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Sensitive detection of CD147/EMMPRIN and its expression on cancer cells with electrochemical technique

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ABSTRACT

Cluster of differentiation 147 (CD147), also known as extracellular matrix metalloproteinase inducer (EMMPRIN), plays an essential role in tumor progression and metastasis, the expression of which on cell surface is a critical clinical testing index for cancer therapy. In this work, an electrochemical method to assay CD147/EMMPRIN expression on tumor cell surface is proposed. While the oxidation of 3,3′,5,5′-tetramethylbenzidine (TMB) catalyzed by horseradish peroxidase (HRP) can be employed for electrochemical measurement, the signal enhancement amplified by gold nanoparticles (GNPs) can be also utilized in this study. Therefore, under optimized conditions, the fabricated biosensor responds linearly to the CD147/EMMPRIN concentration from 125 to 1000 pg/mL with a detection limit as low as 52 pg/mL. High sensitivity can also be achieved for the quantification of breast cancer cells in a linear range from 6.2×10^4 to 6.25×10^5 cells/mL. Moreover, the CD147/EMMPRIN expressed on a single breast cancer cell can be calculated as 2.57×10^4 molecules/cell. The proposed strategy in this study is considerably potential for monitoring the dynamic protein expression on cancer cells and for the effective cancer diagnosis and treatment in the future.

1. Introduction

CD147/EMMPRIN, as an integral plasma membrane glycoprotein [1], is a critical player in intercellular recognition involved in tumor invasion and progression [2–4]. It has also been considered as an attractive marker on cancer cell surface, since it can be found on the surfaces of 90% of micrometastatic tumor cells with high expression, indicating a poor prognosis for cancers [5–9]. Thus, the effective determination of its expression on cell surface is desirable not only for monitoring its role in cancer development but also for cancer diagnosis and treatment.

So far, the semi-quantitative or quantitative data of CD147/EMMPRIN expressed on cancer cell surface are among the crucial testing indices for carcinogenesis. Several approaches including enzyme-linked immunosorbent assay (ELISA), radio immunoassay (RIA) and immunohistochemistry (IHC) have been developed and used for the assay of the protein. Nevertheless, the three traditional methods may have some drawbacks. For instance, the interruption of the intrinsic color of analytes can lead to false results in ELISA, RIA is risky for its radioactivity, while IHC is quite a complicated technique. Therefore, considerable efforts should be made to develop new methods to assay CD147/EMMPRIN expression on cancer cell surfaces, thus we have tried electrochemical technique in this study due to its advantages of simplicity, sensitivity, which is also very safe and easy to be operated.

In this paper, we report a novel method to assay CD147/EMMPRIN expression on cancer cell surfaces by using electrochemical technique coupled with a "sandwich" format. The "sandwich" structure can be formed on the electrode surface only when the CD147/EMMPRIN protein exists, yielding detectable electrochemical signals from the oxidation of TMB catalyzed by HRP. Thus, the proposed method may detect CD147/EMMPRIN expression on cancer cell surfaces with high sensitivity and selectivity, indicating potential applications in clinical diagnosis and prognosis in the future.

2. Experimental

2.1. Chemicals and materials

Recombinant human EMMPRIN Fc Chimera (47.4 kDa, CD147/EMMPRIN), goat polyclonal antibody raised against recombinant

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extracellular domain of human EMMPRIN (anti-CD147), human EMMPRIN goat polyclonal biotinylated antibody (biotinylated-anti-CD147) were obtained from R&D Systems. Bovine serum albumin (BSA), hydrogen tetrachloroaurate (III) trihydrate ($HAuCl_4 \cdot 3\,H_2O$, 99.9%), were purchased from Shanghai Sangon Biotech Co., Ltd. TMB, 4-aminothiophenol (4-AT), trisodium citrate and streptavidin–HRP were ordered from Sigma-Aldrich. Dulbecco's modified Eagle medium (DMEM) was from Nanjing Sunshine Biotechnology Co., Ltd. human breast cancer cells (MCF-7) were provided by Institute of Biochemistry and Cell Biology, Chinese Academy of Science. Other reagents were of analytical reagent grade and used as received. Unless otherwise noted, all solutions were prepared with water that was purified with a Milli-Q purification system (Barnstead, USA) to a specific resistance of >18 MΩ cm.

2.2. Preparation of GNPs

GNPs were prepared by reducing $HAuCl_4$ with trisodium citrate [10]. Briefly, a 100 mL aqueous solution of 0.01% (w/v) $HAuCl_4$ was added into a round-bottom flask and stirred to boil, followed by the rapid addition of 3.5 mL 1% trisodium citrate into the boiling solution, while the color of which became wine red from colorless after boiling for another 15 min with vigorous stirring. The size of the nanoparticles was 12.5 ± 2.3 nm, and the concentration of GNPs was 2.3 nM [11].

2.3. Fabrication of "sandwich" assay system

The substrate gold electrode (3 mm diameter) was first cleaned with piranha solution (98% H_2SO_4:30% H_2O_2 (v:v)=3:1) for 10 min, followed by being rinsed with double-distilled water (*Caution*: *Piranha solution reacts violently with organic solvents and should be handled with great care*!). Then, the electrode was abraded with successively finer grades sand papers and polished with alumina slurry of various particle sizes (1 μm, 0.3 μm, 0.05 μm) on silk. After that, the electrode was sonicated in both ethanol and double-distilled water for about 5 min to remove the residuals. Finally, the electrode was electrochemically cleaned with 0.5 M H_2SO_4 to remove any remaining impurities. After being dried with nitrogen, the pre-treated gold electrode was firstly immersed in 0.1 mM 4-AT solution for 8 h, then the above GNPs solution for 12 h. Subsequently, the electrode was incubated with 100 μg/mL anti-CD147 at 37 °C for 1 h, followed by being washed with 0.01 M phosphate buffered saline (PBS) containing 1% BSA and 5% sucrose to block the possible binding sites on the electrode surface. After that, the electrode was incubated with different concentrations of CD147/EMMPRIN or MCF-7 with CD147/EMMPRIN expressed on the surface at 37 °C for 1 h. After being washed with 0.01 M PBS buffer, the electrode was further immersed in 1 μg/mL biotinylated anti-CD147 at 37 °C for 1 h and 10% streptavidin–HRP (pH 6.0, 25 °C) sequentially, thus the "sandwich" sensor system was finally fabricated.

2.4. Cell culture

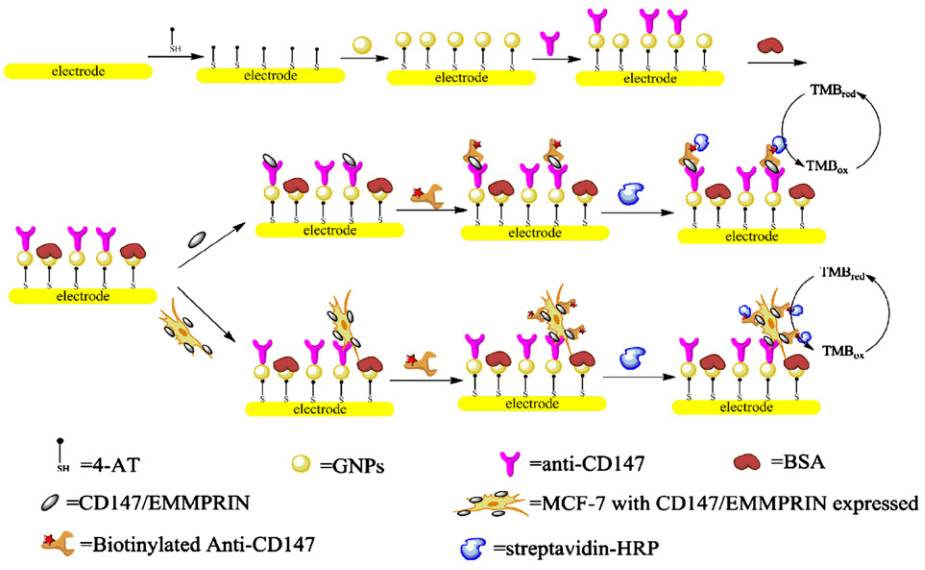
The cell lines MCF-7 were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum at 37 °C in a water-saturated incubator with 5% CO_2. The cells were collected and separated from the medium by centrifugation at 1000 rpm for 5 min after being washed with PBS twice and detached by Trypsin–EDTA for 1–3 min. The cells were then resuspended in the same culture medium with the concentration of 6.2×10^4–1.6×10^6 cells/mL.

2.5. Electrochemical measurements

All the electrochemical measurements were performed on an electrochemical analyzer CH I660C (CH Instruments, USA) at room temperature. The three-electrode system consisted of the "sandwich" structure modified gold electrode as the working electrode, a saturated calomel electrode (SCE) as the reference electrode and a platinum auxiliary electrode as the counter electrode. The test solution for cyclic voltammetry (CV) experiments which was freshly prepared before use contained 20 mM HEPES, 0.2 mM TMB, 0.1 mM H_2O_2, pH 6.0, while 5 mM $Fe(CN)_6^{3-/4-}$ with 1 M KCl was employed for electrochemical impedance spectra (EIS) measurements. Cyclic voltammograms were obtained in the potential range of 0.1–0.6 V at the scan rate of 100 mV/s. For EIS, spectra were recorded by applying a bias potential of 0.259 V vs. SCE and 5 mV amplitude in the frequency range of 0.1–100 kHz.

3. Results and discussion

Scheme 1 illustrates the construction of the "sandwich" structure system for the detection of CD147/EMMPRIN and its expression on cell surfaces. As is shown in the scheme, after the



Scheme 1. Schematic illustration of the "sandwich" biosensor for the detection of CD147/EMMPRIN and those expressed on cancer cells. .

electrode surface has been pre-modified with 4-AT which contains an amino moiety at one end and a thiol moiety at another end [12] so that 4-AT can be covalently linked to the electrode surface by Au–S bond, GNPs are electrostatically adsorbed onto the positive-charged amino moiety of 4-AT. Subsequently, anti-CD147 is covalently immobilized on GNPs via the thiol groups of the antibody and GNPs surfaces [13]. After being further treated with BSA to block the nonspecific binding, the electrode can be then employed to specifically recognize the CD147/EMMPRIN proteins or cancer cells with CD147/EMMPRIN expressed due to antigen–antibody interactions. After the introduction of biotinylated anti-CD147 into the system to form the "sandwich" structure, streptavidin–HRP is further immobilized onto the electrode surface due to the biotin–streptavidin interaction. Therefore, the oxidation of TMB catalyzed by HRP can be used for the electrochemical measurement, thus sensitive assay can be achieved [13,14]. Moreover, since the sensing system is fabricated by exploiting the amplification effects of GNPs for the mass-loading of CD147 antibody and signal amplification [15,16], this method can have ultra-high sensitivity in detecting the CD147/EMMPRIN protein, which may be further employed to assay the expression of CD147/EMMPRIN on cell surfaces. In fact, as is shown in Fig. 1, two pairs of peaks which reflect two successive one-electron reduction–oxidation processes in HRP-catalyzed TMB reactions can be obtained, because TMB firstly yields an intermediate TMB-free radical and then forms the completely oxidized product quinone diimine [13]. Meanwhile, the peaks with high peak currents can be observed in the presence of CD147/EMMPRIN, while only small peaks occur in the absence of CD147/EMMPRIN, indicating the feasibility of the sensing system for the detection of the protein.

The stepwise modification processes have been confirmed by EIS measurements. Fig. 2 shows the Nyquist plots of EIS for the electrode at different modification stages. The Nyquist plot of the EIS includes a semicircular portion and a linear portion. The semicircular portion at higher frequencies corresponds to the electron-transfer-limited process and its diameter is equal to the electron transfer resistance. The linear part at lower frequencies corresponds to the diffusion process [17]. So, the semicircle diameter positively responds to the increase in the interfacial charge transfer resistance [18]. It can be observed that the EIS of the bare gold electrode is similar to a straight line (Fig. 2a), while a semicircle is obtained after the modification of 4-AT, indicating that 4-AT has been successfully assembled onto the electrode surface (Fig. 2b). After the further treatment of the electrode with GNPs, the diameter of the semicircle decreases evidently (Fig. 2c) with a reasonable explanation that the nanoparticles can promote

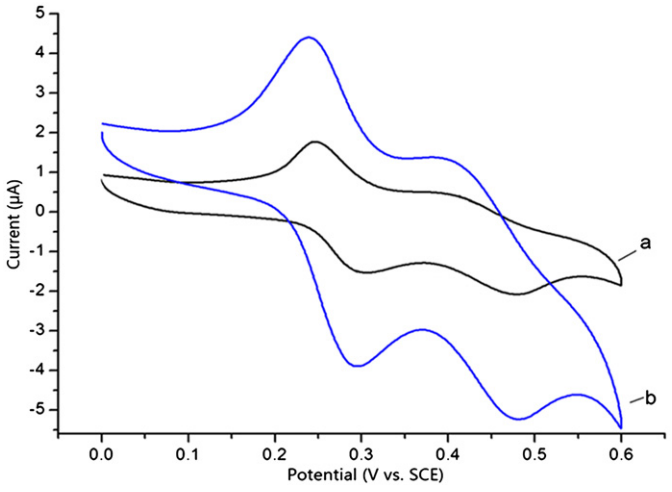


Fig. 1. CV response in the absence (a) and presence (b) of target protein (1000 pg/mL). Scan rate: 100 mV/s.

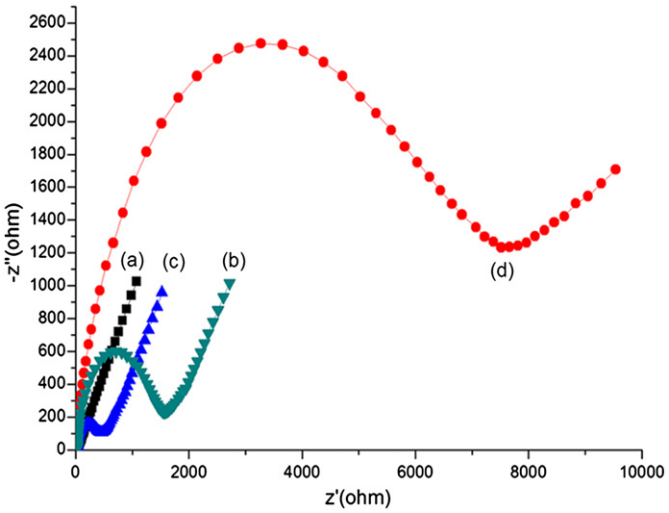


Fig. 2. Nyquist plots of electrochemical impedance spectra for (a) the bare gold electrode, (b) 4-AT modified electrode, (c) 4-AT/AuNPs modified electrode, (d) 4-AT/AuNPs/anti-CD147 modified electrode. Buffer: 1 M KCl containing 5 mM $Fe(CN)_6^{3-/4-}$. Biasing potential: 0.259 V. Amplitude: 5 mV. Frequency range: 0.1 Hz to 100 kHz.

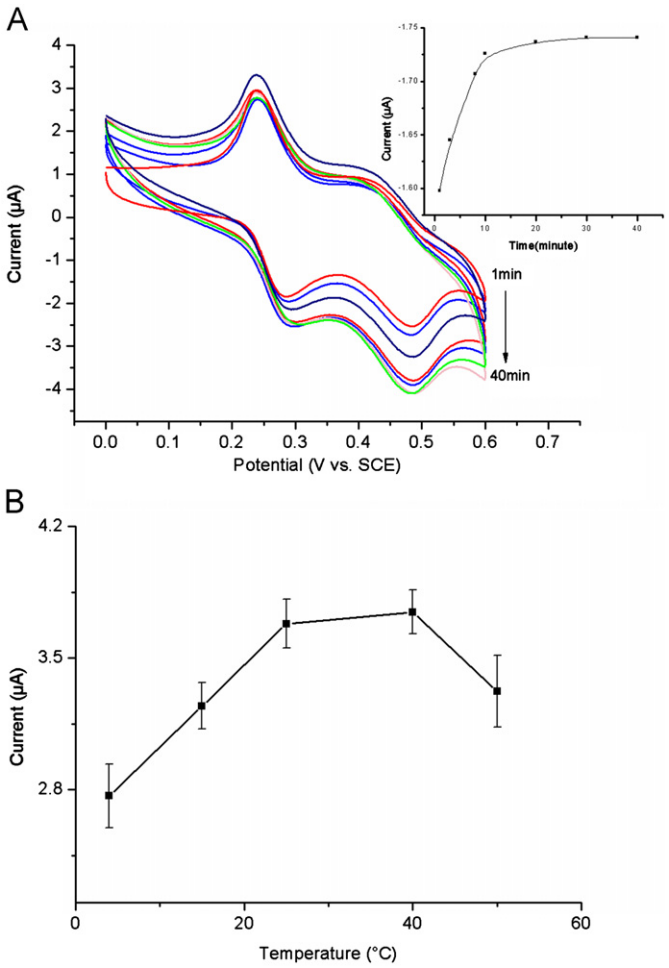


Fig. 3. (A) Cyclic voltammograms for the kinetic of enzymatic reaction. Scan rate: 100 mV/s. (B) Effect of reaction temperature on the enzymatic reaction.

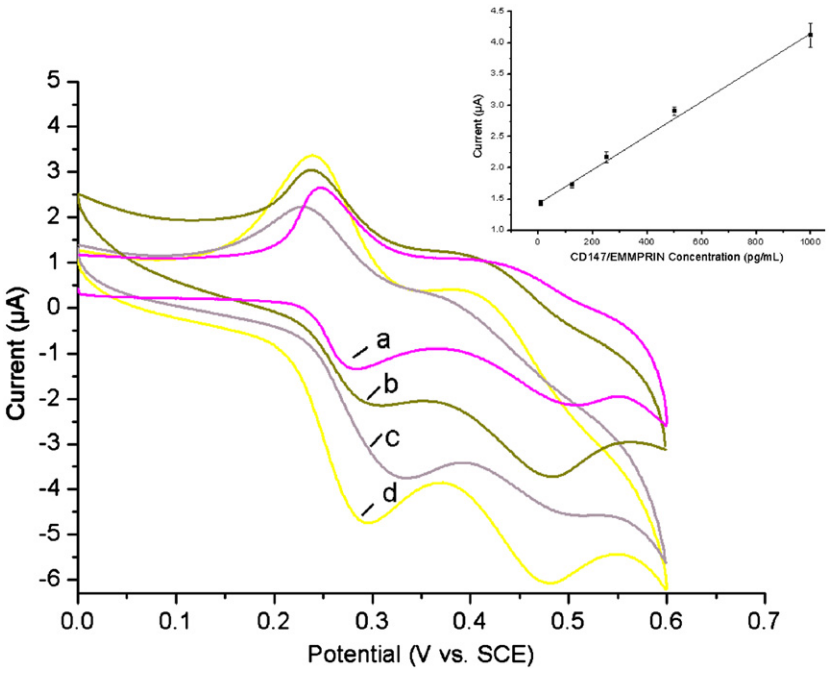


Fig. 4. Cyclic voltammograms corresponding to (a) 10, (b) 125, (c) 500, (d) 1000 pg/mL CD147/EMMPRIN. Scan rate: 100 mV/s. Inset shows the calibration curve for the electrochemical detection of CD147/EMMPRIN. Error bars represent standard deviations of measurements ($n=3$).

the electron transfer rate, indicating the amplification effect of GNPs. Furthermore, after the antibody is introduced into the system, a dramatic increase of electrochemical impedance is obtained (Fig. 2d), suggesting that an orderly negatively charged interface that more strongly repelled the negative redox species $Fe(CN)_6^{3-/4-}$ has been successfully constructed.

It should be mentioned that the measurement is based on the catalytic reaction of HRP towards TMB, thus the assay can be achieved with high sensitivity [14,15]. On the other hand, GNPs are used not only for the better immobilization of anti-CD147, but also for the signal amplification of the detection [13,16], ultrasensitive assay can be thus achieved in this work. By the way, we have optimized the experimental conditions for HRP enzymatic reactions including time and temperature in this work. As is shown in Fig. 3A, the current response keeps increasing until the time is prolonged to 15 min, suggesting 15 min as the optimal time for the enzymatic reaction on the electrode surface. Moreover, 25 °C is chosen as the approximate optimum reaction temperature for the enzyme in this assay (Fig. 3B) with a reasonable explanation that higher temperature might destroy the orderly "sandwiches" modified on the electrode surface, and the experiments can be more easily and conveniently performed at the room temperature around 25 °C.

Fig. 4 indicates the CV response to different concentrations of CD147/EMMPRIN. It can be observed that the anodic peaks at 0.30 V increase along with the concentrations of CD147/EMMPRIN. The reason is clear, since more analytes are recognized by the "sandwich" biosensor, more HRP molecules can be loaded onto the electrode surface to catalyze the oxidation of the signal molecules. The current saturation occurs at the concentration of 10,000 pg/mL when all the binding sites for CD147/EMMPRIN are occupied. Fig. 4 inset shows the linear relationship between the peak current (i_p) and the protein concentration (c_{CD147}) in the range from 125 to 1000 pg/mL. For each concentration of CD147/EMMPRIN, the electrochemical experiments are repeated for at least three times independently. The linear equation is

$$i_p(\mu A) = 0.12902 c_{CD147}(pM) + 1.45834 \quad (1)$$

The correlation coefficient of the linear equation is 0.994, indicating the acceptable precision and reproducibility of the proposed biosensor. The detection limit is calculated as 52 pg/mL, which is the lowest to the best of our knowledge. Furthermore, the recovery of the biosensor has also been evaluated, and the relative standard deviation (RSD) for series of repetitive measurements with different concentrations of CD147/EMMPRIN is all less than or at about 6%.

The proposed biosensor can be also used for the detection of cancer cells. As is shown in Fig. 5A, the peak currents increase with the elevated amount of cells. Further studies reveal that MCF-7 cells can be linearly quantified in the range of 6.2×10^4 to 6.25×10^5 cells/mL (Fig. 5B), with a detection limit of 4.5×10^4 cells/mL. The linear equation is

$$i_p'(\mu A) = 3.7147 \times 10^{-6} c_{MCF-7}(cells/mL) + 1.95442 \quad (2)$$

It has been known that CD147/EMMPRIN expressed on cell surfaces plays a significant role in cancer cell behavior, and its expression on living cancer cells can provide essential information of cancer progression and development. Therefore we have extended our studies to get more information on a single cancer cell. With the above two equations, the amount of CD147/EMMPRIN expressed on single breast cancer cell surface (n_{CD147}) can be obtained with the following equation [19]:

$$n_{CD147}(molecules/cell) = \frac{(i_p - 1.45834) \times 10^{-12} \times \left(6.02 \times 10^{23}\right)}{0.12902 \times 10^3 \times c_{MCF-7}(cells/mL)} \quad (3)$$

So, the amount of CD147/EMMPRIN on each cell surface can be calculated as 2.57×10^4 molecules/cell.

4. Conclusion

In this work, we have introduced a strategy to detect CD147/EMMPRIN and the protein expressed on cancer cell surfaces. The proposed method has a remarkable sensitivity, due to the

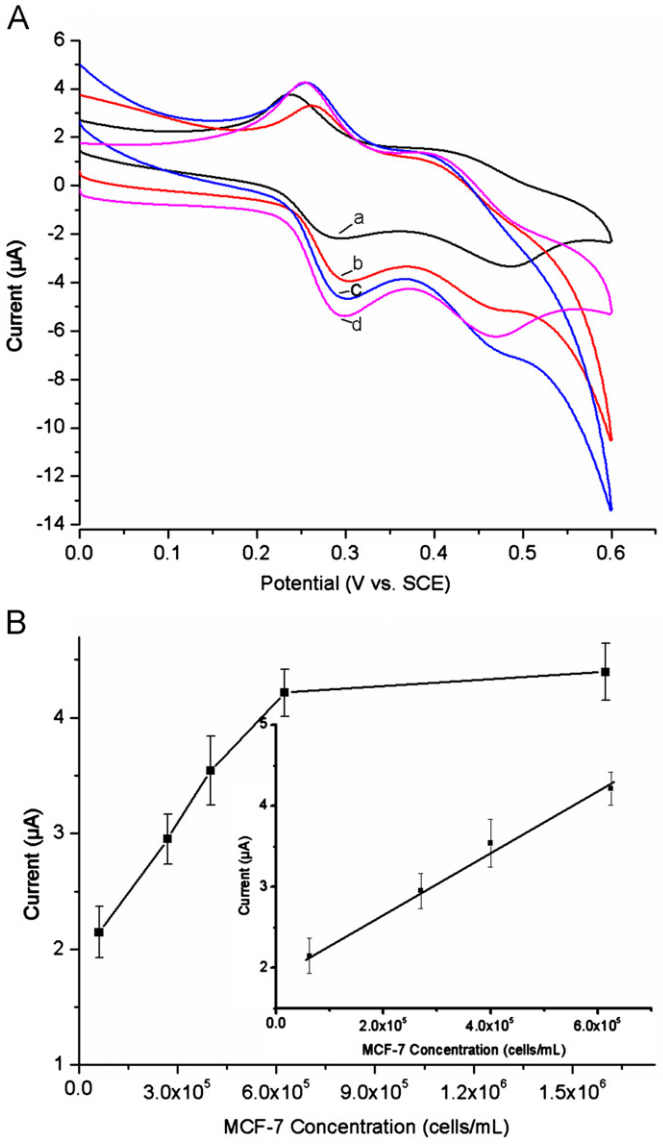


Fig. 5. (A) Cyclic voltammograms for (a) 6.2×10^4, (b) 2.7×10^5, (c) 4×10^5, (d) 1.6×10^6 cells/mL MCF-7. (B) The relationship between the background-subtracted CV anodic peaks at 0.3 V and the MCF-7 concentrations. Inset shows the linear part of the calibration. Error bars represent standard deviations of the measurements ($n=3$).

catalytic reaction of HRP and signal amplification of GNPs. Since CD147/EMMPRIN antigens are widely overexpressed on the surface of many types of tumor cells, the method may be potential for studying protein expression in cancer development, and promising for clinical prognosis and diagnosis of cancers in the future.

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References

[1] C. Biswas, Y. Zhang, R. Decastro, H.M. Guo, T. Nakamura, H. Kataoka, K. Nabeshima, Cancer Res. 55 (2) (1995) 434–439.
[2] S. Kim, H. Takahashi, W.W. Lin, P. Descargues, S. Grivennikov, Y. Kim, J.L. Luo, M. Karin, Nature 457 (7225) (2009) 102–106.
[3] L.A. Liotta, E.C. Kohn, Nature 411 (6835) (2001) 375–379.
[4] C.J.R. Stewart, M.L. Crook, Pathol. Res. Pract. 207 (1) (2011) 30–36.
[5] Z.M. Du, C.F. Hu, Q. Shao, M.Y. Huang, C.W. Kou, X.F. Zhu, Y.X. Zeng, J.Y. Shao, Int. J. Cancer 125 (8) (2009) 1832–1841.
[6] H.M. Guo, R.S. Li, S. Zucker, B. Toole, Cancer Res. 60 (4) (2000) 888–891.
[7] X.Z. Ju, J.M. Yang, X.Y. Zhou, Z.T. Li, X.H. Wu, Clin. Cancer Res. 14 (2) (2004) 494–501.
[8] H.C. Zheng, H. Takahashi, Y. Mural, Z.G. Gui, K. Nomoto, S. Miwa, K. Tsuneyama, Y. Takano, Br. J. Cancer 95 (10) (2006) 1371–1378.
[9] N. Reimers, K. Zafrakas, V. Assman, C. Egen, L. Riethdorf, S. Riethdorf, J. Berger, S. Ebel, F. Janicke, G. Sauter, K. Pantel, Clin. Cancer Res. 10 (10) (2004) 3422–3428.
[10] J. Wang, Y. Cao, Y. Li, Z.Q. Liang, G.X. Li, J. Electroanal. Chem. 656 (1-2) (2011) 274–278.
[11] J. Wang, W.Y. Meng, X.F. Zheng, S.L. Liu, G.X. Li, Biosens. Bioelectron. 24 (6) (2009) 1598–1602.
[12] J. Wang, Y. Cao, Y.Y. Xu, G.X. Li, Biosens. Bioelectron. 25 (2) (2009) 532–536.
[13] Y. Wu, J.W. Zheng, Z. Li, Y.R. Zhao, Y. Zhang, Biosens. Bioelectron. 24 (5) (2009) 1389–1393.
[14] C.J. Orendorff, A. Gole, T.K. Sau, C.J. Murphy, Anal. Chem. 77 (10) (2005) 3261–3266.
[15] X.L. Zhu, Y. Cao, Z.Q. Liang, G.X. Li, Protein Cell 1 (9) (2010) 842–846.
[16] Q. Fan, J. Zhao, H. Li, X.L. Zhu, G.X. Li, Biosens. Bioelectron. 33 (1) (2012) 211–215.
[17] H.L. Zhang, G.S. Lai, D.Y. Han, A.M. Yu, Anal. Bioanal. Chem. 390 (3) (2008) 971–977.
[18] Y. Cao, S. Zhu, J.C. Yu, X.J. Zhu, Y.M. Yin, G.X. Li, Anal. Chem. 84 (10) (2012) 4314–4320.
[19] X.N. Zhang, Y.Q. Teng, Y. Fu, L.L. Xu, S.P. Zhang, B. He, C.G. Wang, W. Zhang, Anal. Chem. 82 (22) (2010) 9455–9460.