Battery-triggered Paper-based Multiplex Electrochemiluminescence Immunodevice Based on Potential-Resolution Strategy

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Supplemental Information

Reagents and Materials

All reagents were of analytical-reagent grade and directly used for the following experiments without purification, and all solutions were prepared using Millipore (model milli-Q) purified water. Antigens (AFP, CA153, CA199 and CEA), mouse monoclonal capture antibodies ($McAb_1$) and signal antibodies ($McAb_2$) of these antigens were purchased from Shanghai Linc-Bio Science Co. Ltd. Tris-(bipyridine)-ruthenium (II)-NHS ($Ru(bpy)_3^{2+}$-NHS) ester were obtained from IGEN (Gaithersburg, MD). Tripropylamine (TPrA), 1-ethyl-3-(3 dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were from Alfa Aesar China LTd. Multi-walled carbon nanotubes (MWCNTs) were purchased from Nanoport Co. Ltd. (Shenzhen, China). Bovine serum albumin (BSA) was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Whatman chromatography paper #1 (58.0 cm × 68.0 cm, pure cellulose paper) was obtained from GE Healthcare Worldwide (Pudong Shanghai, China) and used with further adjustment of size (A4 size). Blocking buffer for the residual reactive sites on

the antibody immobilized paper was phosphate buffer solution (PBS) containing 0.5 % bovine serum albumin and 0.5 % casein, 0.05 % Tween-20 was spiked into 0.01 M pH 7.4 PBS as wash buffer to minimize unspecific adsorption. The human serum samples were from Shandong Tumor Hospital. Carbon nanodots (CNDs) were synthesized according to a previous work with some modifications [1].

Fabrication of this microfluidic paper-based ECL device

Wax printing is rapid, inexpensive, and particularly well-suited for producing large lots (hundreds to thousands) of prototype μ-PADs, therefore, it was used as the pattern method in this work [2]. For wax printing, we designed a pattern of hydrophobic barrier as purple area on a white background in Adobe illustrator CS4. Briefly, the wax-patterns were printed on paper sheet in bulk using the wax printer (Xerox Phaser 8560N color printer) set to the default parameters for photo-quality printing (the most inkjet quantity) in a high-resolution printing mode (Figure S2). Then, the wax-printed paper sheet was placed on a digital hot plate set at 150 ℃ for 130 s. The wax melted and penetrated through the thickness of the porous-structured paper sheet to form the hydrophobic barrier (Figure S3). This fabrication process can be finished within 2 min.

Electronic Supplementary Material (ESI) for Lab on a Chip

Detail washing procedure for this μ-PECLI

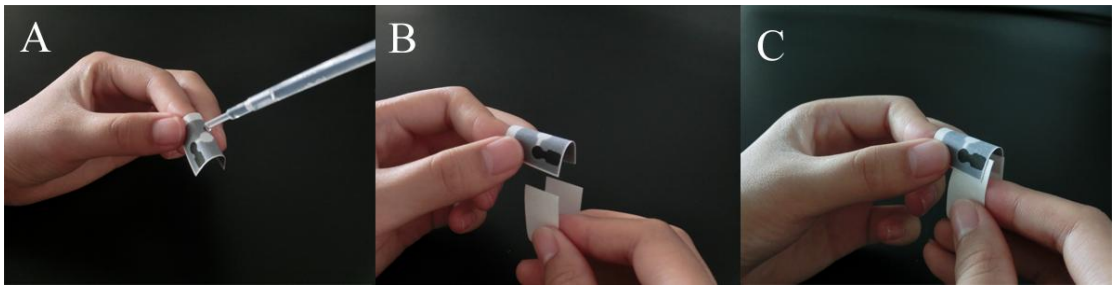


Figure S1 Pictures of the washing procedures for this μ-PECLI. A) Adding washing buffer to the back of the paper auxiliary zone while bending the paper to a inverted-U type; B, C) Contacting the two paper working zones with a piece of U-type blotting paper.

The detailed washing procedures were as follows: Due to the front and back surfaces of the wax-patterned paper electrochemical cell are open to atmosphere, thus the working zone can be washed by applying washing buffer to the back of the paper auxiliary zone while bending the paper layer to an inverted-U type as shown in Figure S1A. Then a piece of U-type blotting paper was contacted with the two paper working zones simultaneously (Figure S1B, C). The washing buffer could go through the paper auxiliary zone and migrate along the paper channels by the capillary and gravity action to wash the paper channels and paper working zones and carries the unbound reagents with it into the blotting paper. This effective washing procedure was used in this work consistently and acquiescently. The washing process was important for preventing the nonspecific binding and for achieving the best possible signal-to-background ratio. Another purpose for this washing procedure was to stop the incubation reaction at exactly same time. The washing procedure was repeated twice to make sure the washing was performed completely. These washing procedures could be finished within 2 min.

Preparation of CNDs and CNDs labeled $McAb_2$.

The electrochemical preparation of CNDs was performed in an electrochemical cell consisting of a graphite rod (GR) working electrode, an Ag/AgCl reference electrode, a Pt mesh counter electrode, and pH 7.0 PBS. The applied potential at the GR electrode was cycled between -3.0 and 3.0 V at 0.1 V/s. With increasing of oxidation time, the electrolyte solution changed from colorless to yellow and finally to dark brown. The dark brown solution was centrifuged (12 000 rpm) for 15 min to remove the non-fluorescent deposit. The supernatant was further dialyzed against water through a dialysis membrane (MWCO of 1 kDa) to remove inorganic salt. Then the CNDs were obtained by ultrafiltering the solution through centrifugal filter devices with a 10 kDa MWCO membrane. The size of the as-prepared CNDs was 2.0 ± 0.3 nm in diameter.

The as-prepared CNDs, with high-density carboxyl functional groups [1], were much easier to facilitate biomolecules binding via EDC chemistry. Briefly, the as-prepared 2.0 nm CNDs were mixed with 200 mM EDC and 50 mM NHS in 1.0 mL of pH 7.4 PBS buffer and activated for 40 min in the dark. The mixture was centrifuged at 13 000 rpm for 15 min. Subsequently, the resulting soft sediment was dispersed in 1.0 mL of pH 7.4 PBS. Then, 50 μL of $McAb_2$ (Anti-AFP and Anti-CA199, respectively) at 1 $mg \cdot mL^{-1}$ were added to the dispersion, and the mixture was stirred overnight. The conjugate was washed with PBS and centrifuged at 13 000 rpm for 15 min three times. The resulting soft sediment was redispersed in 1.0 mL of pH 7.4 PBS. The obtained CNDs labeled $McAb_2$ can be used directly or stored at 4 ℃ for months.

Preparation of $Ru(bpy)_3^{2+}$ labeled $McAb_2$.

Similarly, the $Ru(bpy)_3^{2+}$-labeled $McAb_2$ was obtained by adding 75 μg $Ru(bpy)_3^{2+}$-NHS ester predissolved in 1.5 μL DMSO to 50 μL of $McAb_2$ (Anti-CA153 and Anti-CEA, respectively) PBS (pH 7.4) at 1 $mg \cdot mL^{-1}$. After 40 min incubation at room temperature in the dark, the mixture was filtered through a Sephadex G-25 PD-10 desalting column and eluted with PBS (pH 7.4). The purified $Ru(bpy)_3^{2+}$-labeled $McAb_2$ was further diluted with PBS (pH 7.4) to 1.0 mL and kept at 4 ℃ until use.

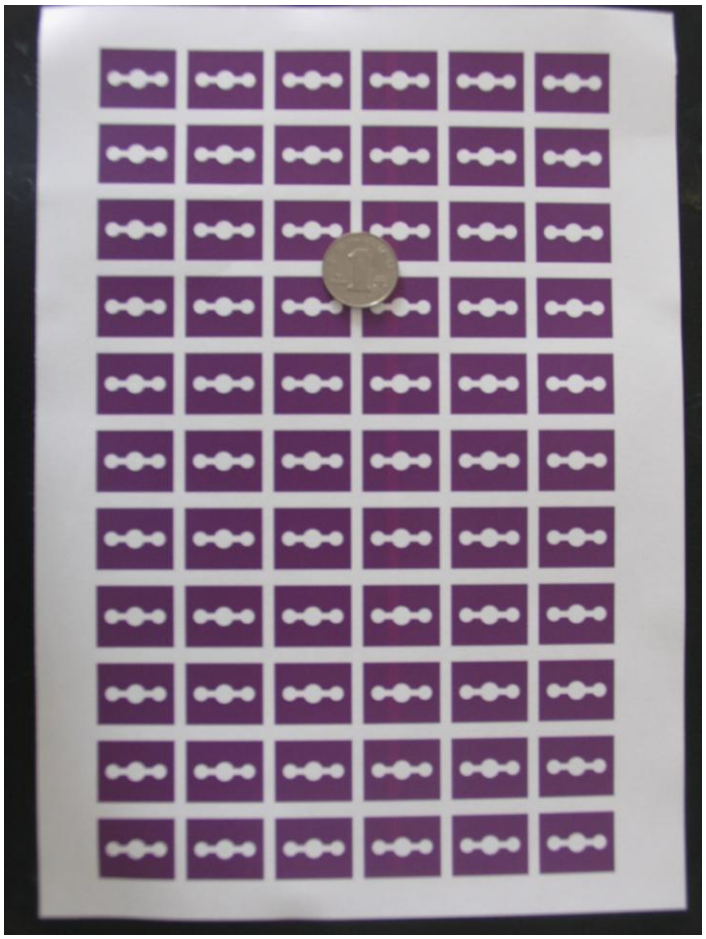


Figure S2. Wax-printed μ-PECLI on a paper sheet (A4) before baking.

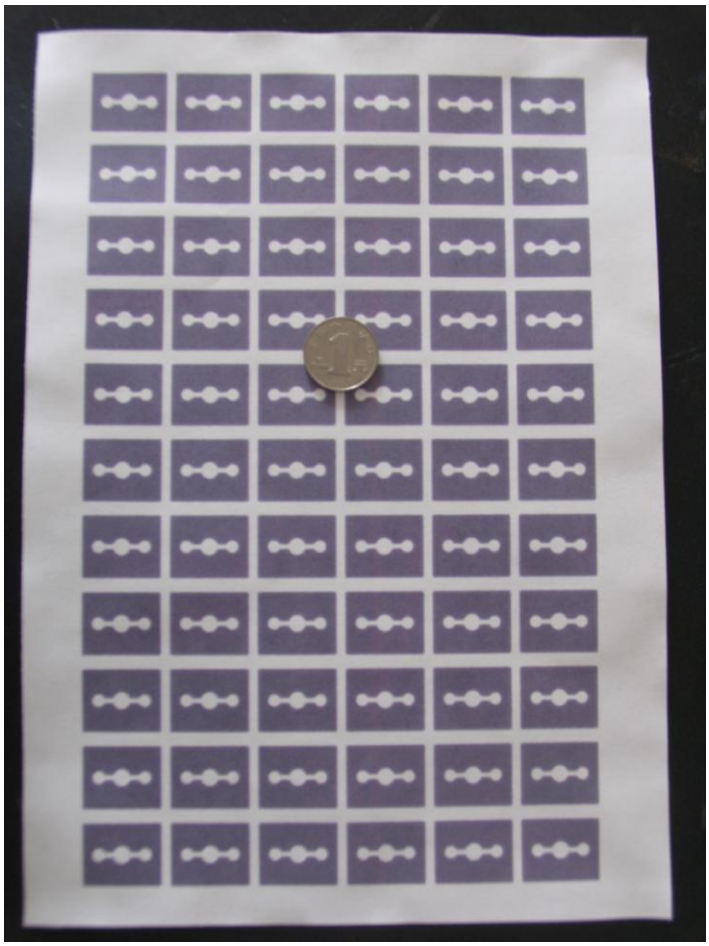


Figure S3. Wax-printed μ-PECLI on a paper sheet (A4) after baking.

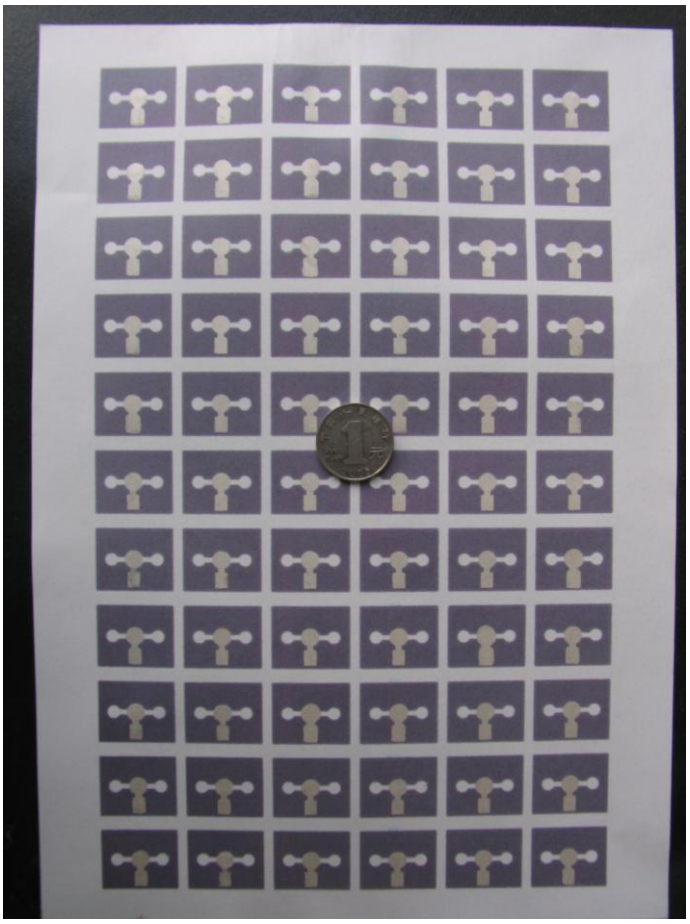


Figure S4. μ-PECLIs on a paper sheet (A4) after screen-printing of Ag/AgCl auxiliary electrode on one surface of paper.

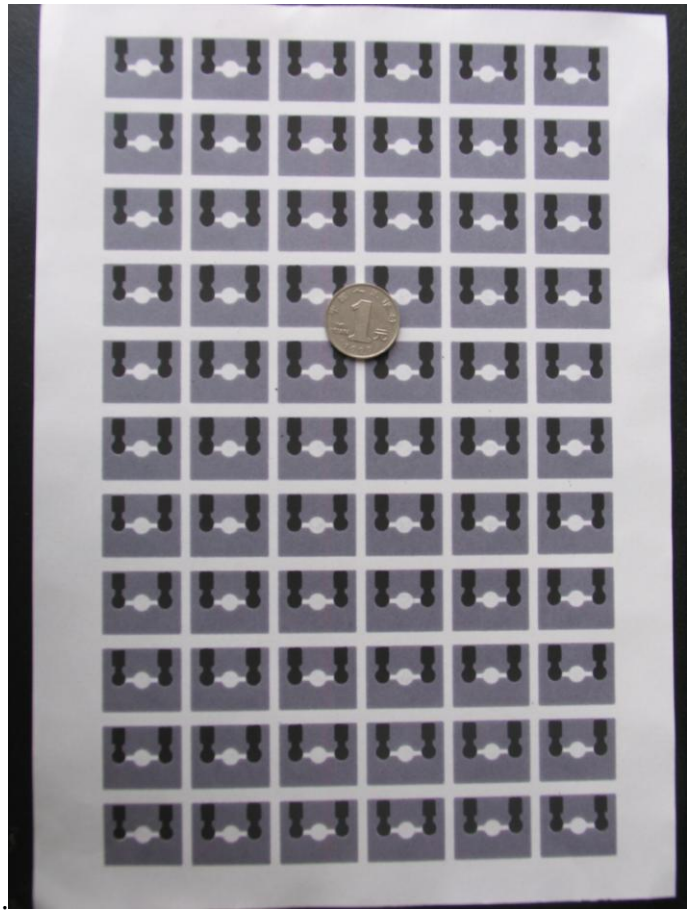
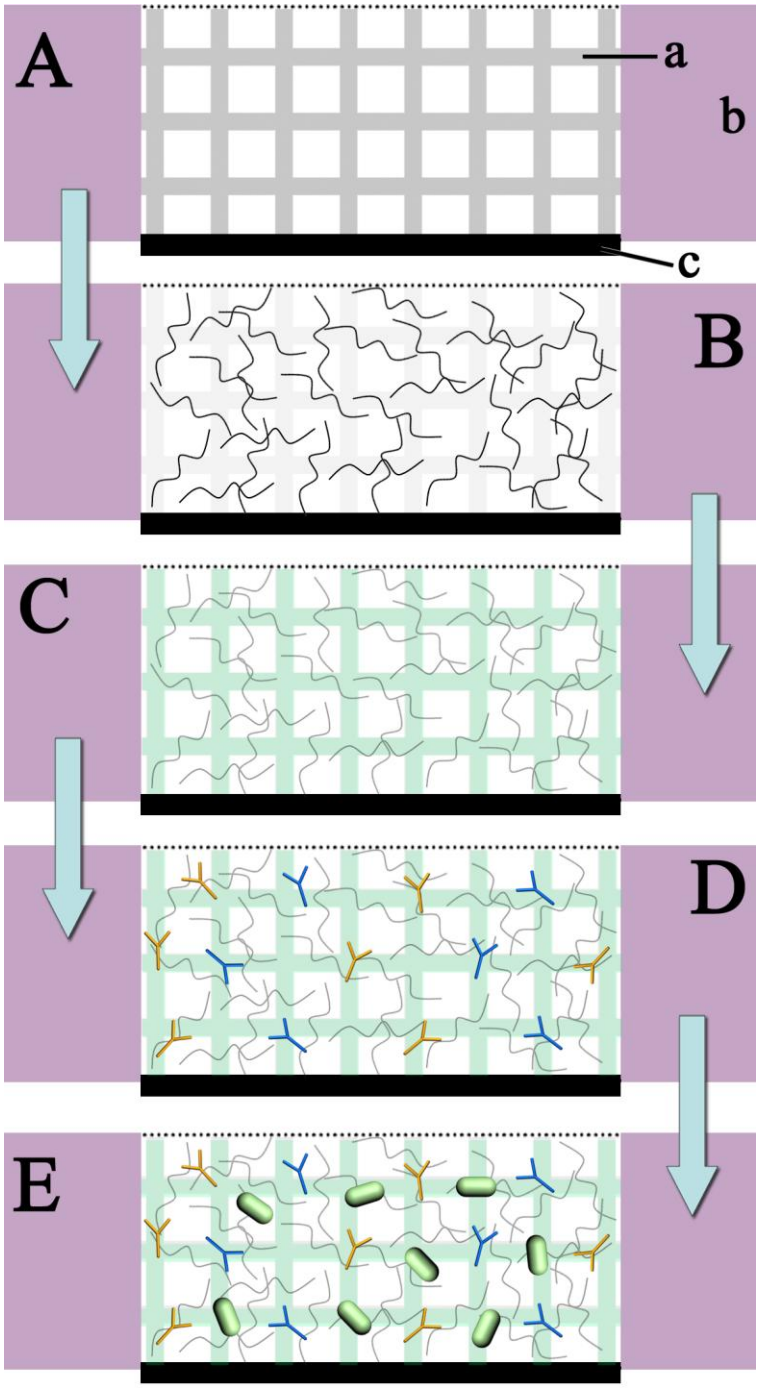


Figure S5. μ-PECLIs on a paper sheet (A4) after screen-printing of counter two working electrodes on another surface of paper.



Scheme S1 Schematic representation of the fabrication procedure for this μ-PECLI. (A) wax-patterned paper working zone on the back of screen-printed working electrode, (a) porous paper network, (b) wax-penetrated paper, (c) screen-printed carbon working electrode; (B) After modification with MWCNTs and washing; (C) After modification with chitosan and washing; (D) After immobilization with two kind of capture antibodies in (C) and washing; (E) After blocking with BSA and washing.

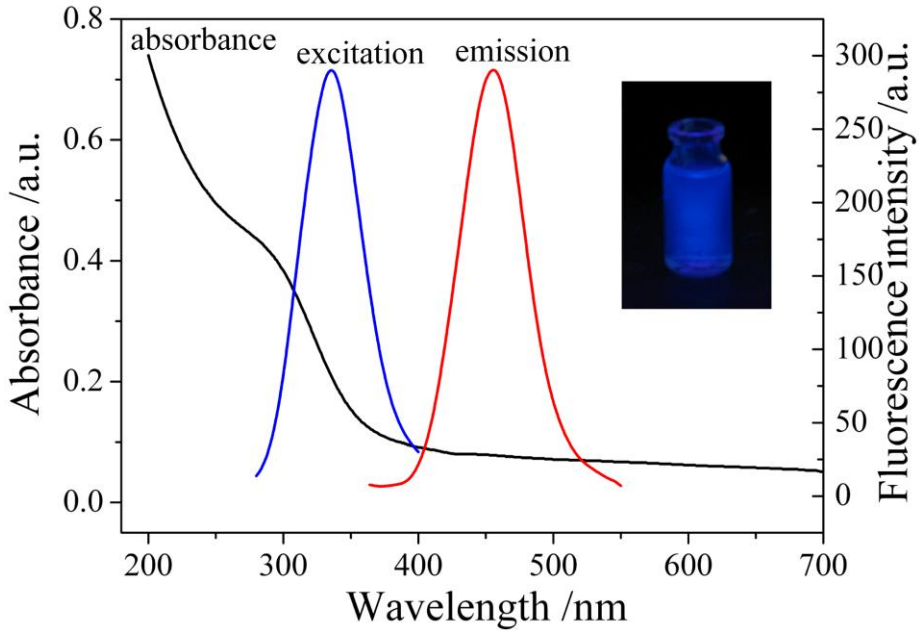


Figure S6. UV-Vis absorption and fluorescence spectra of ~2.0 nm CNDs in aqueous solution. The emission spectrum was obtained under the maximum excitation wavelength of 335 nm, and the excitation spectrum was obtained at the maximum emission wavelength of 457 nm. Inset is the fluorescent photo of a CNDs solution illuminated by an UV beam of 335 nm.

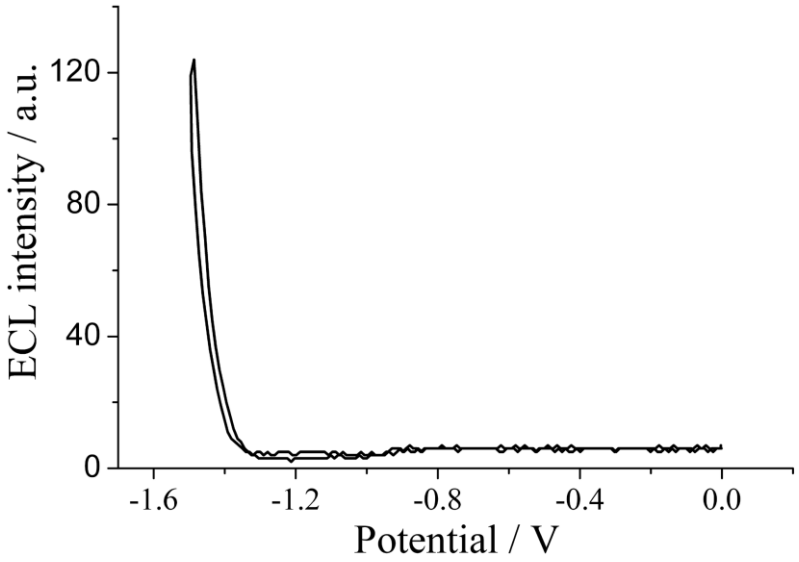


Figure S7. ECL-potential curver of 66 nM $Ru(bpy)_3^{3+}$ in the present of 1 mM $K_2S_2O_8$ in 0.01 M pH 7.4 PBS. Scan rate: $0.1V \cdot s^{-1}$. Initial potential = 0 V; scan direction, from 0 V to -1.5 V to 0 V.

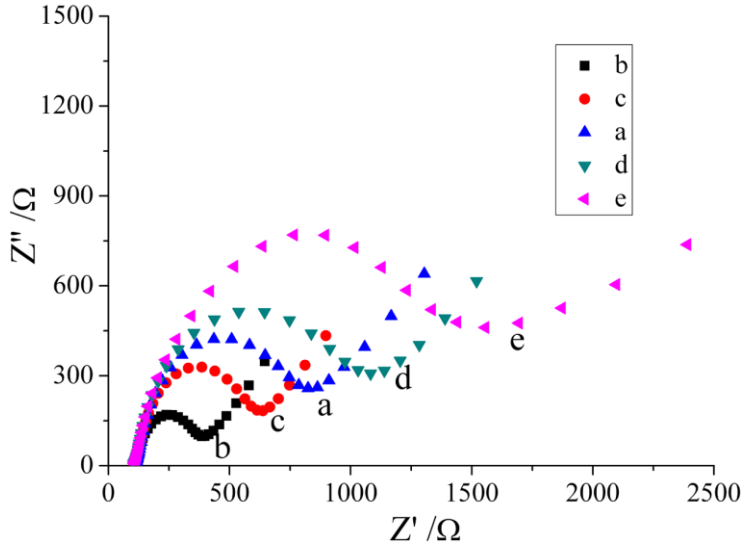


Figure S8. EIS of paper working zones upon the stepwise fabrication processes in 0.1 M KCl containing 5.0 mM $[Fe(CN)_6]^{3+/4+}$.

Characterization of this μ-PECLI

This proposed μ-PECLI using the hybrid ECL system as ECL labels can be used to detect many other protein biomarkers. In this work, AFP, CA 199, CA 153, and CEA were chosen as the model antigens. Covalent bonding has been used to achieve the immobilization of capture antibodies into the paper working zone on the back of the screen-printed working electrode in our previous works [3, 4]. In this μ-PECLI, GA was used as a cross-linking agent to immobilize capture antibodies into the chitosan and multi-walled carbon nanotubes (MWCNTs) modified paper working zone by means of Schiff base reaction between the aldehyde groups of glutaraldehyde and the free amino sites of the antibodies and chitosan.

The fabrication process of ECL immunoarrays on μ-PECLI was characterized by electrochemical impedance spectroscopy (EIS), which was performed on an IM6x electrochemical workstation (Zahner Co., Germany). EIS is one of the most powerful and sensitive techniques for investigating the whole procedure in preparing immunoarrays. The electron transfer of $[Fe(CN)_6]^{3+/4+}$ can be blocked by the formation of BSA/antibodies/chitosan/MWCNTs composites in the paper working zone, which results in the increase in the electron transfer resistance. Figure S8 shows the EIS of the modified paper working zone in each step. The response of EIS in the bare paper working zone exhibited a relatively small semicircular domain (curve a), which implied a low-electron-transfer resistance of the redox couple. After the bare paper working zone was modified with MWCNTs, the resistance was decreased (curve b) mainly due to the effective conductivity of MWCNTs, illustrating the successful immobilization of MWCNTs into the paper working zone. Obviously, the resistance for the redox probe in the chitosan-MWCNTs modified paper working zone (curve c) is much lower, the reason of which may be the abundant amino

groups of chitosan can adsorb much more negatively charged $[Fe(CN)_6]^{3+/4+}$ and it can be easily reached at the fiber surface to accelerate electron transfer. However, for the capture antibodies modified paper working zone through GA linking, the resistance increased markedly (curve d). This is attributed to the fact that the association of GA with chitosan obturated many amino groups of chitosan and the immobilization of capture antibodies formed protein barriers for electron transfer. BSA was employed to occupy the left active sites in the paper working zone for eliminating the unspecific adsorption, which led to the further increase of resistance (curve e). The above results indicate that the immunoarrays on μ-PECLI was constructed.

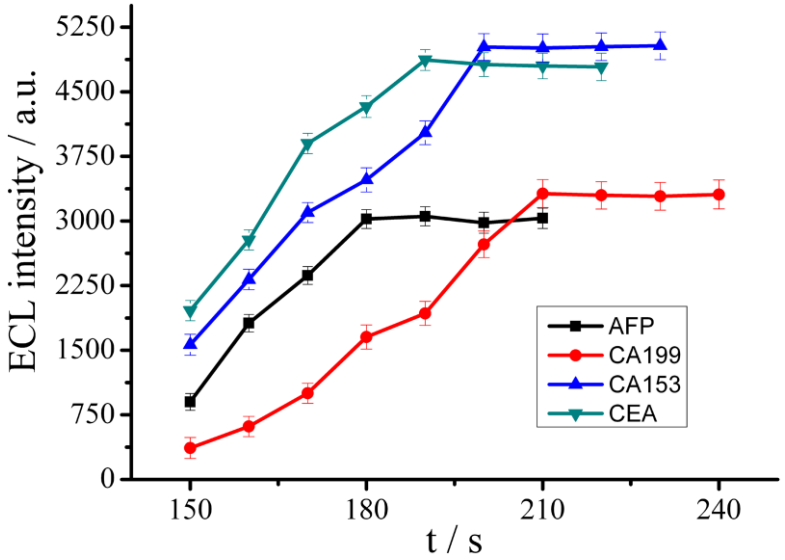


Figure S9. Effects of incubation time on ECL intensities at 50 $U \cdot mL^{-1}$ CA153, CA199 and 50 $ng \cdot mL^{-1}$ AFP, CEA concentration, where n = 11 for each point.

Choice of incubation time

The incubation time is an important parameter to the improvement of immunoassay

efficiency, which is usually controlled by mass transport of immunoreagents and kinetics of immunoreaction. Fast immunoassay is highly desired for paper-based POCT. Several technologies have been explored to accelerate the immunoreaction in the immunoarrays [5-7]. Compared to the micro-well used in conventional ELISA, the incompact porous structure and high surface-to-volume ratio of paper immunozones and the small volume (0.18 mm in thickness and 4 mm in diameter) of the paper immunozones were beneficial to the mass transport of the immunoreagents for further accelerating the immunoreaction [8].

Thus, in this work, to accelerate the immunoreactions in this μ-PECLI, as shown in Scheme 2 and Scheme S1, the immunoarrays were assembled into porous paper working zones on the back of screen-printed working electrodes. The incubation process was performed on-line at room temperature. As shown in Figure S9, all the ECL signals for 50 ng·mL^{-1} AFP, 50 U·mL^{-1} CA199, CA153, and 50 ng·mL^{-1} CEA increased with the increasing of incubation time used in sandwich-type immunoassay and then leveled off, indicated the saturated binding in the immunoreaction. The optimal incubation time of AFP, CA199, CA153, and CEA immunocomplex was 180 s, 210 s, 200 s and 190 s, respectively. However, the successful development of the multiplex paper-based ECL immunoassay required that the common incubation time must be suitable for all analytes. Thus, considering the optimal analytical performance, 210 s of incubation time was used in this immunoassay.

Pictures for running an assay

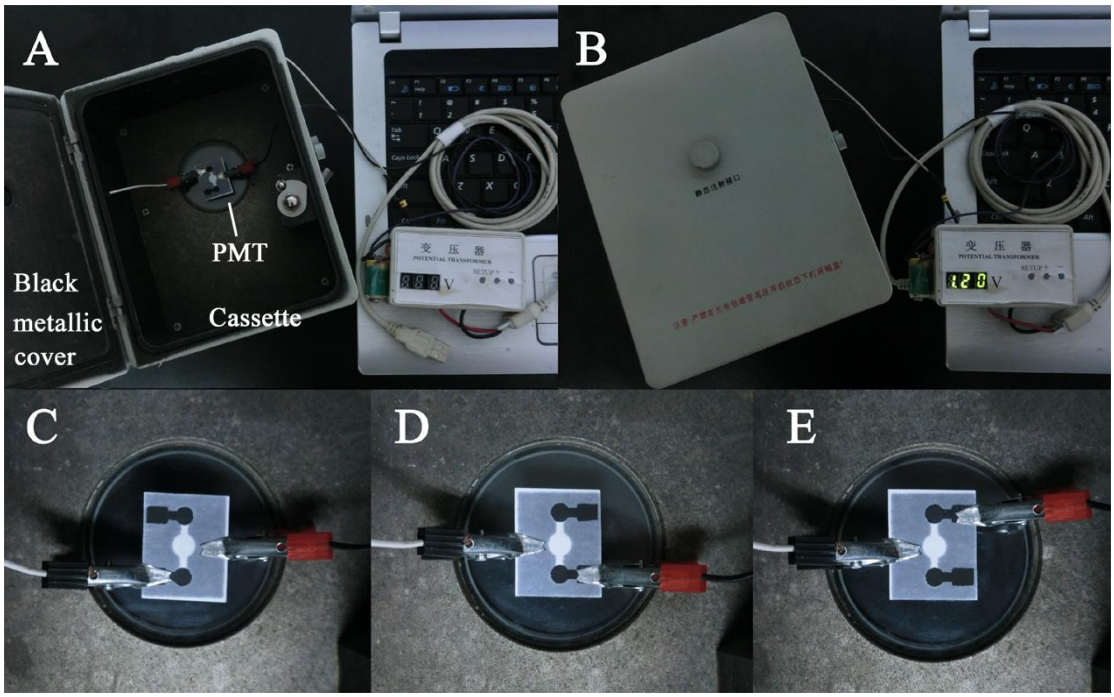


Figure S10 The operation pictures of this μ-PECLI for running an assay. After incubation and washing, paper immunodevice, connected with the voltage-tunable power device (Power off) through two alligator clips, was put on the top of the photomultiplier tube in a cassette with a black metallic cover (A). Then, for ECL detection, the cassette was shut with the black metallic cover, and the voltage-tunable power device was connected with the portable/mobile computer to trigger the ECL assay on the paper immunodevice to detect the first cancer marker (B). Subsequently, for the determination of the other three cancer markers on this paper immunodevice (C, D, E), the connection modes between the paper immunodevice and the voltage-tunable power device were changed successively to repeat the procedures of (A) and (B).

Reference

(1) Zheng, L.; Chi, Y.; Dong, Y.; Lin, J.; Wang, B. *J. Am. Chem. Soc.* **2009**, *131*, 4564-4565.
(2) Carrilho, E.; Martinez, A. W.; Whitesides, G. M. *Anal. Chem.* **2009**, *81*, 7091-7095.
(3) Wang, P.; Ge, L.; Yan, M.; Song, X.; Ge, S.; Yu, J. *Biosens. Bioelectron.* **2012**, *32*, 238-243.
(4) Zang, D.; Ge, L.; Yan, M.; Song, X.; Yu, J. *Chem. Commun.* **2012**, *48*, 4683.
(5) Previte, M. J. R.; Aslan, K.; Malyn, S. N.; Geddes, C. D. *Anal. Chem.* **2006**, *78*, 8020-8027.
(6) Driskell, J. D.; Uhlenkamp, J. M.; Lipert, R. J.; Porter, M. D. *Anal. Chem.* **2007**, *79*, 4141-4148.
(7) Wu, J.; Yan, Y.; Yan, F.; Ju, H. *Anal. Chem.* **2008**, *80*, 6072-6077.
(8) Cheng, C.-M.; Martinez, A. W.; Gong, J.; Mace, C. R.; Phillips, S. T.; Carrilho, E.; Mirica, K. A.; Whitesides, G. M. *Angew. Chem. Int. Ed.* **2010**, *49*, 4771-4774.