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Development of Polydiacetylene-Based Testosterone Detection as a Model Sensing Platform for Water-Insoluble Hormone Analytes

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Abstract: We have developed a polydiacetylene (PDA)-based sensing platform to detect testosterone (T) as a potential biomarker of preterm birth. The insolubility of the steroid hormone in water, where PDA assemblies are dispersed, poses a major issue, since they can hardly interact with each other. To overcome this challenge, acetonitrile was used as a suitable solvent. In addition, to minimize false signals of PDA assemblies caused by the solvent, a mixture of acetonitrile and distilled water was selected. To prove a concept of PDA-based sensing platform for targeting T hormone, we conjugated anti-T antibodies to surface of PDA assemblies to induce selective binding between T and anti-T antibodies. The fluorescence sensory signaling of the PDA-anti-T antibody conjugate was selectively generated for T, over 3.4 times higher sensitivity of the signaling compared to that from other sex steroid hormones studied (β-estradiol and progesterone).

Keywords: polydiacetylene; preterm birth; steroid hormone detection

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1. Introduction

The low birth rate of developed countries is a growing concern, mainly because it will cause national productivity to plummet in the near future. Preterm birth—early delivery of a baby before 37 weeks of pregnancy—is considered a reason for low birth rates [1,2]. Globally, approximately 15 million infants are born preterm every year, and this number is increasing [3]. Preterm birth not only causes health risks, such as deformities or infant death, but also raises the country's economic burden of newborn care.

Placental alpha macroglobulin-1 (PAMG-1) levels, fetal fibronectin levels, and ultrasound have been generally used to diagnose preterm birth [4,5]. PAMG-1 is found in amniotic fluid during pregnancy, and its concentration is 1000 times higher than that in normal vaginal discharge or maternal blood [6,7]. PAMG-1 can be detected using a lateral flow immunoassay, which are typically tests strips containing monoclonal anti-PAMG-1 antibodies with gold nanoparticles as detection label that can be visualized in the presence of PAMG-1. Fetal fibronectin is a glycoprotein of the basal decidual membrane, located near the amniotic fluid and the space between the placental tissue and placenta. It is released by mechanical or inflammation-mediated damage to the membrane or placenta prior to birth. The fetal fibronectin in cervical or posterior vaginal fornix can be detected by enzyme-linked immunosorbent assay, including monoclonal antibodies [8]. However, the

detection of PAMG-1 or fetal fibronectin is usually available at the earliest at 7–10 days before birth delivery. Although obstetric ultrasound can be used to predict the risk of preterm delivery, it requires expensive equipment and trained operators. Therefore, developing low-priced and user-friendly diagnostic technology for the early prediction of preterm birth is essential.

Polydiacetylene (PDA) is a remarkable sensing material due to its dual-mode optical transitions, which produce easily detectable colorimetric and fluorogenic signaling responses. The optical transition of PDA responds to external stimuli, such as biomolecules [9–11] and chemical analytes [12–14]. Assemblies of PDA, such as PDA liposomes in aqueous phase, react or bind with target analytes, which generates an optical transition by distorting the conjugated backbone of PDA. The liposomes consisting of amphiphilic diacetylene or/and lipid molecule are readily formulated in aqueous and the surface of the liposomes could be immobilized with various biomolecules such as peptide, enzyme and antibody [15–20]. These characteristics of simple formulation and easy-to-surface modulation enable for the PDA liposomes to be used as easily accessible and economical sensing platform.

Based on previous studies, including our research [21–23], in patients showing symptoms of preeclampsia, the level of sex steroid hormones, such as estradiol, is 5-fold lower than that in normal pregnant women [21]. Also, there is a study that very low birth weight (less than 1500 g) preterm infants could be influenced by prenatal exposure to high levels of testosterone (T) [24]. Thus, sex steroid hormones could be potential biomarkers of preterm birth, and their early detection could be vital to prevent premature deliveries. As a detection of T in clinical practice, mass spectrometry-based technique is commonly used. The analytical methods such as gas chromatography–mass spectrometry and liquid chromatography–mass spectrometry provide an accurate quantification but require time-consuming and expensive instruments [25]. To our knowledge, the development of PDA-based sensors for hormone detection has rarely been explored [26,27]. Cho et al. studied the selective detection of progesterone using phospholipid-incorporated PDA assembly [26]. This method was inspired by the interactions between steroids and phospholipids of the cellular membrane. In addition, Jung et al. developed a glutathione substrate-tagged PDA assembly for the detection of glutathione S-transferase enzyme-based human growth hormone [27]. However, the main challenge in developing PDA-based hormone detection is that PDA assemblies in aqueous solution cannot efficiently meet and bind with water-insoluble hormones.

Herein, we investigated a suitable co-solvent medium for solubilizing the hydrophobic hormones and dispersing them in water simultaneously while minimizing optical false signaling by the medium. Sex steroid hormones, namely T, progesterone, and β-estradiol, were screened and incubated to generate sensory signals with PDA assembly of albumin or anti-T antibody conjugates via colorimetric and fluorogenic transitions.

2. Experimental Details

2.1. Materials

All solvents were purchased from Daejung Chemicals (Seoul, Korea). 10,12-Pentacosadiynoic acid (PCDA) was purchased from Alfa Aesar (Waltham, MA, USA). 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), and T were purchased from Tokyo Chemical Industry (Tokyo, Japan). Progesterone and Pierce™ anti-T antibodies (T Ab, Product # MIT0103) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). β-estradiol and albumin from human serum were purchased from Sigma Aldrich (Darmstadt, Germany). Phosphate buffered saline (1 × PBS) was purchased from Biosesang (Seoul, Korea).

2.2. Preparation of PDA Assemblies

To prepare PDA liposomes consisting of PCDA monomers, PCDA (3.75 mg) was dissolved in acetone (300 μL) and then injected into distilled water (DI water, 20 mL). The

suspension was sonicated using a bath sonicator (P0000VUV, Kodo, Seoul, Korea) for 1 min and kept at 4 °C overnight.

We modified a protocol by Kim et al. that enables conjugating albumin or T Ab to PDA [28]. 50 mM EDC in DI water (1 mL) was added to an aqueous solution of 0.5 mM PDA (1 mL), after which 50 mM NHS in DI water (1 mL) was added. The mixture was stirred at room temperature for 2 h. The solution was centrifuged (Centurion Scientific, West Sussex, UK) at 15,000 rpm (1411× *g*) for 15 min to remove residual EDC/NHS. The supernatant of the centrifuged solutions was removed and re-dispersed in 1 × PBS (1 mL). Later, albumin or T Ab were added to the dispersion at a concentration of 0.1 mg/mL, and were stirred at room temperature for 2 h. To deactivate residual NHS, 1 mM ethylenediamine in 1 × PBS (1 mL) was added to the dispersion and stirred at room temperature for 2 h. The solution was centrifuged at 15,000 rpm (1411× *g*) for 15 min to remove unreacted residues. After washing three times with 1 × PBS by centrifugation, it was re-dispersed in 1 × PBS (1 mL) with sonication. The PDA assemblies were polymerized for 5 min with a UV lamp (254 nm, 1 mW·cm^{-2}, Vilber, Marne-la-Vallée, France).

2.3. Characterization of PDA Assemblies and Steroid Hormones

The morphology of the PDA assemblies was observed using an ultra-high resolution low-voltage-scanning electron microscope (JSM-7900F, JEOL, Tokyo, Japan) at an accelerating voltage of 5.0 kV. The specimens were coated with platinum with an 8 nm thickness. A Zetasizer (Zetasizer Nano ZS90, Malvern Panalytical, Worcestershire, UK) was used to measure the alteration in zeta potential and size of the PDA assemblies during the conjugation of albumin or T Ab to PDA. The chemical bonding or interaction of the PDA assemblies was monitored using Fourier transform infrared (FT-IR) spectroscopy (Vertex 80 v, Bruker Optics Co., Leipzig, Germany). To check the solubility of steroid hormones in the solvents, 3 mg/mL of hormone (T, progesterone, and β-estradiol) was added to candidate solvents (dimethyl sulfoxide [DMSO], acetonitrile [ACN], ethanol [EtOH], and methanol [MeOH]), and the transmittance of the solutions was measured using UV-Vis spectroscopy (Libra S70, Cambridge, UK)

2.4. Hormone Detection Tests Using Colorimetric Response (CR) and Fluorescence Measurement

To measure the *CR* of the PDA assemblies by adding medium (mixture of ACN:DI water, EtOH:DI water, MeOH:DI water) or hormone (T, progesterone, and β-estradiol) solution (0.1, 0.2, 0.5, 1, 2, and 3 mg/mL), the solution of PDA assemblies (120 μL) and the medium/the hormone solution (80 μL) were incubated for 1 min, and the absorption spectra were measured using UV-Vis spectroscopy.

The *CR* was calculated as follows:

$$CR(\%) = \frac{PB_{before} - PB_{after}}{PB_{before}} \times 100\,,\ PB = \frac{A_{blue}}{A_{blue} + A_{red}} \quad (1)$$

where A_{blue} is the absorbance intensity at 650 nm, and A_{red} is the absorbance intensity at 550 nm. PB_{before} and PB_{after} are the values before and after incubating with medium or hormone samples, respectively.

To measure the fluorescence (FL) intensity of the PDA assemblies, a solution of these assemblies (120 μL) was incubated with hormone solution (80 μL) for 1 min, and the emission spectra were measured using a fluorescence reader (iD5 Multi-Mode Microplate Reader, Molecular Devices, San Jose, CA, USA). The measurement was set to excitation at 485 nm, and the emissive intensity was recorded at 630 nm.

The data of FL intensity at each concentration of analyte hormone were expressed as mean value with error bar (n = 3). The sensitivity of sensing platform for each hormone was calculated from value of slope in the linear fitting. We analyzed limit of detection (*LOD*) was calculated as follows:

$$LOD = Standard\ Error\ (SE) \times \sqrt{N} \times 3.3\ \div Slope \quad (2)$$

where N is the number of data elements (herein $N = 5$, five data point of hormone concentrations: 0.2, 0.5, 1, 2, 3 mg/mL), *Slope* is the value of linear fitting, and standard error (*SE*) is a standard deviation of the regression line (red line in the graphs) calculated using the OriginPro 8 software (Northampton, MA, USA).

2.5. T Hormone Detection Tests in Human Serum (1%)

To prevent the interfering sensory signals of PDA assemblies from human serum proteins in human serum, it was diluted with Tris-HCl buffer (20 mM, pH 7.0) and filtered using centrifugal filter (MWCO of 100–150 kDa) or centrifuging at 1500 rpm for 20 min. Then T hormone was spiked into 50% v/v of the filtered human serum (1%) and ACN, making various concentrations (0.1, 0.2, 0.5, 1, 2 and 3 mg/mL).

3. Results and Discussion

In mammalian blood plasma, water-insoluble sex steroid hormones (Figure 1a) exist as water-soluble complexes via binding to serum albumin or sex hormone-binding globulin [29,30]. Accordingly, these complexes transport and maintain the affinity of hormones in the aqueous phase. Few studies have been conducted to develop PDA-based sensors that target steroid hormones because water-insoluble hormones hardly meet and bind to PDA assemblies in aqueous media. Inspired by the hormone-albumin and hormone-globulin complexes in aqueous plasma, we utilized albumin or hormone-specific binding antibodies that could form a mediator of intermediate solubility (between water-insoluble hormones and water-dispersible PDA assemblies).

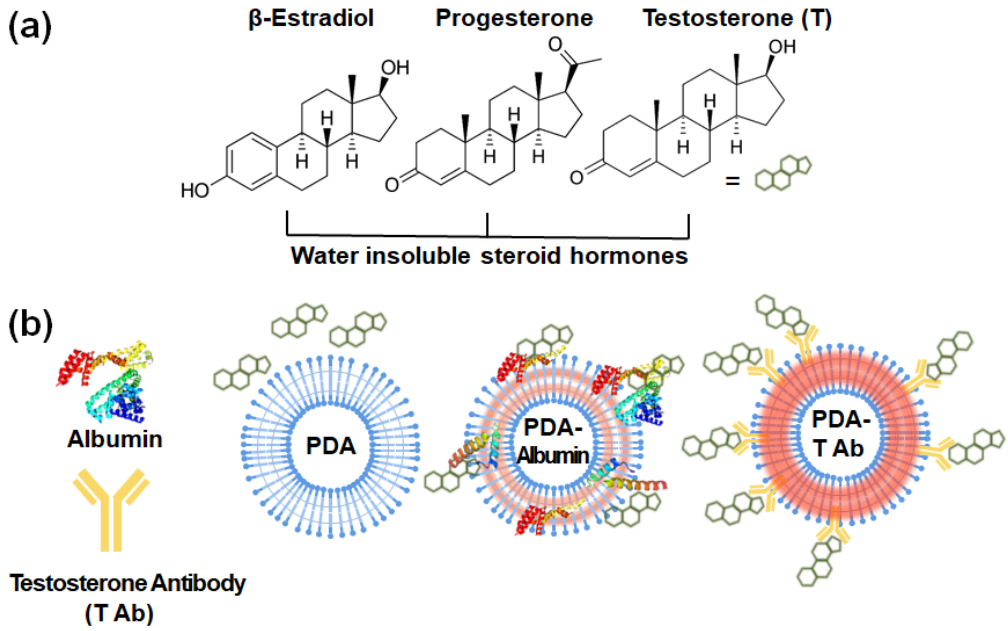

Figure 1. (**a**) Chemical structure of three sex steroid hormones (β-estradiol, progesterone, testosterone (T)) used as preterm biomarkers. (**b**) Schematic scenario of interaction of T on three types of polydiacetylene assemblies; liposome consisting of 10,12-pentacosadiynoic acid monomers (PDA), albumin-conjugated PDA (PDA-Albumin), and anti-T antibody-conjugated PDA (PDA-T Ab), when incubated with T. Depending on the interaction of T and PDA assemblies, colorimetric and fluorogenic sensory signal (red color) generated was drawn in the middle of the PDA assemblies.

Sex steroid hormones such as β-estradiol, progesterone, and T are thought to be pregnancy/preterm birth-related biomarkers [21–23]. Based on the current study that preterm infants could be influenced by T level [24], the PDA sensory platform was designed to target T for preterm birth prediction. We developed three types of PDA-based sensing platforms to compare sensory signals in the presence of T: (1) PDA liposomes consisting of PCDA monomers (PDA), (2) albumin conjugated to PDA (PDA-Albumin), and (3) anti-T

antibody conjugated to PDA (PDA-T Ab) (Figure 1b). We assumed that three types of PDA assemblies could have different numbers of binding and binding affinities of T—the highest binding available onto the PDA-T Ab by pairing interactions between T and T Ab.

The morphology of PDA assemblies was observed using SEM (Figure 2a). While PDA resulted in ~5 μm particle-shaped fragments, PDA-Albumin and PDA-T Ab presented an angular shape of 10–100 μm. Similar aggregated or angular structures of polydiacetylene-antibody conjugates have been reported in the literature [28,31]. The changes in surface charge and size distribution of PDA assemblies during conjugation to albumin or T Ab were measured by dynamic light scattering measurements (Figure 2b,c and Figure S1). PDA has a zeta potential of -46.9 ± 1.4 mV. During EDC/NHS activation of carboxylic acid on PDA, the zeta potential became less negatively charged, resulting in a value of -17.5 ± 0.3 mV (Figure S1). After conjugation with albumin and T Ab, the zeta potential values were increased to -5.8 ± 0.1 and -8.6 ± 0.5 mV, respectively. We hypothesize that positively charged albumin and T Ab were conjugated to the negatively charged PDA, inducing a change in the zeta potential after conjugation [32]. Based on the light scattering measurement, PDA has a size of 130 ± 10 nm, and after conjugation with albumin and T Ab, the size of PDA-Albumin and PDA-T Ab increased to 4400 ± 400 and 3800 ± 500 nm, respectively. Since PDA-Albumin and PDA-T Ab became less negatively charged than PDA, less repulsion between the assemblies would make being aggregated or bigger sized. In Figure S2, the transmittance of PDA-Albumin and PDA-T Ab at 1637 cm^{-1} (-COOH) recorded by FT-IR spectroscopy was relatively lower compared to that of PDA, implying that the carboxylic acid of PDA was consumed by the chemical reaction with EDC/NHS.

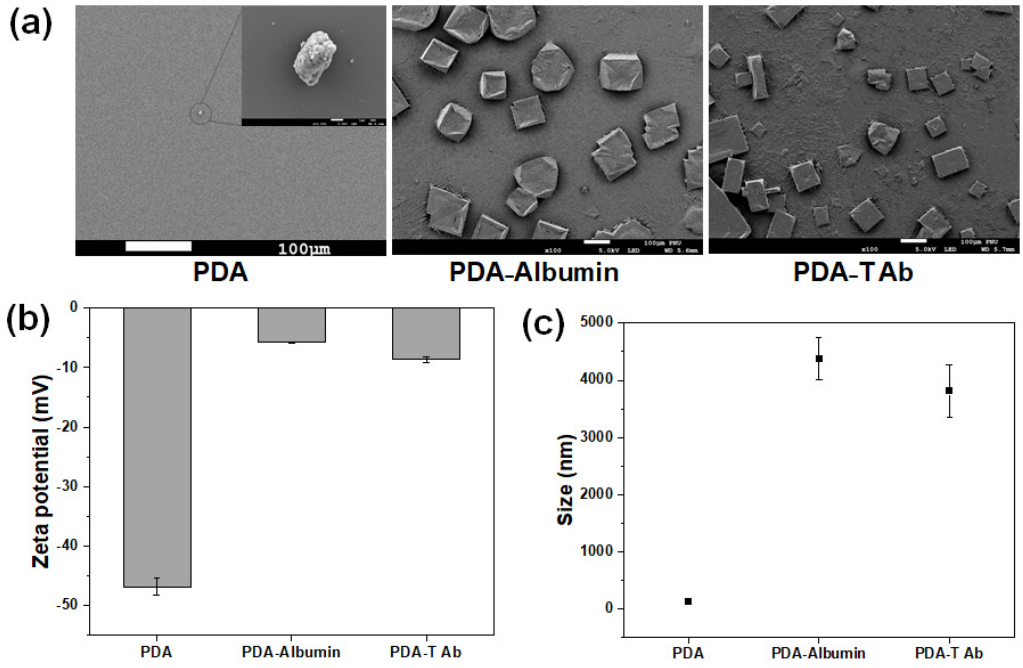

Figure 2. (**a**) SEM image of PDA, PDA-Albumin and PDA-T Ab. Scale bars represent 100 μm. The scale bar of the inset (magnified image) represents 1 μm. (**b**) Zeta potential values and (**c**) size of PDA, PDA-Albumin, and PDA-T Ab.

Since the solubility of steroid hormones in aqueous solution is low, we first aimed to find a suitable solvent medium that would both solubilize the hormones and enable them to encounter and react with PDA assemblies in aqueous solution. It is also important that the solvent minimize false signals from the PDA assemblies when added, since solvents, except for water, usually tend to generate a colorimetric transition [33,34]. Relatively polar solvent candidates, DMSO, ACN, EtOH, and MeOH, were screened for their ability to solubilize hormones by measuring the transmittance at 400 nm using a UV-Vis spectrophotometer

(Figure 3a). DMSO showed the lowest transmittance, which translates as the poorest solubility, and was thus excluded from the solvent candidates. We then assessed the *CR* of the PDA assemblies when introducing the candidate solvents (Figure 3b). Pure solvents (100% ACN, EtOH, and MeOH) induced a certain degree of *CR* (ACN: 15.4%, EtOH: 15.2%, and MeOH: 15.6%, calculated from absorption intensity at 650 nm and 550 nm) with pale violet colorimetric change (see inset image of Figure 3b). Therefore, the solvent ratio was reduced to 50% (*v*/*v*) (mixed with DI water), which decreased the false signals (ACN: 6.7%, EtOH: 7.7%, and MeOH: 4.1%). ACN was selected as the most suitable solvent because it has higher solubility than MeOH and causes fewer false signals than EtOH.

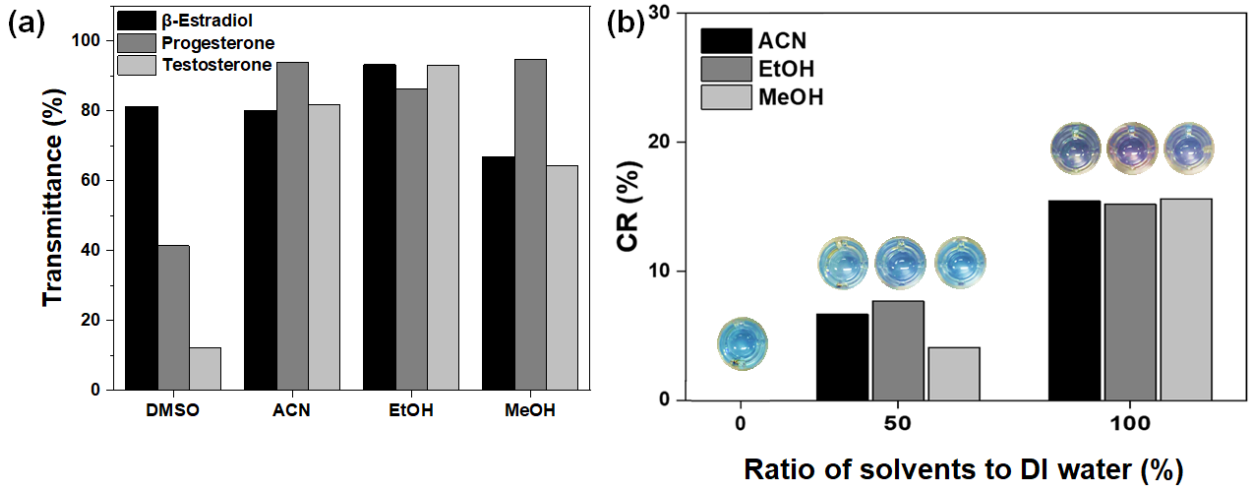

Figure 3. (**a**) Solubility test of sex steroid hormones in solvent medium (Dimethyl sulfoxide (DMSO), acetonitrile (ACN), ethanol (EtOH), methanol (MeOH)). (**b**) Colorimetric response (*CR*) of PDA when adding candidate solvent to the medium. Inset: photograph of the PDA solution after adding solvent to the medium.

The *CR* values of PDA-based sensors are commonly used as sensory signals. In all three types of PDA assemblies (PDA, PDA-Albumin, PDA-T Ab), hormone concentration-dependent *CR* values were observed with a trend of gradual increase (Figure 4a–c), indicating non-specific optical signals from the hormones. In particular, the *CR* of PDA-T Ab for T hormone was not significantly different from that of PDA or PDA-Albumin (Figure 4c), even though PDA-T Ab was designed to selectively react with or bind to T. The *CR* values were calculated by comparing the absorption intensity at 550 nm (wavelength of red absorption) and 650 nm (wavelength of blue absorption). In this study, estimating the correct *CR* values proved difficult since the overall spectral intensity was interfered by the absorption of water-insoluble hormone (Figure 4d–f). A possible explanation is that the instability and insolubility of hormones in aqueous solution inhibited the absorption-based colorimetric sensory signals, and no significant difference was observed in any of the three types of PDA assemblies as a result.

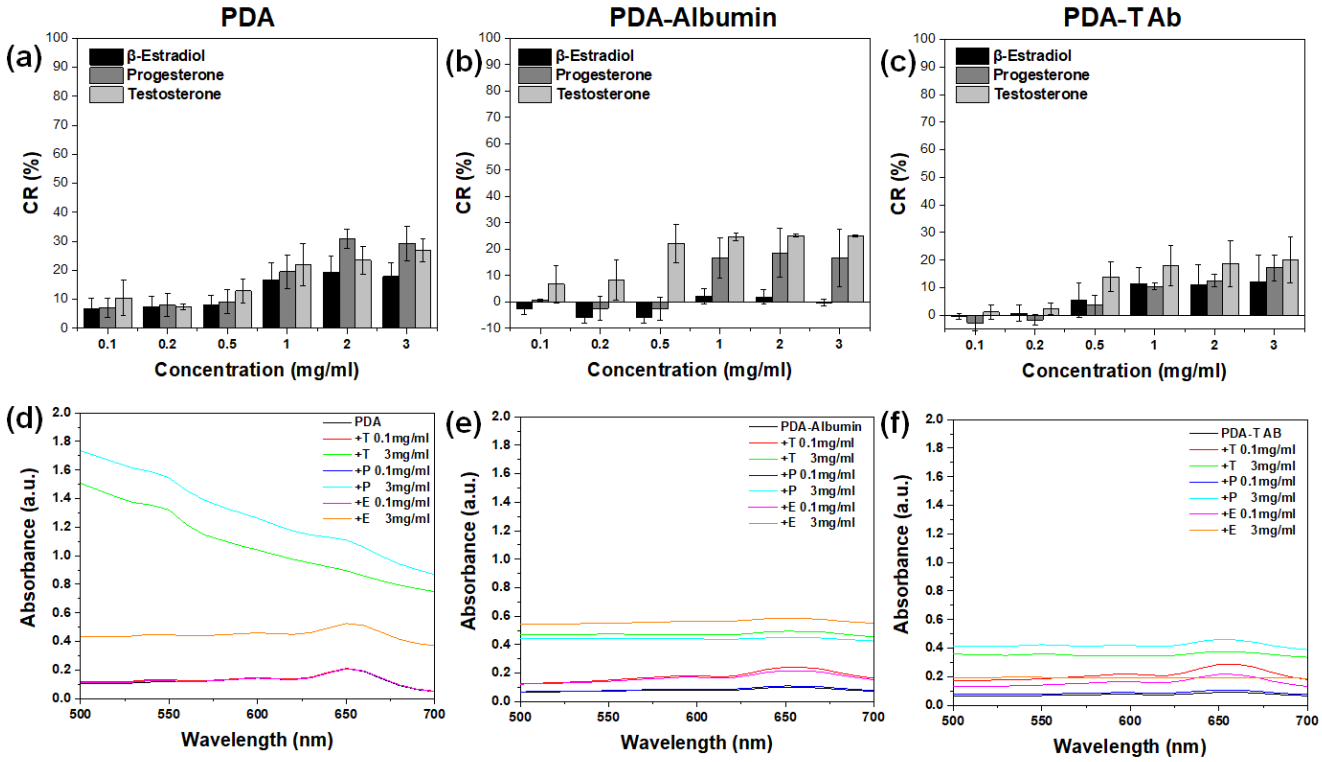

Figure 4. *CR* of (**a**) PDA, (**b**) PDA-Albumin and (**c**) PDA-T Ab after incubating with sex steroid hormones (β-estradiol, progesterone and T). UV-Vis absorption spectra of (**d**) PDA, (**e**) PDA-Albumin and (**f**) PDA-T Ab when incubating with sex steroid hormones at low (0.1 mg/mL) and high (3 mg/mL) concentrations.

To avoid the interruption of signals from the absorbance of the hormones, the FL response of the PDA assemblies was measured using a fluorescence reader (λ_{ex} = 485 nm, λ_{em} = 630 nm). The FL of polydiacetylene is not generally affected by the optical properties of other materials in the same environment, and PDA shows higher sensitivity in fluorogenic mode than in colorimetric transition [35]. As shown in Figure 5a, none of the three hormones (β-estradiol, progesterone, and testosterone) produced FL when incubated with PDA. As shown in Figure 5b, the presence of T increased the FL of PDA-Albumin in a concentration-dependent manner, starting from 0.5 mg/mL of T. When incubated with progesterone, the FL of PDA-Albumin only increased from a concentration 2 mg/mL. In contrast, no FL changes were found in PDA-Albumin when incubated with β-estradiol. The sensitivity of the PDA-Albumin for T and progesterone was 630,000 a.u./mg·mL^{-1} and 130,000 a.u./mg·mL^{-1}, respectively, when considering a range of 0.2–3 mg/mL of either hormone (Figure 5e). In general, progesterone and T have considerable, but non-specific affinity towards albumin in blood plasma [36]; for example, 53–55% of T binds to serum albumin and is transported as a complex in human blood [37]. Therefore, introducing progesterone or T could generate a certain of FL signals originated from distorting conjugated backbone of PDA by binding of progesterone or T onto albumin at the surface of PDA-Albumin.

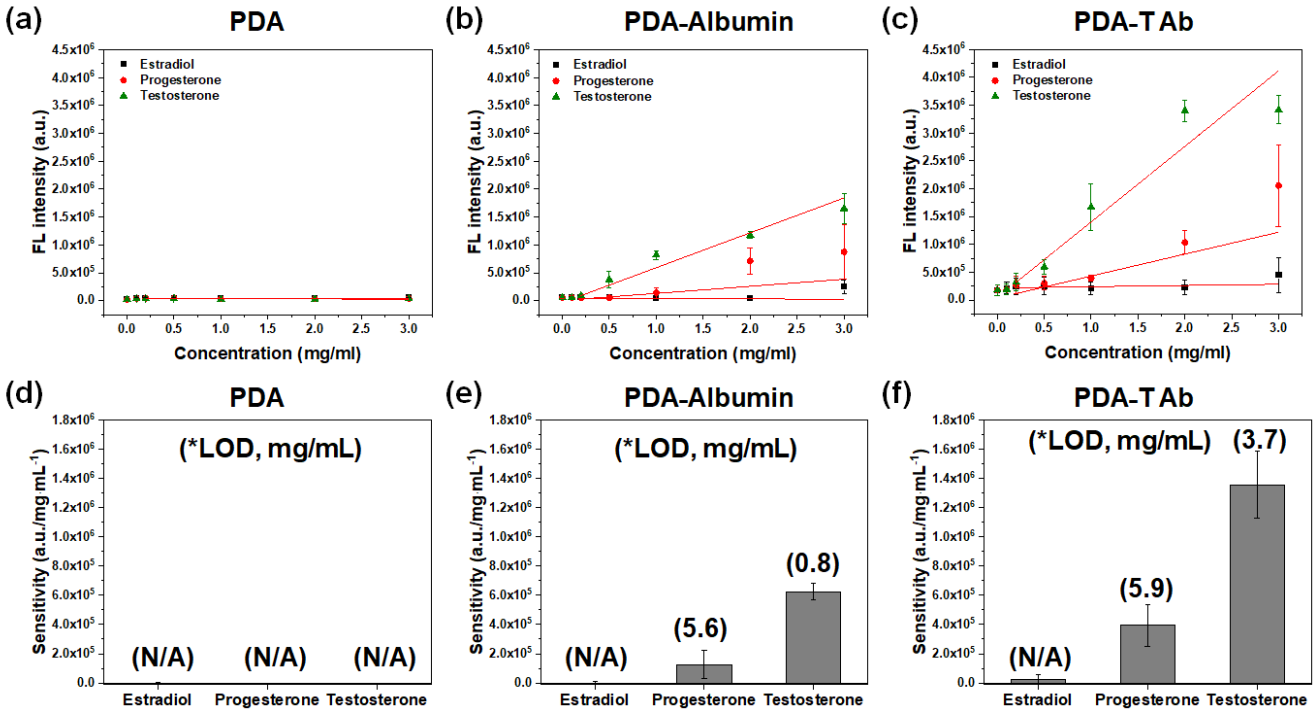

Figure 5. Fluorescence (FL) intensity of (**a**) PDA, (**b**) PDA-Albumin and (**c**) PDA-T Ab after incubation with sex steroid hormones (β-estradiol, progesterone and T). Sensitivity of sensing platforms based on (**d**) PDA, (**e**) PDA-Albumin and (**f**) PDA-T Ab for each sex steroid hormone. * *LOD*: limit of detection.

In order to prove a concept of PDA-based sensing platform for targeting T, FL signals of PDA-T Ab by introducing T was compared with those of PDA-T Ab in presence of β-estradiol and progesterone. As shown in Figure 5c, the PDA-T Ab did not generate FL after incubation with β-estradiol. The sensitivity of PDA-T Ab for T and progesterone was 1,400,000 a.u./mg·mL^{-1}, sensitivity for progesterone: 400,000 a.u./mg·mL^{-1}, in the range of 0.2–3 mg/mL of either hormone (Figure 5f). These results suggest that PDA-T Ab showed more discernable and selective sensory signaling than PDA-Albumin. In both PDA-Albumin and PDA-T Ab, *LOD* values for T and progesterone was similar, respectively. In summary, PDA-T Ab displayed a more selective fluorescent sensory signaling in the presence of T compared to PDA and PDA-Albumin.

To demonstrate T detection using the PDA-T Ab in any biologically complex matrix, we conducted spike tests with filtered human serum. In the spike test, the T hormone concentration-dependent sensory signaling was observed (Figure S3) as a similar trend in Figure 5f, however, the sensitivity was reduced comparing with that without biologically complex matrix. We supposed that non-filtered human serum proteins and small molecules of hormones could interrupt the sensitivity of the sensory signaling.

4. Conclusions

T Ab was conjugated to PDA to selectively detect T as a biomarker of preterm birth. The PDA-based sensory signals were evaluated by comparing with those of other two sex steroid hormones (progesterone and β-estradiol). To overcome the issue of the poor hormone solubility in aqueous solution, we adjusted a co-solvent medium of ACN and DI water (50% v/v) that enables solubilizing the hydrophobic steroid hormones and minimizing false signals from the medium. Although an accurate colorimetric response of PDA assemblies could not be determined due to the interfering absorption of the hormones, we demonstrated that fluorescence sensory signaling of PDA-T Ab was dose-dependent on T concentration and was selective for T hormone. This finding would give an insight for designing PDA-based sensors to detect broad spectrum of water-insoluble target analytes.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/chemosensors9070176/s1, Figure S1: Zeta potential values and size of PDA during conjugating albumin to PDA, Figure S2: FT-IR spectra of PDA, PDA-Albumin and PDA-T Ab, Figure S3: Fluorescence intensity of PDA-T Ab after incubation with testosterone hormones in filtered human serum.

Author Contributions: Conceptualization, S.-C.K., B.-S.A. and S.S.; validation, J.J. and S.-M.A.; formal analysis, investigation, data curation, J.J.; writing—original draft preparation, J.J. and S.S.; writing—review and editing, E.-K.L., S.-C.K., B.-S.A. and S.S.; supervision, project administration, S.S. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: There are no conflicts of interest to declare.

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