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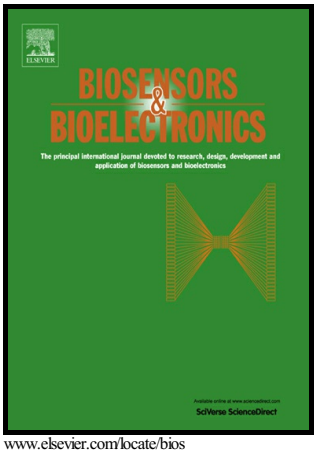

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A novel biosensor based on competitive SERS immunoassay and magnetic separation for accurate and sensitive detection of chloramphenicol

Kang Yang, Yongjun Hu*, Ning Dong

MOE Key Laboratory of Laser Life Science & Institute of Laser Life Science, College of Biophotonics, South China Normal University, Guangzhou 510631, P. R. China

*Corresponding author Telephone: (+86-20)8521-1436 EXT 8107. Fax:(+86-20)8521-6052. Email: yjhu@scnu.edu.cn

ABSTRACT:

The accurate and sensitive detection of chloramphenicol (CAP) is particularly imperative to public health and safety. Here, we present a novel sensor for residual CAP detection based on competitive surface-enhanced Raman scattering (SERS) immunoassay and magnetic separation. In this nanosensor, functionalized Au nanoparticles (AuNPs) were labeled with the Raman reporter molecule (e.g. 4,4′-dipyridyl). With the addition of free CAP, a competitive immune reaction was initiated between free CAP and above AuNPs for conjugating with CAP antibody-modified magnetic nanoparticles (MNPs). Instead of the solid substrate, the antibody conjugated-magnetic beads were used as supporting materials and separation tools in the present sensor. With the aid of a magnet, the

mixture was removed from the supernatant for concentration effects. This caused obvious change of SERS signal intensity obtained from supernatant. The SERS signals were collected from the supernatant directly, which made the SERS measurements more stable, repeatable and reliable. The proposed SERS-based magnetic immunosensor allows us to detect CAP in a fast, selective and sensitive (1.0 pg/mL) manner over a wide concentration range (1-1 $\times 10^4$ pg/mL). In addition, these results demonstrate that this immunosensor holds great potential for the detection of antibiotics in real aquatic environment, which is crucial to our life.

Keywords: chloramphenicol, surface-enhanced Raman scattering, competitive, magnetic separation

1. Introduction

As a powerful technique discovered in the 1970s (Hohenberg and Kohn, 1964; Nie and Emery, 1997), surface-enhanced Raman spectroscopy (SERS) has numerous advantages (Xu et al., 2005; Balabin, 2009, 2010a, b; Clifford et al., 2007) and has been widely used in biochemistry and life sciences (Alvarez-Puebla and Liz-Marzan, 2010; Xie et al., 2011; Kneipp et al., 2010; Feng et al., 2010). In recent years, by combining noble metallic (e.g. Au and Ag) NPs and specific organic Raman reporter molecules, various novel

nanoprobes named “SERS tags” have been designed. Numerous novel immunosensors utilizing these tags have also been developed. Meanwhile, it has attracted many researchers to combine the competitive concept with immunosensors (Brady et al., 2009; Ho et al., 2013; Huang et al., 2011), e.g. Ho’s paper combined the competitive concept with immunoassay and developed a rapid bead-based immunoassay platform. The studies on the development of detection with conventional SERS-based immunoassay mainly focused on the DNA, protein, microbe detection and biomedical science (Allain and Vo-Dinh, 2002; Cao et al., 2002; Cheng et al., 2009; Driskell et al., 2005; Han et al., 2010; Hu et al., 2010; Jehn et al., 2009; Qian et al., 2008; Schluecker et al., 2006). However, the conventional sandwich structure in SERS immunosensors is not suitable to detect small molecules for the small size and non-multiple recognition sites of these targets.

Generally, the solid slide was used as the substrate in the conventional immunoassay (Porter et al., 2008; Lee et al., 2011). There are several drawbacks with this approach, including the requirement of many repeated washing steps, long incubation time and low reaction efficiency. Recently, the functionalized magnetic beads were widely used in the immunoassay and DNA detection (Gao et al., 2012; Kuang et al., 2013; Jing et al., 2013; Jiang et al.,

2015; Moral-Vico et al., 2015). For instance, Kuang's method developed a sensitive and simple detection system for Salmonella detection based on MNPs and QDs. Gao's paper proposed a new approach for enhancing the sensitivity of SERS detection by the combination of CSRP with silver enhancement. In his research, the dual signal amplification was performed on the surfaces of MB modified MNPs, which brought not only the specific recognition of MB for target DNA but also the convenient separation of the CSRP product from the reaction mixture. Since the reactions occurred in aqueous solution and functionalized MNPs were used as supporting materials and separation tools in above researchs, these reactions could be greatly improved due to an increase in the surface area as well as the faster assay kinetics achieved (Zacco et al., 2006). Those overcame the slow reaction problems due to the diffusion-limited kinetics on a solid substrate. It can also provide some advantages such as easy of separation, shorter analytical time and wider linear range.

As the widely used therapeutic agents in humans and growth promoters in aquaculture and agriculture (Cabello, 2006; Kummerer, 2009; Gulkowska et al., 2007), antibiotics are of great importance to human society. CAP is a typical kind of amide alcohols antibiotic. However, CAP residues may cause aplastic anemia and other

adverse reactions (Rajchgot et al., 1983; Shepard and Samaras, 1970). Many analytical methods for CAP detection have been developed, such as ELISA (Kolosova et al., 2000), chromatography-based, including liquid chromatography mass spectrometry (LC-MS) (Impens et al., 2003) and high performance liquid chromatographic (HPLC) (Ali et al., 2009; Tajik et al., 2010). However, these methods are usually time-consuming and labor intensive. What's more, complicated sample preparation processes and highly skilled operators are also needed (Bogusz et al., 2004; Gikas et al., 2004). Recently, Ji's paper developed a rapid SERS method for CAP detection, but the limit of detection (LOD) was not satisfactory (Ji and Yao, 2015). Hence, it is of interest and necessity to develop a quicker, simpler and more sensitive method for CAP detection.

Herein, we developed a new, simple and highly sensitive sensor based on SERS competitive immunoassay and magnetic separation for CAP detection. It overcame the drawback of the conventional SERS immunosensors for the typical sandwich structure was not suitable to realize small molecules detection. With a low LOD, excellent recovery and relative standard deviation (RSD), the proposed SERS-based magnetic sensor provides a new solution for small molecules detection and a potential application in

environmental monitoring and protection.

2. Experimental

2.1 Reagents and materials

4,4′-dipyridyl (DP), bovine serum albumin (BSA) and trisodium citrate were acquired from Sigma-Aldrich, USA. Chloroauric acid ($HAuCl_4$) was received from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). CAP antibody, CAP-BSA and CAP standard solution were purchased from Otwo Biotech (Shenzhen) Inc. N-Hydroxysuccinimide (NHS), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), carboxyl-functionalized magnetic beads (average diameter 500 nm), sulfamethoxazole and norfloxacin were purchased from Aladdin-Reagent Co., Ltd. (Shanghai, China). The following buffer solutions were used in this study: borate buffer (pH 8.2) and phosphate-buffered saline (PBS, pH 7.4). The washing buffer was prepared by adding 0.05% Tween-20 to the PBS. All other reagents were of analytical grade or better. Ultrapure water processed by an ultrapure water system (ELGA, London) was used in all aqueous solutions. The natural water samples were obtained from the Pearl River at Guangzhou, South China.

2.2 Equipment

The Raman spectra were obtained with a microscopic Raman spectrometer (Nippon Optical System Co., Japan). The excitation wavelength was 632.8 nm from a He-Ne laser (Melles Griot, U.S.) with a power of 1 mW at the sample location. All spectra were calibrated with the reference of the 520 cm^{-1} line of silicon. In this study, the typical accumulation time was 30 s. The spectrum was collected by WinSpec/32 software, which could convert the Raman spectra into digital data. Origin 7.0 software was used to process those data. The diameter of AuNPs was measured by a UV/vis spectrometer (Perkin-Elmer, Waltham, MA).

2.3 Synthesis of AuNPs

The AuNPs were prepared as previously described in the literature (Frens, 1973), with slight improvement (Xie et al., 2011). Briefly, 100 mL of 1 mM $HAuCl_4$ aqueous solution was heated to boiling, to which 6 mL of freshly prepared 38.8 mM trisodium citrate solution was added rapidly. The solution color changed from colorless to deep red quickly. After that, the solution was boiled for another 20 min. Afterwards, the solution was cooled down to the room temperature with continuous stirring. According to the UV-vis spectrum for AuNPs in aqueous solution (Njoki et al., 2007), the resulting red AuNPs were about 30 nm in diameter (Fig. S1).

2.4 Preparation of SERS tags

The SERS tags were prepared according to the method reported by Gu and co-wokers with slight modification (Jiang et al., 2006; Yan et al., 2007). Typically, it was prepared in the following process. Firstly, 30 μL of 0.01 mM DP was added to 1.0 mL of above AuNPs and the resultant mixture was allowed to gently shake for 10 min. Subsequently, the reporter-labeled nanoparticles were then separated from the solution by centrifugation at 12,000 rpm for 5 min and dispersed with 1.0 mL of borate buffer (pH 8.2). After that, 6 μL of CAP-BSA （0.5 mg/mL） was added to 1.0 mL of the reporter-labeled nanoparticles. The mixture was incubated at room temperature for 2 h. Then the DP-labeled immunogold nanoparticles were purified by centrifugation at 12,000 rpm for 10 min and resuspension with 1.0 mL of borate buffer (pH 8.2). To make sure that no bare sites on these nanoprobes were left, 20 μL of BSA (2.5%) was added into the above nanoparticles, the mixture was incubated for 1.0 h at room temperature. Finally, the SERS tags were obtained after centrifugation and resuspension with 1.0 mL of PBS (pH 7.4). These tags could be stored for several days at 4℃ to keep its stability and biological activity. This will reduce the time consumption and provide better repeatability.

2.5 Functionalization of MNPs with Antibodies

A stable link between antibody and MNPs is needed in a subsequent detection step. By utilizing the primary amine (-NH_2) groups of the amino acid side chains (Lys) of protein molecules for crosslinking on a carboxyl (-COOH) modified surface, EDC and NHS provide an easy and simple immobilization strategy for above process. The CAP antibody-modified magnetic beads were prepared according to the method reported by Baniukevic with slight modification (Baniukevic et al., 2013). Firstly, the carboxyl-functionalized MNPs were needed to be washed twice with PBS (pH 7.4) by holding the particles with a magnet and then resuspended with 0.5 mL of PBS (pH 7.4). Then the nanoparticles were activated by incubating with 30 μL of EDC (4 mg/mL) for 10 min with gentle shake. After that, 30 μL of NHS (1 mg/mL) was added into the above nanoparticles for another 30 min. After incubation with EDC and NHS, the activated carboxyl-functionalized particles were separated by a magnet and resuspended in PBS (pH 7.4). The CAP antibody solution (0.5 mg/mL) was then added into the activated MNPs for 2 h under continuous stirring. After that, the supernatant was removed from the nanoparticles with a magnet and 50 μL of 2.5% BSA was added to block bare sites on the MNPs, the mixture was incubated for 1h at room temperature. After separated by a magnet and washed twice,

the antibody-modified MNPs dispersing in PBS (pH 7.4) were finally obtained.

2.6 Assay Procedure

As shown in Fig. 1, the MNPs were modified with CAP antibody. SERS tags were labeled with DP and CAP-BSA. 100 μL SERS tags and 20 μL various concentrations of CAP standard solutions were simultaneously dropped into the antibody-modified MNPs. Then the mixture was incubated for 2 h with gentle shake before the supernatant was separated from the mixture with a magnet. Finally, we obtained SERS signal from the supernatant.

3. Results and discussion

3.1 Mechanism of the proposed sensor

CAP antibody-modified magnetic beads can simultaneously capture free CAP and CAP-BSA. In the sensing system, there was a competitive immune reaction between free CAP and the SERS tags for conjugating with CAP antibodies, which were modified on MNPs. After separated by a magnet, the quantity of SERS tags existing in the supernatant would decrease. This would result in an obvious reduction of SERS signal intensity obtained from supernatant. The higher concentrations of free CAP solutions were, the less SERS tags would bind with antibodies modified on MNPs.

Thus, the concentration of SERS tags remaining in supernatant would be higher and the SERS signal obtained from the supernatant would be more intense. Because of the underlying relationship between the SERS intensities and their corresponding CAP concentrations, the quantitative detection of CAP using this SERS-based magnetic immunosensor would be feasible.

3.2 Optimization of the sensing system

3.2.1 Quantity of CAP antibody

It is necessary to optimize the relevant experiment terms. To determine the proper quantity of CAP antibody modified on the activated carboxyl-functionalized MNPs, 2, 4, 6, 8, 10 μL 0.5 mg/mL CAP antibody were added into 1.0 mL MNPs, respectively. The mixtures were incubated with gently shake for 2 h. In the following steps, the amount of SERS tags was sufficient and no free CAP was added. Fig. S2 shows that Raman signal intensities obtained from the supernatant decrease gradually as the quantity of the added antibody solutions increase and reach the minimum when the volume of added antibody is 8 μL. However, when it is more than 8 μL, the signal intensity remains unchanged. This indicates that the antibodies are excessive. Consequently, the proper volume of the added antibody solution should be 8 μL.

3.2.2 Quantity ratio of SERS tags to functionalized MNPs

The quantitative ratio of SERS tags to antibody-modified MNPs and proper incubation time of them were also studied. In this process, the volume of SERS tags was 100 μL. As shown in Fig. S3, it can be seen that SERS signal intensities decrease as the mixture ratio of above two kinds of nanoparticles varies from 10:0 to 10:8 and remain unchanged with more MNPs were added, which indicates that the antibody coupled MNPs are excessive. So the proper volumetric ratio of SERS tags to antibody-modified MNPs should be 10:8.

When the mixture ratio of above mixtures is fixed, we can find that signal intensities decrease as the incubation time increase from 1 h to 2 h (Fig. S3). However, when the incubation time is more than 2 h, the signal intensities remain unchanged, which suggests that the proper incubation time would be around 2 h.

3.3 Sensitivity of the sensing system

After SERS tags and CAP standard solutions have simultaneously incubated with antibody coupled MNPs for 2 h, the Raman spectra of residual SERS tags in the supernatant were obtained. The different concentrations of CAP standard solutions (1, 10, 100, 1×10^3, and 1×10^4 pg/mL, respectively) were used in this approach. A control experiment was also carried out with the

solution containing SERS tags and antibody-modified MNPs but no CAP. The relationship between intensities of SERS signals and CAP concentrations is shown in Fig. 2A. It can be seen that intensities of SERS signal increase as the concentrations of CAP increases from 0 pg/mL to 10 ng/mL. This trend follows the expectation for a competitive immunoassay, which implies that the CAP-BSA coated on AuNPs and the free CAP existing in standard solutions have competitively bound with the CAP antibodies modified on functionalized MNPs. Fig. 2B is a histogram with the band intensity at 1612 cm^{-1} and it shows that the SERS signal intensity of the control experiment (0 pg/mL free CAP) is the lowest, ca. 157 ± 34.5 Counts/30s, so the detection threshold value should be set as 226 Counts/30s (mean of the control intensity minus 2 times standard deviation) (Dufek et al., 2010). It can be seen that the SERS signal still can be identified from the threshold value when the concentration of CAP is as low as 1.0 pg/mL. Thus, it is acceptable and reasonable that the LOD of CAP with this biosensor should be set as 1.0 pg/mL. What is more, SERS signal intensities of any two concentrations in this range can be easily identified and the difference between the two is distinct. Moreover, a good linear correlation, which obeys the equation $Y=275.48+515.96X$ ($R^2=0.996$), can be obtained between the SERS signal intensities and

CAP concentrations (Fig. 4B).

3.4 Specificity for CAP detection

The sensor combining SERS technology and competitive reaction demonstrates the feasibility study to detect the presence of CAP in standard solutions. To confirm the specificity of our SERS-based immunosensor, we investigated two kinds of the potential interfering substances. The first is sulfamethoxazole, a prototypical broad-spectrum antibiotic. The second is norfloxacin, which is another kind of synthetic chemotherapeutic antibacterial agent (Nelson et al., 2007). The molecular structures of these three reagents are seen in Fig. 3A. The proposed immunoassay with CAP, sulfamethoxazole and norfloxacin was carried out with three different concentrations of these reagents, respectively. With the solution containing only SERS tags and antibody-modified MNPs, control experiments were also conducted at the same time. As shown in the histogram (Fig. 3B), the SERS signal intensities increase with increasing concentrations of CAP from 0 pg/mL to 1×10^{3} pg/mL. In contrast, there are no obvious changes in SERS intensities for the sulfamethoxazole and norfloxacin with different concentrations, respectively. In summary, these experimental results reveal that there is no serious cross reaction in the present biosensor. Instead, it confirms good selectivity and sensitivity of the SERS-based

competitive system.

3.5 Application in real samples

The proposed immunosensor has been applied to detect CAP residual in the real samples. First of all, it is necessary to alleviate the matrix effect caused by contaminants in real samples. A series of comparative experiments were conducted to evaluate the effects by diluting the water samples with ultrapure water at different dilution ratio. The results reveal the dilution effects would be obviously observed with the continuous addition of ultrapure water. The matrix effects had little impact on our magnetic immunosensor when the samples were 40 times diluted or more. To further eliminate the matrix effect, filtration and centrifugation were also required. Under these conditions, we detected spiked water samples, and the dependence was measured in the CAP concentration range of 0 - 5×10^5 pg/mL. As shown in Fig. 4A, a good linear correlation is obtained between the SERS signal intensities and CAP concentrations ranging from 5 pg/mL to 5 ng/mL. From Fig. 4B, we can find that the average and standard deviation of signals from real samples show a perfect match with the standard curve. To validate the accuracy and feasibility of this immunosensor, the recoveries of CAP in samples were calculated when its concentrations increased from 5 to 5×10^3 pg/mL. As we can see from Table 1, all their

recoveries are between 81.42% and 96.92% (n=3) and RSD% value, are also obtained for different CAP concentrations, implying a good repeatability.

All these results demonstrate that this SERS-based magnetic immunosensor could be applied for the quantitative analysis of CAP in water with no sophisticated sample processing, beyond simple filtration and centrifugation to remove sundries in the aquatic environment.

4. Conclusion

In summary, a novel SERS-based magnetic immunosensor suitable for the detection of small molecule (such as CAP) is proposed. In this novel sensor, free CAP antigens and CAP-BSA conjugated on the AuNPs competitively react with CAP antibodies coated on the magnetic beads. In this case, the SERS signals were collected from the supernatant directly and were more reproducible, repeatable and reliable. All the results in this paper show that this immunosensor can be applied for the detection of CAP in aquatic environment in a fast, sensitive and selective manner. This SERS-based immunosensor using functionalized MNPs overcomes the drawbacks of the conventional sandwich structure in SERS immunsensors and has multiple superiorities including a wide

dynamic concentration range, low LOD and good recovery. Moreover, it is expected that the proposed approach which combining competitive SERS immunoassay with magnetic separation can be appropriate for the detection of other small molecules.

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Figure captions

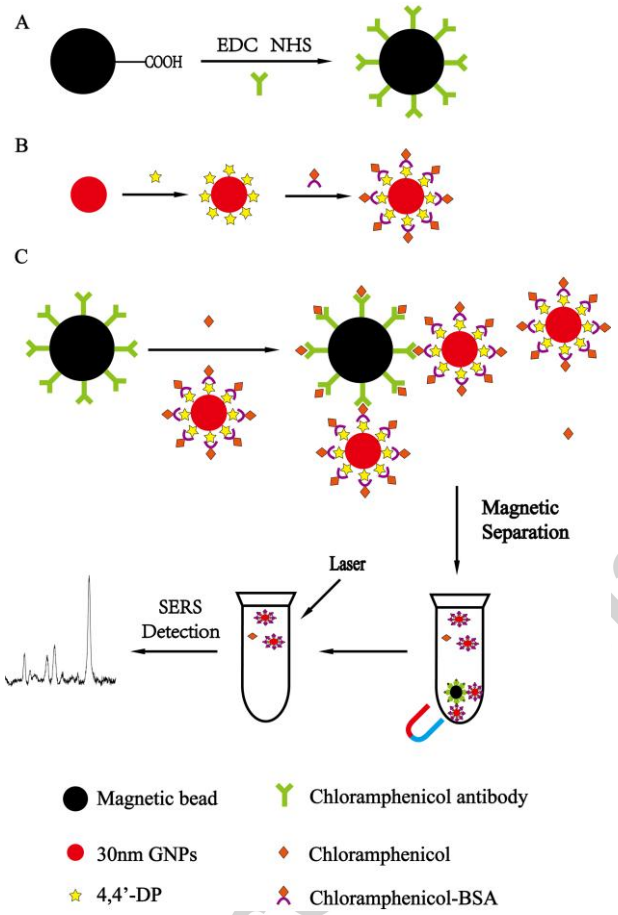
Fig. 1. (A) Preparation of MNPs modified with CAP antibody. (B) Preparation of SERS tags. (C) SERS-based magnetic immunosensor for CAP.

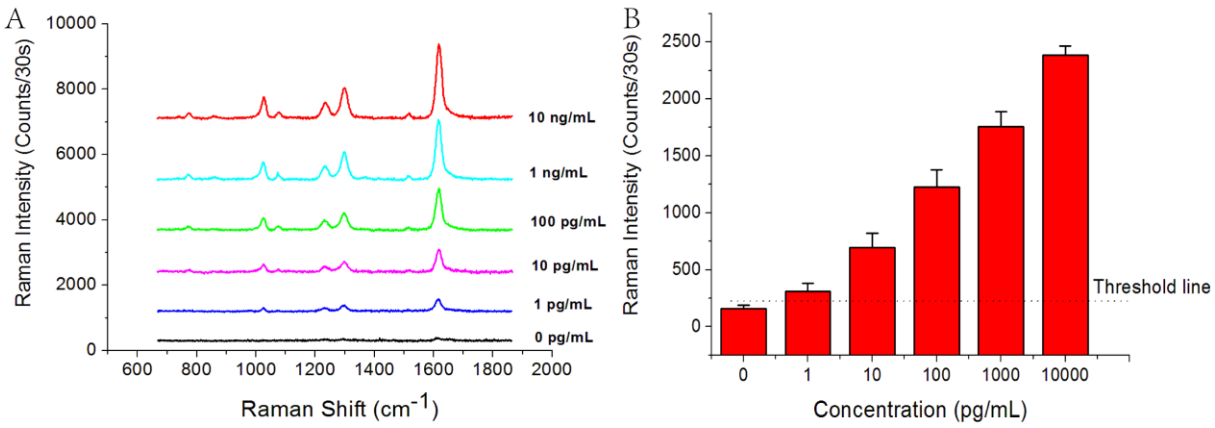
Fig. 2. (A) SERS spectra acquired with various CAP concentrations. (B) Concentration-dependent SERS intensity of the band at 1612 cm^{-1} of DP using the SERS-based magnetic immunosensor. Error bars indicate the standard deviations of three independent measurements.

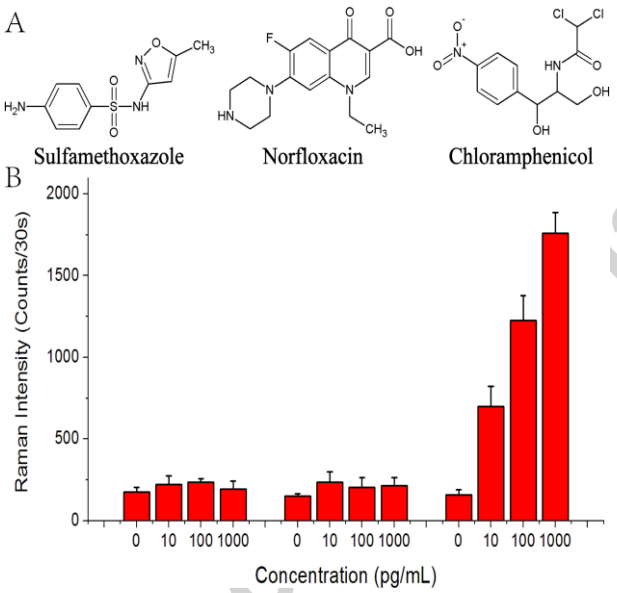
Fig. 3. Specificity of CAP detection. (A) The molecular structures of sulfamethoxazole, norfloxacin and CAP. (B) Concentration-dependent SERS intensity of the band at 1612 cm^{-1} of DP using the SERS-based magnetic immunosensor. Error bars indicate the standard deviations of three independent measurements.

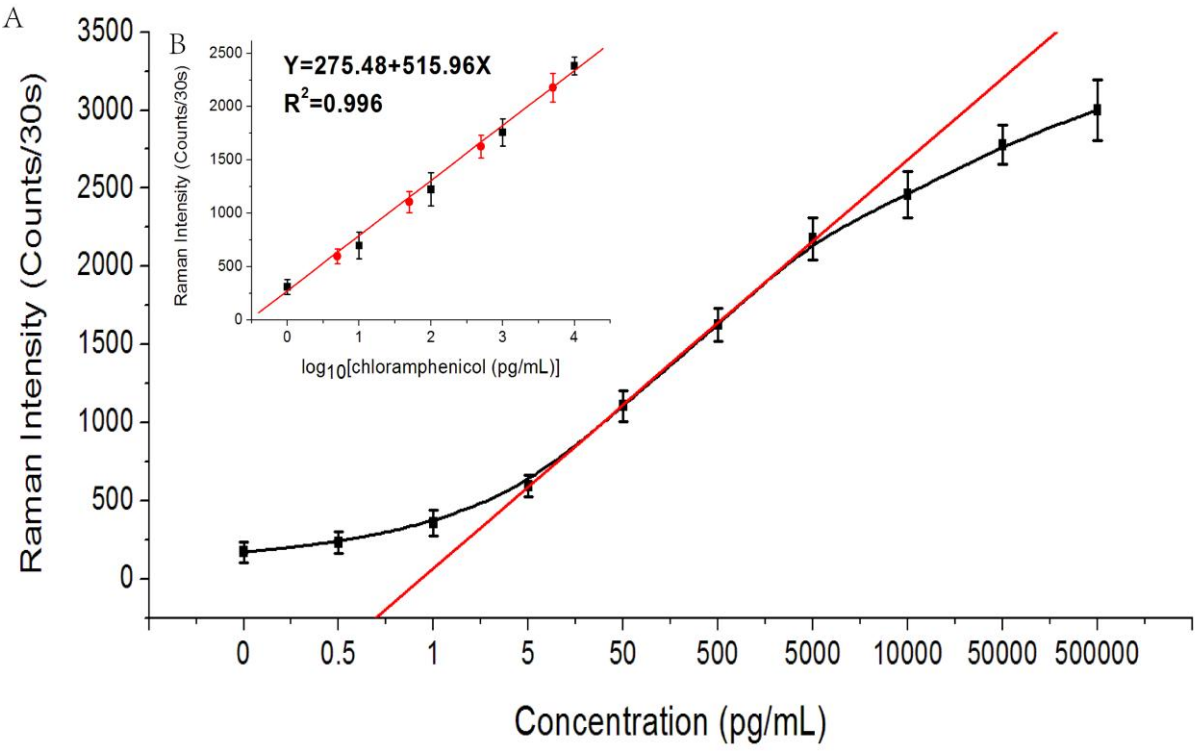
Fig. 4. The relationship between the SERS signal intensity and concentration of CAP. (A) The curve of the SERS signal intensity versus concentration of CAP in diluent samples. (B) The squares represent the average and standard deviation in signal collected from three independent measurements in standard solution and the circles

represent the average and standard deviation in signal collected from three independent measurements in diluent samples.









highlight

1. The biosensor combines competitive SERS immunoassay with magnetic separation.

2. SERS signals were obtained from supernatant instead of solid substrate.

3. SERS signal intensity and CAP concentration was positively correlated.

4. The proposed sensor has a low limit of detection of 1 pg/mL.

5. This novel sensor is low-cost with no sophisticated sample processing.

Table 1. Recovery of CAP in diluent water samples using the SERS-based magnetic immunosensor.

Added concentration (pg/mL)	Deteced concentration (pg/mL)	Recovery ratio (%)	RSD （%）
5	**4.18**	**83.6**	**14.4**
50	**40.71**	**81.42**	**11.8**
500	**414.47**	**82.89**	**9.9**
5000	**4846**	**96.92**	**9.7**