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Immunosensor for trace penicillin G detection in milk based on supported bilayer lipid membrane modified with gold nanoparticles

Han Li[a,1,2], Bing Xu[a,1,2], Danqi Wang[a,2], Yin Zhou[b,3], Hongling Zhang[b,3], Wei Xia[a,2], Shunqing Xu[a,4], Yuanyuan Li[a,*]

[a] Key Laboratory of Environment and Health, Ministry of Education & Ministry of Environmental Protection, and State Key Laboratory of Environmental Health (Incubating), School of Public Health, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, Hubei, China
[b] College of Health Science and Nursing, Wuhan Polytechnic University, Wuhan 430023, Hubei, China

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ABSTRACT

In this work, we developed an immunosensor for electrochemical detection of penicillin G at trace level. The biosensor was fabricated by immobilizing anti-penicillin G in a supported bilayer lipid membrane (s-BLM) modified with gold nanoparticles, and the modified electrodes were characterized by the scanning electron microscope (SEM), cyclic voltammetry and electrochemical impedance spectroscopy. The biosensor was able to detect penicillin G with a linear correlation ranging from 3.34×10^{-3} ng/L to 3.34×10^{3} ng/L and a detection limit of 2.7×10^{-4} ng/L, much lower than the maximum residue limit (MRL) of penicillin G in milk (4 ppb, equal to 4×10^{3} ng/L) set out by the European Union. The mean coefficient variation (CV) of the intra-assays and the inter-assays were 5.4% and 7.7%, respectively. In addition, the concentration of penicillin G in milk samples determined by this biosensor was in good agreement with that determined by high performance liquid chromatography (HPLC) assay.

1. Introduction

Penicillin G is widely used to treat dairy cows infected with mastitis. However, the overuse of penicillin G can lead to penicillin residues in dairy products, which could induce allergic reactions in sensitive individuals as well as the development of drug resistant bacteria (Aarestrup, 1999; Mitchell et al., 1998). European Union (EU) has established EU Regulation 508/1999 to avoid these hazards. It sets the maximum residue limit (MRL) for penicillin G in milk at 4 ppb or 4×10^{3} ng/L. As a consequence, analytical methods with detection limit under the MRL are urgently demanded.

A number of methods have been developed for the detection of penicillin G residues in dairy products. However, conventional microbial inhibition assays fail to identify and quantify the individual residues. The immunoassay methods are also widely used, for example enzyme-linked immunosorbent assays (ELISA), but they require specific antibodies and would be expensive and time-consuming when large numbers of samples need to be analyzed. Thus, a simple, highly specific and sample-saving method needs to be developed to overcome the shortcomings of these existing methods.

The biosensor technologies have been widely used for detection of target biomolecules. They show excellent performance with good selectivity and high sensitivity (Liu et al., 2015; Lv et al., 2015). Recently some biosensors have been reported for the analysis of penicillin G, which usually based on enzyme or ion selectivity (Dawan et al., 2011; Santos et al., 2004; Wu et al., 2014; Zhang et al., 2013). However, most of them rely on complex equipment, and do not have a low enough detection limit. Immunosensors are affinity ligand-based biosensor devices in which the immunochemical reaction is coupled to a transducer (Thakur and Ragavan, 2013). The fundamental of immunosensors is the specificity of the antigen–antibody reaction. This reaction shows high sensitivity, and forms stable immune complexes. In recent years, modern transducer technology enables the label-free detection and quantification of the immune complex. Therefore, a label-free immunosensor could detect signals directly and might provide a better detection limit.

When fabricating an immunosensor, it is very important to choose a suitable matrix to immobilize the antibody. In recent

* Corresponding author. Tel.: +86 27 83693417.
E-mail addresses: leehan1988@126.com (H. Li), xb1993@163.com (B. Xu), wangdanqi0522@163.com (D. Wang), zhyywfm@126.com (Y. Zhou), zhlwjb@sina.com (H. Zhang), xiawei@hust.edu.cn (W. Xia), xust@hust.edu.cn (S. Xu), liyuanyuan@hust.edu.cn (Y. Li).
[1] These authors contributed equally to this paper.
[2] Tel.: +86 27 83693417.
[3] Tel.: +86 27 83956940.
[4] Tel.: +86 27 83657705.

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years, the supported bilayer lipid membrane (s-BLM) has been widely used in electrochemical biosensor and molecular devices as a matrix (Dong and Chen, 2002; Moradi-Monfared et al., 2012; Xia et al., 2010). Bilayer lipid membranes (BLMs) have been used as models of actual living cell membranes (Dolfi et al., 2002; Eray et al., 1995). It can also provide a natural environment for immobilizing various kinds of substances such as proteins, antibodies, and even whole cells (Abdel-Hamid et al., 1999; Hu et al., 2001; Lamken et al., 2004). Several biosensors based on BLMs have provided very low detection limits (Eray et al., 1995). Thus, BLMs would be useful to further stretch the detection limit and enhance the stability of the material. Compared with pure s-BLM, the mechanical, electrical and optical properties of s-BLM doped with nanoparticles change significantly (Rieck et al., 2010). The nanoparticles, which act as a nanoelectrodes array, with good biocompatibility, increase the electrochemical signals alteration of the s-BLM. In addition, because of their good biocompatibility in cells and animals, the nanoparticles retain the biocompatibility of the s-BLM (Hicks et al., 2001; Xu et al., 2005; Yin et al., 2005). In short, the sensitivity of the biosensor based on s-BLM will be improved when modified with nanoparticles.

In this study, we fabricated an electrochemical immunosensor, using gold nanoparticles modified s-BLM as the matrix to immobilize the anti-penicillin G antibody. The s-BLM was formed on a cleaned glassy carbon electrode surface, and the gold nanoparticles were electrodeposited in the s-BLM. Then we immobilized the anti-penicillin G antibodies in the s-BLM. The specific binding between anti-penicillin G and penicillin G lead to the alteration of the impedance. The gold nanoparticles in s-BLM can provide a better microenvironment for s-BLM to maintain the bioactivity of anti-penicillin G. Meanwhile, the high conductivity of the gold nanoparticles can improve the electrical resistance and capacitance of the s-BLM. (Liu and Lin, 2007; Sperling et al., 2008; Voevodin et al., 2007).

2. Materials and methods

2.1. Reagents and chemicals

Gold nanoparticles (16 nm) were purchased from Sigma (St. Louis, MO, USA). Penicillin G sodium salt was purchased from Amresco (USA). The mouse anti-penicillin G (Glycerol Free) antibodies were obtained from United States Biological, Inc. (Swampscott, MA, USA). Egg phosphatidylcholine (PC, ≥99%, TLC) and cholesterol (CH) were bought from Shanghai Chemical Reagent Company (Shanghai, China). N-decane, $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$ were obtained from Tianjin Kermel Chemical Reagent Corporation (Tianjin, China). Tetrabutylammonium bromide solution, acetonitrile (HPLC grade), benzoic anhydride, 1,2,4-triazole and mercuric chloride were purchased from Thermo Fisher Scientific (Waltham, MA, USA). All the glassy carbon electrodes (GC, 3 cm diameter) were purchased from Tianjin Scientific Apparatus Company (Tianjin, China). All other chemicals and solvents used were of analytical grade. Double distilled water was used throughout the work.

The s-BLM forming solution was n-decane containing 4% PC and 1% CH, stored at 4 °C. The working solution (redox mediator) consisted of 10 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ and 0.1 M KCl.

2.2. Apparatus

Cyclic voltammetric and AC impedance measurements were carried out with a CHI 660C electrochemistry workstation. All the experiments were performed with a three-electrode system in which a modified glassy carbon electrode acts as the working electrode, and a platinum wire acts as the counter electrode and a saturated calomel electrode (SCE) acts as the reference electrode.

2.3. Electrode preparations

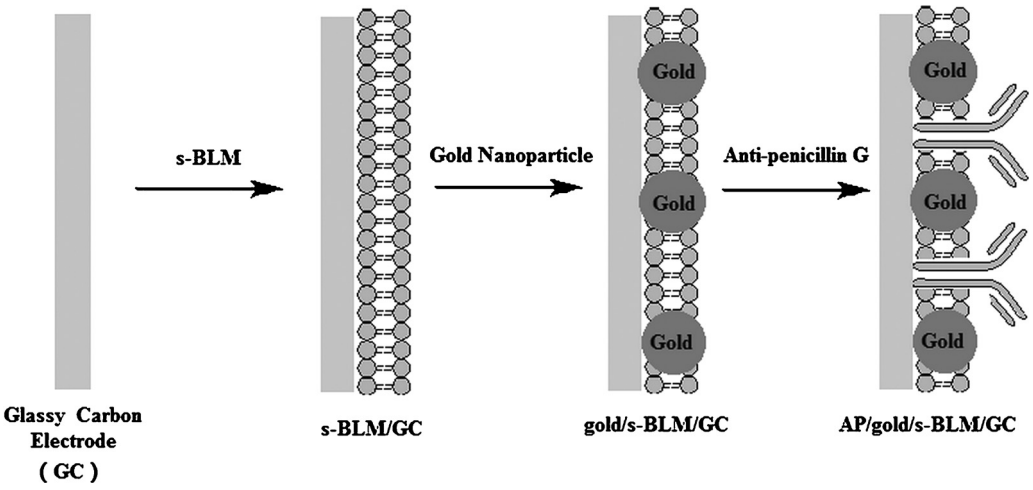
The modified glassy carbon electrodes were prepared as follows: a glassy carbon electrode was firstly polished respectively with 1.0, 0.3, and 0.05 μm alumina slurry, followed by sonication in nitric acid (1:1), ethanol and bi-distilled water for 5 min successively. The electrodes were dried in the air. Then a 10 μL aliquot of the s-BLM forming solution was dropped onto the surface of the cleaned electrode by a microsyringe, and the electrode was kept at room temperature for 15 min. Next, the electrode was immersed into Phosphate Buffered Saline (PBS) for another 15 min, during which the s-BLM was formed spontaneously on the surface of the electrode (s-BLM modified electrode was described as s-BLM/GC). Subsequently, the prepared s-BLM/GC was immersed into the gold nanoparticle solution under a potential of 1.5 V for 20 min for the electrodeposition of gold nanoparticle through the s-BLM (gold nanoparticles modified s-BLM/GC electrode was described as gold/s-BLM/GC). For comparison, gold nanoparticles were electrodeposited on the surface of a bare glassy carbon electrode following the same procedure (gold nanoparticles modified bare glassy carbon electrode was described as gold/GC). After that, a 2 μL aliquot of anti-penicillin G solution (150 ng/mL) was dropped onto the surface of gold/s-BLM/GC electrode for the immobilization of anti-penicillin G, and the electrode was incubated in a wet box for 12 h at 4 °C (Scheme 1). Then the incubated electrode was gently washed with PBS to remove the remaining free anti-penicillin G (the anti-penicillin G modified electrode was described as AP/gold/s-BLM/GC). The obtained modified electrode (working electrode) was stored at 4 °C before using.

2.4. TEM and SEM imaging

The shape and size of gold nanoparticles were confirmed by a transmission electron microscope (TEM) (JEM 2100F STEM/EDS, JEOL Japan, Inc.). In addition, a scanning electron microscope (SEM) (S-3000N, Hitachi High Technologies Japan, Inc.) was used to visualize the electrode's surface, before and after the modification of bilayer lipid membrane and gold nanoparticles.

2.5. Electrochemical measurements

Electrochemical experiments were performed with a conventional three-electrode system in 5 mL working solution at room temperature. Cyclic voltammetry (CV) swept from −0.4 to 0.6 V (vs. SCE) at a sweeping rate of 0.10 V/s was used to monitor the electrochemical characters of the modified electrode during the self-assembling process. Electrochemical changes during the detection phase was characterized by electrochemical impedance spectroscopy (EIS) with a frequency range from 10^{-1} to 10^5 Hz at the formal potential of 330 mV, and an alternating voltage of 10 mV. Impedance data were analyzed by ZVIEW software (Version 3.1C) (Romer and Steinem, 2004; Vockenroth et al., 2007). The change in the electron-transfer resistance is calculated with the following equation: $\Delta R_{ct} = R_{ct(P\text{-}AP)} - R_{ct(AP)}$, where $R_{ct(AP)}$ is the value of the electron transfer resistance produced by AP/gold/s-BLM/GC scanned in working solution, and $R_{ct(P\text{-}AP)}$ is the value of the electron transfer resistance after penicillin G binding to AP/gold/s-BLM/GC. In essence, the value of ΔR_{ct} is correlated to the concentrations of analyzed penicillin G.



Scheme 1. The schematic representation of the electrode surface of the biosensor.

2.6. Sample pretreatment for biosensor

Raw milk samples were purchased from local markets in Wuhan. Before spiking the raw milk samples with penicillin G, the samples were confirmed penicillin G free by HPLC analysis. For spiking, 20 mL of raw milk sample was spiked with different concentrations of penicillin G. The mixtures were centrifuged for 20 min at 14,000 × *g* at room temperature. The fat layer was discarded and the supernatant was diluted 40 times with PBS (pH 5.7) and used for analysis.

2.7. HPLC analyses

HPLC assay was performed as described by Sorensen et al. (1997). Briefly, milk samples were extracted by tetrabutylammonium bromide solution, and purified by C18 solid phase extraction cartridges (AccuBOND ODS-C_{18}, Agilent, Santa Clara, CA, USA), then eluted with the acetonitrile solution. The eluent was derivatized by benzoic anhydride and 1,2,4-triazole containing mercuric chloride, and then determined by HPLC (Agilent 1220 Infinity, Agilent, Santa Clara, CA, USA). A sample amount of 100 μL was injected on a C_{18} column (HC-C_{18}, 5 μm, 150 mm × 4.6 mm, Agilent, Santa Clara, CA, USA), and separated by gradient elution at 1 mL/min flow rate and 30 °C. The penicillin G was detected at 325 nm.

3. Results and discussion

3.1. Surface morphology of the modified electrode

TEM micrograph was used to observe the distribution and general morphology of the particles. TEM examination of the gold nanoparticle revealed the mean particle size to be 16 nm (Fig. 1). The particles were uniform in distribution and mostly spherical in shape.

SEM was used to observe the surface of the electrode and verify the binding of the bilayer lipid membrane and gold nanoparticles. Fig. 2(A) shows the surface of a bare GC electrode at a 2 μm scale. Modification of the bare surface with bilayer lipid membrane (s-BLM/GC electrode) is demonstrated in Fig. 2(B). Then, Fig. 2(C) proves that gold nanoparticles are bound on the s-BLM/GC successfully (gold/s-BLM/GC electrode).

3.2. Cyclic voltammetric behavior of the modified electrode

The cyclic voltammogram (CV) is performed as the electron transfer kinetics of $Fe(CN)_6^{4-/3-}$. It will be changed when the electrode surface was modified by some materials. Therefore, the CV was used to confirm that s-BLM and gold nanoparticles were successfully assembled on the electrode. The CVs of the modified electrodes in working solution are shown in Fig. 3. According to Liu and Wei (2008), gold nanoparticles with a diameter of 16 nm were selected to modify the s-BLM. Compared with the CV of the bare GC electrode (Fig. 3b), which has a redox current with a large peak, the CV of s-BLM/GC electrode shows a decreased peak in response (Fig. 3d), demonstrating that the s-BLM has been formed on the electrode surface and acted as an insulated layer on the electrode surface. However, the s-BLM was not completely ion-impermeable. It was found to be loosely packed on the electrode surface with some pinholes in it. Such a surface was conducive to the electrodeposition of nanoparticles. As anticipated, after the gold nanoparticles were electrodeposited through s-BLM (Fig. 3c) or directly on the bare glassy carbon surface (Fig. 3a), an increase of current response of the respective CV was observed, indicating the enhancing effect of the gold nanoparticles on the electron transfer of the electrodes (Liu and Lin, 2007; Vockenroth et al., 2007).

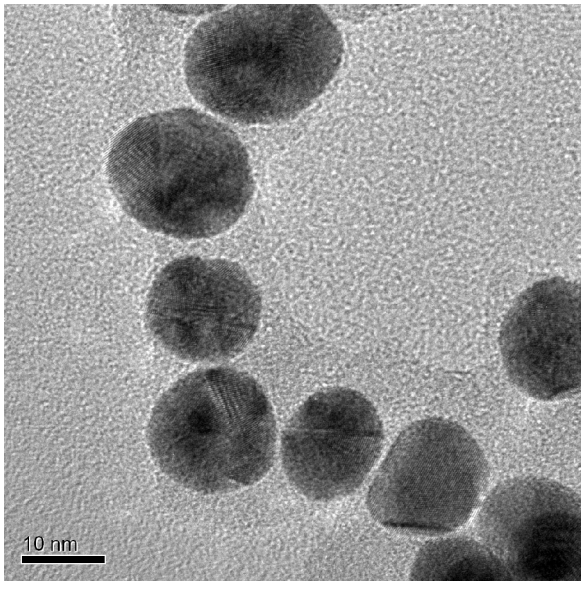


Fig. 1. TEM images of gold nanoparticles at 10 nm.

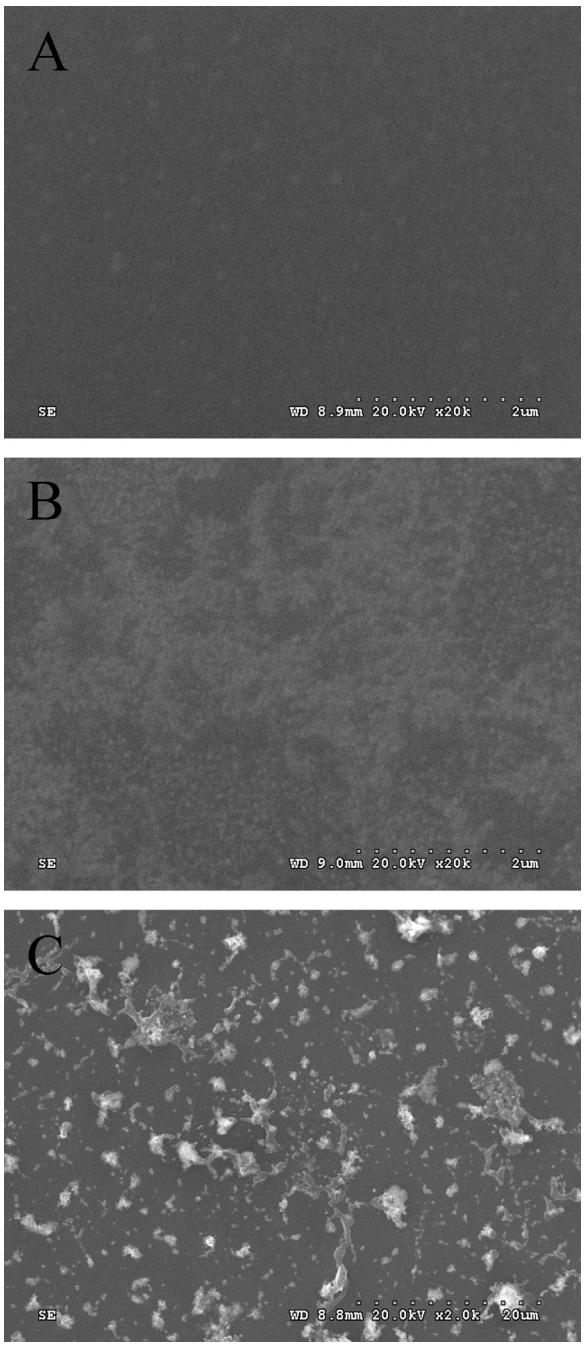


Fig. 2. SEM images of the electrodes. (A) Bare electrode surface; (B) the surface coated with the bilayer lipid membrane at 2 μm; (C) the surface coated with the bilayer lipid membrane and modified with gold nanoparticles at 20 μm.

3.3. Immobilization of anti-penicillin G in gold nanoparticles modified s-BLM

S-BLM has been widely used as an excellent matrix for protein immobilization, since it could maintain the biological activities of proteins well (Janshoff and Steinem, 2005). In order to investigate the optimized concentration of anti-penicillin G, the AP/gold/s-BLM/GC electrodes immobilized with different concentration of anti-penicillin G were incubated with Goat anti-penicillin G and then ALP-labeled Rabbit anti-Goat IgG antibody. The electrodes were scanned at the potential between 0 and 600 mV (vs. SCE) in Tris–HCl buffer (pH 8.0) containing 5 mM α-naphthyl phosphate, and the scanning rate was 100 mV/s. The α-naphthyl was

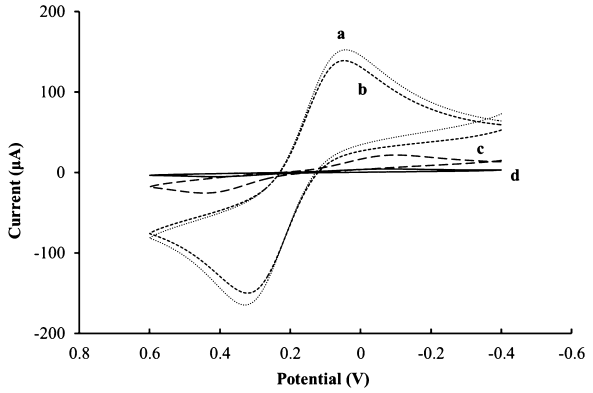


Fig. 3. Cyclic voltammograms of different electrodes in working buffer. (a) Gold/GC electrode; (b) bare GC electrode; (c) gold/s-BLM/GC electrode; (d) s-BLM/GC electrode.

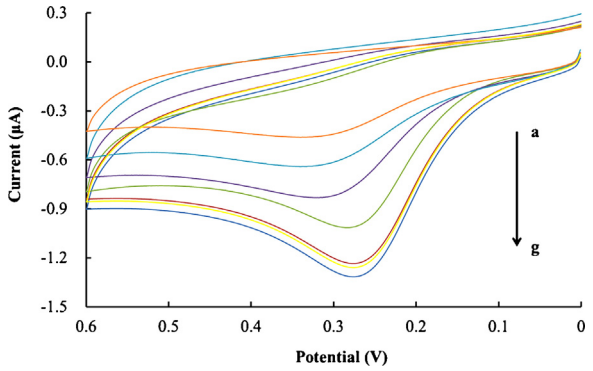


Fig. 4. Cyclic voltammograms of AP/gold/s-BLM/GC electrodes prepared with different concentrations of penicillin G after incubated with goat anti-penicillin G IgG and ALP-labeled rabbit anti-goat IgG in Tris–HCl buffer (pH 8.0) containing 5 mM α-naphthyl phosphate. Concentration of penicillin G: (a) 60 ng/mL; (b) 90 ng/mL; (c) 110 ng/mL; (d) 130 ng/mL; (e) 150 ng/mL; (f) 160 ng/mL; (g) 170 ng/mL.

hydrolyzed under the catalytic reaction of ALP labeled on the IgG in the s-BLM. The CVs of the electrodes are shown in Fig. 4. The well-defined oxidation peaks were observed in the region around 300 mV indicated that the anti-penicillin G had been immobilized in the S-BLM modified by gold nanoparticles. When the anti-penicillin G amount approached 150 ng/mL, the peak current reached an activity peak (Fig. 4). Therefore, we chose 150 ng/mL as the concentration of anti-penicillin G for the preparation of the biosensor.

3.4. Detection of penicillin G by impedance spectroscopy

Electrochemical impedance spectroscopy (EIS) is an efficient and sensitive method for investigating the electron transfer kinetics, and it is usually used for probing the feature of surface-modified electrode (Haas et al., 2001; Ye et al., 2003). Machius et al. characterized the electrical properties of s-BLM on semiconductor and gold surfaces in details (Machius et al., 2003). In order to obtain more detailed information about the modified electrodes, a modified Randles equivalent circuit was applied on them (Ye et al., 2003). The total impedance was determined by several parameters: the electrolyte resistance (R_s); the charge transfer resistance (R_{ct}); the Warburg element (Z_w); the lipid bilayer capacitance (C_{dl}), which consisted of the unmodified glassy carbon electrode (C_{GC}) and a capacitance originated from the s-BLM (C_m) on the surface of the electrode. The complex impedance can be presented as the sum of the real Z_{re} and imaginary Z_{im}, and it mainly originates from the resistance and capacitance of the cell. In the Nyquist

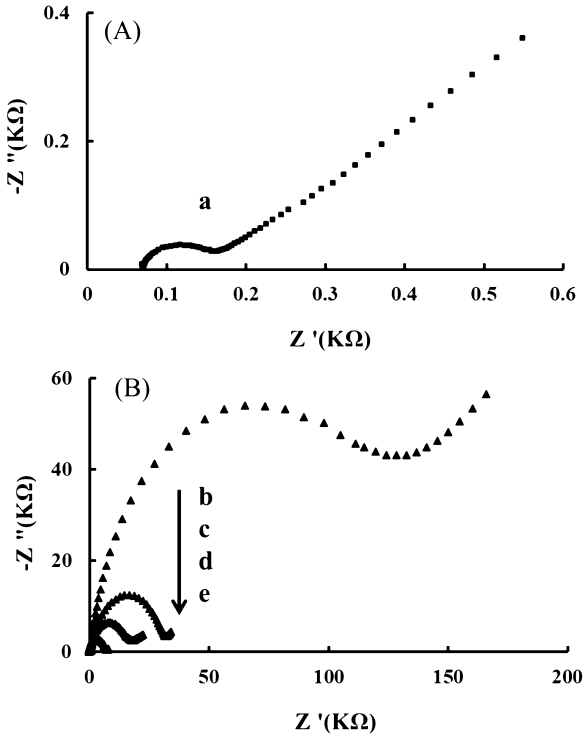


Fig. 5. Electrochemical impedance spectroscopy in working solution. (A.a) Bare GC electrode; (B.b) s-BLM/GC; (B.c) after penicillin G (33.4 ng/L) binding to AP/gold/s-BLM/GC; (B.d) AP/gold/s-BLM/GC; (B.e) gold/s-BLM/GC.

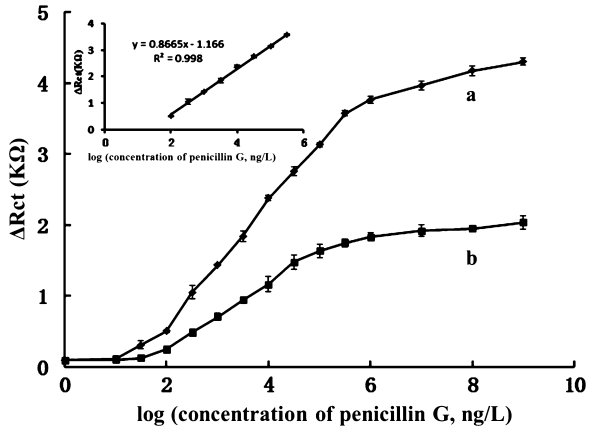


Fig. 6. Calibration curves of the electron transfer resistance change ΔR_{ct} with different concentrations of penicillin G based on the AP/gold/s-BLM/GC electrode (curve a) and the AP/s-BLM/GC electrode (curve b). Inset: the linear part of the calibration curve of AP/gold/s-BLM/GC electrode.

Table 1
Intra-assay and inter-assay precision for the biosensor.

Penicillin G concentration (ng/L)	CV (%)[a]	
	Intra-assay	Inter-assay
3.34	5.8	7.9
33.4	4.8	7.6
3.34×10^2	4.9	7.4
3.34×10^3	5.7	8.0

[a] $n = 6$.

diagram, the diameter of the semicircle of the AC impedance spectrum is equal to the charge transfer resistance on the surface of the electrode. The data were fitted for the parameters with ZVIEW software, and the fitting errors were less than 5%. The impedances of the bare GC electrode and the modified electrode were investigated at a frequency range from 10^{-1} to 10^5 Hz. Fig. 5 illustrates the impedance spectroscopy on a bare glassy carbon electrode (A.a), s-BLM/GC electrode (B.b), gold/s-BLM/GC electrode (B.e), AP/gold/s-BLM/GC electrode (B.d) and after penicillin G binding to AP/gold/s-BLM/GC electrode (B.c) in the working solution. It is shown in Fig. 5(A.a) that the bare glassy carbon electrode exhibits an almost straight line that was characteristic of a diffusional limiting step of the electrochemical process (Feng et al., 2000). With respect to the modified electrodes, apparent differences in impedance spectra were observed (Fig. 5(B)). Compared with the bare GC electrode, the charge transfer resistance of the s-BLM/GC electrode increased greatly, indicating that the s-BLM blocked electron transfer on the electrode surface (Fig. 5(B.b)). Electrodeposition of gold nanoparticles through the s-BLM produced a lower interfacial electron transfer resistance (Fig. 5(B.e)) than that of s-BLM/GC, which indicated that the deposition of gold nanoparticles resulted in an increase of the membrane capacitance. That was due to the gold nanoparticles acted as nanoscale conductors on the electrode surface, which facilitated the electron transfer (Liu and Lin, 2007; Sperling et al., 2008; Voevodin et al., 2007). An increase of electron transfer resistance was observed when AP/gold/s-BLM/GC electrode was obtained (Fig. 5(B.d)). After binding penicillin G to AP/gold/s-BLM/GC electrode, the resistance increased again (Fig. 5(B.c)). The change of charge transfer resistance could be used as the detection signal for penicillin G in this work. The change of electron transfer resistance was calculated using the following the equation: $\Delta R_{ct} = R_{ct(AP\text{-}P)} - R_{ct(AP)}$, where $R_{ct(AP\text{-}P)}$ was the value of electron transfer resistance after penicillin G binding to anti-penicillin G in AP/gold/s-BLM/GC and $R_{ct(AP)}$ was the value of electron transfer resistance of AP/gold/s-BLM/GC in working solution.

To detect penicillin G, the biosensor was incubated with different concentrations of penicillin G and then monitored by the impedance spectroscopy. Fig. 6 shows the electron transfer resistance change (ΔR_{ct}) of the AP/gold/s-BLM/GC electrode (curve a) and the AP/s-BLM/GC (curve b) after reacted with different concentrations of the penicillin G in the range of 3.34×10^{-8} to 3.34×10^6 ng/L. It indicated that the gold nanoparticles modified s-BLM electrode was more sensitive than s-BLM electrode. The biosensor exhibited an acceptable linear response to penicillin G concentration ranging from 3.34×10^{-3} to 1.0×10^3 M with R^2 at 0.998. The detection limit was 2.7×10^{-4} ng/L at the signal-to-noise ratio of 3. Therefore, the biosensor was suitable for quantitative analysis of penicillin G in milk, since the MRL of penicillin G was 4 ppb (equal to 4×10^3 ng/L) which was much higher than the detection limit.

3.5. Precision, selectivity and stability of the biosensor

In order to test the precision, we evaluated the biosensor by monitoring the impedance change using the same concentration of standard penicillin G. We assessed intra-assay precision by using six different AP/gold/s-BLM electrodes prepared following the same procedure to test the same samples on the same day. And all the tests were repeated in three consecutive days in a consistent way for inter-assay. The mean coefficient variation (CV) of intra-assay and inter-assay were 5.4% and 7.7% (Table 1).

The impedance change was measured when the proposed biosensor was reacting with two kinds of antibiotics at the same concentration range (1.0×10^{-19} to 1.0×10^{-5} M), in order to evaluate the selectivity. These two kinds of antibiotics used for evaluation were the penicillin group ampicillin, which has the similar physical and chemical characteristics as penicillin G, and the streptomycin, respectively. Both tested antibiotics generated very low signals of

Table 2
Comparison of results obtained from AP/gold/s-BLM/GC biosensor and HPLC assay for the determination of penicillin G in milk.

Samplings	AP/gold/s-BLM/GC biosensor assay		HPLC assay		*P*
	Penicillin G concentration (ng/mL)	CV (%)[a]	Penicillin G concentration (ng/mL)	CV (%)[a]	
1	15	2.0	20	1.9	>0.05
2	9	1.9	10	1.7	>0.05
3	23	1.8	19	1.6	>0.05
4	12	1.9	15	1.5	>0.05

[a] $n = 6$.

responses compared with those obtained from penicillin G. The responses of the two other antibiotics were the same for all concentrations and were the same as working solution. The response of working solution was lower than the response at the detection limit of penicillin G. These are very different from the responses obtained with penicillin G which increased with concentrations.

We tested the stability of the biosensor by exposing AP/gold/s-BLM/GC electrodes to 33.4 ng/L penicillin G at day 7 after storing in a wet box in the refrigerator at 4 °C. We found that an average response to 33.4 ng/L penicillin G on day 7 was 84% of the initial response. The stability of AP/s-BLM/GC electrode was also studied. The signals retained 75% of its initial response to 33.4 ng/L penicillin G on day 7. The better long-term stability of AP/gold/s-BLM/GC electrode compared with AP/s-BLM/GC demonstrated that the incorporated gold nanoparticles could improve the biocompatibility of s-BLM, prevent the leakage of biomolecules and maintain their biological activities.

3.6. Determination of penicillin G in real milk samples

To evaluate the possible application of our biosensor for real sample analysis, the concentration of penicillin G in milk was determined. Meanwhile, a comparison was made between our assay and HPLC assay, in order to further validate the biosensor penicillin G assay result. We selected four milk samples at local markets in Wuhan, each milk sample is quantified by 6 individual measurements in order to ensure test accuracy. The sample pretreatment was described in Section 2.6 sample pretreatment for biosensor. The results obtained from AP/gold/s-BLM/GC biosensor were consistent with those from HPLC assay (Table 2). The result demonstrated that our biosensor can be used for determination of penicillin G residues in milk samples.

4. Conclusions

In this study we developed a sensitive and easy-to-use nanostructured biosensor for the screening and the detection of penicillin G at trace level. It is based on anti-penicillin G immobilized in s-BLM modified with gold nanoparticles. It can selectively detect penicillin G in milk with a very low detection limit of 2.7×10^{-4} ng/L which is much lower than the MRL (4 ppb, equal to 4×10^{3} ng/L) officially adopted by the EU. Furthermore, gold nanoparticles deposited through the s-BLM have the effect of enhancing the sensitivity and stability of the biosensor. Comparing with the HPLC method, the biosensor is more convenient for manipulations and time-saving since the preparation of the milk sample is very simple. This biosensor can provide a promising alternative approach for the rapid screening of penicillin G in milk samples in the future.

Acknowledgements

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