



# A self-assembled monolayer based electrochemical immunosensor for detection of leukemia *K562A* cells

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## Abstract

A novel strategy to quantify the cell number of leukemia *K562A* cells using electrochemical immunosensor was developed by effective surface immunoreaction between P-glycoprotein (P-gp) on cell membrane and P-gp mouse monoclonal antibody bound on an epoxysilane monolayer modified glassy carbon electrode. The surface morphologies of the epoxysilane monolayer and the bound antibodies were studied with atomic force microscopy. The binding of target *K562A* cells onto the immobilized antibodies increased the electron-transfer impedance of electrochemical probe, which depended linearly on the cell concentration in the range of  $5.0 \times 10^4$ – $1.0 \times 10^7$  cells mL<sup>-1</sup>. The detection limit of the immunosensor was  $7.1 \times 10^3$  cells mL<sup>-1</sup>. The proposed strategy showed acceptable reproducibility with an RSD of 3.4% for the linear slope and good precision with the RSD of 3.7% and 3.0% examined at the cell concentrations of  $2.0 \times 10^6$  and  $1.0 \times 10^7$  cells mL<sup>-1</sup>.

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## 1. Introduction

The detection of cells and their activity monitoring are very significant for life science research, toxicity monitoring, clinical diagnostics, and public health protection. Some techniques such as spectrophotometric method, fluorescent microscopy, amperometric or potentiometric scanning probe technique and flow cytometry have been developed for monitoring cell viability and proliferation [1–3]. The use of cell-based biosensor platforms and study on cytosensing based on cellular electron transfer and signal recognition and transduction have attracted considerable attention [4] due to the remarkable advantages of the electrochemical monitoring of cell viability and proliferation [3], such as low cost, convenient operation, rapid detection and good sensitivity. The amperometric detection

of cell number based on a surface immunoreaction of a cellular membrane protein with its labeled antibody has been proposed in our previous work [5]. With the immunoreactions two quartz piezoelectric crystal immunosensors for *Salmonella paratyphi* A and *S. typhimurium* have been developed by immobilizing their corresponding antibodies on alkanethiols self-assembled monolayer (SAM) modified gold electrodes to recognize these cells [6,7].

Impedance technique is an alternative to develop biosensors for detection of cells [8]. It is reported that the membranes of natural biological cells (thickness 5–10 nm) show a capacitance of 0.5–1.3  $\mu\text{F cm}^{-2}$  and a resistance of 102–105  $\Omega \text{cm}^{-2}$  [9]. When cells attach to an electrode surface, a barrier for the electrochemical process is produced, thereby hindering the access of the redox probe to the electrode surface, resulting in an increase in the electron-transfer resistance. Combining the surface immunoreaction and impedance technique two impedance cell sensors for *Escherichia coli* O157:H7 with simple manipulation and

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low equipment cost have been prepared [10,11]. These works used immobilized anti-*Escherichia coli* O157:H7 antibody to recognize corresponding cells, thus showing good selectivity. In this work we suggested a novel strategy to prepare an impedance sensor for quantifying the cell number of leukemia *K562A* cells. The recognition for cytosensing was based on the immunoreaction between the immobilized P-glycoprotein (P-gp) antibody and the P-gp overexpressed on leukemia *K562A* cell membrane.

The immobilization of antibody on electrode surfaces can commonly be performed by the specific interaction between protein A and antibody [12], the self-assembly of Langmuir-Blodgett film [13], the embedment of polymer or gel matrices [14,15], the entrapment of nanoparticles and the covalent binding to a SAM [6,7,16,17]. In the biomedical sciences silane monolayers have been used for chemical immobilization of antibodies [18]. Among these silanes epoxysilanes are classical compounds to form SAMs for providing a strong binding of biological molecules to different supports [19]. Using an epoxysilane monolayer to serve as a template the immobilization of anti-*Escherichia coli* O157:H7 antibodies onto ITO electrode chips has been carried out [10]. This study formed an epoxysilane monolayer on an electrochemically pretreated glassy carbon electrode (GCE) to chemically anchor P-gp antibody. The P-gp overexpressed leukemia *K562A* cells could thus be recognized for impedance detection of cell number. This proposed sensor showed better sensitivity than those for *E. coli* O157:H7 based on impedance [10,11] and SPR [20] techniques, and *S. typhimurium* based on piezoelectric technique [7,21].

## 2. Material and methods

### 2.1. Chemicals and reagents

(3-Glycidoxypropyl)trimethoxysilane was obtained from Nanjing Yudeheng Coupling Agent Co, LTD (China). P-gp mouse monoclonal antibody (P-gp MAb, 200  $\mu\text{g mL}^{-1}$ ) was purchased from Neomarkers, Fremont, CA, and bovine serum albumin (BSA) was obtained from Sigma (USA). Potassium ferrocyanide and potassium ferricyanide were from Shanghai Chemical Reagent Co, LTD (China). All the other reagents were of analytical grade and used without further purification. Phosphate buffer solution (PBS, pH 7.4) containing NaCl (137 mM), KCl (2.7 mM),  $\text{Na}_2\text{HPO}_4$  (87 mM) and  $\text{KH}_2\text{PO}_4$  (14 mM) was used as the electrolyte in the measuring system. All the solutions were prepared with doubly distilled water.

### 2.2. Cell culture and collection

The leukemia *K562A* cell lines derived from human leukemia were obtained as a gift from the Affiliated Zhongda Hospital of Southeast University (Nanjing, China). The *K562A* cells were cultured in a flask in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% fetal

bovine serum (FCS, Sigma, UK), penicillin (100  $\mu\text{g mL}^{-1}$ ) and streptomycin (100  $\mu\text{g mL}^{-1}$ ) in an incubator (5%  $\text{CO}_2$ , 37 °C). At the growth retardation stage (3 days) the cells reached a number of  $1.0 \times 10^7$  cell  $\text{mL}^{-1}$ , which was determined using a Petroff-Hausser counter. They were collected and separated from the medium by centrifugation at 1000g for 10 min, and then washed twice with a sterile PBS (pH 7.4) [22]. The sediments were re-suspended in PBS to obtain 0.2 mL cell suspensions with the final concentration of cells equal to  $5.0 \times 10^7$  cell  $\text{mL}^{-1}$  for electrochemical measurements. Various contents of cell suspensions were prepared from this stock.

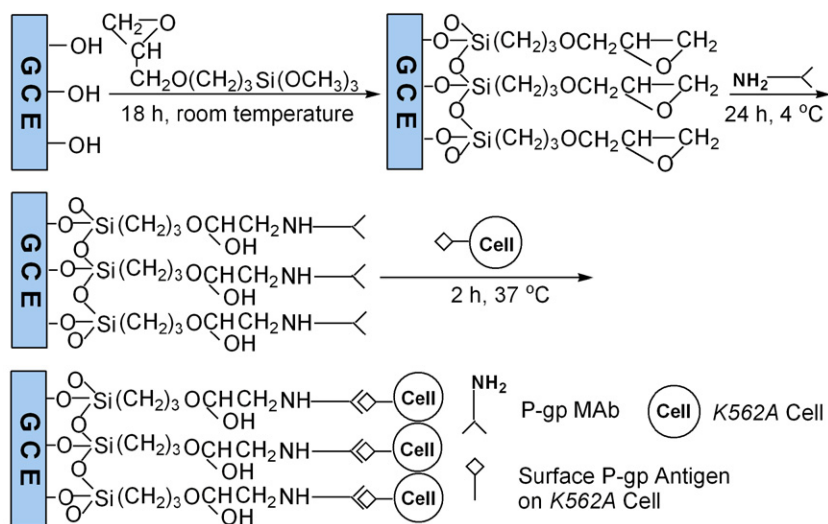
### 2.3. Electrode preparation and cell immobilization

The fabrication and recognition processes of the immunosensor were shown in Scheme 1. Firstly, the GCE (3 mm in diameter) was polished to a mirror-like finish and pretreated electrochemically to create a high number of hydroxy groups on the surface of GCE [23]. After dried under a stream of nitrogen, the electrode was immersed into a 1% solution of 3-glycidoxypropyl trimethoxysilane in acetone for 18 h at room temperature to form an epoxysilane monolayer that had many active tail epoxy groups for reacting readily with protein amino groups [24]. The P-gp MAb was then introduced onto the epoxysilane modified electrode by dropping 2  $\mu\text{L}$  of 20  $\mu\text{g mL}^{-1}$  antibody on the surface and incubating at 4 °C for 24 h. After rinsing with doubly distilled water and blocking the non-occupied binding sites with 3% BSA, 2  $\mu\text{L}$  leukemia *K562A* cells suspension was dropped onto the obtained cell immunosensor and incubated at 37 °C for 2 h, which achieved the recognition of the immunosensor to *K562A* cells by means of the immunoreaction between the cellular membrane P-gp of *K562A* cells and P-gp MAb.

### 2.4. Measurements

The electrochemical behavior of the immunosensor was studied using cyclic voltammetry and impedance measurements. Cyclic voltammetric experiments were performed using a CHI 660B electrochemical analyzer with conventional three-electrode system comprising a platinum wire as auxiliary electrode, saturated calomel electrode as reference and the modified electrode as working electrode. Electrochemical impedance experiments were performed in 1.0 M KCl containing 10 mM  $\text{K}_3\text{Fe}(\text{CN})_6/\text{K}_4\text{Fe}(\text{CN})_6$  (1:1) utilizing an Autolab Electrochemical Analyzer (Eco Chemie, Netherlands), and employing an alternating current voltage of 5.0 mV. The impedance spectrograms were collected within the frequency range of 1–10<sup>6</sup> Hz.

FT-IR spectra were recorded on a Nexus model 870 Fourier Transform IR Spectrophotometer. Samples were thoroughly ground with exhaustively dried KBr and discs were prepared by compression under vacuum. The XPS experiments were performed with an ESCALB MK-II. AFM images were obtained on SPA-300 HV with an SPI



Scheme 1. Fabrication and recognition processes of the immunosensor for K562A cells.

3800 controller (Seiko). All measurements were performed at room temperature.

### 3. Results and discussion

#### 3.1. Characterization of the modified electrode

The silylation reaction of GCE was confirmed by FT-IR spectra (Fig. 1A). No obvious signal was observed at a bare GCE, while the silylated GCE surface displayed several characteristic absorption bands at 1191, 1090 and 823  $\text{cm}^{-1}$ . The peak at 1191  $\text{cm}^{-1}$  was attributed to Si–O–C stretching vibration. The absorption band at 1090  $\text{cm}^{-1}$  resulted from the stretching vibrations of Si–O–C and Si–O–Si together. The peak of C–O–C stretching vibration at 823  $\text{cm}^{-1}$  verified the existence of cyclopropyl. The Si 2p XPS spectra also proved the successful silylation of GCE surface (Fig. 1B). Compared to the bare GCE the silylated GCE displayed a peak of Si 2p from –Si–O– group at 103.1 eV, close to that of Si(2p)<sup>IV</sup> (103.9 eV) in  $\text{SiO}_2$  [25].

The AFM image of the epoxysilane modified GCE showed a uniform distributed and intact monolayer film

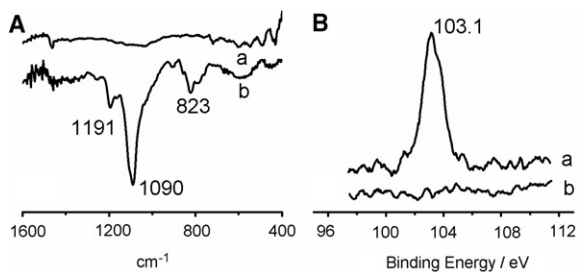


Fig. 1. (A) Infrared spectra of bare GC slice (a) and epoxysilane monolayer (b). (B) XPS spectra of epoxysilane monolayer (a) and bare GC slice (b).

almost without any coagulates on the visible area (Fig. 2A). The monolayer thickness was only about 1.5 nm. The result was identical to those of epoxy-terminated self-assembled monolayers on an ITO surface hydroxylated by HCl [10] and a single crystal silicon surface [19]. When comparing the AFM image of the monolayer modified GCE with that of antibody-immobilized epoxysilane monolayer, obvious difference could be observed. The latter showed a smoothing effect and a height of about 6 nm, indicating the formation of an antibody monolayer (Fig. 2B).

#### 3.2. Voltammetric behavior of recognized leukemia K562A cells

After a bare GCE was electrochemically pretreated the background current increased greatly due to the formation of rougher surface and oxygen-containing groups (Fig. 3, curves b vs. a), The modification of epoxysilane monolayer resulted in an obvious decrease in background (Fig. 3, curve c), and the further binding of antibodies displayed a lower background current (Fig. 3, curve d). After the electrode recognized leukemia K562A cells it showed a well-defined anodic peak at +0.796 V at 50  $\text{mV s}^{-1}$  (Fig. 3, curve e), which could be attribute to the oxidation of guanine within the cytoplasm of the living cells [26]. The guanine molecules were able to cross the cell membrane rapidly during the electrochemical process [22,27]. From the cyclic voltammogram with two continuous sweeps (inset in Fig. 3), it could be concluded that the oxidation process of guanine was irreversible.

#### 3.3. Optimization of the impedance immunosensor for cytosensing

The cyclic voltammograms of 10 mM  $\text{K}_3\text{Fe}(\text{CN})_6/$   $\text{K}_4\text{Fe}(\text{CN})_6$  (1:1) as redox probe in 1.0 M KCl showed a

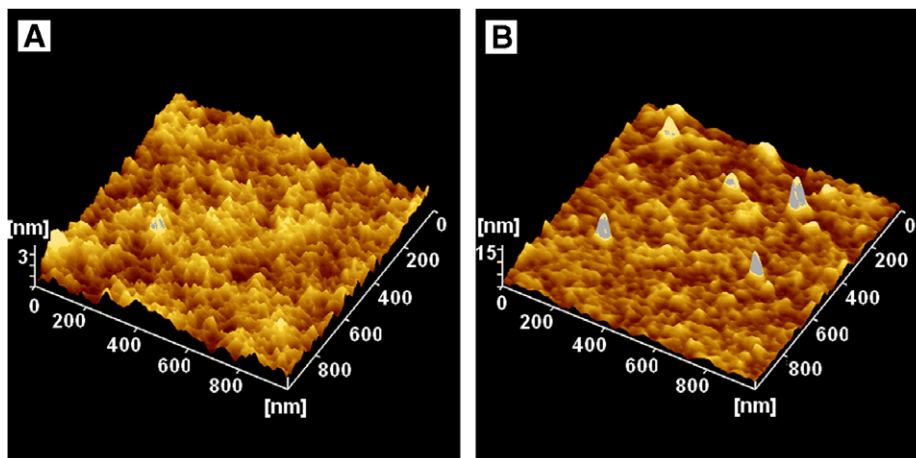


Fig. 2. AFM images of GCE coated with (A) epoxysilane monolayer and (B) antibody-immobilized epoxysilane monolayer.

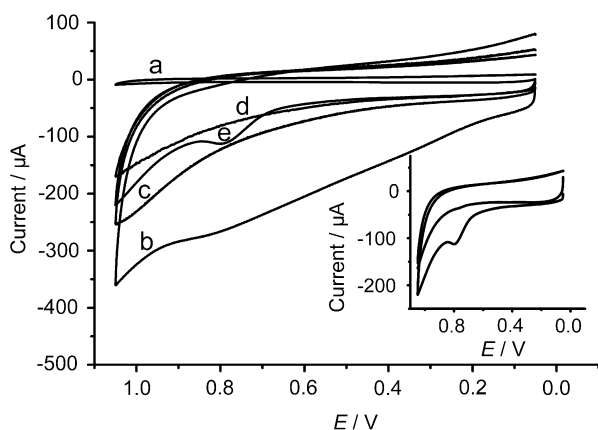


Fig. 3. Cyclic voltammograms of bare GCE (a), GCE pretreated electrochemically (b), GCE coated with (c) epoxysilane monolayer, (d) antibody-immobilized epoxysilane monolayer, and (e) *K562A* cells/antibody-immobilized epoxysilane monolayer in pH 7.4 PBS. Inset: cyclic voltammogram of GCE coated with *K562A* cells/antibody-immobilized epoxysilane monolayer. Scan rate: 50 mV s<sup>-1</sup>.

couple of redox peaks at the bare, epoxysilane monolayer and antibody-immobilized epoxysilane monolayer modified electrodes and *K562A* cells recognized immunosensor. With the further modification and then incubation the redox peak currents decreased (not shown). The peak currents depended on the amount of cells anchored to the antibody-immobilized epoxysilane monolayer and thus the concentration of cells used for immunological recognition. With the increasing amount of cells the response of the probe decreased. Thus the efficient immunological recognition of the P-gp Mab to P-gp on cellular membrane of *K562A* cells could be monitored with the cyclic voltammetric response of this system at the immunosensor incubated in  $4.0 \times 10^5$  cells mL<sup>-1</sup> *K562A* cells. As shown in Fig. 4A with the increasing concentration of silane used for the formation of the epoxysilane monolayer the peak current of the probe decreased and trended to a minimum value after the silane concentration of 1.0% (V:V), indicating the amount of cells anchored to the formed immunosensor

reached the maximum value, and the best immunological recognition of the obtained immunosensor occurred at the silane concentration, which was selected for the preparation of the immunosensor for *K562A* cells.

The silylation time was also an important factor for the formation of best epoxysilane monolayer. With the increasing silylation time the peak current of the probe decreased and reached a minimum value at 18 h (Fig. 4B), which was used for the preparation of the optimal epoxysilane monolayer for binding of P-gp antibody. The presence of P-gp antibody was a key to immunologically recognize *K562A* cells. With the increasing concentration of 2 µL antibody the peak current of the redox probe at the *K562A* cells anchored immunosensor decreased and then increased (Fig. 4C). The decrease of the response was due to the increasing amount of cells anchored at the immunosensor, and the increase resulted from the superabundant agglomerations of antibodies, which induced a coarse and uneven electrode surface. The optimal concentration of antibody was 20 µg mL<sup>-1</sup>, at which the binding time of antibody to the epoxysilane monolayer would affect the formation of antibody-immobilized epoxysilane monolayer. As shown in Fig. 4D 24 h should be used for the binding of antibody for the preparation of immunosensor with the best sensing performance.

### 3.4. Impedance characteristics of modified electrodes

Similar to the cyclic voltammetric characteristics the electron-transfer impedance ( $R_{et}$ ) of the redox probe at the electrodes relied on the modification and the presence of living cells. The electrochemical impedance spectra (EIS) of the modified electrodes were shown in Fig. 5. Upon the electrochemically pretreatment of GCE the  $R_{et}$  decreased from 261.6 to 94.3 Ω (Fig. 5, curves a and b). The formation of epoxysilane monolayer increased the  $R_{et}$  to the value of 680.3 Ω (Fig. 5, curve c). The binding of P-gp Mab and the anchor of *K562A* cells by the immunoreaction between the P-gp Mab and P-gp on cellular

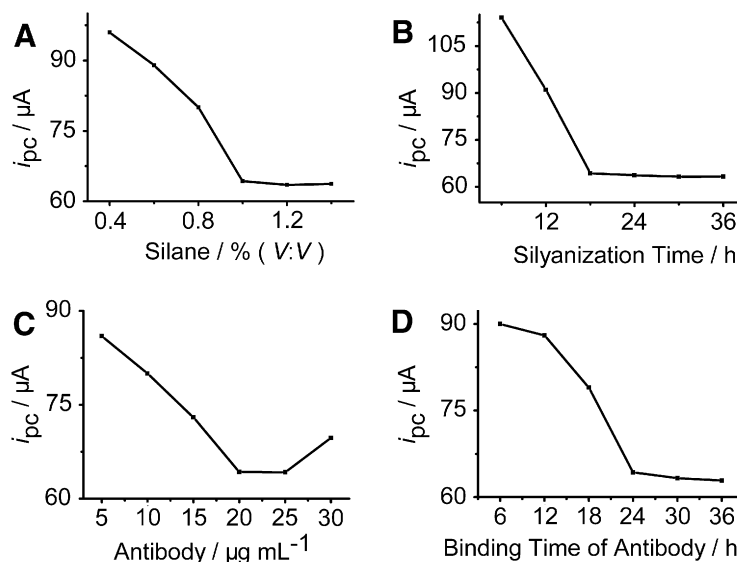


Fig. 4. Effects of epoxysilane concentration in acetone (A), silylanization time (B), antibody concentration (C) and binding time of antibody to epoxysilane monolayer (D) on the cyclic voltammetric response of  $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$  (10 mM, 1:1) in 1.0 M KCl at the immunosensor incubated in  $4.0 \times 10^5$  cells  $mL^{-1}$  *K562A* cells. Scan rate: 50  $mV s^{-1}$ .

membrane resulted in the further increase of the  $R_{et}$  (Fig. 5, curves d and e). The values of  $R_{et}$  at the antibody-epoxysilane monolayer and *K562A* cells anchored monolayer obtained at  $5.0 \times 10^4$  cells  $mL^{-1}$  were 1016.1 and 1170.1  $\Omega$ , respectively.

### 3.5. Impedance immunosensor for detection of *K562A* cells

With the increasing concentration of *K562A* cells the  $R_{et}$  value increased (Fig. 5, curves e–i). The  $R_{et}$  value was proportional to the logarithm of *K562A* cells concentration in the range of  $5.0 \times 10^4$  to  $1.0 \times 10^7$  cells  $mL^{-1}$  (inset in Fig. 5). The detection limit was  $7.1 \times 10^3$  cells  $mL^{-1}$ , which

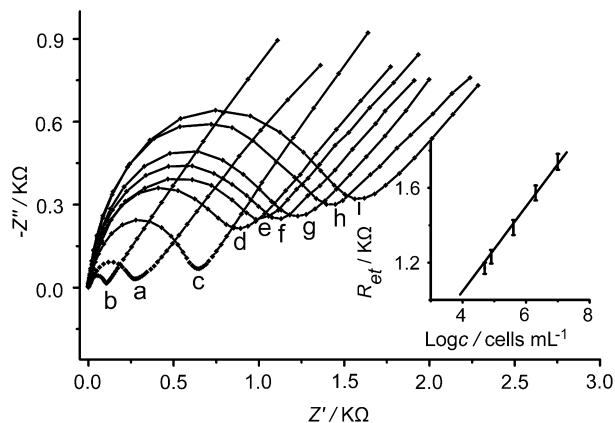


Fig. 5. (a) Nyquist diagrams of electrochemical impedance spectra recorded from 1– $10^6$  Hz for  $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$  (10 mM, 1:1) in 1.0 M KCl at an untreated GCE (a), electrochemically pretreated GCE (b), and GCE coated with epoxysilane monolayer (c), antibody-immobilized epoxysilane monolayer (d) and *K562A* cells/antibody-immobilized epoxysilane monolayer with the cell concentrations of  $5.0 \times 10^4$ ,  $8.0 \times 10^4$ ,  $4.0 \times 10^5$ ,  $2.0 \times 10^6$  and  $1.0 \times 10^7$  cells  $mL^{-1}$  (from e to i). Inset: calibration curve of the immunosensor for *K562A* cells.

was lower than those of  $3.2 \times 10^6$  at a piezoelectric immunosensor for *Salmonella* [21],  $10^7$  cfu  $mL^{-1}$  at an SPR sensor for *Escherichia coli* O157:H7 [20] and  $10^6$  cfu  $mL^{-1}$  at an impedance sensor for *E. coli* O157:H7 based on indium–tin oxide-coated interdigitated array microelectrode [11]. The proposed sensor also showed comparable sensitivity with that of  $6 \times 10^3$  cells  $mL^{-1}$  at an impedance sensor for *E. coli* O157:H7 based on the immobilization of anti-*Escherichia coli* O157:H7 antibodies onto an indium–tin oxide electrode [10].

The fabrication reproducibility of the immunosensors for *K562A* cells was estimated with the slopes of six calibration plots obtained with freshly prepared immunosensors. The RSD of these slopes (inter-assay) was 3.4%, showing acceptable reproducibility. At the cells concentrations of  $2.0 \times 10^6$  and  $1.0 \times 10^7$  cells  $mL^{-1}$ , the immunosensors showed the intra-assay RSD of 3.7% and 3.0% examined for six determinations, respectively, showing good precision.

## 4. Conclusions

The epoxysilane monolayer is successfully formed on an electrochemically pretreated GCE surface containing hydroxyl groups. The introduced active tail epoxy groups make P-gp Mab be easily bound to the monolayer. With a surface immunological recognition process of the P-gp Mab to P-gp on cellular membrane the obtained immunosensor can be used for monitoring the adhesion of leukemia *K562A* cells with cyclic voltammetry and electrochemical impedance spectroscopy, producing a sensitive impedance immunosensor for *K562A* cells. The proposed cell sensor showed good fabrication reproducibility and detection precision and could be further developed as a convenient means for the study of cells proliferation.

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