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Short communication

The strategy of signal amplification for ultrasensitive detection of hIgE based on aptamer-modified poly(di-acetylene) supramolecules

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ABSTRACT

Herein, we demonstrate three strategies of signal amplification for ultrasensitive detection of human immunoglobulin E (hIgE) based on poly(di-acetylene) supramolecules. To fabricate the ultrasensitive PDA biosensor, ethylenediamine as an interlinker and aptamer as a receptor were introduced into the chip fabrication process. Using the prepared PDA liposome biosensor, the hIgE could be detected up to below 1.0 ng/ml by a primary response. In order to accomplish more ultrasensitive detection of protein on a PDA biosensor, polyclonal hIgE antibody was employed as an external mechanical force for the inducement of a secondary response. As a result, a PDA liposome biosensor sensitivity as high as 0.01 ng/ml for the target hIgE was obtained, with a sensitivity which is one hundred times of that of the method without signal amplification. These results indicate that the proposed strategies were capable of ultrasensitive quantitative and qualitative analyses of biomolecules without non-specific binding of non-target proteins.

1. Introduction

Currently, aptamers are used as alternative receptors. Aptamers are oligonucleotides that can bind to a wide range of target molecules, such as drugs, proteins, and other organic or inorganic molecules, with high affinity and specificity. The systematic evolution of ligands by the exponential enrichment (SELEX) method has been used to isolate ligands with high affinities for IgE, D17.4, which competitively inhibits the IgE/Fcε RI interaction (Liss et al., 2002). The application of aptamers as bio-components in biosensors offers many advantages over classical affinity sensing methods primarily based on antibodies. Since the aptamers can easily be engineered *in vitro*, their mass production is relatively cost-effective. Moreover, while antibodies are sensitive to temperature and denature easily upon contact with surfaces, leading to limited shelf lives, aptamers are stable for long-term storage (You et al., 2003).

As biosensors using conjugated polymers, poly(di-acetylenes) (PDAs) biosensors have been intensively investigated for the detection of chemical and biological molecules (Yang and Swager, 1998; Ho et al., 2002; Kim and Bunz, 2006). PDAs are polymers of diacetylene monomers, a class of single chain lipid molecules characterized by alternating triple bonds. Polymerized PDAs such as liposomes or layered films show extreme color which is easily distinguishable by the naked eye (Charych et al., 1993; Reicher et al., 1995; Ma et al., 1998; Wei et al., 2005; Lee et al., 2007; Choi et al., 2008; Yu et al., 2008; Zhang et al., 2009). Most PDA-based assemblies exhibit rapid blue-red colorimetric transitions due to a wide range of stimuli, such as temperature, pH, mechanical perturbations, solvents, and interfacial ligand–receptor binding. (Okada et al., 1998; Nallicheri and Rubner, 1991; Mino et al., 1992; Chance et al., 1977; Chance, 1980; Charych et al., 1993; Ahn et al., 2003). When the polymerized PDA liposomes undergo color shift from blue to red, strong fluorescence (excitation: 530 nm; emission: 590 nm) is indicated by a red phase (Carpick et al., 2000) that is detectable using a fluorescence microscope or microarray reader with proper filters.

In this paper, we report the strategy of fluorescent signal amplification by an interlinker, an aptamer, and a polyclonal antibody on the PDA liposome biosensor for ultrasensitive detection of human hIgE. First, experiments in which the PDA liposomes were stably immobilized onto a glass substrate by an 'ethylenediamine' interlinker were performed. Moreover, after introduction of the anti-hIgE aptamer in lieu of the hIgE antibody as the receptor on the PDA liposome biosensors, the aptamer-modified PDA liposomes were compared with antibody-modified PDA liposomes. Finally, the 'sandwich method' using polyclonal hIgE antibody was introduced onto the PDA liposome biosensor in order to enhance the fluorescent signal of PDA liposomes. Then, the response of the PDA liposome spots were monitored after introduction of target hIgE at various concentrations (1.0 pg/ml–10 μg/ml).

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2. Materials and methods

2.1. Materials

Monoclonal human IgE antigen used in the PDA liposome biosensor was purchased from DIATEC.com (Norway). Anti-IgE DNA aptamer (D17.4ext) with 3′-biotinylation was custom-synthesized by Bioneer Co. (Korea), and its base sequence was as follows:

D17.4ext (45 mer): 5′-GCG CGG GGC ACG TTT ATC CGT CCC TCC TAG TGG CGT GCC CCG CGC–NH_2-3′

10,12-pentacosadiynoic acid (PCDA) was purchased from GFS Chemicals (OH, USA). 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), monoclonal human IgE antibody, polyclonal human IgE antibody, bovine serum albumin (BSA), fibrinogen from human plasma, human immunoglobulin G (hIgG), *N*-hydroxysuccinimide (NHS), *N*-ethyl-*N*-(3-diethylaminopropyl) carbodiimide (EDC), dimethyl sulfoxide (DMSO), ethylenediamine, and absolute ethanol were purchased from Sigma-Aldrich (USA). HPLC-grade chloroform was purchased from Fluka. Amine-coated glass was prepared by Nuricell (Korea). Deionized water (DI, resistance of water was 18.2 MΩ cm), obtained from a water purification system (Human Corporation, Korea), was used for preparation of the washing and buffer solutions.

2.2. Immobilization of PDA liposomes on amine-coated glass surface (Fig. 1(A))

Chloroform solutions of PCDA and DMPC were prepared separately in amber glass vials at −4 °C. Solutions of the two lipid monomers were mixed at 4:1 molar ratios (PCDA:DMPC) for a final lipid concentration of 1.0 mM. After chloroform was removed by N_2 gas, the remaining dry film of mixed diacetylenes was resuspended in 1.0 ml of 10 mM PBS buffer (pH 7.4) by heating in a circulating water bath set at 80 °C with gentle stirring about 15 min. The prepared solution was extruded through prefilter–100 nm membrane–prefilter complex repeatedly. The extruder system was kept at 85 °C for PCDA lipid formation, on the dry bath. Then, the vesicles solution was cooled to room temperature (25 °C) for 20 min. NHS and EDC were separately dissolved in PBS buffer to a total concentration of 200 mM. Then prepared ethylenediamine (total concentration of 1 mM) and an NHS/EDC (equal volume ratios) solution were added to the vesicle solution (Park et al., 2009). The PDA liposome solution, including the interlinker and NHS/EDC, was arrayed by an automated liquid handling system (Aurora biomed, Korea) at room temperature at 9 spots/well, with the 8 wells/amine-coated glass (Nuricell, Korea). The spot out of 8 wells was designated as 'control areas'. The PDA liposome-arrayed glass was then incubated in a chamber at a constant temperature of 4 °C for 2 h. Interlinking and immobilization of the liposome occurred during incubation, after which the PDA liposome-arrayed glass was repeatedly washed with deionized water and 0.1% Tween-20 in water, followed by soft drying under a stream of pure N_2. The arrayed glass was then covered with a multiple frame-seal incubation chamber, forming the final microwell for the multiple chip system.

2.3. Conjugation of biomolecules onto the PDA liposome chip (Fig. 1(B))

After PDA liposomes immobilization, the PDA liposome spots were polymerized under 254-nm UV light at a 1.0 mW/cm^2 intensity for 10 min. After NHS/EDC was dissolved in the PBS buffer, the amine-functionalized anti-hIgE aptamers were added to the solution. These mixed solutions were spotted by an automated liquid handling system on all PDA liposome spots. After spotting the anti-hIgE aptamers, the PDA liposomes-arrayed glass was incubated in a constant-temperature chamber at 4 °C for 3 h, then washed gently with deionized water and dried with pure N_2 gas. The analytes were injected onto each well (from 1 to 8) and the PDA liposome biosensor was incubated at 37 °C for 30 min. Then, the color change was analyzed by fluorescent microscopy after 30 min.

2.4. Detection of human IgE by signal amplification using polyclonal hIgE antibody

Amplification of the fluorescent signal was investigated using a sandwich immunoassay. After reaction between the target human IgE and anti-hIgE aptamer, the polyclonal hIgE antibody (100 ng/ml in PBS buffer) was subsequently injected into the spots and incubated at 37 °C for 30 min during the enhancement step. The subtracted fluorescent signals (a.u.) were corrected by subtracting the control (only PBS buffer) values of the PDA liposome, obtained by flowing polyclonal hIgE antibody over the surface without target hIgE, from the enhanced fluorescent signal values.

2.5. Specificity and selectivity test of target hIgE on aptamer-modified PDA liposome biosensors

Using a PDA liposome biosensor treated with anti-hIgE aptamer, the specificity and selectivity of the target proteins were tested on a prepared PDA liposome biosensor. First, the hIgE, hIgG, fibrinogen, and BSA were diluted to 1.0 μg/ml concentrations. Then, each protein was dropped onto the wells of the PDA liposome biosensors and incubated at 37 °C for 30 min. The PBS buffer was then added onto the aptamer-modified PDA liposome spots as a baseline.

2.6. Fluorescence analysis for hIgE detection

The fluorescent microscope consisted of four major parts: a microscope; a fluorescence unit (composed of a mercury arc lamp as a light source, fluorescent attachment and Nikon G2A filter, suitable for analysis of red phase PDA fluorescence); a digital camera unit (Infinity, USA); image analysis software (i-Solution, Korea). Fluorescence signals from the PDA liposome spots were visualized with this system and digital micrographs taken and spot intensities calculated with the image analysis software. Intensity denotes average intensity value of each pixel.

3. Results and discussion

3.1. Introduction of the interlinker for stable immobilization among liposomes on the PDA liposome biosensors

PDA biosensor chips had a serious problem involving loss of PDA liposomes due to weak immobilization when the PDA liposomes were washed out on the sensing spot during the fabrication process of the PDA biosensor chip. At that time, prior PDA liposome-based solid biosensors showed weak signal and sensitivity of immobilized the PDA spots on PDA liposome chips. To solve this serious problem, the interlinker 'ethylenediamine' was introduced into the fabrication process of the PDA biosensor chip. Using ethylenediamine (1.0 mM) brought strong cross-linking between liposomes through covalent bonding (Park et al., 2009).

To investigate this phenomenon in more detail, fluorescence and electron microscopy was employed to observe the morphology of the PDA layers on glass substrates. Fig. 2(A) shows fluorescence and SEM images of immobilized PDA liposomes both without (i) and with an interlinker (ii), after serial reaction of the aptamer and target hIgE (1.0 μg/ml). The effect of the interlinker is clearly shown in Fig. 2(A) as the PDA liposome was bound more to the surface

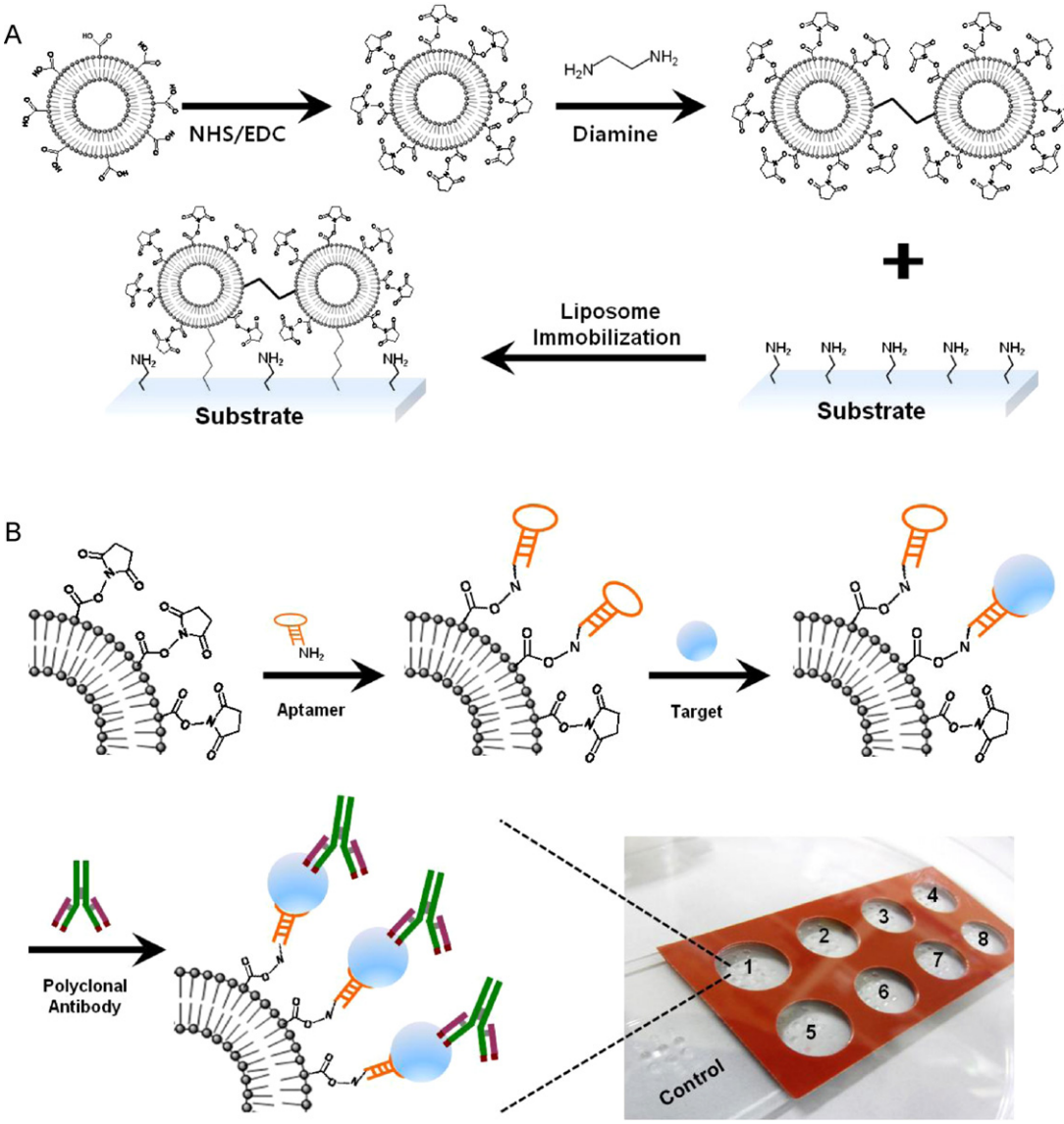

Fig. 1. Schematic diagram illustrating the fabrication steps in the biosensor based on PDA liposomes for detection of hIgE. (A) Preparation of the PDA liposome included PCDA and DMPC and immobilization of PDA liposome onto the amine-coated glass through NHS-activated carboxylic acid-amine coupling. Ethylenediamine is then added to cross-link the PDA liposomes. (B) After treatment of the liposome surface with NHS/EDC, the amine-functionalized anti-hIgE aptamers were immobilized onto the PDA liposome surface. The analytes (hIgE) were then combined on the PDA liposome surface. Finally, hIgE pAb for signal amplification was added onto the PDA liposome surface.

and with greater aggregation. A PDA liposome spot on the biosensor chip showed weak fluorescent intensity because PDA liposomes were washed outside the spot area of the chip in the case of no interlinkers. However, a PDA liposome spot of the biosensor chip in case of an interlinker exhibited a much stronger fluorescent intensity than without an interlinker as the concentration of the immobilized PDA liposomes was dramatically increased within the same spot area.

3.2. Comparison between antibody- and aptamer-modified PDA liposome biosensors

When target hIgE was bound to the antibody-modified liposome surface without the interlinker, subtracted fluorescence intensity increased minimally (below 10 a.u.). Conversely, a PDA liposome spot with an interlinker showed a slight increase (about 30 a.u.) in fluorescence intensity relative to the PDA liposome spot lacking the interlinker. However, the sensitivity of this PDA liposome biosensor was insufficient for allergy diagnosis. There is an important reason for the insignificant change of fluorescent intensity on the hIgE antibody-modified PDA liposome. In this case, the fluorescent signal of the PDA liposome was already saturated by the mass stress on the hIgE antibodies prior to introduction of the analyte due to the molecular weight of the hIgE antibody receptor for detection of the target hIgE being higher (188 kDa) than other receptors (Janeway et al., 2001).

To solve the aforementioned problems of the antibody-based PDA liposome, the anti-hIgE aptamer (receptor) was introduced onto the PDA liposome chips. As shown in Fig. 2(A), in the case of the aptamer-modified liposomes, the subtracted fluorescent signal was much stronger than the antibody-modified liposomes in both cases of without and with interlinkers. The improved sensitivity can be explained by the small size of the aptamer. Since the size of the aptamer was far smaller than the whole antibody, using aptamers on a PDA liposome biosensor can prevent receptors of substantial size in the immobilization step of receptors from saturating the PDA fluorescence signal prior to the target proteins are combining with the receptors on the liposome. Moreover, it is further considered to

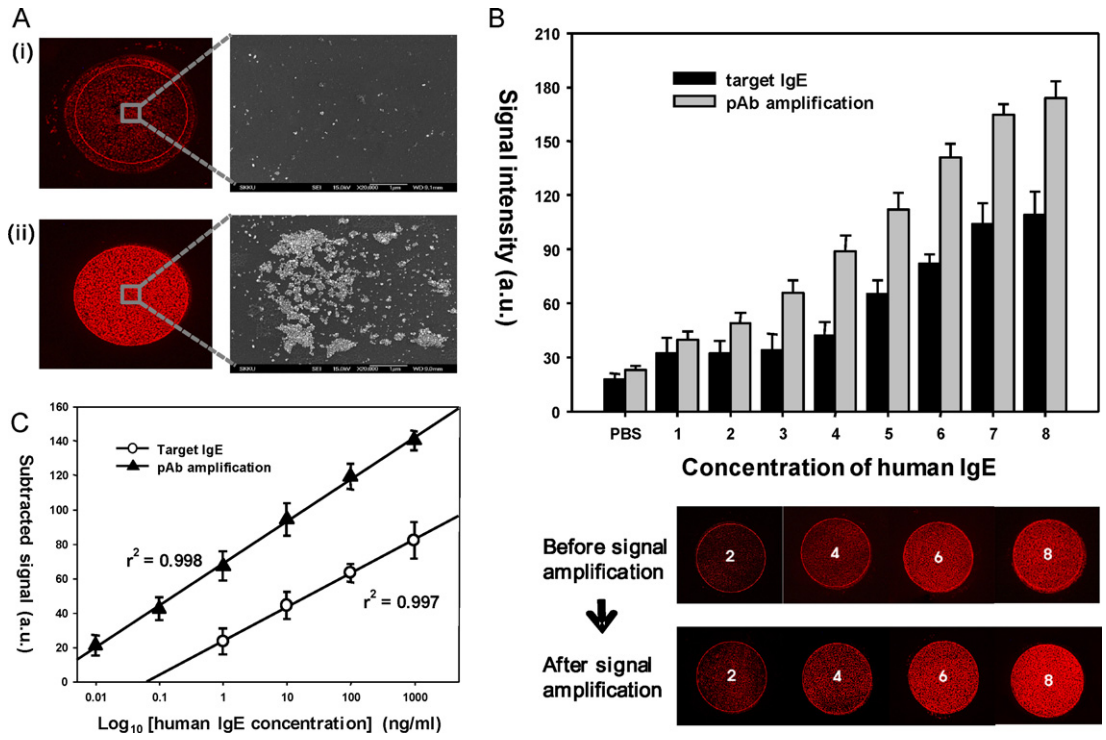

Fig. 2. Fluorescence and SEM images of immobilized PDA spots when the target hIgE, after aptamer immobilization, were conjugated on the PDA liposomes (i) without ethylenediamine cross-linking and (ii) with cross-linking. (B) Diagram representing the fluorescent signals with primary response (black bars) and enhanced response (gray bars) and fluorescent microscopy images after a chip incubation for 30 min at 37 °C. (C) A linear range for the target hIgE obtained from the fluorescence signals of the anti-hIgE aptamer modified-PDA liposome. (Error bars illustrate relative standard deviation (R.S.D.) for five replicates. The subtracted signal is the resulting value from the output signal subtracted from the baseline signal derived from the reaction with PBS buffer.)

improve the efficiency of receptor immobilization and reduce the steric hindrances between approaching proteins and receptors.

3.3. Detection of hIgE on an aptamer-modified PDA liposome biosensor with interlinker

The surface of the PDA liposomes was functionalized with amine-terminated aptamers, very small artificial oligonucleotides (approximately 2 nm). The optimal concentration of the anti-hIgE aptamer on the PDA liposome was 10 nM, as the fluorescence signal indicated the highest and most efficient responses at an anti-hIgE aptamer concentration of 10 nM (data not shown).

After the PDA liposome was conjugated with the anti-hIgE aptamer, their fluorescence signals were monitored with the target hIgE solution at concentration between 0.001 ng/ml and 10 μg/ml in PBS buffer. The black-colored bars in Fig. 2(B) show the fluorescent intensity and images of PDA vesicle spots after interaction between the target hIgE and anti-hIgE aptamer. In this case, the fluorescent signals increased stepwise upon contact with hIgE at concentrations increasing from 1 to 1000 ng/ml. However, the analytes of concentrations below 0.1 ng/ml could not be determined. At a concentration of 10 μg/ml, the change in the fluorescent signal was not much greater than that of the 1.0 μg/ml hIgE analyte. The linear regression equation for hIgE detection by the interaction of hIgE and anti-hIgE aptamer was: $y = 19.6x + 23.6$ ($R^2 = 0.997$), where y and x are the fluorescent signals of the PDA liposome and analyte (hIgE) concentration (ng/ml), respectively. Thus, as shown in Fig. 2(C), the linear dynamic range was demonstrated from 1.0 ng/ml to 1000 ng/ml and the detectable minimum concentration was 1 ng/ml in an aptamer-modified PDA liposome biosensor. The fluorescence signal derived from the anti-hIgE aptamer-hIgE reaction was calculated by subtracting the base signal (fluorescence induced by buffer exposure) from the fluorescence signal induced by the samples, which implied that the lower limit of hIgE detection was below 0.1 ng/ml.

Since allergic patients show high total serum hIgE (greater than 290 ng/ml), only this aptamer-modified PDA liposome system by primary response is feasible for real detection of hIgE. However, this aptamer-based PDA liposome system could detect hIgE at far lower concentrations by a secondary response.

3.4. Fluorescent signal amplification by polyclonal hIgE antibody on an aptamer-modified PDA liposome biosensor with interlinker

In the case herein, in order to accomplish ultrasensitive detection of proteins with an aptamer-modified PDA biosensor, polyclonal hIgE antibody (hIgE pAb) as an external mechanical force for the inducement of secondary response was employed. After the reaction between target hIgE and anti-hIgE aptamer-modified PDA liposome occurred, hIgE pAb (1.0 μg/ml in PBS buffer) was dropped onto the PDA liposome spots for signal amplification. In the case of enhanced signal, the fluorescent signals also increased stepwise upon contact with hIgE concentrations increasing from 0.01 to 1000 ng/ml. However, the analytes at lower concentrations (below 0.001 ng/ml) were not determined as in the case of primary response. At a concentration of 10 μg/ml, the change in the fluorescent signal was not much greater than that of 1 μg/ml hIgE analyte in the primary response. At this time, a linear regression equation for the enhanced response was calculated as: $y = 24.37x + 68.314$ ($R^2 = 0.983$), as shown in Fig. 2(C). In the case of the enhanced response, the linear dynamic range was from 0.01 to 10,000 ng/ml and the detectable minimum concentration was 0.01 ng/ml. These results indicate that the sensitivity and dynamic range of PDA liposome biosensors could be effectively improved by the secondary reaction using hIgE pAb. Therefore, this amplification strategy using a secondary reaction in aptamer-modified PDA biosensors can be

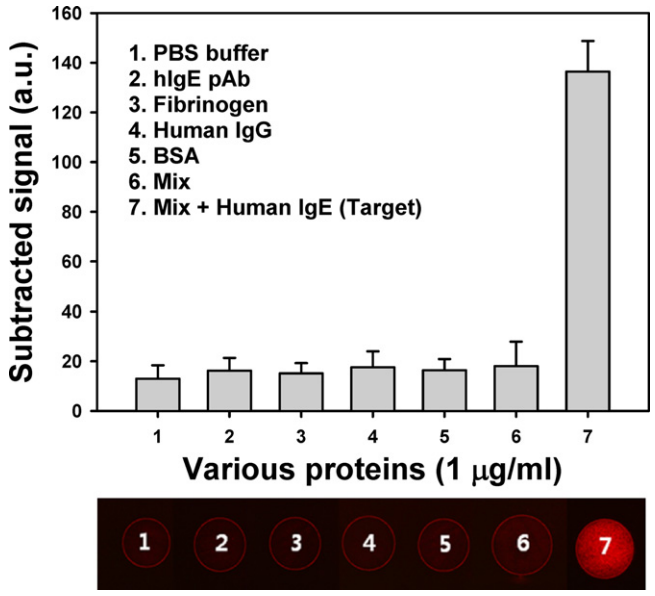

Fig. 3. Specificity and selectivity of the anti-hIgE aptamer for target hIgE detection. The adsorption of fibrinogen, human immunoglobulin G (hIgG), and bovine serum albumin (BSA) was added independently and negligible, as with PBS buffer. (The subtracted signal is the resulting value from the output signal subtracted from the baseline signal derived from the reaction with PBS buffer.)

applied to the detection of various proteins, such as a cancer diagnosis, as well as an allergy diagnosis because this PDA liposome biosensor can detect biomolecules even at very low concentrations.

3.5. Specificity and selectivity of PDA liposome biosensors based on the anti-hIgE aptamers

To investigate non-specific binding of the liposome surface against hIgE pAb, hIgE pAb was injected into the PDA liposome surface prior to reaction of the target proteins. As shown in Fig. 3, without any reaction between the aptamer and target (bar 2), the fluorescent intensity increased insignificantly. This result shows that the adsorption of the hIgE pAb was negligible, even at very high concentrations (1.0 μg/ml). This indicates that this amplification strategy can be applied as a valuable tool for detection of low concentrations of the PDA liposome biosensor.

Finally, an aptamer-modified PDA liposome biosensor was examined in a mixed solution containing BSA, fibrinogen, and hIgG to investigate nonspecific absorption and selectivity of target proteins. These undesired proteins (1.0 μg/ml) and PBS buffer (pH 7.4) were respectively introduced onto the aptamer-conjugated PDA liposome spots to check for nonspecific binding of the chip. Moreover, in order to investigate selectivity of the assay, the experiment was performed by mixing solutions of various proteins (1.0 μg/ml, respectively), target IgE (1.0 μg/ml), and PBS buffer (pH 7.4). As shown in Fig. 3, after the undesired proteins were injected onto the PDA liposome surface, the fluorescent intensities of all proteins slightly increased, similar to the case of only PBS buffer. These results show that nonspecific adsorption of the undesired proteins was negligible, even at very high concentrations. Interestingly, the fluorescent intensity was highly increased in the mixture solution with hIgE target proteins, indicating that the target protein can be selectively detected at low levels in the presence of high concentrations of non-target proteins by aptamer-modified PDA liposome biosensors.

As a result, sensitivity as low as 0.01 ng/ml for detection of hIgE was obtained for the PDA liposome biosensor. In this way, a sensitivity that one hundred times lower than that of the general detection method without signal amplification could be attained. This strategy is capable of performing ultrasensitive quantitative and qualitative analyses of biomolecules.

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