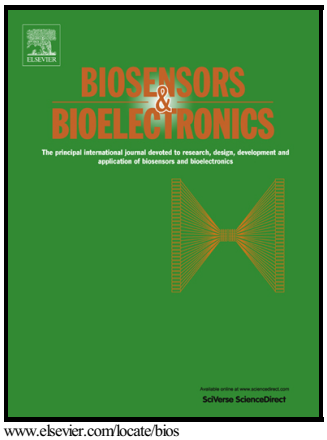

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Jieon Lee, Il-Soo Park, Euihan Jung, Younghoon Lee, Dal-Hee Min

PII: S0956-5663(14)00441-2
DOI: http://dx.doi.org/10.1016/j.bios.2014.06.028
Reference: BIOS6862

To appear in: *Biosensors and Bioelectronic*

Received date: 11 April 2014
Revised date: 6 June 2014
Accepted date: 15 June 2014

Cite this article as: Jieon Lee, Il-Soo Park, Euihan Jung, Younghoon Lee and Dal-Hee Min, Direct, sequence-specific detection of dsDNA based on peptide nucleic acid and graphene oxide without Requiring denaturation, *Biosensors and Bioelectronic,* http://dx.doi.org/10.1016/j.bios.2014.06.028

Direct, Sequence-Specific Detection of dsDNA Based on Peptide Nucleic Acid and Graphene Oxide without Requiring Denaturation

Jieon Lee [a,b], *Il-Soo Park* [a,b], *Euihan Jung* [c], *Younghoon Lee* [c], *Dal-Hee Min** [a,b]

a Center for RNA Research, Institute for Basic Science, Seoul National University, Seoul, 151-747, Korea.

b Department of Chemistry, Seoul National University, Seoul, 151-747, Korea.

c Department of Chemistry, Korea Advanced Institute of Science and Technology (KAIST), Daejeon, 305-701, Korea.

Corresponding author: Prof. Dal-Hee Min

Fax:+82-2-889-1568, Tel:+82-2-880-4338

e-mail: dalheemin@snu.ac.kr

ABSTRACT

Sequence-specific detection of double stranded DNA (dsDNA) is important in various research fields. In general, denaturation of dsDNA into single strands is necessary for the sequence–specific recognition of probes to target DNA, posing several drawbacks which decrease the efficiency as a DNA sensor. Herein, we report a direct, sequence-specific dsDNA detection system without requiring any thermal denaturing step. Our strategy utilizes peptide nucleic acid (PNA) and graphene oxide (GO) as a probe and as a fluorescence quencher, respectively. The PNA first binds to the end of dsDNA strand due to the relatively easily dissociable terminal base pairs of DNA duplex. Next, superior binding affinity of PNA towards complementary DNA induces branch migration for gradual strand replacement, resulting in the formation of PNA/DNA duplex. Unlike other dsDNA sensors based on complementary DNA probes, PNA in combination with GO enabled hybridization with the target sequence hidden as a duplex form without denaturing step and thus, the formation of PNA/DNA duplex was translated into selective fluorescence signal. Moreover, it provided tighter turn-on signal control with very low background signal and high sensitivity and sequence selectivity even in the presence of serum proteins.

KEYWORDS: biosensor, DNA detection, DNA duplex, FRET, graphene oxide, peptide nucleic acid

1. Introduction

DNA detection is important in diagnosis and monitoring the fatal infections caused by viruses such as hepatitis virus, human immunodeficiency virus (HIV), Epstein–Barr virus (EBV) and human papillomavirus (HPV) as well as diseases associated with genetic alterations (Avettand-Fenol et al., 2009; Chu et al., 1998; Dejean et al., 1984; Manos et al., 1999). Conventional DNA detection methods, such as southern blot (Southern, 1975), fluorescence in situ hybridization (FISH) (Levsky and Singer, 2003), polymerase chain reaction (PCR) (Chen et al., 2002) and DNA microarray (Heller, 2002; Hatakeyama et al., 2011) are based on the sequence specific recognition of single stranded nucleic acid through Watson-Crick base pairing. Recently, alternative approaches based on nanomaterials have been developed for simple and fast DNA detection (Elghanian et al., 1997; Lu et al., 2009; Song et al., 2010).

In general, denaturing procedure is necessary to separate two DNA strands prior to sensing of the target DNA by hybridization with probe molecule or sequence because target DNA usually exists in duplex form with complementary strand. In such a case, spontaneous reannealing of original two DNA strands could disturb the hybridization of the target DNA with probe and lower the efficiency of target detection. Recently, several approaches that do not require denaturation and/or rehybridization have been proposed. These direct detection methods utilized double-stranded DNA (dsDNA) binding probes such as zinc finger DNA binding proteins (Segal and Barbas III, 2001), pyrrole-imidazole polyamides (PAs) (Mrksich et al., 1992; Dervan, 2001) for binding at minor groove of dsDNA and triplex-forming

oligonucleotides (TFOs) (Chan and Glazer, 1997) for Hoogsteen hydrogen bonding at major groove of dsDNA. However, these approaches have drawbacks such as low chemical stability of protein as a probe and limitation in recognition length or sequence. Therefore, it is important to establish a robust, versatile and efficient sensing platform for direct dsDNA detection.

Graphene is a 2D carbon material with remarkable electronic, thermal, and mechanical properties (Novoselov et al., 2004; Allen et al., 2010; Kim et al., 2010). Graphene oxide (GO), a water-soluble derivative of graphene, has been recently harnessed in diverse biological applications such as drug delivery, catalysis, enzyme activity assay and biosensor (Liu et al., 2008; Lu et al., 2009; Jang et al., 2010; Lee et al., 2010; Zhu et al., 2010; Pyun, 2011; Chung et al., 2013; Kim et al., 2013; Na et al., 2013;). General strategy in these applications relies on the strong binding of single stranded nucleic acid and/or hydrophobic molecules with GO through pi-pi stacking and/or hydrogen bonding interactions and the fluorescence-quenching capability of GO (Swathi and Sebastian, 2008a, 2009b; He et al., 2010; Wu et al., 2011).

Here, we report a direct dsDNA detection method based on the end invasion of peptide nucleic acid (PNA) towards dsDNA and preferential binding of GO to single-stranded PNA (ssPNA) over DNA/PNA duplex. PNA is a DNA analogue which has amide bonds instead of phosphate backbone (Nielsen et al., 1991; Hyrup and Nielsen, 1996). It hybridizes with complementary DNA or RNA with high sequence specificity in short time. The duplex of PNA with natural nucleic acid has high stability in various buffered conditions and at high temperature, basically due to the uncharged neutral backbone of PNA. Typically, DNA/PNA duplex shows at least 1°C higher melting temperature per base pair than DNA duplex (Jang et al., 2010). In addition to its widely known favorable properties as a biological probe, PNA

could also interact with negatively charged GO stronger than negatively charged nucleic acids and construct stable PNA/GO complex, showing lower background signals due to less non-specific desorption of probe nucleic acid from GO (Kotikam et al., 2012; Guo et al., 2013; Ryoo et al., 2013).

The present strategy starts with preparation of a fluorescent dye conjugated PNA probe and a target dsDNA which consists of an upper strand as an actual target DNA for PNA probe recognition and a bottom strand complementary to target DNA. Upon incubation of dsDNA with PNA probe, the PNA binds at the end of upper strand because the hydrogen bonding at the terminal base pair of DNA duplex is weaker than internal one. Due to the superior binding affinity of PNA to complementary DNA, branch migration for strand replacement occurs gradually. Finally, the bottom DNA strand is dissociated from original DNA duplex and PNA/DNA duplex is formed (Lesignoli et al., 2001; Smolina et al., 2005; Kuhn and Frank-Kamenetskii, 2008). Upon addition of GO to this mixture, free PNA probe, not PNA/DNA duplex, preferentially binds onto GO through pi-pi stacking interaction and hydrogen bonding formation, and subsequently, the fluorescence of the dye conjugated PNA probe is quenched by energy transfer to GO. Therefore, only PNAs forming duplexes with target DNA emit the fluorescence signal, and thus, the initial concentration of the target dsDNA could be quantitatively analyzed (Fig. 1a).

2. Material and methods

2.1. Materials. Graphite nanofibers was 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). Fatal bovine serum (FBS) was purchased from Welgene (Daegu, Korea). DNA strands were purchased from Genotech (Daejon, Korea). PNA probes were purchased from Panagene (Daejon, Korea). Fluorescence intensity and fluorescence image were obtained by 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 bottom 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 hours. 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) for removing 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 35 °C water bath for 12 hours. 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. After that, 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 get rid of 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. Detection of dsDNA using Peptide Nucleic Acid and Graphene Oxide. To prepare duplex DNA target, 2.5 μL of 100 μM upper strand DNA was mixed with and a 1.1-fold excess of bottom strand DNA in pH 8.0 buffer containing 50 mM Tris-HCl and 50 mM NaCl. Then, the mixture was annealed by heating to 90℃ for 5 min and followed by slow cooling at room temperature for 1 hr. For dsDNA detection, reaction mixture was prepared by 100 nM FAM labeled PNA probe incubated with various concentration of annealed target dsDNA in 1XPBS (pH 7.2 buffer containing NaCl 137 mM, KCl 2.7 mM). After incubation at room temperature for 1hr, 1 μL of 500 μg/ml GO stock was added to mixture. Fluorescence intensity was measured at 520 nm.

2.4. Multiple target detection using Peptide Nucleic Acid and Graphene Oxide. For multiple target detection, dsDNA target was incubated with the PNA probes mixture containing each 10 pmol of FAM-HVA probe, ROX-HIV probe and Cy5-HVB probe in 1XPBS. After incubation at room temperature for 1hr, 1.25 μL of 500 μg/ml GO stock was added to mixture. FAM-PNA, ROX-PNA and Cy5-PNA were excited at 492 nm, 587 nm and 650 nm in a homogeneous solution. Fluorescence intensities of each probes were measured at 518, 608, and 670 nm, respectively.

3. Results and discussion

Before the demonstration of the present dsDNA sensor based on PNA and GO, the DNA strand replacement from dsDNA by PNA was first examined by resolving the PNA/DNA duplex and intact dsDNA using nondenaturing polyacrylamide gel electrophoresis (native - PAGE). Due to less negative charge of PNA/DNA duplex compared to DNA/DNA

duplex, the band of the hetero duplex appeared at upper region, implying slower migration than DNA duplex in the gel. When the PNA probe was added to dsDNA, PNA was expected to first bind to the end site of dsDNA, followed by branch migration and complete hybridization between PNA and DNA along with separation of bottom strand DNA (Fig. 1b). Next, we determined the optimal GO concentration for quenching the fluorescence of fluorescein amidite (FAM) labeled ssPNA probe (5' - FAM–TTT GAG GGA AGT TAC GCT TAT - 3'). The aqueous suspension of GO was prepared by using a modified Hummers method (Fig. S1) (Luo et al., 2010). We found that the fluorescence of FAM-PNA probe was quenched up to ~98% in the presence of GO (0.5 µg), giving maximum quenching efficiency under the experimental condition (Fig. 1c).

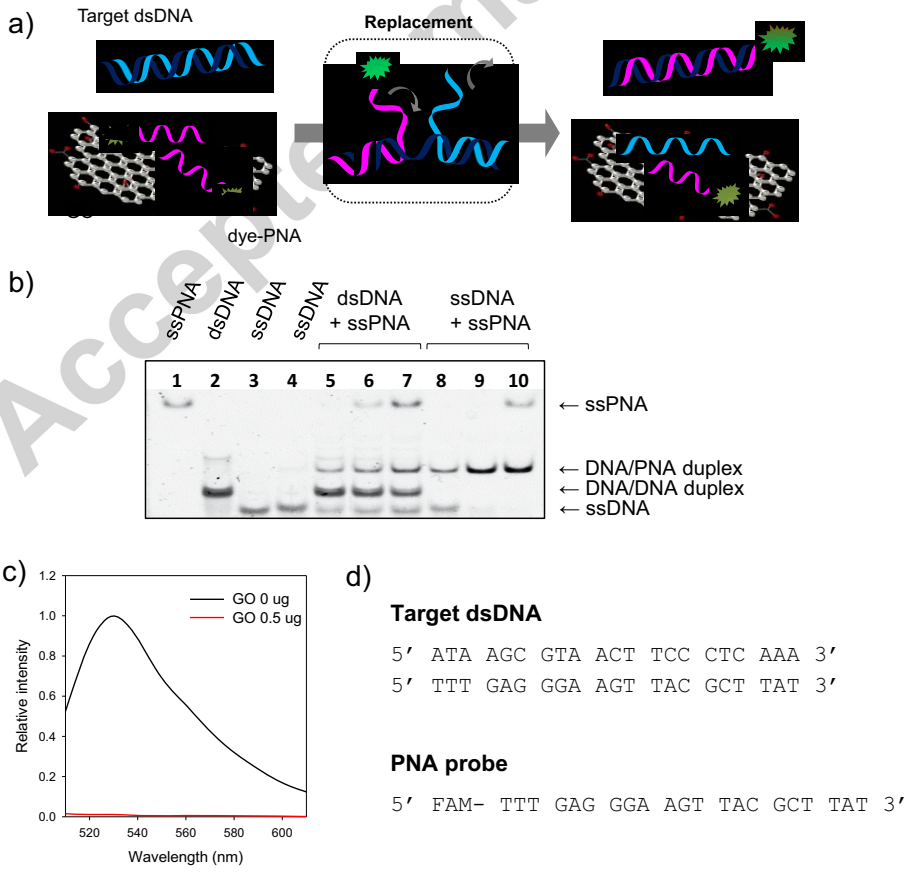

Fig. 1. (a) Scheme of the direct detection of dsDNA using PNA and GO. (b) Gel electrophoresis was performed on a 15% native -PAGE. Lanes 1-4 show each 10 pmol of ssPNA, dsDNA, ssDNA (bottom strand) and ssDNA (upper strand). Lanes 5-7 show 10 pmol of dsDNA incubated with 5, 10, 20 pmol of PNA. Lanes 8-10 show 10 pmol of ssDNA (upper strand) incubated with 5, 10, 20 pmol of PNA. (c) Fluorescence spectra of FAM labeled PNA in the presence of GO (0 μg and 0.5 μg, respectively). (d) Sequences of target dsDNA and FAM labeled PNA probe.

We then performed PNA/GO based fluorometric detection of dsDNA. Various concentrations of target dsDNA solutions ranging from 0 to 400 nM were mixed with FAM-PNA probe and then, GO solution was added. The fluorescence emission spectra of the mixed solutions of dsDNA, FAM-PNA and GO showed the dsDNA concentration dependent fluorescence enhancement with high sequence specificity showing no notable fluorescence intensity from a mixture containing scrambled dsDNA in place of target dsDNA (Fig. 2a). This sensor showed a linear fluorescence increase between 0 and 2000 pM with the detection limit of 260 pM according to the equation, LOD = 3.3 (SD / S) (LOD: limit of detection, SD: standard deviation, S: slope of the calibration curve) (Fig. 2b). As a control, we performed same experiment with FAM-DNA probe in place of FAM-PNA probe. FAM-DNA probe showed significant signal increase upon addition of scrambled DNA followed by addition of GO, which is estimated as ~50% of the fluorescence intensity measured from the mixture of FAM-DNA probe, perfect matched target DNA and GO. The control data suggested that DNA probe is much less attractive for this type of direct, sequence-specific, quantitative detection of dsDNA due to high background fluorescence (Fig. S2). Furthermore, to investigate the applicability for direct sensing of dsDNA in serum containing biological samples, we repeated the dsDNA detection in buffered solutions containing 10% fetal bovine serum (FBS). Under the condition, the sensor also exhibited a linear fluorescence increase in the same

concentration range of target DNA (0 ~ 2000 pM) with a slightly higher detection limit of 400 pM according to the equation, LOD = 3.3 (SD / S) (Fig. S4).

Next, we performed discrimination test between perfect matched target sequence and mismatched DNAs possessing one or three base pair (bp) mismatches. Precise recognition of few base mismatches in DNA sequences having very high sequence similarity is important because it provides information on genetic mutation, DNA damage and single-nucleotide polymorphism (SNP) (Harfe and Jinks-Robertson, 2000; Fedier and Fink, 2004; Barreiro et al., 2008). One and three bp mismatched DNAs were designed based on transversion mismatch at internal bases (A↔T). As expected, the mismatched target DNA showed lower fluorescence intensity than perfect matched target upon addition of FAM-PNA and GO (Fig. 2c). Specifically, single bp mismatch in the middle of the upper target strand induced the decrease of the F/F0 value down to 57% compared to that from the perfect matched target DNA. Furthermore, the F/F0 values decreased to ~35% with three bp mismatches, and down to below ~1% with scrambled dsDNA (in the mixture of 100 nM each target and 100 nM PNA probe) (Fig. 2d).

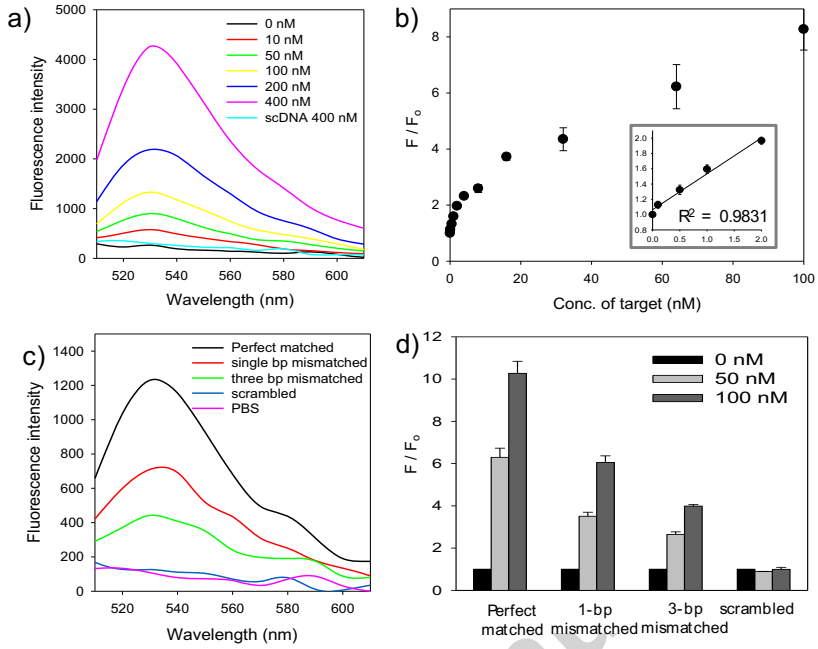

Fig. 2. (a) Fluorescence spectra of FAM-PNA probe in the presence of various concentrations of dsDNA target and scrambled dsDNA 400 nM in buffered solution with addition of GO. (b) Fluorescence intensity enhancement (F/F0) with a wide concentration ranges of target dsDNA from 0 to 100 nM (F: fluorescence intensity of PNA probe hybridized with target, F0: fluorescence intensity of PNA probe without target). Higher concentrations of dsDNA (up to 500 nM) also showed concentration dependent fluorescence increase (Fig. S3). (c) Fluorescence spectra of PNA probe in the presence of target dsDNA, single base pair mismatched, three base pair mismatched dsDNA and scrambled sequence dsDNA with addition of GO. (d) A bar graph showing fluorescence intensity enhancement (F/F0) with matched, mismatched dsDNA target (0, 50, 100 nM, respectively).

Finally, we demonstrated the capability of multiple target detection in a single solution. We chose three target sequences related to viral diseases for multiplex sensing: hepatitis A virus Vall7 polyprotein gene (HVA), HIV and hepatitis B virus surface antigen gene (HVB). Target dsDNAs were prepared by annealing each unique 15 nucleotide sequences of the viruses with complementary strands and PNA probes were conjugated with three different fluorescent dyes—FAM, 6-carboxyl-X-rhodamine (ROX) and cyanine 5 (Cy5)

which showed emission maxima of 518 (green), 608 (orange) and 670 nm (red) with excitation at 492, 587 and 650 nm, respectively. For multiple dsDNA detection, each target dsDNA or different combinations of three dsDNA (200 nM) were added to the mixture of the three dye labelled PNA probes (each concentration=100 nM), followed by addition of GO (Fig. S5). As expected, green, orange and red fluorescence emission spectra and images were obtained only in the sample containing each corresponding HVA, HIV and HVB target dsDNA (Fig. 3).

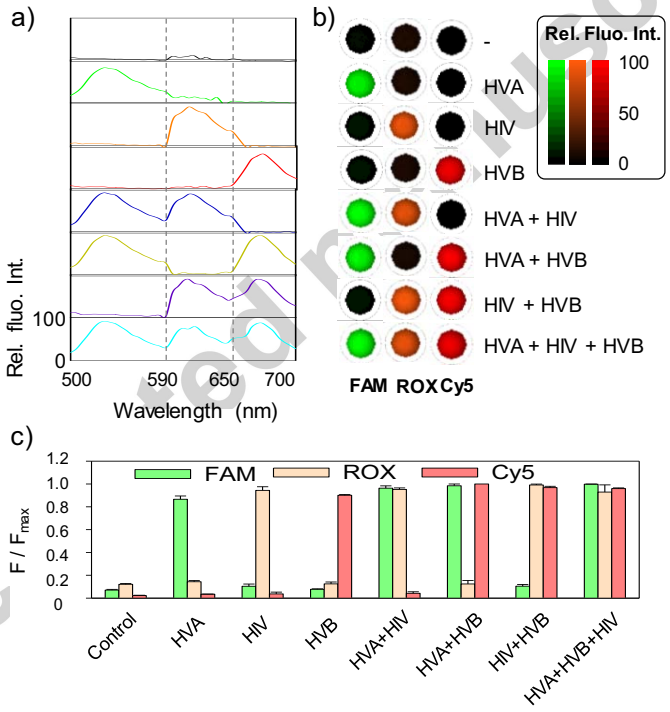

Fig. 3 Multiple target detection of three viral genes in a single solution. (a) Fluorescence spectra of three dye labeled PNAs in the presence of 200 nM target dsDNA in a buffered solution. (b) Fluorescent images of reaction mixtures for multiple detection. Column 1: fluorescence signal of FAM-HVA; Column 2: fluorescence signal of FAM-HVB; Column 3: fluorescence signal of FAM-HIV in the presence of 200 nM of each target. (c) A bar graph showing relative fluorescence intensity of three dye labeled PNAs in the presence of each target dsDNA (200 nM) (F : fluorescence intensity of PNA probe with target, Fmax :

Maximum fluorescence intensity of each PNA probe observed in the presence of perfect matched duplex target).

4. Conclusion

In conclusion, we demonstrated a new GO-based platform for direct and robust dsDNA detection using PNA probe and artificial dsDNA targets without denaturation. To the best of our knowledge, this is first study on sequence-specific, quantitative and direct detection of dsDNA based on GO. The present dsDNA sensor utilized the unique properties of PNA and GO. PNA could invade at the temini of the complementary strand of target dsDNA without sequence limitations, followed by strand replacement based on its higher binding affinity to complementary DNA than DNA probes. By combining PNA probe with GO, quantitative fluorometric dsDNA sensing was enabled even in the serum containing samples without a wide variation of LOD, which was impossible to achieve by using DNA probe. Currently, we are investigating possibility of applying the present strategy to real biological samples containing long dsDNA by utilizing restriction endonuclease to expose a DNA sequence for PNA recognition and invasion. We believe that this new direct GO-based dsDNA sensor using PNA probe will become an important tool in the field of personalized medicine and diagnostics of viral diseases and genetic disorders in the future.

Acknowledgment

This work was supported by the Basic Science Research Program (2011-0017356, 2011-0020322) and by the Research Center Program (EM1302) of IBS (Institute for Basic Science) through the National Research Foundation of Korea (NRF) funded by the Korean government (MEST).

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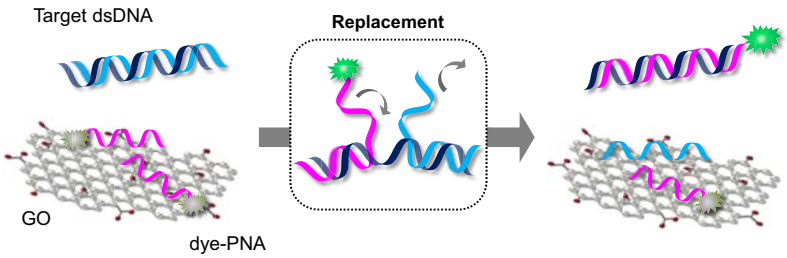
Graphical Abstract



Highlights

▶Multiplexed optical sensing of double stranded DNAs without any denaturing step was achieved. ▶Peptide nucleic acid and graphene oxide were used as a probe and as a fluorescence quencher. ▶Presence of serum proteins did not hamper the performance of the sensor such as low background signal and high sensitivity.