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An ultrasensitive "turn-off" SERS sensor for quantitatively detecting heparin based on 4-mercaptobenzoic acid functionalized gold nanoparticles†

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An ultrasensitive "turn off" Surface Enhanced Raman Spectroscopy (SERS) sensor was developed for the detection of heparin based on the anti-aggregation of 4-mercaptobenzoic acid stabilized gold nanoparticles. In this paper, protamine, a small protein molecule with positive charges, could induce the aggregation of 4-MBA functionalized gold nanoparticles *via* surface electrostatic interaction. However, in the presence of heparin, the aggregation of 4-MBA functionalized gold nanoparticles decreased due to the fact that heparin has a strong affinity toward protamine, further causing the loss of the Raman enhanced effect. The normalized SERS intensity of the Raman reporter was proportional to the concentration of added heparin and a good linear detection range was obtained from 0.05 to 20 ng mL^{-1} (R^2 = 0.999) with a calculated detection limit of 0.03 ng mL^{-1}. Moreover, the developed highly selective method is also successfully demonstrated in fetal bovine serum. Our method is specific, simple and cheap, which could be applied to other further research studies of heparin.

Introduction

Heparin is a kind of natural anticoagulant substance[1] which has large negative charges,[2] and now it is being widely used as an anticoagulant in the surgery of extracorporeal blood circulation[3,4] such as thrombosis and surgical treatment of thrombotic disorders. However, heparin overdose usually leads to some potentially fatal bleeding complications and other adverse reactions.[5,6] Therefore, heparin detection plays a crucial role, especially for pediatric patients. In recent years, researchers have developed many new heparin detection methods, such as colorimetry,[7–9] fluorescence spectroscopy,[10–12] spectrophotometry[13–15] and other electrochemical methods.[16–18] But there are still difficulties in the quantitative detection of heparin because of its natural polydispersity[19] and chemical heterogeneity,[20] as well as weak fluorescence.[21] Hence there is an urgent need to develop fast, simple, cheap and sensitive detecting methods.

Raman spectroscopy is based on inelastic light scattering by molecular vibrations, which could provide molecular fingerprint information. However, its widespread applicability is limited by the intrinsically low scattering intensity due to the small Raman scattering cross section of most molecules.[22] Surface Enhanced Raman Spectroscopy (SERS)[23] has attracted more and more attention because it has an ultrahigh detection sensitivity that is 10–14 orders of magnitude higher than that of Raman spectroscopy.[24] Over the last few decades, SERS has become a powerful analytical technique and its two theoretical mechanism models, electromagnetic mechanism (EM) and chemical mechanism (CM), have been well developed. In general, the EM is the dominant contributor to SERS, which can be largely influenced by various factors, such as the nanoparticles' composition, their size and shape, the surrounding medium, and their aggregation state. Recently, the SERS sensor based on the aggregation state of Au/Ag nanomaterials has gained popularity in the detection of protein, DNA and heavy ions.[25,26] Li's group developed a method for the ultrasensitive and selective detection of copper(II) and mercury(II) ions utilizing coordination between the SERS probe on the surface of the silver nanoparticles and the metal ions present.[27] Liang and his coworkers introduced aggregated silver nanoparticle (AgNP)-based SERS into an ELISA (enzyme-linked immunosorbent) signal generation system and achieved an ultrasensitive detection of proteins and small molecules.[28]

In this paper, we report a simple and efficient method for quantitatively detecting heparin using surface enhanced Raman scattering. The method utilizes a "turn off" SERS sensor with 4-mercaptobenzoic acid (4-MBA) modified gold nanoparticles.

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Protamine, a positively charged protein, can lead to the aggregation of 4-MBA modified gold nanoparticles due to electrostatic interaction. But, heparin has a stronger ability to bind protamine *via* electrostatic interaction,[29] which has been applied to clinical and experimental practice. So in the presence of heparin, the aggregation effect of gold nanoparticles would recede, and then the Raman signals would decrease. Meanwhile, there is also experimental evidence to prove that the method provides an ultrasensitive, selective and practical strategy for heparin detection.

Experimental

Reagents and chemicals

Chloroauric acid ($HAuCl_4$) and protamine sulfate salt were purchased from Sigma-Aldrich (Shanghai, China). Heparin (sodium salt, 185 U mg^{-1}) and Rhodanine 6G (R6G) were obtained from Aladdin Chemistry Co. Ltd. (Shanghai, China). Sodium chloride (NaCl), potassium chloride (KCl), magnesium chloride ($MgCl_2 \cdot 6H_2O$) and D-Glucose were acquired from Beijing Chemical Works (Beijing, China). Ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) was obtained from Tianjin Jinke Fine Chemical Research Institute (Tianjin, China). Aluminium chloride hexahydrate ($AlCl_3 \cdot 6H_2O$) and L-ascorbic acid were purchased from Xilong Chemical Co. Ltd. (Guangzhou, China). 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was purchased from J&K Scientific Co. Ltd. (Beijing, China) and HEPES buffer solution was prepared from ultrapure water and adjusted with 1 M NaOH to pH 7.4. Adenosine 5′-triphosphate (ATP), folic acid (FA) and chondroitin sulfate (Chs) were purchased from Sangon Biotech (Shanghai, China) Co., Ltd. Fetal bovine serum was obtained from Tianhang Biological Technology Co. Ltd. (Zhejiang, China) and the stock solution was cryopreserved.

Instruments

SERS measurements were performed using a confocal Raman Spectroscopy instrument (Jobin Yvon HR-800, France) equipped with a 50× long working distance lens and a 647 nm diode He–Ne laser source. The system uses 600 lines per mm gratings and its laser power is 30 mW. All Raman spectra were recorded in the range of 600–2000 cm^{-1} with an exposure time of 3s. Transmission electron microscopy (TEM) images were recorded on a JEM-2100 (HR, Japan) with an accelerating voltage of 200 kV. The UV-visible transmission spectra were obtained using a Hitachi U-3900H spectrophotometer (Hitachi Ltd., Japan). The zeta potential was acquired using a Malvern ZetasizerNano-ZS90 (ZEN3590). The ultrapure water used throughout the experiments was purified using a Milli-Q A10 filtration system (Millipore, Billerica, MA, USA).

Preparation of AuNPs

The 41 nm diameter AuNPs were synthetized by citrate-mediated reduction of $HAuCl_4$ on the basis of the literature.[30] For the uniformity of the colloid, all glassware used in the synthesis experiment was cleaned with freshly prepared aqua regia, rinsed thoroughly with water and dried in an oven. First, 50 mL of $HAuCl_4$ (0.01% m m^{-1}) was heated with stirring. When boiling, 0.50 mL of 1% (m m^{-1}) sodium citrate solution was added quickly, resulting in a change in the color of the solution from pale yellow to purple, and then the solution was kept boiling for another 30 minutes. Finally, the colloidal gold was cooled naturally to room temperature under stirring and stored in a glass reagent bottle at 4 °C. The properties of the obtained AuNPs were measured by UV-vis spectrophotometry in conjunction with TEM images.

4-Mercaptobenzoic acid functionalized AuNPs

50 μL of 4-mercaptobenzoic acid was added into 4.95 mL of AuNPs for a final concentration from 10 μM to 100 μM. The solution was kept under continuous stirring for 1 h and then left overnight at room temperature for further testing.

Sensitive detection of heparin

For heparin detection, 10 mM, pH = 7.4 HEPES buffer solution was used to make standard solutions of heparin and protamine. First, heparin solutions of various concentrations (0–100 ng mL^{-1}) were mixed with 0.6 μg mL^{-1} protamine several times. And then 5 mL of 4-mercaptobenzoic acid functionalized AuNP solution was added into the mixture and incubated for a few minutes. Finally, the Raman spectra were recorded with an exposure time of 3 s at ambient temperature.

Selectivity assay toward heparin

To prove the good selectivity of this method towards heparin, we investigated the response of the SERS sensor toward some interfering ions and substances under the same optimized conditions, including Na^+, K^+, Mg^{2+}, Ca^{2+}, Al^{3+}, $H_2PO_4^-$, $S_2O_7^{2-}$, glucose, AA, ATP, folic acid (FA) and chondroitin sulfate (Chs).

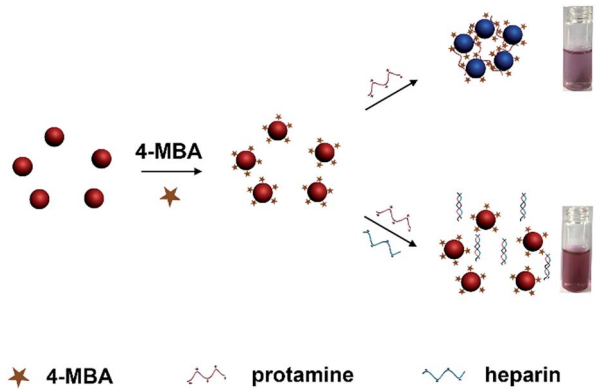
Detection of heparin in FBS

We studied the practical performance of our approach by detecting heparin in 0.1% FBS diluent (v/v, diluted with 10 mM HEPES buffer). We used FBS diluent to prepare heparin solutions by the standard addition method.

Results and discussion

The mechanism of the heparin detection sensor

The project rationale of heparin detection is illustrated in Scheme 1. It's well known that there is a strong non-covalent interaction between protamine and heparin. During the detection process of heparin, we employed 4-MBA as the Raman probe molecule for obtaining simple, narrow and characteristic peaks. During normal SERS measurement, we can observe that gold nanoparticle functionalized 4-MBA still remained dispersed and the colour was amaranth, the same as that of the AuNPs (see Fig. 1 and S-1(a)†). After the addition of protamine, which is a low molecular weight protein with 20 positive charges and rich in basic arginine residues, negatively charged 4-MBA functionalized AuNPs would be reunited, resulting in



Scheme 1 Quantitative detection of heparin utilizing electrostatic interaction between protamine and 4-MBA functionalized AuNPs.

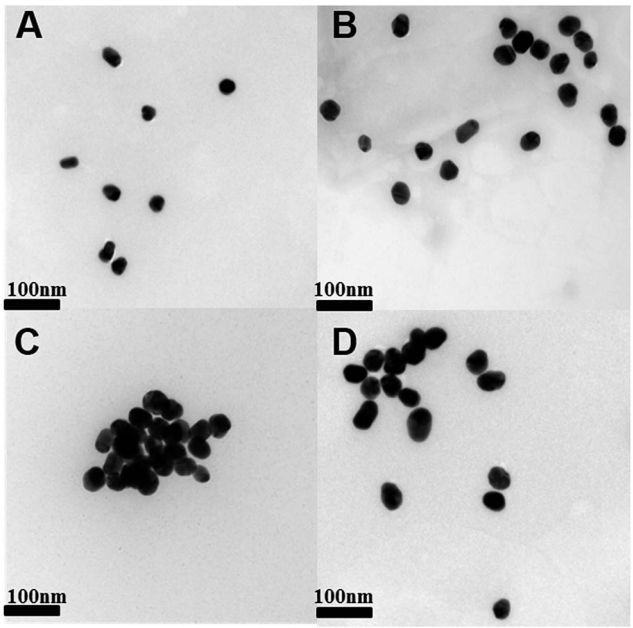


Fig. 1 TEM images of (A) AuNPs; (B) 4-MBA functionalized AuNPs; (C) 4-MBA functionalized AuNPs + protamine (0.6 μg mL^{-1}); and (D) 4-MBA functionalized AuNPs + protamine (0.6 μg mL^{-1}) + heparin (20 ng mL^{-1}).

electrostatic interaction. However, in the presence of heparin, protamine would preferentially bind with heparin due to their strong affinity, and then the aggregation of 4-MBA stabilized AuNPs receded, displaying a decrease of the SERS signal. Meanwhile, we also measured the zeta potentials of 4-MBA modified gold nanoparticles before and after the addition of protamine and heparin (see Table S-2†). From Table S-2,† we can see that there is a lowest zeta potential in the presence of protamine, which indicated the aggregation of 4-MBA functionalized AuNPs induced by protamine. Furthermore, the zeta potential increased after the addition of heparin, suggesting that the dispersal of 4-MBA modified gold nanoparticles occurred due to a stronger interaction between protamine and heparin.

In Fig. 2, the Raman spectra of 4-MBA and UV-vis spectra of the different samples are presented to verify the feasibility of our sensor. As shown in Fig. 2A, a strong SERS signal appears when 4-MBA, gold nanoparticles and protamine exist simultaneously, whereas, there was a signal decrease when heparin replaced protamine (the red line). Moreover, the Raman signal would be reduced after the addition of heparin and protamine. The same phenomenon was also evidenced by the UV-vis spectra (See Fig. 2B). The UV-vis spectrum of the mixture of AuNPs and 4-MBA was similar to that of AuNPs, which indicates that AuNPs functionalized with 4-MBA still remained dispersed and stable. When protamine was mixed with AuNPs and the probe, the absorbance of AuNPs would be lower, which resulted from protamine inducing the aggregation of 4-MBA functionalized AuNPs. Upon the coinstantaneous presence of heparin (20 ng mL^{-1}) and protamine, 4-MBA modified AuNPs would disperse again and the absorbance becomes higher than that when protamine was present alone on account of the concentration of protamine decreasing due to binding interaction between protamine and heparin instead of protamine and AuNPs.

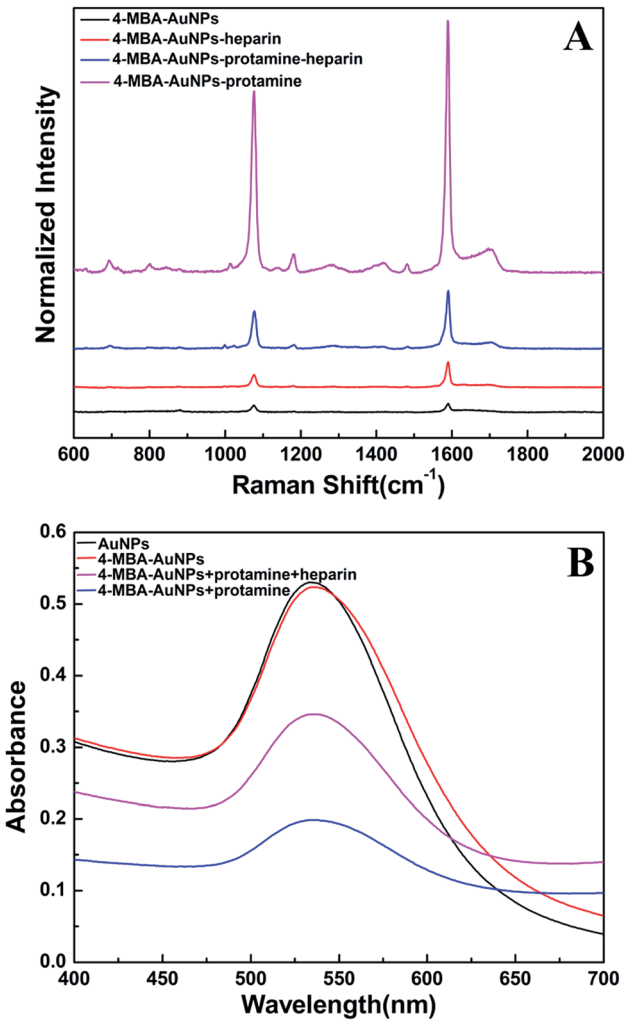


Fig. 2 (A) Raman spectra and (B) UV-vis spectra of the different samples.

The optimization of experimental conditions

For the sake of the best detection effect, several experimental conditions were investigated including concentrations of Raman probes and protamine and the molecular type of Raman reporters. However, the influence of nanoparticles' size should be investigated before discussing the optimization of 4-MBA and protamine concentration. As shown in Fig. S-2,† the Raman signal of 13 nm AuNPs is almost zero in the presence of 0.4 μg mL^{-1} protamine compared with that of 41 nm AuNPs. In addition, other researchers have observed that the optical absorption of a small particle has a dominant effect and larger particles would limit biomedical therapeutic applications.[31] Therefore, gold nanoparticles with the size of 41 nm were used in the following experiment.

The concentration influence of 4-MBA and protamine in this SERS assay of heparin has been shown in Fig. 3 and 4 and the comparison results of different Raman reporters were plotted in Fig. S-4.†

Fig. 3 shows the Raman normalized intensity (I/I_0) of the characteristic band at 1590 cm^{-1} in the presence of protamine that changes with concentrations of 4-MBA. As shown in Fig. 3, the relative intensity reaches a maximum value when the concentration of 4-MBA is 40 μM. This is because further increasing 4-MBA concentration would also induce AuNP aggregation. In addition, we also present a relationship between concentrations of 4-MBA and UV-vis absorbance in the presence of 0.6 μg mL^{-1} protamine (Fig. S-3†). Likewise, there is a turning point at 40 μM because a concentration lower than 40 μM would be less efficient and unfavorable to quantitative detection and a higher concentration would lead to the decrease of the amounts of protamine due to the formation of a peptide from protamine and the carboxyl group of the redundant 4-MBA. Thus, we chose 40 μM 4-MBA as the optimal concentration.

As illustrated in Fig. 4, the effect of protamine concentrations was investigated and 0.6 μg mL^{-1} protamine generated the most obvious aggregation of AuNPs due to electrostatic interaction between protamine and 4-MBA modified gold nanoparticles, resulting in increasing of the relative intensity.

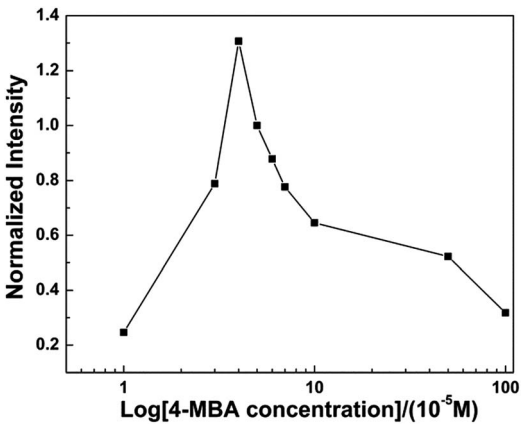


Fig. 3 The curve of the relationship between the concentration of 4-MBA and normalized intensity of the characteristic band at 1590 cm^{-1} in the presence of protamine.

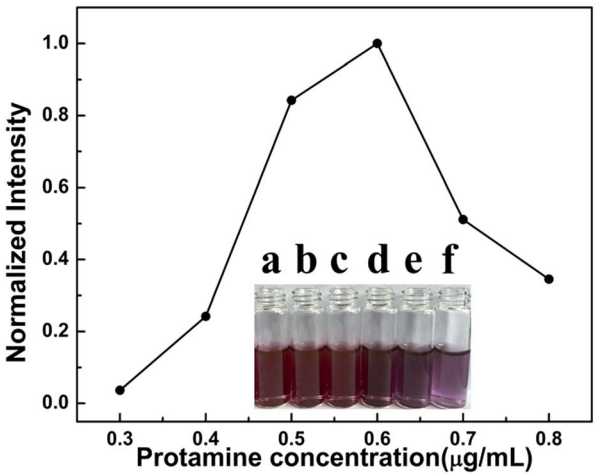


Fig. 4 SERS intensity at 1590 cm^{-1} *versus* the concentration of 4-MBA in the presence of protamine. Inset: photographs of 4-MBA-AuNPs/protamine in aqueous solution containing protamine with different concentrations ((a): 0.3 μg mL^{-1}; (b): 0.4 μg mL^{-1}; (c): 0.5 μg mL^{-1}; (d): 0.6 μg mL^{-1}; (e): 0.7 μg mL^{-1}; (f): 0.8 μg mL^{-1}).

The increase could be clarified by electrostatic interaction between 4-MBA modified gold nanoparticles and protamine. Further increasing protamine concentration would lead to a gradual decrease because protamine was a stabilizer towards 4-MBA modified AuNPs at high concentration.

Ultrasensitive sensing of heparin

To quantitatively detect heparin utilizing the proposed strategy, the SERS spectra of 4-MBA modified AuNPs in the presence of heparin with different concentrations were recorded under the optimized conditions. Fig. 5A depicts some Raman spectra of 4-MBA functionalized AuNPs with the addition of different concentrations of heparin (0–20 ng mL^{-1}). As shown in Fig. 5A, there are two obvious SERS peaks at 1077 cm^{-1} and 1590 cm^{-1}, which correspond to the $\nu_{(c-c)}$ ring-breathing modes of 4-MBA.[32] The normalized intensity (I/I_0) of these bands increased gradually with the increasing concentration of heparin as well as a colour change of the solution (Fig. S-1†). In our work, the intensity of the Raman band around 1590 cm^{-1} was chosen as

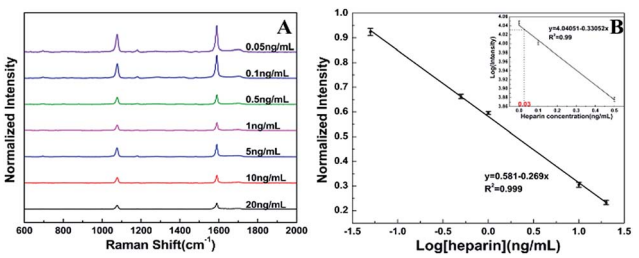


Fig. 5 (A) SERS spectra of 4-MBA-AuNPs with different concentrations of heparin in the presence of protamine (0.6 μg mL^{-1}). (B) The Raman relative intensity at 1590 cm^{-1} varies with different concentrations of heparin under the optimized conditions. The inset of B shows the limit of detection of heparin.

the response signal for quantitatively detecting heparin. There is a good linear relationship for heparin in the concentration range from 0.05 to 20 ng mL^{-1} as shown in Fig. 5B. The linear regression equation of the normalized intensity values at 1590 cm^{-1} to the logarithms of the concentrations of heparin is $y = 0.581 - 0.269x$ with a squared correlation coefficient of 0.999, which is qualified for quantitative detection by this method. As shown in the inset of Fig. 5B, the limit of detection of the SERS sensor for heparin is as low as 0.03 ng mL^{-1} (S/N = 3), which is lower than those of the reported methods for a large measuring range for heparin, and the linear response ranges are wider than the lower LOD (Table S-1†). The developed method was proved to be ultrasensitive and highly reliable for the detection of heparin levels with the excellent low LOD. In addition, the UV-vis spectra also showed the rise of absorbance along with the increase of concentration of heparin (as shown in Fig. S-5†).

Selectivity of the heparin assay

Subsequently, it's necessary to investigate whether this sensor has a specific response to heparin for practical application. Therefore, we utilized our method under the same optimized conditions to detect some general interfering agents, including Na^+, K^+, Mg^{2+}, Ca^{2+}, Al^{3+}, $H_2PO_4^-$, $S_2O_7^{2-}$, glucose, AA, ATP, FA and Chs. For discussing the possibility of biological application, the concentrations of ATP, FA and Chs were consistent with heparin at 20 ng mL^{-1} while the concentrations of other interfering substances were 5 mM. As manifested in Fig. 6, none of these interfering ions and substances could cause an obvious change of the Raman signal as heparin did. Hence, our SERS sensor has a high specificity toward heparin.

Practical analysis in fetal bovine serum

To investigate potential application of the designed assay in practical detection, we tested fetal bovine serum (FBS) diluent samples with different concentrations of heparin and evaluated the recoveries. As we all know, FBS is a complicated practical environment. To avoid the possible matrix interference, we diluted FBS with HEPES buffer to reduce the concentrations of possible interferents firstly as reported in various manuscripts.[33,34] Secondly, it's proved that FBS has no effect on detecting heparin in biological systems in another manuscript.[35] Table 1 shows a satisfactory result of the recoveries varying from 90.99% to 104.20% with a low RSD less than 3.21%. The result indicates that there is a promising future for the proposed method to perform in real samples, and the possibility of detecting heparin in human blood could be investigated.

Table 1 The performance of the developed method in 0.1% FBS

Sample	Added (ng mL^{-1})	Calculated (ng mL^{-1})	Recovery (%)	RSD (%)
1	0.05	0.048	95.57	3.21
2	0.10	0.095	95.25	2.89
3	0.50	0.521	104.20	2.78
4	10.00	9.099	90.99	2.05

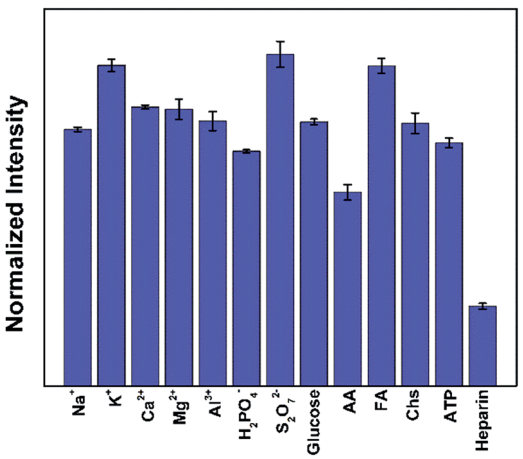


Fig. 6 Selectivity of the detection assay of heparin (20 ng mL^{-1}) (normalized intensity of the characteristic Raman band at 1590 cm^{-1}) over other potentially coexisting species.

Conclusions

In summary, we have carried out a novel "turn off" strategy for quantitative SERS detection of heparin with high sensitivity and selectivity based on the specific interaction between heparin and protamine, which is characterized by spectral changes of 4-MBA as the Raman signal reporter. Our developed sensor demonstrates several analytical virtues. Firstly, the proposed sensor achieves high sensitivity with an extremely low detection limit of 0.03 ng mL^{-1}. Secondly, high selectivity towards heparin can be obtained due to the particular affinity of protamine and heparin. Furthermore, the method here provides a satisfying result to detect heparin in fetal bovine serum. Therefore, this novel biosensor-based SERS sensing approach presents a general platform for the simple, ultrasensitive, selective and practical detection of heparin.

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