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Highly Sensitive Surface-Enhanced Raman Scattering Sensing of Heparin Based on Antiaggregation of Functionalized Silver Nanoparticles

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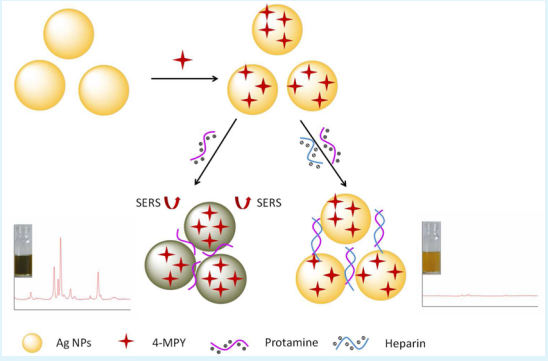
Supporting Information

ABSTRACT: We report a simple and sensitive surface-enhanced Raman scattering (SERS) platform for the detection of heparin, based on antiaggregation of 4-mercaptopyridine (4-MPY) functionalized silver nanoparticles (Ag NPs). Here, protamine was employed as a medium for inducing the aggregation of negatively charged 4-MPY functionalized Ag NPs through surface electrostatic interaction, which resulted in significantly enhanced Raman signal of the Raman reporter. However, in the presence of heparin, the interaction between heparin and protamine decreased the concentration of free protamine, which dissipated the aggregated 4-MPY functionalized Ag NPs and thus decreased Raman enhancement effect. The degree of aggregation and Raman enhancement effect was proportional to the concentration of added heparin. Under optimized assay conditions, good linear relationship was obtained over the range of 0.5−150 ng/mL (R^2 = 0.998) with a minimum detectable concentration of 0.5 ng/mL in standard aqueous solution. Furthermore, the developed method was also successfully applied for detecting heparin in fetal bovine serum samples with a linear range of 1−400 ng/mL.



KEYWORDS: *heparin, surface-enhanced Raman scattering, silver nanoparticles, antiaggregation*

INTRODUCTION

Heparin is a highly negatively charged glycosaminoglycan, which has a molecular weight range from 5000 to 40 000 with average charge up to ∼70.[1] It plays a vital role in the regulation of various normal physiological and pathological processes such as venous thromboembolism, lipid transport and metabolism, cell growth and differentiation, and blood coagulation.[2−5] On the contrary, the overdose of heparin often induces hemorrhage, thrombocytopenia, or other bleeding complication.[6,7] So, the United States, European Union, and China have upgraded inspection standards of heparin after the "heparin incident" in 2008.[8] The therapeutic dosing level of heparin is 2−8 U/mL (17−67 μM) during cardiovascular surgery and 0.2−1.2 U/mL (1.7−10 μM) for postoperative and long-term care.[9] However, quantitative measurement of heparin seems to be difficult due to its natural polydispersity, chemical heterogeneity, and lack of fluorescent properties or significant absorbance.[10] Therefore, the development of sensitive, selective, and simple sensing strategy for the detection of heparin has attracted considerable research efforts in recent years.

To date, various methods had been used for detection of heparin, such as, fluorescent,[11,12] electrochemistry,[13] light scattering,[14] spectrophotometry,[15] and colorimetry.[16,17] Typically, Li's group developed a simple and sensitive method for visual detection of heparin using positively charged gold nanoparticles as colorimetric probes with a detection limit of 0.03 μg/mL.[17] Wang's group provided the ratiometric fluorescence sensor based on a pyrene derivative and quantification detection of heparin in aqueous solution and serum. The detection limit of the sensor is 0.157 μM, and its linear range is 5−30 μM.[18] Li's group reported the electrochemical sensor for heparin based on a poly(thionine) modified glassy carbon electrode.[19] Although these existing methods can measure heparin to microgram per liter concentrations, there is still urgently necessary to explore simpler and more sensitive methods for field assays.

Surface-enhanced Raman scattering (SERS) technique, which extraordinary sensitivity for applications in numerous chemical and biological systems, has attracted great attention and become a powerful spectroscopy technique.[20,21] Its two well-known primary theoretical mechanism models are long-

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range electromagnetic (EM) enhancement and short-range chemical enhancement (CE). EM enhancement emphasizes the effect of the nanosubstrate providing the long-range electromagnetic fields, which determined by the nanosubstrate's inherent properties (material type, shape, and size). And CE is achieved by changing the scattering cross section of the analytes attached on a metal surface, thus depends on the chemical features of the analytes themselves.[21,22] It is acknowledged that EM enhancements always considered as the major contribution to the SERS phenomenon.[23,24] Since its discovery in the late 1970s, SERS has been applied to many analyses. Specifically, it possesses great potential in nucleic acid and metal ions detection systems, immunoassay, cell imaging, and cancer therapeutics, which are mainly due to its nearly 10^4-10^6 fold enhancement in comparison with normal Raman spectra, lack of photobleaching and self-quenching, as well as the use of a single laser wavelength for multiple species.[25,26] Recently, various nanoparticles-based SERS applications have been reported, based on the fact that SERS signal of Raman reporter tagged on the nanoparticles can be significantly enhanced by the interparticle plasmonic coupling induced by the aggregation of nanoparticles. For example, Zamarion's group reported ultrasensitive SERS nanoprobes for hazardous metal ions based on trimercaptotriazine-modified gold nanoparticles (Au NPs).[27] Wang's group developed a direct SERS technique for Hg^{2+} detection based on the investigation of the interaction between silver nanoparticles (Ag NPs) and Hg^{2+}.[28] Our group has also developed an SERS approach to detect As^{3+} by using glutathione-functionalized Ag NPs and 4-MPY as the Raman reporter.[29] Au NPs and Ag NPs are both widely used in the SERS-based assay but, generally speaking, Ag NPs is a much more efficient Raman substrate than Au NPs. It can provide 10-fold to 100-fold higher SERS signals than the similar Au NPs, which is mainly due to its d-s band gap, is in the ultraviolet (UV) region, giving rise to less damping of the plasmon mode.[30,31]

Accordingly, in the present study, we applied SERS technique to the sensitive and selective detection of heparin based on antiaggregation of 4-MPY functionalized Ag NPs upon the presence of protamine. Protamine is a kind of natural peptide with polypositive ion, which has ∼20 charges;[32] its special structure could lead to the aggregation of 4-MPY functionalized Ag NPs, thus resulting in the changes in colors, surface plasmon resonance absorption and Raman intensity of 4-MPY. Meanwhile, there is strong affinity between protamine and heparin, which has been used in clinical and experimental observation.[33] In the presence of heparin, the Raman enhancement effect of 4-MPY functionalized Ag NPs induced by protamine would be decreased. The experiment results proved that this sensing strategy provided a good performance in terms of sensitivity, selectivity, linearity, and limits detection of heparin in both standard aqueous solution and fetal bovine serum sample.

EXPERIMENTAL SECTION

Reagents. Hydroxylamine hydrochloride ($NH_2OH{\cdot}HCl$) was obtained from Tianjin Kermel Chemical Reagent Co. Ltd. (Tianjin, China). Protamine sulfate salt, hyaluronic acid (HA) salt, and 4-mercaptopyridine (4-MPY) were obtained from Sigma-Aldrich. Heparin sodium salt (185 U/mg) from bovine intestinal mucosa, chondroitin sulfate (Chs), silver nitrate ($AgNO_3$), Rhodanine 6G (R6G), and thymine (T) were purchased from Aladdin Chemistry Co. Ltd. (Shanghai, China). Bovine serum albumin (BSA), glucose, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Shanghai Shenggong Co. (Shanghai, China). HEPES buffer solution used in the experiment was adjusted with 1 M sodium hydroxide (NaOH) to pH 7.4. Fetal bovine serum (FBS) stock solution (HyClone, Thermo Scientific, Australia) and blood samples (Collected from three adult Kunming male mice) were stored at −20 °C and were thawed at room temperature before use. All other chemicals were provided by Sinopharm Chemical Reagent (Shanghai, China). Deionized water with 18.2 MΩ specific resistances obtained from a Pall Cascada laboratory water system. All of the reagents were of analytical grade and used without future purification.

Apparatus. All measurements of SERS spectra were recorded using Thermo Scientific RFS 100 Raman system equipped with a microscope and a 632.8 nm diode-pumped He−Ne laser source. Transmission electron microscopy (TEM) images were acquired on a JEM-1230 electron microscope (JEOL Ltd., Japan) operating at 100 kV. UV−vis absorption spectra were performed on a Thermo Scientific NanoDrop 2000C spectrophotometer (Gene Company Ltd.). Zeta potential measurements were performed on Malvern Zetasizer Nano-ZS90 (ZEN3590, Malvern Instruments Ltd., U.K.). All glassware used in the experiment were washed with freshly prepared aqua regia, rinsed thoroughly in deionized water and dried in air.

Preparation of Ag NPs. Ag NPs were prepared by reducing $AgNO_3$ using $NH_2OH{\cdot}HCl$ at room temperature with slight modifications.[34] Briefly, 10.44 mg of $NH_2OH{\cdot}HCl$ was added to 89.0 mL of H_2O and was mixed with 1 mL of 0.30 M NaOH to maintain an alkaline pH. Then, 10 mL of 0.01 M $AgNO_3$ was added to the mixtures under stirring. The above solution was continuously stirred for an additional 1 h. The prepared silver colloid was stored at room temperature. TEM and UV−vis spectroscopy were used to characterize the typical surface plasmon resonance peak and morphology of the produced silver colloid. The size of prepared Ag NPs was estimated to 35 ± 5 nm (size ranging from 30 to 55 nm is widely used in SERS-based method), and zeta potential of Ag NPs was measured as −38.9 mV.

Functionalization of Ag NPs with 4-MPY. 4-MPY of 250 μMwas prepared in double-distilled water. Then 50 μL of 4-MPY was added to 4.95 mL of the prepared Ag NPs with stirring for 1 h, and the mixture was subsequently left for overnight without disturbance at room temperature. The zeta potential of 4-MPY functionalized Ag NPs was measured as −38.1 mV. The zeta potential of Ag NPs was measured as −38.9 mV, it was found that 4-MPY had little effect on the negatively charged Ag NPs.

SERS Detection of Heparin. The SERS detection of heparin was performed at ambient temperature. Various concentrations of protamine and heparin were prepared by 10 mM HEPES buffer solution. The samples for heparin detection were prepared by adding different amounts of heparin (0−150 ng/mL) to the solution of 0.5 μg/mL of protamine for 2 min, then 80 μL of 4-MPY functionalized Ag NPs were added to the above mixture and incubated for another 2 min. Finally, about 5 μL of the above mixture in the capillary tube was detected by Raman spectrometry with an exposure time of 4 s.

Selective Detection of Heparin. In the experiments of selectivity and practical assay, all samples were tested in a similar way. We investigated the selectivity of the our approach for other potentially competing substances (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, PO_4^{3-}, HPO_4^{2-}, $S_2O_4^{2-}$, glucose, BSA, ATP, HA, Chs) under the same optimized conditions.

Analysis of Real Samples. FBS samples at 0.2 % (v/v) were prepared by diluting the stock solutions with HEPES buffer (pH 7.4, 10 mM). Blood samples were added sodium citrate to prevent from clotting, centrifuged, and then diluted to 30-fold with 10 mM HEPES (pH 7.4). The real samples were spiked with standard protamine and heparin at certain concentrations. Then the same procedure was prepared as that of the standard solution detection mentioned above.

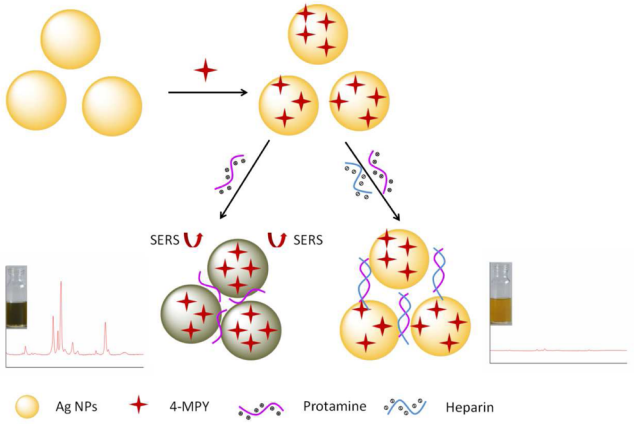
Safety Consideration. Aqua regia has strong oxidizing capacity and adverse effects on human health; thus, all of the experiments involving aqua regia should be performed with gloves and protective glasses. The waste solution of the experiment should be collectively reclaimed to avoid polluting the environment.

RESULT AND DISCUSSION

Sensing Mechanism of Heparin Sensor. A schematic representation of the mechanism of SERS sensing heparin based on antiaggregation of the 4-MPY functionalized Ag NPs is illustrated in Scheme 1. During the process of SERS probe synthesis, as a Raman reporter, 4-MPY, could replace parts of the hydroxylamine ions, and contact with Ag NPs mainly through Ag−S or Ag−N bond.[35,36] As shown in Figures 1b and 2d, by adjusting the adding amount of Raman reporter 4-MPY, the color of negatively charged Ag NPs colloid was still bright yellow, which indicated that the 4-MPY functionalized Ag NPs were well stabilized. Protamine, a low molecular weight protein, has ∼20 positive charges in physiological condition and is rich in basic arginine residues, and could be absorbed on the surface of negatively charged 4-MPY functionalized Ag NPs through electrostatic interaction. Thus protamine acted as a medium for inducing the aggregation of negatively charged 4-MPY functionalized Ag NPs as well as an obvious color change, as seen from Figures 1c and 2g. And, as shown in Figure 2g, the aggregated 4-MPY functionalized Ag NPs could produce great SERS signals. However, in the simultaneous presence of protamine and heparin in the sensing solution, the aggregation of 4-MPY functionalized Ag NPs would be prevented because of that protamine was hybridized with heparin first, as seen from Figures 1d and 2f. As we know, their strong electrostatic binding has been used in experimental observation and clinical application.[32] Herein, we took advantage of their strong affinity to develop a SERS detection of heparin based on antiaggregation of 4-MPY functionalized Ag NPs.

To demonstrate the feasibility of this sensor strategy, Raman spectra, UV−vis absorption spectra, and TEM images were also used to characterize the system. As shown in Figures 1 and 2, 4-MPY functionalized Ag NPs were remained stable, and there were strong SERS signals only in the simultaneous presence of Ag NPs, 4-MPY, and protamine. On the contrary, there were almost no SERS signals in the presence of heparin alone. And with the adding of heparin, there was a significant decrease in the SERS intensity. In addition, the UV−vis spectra were added to show the changes of typical surface Plasmon resonance peak (see Figure S1 in the Supporting Information). And, only in the presence of protamine alone could induce the aggregation of 4-MPY functionalized Ag NPs. In the simultaneous presence of protamine and heparin, 4-MPY functionalized Ag NPs were still monodisperse.

Scheme 1. Schematic Diagram of the Proposed SERS Method for Measuring Heparin Using 4-MPY Functionalized Ag NPs



Optimization of the Responsive Conditions. For the best performance of our proposed sensing assay, several responsive conditions were investigated including Raman reporters and concentrations of protamine.

As seen from Figure S2 in the Supporting Information, three kinds of Raman reporters were tested to optimize the suitable reporter. As the Raman reporter, 4-MPY exhibited the strongest SERS signals, which mainly due to their types of bindings with Ag NPs. 4-MPY bonded with Ag NPs mainly through Ag−S or Ag−N bond, and the stronger Ag−S bond played a major role in the chemisorption process.[37] The zeta potential of 4-MPY functionalized Ag NPs was −38.1 mV, which was little affected by 4-MPY. R6G contacted with Ag NPs mainly through weak electronic interaction, it could greatly affect the surface negative charges of Ag NPs and might have powerful competitive ability with protamine, and then the zeta potential was reduce to −27.0 mV. Thymine (T) contacted with Ag NPs through Ag−N bond and the zeta potential was −38.3 mV. However, as seen from Figure S2, T and R6G exhibited weak SERS signals while 4-MPY exhibited strong SERS signals in this method. Considering all these reasons, we chose 4-MPY as Raman reporter in the developed method.

As shown in Figure 2b, although Ag NPs could aggregate after addition of protamine, there was no SERS signal. Therefore, the Raman reporter played an important role in this SERS based assay; the effect of the concentration of 4-MPY on the SERS intensity was investigated with five different concentrations ranging from 1.0 to 3.0 μM. The corresponding Raman spectra are shown in Figure 3A; the largest signal was achieved with 2.5 μM 4-MPY, and further increasing the concentration of 4-MPY did not induce obviously enhancement of SERS signal. Generally speaking, higher concentration of the Raman reporter would induce Ag NPs aggregation, and thus, the concentration of 2.5 μM was chosen in this work.

Effect of the concentration of protamine added in the system was further investigated to optimize the detection sensitivity toward heparin, and the corresponding Raman spectra were performed in Figure 3B. We noted that the concentration lower than 0.5 μg/mL would be less efficient and unfavorable to lower detection limit, whereas protamine concentration higher than 0.5 μg/mL did not induce obviously enhancement of signal, which was mainly due to that protamine play a role of stabilizer for 4-MPY functionalized Ag NPs at high concentrations. Therefore, the concentration of 0.5 μg/mL was employed for the sensing of heparin.

Sensitivity and Selectivity of the Sensor. Under the above optimized conditions, the SERS spectra of the 4-MPY functionalized Ag NPs in different concentrations of heparin were recorded. As shown in Figure 4A, there were many spectra features that were attributed to 4-MPY bands which could be used for indirect quantitative determination of heparin, such as those at 712, 1015, 1066, 1099, 1227, and 1584 cm^{-1}. The band at 1015 cm^{-1} was related to ring-breathing vibrations.[38] The band at 1066 and 1227 cm^{-1} corresponded to the C−H stretching mode, and 1584 cm^{-1} was assigned to C−C stretching mode.[39,40] The strongly enhanced band at 1099 cm^{-1} corresponding to the ring-breath/C−S stretching mode indicated that 4-MPY was adsorbed onto the surfaces of the Ag NPs through the sulfur atom.[41,42] It was also supported by the C−S stretching mode at 712 cm^{-1}.[39,42] We could clearly note that the peak around 1099 cm^{-1} was the most prominent one,

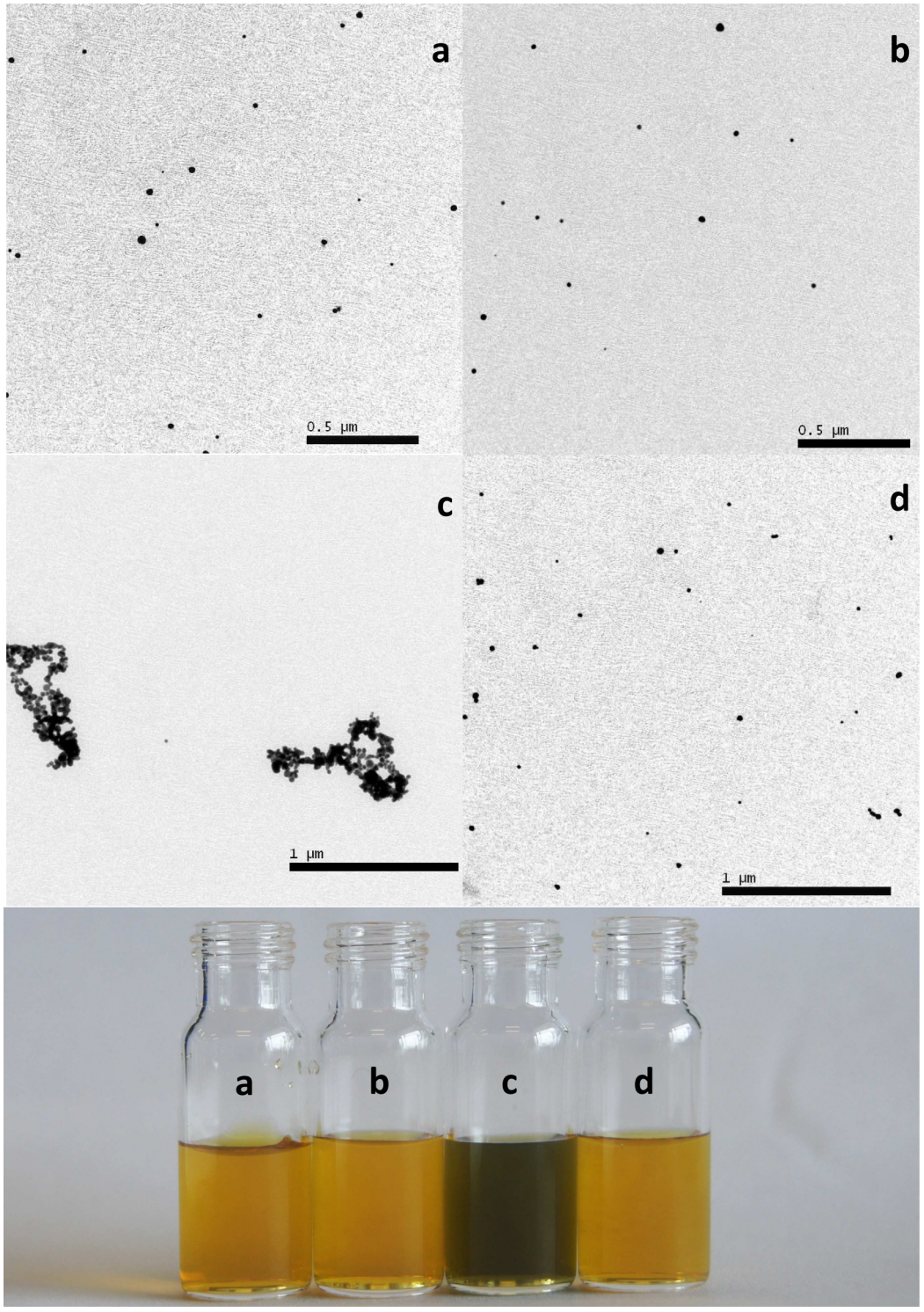


Figure 1. TEM images and color changes (bottom panel) corresponding to (a) Ag NPs; (b) 4-MPY-Ag NPs; (c) 4-MPY-Ag NPs + protamine (0.5 μg/mL); and (d) 4-MPY-Ag NPs + protamine (0.5 μg/mL) + heparin (150 ng/mL).

and its intensity was very sensitive to the concentration of heparin. Therefore, it was selected as an optimum peak for the quantitative detection of heparin. This relationship is expounded in Figure 4B; a satisfying linear relationship was obtained over the range of 0.5−150 ng/mL with the correlation coefficient of 0.998. The minimum detectable concentration was 0.5 ng/mL, which confirmed the exquisite sensitivity of our sensing assay.

To investigate the selectivity of our developed approach toward heparin, the potential competing substances, including 10 mM of Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, PO_4^{3-}, HPO_4^{2-}, $S_2O_4^{2-}$, glucose, 0.5 μg/mL of BSA, 100 ng/mL of ATP, 1000 ng/mL of HA and Chs were examined under optimized conditions. As shown in Figure 5, only the addition of 100 ng/mL of heparin could obviously prevent the aggregation of Ag NPs through the strong affinity of protamine and heparin, resulting in a signification decrease of SERS signals. As shown in Figure S3

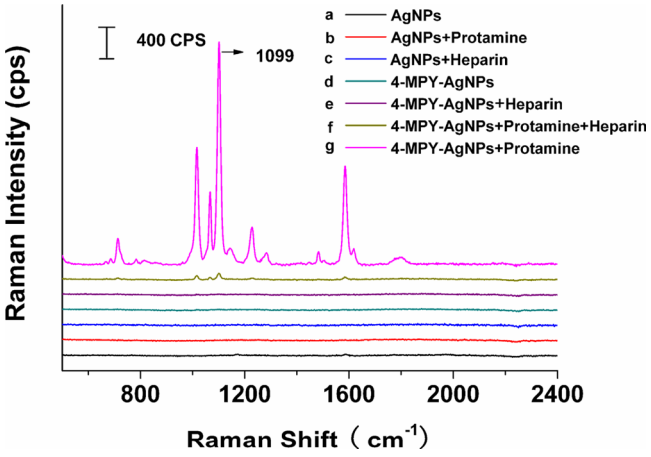


Figure 2. Raman spectra of (a) Ag NPs; (b) Ag NPs + protamine (0.5 μg/mL); (c) Ag NPs + heparin (150 ng/mL); (d) 4-MPY-Ag NPs; (e) 4-MPY-Ag NPs + heparin (150 ng/mL); (f) 4-MPY-Ag NPs + protamine (0.5 μg/mL) + heparin (150 ng/mL); (g) 4-MPY-Ag NPs + protamine (0.5 μg/mL).

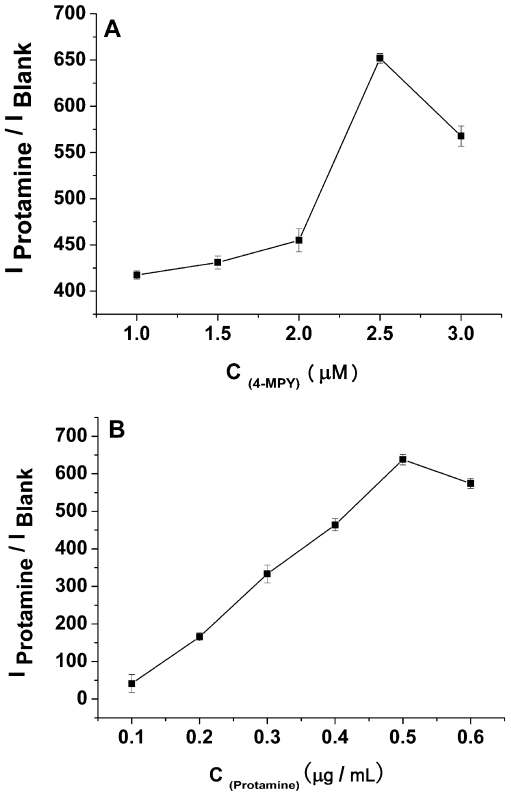


Figure 3. (A) Effect of the concentration of 4-MPY (from 1.0 to 3.0 μM) of the sensing system on the Raman signal intensity in the presence of 0.5 μg/mL protamine. (B) Effect of the concentration of protamine (from 0.1 to 0.6 μg/mL) of the sensing system on the Raman signal intensity in the presence of 2.5 μM 4-MPY. The error bars represent the standard deviations based on three independent measurements.

in the Supporting Information, we have also investigated different concentrations of HA and Chs, analogues of heparin, at the same concentration, as well as 10-fold concentration of heparin, the selectivity of this method was still ideal. It is mainly due to their low charge density per repeat unit. Generally speaking, heparin possesses one carboxylate per repeat unit and

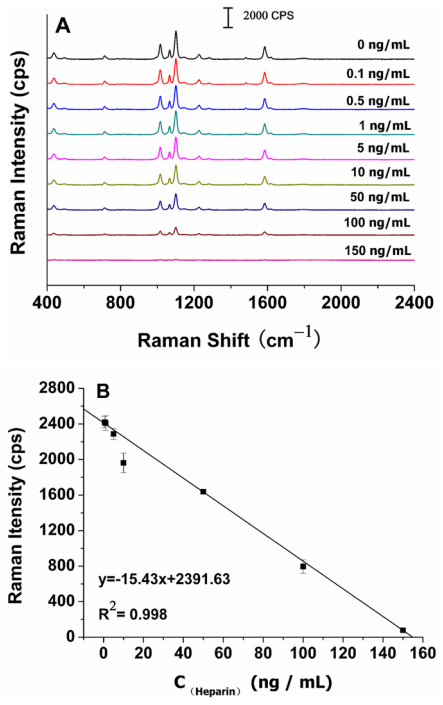


Figure 4. (A) SERS spectra changes of proposed probe upon addition of different concentrations of heparin (0–150 ng/mL). (B) Plot of corresponding intensity of the Raman band at 1099 cm^{-1} versus the concentration of heparin range from 0.5 to 150 ng/mL (R^2 = 0.998).

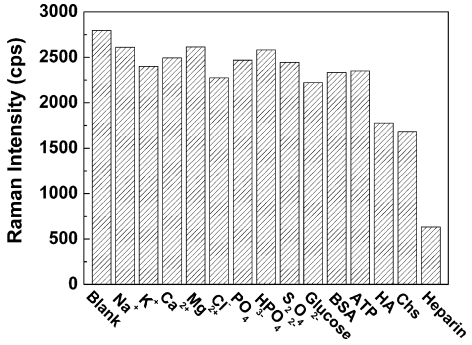


Figure 5. Raman corresponding intensity responses of proposed probe in 10 mM HEPES solutions (pH 7.4) upon the addition of heparin and various potentially coexisting species respectively: 10 mM Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, PO_4^{3-}, HPO_4^{2-}, $S_2O_4^{2-}$, glucose; 0.5 μg/mL BSA; 1000 ng/mL HA and Chs; 100 ng/mL ATP and heparin.

three sulfate groups; however, HA has only one carboxyl group, and Chs has one sulfate and one carboxylate group. Thus, the electrostatic attraction between protamine and HA or Chs was obviously weaker than that between protamine and heparin.[18] All these results indicated that the developed sensor program possessed excellent selectivity and reliability toward heparin.

Sensing Performances in Real Samples. To test the practicality of the developed sensing strategy for practical sample analysis, heparin was measured in FBS and blood samples. Here, heparin was complicated biological fluids, suffering from matrix effect. As seen from Figure 6, 0.8 μg/mL protamine was selected for eliminating the possible matrix interference of FBS samples. The peak around 1099 cm^{-1} remained as an optimum peak for the quantitative detection of heparin in FBS samples. SERS intensity response versus heparin concentrations (0–400 ng/mL) was shown in Figure

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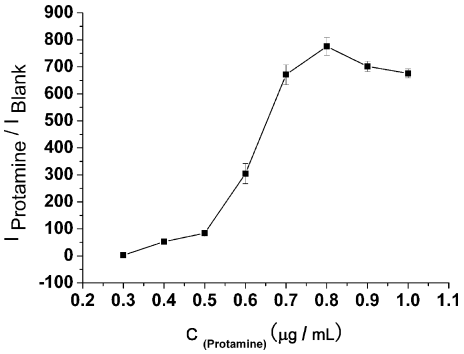

Figure 6. Effect of the concentration of protamine (from 0.3 to 1.0 μg/mL) of the FBS samples sensing system on the Raman signal intensity in the presence of 2.5 μM 4-MPY.

7A, and a satisfying linear relationship was obtained over the range of 1−400 ng/mL with the correlation coefficient of 0.987,

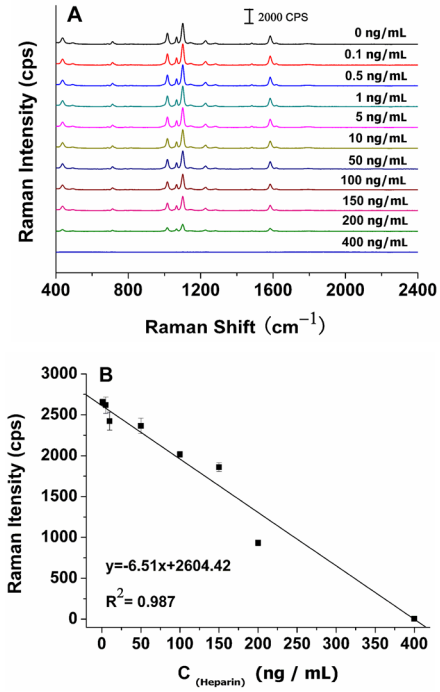

Figure 7. (A) SERS spectra changes of proposed probe in spiked FBS sample upon addition of different concentrations of heparin (0−400 ng/mL). (B) Plot of corresponding intensity of the Raman band at 1099 cm^{-1} versus the concentration of heparin range from 1 to 400 ng/mL (R^2 = 0.987).

as seen from Figure 7B. SERS spectra change between 10 and 50 ng/mL was small. However, we could distinguish easily through the magnified image, as seen from Figure S4 in the Supporting Information. We noted that the minimum detectable concentration value was similar to that in the standard solution of HEPES. As shown in Figure S5 in the Supporting Information, the matrix interference of blood samples was not able to eliminate very well even at 80 μg/mL of protamine spiked. As far as we know, blood is very complicated biological fluids. Compared with serum, blood contains abundant fibrinogen and other enzymes, and the complicated matrix might disturb electrostatic interaction of the detection system; thus, we think this probe is more suitable for determination heparin of in FBS samples.

CONCLUSIONS

A novel SERS sensing strategy has been developed for the detection of heparin in aqueous media based on antiaggregation of 4-MPY functionalized Ag NPs. By taking advantage of the strong affinity between protamine and heparin, excellent analytical performance in terms of sensitivity, selectivity, linearity, and limits detection of heparin was attained. Compared with other methods for the detection of heparin, it provided good performance as well (as seen from Table S1 in the Supporting Information), and materials used in our method were inexpensive and available commercially. Furthermore, this sensing method exhibits satisfying results in FBS samples, which indicates a great practicality for application in real analysis/monitoring and other related fields.

ASSOCIATED CONTENT

Supporting Information

UV−vis spectra of Ag NPs in different conditions; Raman spectra of different types of Raman reporters functionalized Ag NPs; Raman intensity responses of the developed method for heparin and different concentrations of HA and Chs; SERS spectra changes of proposed probe in spiked FBS sample upon addition of 10 ng/mL and 50 ng/mL of heparin; Raman spectra of 4-MPY-Ag NPs with different concentration of protamine in blood samples; and a comparison of other methods for the detection of heparin with our proposed method. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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