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ARTICLE TYPE

Aptamer-Based Competitive Binding Assay for One-Step Quantitation of Hepatitis B Surface Antigen

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An aptamer-based competitive binding assay for one-step (i.e. no requirement of pretreatment) quantitation of target molecules of interest has been developed. This method has been successfully employed for the fast and sensitive detection of hepatitis B virus surface antigen (HBsAg). Key features of our method include its low intrinsic background noise, low costs, high resolution, and high sensitivity, enabling detection of as low as 1.25 mIU/mL, approximately 40-fold better than that of the most widely used Abbott Architech assay for HBsAg detection, without the tedious extraction and/or washing procedures. Moreover, this assay has better recovery and accuracy than that of conventional competitive binding assay or others for HBsAg quantitation.

Introduction

It is estimated that approximately 350 million individuals are chronically infected with hepatitis B virus (HBV) worldwide.[1] HBV is classified in the family *Hepadnaviridae*, and without intervention, 15% to 40% of chronic HBV-infected individuals will develop cirrhosis, end-stage liver disease, hepatocellular carcinoma, or require liver transplantation over the course of several decades, resulting in over a half million deaths annually.[2]

HBV has a small (3.2 kb) partially double-stranded DNA genome of approximately 3200 base pairs organized into four partially overlapping open reading frames, which encode the envelope, core (precore/core), polymerase and X proteins.[3] Apart from the pathology of HBV itself, recombinant hepatitis B surface antigen (HBsAg) is used for HBV vaccination and the development of antibody to HBsAg is typically associated with protective immunity.[4] Moreover, a number of antiviral drugs have been introduced recently for the treatment of chronic HBV infection, and viremia is an important component in the decision to initiate treatment and in monitoring therapeutic response.[5]

Accordingly, it is of great importance to quantitatively detect HBV and/or its biomarkers. First, quantitation of HBV DNA in blood or serum became a conventional tool among the quantitative methods developed so far, in the assessment and management of chronic infection.[6] And along with serologic tests for HBV and measurement of serum transaminases, HBV viral load testing is also used to determine the phase of chronic HBV infection and is known to be useful in distinguishing active from inactive disease in individuals even with no detectable hepatitis B e antigen (HBeAg).[7] Importantly, however, the detection of hepatitis B surface antigen (HBsAg) in serum was additionally pivotal to the discovery and/or quantitation of HBV more than 4 decades ago, because in virus-infected liver cells, HBsAg is produced in excess and secreted into the blood, where it can serve as a marker for active infection and infectivity, remaining the cornerstone of diagnosis today.[4] Since the methods for HBsAg detection using radioimmunoassay and enzyme immunoassays were first reported in 1970s,[8,9] significant advances have been made in the development of quantitative HBsAg assays, including Architech HBsAg QT (Abbott Diagnostic, Wiesbaden, Germany) and Elecsys HBsAg II (Roche Diagnostics, Indianapolis, IN, USA).

While these advanced methods are sufficient for many clinical and research diagnoses, the methods may be inadequate for some molecular HBsAg implementations and development, because antibodies are playing very crucial roles in these immunoassays. In consideration of the drawbacks of using antibodies for HBsAg quantitation, it would be highly advantageous to replace antibodies with aptamers in the immunoassays. Aptamers are a class of nucleic acids that can selectively bind, with high affinity, to a wide array of target molecule.[10-15] They are able to fold into a well-defined three-dimensional structure, enabling the molecular recognition and specific binding of the corresponding target. Thus, emerging successful alternatives to antibodies, a variety of DNA-, RNA-, and PNA (peptide nucleic acid)-based aptamers have been found to bind with the dissociation constants ranging from nanomolar to picomolar level.[15]

Aptamers have a number of unique features which make them a more effective choice than antibodies, including low costs, large-scale production, increased stability against high temperature and extreme pH, long half-life and reversible denaturation.[10,16] In these regards, it is desirable to employ them for HBsAg quantitation in combination both with the conventional competitive binding assays and fluorescence resonance energy transfer (FRET), because the conventional competitive binding assays have proved to be powerful tools for diagnostic assays,[17,18] and because FRET-based probes for quantitation assays have recently been developed to overcome some disadvantages of traditional fluorogenic probes.[19,20]

We have combined in the present study the conventional competitive binding assay with FRET and aptamers, in order to develop an assay for specific detection and quantitation of HBsAg. As Fig. 1 highlights the principle of our detection system,

Cy3 as FRET donor is covalently attached to anti-HBsAg aptamer and Cy5 as FRET acceptor to HBsAg, respectively. Because FRET is a state-to-state electronic energy transfer between the excited states of the fluorogenic dyes, in which the excitation energy is transferred from a donor to an acceptor without the emission of a photon, with inverse sixth-power dependence on the distance between the two dyes,[18,19] the fluorescence intensity of Cy5 can be the highest (i.e. emission from the FRET donor is suppressed and emission from the FRET acceptor is enhanced, leading to a shift in the color of the emitted light toward light of a longer wavelength) in the absence of unlabeled-HBsAg (analyte), whereas the fluorescence intensity of Cy5 can be decreased with the amount of unlabeled-HBsAg increased in the system. Such energy transfer processes could provide us with significant information on the distance and interaction between two dyes, based on the intensities and wavelength shifts of the absorption and emission,[19] allowing us to specifically detect and quantitate the target molecule, HBsAg.

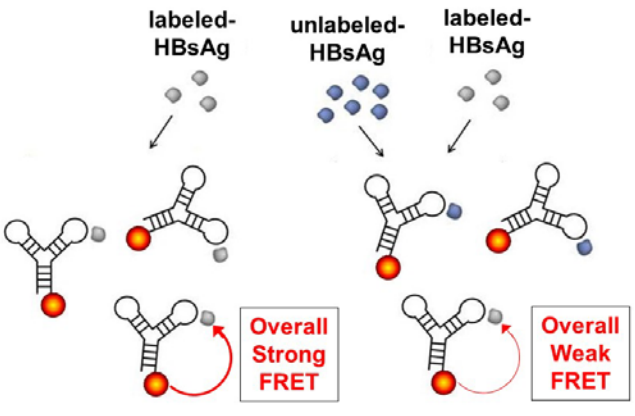

Figure 1. Schematic illustration of aptamer- and FRET-based competitive binding assay. Cy3 as FRET donor is covalently attached to anti-HBsAg RNA aptamer and Cy5 as FRET acceptor to HBsAg, respectively. With the fixed concentrations of the two labeled probes, FRET between the donor and the acceptor is enabled to strongly occur in the absence of the target, while the presence of the target disrupts the formation of a labeled RNA-labeled HBsAg hybrid, removing the donor from the vicinity of the acceptor, and gradually restoring the donor's fluorescence in a concentration-dependent fashion. The target concentration can thus be quantified based on the emission change at either donor's or acceptor's characteristic wavelength or both.

Materials and methods

Chemicals. Unless otherwise noted, reagents were obtained from commercial suppliers and were used without further purification, and deionized and depc-treated water was used for all experiments. All experiments were performed in triplicate.

Preparation of Cy3-labeled RNA aptamers. Sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC, Sigma) was used as cross-linker. RNA-based aptamers[21] for specific binding to HBsAg (the only reported anti-HBsAg aptamer, to our best knowledge) were synthesized, using the antisense oligonucleotide containing the T7 promoter sequence at the 3'-end (i.e. 5'-GTATG TGGGC TGAAC TCAAT CAGGT CCCAA TCCCC AACAT ACACA TGACC CGTCG TTTAC GATCA TTATA GACGG CCATG ATTGA CACGC AATCA ACCCC CTATA GTGAG TCGTA TTA-3'), as previously described,[12] and the resultant GSMP-containing 99-mer RNA was modified to contain a 5' thiol group, via an enzymatic method for the introduction of 5'-terminal sulfhydryl group at the 5'-termini of RNA molecules according to the literature.[12,22] Prior to the thiol-functionalized RNA preparation, the 5'-deoxy-5'-thioguanosine-5'-monophosphorothioate (GSMP) was synthesized,[22] as a substrate for T7 RNA polymerase that requires guanosine for efficient transcription initiation. The *in vitro* transcription after treatment of alkaline phosphatase was used to incorporate a sulfhydryl moiety to 5'-end of RNA molecule. After conjugation of amine-functionalized Cy3 (AAT Bioquest) with sulfo-SMCC according to the manufacturer's protocols, the resulting product was covalently attached to the thiol-functionalized RNA aptamer.

Conjugation of the target protein with NHS-activated Cy5. The NHS ester reaction was used for the protein labeling. Cy5-NHS ester (Lumipore) was chosen as the labeling dye. 1 nmol of HBV surface antigen stock (Abcam) was diluted in 0.1 M sodium carbonate buffer (pH 9.3). Cy5-NHS ester in DMSO was mixed in a 8:1 ratio with protein solution in order to be randomly introduced to HBsAg according to the literature.[23-25] It should be noted that there was no solid position of Cy5 in the resulting Cy5-labeled HBsAg when employing this conjugation method. After the mixture was shaken at room temperature for at least 4 hours in the dark, the sample was transferred to vivaspin (Sartorius), washed with the reaction buffer at least 3 times, 0.1 M sodium acetate pH 9.3, and eluted with 50 mM of Tris·HCl pH 7.5. After UV measurement with an Agilent 8453 spectrometer, the labeled protein was stored at 4 °C in the dark.

Quantitation of unlabeled target protein. Fluorescence spectra of the two probes (5 pmol each) in the absence or presence of unlabeled HBsAg were measured in a Tris-buffered solution (20 mM Tris·HCl, 140 mM NaCl, 5 mM $MgCl_2$, pH 8.2, final volume 200 μL) at 4 °C for 24 h according to the literature.[21,26] Samples containing bovine serum albumin (BSA) instead of unlabeled HBsAg were used as negative controls. The range of HBsAg concentrations assayed was 2.5 pmol to 250 pmol in 200 μL or 1.25 mIU/mL to 125 mIU/mL. To precisely quantify the level of unlabeled HBsAg, fluorescence signals were obtained at 570 nm and 670 nm with an excitation at 550 nm, because as the level of the acceptor emission increases, and the decay of the donor emission increases as well in any FRET process. Fluorescence spectra were recorded using a Synegy Mx spectrofluorophotometer (BioTek). Individual spectra were deconvoluted using OriginLab (OriginLab Corp.) or SigmaPlot (Systat Software) by comparison to spectra collected from the individual components alone.

Detection of target protein in clinical samples. 25 HBsAg-positive and 19 HBsAg-negative samples from National Cancer Center were determined by the Abbott Architech HBsAg assay and Abbott RealTime HBV Quantification Kit. The study protocol conformed to the 1975 Declaration of Helsinki and was approved by the ethics committees of National Cancer Center in South Korea. The results using the aptamer- and FRET-based competitive binding assay were compared with those obtained from Abbott Architech HBsAg assay.

Results

Cy3-labeled RNA aptamers and Cy5-labeled HBsAg.

An absorbance of the Cy5-labeled HBsAg eluted through vivaspin was analyzed by UV spectrometer, and both the dye-to-protein ratio of the conjugated protein and the molar

concentrations of the sample were calculated based on the Beer's law. The molar extinction coefficient was extrapolated on the basis of the amino acid sequence of the surface antigen.[27] Because the absorbance of HBsAg is near at 210 and 280 nm (refer ESI†, Fig. S1A), the presence of the fluorescent label was verified by absorption measurements at 650 nm for Cy5 (ε_{Cy5}(650 nm) = 250,000 $M^{-1}cm^{-1}$) (Fig. S1B, ESI†).[26] The separated peaks of HBsAg were probably due to their conformation difference at the solution.[28] The average dye-to-protein ratio was estimated to be 1.01. In a similar manner, the concentrations of the RNA stock solutions were calculated from absorption measurement at 260 nm (1 OD_{260} = 40 μg/ml), where the absorption of the Cy3 label at 260 nm was taken into account (Fig. S2, ESI†). The presence of the fluorescent labels was verified by absorption measurements at 550 nm for Cy3 (ε_{Cy3}(550 nm) = 150,000 $M^{-1}cm^{-1}$).[26] The dye-to-RNA ratio of Cy3-labeled RNA aptamers was found to be 1.04.

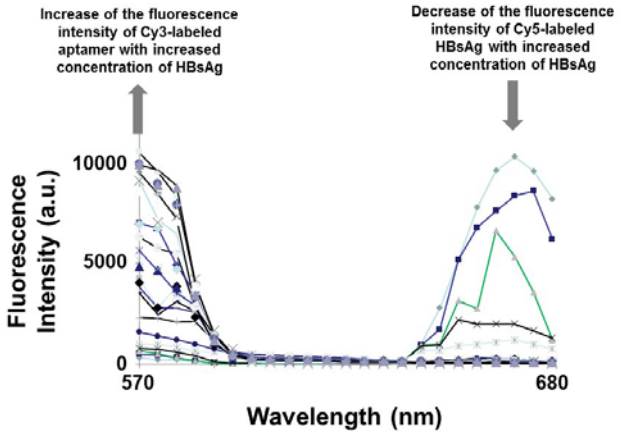

Figure 2. Fluorescence spectra changes of 5 pmol of Cy3-labeled anti-HBsAg aptamer (as FRET donor) and 5 pmol of Cy5-labeled HBsAg (as FRET acceptor) with the amount of unlabeled HBsAg (as analyte) increased (0 – 250 pmol), showing that the two dye-labeled molecules in a buffer quantitatively produce a significant FRET signal change as the amount of analyte increases.

In vitro quantitation of unlabeled HBsAg based on FRET between Cy-3labeled RNA and Cy5-labeled HBsAg.

To verify the proof-of-concept, equimolar amounts (5 pmol) of both dye-labeled probes were mixed in a Tris-buffered solution in the absence or presence of unlabeled HBsAg, and the emission fluorescence spectrum of each sample for quantitation of unlabeled HBsAg was scanned using a fluorimeter upon excitation at 550 nm.

As clearly demonstrated in Fig. 2, our aptamer- and FRET-based quantitative assay relies on the competitive binding of unlabeled HBsAg to Cy3-labeled RNA with Cy5-labeled HBsAg, while Fig. S3 (ESI†) reveals that there is no change in the fluorescence intensity of Cy3-labeled aptamer in the absence of Cy5-labeled HBsAg as the analyte concentration increases, indicating that FRET or the decrease of Cy3 fluorescence intensity is caused only by the presence of Cy5-labeled HBsAg within the Föster radius. With the fixed concentrations of the two labeled probes, FRET between the donor and the acceptor is enabled to strongly occur in the absence of the target. It should be noted that the presence of non-specific target (bovine serum albumin, BSA, in this study) does not interrupt the binding between the donor-labeled RNA and the acceptor-labeled HBsAg, leading to no effect on the average FRET between the two dyes. The presence of the target, however, disrupts the formation of a labeled RNA-labeled HBsAg hybrid, removing the acceptor from the vicinity of the donor, gradually restoring the donor's fluorescence in a concentration-dependent fashion, and thus revealing the presence of the target.[26] The target concentration can thus be quantified based on the emission change at either donor's or acceptor's characteristic wavelength or both (Fig. 3).

Fig. 3A shows that the fluorescence intensities of the FRET donor, Cy3, are increased as the amounts of unlabeled HBsAg are increased, allowing for highly specific and wide-range detection (from 2.5 pmol to 250 pmol or from 1.25 mIU/mL to 125 mIU/mL) of unlabeled HBsAg. To our best knowledge, this is the first demonstration of an aptamer- and FRET-based competitive binding assay for sensing HBsAg wherein unlabeled HBsAg as a analyte is quantitated. The limit of detection (LOD) can be defined as the lowest measured value for which the measured value minus 2 SDs is higher than the blank mean value plus 2 SDs. Based on this criterion, the insetresults shown in Fig. 3A demonstrate successful detection of as low as 2.5 pmol of unlabeled HBsAg, thereby illustrating the ultrasensitive capability of our method. Interestingly, Fig. 3B shows that the fluorescence intensities of the FRET acceptor, Cy5, are decreased in a two-phase fashion as the amounts of unlabeled HBsAg are increased, which is attributed presumably to the differentiated FRET efficiency at the given concentrations of unlabelled HBsAg between Cy3 at the 5'-terminus of aptamer and Cy5 randomly introduced to HBsAg.[19,26] The linear range outlined in the inset of Fig. 3B suggests that the fluorescence measurement at 670 nm may be applied to quantitation of HBsAg in a range between 50 pmol and 250 pmol. In the following experiments, fluorescence intensities at 570 nm have been used for quantitation of HBsAg.

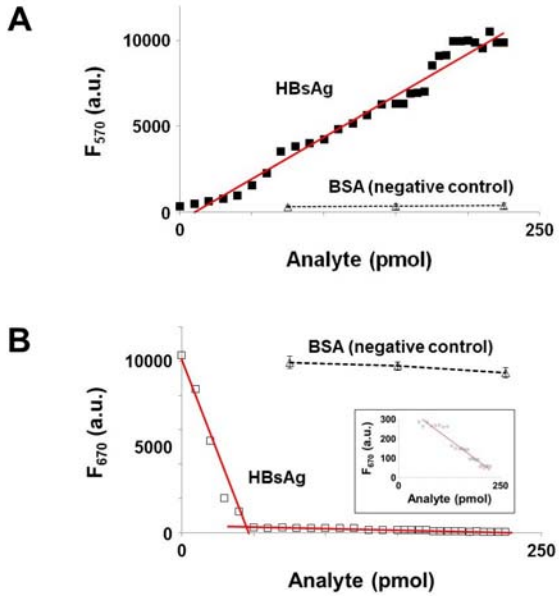

Figure 3. Experimental results of aptamer- and FRET-based quantitative assay. The thick line and dotted line represent quantitation data for unlabeled HBsAg (as analyte) and for unlabeled bovine serum albumin (BSA, as non-specific analyte). (A) Linear increase of fluorescence emission intensity at 570 nm with the amount of unlabeled-HBsAg increased. (B) Two-phase linear decrease of fluorescence emission intensity at 670 nm with the amount of unlabeled-HBsAg increased. The linear range outlined in the inset indicates that the fluorescence measurement at 670 nm can be applied to quantitation of HBsAg in a range between 50 pmol and 250 pmol.

Evaluation of the performance of the aptamer- and FRET-based competitive binding assay in comparison with the conventional methods

Fig. 4 shows that our assay and the Abbott Architech HBsAg assay have a good correlation (R^2 = 0.954), suggesting that a direct relationship exists between the amount of HBsAg in the sample and the relative light units (RLUs) detected by the Architect Immunoassay System optics. Interestingly, the lowest point is observed to be far from (0,0), which is attributed probably to the facts that the Architect HBsAg is a fully automated system and can detect as low as 0.2 ng/mL of HBsAg with a dynamic range of 0.05-250.0 IU/mL,[29] and that the sensitivity of our assay is approximately 40-fold higher than that of the Abbott Architech assay (our assay's cutoff value, 12.5 pmol/mL or 1.25 mIU/mL). This can be supported by the fact that 19 HBsAg-negative samples are undetectable by the Abbott Architech HBsAg assay, while our assay can discriminate the levels of HBsAg in the samples (Figs. S4 and S5, ESI†). It should be noted, however, that our method has a lower upper-limit of detection than that of the Abbott Architech HBsAg assay, because the amounts of HBsAg in 6 samples (sample #: 3, 12, 13, 14, 15, and 17) produce overflowed fluorescence intensities at 570 nm, i.e. the amount of HBsAg in the sample exceeds 1.25 nmol/mL or 125 mIU/mL. This drawback of our method is expected to be overcome by changing the fixed concentrations of the two probes.

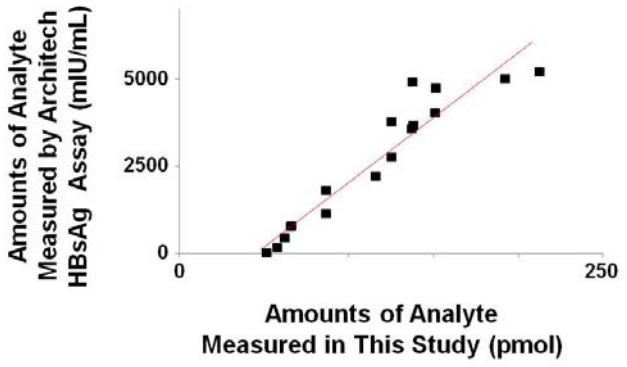

Figure 4. Comparison between our aptamer- and FRET-based competitive binding assay and the conventional Abbott Architech HBsAg assays. Samples from 25 HBsAg-positive patients were measured by the two methods. The label of x-axis is the amount (pmol) of HBsAg in 200 μL estimated by the aptamer- and FRET-based competitive binding assay, while that of y-axis is the amount (mIU/mL) of HBsAg measured by the Abbott Architech HBsAg assays.

Discussion

The Architect HBsAg assay was compared to our aptamer- and FRET-based competitive binding assay. The Architect HBsAg is a chemiluminescent immunoassay, most widely used at present in clinical studies.[29] The Architect HBsAg assay is a two-step assay for quantitatively determining human serum and plasma HBsAg concentrations. In the first step, the sample and hepatitis B surface antigen (anti-HBsAg) antibody coated with paramagnetic microparticles are combined, allowing HBsAg present in the sample to bind to the anti-HBsAg antibody-coated microparticles. After washing, acridinium-labeled anti-HBsAg antibody conjugate is added. Following another wash cycle, pre-trigger and trigger solutions are added to the reaction mixture and the resulting chemiluminescent reaction is measured as RLUs.

In contrast, our method allows for a one-step (no requirement for pre-treatment) assay for quantiation of HBsAg. The assay took advantage of aptamers over antibodies and showed improved sensitivity even without disrupting HBV particles. Moreover, the sensitivity of our assay is approximately 40-fold higher than that of the Abbott Architech assay (cutoff value, 12.5 pmol/mL or 1.25 mIU/mL) with good accuracy, reproducibility, and specificity. Highly sensitive HBsAg detection of our method might be useful for several clinical applications, including the possible replacement of HBV DNA monitoring by a PCR-based method for blood screening with the sensitive HBsAg detection method because the aptamer- and FRET-based method is simpler, more convenient, and less expensive than HBV DNA quantitation by real-time PCR. It should be noted, in addition, that because the conventional HBsAg detection methods including the Abbott Architech assay use monoclonal or polyclonal antibodies against external structural regions within the determinant "a" loop as epitopes, HBsAg mutants, such as G130D, T131N, M133T and G145R are found to escape detection for HBsAg by the current HBsAg assays.[30,31] In consideration of the advantageous properties of the aptamer- and FRET-based HBsAg assay, our future work will be focused on determination of the hepatitis B surface amino acid sequences of all possible cases with detectable amounts of HBV DNA.

To summarize, we report a robust quantitation assay that is capable of sensitive detection of HBsAg by FRET measurement between FRET donor-labeled RNA and FRET acceptor-labeled HBsAg. Our developed assay allows for a one-step measurement without the tedious extraction and/or washing procedures. In consideration of the advantageous properties of aptamers over antibodies and sensitive and precise FRET measurement, our developed assay can be generally utilized to detect other biomarkers and to potentially benefit the scientific and clinical community.

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Notes and references

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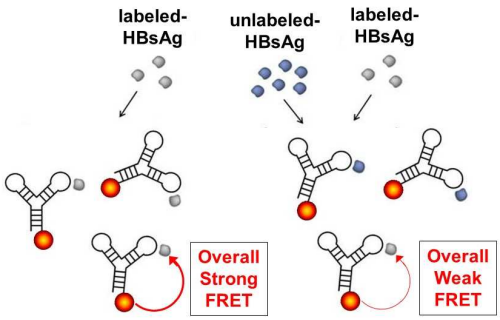
Table of contents entry



A robust aptamer- and FRET-based competitive binding assay for one-step quantitation of hepatitis B surface antigen is reported.