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BSA as additive: A simple strategy for practical applications of PNA in bioanalysis

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ABSTRACT

Application of peptide nucleic acid (PNA) in bioanalysis has been limited due to its nonspecific adsorption onto hydrophobic surface in spite of favorable properties such as higher chemical/biological stability, specificity and binding affinity towards target nucleic acids compared to natural nucleic acid probes. Herein, we employed BSA in PNA application to enhance the stability of PNA in hydrophobic containers and improve the sensing performance of the DNA sensor based on graphene oxide (GO) and PNA. Addition of 0.01% BSA in a PNA solution effectively prevented the adsorption of PNA on hydrophobic surface and increased the portion of the effective PNA strands for target binding without interfering duplex formation with a complementary target sequence. In the GO based biosensor using PNA, BSA interrupted the unfavorable adsorption of PNA/DNA duplex on GO surface, while allowing the adsorption of ssPNA, resulting in improvement of the performance of the DNA sensor system by reducing the detection limit by 90-folds.

1. Introduction

Peptide nucleic acid (PNA) is a DNA mimic composed of natural nucleobases and pseudo-peptide backbone (Hyrup and Nielsen et al. 1996; Ray and Norden, 2000). Being a polyamide based molecule, PNA exhibits high thermal/chemical stability as well as resistance to nuclease-mediated degradation. In addition, the electrically neutral backbone enables PNA to hybridize with complementary DNA or RNA with high affinity and specificity. Therefore, PNA can be employed as not only a potent tool for molecular biology and genetic diagnostics, but also a powerful anti-sense drug candidate. Recently, many researchers demonstrated various nanomaterial based biosensors in combination with PNA (Gaylord et al., 2005; Ryoo et al., 2013; Soleymani et al., 2009; Zhang et al., 2008). For example, a graphene oxide (GO) based sensing system using PNA probe was developed for target specific bio-detection (Kotikam et al., 2012; Guo et al., 2013), enzyme activity assay (Park et al., 2013), duplex nucleic acid detection (Lee et al., 2014) as well as live cell imaging of biomolecules (Ryoo et al., 2013). However, despite of a lot of merits of PNA, its applications have been limited by its poor solubility in aqueous solution which induces self-aggregation or nonspecific adsorption on hydrophobic solid supports that are commonly used in biological applications such as polystyrene (PS) plates and plastic tubes (Ray and Norden, 2000; Tackett et al., 2002). Generally, a mixed solution of PNA and solvent should be heated over 60 °C to dissolve PNA to make a stock solution. PNA at nanomolar concentrations prepared for working solutions easily loses its function since the PNA in diluted solutions get adsorbed on vessel walls over time. Thus, to enhance the solubility of PNA and prevent self-aggregation, many approaches have been developed such as direct backbone modifications or conjugation of solubility enhancers utilizing charged amino acids, hydrophilic linkers or natural nucleic acids (Efimov et al., 1998; Gildea et al., 1998; Kuwahara et al., 1999; Sahu et al., 2011). Moreover, organic solvents such as dimethylformamide and dimethyl sulfoxide have been used for dissolving PNA (Lateef et al., 2007; Narita et al., 1984; Uhlmann et al., 1998). However, apart from the self-aggregation of PNA, nonspecific adsorption of PNA on hydrophobic support has been relatively overlooked during PNA applications and could not be adequately overcome with structural modifications of PNA or usage of organic solvents. In addition, the excessive modifications or the high volume ratio of organic solvents could induce poor

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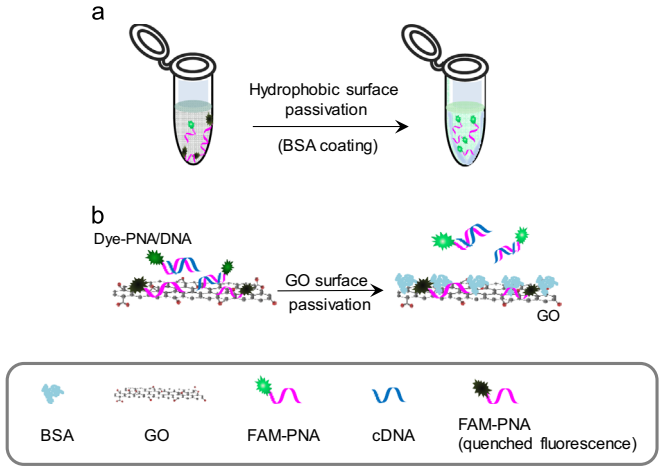

Scheme 1. BSA blocking to (a) prevent the nonspecific adsorption of PNA on hydrophobic surface and (b) inhibit the adsorption of PNA/DNA duplex on GO surface in GO-based biosensing platform using PNA probe.

performance of PNA and other side effects in biological experiments (Abibi et al., 2004; Sen and Nielsen, 2006).

Here, we employed bovine serum albumin (BSA) as an additive to prevent the nonspecific adsorption of PNA on a hydrophobic surface and to enhance its solubility in aqueous media and thus, improved the yield of target-specific hybridization (Scheme 1a). BSA is one of the serum albumin proteins, which is widely used blocking agent due to its high efficiency, cost effectiveness and biocompatibility in various bioanalytical experiments (Brorson, 1997). Because BSA has both hydrophobic and hydrophilic parts on the surface (Brown, 1975; Jeyachandran et al., 2009), it can prevent nonspecific protein–surface interactions or protein–protein interactions in diverse biochemical applications such as ELISA and immunoblottings (Sawasdikosol, 2010; Xiao and Isaacs, 2012). Therefore, the usage of BSA can be a simple, effective method as a remedy to reduce a main shortcoming of PNA. Furthermore, we extended the application of BSA to GO/PNA based oligonucleotide sensors to improve sensing performance by preventing the interaction between PNA/DNA duplex and GO as well as the adsorption of PNA on hydrophobic surface (Scheme 1b).

2. Materials and methods

2.1. Materials

Graphite nanofibers were purchased from Catalytic Materials LLC (USA). Sulfuric acid (H_2SO_4) was purchased from Samchun chemical (Seoul, Korea). Hydrogen peroxide (30% in water) (H_2O_2) was purchased from Junsei (Japan). Potassium permanganate ($KMnO_4$), potassium persulfate ($K_2S_2O_8$) and phosphorus pentoxide (P_2O_5) were purchased from Sigma-Aldrich (MO, USA). Bovine serum albumin (BSA) was purchased from Bovogen Biologicals (Victoria, Australia). DNA strands were purchased from Genotech (Daejon, Korea). Dye labeled peptide nucleic acid (F-PNA) strand (5′-FAM-OO- TTT GAG GGA AGT TAC GCT TAT-3′) was purchased from Panagene (Daejon, Korea). Dye labeled DNA (F-DNA) strand (5′-FAM-TTT GAG GGA AGT TAC GCT TAT-3′), complementary DNA strand (5′-ATA AGC GTA ACT TCC CTC AAA-3′) and scrambled sequenced DNA strand (5′-TAG CTT ATC AGA CTG ATG TTG A-3′) were purchased from Genotech (Daejon, Korea). Microtube was purchased from Axygen (Tewksbury, USA). 96 well black plate was purchased from SPL (Pocheon, Korea). 96 well cell culture plate with the physical surface treatment was purchase from Greiner Bio-One (Frickenhausen, Germany). Fluorescence intensity and fluorescence image were obtained by a fluorometer SynergyMx (Biotek, UK).

2.2. Preparation of graphene oxide

Graphene oxide (GO) was synthesized by using Hummers method with pre-oxidation treatment, using graphite nanofiber as starting material. First, concentrated H_2SO_4 (5 ml) was heated to 80 °C in a round bottomed flask, then $K_2S_2O_8$ (0.15 g) and P_2O_5 (0.15 g) were added in a round bottom flask, keeping at 80 °C for 4.5 h. After the mixture was cooled, the reaction mixture was diluted with deionized (DI) water. This solution was filtered and rinsed with DI water (100 ml) to remove remained reactants. Obtained pre-oxidized graphite nanofibers were dried in air. Then, the dried solid was transferred into a 50 ml round bottom flask and concentrated H_2SO_4 was added (25 ml). Then the mixture was chilled to 0 °C using an ice bath. $KMnO_4$ (1 g) was added to the mixture slowly with stirring, keeping the temperature below 10 °C. After mixing, the flask was placed in a 35 °C water bath for 12 h. The mixture was transferred into an Erlenmeyer flask (500 ml) in an ice bath. DI water (100 ml) was added to the flask slowly with stirring. During this step, keeping the temperature below 55 °C was needed. Then, 5 ml of 30% H_2O_2 solution was added to the mixture, of which the color turned into bright yellow. This mixture was collected, centrifuged and then, rinsed with 3.4% HCl solution and acetone to remove residual salts and acid. Obtained solid GO was dried under vacuum before use. To get the GO solution, GO was dissolved in water, then the solution was centrifuged at 3000 rpm for 30 min to remove large chunks.

2.3. DNA detection using PNA and Graphene Oxide in the presence of BSA

To prepare PNA/GO complex, 20 pmol of PNA strand was incubated with GO 1 μg in 30 μl of PBS (pH 7.2 buffer containing 137 mM NaCl, 2.7 mM KCl) containing 0 or 0.01% BSA for 30 min. Then, 30 μl of various concentrations of DNA target in PBS were added to the mixture. After incubation at room temperature for 2 h, fluorescence intensity was measured at 520 nm.

3. Results and discussion

3.1. BSA as an additive for blocking the nonspecific adsorption of PNA on hydrophobic surface

In the present study, the degree of PNA adsorption on a hydrophobic surface was judged by monitoring changes in the fluorescence emission intensity of the dye-labelled PNA in solution. In the PNA structure, ethylene glycol linker (O), one of the solubility enhancers, was utilized to minimize the possibility of self-aggregation of PNAs as well as to give space between the fluorescein (FAM) moiety and the PNA strand. The 200 nM F-PNA (FAM–OO–TTT GAG GGA AGT TAC GCT TAT) in a phosphate buffered saline (PBS, pH 7.4) was prepared by dilution of a 100 μM F-PNA stock solution. The fluorescence intensity of the diluted F-PNA solution gradually decreased down to ~25% of the original intensity over first 2 h, showing no more significant decrease with additional 3 h of incubation when the solution was incubated in a 96 well black PS plate (Fig. 1a, b). On the other hand, the intensity of F-PNA incubated with 0.01% BSA very slightly decreased for initial 1 h and maintained at ~90% of initial intensity in the same PS plate. As a control, when DNA probe was used in place of PNA, ~90% of initial fluorescence intensity was maintained over time regardless of BSA addition (Fig. S1). The degree of fluorescence intensity decrease of F-PNA was dependent on BSA concentration

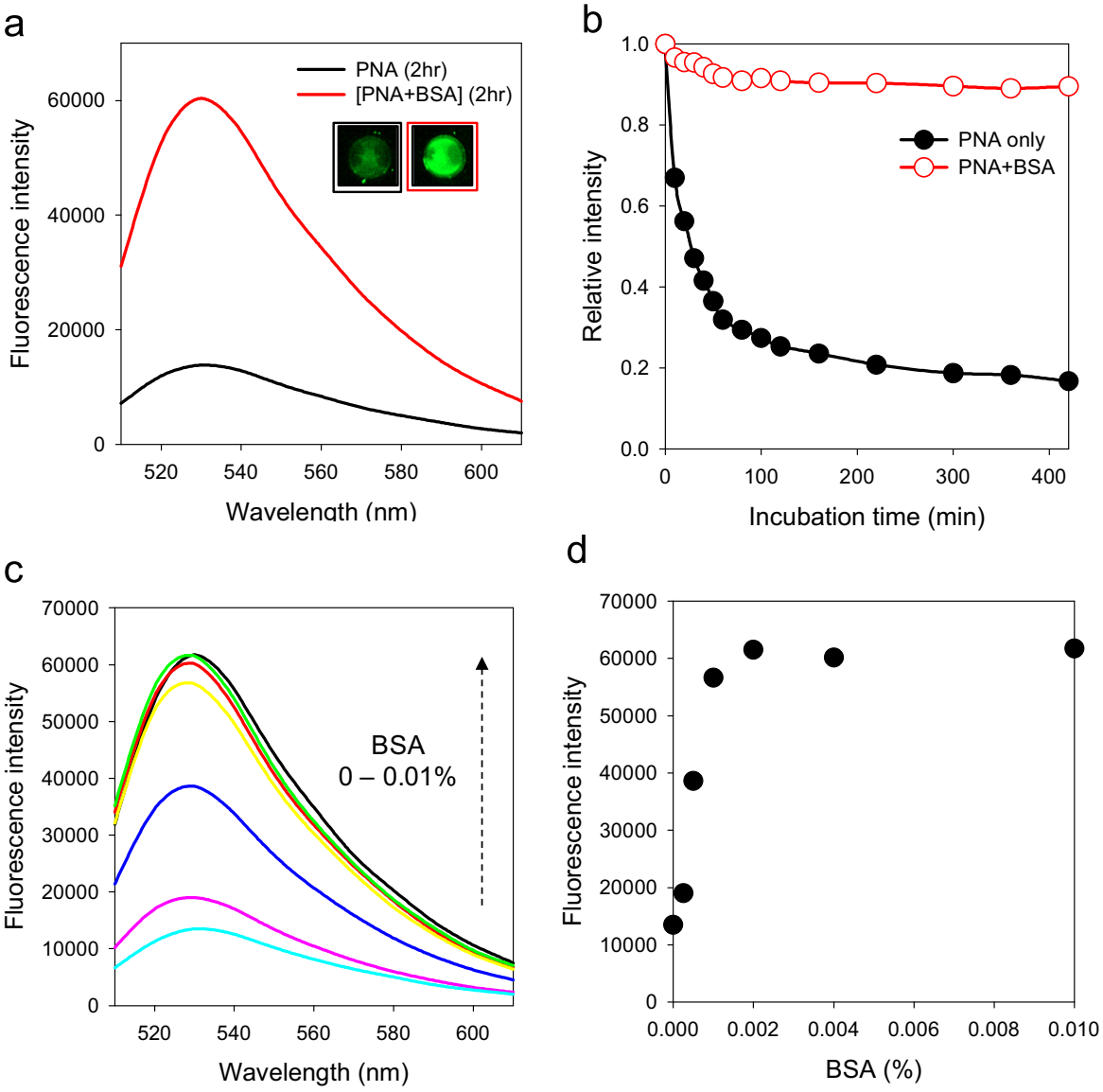

Fig. 1. (a) Fluorescence spectra and (b) time-dependent relative fluorescence intensity of F-PNA in buffered solutions containing 0 and 0.01% BSA. Fluorescence spectra of F-PNA in a solution (c) fluorescence spectra and (d) intensity of F-PNA in presence of various concentration of BSA.

and maximum prevention of nonspecific adsorption was observed above as little as 0.002% BSA (Fig. 1c, d). The decay of fluorescence intensity of F-PNA solution was accompanied by slight red-shift of emission spectrum, suggesting that the decline of intensity could be due to the aggregation of F-PNA on a container surface by adsorption (Fig. S2).

To investigate the adsorption of F-PNA on the surface, we compared the fluorescence signal from the container wells (96 well black plate) before and after removing the F-PNA solution following 3 h incubation. In the case of F-PNA solution omitting BSA, the similar fluorescence intensity to the incubated solution was measured in the vacant container even after transferring out the solution and several washing. However, F-PNA solutions with BSA showed much lower fluorescence intensity from the empty container, BSA concentration dependently, after removing the solution. The tendency was also correlated with surface coverage of BSA on a PS surface characterized by AFM (Fig. S3). The result indicated that the nonspecific adsorption of PNA on hydrophobic surface can be prevented by BSA blocking. Furthermore, a post-addition of BSA to the adsorbed F-PNA on the hydrophobic support resulted in the gradual recovery of fluorescence intensity until the equilibrium was reached, indicating that BSA could induce desorption of the F-PNA from a solid surface into the solution as well as the inhibition of the adsorption of F-PNA to a solid surface (Fig. S4).

Next, we utilized GO for identifying how much percentage of PNA strands were available as a probe in a hydrophobic container in the presence of BSA. GO, a water-dispersible graphene derivative, having a hexagonal lattice of sp2 carbons and oxygen containing functional groups was prepared by using a modified Hummers method (Fig. S5) (Allen et al., 2010; Cote et al., 2009; Kim et al., 2010; Zhu et al., 2010). GO has high affinity to hydrophobic molecules as well as efficient fluorescence quenching of dyes near its surface, which have been employed in various bioanalytical applications (Kotikam et al., 2012; Lee et al., 2011; Li et al., 2013; Lu et al., 2009; Pu et al., 2011; Zhang et al., 2011a; Zhang et al., 2011b). Thus, the nucleobases of single stranded PNA (ssPNA) can be adsorbed on the GO surface, inducing the fluorescence quenching of the dye conjugated to PNA. Upon the addition of GO to F-PNA solutions incubated in a hydrophobic container without and with 0.01% BSA showed the decrease in the fluorescence intensity, corresponding to 18 and 91% of the initial

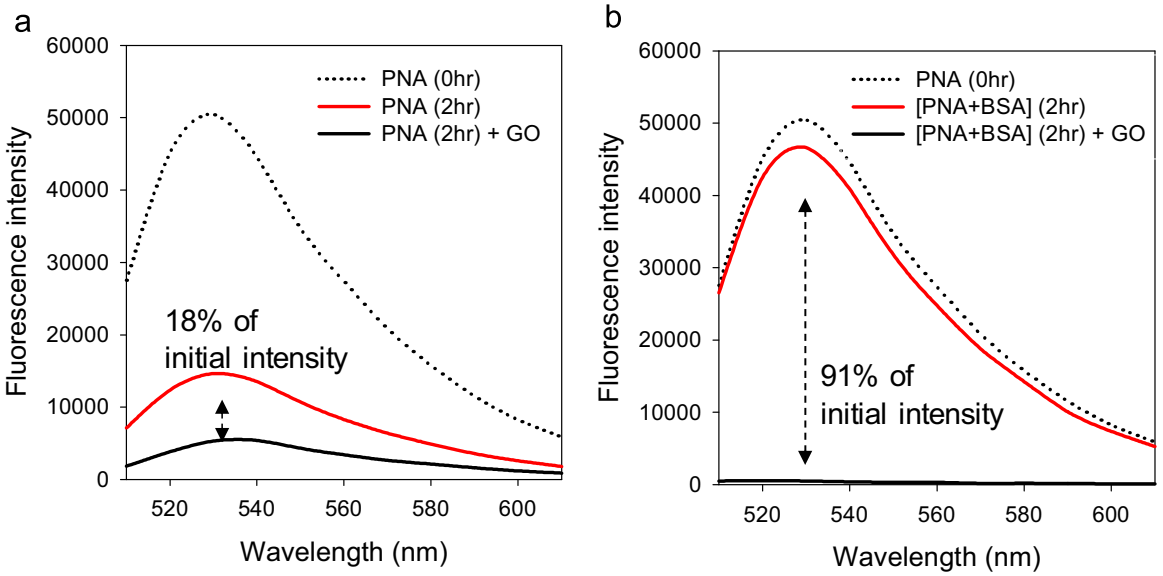

Fig. 2. Fluorescence spectra of F-PNA (a) in buffered solution and (b) in BSA containing buffered solution, in the presence of GO.

intensity of F-PNA, respectively (Fig. 2). We could assume that each percentage represented the proportion of ssPNA in each solution that was able to interact with GO, exhibiting exposed bases, and that the PNA adsorbed on the hydrophobic surface cannot function as a probe for hybridization. To examine capability of F-PNA for sequence specific hybridization with target nucleic acid, we next performed an experiment in which equal molar amount of complementary DNA (cDNA) was added before and after F-PNA incubation in a hydrophobic container. The pre-hybridized F-PNA/DNA duplex was highly soluble due to the polyanionic backbone of DNA and maintained the almost uniform fluorescence intensity, which was nearly identical with F-PNA solution containing 0.01% BSA. However, the F-PNA solution that was incubated in the container for 2 h, followed by the addition of cDNA, showed much lower fluorescence intensity compared to the pre-hybridized F-PNA/DNA duplex (Fig. S6). These results suggested that the adsorbed F-PNA on a hydrophobic support cannot play a role as a probe for hybridization with complementary target sequence.

To further investigate the problems occurred by nonspecific adsorption of PNA in biosensor application, we carried out a dsDNA detection using GO and PNA probe in the presence of BSA on the basis of end invasion of PNA towards dsDNA (Fig. S7) (Lee et.al., 2014). In this method, a dye labeled PNA probe was incubated with target dsDNA for the formation of PNA/DNA duplex, followed by GO addition for the adsorption of excess ssPNA and subsequent fluorescence quenching of the dye conjugated to PNA. In contrast to BSA containing samples, BSA free sample showed a high background signal and target concentration independent F/F0 values, presenting the unfeasibility of dsDNA detection without BSA. This result indicated that nonspecifically adsorbed dye-labeled PNA could give high background signal in a fluorometric biosensing platform.

We next performed an experiment to reveal BSA effect on the adsorption of F-PNA on a hydrophobic solid surface by varying the concentration of F-PNA. Lower concentration of F-PNA showed more significant decreases in fluorescence intensities after incubation in the absence of BSA. It indicated that the portion of adsorbed F-PNA was higher with low concentration of F-PNA on

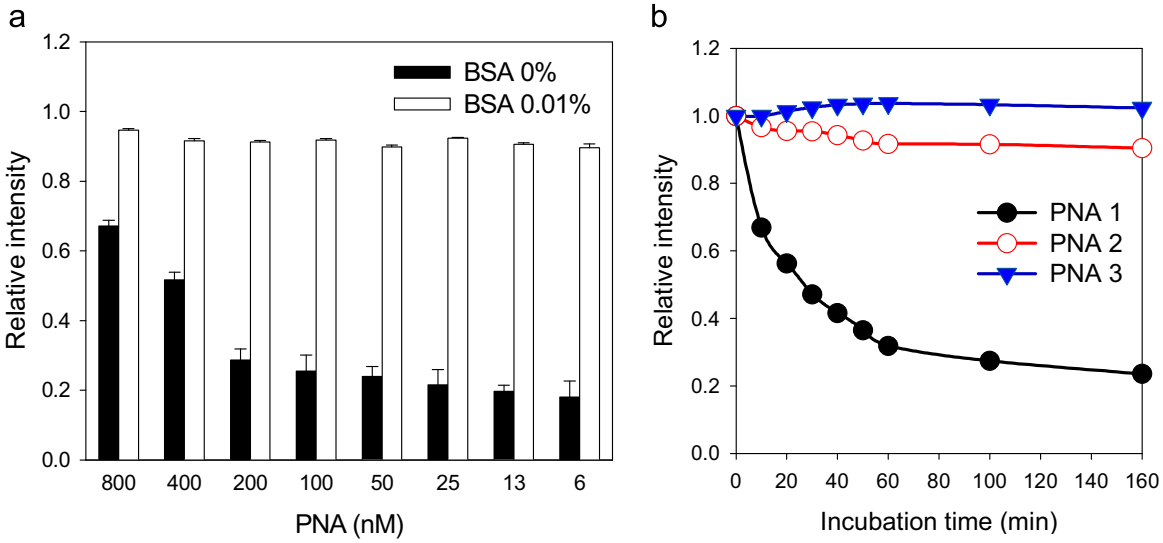

Fig. 3. (a) Relative fluorescence intensity (F_0 min = 1) of various concentrations of F-PNA with and without 0.01% BSA. (b) Time dependent fluorescence intensity of three types of PNAs (20 nM), PNA1: diluted from a 20 μM PNA stock, PNA2: diluted from a 20 μM PNA stock, followed by addition of BSA, PNA3: diluted from 20 μM PNA stock containing 0.5% BSA.

the hydrophobic support probably due to the relatively larger surface contact area per PNA. However, the F-PNA solution containing BSA maintained the fluorescence signal over 80% of initial intensity regardless of F-PNA concentration (Fig. 3a). Because the concentration of F-PNA probes used in actual experiments is usually in nanomolar range, simply adding 0.01% BSA to a PNA solution can be very useful to various F-PNA applications by preventing the adsorption of PNA on a hydrophobic support. To be practical, it will be more convenient if a concentrated stock solution of PNA is made as a mixture with BSA at a proper concentration and used directly for dilution to make each working solution since additional pipetting of BSA solution for each sample every time could be cumbersome and inefficient. We found that the 100-fold diluted F-PNA solution from the 20 μM F-PNA stock solution containing 0.5% BSA exhibited a constant fluorescence intensity without notable decrease in fluorescence intensity (Fig. 3b). We then investigated the sequence-specific target DNA binding ability of F-PNA probe with varying concentrations of BSA. The PNA/DNA duplex was first prepared by mixing equal molar amount of both strands in a buffered solution containing various concentrations of BSA. The duplex was characterized by using non-denaturing polyacrylamide gel electrophoresis (native-PAGE). Due to less negative charge of F-PNA/DNA duplex compared to DNA/DNA duplex, the band of hetero duplex appeared at upper region showing slower migration than DNA duplex in the gel. The functionality of F-PNA wasn't affected by BSA even though the concentration of BSA was raised up to 1% in F-PNA solution (Fig. S8a). The co-incubation of F-PNA with BSA in a stock solution was similarly effective in preventing F-PNA adsorption with the typical blocking method in that a high concentration of BSA is treated on the support and rinsed away before assay. Both of the methods allowed much higher fluorescence of F-PNA compared to F-PNA solution without BSA, regardless of the type of surface materials (Fig. S8b). Taken together, we found that BSA in a PNA stock solution can play an important role in a simple and practical usage of PNA.

3.2. BSA as an additive in nucleic acid sensing platform using PNA and GO

We extended the application of BSA blocking to GO based sensing system using PNA. The fluorometric biosensing system was built on the preferential binding of GO to ssPNA over PNA/DNA duplex and the fluorescence quenching capability of GO. In case of the duplex, the nucleobases are shielded within negatively-charged DNA backbone and thus, they existed preferentially apart from GO. Compared to original GO based sensing system using DNA probe, PNA/GO based sensing systems showed improved sensing performance and suitability for more practical purposes such as target sensing in live cells, quantitative assay of polymerase activity and sequence specific detection of dsDNA, which were very difficult to achieve by using DNA probe (Guo et al., 2013; Kotikam et al., 2012; Park et al., 2013; Ryoo et al., 2013). However, unfavorable experimental conditions such as very high concentration of GO (Zhao, 2011) or presence of divalent metal ions (Lei et al., 2011; Yun et al., 2012) can cause the nonspecific adsorption of the duplex on GO surface. This phenomenon makes the increase of the fluorescence intensity smaller associated with fluorescence recovery of probe by forming a duplex with target relative to the quenched fluorescence intensity of probe by GO. As a result, the target responsive fluorescence signal gets lower, resulting in poor performance as a sensor. Therefore, it is necessary to minimize the adsorption of the duplex on GO as well as adsorption of PNA on a hydrophobic support for better efficiency. Because GO is known to have affinity for various proteins including BSA due to its amphiphilicity (Cote et al., 2011; Liu et al., 2010), we applied BSA in GO/PNA sensing system to prevent the adsorption of the PNA/DNA duplex on GO (Scheme 1b).

The interaction of BSA on GO was first confirmed by using isothermal titration calorimetry (ITC) analysis (Fig. S9). We then compared the adsorption of ssPNA and PNA/DNA duplex onto GO surface in the presence of BSA to verify the selective blocking of the adsorption of PNA/DNA duplex. We measured the degree of fluorescence quenching of F-PNA and F-PNA/DNA duplex with varying GO concentrations in the absence of and in the presence of 0.01% BSA. The concentration of BSA which maximized the FPNA/DNA/FPNA ratio was determined as 0.01% (Fig. S10). In case of 10 pmol F-PNA, 0.10 and 0.13 μg of GO were required to quench the 50% fluorescence signal in a buffered solution without BSA and with BSA, respectively. However, F-PNA/DNA duplex (10 pmol) required 3.9 μg of GO for quenching the 50% fluorescence signal in a BSA containing buffered solution whereas 1.0 μg of GO was needed in a buffered solution omitting BSA (Fig. 4a). It is notable that BSA in PNA/GO based sensor prevents the adsorption of PNA/DNA duplex on GO surface while it has little effect on the interaction between ssPNA and GO surface. We then calculated the FPNA/DNA/FPNA at varying concentrations of GO to confirm the improved efficiency of the GO based sensor by BSA. When BSA was added in the PNA/GO mixture, the histogram of FPNA/DNA/FPNA shifted toward slightly higher GO concentration. However, meaningful values of FPNA/DNA/FPNA were obtained in much broader range of GO and a higher maximum value of FPNA/DNA/FPNA was observed in the presence of 0.01% BSA, indicating that BSA enabled the sensor to detect the target DNA with higher sensitivity in wider range of GO concentrations (Fig. 4b).

The BSA effect for selective blocking of GO could also be verified by elevating the concentration of metal ions in sensing condition. Divalent cations such as Mg^{2+} and Ca^{2+} required for various biofunctions generally facilitate the adsorption of PNA/DNA duplex onto the GO surface by neutralizing the electrostatic repulsion between the duplexes and GO. However, addition of BSA to GO in a solution containing Mg^{2+} prevented the adsorption of the duplex on GO and thus, the fluorescence of the dye conjugated to PNA did not diminish over time even under high concentrations of Mg^{2+} (Fig. 4c, Fig. S11). Next, we carried out DNA detection in a buffered solution containing 2.5 mM $MgCl_2$ in the presence of BSA. Under the condition, the sensor with BSA exhibited a detection limit of 0.5 nM according to the equation, LOD = 3.3 (SD/S), which was 90-fold lower than that of the conventional PNA/GO based DNA sensing system omitting BSA of which detection limit was 45.2 nM (Fig. 4d, Fig. S12).

4. Conclusion

In summary, BSA was employed in PNA application to enhance the stability of PNA in hydrophobic containers and improve the sensing performance of GO/PNA based DNA sensor. Although PNA has favorable properties such as higher chemical/biological stability, specificity and binding affinity for target nucleic acids compared to natural nucleic acid probes, its application has been limited due to nonspecific adsorption on hydrophobic surface. By incorporating 0.01% BSA in a PNA solution, the adsorption of PNA on hydrophobic surface was easily prevented and the portion of the effective PNA strands for target binding could be increased without any disturbance on hybridization with a complementary target. Moreover, high concentration of BSA in a PNA stock showed same effect in preventing the nonspecific adsorption during the usage of diluted PNA stock. Also, in the GO based biosensor using PNA, BSA interrupted the unfavorable adsorption of PNA/DNA duplex on GO surface, while allowing the adsorption of ssPNA. The selective blocking on GO can improve the performance of the

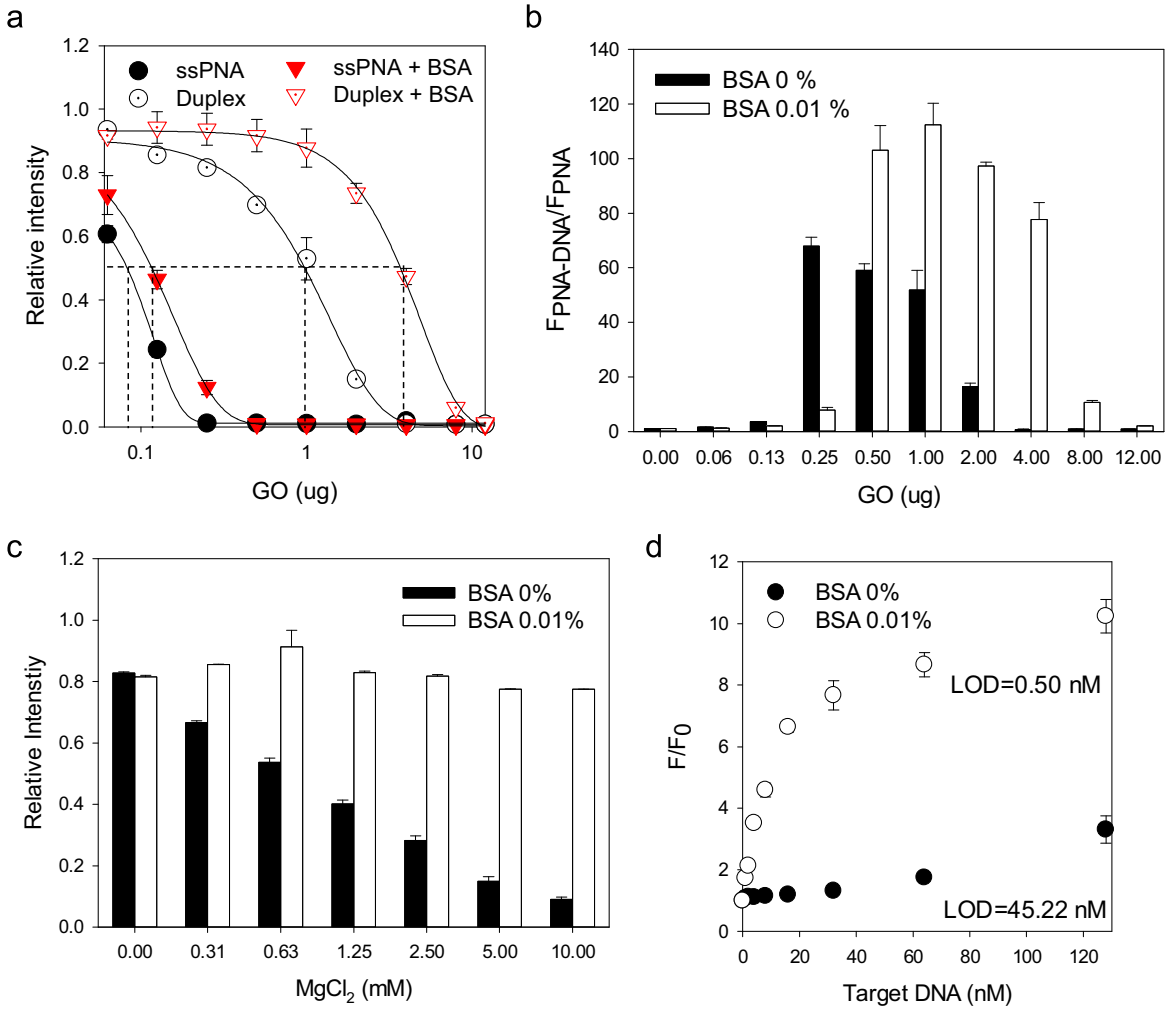

Fig. 4. (a) GO concentration-dependent fluorescence intensity of F-PNA and F-PNA/DNA duplex in a buffered solution with or without 0.01% BSA. (b) GO concentration-dependent FPNA-DNA/FPNA. (c) Mg^{2+} concentration-dependent relative fluorescence intensity of F-PNA/DNA duplex in the presence of GO. (d) Fluorescence recovery of F-PNA/GO upon addition of target DNA in a buffered solution containing 2.5 mM Mg^{2+} and BSA.

system by reducing the detection limit by 90-folds. This work is the first to utilize BSA for high yield of available PNA in its application by preventing the nonspecific adsorption and for signal enhancement in GO/PNA based sensor system. We believe that the present study will provide great opportunity to widen PNA application as useful tools in basic life science and biomedical research as well as therapeutics in gene therapy.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2015.02.030.

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