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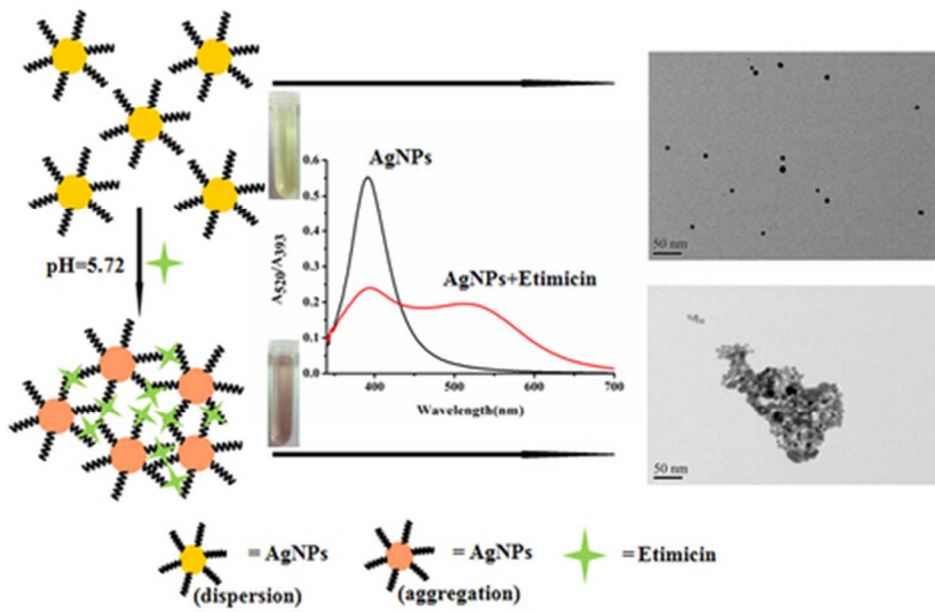


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Graphical Abstract
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4 1 **Label-free silver nanoparticles for visual colorimetric detection of**
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7 2 **etimicin**
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Abstract

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.

51 1.Introduction

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

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

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

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

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

103 **2.Experimental**

104 *2.1 Reagents*

105 Etimicin and sodium borohydride ($\text{NaBH}_4 \geq 96.0\%$) was purchased from

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4 106 Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Silver nitrate ($\text{AgNO}_3 \geq$
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6 107 99.8%) was received from Nanjing Chemical Reagent Co., Ltd. (Nanjing, China).
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8 108 Sodium citrate was obtained from Shanghai Rongrun Chemical Reagent Co., Ltd.
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10 109 (Shanghai, China). All chemicals used were of analytical reagent grade and used as
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12 110 received without further purification. Milli-Q-purified distilled water was used
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14 111 throughout the experiments.

17 112 2.2 Apparatus

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20 113 TEM analysis was performed on a FEI Tecnai G2 F20 transmission electron
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22 114 microscope (TEM). UV–Vis absorption spectra were measured on Shimadzu
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24 115 UV-1800 spectrophotometer (Shimadzu, Japan) equipped with 1.0 cm quartz cells.
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26 116 All pH measurements were handled with a pHs-25 digital pH-meter (Shanghai Wei
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28 117 Ye Instrument Factory, China).

31 118 2.3 Preparation of citrate-capped AgNPs

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35 119 Prior to use, all glassware was soaked in aqua regia (1:3 HNO_3/HCl) and rinsed
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37 120 thoroughly with Milli-Q water and dried in air. AgNPs were synthesized by reducing
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39 121 AgNO_3 with sodium borohydride according to the method in the literature.³² Briefly,
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41 122 25 mL AgNO_3 solution (1.0 mM) was added dropwise into 75 mL NaBH_4 solution
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43 123 (2.0 mM) under vigorous stirring. Ten minutes later, 5 mL 1% (w/w) sodium citrate
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45 124 aqueous solution was added to stabilize the colloid. The colloid was stirred for
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47 125 another 20 min and then left for 2 days at 4°C. Finally, AgNPs were washed by
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49 126 Milli-Q water and centrifuged for three times to remove the excess sodium citrate. At
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51 127 last, AgNPs were dispersed in 100 mL water for further investigation. The size of
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53 128 AgNPs was verified through TEM image which showed about 22 nm and the
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55 129 concentration (1.15 nM) was calculated according to Beer's law using a molar
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57 130 extinction coefficient of $\epsilon = 3.35 \times 10^9 \text{ M}^{-1}\text{cm}^{-1}$.

59 131 2.4 Colorimetric detection of etimicin

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4 132 In a typical experiment for detecting etimicin, 1 mL appropriate concentration of
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6 133 etimicin, 0.5 mL of Britton–Robinson buffer solution (pH5.72) and 0.4 mL of the
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8 134 prepared cit-AgNPs aqueous solution were sequentially added into a 5.0 mL
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10 135 calibrated test tube. Then, the mixture was diluted to 2.5mL with Milli-Q water and
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12 136 mixed thoroughly to incubate for a certain time. Finally, the reaction solution was
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14 137 transferred into a 1 cm spectrometric cell to record the absorbance. And the
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16 138 concentration of etimicin was quantified based on the absorption ratio(A_{520}/A_{393}) or
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18 139 naked eyes observation. The color change of the sensing system was also recorded by
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20 140 digital camera.

21 22 23 141 **3. Results and discussion**

24 25 26 142 *3.1 Detection principle*

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29 143 Citrate-capped AgNPs have electronegative charged surface and can be dispersed
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31 144 from each other in the water symmetrically by the electrostatic repulsion exhibiting
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33 145 yellow color for the plasmon resonance absorption.³³ As shown in Fig. 1A, the stable
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35 146 AgNPs (22 nm in diameter) solution had a surface plasma resonance absorption peak
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37 147 at 393 nm (Fig. 1A, a). After adding etimicin into AgNPs solution, it was observed
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39 148 that the AgNPs quickly aggregated in solution. This aggregation was identified by the
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41 149 TEM images (Fig. 1B). As a result of aggregation, the color of AgNPs changed from
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43 150 yellow to wine-red (Fig. 1A) and the absorbance at 393 nm decreased and a new
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45 151 absorption band around 520 nm appeared (Fig. 1A, b). The color and absorption
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47 152 spectra changes of AgNPs suspension can be developed into a simple, rapid and
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49 153 sensitive colorimetric method for the detection of etimicin by naked eyes or UV–vis
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51 154 spectroscopy.

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55 155 The reasons for the aggregation of AgNPs were also investigated in this paper. As
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57 156 shown in Fig. 2, etimicin molecule contains five amino groups and three hydroxyls.
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59 157 The amino groups of etimicin can make the molecule carry high positive charge at
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158 certain pH, which would absorb onto the surface of AgNPs owing to the electrostatic

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4 159 attraction. Hydroxyls of etimicin can also interact with the undissociated –COOH on
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6 160 the surface of AgNPs through hydrogen-bonding recognition. In short, etimicin has
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8 161 strong affinity towards AgNPs. As a result, the negative charge density on AgNPs
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10 162 surface decreases and the electrostatic stability is broken, which may lead to the
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12 163 aggregation of AgNPs.³⁴ Since the aggregation is strongly correlated with the
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14 164 remarkable property known as LSPR³⁵, the distribution state of AgNPs altered by
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16 165 etimicin can be observed by a common UV–Vis spectrophotometer or even naked
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18 166 eyes. Thus etimicin could be sensitively detected by this simple and rapid colorimetric
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20 167 assay.

21 22 23 168 *3.2 Factors influencing of colorimetric detection*

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26 169 The performance of the as-developed etimicin detection is strongly influenced by
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28 170 the experimental conditions such as pH, AgNPs concentration, reaction temperature,
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30 171 reaction time. Therefore, each detection parameter was optimized to establish the
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32 172 optimum analytical conditions for the detection of etimicin, while keeping the other
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34 173 parameters constant.

35 36 37 174 *3.2.1 Effect of pH*

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41 175 The pH of the solution can affect the form of etimicin in aqueous solution, then
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43 176 interfere with the interaction between AgNPs and etimicin. So we investigated the
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45 177 effect of the pH of the solution in the range from 3.29 to 10.38. The pH of the AgNPs
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47 178 solution was adjusted with B-R buffer. From Fig. 3A, we can see the AgNPs solution
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49 179 is stable at pH3-9. It is attributed to that the citric acid is a tribasic acid with three pKa
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51 180 values of 3.13, 4.76 and 6.40, respectively.³⁶ At pH<3, the ionization of the carboxyl
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53 181 groups of citric acid was suppressed which decreased the electrostatic repulsion
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55 182 among AgNPs and induced the aggregation of AgNPs. Citrate-capped AgNPs were
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57 183 unstable in strong basic media, and easily aggregated even without etimicin. To take
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59 184 5.75×10^{-7} mol/L etimicin for example, the absorption ratio (A_{520}/A_{393}) versus the
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185 pH of the solution was obtained, as shown in Fig. 3A. It could be seen that the

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4 186 absorption ratio (A_{520}/A_{393}) was very low in strong acidic and strong basic media, and
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6 187 the highest absorption ratio (A_{520}/A_{393}) was obtained at pH 5.72. When pH was 5.72,
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8 188 the carboxyl groups of citric acid partly existed in the form of anions, and others
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10 189 didn't dissociate. $-\text{NH}_2^+$, $-\text{NH}_3^+$ and $-\text{OH}$ on the surface of protonated etimicin could
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12 190 absorb onto AgNPs through electrostatic attraction and hydrogen-bonding interaction,
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14 191 which would partly decrease the surface charges of the AgNPs and induce their
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16 192 aggregation. Thus, pH 5.72 was chosen in the following experiments.
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18 19 193 *3.2.2 Effect of AgNPs concentration*

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22 194 AgNPs concentration has great effects on the interaction of AgNPs with etimicin.
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24 195 The absorption ratio (A_{520}/A_{393}) of reaction system with a variety volume of AgNPs
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26 196 solutions was given in Fig. 3B. High sensitivity was obtained with low AgNPs
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28 197 concentration. However, the absorption ratio (A_{520}/A_{393}) dropped down steeply when
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30 198 the AgNPs was more than 0.4 ml, which was difficult for accurate etimicin detection
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32 199 with good performance. Thereby, 0.4ml of AgNPs was chosen as the optimum value.
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34 35 200 *3.2.3 Effect of reaction temperature*

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39 201 The reaction temperature can also affect the electrostatic attraction and
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41 202 hydrogen-bonding interaction between AgNPs and etimicin. As displayed in Fig. 3C,
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43 203 the absorption ratio (A_{520}/A_{393}) was the highest at 18°C. When the temperature was
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45 204 above or below 18°C, the absorption ratio declined. Because the movement of AgNPs
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47 205 and etimicin speeded up with the increase of reaction temperature, which made it
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49 206 difficult to establish the interaction between AgNPs and etimicin. But the reaction
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51 207 slowed down under too low temperature. Therefore, the subsequent experiments were
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53 208 performed at room temperature.
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55 56 209 *3.2.4 Effect of reaction time*

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59 210 The reaction time between AgNPs and etimicin is a key point that affects the
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211 colorimetric assays. The absorption spectra of the reaction mixture were recorded at

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4 212 different times. As shown in Fig. 3D, the absorption ratio (A_{520}/A_{393}) quickly
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6 213 increased within the initial 30 min, whereas it became weak and slow when the
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8 214 incubation time was more than 30 min. This result indicated that the aggregation of
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10 215 AgNPs almost completed within 30 min. For detective convenience, 30 min was
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12 216 selected as reaction time.

13 14 15 217 *3.3 Colorimetric assay of etimicin*

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18 218 As shown in Fig. 4, a colorimetric sensor is developed for the quantitative
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20 219 determination of etimicin, based on the label-free AgNPs as a probe under the
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22 220 optimum conditions. The picture of AgNPs solution in the presence of different
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24 221 concentrations of etimicin in the range from 3.5×10^{-7} to 6.5×10^{-7} mol/L was shown in
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26 222 Fig. 4A. The color of reaction system got changed as the order of yellow \rightarrow orange \rightarrow
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28 223 wine-red \rightarrow purple with the increase of etimicin concentration. So the naked eye
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30 224 alone could judge the presence of etimicin without the use of any advanced
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32 225 instruments. Furthermore, to quantitatively detect etimicin with the developed method,
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34 226 UV-vis absorption spectra were recorded with different concentrations of etimicin.
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36 227 As shown in Fig. 4B, on the addition of etimicin, the absorption peak at 393nm
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38 228 gradually decreased, and a new peak located around 520 nm increased obviously. The
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40 229 absorption ratio (A_{520}/A_{393}) linearly increases with the increase of etimicin in the
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42 230 range from 3.75×10^{-7} to 5.75×10^{-7} mol/L (Fig. 5). The standard regression equation is
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44 231 $A_{520}/A_{393} = 0.491c - 1.762$, $R^2 = 0.995$. The detection limit is 3.59×10^{-7} mol/L ($S/N = 3$).
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46 232 The relative standard deviations (RSD) for determination of 4.25×10^{-7} mol/L,
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48 233 4.75×10^{-7} mol/L and 5.25×10^{-7} mol/L of etimicin was 1.95%, 1.87% and 0.68% ($n=6$),
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50 234 respectively, demonstrating that the precision of the proposed method was acceptable.
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52 235 In addition, a comparison of the proposed method for the determination of etimicin
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54 236 with some other reported detection methods is represented in Table 1. we can see that
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56 237 the suggested method exhibited higher sensitivity with lower detection limit. Even
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58 238 more important, our assay has distinctive advantages such as simplicity, rapidness and
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60 239 low cost.

240 3.4 Selectivity for etimicin detection

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

251 3.5 Analysis of real samples

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

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4 268 analytical precision and good recovery indicated that this colorimetric method was
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6 269 reliable and could be widely applied in the biological samples testing.
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9 270 **Conclusion**

10 271 In summary, a novel AgNPs-based sensor was proposed for the highly sensitive
11 272 and selective detection of etimicin. Etimicin can induce the aggregation of AgNPs
12 273 owing to the electrostatic attraction and hydrogen-bonding interaction, resulting in
13 274 changes in color and absorption spectra of AgNPs suspension. Thus the concentration
14 275 of etimicin could be monitored by a UV–vis spectrometer or even naked eyes .The
15 276 proposed method has several substantial superiorities compared with the previously
16 277 reported methods. First, this method is simple in design, and as low as 4×10^{-7} mol/L
17 278 etimicin can be visualized by the naked eye without the requirement of any
18 279 complicated or expensive instruments. Second, this strategy is fast in manipulation,
19 280 and the detection can be completed within 30 min. Third, the labo-intensive and
20 281 cumbersome AgNPs modification steps are avoided, which is facile, low-cost and
21 282 particularly useful for resource-limited conditions. Finally, this approach with high
22 283 sensitivity and selectivity has been successfully applied for the detection of etimicin
23 284 in human urine. We hope that this method may be exploited as an effective means of
24 285 detecting etimicin in clinical monitoring.
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Figure Captions

382 **Fig. 1** UV–vis spectra, photo images (A) and TEM micrographs (B) of AgNPs
383 without etimicin (a) and with 5.25×10^{-7} mol/L etimicin (b). Experimental condition:
384 pH=5.72; $V_{\text{AgNPs}}=0.4$ mL; reaction temperature, room temperature; incubation time,
385 30 min.

386 **Fig. 2** Molecular structure of etimicin.

387 **Fig. 3** (A) Effect of pH of the solution on the absorption ratio (A_{520}/A_{393}) in the
388 absence (1) and in the presence (2) of 5.75×10^{-7} mol/L etimicin. Experimental
389 condition: $V_{\text{AgNPs}}=0.4$ mL; reaction temperature, room temperature; incubation time,
390 30 min. (B) Effect of AgNPs concentration on the absorption ratio (A_{520}/A_{393}) in the
391 presence of 5.75×10^{-7} mol/L etimicin. Experimental condition: pH=5.72; reaction
392 temperature, room temperature; incubation time, 30 min. (C) Absorption ratio
393 (A_{520}/A_{393}) of the AgNPs suspension at different reaction temperature in the presence
394 of 5.75×10^{-7} mol/L etimicin. Experimental condition: pH=5.72; $V_{\text{AgNPs}}=0.4$ mL ;
395 incubation time, 30 min. (D) Time-dependent absorption ratio (A_{520}/A_{393}) change of
396 the AgNPs suspension upon addition of 5.25×10^{-7} mol/L etimicin. Experimental
397 condition: pH=5.72; $V_{\text{AgNPs}}=0.4$ mL; reaction temperature, room temperature.

398 **Fig. 4** (A) Photograph showing colorimetric change of AgNPs with the increase of
399 etimicin concentrations from 3.25×10^{-7} to 6.75×10^{-7} mol/L in B–R buffer (pH 5.72).
400 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} ,
401 5.25×10^{-7} , 5.75×10^{-7} , 6.25×10^{-7} , 6.75×10^{-7} mol/L, respectively. (B) UV–vis
402 absorption spectra of citrate-capped AgNPs in the presence of different concentrations
403 of etimicin. The arrows indicate the signal changes with the increase of etimicin
404 concentration.

405 **Fig. 5** Plots of the absorption ratio (A_{520}/A_{393}) versus etimicin concentration ranging
406 from 3.25×10^{-7} to 6.75×10^{-7} mol/L. Experimental condition: pH=5.72; $V_{\text{AgNPs}} =$
407 0.4 mL ; reaction temperature, room temperature; incubation time, 30 min.

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

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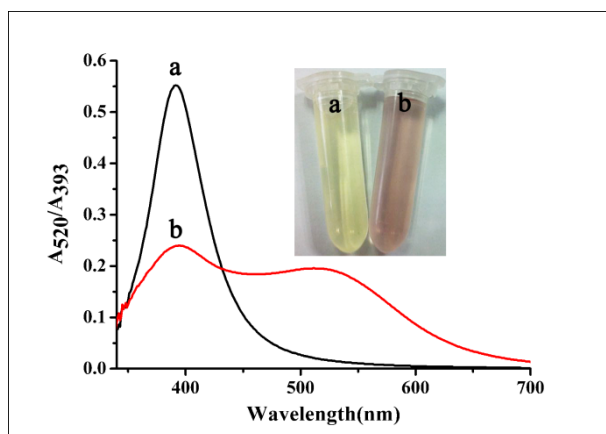
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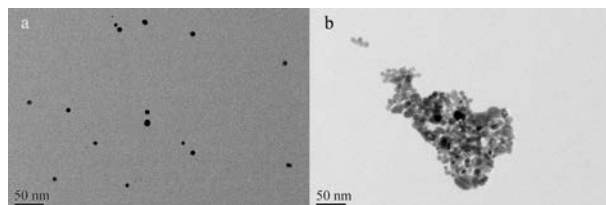
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Fig. 1

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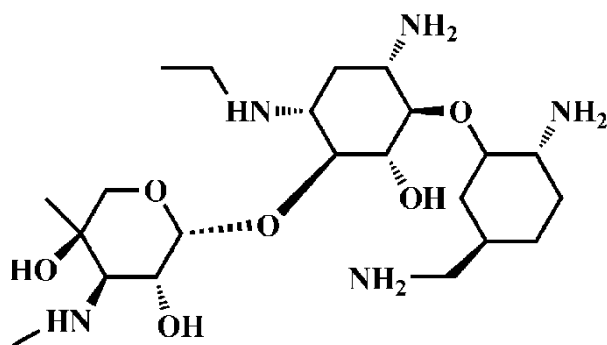
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Fig. 2



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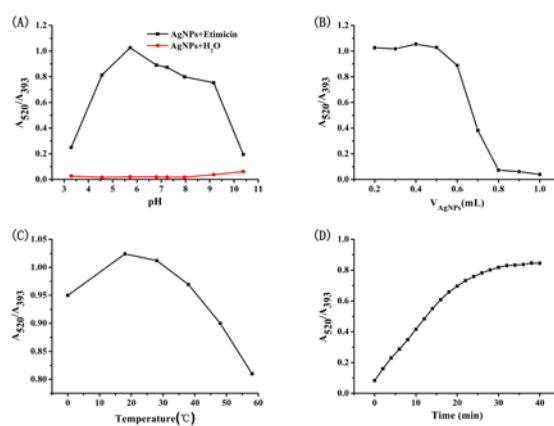
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Fig. 3



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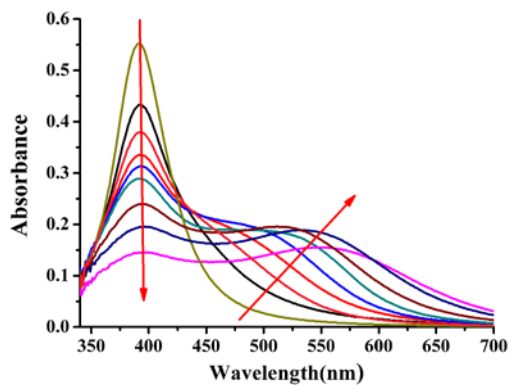
Fig. 4

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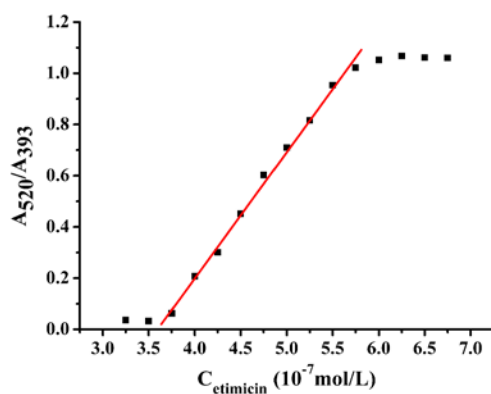
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Fig. 5



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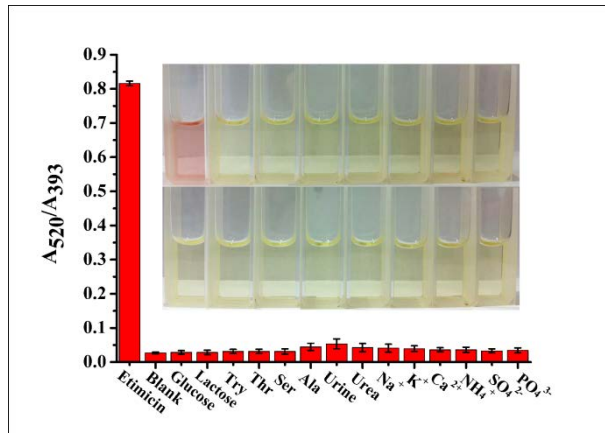
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Fig. 6



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614 **Table 1** Comparison of the proposed method for the determination of etimicin with
 615 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

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4 647 **Table 2** Determination results of etimicin in urine sample using the proposed method.
5 648 The experiments were performed at pH of 5.72, V_{AgNPs} of 0.4mL, room temperature;
6 649 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

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