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Aptasensors modified by antimony tin oxide nanoparticles-chitosan based on the interdigitated array microelectrodes for tetracycline detection

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Aptasensors modified by antimony tin oxide nanoparticles-chitosan(nano ATO-CS) based on the interdigitated array microelectrodes (IDAMs) were developed for the detection of tetracycline. The nano ATO-CS film was fabricated onto the microelectrode surface, and then tetrecycline aptamer was modified onto the film to prepare an aptasensor. The results showed that the chitosan can disperse nano ATOs evenly and make them fixed on the microelectrode surface firmly. Nano-ATOs being incorporated into chitosan film can effectively promote electron transfer reaction and enhenced the electrochemical response.. The electrochemical properties of the fabricated processes were characterized by electrochemical impedance spectroscopy (EIS). Parameters affecting the aptasensor response such as pH of the base solution, the concentration of the aptamer and incubation time were optimized. Under optimum conditions, different concentration of tetracyclines was detected with the aptasensor. Based on the contributions of nano ATO-CS solutions, the proposed aptasensor displayed high sensitivity, high specificity, a low detection limit(3.0×10^{-9} g/mL). It could be successfully applied to the detection of tetracyclines in real milk spiked samples.

Introduction

Tetracycline antibiotic, a kind of broad spectrum antibiotics, is not only used for the prevention and treatment of animal diseases in livestock and poultry industry, but also has effect on promoting growth[1]. Unfortunately, it will exhibit high acute toxicity when it is abused, with the majority being hazardous to human body and the environment.

Recently, many analytical methods, such as the microbiological methods [2-4], high performance liquid chromatography [5],gas chromatography [6-8], immunoassays [9-10], have been reported for the detection of tetracycline. However, most of the above-mentioned methods are often tedious, time-consuming and require expensive apparatus. Biosensors can substitute the current analytical methods by simplifying or eliminating sample preparation, especially aptasensors, which has emerged as a promising alternative to detect antibiotics because of their rapid response and high specificity[11].

The characteristics of electrodes have significant effects on the detection of target analyte by impedance measurement. Macroelectrodes used in impedance biosensors do not have enough sensitivity to measure the weak signal produced by the biological reaction. However, by using IDAMs as the electric-signal transducer, the sensitivity of impedance immunosensor can be improved significantly; this results in the development of sensitive, rapid, specific, miniature, and easy-to-operate biosensing devices[15-16]. Particularly, IDAMs offeres promising advantages, such as low ohmic drop, quick establishment of the steady-state condition, and increased signal-to-noise [17] and it has demonstrated particular advantages for applications in the fields of chemical and biochemical processing and environmental monitoring [18-20].

Electrochemical impedance spectroscopy (EIS) is a relatively sensitive technique depending on the frequency of the alternating current employed (typically, from 1Hz to 100 kHz) [21-23]. EIS is superior to other analysis methods mainly because that any intrinsic property of a material or a specific process that could affect the conductivity of an electrochemical system can potentially be studied by EIS.

To enhance the sensitivity of the electrochemical sensors, nanomaterials and conducting polymers have also been widely employed. Antimony tin oxide nanoparticles, namely antimony doped tin oxide, pure SnO_2 is a typical insulator, its resistance value is relatively large and reached to MΩ. Tin oxide crystal is tetrahedral rutile structure, which provides a combination space for biological molecules. After incorporating a certain amount of antimony, the resistance values were significantly decreased by one order of magnitude, which is due to a certain proportion of donor doping of Sb elements, sb^{5+} ion substitued for sn^{4+} ions in the lattice position, formating the center of a monovalent positive charge and an excess of valence electron, Then, the valence electrons will become a conductive electron. The effect is caused by the escape of valence electrons from the

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† Electronic Supplementary Information (ESI) available: Additional Figures and PXRD patterns for all co-crystals. Crystallographic files for **1-4** in cif format. See DOI: 10.1039/x0xx00000x

shackles.Thus the incorporation of Sb results in an increase of the net electron, increasing the carrier concentration and improving the electric conductivity. Thus, nano ATO has good electrical conductivity mainly because Sn^{4+} was replaced by the doped Sb^{5+} and formed defects, and SnO_2 produce double ionized oxygen vacancy defects at high temperatures[24-26]. In this work, we just used this single nanomaterial to reach an excellent sensitive effect, avoided the inconvenience of layer by layer assembly and also improve the sensitivity of the aptasensor. Chiosan, has also been introduced into the modified layers to immobilize the aptamers due to its excellent biocompatible, film forming ability, and a susceptibility to chemical modifications[27-28]. Moreover, chiosan solution also can be used as a good solvent.

In this work, the electrochemical impedance spectroscopy (EIS) technique was applied to study the reaction of the fabricated IDAMs. Moreover, considering the benefits of nano ATO-CS, we integrated them into an aptasensor to exploit the synergy contributions on the improvement of aptasensor characteristics. To the best of our knowledge, such an aptasensor has not been reported. The proposed aptasensor has the advantages of high sensitivity and it solves the problem of fixing aptamers inconveniently to microelectrodes and weak signal. It was developed for the sensitive detection of tetracycline in real milk samples.

Experimental

Reagents and chemicals

$NaH_2PO_4 \cdot 2H_2O$ and $Na_2HPO_4 \cdot 12H_2O$ were purchased from Beijing Chemical Technology Co., Ltd. (Beijing, China). The 0.1 M pH 7.5 phosphate buffer solutions (PBS) were prepared by mixing the stock solutions of $NaH_2PO_4 \cdot 2H_2O$ and $Na_2HPO_4 \cdot 12H_2O$. Antimony tin oxide was obtained from Beijing gold deco island co., LTD.(Beijing,China). $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$ were purchased from Yongda Chemical Reagent Co., Ltd.(Tianjin,China). The aptamer sequences specific for tetracycline were identified by Aniela Wochner et al[29]，DNA oligonucleotides modified with mercapto groups (5'-SH-$(CH_2)_6$-GTC TCT GTG TGC GCC AGA GAA CAC TGG GGC AGA TAT GGG CCA GCA CAG AAT GAG GCC C-3') were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The 5 mM tetracycline was obtained from the Sigma company (USA). All other chemicals were of analytical reagent grade. All the solutions were prepared with ultrapure water which was purified with a Milli-Q purification system (Branstead, USA).

Apparatus

Electrochemical measurements were performed with a CHI660D workstation (China). The solution pH values were measured using an FE 20K Mettler-Toledo pH meter (Switzerland). Ultrasonication was performed using a SK3300H ultrasonic cleaner (Shanghai, China). The solution was blended using a PTR – 35 SPC vortex mixer (Britain). The interdigitated array microelectrode (IDAM) was selected for the operation platform of this work(as shown in Fig.1). All electrochemical experiments were carried out at room temperature (RT).

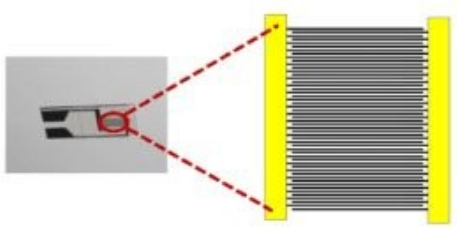


Fig.1 IDAM

Preparation of nano ATO-CS nanocomposites

The nano ATO-CS nanocomposites were prepared as follows: all glassware used in the preparation was thoroughly cleaned in aquaregia(3 parts HCl, 1 part HNO_3), rinsed in triply ultrapure water and oven-dried prior to use. 0.1g of chitosan was added into 50mL of 1.0% acetic acid solution, after stirring until it became translucent with no visible particulate matter. In this work, we found that the chitosan solution can be used as good solvent of antimony tin oxide, thus preparing nano ATO-CS mixture can simplify modification process of microelectrodes. 1.2g antimony tin oxide powder was dispersed into the prepared 4 mL 0.2% chitosan solution and sonicated for 2h until it became stable dispersion. The resulting nanocomposites were used for all the characterizations and experiments.

Preparation of the aptamer

According to the illustrations of the tetracycline aptamer, 14 μL buffer was added into an OD primer and formated into 100 μM storage liquid. The aptamers were easily attached to the wall of tubes, so, it needed to centrifuged(10000 rpm) for 5 minutes before opening the tube. In this work, 140 μL of 0.1 M PBS (pH 7.0) was respectively added into each OD tube and configured to 10μM concentration of tetracycline aptamers. Various concentrations of tetracycline aptamer was obtained by the diluted 10μM of tetracycline aptamer for the follow-up experiments using. The resulting aptamer was preserved in -20℃when not in use.

The fabrication of the aptasensor

Prior to modification, the microelectrode were sequentially soaked in 0.1 M NaOH solution and HCl solution for 10 min, then the electrode surface was wiped respectively with lens wiping paper dipping absolute ethyl alcohol and finally washed with ultrapure water and blowed under nitrogen[30-32]. After being dried at RT, 3 μL of the antimony tin oxide-chiosan nanocomposites was pipetted onto the surface of the microelectrode, the modified microelectrode was dried at RT. Then, 6 μL of the aptamer was assembled on the above-modified IDAM surface. Finally, 3 μL of the prepared tetracycline was added onto the modified electrode surface. After every adsorption step, the modified electrode was thoroughly rinsed with ultrapure water and dried with nitrogen. The stepwise preparation of the aptasensor was shown in Fig.2.

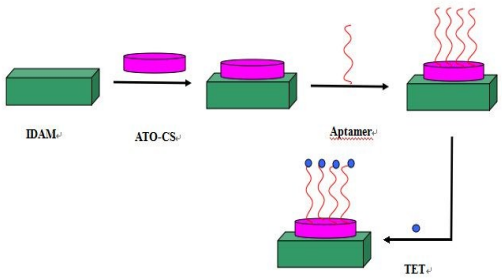


Fig.2 Combination process of aptasensor

The working principle of the aptasensor

In this experiment, the composite of nano ATO-CS is used to improve the electronic conductivity of the electrode surface and accelerate the electron transfer rate. Chitosan as membrane material contains large groups of -NH_2 and -OH which is preferable to maintain the high biological activity of the immobilized biomolecules. The substrates of interdigitated array microelectrodes are gold-plated. The nano ATO-CS can be modified onto the microelectrodes surface by electrostatic adsorption of Au-NH_2 non-covalent bond. Then, dropping tetracycline aptamers sulfhydrylated(-SH) onto the above microelectrodes surface. The aptamers can be effectively immobilized on the microelectrodes surface due to the interaction force molecules between thiol(-SH)and amino(-NH_2) groups of molecules and tetrahedral rutile structure of tin oxide in nano ATO. Finally, in the presence of target tetracycline, specific reactions were conducted and the response compounds were fixed by covalent binding. Further more, the response compound of tetracycline and probe hindered the electron transfer between $[Fe(CN)_6]^{3-}$ and $[Fe(CN)_6]^{4-}$, causing resistance response increased. By establishing the standard curve of the relationship between the change of electrical signal and the different concentration, the concentration of tetracycline in the unknown sample can be inferred.

Electrochemical measurements

Electrochemical measurements were carried out in a conventional electrochemical cell at 37 ℃ . Various steps of electrode surface modification including nano ATO-chitosan, tetracycline aptamer and aptamer combing with tetracycline were characterized by electrochemical impedance spectroscopy. Capture antibiotic target detection objects: in this experiment, specific aptamer was fixed to the electrode surface, the tetracycline aptamer was fixed on the microelectrode surface in this work, and then 3 μL different concentrations of tetracycline was added and incubated for 30 min at room temperature, making the antibiotic target and aptamers immuned and generated response compound. In impedance analysis, corresponding resistance will increase because that reaction of tetracycline aptamer and tetracycline will impede electron transfer of electrode surface, leading to electrochemical signals is abated. Thus, the different concentration of tetracycline can be quantitatively detected by the impedance difference before and after reaction of aptamer and the target. Computation formula is as follows:

$NIC = (Z_{sample} - Z_{control})/Z_{control} \times 100\%$

Z_{sample} represents the impedance value after the aptasensor contacting sample,

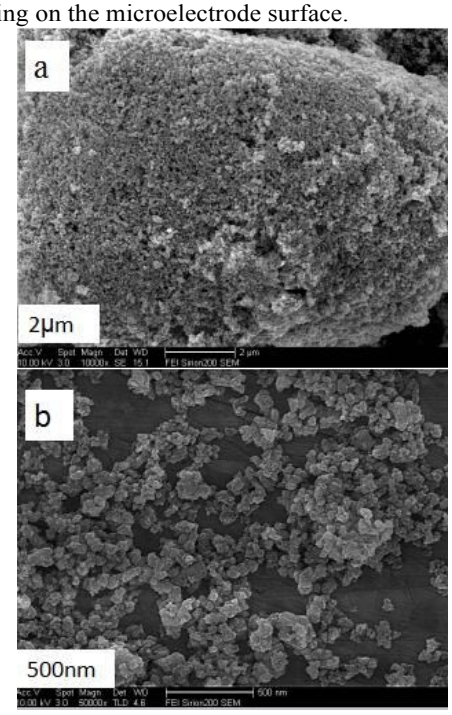
$Z_{control}$ represents the impedance value before the aptasensor contacting sample.

Electrochemical impedance spectroscopy （ EIS ） measurements of the modified microelectrodes were carried out in PBS (pH 7.0) containing 5 mM $K_3[Fe(CN)_6]$ / $K_4[Fe(CN)_6]$ (1:1) and 0.1 M KCl. The frequency was measured from 1 Hz to 100 KHz and the ac voltage amplitude was 55 mV. Record the Nyquist impedance spectrum, the impedance-frequency curve of Bode diagram and phase frequency curve in the process ofreaction. The sensitivity and specificity of the proposed aptasensor were investigated by EIS.

Results and discussion

SEM characterization of modified microelectrodes

The morphologies of the films were investigated by SEM. As seen from Fig.3 a and b, the ATO-CS film was formed on the microelectrode surface. The thin film surface had nanoscale cracks and holes, this phenomenon is due to the increase of membrane thickness and grain growth tend to be more complete and tight. There exists a phenomenon of oxygen vacancy in membrane layer, which improved the conductivity of ATO membrane. The conductive mechanism related as shown in Fig. 4a and b. Moreover, we can see that the density of membrane layer was increased and it has good uniformity. Can be seen from Fig.3 c and d, aptamers(white spots in picture) attached on the microelectrode surface evenly. Furthermore, aptamers and nano ATO-CS have a good connection due to tetrahedral structure of SnO_2. Thus, aptamers can be more stable fixing on the microelectrode surface.



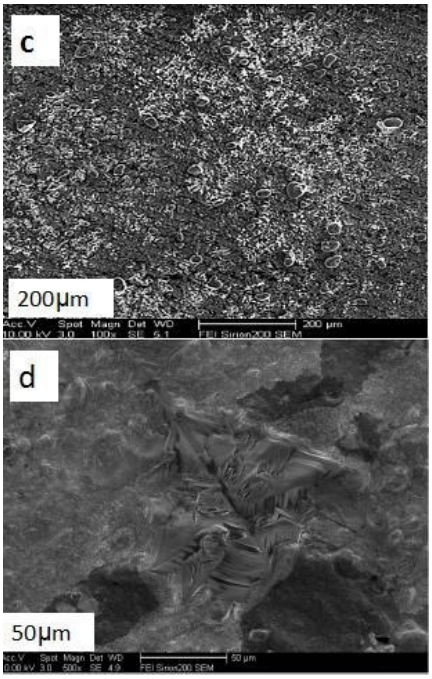


Fig.3 a and b. SEM of ATO-CS/IDAM, c and d. SEM of ATO-CS/Aptamer/IDAM

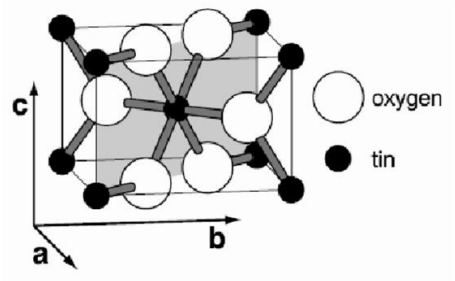


Fig.4a tetrahedral structure of SnO_2

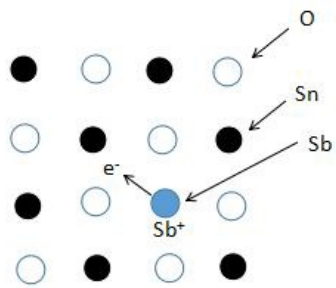


Fig.4b Schematic diagram of Sb doped

Electrochemical behavior of the modified microelectrodes

The stepwise assembly of the aptasensor was characterized by EIS, and the results were shown in Fig.5. As we can be seen, curve a presented a small semicircle domain implying a low electron transfer resistance (R_{et}) about 700 Ω on the bare microelectrode. After nano ATO-CS composites was dropped onto the electrode surface, the R_{et} decreased dramatically to 80 Ω (curve b). The reason maybe that the excellent conductivities of the nano ATO-CS composites, and larger effective surface area. After the tetracycline aptamer was modified onto nano ATO-CS / IDAM, the EIS presented an apparent increase and the R_{et} was about 150 Ω (curve c), which was due to the inhibition effect of the macromolecules for electron transfer. The R_{et} further increased to about 210 Ω when non-conductive tetracycline was covered onto the aptamer/nano ATO-CS/IDAM (curve d).

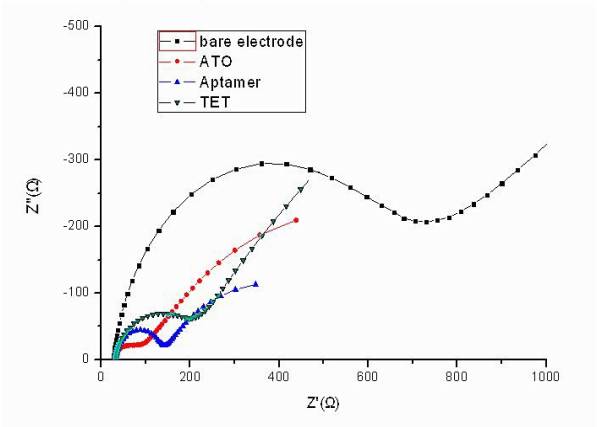


Fig.5 EIS of aptasensor (a) bare electrode (b)nano ATO-CS (c) TET aptamer (d)TET; under testing solution of 5 mM [Fe(CN)6]3-/4- and 0.1 M KCl(pH 7.0 PBS)

Optimization parameters of the biosensor performance

The pH plays an important role in achieving good analytical performance. The response R_{et} of the modified electrodes were investigated in a series of PBS (0.1 M, pH from 6.0 to 8.5) including 0.1M KCl (as shown in Fig.6a). Can be seen from the graph, the aptasensor impedance value increases with the increase of pH value, the maximum value of the response R_{et} was at pH 7.5. The aptasensor impedance showed a trend of decrease when the pH value of base solution was continues to rise, which indicated that the pH of base solution has a great influence on the performance of aptasensor. This is because that the target molecule is a kind of protein and its activity could be greatly reduced in alkaline environment or weak acid environment. Thus, a pH 7.5 of PBS was used in the subsequent experiment.

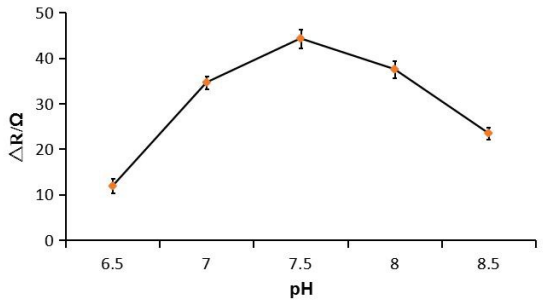


Fig.6a Optimization of pH

The influence of aptamer concentration on the response of aptasensor was also studied (as shown in Fig.6b). The results showed that the peak R_{et} increased gradually as the aptamer concentration increasing and reached the maximal value at 6 μM. After that, the response was almost stable as the

concentration of aptamer further increased, which shows that the aptamer account fixed on the aptasensor has reached saturation point. Therefore, 6 μM of aptamer was chosen as the optimum aptamer concentration for fabrication of the aptasensor.

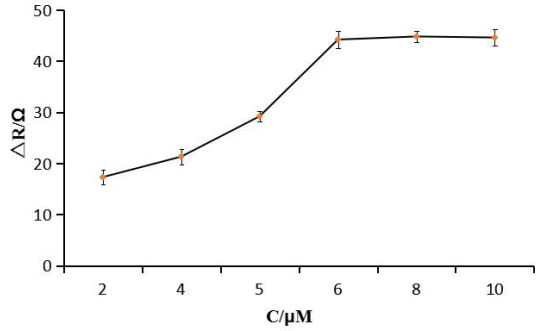


Fig.6b Optimization of aptamer concentration

The effect of incubation time was investigated under the above optimal experimental parameters. The results showed that the peak R_{et} increased greatly with the increase of incubation time (as shown in Fig.6c). When the time was longer than 30 min, the incubation curve trended to a stable value, which indicated that the interaction between tetracycline and tetracycline aptamers have reached equilibrium. Thus, the time of 30 min was selected as the optimal incubation time.

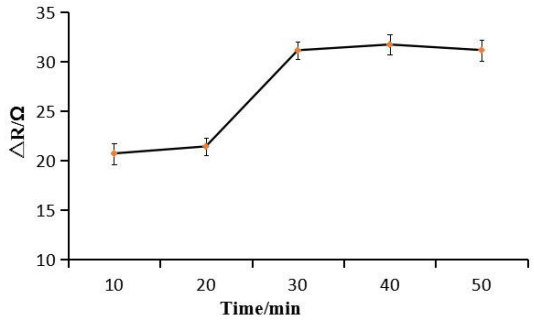


Fig.6c Optimization of incubation time

Calibration curve

Different concentrations of tetracycline were detected by the prepared aptasensor under the above optimal experimental parameters. Impedance change with 10^{-10} to 10^{-3}g/mL of tetracycline concentration was shown in Fig.7. The calibration plots for tetracycline detection with the prepared aptasensor under the optimal experimental parameters were showed in Fig.8. A gradual increase in R_{et} was observed with increasing tetracycline concentration and the corresponding calibration curve exhibited good linearity. The changes of the impendence response of the aptasensor were found to be proportional to tetracycline concentration in the linear range from 10^{-10}-10^{-3}g/mL, with a detection limit of 3.0×10^{-9}g/mL(S/N=3). The linear slope was 12.939 and the correlation coefficients were 0.98643, respectively. Compared with other previously reported methods of detecting tetracycline, the proposed aptasensor exhibited a higher sensitivity with a lower detection limit(Table 1).

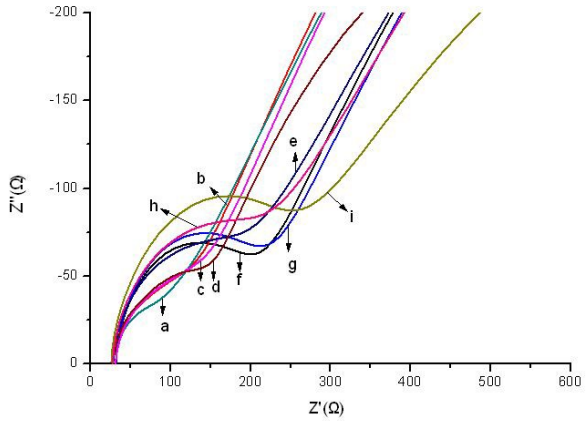


Fig.7 Optimaiton of tetracycline concentration: impedance change with 10^{-10} to10^{-3}g/mL a.aptamer, b.10^{-10}g/mL, c.10^{-9}g/mL, d.10^{-8}g/mL, e.10^{-7}g/mL, f.10^{-6}g/mL, g.10^{-5}g/mL, h.10^{-4}g/mL, i.10^{-3}g/mL

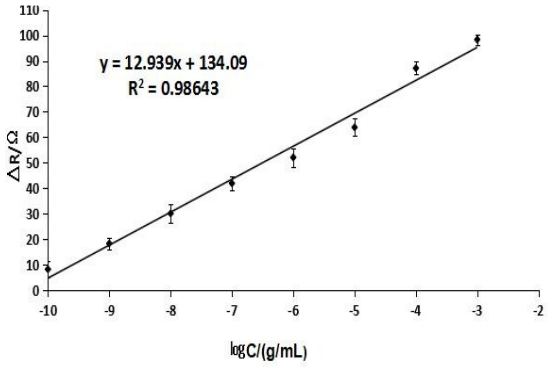


Fig.8 Detection standard curve of aptasensor to tetracycline

Comparison with the aptasensor without nano ATO-CS

In order to verify the addition of nano materials can improve the sensitivity of the aptasensor, we made a comparative test. The assembly process is as follows: prior to modification, the microelectrode were sequentially soaked in 0.1 M NaOH solution and HCl solution for 10 min, then the electrode surface was wiped respectively with lens wiping paper and finally washed with ultrapure water. After being dried at RT, 6 μL of the aptamer was assembled on the IDAM surface by Au-S covalent bond. Then, 3 μL of the prepared tetracycline was added and specific binding was reacted. Different concentrations of tetracycline were detected by the contrapositive aptasensor. When the tetracycline concentration is low (10^{-10}g/mL), it can not be detected by this aptasensor. Impedance change with 10^{-9} to 10^{-3}g/mL of tetracycline concentration was shown in Fig.9. The calibration plots were showed in Fig.10. The sensitivity can be compared by the slope of the linear relationship of the calibration curve, the greater the slope, the higher the sensitivity, which means that the change of the concentration of the detected objects can

cause a higher signal change. As can be seen from Fig.10,the linear slope was 9.9. Thus, the sensitivity of the aptasensor without nano ATO-CS is lower than that of the aptasensor in this work.

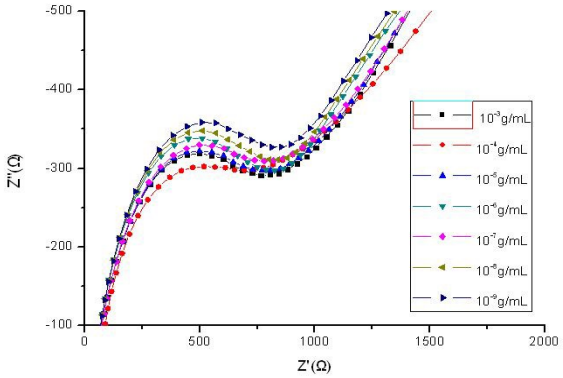


Fig.9 Optimaiton of tetracycline concentration of aptasensor without nano ATO-CS

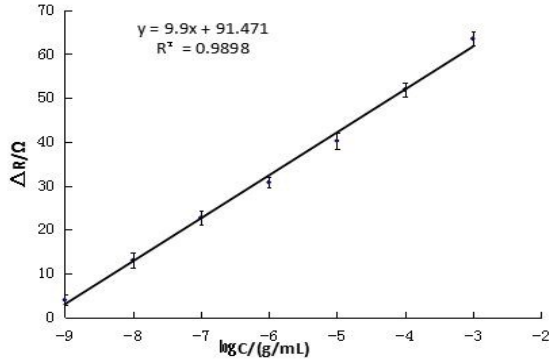


Fig.10 Standard curve of aptasensor without nano ATO-CS

Specificity, repeatability, regeneration and stability of the aptasensor

Specificity is an important property of the aptasensor. The IDAMs-based aptasensor was evaluated for specificity in 0.1 M pH 7.0 PBS containing 5 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M KCl by testing other non-target small molecule antibiotics such as streptomycin, oxytetracycline, kanamycin. The testing concentration was 100 ng/mL. The impedance responses of different antibiotics were obtained (Fig.11). It was observed that the impedance change of other three antibiotics was negligible, indicating that the specificity of the developed aptasensor for tetracycline was good.

To investigate the repeatability of the aptasensor, five aptasensors fabricated independently under the same conditions were examined. The testing tetracycline concentration was 100 ng/mL. Each of the five aptasensors was used for 3 times continuously, and the average value of the obtained impedance difference in five group was analyzed. The inter-and intra-group coefficient of variation were 3.7% and 4.62%, which indicated that the aptasensor had a good repeatability.

In this experiment, glycine-HCl buffer(pH2.8) was used as eluent. After 100ng/mL tetracycline solution was detected using the same microelectrode, making the microelectrode soaked in eluent for 5 min and the tetracycline was dissociated from the aptamers. After being resurrected 5 times, the impedance response of the aptasensor is more than 85% of the original impedance, and the relative standard deviation is only 6.4%, which indicates that the aptasensor has better regeneration performance.

Stability is a key parameter for the application and development of the sensor. we prepared five aptasensors and stored them for 15 days in refrigerator(4℃).Then we used them to detect the 100 ng/mL tetracycline. The impedance response decreased by about 7.2 %, demonstrating that the aptasensor had good stability.

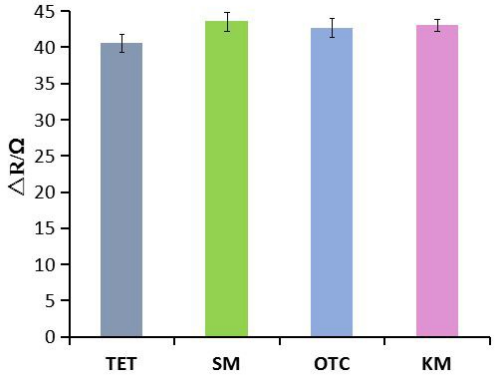


Fig.11 Specificity of the aptasensor

Determination of tetracycline in real samples

Although the proposed sensor showed good selectivity towards tetracycline, it is worth exploring the analytical utility of the sensor for practical application. The milk samples used in this work were all purchased from a supermarket in China. The proportion of the original fat in the milk was 6%. Preprocessing: the milk sample was diluted according to dilution ratio of 1:10, and then centrifuged in 20000 rpm for 90 min, finally, the milk was divided into three layers. Remove the macromolecular material in upper and lower layer, such as fat and casein. Further, tetracycline standard solution was spiked into the diluted milk, making the concentrations of 5×10^{-9} g/mL, 5×10^{-8}g/mL, 5×10^{-7}g/mL, and then experiments were carried out according to the aforementioned optimized conditions for tetracycline detection with the developed aptasensor. The tetracycline concentration recoveries were between 94.9% and 104.2% (Table 2), which clearly indicated that the aptasensor was suitable for the detection of tetracycline in real milk samples.

Conclusions

In this paper, we have developed an ultrasensitive and highly specific electrochemical aptasensor for tetracycline detection based on the nano ATO-CS / aptamer / target configuration. A detection limit down to 3.0×10^{-9} g/mL for tetracycline has been achieved. This could be ascribed to improvement of the conductivity of nano ATOs and the good immobilization ability of CS. The aptasensor possessed high sensitivity, good reproducibility and stability. In addition, the proposed sensor

was successfully applied for tetracycline detection in milk samples. It could be a promising tool for use in food analysis and clinical diagnosis.

Acknowledgements

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Table 1 Comparison with other electrochemistry methods of detecting tetracycline

Method of detection	Limit of detection/M	Linear range/M	Ref
ELISA Multi-detection	3.0×10^{-7}	3×10^{-7}-3×10^{-6}	[33]
Aptasensor	1×10^{-6}	5×10^{-6}-5×10^{-3}	[34]
ELAA	2.5×10^{-9}	1×10^{-9} -1×10^{-4}	[35]
Fluorescence Detection	2.5×10	1.0×10^{-6}-1.0×10^{-5}	[36]
Aptasensor detection	3.0×10^{-9}	1×10^{-10}-1×10^{-3}	this work

Tab.2 Testing results of TET in milk samples

Milk Samples	Blank Detection(M)	Added(M)	Standard Value(Ω)	Detected Value(Ω,av)	Recovery(%)	RSD(%, n=3)
1	Not detected	5×10^{-9}	26.68	25.9	97.08%	4.3%
2	Not detected	5×10^{-8}	39.62	41.3	104.24%	3.6%
3	Not detected	5×10^{-7}	52.56	49.9	94.94%	5.0%