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Short Communication

An Electrochemical Benzylpenicillin Biosensor Based on β-Lactamase and Fullerene Supported by A Bilayer Lipid Membrane

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A novel biosensor with high sensitivity for the electrochemical detection of benzylpenicillin was successfully constructed by immobilizing β-Lactamase on a fullerene (C_{60}) supported bilayer lipid membrane (C_{60}-s-BLM) modified gold electrode (GE). β-Lactamase was well immobilized on the electrode surface because of the good biocompatibility of the BLM. C_{60} was used as an electron transfer enhancer and an efficient immobilization matrix for the sensitivity enhancement. Transmission electron microscope (TEM) and cyclic voltammetry (CV) were used to characterize the surface morphology and the catalytic performance of the electrode, respectively. The results showed that the sensor had a good linearity in the range of 1.9-223.3 ng/L and a detection limit of 0.6 ng/L.

Keywords: Benzylpenicillin; β-Lactamase; Fullerene; Bilayer lipid membrane; Biosensor

1. INTRODUCTION

Benzylpenicillin (Penicillin G) is a kind of β-lactam antibiotic. It contains penicillin, which can destroy bacterial cell walls and play a bactericidal role during reproductive period of bacterial cells [1]. Because of this characteristic, benzylpenicillin has been widely used in the treatment of hemolytic Streptococcus infection, Streptococcus pneumoniae infection, anthrax infection, syphilis, etc [2-5]. In the dairy industry, benzylpenicillin is also widely used in veterinary medicine and even dietary supplements to improve the milk production [6,7]. However, its excessive use often leads to antibiotic contamination in milk, which may result in failure to meet food safety standards [8]. Since the 1980s, to meet these standards before entering the market, people began using β-Lactamase to catalyze the hydrolysis and degradation of benzylpenicillin and destroy the antibiotic residues [9].

Although penicillin is generally not toxic to humans, including children and pregnant women, and it can be used to treat symptoms in medical clinical practice, its residue may will have adverse effects on animals and humans, such as increasing bacterial resistance and toxicological effects [10,11]. Long term consumption of dairy products containing antibiotics can lead to drug resistance of some pathogens, such as Staphylococcus aureus [12]. Antibiotic resistant pathogens can be transferred through the food chain, leading to the transfer of some resistant genes from animals to human bacterial (intestinal) fauna, and finally excretion into the environment, thus polluting the environment [13].

Therefore, to avoid the harm caused by benzylpenicillin residues, several methods to detect these residues have been developed, among which the most important is based on liquid chromatography (LC) [14-16]. Although benzylpenicillin detection method based on LC has high specificity, its experimental steps are too complicated and instruments are expensive [17]. In contrast, use of a biosensor that can detect benzylpenicillin specifically is a simple and easy alternative method.

When fabricating a biosensor, it is very important to choose a suitable matrix to immobilize the enzyme, antibody or other recognition factor. A bilayer lipid membrane (BLM) is a kind of biomembrane composed of lipid molecules and formed by supramolecular self-assembly [18]. The BLM has the characteristics of fluidity and self-sealing. It can provide an environment for receptor ligand interaction and maintain its biological activity [19]. It can also significantly reduce the background noise, and prevent the hydrophilic electroactive substances from reaching the sensing membrane and producing undesirable reactions. These characteristics are very beneficial for biosensors.

In this study, we fabricated an electrochemical benzylpenicillin sensor, using C_{60} supported BLM (C_{60}-s-BLM) as the matrix to immobilize the β-lactamase. As a kind of biomaterial, BLM has good biocompatibility. It can be used as a biological matrix to fix β-lactamase and maintain its activity. In addition, C_{60} can substantially increase the stability of BLM, which is helpful for providing a stable substrate for β-lactamase. Moreover, C_{60}, as a good electron mediator, can dramatically alter the electrical properties of BLM and increase its sensitivity to a number of redox species in solution.

2. EXPERIMENTAL SECTION

2.1. Apparatus and materials

Electrochemical measurements were performed by a computer-controlled electrochemical workstation (CHI660D, Shanghai Chenhua Co., China). A modified electrode was used as the working electrode. A saturated calomel electrode (SCE) and a platinum wire electrode were used as the reference and auxiliary electrode, respectively. The assembling interface was tracked by Transmission electron microscope (TEM, (Philips Fei Co., Hillsboro, OR).

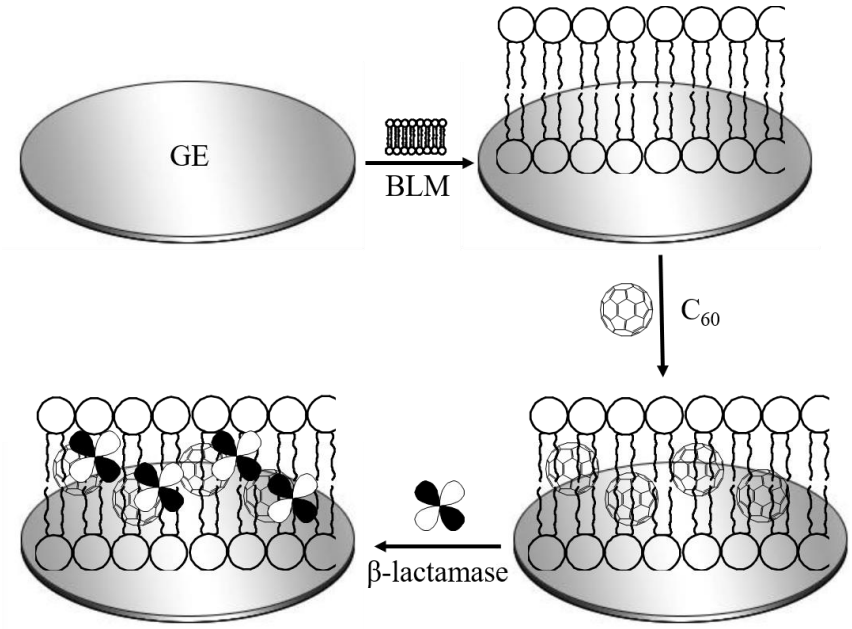
β-lactamase, lecithin, cholesterol and n-hexane were obtained from Aladdin Chemical Reagents Co. Ltd. (Shanghai, China). C_{60} was purchased from XFNANO technology Co., Ltd. (Shanghai, China). Phosphate buffer solutions (PBS) with various pH values were prepared using 0.1 M Na_2HPO_4, 0.1 M KH_2PO_4, and 0.1 M KCl and kept at 4 °C before use. Distilled water was used throughout this study. All other chemicals were of analytical grade and used as received without further purification.

2.2. The preparation of the film-forming solution and the β-lactamase solution

The film-forming solution was prepared as follows: 1.0 g cholesterol and 4.0 g lecithin were dissolved in 100 mL n-hexane under stirring. Then, 5 mg C_{60} was added into the n-hexane and dispersed by ultrasonic waves. β-Lactamase was dissolved in a 0.1 M PBS solution at pH 6.0, and because the isoelectric point of the β-lactamase solution was 6.0.

2.3 Fabrication of the proposed biosensor

Before use in the modification process, a gold electrode (GE) with a 4 mm diameter was first polished with 0.3 and 0.05 μm alumina slurries to obtain a mirror-like surface, respectively. 5 μL film forming solution and C_{60} were added onto the surface of the GE and kept at room temperature for 15 min. Then the modified electrode was immersed in PBS for another 15 min, during which the C_{60}-s-BLM was formed spontaneously on the surface of the electrode. This means that the C_{60}-s-BLM modified GE (C_{60}-s-BLM/GE) was successfully constructed. After that, 5 μL β-lactamase solution (1μg/mL) was dropped onto the surface of the C_{60}-s-BLM/GE (β-lactamase/C_{60}-s-BLM/GE). The obtained biosensors were stored under refrigeration at 4°C. A schematic illustration of the stepwise procedure of the biosensor fabrication is shown in Scheme 1.



Scheme 1. The schematic illustration of the stepwise procedure of the biosensor fabrication.

3. RESULTS AND DISCUSSION

3.1 Characterizations of the C_{60}-s-BLM

The morphology of the C_{60}-s-BLM was characterized by TEM, as shown in Fig. 1. It was found that C_{60} was successfully embedded into the bilayer lipid membrane.

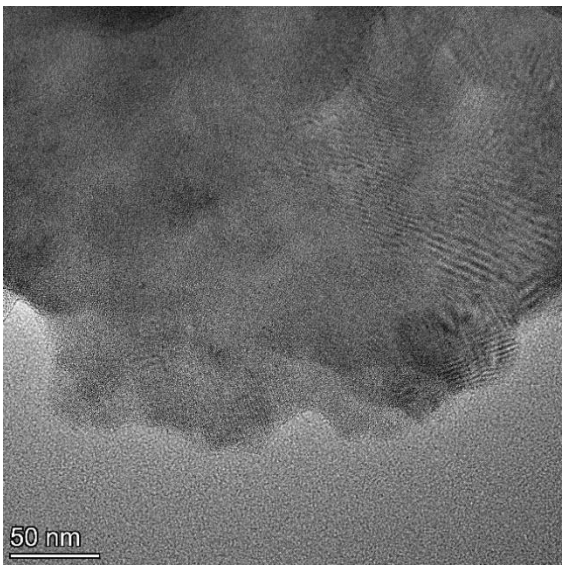


Figure 1. TEM of C_{60}-s-BLM.

3.2 The electrochemical characterization of the stepwise modified electrodes

The CVs of the fabricated biosensor during stepwise modification conducted in a 1 mM $[Fe(CN)_6]^{3-/4-}$(1:1) solution with a scan rate of 100 mV s^{-1} are shown in Fig. 2. A well-defined redox peak of $[Fe(CN)_6]^{3-/4-}$ was observed in the CV of the bare GE (curve a). Curve b shows the CV of BLM/GE, in which the peak current decreased significantly, indicating that the BLM could hinder the transmission of electrons. After assembling C_{60}, the peak current increased (curve c). The results illustrated that that BLM and C_{60} had been successfully assembled on the electrode and C_{60} could promote the electron transfer. The current response of $[Fe(CN)_6]^{3-/4-}$ on the β-lactamase/C_{60}-s-BLM/GE was further decreased (curve d), which is mainly because the enzyme is a macromolecular substance, and the active site is deeply buried in the interior, so the addition of enzyme reduces the redox peak current.

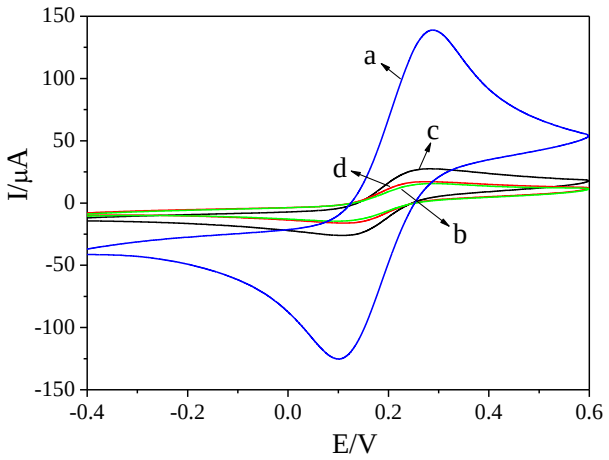


Figure 2. CVs of the bare GE, BLM/GE, C_{60}-s-BLM, β-lactamase/C_{60}-s-BLM/GE in 1 mM $[Fe(CN)_6]^{3-/4-}$(1:1) solution with a scan rate of 100 mV s^{-1}.

3.3 Electrochemical response of benzylpenicillin on the biosensor

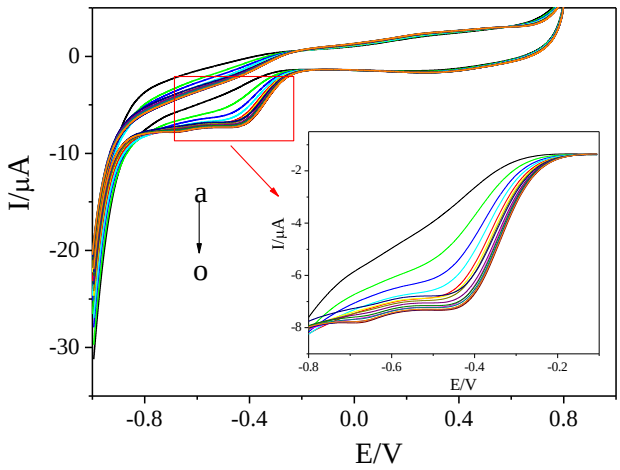


Figure 3. The CV behaviors of the β-lactamase/C_{60}-s-BLM/GE toward different concentrations of benzylpenicillin (a: 1.9 ng/L, b: 4.0 ng/L, c: 6.0 ng/L, d: 8.0 ng/L, e: 9.7ng/L, f: 15 ng/L, g: 24.9 ng/L, h: 39.9 ng/L, i: 59.8 ng/L, j: 84.6 ng/L, k: 94.5 ng/L, l: 114.4 ng/L, m: 144.2 ng/L, n: 183.9 ng/L, o: 223.3 ng/L) in 0.1 M pH 6.0 PBS (Scan rate: 100 mV s^{-1}).

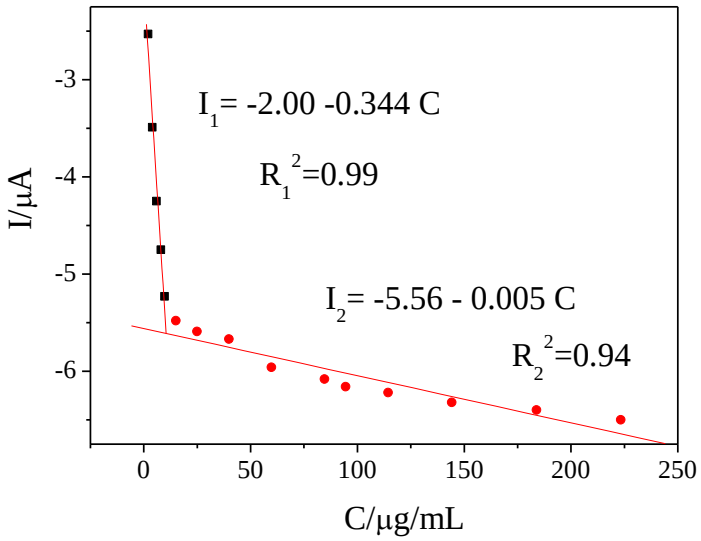


Figure 4. Corresponding linear calibration curve of peak current vs. benzylpenicillin concentration.

Fig. 3 showed the electrocatalytic behavior of the β-lactamase/C_{60}-s-BLM/GE toward different concentrations of benzylpenicillin in 0.1 M PBS (pH 6.0). The peak currents gradually increase with the increase of the concentration of benzylpenicillin. The linear relationship between the peak current and concentration were shown in Fig. 4. The result showed that the peak current is proportional to the concentration of benzylpenicillin from 1.9 to 9.7 ng/L and from 15 to 223.3 ng/L. The detection limit is 0.6 ng/L. At low penicillin levels, the local concentration at the electrode surface is rapidly depleted as the substrate is converted into product by the catalytic action of modified electrode, which leads to high sensitivity of the electrode response. When the concentration of penicillin was high, the nano-material

is supplied with substrate for a longer period of time and the reaction proceeds over a larger time window. This, together with the possibility of fouling of the electrode surface by the reaction products, results in a lower slope. Also, it attains a saturation level at higher concentration. Therefore, in different concentration range, the sensor shows different linear correlation [20]. The performance of the modified electrode is compared with that other sensors for benzylpenicillin detection, and the results are shown in Table 1. It can be seen that most of the detection methods are impedance method (EIS) and differential pulse voltammetry (DPV), which have high sensitivity. In this work, the CV is used to detect, and the performance is well.

Table 1. Comparison between the detection limit of the proposed method with the other reported methods.

Modified electrode	Linear range (ng/L)	Detection limit (ng/L)	Technique used	Reference
a CDI/graphite	1.04×10^{5}-2.66×10^{5}	3.2×10^{4}	DPV	[11]
[b]PAMAM/SWNT/GCE	2.2×10^{6}-1.0×10^{10}	-	EIS	[21]
[c]gold/s-BLM/GC	3.34×10^{-3}-3.34×10^{3}	2.7×10^{-4}	EIS	[22]
[d]PVA/-P'nase/PE	3.02×10^{6}-1.14×10^{8}	6.85×10^{5}	DPV	[23]
C_{60}-s-BLM/GE	0.24-1230	0.08	CV	This work

[a] N-Cyclohexyl- N'-(2-morpholinoethyl) carbodiimide
[b] polyamidoamine/single-walled carbon nanotubes layer-by-layer film
[c] gold/supported bilayer lipid membrane/glassy carbon electrode
[d] Poly (vinyl alcohol)/Penicillinase/platinum electrode

The linear equations of the two sections are as follows: I_1= -2.00-0.34C, R^2=0.99; I_2= -5.56-0.005C, R^2=0.94. The main principle is that β-lactamase can hydrolyze the β-lactam ring in penicillin to produce hydrogen ions, which causes the change of pH in the solution, and then the chemical signal is converted into an electrical signal through the modified electrode. The different degree of hydrolysis of penicillin by β-lactamase results in different concentration of H^+ and different detected electric signals, which finally realizes the quantitative detection of penicillin. The reaction of the hydrolytic degradation of the four-membered β-lactam ring is shown in Fig. 5 [24].

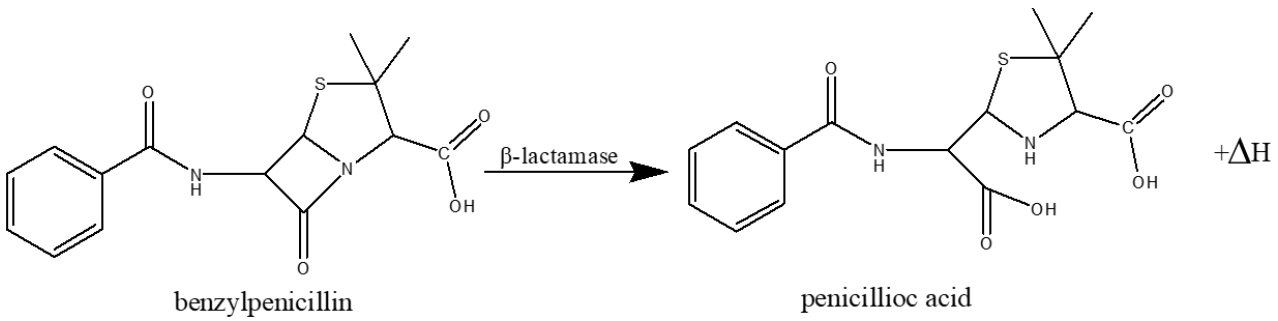


Figure 5. The reaction of the hydrolytic degradation of the four-membered β-lactam ring.

3.4 Anti-interference ability and stability

The interference of bovine serum albumin and inorganic ions (Ca^{2+}, Na^{+}, K^{+}, Cl^{-}, SO_4^{2-}) on the determination of benzylpenicillin was investigated. Compared with benzylpenicillin, it is found that the tested substances have no obvious interference to the determination, which indicates that the biosensor has good anti-interference ability. The stability of the biosensor was investigated by detecting the CV response to the same concentration of benzylpenicillin every few days. The peak current retained 96.2 % after 3 days.

3.5 Applicability to samples

CV is used to determine the content of benzylpenicillin (using the standard addition method). Table 2 shows that the percentage recoveries obtained for benzylpenicillin in milk ranged from 75-110%.

Table 2. Recovery of benzylpenicillin in milk samples.

Samples	Penicillin concentration in milk/(ng/L)	Penicillin concentration recovered with biosensor/(ng/L)	Recovery/%
1	5.0	5.5 ± 3.5	110 ± 30
2	10.0	8.0 ± 4.0	80 ± 22
3	20.0	15.0 ± 2.0	75 ± 8

[a] Mean ± SD of three measurements.

The results show that samples that contained lower benzylpenicillin concentrations reported higher standard deviations than samples that contained higher benzylpenicillin concentrations. This suggests that the reproducibility of the response was better for higher benzylpenicillin concentrations than for lower penicillin concentrations.

4. CONCLUSIONS

In summary, an electrochemical benzylpenicillin biosensor based on β-lactamase and C_{60} supported by BLM was successfully constructed in this paper. β-lactamase was well immobilized on the electrode surface because of the good biocompatibility of the BLM. And C_{60}, as an electron transfer enhancer and efficient immobilization matrix effectively improved the sensitivity of the sensor.

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