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Short communication

Paper supported immunosensor for detection of antibiotics

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ABSTRACT

Paper supports were used to develop a simple, inexpensive, fast and sensitive electrochemical immunosensor for the analysis of antibiotic residues in milk samples, where single-walled carbon nanotubes (SWNTs) and a simple dip–dry coating method were employed to prepare the highly sensitive biosensor. Well-dispersed SWNTs were impregnated with an antibody against neomycin to obtain a composite coating solution, followed by dipping the filtration paper in the solution to fabricate the sensitive biosensor which had high electrical conductivity. Based on the impedance change in the entire paper supported biosensor with increased concentrations of neomycin, the limit detection of the optimized method was 0.04 ng mL^{-1} and a linear detection range from 0.2 to 125 ng mL^{-1}, well below the European Union regulations for neomycin in this matrix. This paper supported biosensor was applied to determine neomycin in milk samples after a simple sample treatment, with spiked recoveries which ranged from 93.25 to 110.47%. A variety of antibiotic residues in milk samples could be determined following similar sensor preparation.

1. Introduction

Aminoglycoside antibiotics are widely used in veterinary medicine to treat infectious diseases (Riviere and Spoo, 2001; Silva and Carvalho, 2007; Vakulenko and Mobashery, 2003). However, despite of their excellent clinical success, extensive use and misuse may lead to antimicrobial resistance and other adverse health effects such as ototoxicity and nephrotoxicity (Heeschen, 1993; Smith and Baker, 2002). Moreover, neomycin is extremely nephrotoxic compared to other aminoglycosides (Fuentes et al., 1997). In the dairy industry, antimicrobial residues may inhibit starter cultures for cheese and yogurt production (Suhren, 1996). Thus, it is essential to effectively control and analyze aminoglycoside antibiotic residues in milk. The maximum residue limits (MRLs) for antibiotics in milk are laid down in the commission regulation No. 2377/90 within the European Union, as well as similar regulations in other countries (Di Corcia and Nazzari, 2002).

To date, LC and HPLC methods as well as immunoassays have been used in the quantification of aminoglycoside antibiotics, but these methods are time-consuming or expensive, and not suitable for fast on-site analysis (Zawilla et al., 2006; Knecht et al., 2004; Jin et al., 2006; Stead, 2000). Modified-RNA aptamer-based sensors have also been developed for the determination of aminoglycoside antibiotics, however, the sensitivity of this approach is low (De-los-Santos-Alvarez and Lobo-Castanon, 2007).

Electrochemical immunosensors based on specific antigen–antibody recognition have proved to be rapid, sensitive, inexpensive and convenient, and have been widely used in the fields of drug diagnosis, environmental analysis, and veterinary residues in food (Wilson and Hu, 2000). Amperometric immunosensors have been reported to have satisfactory sensitivity, however, the preparation of such sensors involves multiple immobilization steps and high cost (Zhu et al., 2010), which makes the method applied for the detection of aminoglycoside antibiotics a challenging problem.

In this study, we developed a sensitive, rapid, simple, and low-cost electrochemical immunosensor to detect aminoglycoside antibiotics based on a paper supported sensor according to our earlier study (Wang et al., 2009; Shim et al., 2008), which has not been reported until now. The method only required the SWNT–antibody composites dip–dry cycles to coat the filtration paper. Moreover, this method also showed good sensitivity with a detection limit of 0.04 ng mL^{-1} for neomycin and satisfactory recoveries from spiked milk samples. Notably, it can also be used for the analysis of other antibiotics following similar procedures.

2. Experimental

2.1. Reagents and materials

Neomycin, gentamicin and poly(sodium 4-styrenesulfonate) (PSS) were purchased from Sigma Chemical Company (St. Louis,

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MO, USA). Carboxylated SWNTs were obtained from the Chengdu Organic Chemicals Co. Ltd., Chinese Academy of Sciences and used as received. The filtration paper strips used for sensor fabrication were bought from Hangzhou Xinhua Paper Industry Co., Ltd. Rabbit polyclonal antibodies against neomycin were produced in our laboratory through the immunization of a New Zealand rabbit.

All buffer solutions used in this study were prepared in our laboratory. Other chemicals were purchased from standard commercial reagents companies and were of analytical grade purity.

2.2. Apparatus and equipment

All electrochemical measurements were performed using a standard cell with a conventional three-electrode system on a CHI 660 B electrochemical workstation (Chenhua Instrument Company, Shanghai), in which Pt wire and Ag/AgCl were used as counter and reference electrodes, respectively. And the fabricated paper supported sensor strips functioned as working electrode, which were clamped through the electrical connector of the electrochemical workstation. To avoid the interference from the device, the sensor strips were fixed in the same position, at one centimeter of strips in this study. Scanning electron microscopy (SEM) images of the strip sensors before and after immune detection were obtained using a JSM 6380LV scanning electron microscope.

2.3. Fabrication of paper supported immunosensor

Prior to incubation with antibody, carboxylated SWNTs at a concentration of 3 mg/mL, were dispersed in a PSS–water solution by sonication. As a splendid stabilizer of proteins, PSS proved to be very efficient, biocompatible and appropriate for dispersing carbon nanotubes (CNT) when using immunosensors in our previous work (Wang et al., 2009). The antibodies against neomycin, purified according to the saturated ammonium sulfate (SAS) method (Song et al., 2010), were added into the well dispersed CNT solution, and the weight ratio of antibody to SWNTs was approximately 1:2000. After 30 min incubation, the filtration paper strips with dimensions of 4 cm × 0.5 cm were dipped into this coating solution, and then freeze-dried under vacuum to minimize denaturation of the antibody. The composites of SWNTs and proteins were coated on the strips through a simple layer-by-layer method in a cyclical dip–dry procedure.

2.4. Analysis of aminoglycoside antibiotics using the immunosensor

The fabricated paper immunosensor functioned as the working electrode on the three-electrode electrochemical system. Before the measurement of current, it was incubated for 5 min in the buffer solution containing neomycin, which was stirred to prompt the incubation. Then, the current values against time (*i*–*t* curves) were recorded by a chronoamperometry method performed on the electrochemical workstation with an initial potential of 0.5 V (vs. Ag/AgCl). To sense the current change with relevant concentration of neomycin, the transient plateaus of the current on the *i*–*t* curves were used as the signal currents.

Considering that the immune reaction would dramatically influence the sensitivity of the immunosensor, measurements were carried out to optimize the analytical parameters. Optimization of the buffer solutions (0.1 M PBS, 0.1 M Tris–HCl, 0.1 M HEPES and 0.1 M Borate buffer solution), pH values (PBS pH 6.8, 7.4 and 8.0) and reaction temperatures (4, 25, 37 and 45 °C) were performed.

A nontarget analyte (gentamicin as the control) was employed to test the specificity of the immunosensor, following the same procedure as for neomycin.

Cyclic voltammograms were measured for different concentrations of neomycin with the fabricated paper supported sensor as the working electrode. They were performed on the three-electrode electrochemical system with potential from 0.5 V to −0.4 V (vs. Ag/AgCl) and the scan rate of 0.1 v/s in 0.1 M PBS solution containing 5 mM $[Fe(CN)_6]^{3-/4-}$.

2.5. Testing of milk samples

Prior to analysis, 10 mL milk samples obtained from the local supermarket were treated with 1 mL of acetic acid (20 vol% ratio) and shaken for 1 min. The samples were diluted with Milli-Q water (twice), shaken again, incubated at 4 °C for 1 h, centrifuged (5000 × *g* for 20 min) to remove the proteins, and filtered through a 0.22 μm filter membrane before analysis. For the recovery studies, blank milk samples were spiked in triplicate at three different levels (0.5, 5, 50 ng mL^{-1}).

3. Results and discussion

3.1. Verification of the immunosensor detection method and optimization of the analytical parameters

Considering the significance of the development of a quick, simple, inexpensive and sensitive analytical method for the determination of aminoglycoside antibiotics in milk as well as the feasibility of establishing a rapid biosensor based on fibrous materials according to our previous studies (Wang et al., 2009; Shim et al., 2008), we thus prepared an electrochemical paper-sensor for the sensitive determination of neomycin in milk. In this study, the signal sensing of the sensors was attributed to the current change in the biosensors before and after the immune reaction following the addition of different concentrations of analyte. Thus, the factors which influenced the immune reactions would greatly affect the sensitivity and accuracy of the sensors. Therefore, we optimized the analytical parameters to obtain the best signal-to-noise ratio before using the sensor to analyze neomycin.

As shown in Fig. 1, it was clear that the current values decreased with increased concentrations of neomycin, regardless of the type of buffer solution, pH value or reaction temperature. This was likely due to the antibody–antigen complex moving away from the adjacent nanotubes, widening the gap junctions, slackening the charge transfer and increasing the nanotubes contact resistance, resulting in a decrease in current passing through the paper. It is notable that the trend in current change was consistent with that described in our previously reported paper (Wang et al., 2009). It can also be seen from Fig. 1 that the current dropped more rapidly in low concentrations of analyte compared with high concentrations. In particular, the current only dropped a little when the analyte reached the highest concentration. However, this was favorable for obtaining a high sensitivity in the trace detection of neomycin in this study.

The specific optimization results are shown in Fig. 1A–C. Considering that an adequate ionic strength was needed for suitable conductivity and the activity of the antibody, a concentration of 0.1 M was employed for all the buffer solutions to explore the strongest current response prior to sensor application. The results in Fig. 1A show that PBS buffer solution exhibited much higher current than the other three buffers, and the best signal-to-noise ratio among these buffer solutions also indicated the potential to obtain the highest sensitivity. Therefore, it was reasonable to observe that the signal current measured in PBS buffer solution decreased faster than that in the other buffers following the addition of more neomycin. Hence, PBS buffer solution was adopted in subsequent experiments in this study.

Since the pH of buffers not only affects electron transduction in the electrochemical measurement cells, but also impacts on the

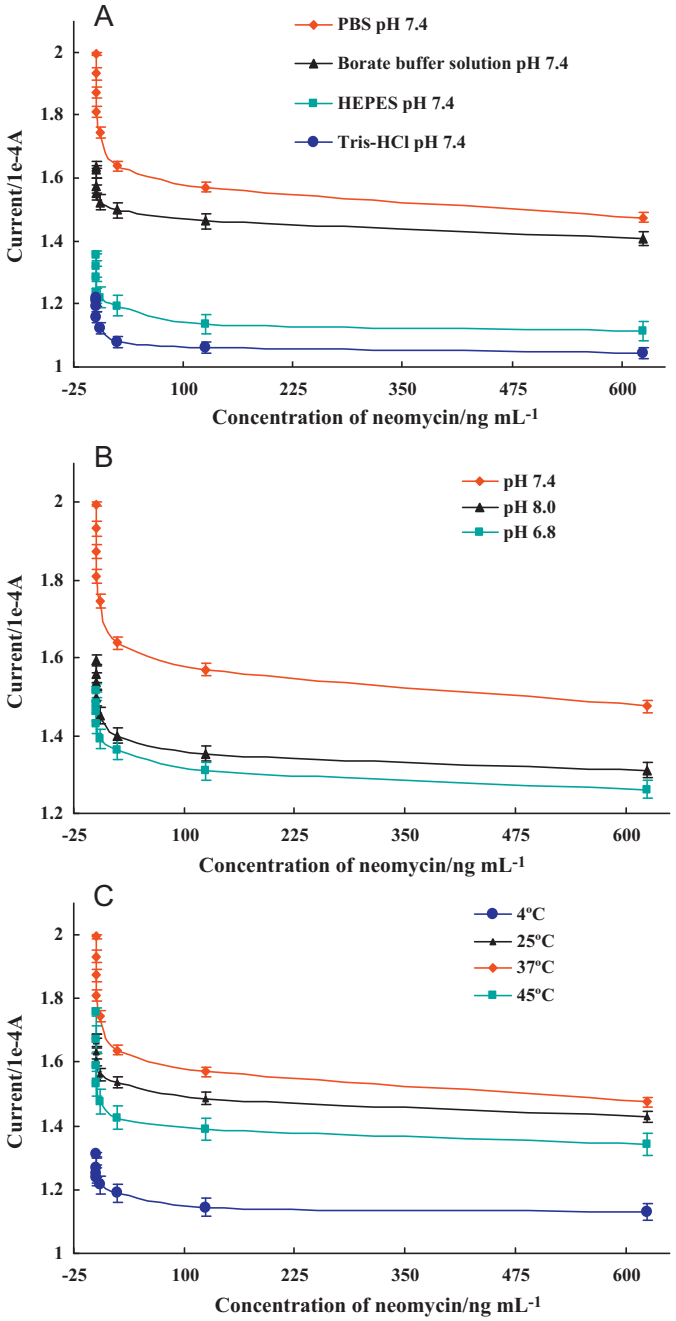

Fig. 1. The conditions optimization of the sensing conditions for neomycin. (A) water buffer solutions; (B) pH for PBS buffer solution; (C) temperature in PBS buffer at pH 7.4.

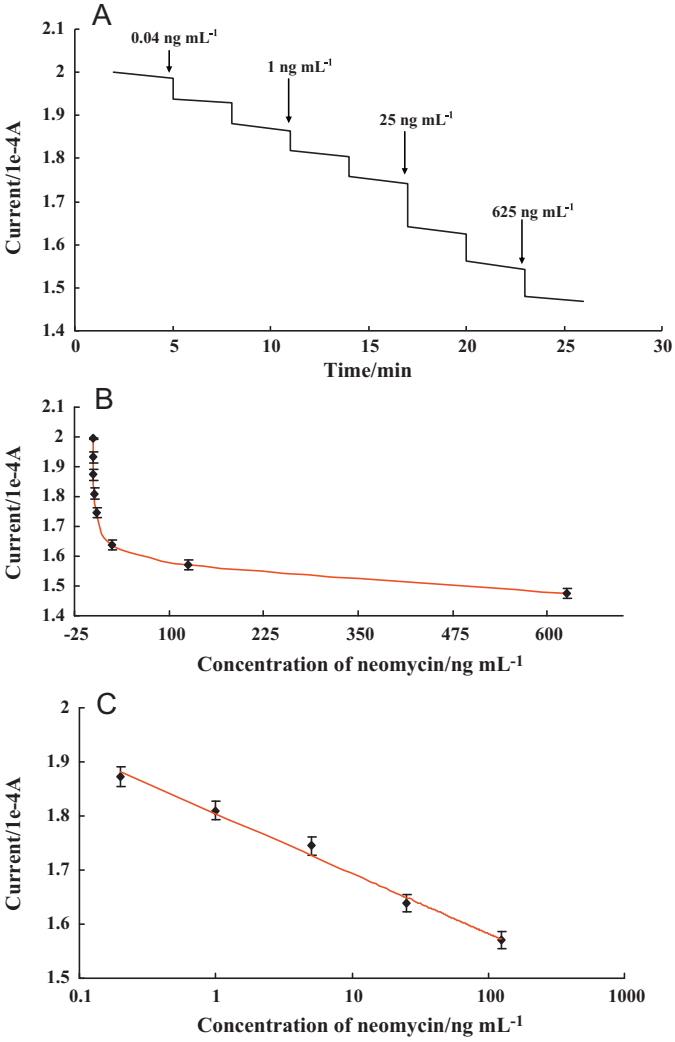

Fig. 2. The sensing results of neomycin (A) and the calibration curve of the determination (B and C). The incubation time was not included in panel A.

immune reaction, it is essential to optimize the pH in electrochemical immunosensor research. In this study, we explored the current under three different pH values (pH 6.8, 7.4 and 8.0). From Fig. 1B we can conclude that the neutral system was much more suitable for measuring neomycin in this study, as pH 7.4 showed maximum responses at all concentration of neomycin when comparing these three pH values.

The optimum temperature was also investigated, and this temperature would mostly likely be that which achieved the highest activity in the antibody–antigen immune reaction. As shown in Fig. 1C, experiments carried out at 37 °C showed the best sensitivity for the analysis of neomycin. Thus, we chose 37 °C and PBS at pH 7.4 for subsequent experiments.

3.2. Sensitivity of the paper immunosensor

In this system, the current signal was responsive to the amount of neomycin hybridized with antibody incorporated in the SWNTs, with which it formed a composite of an immunocomplex integrated with SWNTs. The variations in the paper sensors were also determined by observing the changes in the surface morphology before and after the immune reaction, which were characterized using SEM images (Fig. S1).

Taking into consideration the current transient balance after 180 s, the current values in the region of $t = 0$ to 180 s were used to investigate the current evolution during amperometric measurements, which indicated a satisfactory sensitive analysis (Fig. 2A and Fig. S2).

And the limit of the detection (LOD) was calculated as LOD = 3SD/S, where SD is the standard deviation of the blank noise based on five measurements and S indicates the slope of the calibration curve. Therefore, the limit of detection of neomycin was determined to be 0.04 ng mL^{-1} in this study, which is much lower than the maximum residue limits defined by the food administration authorities. Furthermore, a superb linear calibration curve of neomycin determination was obtained with an equation of $Y = 1.805 - 0.0483X$, and a good correlation coefficient of 0.9907 for the experiments. Specifically, the excellent quantitative results

Table 1
Recoveries of neomycin from spiked milk samples ($n = 3$).

Samples (negative samples from supermarket)	Fortification level (ng mL^{-1})	Mean ± SD (ng mL^{-1})	Recovery (%)	CV (%)
1	0.5	0.47 ± 0.04	93.25 ± 8.96	9.61
2	5	5.52 ± 0.63	110.47 ± 12.67	9.33
3	50	47.76 ± 3.05	95.51 ± 6.10	6.39

were in the concentration range from 0.2 to 125 ng mL^{-1}, which is satisfactory in the determination of neomycin according to the authoritative regulations (Gooding, 2006; Lu et al., 2009; Wan et al., 2011).

The results of chronoamperometry were confirmed by cyclic voltammograms. As shown in Fig. S3, the current decreased upon the increasing concentration of neomycin, which indicated a clear decrease of conductivity of the paper sensor strips upon the increasing concentration of neomycin (in the range of high concentration). While this effect was clearly absent when no analyte was added into the system. As expected, the result of CV data was consistent with that of chronoamperometry discussed above.

3.3. Specificity of the paper immunosensor

In the present research, we also investigated the specificity of the immunosensor. Gentamicin, as the control in this study, belongs to the same group of aminoglycoside antibiotics as neomycin. As shown in Fig. S4-5, Supplemental information, the current variations in the control were negligible. These variations may be attributed to slight fluctuations in the solutions during the measurements, and there was no correlation between the current variations and the concentration of gentamicin, indicating high specificity.

3.4. Analysis of the spiked milk samples

Neomycin, an aminoglycoside antibiotic widely used in veterinary treatments, is a contaminant in milk and milk products. The monitoring of milk is becoming more and more rigorous as more attention is paid to food safety by consumers. Therefore, we analyzed real milk samples (negative confirmed by ELISA) to test the repeatability and accuracy of the strip sensors for practical applications. Three different concentrations of neomycin (0.5, 5, 50 ng mL^{-1}) were spiked into each sample (three parallel tests for each concentration), respectively. From the recovery data shown in Table 1, it can be seen that fortified recoveries ranged from 93.25 to 110.47%, and the coefficient of variation was less than 10%. Notably, even at the low concentration, recovery was also good. These results indicate the high sensitivity and reliability of the sensor in the analysis of neomycin in milk samples.

4. Conclusions

In this study, a simple, cheap, quick and sensitive paper-sensor was developed for the analysis of aminoglycoside antibiotic residues in milk. The sensors showed a low detection limit of 0.04 ng mL^{-1}, a wide linear detection range of 0.2–125 ng mL^{-1} and satisfactory recoveries from spiked milk samples, as well as high specificity. Other advantages of this sensor were low cost and simplicity of preparation, as only filtration paper and the dip–dry cycle method were used making it possible to manufacture large batches for future practical applications. However, further work is needed to validate this assay for the analysis of other antibiotics and for simultaneous multiple detection.

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