Detection of Chlortetracycline Hydrochloride in Milk with Solid SERS Substrate Based on Self-assembled Gold Nanobipyramids

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Abstract

In this paper, a high-yield, monodisperse Au nanobipyramids (Au NBs) sol was prepared by seed-mediated method, and gold nanoparticles were assembled on the surface of the silicon wafer by self-assembly technology to obtain a solid SERS substrate. Scanning electron microscopy (SEM) showed that the average length of Au NBs was 34.31 nm, and the analysis enhancement factor (AEF) was approximately $7.3 \times 10^5$ with rhodamine 6G (R6G) as a probe. SERS detection of chlortetracycline hydrochloride (CCH) in milk was performed utilizing the prepared Au NBs substrate, and the limit of detection was 0.01 mg/mL. In the range of 0.01~1 mg/mL, the mass concentration of CCH and SERS signal intensity satisfied the linear relationship of $y=258.467x+150.501$ and the value of correlation coefficient was 0.9785. In addition, the recovery of spiked samples fluctuated between 96.80% to 111.38%. These results proved that the method is simple and fast, and it is promising to be applied to the field detection of antibiotics in milk.

Key words: Surface enhanced Raman spectroscopy; Au nanobipyramids; self-assembly; chlortetracycline hydrochloride; milk
**Introduction**

Chlortetracycline hydrochloride (CCH) is a tetracycline broad-spectrum antibiotic that inhibits a variety of pathogenic bacteria and is widely used as a veterinary drug in animal husbandry. Non-standard use of antibiotics or negligence of supervision can lead to neutral antibiotic residues in animal-derived foods and dairy products with excessive antibiotics can cause damage to human bodies. Therefore, countries around the world have strict testing standards for antibiotic residues in milk. At present, the commonly used detection methods for antibiotic residues in animal products are liquid chromatography-tandem mass spectrometry (LC-MS/MS), high performance liquid chromatography equipped with UV detector (HPLC-UV), gas chromatography tandem mass spectrometry (GC-MS/MS). Although the above methods have accurate detection results, these both require complicated instrument operation processes, cumbersome sample processing and data analysis processes. Therefore, it is of great significance to provide a rapid and simple method for detecting CCH in milk.

Surface Enhanced Raman Scattering (SERS) is a highly sensitive vibrational spectroscopy technique for low-concentration analyte detection. Compared with Raman spectroscopy, highly sensitive analysis down to the single molecule can be achieved with SERS. The SERS effect is due to noble metal (gold or silver) nanoparticles as SERS substrate generate local surface plasmons by electromagnetic wave excitation, thereby amplifying the electric field near the particle surface and increasing the intensity of the SERS signal. In addition, gold nanoparticles also possess chemical inertness and biocompatibility, which are widely used in the field of SERS detection. The morphology of Au nanoparticles is mainly composed of Au nanospheres (Au NSs), Au nanorods (Au NRs), and Au nanobipyramids (Au NBs). Among them, Au NBs consist of two cones connected at the bottom, which can tune the plasmon resonance wavelength over a large range by controlling the size. And the sharp tips of Au NBs can provide local enhanced electric fields several times higher than Au NSs and Au NRs. However, the yield of Au NBs is poor during the preparation process. Previously, it was necessary to obtain monodisperse Au NBs sols through a purification process. However, subsequently it was found that ripening the seeds can effectively improve the yield of Au NBs. In recent years, in order to enhance the SERS performance of substrates, researchers have used
self-assembly technology to adsorb noble metal nanoparticles on the surface of solid materials and improved the stability of the substrate by creating "hot spots" between the nanoparticles. Various morphologies of Au NPs have been used as assembly units to prepare solid SERS substrates.

In this study, a high-yield, monodisperse Au NBs was prepared by seed-mediated method. Then a SERS substrate was constructed on the silicon wafer with Au NBs particles used as assembly units by surface replacement and interfacial self-assembly techniques. The signal enhancement effect of the SERS substrate was studied by using rhodamine 6G (R6G) as a probe molecule. The results showed that the substrate coated with Au NBs had a good SERS enhancement effect. Finally, the substrate was used to rapidly detect CCH in milk. Compared with conventional large-scale instrument-based detection methods, this method is simple and rapid, and is suitable for rapid detection of antibiotics in milk.

**Experimental**

**Reagents and Instrumental**

Sodium borohydride (NaBH₄), cetyltrimethylammonium bromide (CTAB) and polyvinyl pyrrolidone (PVP) were obtained from Sigma-Aldrich Co., Ltd., and ascorbic acid (AA) was obtained from Acros Organics Co., Ltd. Cetyltrimethylammonium chloride (CTAC, 97%) and rhodamine 6G (C₂₈H₃₁ClN₂O₃) was obtained from TCI Shanghai Chemical Industry Development Co., Ltd. Chlortetracycline hydrochloride (C₂₂H₂₃Cl₄N₂O₈·HCl), tetrachloroauric (III) acid tetrahydrate (HAuCl₄·4H₂O), trisodium citrate (C₆H₃Na₃O₇), silver nitrate (AgNO₃), hydrochloric acid (HCl), n-hexane and chloroform were obtained from Sinopharm Chemical Reagent Co., Ltd. Ethanol (AR) was purchased from Longxi Chemical Industry Co. Ltd., and milk was obtained from a local supermarket. Milli Q water (>18.0 MΩ cm) was purified with a Sartorius Arium 611 UV ultrapure water system. All chemicals were used without further purification.

Raman spectra and SERS spectra were recorded by a portable Raman Spectrometer BWS415-785H (B&W Tek, Inc.) with Raman spectrum over the range of 175 to 3200 cm⁻¹.
The excitation source of Raman Spectrometer was 785 nm laser. The typical laser power is 150 mW and the integral time is 5 s unless otherwise stated.

**Synthesis of Au NBs**

Au NBs were synthesized using the seed-mediated growth method\(^9\). Briefly, 0.25 mL of NaBH\(_4\) (25 mmol/mL) prepared with ice water rapidly injected into a 10 mL of CTAC (50 mmol/mL) aqueous solution containing HAuCl\(_4\) (0.25 mmol/mL), citric acid (5 mmol/mL) under vigorous stirring at room temperature. Vigorously stirred for 2 minutes, the seed solution was heated in an 80 °C oil bath for 7 hours under gentle stirring, and finally, the seed solution was stored at room temperature.

Growth solution prepared by successively mixing 40 ml of CTAB (100 mmol/mL) and 0.4 ml of H\(_2\)AuCl\(_4\) (50 mmol/mL), 10 ml of AgNO\(_3\) (10 mmol/mL), 0.8 ml of HCl (1 mol/mL), and 0.32 ml of AA (100 mmol/mL). Au NBs were prepared by injecting 0.5 ml of a seed solution to growth solution at room temperature and gently stirring for 2 hours. Because CTAB solution crystallizes at room temperature, the Au NBs solution is centrifuged at 9000 rpm for 15 min and precipitate was redispersed in 40 ml of water for further use.

The Au NBs particles were assembled on the surface of the silicon wafer by the method of Lin\(^20\) et al. The preparation process was shown in Fig. S1 (Supporting Information). 10 mL of Au NBs solution was centrifuged and re-dispersed in 10 ml of 1% PVP in ethanol, and centrifuged again and redisposed in 1 ml of ethanol solution. Then, 400 µl of the treated Au NBs were added to a 5 ml centrifuge tube containing a certain amount of water and n-hexane, and a dense monolayer was formed by shaking. At last, the gold film was assembled into the silicon wafer by the Langmuir-Schaefer method\(^21\).

**Sample preparation and detection**

The spiked milk sample was obtained by dissolving the solid powder of CCH in milk. First, prepare 1 mg/mL (1.94 mmol/L) CCH spiked milk sample, and then the milk samples were then diluted in milk to a gradient mass concentration of 0.75, 0.5, 0.25, and 0.01 mg/mL (the corresponding molarity is 1.46, 0.7, 0.49, and 0.02 mmol/L), respectively. The blank sample
was milk without CHH. The CCH solid powder was dissolved in water to prepare a 5 mg/mL standard aqueous solution of CCH, the ultraviolet-visible (UV-Vis) absorption spectrum of the CCH solution shown in Fig. S2 (Supporting Information).

The spiked milk was pretreated with trichloroacetic acid to precipitate the protein, and after centrifugation and filtration, the filtrate was collected for SERS detection. First, take 5 mL of different concentrations of milk samples, and then add 0.5 mL of 15% trichloroacetic acid. After thorough mixing, the mixtures were centrifuged at 1000 rpm for 10 minutes, and the supernatant was filtered through a 0.22 μm filter membrane, and finally, the filtrate was collected for testing.

The filtrate was dropped on the solid SERS substrate for spectroscopic detection. In detail, 10 μL of the sample was dropped on the surface of the silicon wafer coated with Au NBs, and waiting 30 seconds to make the droplets to be measured fully contacted with the Au NBs. After that, the SERS detection was performed. For comparison, CCH solution was also detected using unmodified silicon wafer, the measured spectrum was shown in Fig. S3 (Supporting Information). Unless otherwise specified, the laser power was 150 mW and the integration time was 5 seconds. Spectral data acquisition and related processing employed the software Bwram 1.01.20 that comes with the spectrometer. A schematic diagram of the SERS detection process is shown in Fig. 1.

Results and discussion

Characterization of the Au NBs

Au NBs were characterized by scanning electron microscopy, and the result was shown in Fig. 2(a). The Au NBs possessed a biconical shape and a uniform size distribution. The morphology of the sol nanoparticles prepared by the seed-mediated method distributed more uniformly and have the ability to regulate the size of the nanoparticles, compared with the Au NBs obtained by the conventional one-step method. The size distribution of Au NBs were shown in Fig. 2(a). The length of Au NBs was between 26 and 40 nm, and the average was 34.31 nm.
It can be seen from the ultraviolet-visible (UV-Vis) absorption spectrum of the gold nano sol shown in Fig. 2(b) that the sol had a visible absorption peak at 683 nm, which is attributed to the plasma single-mode vibration of the Au NBs. The UV absorption peak of the gold or silver sol prepared by the conventional one-step method is concentrated between 400 and 550 nm\textsuperscript{22,23}, while the UV absorption peak of Au NBs is located at 683 nm, making the UV absorption peak is red-shifted and more closely matches the excitation source of 785 nm compared to the spherical sol substrate. The reflection spectrum of the solid SERS substrate was shown in Fig. S4 (Supporting Information).

Enhanced performance and repeatability of the SERS substrate

The analysis enhancement factor (AEF) of the substrate for SERS detection was calculated for characterizing the Raman signal enhancement effect of the substrate decorated with Au NBs. The R6G solution (10\textsuperscript{-7} mol/mL) was added dropwise on the silicon wafer substrate, and then the SERS spectrum was detected. Combined with the normal Raman spectrum of R6G solution (10\textsuperscript{-1} mol/mL) dropped on the surface of a blank silicon wafer, the AEF of the substrate was calculated with a characteristic peak at 1361 cm\textsuperscript{-1}. The formula for AEF of the substrate is as follows\textsuperscript{24,25}:

\[ AEF = \frac{I_{\text{SERS}}}{I_{\text{normal}}} \times \frac{C_{\text{normal}}}{C_{\text{SERS}}} \]  

(1)

Among them, \(I_{\text{SERS}}\) and \(I_{\text{normal}}\) are the Raman characteristic peak intensities of R6G molecules at 1361 cm\textsuperscript{-1} adsorbed on the substrate surface and the blank silicon wafer surface, respectively, as shown in Fig. 3. \(C_{\text{normal}}\) and \(C_{\text{SERS}}\) are the R6G solution concentrations used for normal Raman detection and SERS detection, respectively. According to formula (1), the AEF of the Au NBs substrate was calculated to be about 7.3×10\textsuperscript{5}.

The reproducibility and stability of Raman signal are important parameters for evaluating the performance of SERS substrates\textsuperscript{26}. In order to verify the signal repeatability and temporal stability of the SERS substrate, we prepared 15 batches of Au NBs SERS substrate, and then tested the R6G solution (10\textsuperscript{-6} mol/mL) separately. Five points were randomly selected in each silicon substrate for SERS detection and averaged to evaluate the signal repeatability of 15
batches of SERS substrates. The measured spectrum was shown in Fig. S5 (a) (Supporting Information), and the SERS signals of different batches changed similarly. The signal repeatability of the SERS substrate was characterized by the strongest characteristic peak of R6G at 1361 cm\(^{-1}\). The relative standard deviation (RSD) was 5.09%, as shown in Fig. S5 (b) (Supporting Information), which showed that the SERS substrate had a higher signal repeatability. In addition, we used R6G solution (10\(^{-6}\) mol/mL) to test the time stability of the Au NBs SERS substrate, SERS spectra were collected every 3 days within 21 days. As shown in the Fig. S6 (Supporting Information). As a result, there was no apparent change in the intensity of the SERS signal even after 21 days of SERS substrate fabrication, indicating pretty temporal stability of this SERS substrate.

**Raman peak assignment of chlortetracycline hydrochloride**

The Optimized structure of CCH was shown in Fig. 3(a). We used Gaussian 09 to optimize the molecular structure of CCH, and based on the density functional theory, using the RB3LYP and 6-31G (d) basis set and using the SMD model with water as the solvent were performed based on the optimized gas-phase geometries of all species to calculate the Raman spectrum of CCH. The normal Raman spectrum of solid powder and SERS spectrum of the standard aqueous solution collected by experiments were compared with theoretical Raman spectrum shown in Fig. 3 (b). The comparison showed that the theoretical Raman characteristic peaks of CCH were well consistent with the Raman characteristic peaks of the solution and solid powder, and part of the deviation was caused by the polarity of water in the solution\(^{27}\). The attribution of main Raman characteristic peaks is shown in the table 1.

**Detection of chlortetracycline hydrochloride in milk with Au NBs substrates**

First, the spiked milk containing different mass concentrations of CCH was performed pretreatment to obtain the supernatant, and drop the supernatant on the surface of a silicon wafer SERS substrate for detection. Each concentration was repeated 5 times and averaged to calculate the corresponding error bars, shown in Fig.4(a). The SERS spectra at different mass concentrations both had obvious characteristic peaks at 759, 1306, and 1442 cm\(^{-1}\), which
determined that the SERS spectra measured belonged to CCH. When the concentration is as low as 0.01 mg/mL, characteristic peaks can still be seen in the SERS spectrum and the intensity conformed to the principle of 3 times signal-to-noise ratio. Therefore, it confirmed that the limit of detection of CCH in milk with SERS substrate was 0.01 mg/mL. Utilizing the characteristic peak intensity of CCH at 759 cm\(^{-1}\) as a reference, the relationship between the Raman peak intensity and the concentration change was obtained (Fig. 4 (b)). As can be seen from Fig. 4 (b), the linear relationship, \(y = 258.467x+150.501\), between the SERS spectral intensity of CCH and concentration was obtained by fitting in the mass concentration range of 0.01–1 mg/mL, and the correlation coefficient \(R^2 = 0.9785\).

To verify the reliability and accuracy of the detection method, we measured the recovery of this method by detecting milk samples spiked with CCH. Spiked milk samples were configured at three mass concentrations of 0.1 mg/mL, 0.4 mg/mL, and 0.8 mg/mL, and the SERS spectra were collected three times. Then the mass concentration of CCH in milk was calculated based on the fitted curve and the intensity of the Raman peak at 759 cm\(^{-1}\), and the recovery rate, average recovery rate, and relative standard deviation of the method were consecutively obtained, shown in Table 2. The recovery rate of CCH in milk using substrate coated with Au NBs fluctuated between 96.80% and 111.38%, and the RSD was less than 7%. The detection of antibiotics in milk using SERS technology is inferior to instrumental analysis method, like LC-MS/MS\(^{28}\), HPLC-UV\(^{29}\) in terms of detection limit and quantitative detection. Despite its disadvantages, SERS technology still has great advantages as a preliminary screening and on-site detection for antibiotics. The SERS technique is simple, rapid, and sensitive, while the portable Raman spectrometer enables the detection of antibiotics not limited in the laboratory. The specific comparison information is summarized in Table S1 (Supporting Information).

**Conclusion**

In this paper, a high-yield, monodisperse Au NBs sol was prepared using the seed-mediated method. The ultraviolet absorption spectrum showed that the absorption peak of the Au NBs was located at 683 nm. Compared with the traditional spherical sol, the red shift of the
absorption peak made the gold sol more match long-wavelength excitation light source. The Au NBs were regarded as assembly units through interface replacement and self-assembly technology to prepare SERS substrates. The enhancement factor of the prepared SERS substrate to the R6G solution was about $7.3 \times 10^3$, which had a good Raman signal enhancement effect and signal repeatability. At last, the SERS spectrum collected from chlortetracycline hydrochloride in milk showed that the minimum detection limit was 0.01 mg/mL, the characteristic peak intensity and concentration met a linear relationship of $y = 258.467x + 150.501$, and the linear correlation coefficient was 0.9785. The method is simple and fast, and it is expected to be applied to the rapid detection of antibiotics in milk on site.

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Table 1. Theoretical and experimental vibration frequencies of CCH

| Theoretical Calculation/ cm⁻¹ | Solid Powder/ cm⁻¹ | SERS/ cm⁻¹ | Adscription |
|------------------------------|-------------------|-----------|-------------|
| 761                          | 761               | 759       | δ(C21-C22) + δ(N4-Hx) + δ(C33-Hx) |
| 1198                         | 1198              | 1195      | δ(C25-H49) + δ(O31-H51) + δ(C16-H30) |
| 1324                         | 1311              | 1306      | δ(C16-H30) + δ(Ring 1) + δ(C15-H7) + δ(Ring 3) |
| 1448                         | 1448              | 1442      | ν(C1-C3) + δ(Ring 4) + δ(N4-Hx) |

Note: ν-stretch vibration; δ-bend vibration

Table 2. Mass concentration, recoveries and RSD spiked at 0.1, 0.4 and 0.8 mg/mL

| Spiked Mass Concentration/ mg/mL | Number | Mass Concentration/ mg/mL | Recovery/ % | RSD/ % |
|---------------------------------|--------|---------------------------|-------------|--------|
| 0.1                             | 1      | 0.111                     | 111.38      |        |
|                                 | 2      | 0.106                     | 105.58      | 6.28   |
|                                 | 3      | 0.098                     | 98.21       |        |
| Mean                            |        | 0.105                     | 105.06      |        |
| 0.4                             | 1      | 0.413                     | 103.28      |        |
|                                 | 2      | 0.387                     | 96.80       | 3.53   |
|                                 | 3      | 0.391                     | 97.73       |        |
| Mean                            |        | 0.397                     | 99.27       |        |
| 0.8                             | 1      | 0.816                     | 102.01      |        |
|                                 | 2      | 0.793                     | 99.11       |        |
|                                 | 3      | 0.821                     | 102.64      | 1.86   |
| Mean                            |        | 0.810                     | 101.25      |        |
Figure Captions

**Fig. 1** Schematic diagram of the SERS method proposed for CCH in milk detection.

**Fig. 2** (a) The scanning electron microscopy image and size distribution of Au NBs, (b) The ultraviolet-visible absorption spectra and physical photo of Au NBs.

**Fig. 3** R6G solution normal Raman spectrum (10^{-1} mol/mL) and SERS spectrum (10^{-7} mol/mL). The laser energy is 30 mW and the integration time is 3 s.

**Fig. 4** (a) Optimized structure of CCH, (b) Raman spectrum of the theoretical calculations, solid powder of CCH and SERS spectrum of standard aqueous solution

**Fig. 5** (a) SERS spectra of different mass concentrations of CCH in milk using the silicon wafer substrates, (b) Fitting curve relationship of characteristic peak (759 cm^{-1}) intensity with CCH mass concentration. The laser energy is 150 mW and the integration time is 5 s.
Fig. 1 Schematic diagram of the SERS method proposed for CCH in milk detection.

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Fig. 4 (a) Optimized structure of CCH, (b) Raman spectrum of the theoretical calculations, solid powder of CCH and SERS spectrum of standard aqueous solution.
Fig. 5 (a) SERS spectra of different mass concentrations of CCH in milk using the silicon wafer substrates, (b) Fitting curve relationship of characteristic peak (759 cm$^{-1}$) intensity with CCH mass concentration. The laser energy is 150 mW and the integration time is 5 s.