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Electrostatic Interaction Based Approach to Thrombin Detection by Surface-Enhanced Raman Spectroscopy

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We have developed an electrostatic interaction based biosensor for thrombin detection using surface-enhanced Raman spectroscopy (SERS). This method utilized the electrostatic interaction between capture (thrombin aptamer) and probe (crystal violet, CV) molecules. The specific interaction between thrombin and aptamer could weaken the electrostatic barrier effect from the negative charged aptamer SAMs to the diffusion process of the positively charged CV from the bulk solution to the Au nanoparticle surface. Therefore, the more the bound thrombin, the more the CV molecules near the Au nanoparticle surface and the stronger the observed Raman signal of CV, provided the Raman detections were set at the same time point for each case. This procedure presented a highly specific selectivity and a linear detection of thrombin in the range from 0.1 nM to 10 nM with a detection limit of about 20 pM and realized the thrombin detection in human blood serum solution directly. The electrostatic interaction based technique provides an easy and fast-responding optical platform for a "signal-on" detection of proteins, which might be applicable for the real time assay of proteins.

Thrombin (activated Factor II) is a specific serine protease involved in the coagulation cascade, which converts soluble fibrinogen into insoluble strands of fibrin and catalyzes many other coagulation-related reactions.[1] Thrombin is crucial in physiological and pathological coagulation, and it regulates many processes in inflammation and tissue repair at the vessel wall.[2] Normally, the concentration of thrombin in blood during the coagulation progress varies from nM to low μM levels,[3] while the detection at high pM range is important for related diagnoses.[4] Therefore, it is necessary to develop a sensor toward thrombin detection with high sensitivity, selectivity, and simplicity.

In recent years, electrochemical[5−11] and optical techniques[12−17] were widely applied in the field of thrombin detection with various methods. Among the optical detection methods, surface-enhanced Raman spectroscopy (SERS) distinguishes itself with several advantages: the unique Raman spectrum offers enormous structure information content about analyte, and the narrow peak width avoids peak overlapping in complex systems; the Raman signal is free from problems associated with photobleaching and self-quenching, while it can be excited at any wavelength;[18] the single-molecule detection ability makes SERS comparable to fluorescence for trace amount detection.[19−21] SERS has been successfully applied to qualitatively and quantitatively analyze chemical or biological species from small biomolecules[22] to DNA,[23] proteins,[16,17,24] and even cells[25] with high sensitivity and selectivity. Recently, by combining the sandwiched bioassay format with structure and Raman labeled probes obtained by either coadsorption[16] or conjugation of Raman label and capture probes on Au NPs, SERS

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has realized the thrombin detection with a detection limit at 0.1 pM.[17] Among the above SERS based detections either for thrombin or for other proteins and DNA, the main focus is to design Raman labeled probes with high sensitivity and stability while there is only one paper that has mentioned the advantage of the electrostatic interaction between target, capture, and probe molecules in connection with the SERS technique.[23h]

Therefore, in this paper, taking thrombin as the model protein, we developed a novel SERS biosensor on the basis of the electrostatic interaction. Both the electrostatic interaction between the positively charged probe molecule (crystal violet, CV) and the negatively charged thrombin aptamer SAMs, and the specific interaction between thrombin and aptamer, were involved for the procedure. It was observed that provided the Raman signal was recorded at the same time point, the more the thrombin was bound with the aptamer, the stronger the SERS signal of CV observed, which showed a linear relationship in the range of thrombin concentration from 0.1 nM to 10 nM. Other interference, IgG, BSA, and Human Serum were selected to investigate the selectivity of the assay. The proposed procedure was demonstrated as a convenient "signal-on" detection platform for thrombin with high sensitiviy and selectivity.

EXPERIMENTAL SECTION

Materials and Apparatus. Human thrombin (37423 mg/mol) was purchased from Sigma-Aldrich Co., Ltd. The 27-base thrombin aptamer modified at the 3′ end with the thiol linker, 5′-GGT TGG TGT GGT TGG CCA ACC TTA AGG-$(CH_2)_6$-SH-3′, 5′-FITC (Fluorescein-5-isothiocyanate)-GGT TGG TGT GGT TGG CCA ACC TTA AGG-$(CH_2)_6$-SH-3′, and the two sites mismatched aptamer sequence 5′-GGT TGG TGT TGT TTG CCA ACC TTA AGG-$(CH_2)_6$-SH-3′ were from Takala Biotechnology Co., Ltd. (Dalian, China). $HAuCl_4 \cdot 3H_2O$, NH_2OH, trisodium citrate, CV, and other chemicals were all of analytical grade and used as obtained. Ultrapure water with an electrical resistance larger than 18.2 MΩ was used throughout.

The buffers for the experiments included the following: (a) immobilization buffer (TE), 10 mM Tris, 1 mM EDTA, and 1 M NaCl; (b) phosphate buffer solution (PBS), 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl; (c) binding buffer, 100 mM Tris, 140 mM NaCl, 20 mM $MgCl_2$, 20 mM KCl. The pH value of all buffers was 7.4.

For Raman measurements, a confocal microprobe Raman instrument (RamLab-010, Jobin Yvon Horiba, France) was used. A 632.8 nm He−Ne laser excitation (0.1mW) and a 50 × long working-distance objective (8 mm) were used in this work. The width of the slit and the size of the pinhole were set as 100 and 1000 μm, respectively. Raman mapping was carried out with 5 × 5 points in an area of 40 μm × 40 μm.

Preparation of SERS Active Substrates. Gold nanoparticles (Au NPs) with an average diameter of 56 nm (Figure S1a, Supporting Information) were prepared with a step-by-step growth procedure.[26,27] In brief, 9 mL of 1% sodium citrate was quickly added in 94 mL of 1 mM $HAuCl_4$ boiling water solution and kept boiling for 10 min under vigorous stirring to obtain a wine red Au seeds solution. The size of the obtained Au NPs is about 15 nm. Then, Au NPs centered at about 26 nm were generated by dropping 0.8 mL of 1% $HAuCl_4$ into a mixture containing 20 mL of Au seeds, 24 mL of 25 mM NH_2OH, and 56 mL of H_2O at room temperature. Finally, for Au NPs at about 56 nm, 0.9 mL of 1% $HAuCl_4$ was similarly added to a solution mixed with 10 mL of 26 nm Au NP solution, 9 mL of 25 mM NH_2OH, and 80 mL of H_2O.

The SERS substrate was obtained as follows: the so-prepared Au NPs were concentrated by centrifugation, and then 1 μL of the obtained solution was dropped on a cleaned Si (111) surface and dried in air slowly to form a uniform SERS active surface (Figure S1b, Supporting Information). It should be mentioned that the concentration of the Au NPs is very important to prepare the uniform substrate. Too high or too low a concentration of the sol−gel will reduce the uniformity of the substrate for Raman detection. The surface morphology of the substrate was determined by SEM images using a JSM-6700F microscope (JEOL, Ltd., Japan).

Thiolated Aptamer Immobilization. The thiolated aptamer was dissolved in TE buffer to a concentration of 1 μM. Immediately before use, the buffer containing aptamer was heated to 95 °C for 5 min, and then cooled to 72 °C followed by remaining at this temperature for 10 min to ensure proper intramolecular folding

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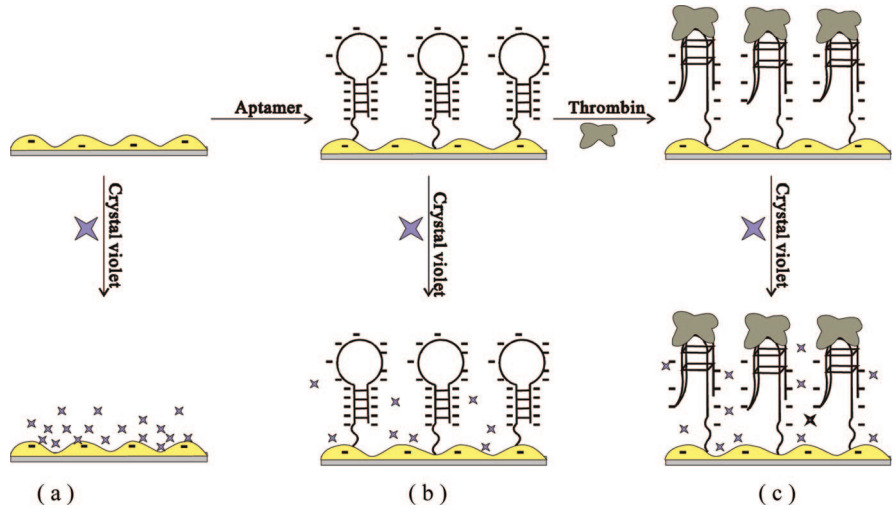

Figure 1. Schematic illustration of the procedure for thrombin detection according to the different amount of CV molecules contributed to the SERS signal due to the different electrostatic effects from the changing structure of the anchored thrombin aptamer.

into hairpin-loop conformations. The resulting solution (20 μL) was dropped on the Si (111) substrate and incubated at room temperature for 15 h to yield a self-assembled layer of the aptamer. The resulting surface with the aptamer anchored was rinsed thoroughly with PBS three times to remove the unbound aptamer.

Determination of Surface Coverage of the Aptamer. The surface coverage of the aptamer on the SERS surface was obtained by a traditional fluorescence technique,[28] that is, the FITC labeled aptamer was used instead, and the so-obtained Au NPs surface was incubated in the TE buffer containing 10 mM mecaptohexanol overnight to displace the modified aptamer; then, the solution was collected for fluorescence detection. The fluorescence maximums (measured at 520 nm) were converted to molar concentrations of the FITC labeled aptamer with reference to a standard FITC-labeled aptamer solution with the known concentration under the same conditions (Figure S2a, Supporting Information). Finally, the coverage of the aptamer on the surface was obtained by dividing the measured aptamer molar concentration by the surface area. The surface coverage obtained is about 29.8 pmol/cm,^{2} which is similar with the value previously reported.[28] It should be noted that the surface coverage could be varied from 29.8 to 11.7, 1.7, and 0.3 pmol/cm^2 by controlling the initial concentration of the aptamer solution at 1, 10^{-1}, 10^{-2}, and 10^{-3} μM, respectively (Figure S2b, Supporting Information).

Thrombin-Binding Assay Using SERS. The aptamer-functionalized surface was immersed in 20 μL thrombin binding buffer solution containing different concentrations in the range of 0.1 nM−1000 nM. After reacting 3 h, the surface was washed with the same binding buffer to remove the unbound thrombin. Finally, the aptamer-thrombin-modified surface was immersed in the binding buffer solution containing 1 μM CV to retain the stabilization of the 3D conformation between aptamer and thrombin[6] and obtain a strong SERS signal of CV molecules.[29]

RESULTS AND DISCUSSION

Analytical Principle. It is well-known that the SERS enhancement is very sensitive to the distance between the SERS-active substrate and the target molecules:[30−32] only the first layer of molecules directly adsorbed on the surface have the main contribution to the SERS signal obtained, although those at a distance of 10 nm near the surface also have a contribution. It indicates that the SERS signal is tunable by controlling the amount of the probe molecule near the SERS-active substrate, which could be realized through change of either the steric effect or the electrostatic interaction from other surrounding species. The effect of the latter one is demonstrated in Figure 1.

Here, by utilizing the fact that the SERS signal of the probe molecule could be tuned by changing the charging state of the SERS substrate by the electrostatic effect from the charged biological species modified,[23h,33] we tried to develop a simple biosensor for thrombin detection. A simple model describing the principle and procedure of thrombin detection and the related SERS spectra are shown in Figures 1 and 2, respectively.

When a Au NP surface free of aptamer is immersed in a buffer solution containing 1 μM CV with a solution depth of about 10 μm, a strong SERS signal of CV molecules is obtained as the Raman spectrum (a) in Figure 2. The molecular structure of CV is shown in the inset. The Raman spectrum clearly displays several strong Raman peaks at 912 cm^{-1} (ring skeletal vibration of radial orientation), 1168 cm^{-1} (in plane ring C−H bending), 1300 cm^{-1} (ring C−C stretching), 1377 cm^{-1} (N-Ph stretching) and 1530, 1582, 1617 cm^{-1} (ring C−C stretching).[34] Considering the characteristic of the SERS spectrum, that is, the positions and the relative intensities of Raman peaks, it is very similar to those reported before;[34,35] we deduce that CV molecules also parallel adsorb on the present surface. In the following discussion, the strongest Raman band at 1617 cm^{-1} is chosen as a characteristic SERS peak of CV for the quantitative analysis.

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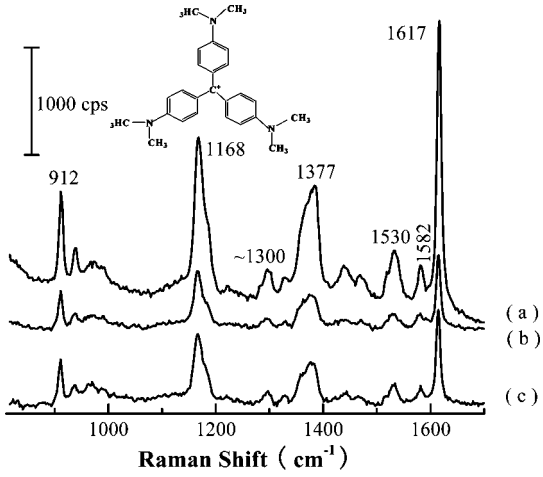

Figure 2. SERS spectra of CV on the Au NP surfaces: (a) naked one, (b) thrombin aptamer modified, (c) after incubated in 0.1 nM thrombin solution in the binding buffer solution containing 1 μM CV. The inset displays the molecular structure of CV.

After the surface was functionalized with the SAMs of the negatively charged thrombin-binding aptamer, one would observe from the diagram in Figure 1 that not only some adsorption sites of CV are occupied by the modified aptamer but also the electrostatic attraction from the aptamer drastically decreases the diffusion rate of CV molecules from the bulk solution to the interface. The latter will decrease the amount of CV molecules contributed to the SERS signal before the diffusion finishes. It has been observed that the diffusion might last for 1 h (vide infra). Therefore, both the occupation and the electrostatic attraction from the modified aptamer will induce the decreased SERS signal of CV molecules. As shown in Figure 2b, the SERS signal was only 0.25 for the CV upon modifying ss-DNA on the Au NP surface as compared to the case free of aptamer (Figure 2a).

The electrostatic attraction from the aptamer would decline because of its decreased negative charge upon binding thrombin (Figure 1),[7,11] which will neutralize part of the negative charge of the aptamer. Therefore, the diffusion process is accelerated and more CV molecules will be involved in the SERS signal observed, and then induce an enhanced SERS signal evidenced by the spectrum (c) shown in Figure 2, given that the Raman signal recording for both cases were set at the same time point. It clearly displays that after being incubated in 0.1 nM thrombin solution, the Raman intensity of the obtained SERS spectrum increased to a value of about 1.4 times that of the case before the introduction of thrombin as shown in Figure 2b.

It should be emphasized that the specific interaction between aptamer and thrombin also plays an important role for the diffusion process of CV. The SERS signal will decrease slightly but not increase obviously because of the nonspecific adsorption of thrombin on the substrate either before or after another non-totally matched SS-DNA, such as the one with two G bases that was replaced by T, being modified on the substrate (Figure S3, Supporting Information), which indicates that the nonspecific adsorption of thrombin is insignificant for the observed SERS signal of CV.

Since the electrostatic attraction from the modified aptamer before and after thrombin binding only decreases the diffusion rate of CV molecules from the bulk solution to the interface and would be gradually decreased with the prolonged incubation time in the CV solution, the SERS signal of CV would increase accordingly until a stable state is reached. Figure 3(a) shows the time dependent Raman intensity of the 1617 cm^{-1} peak on the aptamer modified surface free of thrombin. It clearly displays that the Raman intensity quickly increased about 550% (from 0.1 to 0.65) in the initial 20 min, and then increased another 30% in the next 30 min.

Meanwhile, the SERS spectrum difference between the conditions with different amounts of the bound thrombin is also decreased accordingly with the prolonged incubation period. Figure 3b shows the normalized Raman intensities at 5 min, 45 min, and 1 h for the cases of blank, 10 nM, and 100 nM binding thrombin, respectively. One easily observes that at 5 min, the difference between blank and 10 nM, or 10 nM and 100 nM, was about 30% while the difference is only about 5% for them at 1 h, respectively. The time dependent SERS signal indicates that the earlier the SERS detection, the more obvious the electrostatic interaction and the SERS signal difference between different systems.

The above results indicate that the effect from the electrostatic attraction of the modified thrombin aptamer on the adsorption of CV on the Au NP surface could be continuously changed through binding different amounts of thrombin, which provided a quantitative detection of thrombin on the basis of the tunable electrostatic interaction: the more the amount of the bounded thrombin, the stronger the SERS signal of CV molecules, that is, the SERS intensity of CV could be used as the indicator of the concentration of thrombin molecules in the system. Furthermore, to avoid the time dependent effect and optimize the difference of SERS signal between different systems, that is, the reproducibility and reliability of the experiment, the time for all the systems incubated in the CV solution was controlled at 5 min before SERS detection.

It should be noted that although the electrostatic interaction between protein and molecules has been widely utilized for protein detection,[7,11,36] both the near neutral of human thrombin with an isoelectric point at 7.0−7.6[11,37] in the binding solution (pH = 7.4) and the minor difference between different concentrations at 1 h (Figure 4b) indicate that the electrostatic interaction between thrombin and CV is negligible or the interaction has little effect on the observed SERS signal in our case.

Quantification of Thrombin Analyte. Before the quantification of thrombin, both the SEM image and the Raman mapping were applied to monitor the surface morphology change of the prepared Au NPs substrate under each step to testify to the robustness and reproducibility of the substrate. The similar surface morphology of the aptamer modified substrate before and after thrombin binding shown in Figure 4, panels a and b, respectively, clearly displays that the Au NPs physically adsorbed on the Si(111) surface are strong enough to endure the incubation and washing processes before SERS detection. Furthermore, considering that the laser illuminated area is about 2 μm × 2 μm (shown as the dashed frames in each Figure) for SERS detection, the obtained surface morphology is rather uniform at this scale, which is

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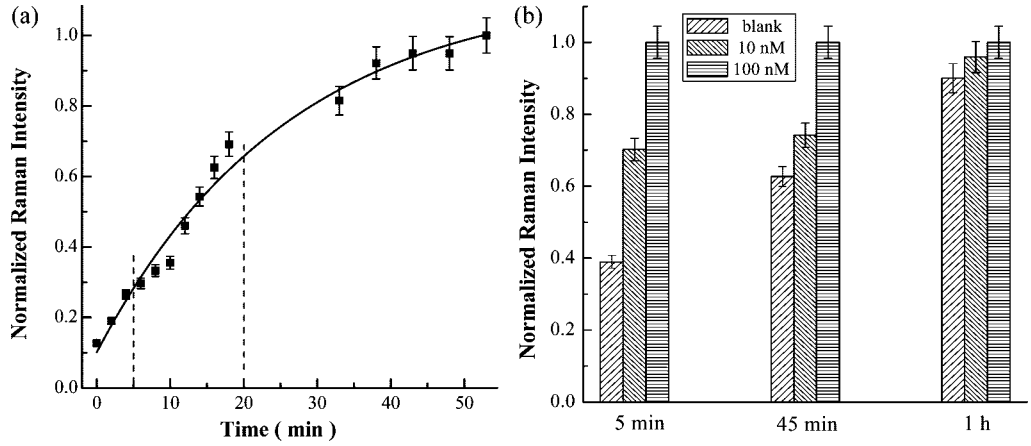

Figure 3. (a) Time dependent normalized Raman intensity of the 1617 cm^{-1} peak on the aptamer modified Au NP surface in the binding buffer solution containing 1 μM CV. (b) The normalized Raman intensity of this peak at different time intervals in the binding buffer solution containing 1 μM CV for the cases of 0 (blank system), 10 nM, and 100 nM thrombin bound with the aptamer on surfaces.

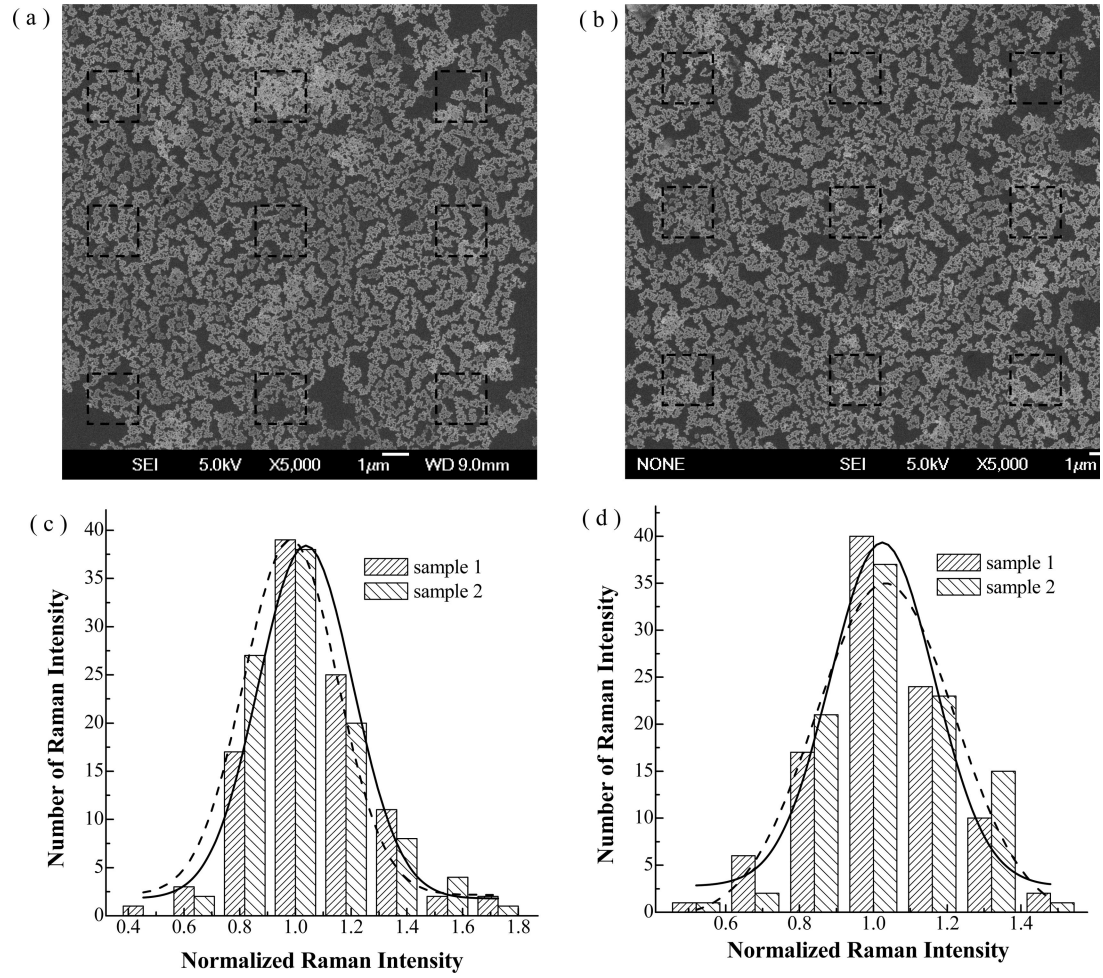

Figure 4. SEM images of the aptamer modified Au NPs surface before (a) and after thrombin binding (b). Statistic distributions of the normalized Raman intensity of the 1617 cm^{-1} peak on the aptamer modified Au NPs surface before (c) and after thrombin binding (d); each of the two curves are the statistical result from the two parallel substrates, respectively.

demonstrated by the very similar curve shape and peak position of the statistic distribution of SERS signal by Raman mapping at different sites on one surface (Figure S3, Supporting Information).

Panels c and d of Figure 4 give the statistic distribution of the normalized SERS signals on the two parallel substrates according to panels a and b of Figure 4, respectively. Each distribution curve was a statistical result of 100 Raman lines obtained by Raman mapping four times at different sites. It clearly shows that each curve displays a rather sharp fwhm (full width of half-maximum), and the deviation of the peak positions of the two parallel results

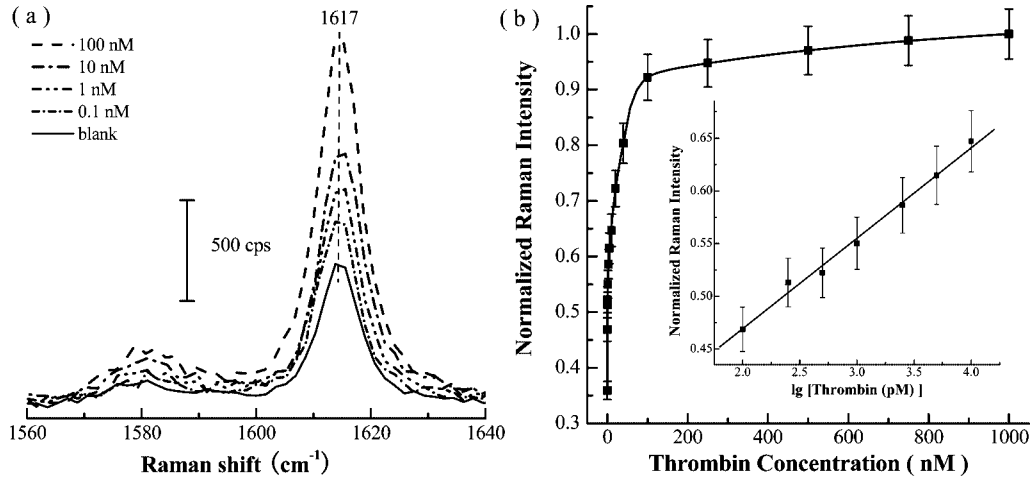

Figure 5. (a) SERS spectra of CV in the range from 1560 to 1640 cm^{-1} on the aptamer modified Au NP surface after binding thrombin with varying concentrations as mentioned for the binding buffer solution containing 1 μM CV;(b) Thrombin concentration (0−1000 nM) dependent normalized Raman intensity of 1617 cm^{-1} peak. The inset shows the calibration curves of thrombin in binding buffer (0.1−10 nM).

is less than 5%, although there are certain possibilities of observing either too weak or too strong SERS signals on some sites because of the random distribution of defects and multilayer structure on the prepared SERS substrates shown in Figure 4a,b.

Therefore, the robustness of the prepared SERS substrate and the excellent reproducibility from site to site and from sample to sample enable the quantitative detection of thrombin. In the following discussion, each data below represented an average result of at least 15 measurements with three parallel assays.

Figure 5a shows the SERS spectra of CV from 1560 to 1640 cm^{-1} on the aptamer modified Au NP surface in the binding buffer solution containing 1 μM CV after binding 0, 0.1, 1, 10, and 100 nM thrombin, respectively. One clearly observes that the Raman intensity of the 1617 cm^{-1} peak drastically increases with increasing thrombin concentration from 0 to 100 nM. Such a tendency obviously declines with the thrombin concentration as it further increases from 100 nM to 1000 nM (not shown). Meanwhile, we notice that the peak position does not change with the increasing thrombin concentration, which might indicate the existence of the modified aptamer (or the bounded thrombin), only displays the physical electrostatic effect as mentioned above, and has little effect on the adsorption behavior of CV.

Figure 5b shows the normalized Raman intensity of the 1617 cm^{-1} peak varying with the thrombin concentration in the range from 0 nM to 1000 nM. It clearly displays an almost exponential increasing curve in the tested concentration range: the Raman intensity increases by about 160% in the range from 0 to 100 nM and then only increases by less than 10% from 100 nM to 1000 nM. The increasing SERS signal with the thrombin concentration suggests that a "signal-on" biosensor is able to operate in this system.

The inset in Figure 5b shows a linear relationship for the natural logarithm over concentrations of thrombin ranging from 0.1 nM to 10 nM. The regression equation is $y = 0.30 + 0.08 \log x$ (where x is the target protein concentration in pM and y is the normalized Raman intensity of the 1617 cm^{-1} peak) with a correlation coefficient of 0.995. The response toward the target protein gave a relative standard deviation of 4.5%. By evaluating the average response of the blank plus three times the standard deviation,[38] a detection limit of 20 pM was calculated. Meanwhile, considering that every 20 μL thrombin solution was added in each test, the absolute detection limit of thrombin was at the level of 0.4 fmol. Furthermore, such a detection procedure has successfully realized thrombin detection in a complex matrix containing 0.1 nM thrombin and 50% human blood serum directly without any further analytical steps (vide infra).

It should be noted that although the obtained absolute detection limit (20 pM) has not been obviously improved compared with those reported for approaches with electrochemistry (0.5 nM)[9] or fluorescence (10^2 molecules)[13] techniques, the present procedure demonstrates a much easier, reproducible, and manipulable platform, considering the simple pretreatment of the sensor and the thousands of samples on one prepared surface since the laser illuminated area is about 2 μm × 2 μm and the area of the so-prepared SERS active surface is about 2000 μm × 2000 μm.

Selectivity of Sensing System. To determine the specificity of the procedure, a series of comparative studies between the thrombin and other proteins was performed. Other interference: BSA, human IgG, and Serum (human serum) were selected and detected individually with the same procedure as that of the thrombin. The related results are shown in Figure 6.

For the case of a naked Au NP surface free of aptamer modification, the SERS signal is the strongest. After the surface was modified by aptamer, a low background signal was observed at the blank one where the binding buffer is free of target proteins, whereas the introduction of 0.1 nM thrombin induced the SERS signal to increase about 28% in comparison with the blank one.

Interestingly, one observes that all the SERS signals obtained from these cases of 7 μM IgG, 150 nM BSA, and 50% Serum were rather lower than that from the blank one, which decreased about 12%, 28%, and 12%, respectively. Such a tendency of the SERS signal of CV to decline with the introduction of the other proteins might be a result of the nonspecific adsorption of these proteins, which might induce somewhat a steric hindrance effect to block some adsorption sites of CV on the Au NP surface, and the

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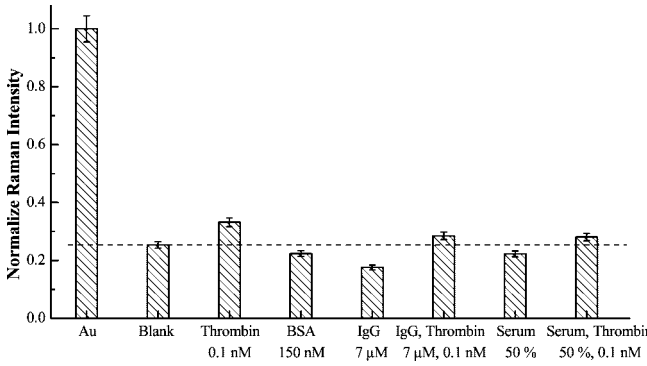

Figure 6. Specificity analysis of the SERS assay under different conditions: Au (naked Au surface), blank (aptamer modified Au surface), and treated by 0.1 nM thrombin, 150 nM BSA, 7 μM IgG, mixture of 0.1 nM thrombin and 7 μM IgG, 50% Serum, and mixture of 0.1 nM thrombin and 50% Serum, respectively.

electrostatic repulsion with the negative charge in the binding solution (the isoelectric point is ca. 7.0, 5.4, and 5.3 for BSA,[39] IgG[40] and Serum,[41] respectively).[37] When these proteins were mixed with thrombin and incubated together, the SERS signal will decrease slightly compared to the case containing only 0.1 nM thrombin. The SERS signal decreased to 86% and 85% for the case of 7 μM IgG or 50% Serum mixed into 0.1 nM thrombin solution, respectively, as shown in Figure 6. The result indicates that the existence of other nonspecific proteins at high concentration may induce an underestimation of the real concentration of thrombin. Nevertheless, the negative signal from these proteins with at least 10^4 higher concentration than that of thrombin still indicates the proposed sensor has a high selectivity for the thrombin assay.

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CONCLUSIONS

In summary, the present study described a novel and efficient detection procedure of thrombin using the SERS technique based on the changing electrostatic effect from the structure-switching aptamer upon thrombin binding. In the sensing system, SERS signals with the concentration of thrombin over a range from 0 to 1 μM were detected, and the linear range and detection limit was 0.1 nM−10 nM and 20 pM, respectively. Besides the high reliability and reproducibility of the system, the special selectivity of the sensor was illustrated. Furthermore, the proposed approach provided an easy and fast-responding procedure, which might hold a promising potential for application in biological or clinical thrombin detection.

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SUPPORTING INFORMATION AVAILABLE

Further details are given in Figures S1−S4. This material is available free of charge via the Internet at http://pubs.acs.org.

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