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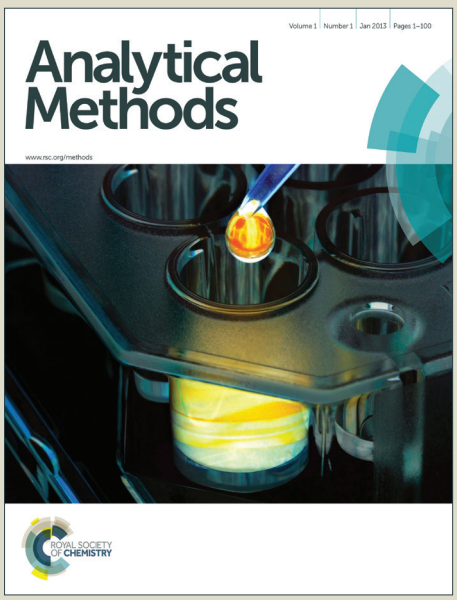

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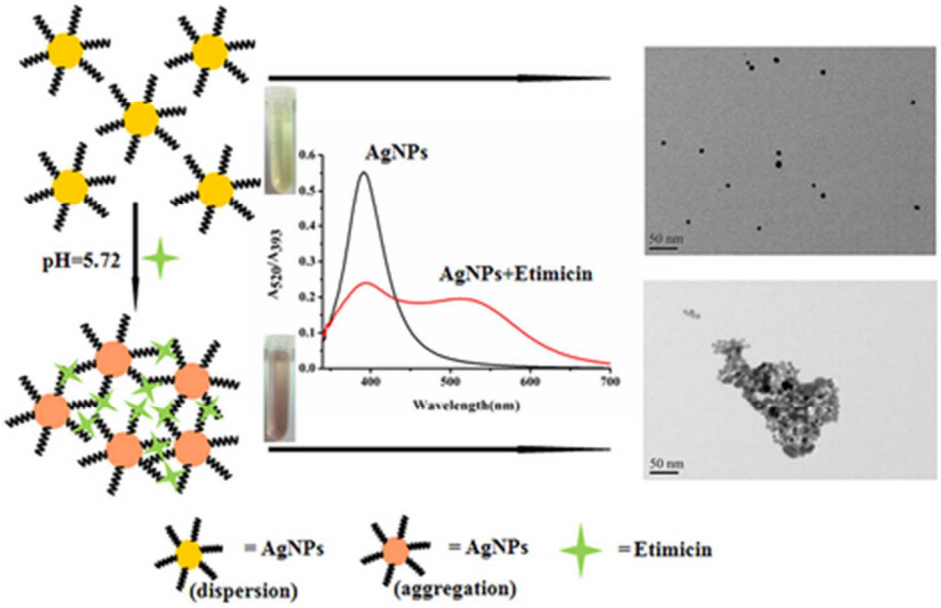

Graphical Abstract
39x25mm (300 x 300 DPI)

Label-free silver nanoparticles for visual colorimetric detection of etimicin

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Abstract

A simple, rapid and ultrasensitive method for visual colorimetric detection of etimicin based on the label-free silver nanoparticles (AgNPs) has been developed in this paper. Etimicin can induce the aggregation of AgNPs owing to the electrostatic attraction and hydrogen-bonding interaction, causing changes in absorption spectra and color of AgNPs suspension. The concentration of etimicin can be determined by a UV–Vis spectrophotometer or even naked eyes. The effect of different factors such as pH, the concentrations of AgNPs, reaction temperature and reaction time were investigated. Under the optimum conditions, this analytical method showed an ultralow detection limit of 3.59×10^{-7}mol/L for etimicin. Furthermore, as low as 4×10^{-7} mol/L etimicin can be visualized by the naked eye without the requirement of any complicated or expensive instruments. The AgNPs sensor also showed good selectivity in the presence of potential interfering substances. The proposed method has been successfully applied to determine the concentration of etimicin in human urine, and may provide new opportunities in the development of sensors for clinical monitoring etimicin in the future.

1.Introduction

Etimicin (ETM) is a semi-synthetic aminoglycoside antibiotic prepared from gentamicin C_{1a} by introduction of an ethyl group at the 1-N-position. [1] It has a broad spectrum of activity against both Gram-positive and Gram-negative bacteria, including strains which are resistant to other aminoglycosides and similar as netilmicin. [2–4] The oto- and nephro-toxicity of etimicin are lower than those of other aminoglyciside antibiotics and even lower than netilmicin. So it has been widely used in clinical treatment. Nevertheless, etimicin still has a narrow therapeutic range. Therefore, the monitor of etimicin is usually required in clinical application.

Different analytical methods for the determination of etimicin have been described such as evaporative light-scattering detection (ELSD), [5] microbiological assay, [6] electrochemiluminescence (ECL), [7] liquid chromatography with pulsed amperometric detection, [8,9] high-performance liquid chromatography with pre-column derivatization with detection of UV [10,11] and fluorescence. [12,13] A volatile mobile phase is required for ELSD detection and it is low sensitive in detection. [8] Pulsed amperometric detection suffers from some stability problems and some experience is required to obtain a good reproducibility. [8] The reproducibility of ECL should also be improved. Since etimicin does not contain a significant UV absorbing chromophore, most determinations have to derivate etimicin at first. However, pre-column derivatization is cumbersome, time-consuming and gives some problems with quantitation or results in unstable derivatives. Thus, a simple, rapid strategy with good sensitivity and selectivity is needed to overcome these problems for etimicin determination.

Precious metal nanomaterials (e.g. Au and Ag) have gained a great deal of attention in the past decade because of their unique electronic and optical properties. [14] In particular, the surface plasmon resonance (SPR) absorption of metal nanoparticles is extremely sensitive to their size, shape, distances, and the surrounding media. [15] When the nanoparticles approach each other and aggregate, the color of the nanoparticles change due to the shift of the surface plasmon band to longer wavelength ,based on

which metal nanoparticles have recently been used as suitable probes in colorimetric determinations. The major advantage of Au/AgNP-based assays is that the molecular recognition events can be transformed into color changes, which can be observed by the naked eye. Apparently, no sophisticated instruments are required in the detection procedures. However, AgNPs have shown some unique characteristics and advantages over AuNPs to a certain degree since they possess much higher absorption coefficients than AuNPs of the same size, [16,17] which allows sensitive colorimetric detection with minimal material consumption. Additionally, Ag nanomaterials are more cost-effective in their preparation compared to Au nanomaterials. Sensors based on the color change of AgNPs have been applied to determine many substances such as metal ions, [18-21] drugs, [22-24] small molecular, [25-27] proteins [28,29] and chiral compounds. [30,31]

Herein, a etimicin colorimetric sensor was developed based on a rapid color change from yellow to purple when label-free AgNPs was mixed with etimicin. Citrate-capped AgNPs have electronegative charged surface and can be dispersed from each other by the electrostatic repulsion. However, the presence of etimicin would induce the aggregation of AgNPs owing to the electrostatic attraction and hydrogen-bonding interaction, causing color and absorption spectra changes of AgNPs suspension. Etimicin can be directly detected by monitoring the color change , scanning UV−vis spectroscopy, or even with naked eyes. This method has been successfully applied to determine etimicin in human urine. To the best of our knowledge, it was the first attempt to use visual colorimetric method to detect aminoglycosides in human urine, which may provide new opportunities in the development of sensors for guiding clinical monitoring.

2.Experimental

2.1 Reagents

Etimicin and sodium borohydride ($NaBH_4 \geqslant 96.0\%$) was purchased from

Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Silver nitrate ($AgNO_3 \geqslant$ 99.8%) was received from Nanjing Chemical Reagent Co., Ltd. (Nanjing, China). Sodium citrate was obtained from Shanghai Rongrun Chemical Reagent Co., Ltd. (Shanghai, China). All chemicals used were of analytical reagent grade and used as received without further purification. Milli-Q-purified distilled water was used throughout the experiments.

2.2 Apparatus

TEM analysis was performed on a FEI Tecnai G2 F20 transmission electron microscope (TEM). UV–Vis absorption spectra were measured on Shimadzu UV-1800 spectrophotometer (Shimadzu, Japan) equipped with 1.0 cm quartz cells. All pH measurements were handled with a pHS-25 digital pH-meter (Shanghai Wei Ye Instrument Factory, China).

2.3 Preparation of citrate-capped AgNPs

Prior to use, all glassware was soaked in aqua regia (1:3 HNO_3/HCl) and rinsed thoroughly with Milli-Q water and dried in air. AgNPs were synthesized by reducing $AgNO_3$ with sodium borohydride according to the method in the literature.[32] Briefly, 25 mL $AgNO_3$ solution (1.0 mM) was added dropwise into 75 mL $NaBH_4$ solution (2.0 mM) under vigorous stirring. Ten minutes later, 5 mL 1% (w/w) sodium citrate aqueous solution was added to stabilize the colloid. The colloid was stirred for another 20 min and then left for 2 days at 4℃. Finally, AgNPs were washed by Milli-Q water and centrifuged for three times to remove the excess sodium citrate. At last, AgNPs were dispersed in 100 mL water for further investigation. The size of AgNPs was verified through TEM image which showed about 22 nm and the concentration(1.15 nM) was calculated according to Beer's law using a molar extinction coefficient of $\varepsilon = 3.35 \times 10^9\ M^{-1}cm^{-1}$.

2.4 Colorimetric detection of etimicin

In a typical experiment for detecting etimicin, 1 mL appropriate concentration of etimicin, 0.5 mL of Britton–Robinson buffer solution (pH5.72) and 0.4 mL of the prepared cit-AgNPs aqueous solution were sequentially added into a 5.0 mL calibrated test tube. Then, the mixture was diluted to 2.5mL with Milli-Q water and mixed thoroughly to incubate for a certain time. Finally, the reaction solution was transferred into a 1 cm spectrometric cell to record the absorbance. And the concentration of etimicin was quantified based on the absorption ratio(A_{520}/A_{393}) or naked eyes observation. The color change of the sensing system was also recorded by digital camera.

3. Results and discussion

3.1Detection principle

Citrate-capped AgNPs have electronegative charged surface and can be dispersed from each other in the water symmetrically by the electrostatic repulsion exhibiting yellow color for the plasmon resonance absorption.[33] As shown in Fig. 1A, the stable AgNPs (22 nm in diameter) solution had a surface plasma resonance absorption peak at 393 nm (Fig. 1A, a). After adding etimicin into AgNPs solution, it was observed that the AgNPs quickly aggregated in solution. This aggregation was identified by the TEM images (Fig. 1B). As a result of aggregation, the color of AgNPs changed from yellow to wine-red (Fig. 1A) and the absorbance at 393 nm decreased and a new absorption band around 520 nm appeared (Fig. 1A, b). The color and absorption spectra changes of AgNPs suspension can be developed into a simple, rapid and sensitive colorimetric method for the detection of etimicin by naked eyes or UV–vis spectroscopy.

The reasons for the aggregation of AgNPs were also investigated in this paper. As shown in Fig. 2, etimicin molecule contains five amino groups and three hydroxyls. The amino groups of etimicin can make the molecule carry high positive charge at certain pH, which would absorb onto the surface of AgNPs owing to the electrostatic

attraction. Hydroxyls of etimicin can also interact with the undissociated –COOH on the surface of AgNPs through hydrogen-bonding recognition. In short, etimicin has strong affinity towards AgNPs. As a result, the negative charge density on AgNPs surface decreases and the electrostatic stability is broken, which may lead to the aggregation of AgNPs.[34] Since the aggregation is strongly correlated with the remarkable property known as LSPR[35], the distribution state of AgNPs altered by etimicin can be observed by a common UV–Vis spectrophotometer or even naked eyes. Thus etimicin could be sensitively detected by this simple and rapid colorimetric assay.

3.2 Factors influencing of colorimetric detection

The performance of the as-developed etimicin detection is strongly influenced by the experimental conditions such as pH, AgNPs concentration, reaction temperature, reaction time. Therefore, each detection parameter was optimized to establish the optimum analytical conditions for the detection of etimicin, while keeping the other parameters constant.

3.2.1 Effect of pH

The pH of the solution can affect the form of etimicin in aqueous solution, then interfere with the interaction between AgNPs and etimicin. So we investigated the effect of the pH of the solution in the range from 3.29 to 10.38. The pH of the AgNPs solution was adjusted with B-R buffer. From Fig. 3A, we can see the AgNPs solution is stable at pH3-9. It is attributed to that the citric acid is a tribasic acid with three pKa values of 3.13, 4.76 and 6.40, respectively.[36] At pH$<$3, the ionization of the carboxyl groups of citric acid was suppressed which decreased the electrostatic repulsion among AgNPs and induced the aggregation of AgNPs. Citrate-capped AgNPs were unstable in strong basic media, and easily aggregated even without etimicin. To take 5.75×10^{-7} mol/L etimicin for example, the absorption ratio (A_{520}/A_{393}) versus the pH of the solution was obtained, as shown in Fig. 3A. It could be seen that the

absorption ratio (A_{520}/A_{393}) was very low in strong acidic and strong basic media, and the highest absorption ratio (A_{520}/A_{393}) was obtained at pH 5.72. When pH was 5.72, the carboxyl groups of citric acid partly existed in the form of anions, and others didn't dissociate. $-NH_2^+-$, $-NH_3^+$ and -OH on the surface of protonated etimicin could absorb onto AgNPs through electrostatic attraction and hydrogen-bonding interaction, which would partly decrease the surface charges of the AgNPs and induce their aggregation. Thus, pH 5.72 was chosen in the following experiments.

3.2.2 Effect of AgNPs concentration

AgNPs concentration has great effects on the interaction of AgNPs with etimicin. The absorption ratio (A_{520}/A_{393}) of reaction system with a variety volume of AgNPs solutions was given in Fig. 3B. High sensitivity was obtained with low AgNPs concentration. However, the absorption ratio (A_{520}/A_{393}) dropped down steeply when the AgNPs was more than 0.4 ml, which was difficult for accurate etimicin detection with good performance. Thereby, 0.4ml of AgNPs was chosen as the optimum value.

3.2.3 Effect of reaction temperature

The reaction temperature can also affect the electrostatic attraction and hydrogen-bonding interaction between AgNPs and etimicin. As displayed in Fig. 3C, the absorption ratio (A_{520}/A_{393}) was the highest at 18°C. When the temperature was above or below 18°C, the absorption ratio declined. Because the movement of AgNPs and etimicin speeded up with the increase of reaction temperature, which made it difficult to establish the interaction between AgNPs and etimicin. But the reaction slowed down under too low temperature. Therefore, the subsequent experiments were performed at room temperature.

3.2.4 Effect of reaction time

The reaction time between AgNPs and etimicin is a key point that affects the colorimetric assays. The absorption spectra of the reaction mixture were recorded at

different times. As shown in Fig. 3D, the absorption ratio (A_{520}/A_{393}) quickly increased within the initial 30 min, whereas it became weak and slow when the incubation time was more than 30 min. This result indicated that the aggregation of AgNPs almost completed within 30 min. For detective convenience, 30 min was selected as reaction time.

3.3 Colorimetric assay of etimicin

As shown in Fig. 4, a colorimetric sensor is developed for the quantitative determination of etimicin, based on the label-free AgNPs as a probe under the optimum conditions. The picture of AgNPs solution in the presence of different concentrations of etimicin in the range from 3.5×10^{-7} to 6.5×10^{-7} mol/L was shown in Fig. 4A. The color of reaction system got changed as the order of yellow → orange → wine-red → purple with the increase of etimicin concentration. So the naked eye alone could judge the presence of etimicin without the use of any advanced instruments. Furthermore, to quantitatively detect etimicin with the developed method, UV–vis absorption spectra were recorded with different concentrations of etimicin. As shown in Fig. 4B, on the addition of etimicin, the absorption peak at 393nm gradually decreased, and a new peak located around 520 nm increased obviously. The absorption ratio (A_{520}/A_{393}) linearly increases with the increase of etimicin in the range from 3.75×10^{-7} to 5.75×10^{-7} mol/L (Fig. 5). The standard regression equation is $A_{520}/A_{393}=0.491c - 1.762$, $R^2=0.995$. The detection limit is 3.59×10^{-7} mol/L (S/N = 3). The relative standard deviations (RSD) for determination of 4.25×10^{-7} mol/L, 4.75×10^{-7} mol/L and 5.25×10^{-7}mol/L of etimicin was 1.95%, 1.87% and 0.68% (n=6), respectively, demonstrating that the precision of the proposed method was acceptable. In addition, a comparison of the proposed method for the determination of etimicin with some other reported detection methods is represented in Table 1. we can see that the suggested method exhibited higher sensitivity with lower detection limit. Even more important, our assay has distinctive advantages such as simplicity, rapidness and low cost.

3.4 Selectivity for etimicin detection

The selectivity of this method was investigated following the general procedure in the presence of 5.25×10^{-7} mol/L etimicin or some potential interfering substances such as sugars (glucose, lactose), amino acids (Try, Thr, Ser, Ala), common ions (Na^+, K^+, Ca^{2+}, NH_4^+, Cl^-, SO_4^{2-} and PO_4^{3-}) at concentration of 5.25×10^{-5} mol/L. In order to detect etimicin in human urine sample, human urine and urea were also detected. The experimental results were shown in Fig. 6. It was clear that only etimicin showed a remarkable higher absorption ratio (A_{520}/A_{393}) and obvious color change. Above results indicated that 100-fold interfering substances did not interfere with the detection of etimicin, and label-free AgNPs can be applied to detect trace etimicin in human urine sample.

3.5 Analysis of real samples

To further explore the practical application of this colorimetric method, the detection of human urine sample was carried out by standard addition method. At first, the urine sample was diluted 150 times with Milli-Q water to reduce the effect of matrix before detection. No other pretreatment was performed. Then different amounts of known concentrations of etimicin were added into the sample to obtain the demanded concentrations from 0.75×10^{-7} to 5×10^{-7} mol/L. And then operated according to the procedure described in Section 2.4. The results were shown in Fig. S1. The color change can be clearly differentiated when the concentration of etimicin was 1.5×10^{-7} mol/L (Fig. S1), indicating that the proposed method can be used to detect as low as 1.5×10^{-7} mol/L of etimicin in urine sample by naked eye observation. The absorption ratio (A_{520}/A_{393}) exhibited a good linear correlation (R^2=0.995) with etimicin concentration in the range from 1.25×10^{-7} to 4.25×10^{-7} mol/L (Fig. S1), and the detection limit ($3\sigma/S$) was calculated to be 1.06×10^{-7} mol/L. The results of the determination and recovery were shown in Table 2. It displayed that the average recoveries for etimicin ranged from 95% to 105% at three spiked levels. The relative standard deviation (RSD) of three parallel experiments were all below 5%. The high

analytical precision and good recovery indicated that this colorimetric method was reliable and could be widely applied in the biological samples testing.

Conclusion

In summary, a novel AgNPs-based sensor was proposed for the highly sensitive and selective detection of etimicin. Etimicin can induce the aggregation of AgNPs owing to the electrostatic attraction and hydrogen-bonding interaction, resulting in changes in color and absorption spectra of AgNPs suspension. Thus the concentration of etimicin could be monitored by a UV–vis spectrometer or even naked eyes .The proposed method has several substantial superiorities compared with the previously reported methods. First, this method is simple in design, and as low as 4×10^{-7} mol/L etimicin can be visualized by the naked eye without the requirement of any complicated or expensive instruments. Second, this strategy is fast in manipulation, and the detection can be completed within 30 min. Third, the labo-intensive and cumbersome AgNPs modification steps are avoided, which is facile, low-cost and particularly useful for resource-limited conditions. Finally, this approach with high sensitivity and selectivity has been successfully applied for the detection of etimicin in human urine. We hope that this method may be exploited as an effective means of detecting etimicin in clinical monitoring.

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Figure Captions

Fig. 1 UV–vis spectra, photo images (A) and TEM micrographs (B) of AgNPs without etimicin (a) and with 5.25×10^{-7} mol/L etimicin (b). Experimental condition: pH=5.72; V_{AgNPs}=0.4 mL; reaction temperature, room temperature; incubation time, 30 min.

Fig. 2 Molecular structure of etimicin.

Fig. 3 (A) Effect of pH of the solution on the absorption ratio (A_{520}/A_{393}) in the absence (1) and in the presence (2) of 5.75×10^{-7} mol/L etimicin. Experimental condition: V_{AgNPs}=0.4 mL; reaction temperature, room temperature; incubation time, 30 min. (B) Effect of AgNPs concentration on the absorption ratio (A_{520}/A_{393}) in the presence of 5.75×10^{-7} mol/L etimicin. Experimental condition: pH=5.72; reaction temperature, room temperature; incubation time, 30 min. (C) Absorption ratio (A_{520}/A_{393}) of the AgNPs suspension at different reaction temperature in the presence of 5.75×10^{-7} mol/L etimicin. Experimental condition: pH=5.72; V_{AgNPs}=0.4mL ; incubation time, 30 min. (D) Time-dependent absorption ratio (A_{520}/A_{393}) change of the AgNPs suspension upon addition of 5.25×10^{-7} mol/L etimicin. Experimental condition: pH=5.72; V_{AgNPs}=0.4 mL; reaction temperature, room temperature.

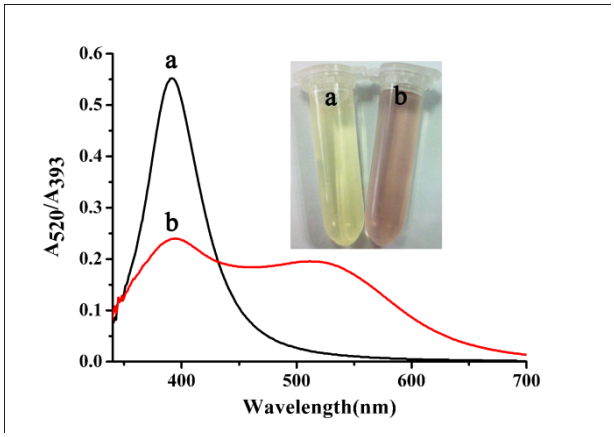
Fig. 4 (A) Photograph showing colorimetric change of AgNPs with the increase of etimicin concentrations from 3.25×10^{-7} to 6.75×10^{-7} mol/L in B–R buffer (pH 5.72). From 1 to 10, they are 3.25×10^{-7}, 3.75×10^{-7}, 4×10^{-7}, 4.25×10^{-7}, 4.5×10^{-7}, 4.75×10^{-7}, 5.25×10^{-7}, 5.75×10^{-7}, 6.25×10^{-7}, 6.75×10^{-7} mol/L, respectively. (B) UV–vis absorption spectra of citrate-capped AgNPs in the presence of different concentrations of etimicin. The arrows indicate the signal changes with the increase of etimicin concentration.

Fig. 5 Plots of the absorption ratio (A_{520}/A_{393}) versus etimicin concentration ranging from 3.25×10^{-7} to 6.75×10^{-7} mol/L. Experimental condition: pH=5.72; V_{AgNPs} = 0.4mL ; reaction temperature, room temperature; incubation time, 30 min.

Fig. 6 Visual color changes and the absorption ratio of the AgNPs solution in the presence of 5.25×10^{-7} mol/L etimicin or 5.25×10^{-5} mol/L other interfering substances. The experiments were performed at pH of 5.72, V_{AgNPs} of 0.4 mL, room temperature, incubation time of 30 min.

Fig. 1

(A)



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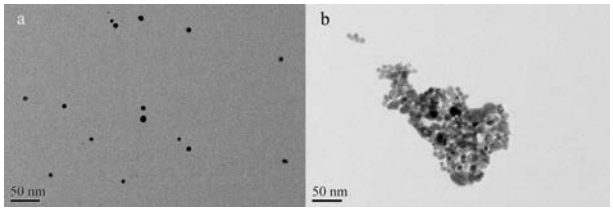

Fig. 2

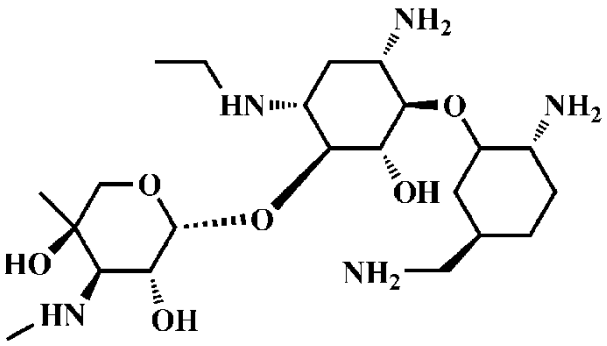

Fig. 3

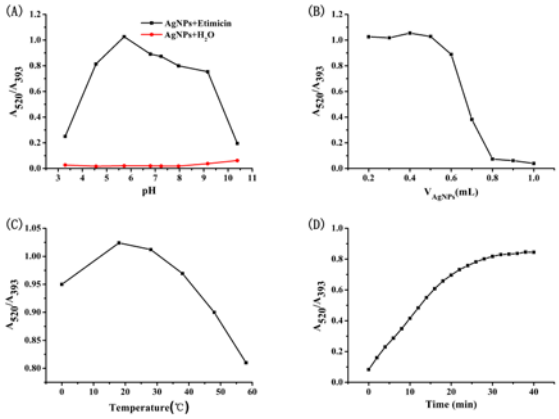

Fig. 4

(A)

(B)

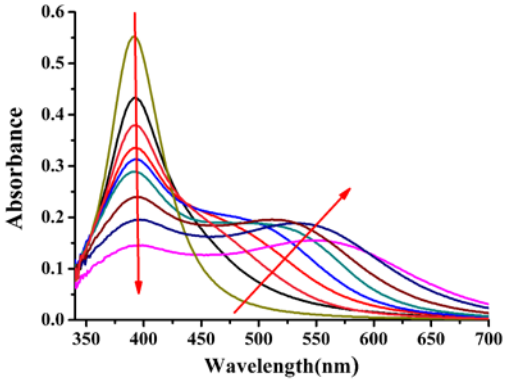

Fig. 5

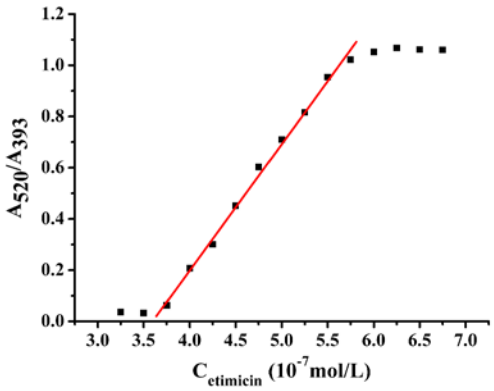

Fig. 6

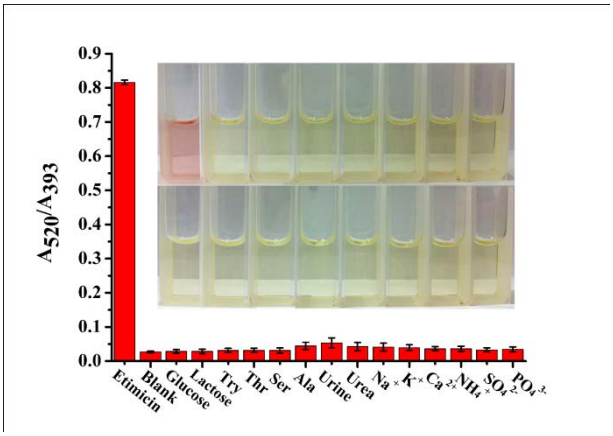

Table 1 Comparison of the proposed method for the determination of etimicin with some other reported detection methods.

Analytical method	Reagents/detection or technique	Sample matrix	Analytical range, (μg/ml)	LOD (ng/mL)	References
-	Microbiological assay	Plasma	0.5-16	-	6
-	Electrochemiluminescence detection	Injections	0.008-0.16	6.7	7
LC	Pulsed amperometric detection	Commercial samples of etimicin	5-125	600	8
RP-HPLC	UV detection	-	100-1000	-	10
HPLC	UV detection	-	40-200	-	11
HPLC	Fluorescence detection	Rat Plasma	0.038-9.69	10	12
-	Fluorescence detection	Injections, urine	1-10	-	13
-	Label-free AgNPs	Urine	0.179-0.247	171.5	This work

Table 2 Determination results of etimicin in urine sample using the proposed method. The experiments were performed at pH of 5.72, V_{AgNPs} of 0.4mL, room temperature; incubation time of 30 min.

Sample	Added amount (10^{-7} mol/L)	Found amount (10^{-7} mol/L)	R.S.D. (%, n=3)	Recovery (%, n=3)
1	2	1.97	4.74	98.5
2	3	3.05	3.03	101.7
3	4	3.86	3.25	96.5