEX) to efficiently develop DNA and RNA aptamers.[1,2] Thus, over the last 25 years, numerous DNA or RNA aptamers with high affinity and selectivity have been developed for proteins, peptides, small molecules, bacteria, and mammalian cells. One of the most successful aptamers is "Macugen®", which is the first aptamer-based drug to be used for treating age-related macular degeneration. Macugen® is an anti-vascular endothelial growth factor (VEGF)-165 RNA aptamer with substitution of modified nucleotides.[3] The binding affinity of Macugen® for VEGF-165 (K_d) is 0.14 nM, which corresponds to the affinity of highly effective antibodies. Thus, researchers in many areas aiming at medical application are currently focusing on the development and engineering of aptamers.

Aptamers have been of interest as useful tools for target recognition in the field of biosensor development. Because of their ease of synthesis and modification, aptamers tend to be combined with nanomaterials such as gold nanoparticles, magnetic nanoparticles and quantum dots.[4] Functional groups and fluorescent dyes can be easily introduced into aptamers at the end and/or middle of their sequences, which enables the manufacture of well-designed aptamer-based materials for detecting the target molecules. Target detections based on fluorescent assay, colorimetric assay, and enzyme-based assay were achieved using such aptamer-modified nanomaterials.[4] Furthermore,

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a variety of aptamer-based electrochemical biosensors have also been constructed. Covalently immobilized aptamers via a thiol group can recognize target molecules on the electrode. As a result of the binding to target molecules, electrochemical signals (such as current) are generated via redox compounds.[5] Moreover, measurement by impedance spectroscopy or the use of a field effect transistor was also integrated to confirm the binding of aptamers to target molecules.[5] Consistent with the growing number of applications of aptamers, it is well known that in the field of biosensor development, aptamers have advantages over antibodies because of their ease of use.

In addition, the most unique and impressive feature of aptamers is that we can create aptamer-based biosensors by predicting the secondary structures via the observation of aptamer sequences. A biosensor is composed of at least two elements: a molecular recognition element and a signal transducer. In general, techniques for detection systems included in the biosensors can be divided into two approaches, with or without bound/free separation that removes nonreactive molecules from the sensor. We call the detection format of specific molecules without washing or separation, homogeneous detection. Aptamer-based biosensors can be produced by the fusion of a target-recognition element to a module to produce signal(s) that inform us of the presence of the target molecules. Therefore, on the basis of the engineering of oligonucleotide sequences, including aptamers, oligonucleotide aptamers strongly contribute to the development of various homogeneous assay formats.

A demand for the development of biosensors to be used in conduct homogeneous sensing systems for point-of-care testing (POCT) based on biomarkers is growing. For example, for the appropriate administration of a molecular target drug, it is essential to know the concentration of the target. The method of diagnosis by POCT should be simple, quick and easy to use for “on the spot” detection. However, from the point of view of diagnostics supporting low concentrations of targets, homogeneous sensing platforms require not only simplicity of the method, but also sensitivity.

Therefore, in this review, we introduce recent advances in the development of aptamer-based biosensors involving the fusing of a signal transducer to the aptamer, which results in a highly sensitive homogeneous assay.

2. Target Sensing with Detection of Specific Nucleic Acid Sequences

One of the most robust methods of signal amplification is through the polymerase chain reaction (PCR) of a certain DNA sequence. DNA aptamers have the advantage that PCR can be used for the sensitive detection of targets. Rolling circle amplification (RCA) is another method of amplifing specific DNA from circular template oligonucleotide by polymerase-mediated isothermal reaction, and has been utilized in the development of aptamer-based biosensors.[6] Moreover, proximity ligation assay (PLA) is known as a detection method based on the amplification of a specific DNA sequence to achieve the detection of proteins without bound/free separation.

PLA requires two ligands, two affinity probe oligonucleotides, and one connector oligonucleotide. The two ligands are each modified by one of the two probes. Binding the probe-modified ligands to the same target molecule causes the attached oligonucleotide probes to be in close proximity. A connector oligonucleotide can be hybridized with the end of two affinity probe oligonucleotides. Then a nick between two affinity probe oligonucleotides can be ligated by a DNA ligase. Finally, the ligated oligonucleotides can act as PCR template and we can read out the presence of the target molecule by PCR experiments with, for example, real-time PCR [Fig. 1(a)].

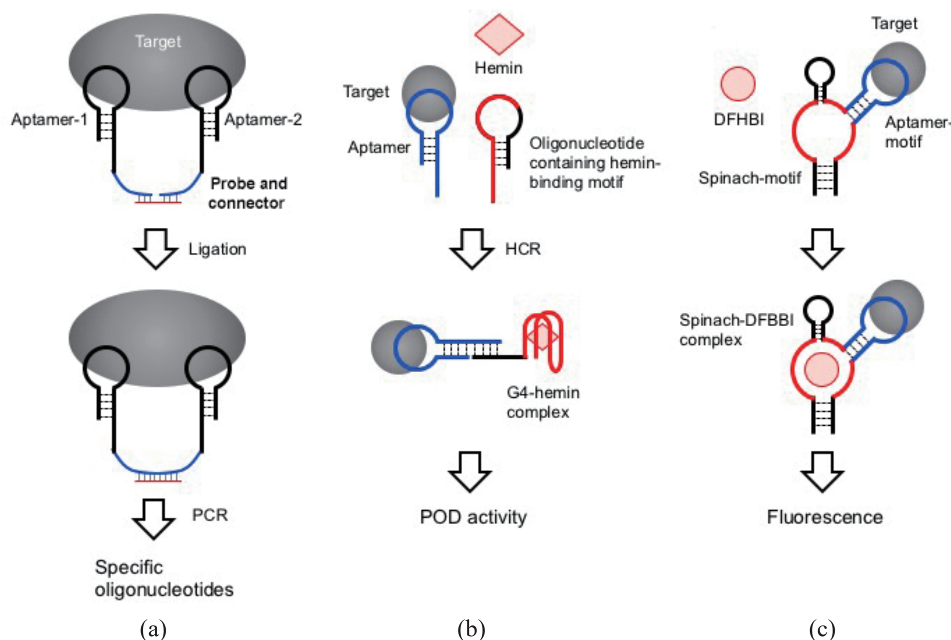

Fig. 1. (Color online) Schematic diagram of the homogeneous detection systems described in this review. (a) Target sensing with detection of specific oligonucleotide, (b) target sensing with POD activity derived from complex of G4 structure and hemin, and (c) target sensing with fluorescence derived from Spinach.

In the first report of PLA using aptamers for protein detection, Fredriksson *et al*. described a homogeneous assay for detecting the homodimer of the platelet-derived growth factor B-chain (PDGF-BB)[7] using a DNA aptamer bound to PDGF-BB. Probe oligonucleotides were attached by adding extension sequences at either the 5' or 3' end of an anti-PDGF-BB DNA aptamer. Because PDGF-BB is a homodimeric protein, one DNA aptamer was sufficient to perform the PLA homogeneous assay. The authors detected 24000 PDGF-BB molecules (40 zmol of PDGF-BB) using the PLA system. The authors demonstrated that the sensitivity of the homogeneous PLA system was ~1000-fold higher than that of sandwich ELISA for PDGF-BB. In addition, Fredriksson *et al.* reported an example in which two types of DNA aptamers were used in the PLA system for the detection of human thrombin.[7] Moreover, a PLA system, developed by Niu *et al*., with four DNA aptamers for certain tripeptides detected mouse cathepsin D at a concentration as low as 100 amol.[8]

3. Target Sensing with Enzymatic Activity

3.1 Signal transduction based on peroxidase (POD) activity derived from a complex of guanine-quadruplex structure and hemin

The use of enzymes is generally applied in the development of biosensors for the sensitive detection of various target molecules because enzymes can amplify the detection signal(s) via enzymatic activity. Therefore, it is accepted that an enzyme is a valuable signal transducer for constructing a sensitive detection system.

In 1998, Travascio *et al.* found that DNA aptamers bound to hemin, and that the aptamer-hemin complex showed enhanced POD activity.[9] The hemin-binding aptamer folds into a guanine-quadruplex (G4) structure,[10] which indicates that G4-forming oligonucleotides can be considered to be the "apoenzyme" that catalyzes the H_2O_2-mediated oxidation. The POD activity derived from G4-hemin complexes can be followed by a colorimetric change or a chemiluminescent emission. In recent years, high-sensitivity aptasensors containing hemin-binding G4 motifs have been developed, some of which include the homogeneous detection system [Fig. 1(b)].

Wang *et al*. reported a colorimetric aptasensor based on POD activity from a G4-hemin complex to detect ochratoxin A, a mycotoxin found in food, by employing the hybridization chain reaction (HCR).[11] A single-stranded DNA (ssDNA) having a hemin-binding sequence was partially hybridized at the 5' end and 3' end of the oligonucleotide and did not show catalytic activity in the absence of ochratoxin A. Additionally, another oligonucleotide containing the aptamer sequence for ochratoxin A was designed to input the binding signal. The binding of the oligonucleotide containing the aptamer to ochratoxin A could unlock the hybridization of DNA, and the hemin-binding G4-forming sequence was exposed, and catalyzed the tetramethylbenzidine (TMB) oxidation. This aptasensor produced a colorimetric signal in an ochratoxin A-concentration-dependent manner. The authors obtained a limit of detection (LOD) of 0.01 nM, which is superior or equal to the previous results of fluorescent assay, electrochemical assay and colorimetric assay.

Recently, two reports introduced very sensitive biosensing platforms using G4 oligonucleotide-enhanced POD activity combined with exonuclease III (Exo III)-assisted signal amplification, resulting in a LOD of sub-picomolar concentration.[12,13] Those systems needed at least two oligonucleotides; a partially duplexed aptamer-containing oligonucleotide, and an ssDNA folded into a hairpin structure, which is an intramolecular displacement probe with hemin-binding motifs. Hemin-binding DNA was caged in the duplex of the hairpin structure. When the aptamer was bound to the target molecule, the oligonucleotide that could be displaced by an intramolecular displacement probe was exposed. This intramolecular displacement probe can hybridize with the aptamer-containing oligonucleotide. The generation of hemin-binding oligonucleotides was assisted by Exo III, which selectively digests the 3'-terminus of double-stranded DNA (dsDNA) with a blunt or recessed 3'-end. Exo III digested the hybridized dsDNA of the intramolecular displacement probe, yielding hemin-binding oligonucleotides. Importantly, since the digestion by Exo III occurs at only the 3'-terminus of the intramolecular displacement probe, the aptamer-containing oligonucleotide can fold into a partially duplexed structure and can again bind to the target. Therefore, hemin-binding oligonucleotides can be continuously produced via the binding of a target molecule, giving rise to highly amplified signals derived from POD activity.

On the basis of the above principle, Zou *et al.* detected human thrombin with a LOD of 0.92 pM.[13] Bi *et al*. performed the Exo III-assisted signal amplification using two intramolecular displacement probes to detect PDGF-BB.[12] Their system achieved high sensitivity with a LOD of 0.68 pM. Remarkably, the developed aptasensor platforms were effective in diluted blood plasma samples.

3.2 Proof of concept of using various proteinaceous enzymes for homogeneous detection with aptasensor

To address the construction of more aptasensors that can induce various enzymatic activity, we have shown a homogeneous sensing system in which DNA aptamer(s) and a proteinaceous enzyme were combined, termed an aptameric enzyme subunit (AES).[14] An AES is composed of

two aptamers. The first aptamer is a target-binding aptamer, and the second aptamer is an enzyme-inhibiting aptamer. We have established some strategies for creating AES. For example, a target-binding aptamer is inactivated by the hybridization of a partially complementary strand attached at an enzyme-inhibiting aptamer.[14,15] Thus, in the absence of target molecules, the enzyme-inhibiting aptamer of AES folds into the structure, binds to the enzyme, and inhibits its enzymatic activity. In the presence of target molecules, the target-binding aptamer folds into the original structure, which leads to the disruption of the structure of the enzyme-inhibiting aptamer and a decrease in the inhibitory activity. We can observe the enzymatic activity as the read out of AES, which indicates the presence of the target molecules.

Since it is known that some thrombin-binding aptamers have a highly inhibitory effect on the coagulation activity of thrombin, including a 31-mer thrombin aptamer we previously developed,[16] the thrombin-binding aptamers were used as enzyme-inhibiting aptamers. We have developed two types of AES for the detection of adenosine[14] and insulin.[15] We found the lower limit of adenosine detection was 25 μM. Given that the enzymatic activity of thrombin is not very high, it is possible to construct more sensitive AES by utilizing other enzymes. Thus, the key consideration is to find a pair comprizing an enzyme having high enzymatic activity (such as a horseradish POD or an alkaline phosphatase) and an aptamer that inhibits its enzymatic activity.

4. Target Sensing with Fluorescence Derived from RNA-Fluorophore Complex

In 2011, Paige *et al.* found an RNA aptamer-fluorophore complex termed Spinach, which is an RNA mimic of green fluorescent protein (GFP).[17] Spinach is an RNA that binds to and activates 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI), a fluorophore of GFP, resulting in the fluorescence. The structure of Spinach itself and the structure of Spinach-DFHBI have been solved by X-ray crystal structure analyses.[18,19] Importantly, the topology of the Spinach core is a G4 motif that plays a role in the binding of fluorophore.

An advantage of Spinach-based sensing strategy is low levels of background signal, because Spinach and DFHBI itself are nonfluorescent. Moreover, tagging Spinach with RNA aptamers is probably the easiest way to develop a fluorescence-based aptasensor among the modification methods of using fluorescent dye to RNA aptamers. Strach *et al*. have described the basic idea of the experimental design for generating Spinach-based biosensors.[20] Spinach has four stem-loop structures and one stem-loop can be replaced with additional sequences. Therefore, by inserting a recognition module such as an aptamer at the stem-loop, we can generate a target-inducible Spinach-based aptasensor [Fig. 1(c)].

Nakayama *et al.* reported an aptasensor involving Spinach and an RNA aptamer for cyclic-di-guanosine monophosphate (GMP).[21] The aptamer bound to cyclic-di-GMP was inserted into Spinach RNA. Upon cyclic-di-GMP binding, the aptamer folded, induced the folding of Spinach, and activated the fluorescence of DFHBI. Moreover, after the identification of the G-quadruplex motif of Spinach, which is the binding site for DFHBI, DasGupta *et al.* developed a Spinach-based RNA aptasensor to detect Pb^{2+} by means of Pb^{2+}-induced stabilization of the G4 structure.[22] The authors created truncated Spinach that showed no fluorescence in the presence of DFHBI owing to the unfolding of G4. However, in the presence of DFHBI and Pb^{2+}, Pb^{2+} supported the folding of G4, and therefore, the fluorescence derived from truncated Spinach occurred in a Pb^{2+}-concentration-dependent manner. These Spinach-based aptasensors detected cyclic-di-GMP and Pb^{2+} with LODs of 320 nM and 5 nM, respectively.

Table 1
Summary of detection methods described in this review.

Signal transducer	Target	Detection method	LOD	Amplification	Reference
Specific DNA	PDGF-BB	Real-time PCR	40 zmol	PCR	(7)
	Mouse cathepsin D	Electrophoresis	100 amol	PCR	(8)
POD activity derived from G4-hemin complex	Ochratoxin A	Colorimetry	0.01 nM	HCR	(11)
	Human α-thrombin	Chemiluminescent detection	0.92 pM	Exo-III-assisted recycling	(13)
	PDGF-BB	Chemiluminescent detection	0.68 pM	Exo-III-assisted recycling	(12)
Fluorescence derived from Spinach	c-di-GMP	Fluorescent detection	320 nM	Not performed	(21)
	Pb^{2+}	Fluorescent detection	5 nM	Not performed	(22)

5. Conclusions and Perspective

Here, we reviewed aptamer-based homogeneous sensing platforms with high sensitivity. A summary of the homogeneous sensing methods described in this review is shown in Table 1. The amplicon of specific DNA, POD activity, and GFP-like fluorescence derived from DNA/RNA directly attached at the aptamers are utilized as signals to identify the presence of target molecules (Fig. 1). In five studies, except for the Spinach-based assay, signal amplification was adopted. Notably, PCR or other nucleic acid amplification methods assist the ultrasensitive detection (from amol to zmol) of the target proteins after PLA reaction, which results in the highest sensitivity among homogeneous sensing methods. HCR and Exo-III-assisted recycling are included to generate the G4-hemin complex. These methods enable one input associated with the binding of an aptamer to a target to give rise to the designed G4 structures that bind to hemin and cause the oxidation activity. In addition, to create other enzyme-based detection methods, we believe the strategy of AES might be promising.

RNA Spinach-based sensors producing fluorescence were constructed via the observation of the structural characteristics of Spinach, that is stem-loops and a G4 motif that binds to a fluorophore. Two strategies were reported, which are based on adding a target-recognizing aptamer, and modulation of the G4 stability. No signal amplification was carried out, but the Spinach-based sensors were very simple in design, with GFP-like fluorescence, and do not involve materials other than a fluorophore.

Importantly, almost all the papers we mentioned herein described excellent specificity to the targets. The developed homogeneous detection methods worked well for real samples such as plasma and food extracts, which is attributed to the specific binding of aptamers to targets. In conclusion, the interesting characteristics of aptamers have rendered aptamers attractive and promising ligands for the construction of biosensors, especially those for homogeneous target sensing in clinical applications.

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