Supporting Information

Electrochemiluminescent Imaging for Immunoassay of Prostate Specific Antigen Utilizing AIE-active Polymer Dots as Probe

Hang Gao,* Jia-Yuan Zhu, Lingling Xu, Qin Xu*

School of Chemistry and Chemical Engineering, Yangzhou University, Yangzhou 225002, China

Corresponding author: gaohang@yzu.edu.cn (H. Gao); xuqin@yzu.edu.cn (Q. Xu)

Table of contents:

Experimental Section

Materials and Reagents

Triethylamine (TEA, ≥99%), tri-*n*-propylamine (TPrA), sodium oxalate ($Na_2C_2O_4$), tetrahydrofuran (THF, anhydrous, ≥99.9%, inhibitor-free), sodium carbonate (Na_2CO_3), and sodium hydroxide (NaOH) were purchased from Shanghai Aladdin Bio-Chem Technology Co., Ltd. Tetrakis(triphenylphosphine)palladium ($Pd(PPh_3)_4$) was bought from Shanghai J&K Scientific Ltd. Streptavidin (SA), bovine serum albumin (BSA), glutaraldehyde (GA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), polyethylene glycol (PEG, average Mw: 3350), and 3-Aminopropyltriethoxysilane (APTES) were obtained from Sigma-Aldrich Co., Ltd. (Shanghai, China). Prostate-specific antigen (PSA) and its mouse monoclonal antibodies labeled with biotin were purchased from Beijing Key-Bio Biotech Co., Ltd. (Beijing, China). CEA and CA199 were purchased from Beijing Key-Bio Biotech Co., Ltd. (Beijing, China). NSE were purchased from Shanghai Anyan Trading Co., Ltd. (Shanghai, China). Phi29 DNA polymerase, T4 DNA ligase, Exonuclease I (Exo I), Exo III, and dNTPs were purchased from Sangon Bioengineering Co., Ltd. (Shanghai, China). Oligonucleotides were synthesized and purified by Sangon Bioengineering Co., Ltd. (Shanghai, China), and their sequences were listed in the following Table. All other reagents were of analytical grade and directly used without further purification. Phosphate buffer saline (PBS, 0.1 M) was prepared by mixing stock solutions of NaH_2PO_4 and Na_2HPO_4. Ultrapure water obtained from a Millipore water purification system (≥18 MΩ cm, Milli-Q, Millipore) was used in all tests.

Apparatus

Transmission electron microscopic (TEM) images were acquired on a FEI Tecnai F20 transmission electron microscope (Thermo Fisher Scientific, U.S.A.). Zeta potential analysis was performed on a 90 Plus/BI-MA Sequipment (Brookhaven, U.S.A.). The UV-vis absorption spectra were obtained using a UV-3600 UV-vis-NIR spectrophotometer (Shimadzu Co.). Fluorescence measurements were conducted on an F-7000 fluorescence spectrometer (Hitachi Co., Japan) equipped with a xenon lamp. The absolute photoluminescence quantum yields (Φ_{PL}) and the decay lifetimes were measured with HORIBA FL-3 fluorescence spectrometer. The NMR spectra were obtained from a Bruker Advance 400 spectrometer (Bruker, German) with 400 MHz for

^{1}H NMR reported as parts per million (ppm) from the internal standard tetramethylsilane (TMS). Electrochemical experiments were performed on a CHI 660a electrochemical workstation (CH Instruments Inc., China). Electrochemical impedance spectroscopic (EIS) measurements were performed on a PGSTAT30/FRA2 system (Autolab, the Netherlands) in 0.1 M KCl aqueous solution containing 5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1). ECL measurements were carried out in a self-made cell on MPI-E multifunctional electrochemical and chemiluminescent analytical system (Xi'an Remex Analytical Instrument Co., Ltd. China). The visual ECL images of these Pdots were collected by Olympus DP71 CCD. Ultrasonic synthesis experiments were conducted on the sonicator (Elmasonic P30H, Germany).

Preparation of Electrodes

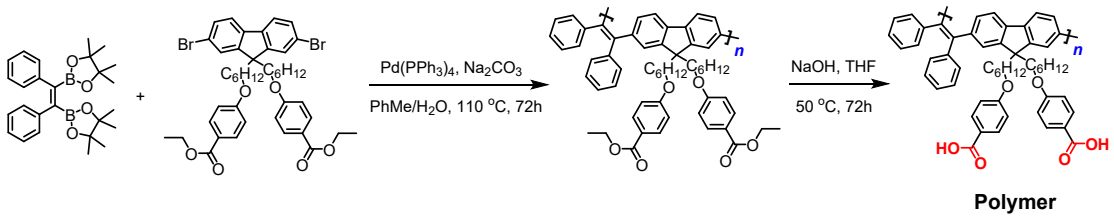
Glassy carbon electrode (GCE, 3 mm in diameter) was polished on a microcloth to a mirror surface using 0.02 - 0.05 μm alumina slurry, followed by sonication in water, water/ethanol (1:1), and water, successively. These electrodes were washed thoroughly with ultrapure water and dried in a steam of nitrogen for next experiments.

Electrochemical and ECL Measurements

The cyclic voltammograms (CVs) were examined using a conventional three-electrode system, where the modified GCE was used as a working electrode, a platinum wire was used as an auxiliary electrode, and an Ag/AgCl electrode (saturated KCl) was used as the reference electrode.

The glass carbon electrodes modified with Pdots were prepared by dropping 10 μL of 60 μM corresponding Pdots solution on an activated GCE and dried under 35 ^{o}C. The ECL curves were obtained by scanning from 0 to +1.50 V in 0.1 M PBS (pH 7.4) containing 25 mM TEA as anodic co-reactant. Unless otherwise stated, the ECL window was placed in front of the photomultiplier tube (PMT) biased at 400 V with a scan rate of 100 mV s^{-1}. The ECL emission spectrum of Pdots was obtained by a self-made ECL spectrum analyzer consisting of a CHI-660D electrochemical workstation and a FLS-980 fluorescence spectrophotometer. The ECL spectrum of Pdots modified electrode was recorded in 0.1 M pH 7.4 PBS containing 25 mM TEA at +1.4 V for 10 s.

Synthesis Procedures of the Polymers

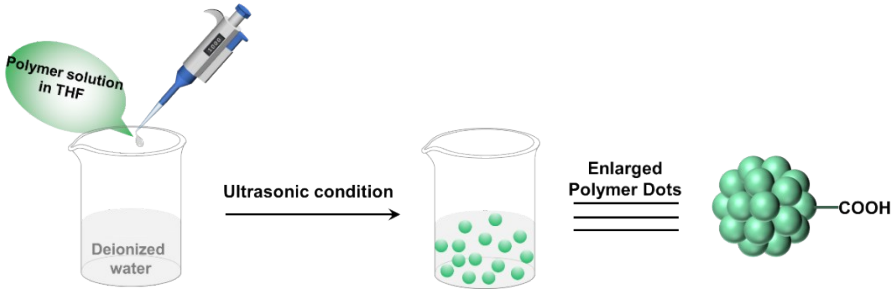


Scheme S1. The synthetic routes of polymer.

Under argon protection conditions, tetrakis(triphenylphosphine)palladium (39.9 mg, 3.5% mmol) was added to a mixture of (*Z*)-1,2-diphenyl-1,2-bis(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)ethene (150.0 mg, 0.35 mmol), diethyl 4,4'-(((2,7-dibromo-9H-fluorene-9,9-diyl)bis(hexane-6,1-diyl))bis(oxy))dibenzoate (284.9 mg, 0.35 mmol), and sodium carbonate (365.7 mg, 3.5 mmol) in toluene/water (10 mL: 1 mL). Afterward, the mixture was vigorously stirred at 110 °C for 72 h. After that, the reaction mixture was cooled to room temperature, and extracted with dichloromethane (DCM; 20 mL) for three times. The combined organic solution was successively washed with water and saturated salt solution. Then, the organic solution was dried over Na_2SO_4 and filtered. Subsequently, the solution was concentrated to 2 mL under reduced pressure, and purified by precipitation method in methanol (100 mL). Through filtration, the resulting polymer was afforded as green solid.

The polymer (80 mg, 0.09 mmol) mentioned above was dissolved in THF (10 mL), and NaOH (17.6 mg, 0.44 mmol) was added under stirred conditions. Subsequently, the reaction mixture was heated at 50 °C for 72 h. After cooling to room temperature, 0.1 M HCl was dropwise added to the mixture until pH value of the solution reaches 2. Then, the solvent was removed by evaporation under vacuum, and the crude product is re-dissolved in THF. After filtration to remove inorganic salt, the filtrate was injected into methanol (100 mL) and the precipitate was filtrated as desired polymer, a green powder.

Preparation of Pdots



Scheme S2. The preparation of Pdots.

Polymer (5.0 mg) was dissolved in THF (10.0 mL), and then 1 mL of the solution was diluted to 10 mL with THF, thus obtaining polymer solution of 50.0 μg mL^{-1}. Subsequently, 2 mL of solution mentioned above was quickly injected into 10 mL of ultrapure water under ultrasonic conditions, and continued ultrasound for 5 min. Afterward, through rotary evaporation under reduced pressure, the mixture was concentrated to 2 mL, and then filtrated through a 0.22 μm pore membrane, eventually obtaining 50.0 μg mL^{-1} Pdots.

Preparation of Pdots-BHQ

The Pdots dispersion (50 μg mL^{-1}, 500 μL) was mixed completely with HEPES buffer (1 M, 6 μL) and PEG (5% w/v, Mw 3350, 6 μL), and the pH was adjusted to 7.1. After that, 60 μL DNA1 (100 μM) and 60 μL EDC (5 mg mL^{-1}) were added to the mixture and vibrated for 3 h at room temperature. The resulting Pdots-DNA was separated by ultrafiltration for three times to remove free DNA1. Subsequently, the purified Pdots-DNA1 hybridized with molar equivalent BHQ-DNA2 in hybridization buffer (10 mM PBS, 0.25 M NaCl, pH 7.4) at 37 °C for 30 min to obtain the self-quenched probe, which was stored at 4 °C for further use.

Preparation of Circle DNA Template

The circle DNA template was prepared according to previous work with small modifications.[S1] 5 μL of phosphorylated linear DNA (100 μM), 10 μL of ligation DNA (100 μM), 10 μL of ultrapure water and 3 μL of 10×T4 buffer were mixed and annealed at 95 °C for 5 min, and then slowly cooled to room temperature. 2 μL of T4 DNA ligase (400 U μL^{-1}) was added to the mixture and incubated at 37 °C for 2 h. After the T4 DNA ligase was inactivated at 65 °C for 10 min, 5 μL 10×exonuclease I buffer, 5 μL 10×exonuclease III buffer, 4 μL of exonuclease I (20 U μL^{-1}) and 4 μL of exonuclease III (100 U μL^{-1}) were added in the mixture to incubate at 37 °C for 2 h for forming circular DNA template. The mixture was finally heated at 80 °C for 15 min to denature the exonuclease I and exonuclease III, and stored at 4 °C.

Gel Electrophoresis Analysis

Agarose hydrogel was prepared with 0.7% agarose, 1×TBE buffer and 1×GelRed dye. The agarose gel electrophoresis experiment was performed by injecting the loading samples containing 6 μL RCA product with and without primer DNA and 1.5 μL 6×loading buffer into the hydrogel and running at 110 V for 80 min. 15% native polyacrylamide gel was prepared using 1×TBE buffer, which was injected with the mixture of 5 μL DNA and 1.5 μL 6×loading buffer to

perform PAGE analysis by running at 120 V for 80 min, and then staining with GelRed dye for 30 min. Molecular Imager Gel Doc XR was used to obtain the resulting boards.

Preparation of ECL Immunosensors Array

Before experiments, aminated ITO electrodes were first prepared. In brief, the cut ITO electrodes were immersed in acetone and ethanol for ultrasonic cleaning for 20 min each time; then they were immersed in a mixture (60 mL of water, 22.5 mL of 30% hydrogen peroxide, and 22.5 mL of concentrated ammonia solution) and heated at 60 °C for 1 h to form hydroxyl group on the electrode surface. Subsequently, the hydroxylated ITO was immersed in a dichloromethane solution containing 2% aminopropyltriethoxysilane (APTES) at room temperature for 2 h, and amino groups were modified by silane coupling. Finally, ITO was washed with DCM twice and dried with nitrogen gas, thus obtaining an aminated ITO electrodes.

An insulating sticker with 1 × 7 well array was pasted on the aminated ITO electrodes that act as working electrode, in which each well has the diameter of 2 mm and depth of 1 mm. 2 μL of GA (25%) was dropped on the well electrodes to bond with amino group through condensation reaction. After incubation for 1 h, 10.0 μg mL^{-1} capture antibody (Ab1; 2 μL) was added to well electrodes. To fully bind the Ab1, the incubation time was set to overnight at 4 °C. After that, to block the nonspecific binding sites, 5% bovine serum albumin (2 μL) was added into the electrodes array and incubated for 30 min. Subsequently, the array was placed vertically and washed for three times, thus obtaining the immunosensors array applied for further imaging analysis. Note: after each modification step, excess nonspecific adsorbed materials were washed away with PBS (0.01 M, pH 7.4).

ECL Imaging Detection

Various concentration of PSA solution (2 μL) was added into wells. After incubation at 37 °C for 1 h, 10.0 μg mL^{-1} biotinylated detection antibody (Ab2) was added to recognize PSA. Subsequently, streptavidin (SA) was added into wells to bind Ab2. To activate RCA process, 5.0 μM biotin labeled primer DNA (2 μL) was added to wells, and incubated at 37 °C for 30 min for the recognition of SA moiety. At the same time, a mixture of circular DNA (7.5 μL), dNTPs (1 μL, 100.0 mM), phi29 DNA polymerase (10 U), 10× reaction buffer (2.5 μL), and 15 μL of ultrapure water was prepared. Subsequently, 2 μL of the mixture described above was injected into wells quickly, and incubated at 37 °C for 2.5 h. Afterwards, 2 μL of “off” probe Pdots-DNA1-DNA2 was

added to wells and incubated at 37 °C for 2 h. After that, the array electrodes were dried at 37 °C in dark, and the generated film on electrode surface was applied to perform ECL imaging. The measurements were conducted in 0.1 M PBS (pH = 7.4) containing 25 mM TEA as oxidative-reductive co-reactants, and the potential was set to +1.2 V. The acquisition time was set to 5 s.

2. Supplementary Figures and Tables

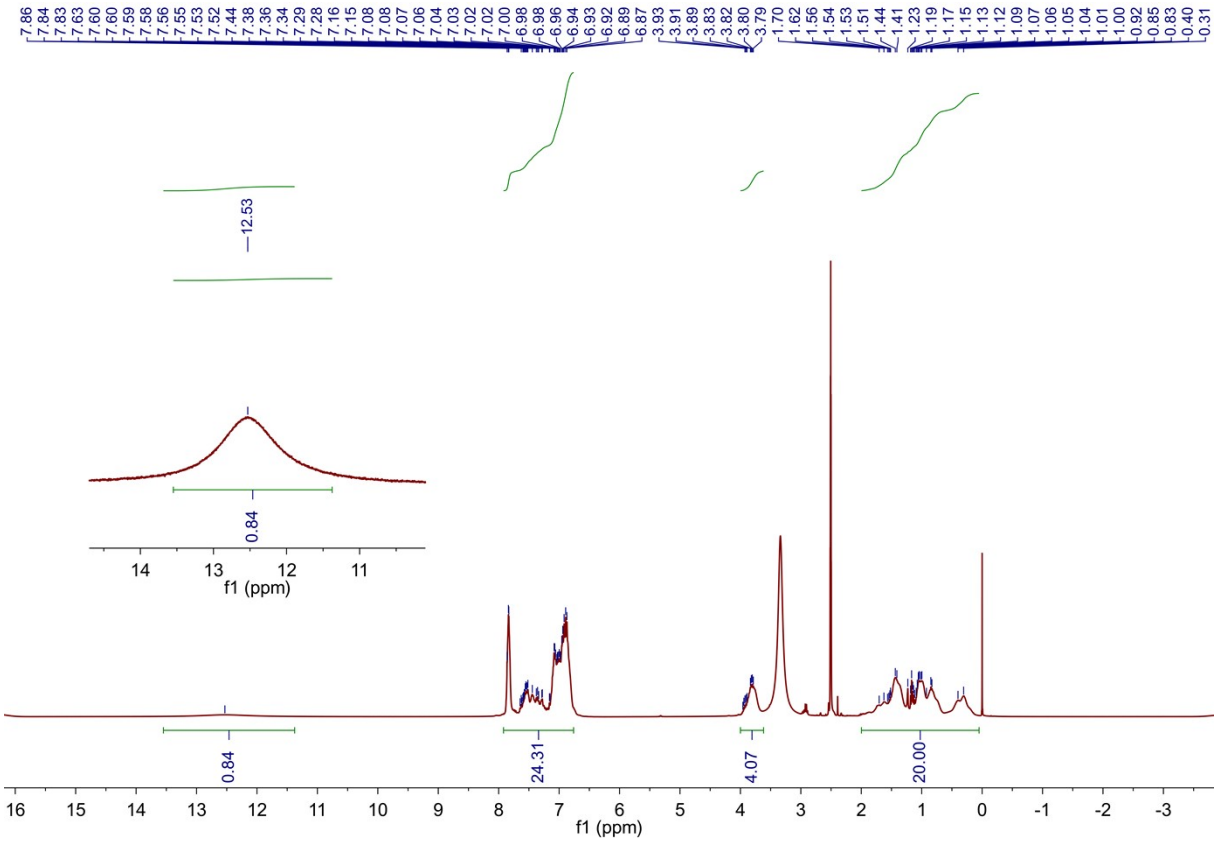


Fig. S1 ^{1}H NMR of Polymer.

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 12.53 (s, 2H), 7.86 - 6.87 (m, 24H), 3.93 - 3.79 (m, 4H), 1.70 - 0.31 (m, 20H).

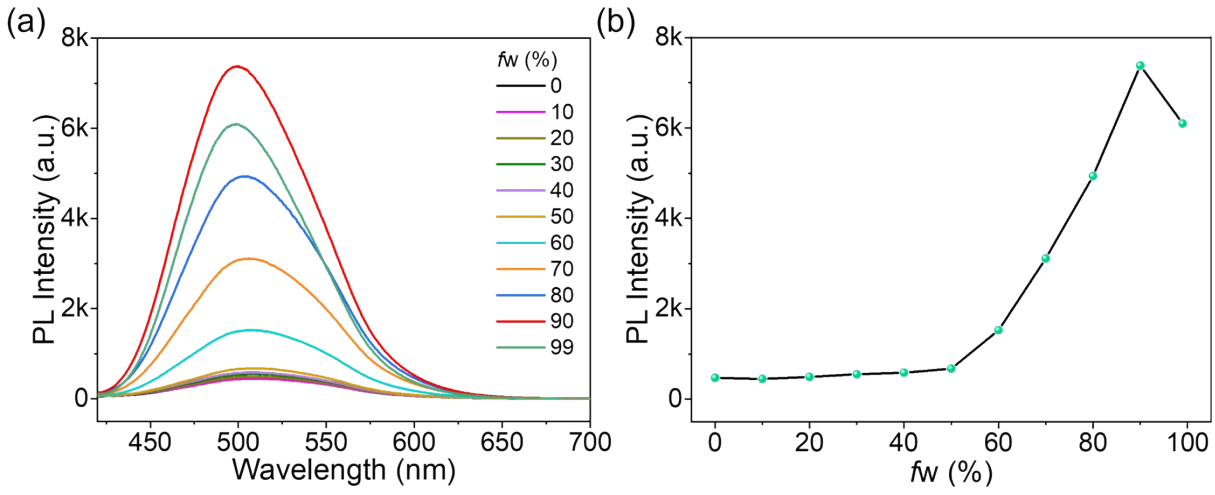


Fig. S2 PL spectra (a) and PL trends (b) of polymer in THF/H_2O mixtures with different water fractions (f_ws).

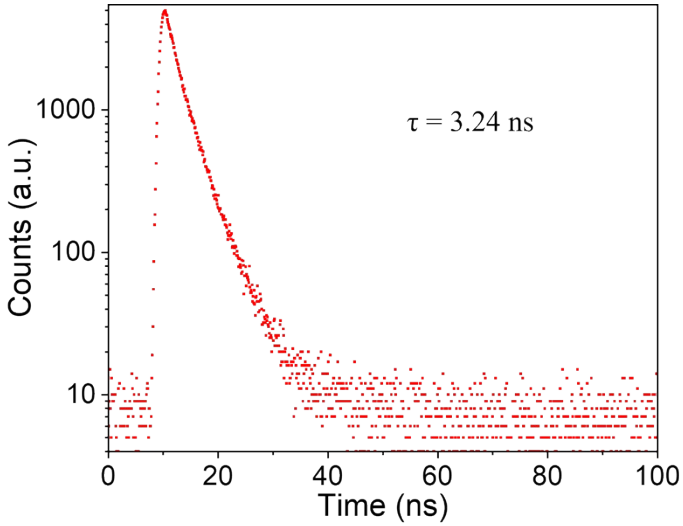


Fig. S3 Photoluminescent lifetime of Pdots measured at 298 K.

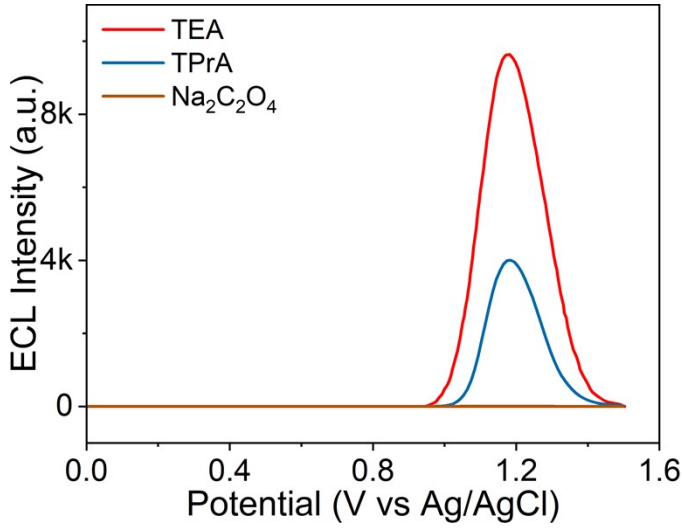


Fig. S4 ECL-potential curves of Pdots/GCE in 0.1 M PBS with different co-reactants (25 mM).

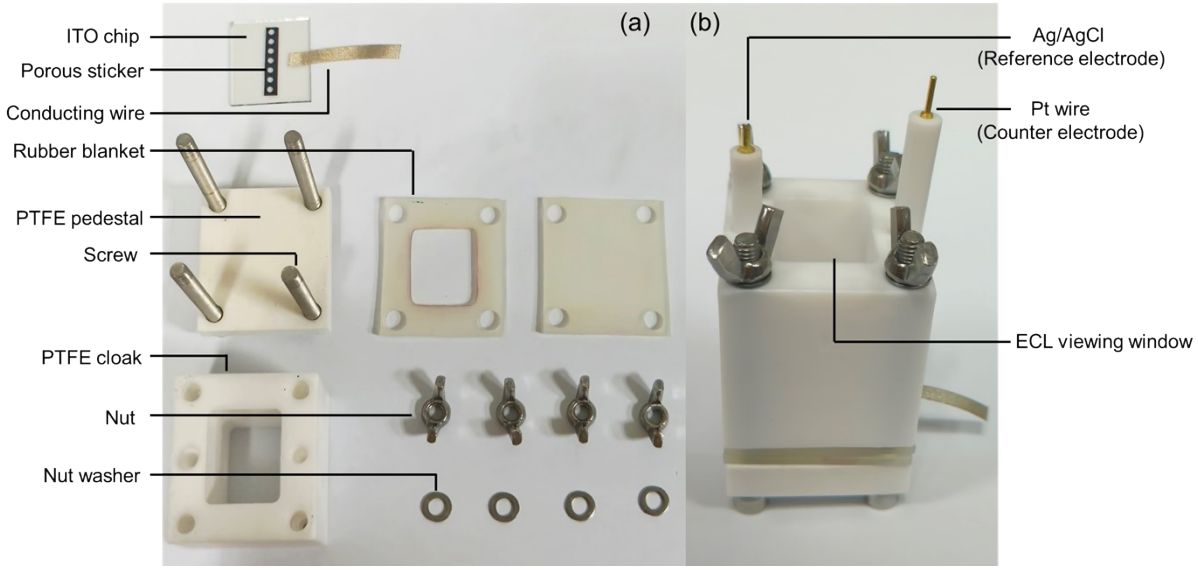


Fig. S5 Electrolytic cell for ECL imaging.

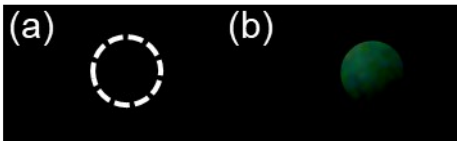


Fig. S6 ECL images of this sensor measured with probe Pdots-DNA1-DNA2 (a) and Pdots-DNA1 (b) in the absence of PSA.

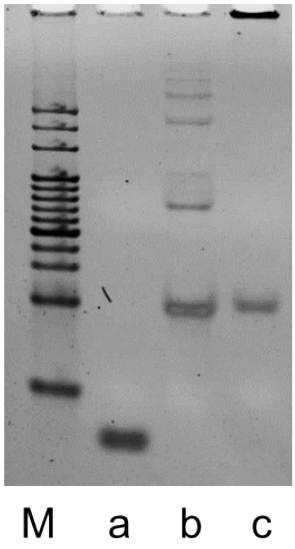


Fig. S7 PAGE analysis: Lane M: DNA ladder; Lanes a-c represent bio-primer DNA, circular DNA, and bio-primer DNA + circular DNA, respectively.

Table S1. DNA sequences used in this work.

DNA Name	Sequence (5'-3')
NH_2-DNA1	5'-NH_2-$(CH_2)_6$-TTTTTTTTTT-AGCAACCTCAAACAGACACTTAGG-3'
BHQ-DNA2	5'-GTTTGAGGTTGCTGGGGTTTTTTTTTT-BHQ-3'
Bio-primer DNA3	5'-biotin-AATCTGGACGCGCATGAA-3'
DNA3	5'-GATCCTAAGTGTCTGTTTGAGGTTGCTTTTCCTAAGTGTCTGTT TGAGGTTGCTTTTCCTAAGTGTCTGTTTGAGGTTGCTTTT-3'
Linear DNA	5'-P-TCCAAAGGAAAGCAACCTCAAACAGACACTTAGGAATTCA TGCGCGTCCAGATT-3'
Ligation DNA	5'-TTCCTTTGGAAATCTGGACG-3'

Table S2. Comparison of the linear range and detection limits of different methods for detection of PSA.

Methods	Linear range （ng/mL）	Detection limit (pg/mL)	Ref.
DPV	0.2 - 40	20	S2
DPV	0.05 - 80	1	S3
SWV	0.005 - 10	3	S4
SWV	0.001 - 10	0.4	S5
CV	0.1 - 100	1	S6
CV	0.05 - 50	15	S7
LSV	0.01 - 100	7	S8
PEC	0.1 - 50	76	S9
ECL	0.1 - 8	38	S10
ECL	0.005 - 5	0.17	S11
ECL	0.005 - 200	0.1	S12
ECL	0.001 - 100	0.44	S13
ECL	0.005 - 500	4.2	**This work**

Note: DPV, SWV, CV, LSV, PEC, and ECL represent different pulse voltammetry, square wave voltammetry, cyclic voltammetry, linear sweep voltammetry, photoelectrochemistry, and electrochemiluminescence, respectively.

References:

[S1] Ren, K.; Liu, Y.; Wu, J.; Zhang, Y.; Zhu, J.; Yang, M.; Ju, H. A DNA dual lock-and-key strategy for cell-subtype-specific siRNA delivery. *Nat. Commun*. **2016**, *7*, 13580

[S2] Salimi, A.; Kavosi, B.; Fathi, F.; Hallaj, R., Highly Sensitive Immunosensing of Prostate-Specific Antigen Based on Ionic Liquid-Carbon Nanotubes Modified eElectrode: Application as Cancer Biomarker for Prostatebiopsies. *Biosensors and Bioelectronics* **2013**, *42*, 439-446.

[S3] Kavosi, B.; Salimi, A.; Hallaj, R.; Amani, K., A Highly Sensitive Prostate-Specific Antigen Immunosensor Based on Gold Nanoparticles/PAMAM Dendrimer Loaded on MWCNTS/Chitosan/Ionic Liquid Nanocomposite. *Biosensors and Bioelectronics* **2014**, *52*, 20-28.

[S4] Yang, M.; Javadi, A.; Gong, S., Sensitive Electrochemical Immunosensor for the Detection of Cancer Biomarker Using Quantum Dot Functionalized Graphene Sheets as Labels. *Sensors and Actuators B: Chemical* **2011**, *155*, 357-360.

[S5] Akter, R.; Rahman, M. A.; Rhee, C. K., Amplified Electrochemical Detection of a Cancer Biomarker by Enhanced Precipitation Using Horseradish Peroxidase Attached on Carbon Nanotubes. *Analytical Chemistry* **2012**, *84*, 6407-6415.

[S6] Zhang, J.; Ting, B. P.; Khan, M.; Pearce, M. C.; Yang, Y.; Gao, Z.; Ying, J. Y., Pt Nanoparticle Label-Mediated Deposition of Pt Catalyst for Ultrasensitive Electrochemical Immunosensors. *Biosensors and Bioelectronics* **2010**, *26*, 418-423.

[S7] Wang, H.; Zhang, Y.; Yu, H.; Wu, D.; Ma, H.; Li, H.; Du, B.; Wei, Q., Label-Free Electrochemical Immunosensor for Prostate-Specific Antigen Based on Silver Hybridized Mesoporous Silica Nanoparticles. *Analytical Biochemistry* **2013**, *434*, 123-127.

[S8] Qu, B.; Guo, L.; Chu, X.; Wu, D. H.; Shen, G. L.; Yu, R. Q., An Electrochemical Immunosensor Based on Enzyme-Encapsulated Liposomes and Biocatalytic Metal Deposition. *Analytica Chimica Acta* **2010**, *663*, 147-152.

[S9] Z. Lv , R. Zeng , L. Zhu , Z. Qiu , M. Li and D. Tang , Photoelectrochemical immunoassay for prostate-specific antigen based on gasochromic reaction of Pd-MoO_3. *Sens. Actuators B Chem.* **2022**, *370*, 132440.

[S10] Qi, H.; Wang, C.; Qiu, X.; Gao, Q.; Zhang, C., Reagent-Less Electrogenerated Chemiluminescence Peptide-Based Biosensor for the Determination of Prostate-Specific Antigen. *Talanta* **2012**, *100*, 162-167.

[S11] Cao, J. T.; Yang, J. J.; Zhao, L. Z.; Wang, Y. L.; Wang, H.; Liu, Y. M.; Ma, S. H., Graphene Oxide@Gold Nanorods-Based Multiple-Assisted Electrochemiluminescence Signal

Amplification Strategy for Sensitive Detection of Prostate Specific Antigen. *Biosensors and Bioelectronics* **2018**, *99*, 92-98.

[S12] Khoshfetrat, S. M.; Khoshsafar, H.; Afkhami, A.; Mehrgardi, A.; Bagheri, H. Enhanced Visual Wireless Electrochemiluminescence Immunosensing of Prostate-Specific Antigen Based on the Luminol Loaded into MIL-53(Fe)-NH2 Accelerator and Hydrogen Evolution Reaction Mediation. *Anal. Chem*. **2019**, *91*, 6383– 6390.

[S13] Yang, C.; Guo, Q.; Lu, Y.; Zhang, B.; Nie, G. J. S.; Chemical, A. B. Ultrasensitive "signal-on" electrochemiluminescence immunosensor for prostate-specific antigen detection based on novel nanoprobe and poly (indole-6-carboxylic acid)/flower-like Au nanocomposite. *Sens. Actuators B Chem.* **2020**, *303*, 127246.