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Colorimetric detection of clinical DNA samples using an intercalator-conjugated polydiacetylene sensor

Yun Kyung Jung [a,b], Hyun Gyu Park [a,*]

[a] Department of Chemical and Biomolecular Engineering (BK21+Program), KAIST, 291 Daehak-ro, Yuseong-gu, Daejeon 305-701, Republic of Korea
[b] School of Natural Science, Ulsan National Institute of Science and Technology, Ulsan 689-798, Republic of Korea

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ABSTRACT

We herein developed a novel colorimetric polydiacetylene (PDA) sensor for very convenient detection of clinical DNA samples based on the interaction between an intercalator and dsDNA. We modified the terminal carboxyl group of a diacetylene monomer (10,12-pentacosadiynoic acid; PCDA) with the intercalator 9-aminoacridine (9AA) and prepared 9AA-modified PDA liposomes containing PCDA-9AA/PCDA/phospholipid (1,2-dimyristoyl-rac-glycero-3-phosphocholine) at a molar ratio of 1.5:6.5:2.0. The PDA sensor underwent an obvious color transition from blue to red in the presence of dsDNA molecules that were PCR-amplified from genomic DNA due to the insertion of the 9AA head group of PDA into the dsDNA. DNA concentrations as low as 20 nM and relatively small molecules (around 100 base pairs) could be detected by the sensor within 1 h without DNA electrophoresis. This novel colorimetric method is simple, does not require any instrument, and is therefore appropriate for POCT or portable molecular diagnostic kit.

1. Introduction

DNA biosensors that can be used to analyze clinical samples have been intensively investigated as potential tools for examining diseases caused by genetic defects (Chua et al., 2011; Thaitrong et al., 2010; Sassolas et al., 2008; Teles and Fonseca, 2008; Turner, 2013; Wang, 2000). Genetic analyses are conducted by nucleic acid hybridization (Wang, 2000) or by identifying the product of PCR amplification based on a genomic DNA template (Skuridin et al., 1996). Among the most frequently used methods for DNA detection is agarose gel electrophoresis, in which double-stranded (ds) DNA is visualized in the gel by adding a fluorescent intercalating dye such as ethidium bromide (EtBr) that is inserted between base pairs (Aaij and Borst, 1972; Vardevanyan et al., 2003). However, this method requires skill, time, and specialized equipment to resolve and monitor DNA fluorescence, thereby restricting its utility in point-of-care testing (POCT) or portable diagnostic kits.

Recent advances in DNA detection systems include the development of optical, electrochemical, and mass-sensitive methods (Sassolas et al., 2008; Teles and Fonseca, 2008; Wang, 2000). In particular, optical colorimetric sensors have the advantage of decreasing experimental costs and circumventing the relative complexity inherent in optical imaging and other detection methods by relying on an unaided visual readout instead of complicated instruments, making them amenable to on-site detection in real time (Chua et al., 2011; Sassolas et al., 2008; Teles and Fonseca, 2008). One of the most widely studied colorimetric techniques uses unmodified gold nanoparticles (AuNPs) as a sensor; dsDNA can be distinguished from unfolded, single-stranded (ss) DNA by aggregation-dependent color changes (Li et al., 2009; Li and Rothberg, 2004). However, this approach is still time-consuming and requires extensive training in the synthesis of AuNPs, and is less tolerant to interference than ligand-stabilized AuNPs.

With regards to portable and more efficient analytic sensors for detecting dsDNA in clinical samples, conjugated polymer-based sensors (Ho et al., 2002, 2005; Jung et al., 2008, 2010; Lee et al., 2007; Wang et al., 2006; Wang and Ma, 2005) have the important advantage of amplifying signals in response to external stimuli as compared to conventional sensors that are based on small molecules (Ali and Li, 2009; Du and Tang, 2011; Li et al., 2009; Li and Rothberg, 2004). The versatile and stable conjugated polydiacetylene (PDA) has useful structural and sensing properties when its head group is linked to ligands that can recognize external stressors such as pH, organic solvents, mechanical stress, and specific molecules. The unique and rapid chromatic change from blue to red can be directly perceived by the naked eye, which makes PDA a promising material for dsDNA detection that has the desired characteristics of simplicity and portability. Few studies have detected nucleic acids by exploiting the colorimetric properties of PDA. One group described a colorimetric method for

* Corresponding author.
E-mail address: hgpark@kaist.ac.kr (H.G. Park).

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detecting oligonucleotides that involved hybridization with probe oligonucleotide-functionalized PDA vesicles (Wang et al., 2006; Wang and Ma, 2005). However, synthetic oligonucleotides—and not clinical targets—were detected in this study. Only one study, carried out by our group, has directly detected clinical dsDNA amplified from genomic DNA using a primary amine-conjugated PDA colorimetric sensor (Jung et al., 2008). The main shortcoming of this sensor was low specificity for nucleic acids, because the principal sensing mechanism is based on relatively nonspecific ionic interactions between the positively charged PDA liposome and negatively charged nucleic acid.

We present here a novel approach based on intercalation that has greater specificity for clinical dsDNA detection. The positively charged planar aromatic dye 9-aminoacridine (9AA) was selected as a ligand for specific interaction with dsDNA. The 9-AA is inserted orthogonally into dsDNA without forming covalent bonds, and are sandwiched between two adjacent base pairs by both dispersive forces and electrostatic interactions (Ihmels and Otto, 2005; Medhi et al., 1999; Rao and Kollman, 1987). We modified the terminal carboxyl group of a diacetylene monomer with 9AA, and 9AA-functionalized liposomes consisting of diacetylene monomer/phospholipid assemblies were prepared and polymerized. The insertion of the 9AA head group attached to the surface of the resulting PDA vesicles into the PCR products of the Breast Cancer 1 gene (*BRCA1*) which is the best-known gene associated with breast cancer risk (Levy-Lahad and Friedman, 2007) created stress force on the PDA backbone, resulting in a color transition that was detectable by the naked eye. The sensor was used to assay dsDNA, yielding a linear relationship from 1 to 100 nM and a detection limit of 20 nM in 1 h, indicating satisfactory sensitivity. Morphologic transformations of PDA vesicles caused by interactions with DNA molecules were characterized based on images acquired by transmission electron microscopy (TEM). Importantly, our sensor satisfies the requirements of simplicity, rapidity, and low cost for potential use in POCT.

2. Material and methods

2.1. Materials

The diacetylene monomer 10,12-pentacosadiynoic acid (PCDA; Fig. 1) was purchased from GFS chemicals (Powell, OH, USA). The phospholipid 1,2-dimyristoyl-rac-glycero-3-phosphocholine (DMPC) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and the intercalator 9-aminoacridine (9AA) was obtained from Merck (Darmstadt, Germany). Solvents used in the experiments were of reagent grade. Sequences of primers used for PCR amplification of *BRCA1* are shown in Table S1.

2.2. Synthesis of the diacetylene monomer PCDA-9AA

The intercalator-modified diacetylene monomer investigated in this study was prepared by coupling 9AA linked to a 6-aminocaproic acid spacer with PCDA (Fig. S1). To synthesize PCDA-9AA, PCDA-*N*-hydroxysuccinimide (NHS) was obtained by activating PCDA with NHS in CH_2Cl_2 in the presence of 1-[3-(dimethylamino) propyl]-3-ethylcarbodiimide. The amine group of 6-aminocaproic acid that was used as a linker was protected with di-tert-butyl dicarbonate, and the carboxylic acid on the other side was activated with NHS. The activated ester group of the linker was coupled to the free amine of 9AA, and the protected amine group of the resultant spacer-9AA conjugate was deprotected using trifluoroacetic acid. The exposed amine group of the deprotected compound was then reacted with PCDA-NHS to form the intercalator-conjugated monomer PCDA-9AA (Fig. S1). The products at

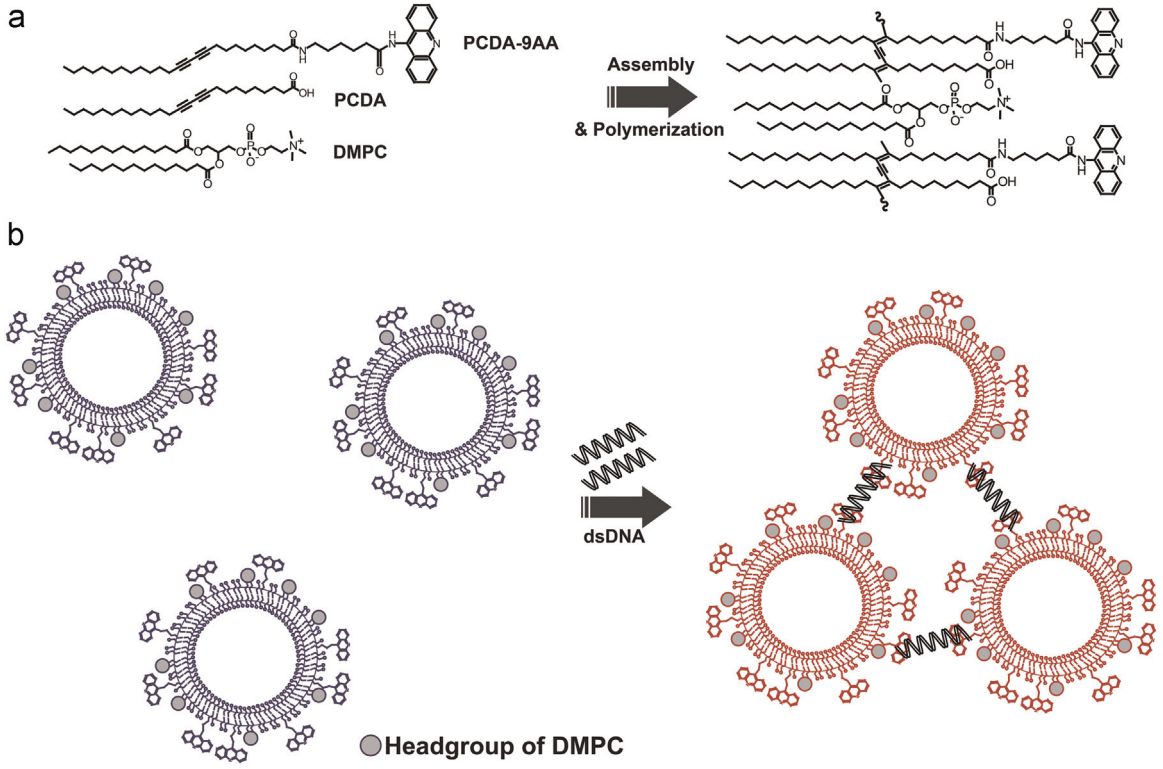

Fig. 1. Schematic illustration of colorimetric detection of double-stranded (ds) DNA based on intercalation of a polydiacetylene (PDA) head group into the double helix of dsDNA. (a) The polyconjugated ene-yne structure of 9AA-functionalized vesicles composed of 10,12-pentacosadiynoic acid-9-aminoacridine (PCDA-9AA), PCDA, and 1,2-dimyristoyl-rac-glycero-3-phosphocholine (DMPC). (b) Color transition of the PDA sensor caused by the intercalation of the 9AA head group of PDA into dsDNA. (For the interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

each step were isolated and purified by silica gel column chromatography (Sigma-Aldrich, St. Louis, MO, USA) with appropriate elution buffers; the final product was obtained as a solid with a slight green-blue color, and was characterized by nuclear magnetic resonance (NMR) spectroscopy on an AMX FT500 MHz instrument (Bruker, Coventry, UK) (Fig. S2) as follows: yield, 0.19 g (23.89%); ^{1}H NMR (300 MHz, $CDCl_3$), δ=8.25 (d, 2H), 8.01 (d, 2H), 7.76 (t, 2H), 7.56 (t, 2H), 5.72 (s, 1H), 3.33 (m, 2H), 2.85 (m, 2H), 2.23, (m, 2H), 2.72 (m, 2H), 1.83–1.60 (m, 6H), 1.62–1.41 (m, 14H), 1.39–1.18 (m, 20H), 0.85 (t, 3H). The light-sensitive, modified diacetylene monomer was stored in a dark bottle at −20 °C until use.

2.3. Preparation of PDA liposomes for DNA detection

To optimize the conditions of heterogeneous liposomes to detect PCR-amplified dsDNA (Fig. 1), different molar ratios of lipid mixtures containing the functionalized diacetylene monomer PCDA-9AA, unmodified diacetylene monomer PCDA, and phospholipid DMPC were dissolved in chloroform and dried with a stream of N_2 gas. DNase/RNase-free distilled water was added to the dried monomer/lipid films for a total mixed lipid concentration of 1 mM. To make monodisperse and homogeneous vesicles, the suspension was sonicated using a model 1510 instrument (Branson Ultrasonics, Danbury, CT, USA) for 20 min at approximately 80 °C and immediately filtered to remove dispersed lipid aggregates using a Minisart 0.8-μm syringe filter (Sartorius AG, Goettingen, Germany). The vesicle solution was cooled and stored at 4 °C for at least 6 h to induce the self-assembly of the diacetylene monomer/lipid mixture. Aligned, 9AA-functionalized liposomes were polymerized by UV irradiation at 400 μW/cm^2 (Vilber Lourmat, Marne La Vallée, France) at 254 nm for 6 min, yielding deep blue-colored PDA liposomes.

2.4. PCR amplification of BRCA

Genomic DNA of breast cancer patients was amplified to detect *BRCA* by conventional PCR. Each amplification reaction using forward primer and reverse primers 1 or 2 (Table S1) was carried out on a Model 9200 thermocycler (Perkin-Elmer, Norwalk, CT, USA) in a 50-μl reaction volume containing 100 ng of human genomic DNA extracted from the blood of breast cancer patients, 0.25 μM each primer, 1× PCR reaction buffer (10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$), 0.2 mM dNTP, and 1.25 U *Taq* DNA polymerase (Takara Bio Inc., Otsu, Japan). The cycling program was as follows: 94 °C for 5 min; followed by 35 cycles of 30 s at 94 °C, 30 s at 58 °C, and 1 min at 72 °C; and 5 min at 72 °C. PCR products were purified using the GeneAll PCR SV kit (General Biosystem, Seoul, Korea) and their sizes were determined by agarose gel electrophoresis. DNA concentrations were determined by measuring the absorbance at 260 nm on a Cary 100 UV–visible spectrophotometer (Varian, Palo Alto, CA, USA). The amplicons were 605 and 124 bp.

2.5. Colorimetric and spectrophotometric detection of DNA

Purified PCR products of two different sizes (605 and 124 bp) and a 26-mer oligonucleotide were used at a concentration of 100 nM; 3 μl of each DNA sample was mixed with 20 μl of polymerized PDA liposome composed of PCDA-9AA/PCDA/DMPC at a molar ratio of 1.5:6.5:2.0 (Fig. 2). In all PDA solutions, the pH was maintained at around 5.0. Chromatic transitions of 9AA-functionalized PDA liposomes were compared to those of PDA liposomes consisting of PCDA/DMPC at a molar ratio of 7:3, which were used as a negative control (Fig. S3). Color changes of the mixed vesicles were imaged and analyzed using a two-beam Cary 100 Conc UV–visible spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) after a 1-h reaction with DNA. All measurements were

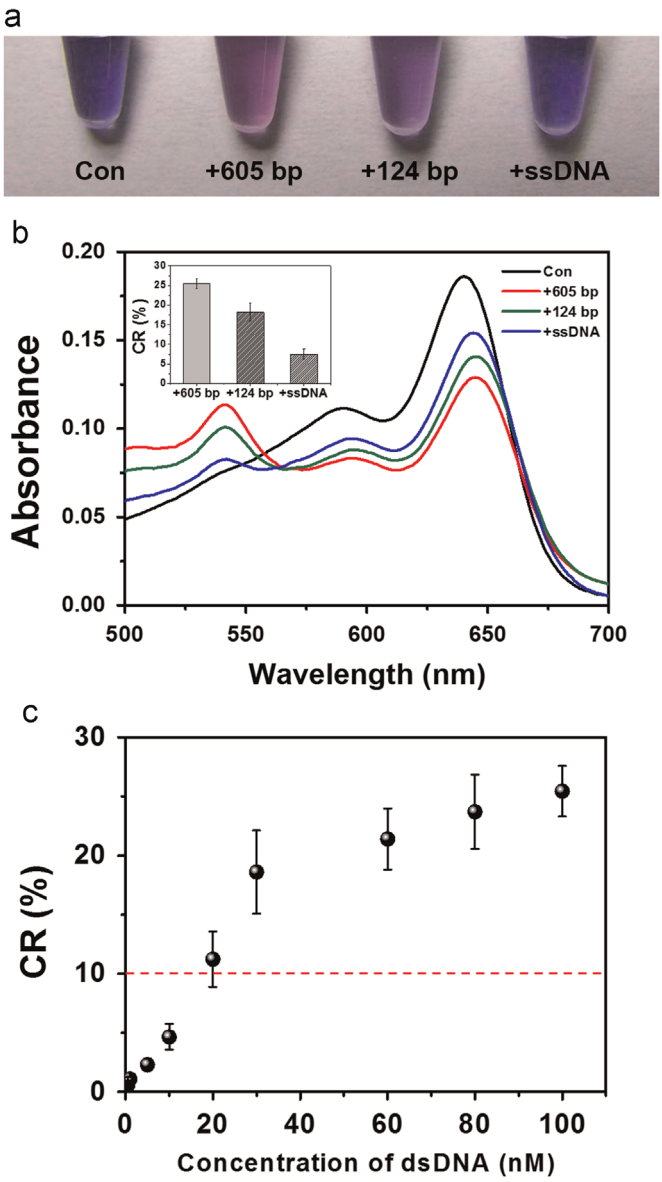

Fig. 2. Colorimetric detection of dsDNA by 9AA-functionalized PDA vesicles. (a) Chromatic and (b) spectroscopic changes (Inset: colorimetric response (CR, %)) of PDA liposomes in the presence of 605- (100 nM) or 124-bp (100 nM) dsDNA or ssDNA (100 nM). PDA liposomes without DNA were used as a control (Con). (c) Plot of the relationship between indicated concentrations of a 605-bp PCR amplicon and CR (%). Broken red line indicates a CR value of 10%. (For the interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

carried out at 25 °C in a cell with an optical path length of 1 cm. To quantify the blue-to-red color transition of PDA vesicles interacting with DNA, the colorimetric response (CR) was calculated as follows: CR (%)=$[(PB_0-PB_1)/PB_0]\times 100\%$, where $PB=A_{blue}/(A_{blue}+A_{red})$; A is the absorbance of the blue (~645 nm) or red (~541 nm) component in the UV–visible absorption spectrum; PB_0 is the blue/red ratio of the control sample in the absence of DNA; and PB_1 is the value of the sample after the color change in the presence of DNA (Jung et al., 2010). To determine the limit of detection (LOD) of our sensor with clinical dsDNA samples, various concentrations of 605-bp amplicons were mixed with 9AA-conjugated PDA vesicles. Samples were incubated for 1 h and the relationship between dsDNA concentration and quantitative CR value was plotted to determine the LOD. Previous studies have suggested that a change in CR of 10% is readily discernible by the

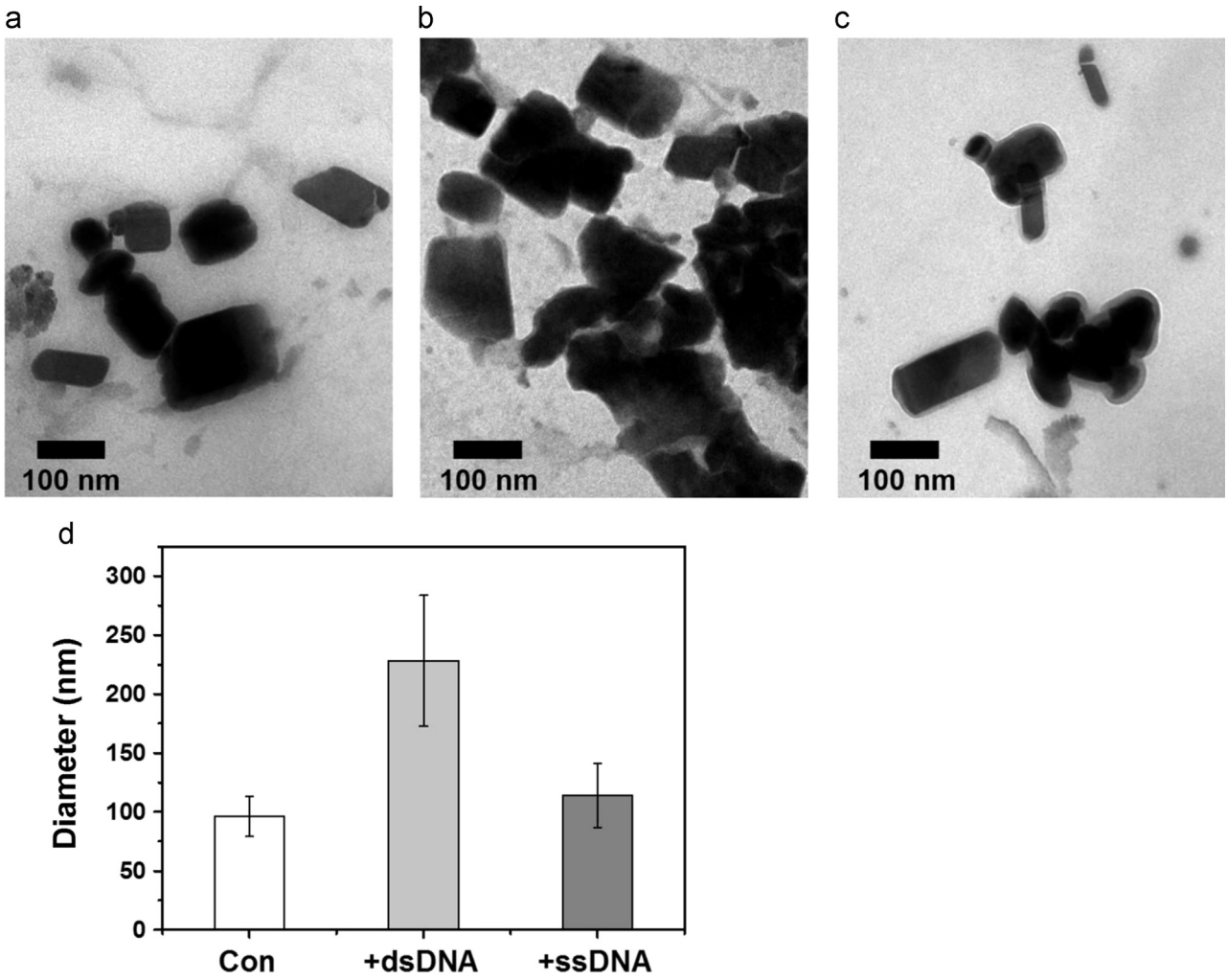

Fig. 3. Negatively stained TEM images showing morphologic changes of 9AA-functionalized PDA vesicles before (a) and after the addition of dsDNA (b) or ssDNA (c). Images are shown at 50,000 × the actual size. (d) Size of PDA vesicles before and after the addition of DNA molecules.

unaided human eye (Jung et al., 2010). Using this metric, we estimate the limit of visual detection of our sensor.

2.6. Structural analysis

To evaluate the size and morphology of the PDA vesicles, images of vesicle samples before and after the addition of DNA molecules were obtained by energy-filtering TEM (EM912 Ω; Carl Zeiss, Jena, Germany) at an accelerating voltage of 120 kV, on an instrument equipped with energy-dispersive spectroscopy, scanning TEM, and top/bottom charge-coupled device camera (Fig. 3). Samples were prepared by incubating vesicle solutions on carbon-coated films that were deposited onto a Cu grid (300 mesh) for 5 min and negatively stained with a 2% uranyl acetate solution for 2 min.

3. Results and discussion

3.1. Principle

The unique chromatic properties of PDA in response to biological stimuli were used to analyze genomic DNA samples obtained from breast cancer patients. The underlying principle is similar to that of agarose gel electrophoresis, in which DNA can be visualized with an intercalator dye such as EtBr (Aaij and Borst, 1972; Vardevanyan et al., 2003). The insertion of an intercalator-modified PDA head group into the DNA double helix causes stress-induced structural transformations of the PDA backbone with consequent blue-to-red color transition. To generate a PDA sensor specific to dsDNA, the terminal carboxylic group of the diacetylene monomer was linked to the intercalator 9AA to produce PCDA-9AA (Fig. S1), which formed the diacetylene assembly, composed of the unmodified monomer PCDA and phospholipid DMPC (Fig. 1a). Taking into consideration flexibility as well as accessibility to DNA, 6-aminocaproic acid was employed as a linker moiety. When photopolymerized by 254-nm UV radiation, the 9AA of the PDA sensor is exposed to hydrophilic solution. The 9AA-modified PDA sensor would bind to PCR-amplified dsDNA through intercalation and undergo a dramatic color transition that can be detected by the naked eye (Fig. 1b).

3.2. Colorimetric and spectrophotometric detection of dsDNA with 9AA-functionalized PDA vesicles

Of the various 9AA-modified PDA liposomes, those composed of PCDA-9AA/PCDA/DMPC at a molar ratio of 1.5:6.5:2.0 (Fig. 2a) showed the greatest color change from deep blue to red when mixed with 100 nM of a 605-bp amplicon obtained using the forward primer and reverse primer 1 (Table S1). The blue-colored control solution had a maximum absorption of ~645 nm (Fig. 2b). Upon interaction with dsDNA, the liposome suspension changed to

a red color with a maximum absorption of ~541 nm (Fig. 2b). The color change was quantified as CR (%), which reflects the extent of PDA colorimetric transition within the visible spectrum after incubation with dsDNA; the calculated value for the red liposome solution was ~24% (Fig. 2b, inset). Likewise, a shorter 124-bp amplicon obtained using the forward primer and reverse primer 2 (Table S1) caused a color transition in PDA vesicles corresponding to ~18% CR. These results showed that the intercalator moiety of the PDA liposome was open to the hydrophilic water surface, with its insertion between base pairs of DNA causing stress to the polyconjugated diacetylene backbone of a PDA liposome.

A quantitative comparison was made between the chromatic transition of PDA liposome in the presence of ds- and ssDNA (100 nM of a 26-mer oligonucleotide that served as a negative control). A negligible color change with a CR value of ~7% was observed upon addition of ssDNA (Fig. 2a and b), since intercalation was not possible. However, electrostatic interactions between the cationic amine group of 9-AA and the negatively charged phosphate backbone of ssDNA (Medhi et al., 1999) can result in an increase in the CR value to ~7%. These results suggest a significant difference in the stresses imposed on the poly(ene-yne) backbone by dsDNA and ssDNA owing to their distinct capacities for intercalation. Given that 9AA is a positively charged planar aromatic dye, dispersive forces between the aromatic moieties and DNA bases, as well as electrostatic interactions between the cationic amine of 9AA and anionic phosphate backbone of DNA, are expected to enhance this color transition in the detection of dsDNA.

With the exception of PCDA-9AA, PDA vesicles derived from a PCDA/DMPC assembly did not change color after incubation with 605- or 124 bp-dsDNA or ssDNA, as evidenced by the CR values of 3%, 3%, and 0.8%, respectively, which are insufficient to identify color changes (Fig. S3). We therefore concluded that the 9AA functional group plays an essential role in specifically detecting dsDNA.

3.3. Detection limit

To determine the detection sensitivity of the PDA colorimetric sensor, a calibration curve was generated from various concentrations of the 605-bp *BRCA* gene amplicon (Fig. 2c). As predicted, the CR value increased as a function of target DNA concentration (0, 1, 5, 10, 20, 30, 60, 80, or 100 nM). Since the color change was readily discernible by naked eye for CR values $>10\%$ (Jung et al., 2010), we estimated the LOD of our sensor as ~20 nM (Fig. 2c). The excellent sensitivity of our colorimetric sensor is attributable to signal amplification by PDA intercalation into the dsDNA molecule (Fig. 1b), which has an abundance of available binding sites that enable maximum reaction at low concentrations of target analyte.

Since the acidic 9AA-functionalized PDA liposome is susceptible to the basic pH and salts present in the PCR reaction buffer, amplicons used for detection should be purified to remove these components. Although this step decreases sample yield, the PDA-based DNA sensor has the advantage of a short purification time (i.e., <1 h) and the color transitions induced by different sizes of dsDNA (124 and 605 bp) demonstrated that the sensor can detect DNA molecules as small as ~100 bp.

3.4. Structural studies of interactions between DNA and 9AA-functionalized PDA vesicles

Because chromatic transformations of PDA are closely related to conformational alterations of the backbone induced by external stimuli, structural changes in PDA vesicles—such as shape, size, and aggregation—upon addition of DNA were characterized by TEM. The 9AA-functionalized PDA liposome had a rectangular shape and was around 96 nm in size (Fig. 3a and d). Upon interaction with dsDNA, the rectangular shape was maintained but the vesicles assembled into clusters of ~228 nm (Fig. 3b and d), likely due to cross-linking between 9AA-functionalized PDA liposomes resulting from the interaction of one dsDNA with two 9AA moieties (Fig. 1b). The greater force exerted on the conjugated backbone of PDA vesicles by cluster formation may cause the prominent color change. In contrast, in the presence of ssDNA, PDA vesicles showed negligible conformational changes and their size remained around 114 nm (Fig. 3c and d).

The TEM data were consistent with results obtained from the colorimetric studies. The color transitions reflected the interconnection of PDA vesicles by dispersive forces or electrostatic interactions in the presence of dsDNA, providing evidence for the existence of dsDNA-PDA interactions based on intercalation. This was not observed for ssDNA, since 9AA was unable to intercalate into a single DNA strand.

4. Conclusions

We developed a simple, rapid, and sensitive colorimetric PDA sensor to detect DNA obtained from clinical samples based on a modified intercalating dye. The terminal group of a PCDA monomer conjugated with the 9AA intercalator was used to prepare PDA vesicles composed of PCDA and DMPC. The successful detection of dsDNA by the chromatic sensor was visualized as a distinct color change caused by the insertion of the 9AA head group of PDA into the dsDNA molecule. This method has an LOD of 20 nM and can be used to detect dsDNA molecules as short as ~100 base pairs in 1 h. Due to its technical simplicity, rapidity, high selectivity, and sensitivity, this newly developed PDA has potential clinical applications in POCT.

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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.2015.04.093.

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