Specific Colorimetric Detection of Proteins Using Bidentate Aptamer-Conjugated Polydiacetylene (PDA) Liposomes

By Yun Kyung Jung, Tae Won Kim, Hyun Gyu Park, and H. Tom Soh*

The development of a bidentate aptamer-functionalized polydiacetylene (PDA) liposome sensor that is capable of specific colorimetric detection of proteins, directly in complex mixtures (e.g., serum), at sub-micromolar concentrations within 15 min, is reported. In comparison to sensors fabricated with a single aptamer reagent, the conjugation of bidentate aptamer pairs that recognize two distinct exosites of the target protein (thrombin) to the liposome results in significant enhancements of the sensitivity and the specificity. To elucidate the mechanism behind this enhancement, experimental evidence is presented that suggests that the liposomic aggregation triggered by specific, multi-site binding to the target protein is responsible for the improved colorimetric response. Since the colorimetric protein sensor does not require any power or instrumentation, it offers a promising approach towards molecular diagnostics at point-of-care (POC), especially in low-resource settings.

1. Introduction

Point-of-care (POC) diagnostics hold the promise to play a pivotal role in public health by significantly decreasing the time between diagnosis and treatment.[1] Among the many approaches that have been developed, colorimetric biosensors possess an inherent advantage in that they do not require any instrumentation or power supply, making them ideal for low-resource settings.[2–12] Previously, colorimetric protein detection has been achieved by methods such as the enzyme-linked immunosorbent assay (ELISA),[3] colorimetric metal nanoparticles (e.g., silver[4] and gold[5]), bio-barcode amplification (BCA),[6] and the colorimetric immuno-protein phosphatase inhibition assay (CIPPIA).[6] Recently, there has been significant interest in using conjugated polymers as biosensors,[7–12] because the polymer backbones in such systems can directly function as optical reporters for molecular binding interactions and interfacial biological processes. For example, in systems based on polythiophene,[7,8] or polydiacetylene,[7,9–12] binding interactions induce the delocalization of π-electrons along the polymer backbone, resulting in a distinct optical response without the need for secondary color-developing reagents.

Polydiacetylene (PDA)[7,8–12] is particularly interesting because it yields a significant chromatic change in response to a variety of external stimuli such as temperature,[9] pH,[9,10] and molecular recognition.[11,12] The PDA structure, which is conjugated through 1,4-addition of diacetylenic monomers upon UV irradiation at 254 nm, appears blue to the unaided eye, due to the formation of an alternating triple-/double-bond backbone structure.[9–12] However, external stimuli induce a change in the effective conjugation length and angle of the backbone,[9–12] resulting in a blue-to-red colorimetric transition. PDA offers additional advantages in that it can be self-assembled into liposomes in solution, or thin films immobilized onto various solid supports via standard methods. Despite these attributes, it has been a significant challenge to utilize PDA materials as colorimetric biosensors that can effectively function in clinically relevant, complex samples (e.g. serum), due to the sensor’s non-specific response to background molecules.

To address this important need, we have integrated DNA aptamers,[13–15] with PDA sensors to achieve direct colorimetric detection of target proteins at sub-micromolar concentrations with high specificity. With respect to previous work,[16] our approach is unique in a number of attributes. First, instead of using a single aptamer reagent, we employed bidentate aptamer pairs (15-mer BOCK[13] and 29-mer TASSET[14]) that recognize two distinct binding sites of a target protein (thrombin).[15] As previously described by Schrader and others[17] this strategy is highly effective, and in our work, we show that it yields significantly higher sensitivity and specificity, and a striking improvement in the colorimetric response. Second, we directly conjugated the aptamers to NHS-modified diacetylene monomers (Scheme 1a) and employed 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine (DMPE)[18] for fabrication of functional PDA liposomes (Scheme 1b). This allowed precise control of the molecular ratio between the diacetylene lipids and phospholipids, which affects the shape, size, membrane fluidity, and ultimately, the chromatic sensitivity of the vesicles. Finally, we provide direct experimental evidence to elucidate the mechanism behind the enhanced colorimetric change via scanning electron microscopy (SEM),[19] optical microscopy[20] and dynamic light scattering (DLS).[21]
we believe our synthesis strategy of direct conjugation offers a more efficient and reproducible means of tethering the aptamers to diacetylene lipids because it avoids hydrolysis of NHS groups in the lipid headgroup during the conjugation process.

Next, we challenged the three types of sensors with 3 μM thrombin, and visually observed the color change. After a 15 minute incubation, type I and type II sensors exhibited minimal color change (Figure 1a, top and middle). However, type III sensors, which contain both aptamers, showed a clear color transition from blue to red (Figure 1a, bottom). To quantify the degree of color change, we calculated the colorimetric response (CR, %) of each sample after incubation with thrombin by determining the change in the ratio of the absorbance (A) measurements at 536 nm (appears red) and 640 nm (appears blue) in the absence (PB0) and presence (PB) of thrombin, as previously reported.11 We used the following formula:

\[
\text{CR(\%)} = \frac{([P_B - P_{B0}]/P_{B0}) \times 100}{A_{640nm}}
\]

where \(P_B = A_{640nm}/(A_{640nm} + A_{536nm})\) (1)

Thus, higher CR values represent more efficient transition to the red color. After the addition of thrombin, the CRs of type I and type II sensors were approximately 9% and 10%, respectively (Figure 1b). On the other hand, the PDA type III sensor exhibited a two-fold higher CR (~21%) upon interaction with thrombin, presumably because the BOCK/thrombin/TASSET sandwich complex22 impose higher stress on the structure of PDA backbone.

To quantify the sensitivity of the type III colorimetric sensor, we used the absorption spectra to generate a calibration curve. As the sensor was challenged with an increasing concentration of thrombin (0, 0.05, 0.1, 0.5, 1, 3, 5 or 10 μM), we observed a decrease in absorbance at 640 nm concurrently with an increase in absorbance at 536 nm (Figure 2a), which results in a monotonic increase in the CR value (Figure 2b). Previous studies have suggested that a change in CR of 10% is readily discernible by the unaided human eye.13 Using this metric, we estimate the limit of visual detection (LOD) of our sensor at ~0.5 μM (Figure 2b).

In order to validate that the signal from the type III sensor is specifically generated in response to thrombin, we challenged it with unrelated proteins including bovine serum albumin (BSA), immunoglobulin G (IgG), streptavidin (STA), and fetal bovine serum (FBS, 10%) which contains a rich variety of proteins. Compared to the signal from thrombin, other proteins and mixtures produced negligible color change (Figure 3a). Furthermore, we investigated the response of the type III sensor to the non-target proteins in a concentration dependent manner.
were carried out in triplicate to generate the error bars. The experiments were carried out in triplicate to generate the error bars.

Figure 2. Absorbance spectrum for the type I, II, and III PDA sensors after interaction with thrombin at various concentrations (0, 0.05, 0.1, 0.5, 1, 3, 5, 10 μM). a) Spectroscopic response of the sensor when challenged with varying concentrations of thrombin (0, 0.05, 0.1, 0.5, 1, 3, 5, 10 μM). b) Colorimetric response as a function of thrombin concentration (μM). The experiments were carried out in triplicate to generate the error bars.

Figure 3. Colorimetric response of the type III PDA sensor is specific to thrombin. a) The sensor was challenged for 1 h with a negative control without protein (NC), a positive control containing thrombin (3 μM), or non-target proteins including bovine serum albumin (BSA, 3 μM), immunoglobulin G (IgG, 3 μM), streptavidin (STA, 3 μM), and fetal bovine serum (FBS, 10%). b) As an additional control for specificity, we examined a PDA liposome modified with a random DNA sequence with thrombin (3 μM). c) The sensor displays a robust color change when challenged with thrombin (3 μM) in FBS (10%).

We found that, compared to thrombin, even ~15-fold higher concentrations of BSA, IgG, and STA did not elicit a significant sensor response (Figure S2a–c). This clearly illustrates that the sensor does not respond to the wide range of background molecules, and that the colorimetric signal is specific to the target protein.

To further verify that the color change is indeed the result of specific binding of thrombin to the BOCK and TASSET aptamers (see the sequences in Experimental section), we fabricated a PDA sensor containing an unrelated, random sequence of DNA [5′-CGTTACAGTGGTGTAACGGGGTTAT-GGGGTG-3′]. In this case, the sensor produced insignificant color change when challenged with thrombin (Figure 3b), indicating that the chromatic transition is indeed induced by the specific binding of the aptamers to their target. Finally, in order to validate the utility of the type III sensor in complex mixtures, we challenged the sensor with 3 μM thrombin in 10% FBS. We observed a robust color change, as shown in Figure 3c.

Finally, to elucidate the mechanism behind the apparent difference in chromatic response between type III sensors and the single-aptamer sensors, we investigated changes in sensor morphology before and after the addition of target via SEM, optical microscopy, and DLS. We observed that in the absence of target, type III sensors are spherical in shape with an approximate diameter of ~330 nm (Figure 4a, 4g and Table 1). However, the addition of thrombin dramatically changed their morphology, leading to formation of aggregate clusters that range up to ~10 μm in size (Figure 4b, 4h, and Table 1). The presence of non-target proteins did not induce an equivalent change in morphology (Figure 4c–f, 4i–l, and Table 1).

Based on these observations, we suspect that the higher CR response of the type III sensor originates from the apparent aggregation of the liposomes wherein thrombin serves as a cross-linking agent in the manner shown in Scheme 1c (bottom). Considering that the color change is proportional to the thrombin concentration without any chemical change in...
aptamers to NHS-activated diacetylene monomers in order to prevent hydrolysis of NHS groups of the diacetylene lipids during the conjugation process, which enables controllable and reproducible sensor fabrication. Using this process, we have generated liposomes containing bidentate aptamers that yield significantly higher sensitivity and specificity relative to single-aptamer-based sensors, with a pronounced improvement in the colorimetric response. By analyzing the morphology of these sensors with SEM, optical microscopy and DLS, we have discovered a possible mechanism for this phenomenon, in which target proteins serve as a crosslinking agent to aggregate liposomes containing bidentate aptamers. We postulate that such aggregation would modulate the electron structure of the PDA, we postulate that the aggregation process induces significant tilting of the PDA backbone that decreases the effective conjugation length and modulate the \( \pi-\pi^* \) band gap which would affect the absorption spectrum with enhanced absorption at 540 nm, resulting in a color shift to red.

3. Conclusions

In this work, we demonstrate the first synthesis of PDA liposome structures functionalized with bidentate aptamers, and used these as sensors for the colorimetric detection of proteins. We have developed a process to covalently attach the DNA aptamers to NHS-activated diacetylene monomers in order to prevent hydrolysis of NHS groups of the diacetylene lipids during the conjugation process, which enables controllable and reproducible sensor fabrication. Using this process, we have generated liposomes containing bidentate aptamers that yield significantly higher sensitivity and specificity relative to single-aptamer-based sensors, with a pronounced improvement in the colorimetric response. By analyzing the morphology of these sensors with SEM, optical microscopy and DLS, we have discovered a possible mechanism for this phenomenon, in which target proteins serve as a crosslinking agent to aggregate liposomes containing bidentate aptamers. We postulate that such aggregation would modulate the electron structure

![Figure 4](image-url) SEM images (1000×) show the morphology of the Type III sensor after drying a) in the absence of proteins and in the presence of b) thrombin, c) bovine serum albumin (BSA), d) immunoglobulin G (IgG), e) streptavidin (STA), and f) 10% fetal bovine serum (FBS). Optical micrographs (200×) display the morphology of type III sensors in liquid g) before addition of proteins and upon addition of h) thrombin, i) BSA, j) IgG, k) STA, and l) 10% FBS.

Table 1. Particle size measurements (nm) based on dynamic light scattering before and after the addition of proteins.

<table>
<thead>
<tr>
<th>Without proteins</th>
<th>Thrombin treated</th>
<th>BSA treated</th>
<th>IgG treated</th>
<th>STA treated</th>
<th>FBS treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>329 ± 37</td>
<td>9477 ± 420</td>
<td>696 ± 54</td>
<td>498 ± 49</td>
<td>490 ± 66</td>
<td>940 ± 187</td>
</tr>
</tbody>
</table>

© 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim
of the π-conjugated polymer system, which leads to a change in the absorption spectrum and thus the chromatic response. Although further improvements in sensitivity and specificity will be necessary for clinical applications, we believe that the use of conjugated polymer materials with multi-dentate aptamers presents an interesting alternative for simple, low-cost colorimetric detection of proteins in complex biological samples.

4. Experimental Section

Materials: The diacetylene monomers, 10,12-tricosadiynoic acid (TCDA) and 10,12-pentacosadiynoic acid (PCDA) were purchased from GFS chemicals (Powell, OH, USA). Human alpha-thrombin was purchased from Haematologic Technologies Inc. (Essex Junction, VT, USA). Human IgG was purchased from Invitrogen (Carlsbad, CA, USA). STA from Streptomyces avidini (affinity-purified, lyophilized powder, salt-free), BSA, FBS, dimethyl sulfoxide (DMSO) and DMPE were purchased from Sigma (St. Louis, MO, USA). The two thrombin-binding aminodna oligonucleotides (BOCK and TASSSET) were obtained from Integrated DNA Technologies, Inc. (IA, USA). The sequence of the BOCK 15-mer (5′-GGTTACAGTTGGGTAACGGGGTTATGGGGTG-3′) was screened out by Bock et al. [13] in 1992, while that of the TASSSET 29-mer (5′-AGTCTCGTGTACCGACAGCTGGTGTGGTGTGGTG-3′) was selected by Tasset et al. [14] in 1997. We selected another random DNA oligonucleotide (X, 5′-CGTTACAGTTGGGTAACGGGGTTATGGGGTG-3′) as a control, nonbinding sequence. All solvents used in this study were of analytical grade. Millipore Microcon (molecular weight cut off (MWCO) 3000 Da and 10000 Da) were purchased from Fisher Scientific (Pittsburgh, PA, USA) and Vivaspin centrifugal concentrators (MWCO 5 000 Da) were purchased from Cole-Parmer (Vernon Hills, IL, USA).

Synthesis of NHS-Modified Diacetylene Monomers (TCDA-NHS): We added 2.07 g (10.82 mmol) of N-hydroxysuccinimide (NHS) and 1.24 g (10.82 mmol) of N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC-HCl) to a solution containing 2.50 g (7.21 mmol) of TCDA in 20 mL of methylene chloride at room temperature. The resulting solution was stirred at room temperature for 2 h. We removed the solvent in vacuo, and purified the residue by extraction with ethyl acetate to obtain 2.78 g (86.7%) of the desired diacetylene monomer, 10,12-tricosadiynoic acid-N-hydroxysuccinimide (TCDA-NHS, see Scheme 1), as a white solid. Since the modified diacetylene monomers are light-sensitive, they were stored in a dark bottle at room temperature.

Quantifying Thrombin Concentration-Dependent Chromatic Transitions of Bidentate Aptamer-Conjugated PDA Liposomes: The bidentate aptamer pair-modified PDA liposomes were incubated with various concentrations of thrombin (0, 0.05, 0.1, 0.5, 1, 3, 5, 10 μM) at 25 °C for 1 h. We recorded the absorbance profiles using absorption spectroscopy, and quantitatively analyzed the resulting data with the CR (%) equation. We collected data from three independent experiments, and each plotted point indicates the mean value with the error bars indicating standard deviation.

Liposome Structural Analysis Using SEM, Optical Microscopy, and DLS: We performed structural characterization of the aptamer-conjugated PDA vesicle suspensions before and after interaction with protein samples using SEM [19], optical microscopy [20], and DLS [21]. We quantitatively analyzed the resulting data with the CR (%) equation. We performed absorbance measurements using UV-VIS spectrophotometry, and quantitatively calculated the CR (%) as described above.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.
Acknowledgements

We are grateful for the financial support of the ARO Institute for Collaborative Biotechnologies, and National Institutes of Health. Y. K. Jung was partially supported by the Korea Research Foundation (KRF-2008-357-D00086).