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A simple and rapid electrochemical strategy for non-invasive, sensitive and specific detection of cancerous cell

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ABSTRACT

Developing non-invasive, sensitive and specific sensing strategies for cancerous cell detection with simple and low cost instrumentations provide great advantages in cancer research and early diagnosis of diseases. In the present work, gold nanoparticles (Au NPs) functionalized with recognition components (folic acid) and signal indicator (ferrocene) was designed to fabricate electrochemical cytosensor. The Au NPs can not only accelerate electron transfer between signal indicator and the underlying electrode but also accumulate more ferrocene on the cytosensor surface to magnify signal for improving detection sensitivity. The surface-tethered folic acid plays a key role in specific binding folate receptor-riched HeLa cells on the cytosensor surface, resulting in corresponding current signal change measured by differential pulse voltammetry method. A wide detection range from 10 to 10^6 cells/mL with a detection limit as low as 10 cells/mL for cancerous cells was reached in the presence of a large amount of normal ones with fast differential pulse voltammetry measurement. Detection of the captured cells can be finished within 1 min. The developed strategy provides a new way for operationally simple, rapid, sensitive and specific detection of cancerous cells.

1. Introduction

Cancer has become one of the most likely causes of death worldwide. The chance of being cured increases with early diagnosis and treatment of the cancer disease [1]. Most of conventional approaches including fluorescent, radioactive and spectrophotometric procedures need for stringent laboratory conditions and expensive instrumentation and are usually high-cost and time-consuming in preparation steps. Moreover, some approaches may couple with radioactive risk. Therefore, it is highly desirable to develop simple, rapid and non-destructive methods for early detection of cancer with low cost [2]. To meet these specific requirements, electrochemical techniques have attracted increasing attention due to the high sensitivity, rapidity, simple operation, broad dynamic range, minimal power requirement and excellent reproducibility [3–6]. Electrochemical cytosensors are generally fabricated based on measuring the changes in current or resistance at the cytosensor interface that are related to the biological status of the cells, including cellular viability, proliferation, apoptosis and immobilized cell number [3]. Some cytosensors are developed based on electrostatic attraction between the cells and the cytosensor surface, which are used to investigate cell adhesion, proliferation and apoptosis [7–9]. While, the normal cells are also negatively charged, therefore, such cytosensors lack detection specificity and could not distinguish cancerous cells from nonconcerous ones. To meet the requirements of clinical diagnosis and therapy, an increasing interest has focused on "target-binding" technology to develop cytosensors with selectivity [10]. Owing to the highly specific recognition between antibody and antigen, electrochemical immunoassay has been developed to evaluate cell surface carbohydrates and glycoprotein [11,12]. Based on high affinity of folic acid for folate receptor, several electrochemical cytosensors were developed to selectively detect cancerous cells from normal ones [13,14]. To accurately determine cancerous cells at ultralow level for the early stage detection of diseases, various signal amplification approaches have been developed to enhance the electrochemical current signal of recognition events [15–18]. Gold nanoparticles functioned with both

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recognition component and signal amplification component were used to fabricate cytosensor for cytosensing and evaluating cell surface carbohydrate and glycans [19,20]. By binding aptamer on graphene modified electrode surface, Qu's group developed a cytosensor to distinguish cancerous cells from normal ones [21].

From a clinical point of view, one key goal of developing reliable cytosensing technology is to treat sample without introducing of extraneous reagents and reduce time consumption [22]. To decrease the risk of false-positive results, any possible contamination on the cells should be decreased as low as possible. Though the developed electrochemical cytosensors present advantages on cytosensing, these studies mainly suffer from the following drawbacks. (1) Electrochemical impedance spectroscopy (EIS) is a commonly used method to monitor live cells. Recently; however, Cheng's group found that a larger resistance change occurred during EIS measurement [23]. They speculated the possible reason might be detachment of some cells from the cytosensor film due to deterioration of cell vitality under a longer time of electrical field effect or accumulation of external redox probe within the film. Thus a fast response electrochemcial technique, differential pulse voltammetry (DPV), was used as an alternative method. (2) To produce detection signal, enzymatic substrates, for example H_2O_2, or external redox probe, for example $Fe(CN)_6^{4-}/Fe(CN)_6^{3-}$, are often required to be added in detection solution. While, it was reported that H_2O_2 might induce cell apoptosis [24]. $Fe(CN)_6^{4-}/Fe(CN)_6^{3-}$ can be easily metamorphic in biological condition, producing unexpected effects on viability of tissue [25,26]. So the detection results might be influenced by the added substrates in detection solution. It remains a challenge to develop approaches for non-invasive cell detection.

Keeping that in mind, we have developed a novel electrochemical cytosensor which can sensitively differentiate cancerous cells from control ones by making use of the advantages of tumor marker and gold nanoparticles. To avoid the potential contamination, the current signal indicator did not dissolve in detection solution while it was immobilized on the electrode surface and did not directly contact with cancerous cells. With fast response DPV approach, the immobilized cells can be quickly detected within 1 min with simple instrumentation. The rapid detection can further avoid possible contamination and reduce the time of electrical field effect on the cells and thus reduce the loss of cell viability.

2. Experimental

2.1. Materials

1,6-Hexanedithiol and N-hydroxysuccinimide (NHS) were purchased from Acros Organics. 16-Mercaptohexadecanoic acid, 6-(Ferrocenyl) hexanethiol and N-(3-Dimethylaminoprophyl)-N′-ethylcarbodimide hydrochloride (EDC) were purchased from Sigma-Aldrich Company. Folic acid was obtained from Beijing Dingguo Changsheng Biotech Co. Ltd. Chloroauric acid was obtained from Shanghai Chemical Reagent Co. Ltd. Sodium citrate was obtained from Beijing Chemical Works. Physiological saline (0.9%) was used as the buffer solution. The water used was purified through a Millipore system. All chemicals were used as received.

2.2. Preparation and characterization of gold nanoparticles

Au NPs were prepared by a conventional citrate-reduction method [27,28]. After sodium citrate solution (1% by weight, 0.5 ml) was added to a boiling chloroauric acid solution (0.01% by weight, 50 ml), the mixture was kept boiling and stirring for another 20 min. After being cooled to room temperature, the resultant Au NPs solution was stored at 4 °C.

The gold hydrosol was characterized from 350 to 800 nm by a VARIAN-50 Conc UV–Visible spectrophotometer (Fig. S1 in Supporting Information (SI)) and Transmission electron microscopy (Fig. S2 in SI). The diameter was evaluated as 15 nm.

2.3. Fabrication of the sensing interface

A fresh gold bead electrode (0.11 cm^2) was firstly immersed into 1 mM 1,6-Hexanedithiol ethanol solution (1.0 mM) for 2 h. After being taken out, the electrode was rinsed with large amount of ethanol and Milli-Q water and then immersed into the Au NPs solution for 1 h. The resultant modified electrode was washed thoroughly with Milli-Q water and then immersed in a mixture solution of 16-mercaptohexadecanoic acid (1 mM) and 6-(ferrocenyl) hexanethiol (1 mM) with volume ratio of 1:5 for 8 h. To active the carboxylate terminal group of 16-mercaptohexadecanoic acid the obtained electrode was then immersed in the mixture of EDC (75 mM)/ NHS (15 mM) for 1 h. At last, the activated modified electrode was immersed into folic acid solution (0.5 M) for 2 h. Thus a cytosensor was prepared for selective detection of cancerous cells.

2.4. Cell culture and cell immobilization

The cell lines, HeLa cells (human cervical carcinoma cell) and normal cells (HEK 293), were obtained from the Kunming Institute of Zoology, Chinese Academy of Sciences. The cell lines were cultured cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin (100 mg mL^{-1}) in a 5% CO_2 humidified chamber at 37 °C. After the concentration of cells reached about 1×10^6 cells mL^{-1}, HeLa and HEK 293 cells were trypsinized in the presence of 0.25% trypsin solution and collected from the medium by centrifugation at 3000 rpm for 5 min. Then the collected cells were washed twice with a sterile PBS (pH 7.2). The cell sediment was re-suspended in the physiological saline (0.9% NaCl) solution to obtain a homogeneous cell suspension. Then the as-prepared cytosensor was incubated in the cell suspensions at different concentrations at 37 °C for 10 min.

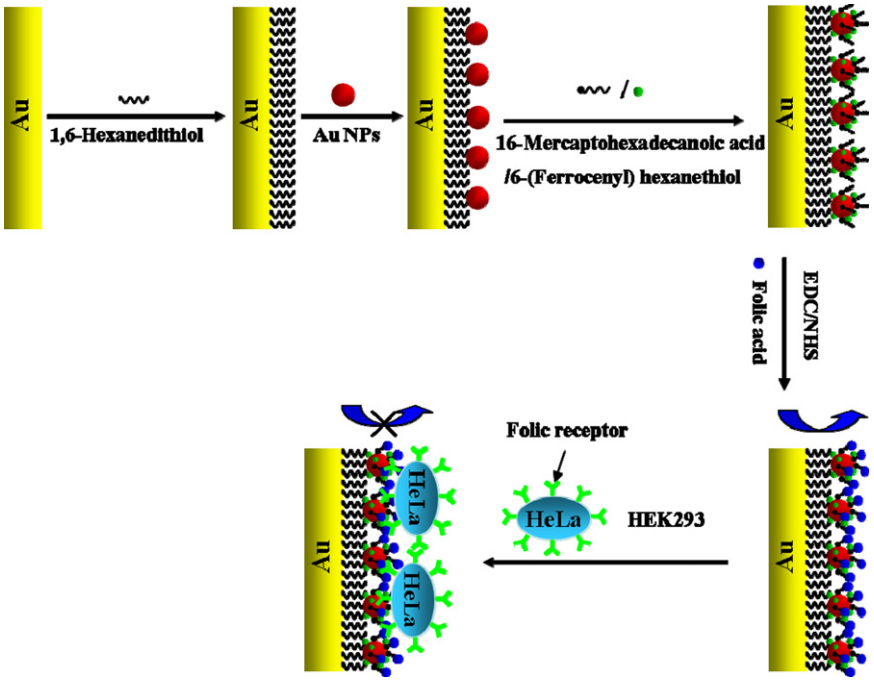
2.5. Electrochemical measurements

Electrochemical measurements were conducted with a conventional three-electrode system by CHI 832B electrochemistry workstation (Co. Chenhua, China). Platinum foil and Ag/AgCl electrode worked as counter and reference electrode, respectively. $NaClO_4$ solution (0.1 M) was used as supporting electrolyte.

3. Results and discussion

3.1. Fabrication and characterization of the cytosensor

The excellent biocompatibility and the ability to facilitate electron transfer enable gold nanoparticles (Au NPs) to be a promising platform for construction of biosensors and improving the sensing performance [29]. As shown in Scheme 1, the electrochemical cytosensor was prepared by first assembling dithiol molecules, 1,6-hexanedithiol, on a gold electrode surface to produce a monolayer with –SH as terminal group. Thus, Au NPs can be easily assembled on the 1,6-hexanedithiol layer through Au-S bond. After that, the Au NPs was functionalized with a mixed monolayer of 16-mercaptohexadecanoic acid and 6-(ferrocenyl) hexanethiol. The 16-mercaptohexadecanoic acid was further used to covalently bond folic acid onto the electrode surface to



Scheme 1. The preparation process of the developed electrochemical cytosensor and the mechanism for cancer cell detection. The surface-confined ferrocene acts as current signal indicator for cell detection, which can be significantly magnified by the surface-confined gold nanoparticles. The current response of the cytosensor is decreased once HeLa cells are selectively captured. The decreased current signals is directly related to the amount of the captured cancer cells.

specifically recognize folate receptor riched cancerous cells according to the high affinity of folic acid for folate receptor [30]. Folate receptor is usually over-expressed on cancerous cells while is limited expressed on normal ones [31,32]. Hence, the surface-tethered folic acid can act as target molecule in selective detection of cancerous cells [1]. Here, the surface-tethered ferrocene acted as electrochemical current signal indicator. After binding folic acid the current response of the surface tethered ferrocene was reduced (Fig. S3 in SI), which can be further decreased by the captured cells.

Fig. 1A (a) shows current response of the as-prepared cytosensor in supporting electrolyte. One pair of redox peaks with high magnitude were observed obviously. The apparent formal potential was calculated as 0.37 V from the average of oxidation and reduction peak potentials. As a control experiment, a gold electrode was immersed into mixture of 16-mercaptohexadecanoic acid and 6-(ferrocenyl) hexanethiol, producing a gold electrode modified with mixed monolayer of 16-mercaptohexadecanoic acid and 6-(ferrocenyl) hexanethiol (named as Au/(MHDA-HT-Fc)). In this case, the 6-(ferrocenyl) hexanethiol directly linked on the gold electrode surface and was much closer to the underlying electrode than that on the as-prepared cytosensor. However, the current response of the Au/(MHDA-HT-Fc) electrode, Fig. 1A (b), was significantly lower than that of the cytosensor, Fig. 1A (a). It was considered that the significant current response of the cytosensor was ascribed to the magnification function of the assembled gold nanoparticles. On the one hand, nanoparticles can accelerate electron transfer [33,34]. As confirmed by Pinagarron et al. that the gold nanoparticle can facilitate electron transfer between surface-immobilized protein and the underlying electrode [35]. On the other hand, thanks to the large surface-to-volume ratio and three dimensional structure of nanoparticles large amount of 6-(ferrocenyl) hexanethiol was accumulated on the Au NPs surface, leading to the magnified current response. Additionally, the peak-to-peak potential difference was quite small and did not change obviously with increasing scan rate, Fig. 1(B). It was about 20 mV at scan rate of 50 mV/s and slightly increased to 32 mV at scan rate of 500 mV/s,

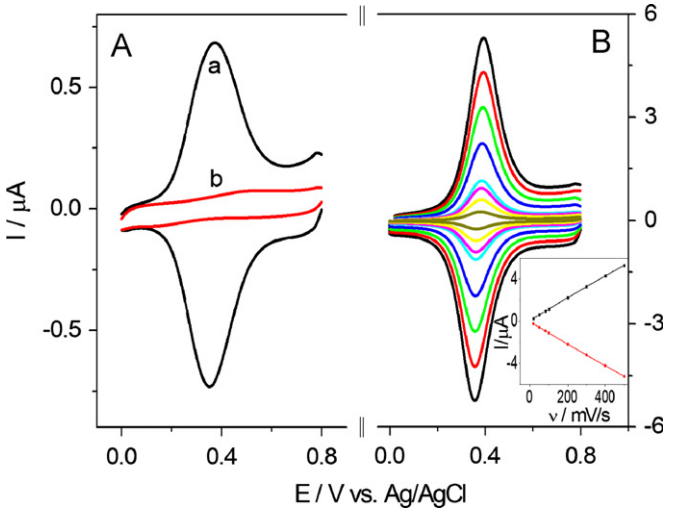


Fig. 1. (A) Current responses of the as-prepared cytosensor (a) and the Au/(MHDA-HT-Fc) modified electrode (b) in supporting electrolyte with a scan rate of 80 mV/s. (B) Current responses of the as-prepared cytosensor with different scan rate. From inner to outer: 20, 50, 80, 100, 200, 300, 400, 500 mV/s. The inset is peak currents as a function of scan rate.

indicating that electron can be facilely transferred to the underlying electrode under function of the Au NPs. The peak current of the as-prepared cytrosensor increased linearly with increasing scan rate (the inset of Fig. 1B), suggesting a surface-controlled electron transfer process of the ferrocene.

The stability of the as-prepared cytosensor was investigated by continued potential scan of 20 segments in supporting electrolyte. Then the cytosensor was immersed in physiological saline (0.9% NaCl) solution for 1 h. No obvious current change was monitored in both cases, Fig. 2. The results validate that the as-prepared modified electrode presents good stability and is qualified to be a cytosensor for cancerous cell detection.

3.2. Detection principle of the developed cytosensor

Since folic receptor is over-expressed on membrane of epithelial cancers, human cervical carcinoma cell (HeLa cell) was used as a model to validate the developed cytosensoring system. Owing to the high affinity of folic acid for folate receptor, the folic acid functionalized cytosensor can specifically recognize HeLa cells. The principle of the electrochemical detection cancerous cells is described as following. In an electrochemical system, redox process of the surface-tethered ferrocene involves two steps [36–38]. One is the electron transfer between the ferrocene and the underlying electrode. The other is anion transfer between the supporting electrolyte solution and the modified molecular layer to form ion pairs between the ferrocenium cations and anions. The redox process of the surface-confined ferrocene in the supporting electrolyte could be described as follows:

$$-\mathrm{Fc}+\mathrm{X_s^-}\rightarrow-\mathrm{Fc}+\mathrm{X^-}+\mathrm{e^-}+\mathrm{solv}$$

where, $-\mathrm{Fc}$ and $-\mathrm{Fc^+}$ represent the neutral and oxidized states of the ferrocene group, respectively. $-\mathrm{Fc^+X^-}$ means an ion pair between the ferrocenium cation and anion from electrolyte. $\mathrm{X_s^-}$ and 'solv' represent anion and solvent molecule in electrolyte, respectively. Due to the large size (the diameter is about 10 μm) and high resistance (10^2–10^5 Ω) cell is considered as one kind of dielectric material [39,40]. Therefore, the solvated anion transfer would be hindered upon capturing cells on the cytosensor surface, resulting a decreased current response. The degree of current decrease is directly related to the amount of the captured cells.[3] Since surface-tethered ferrocene acted as current signal indicator, the experiments were performed in supporting electrolyte without introducing external signal indicator such as potassium ferricyanide, which can reduce the possible contamination during cell detection [25,26].

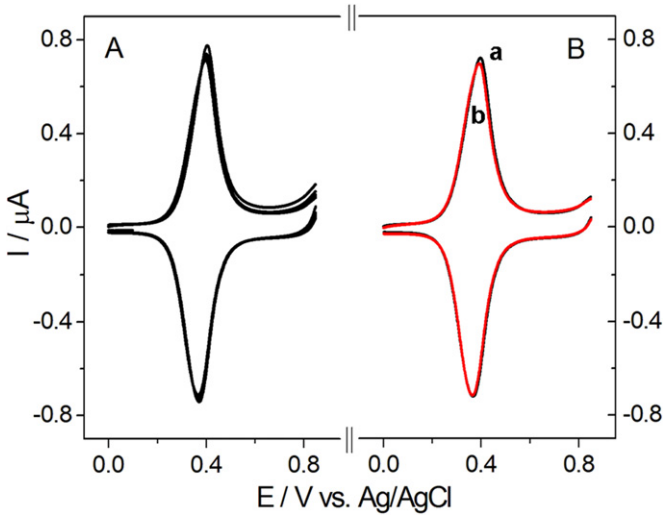


Fig. 2. (A) Current response of the modified electrode in supporting electrolyte by continued potential scan of 20 segments. (B) Current response of the modified electrode before (a) and after being immersed in 0.9% NaCl solution for 1 h (b). Scan rate: 100 mV/s.

3.3. Detection of cancerous cells

In our investigations, DPV was performed for cell detection since it is very sensitive to current signal change. Furthermore, DPV is a fast response technique, which avoids a long time of electrical field effect on the cells and thus reduces the loss of cell viability [23]. Learned from Fig. 3(A), the current response of the cytosensor is related to the incubation time in HeLa cell suspension. The peak current decreased significantly after incubation for 5 min and reached a plateau from 10 min to 30 min, Fig. 3(B). Therefore, 10 min was used as the optimal incubation time for HeLa cell detection.

Fig. 4(A) shows the DPV responses of the cytosensor after being incubated in HeLa cell suspensions with different concentrations. The repeatability of the cytosensors was evaluated by plotting the degree of current decrease $(I_{Blank}–I_{HeLa})/(I_{Blank}–I_{10^6HeLa})$ against the logarithm of HeLa cell concentration with error bar, where I_{Blank}, I_{HeLa} and I_{10^6HeLa} are the current intensities in the absence of cells, different concentrations of HeLa cells and 10^6 HeLa cells/mL,

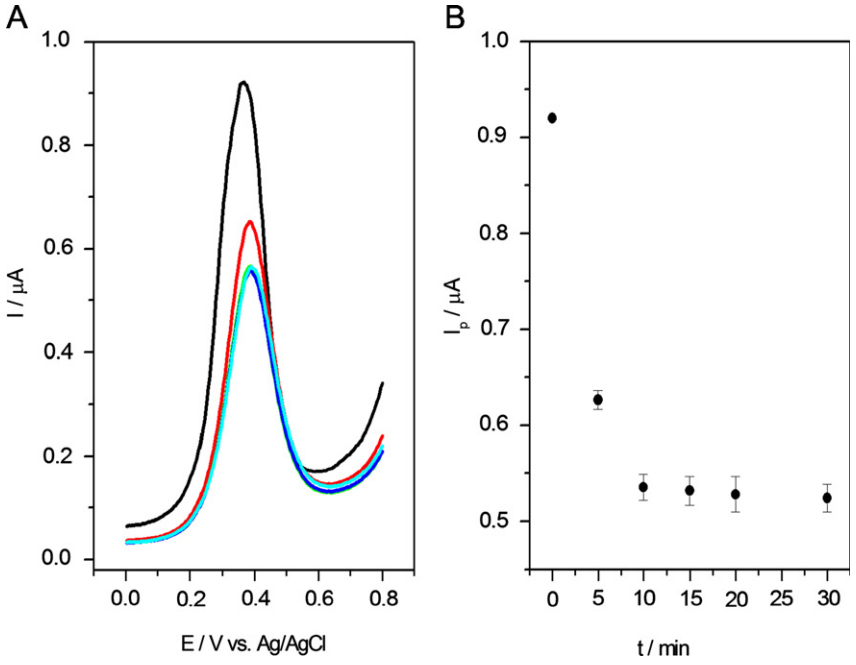


Fig. 3. (A) DPV responses of the as-prepared cytosensor against the immersing time in HeLa suspension (10^4 cells/mL): 0, 5, 10, 15, 20, 30 min from up to down. (B) Peak current intensity as a function of immersing time.

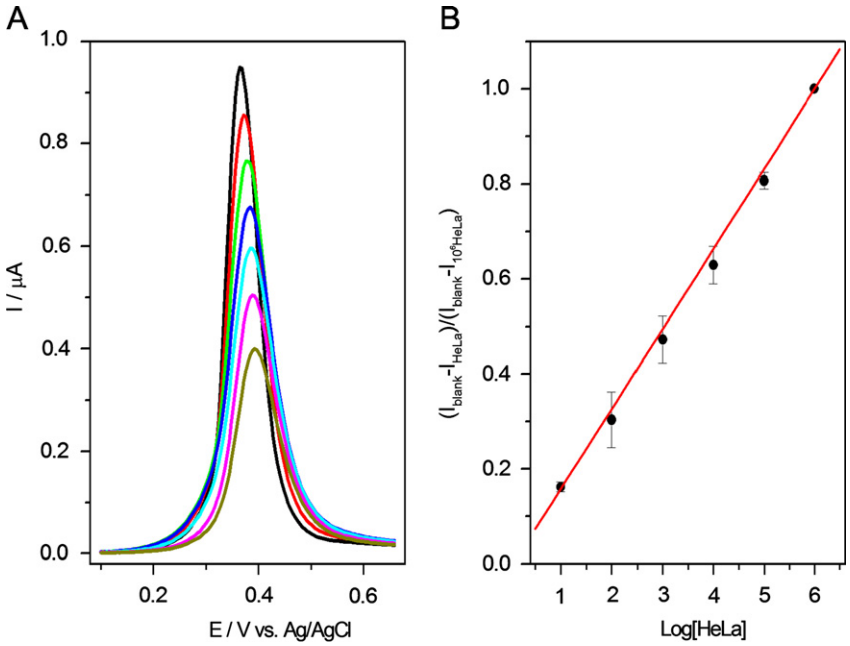


Fig. 4. (A) DPV responses of the as-prepared cytosensor after being incubated with different concentrations of HeLa cells in physiological saline: 0, 10, 10^2, 10^3, 10^4, 10^5, 10^6 cells/mL (from outer to inner). (B) The current change with error bars against the logarithm of HeLa cell concentration.

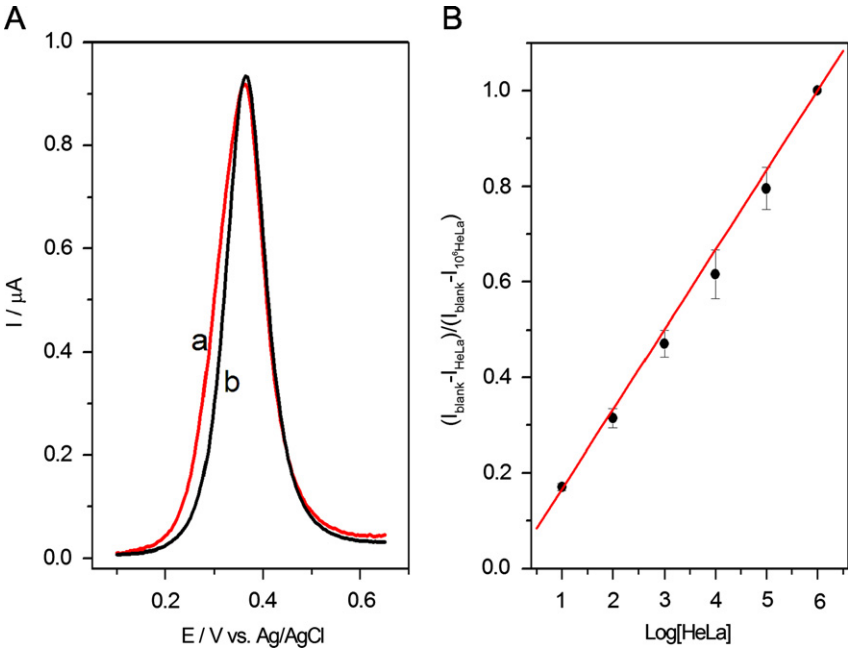


Fig. 5. (A) DPV responses of the as-prepared cytosensor in supporting electrolyte before (a) and after (b) incubation in normal cell suspension (HEK 293, 10^4 cells/mL) for 10 min. (B) The current change with error bars against the logarithm of HeLa cell concentration containing normal cell (HEK 293, 10^4 cells/mL).

respectively. It was found that the degree of current change caused by the captured cells depended linearly on the logarithm of HeLa cell concentration from 10 to 10^6 cells/mL ($R^2=0.99$), Fig. 4(B), suggesting a wide detection range and a detection limit as low as 10 cells/mL with good repeatability.

3.4. Selective detection of cancerous cells

Cancerous cells are coexisted with normal ones in the body. Therefore, it is important to sensitively differentiate cancerous cells from normal ones for the early diagnosis. Since folate receptor is over-expressed on HeLa cells while limit-expressed on normal tissues [31,32], no obvious current change was monitored after incubating the cytosensor in normal cell suspension (HEK 293, 10^4 cells/mL), Fig. 5(A). Hence, it is expected that the as-prepared cytosensor could selectively detect HeLa cells according to the high affinity of folic acid for folate receptor. Similar as the detection of the cytosensor on HeLa cells, Fig. 4(A), the DPV responses of the cytosensor after being incubated in HeLa cell suspensions containing normal cells decreased with increasing concentration of HeLa cell suspension (not shown here). The degree of current decrease $(I_{Blank}-I_{HeLa})/(I_{Blank}-I_{10^6HeLa})$ against

the logarithm of HeLa cell concentration with error bar was plotted to evaluate the detection repeatability, Fig. 5(B), which shows a linear relationship in the range of 10–10^6 cells/mL (R^2=0.99). The current change of the cytosensor to HeLa cell as low as 10 cells/mL was obviously monitored even in the presence of a large amount of normal cells, confirming the high selectivity as well as high sensitivity of the developed strategy.

Recently, a detection limit of 6 cells/mL was realized with EIS approach, while, the linear detection range of 6–10^3 cells/mL was narrow [41]. Weng et al. reported that a detection limit of 10 cells/mL with detection range of 10–10^5 cells/mL could be realized with AC impedimetric approach [13], which takes about 50 min for each measurement. In our investigation, however, the detection can be finished within 1 min with DPV approach. The short time of electrical field effect can reduce the loss of cell viability. Meanwhile, the rapid measurement can avoid any possible contamination on the cells during a long time detection and thus decrease the possible risk of false results. Compared with our previous report [14,42], this work developed different strategies for signal magnification and redox probes immobilization. Au NPs were used to facilitate electron transfer and immobilize large amount of redox probe, leading to the magnified current response. Additionally, both the assembling of gold nanopaticles on Au electrode and their functionalization with folic acid and redox probe are through stable Au–S covalent bond, which can improve the stability of the cytosensors.

4. Summary

We have successfully developed an electrochemical cytosensor with good stability and repeatability for cancerous cell detection. Folate receptor riched HeLa cells can be sensitively and specifically differentiated from normal ones. A detection limit as low as 10 cells/mL with a wide detection range from 10 to 10^6 cells/mL was reached in the presence of a large amount of normal cells (HEK 293). The Au NPs functionalized with recognization components and signal indicators plays a key role in both specific cell detection and signal magnification for improving detection sensitivity. Ultilizing surface-tethered ferrocene as current signal indicator can avoid the addition of extraneous reagents in detection solution, thus reducing possible contamination on cell detection. The detection process is simple, rapid and non-invasive on the captured cells and hence decreases the risk of false results. The developed strategy might be beneficial to early diagnosis of diseases, which will improve successful treatment.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2012.11.040.

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