Supporting Information

A Novel Electrochemiluminescence Immunosensor *via* Polymerization-Assisted Amplification

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Experimental Section

Chemicals and reagents. Gold electrodes were purchased from CHI Instruments, Inc. Au sbustrates (50-Å chrome followed by 1000-Å gold on float glass) were obtained from Evaporated Metal Films (Ithaca, NY). Carcinoembryonic antigen (CEA), monoclonal (mouse) antibody to CEA (Ab1) and rabbit antibody to CEA (Ab2) were purchased from Biodesign (Saco, MA). Tris(2,2'-bipyridine)ruthenium chloride hexahydrate ($Ru(bpy)_3Cl_2 \cdot 6H_2O$), bovine serum albumin (BSA), Tween-20, N-hydroxysuccinimide (NHS), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), ascorbic acid, 2,2'-bipyridyl (bpy), $CuCl_2$, N,N-dimethylformamide, 2-(Diisopropylamino)ethylamine (DPEA), 11-mercapto-1-undecanoicacid (MUA), magnesium sulfate ($MgSO_4$), and sodium hydrogen carbonate ($NaHCO_3$) were purchased from Sigma-Aldrich (St. Louis,

MO). Glycidyl methacrylate (GMA) was from Alfa Aesar (Ward Hill, MA) and purified at home to remove the inhibitor.[S1] Clinical serum samples were collected by Jiangsu Institute of Cancer Prevention and Cure (Nanjing, China). *o*-Aminobenzoic acid (*o*-ABA) was a gift from Xingshengchem (Yancheng, China). 0.1 M phosphate buffer solutions (PBS) were prepared by mixture of 0.1 M NaH_2PO_4 and Na_2HPO_4. Twice-distilled water was used throughout the study.

Apparatus. The electrochemical and ECL measurements were carried out on a MPI-E multifunctional electrochemical and electrochemiluminescent analytical system (Xi'an Remex Analytical Instrument Ltd. Co., China). All the ECL measurements were performed in a 5 mL glass cell composed of a modified Au working electrode, a platinum counter electrode, and an Ag/AgCl (saturated KCl solution) reference electrode.

The morphology of the polymers on the Au substrate was characterized by a scanning electron microscope (SEM) instrument (LEO 1530 VP, Germany) with an acceleration voltage of 10 kV. A thin gold film was sputtered atop the samples by argon plasma sputtering for 45 s prior to SEM measurements.

The XPS measurements were performed on a Thermo ESCALAB 250 spectrometer that used a monochromatized Al K_α X-ray source (1486.6 eV photons) at a constant dwell time of 100 ms and a pass energy of 40 eV. The samples were mounted on the standard sample studs by means of double-sided adhesive tape. The core-level signal was obtained at a photoelectron takeoff angle (α, measured with respect to the sample surface) of 90°. The X-ray source ran at a reduced power of 150

W (15 kV and 10 mA). The pressure in the analysis chamber was maintained at 10^{-8} Torr or lower during each measurement. All binding energies (BE's) were referenced to the C1s hydrocarbon peak at 284.6 eV. Surface elemental stoichiometries were determined from the spectral area ratios, after correction with experimentally determined sensitivity factors, and were reliable to within 10%. The elemental sensitivity factors were calibrated using stable binary compounds of well-established stoichiometries.

Synthesis of initiator coupled with polyclone CEA antibody. The N-hydroxysuccinmidyl bromoisobutyrate (initiator) was prepared according to a previous report.[S2] In brief, 1.34 mL bromoisobutyryl bromide was dissolved in 50 mL diethyl ether (cool in ice bath). A solution of N-hydroxysuccinimide (0.43 M) and triethylamine (0.65 M) was then dropwise added into this solution. When the addition was completed, the mixture was stirred for 1 h, followed by filtration to remove any precipitates. The solution was thoroughly washed with saturated $NaHCO_3$, water, dried over $MgSO_4$ and evaporated in vacuum to obtain NHS active ester, a crude white solid product (note: all solution must be anhydrous). To synthesize initiator-conjugated polyclone CEA antibody ($Ab2^*$), the prepared NHS-coupled initiator (10 mg mL^{-1} in DMF, 10 μL) was added to a polyclone CEA antibody solution at 10 mg mL^{-1}. The molar ratio of initiator to antibody was controlled at 8:1. The mixture was stirred overnight to allow the coupling reaction to reach completion and the excess NHS ester to hydrolyze. The concentration of the solution was determined by the UV absorbance at 280 nm, then diluted with PBS buffer (0.1 M, pH 7.4) to 1 mg mL^{-1}.

Activator generated electron transfer for atom transfer radical polymerization (AGET ATRP) and DPEA coupling. Prior to each experiment, the Au electrodes were polished with diamond paste and alumina slurry of 0.05 μm particles on a polishing cloth (Buehler, LakeBluff, IL), followed by sonication in water and ethanol. The electrodes were then rinsed with deionized water and dried under a stream of N_2, followed by subsequent surface modification. For attachment of monoclone CEA antibody (Ab1), a poly(*o*-ABA) film (PAB) was first electropolymerized on the gold electrode surface by dipping the previously mentioned clean electrode in a 1 M H_2SO_4 solution that contained 50 mM *o*-ABA and was scanned in a potential range of 0-1.0 V for 10 cycles at a scan rate of 40 mV s^{-1}. The PAB-modified electrodes were then removed from the solution, rinsed with water three times and immersed in water for 30 min. The modified gold electrode was then dipped into a freshly prepared aqueous solution that contained EDC (50 mM) and NHS (15 mM) for 30 min. This was immediately followed by 30 min of incubation with 1 mg mL^{-1} Ab1 in pH 7.4 PBS with 0.05% Tween-20. The resultant Ab1 modified gold electrode (Ab1/Au) was then incubated for 30 min with 1 mg mL^{-1} BSA and 0.05% Tween-20 to block non-specific binding sites. A sandwiched immunoassay was used for protein detection and introduction of $Ab2^*$ onto the gold surface. 6 μL CEA containing samples in buffer was spotted on the Ab1 modified substrate at 37 ^oC for 30 min to capture antigen and form Ag-Ab1 immunocomplexes on surface through the first immunoreaction. After being washed thoroughly with PBS, 6 μL $Ab2^*$ was spotted on the Ag/Ab1/Au surface at 37 ^oC for 30 min to immobilize $Ab2^*$ on the electrode surface with the second immunoreaction. The electrode was

thoroughly rinsed again with PBS to obtain the $Ab2^*$ modified electrode ($Ab2^*$/Ag/Ab1/Au). The polymerization was performed by immersing $Ab2^*$ modified electrode in a mixture of $CuCl_2$ (4 mg), bpy (9.38 mg), GMA (800 μL), DMF (400 μL), DI H_2O (400 μL) in a glass container. Then, 50 μL ascorbic acid was added to reduce Cu (II) ions and start the AGET ATRP reaction. Following that, the electrode was thoroughly rinsed, bathed in acetone and shaken for 2 h to remove nonspecifically adsorbed monomers. It was then immersed into DPEA solution for 5 h at room temperature to bring DPEA onto the polymer materials. To ensure the signal had not originated from nonspecific absorption and cross reaction, control experiments were conducted using Ab2 without initiator coupling or incubation of Ab1-Au in 0 ng mL^{-1} CEA, or using IgG and AFP instead of CEA to complete the immunoreactions. For comparative experiments, Ab2, pre-conjugated with DPEA through EDC and NHS at a 1:100 reaction ratio to reach its maximum loading capacity, was used to complete the sandwiched immunoassay.

ECL detection. The ECL measurements were performed in a 5 mL glass cell. The electrolyte was 1 mM $Ru(bpy)_3^{2+}$ in 0.1 M phosphate solution (pH 7.5). The potential range applied on the working electrode in CV measurement was from 0.5 to 1.3 V at 50 mV s^{-1}. The ECL emission intensity corresponding to CV measurements was recorded by the MPI-E multifunctional chemiluminescence analyzer. The emission window was placed in front of the photomultiplier tube biased at 600 V.

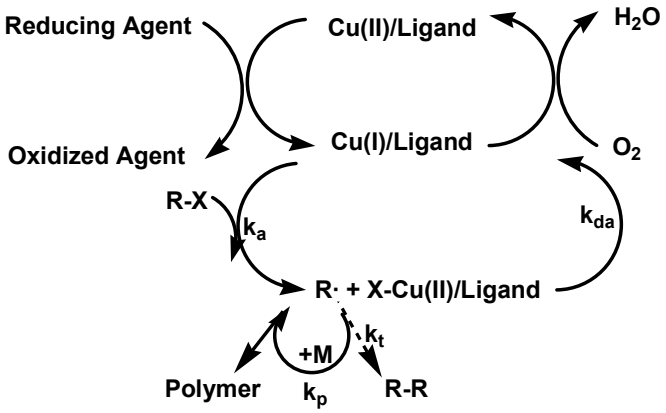
The optimization of the experiment. The ECL intensity was associated with the accessibility and steric hindrance of epoxy groups on the electrode that were available for DPEA coupling. The ECL

intensity of DPEA/PGMA/Ab2/Ag/Ab1/Au electrode initially increased with the reaction time and reached its maximum at AGET ATRP time of 2 h. At that time the accessibility of epoxy groups on the electrode was the largest and the steric hindrance was the smallest (Fig. S2), thus the AGET ATRP time was fixed at 2 h. At 2 h polymerization, the polymer film (PGMA/Ab2/Ag/Ab1/Au) thickness reached a maximum of about 14.3 nm, detected by ellipsometry (Fig. S3).

The ECL intensity also depended on the incubation time of the polymer materials modified electrode in DPEA containing solutions. The ECL intensity of DPEA/PGMA/Ab2/Ag/Ab1/Au electrode increased initially with the incubation time. The signal reached its maximum at an incubation time of 5 h. A further increase of incubation time resulted in no signal increase, attributed to close-to-completion coupling of DPEA to the accessible PGMA side chains (Fig. S4). Therefore, the incubation time of 5 h was used throughout our study.

The ECL intensity was observed to be related to the pH of the detection solution. At low pH values, the radical cation of the tertiary amine group was difficult to deprotonate into a high-reducing free radical intermediate. Meanwhile, at high pH values, $Ru(bpy)_3^{3+}$ competitively reacted with OH^- ions in the system, which reduced the availability of $Ru(bpy)_3^{3+}$ and decreased the ECL intensity. In this study, pH of 7.5 was found to obtain the largest ECL response (Fig. S5).

Scheme S1. The mechanism of AGET ATRP.



Scheme S2. Reaction mechanism of $Ru(bpy)_3^{2+}$/TA ECL system.

$$Ru(bpy)_3^{2+} \rightarrow Ru(bpy)_3^{3+} + e \quad (1)$$

$$TA \rightarrow TA^{\bullet+} + e \quad \text{(oxidized on electrode surface directly)} \quad (2a)$$

$$Ru(bpy)_3^{3+} + TA \rightarrow TA^{\bullet+} + Ru(bpy)_3^{2+} \quad \text{(oxidized by } Ru(bpy)_3^{3+}\text{)} \quad (2b)$$

$$TPA^{\bullet+} \rightarrow TPA^{\bullet} + H^{+} \quad \text{(radical rests on a carbon atom)} \quad (3)$$

$$Ru(bpy)_3^{3+} + TPA^{\bullet} \rightarrow [Ru(bpy)_3^{2+}]^{*} + \text{TPA fragment} \quad (4)$$

$$[Ru(bpy)_3^{2+}]^{*} \rightarrow Ru(bpy)_3^{2+} + h\nu \quad (5)$$

Both $Ru(bpy)_3^{2+}$ and TA can be oxidized directly to its oxidized state of $Ru(bpy)_3^{3+}$ and TPA radical cation ($TPA^{\bullet+}$) at a certain applied potential. The $TPA^{\bullet+}$ was unstable, short-lived, and believed to lose a proton from an α-carbon to form the strong reduction intermediate $TPA^{\bullet}$. This reduction intermediate was then reacted with $Ru(bpy)_3^{3+}$ via an electron transit reaction to generate emitting species of $[Ru(bpy)_3^{2+}]^{*}$.[S3,S4]

Fig. S1. The surface reflectance FT-IR spectrum of (a) Ab2*/Ag/Ab1/Au and (b) PGMA/Ab2/Ag/Ab1/Au.

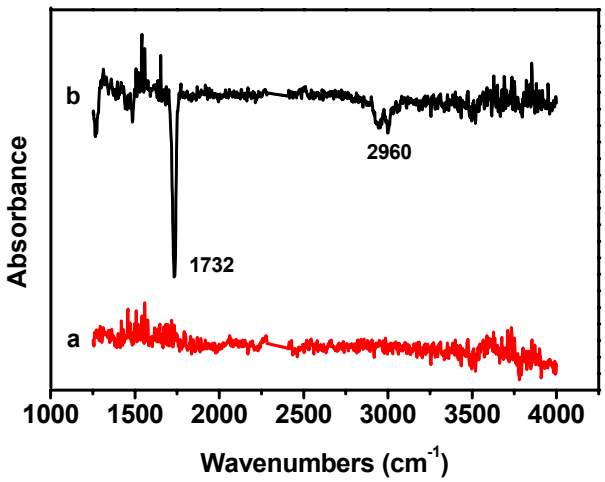


Fig. S2. Plots of the ECL response of DPEA/PGMA/Ab2/Ag/Ab1/Au as a function of the AGET ATRP reaction time. The concentration of CEA was 1 ng mL^{-1}, detection solution was 1 mM $Ru(bpy)_3^{2+}$ in 0.1 M phosphate solution (pH 7.5) and scan rate of 50 mV s^{-1}.

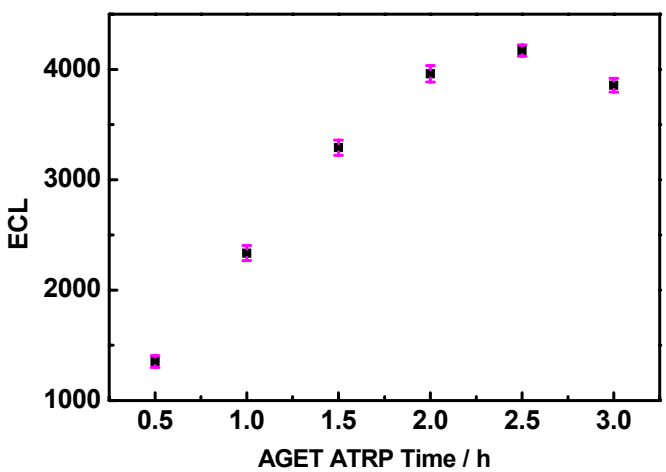


Fig. S3. Polymer film thickness as a function of the AGET ATRP reaction time. The conditions are as same as Fig. S2.

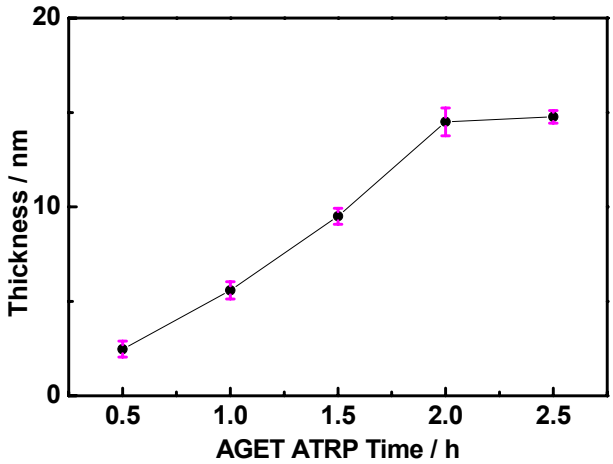


Fig. S4. Plots of the ECL response of DPEA/PGMA/Ab2/Ag/Ab1/Au against the coupling time of DPEA with PGMA. The conditions are as same as Fig. S2.

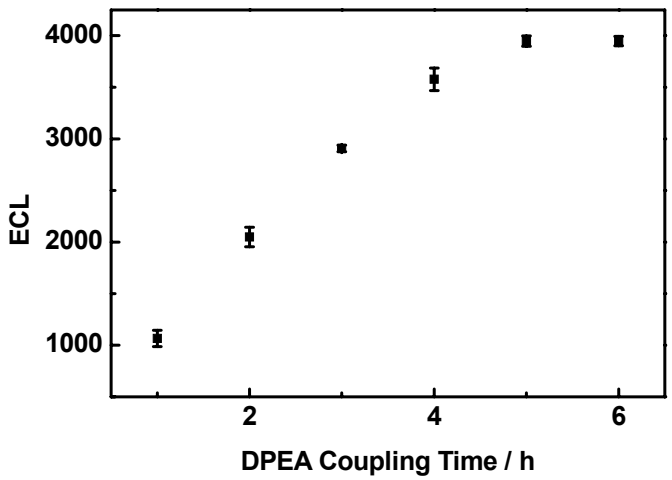


Fig. S5. Plots of the ECL response of DPEA/PGMA/Ab2/Ag/Ab1/Au as a function of the pH of detection solution. The conditions are as same as Fig. S2.

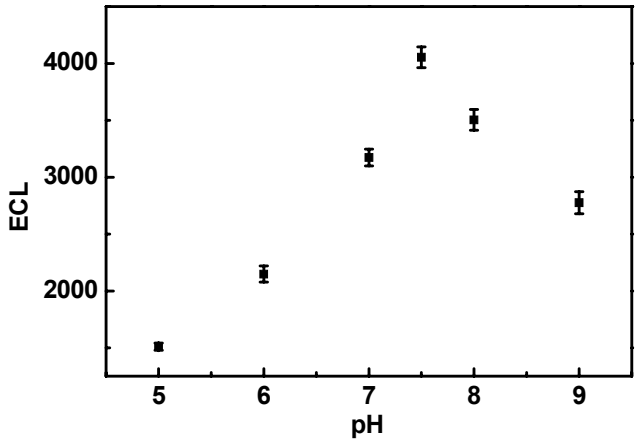


Fig. S6. The ECL response of electrode Ab1/Au was incubated in 1 ng mL^{-1} AFP solution, the other conditions are the same with Fig. S2.

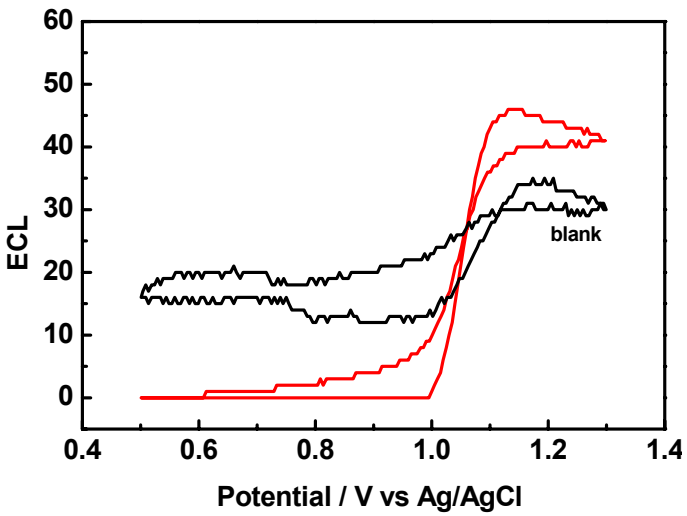
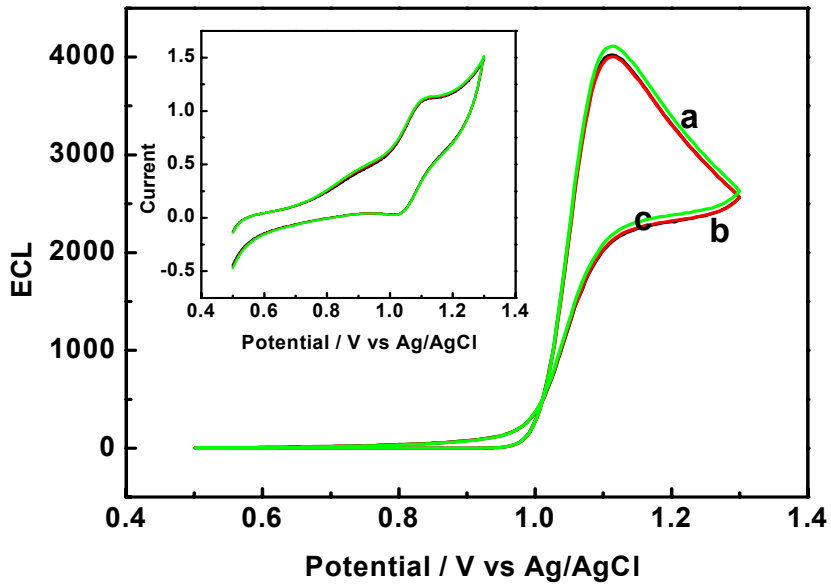


Fig. S7. Reproducibility of the biosensor for detection of CEA. The experiments were conducted on three different Au substrates (a, b, c) in the detection of CEA at 1 ng mL^{-1}.



Reference

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