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An ultra-sensitive detection of a whole virus using dual aptamers developed by immobilization-free screening

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ABSTRACT

In this study, we successfully developed a ssDNA aptamer pairs by using an advanced immobilization-free SELEX method with affinity-based selection and counter-screening process at every round. By implementing this method, two different aptamers specifically binding to bovine viral diarrhea virus type 1(BVDV type 1) with high affinity were successfully screened. This aptamer pair was applied to ultrasensitive detection platform for BVDV type 1 in a sandwich manner. The ultrasensitive detection of BVDV type 1 using one of aptamers conjugated with gold nanoparticles was obtained in aptamer–aptamer sandwich type sensing format, with the limit of detection of 800 copies/ml, which is comparable to a real-time PCR method.

1. Introduction

Aptamer, as a recognition molecule, is a ssDNA or RNA oligonucleotide that binds to various targets, including bacteria, small molecules and so forth, with high specificity and affinity (Ellington and Szostak, 1990; Tuerk and Gold, 1990). Aptamers can specifically bind and discriminate their targets in a heterogeneous environment and can be applied in many fields, including biosensors, pharmaceutics, drug delivery, imaging and purification (Bagalkot et al., 2006; Kim et al., 2010; Lin et al., 2011; Said et al., 2009). For obtaining the aptamers, many different forms of the systematic evolution of ligands by exponential enrichment (SELEX) (Ellington and Szostak, 1990; Tuerk and Gold, 1990), in which binding, separation, and amplification can be altered to accommodate the various needs, have been modified and developed. Significant technological progress has been made in improving the methodologies for the efficient separation of target-bound ssDNAs from the unbound ssDNAs (Berezovski et al., 2005; Mendonsa and Bowser, 2004; Miyachi et al., 2009; Mosing et al., 2005; Oh et al., 2011; Park et al., 2009) in SELEX. However, the most SELEX processes still require complicated immobilization steps for the targets, especially for the small molecules which need different chemistry to be immobilized, in addition to its labor intensive feature.

Hence, a new immobilization-free SELEX method without target immobilization step by using graphene oxide (GO) was established in our previous study (Park et al., 2012) based on many extensive studies regarding interaction between oligonucleotides and graphene (Liu et al., 2010b; Patil et al., 2009; Wang et al., 2010; Wu et al., 2011). Here, we successfully applied the advanced GO based immobilization-free SELEX method and obtained ssDNA aptamers including the aptamers having different binding sites, which enable sensitive detection of the target in a sandwich fashion using a aptamer pair obtained in this method. In this advanced SELEX, every round begins from the counter-screening step followed by the target-induced screening step based on affinity-based release. The counter-screening step refers to the screening process, which is intended for removing of ssDNAs that are bound to the counter targets, which are unwanted to be bound or similar to the target in terms of molecular structure. The counter step at every round enables screening the aptamer candidates highly specific to only the target and the affinity-based release method at every round enable considerably more stringent selection conditions for high affinity aptamer screening.

We used this advanced SELEX method for obtaining aptamers that bind to a whole bovine viral diarrhea virus (BVDV) type 1 specifically. The BVDV is world-widely distributed and an important cattle pathogen causing variety of syndromes including abortions, respiratory disease, congenital abnormalities, persistently infected (PI) cattle, mucosal disease, and acute infections (Baker, 1995; Houe, 1995), and infection with this virus results in reduced productivity and increased mortality (Kampa et al., 2009). Hence, there have been many different sensitive detection methods for the of BVDV reported, including reverse transcription polymerase chain reaction (RT-PCR) (Drew et al., 1999; Karaoglu et al., 2003; Moussa, 1995; Pfeffer et al., 2000; Young et al., 2006;

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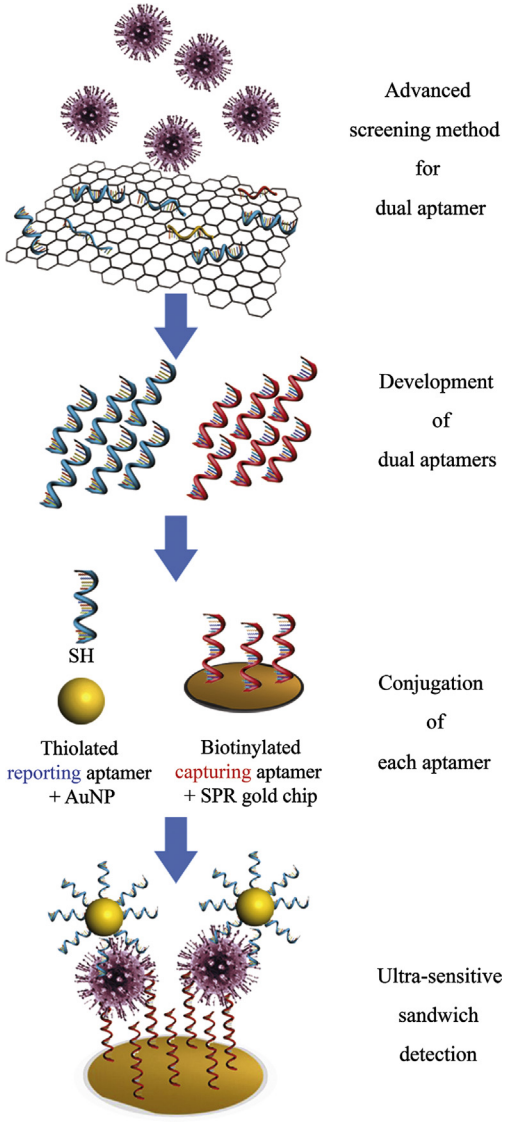
Zhang et al., 2012), antigen capture enzyme-linked immunosorbent assay (ACE) (Saliki et al., 1997; Sandvik and Krogsrud, 1995), virus isolation (VI) (Gao et al., 2011; Goyal et al., 2002; Lecomte et al., 1996; Petrovic et al., 2004), or immunohistochemistry (IHC) (Baszler et al., 1995; Bedekovic et al., 2011; Lertora et al., 2003; Luzzago et al., 2006). Since the ACE assay protocol must be validated for each individual assay (Vander Ley et al., 2012), RT-PCR has been widely accepted because of its high sensitivity even though it requires complicated equipment and relatively long time. Regarding the most important counter target classical swine fever virus (CSFV), both CSFV and BVDV are species of *Pestivirus* genus, which infect mammals, including ruminant animals and various species of swine with similar clinical symptoms (Terpstra and Wensvoort, 1991). Moreover, because the detection and epidemiology of CSFV can be interfered by BVDV in pigs (Wieringa-Jelsma et al., 2006), it is essential to develop selective recognition molecule for accurate detection of BVDV.

For selective and sensitive detection of BVDV, a DNA aptamer pair was successfully screened out within 15 days including cloning and sequencing steps in this study. These results are not only the first dual aptamers developed for the whole virus particle, but also the first aptamer-based sandwich type detection of the whole virus (Scheme 1). With this aptamer-based sandwich detection method using aptamer pairs obtained in this study, the detection limit for BVDV type 1 whole virus particle was decided to be 5×10^2 $TCID_{50}$/ml which is estimated to be 800 copies/ml, similar to the real-time PCR based detection.



Scheme 1. Schematic illustration for sandwich-type application of selected aptamer pairs. After selection of dual aptamers, one aptamer was used as capturing probe and the other aptamer labeled with gold nanoparticles was used as reporting probe in the signal enhanced SPR biosensor platform.

2. Material and methods

2.1. Reagents

2.1.1. Chemicals

3,3′-Dithiodipropionic acid (DTPA) and ethanolamine were purchased by Sigma Aldrich (U.S.A.), N-ethyl-N'-(dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were purchased by Sigma Aldrich (U.S.A.). Hydrogen tetrachloroaurate (III) (HAuCl4), trisodium citrate dehydrate, MCH (6-mercapto-1-hexanol) and Tris-(2-carboxyethyl)phosphine hydrochloride (TCEP) were purchased from Sigma (U.S.A.).

2.1.2. Oligodeoxynucleotides

The ssDNA library comprised a randomized region of 30 (N30) nucleotides flanked by two constant regions allowing primer annealing and PCR amplification (primer sequences: FP 5′-CGTACGGAATTCGCTAGC-3′ and RP 5′-CACGTGGAGCTCGGATCC-3′. 5′-CGTACGGAATTCGCTAGC-N30-GGATCCGAGCTCCACGTG-3′. For PCR amplification, FP: 5′-fluorescein-CGTACGGAATTCGCTAGC-3′ and RP: 5′-CACGTGGAGCTCGGATCC-3′ were used. All sequenced aptamers were labeled with 5′-biotin for immobilization on SPR chips. All oligodeoxynucleotides sequencing and synthesis services were provided by Genotech, Korea.

2.2. Screening of dual aptamers for BVDV type 1

The SELEX process round (Fig. 1a) was performed by initially heating 200 pmoles (Tris HCl 20 mM, pH 7.4) of random library for 15 min and then cooling for 5 min on ice for the best conformational structure of oligonucleotides. For the 1st round of SELEX, 200 pmoles of denatured ssDNA library was incubated with a counter-target mixture that included 10^6 $TCID_{50}$ of BVDV type 2, classical swine fever virus (CSFV), which is in the same family as BVDV, MDBK cells which used to culture BVDV and 600 pmoles of BSA for 30 min. The counter screening is intended for removing of ssDNA that is bound to the counter targets, which are very similar to the target in terms of molecular structure or are unwanted to be bound. After incubation, the 4 mg of GO which was synthesized by previously reported procedures (Hummers and Offeman, 1958) (Supplementary information) was added and incubated for 2 h (final volume 1 mL) in order to separate the oligonucleotides that did not bind to the counter targets. The oligonucleotides that did not bind to the counter targets are adsorbed to the surface of GO by π–π stacking interactions and separated by centrifugation while oligonucleotides that bound to the counter target is suspended in the binding buffer. Subsequently the solution was centrifuged for 3 min at 10,000 rcf to precipitate the GO on which oligonucleotides were adsorbed and discard supernatants. After discarding supernatants in the separation process, the centrifuged GO is suspended and washed out with same buffer 3 times for harsher condition. Then, the ssDNA was recovered from the GO with the addition of target BVDV type 1 (10^6 $TCID_{50}$) and incubated further for 2 h. The mixture solution was centrifuged and ssDNA library pool was recovered by ethanol precipitation. The recovery amount of ssDNA was measured by (Nanodrop, ND-1000, spectrophotometer) and the obtained library pool was amplified by PCR and ssDNA was again generated by PAGE separation and purification.

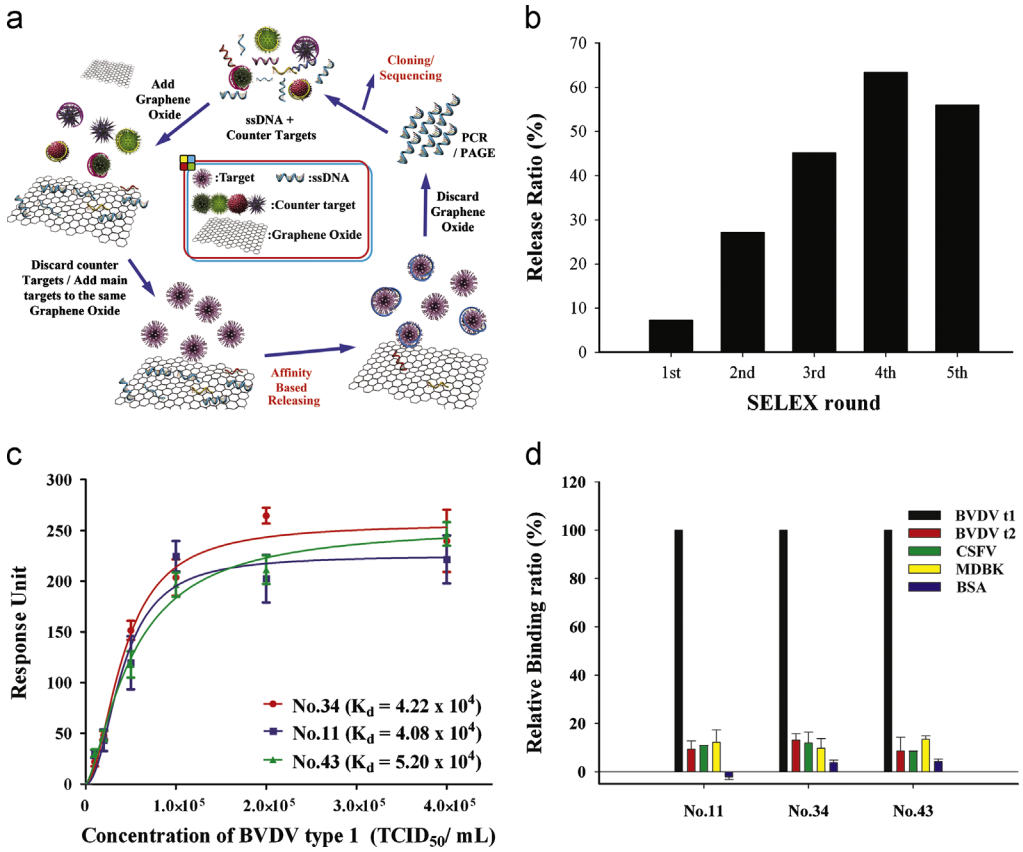


Fig. 1. (a) The schematic diagram of advanced screening method. (b) Ratio of released DNA from GO in each SELEX round. (c) Analysis of the selected aptamer candidates that shows specificity for counter targets (BVDV type 2, CSFV, MDBK, BSA) through SPR. The change in the response unit for various concentrations of BVDV type 1 was monitored. (d) The specificity of selected aptamers. A surface plasmon resonance (SPR) assay showed selected No. 11, No. 34, and No. 43 bound only to BVDV type 1.

2.3. Characterization of selected aptamers by SPR

To immobilize aptamers on SPR gold chip, the chip was washed with ethanol and distilled water (DW), the clean chip was immersed in 100 mM DTPA solution at room temperature overnight. Then carboxyl functional groups on chip surface were activated with 0.1 M EDC and 0.05 M NHS for 30 min. After this, chip was incubated with 100 μg/mL (1.9 μM) of streptavidin for 90 min on ice. The un-reacted functional groups were blocked by addition of 50 mM ethanolamine solution for 30 min. Then biotin labeled aptamers were incubated (1 μM) for 60 min at room temperature. Finally, chip was blocked with 50 μg/ml BSA solution for 30 min and washed with DW. The purpose of the second blocking with the bovine serum albumin is not only to block non-occupied sites on the surface but also to space out and stabilize biotinylated aptamers immobilized on the surface to reduce steric hindrance.

By using this aptamer modified gold chip, SPR (Eco Chemie, Netherlands) analysis was performed after injection of 50 μl of each counter targets (BVDV type 2, CSFV, MDBK, BSA) and main target as well. The binding reaction was performed for 30 min with a 5 min dissociation time at room temperature. After selecting the most highly specific aptamer, dissociation constant was determined with the nonlinear regression analysis.

2.4. Application of dual aptamers conjugated with gold nanoparticles for sandwich assay

After immobilization of 1 μM capturing aptamers No. 11 on the SPR gold chip, the various concentrations of BVDV type 1 was first injected to SPR cell and incubated for 30 min followed by washing with buffer. Sequentially, the 1 nM of reporter aptamer No. 43-AuNP conjugates were added to the various concentration of BVDV type 1 which is already captured by aptamer No. 11 and real-time angle shift was recorded. For the specificity test of signal enhancement assisted by aptamer conjugated with AuNP sandwich system, the counter targets including BVDV type 2, CSFV, BSA, MDBK cells were injected instead of main target BVDV type 1.

3. Results and discussion

3.1. Screening of BVDV type 1 binding aptamers

To develop ssDNA aptamers to BVDV type 1, 200 pmoles of a random DNA library (1.2×10^{14} molecules, 4 μg) was incubated with a counter-target mixture that included 10^6 $TCID_{50}$ (50% tissue culture infectious dose) of BVDV type 2, a classical swine fever virus (CSFV), which is the same family of BVDV, MDBK cells which were used to culture BVDV, and 600 pmoles of BSA and then followed by the addition of 4 mg of GO. According to our previous study, the maximum weight ratio of adsorbed 70-mer ssDNA/GO was 10 μg/mg and the experimental ratio was maintained at 1:1000 at every stage (Park et al., 2012). In this counter step, the counter targets are known structurally very similar to the main target virus, belong to the same family, or likely to co-exist with the target virus in a heterogeneous environment. This counter-step is repeated at every round for increasing the specificity by the efficient removal of non-specific sequences at each round. Unbound ssDNAs in this counter selection step are adsorbed onto GO surface through π–π stacking interactions between the ring structure of the nucleotide bases and the hexagonal structure of GO. Then, the ssDNAs bound to counter targets were removed by centrifugation, and only ssDNAs adsorbed GOs are used for the

next step, which is target-induced detachment of ssDNAs from GO due to affinity-based conformational change of the adsorbed ssDNAs. This conformational change-based release of ssDNAs with the target virus BVDV type 1 offers the stringency of selection directly. Depending on the net charge or hydrophobicity of protein, it can be non-specifically adsorbed to the GO (Laaksonen et al., 2010; Liu et al., 2010a; Zhang et al., 2010), however the main target BVDV type 1 did not show non-specific adsorption to the surface of GO due to the neutral pH range of binding buffer. In this study, 10^6 $TCID_{50}$ of the target BVDV type 1 was added to the solution containing the GOs on which the ssDNAs adsorbed. Compared to the number of total counter targets used in the counter step, this smaller number of the target BVDV type 1 also contributed to the stringency of the protocol, in addition to the target induced affinity-based release. The ssDNAs obtained from the target virus BVDV type 1, after the release from GO, were purified and amplified by using both ethanol precipitation and PCR, respectively. After the separation of PCR products, the separated ssDNAs were subjected to the additional rounds of selection. The recovery ratio of the released ssDNAs was observed to be linearly increasing until the 4th round. The amount of ssDNAs released in the 5th round no longer increased, indicating that the sufficient enrichment is accomplished for the target, BVDV type 1 (Fig. 1b).

Finally, after the 5th round of the highly stringent immobilization-free SELEX, followed by cloning and sequencing, total 42 sequences of aptamer candidates were obtained. The surface plasmon resonance (SPR)-based binding assay was performed to characterize the affinity and specificity of the 10 candidate sequences selected from the 42 sequences, according to Gibb's free energy values (Supplementary Table. S1 and Fig. S2). From the specificity assay with four counter-targets, three aptamer candidates (Nos. 11, 34, and 43) were finally found to be highly specific to BVDV type 01 (Fig. 1d and Supplementary Fig. S1). Subsequently, the binding affinities of the three candidates were evaluated by using a dose-dependent assay and their dissociation constants (K_d values) were calculated to 4.08×10^4, 4.22×10^4, and 5.2×10^4 $TCID_{50}$/mL, for Nos. 11, 34, and 43, respectively (Fig. 1c).

3.2. Characterization of the dual aptamers for BVDV type 1

The binding of three aptamers with BVDV type 1 was shown to be concentration dependent in a range from 10^4 $TCID_{50}$/ml to 10^5 $TCID_{50}$/ml (Fig. 1). Below 10^4 $TCID_{50}$/ml, it was not possible to distinguish the differences in angle shift data of SPR. Moreover, the detection range based on SPR analysis was not sufficient for the real detection of BVDV type 1, compared to the various conventional detection methods (Da Silva et al., 1995; Letellier and Kerkhofs 2003; Mahlum et al., 2002).

Therefore, possible secondary interactions among three selected aptamers were investigated with an aim of finding a novel aptamer pair, which bind to the target at two different sites, and so could be applied to a sandwich-type assay. In order to test the secondary interaction, a 1 μM of a capturing aptamer was immobilized onto a SPR chip, and 5×10^4 $TCID_{50}$/mL of BVDV type 1 was injected. Subsequently, the second aptamer was injected to find if the secondary binding is occurred. After the analysis of all possible 9 combinations from selected three aptamers, we found that the aptamer No. 43 is capable of participating in the secondary binding interaction when the aptamer No. 11 was immobilized as the capturing probe, indicating that two aptamers' binding sites are different and the combination is in harmony. Regarding reverse combination, no binding was observed mainly due to the affinity differences and other subtle parameters, such as different ranges of optimum concentrations of both aptamers, affecting on the sandwich binding steps within these limited experiments aimed to confirm aptamer-based sandwich bindings with fixed concentration (1 μM).

As can be seen in Fig. 2a, an increasing signal was observed with the increasing concentration of the secondary reporting aptamer No. 43, until the binding site of the target virus is saturated. Due to electric repulsion of DNA and saturation of binding sites, the signal is not linearly correlated over 1 μM of reporting aptamers.

3.3. Application of dual aptamer for the sandwich type detection of BVDV type 1

After measuring the small increment in the SPR signal with the use of the secondary reporting aptamers un-functionalized in a sandwich-type manner, the secondary reporting aptamer No. 43 was functionalized with gold nanoparticles (AuNPs) for further signal enhancement. The AuNP is one of the leading nano-materials frequently used for the signal enhancement in the sandwich-type systems (Bek et al., 2008; Kim and Lee, 2012; Park and Hamad-Schifferli, 2010; Rand et al., 2011; Su et al., 2011; Yang et al., 2012). This nano-material based sandwich detection method in SPR sensing platform is also well established elsewhere (Beccati et al., 2005; Cao and Sim, 2007; Kwon et al., 2012; Wang et al., 2009). In this study, the primary capturing aptamer No. 11 was immobilized on the surface of SPR gold chips, followed by injection of various concentrations of BVDV type 1 into SPR cell for

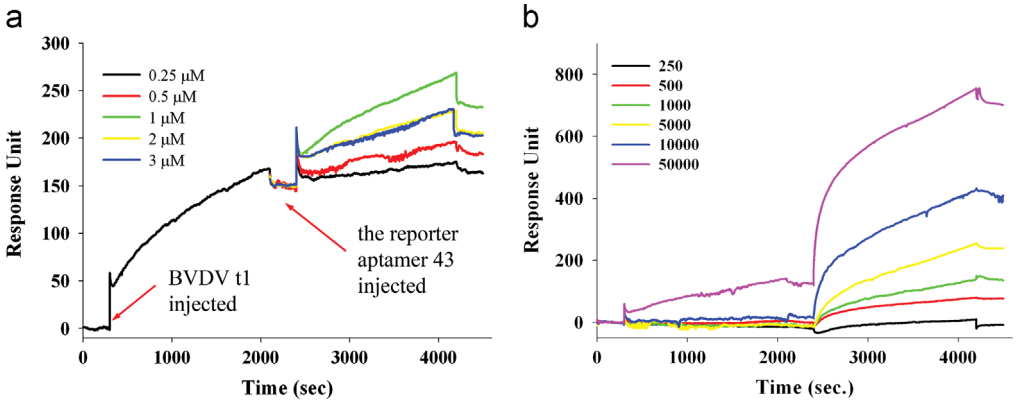


Fig. 2. Aptamer-based sandwich assay. (a) Aptamer No. 11, which possess the highest affinity (the lowest K_d), was used as a capturing aptamer for BVDV type 1 and aptamer No. 43 was used as reporting aptamer. Different concentrations of reporting aptamer No. 43 was injected to 5×10^4 $TCID_{50}$/ml of BVDV type 1 already captured by capturing aptamer No. 11. This real-time signal demonstrates these candidate pair can be applied to sandwich based assay for the sensitive detection of BVDV type 1. (b) Aptamer conjugated with AuNP sandwich system shows dose dependent result of various concentration of BVDV type 1 ($TCID_{50}$/ml).

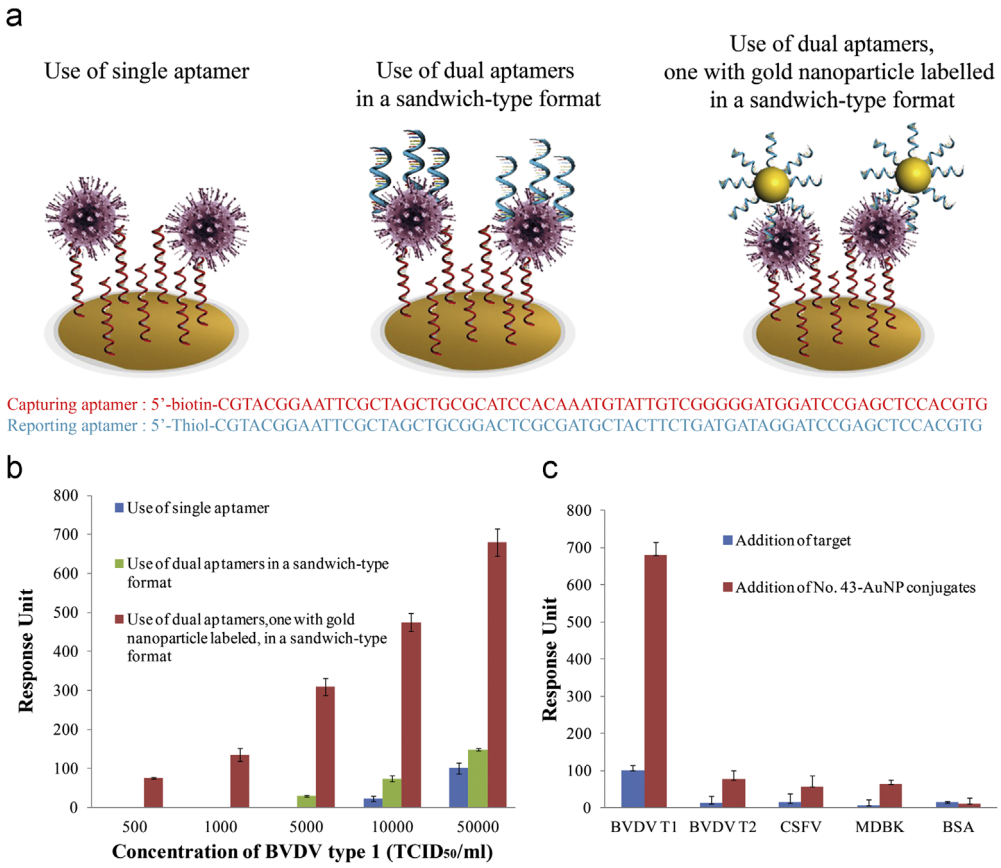


Fig. 3. (a) Schematic illustration of detection platform for BVDV type 1 including aptamer-based sandwich assay with or without AuNP labeling. (b) Signal enhancement with aptamer-based sandwich with or without AuNP labeling. The results without the use of non-labeled aptamer (green) and with AuNP labeled aptamer (black) showed enhanced signal depending on various concentration of BVDV type 1. (c) The specificity of selected reporter aptamer conjugated with AuNP sandwich systems when the same amount of virus particles (50,000 $TCID_{50}$/ml) were injected. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

30 min, and then the reporting aptamer No. 43 conjugated with AuNPs were added to enhance the angle shift on SPR sensor platform (Fig. 3a). In other words, two different ssDNA aptamers as capturing and reporting probes were used in this SPR-based sandwich type binding assay. In addition, due to the inherent characteristic of DNA, the modification of reporting aptamer as the signaling moiety is much more flexible and the stability and consistency should be much more improved than the other reporting probes. The signal enhancement assisted by aptamers-conjugated with or without AuNPs in SPR platforms are shown in Fig. 3b. The angle shift with the aptamers conjugated with AuNPs was considerably increased within the range from 5×10^2 $TCID_{50}$/ml to 5×10^4 $TCID_{50}$/ml (Fig. 2b), which is more than 20- or 10-fold enhanced than single-aptamer based SPR assay or aptamer sandwich without AuNP conjugation, respectively. In addition, the detection limit using dual aptamers obtained in this study was 5×10^2 $TCID_{50}$/ml, which can be converted to 800 copies/ml according to the results of real-time PCR based calculation (Supplementary data). Moreover, regarding the volume used in this SPR platform (50 μl, the real detectable number of BVDV type 1 is 40 copies, which is similar to the RT-PCR based detection system (Yan et al., 2011). More importantly, when this secondary aptamers were conjugated with AuNPs, the limit of detection was enhanced from 10^4 $TCID_{50}$/ml to 500 $TCID_{50}$/ml, which is similar to the commercialized conventional real-time PCR kit (VetMAXTM-Gold BVDV Detection Kit, Applied Biosystems) (Table 1).

The selectivity of this aptamer conjugated with AuNP based sandwich system is additionally examined. The three different counter targets and BSA were tested to find if the change in SPR angle shift was occurred. The binding signals for these counter targets were all very small even at the highest concentrations equivalent to the BVDV type 1 (Fig. 3c and Supplementary Fig. S3).

Table 1
The comparison of limit of detection of each sensing platform for detection of BVDV type 1.

Platform	Limit of detection ($TCID_{50}$/ml)	Note
Sandwich with AuNP	500	This study
Sandwich without AuNP	5000	This study
Without sandwich	10,000	This study
Other PCR based method	0.4–1400	– Da Silva et al. (1995)

4. Conclusion

In conclusion, an advanced immobilization-free SELEX method was successfully adopted for screening aptamers against whole-virus particle of BVDV type 1. This advanced method provides a simplified process for aptamer screening with high affinity and specificity within 5 SELEX rounds. For the first time, the aptamer pair was successfully found for a whole virus without any immobilization process. In addition, by applying the selected aptamer pair, highly sensitive detection of 500 $TCID_{50}$/ml or maximum 800 copies/ml (around 40 copies) of BVDV type 1 was successfully conducted with the aptamer conjugated AuNP based

sandwich-type SPR method. This ultra-sensitive detection limit is similar to the commercialized real-time PCR detection kit. Furthermore, this novel aptamer pairs can be applied to variable sandwich format biosensors.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2013.07.052.

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