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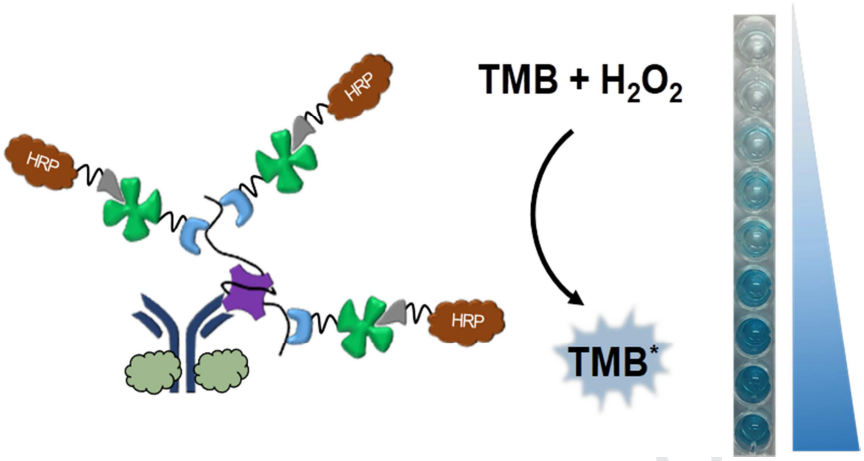
Graphical Abstract



Highly sensitive and universal detection strategy based on a colorimetric assay using target-specific heterogeneous sandwich DNA aptamer

Juyoung Kang[†], Gyuho Yeom[†], Hyungjun Jang,
Chin-Ju Park* and Min-Gon Kim*[1]

ABSTRACT: A simple, universal, and sensitive colorimetric biosensor for detecting of various biomarkers was devised using a target-specific DNA aptamer, as the recognition element, and engineered with streptavidin-fusion replication protein A 70kDa (RPA70A) linked to biotin-horseradish peroxidase, as the colorimetric element. To improve sensitivity and stability compared to other colorimetric detection techniques, we developed a novel detection strategy by integrating a newly selected heterogeneous sandwich DNA aptamer and protein engineering in this study. The proposed assay is based on a change in color from colorless to blue due to the interaction of the aptamer with RPA70A in the presence of the target; this color change could be observed by the naked eye or measured with a UV-vis spectrometer. We confirmed its high sensitivity and specificity for two model targets using their aptamers under optimal experimental conditions. In addition, the feasibility of the assay was tested in clinical samples containing NPs of influenza A or B virus. These results suggest that our detection system developed herein can be universally applied to the diagnosis of various diseases owing to its stability, sensitivity, and specificity.

Keywords

Colorimetric assay, Replication Protein A, Aptamer, Heterogeneous sandwich, Universal detection

Department of Chemistry, School of Physics and Chemistry, Gwangju Institute of Science and Technology (GIST), 123 Cheomdangwagi-ro, Buk-gu, Gwangju, 61005, Republic of Korea

* Corresponding author.
C.-J. Park: Tel: +82-62-715-3630; Fax: +82-62-715-2866; E-mail address: cjpark@gist.ac.kr
M.-G. Kim: Tel: +82-62-715-3330; Fax: +82-62-715-3419; E-mail address: mkim@gist.ac.kr
† These authors contributed equally to this work

ORCID
Min-Gon Kim: 0000-0002-3525-0048

1. INTRODUCTION

The development of highly sensitive and accurate detection techniques for infectious viral diseases that pose a major threat to weakly immunized children and the elderly is of paramount importance to public health issues [1]. In particular, accurate and sensitive diagnosis is essential for the appropriate treatment of many diseases caused by viruses, such as influenza A and B virus, which have become widespread globally with high infection rates [2–4]. Several techniques for detecting these viruses have been reported; among these, the most commonly used method is a polymerase chain reaction (PCR)-based technique for amplifying and detecting viral genes in human biological samples [5–9]. This method is more accurate than other methods but requires complicated sample preparation and sophisticated equipment.

Among the various methods for biomarker detection including PCR, colorimetry-based assay is an attractive method with the following advantages: use of visible light for detection, ease of operation, and fast reading [10–12]. Biosensors using colorimetric detection convert colorless signals into visible color changes. Various strategies for generating colorimetric signals have been successfully studied; however, several challenges, such as poor stability and low sensitivity, remain unresolved [13,14]. For example, horseradish peroxidase (HRP) is widely used in biosensors based on colorimetric detection because it is capable of reacting with various substrates to exhibit characteristic color changes [15–17]. These methods have been reported to enable the recognition and detection of color changes based on the presence or absence of a target, through conjugation with an antibody or elements capable of recognizing the target. However, they have limitations regarding the detection of biomarkers requiring high sensitivity because of the poor stability or the need for complex steps for signal amplification [18]. Moreover, depending on the conjugation method, a low recognition rate and false-positive signals may be obtained.

To address these limitations, several techniques that change target-recognition elements into aptamer or add simple signal amplification steps to the analysis have been developed [19–22]. Aptamers are single-stranded DNA, RNA, or peptide molecules that interact with a desired target with high affinity and specificity [23,24]. They are widely used as clinical diagnostic tools and therapeutic agents because they possess several advantages over antibodies, such as lower manufacturing costs and shorter production times [25–27]. In addition, when an aptamer is used as a recognition agent, it enables highly sensitive detection by incorporating various signal amplification methods that utilize the favorable characteristics of nucleic acids [28]. We previously developed an aptamer-based colorimetric biosensor using HRP-encapsulated liposomes with signal amplification for highly sensitive detection of disease biomarkers [29]. This method achieved high sensitivity but is restricted in practical

diagnosis applications due to some limitations. These constructs are difficult to store for a long time based on the characteristics of liposomes, and the synthesis of HRP-loaded liposomes involves markedly complex steps.

Herein, we devised a novel detection platform based on a colorimetric assay using heterogeneous sandwich DNA aptamers and engineered replication protein A for amplification of the colorimetric signal. Replication protein A (binding domain A of human replication protein A 70kDa; RPA70A) is a single stranded-DNA (ssDNA) binding protein that binds universally with aptamers (i.e. ssDNA) [30]. Lyophilized streptavidin-fusion RPA70A linked with biotin-HRP (STA-RPA70A@biotin-HRP) used as a colorimetric agent in the proposed detection method is more stable than enzymes encapsulated in liposomes or chemical reagents used in other colorimetric assays. We additionally developed a new DNA aptamer specific to influenza B virus nucleoprotein (NP) using the previous strategy for the selection of a heterogeneous sandwich DNA aptamer. Using these materials, we successfully applied the aptamer to the detection of influenza A and B virus NPs in the devised colorimetric biosensor, with a femtomolar limit of detection (LOD), and demonstrated its potential for application in diverse clinical fields.

2. EXPERIMENTAL SECTION

2.1 Materials and reagents

Histidine (His)-tagged recombinant NP (A/Puerto Rico/8/34/Mount Sinai (H1N1)) and Influenza B (B/Florida/4/2006) NP expressed in a baculovirus-insect cell expression system were purchased from Sino Biological Inc. (Beijing, China). Anti-influenza A NP antibody (capture and detection) and anti-influenza B NP antibody ("immobilized Ab") produced in a murine model were purchased from HyTest Ltd. (Turku, Finland) and Fitzgerald Industries International (Acton, MA, USA), respectively. Peroxidase Labeling Kit-NH2 was purchased from Dojindo molecular technologies, Inc. (Rockville, MD, USA). The oligonucleotide sequence of the influenza A NP aptamer employed in this study is 5′-TAG GGA AGA GAA GGA CAT ATG ATT GGC TTG CAT GCT GGA CTT CCT ACT GGT TTT TGA CTA GTA CAT GAC CAC TTG A-3′. Bovine serum albumin (BSA) and surfactant 10G (95R-103) were purchased from Fitzgerald Industries International. Neo protein saver was obtained from TOYOBO Co., Ltd (Osaka, Japan) and 96-well plates were purchased from Greiner Bio-One Inc. (Kremsmünster, Austria). STA-HRP and 3,3',5,5'-tetramethylbenzidine (TMB) substrate reagent sets were purchased from BD Biosciences (San Diego, CA, USA). Tris-HCl, potassium chloride, magnesium chloride, sodium chloride, Triton X-100, and Tween 20 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Human nasal fluid from a single human donor (991-13-S) was purchased from LEE Biosolutions Inc. (Maryland

Heights, MO, USA). dNTP mixture, lambda exonuclease and nTaq polymerase were obtained from Enzynomics (Daejeon, Korea). The PCR Product Purification Kit was purchased from Gene All (Daejeon, Korea). All oligonucleotides employed in this study were purchased from Integrated DNA Technologies Inc. (Coralville, IA, USA). All buffers and reagent solutions were prepared using distilled water (DW) generated on an ELGA water purification system (Lane End, UK).

2.2 H-sandwich SELEX

The H-sandwich systematic evolution of ligands by exponential enrichment (SELEX) method introduced in the previous study was used to develop a heterogeneous sandwich DNA aptamer specific to influenza B virus NP [31]. To immobilize the antibody for capturing the NP and aptamer, anti-influenza B NP antibody (1 $\mu g \cdot mL^{-1}$) was incubated in 0.1 M carbonate buffer (pH 9.5) in a 96-well plate at 4 °C overnight. After incubation and washing, the wells were subsequently blocked with 1% (w/v) BSA at 37 °C for 1 h. Recombinant NP (1 $\mu g \cdot mL^{-1}$) (i.e., positive well) or PBS-T (i.e., counter well) with coated antibody were prepared at 37 °C for 2 h. The ssDNA random library was denatured by heating at 90 °C for 5 min in binding buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$) and immediately cooled on ice. After that, the ssDNA library was added to BSA-blocked counter wells for 30 min to exclude ssDNA bound to antibodies or BSA. The supernatants containing unbound ssDNA were incubated in wells containing the antibody-NP complex (positive well) for 30 min. The wells were then washed with PBS-T, and binding buffer was added and incubated at 80 °C for 10 min to elute antibody-NP complex-bound ssDNA. The mixture was purified using the PCR purification kit according to the manufacturer's protocol. The precipitate was dissolved in 30 µL of sterile water and stored at 4 °C for subsequent experiments.

In order to amplify the bound ssDNA, PCR was performed using a forward primer and phosphorylated reverse primer. The PCR was carried out in 50 µL of solution containing purified template DNA, two primers (1 µM each), 2.5 mM dNTP mixture, and nTaq polymerase. PCR conditions were as follows: 95 °C for 5 min; 30 cycles of 95 °C for 30 s, 56 °C for 20 s, and 72 °C for 20 s; and final extension at 72 °C for 5 min. The phosphate-labeled PCR product was then cleaved into ssDNA by lambda exonuclease before the next round of selection. Furthermore, the H-sandwich SELEX procedure included additional rounds of counter-SELEX using human nasal fluid and NP of influenza A virus to increase specificity (4^{th} and 9^{th} rounds). A total of 12 sequential rounds of selection were performed in accordance with the abovementioned protocol.

2.3 Purification of STA-fusion RPA70A protein

STA-fusion RPA70A (STA-RPA70A) was subcloned into the pET His6 TEV LIC cloning vector (1B), which

was a gift from Scott Gradia, and transfected into BL21 (DE3) cells. Proteins were overexpressed and purified as described previously [30]. Cells were grown at 37°C to an OD 600 of 0.5–0.6, following which IPTG was added to a final concentration of 0.5 mM. Cells were incubated overnight at 18°C. His-tagged STA-RPA70A proteins were purified using an Ni-NTA column (Elpis Biotech, Daejeon, Korea) and eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 300 mM imidazole). Further purification was performed by size exclusion chromatography using AKTA pure and Hi-Load 16/600 200pg (GE Healthcare) with PBS buffer at a flow rate of 1 mL/min (**Fig. S1**).

2.4 Preparation of STA-fusion RPA70A linked to biotin-HRP

To synthesize STA-fusion RPA70A linked to biotin-HRP (STA-RPA70A@biotin-HRP), purified STA-RPA70A was mixed with biotin-HRP and incubated at 4 °C for 1 h. After incubation, size exclusion chromatography was performed to remove the remaining uncoupled biotin-HRP. The final concentration of STA-RPA70A@biotin-HRP was 0.5 $mg \cdot mL^{-1}$, and it was lyophilized for 12 h using an Ilshin freeze dryer (FD5520S; Ilshin Biobase Co., Dongducheon, Korea) for further experiments.

2.5 Direct enzyme-linked oligonucleotide assay (ELONA)

To determine the binding affinities of the selected aptamers, we performed direct enzyme-linked oligonucleotide assay (ELONA) as described previously [29]. Recombinant NP of influenza B virus (1 $\mu g \cdot mL^{-1}$) was immobilized in a 96-well plate at 4 °C overnight. After blocking with 1% (w/v) BSA in PBS-T for 1 h, four biotin-labeled aptamers (0–10 µM) were used as the detection probes. The binding affinities between the aptamers and influenza B virus NP were determined by measuring absorbance values at 650 nm using streptavidin-HRP and TMB solution and calculating K_d using GraphPad Prism version 7.01 software.

2.6 Measurement of NPs of influenza A and B virus

The STA-RPA70A@biotin-HRP, in the form of lyophilized powder, used in this study was defined as “1 X” by adding 50 µL of DW. Anti-NP antibodies (of influenza A or B virus) were diluted to 1 $\mu g \cdot mL^{-1}$ in 0.1 M carbonate buffer (pH 9.5) and coated on a 96-well plate at 4 °C overnight. After incubation, the wells were blocked with 1% (w/v) BSA including 2% (w/v) protein saver in PBS-T for 1 h. After the washing steps, NPs and NP-specific aptamers (10 nM) were mixed in binding buffer in concentrations ranging from 0.1 $pg \cdot mL^{-1}$ to 1 $\mu g \cdot mL^{-1}$ and incubated in the antibody-captured wells at 50 µL/well for 1 h. The wells were then washed using 200 µL PBS-T, and STA-RPA70A@biotin-HRP was added to the wells and incubated for 10 min. After washing to remove residual reactants, a mixture of TMB reagents (100 µL) was added to induce a catalytic reaction in the wells. The HRP of the conjugates catalyzed the oxidation of TMB, and the color changed from colorless to dark

blue.

2.7 Sandwich enzyme-linked immunosorbent assay (ELISA)

To compare the sensitivity with the developed method, sandwich ELISA was performed using the same capture antibody, and HRP-conjugated detection antibody for colorimetric signal generation. Except for using the detection antibody that binds NP in the form of sandwich, assay procedures were similar the developed method mentioned above. The detection antibody-conjugated HRP was made following the kit's user protocol. All reagent concentrations were the same as those analyzed in the developed method.

2.8 Evaluation of clinical samples

We obtained patient samples containing NPs of influenza A and B viruses from Discovery Life Sciences, Inc (Huntsville, AL, USA). The study protocol was explained in detail to patients from whom samples were obtained, and signed informed consent forms were obtained in accordance with the institution's review board's approved guidelines and relevant regulations. Biological fluid samples were stored at −80 °C until subsequent analysis. The analysis of NPs in the clinical samples for the proposed method was performed according to the procedure described above.

3. RESULTS AND DISCUSSION

3.1 Principle of the detection system using STA-RPA70A@biotin-HRP

Our proposed detection system comprises a colorimetric assay that utilizes heterogeneous sandwich DNA aptamers and RPA70A protein binding to ssDNA (**Fig. 1**). RPA70A interacts nonspecifically with single-stranded DNA (ssDNA), binding to the exposed base without altering the structure of the DNA [28]. To apply this property of RPA70A to colorimetric assays, we purified RPA70A fused with STA. Subsequently, HRP tagged with biotin was linked to RPA70A *via* STA-biotin binding.

In a previous study, we developed a method for screening DNA aptamers with heterogeneous sandwich structure [31]. Aptamers selected through this method served as a detection probe, and we selected DNA aptamers for influenza A and B virus NPs as model targets.

When a mixture of the incubated target and aptamer is added to a well containing the immobilized capture antibody, the complex formed a heterogeneous sandwich structure (antibody-target-aptamer) through its other binding site. Next, the STA-RPA70A@biotin-HRP binds to the strand of the exposed aptamer, and the HRP linked to RPA70A reacts with the TMB mixture to cause a color change.

3.2 Binding affinities of selected aptamers

The random nucleotide library, generated from a pool of 10^{16} ssDNA molecules with 40-base random sequences, was screened *via* 12 rounds of H-sandwich SELEX. **Table S1** shows the sequences of the selected aptamers. In addition, the eluted DNA concentration was measured before performing the next round to evaluate the efficiency of enrichment (**Fig. S2A**). The ratio value is calculated as the ratio of the DNA concentration in the positive well to the DNA concentration in the counter well. As the number of rounds increased, the DNA concentration of the positive well gradually increased, and then decreased again after additional counter selection in the 4th and 9th rounds. This low concentration may be due to ssDNA binding to nasal fluid or other NPs during counter selection. H-sandwich SELEX was completed in the 12th round, at which point the concentration ratio showed saturation. A total of four aptamer candidates were obtained, and their binding affinity to the influenza B virus NP was measured by ELONA. As shown in **Fig. S2B**, Inf B-apt1 showed the highest binding affinity to the NP, with a dissociation constant (K_d) value of 0.97 ± 0.6 nM; therefore, we used Inf B-apt1 in subsequent experiments.

3.3 Confirmation of STA-RPA70A@biotin-HRP

After confirmation of STA-RPA70A purification by SDS-PAGE (**Fig. S1**), STA-RPA70A was reacted with biotin-HRP and separated by size exclusion chromatography to use as a colorimetric element in the proposed assay (**Fig. 2A**).

The peak of the purified STA-RPA70A (black) and the peak of the STA-RPA70A@biotin-HRP (red, after incubation with STA-RPA70A and biotin-HRP) were obtained through size exclusion chromatography. The two peaks showed a difference in size; the STA-RPA70A@biotin-HRP peak increased in molecular weight, producing the front peak, and the unreacted STA-RPA70A produced the rear peak. We then performed a plate-based colorimetric assay to measure the absorbance in order to evaluate the quality of the synthesized STA-RPA70A@biotin-HRP. STA was coated onto a 96-well plate and then immobilized with biotin-labeled ssDNA (oligo dT) to confirm linking of RPA70A to the biotin-HRP. As shown in **Fig. 2B**, the wells containing immobilized ssDNA displayed higher absorbance value than the wells containing immobilized STA, BSA, or anti-mouse IgG. These results indicate that STA-RPA70A@biotin-HRP was adequately synthesized via the established protocol.

3.4 Optimization test

To obtain optimal detection performance, factors influencing detection efficiency were investigated. Aptamer concentration is important for induction of STA-RPA70A@biotin-HRP: if the concentration of aptamer is insufficient when binding to the target, sensitive detection by the interaction of the aptamer with RPA70A is not

possible. The NP concentration was 10 ng·mL^{-1} and that of the aptamer ranged from 1 nM to 100 nM (**Fig. S3A**). As the aptamer concentration increased, so did the absorbance values of wells with or without the target. For optimal detection efficiency, the ratio of the absorbance value of the well containing the target to that of the background signal was calculated and indicated the appropriate ratio of on-off (on; w/ NP, off; w/o NP) absorbance values at an aptamer concentration of 10 nM. Thus, 10 nM was chosen as the optimal aptamer concentration for incubation with NP. The dilution ratio of synthesized STA-RPA70A@biotin-HRP is another factor that affects the sensitivity of detection. As shown in **Fig. S3B**, the absorbance value decreased with increasing dilution ratio, and the on-off absorbance ratio was appropriate at 0.1 X (1:10 dilution). These results were used as the basis for the selection of aptamer concentrations and dilution ratios for subsequent experiments.

3.5 Performance of the proposed assay for detection of biomarkers

After confirming the optimum conditions and quality of the synthesized STA-RPA70A@biotin-HRP, we assessed the performance of the present assay for the detection of influenza A and B virus NPs using heterogeneous sandwich aptamers by evaluating its sensitivity under different NP concentrations. To calculate the sensitivity of the proposed method, the absorbance values for various concentrations were measured along the UV spectrum. **Fig. 3** shows the pattern of increase of the absorbance values and color changes with increasing influenza A and B virus NP concentration, from 0.1 pg·mL^{-1} to 1 μg·mL^{-1}, along the UV spectrum. The graph in **Fig. 3A** (bottom) shows that the color at 1 pg·mL^{-1} of NP was easily observed by the naked eye. The inserted linear graph also shows the high linearity of the absorbance values as the NP concentration was increased (R^2 = 0.99). The LOD was calculated as 0.3 pg·mL^{-1} (S/N = 3) using the formula defined by the absorbance value (without NP) + 3.3 SD on a linear graph. Similarly, the color change and absorbance values were measured according to the influenza B NP concentration (**Fig. 3B**). We could observe an obvious color change at the NP concentration of 1 pg·mL^{-1} and the LOD of 0.16 pg·mL^{-1} (S/N = 3). Moreover, we compared the influenza A virus NP detection sensitivity with our developed method and conventional sandwich ELISA using the same capture antibody. As shown in **Fig. S4**, the color at 100 pg·mL^{-1} of NP was observed with the naked eye in conventional ELISA. It is 100 times higher concentration than that is detected with our proposed method, 1 pg·mL^{-1}. These results demonstrate that our proposed method is 100 times more sensitive than the conventional sandwich-based assay. We propose that more colorimetric agents can be combined with detection agents in our approach compared to conventional ones. Even when compared with other colorimetric methods for detecting similar targets, the proposed detection method offers higher sensitivity and simplicity (**Table 1**).

We used aptamers specific to each target and measured the absorbance values in various solutions containing

different substances (PBS, nasal fluid, NP of influenza A virus, NP of influenza B virus, and NP mixture) to determine the specificity of the proposed detection method (**Fig. 4**). In wells with an influenza A NP-specific aptamer, higher absorbance values were observed for the influenza A NP and NP mixed solution than for other solutions. Similarly, high absorbance values were obtained for the influenza B NP and NP mixed solution in wells containing the influenza B NP-specific aptamer. In particular, the low absorbance for nasal fluid and PBS indicated that the aptamer did not bind, and, therefore, no color change occurred. These results proved that our sensing platform using STA-RPA70A@biotin-HRP exhibited high sensitivity and specificity owing to the use of target-specific aptamers and RPA70A.

In addition, we measured the change in absorbance for each aptamer length by adding oligo dT to the aptamer sequence specific for NP of influenza A virus. This allowed us to confirm the improvement in sensitivity according to the length of the aptamer (**Fig. S5**). The findings showed that the absorbance value of the concentration was increased when 20 bases or more were added to the aptamer, and the absorbance of the aptamer with 30 added bases was increased by approximately 1.3 times compared with the original absorbance. Therefore, in cases of target detection requiring high sensitivity, the performance of the method can be improved by increasing the length of the aptamer to an optimal length.

3.6 Clinical application

To confirm the potential for clinical application, we evaluated the utility of the present assay based on STA-RPA70A@biotin-HRP and aptamers for practical detection of influenza A and B virus NPs in patient samples. As shown in **Fig. 5**, we observed significant differences in absorbance values between influenza virus-negative and -positive samples. These results indicate that the method proposed in this study is useful for the simple and sensitive detection of disease biomarkers in human samples and could be clinically applied.

3.7 Confirmation the stability of colorimetric agents

A further experiment was performed to investigate the stability of synthesized STA-RPA70A@biotin-HRP in the freeze-dried powder form. For application in various fields, the colorimetric element must be stable without limitations in regard to storage period or formulation. We verified the performance of the assay by measuring the absorbance according to storage period and formulation. As shown in **Fig. 6A**, the on-off ratio of the absorbance values was almost constant even with increasing storage period, and the values for wells containing NP after 2 months were 93% that of the value immediately after preparation. In addition, we observed obvious differences in on-off ratios both in lyophilized and in solution formulations (**Fig. 6B**). These results indicate that the synthesized STA-RPA70A@biotin-HRP was highly stable and active, regardless of the storage period or

formulation.

CONCLUSIONS

We developed heterogeneous sandwich DNA aptamers specific for the influenza B virus NP and applied them to a novel biosensor platform that improved upon the previously developed liposome-based colorimetric detection method. The newly developed method exhibited high sensitivity and stability through protein engineering and purification of STA-fusion RPA70A protein. We also prepared the STA-HRP and biotin-HRP (STA-RPA70A@biotin-HRP) conjugate *via* the STA-biotin interaction for colorimetric signal detection. The proposed detection system based on STA-RPA70A@biotin-HRP has three advantages. First, long-term storage can be achieved through lyophilization. Second, the STA-RPA70A@biotin-HRP can be combined with the aptamer at higher yields by increasing the aptamer length because the conjugate was synthesized compactly only through protein linkages. Aptamer length can be regulated according to required sensitivity in further study. Finally, this method can be used universally in biosensors with a heterogeneous sandwich format. We confirmed the specificity and universality of the present detection method for influenza A and B virus NPs and achieved LODs of 0.3 and 0.16 pg mL^{-1}, respectively. The efficiency of detection of this system was evaluated using patient samples, and the results support its application for sensitive diagnosis in various clinical fields.

Conflict of interest

The authors declare no competing financial interest.

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ASSOCIATED CONTENT

Supplementary Material

Sequences of aptamer candidates specific to influenza B virus NP; Confirmation of purified STA-fusion RPA70A by SDS-PAGE; Elution profile and binding affinities of aptamer candidates; Optimization of proposed colorimetric assay; Conventional sandwich ELISA for detection of influenza A virus NP; Comparison of

absorbance signal for various aptamer lengths (Word).

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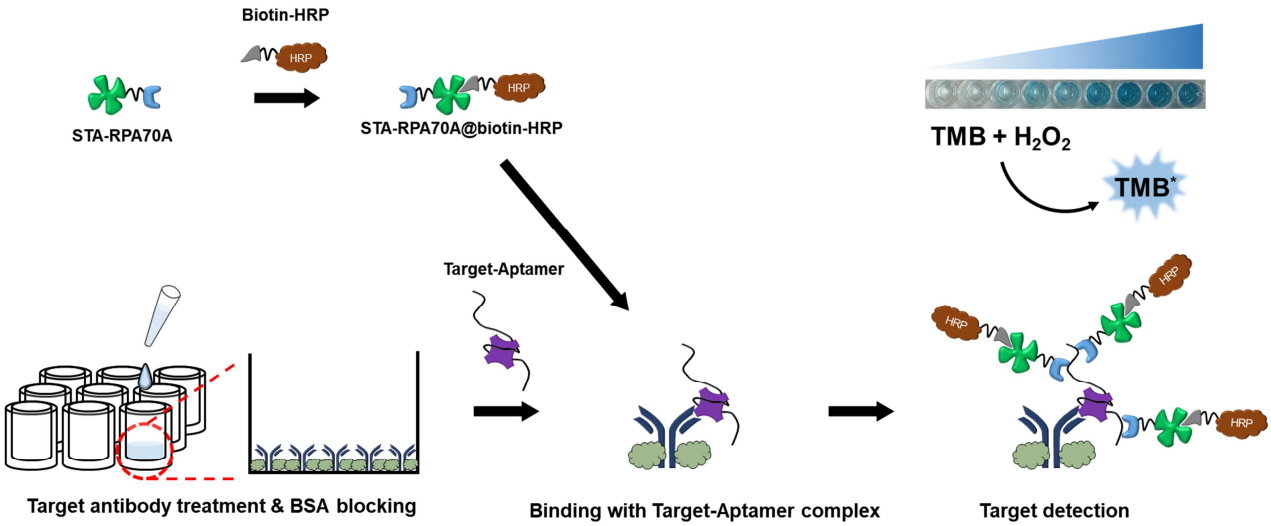
Detection method	Target	LOD	Reference
Enzyme-encapsulated liposome	H5N1	0.04 ng·mL^{-1}	[14]
RPA70A-conjugated liposome with HRP encapsulation	NP of SFTS virus	9 pg·mL^{-1}	[29]
Magnetic nanoparticle	NP of influenza A virus	0.3 nM	[32]
Functionalized magnetic microparticle	Influenza virus	11.16 μg·mL^{-1}	[33]
STA-RPA70A@biotin-HRP	NPs of influenza A and B virus	0.3 / 0.16 pg·mL^{-1}	**This study**



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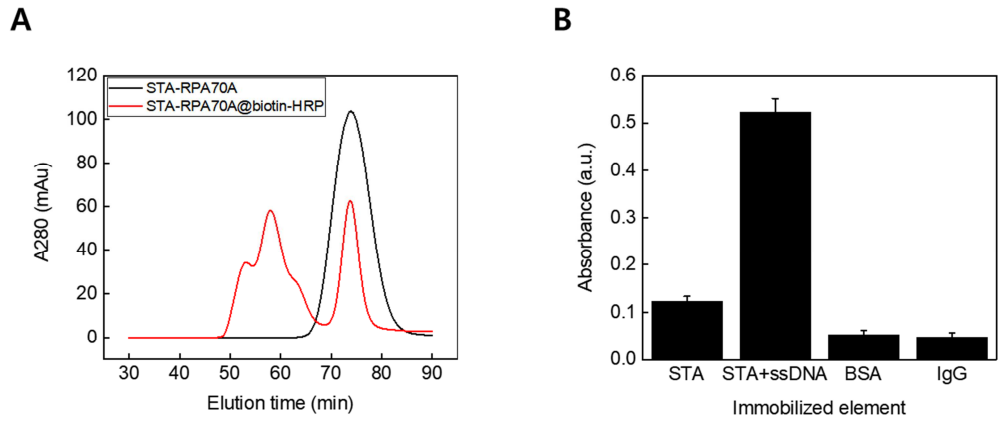

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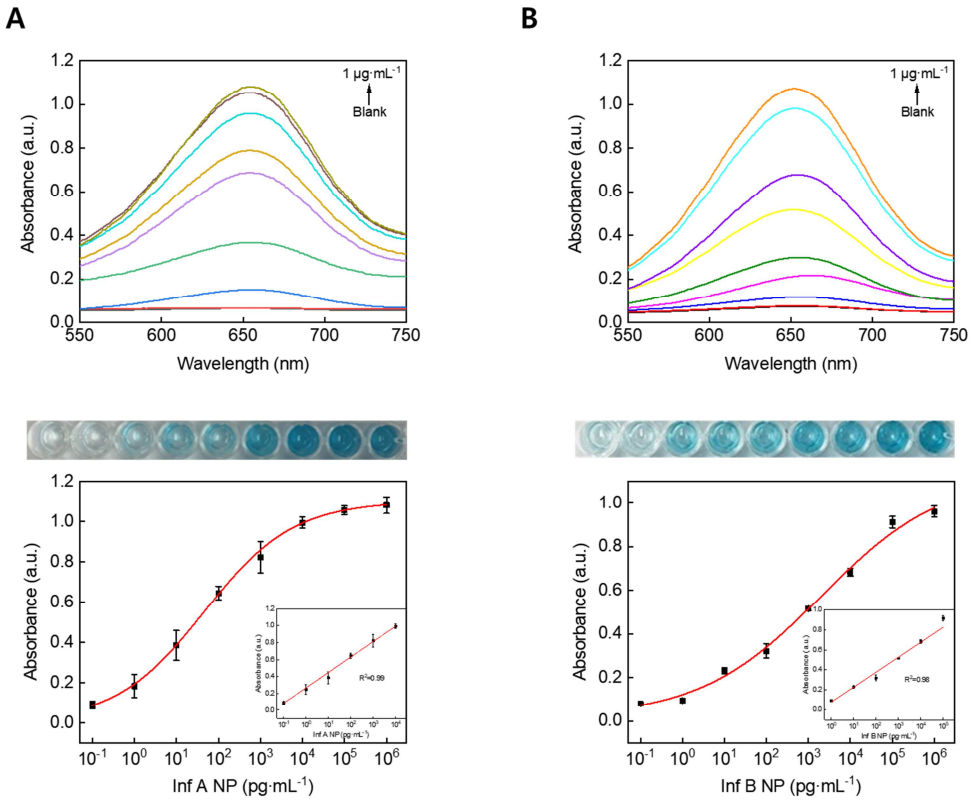

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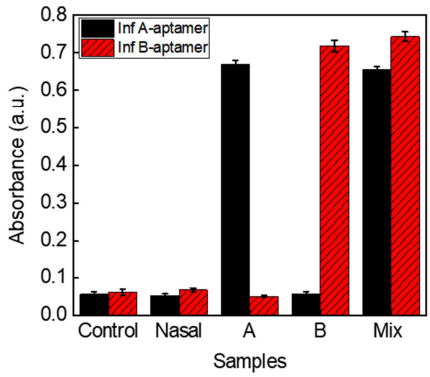

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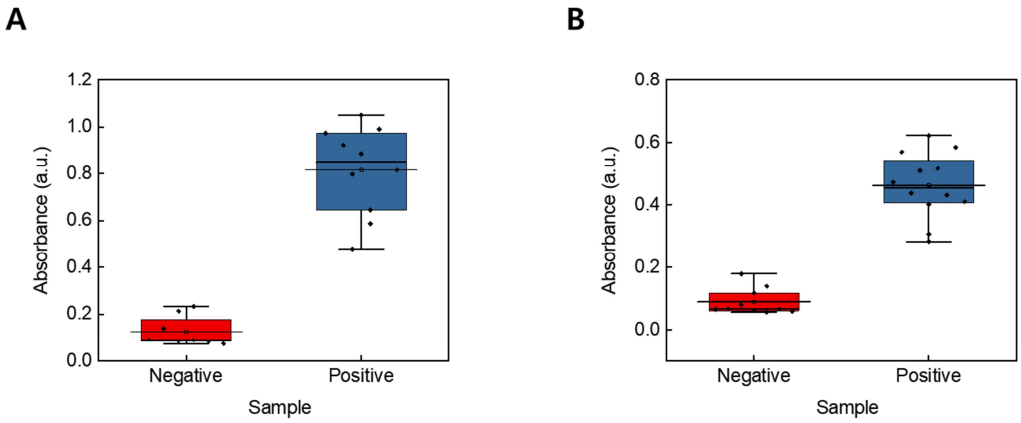

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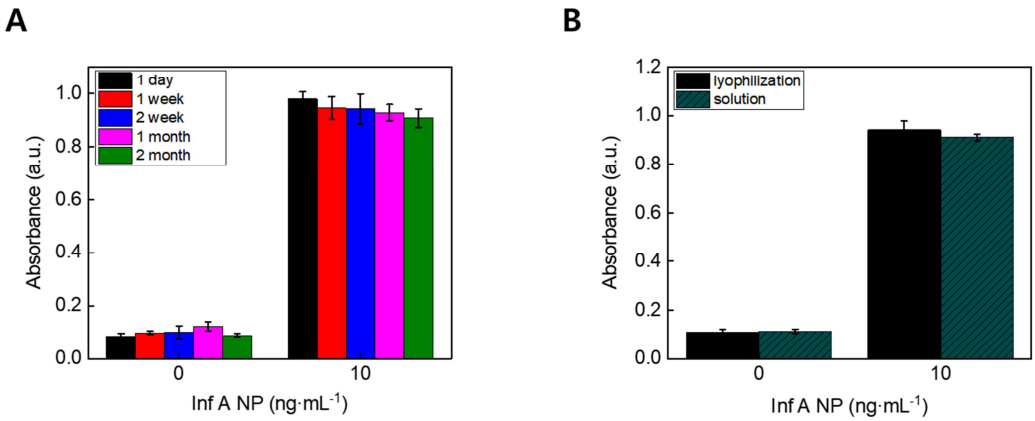

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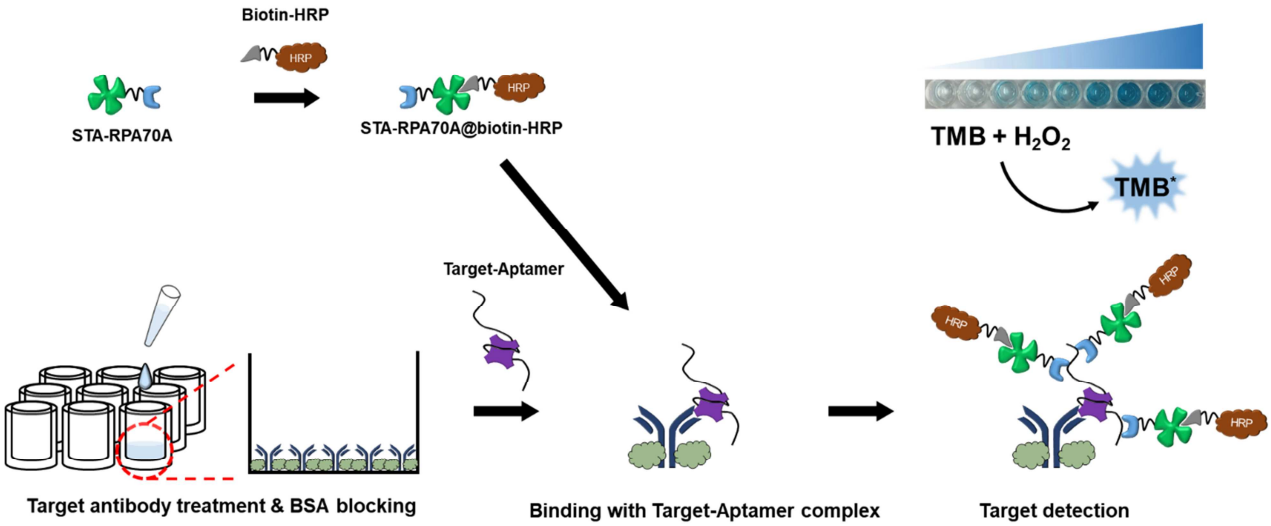

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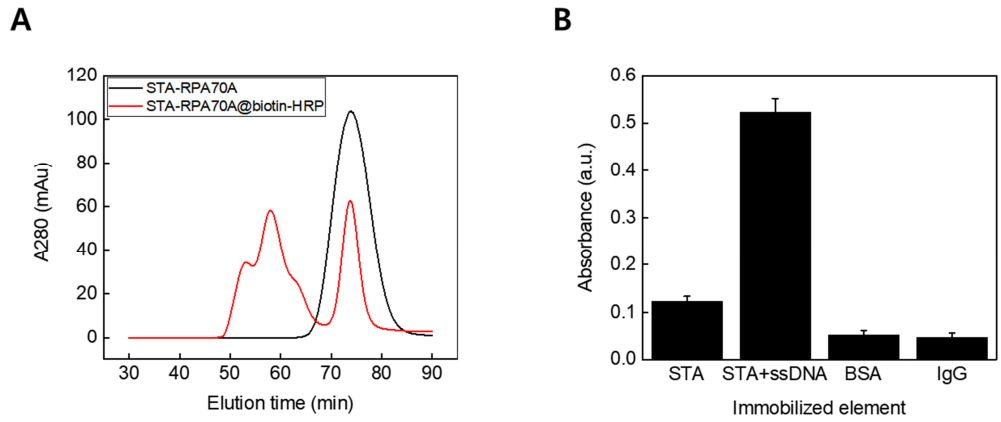

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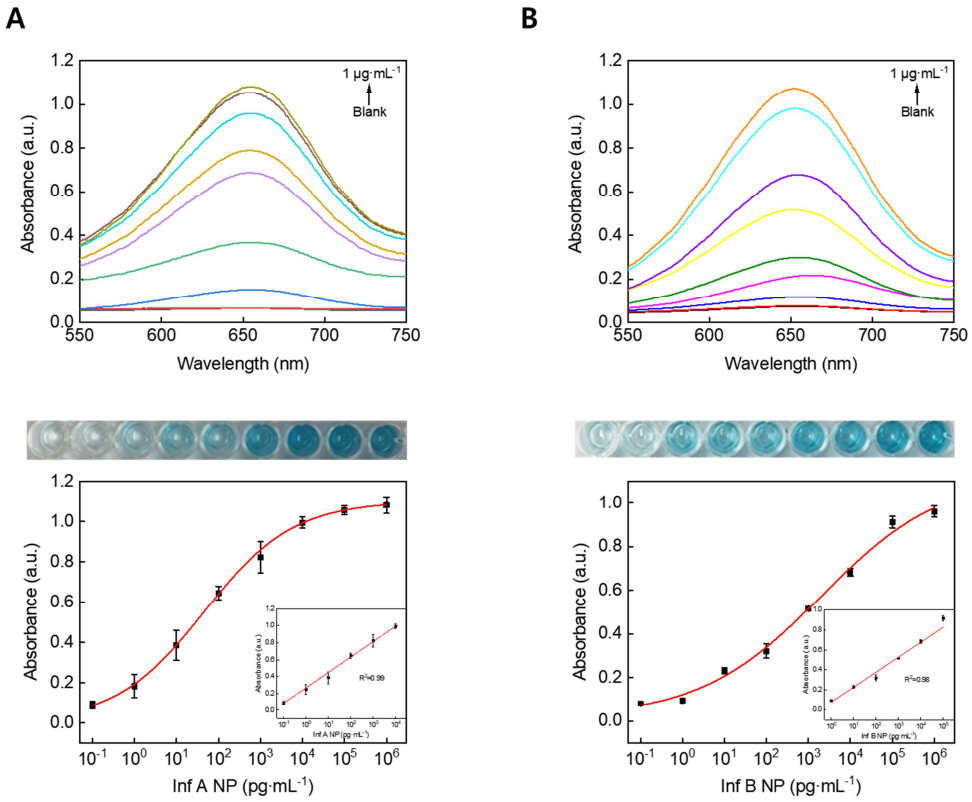

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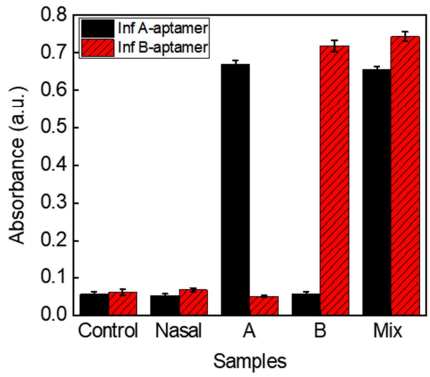

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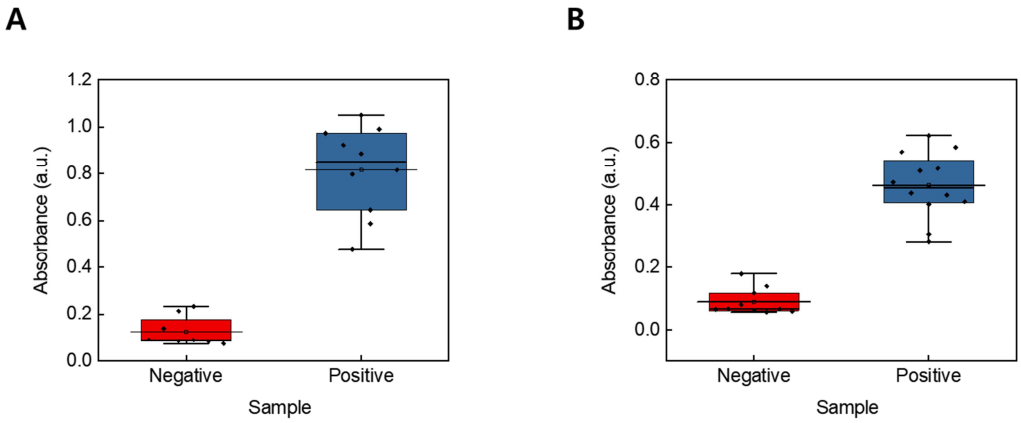

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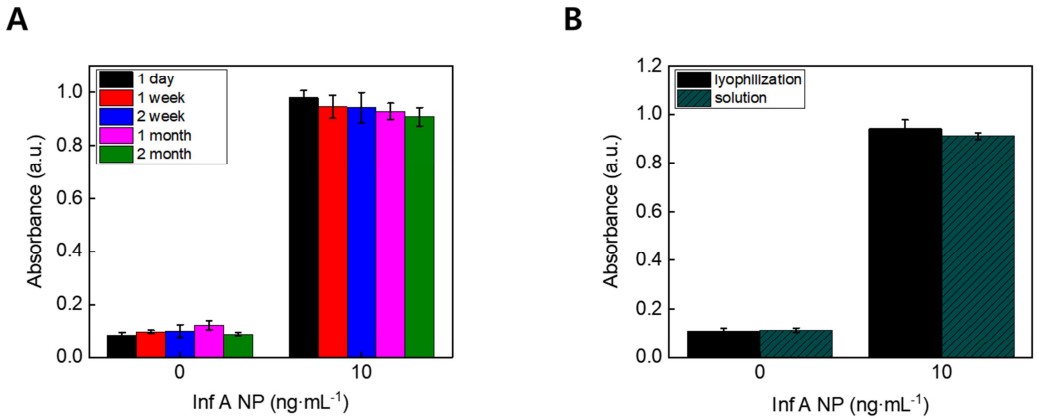

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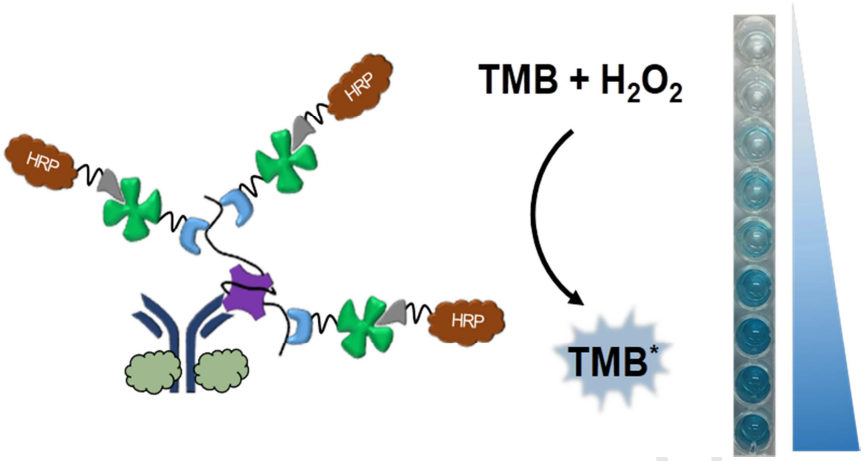
Graphical Abstract



Highlights

- We newly selected heterogeneous sandwich DNA aptamer specific for influenza B virus NP.
- Our sensing platform improves the efficiency of biosensor *via* protein engineering.
- Long-term colorimetric reagent (STA-RPA70A@biotin-HRP) storage can be achieved through lyophilization.
- STA-RPA70A@biotin-HRP can be combined with the aptamer at higher yields by increasing the aptamer length.
- This method is universally useful in biosensor with a heterogeneous sandwich format.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

CRediT author statement

Juyoung Kang: Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Data Curation, Visualization, Writing – Original Draft, Writing – Review & Editing

Gyuho Yeom: Conceptualization, Methodology, Formal Analysis, Investigation, Data Curation, Writing – Original Draft, Writing – Review & Editing

Hyungjun Jang: Methodology, Investigation

Chin-Ju Park: Conceptualization, Writing – Review & Editing, Funding acquisition, Supervision, Project Administration

Min-Gon Kim: Conceptualization, Writing – Review & Editing, Funding acquisition, Resources, Supervision, Project Administration