Biosensors and Bioelectronics 44 (2013) 10–15

Contents lists available at SciVerse ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

A "turn-off" SERS-based detection platform for ultrasensitive detection of thrombin based on enzymatic assays

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ARTICLE INFO

Article history:
Received 22 October 2012
Received in revised form
21 December 2012
Accepted 4 January 2013
Available online 11 January 2013

Keywords:
Thrombin
SERS
Enzymatic amplification

ABSTRACT

Herein we describe a novel "turn-off" biosensing strategy for thrombin detection based on Surface Enhanced Raman scattering (SERS) and the mediation of spacing between 4-mercaptobenzoic acid (4-MBA) labeled gold nanoparticles (AuNPs). The multiple arginine peptides that initiated gold nanoaggregates incorporating Raman reporter molecules due to the formation of "Hot Spots", are induced to disband by the addition of thrombin in which the peptides are catalytically cleaved into fragments and thus SERS signals of 4-MBA are sharply declined because of the weakened ability of fragments to induce aggregation. Through this strategy, a novel "turn-off" SERS biosensor for thrombin based on enzymatic amplification is established with sensitivity, selectivity and simplicity as AuNPs and peptides are easily accessible. Compared with non-enzymatic amplification based methods, this newly proposed method has improved sensitivity. The limit of detection was 160 fM (at the ratio of signal to noise, $S/N=3:1$). Further, controlled experiments showed that the method exhibited good selectivity over other proteases. The method demonstrated the capability and the potential for application in complex matrix and future biomarker development. The results also presented the potential and superiority of SERS biosensor based on signal amplification.

1. Introduction

Highly sensitive and selective methods for the detection of specific protease such as thrombin are important for the future of clinical diagnosis and therapeutic researches (Shuman and Majerus, 1976). For example, concentration of thrombin in blood normally varies from the low micromolar level to the nanomolar level during the coagulation progress, while the detection at picomolar range is crucial to clinical diagnosis (Bichler et al., 1996). surface enhanced Raman scattering (SERS) is an analytical technique that has previously been used for fabrication of biosensors for proteins and DNA, offering advantages over alternative techniques as SERS distinguishes itself with several advantages (Hu et al., 2008): the unique Raman spectra that offers structural information about content, the narrow peak width that avoids peak overlapping, Raman signal that is free from photo bleaching and the single molecule detection ability for ultrasensitive detection of analytes (Nie and Emory, 1997). In recent years, individual nanostructures, single-particle nanomaterial and SERS-active substrates have been reported in the design and fabrication SERS biosensors for biomolecules, heavy metal ions and pesticides (Gong et al., 2012). Cross-linking of nanomaterials such gold nanoparticles, gold nanorods to produce junctions and aggregations is an alternative method in developing powerful tools for ultrasensitive detection of analytes, due to the greatly enhanced electromagnetic (EM) field produced by the surface plasmon resonance (SPR) on metal nanostructures, particularly in "hot spots" of an ensemble of structures (Pieczonka and Aroca, 2005). Nie's group reported SERS nanobeacons by cross-linking of SERS-active gold nanoparticles using target-specific hybridization of DNA, indicating the ability of SERS for sensitive detection of biomolecules (Qian et al., 2008), while Irudayaraj's group fabricated nanorod–nanoparticle junction for protein detection based on aptamers, broadening the use of SERS in biological fields (Wang et al., 2010b). Recently, SERS-based methods for detection of protein have been widely reported based on "hot spots"(Cho et al., 2008; Graham et al., 2011; Wang et al., 2007). Nevertheless, most of these methods focused on the target-induced aggregations and flocculation, which may limit the sensitivity. An alternative approach would lie in catalytic amplification by target thereby amplifying signals and improve sensitivity.

Recently, strategies of signal amplification have been used in fluorescent detection platform for detection of DNA, protein and small molecules (Freeman et al., 2011; Lin et al., 2011; Niu et al., 2012). Techniques based on DNAzymes, exonuclease and magnetic beads based circulations have been deployed in protein

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http://dx.doi.org/10.1016/j.bios.2013.01.006

detection. However, such strategies have their limitations such as the need of specific sequences and co-factors (Dougan et al., 2011), in addition to the necessary design and optimization of DNA sequences. An alternative is the use of commercially available fluorogenic substrates specific to target protease, which is costly and could exhibit photo bleaching. While fluorescence is a robust and mature detection platform, it exhibits broad emission spectra and non-uniform photo bleaching. Compared with fluorescence, SERS provides rich vibrational spectra and is free from photo bleaching. Therefore, it would be advantageous to couple the benefits of a signal amplification approach with a SERS based detection technique for proteins such as thrombin. Even though there are limited reports on catalytic SERS detection platform for DNA and heavy metal ions based on DNAzyme and autonomous DNA machine (Dougan et al., 2011; Wang and Irudayaraj, 2011), few has reported on protein detection by SERS based on enzymatic amplification (Moore et al., 2004; Yazgan et al., 2010). Further, even though there are reported works on detection of thrombin based on "hot spots"(Cho et al., 2008; Hu et al., 2008; Wang et al., 2010a), few focused on detection based on enzymatic amplification. Besides the application of the DNA techniques, the use of protease specific substrate to fabricate SERS based protein detection platform is believed to be promising for its advantages over fluorescence. Further, harnessing the catalytic property of enzymes to amplify analytical signals, enzyme assays are highly sensitive. Therefore, the combination of enzymatic amplification using protease specific substrates and SERS showed great potential for protein detection. It remains essential, however, to prove that the enzymatic process of protein is amenable for SERS analysis.

Gold nanoparticles (AuNPs) have been widely reported in colorimetric and electrochemical biosensors due to their unique optical properties. AuNPs of size greater than 20 nm provide strong Raman enhancement of the reporters owing to the strong electromagnetic field around gold (Njoki et al., 2007). Further, cross-linking or aggregation of AuNPs produces "hot spots" between them and greatly enhances Raman signal. Because of its stability and monodispersity, AuNPs could be easily conjugated with Raman reporters and biomolecules in fabrication of biosensors. As the surface of AuNPs through synthesis by citrate tri-sodium is negatively charged (Munro et al., 1995), peptides consisting of multiple arginines are used herein to facilitate the aggregation and promote SERS of Raman reporters. Such peptides feature the ability to induce the aggregation of AuNPs due to high isoelectric point. Further, based on the catalytic property of protease, these peptides could be hydrolyzed (Fan et al., 2012) into fragments by thrombin, thus having weak interaction with AuNPs to greatly enhance the SERS signal change. Through this strategy, "turn-off" SERS biosensor is believed to establish based on enzymatic cleavage of substrates, and by optimizing parameters relating to AuNPs, selectivity of the peptide is improved without separation steps. Further, the ON/OFF and "turn-on" sensing strategy has been reported (Horvath et al., 2004) and proved to have remarkable applicability in plasmonic fields through switching the signal "on" or "off" by analytes to enhance signal (Cao et al., 2011). It is believed that this concept with SERS sensitivity will provide new means to protein detection based on unique properties of AuNPs and peptides.

Therefore, a novel "turn off" SERS biosensor for thrombin was developed herein for the first time based on the unique optical property of AuNPs, high sensitivity of SERS and the specific catalytic cleavage of designed substrates by thrombin. The results undoubtedly showed the potential and superiority of SERS biosensor based on signal amplification. To demonstrate the general applicability of our strategy, thrombin was chosen as a model protein.

2. Experimental

2.1. Materials

Human α-thrombin, Trypsin, Lysozyme, HSA, Chymotrypsin, BSA, and IgG were provided by Sigma, Human Serum sample was purchased from Y-J Biological, Shanghai. $HAuCl_4 \cdot 3H_2O$, tri-sodium citrate, 4-mercaptobenzoic acid (MBA) and other chemicals were of analytical grade and used without any further purification unless otherwise specified. The peptide, Arg–Arg–Arg–Arg–Arg–Arg–Arg–Arg–Arg (RRRRRRRRR, Arg_9), was 95% pure and purchased from Sangon Biotechnology Inc. (Shanghai, China). Ultrapure water with an electrical resistance larger than 18.2 MΩ was used throughout the experiment. To mimic the physiological condition, PBS was used as the buffer. All solutions and the buffers in the experiments were prepared using sterile water.

2.2. Equipment

UV spectra of AuNPs and AuNPs treated with Arg_9 were obtained by a Shimadzu UV-2550. UV–v is an absorption spectrometer. Transmission Electron Microscope images were obtained with a JEM-2100 (HR) microscope at acceleration voltage at 200 kV. SERS measurements were performed with a confocal microscope (Jobin-Yvon HR-800) equipped with an air-cooled charge-coupled device detector. He–Ne laser with 632.8 nm radiations was used for excitation. An Olympus 50 long working distance lens collected the scattering light to the charge-coupled device (CCD) detector. All the spectra here were the results of 5 s twice accumulations. Illustrated Raman spectra were smoothed once at degree of 4 and size of 9.

2.3. Preparation of gold nanoparticles (AuNPs) and 4-MBA modified AuNPs

Gold nanoparticles with a diameter of ~30 nm were prepared by reduction of gold (III) chloride hydrate using sodium citrate. In brief, 100 ml of 0.01% (w/w) $HAuCl_4$ was heated by boiling under vigorous magnetic stirring. Then, 1.5 ml of 33.8 mM sodium citrate solution was added immediately. The solution was kept boiling and stirring for 20 min after having turned red, followed by cooling to room temperature. The AuNPs concentrations was ~0.33 nM, which was calculated using Beer's law and the extinction coefficients (ε) (30 nm AuNPs is $2.3 \times 1010\ M^{-1}\ cm^{-1}$ (Liu et al., 2012)). After that, 1 mM 4-MBA solution dissolved in ethanol was added to AuNPs for a final concentration from 1 μM to 20 μM. The solution was kept standing for 24 h at room temperature, followed by centrifugation at 4500 rpm for 15 min three times to remove the excess unbound 4-MBA. At last, the oily 4-MBA modified AuNPs' in the bottom was redispersed in phosphate buffer (1 mM, pH 6.4).

2.4. Protocol for thrombin detection

Briefly, Arg_9 was dissolved in PBS (10 mM, pH 7.4). Thrombin standards at various concentrations were prepared by dissolving thrombin in PBS buffer (10 mM, pH 7.4). A total volume of 100 μL of reaction mixture containing Arg_9 (1.5 μM) and thrombin (0–80 nM) was incubated at 37 °C for 45 min. Then, the reaction mixture was added to the buffer solution of AuNPs (0.9 ml) for SERS measurements.

2.5. Specificity comparison and recovery test for thrombin assay

To compare the specificity of the method for thrombin, control experiments were performed. 100 μL of reaction mixture containing Arg_9 (1.5 μM) and trypsin, chymotrypsin, pepsin, lysozyme, HSA, BSA, IgG were separately incubated at 37 °C for 45 min. Then, the reaction mixture was added to AuNPs for SERS measurements. SERS spectra showed that the method exhibited good selectivity.

To test the recovery and applicability of the method for complex matrix, diluted human serum sample was used in the method. The 100-fold diluted human serum containing spiked thrombin (0, 0.01, 0.1, 1, and 10 nM) was analyzed by following the same procedure for the detection of thrombin standards in PBS buffer solution followed by SERS measurements. For comparison, thrombin (0, 0.01, 0.1, 1, and 10 nM) in PBS buffer was analyzed by performing the same protocol as above.

3. Results and discussion

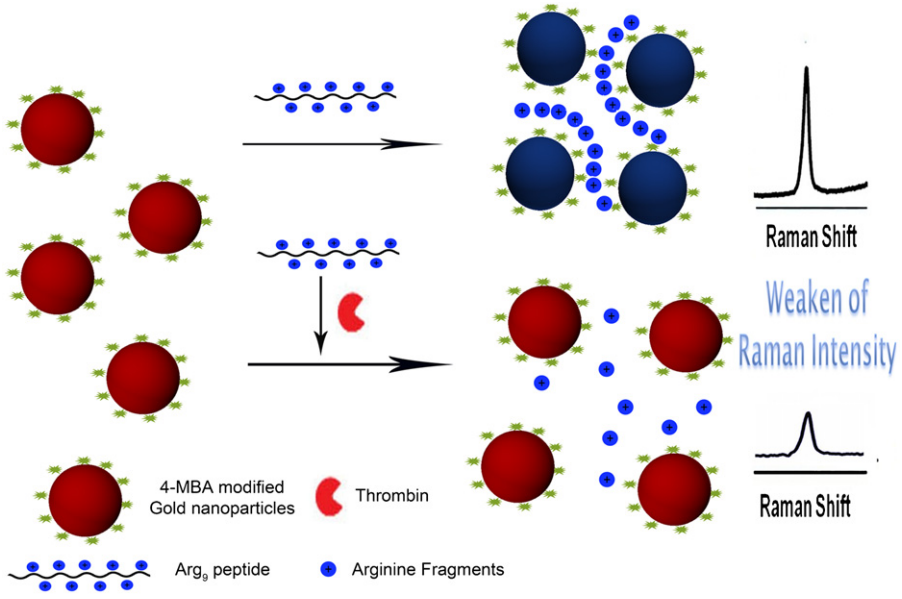
3.1. Sensing mechanism and feasibility of the enzymatic assays

The basic design of the strategy is demonstrated as Scheme 1. AuNPs with the diameter of ~30 nm were synthesized according to literature (Liu and Lu, 2006). The as-synthesized nanoparticles were further functionalized with Raman reporter, 4-mercaptobenzoic acid (4-MBA). A deca-peptide Arg–Arg–Arg–Arg–Arg–Arg–Arg–Arg–Arg–Arg (RRRRRRRRR, Arg_9) was designed as the substrate of thrombin. The peptide was positively charged at pH lower than 11 due to the high isoelectric point of arginine (Zhang et al., 2009). In the absence of thrombin, the addition of Arg_9 to 4-MBA modified AuNPs would induce the aggregation of AuNPs due to the strong electrostatic interaction of arginine residues and the negatively charged AuNPs. In this way, electromagnetic (EM) "hot spots" can be fabricated and large EM coupling effect is produced at the hot spots between AuNPs, which results in the enhancement in SERS intensity of 4-MBA. However, once the peptide is treated with thrombin, it will be hydrolyzed into arginine fragments. As the electrostatic interaction between fragments and AuNPs is relatively weak, the degree of aggregation induced would be reduced, thereby leading to declined SERS intensity. Therefore, the introduction of thrombin to cleave Arg_9 would change SERS intensity and enhancement of SERS intensity recession would be observed, and the amount of thrombin introduced would clearly influence the amount of peptide hydrolyzed under a specified time interval due to the catalytic property of enzymes. The change in SERS intensity can be used as assays for thrombin.

To verify the feasibility of the method, a series of experiments were conducted. TEM images indicated that the 4-MBA modified AuNPs were well dispersed in the absence of Arg_9. Upon the addition of Arg_9, aggregation of AuNPs occurred, according to Fig. S2C. However, once the Arg_9 was first incubated with thrombin at 37 °C for 1 h, the addition of the mixture to AuNPs lead to the reduced aggregation, compared with Fig. S2B. SERS spectrum of the 4-MBA modified AuNPs is demonstrated in Fig. 1 (curve a), showing that the SERS intensity of characteristic peak of 4-MBA at 1077 cm^{-1} is not significant, probably because of the weak enhancement ability of AuNPs. Further, control experiments showed that neither the PBS buffer, nor thrombin would lead to the aggregation (Fig. 1, curves b and c). Furthermore, after incubated with thrombin for periods of time, 4-MBA modified AuNPs were still aggregated when the peptide was added, showing that thrombin would not protect AuNPs from being aggregated (Fig. 1, curve d). When Arg_9 was added to AuNPs, aggregation occurred and SERS intensity at 1077 cm^{-1} was significantly enhanced, according to Fig. 1 (curve e). Further, SERS intensity of 4-MBA is less remarkable upon the addition of Arg_9 treated with thrombin, according to Fig. 1 (curve f). Absorption spectral change was also observed after Arg_9 was added to AuNPs (Fig. S2D). It was obvious that $A_{700\ nm}/A_{528\ nm}$ decreased when Arg_9 treated with thrombin was added to AuNPs. Hence, based on the SERS intensity change due to the peptide-induced aggregation of AuNPs, detection of thrombin was believed to have feasibility through this strategy.

3.2. Optimization of pH and incubation time

We then studied the effect of pH of AuNPs and cleaving solution on the sensitivity of the sensor for thrombin. The pH of AuNPs and cleaving solution were varied respectively from near neutral to basic. The pH of cleaving solution under 7 was not studied as the enzymatic reactivity of thrombin under 7 was



Scheme 1. Strategy for fabrication of SERS biosensor for thrombin based on enzymatic assays.

lowered. We then plotted (Ix_0–Ix) against different pH, where Ix_0 and Ix represent the normalized SERS intensity of 4-MBA AuNPs incubated with Arg_9 in the absence and presence of thrombin, respectively. According to Fig. S3A, it has been found out that the greatest sensitivity occurred when pH of AuNPs and cleaving solution was set to 6.4 and 7.4 respectively (curves b and a). This is mainly because of the pH effect on electrostatic interactions between AuNPs and Arg_9 in addition to the enzymatic reactivity of thrombin under different pH. It can be indicated that the stability of AuNPs was lowered due to the decreasing charges on surface of AuNPs with lowering pH, while Arg_9 were more positively charged under this circumstance. Further, the enzymatic reactivity of thrombin was enhanced with increased pH. Therefore, it would be advantageous to study the pH effect on enzyme assays and electrostatic interactions separately to improve sensitivity.

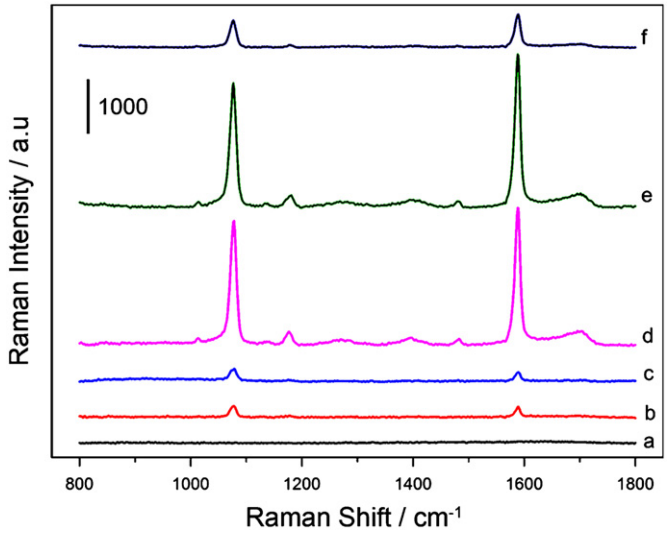


Fig. 1. SERS spectra of 4-MBA modified AuNPs (a) in dispersed form, (b) in PBS buffer, (c) with thrombin (80 nM), (d). (80 nM) and Arg_9 (1.5 μM), (e) with Arg_9 (1.5 μM), and (f) with Arg_9 (1.5 μM) after treated with thrombin (80 nM) for 45 min.

Next, detection of thrombin activity was further studied by treating different amounts of thrombin with 1.5 μM Arg_9 for 1 h. Control experiments showed that the absence of thrombin would lead to the aggregation of AuNPs by Arg_9. As shown in Fig. S3B, itis apparent that normalized SERS intensity decreased by prolonging the incubation time with thrombin. Further, with increasing concentration of thrombin, decreased normalized SERS intensity was observed, indicating that more Arg_9 was hydrolyzed due to the enzymatic cleavage by thrombin and then the electrostatic interaction between arginine residues and AuNPs was weakened. At the same time, the rate of reduction in normalized SERS intensity between different concentrations of thrombin is changed accordingly with the extended incubation period. It has also been founded that after 30 min, one can observe a slower decline in Raman intensity, indicating that such tendencies between concentrations may be attributed to electrostatic interactions between AuNPs and arginine fragments, which could still had an influence on the stability of AuNPs. In addition, the above results suggest that by extension of incubation period, detection at lower concentration would be possible and sensitivity of the method could be achieved. To realize detection of thrombin at both low and high concentrations, the incubation time of Arg_9 and thrombin was optimized at 45 min. The simple preparation steps and no need of washing could make up for the disadvantage of this relatively longer incubation time. However, for the detection of high concentration of thrombin, it's believed that a shorter-time enzymatic reaction could be applied for fast analysis.

3.3. Analysis of thrombin with high sensitivity

Consequently, under the optimized conditions above, quantitatively and sensitive detection of thrombin was achieved by monitoring the SERS peaks of 4-MBA labeled on gold nanoparticles on different spots of three parallel samples at varied concentrations. The ratio between AuNPs and thrombin ranged from 74:1 to 1:26, showing the capability for detection of thrombin at extend concentration range and reasonable detection efficiency. Fig. 2A presented the SERS spectra of 4-MBA modified gold nanoparticles upon the addition of Arg_9 incubated with different amounts of thrombin after reaction for 45 min. One clearly observes that the Raman intensity of peak at 1077 cm^{-1} drastically decreased with increased concentration of thrombin from

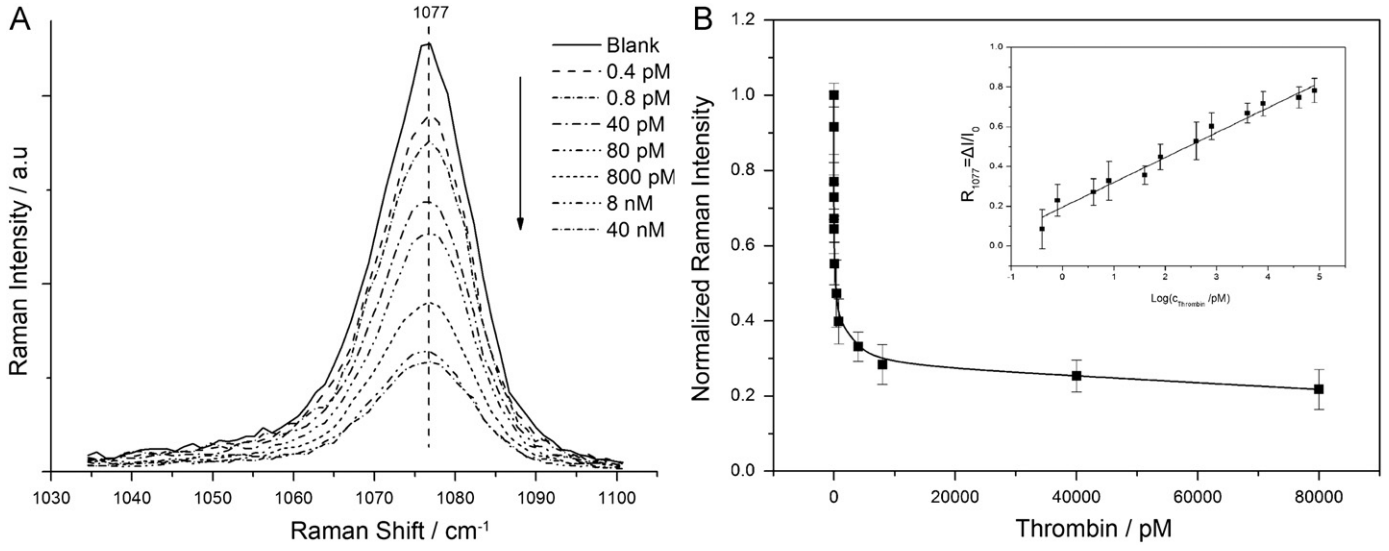


Fig. 2. (A) SERS spectra of 4-MBA modified AuNPs incubated with Arg_9 treated with different amounts of thrombin for 45 min and (B) Thrombin concentration (0–80 nM) dependent normalized Raman intensity of 1077 cm^{-1} peak. The inset shows the calibration curves of R_{1077} at 1077 cm^{-1} against natural logarithm over concentration of thrombin. Each error bar represents the standard deviation in normalized Raman intensity associated with $n=6$.

0 to 40 nM, indicating that with more thrombin introduced, the enzymatic cleavage of Arg_9 was enhanced and more Arg_9 were hydrolyzed. TEM images of AuNPs incubated with Arg_9 after treated with different concentrations of thrombin were consistent with such tendency. It can be found that the degree of AuNPs aggregation has an increasing tendency when different concentrations of thrombin were added starting from 80 nM to 0.4 pM (Fig. S4, panels 'a–h'). It could also be found that the numbers of both AuNPs to form aggregates and the formed gold "gaps" are correspondingly decreased along with addition of excess thrombin (Supporting Information, Fig. S4), which is also consistent with the tendency of Raman intensity. Further, the position of characteristic peak of 4-MBA at 1077 cm^{-1} did not change with the increasing thrombin concentration, indicating the introduction of thrombin only worked as a protease. Fig. 2B showed the normalized SERS intensity at 1077 cm^{-1} varying with the concentration of thrombin from 0 to 80 nM, which presents a nearly exponential decreasing curve in the tested concentration range. To clearly demonstrate the detection for thrombin in this work, an appropriate calibration curve was developed. First, Raman spectra of silica at 520 cm^{-1} (Si_{520}) were used for system calibration. Spectra of control substrate without thrombin were used as a reference for normalization. Six replicate SERS spectra of MBA were obtained at each concentration (from 80 nM to 0.4 pM) and the averaged intensity of the characteristic peaks at 1077 cm^{-1} were used to calibrate the SERS intensity with reference of the same peak got from the control ($R=\Delta I/I_0$, I_0, without thrombin). Based on this concept (Jiang et al., 2012; Wang and Irudayaraj, 2011), the inset of Fig. 2B showed the linear relationship between the natural logarithm over concentration of thrombin ranging from 400 fM to 80 nM and the calibrated normalized SERS intensity ($R=\Delta I/I_0$, I_0, without thrombin). The regression equation is $y=0.193+0.127\,x$ (where x is logarithm of the target protein concentration in pM and y is the normalized SERS intensity ($R=\Delta I/I_{0)}$ of peak at 1077 cm^{-1}) with a squared correlation coefficient of 0.973. Calculation from the standard deviation of the blank was used in determination of LOD. To calculate the limit of detection, the average response of the blank was first calibrated with reference from control and then plus three times the standard deviation (the propagated standard deviation was 0.441), followed by substitution in the regression equation, and therefore a detection limit of 160 fM was calculated. Considering that every 100 μL thrombin solution was added, the absolute detection limit was 0.016 fmol. However, the actual detection limit could be lower regarding the amount of samples probed by laser beam. Compared with reported methods based on aptamers or enzymatic amplification, the proposed method has better or comparable sensitivity. The superiority in sensitivity of the method could be attributed to the combination of advantages of SERS and high sensitivity of enzymatic strategies. Further, this method is much easier and cost-effective.

3.4. Specificity of the enzymatic assays

To determine the specificity of the method, a series of control experiments between thrombin and other interferences were performed. Some interferences including trypsin, pepsin, chymotrypsin, lysozyme, HSA, BSA, IgG and Factor Xa (200 nM) were detected (Fig. 3A). After separate incubation of Arg_9 with these interferences, the mixture was added to 4-MBA modified AuNPs. Results in Fig. 3A showed that the normalized SERS intensity of 4-MBA at 1077 cm^{-1} did not decrease significantly. Further, one observes that the SERS signals obtained from these cases of pepsin, lysozyme, HSA, BSA and IgG were rather lower than that from the blank one. Such a tendency of the SERS signal of 4-MBA to decline with the introduction of the other proteins might be

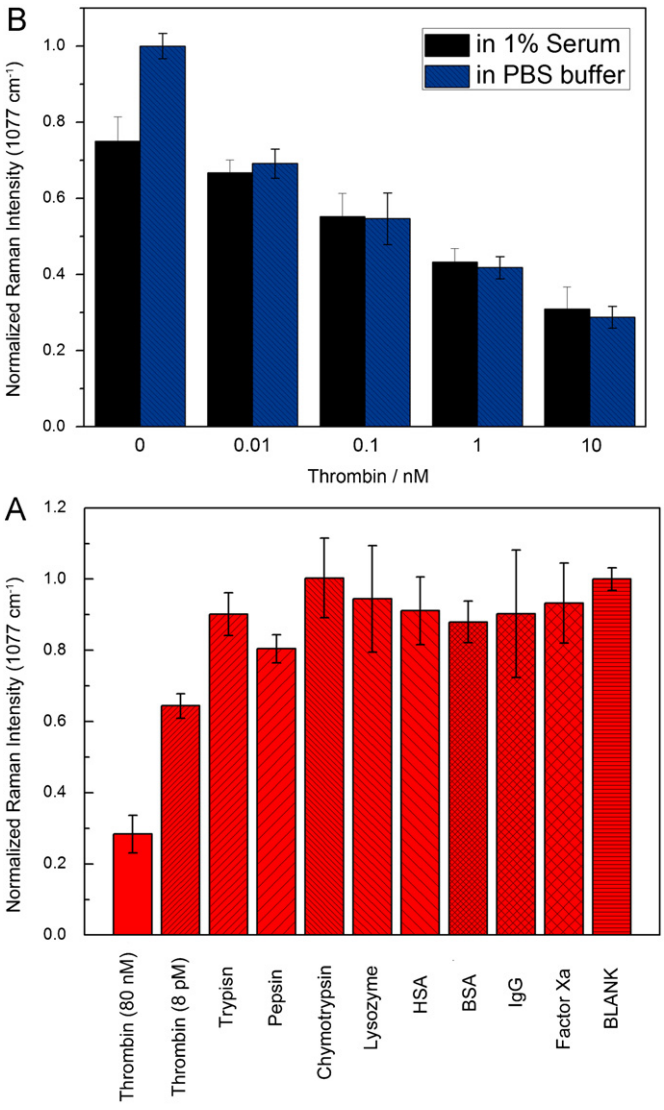


Fig. 3. (A) Evaluation of the specificity of the SERS biosensor. Variation of SERS intensity of 4-MBA modified AuNPs at 1078 cm^{-1} after separate incubation of Arg_9 (1.5 μM) with thrombin (80 nM), trypsin (200 nM), pepsin (200 nM), chymotrypsin (200 nM), lysozyme (200 nM), HSA (200 nM), BSA (200 nM), IgG (200 nM) and Factor Xa (200 nM) and (B) Recovery test of thrombin spiked in 1% serum. Each error bar represents the standard deviation in normalized Raman intensity associated with $n=6$.

due to the nonspecific adsorption of these proteins, which might provide protection against aggregation from Arg_9. However, when thrombin and the Arg_9 was first incubated under the same condition and then added to 4-MBA modified AuNPs, SERS intensity of 4-MBA at 1077 cm^{-1} decreased significantly, indicating that the substrate exhibited good specificity toward thrombin. Even though the existence of other interferences at high concentration could induce a statistically valid reduction in SERS signal, such lower responsivity from these proteins with 10^4 higher concentrations than that of thrombin (8 pM) still indicates the proposed sensor has a high selectivity for the thrombin assay. Further, it is interesting that trypsin, even though having the ability to hydrolyze the Arg_9 according to literature (An et al., 2007) had actually relatively less interference. This was due to that under the readily-optimized pH of 4-MBA modified AuNPs, trypsin was positively-charged with a pI of 10.1–10.6 (Tietze, 1953), thus resulting the aggregation of 4-MBA modified AuNPs. Therefore, the specificity of the peptide over trypsin was improved based on the electrostatic interaction between AuNPs

and trypsin. In addition to the use of aptamers (Zhao et al., 2011) and modification of substrates (Fan et al., 2012), it has been found in this work that based on the electrostatic property of AuNPs, the specificity of enzyme assays could be improved, providing new ways and opportunities for enzymatic assays.

3.5. Analysis of thrombin in human serum

At last, we tested the influence of complex sample matrix on the method using human serum (Fig. 3B). The inhibitors in serum could lead to low recovery of spiked thrombin, and the dilution of the serum could improve the recovery. Therefore, we used diluted human serum as sample matrix. The sample was first 100-folded diluted in PBS buffer, which was then used as the assay medium. Next, this serum-containing medium was detected following the same detection procedure for thrombin as in aqueous solution without human serum. Further, thrombin determination was conducted in in spiked human serum to investigate the ability of the sensor to overcome interference in complex matrix samples. For comparison, aqueous solution of PBS without human serum was used as the medium to determine thrombin concentration (0.01, 0.1, 1, and 10 nM) following the identical determination process for thrombin. The concentration of thrombin in the diluted serum was further determined from the calibration equation obtained by the measurement of thrombin standards. According to Fig. 3B, a near linear decrease for thrombin was observed in the diluted serum, which was identical to that in aqueous buffer, that is, from 0.5 to 20 nM. Besides, good reproducibility was also obtained in the serum matrix, similar with that in PBS buffer. The analysis of thrombin spiked to 100-fold diluted serum showed 107–93% recovery, suggesting little interference from the sample matrix, and the thrombin level in serum sample was about 292 pM, which is similar to previous reports (Zhao et al., 2011). Our method demonstrated the potential of the method for application in complex matrix and future biomarker development.

4. Conclusion

In summary, we have demonstrated for the first time a "turn-off" SERS biosensor for thrombin based on enzyme assays. The results showed that the combination of SERS and enzymatic property of target have enhanced sensitivity and provide new opportunities to signal amplification-based methods. The whole process proposed in this method requires only about 60 min and the limit of detection of the method is as low as 0.016 fmol (160 fM). Compared with other assays for thrombin, this study is meaningful for several reasons: (1) This strategy does not require complicated procedures, making the method simple, fast and sensitive; (2) Based on the response of AuNPs to trypsin under different pH, the method can also be employed as biosensors for trypsin without further optimization, which provides new ways and opportunities for methods based on multi-arginine peptides; and (3) combining the high sensitivity of SERS and enzymatic activity of protease, this strategy had the potential for development of assays for other targets with excellent performance. The effort for simple fabrication of multifunctional SERS biosensor is underway.

Note

Additional experimental results of optimization of AuNPs, TEM images are presented in the Supporting Information.

Acknowledgment

We gratefully acknowledge the support from the Natural Science Foundation of China (NSFC) (Nos. 20805034, 30772058, 20927003, 90913013, and 21175101).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2013.01.006.

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