Preparation of Molecularly Imprinted Composites Initiated by Hemin/Graphene Hybrid Nanosheets and Its Application in Detection of Sulfamethoxazole*

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Summary: Molecularly imprinted polymers (MIPs) exhibit high selectivity resulting from imprinted cavities and superior performance from functional materials, which have attracted much attention in many fields. However, the combination of MIPs film and functional materials is a great challenge. In this study, hemin/graphene hybrid nanosheets (H-GNs) were used to initiate the imprinted polymerization by catalyzing the generation of free radicals. Thus, MIPs using sulfamethoxazole as the template was directly prepared on the surface of H-GNs without any film modification. Most importantly, the template could be absorbed on the H-GNs to enhance the number of imprinted sites per unit surface area, which could improve the selectivity of MIPs film. Thus, the composites could exhibit high adsorption capacity (29.4 mg/g), imprinting factor (4.2) and excellent conductivity, which were modified on the surface of electrode for rapid, selective and sensitive detection of sulfamethoxazole in food and serum samples. The linear range was changed from 5 μg/kg to 1 mg/g and the limit of detection was 1.2 μg/kg. This sensor was free from interference caused by analogues of sulfamethoxazole, which provides a novel insight for the preparation of MIPs-based sensor and its application in food safety monitoring and human exposure study.

Key words: MIP/H-GNs composites; sulfamethoxazole; nanoenzyme-mediated polymerization; sensor; dietary exposure

The antibiotic sulfonamides (SAs) are commonly used as antibacterial drugs in both human and veterinary medicine to fight infectious diseases, and in animal feed to promote livestock growth. However, 95% of the farmers never observed withdrawal periods although 80% of them knew the importance of withdrawal periods[1]. Thus, residue of antibiotics in food samples cannot be ignored, which presents a potential harm to human health on a worldwide scale, especially for the drug resistance. The European Commission (EC), America and other countries have adopted the maximum acceptable limit of residual sulfonamides in different food samples. Among SAs drugs, sulfamethoxazole (SMX) is one of the widely used antimicrobial agents. Therefore, it is necessary to develop a rapid, simple, sensitive and selective method to monitor the SMX residue in food production, packaging and distribution and human exposure information.

Numerous methods have been proposed for determination of SMX in the complex samples, containing chromatography with different detectors, spectrophotometry, capillary electrophoresis and electrochemical sensor[2–4]. Among these methods, electrochemical sensor is a powerful analytical technique, as it is simple and quick to use, low-cost, highly sensitive and environmentally friendly. Different functional materials, such as graphene, carbon tubes and metal oxide, are used to improve the sensitivity of sensor due to the high catalytic performance[5, 6]. The greatest drawback of electrochemical sensor is the weak selectivity, because of the complex samples matrix and interference caused by its analogue. Accordingly, there is a considerable interest in introduction of molecularly imprinted polymers (MIPs). Molecular imprinting is known as an ideal technology to construct tailor-made recognition sites to selectively recognize target molecules[7, 8]. However, the conductivity of MIPs is low, which will decrease the electrocatalytic activity

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of sensor. Thus, different types of carbon materials are introduced to the molecularly imprinted procedure to overcome this weakness[9]. But the combination of MIPs film and functional materials is a great challenge.

In general, carbon materials have no other functional groups but carboxyl groups. Thus, γ-methac ryloxypropyltrimethoxysilane is often modified on the surface of materials to provide the vinyl groups, which can be used to construct imprinted cavities[10]. It should be noted that silica film modification can also decrease the conductivity of electrode. In fact, horseradish peroxidase can be used to initiate the polymerization of methacrylate or vinyl monomers and cross-linkers by catalyzing the generation of free radicals[11]. Thus, hemin/graphene hybrid nanosheets (H-GNs) with peroxidase-like activity can be used for direct preparation of MIPs owing to their striking merits. Compared with the enzyme, the stability and catalytic efficiency of initiator were greatly improved and MIPs can be polymerized in organic solvent. The electrode modified with this composite can selectively recognize target molecules without the decrease of conductivity, which guarantees the selective and sensitive detection.

In this study, H-GNs initiated synthetic approach is described for the preparation of MIPs using SMX as the template and methacrylic acid (MAA) as the monomer. Selective recognition and the initiated mechanism were studied. Then, the composites were modified on the electrode to selectively and sensitively determine SMX. The determination of SMX in the complex samples was also carried out and the results were compared with those obtained by the LC-MS/MS method.

1 MATERIALS AND METHODS

1.1 Chemicals and Apparatus

SMX, sulfamethazine (SMZ), sulfadimethoxine (SDM), sulfamerazine (SMR), sulfameter (SME), sulfamethoxazole (SMO), 2,2-azobisisobutyronitrile (AIBN), MAA, and hemin and ethylene glycol dimethacrylate (EGDMA) were purchased from Sigma (USA). Methanol, acetonitrile, potassium ferricyanide, acetylacetone and hydrogen peroxide were purchased from the Sinopharm Chemical Reagent Co., Ltd (China). Graphene oxide (GO) was purchased from Nanjing Xianfeng Nano Co. (China). EGDMA was purified prior to use via general distillation method and AIBN was recrystallized from methanol. Ultrapure water was obtained from a Milli-R04 purification system (Millipore, Germany). The surface morphology of the MIP composites was evaluated by atomic force microscopy (DI NanoScope IV AFM, Veeco Co. Ltd., USA).

1.2 Preparation of MIP/H-GNs Composites

H-GNs with peroxidase-like activity were synthesized based on the literature[12, 13]. Briefly, 20.0 mL of graphene oxide dispersion (0.5 mg/mL) was mixed with an equal amount of hemin aqueous solution. After introduction of ammonia solution (200.0 μL) and hydrazine solution (30 μL), respectively, the mixture was vigorously shaken for 5 min and then incubated in a water bath (60°C) for 3.5 h. The dispersion was filtered with a nylon membrane (0.22 μm) to obtain H-GNs.

Then, H-GNs initiated synthetic approach is described for the preparation of MIPs (fig. 1). Template molecules (SMX, 1 mmol/L), functional monomer (MAA, 4 mmol/L) and cross-linker (EGDMA, 10 mmol/L) were dispersed in 5 mL acetonitrile/water solution (1/1, v/v) by ultrasonic pretreatment to form a pre-polymerization solution. Then, 200 μL of H-GNs (1 mg/mL), H_2O_2 solution (200 μL) and acetylacetone (3.6 μL) were used as the initiator system, which was added into above pre-polymerization solution to prepare MIPs. After deoxygenating with nitrogen for 5 min, polymerization took place at 60°C for 24 h and the templates were removed with acetic acid/methanol (1/9, v/v). The residue of template was monitored by the HPLC-UV method (λ=266 nm). Respective non-imprinted composites (NIP/H-GNs) were prepared in the same conditions but without SMX in the polymerization solution.

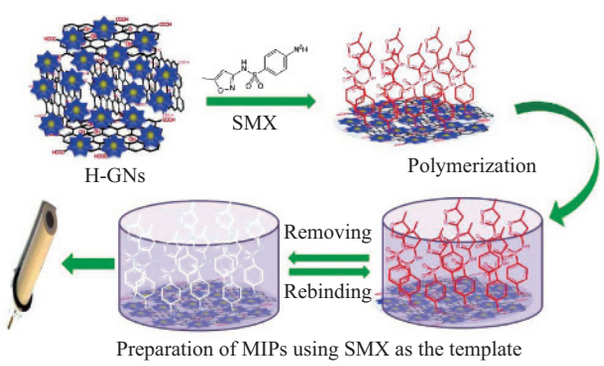

Fig. 1 The preparation procedure of MIP/H-GNs modified electrode

1.3 Electrochemical Detection of SMX in Food and Serum Samples

The GC electrode (3.0 mm diameters) was further polished with 0.05-mm alumina powder on a polishing microcosm and rinsed thoroughly with doubly distilled water prior to modification. MIP/H-GNs composites were dispersed in anhydrous ethanol by ultrasonic pretreatment. Then, 10 μL of MIP composites (5 mg/mL) were dropped on the surface of GC electrode and dried under an infrared lamp. This composition was used as the working electrode.

The food samples (such as milk and honey) purchased from the local retail market were selected for spiked sample analysis. Human serum samples

were kindly provided by a team of volunteers and then stored at –20°C until analysis. After spiked with different levels of SMX, the spiked milk sample was extracted by 30 mL 5% perchloric acid and the mixture was shaken for 30 min. After centrifugation at 3000 r/min for 10 min, the supernatant solution was filtered through a 0.22 μm filter. After concentrating under reduced pressure distillation, the residue was dissolved in 10 mL acetonitrile. The honey and serum samples were performed in the same conditions but the extraction solution was phosphoric acid aqueous solution (pH=2.0).

The electrochemical determination was performed using an electrochemical workstation (CH Instruments 660C, Shanghai Chenhua Co. Ltd., China) and a standard three-electrode system with MIP/H-GNs modified electrode as the working electrode, a platinum wire as the counter electrode and a saturated calomel electrode (SCE) as the reference electrode. The working electrode was firstly inserted into the extraction solution to selectively adsorb SMX with stirring at 400 r/min for 10 min and then immediately placed in the PBS buffer (0.1 mol/L, pH=7.0) containing 5 mmol/L potassium ferricyanide as a probe to indirectly determine SMX in food and serum samples.

2 RESULTS

2.1 Preparation and Characterization of MIP/H-GNs Composites

DaoudAttieh *et al* reported a horse radish peroxidase (HRP)-mediated preparation of MIPs[11], however, the stability of enzyme is very weak and polymerization can only be performed in an aqueous solution. In this study, the initiation system of MIPs preparation contained H-GNs, anoxidant (H_2O_2), and a polymerization mediator (acetylacetone). Hemin can be used as an electron source based on the reversible Fe^{3+}/Fe^{2+} redox couple, which produces hydroxyl free radicals as short-living, highly reactive intermediates in the presence of H_2O_2[14]. These radicals can react with polymerization mediator to produce acetylacetone radicals, which are capable of initiating the polymerization of a wide variety of acrylic or vinyl monomers. Compared with free hemin, graphene as a support can prevent hemin molecules from self-dimerization to form inactive dimers, and H_2O_2 attack for hemin to decrease oxidative ability[15]. More importantly, the maximum adsorption capacity of SMX on the H-GNs was 3.2 mg/g, which increased the number of imprinted sites per unit surface area, and then improved the selectivity of MIPs composites. The results of AFM indicated that MIPs layer of about 30 nm could be directly synthesized on the surface of H-GNs (fig. 2). This core-shell structure can obviously improve the mass transfer rate of the analytes and avoid the leakage of residual templates, which exhibits the advantages of surface-imprinting technology[16].

2.2 Adsorption Performance of MIP/H-GNs Composites

High adsorption capacity was the main feature of MIP composites, which was investigated by the rebinding experiment using 10 mg of MIPs/H-GNs composites and acetonitrile as a solvent. The results indicated that the adsorption capacities were increased with the increase of initial concentrations (fig. 3) and the binding amount of SMX on the MIPs/H-GNs composites was nearly 15.9 mg/g, which was 4.2 times higher than that on the blank control (NIPs/H-GNs composites), due to the successful construction of highly selective imprinted sites. When the initial concentration was lower than 10 μg/mL, almost all of SMX could be adsorbed on the MIP composites (99.1%), however, the collection efficiency was less than 27.8% of SMX on the NIPs/H-GNs composites. It was shown that the MIP composites could be applied to selectively recognize SMA and remove matrix interference. Then, the Langmuir and Freundlich isotherms were performed to study the interaction between the target molecules and the MIP composites. The experimental data were better fit with the Langmuir model than the Freundlich model, implying the homogeneous binding of SMX on the active sites of the adsorbent. The Q_{max} value was 29.4 mg/g on the MIPs composites, which is much more than those reported in the literatures (table 1)[17, 18]. For the MIPs,

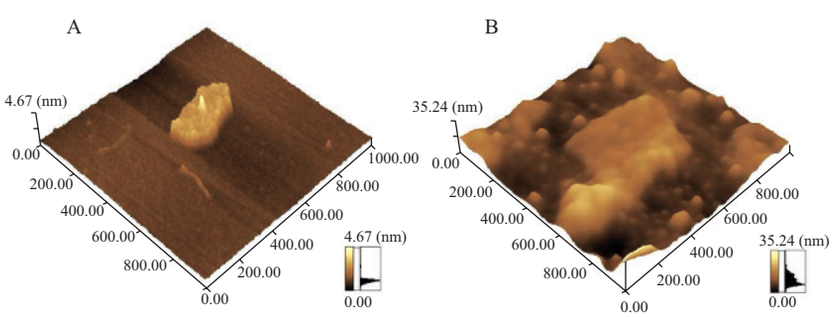

Fig. 2 AFM images of H-GN (A) and MIP/H-GNs composites (B), respectively

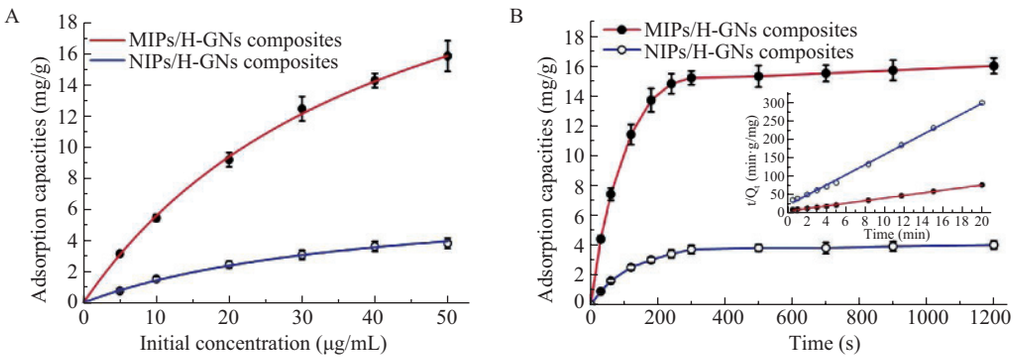

Fig. 3 A: Adsorption isotherms (A) and rebinding kinetic behavior (B) of SMX on the MIP/H-GNs composites and NIP/H-GNs composites, respectively. Inset in B: the pseudo-second-order model for the adsorption of SMX

Table 1 Comparison of different methods using MIPs as the sorbents

MIP composites	Adsorption capacities (mg/g)	Imprinting factor	Detection	Linear range (ng/mL)	LOD (ng/mL)	Ref.
Magnetic carbon nanotubes/MIPs	0.86	10.0	HPLC-UV	50–20000	6.04	[17]
Core-shell magnetic MIPs	0.34	9.5	HPLC-UV	20–20000	14.6	[18]
Molecularly imprinted polydopamine	-	-	ECS	20.24–43010	20.24	[19]
MIPs/boron doped diamondelectrode	-	-	ECS	25.3–25300	6.1	[20]
MIPs/H-GNs	29.4	4.2	ECS	5–1000	1.2	In this study

HPLC-UV: high-performance liguid chromatography-ultraviolet; ECS: electrochemical sensor

both of high adsorption capacity and imprinting factor are very important. The adsorption kinetics test was further used to study the adsorption performance of MIP composites. The results indicated that the SMX adsorption could reach equilibrium at 4 min, because of the surface-imprinting strategy and the large surface area of H-GNs. It was noted that the adsorption kinetics was fitted to the pseudo-second-order model. The adsorption capacity ($Q_{e,calc}$=16.8 mg/g) was the same as the experimental values (16 mg/g) and the correlation coefficients was 0.998.

2.3 Electrochemical Properties of the MIP/H-GNs Composites

Electrochemical properties of bare GC electrode and MIP/H-GNs modified electrodes were studied by the cyclic voltammograms (CV). In general, introduction of MIPs on the surface of electrode will decrease the conductivity and catalytic ability. The results of fig. 4 indicated that pairs of well-defined Fe^{3+}/Fe^{2+} redox peaks were observed and the current of MIP/H-GNs modified electrodes showed a minimal difference compared to the bare electrode. The results could be explained by a greater electroactive surface area and enhanced electrontransfer of H-GNs. Furthermore, direct oxidation and indirect detection using $K_4Fe(CN)_6$ as a probe were both used for the detection of SMX. It was shown that 30 ng/mL SMX could be directly oxidized on MIP/H-GNs modified electrode, however, current responses were found in a linear range of 30–500 ng/mL. For the indirect competitive assay, the imprinted cavities were occupied after rebinding by SMX and the probe reached on the electrode was deceased. We found 5 ng/mL SMX cloud lead to the change of current and it exhibited a wide linear range. Thus, the adsorption time, stirring rate, concentration of probe and electrolyte solution were studied for SMX detection, respectively. The results indicated that adsorption of SMX on the electrode could reach a maximum when the solution was stirred at 400 r/min for 10 min and the optimized electrolyte solution was PBS buffer (0.1 mol/L, pH=7.0) containing 5 mmol/L potassium ferricyanide.

Selectivity is a key parameter for SMX detection in the complex samples. Due to the presence of

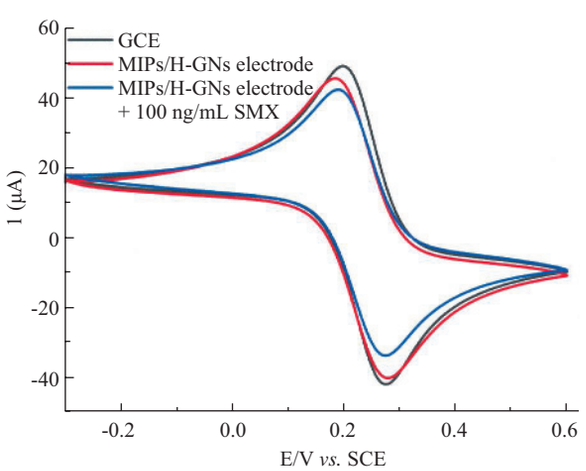

Fig. 4 Cyclic voltammograms of bare GC electrode, MIP/H-GNs modified electrode, and MIP/H-GNs modified electrode incubated with 100 ng/mL SMX at 5 mmol/L $K_4Fe(CN)_6$ in 0.1 mol/L PBS buffer

imprinting cavities, MIP/H-GNs modified electrode exhibited high selectivity for target molecules (fig. 5). When the concentration was 100 ng/mL, the change of current caused by SMX was 3.14 μA and those by its analogues were all below 0.72 μA, which was the same as the current (0.77 μA) caused by 10 ng/mL SMX. It is obvious that the sensor exhibits good selectivity for SMX detection. Furthermore, different types of blank food and serum samples were used to study the anti-interference effect of MIP/H-GNs modified electrode. There was almost no change in current for blank samples (RSD ≤7.4%), implying excellent antifouling properties.

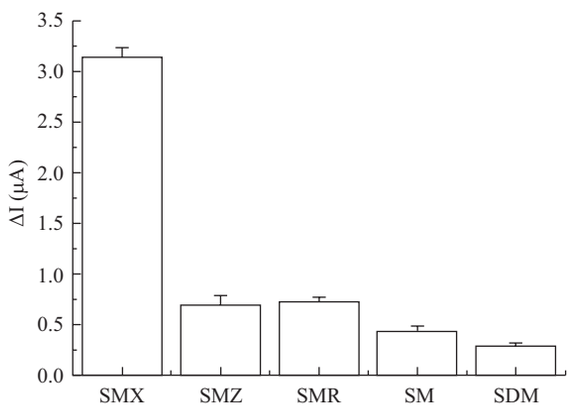

Fig. 5 Responses of differential pulse voltammograms in the presence of SMX, SMZ, SMR, SM and SDM at the MIP/H-GNs modified electrode, respectively. The concentration was 100 ng/mL.

2.4 Detection of SMX in Real Food and Serum Samples

The maximum residue limit assigned by the Commission of the European Communities and Japan was 100 and 25 ng/mL, respectively. However, there is little information about human exposure to SMX through the diet in China. In this study, the indirect competitive assay and the differential pulse voltammetry (DPV) were proposed to determine SMX in real food and serum samples. As showed in fig. 6, DPV responses on the MIP/H-GNs modified

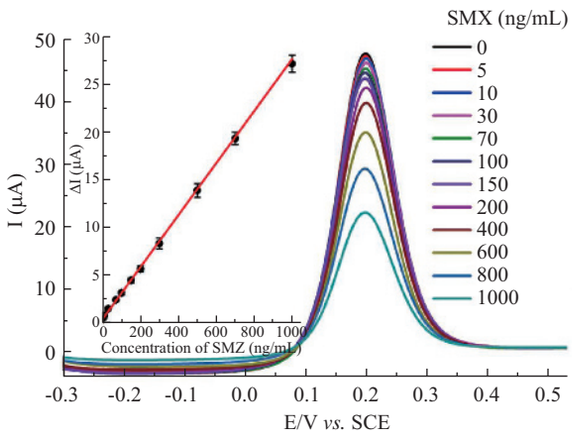

Fig. 6 Differential pulse voltammograms for MIP/H-GNs modified electrode at 0–1000 ng/mL of SMX in PBS buffer (0.1 mol/L, pH=7.0) containing 5 mmol/L $K_4Fe(CN)_6$ as a probe. Insert: peak currents as a function of the SMX concentrations

electrode gradually decrease with the increase of SMX concentration. The results are inconformable with the linear relationship for the concentration range of 5 ng/mL to 1000 ng/mL and the correlation coefficient was 0.994. Thus, this sensor can be used to detect SMX in actual samples. The limit of detection (LOD) for electrochemical determination was calculated to be 1.2 ng/mL (S/N=3), which is lower than other MIP modified electrode[19, 20] (table 1). Yari *et al* reported a silver-filled MWCNT nanocomposite modified electrode and the LOD was 0.01 μmol/L (2.53 ng/mL)[21]. Chen *et al* proposed an ascorbic acid reduced graphene oxide-modified electrode, and the LOD value was 0.04 μmol/L (10.12 ng/mL)[22]. For these nanomaterials modified electrode, matrix interference and antifouling properties were great challenges for the practical application of sensor, because these sensors were only utilized to determine SMX in urine or water samples. Due to the highly selective recognition of MIPs, antifouling properties of electrode were greatly improved, which could be used to determine SMX in complex samples.

Table 2 Recoveries of SMX spiked from food and serum samples

Samples	Spiked level (ng/mL)	Found (ng/mL)	Recovery (%)	RSD (%, *n*=5)
	10	9.8	98.0	4.8
Milk	100	99.6	99.6	6.2
	700	717.5	102.5	5.8
	10	10.4	104.0	5.4
Honey	100	101.4	101.4	6.1
	700	689.5	98.5	4.5
	5	5.1	102.0	3.9
Serum 1	10	9.9	99.0	4.2
	50	51.8	103.6	3.7
	5	5.2	104.0	3.9
Serum 2	10	10.3	103.0	4.5
	50	49.9	102.0	4.1

The reliability and stability of the proposed electrode were further studied by DPV in the buffer solution containing 10 ng/mL SMX. The results indicated that no significant change in DPV response was observed when the electrodes were used in 6 assays every day for one week (RSD ≤6.9%). After storing at a room temperature for a month, the change of current was about 96.2% of the original values, implying the excellent stability.

Under the optimal conditions, this sensor was applied to determine SMX in the complex samples to evaluate its feasibility. We found that the SMX contents in food and serum samples were too low to be detected by this sensor. The results of spiked test indicated that the recovery ranged from 98% to 104%, and the RSD value was no more than 6.2%. Furthermore, all samples were simultaneously determined by both the proposed sensor and the classical LC-MS/MS method. The results of correlation assay indicated that two methods showed a good consistency and the correlation coefficient was 0.998. It is shown that the proposed sensor can be used to determine SMX in the complex samples, which provides an effective technology for the regulation of antibiotic-resistance and human exposure study.

3 DISCUSSION

In this study, H-GNs with peroxidase-like activity were applied to not only initiate the preparation of MIP composites, but also improve the catalytic ability and conductivity of modified electrode. In general, modification with initiator groups is essential for surface-imprinting, but most iniferters are toxic reagents and their synthesized procedure is complex. This study provided an environment-friendly approach to prepare MIPs with core-shell structure. Finally, MIP shell was formed using MAA as the monomer and SMX as the template to selectively recognize SMX. Although the SMX molecule has one primary amine group and one secondary amine group, the pKa value is 5.7 due to the strong acidity of sulfonylamino group. The hydrogen atom in the carboxyl group of MAA could be the proton donor, while the SMX was the proton receptor. Thus, MAA could interact specifically with SMX by a hydrogen bond. It was shown that the adsorption capacity of SMX onto MIP and NIP composites increased with the increase of concentration, and the adsorption ability of MIP was better than that of NIP, because of the formation of imprinting cavities. Subsequently, it was modified on the electrode to determine SMX in the complex samples. Compared with direct oxidation, the sensitivity of the indirect competitive assay was greatly improved and 5 ng/mL SMX could lead to the change of current and it exhibited a wide linear range. There was almost no change in current for the blank samples, implying excellent antifouling properties. Thus, modified electrode could be used for SMX detection avoiding the use of complex cleaning procedure during the measurements. This sensor displayed a high sensitivity (LOD=1.2 ng/mL), excellent recognition selectivity and good reliability. Based on the maximum residue limit values, the proposed sensor can be used for rapid, simple, highly effective and sensitive detection of SMX in the complex samples. It can be used for the regulation of antibiotic-resistance and human exposure study. Bacterial antibiotic resistance has long been a public health concern worldwide. Their uncontrolled applications in the livestock and poultry breeding pose a significant threat to a balanced ecosystem and public health. Analytical methods capable of rapid, easy, convenient, and sensitive screening are necessary for on-site monitoring. For the preparation of MIPs, core-shell surface imprinting materials represent a rather new trend in analytical sciences. Atom transfer radical polymerization as a typical technology can be performed by a biocatalytic system or nanozymes with peroxidase-like activity in the future.

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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