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Spectrum-resolved Dual-color Electrochemiluminescence Immunoassay for Simultaneous Detection of Two Targets with Nanocrystals as Tags

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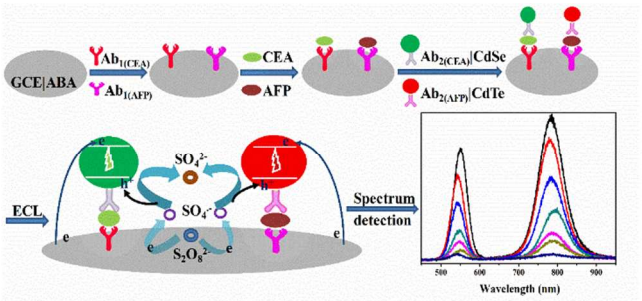
ABSTRACT: A spectrum-resolved dual-color electrochemiluminescence (ECL) immunoassay was designed and implemented to simultaneously detect carcinoembryonic antigen (CEA) and alpha fetoprotein (AFP) with CdTe (λ_{max} = 776 nm) and CdSe (λ_{max} = 550 nm) nanocrystals (NCs) as ECL tags. The CdTe and CdSe NCs were labeled with respective probe antibodies (Ab_2) of CEA and AFP, respectively, and then immobilized onto the working electrode surface via sandwich-type immunoreactions. Both CdTe and CdSe NCs within the NCs-immunocomplexes can be electrochemically reduced and simultaneously give off monochromatic ECL emissions in near-infrared and greenish region, respectively, when $(NH_4)_2S_2O_8$ was used as a cathodic ECL coreactant. The ECL spectra of the two surface-confined NCs were well separated and had no cross energy-transfer interactions, which made the dual-color immunoassay to be highly selective and sensitive towards respective target analytes. With the proposed ECL biosensor, CEA and AFP were simultaneously detected and quantified with an extremely low detection limit of 1 pg/mL for CEA and 10 fg/mL for AFP, respectively. This work demonstrated the probability of performing multi-analyte assays via a spectrum-resolved ECL strategy with improved sensitivity and signal-to-noise ratio as compared to NCs-based fluorescent multi-analyte assays.

Multiplexing analysis permits great throughput, minimizes sample volume and handling, decreases assay cost and increases the amount of information obtainable from each sample. Various approaches for conducting multiplexed assays have been described using, for example, fluorescence (FL) microspheres or semiconductor nanocrystals (NCs, or quantum dots) probes,[1-9] electrochemical microelectrode arrays,[10] and electrochemical redox reactions associated with target biomolecules.[11-13] Electrochemiluminescence (ECL) is a controllable mode of chemiluminescence (CL) and its light generation is initiated by the electron-transfer reactions occurred at electrode surface.[14,15] ECL has many distinct advantages over other spectroscopic-based detection systems,[16] and ECL related bioassay has proven to hold excellent reproducibility, sensitivity, and limit of detection over other assays including enzyme-linked immunosorbent assay (ELISA), CL, FL, and surface plasmon resonance based immunosensors.[17-20] Typically, the commercial ECL detection system based on $Ru(bpy)_3^{2+}$/tri-*n*-propylamine reagent kits has been widely used in biomedical and diagnostics assays.[14,21] However, the developments of ECL based multiplexed analysis is still limited, despite the facts that some promising potential-resolved,[22-26] and imaging or spatial-based[27-32] strategies have been recently proposed. Actually, the ECL-potential profile is usually found to be broad, hence poor resolution is expected when potential-resolved ECL detection method is used. On the other hand, imaging or spatial-based ECL analysis often involves the use of specially designed electrochemically conductive multi-well plates and a highly sensitive imaging system, not to mention that a large volume of test solution is needed for conducting multiple target analysis.



Scheme 1. Schematic illustration of spectrum-resolved dual-color ECL immunoassay with nanocrystals as tags

Recently, our group developed a dual-stabilizers-capped strategy to prepare CdTe and CdSe NCs with monochromatic ECL emission,[33-35] and designed an homemade ECL spectrum acquiring system,[36-38] which could perform spectrum-based ECL immunoassay in near-infrared and greenish waveband with the dual-stabilizers-capped CdTe and CdSe NCs at tags, respectively.[39,40] Inspired by FL based spectroscopic multiplexing assays,[4] herein we proposed a spectrum-resolved dual-color ECL immunoassay for simultaneous detecting two antigens with the dual-stabizers-capped CdTe and CdSe NCs as ECL tags. Scheme 1 shows the principle of the dual-color ECL immunoassay, where individual capture antibodies (Ab_1) for antigen CEA (CEA = carcinoembryonic antigen, Ag) and antigen AFP (AFP = alpha fetoprotein, Ag) are simultaneous and covalently attached to the glassy carbon electrode (GCE) surface via an electrochemically deposited ABA film (ABA = p-aminobenzoic acid) and result in GCE|ABA-$\{^{Ab_{1(CEA)}}_{Ab_{1(AFP)}}$. A sample solution containing both CEA and AFP targets are then al-

lowed to interact with the capture antibodies to form respective antibody<antigen conjugates on GCE, i.e. GCE|ABA-$\{^{Ab_{1(CEA)} < CEA}_{Ab_{1(AFP)} < APF}$. Surface-confined sandwich type immuno-complexes on GCE, i.e. GCE|ABA-$\{^{Ab_{1(CEA)} < CEA > Ab_{2(CEA)}|CdSe}_{Ab_{1(AFP)} < APF > Ab_{2(AFP)}|CdTe}$ were then obtained with NCs labeled probe antibodies (Ab_2), i.e., $Ab_{2(CEA)}$|CdSe conjugate and $Ab_{2(AFP)}$|CdTe conjugate. The spectral ECL wavelength and intensity of GCE|ABA-$\{^{Ab_{1(CEA)} < CEA > Ab_{2(CEA)}|CdSe}_{Ab_{1(AFP)} < APF > Ab_{2(AFP)}|CdTe}$ were directly correlated to the identity and concentration of respective target antigens present in the same solution, which provides a basis for detection and quantification of target analytes.

Ideally, prospective ECL tags used in this study should meet several criteria: (1) high ECL efficiencies for high sensing sensitivity and low detection limits; (2) sufficient and suitable functional groups to label antibodies; (3) spectral distinguishable ECL emission for spectrum-based ECL signal separation and the individual species quantification; (4) simultaneous ECL emission from different tags under the same experimental conditions.

On the basis of our previous studies in monocharomatic electrochemiluminophores and spectrum-based monocharomatic ECL immunoassays,[33-40] dual-stabilizers-capped CdSe (λ_{max} = 550 nm) NCs with monocharmatic ECL in greenish wavebands and dual-stabilizers-capped CdSe CdTe (λ_{max} = 776 nm) NCs with monocharmatic ECL in near-infrared wavebands were chosen as our present dual ECL tags for the spectrum-resolved mutilpexing ECL asasay. These two NCs can simultaneously produce efficient "reductive-oxidation" ECL using $(NH_4)_2S_2O_8$ as ECL coreactant. No overlapping in spectra ECL waves and no energy-transfer between the two NCs were found, making the proposed system to be an excellent model of an ECL spectrum-resolved multiplexing detection biosensor.

EXPERIMENTAL SECTION

Chemicals and Materials. All chemical reagents were of analytical grade or better and used as received, and all aqueous solutions were prepared with doubly distilled (DD) water (see Supporting Information). Carcinoembryonic antigen (CEA, Ag), capture CEA antibody ($Ab_{1(CEA)}$), probe CEA antibody ($Ab_{2(CEA)}$), prostate specific antigen (PSA, Ag), alpha fetoprotein (AFP, Ag), capture AFP antibody ($Ab_{1(AFP)}$), probe AFP antibody ($Ab_{2(AFP)}$), and carbohydrate antigen 125 (CA125, Ag) were purchased from Beijing Biosynthesis Biotechnology Co., Ltd. (Beijing, China) and diluted with 10 mM phosphate buffer solution (PBS, pH 7.4) before use. The CdTe (λ_{max} = 776 nm) and CdSe (λ_{max} = 550 nm) NCs were prepared as reported previously[33,34] (see Supporting Information for details). Both CdSe NCs labeled $Ab_{2(CEA)}$, i.e., $Ab_{2(CEA)}$|CdSe conjugate, and CdTe NCs labeled $Ab_{2(AFP)}$, i.e., $Ab_{2(AFP)}$|CdTe conjugate, were prepared with the previously developed N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) assisted labeling protocol (see Supporting Information).[21,39,40]

Apparatus. The instruments and methods used for characterizing common optical, electrochemical, and ECL properties of the present test system have been detailed in our previous papers.[36,39,40]. Ultraviolet−visible (UV−vis) absorption measurements were carried out on a TU-1901 UV−vis spectrophotometer (Beijing Purkinje General Instrument Co., Ltd., China). Photoluminescence (PL) spectra were recorded using an F-320 spectrofluorimeter (Tianjin Gangdong Sci & Tech Development Co., Ltd., China). The photoluminescence quantum yield (PLQY) was recorded with a fluorescence spectrometer (Model FLS920, Edinburgh Instruments, U.K.). Potential-resolved ECL and cyclic voltammetric (CV) measurements were carried out on an MPI-EII ECL analyzer (Xi'an Remex Analytical Instrument Co., Ltd., China) by using a single chamber quartz ECL cell along with a three-electrode system consisting of a glassy carbon working electrode (GCE, diameter of 5 mm), a Pt coil counter electrode, and a Ag/AgCl (saturated KCl) reference electrode. The voltage of the photomultiplier tube (PMT, 185-850 nm) was biased at 600 V during ECL measurements. Electrochemical impedance spectroscopy (EIS) was conducted with a VersaSTAT 3 electrochemical analyzer (Princeton Applied Research, U.S.A.). ECL spectra acquisition was accomplished with a homemade ECL spectrometer consisting of an Acton SP2300i monochromator equipped with a liquid N_2 cooled PyLoN 400BR-eXcelon digital CCD detector (Princeton Instruments, U.S.A) and a VersaSTAT 3 electrochemical analyzer (Princeton Applied Research, U.S.A.).[37,38] TTL strategy was utilized to simultaneously trigger VersaSTAT 3 electrochemical analyzer and Acton SP2300i monochromator, ECL generated at the electrode surface was collected with an objective lens and then delivered to the Acton SP2300i monochromator.

Procedures for Dual-color ECL Immunoassay. The surface-confined sandwich type immuno-complexes on GCE were fabricated via previously reported route with p-aminobenzoic acid (ABA) as linker mocules.[37,39,40] After polished with 0.3 μm alumina slurry and thoroughly rinsed with DD water, GCE was scanned from 0.40 to 1.20 V (vs Ag/AgCl) at 10 mV/s for three cycles in 10 mM pH 7.4 PBS containing 1.0 mM p-aminobenzoic acid (ABA) and 10 mM KCl to form GCE|ABA.[21] The GCE|ABA was activated in 0.10 M PBS (pH 6.0) containing 100 mg/mL EDAC and 100 mg/mL NHS, and then incubated in 10 mM pH 7.4 PBS containing a mixture of 1 μg/mL $Ab_{1(CEA)}$ and 100 ng/mL $Ab_{1(AFP)}$ at room temperature for 3 h to covalently bind both $Ab_{1(CEA)}$ and $Ab_{1(AFP)}$. The obtained GCE|ABA-$\{^{Ab_{1(CEA)}}_{Ab_{1(AFP)}}$ was rinsed with 10 mM pH 7.4 PBS and DD water to eliminate physically absorbed Ab_1 and then blocked with 1 % bovine serum albumin (in pH 7.4 PBS) for 30 min to remove any remaining activated carboxyl sites. A drop of 20.0 μL sample containing CEA and AFP of various concentrations was casted on GCE|ABA-$\{^{Ab_{1(CEA)}}_{Ab_{1(AFP)}}$ and then incubated at 30 °C for 90 min to form GCE|ABA-$\{^{Ab_{1(CEA)} < CEA}_{Ab_{1(AFP)} < APF}$ conjugates. The resultant electrode was allowed to react with 100.0 μL PBS (10 mM, pH 7.4) containing both $Ab_{2(AFP)}$|CdTe and $Ab_{2(CEA)}$|CdSe conjugates for 1 h to form GCE|ABA-$\{^{Ab_{1(CEA)} < CEA > Ab_{2(CEA)}|CdSe}_{Ab_{1(AFP)} < APF > Ab_{2(AFP)}|CdTe}$. Electrodes used for control experiments were prepared in a similar fashion without adding a specific antigen.

The proposed dual-color ECL immunoassay is carried out on homemade ECL spectrometer,[36,39,40] in which a cyclic potential from 0.0 to -1.60 V vs Ag/AgCl is applied to GCE|ABA-$\{^{Ab_{1(CEA)} < CEA > Ab_{2(CEA)}|CdSe}_{Ab_{1(AFP)} < APF > Ab_{2(AFP)}|CdTe}$ for one cycle in contact with 0.10 M $(NH_4)_2S_2O_8$-0.10 M pH 7.4 PBS at a scan rate of 50 mV/s. All the photons generated in the whole ECL process were collected by the CCD camera and utilized to depict the ECL spectrum.

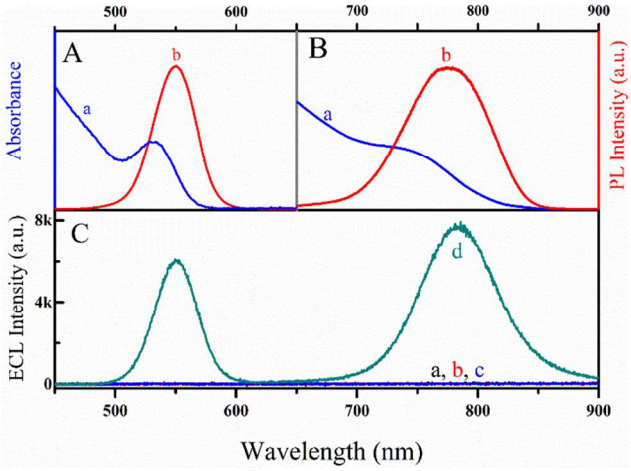


Figure 1. (A) Dual-stabilizers-capped CdSe (λmax = 550 nm) and (B) CdTe (λmax = 776 nm) NCs' (a) absorption and (b) PL spectra; (C) ECL spectra obtained from (a) GCE, (b) GCE|ABA, (c) GCE|ABA-$\{^{Ab_{1(CEA)} < CEA > Ab_{2(CEA)}}_{Ab_{1(AFP)} < APF > Ab_{2(AFP)}}$, and (d) GCE|ABA-$\{^{Ab_{1(CEA)} < CEA > Ab_{2(CEA)}|CdSe}_{Ab_{1(AFP)} < APF > Ab_{2(AFP)}|CdTe}$ in 0.10 M $(NH_4)_2S_2O_8$-0.10 M pH 7.4 PBS upon a potential cycling between 0 and -1.60 V vs Ag/AgCl for one cycle at 50 mV/s. The ECL spectra resulted from the total photons generated during the cyclic potential scanning using a homemade ECL spectrometer. The immune-complexes were formed with 20.0 μL samples containing both 10 ng/mL CEA and 100 pg/mL AFP.

RESULTS AND DISCUSSION

Characterization of the Electrochemiluminophore and Dual-color ECL immunosensor. As depicted in Figure 1, the dual-stabilizers-capped CdSe (λ_{max} = 550 nm) NCs display an absorption peak at ~531 nm and a symmetrical narrow PL peak at ~550 nm (Figure 1A), while dual-stabilizers-capped CdTe (λ_{max} = 776 nm) NCs show an absorption peak at ~735 nm and a nearly symmetrical and relatively broad PL peak at ~776 nm (Figure 1B). These results indicate that the as-prepared CdSe (λ_{max} = 550 nm) and CdTe (λ_{max} = 776 nm) NCs are almost monodisperse.[41,42] Previous research has demonstrated that dual-stabilizers-capped CdSe and CdTe NCs could preserve their absorption and emission features in the Ab_2-NCs conjugates,[21,37] and were promising monochromatic electrochemiluminophores for color-selective ECL immunoassays.[39,40] In the present study, the PLQY of CdSe (λ_{max} = 550 nm) NCs and their Ab_2 conjugates $Ab_{2(CEA)}$|CdSe was determined to be ~11.6% and 6.70%, respectively. On the other hand, CdTe (λ_{max} = 776 nm) NCs and their Ab_2 conjugates $Ab_{2(AFP)}$|CdTe showed a PLQY of ~19.7% and 11.1%, respectively. Our previous studies have shown that the both dual-stabilziers-capped CdTe NCs and CdSe NCs could preserve their ECL nature in corresponding Ab_2|NCs conjugates and were efficient electrochemiluminophores for immunoassay.[21,39,40]

As shown in Figure 1C, in 0.10 M $(NH_4)_2S_2O_8$-0.10 M pH 7.4 PBS, no obvious ECL is observed from bare GCE (curve a), GCE|ABA (curve b), and GCE|ABA-$\{^{Ab_{1(CEA)} < CEA > Ab_{2(CEA)}}_{Ab_{1(AFP)} < APF > Ab_{2(AFP)}}$ (curve c) when the electrode is cycled between 0 and -1.60 V for one cycle, whereas efficient reductive-oxidation ECL[43] is obtained on the GCE|ABA-$\{^{Ab_{1(CEA)} < CEA > Ab_{2(CEA)}|CdSe}_{Ab_{1(AFP)} < APF > Ab_{2(AFP)}|CdTe}$ prepared with 20.0 μL sample containing both 10 ng/mL CEA and 100 pg/mL AFP (curve d). Spectrum resolved ECL immunoassay for determining single analyte has shown that co-existed proteins in the test sample would not compromise the bioactivity of probe antibody in both Ab_2|CdSe NCs and Ab_2|CdTe NCs conjugates,[39,40] the two Ab_2|NCs conjugates could be efficiently immobilized onto the GCE electrode via bio-selective sandwich immunoreactions. It is evident that the ECL spectra obtained from the GCE|ABA-$\{^{Ab_{1(CEA)} < CEA > Ab_{2(CEA)}|CdSe}_{Ab_{1(AFP)} < APF > Ab_{2(AFP)}|CdTe}$ show two well-distinctive emissions peaked at ~547 nm from CdSe NCs and ~783 nm from CdTe NCs, respectively. The individual ECL emission matches well with respective PL emission, confirming that both the dual-stabilizers-capped CdSe and CdTe NCs in respective Ab_2|NCs conjugates were simultaneous immobilized onto GCE, and preserved their monochromatic ECL nature in the immune-complexes.[39,40] Clearly, color-selective ECL of different wavebands is achieved by simultaneous electrochemical reduction of the dual-stabilizers-capped CdSe and CdTe NCs in the presence of cathodic ECL coreactant $(NH_4)_2S_2O_8$. It is well known that the color-selective PL nature of NCs enables them to be excited together with a single excitation source for simultaneous determination of multiple targets.[1,4,44] Compared to the PL method, the present ECL spectrum-resolved multiplexing detection strategy could provide a much higher signal-to-noise ratio hence a much lower detection limit, because unlike PL, ECL has almost no background signals. CV and EIS responses of the stepwise modified GCE (Figures S1 and S2 in Supporting Information), i.e., the bare GCE, GCE|ABA, GCE|ABA-$\{^{Ab_{1(CEA)}}_{Ab_{1(AFP)}}$, GCE|ABA-$\{^{Ab_{1(CEA)} < CEA}_{Ab_{1(AFP)} < APF}$, and GCE|ABA-$\{^{Ab_{1(CEA)} < CEA > Ab_{2(CEA)}|CdSe}_{Ab_{1(AFP)} < APF > Ab_{2(AFP)}|CdTe}$ further confirmed that the probe antibodies preserved their bio-activity in both $Ab_{2(AFP)}$|CdTe and $Ab_{2(CEA)}$|CdSe conjugates, respectively, so that the ECL tags, i.e. both dual-stabilizers-capped CdSe and CdTe NCs, could be simultaneous immobilized onto GCE surface via sandwich typed immune-reactions.[21,37,39,40]

Potential-resolved multiplexed simultaneous detection with the present ECL system was found to be impractical. As shown in Figure S3, the onset as well as the maximum cathodic ECL emission potentials from GCE|ABA-$\{^{Ab_{1(CEA)} <}_{Ab_{1(AFP)} < APF > Ab_{2(AFP)}|CdTe}$ and GCE|ABA-$\{^{Ab_{1(CEA)} < CEA > Ab_{2(CEA)}|CdSe}_{Ab_{1(AFP)}}$ electrodes are very close and largely overlapped.

A typical reductive-oxidation ECL mechanism[14] is believed to be operative for the present dual-color GCE|ABA-$\{^{Ab_{1(CEA)} < CEA > Ab_{2(CEA)}|CdSe}_{Ab_{1(AFP)} < APF > Ab_{2(AFP)}|CdTe}$/$S_2O_4^{2-}$ ECL system. Upon the cathodic potential scanning, strong oxidant $SO_4^{-\bullet}$ (E_0 > 3.15 V vs SCE)[14] and negatively charged NCs radicals ($R^{-\bullet}$), i.e., $R_{(CdSe)}^{-\bullet}$ and $R_{(CdTe)}^{-\bullet}$, are produced via the electrochemical reduction processes, respectively (eqs. 1, 2, and 3).[45] Then, the strong oxidant $SO_4^{-\bullet}$ radical injects holes into the highest occupied molecular orbital (HOMO) of $R^{-\bullet}$, producing an excited state R*, i.e., $R_{(CdSe)}^{*}$ and $R_{(CdTe)}^{*}$ (eqs. 4 and 5). Finally, dual-color ECL is generated along with the light emissions from the excited species $R_{(CdSe)}^{*}$ and $R_{(CdTe)}^{*}$ (eqs. 6 and 7).

$$S_2O_8^{2-} + e \rightarrow S_2O_8^{3-\bullet} \rightarrow SO_4^{2-} + SO_4^{-\bullet} \quad (1)$$

$$CdSe\ NCs + ne \rightarrow nR_{(CdSe)}^{-\bullet} \quad (2)$$

$$CdTe\ NCs + ne \rightarrow nR_{(CdTe)}^{-\bullet} \quad (3)$$

$$SO_4^{-\bullet} + R_{(CdSe)}^{-\bullet} \rightarrow R_{(CdTe)}^{*} + SO_4^{2-} \quad (4)$$

$$SO_4^{-\bullet} + R_{(CdTe)}^{-\bullet} \rightarrow R_{(CdTe)}^{*} + SO_4^{2-} \quad (5)$$

$$R_{(CdSe)}^{*} \rightarrow CdSe + h\nu\,(\sim 547\ nm) \quad (6)$$

$$R_{(CdTe)}^{*} \rightarrow CdTe + h\nu\,(\sim 783\ nm) \quad (7)$$

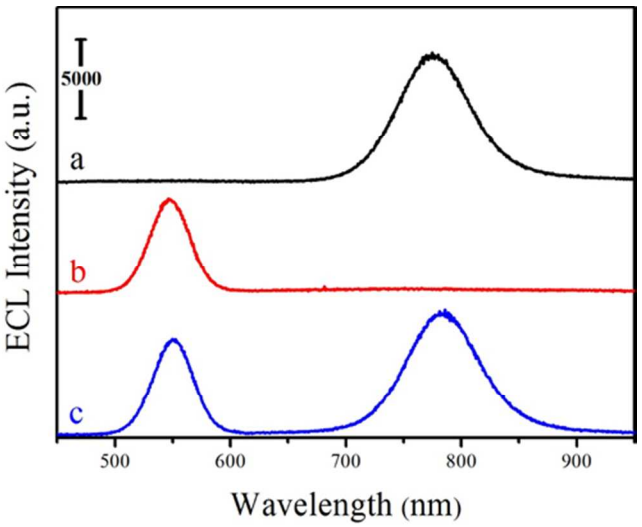


Figure 2. Color-selectivity and cross-reactivity of the dual-color multiplexing ECL immunoassay towards samples containing: (a) 100 pg/mL AFP, (b) 10 ng/mL CEA, and (c) 10 ng/mL CEA + 100 pg/mL AFP. Experimental conditions for ECL generation and collection were the same as in Figure 1C.

Color-selectivity and Cross-reactivity of the Dual-color ECL Sensing Strategy. The color-selectivity and cross-reactivity of the proposed dual-color multiplexing ECL immunoassay were examined with the ECL spectra acquired from samples containing different analytes (Figure 2). When the sample contains only AFP antigen, the ECL spectra obtained from the GCE|ABA-$\{^{Ab_{1(CEA)}}_{Ab_{1(AFP)}} < APF > Ab_{2(AFP)}|CdTe$ shows a single peak at ~783 nm with the full width at half maximum (FWHM) of ~76 nm (curve a); no detectable ECL emission is observed at wavelength shorter than 650 nm. Similarly, a single ECL peak at ~547 nm with the FWHM of ~37 nm is observed from the GCE|ABA-$\{^{Ab_{1(CEA)} < CEA > Ab_{2(CEA)}|CdSe}_{Ab_{1(AFP)}}$ prepared with a sample containing only CEA antigen (curve b); no obvious ECL emission can be seen at wavelength beyond 650 nm. Importantly, as shown in Figure 2c (also curve d in Figure 1C), when the GCE|ABA-$\{^{Ab_{1(CEA)} < CEA > Ab_{2(CEA)}|CdSe}_{Ab_{1(AFP)} < APF > Ab_{2(AFP)}|CdTe}$ prepared with a mixture of CEA and AFP analytes, the obtained ECL spectra demonstrate an additive behavior of individual emissions associated with respective analyte and its ECL tag. More specifically, the ECL intensity at ~547 nm and ~783 nm in Figure 2c is nearly identical to that at the same emission wavelength shown in Figure 2b and Figure 2a, respectively. This is significant, given the fact that the concentration of CEA (10 ng/mL) and AFP (100 pg/mL) used for construction of the GCE|ABA-$\{^{Ab_{1(CEA)} < CEA > Ab_{2(CEA)}|CdSe}_{Ab_{1(AFP)} < APF > Ab_{2(AFP)}|CdTe}$ was exactly the same as that used for fabrication of respective single antigen containing electrodes, namely GCE|ABA-$\{^{Ab_{1(CEA)} < CEA > Ab_{2(CEA)}|CdSe}_{Ab_{1(AFP)}}$ and GCE|ABA-$\{^{Ab_{1(CEA)}}_{Ab_{1(AFP)} < APF > Ab_{2(AFP)}|CdTe}$. These results reveal that the possible energy transfer between the highly excited state CdSe* and the ground state CdTe NCs is negligible; otherwise an increase in ECL emission intensity at ~783 nm and a decrease at ~547 nm would be expected. This could be contributed mainly to the fact that unlike in the solution phase, surface-confined CdSe and CdTe NCs cannot freely move around, thereby eliminating their collision based energy transfer. As a result, quantitative analysis of two individual target antigens simultaneously can be realized with the proposed dual-color ECL immunoassays.

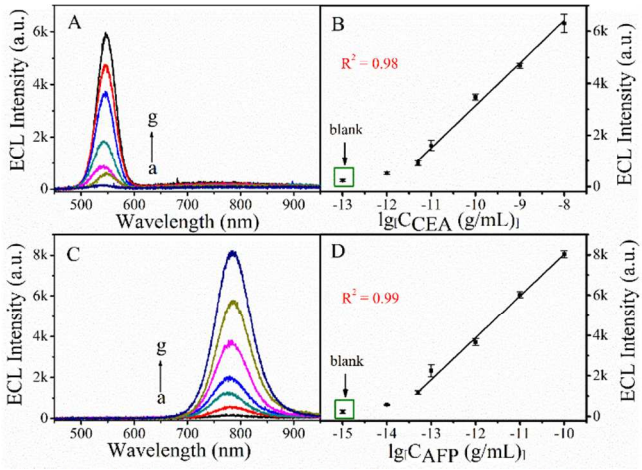


Figure 3. Spectral ECL responses of the dual-color multiplexing ECL immunoassay towards samples only containing (A) CEA with concentrations of (a) 0, (b) 1, (c) 10, (d) 50, (e) 100, (f) 1000, (g) 10000 pg/mL and (C) AFP with concentrations of (a) 0, (b) 0.01, (c) 0.05, (d) 0.1, (e) 1, (f) 10, (g) 100 pg/mL, respectively. Their corresponding calibration curves were shown in (B) for CEA and (D) for AFP, respectively. Experimental conditions for ECL generation and collection were the same as in Figure 1C.

Figure 3 shows the dependence of spectral ECL responses on the concentrations of one target antigen when the other is absent from the sample solution. As expected, the intensity of ECL spectra measured from the GCE|ABA-$\{^{Ab_{1(CEA)} < CEA > Ab_{2(CEA)}|CdSe}_{Ab_{1(AFP)}}$ (Figures 3A and B) and the GCE|ABA-$\{^{Ab_{1(CEA)}}_{Ab_{1(AFP)} < APF > Ab_{2(AFP)}|CdTe}$ (Figures 3C and D) increases with the increase of respective target concentration. A linear relation between the ECL peak intensity and logarithmic concentration of CEA (Figure 3B, 10 pg/mL to 10 ng/mL) or AFP (Figure 3D, 50 fg/mL to 100 pg/mL) is illustrated. A detection limit of 1 pg/mL of CEA or 10 fg/mL of AFP can be also estimated from Figures 3B and 3D, respectively.

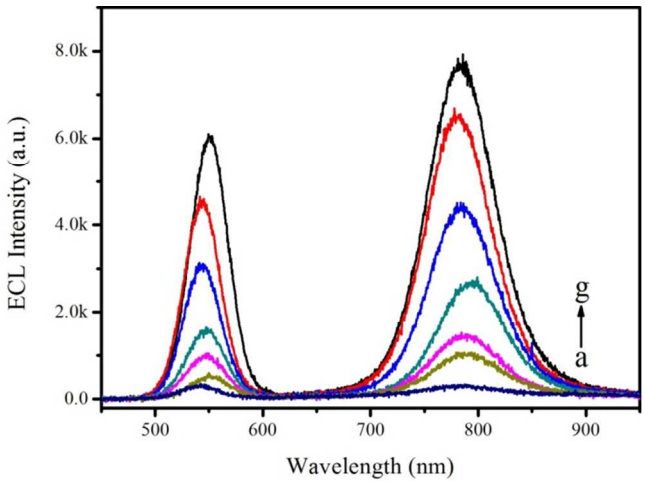


Figure 4. Spectral ECL responses of dual-color multiplexing ECL immunoassay towards 20.0 μL samples containing a mixture of CEA and AFP: (a) 0 pg/mL CEA and 0 fg/mL AFP, (b) 1 pg/mL CEA and 10 fg/mL AFP, (c) 10 pg/mL CEA and 50 fg/mL AFP, (d) 50 pg/mL CEA and 100 fg/mL AFP, (e) 100 pg/mL CEA and

1 pg/mL AFP, (f) 1 ng/mL CEA and 10 pg/mL AFP, and (g) 10 ng/mL CEA and 100 pg/mL AFP.

Spectrum-resolved Dual-color Multiplexing ECL Immunoassay. Figure 4 shows the spectral ECL responses of dual-color multiplexing ECL immunoassays for simultaneous detection of two target analytes (i.e., CEA and AFP) mixed in a sample solution at various concentration combinations, where the intensity of both ECL spectra associated CEA (CdSe) at ~547 nm and AFP (CdTe) at ~783 nm increases gradually with the increase of respective analyte concentration. Further analysis of the spectral data indicates that the coexistence of the second analyte does not affect the linear correlation between the peak intensity of the first ECL spectra and the logarithmic concentration of the first analyte (Figure S4 in Supporting Information). For example, a dynamic range of 10 pg/mL to 10 ng/mL for CEA (Figure S4A) and 50 fg/mL to 100 pg/mL for AFP (Figure S4B) is obtained when the two analytes are present in the same solution. These results are highly coincidence with the data presented in Figures 4B for CEA and 4D for AFP, respectively, in which only one analyte is determined. Therefore, the spectrum-resolved dual-color ECL immunoassay strategy proposed in this work is promising for reliable detection and quantification of two types of antigens simultaneously.

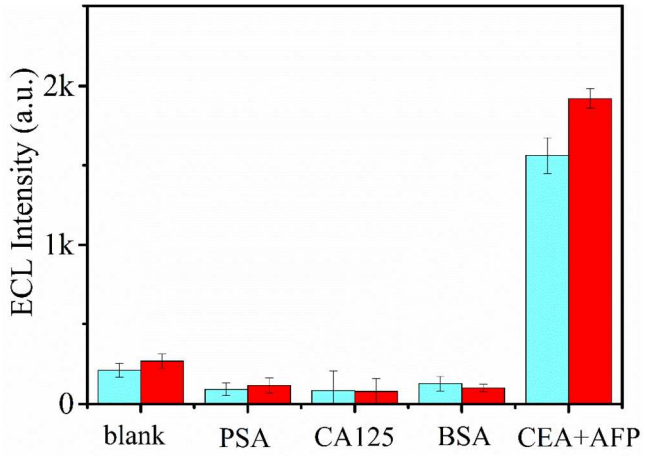


Figure 5. Specificity of the dual-color ECL immunoassay towards interferences. The bars represent the spectral response of blank, 100 pg/mL PSA, 10 μU/mL CA125, 100 pg/mL BSA, and 10 pg/mL CEA + 100 fg/mL AFP samples in greenish (cyan) and NIR (red) region, respectively. The error bars represent the standard deviations from three experiments.

Specificity of Dual-color Multiplexing ECL Immunoassay. Similar to previously reported spectrum-resolved monochromatic ECL immunoassay towards a single target with dual-stabilizers-capped NCs as ECL tags,[39,40] the present dual-color multiplexing ECL immunoassay also demonstrated acceptable specificity towards various interferences. As shown in Figure 5, the ECL intensity obtained from blank, 100 pg/mL prostate specific antigen (PSA), 10 μU/mL carcinoma antigen 125 (CA125), or 100 pg/mL bovine serum albumin (BSA) is less than 5-10% of that obtained from a much low concentration of CEA (10 pg/mL) or AFP (100 fg/mL). The results further suggest that the specificity of ECL immunoassay with dual-stabilizers-capped NCs as ECL tags is predominately controlled by the specific and selective interactions between antibody and antigen,[37,38] whereas the labeling procedures of ECL tags have little effects on the specificity.

CONCLUSION

A spectrum-resolved dual-color ECL immunoassay strategy was developed for simultaneous determination of CEA and AFP, in which dual-stabilizers-capped CdSe (λ_{max} = 550 nm) and CdTe (λ_{max} = 776 nm) NCs were used as the ECL emitters and the ECL signals were generated upon cathodic potential scanning in the presence of persulfate coreactant. The ECL spectra of the two immobilized ECL emitters were well separated and showed no cross interference, resulting in great sensitivity and selectivity towards the analysis of each individual target analyte. The detection approach presented in this paper could be extended to simultaneous detection and qualification of multiple target analytes, such as DNA, RNA, protein, and virus, with using suitable ECL emitters and coreactants. The inherent features of the ECL technology, especially its low background, could make the present multiplexing detection scheme to become much more attractive than NCs-based fluorescent multiplexing assays, especially when limit of detection is a primary factor in considering a suitable analytical technique. Applications of present ECL platform into biomedical diagnosis are also expected.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Chemicals and materials, electrochemical and potential resolved ECL characterizations for fabrication procedures, calibration curves for simultaneously determining CEA and AFP.

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Notes

The authors declare no competing financial interest.

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