Fabrication of a Molecularly Imprinted Electrochemical Sensor for Enrofloxacin and Its Application in Rapid Food Detection (Postprint)

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Abstract

A molecularly imprinted electrochemical sensor capable of specifically recognizing the template molecule and its structural analogs was prepared on the surface of a glassy carbon electrode by electropolymerization using enrofloxacin (ENRO) as the template molecule and o-phenylenediamine (OPD) and o-aminophenol (OAP) as composite functional monomers in NaAc-HAc buffer solution. A mixed solution containing 1 mol/L potassium chloride and 1 mmol/L potassium ferricyanide was selected as the characterization solution, and the electrochemical response characteristics of the sensor were investigated using cyclic voltammetry and square wave voltammetry, with optimization of the preparation and detection conditions. The results demonstrated that under optimal conditions, enrofloxacin exhibited a good linear relationship in the concentration range of 2×10 mol/L to 4×10 mol/L, with a detection limit of 7.0×10 mol/L. The sensor possessed excellent stability and reproducibility, and demonstrated good selectivity for enrofloxacin and its structural analogs. The sensor was employed to detect enrofloxacin in actual samples of milk, chicken, pork, and eggs, with spike recoveries ranging from 83.2% to 92.7% and relative standard deviations (RSD) between 1.0% and 4.8% (n=5). The sensor features strong selectivity, good stability, simple operation, rapid and sensitive detection, low cost, and exhibits promising application prospects.

Full Text

Preparation of Enrofloxacin Molecularly Imprinted Electrochemical Sensor and Its Application in Rapid Food Detection

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Abstract

A novel molecularly imprinted electrochemical sensor was fabricated on the surface of a glassy carbon electrode via electro-polymerization in NaAc-HAc buffer solution, using enrofloxacin (ENRO) as the template molecule and o-phenylenediamine (OPD) and o-aminophenol (OAP) as composite functional monomers. The sensor could specifically recognize the template molecule and its structural analogs. A mixed solution containing 1 mol/L KCl and 1 mmol/L K [Fe(CN)] was employed as the characterization solution, and the electrochemical response characteristics of the sensor were investigated using cyclic voltammetry (CV) and square wave voltammetry (SWV), with optimization of preparation and detection conditions.

The results demonstrated that under optimal conditions, enrofloxacin exhibited a good linear relationship in the concentration range of 2×10 mol/L to 4×10 mol/L, with a detection limit of 7.0×10 mol/L. The sensor showed excellent stability, reproducibility, and selectivity toward enrofloxacin and its structural analogs. When applied to real food samples including milk, chicken, pork, and eggs, the spiked recoveries ranged from 83.2% to 92.7%, with relative standard deviations (RSD) between 1.0% and 4.8% (n=5). The sensor offers strong selectivity, good stability, simple operation, rapid and sensitive detection, low cost, and promising application prospects.

Keywords: enrofloxacin; food; molecularly imprinted polymer; electrochemical sensor; rapid detection

Introduction

In recent years, the abuse and residue of antibiotics have attracted widespread attention. Antibiotic misuse not only contributes to bacterial resistance but also causes poisoning in livestock and allergic reactions in humans [1]. China has implemented strict regulatory systems for antibiotic usage. Enrofloxacin, a chemically synthesized third-generation fluoroquinolone antibiotic (molecular structure shown in Figure 1 [Figure 1: see original paper]), exerts its antibacterial effect by selectively inhibiting bacterial DNA gyrase and topoisomerase IV [2]. As a broad-spectrum antibiotic with significant efficacy, it is widely used for disease prevention and treatment in livestock, poultry, and aquatic products [3]. Currently, enrofloxacin is a designated veterinary drug in China and has been included in national mandatory inspection programs. Therefore, establishing a simple, rapid, sensitive, and accurate method for detecting enrofloxacin in foods is of great importance.

Figure 1 Enrofloxacin molecular structure

Conventional detection methods for enrofloxacin in samples include gas chromatography [4], liquid chromatography [5-7], and chromatography-mass spec-

trometry techniques [8,9]. However, these chromatographic methods suffer from high operational costs and require complex sample pretreatment and long analysis times. Molecularly imprinted electrochemical sensors combine the specific recognition capability of molecularly imprinted polymers with the advantages of rapid and sensitive electrochemical detection, offering simple equipment, low cost, convenient operation, fast detection, strong selectivity, and good stability. This study prepared an enrofloxacin molecularly imprinted electrochemical sensor and applied it to rapid detection in food samples.

Materials and Methods

1.1 Instruments and Reagents

A CHI600E electrochemical workstation (Shanghai Chenhua Instrument Co., Ltd.) was used with a three-electrode system: glassy carbon electrode (GCE) as the working electrode, Ag/AgCl as the reference electrode, and platinum wire as the counter electrode (Shanghai Chenhua Instrument Co., Ltd.).

Enrofloxacin (ENRO, 98% purity), o-phenylenediamine (OPD, 99.5% purity), o-aminophenol (OAP, 98% purity), resorcinol (99% purity) were purchased from Aladdin Reagent Co., Ltd. -methylacrylic acid (99% purity) was obtained from Tianjin Bodi Chemical Co., Ltd. Potassium ferricyanide, potassium chloride, acetonitrile (analytical grade) were from Tianjin Yongda Chemical Reagent Co., Ltd. Disodium hydrogen phosphate, sulfuric acid, sodium hydroxide, nitric acid, phosphoric acid, acetic acid, anhydrous sodium acetate, and anhydrous ethanol were all analytical grade reagents.

Food samples including pork, chicken, eggs, and milk were purchased from local markets.

1.2 Pretreatment of Glassy Carbon Electrode

The glassy carbon electrode (GCE) was sequentially polished with Al O powder of different particle sizes (0.5 m to 0.05 m) on a chamois leather surface until a mirror finish was obtained. After thorough rinsing with ultrapure water, the electrode was ultrasonically cleaned in 0.5% nitric acid, anhydrous ethanol, and ultrapure water (5-6 s each). Following nitrogen drying, the electrode was treated electrochemically in 0.5 mol/L H SO solution to achieve optimal condition. After rinsing with ultrapure water and nitrogen drying, the electrode was subjected to cyclic voltammetric scanning in the characterization solution until symmetrical and reversible redox peaks were obtained (peak potential difference <85 mV, peak current ratio of 1:1) [10].

1.3 Sample Preparation

Five grams of homogenized sample (pork, chicken, milk, or eggs) was placed in a 50 mL centrifuge tube, mixed with 10 mL acetonitrile, vortexed for 2 min, and ultrasonically extracted for 10 min. After centrifugation at 10,000 r/min

for 5 min, the supernatant was collected. The residue was re-extracted with 10 mL acetonitrile following the same procedure. The combined supernatants were diluted to 25 mL with acetonitrile for subsequent analysis [11].

1.4 Experimental Procedure

All experiments were conducted at room temperature. Cyclic voltammetry (CV) and square wave voltammetry (SWV) were employed to optimize experimental conditions and characterize sensor performance, while SWV was used for real sample detection. The three-electrode system was immersed in K [Fe(CN)] solution containing electroactive probes to measure current response. Parameters were set as follows: CV scanning potential -0.2 V to 0.8 V at 50 mV/s; SWV potential range -0.1 V to 0.6 V, potential increment 0.005 V, pulse amplitude 0.025 V, frequency 5 Hz. After each measurement, the GCE was immersed in eluent to remove template molecules, then rinsed with ultrapure water, dried with nitrogen, and prepared for the next measurement.

1.5 Data Processing and Analysis

Origin 8.0 and Excel 2016 were used for plotting and statistical analysis. All experiments were performed in triplicate, and data are presented as mean values of three parallel measurements.

Results and Discussion

2.1 Electrochemical Activity of Enrofloxacin

To verify whether the inherent electrochemical activity of enrofloxacin would affect sensor preparation, CV was employed to investigate its electrochemical properties. As shown in Figure 2 [Figure 2: see original paper], enrofloxacin exhibited an irreversible oxidation peak during cyclic scanning. Direct electrochemical analysis of enrofloxacin suffers from low sensitivity and generates oxidation products that adsorb onto the working electrode surface, affecting electrode performance.

This study combined electrochemical analysis with molecular imprinting technology to prepare an enrofloxacin molecularly imprinted electrochemical sensor for indirect detection of enrofloxacin residues in foods using electroactive probes.

Figure 2 CV curves of enrofloxacin in sodium acetate buffer (pH = 5.2)

2.2 Selection of Functional Monomers

The fundamental principle for selecting functional monomers is based on the template molecule structure and interaction types, with hydrogen bonding being the most common interaction [11,12]. According to this principle, compounds containing hydroxyl and amino groups—including o-phenylenediamine, o-aminophenol, resorcinol, and -methacrylic acid—were preliminarily evaluated

as potential functional monomers. Their physicochemical properties and polymerization characteristics were further investigated to identify the most suitable compound.

During experiments, when -methacrylic acid was used as the functional monomer, no redox peaks appeared during polymerization, indicating that -methacrylic acid did not undergo electrochemical reaction with the template molecule during electro-polymerization, making it unsuitable. However, when pairwise combinations of o-phenylenediamine, o-aminophenol, and resorcinol were tested, OPD/OAP showed the best polymerization performance. As shown in Figure 3-A [Figure 3: see original paper], OPD/OAP could polymerize on the electrode surface to form a non-conductive polymer film with excellent insulating properties and nearly zero current response. Therefore, OPD/OAP was selected as the optimal composite functional monomer. An important advantage of composite functional monomers is their ability to provide more active binding sites than single monomers, resulting in stronger specific binding with template molecules.

The electrode with OPD/OAP polymer film was immersed in deoxygenated 0.5 mol/L H SO -methanol (1:1, V:V) for 12 min, then rinsed with ultrapure water and dried with nitrogen for re-characterization. No redox peaks were observed, and current intensity was nearly zero, confirming the stability of the OPD/OAP polymer film under acidic conditions. This further validated OPD/OAP as the optimal composite functional monomer for electro-polymerization.

Comparison of Figure 3-A and Figure 3-B [Figure 3: see original paper] reveals that under identical conditions, the CV curve of OPD/OAP polymerization without template molecules was essentially the same as that with template molecules, except for a negative shift of the highest peak potential. This suggests that the template molecule lacked electrochemical activity within the scanning potential range and did not undergo electrochemical reactions during polymerization. The hydrogen bonding between enrofloxacin and OPD/OAP formed a polymer film without destroying the molecular structure, ensuring that template molecules in the imprinted film on the GCE could be eluted while maintaining intact imprinted cavity structures.

2.3 Formation of Poly-OPD/OAP Composite Imprinted Film

The formation characteristics of poly-OPD/OAP composite film were investigated by comparing polymerization of different functional monomers in sodium acetate electrolyte. As shown in Figure 4 [Figure 4: see original paper], the cyclic voltammograms during electro-polymerization revealed that OPD and OAP individually exhibited oxidation peaks near 0.5 V and 0.55 V, respectively. With increasing polymerization cycles, the CV curves declined rapidly, indicating an irreversible process forming non-conductive polymer films on the working electrode surface. In contrast, Figure 3-B [Figure 3: see original paper] shows the electro-polymerization curve of OPD/OAP composite functional

monomers, where the peak potentials shifted positively, representing a completely irreversible reaction with significantly increased peak current. This indicates that interactions occurred between the composite functional monomers during polymerization, rapidly generating a dense, non-conductive composite polymer film on the electrode surface. Compared with single monomer polymerization, the composite functional monomers demonstrated faster film formation and stronger insulating capability.

2.4 Optimization of Polymerization Conditions

2.4.1 Determination of Template-to-Functional Monomer Ratio A critical condition for preparing molecularly imprinted electrochemical sensors via electro-polymerization is determining the appropriate amounts of template molecule and functional monomer. Extensive research indicates that excessive functional monomer leads to overly rapid film formation, with monomer molecules occupying excessive electrode surface area and reducing the number of imprinted sites, thereby decreasing stability. Conversely, insufficient functional monomer results in slow film formation and reduced imprinted sites, compromising the imprinting effect. Initially, functional monomer concentration was fixed at 10 mmol/L, and five different template concentrations (0.1, 0.2, 1, 2, and 4 mmol/L) were tested. Results showed optimal imprinting effect at 2 mmol/L template concentration. Since OPD and OAP have similar molecular weights, equal concentrations were used for electro-polymerization [13]. The effects of template-to-composite functional monomer ratios (M:OPD:OAP) of 2:5:5, 2:10:10, 2:15:15, 2:20:20, and 2:25:25 were evaluated. As shown in Figure 5 [Figure 5: see original paper], the best imprinting effect with maximum response current during elution was achieved at M:OPD:OAP = 2:15:15, which was therefore selected as the optimal ratio.

2.4.2 Selection of Polymerization Electrolyte Polymerization was investigated in NaAc-HAc electrolyte solution (pH 5.2) and Na HPO -NaH PO (phosphate buffer saline, PBS) electrolyte solution (pH 7.2). Both systems could form non-conductive OPD/OAP polymer films. However, as shown in Figure 6 [Figure 6: see original paper], polymerization in pH 5.2 NaAc-HAc electrolyte solution exhibited faster film formation, stronger membrane stability, better insulation, and larger film-formation peak currents. Therefore, pH 5.2 NaAc-HAc solution was selected as the electrolyte.

2.4.3 Selection of Polymerization Potential The effects of five different polymerization potential ranges (0–0.8 V, 0–1.0 V, -0.2–0.8 V, -0.2–1.0 V, and 0–1.2 V) on polymerization were examined. As shown in Figure 7 [Figure 7: see original paper], imprinted film detachment occurred after elution at 0–0.8 V and 0–1.0 V. At -0.2–0.8 V, peak potentials shifted positively with decreased redox peak currents. Template molecule elution was difficult in the -0.2–1.0 V range. The 0–1.2 V range provided good template elution and maintained film integrity, making it the optimal polymerization potential.

2.4.4 Selection of Polymerization Cycles Polymerization cycles significantly affect film thickness, imprinting effect, and sensitivity. Insufficient cycles produce thin films with few imprinted cavities and poor imprinting effects, while excessive cycles create overly thick films where template molecules cannot be effectively eluted, blocking access to the electrode surface. Under otherwise identical conditions, different cycle numbers (5, 10, 15, 20, 25, and 30 cycles) were tested for electro-polymerization. After eluting template molecules from the prepared imprinted electrodes, SWV characterization showed maximum current response at 15 cycles (Figure 8 [Figure 8: see original paper]), which was selected as the optimal number.

2.5 Selection of Eluent and Elution Time

Four eluent systems were evaluated: 0.1 mol/L H SO -50% methanol (1:1, V:V), 0.5 mol/L H SO -50% methanol (1:1, V:V), 0.1 mol/L NaOH in ethanol-water (3:1, V:V), and ethanol-water (3:1, V:V). The first three systems showed difficult or poor template elution, while 0.1 mol/L NaOH in ethanol-water (3:1, V:V) demonstrated good elution efficiency.

Elution time was optimized by immersing imprinted electrodes in the eluent and characterizing at 2-min intervals using SWV. Maximum and stable peak current indicated complete template removal. As shown in Figure 9 [Figure 9: see original paper], peak current increased continuously with elution, reaching 12 A at 12 min, after which no further increase occurred, confirming complete template removal. Therefore, 12 min was selected as the optimal elution time.

2.6 Optimization of Adsorption Time

The imprinted sensor prepared under optimal conditions was immersed in 0.01 mmol/L enrofloxacin standard solution for different adsorption periods, then characterized in probe solution to determine optimal adsorption time. As shown in Figure 10 [Figure 10: see original paper], response peak current decreased gradually with increasing adsorption time, stabilizing after 6 min, indicating that enrofloxacin had fully occupied the imprinted cavities. Thus, 6 min was selected as the optimal adsorption time.

2.7 Molecular Imprinting Effect

The imprinting effect was verified using CV and SWV (Figures 11 and 12 [Figure 11: see original paper][Figure 12: see original paper]). The SWV curves showed maximum response peak current for the bare electrode (curve a), indicating unhindered electron transfer of probe ions. After electro-polymerization, the non-eluted imprinted film electrode (curve d) showed negligible current response, demonstrating that the non-conductive polymer film blocked electron transfer and inhibited redox reactions. After template elution (curve b), peak current increased significantly as probe ions accessed the electrode surface through imprinted cavities. Upon re-adsorption of template molecules (curve c), peak cur-

rent decreased compared to the eluted state (curve b), confirming that some cavities were re-occupied, reducing probe ion access. CV and SWV characterization yielded consistent results.

2.8 Selectivity of Molecularly Imprinted Polymer Film

Selectivity was investigated using structural analogs (ciprofloxacin, danofloxacin, norfloxacin) and a structurally different compound (tetracycline) as interferents (structures shown in Figure 13 [Figure 13: see original paper]). As shown in Figure 14 [Figure 14: see original paper], the sensor exhibited the strongest response to the template molecule enrofloxacin, followed by its structural analogs, while showing weak response to tetracycline. Ciprofloxacin, danofloxacin, and norfloxacin have similar molecular structures and weights to enrofloxacin, allowing them to enter imprinted cavities and bind with functional monomers, demonstrating the sensor' s capability to detect enrofloxacin and its analogs. Tetracycline' s structural dissimilarity resulted in poor selectivity, as it could not enter the imprinted cavities and thus did not interfere with enrofloxacin detection. These results confirm good sensor selectivity.

2.9 Linear Relationship and Detection Limit

Different concentrations of enrofloxacin standard solutions (0.002–0.04 mmol/L) were prepared. Using SWV, the response values of the molecularly imprinted polymer electrode were measured in blank solution and after adsorption in various standard solutions. Plotting ΔI versus enrofloxacin concentration yielded the standard curve and linear range. The detection limit was determined at S/N=3. As shown in Figures 15 and 16 [Figure 15: see original paper][Figure 16: see original paper], enrofloxacin showed a good linear relationship with ΔI in the range of 2×10 mol/L to 4×10 mol/L, with the linear equation $\Delta I = 49.566x + 0.1869$, correlation coefficient $R^2 = 0.9990$, and detection limit of 7.0×10 mol/L.

2.10 Recovery and Precision

Spiked recovery tests were performed on food samples at two levels (2×10 mol/L and 2×10 mol/L) under optimal conditions. As shown in Table 1 , average recoveries ranged from 83.2% to 92.7%, with RSD values between 1.0% and 4.8% (n=5), indicating good recovery and precision.

2.11 Real Sample Analysis

Food samples (eggs, pure milk, chicken, pork) were prepared according to section 1.3. Two milliliters of filtered test solution was placed in an electrolytic cup and diluted to 10 mL with NaAc-HAc buffer for detection using the procedure in section 1.4. No enrofloxacin was detected in any of the samples.

2.12 Reproducibility and Stability

Reproducibility was evaluated by using the enrofloxacin-imprinted sensor 20 times under optimal conditions, yielding an RSD of ΔI 6.41% (n=20), demonstrating good reproducibility. Stability was assessed by storing prepared imprinted electrodes at 4°C. After 7 days, the sensor retained 92.6% of its initial response to 0.01 mmol/L enrofloxacin; after 14 days, 87.8%; and after 21 days, 82.0%, confirming good stability.

Conclusion

This study successfully prepared a molecularly imprinted electrochemical sensor for enrofloxacin detection using OPD/OAP as optimal functional monomers, selected based on enrofloxacin molecular structure, physicochemical properties, and hydrogen bonding interactions. The optimal electro-polymerization conditions were established as: template-to-functional monomer ratio of 2:15:15, NaAc-HAc buffer (pH 5.2) as polymerization electrolyte, 15 polymerization cycles, potential range of 0–1.2 V, eluent of 0.1 mol/L NaOH in ethanol-water (3:1, V:V), template elution time of 12 min, and imprinted electrode adsorption time of 6 min. CV and SWV characterization confirmed excellent imprinting performance.

Selectivity studies demonstrated that the sensor exhibited the highest selectivity for the template molecule enrofloxacin, moderate selectivity for its structural analogs, and poor selectivity for structurally dissimilar tetracycline. The sensor showed a linear range of 2×10 mol/L to 4×10 mol/L with a detection limit of 7.0×10 mol/L. Sample recoveries ranged from 83.2% to 92.7% with RSD values of 1.0%–4.8% (n=5), and the sensor maintained good performance after 20 consecutive uses. These results demonstrate that the sensor possesses good stability, high sensitivity, and strong anti-interference capability, making it suitable for detecting enrofloxacin residues in food samples.

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Note: Figure translations are in progress. See original paper for figures.