Improved EIS Performance of an Electrochemical Cytosensor Using Three-Dimensional Architecture Au@BSA as Sensing Layer

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Supporting Information

ABSTRACT: An ultrasensitive electrochemical cytosensor for quantitative determination of carcinoembryonic antigen (CEA)-positive tumor cells was developed using three-dimensional (3D) architecture Au@BSA microspheres as sensing layer with the conjugation of targeting molecule monoclonal anti-CEA antibody (anti-CEA). The prepared Au@BSA microspheres exhibited satisfactory biocompatibility for cell proliferation via evaluation from thiazolyl blue tetrazolium bromide (MTT) assay, providing a suitable platform for cell adhesion study. Attributed to the excellent electroconductivity of Au@BSA, amplified electrochemical signals could be obtained and resulted in the greatly enhanced detection sensitivity. Electrochemical testing techniques including electrochemical impedance spectroscopy (EIS), differential pulse voltammetry (DPV), and cyclic voltammetry (CV) were applied to assess the optimal conditions, specificity, and detection performance of as-fabricated cytosensor. The attachment of CEA-positive BXPC-3 cells onto the anti-CEA immobilized sensing layer led to the increased EIS responses, which changed linearly in the cell concentration range from 5.2 × 10^2 to 5.2 × 10^7 cells mL^{-1} with a detection limit of 18 cells mL^{-1}. This proposed cytosensing strategy revealed high specificity to CEA-positive cells, acceptable intra-assay precision, excellent fabrication reproducibility with the RSD of 3.5%, and good stability owing to the outside BSA biocompatible layer, developing a promising technique for early monitoring of tumor cells at a lower level.

As a serious health problem with high mortality ratio, cancer has become a global concern in recent decades.1 Early diagnosis of cancer plays a vital role during the course of therapy.2,3 Thus, developing a rapid and sensitive technique of detecting tumor cells has aroused wide research interest in recent years.4−6 As yet, electrochemical biosensing approaches, provided with a variety of advantages such as miniaturization, rapid response, low cost and high sensitivity, have been widely applied to the field of bioassay.7−10 To fabricate an effective electrochemical cytosensor, two significant factors involving the electron transfer efficiency11 and biocompatibility12 need to be taken into account. In view of this point, selecting an appropriate biomaterial as the sensing platform is the emphasis in our research. With the advocacy of green nanoscience,13,14 a new generation of nanomaterials synthesized by the template of bovine serum alumin (BSA) has attracted great attention from researchers.15−19 In the previous study, we have reported the Ag@BSA-based electrochemical biosensors for sensitive detection of KB cells20 and retinal-binding protein,21 demonstrating the excellent conductivity and biocompatibility of Ag@BSA microspheres. As a sensing platform, keeping a highly stable property under diverse conditions is fairly essential.22 To further optimize the sensing performance, herein we employed “green”-synthesized Au@BSA microspheres with superb stability from gold nanoparticles (AuNPs)23 combined with BSA layer to investigate the electrochemical behaviors of tumor cells incubated on this platform, which could contribute to the understanding of life process24 and deeper research toward single cell.25,26

Upon the improved synthetic route,27 a benign reductant ascorbic acid has been applied in place of hydrazine monohydrate for the preparation of core−shell nanomaterials, presenting a novel and environmentally friendly method to fabricate sensing layers. In addition, a satisfactory stability under a diversity of electrolytes can be achieved by the introduction of AuNPs and a great part of the electrochemical signals have been retained after long periods,28 creating a suitable living condition with excellent biosecurity for cell adhesion.29 Moreover, the unique three-dimensional (3D) architecture and high hydrophilicity of Au@BSA may strengthen the adhesive force between biomolecules and nanostructured substrates. Meanwhile, the outside BSA layer can not only...
serve as a multifunctional interface to be easily functionalized with targeting molecules, but also maintain the bioactivity of immune substances and improve the water-solubility of the synthesized nanocomposites.30 On the basis of the above advantages, Au@BSA microspheres conform to the high standard of an appropriate sensing platform for further research.

Carcinoembryonic antigen (CEA), a type of glycoprotein with a molecular mass of ~200 kDa, connects with many types of cancer, such as pancreatic cancer, breast cancer, colon cancer and lung cancer.31,32 As a frequently used tumor marker for clinical diagnosis, CEA can be further applied in the development of electrochemical cytosensing strategy to realize the specific detection of tumor cells. Human BXPC-3 pancreatic cancer cells were used as a model of CEA-positive cells, which contain CEA expression patterns on the cellular surface.33,34 For the specific recognition of tumor marker CEA, a monoclonal anti-CEA antibody (anti-CEA) was adopted as the targeting molecule after it was covalently conjugated to the sensing platform via glutaraldehyde (GA). Electrochemical impedance spectroscopy (EIS) is an effective approach to monitor the interfacial properties of modified electrodes and often employed for the analysis of electrochemical process associated with electroactive substances. Therefore, EIS was used for the characterization of each assembly process and cell determination. Through the highly specific immunoreaction, this Au@BSA-based cytosensor exhibited a broad detection range with a fairly low detection limit of BXPC-3 cells, offering a potential alternative method for tumor cell detection in clinical diagnosis with further efforts.

**EXPERIMENTAL SECTION**

**Materials and Reagents.** Chloroauric acid (HAuCl₄), ascorbic acid (AA), glutaraldehyde solution 25% (GA), and ethanol (CH₃CH₂OH) were purchased from Sinopharm Company (China). Lyophilized 99% bovine serum albumin (BSA, molecular mass ~66.4 kDa) was obtained from Sigma-Aldrich Company (China). MTT (C₉H₅N₅SBr, ultra pure grade) was acquired from Solarbio Company. The monoclonal anti-CEA antibody (anti-CEA, affinity purification) was purchased from Shanghai Linc-Bio Science Company limited. The 10 mM phosphate buffer solution (PBS, pH 7.4) containing 14 mM KH₂PO₄, 87 mM Na₂HPO₄, 2.7 mM KCl and 137 mM NaCl was used as the rinsing solution and the diluent of anti-CEA. [Fe(CN)₆]₃⁻/₄⁻ solution containing 10 mM K₃Fe(CN)₆, 10 mM K₄Fe(CN)₆ and 0.1 M KCl (as the supporting electrolyte) was used as redox probe in the measuring system. Redistilled water (18.2 MΩ) was used throughout the experiments. All the other reagents were of analytical grade and used as received without further purification.

**Assembly Process of the Electrochemical Cytosensor.**

The synthetic steps of the sensing layer Au@BSA microspheres were depicted in the Supporting Information. The assembly process of this Au@BSA-based electrochemical cytosensor was visually illustrated in Scheme 1. First, the Au electrodes (2.0 mm in diameter) were polished to a mirrorlike finish with α alumina powder and washed ultrasonically in ethanol and redistilled water. To remove any possible contaminant, the polished electrodes were immersed into a mixture of piranha solution (98% H₂SO₄/30% H₂O₂, 3:1) for 5 min and rinsed with ethanol and redistilled water, respectively. Finally, the Au electrodes were dried under a stream of nitrogen for the following modification. To structure a biomimetic sensing layer, 3 μL of Au@BSA microspheres (1.5 mM) was dropped onto the electrochemically pretreated Au electrode surface, which was preserved in the refrigerator at 4 °C for 2 h. Before further modification, the electrodes were rinsed fully with pH 7.4 PBS buffer to remove unbound Au@BSA microspheres. GA is an optimal reagent for realizing the chemical activation at neutral pHs and it can react quantitatively with primary amino groups to form stable imines.37 To cross-link targeting molecule anti-CEA, 2.5 μL of GA solution at 12.5% was added to the substrate and incubated at room temperature for 1 h. So as to ensure only one aldehyde group of GA reacting with amino groups of Au@BSA, a high molar ratio of GA to Au@BSA was adopted.38,39 After reaction, the electrodes were washed with pH 7.4 PBS buffer. Then, 3 μL of anti-CEA solution was coated onto the modified electrode surface and incubated at 4 °C under a humid circumstance for at least 4 h. Afterward, 10 mM, pH 7.4, PBS was employed to rinse the electrodes thoroughly to remove physically combined biomolecules. Accordingly, the preparation of a layer-by-layer (LBL) assembled cytosensor with the capability for immunological recognition of CEA-positive tumor cells was eventually accomplished.

**Cell Culture, Collection, and Immobilization.** All kinds of cells were obtained from the cell bank of Chinese Academy of Sciences. Human BXPC-3 pancreatic cancer cells were cultured in the cell incubator at 37 °C under a humidified atmosphere containing 5% CO₂. BXPC-3 cells were seeded in a flask containing Dulbecco’s Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), penicillin (100 U/mL) and streptomycin (100 μg/mL). After a period of incubation, BXPC-3 cells were trypsinized in 0.25% trypsin solution and collected from the culture medium by centrifugation at 1200 rpm for 5 min, and then washed twice with pH 7.4 sterile PBS. The sediments were suspended in pH 7.4 sterile PBS to obtain 0.5 mL cell suspensions with a certain concentration for electrochemical measurements. The cell suspensions with different contents were prepared from this stock. At the growth logarithmic stage (3 days), BXPC-3 cells reached a number of 5.2 × 10⁷ cells mL⁻¹, which was determined by using a Petroff-Hausser counter. Then, 5 μL of the cell suspension with various concentrations was dropped onto the modified electrodes and incubated at 37 °C for 2 h to achieve cell immobilization.

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**Scheme 1. Schematic Representation of the Au@BSA-Based Cytosensing Strategy for CEA-Positive Tumor Cell Detection via EIS**
Before measurement, the electrodes were gently rinsed with pH 7.4 sterile PBS to remove uncombined cells.

**Instrumentation, Characterization, and Electrochemical Measurements.** The scanning electron microscopy (SEM) image was obtained by a ZEISS-ULTRA 55 scanning electron microscope and the transmission electron micrograph (TEM) was recorded on a JEOL JEM-2010 transmission electron microscope operating at an accelerating voltage of 200 kV. Atomic force microscope (AFM) measurement was carried out on a MultiMode Nanoscope from digital instruments (Bruker AXS GmbH). The contact angle measurement was performed on an OCA20 optical contact angle analyzer (Dataphys instruments) using a CCD video camera and a horizontal light source to illuminate the liquid droplet. Zeta potential was measured by Malvern Zetasizer Nano ZS 90. Electrochemical measurements containing EIS, cyclic voltammetry (CV) and differential pulse voltammetry (DPV) were performed on a CHI 660B electrochemical workstation with a conventional three-electrode system composed of a modified Au electrode as a working electrode, a platinum wire as an auxiliary electrode and saturated calomel electrode (SCE) as reference. All the electrochemical measurements were carried out in 10 mM, pH 7.4, sterile PBS containing K₃Fe(CN)₆/ K₄Fe(CN)₆ (10 mM, 1:1) and 0.1 M KCl. The impedance spectra were recorded within the frequency range of 10⁻²–10⁵ Hz with signal amplitude of 5 mV.

**RESULTS AND DISCUSSION**

**Characterization of Au@BSA Sensing Layer.** Au@BSA microspheres, as the sensing layer of this cytosensor, have a significant impact on the final detection performance. The morphology of Au@BSA microspheres was characterized by SEM and TEM. Figure 1A depicted the good monodispersity and uniform morphology of Au@BSA with an average size of 500 nm in diameter. Typical TEM image in Figure 1B clearly revealed the core–shell structure of Au@BSA and a thin layer (BSA) wrapped outside. Herein, innumerable tiny AuNPs (with an average diameter of 20 nm) could be observed from the image contrast, which could greatly promote the electron transfer and improve the detection sensitivity of the fabricated cytosensor. Meanwhile, the BSA layer behaved as a multifunctional platform to conjugate targeting molecules and block nonspecific binding sites. The primary functional groups of Au@BSA were characterized by FT-IR (Figure S1 in the Supporting Information).

After the formation of Au@BSA sensing layer onto Au electrode, the surface topography could be explicitly observed from AFM measurement (Figure 1C), which displayed a well-organized distribution and presented a 3D nanostructured platform. This 3D nanostructure could strengthen local topographic interactions between Au@BSA sensing layer and nonspecific binding sites. The primary functional groups of Au@BSA were characterized by FT-IR (Figure S1 in the Supporting Information).

MTT cell viability assay (shown in Figure 1D) was conducted to evaluate the cytotoxicity of Au@BSA with various concentrations at 37 °C for 24 and 48 h. BXPC-3 cells (1 × 10⁴ cells per well) were seeded in each assigned well of a 96-well plate before exposure to Au@BSA. Controls were incubated under the same condition without addition of Au@BSA. The absorbance of formazan (split from MTT through the effect of dehydrogenases in living cells) at 490 nm is proportional to the number of living cells. With the Au@BSA concentration...
increasing from 0.375 to 3 mM at 24 h, the MTT assay showed no significant viability decreases of BXPC-3 cells compared to controls. From the MTT assay at 48 h, only slightly lower absorbance could be observed and more than 85% of BXPC-3 cells were still alive, indicating the good biocompatibility of Au@BSA. In addition, Figure 1E showed that the contact angle of Au@BSA sensing layer onto Au electrode was 21.89° (average), demonstrating the high hydrophilicity of Au@BSA microspheres. Thus, the benign biocompatibility of Au@BSA could be further confirmed.

Optimal Conditions of Immune Recognition Process. As for immunobinding assay, concentration of antibody and binding time of antibody directly affected the electrochemical signals during the assembly process, which were also related to the sensitivity of cell determination. To immunologically recognize BXPC-3 cells, the used concentration of anti-CEA was key to enhance the recognition efficiency. As shown in Figure 2A, CV peak current at the cell adhered cytosensor decreased gradually with the increasing anti-CEA concentration, demonstrating increasingly more BXPC-3 cells were being captured. A minimum CV response obtained at 25 μg mL⁻¹ expounded that cells adhered to the modified electrodes had been saturated. For the sake of maximum electrochemical signals, 25 μg mL⁻¹ of anti-CEA was recommended for target conjugation. Furthermore, binding time of anti-CEA was also a crucial factor in the resulting performance of cell-capture. The electron transfer resistance (Rₑ) presented a rising trend with the increasing binding time of anti-CEA and reached an approximate plateau at 4 h (Figure 2B). It was beneficial for electrodes conjugated adequate antibodies to capture most BXPC-3 cells after sufficient binding time of anti-CEA. Thereby, 4 h was applied as the optimal time for anti-CEA binding.

EIS Monitoring of Cytosensor Fabrication. EIS has been popularly utilized to monitor the LBL assembly process due to its immediate and sensitive response from the electrode surface changes. Figure 3A demonstrated the detailed changes of Rₑ value with LBL modification, using [Fe(CN)₆]³⁻/[Fe(CN)₆]⁴⁻ (10 mM, 1:1) and 0.1 M KCl at (a) an electrochemically pretreated Au electrode, after (b) formation of Au@BSA film, (c) conjugation with GA, (d) immobilization of anti-CEA, and (e) capture of BXPC-3 cells. The equivalent circuit model inset in the histogram (B) was used to fit the impedance data of assembly process into Rₑ values.
41.43 Ω (curve b), implying the accelerated electron transfer rate due to the formation of sensing layer. From the Nyquist diagram of Au@BSA (inset in Figure 3A), this electrochemical process is on the verge of Warburg-type curve and with the characteristic of diffusion-controlled process.\(^{44}\) Besides, the peak current of CVs linearly transformed with the square root of scan rates (Figure S4 in the Supporting Information), also exactly indicating that the electron transfer of Au@BSA-bound surface was diffusion-controlled. To obtain covalent conjugation of anti-CEA, GA was employed as a mediator to achieve the “bridge effect” between Au@BSA and anti-CEA. After rapid and quantitative reaction with GA, the \(R_{et}\) value sharply increased to 597.3 Ω (curve c), which resulted from the steric hindrance of kinetic solvent effect and the formation of stable imines. Under the “bridge effect” of GA, anti-CEA was covalently immobilized onto the surface of Au@BSA through Schiff base structures,\(^{45}\) increasing \(R_{et}\) to the value of 1919 Ω (curve d). The great increment of \(R_{et}\) could be attributed to the optimal concentration and sufficient binding time of anti-CEA. In addition, the assembly process of cytosensor was also characterized by zeta-potential measurements. Based on the different isoelectric points of each assembly layer, the apparent charge of zeta-potential could be observed from Figure S5 in the Supporting Information, providing an assisted evidence for successful assembly steps. Highly specific immune recognition of BXPC-3 cells (5.2 × 10^5 cells mL^-1) drastically increased the \(R_{et}\) value to 2725 Ω (curve e), indicating a greater barrier had been caused due to the cell proliferation on the electrode surface. The blocking effect on redox probe created by the electrochemical behaviors of BXPC-3 cells could be also obviously observed from CVs changes (Figure S6 in the Supporting Information). On the basis of the equivalent circuit model (inset in Figure 3B), the successively changed impedance data were fitted into \(R_{et}\) values, which was visually illustrated by histogram.

**Detection Performance of the Electrochemical Cyto-sensor.** EIS response immediately changed due to the electronic shielding effect from the cell membrane, reflecting the efficiency of electron transfer between the electrode surface and the electroactive substance. Thus, EIS was adopted for quantitative determination of tumor cells. In order to investigate its self-sensing property, Au@BSA was employed alone for BXPC-3 cell detection. As shown in Figure 4A, Nyquist diagrams changed by degrees as increasing concentrations of BXPC-3 cells were adhered, which was proportional to the logarithm of cell concentration in the range of 2.8 × 10^3 to 2.8 × 10^7 cells mL^-1. The \(R_{et}\) increment could be defined as: \(\Delta R_{et} = R_{Amp} - R_{Blank}\), where \(R_{Amp}\) equaled to the value of \(R_{et}\) after amplification and \(R_{Blank}\) equaled to the value of \(R_{et}\) after conjugating anti-CEA but without cell adhesion. The linear relationship could be depicted as \(\Delta R_{et} (\Omega) = 1581.98 \times \lg C_{[BXPC-3]} (\text{cells mL}^{-1}) - 3588.16 (R = 0.9933)\) with a detection limit of 10^3 cells mL^{-1} (signal-to-noise, S/N = 3) (inset in Figure 4A). This obtained analytical performance of Au@BSA sensing layer could be attributed to the good conductivity, stability and biocompatibility of Au@BSA, the enhanced local topographic interactions with the cellular surface, and the electrostatic adhesion with negatively charged tumor cells.

After covalent conjugation of targeting molecule anti-CEA, immunological reaction with tumor marker CEA resulted in highly specific cell-capture onto electrode surface, which revealed the improved EIS response compared to the pure Au@BSA. A few physical factors which may contribute to cell adhesion could be eliminated. The penetration of the redox probe \([\text{Fe(CN)}_6]^{3-/4-}\) was gradually reduced with the increasing BXPC-3 cell concentration ranging from 5.2 × 10^3 to 5.2 × 10^7 cells mL^-1, with a correlation coefficient of 0.994 (Figure 4B). The linear regression equation was \(\Delta R_{et} (\Omega) = 855.43 \times \lg C_{[BXPC-3]} (\text{cells mL}^{-1}) - 697.49\), with a detection limit of 18 cells mL^-1 (S/N = 3), which was much lower than or comparable with those of \(7.1 \times 10^3\) cells mL^-1 at a self-assembled monolayer based electrochemical immunosensor for detection of K562 cells,\(^{46}\) \(1.0 \times 10^5\) cells mL^-1 at an impedance cytosensor for detection of K562 cells,\(^{47}\) \(500\) cells mL^-1 at a cell-based electrochemical enzyme-linked immunoassay for detection of HeLa cells,\(^{48}\) and 10 cells mL^-1 at an electrochemical current rectifier cytosensor for detection of HeLa cells (summarized in Table S1 in the Supporting Information).\(^{49}\) This improved EIS detection performance was largely due to the enhanced electron transfer rate from Au@BSA and highly specific antigen–antibody immunoreaction.

![Figure 4](https://example.com/figure4.png)
**Specificity, Reproducibility, and Stability of the Cytosensing Strategy.** Different kinds of cells at the same concentration (1.0 × 10^5 cells mL^-1) were applied to investigate the specificity of the proposed cytosensing strategy. CEA-negative cell lines containing human K562 leukemia cells, human 293FT embryonic kidney cells, human GES-1 gastric mucosal epithelial cells and human HK-2 proximal tubular cells were, respectively, incubated on the fabricated cytosensor for 2 h and rapidly detected by DPV technique. Meanwhile, CEA-positive BXPC-3 cells were also incubated under the same experimental conditions as a control. As shown in Figure 5, it could be clearly observed that only BXPC-3 cells resulted in an evident DPV change with a sharply decreased peak current of more than 13.5 μA (average). While CEA-negative cells were incubated on the cytosensor, no significant DPV changes could be observed just with a slightly decreased peak current of less than 2.5 μA. Five times greater DPV signal change was originated from BXPC-3 cells, testifying this sensing layer was highly specific to CEA-positive tumor cells. The intra-assay precision of this cytosensor was evaluated by detecting BXPC-3 cells at two levels for five replicate determinations. The relative standard deviations (RSD) of the intra-assay with this method were 3.9% and 4.6% at the cell concentrations of 5.2 × 10^5 and 5.2 × 10^6 cells mL^-1, signifying an acceptable precision. A series of five replicate measurements from the batch were carried out to estimate the interassay reproducibility of freshly prepared cytosensors and resulted in an RSD of 3.5%, indicating the excellent fabrication reproducibility of this strategy. As an important property of fabricated cytosensor, the stability is necessary to be investigated. When the anti-CEA/GA/Au@BSA modified cytosensor was stored in the refrigerator at 4 °C over 20 days, 91.5% of its initial signal response remained, suggesting a quite satisfactory stability which could be attributed to the BSA layer acting as a support to retain the activity of biomolecules.

**CONCLUSIONS**

In this work, we have designed an Au@BSA-based cytosensing strategy for ultrasensitive tumor cell determination via specific recognition of CEA expression patterns on the cellular surface. Three-dimensional architecture Au@BSA microspheres, equipped with convenient and "green" synthesis route, excellent conductivity, stability and biocompatibility, performed as an appropriate sensing layer for cell adhesion study. With the coherent conjugation of anti-CEA, a broader detection range and a much lower detection limit have been achieved by this strategy, also revealing the high specificity to CEA-positive tumor cells. This electrochemical cytosensor with simple operation and rapid response from EIS may provide a feasible technique for early diagnosis of cancer in clinical application.

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