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A simple and universal "turn-on" detection platform for proteases based on surface enhanced Raman scattering (SERS)

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ABSTRACT

We describe herein a novel' "turn-on" SERS-based strategy for protease detection based on surface enhanced Raman scattering (SERS) and the mediation of spacing between 4-mercaptobenzoic acid (4-MBA) labeled gold nanoparticles (AuNPs) through enzyme assays. The method employed non-cross-linking aggregation of 4-MBA-modified AuNPs by peptides after treatment with target protease. Thus, SERS signals of 4-MBA are sharply increased because of the decreased electrostatic stability of AuNPs that initiated gold nanoaggregates incorporating Raman reporter molecules due to the formation of "Hot Spots". Through this strategy, a novel and facile "turn-on" SERS biosensor for trypsin and thrombin based on enzymatic cleavage activity is established with sensitivity, selectivity and simplicity as AuNPs and peptides are easily accessible. Compared with other methods, this newly proposed method has improved sensitivity. The limit of detection was 85 fM (at the ratio of signal to noise, S/N=3:1) for trypsin. Controlled experiments showed that the method exhibited good selectivity over other proteases. We also proved that this principle could be easily adapted to detection of other proteases such as thrombin. The method demonstrated the capability for application in complex matrix samples. The results also presented the potential and superiority of SERS biosensor as a general approach for proteases based on enzyme activity.

1. Introduction

Proteases are vital enzyme catalysis that play important roles in various biological and physiological processes including digestion, blood coagulation, apoptosis and a series of cellular activities (Shuman and Majerus, 1976; Turk, 2006). Further, proteases are also found to be associated with virus and bacterial infections, as abnormal expression of certain proteases are related to diseases such as cancer, Alzeheimer's diesease, etc. (López-Otín and Bond, 2008). Therefore, highly sensitive and selective methods for the detection of specific protease are critical for the future of clinical diagnosis and therapeutic researches. Recently, significant efforts have been made to develop biosensors of proteases with high sensitivity and selectivity, ranging from fluorescence (Zhao et al., 2011), chromatography (Craig et al., 1998), colorimetry (Chen et al., 2010), electrochemistry (Cao et al., 2013) to immunology (Grierson et al., 2010). Despite the superiority of these detection platforms reported, most of them bare such drawbacks as complication, difficulty in material design, requiring labeling steps and lack of sensitivity, which may limit their application in biological and medical fields. Therefore, it is highly desirable to develop a simple, rapid method with satisfactory sensitivity and selectivity for detection of proteases.

Surface enhanced Raman scattering (SERS) has received vast attention as a potentially suitable analytical technique for detection of proteases due to the advantages of SERS over alternative techniques (Bantz et al., 2011; Haynes et al., 2005; Kneipp et al., 1997). Based on the cross-linking or aggregation of SERS-substrates such as gold (Ni et al., 1999), silver nanoparticles (Wang and Chen, 2009) and nano rods (Wang et al., 2010a), ultrasensitive detection method of analytes can be developed 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 (Doering and Nie, 2003; Nie and Emory, 1997; Qian et al., 2008). Recently, SERS-based methods for detection of proteases have been widely reported based on "hot spots" (Welser et al., 2011). Irudayaraj's group fabricated nanorod–nanoparticle junction for thrombin detection based on aptamers, broadening the use of SERS in biological fields (Wang et al., 2010b). Additionally, catalytic signal amplification by target is a promising technique with improved sensitivity compared with the traditional target-induced flocculation mode. Some

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methods employed DNA-involved techniques, DNAzymes, exonuclease and magnetic beads based circulations in protease detection (He et al., 2013; Li et al., 2012). Despite the excellent ultrasensitivity, however, most of the strategies were complicated as they required specific sequences of DNA and co-factors, along with the necessary design and optimization of DNA sequences. Other methods were based on the enzyme assays of SERS-active gold nanoparticles (AuNPs), where the stability of the surface charged AuNPs was destroyed by substrates, inducing AuNPs aggregation and enhancement of SERS intensity when AuNPs were added to the substrates which would cause the change of surface charges of AuNPs (Pan et al., 2012). Until now, different types of protease-specific substrates have been reported to facilitate the enzyme assays of SERS-active substrates such as gold nanoparticles and silver nanoparticles. Our group has previously developed a highly sensitive SERS method for detection of thrombin by using peptide substrates containing multiple arginines (Wu et al., 2013). Li's group has used protamine, a non-synthetic substrate, to establish a sensing platform for the detection of trypsin based on anti-aggregation of SERS-active silver nanoparticles (Chen et al., 2013). Another general mode by controlling distance between reporter and SERS substrate was also reported in detection of chymotrypsin by SPR-SERS spectroscopy (Fu et al., 2013). These methods may pave us a new way for the SERS-based detection of proteases by integrating SERS sensitivity and catalytic property of proteases. Until now, most of the methods reported were using the anti-aggregation of SERS-active substrates or "turn-off" SERS mode by enzyme assay due to the majority of the proteases being hydrolytic types, while there having been no examples based on "turn-on" strategies.

We assume that a novel "turn-on" strategy for SERS-based enzyme assay would be more advantageous in consideration of simplicity, ultrasensitivity and generality, in which the SERS-active substrates would only be aggregated by peptides after the incubation with target protease. Design of the peptide is flexible and the principle can be easily applied to other proteases. Further, this strategy harnesses the catalytic property of enzymes to amplify SERS signal and are highly sensitive for SERS-based protease assays. The "turn-on" mode is easily to prepare and could overcome the major limitations of "turn-off" mode such as suspicious of false-positive responses (Chen et al., 2010). Through this strategy, a simple and universal "turn-on" SERS biosensor would be established based on enzymatic cleavage of substrates. It is believed that this concept with SERS sensitivity will provide new means to protease detection based on unique properties of AuNPs and peptides.

Therefore, a novel "turn-on" SERS strategy was developed herein for the first time based on high sensitivity of SERS and enzyme assay through non-crosslinking aggregation of AuNPs. In this work, a short peptide substrate has been designed to stabilize the AuNPs with lower isoelectric point, and cleavage of the peptides by target protease can greatly enhance the SERS signal through AuNPs aggregation. Trypsin and thrombin were adopted as protease models, considering its importance in clinical diagnosis. The results undoubtedly showed the high sensitivity toward target and capability of accurate quantification, along with the potential as a universal approach for protease sensing and superiority of this "turn-on" SERS strategy based on enzymatic amplification.

2. Experimental section

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. Peptide I, Arg-Cys-Phe-Arg-Gly-Gly-Asp-Asp (RCFRGGDD, RD-8) and peptide II, Arg-Cys-Phe-Leu-Val-Pro-Arg-Gly-Ser-Asp-Asp (RCFLVPRGSDD) were 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, phosphate buffer saline (PBS) was used as the buffer. All solutions and the buffers in the experiments were prepared using sterile water.

2.2. Instrumentation

UV spectra of AuNPs and AuNPs treated with peptides were obtained by a Shimadzu UV-2550 UV–vis absorption spectrometer. 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 ~40 nm were prepared by reduction of gold (III) chloride hydrate using sodium citrate (Elghanian et al., 1997; Storhoff et al., 1998). In brief, 100 ml of 0.01% (w/w) $HAuCl_4$ was heated to boiling under vigorous magnetic stirring. Then, 1.0 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. After that, 1 mM 4-MBA solution dissolved in ethanol was added to AuNPs for a final concentration from 1 μM to 10 μM. The solution was kept standing for 10 h at room temperature, followed by centrifugation at 3800 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 (10 mM, pH 7.4).

2.4. SERS analysis of trypsin

Briefly, RD-8 was dissolved in PBS (10 mM, pH 7.4). Trypsin standards at various concentrations were prepared by dissolving trypsin in PBS buffer (10 mM, pH 7.4). A total volume of 100 μL of reaction mixture containing RD-8 (20 μM) and trypsin (0–100 nM) was incubated at 37 °C for 60 min, respectively. Then, the reaction mixture was added to the buffer solution of AuNPs (100 μL) for SERS measurements.

2.5. Specificity comparison and recovery test for trypsin assay

To compare the specificity of the method for trypsin, control experiments were performed. 100 μL of reaction mixture containing RD-8 (20 μM) and thrombin, chymotrypsin, pepsin, lysozyme, HSA, BSA, IgG were separately incubated at 37 °C for 60 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

trypsin (0, 0.01, 0.1, 1, and 20 nM) was analyzed by following the same procedure for the detection of trypsin standards in PBS buffer solution followed by SERS measurements.

3. Results and discussion

3.1. Sensing mechanism and feasibility of the enzyme assays

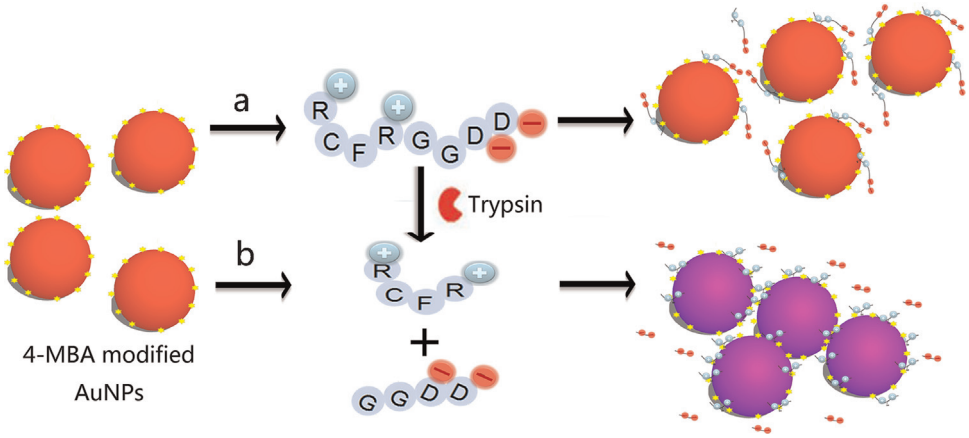
Scheme 1 shows the possible sensing mechanism of SERS detection of trypsin based on aggregation of 4-MBA modified AuNPs through trypsin activity assay. AuNPs with the diameter of ~40 nm were synthesized according to literature. The gold nanoparticles were then functionalized with Raman reporter, 4-mercaptobenzoic acid (4-MBA). Peptide I, Arg-Cys-Phe-Arg-Gly-Gly-Asp-Asp (RCFRGGDD, RD-8) was designed as the substrate of trypsin. The total charge of RD-8 is negative at pH 7.4 because of the two Asp residues in the sequence. The –SH group on cysteine makes it available for the peptide to attach to 4-MBA modified AuNPs through Au–S bonds, and the binding of RD-8 does not induce AuNPs aggregation because of the negatively charged property of the peptide (Scheme 1, path a). Once the peptide is treated with trypsin, it would be hydrolyzed and release mixture of shorter and positively charged peptides such as Arg-Cys-Phe-Arg-NH_2 (RCFR) and Cys-Phe-Arg-NH_2 (CFR). The addition of the cleaved solutions to 4-MBA modified AuNPs would induce the AuNPs aggregation due to the strong electrostatic interaction of positively charged peptides and the negatively charged AuNPs (Scheme 1, path b). In this way, electromagnetic (EM) "hot spots" can be fabricated and large EM coupling effect is produced at the hot spots between AuNPs, resulting in enhancement of SERS intensity of 4-MBA. The SERS intensity change reflects the activity of trypsin, which can be used as assay for trypsin.

To verify the feasibility of the method, a series of experiments were conducted. SEM images indicated that the 4-MBA modified AuNPs were well dispersed when incubated with RD-8 (Supporting information, Fig. S1, a). Upon the enzyme activity of RD-8 with trypsin, aggregation of AuNPs occurred, according to Fig. S1. SERS spectra of the AuNPs and 4-MBA modified AuNPs showed that the SERS intensity of characteristic peak of 4-MBA around 1076 cm^{-1} is not significant (Fig. S2A, a and b), probably because of the weak enhancement ability of AuNPs. When RD-8 was added to AuNPs, AuNPs were still dispersed and SERS intensity at 1076 cm^{-1} remained at low level and unenhanced, according to Fig. S2A (curve c). Control experiments showed that trypsin itself would not lead to the aggregation (Fig. S2A, d), while PBS buffer could aggregate AuNPs and increase SERS intensity (Fig. S2A, e). However, once we added PBS buffer containing peptides to the AuNPs, no aggregation was observed along with little enhancement of SERS spectra, showing that the peptide itself could protect the AuNPs from high ionic strength (Fig. S2A, c). Next, upon the addition of trypsin-treated RD-8 to AuNPs, aggregation occurred and SERS intensity at 1076 cm^{-1} was remarkably enhanced, according to Fig. S2A (curve f). MALDI-TOF spectra proved that the peptide was cleaved after reaction with trypsin (Fig. S5). In the spectrum (Fig. S5A), m/z=924.39 was observed. Trypsin cleaved the peptide at the C-terminus of R, producing RCFR fragments with a calculated molecular weight of 580.71. In the spectrum, m/z=579.81 was observed (Fig. S5B). Therefore, MALDI-TOF experiments proved the cleavage of the peptides. Absorption spectral change was also observed when RD-8 treated with trypsin was added to AuNPs (Fig. S6A). It was obvious that $A_{650\ nm}/A_{530\ nm}$ increased when RD-8 was treated with trypsin and then added to AuNPs. UV–vis spectra also proved that the peptide could protect the AuNPs, probably because the peptide could attach to AuNPs by –SH group and form a negatively charged layer of Asp residues for AuNPs. Hence, based on the SERS intensity change due to aggregation of AuNPs through peptide cleavage with protease, detection of trypsin was believed to have feasibility through this strategy.

3.2. Parameters optimization

We then studied several parameters regarding the peptide sequence, NaCl concentration, AuNPs and reaction time of trypsin. The diameter of AuNPs employed in the experiment and concentration of 4-MBA modified were optimized and shown in Fig. S7. 40 nm-sized AuNPs were selected throughout the study and the concentration of 5 μM for 4-MBA was chosen. Further, we studied the effect of the amount of the RD-8 peptide for detection of trypsin, and 20 μM was chosen as the optimal concentration of peptide as the SERS intensity was the highest for trypsin assay (Fig. S8)

The –SH group of the cysteine residue was essential to realization of the principle, as it enables the peptide to attach to 4-MBA modified AuNPs through Au–S bonds. As shown in Fig. S2B, while peptide without cysteine could not induce the aggregation of AuNPs after cleaved by trypsin (Fig. S2B, b), peptides containing cysteine could observe apparent increase in SERS intensity (Fig. S2B, c). The XPS spectrum in Fig. S4 showed characteristic N 1s peak at 400.07 eV after the addition of RD-8 peptide to AuNPs, which was in accordance with the binding of peptides. Further, increasing the number of Arg would lead to larger extent of aggregation (Fig. S2B, d), and increased Asp residues have little influence on the SERS spectrum of 4-MBA modified AuNPs



Scheme 1. Schematic diagram of SERS-based enzyme assay for trypsin.

(Fig. S2B, e). However, further increase of arginine number would raise the isoelectric point of RD-8 and aggregate AuNPs. Therefore, the numbers of Arg and Asp were optimized as two respectively to ensure the AuNP stability.

Detection of trypsin activity was next studied by treating different amount of trypsin with 20 μM peptide for one hour followed by addition to 4-MBA modified AuNPs (Fig. S9A). As shown in Fig. S9B, it is apparent that normalized SERS intensity increased by prolonging the incubation time with trypsin, indicating that RD-8 was hydrolyzed by trypsin and then the electrostatic interaction between peptide fragments and AuNPs resulted in the aggregation. The rate of increase in Raman intensity was reduced after 45 min, showing that positively-charged peptides were sufficient in the solution to make AuNPs aggregation. Therefore, the incubation time of RD-8 and trypsin was optimized at 60 min. Compared with literatures reported (Xue et al., 2011), this method had a relatively longer incubation time, which could be attributed to the remaining RD-8 unreacted in the solution to stabilize AuNPs. However, our method features simple preparation steps and no need of washing, which could make up for this relatively longer incubation time, Further, it was believed that a shorter-time enzymatic reaction could be applied for fast SERS analysis of trypsin at high concentrations, as increase in Raman intensity could already be observed after 15 min.

3.3. SERS analysis of trypsin

Under the optimized conditions above, quantitatively and sensitive detection of trypsin was achieved by monitoring the SERS peaks of 4-MBA modified on gold nanoparticles on different spots of three parallel samples at varied concentrations. One clearly observes that the Raman intensity of peak at 1076 cm^{-1} drastically increased with trypsin from 0 to 100 nM, indicating that with more trypsin introduced, the enzymatic cleavage of RD-8 was enhanced. Further, the position of characteristic peak of 4-MBA around 1076 cm^{-1} did not change significantly with the increasing trypsin (Fig. 1), indicating that trypsin only worked as a protease. Fig. 2A presented the SERS spectra of 4-MBA modified gold nanoparticles upon the addition of RD-8 incubated with different amounts of trypsin after reaction for 60 min. SEM images of AuNPs incubated with RD-8 after treated with different concentrations of trypsin were consistent with such tendency. It could also be found that the numbers of both AuNPs to form aggregates and the formed gold "gaps" are correspondingly increased along

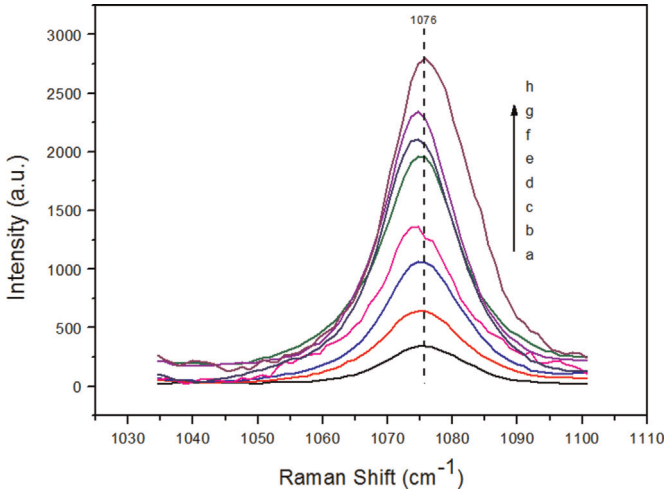


Fig. 1. SERS spectra (from 1035 cm^{-1} to 1100 cm^{-1}) of 4-MBA modified AuNPs incubated with RD-8 treated with different amounts of trypsin for 60 min: a–h: 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1, 20, 50, and 100 nM.

with addition of trypsin (Fig. S1), which is also consistent with the tendency of Raman intensity. Two main bands around 1076 cm^{-1} and 1580 cm^{-1} are attributed to 4-MBA. The strong SERS bands at 1076 cm^{-1} and 1580 cm^{-1} corresponding to aromatic ring vibrations of 4-MBA indicated that 4-MBA was adsorbed on the surface of AuNPs (Talley et al., 2004). It can be clearly seen that the peaks around 1076 cm^{-1} were the most prominent and stable, and its Raman intensity had a significant increase with the increase of trypsin concentration. Thus, the Raman intensity of 4-MBA on AuNPs at 1076 cm^{-1} was assigned for quantitative analysis of trypsin. Fig. 2B showed the normalized SERS intensity at 1076 cm^{-1} varied with the concentration of trypsin from 0 to 100 nM, which presents a nearly exponential increasing curve in the tested concentration range. All the Raman spectra were then baseline-corrected, and their intensities were normalized with respect to the peak of the highest intensity in the spectra. Six replicate SERS spectra of 4-MBA modified AuNPs were obtained at each concentration (from 0 nM to 100 nM), and Fig. 2C showed the linear relationship between the natural logarithm over concentration of trypsin ranging from 0 to 100 nM and the normalized SERS intensity. The regression equation is $y=0.146x+0.712$ (where x is logarithm of the target protein concentration in nM and y is the normalized SERS intensity) with a squared correlation coefficient of 0.994. To calculate the limit of detection, the average response of the blank plus three times the standard deviation (the propagated standard deviation was 0.0240) were substituted in the regression equation, and therefore a detection limit of 85 fM was calculated. As the volume of trypsin was 100 uL, the absolute detection limit was 0.0085 fmol. However, the actual detection limit could be lower as there were fewer amounts of samples probed by laser beam. Compared with reported methods based on enzyme assays (Adjémian et al., 2010; Fan et al., 2012; Xue et al., 2011), the proposed method has better or comparable sensitivity due to the combination of SERS and high sensitivity of enzymatic strategies. Further, this "turn-on" strategy is much easier, direct and cost-effective.

It's notable that two new bands at 1000 cm^{-1} and 1024 cm^{-1} after cleavage of RD-8 with trypsin were found, which were not obvious in the spectrum of 4-MBA modified AuNPs. Further, band at 1580 cm^{-1} were overlapped with broad bands, and two peaks at 1575 cm^{-1} and 1585 cm^{-1} were observed while the peak at 1076 cm^{-1} remained narrow and stable. Such bands at 1000 cm^{-1} and 1024 cm^{-1} could be assigned to phenyl "ring-breathing" stretching vibrations of Phe residue in the peptide. Broad bands from 1400 cm^{-1} to 1600 cm^{-1} could be assigned to the overlaps of C–C stretching modes, $\nu(COO^-)$, amide II and aromatic ring vibration modes of 4-MBA and peptide. Further, characteristic Phe ring-breathing vibrations,ν_{8a}, was observed around 1572–1580 cm^{-1} (Podstawka et al., 2004). As peak at 1000 cm^{-1} were not enhanced for 4-MBA, we assumed that it could be mainly originated from the Phe residue of RCFR and CFR. Therefore, mercaptoacetic acid (MA)-modified AuNPs were introduced as comparison. Fig. S11A showed that unmodified AuNPs did not exhibit any valid Raman signal in SERS spectrum (Fig. S11A, c), and the response was insignificant after peptide was treated with peptide, as bands at 1000 cm^{-1} were weak in intensity (Fig. S11A, b). However, several new bands in SERS spectra of MA-modified AuNPs appeared after incubated with RD-8 in the presence of trypsin (curve a), which was similar when 4-MBA modified AuNPs were employed. Bands assigned to Phe residue (1013 cm^{-1}), deformations of the CH_2 and CH_3 groups (1457 cm^{-1}, 1489 cm^{-1}) and amide vibration (1281 cm^{-1}, 1703 cm^{-1}) were enhanced (Proniewicz et al., 2013). The possible reason for such difference could be the role of thiol-binding molecules that partially blocked the surface of AuNPs. Apparently, differences in both peak positions and relative intensities were observed from the SERS spectra,

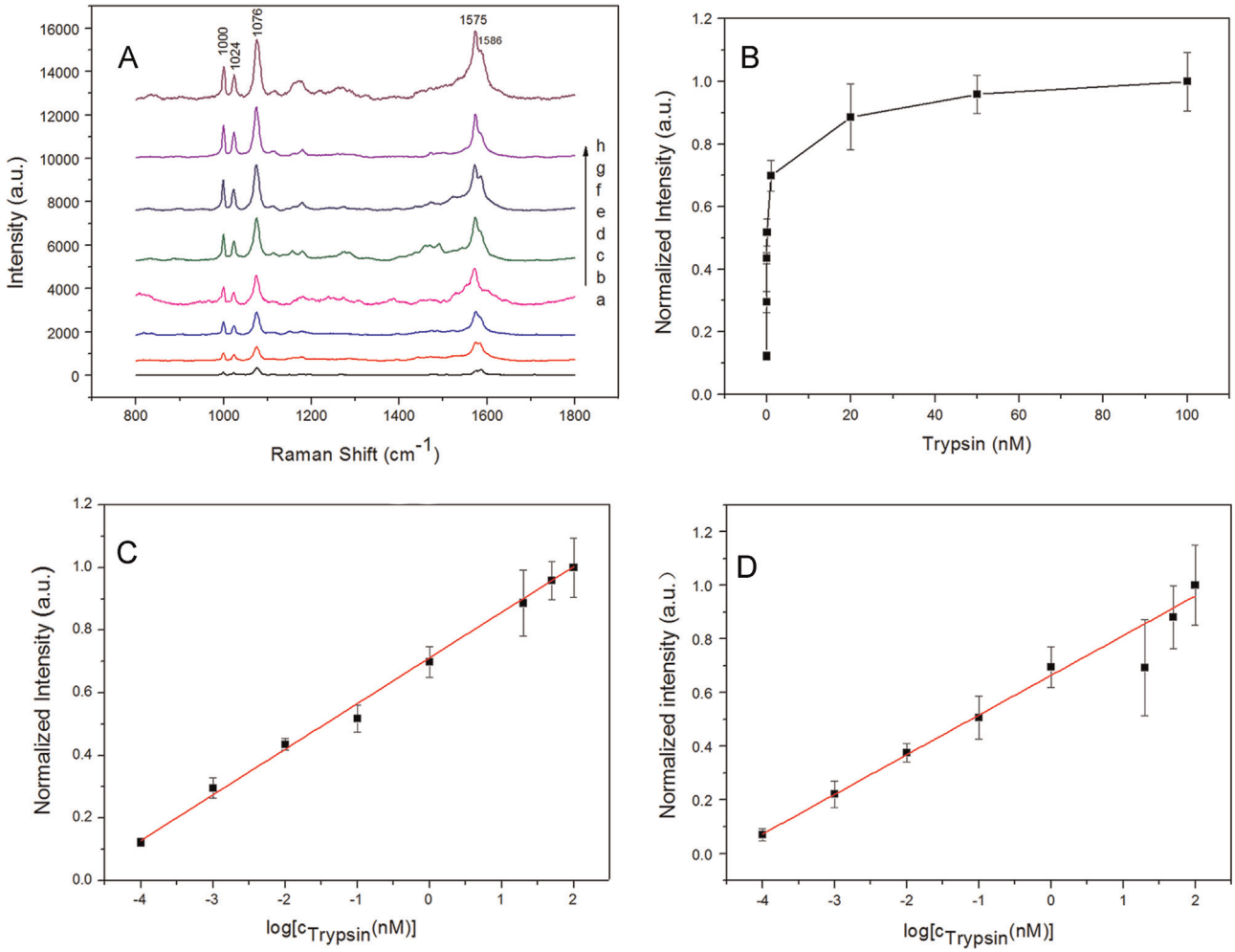


Fig. 2. (A) SERS spectra of 4-MBA modified AuNPs incubated with RD-8 treated with different amounts of trypsin for 60 min: a–h:10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1, 20, 50, and 100 nM. (B) Trypsin concentration (0–100 nM) dependent normalized Raman intensity of 1076 cm^{-1} peak. (C) The calibration curve of normalized Raman intensity at 1076 cm^{-1} against natural logarithm over concentration of trypsin. (D) The calibration curve of normalized Raman intensity at 1000 cm^{-1} against natural logarithm over concentration of trypsin. Each error bar represents the standard deviation in normalized Raman intensity associated with $n=6$.

probably owing to different absorbing states of peptides and aromatic rings.

To further confirm that bands at 1000 cm^{-1} and 1024 cm^{-1} were originated from RD-8, we compared SERS spectra of 4-MBA modified AuNPs under different circumstances (Fig. S11B). First, SERS spectrum of aggregated 4-MBA modified AuNPs without RD-8 presented only two main bands at 1076 cm^{-1} and 1580 cm^{-1} (Fig. S11B, b), and only when trypsin treated RD-8 were introduced, broadened bands around 1580 cm^{-1} and bands at 1000 cm^{-1} and 1024 cm^{-1} appeared (Fig. S11B, c). Therefore, bands at 1000 cm^{-1} and 1024 cm^{-1} were proved from Phe residue of peptides. On the other hand, these new band had rather weak intensities when aggregating the mixture of 4-MBA modified AuNPs and uncleaved peptides by excessive salts (Fig. S11B, d). The $I_{1000cm-1}/I_{1076cm-1}$ value was largest only when trypsin was treated with RD-8 (Fig. S12), indicating that the relative intensity between 1000 cm^{-1} and 1076 cm^{-1} was associated with trypsin and RD-8. When RD-8 were treated with trypsin, the released short peptides induced the AuNPs aggregation and then were absorbed between the gaps of AuNPs. Hence, the "hot-spots" between AuNPs greatly enhanced the Raman signal of Phe. However, without trypsin, the amount of RD-8 absorbed between gold gaps after aggregation was limited due to electrostatic repulsion, resulting the differences in SERS spectra. Therefore, the cause of aggregation of AuNPs can be identified and specificity is improved by incorporating two events: the specific SERS spectra of the peptide and the relative SERS intensity of peptides and dye-modified AuNPs.

Further, as the peak at 1000 cm^{-1} was responsive of trypsin, it is believed that the peak at 1000 cm^{-1} could indicate trypsin concentration as an intrinsic signal source from RD-8. As shown in Fig. 2D, the Raman intensity at 1000 cm^{-1} exhibited a good linear fit to the logarithm of the trypsin concentration in the range of 0–100 nM, and the minimum detectable concentration was 100 fM, indicating that the SERS strategy was ultrasensitive and highly reliable for the detection of trypsin. The regression equation is $y=0.148x+0.664$ (where x is logarithm of the target protein concentration in nM and y is the normalized SERS intensity) with a squared correlation coefficient of 0.992.The limit of detection (LOD) were calculated as 524.8 fM for trypsin according to procedure described above. This result demonstrated the label-free capability of our principle as a highly sensitive approach for proteases assays.

3.4. Interference study and detection of trypsin in serum

The selectivity of the proposed SERS-based strategy toward trypsin was also investigated relative to other potentially competitive enzymes, including pepsin, thrombin, lysozyme, and chymotrypsin (200 nM). As shown in Fig. S13, in the presence of these interferences, SERS intensity increased slightly compared to that

given by the blank, probably due to the adsorption of the proteins. However, when trypsin and the RD-8 was first incubated under the same condition and then added to 4-MBA modified AuNPs, SERS intensity of 4-MBA at 1076 cm^{-1} was enhanced significantly, indicating that the substrate exhibited good specificity toward trypsin.

At last, we tested the influence of complex sample matrix on the method using human serum. Diluted human serum was used as sample matrix to improve the assay recovery. The sample was first 100-fold diluted in PBS buffer, which was then used as the assay medium. Next, trypsin determination was conducted in spiked human serum to investigate the ability of the sensor to overcome interference in complex matrix samples. Trypsin at the concentrations of 0.01, 0.1, 1, and 20 nM has been added in the samples and the final concentration of trypsin is calculated according to the obtained spectral information and the linear regression equation of the standard curve. Table S1 summarizes the recoveries and standard errors of this assay. Results showed that this method presented satisfactory accuracy and precision in serum sample analysis. Hence, our method based on 4-MBA modified AuNPs aggregation activated by trypsin assay has the potential for application of protease detection in complex matrix, and clinical diagnosis.

Repeatability of the proposed procedure was also investigated and the results were given in Table S2. The results of intra-day and inter-day repeatability showed adequate values of precision considering SERS intensity values. The results suggested the SERS-based enzyme assay could be used for protease analysis with acceptable stability.

3.5. Application in thrombin detection

To test the general applicability of our strategy, we detected a second enzyme, human thrombin, a serine protease involved in blood coagulation and several diseases. We designed peptide II with the sequence Arg-Cys-Phe-Leu-Val-Pro-Arg-Gly-Ser-Asp-Asp (RCFLVPRGSDD) by exchanging peptide sequence with thrombin-active cleavage sites. Thrombin could recognize the sequence of LVPRGS and cleave at the C-terminus of the underlined arginine residue (Li et al. 2014). Next, enzyme assays were performed according to established method. We found that the ability of the peptide to stabilize AuNPs were ever stronger than RD-8, possibly attributed to the longer peptide length, and so little aggregation was observed using the optimized parameter for trypsin. One solution is increasing the amount of sodium chloride in solution to break the electrostatic stability of AuNPs (Miao et al., 2013). Aggregation was then successfully observed when the thrombin-digested peptide was added to the 4-MBA modified AuNPs solution. Fig. S15 shows that resulting colors turned from red to dark gray with addition of thrombin, and SERS spectra of AuNPs solution in the presence of thrombin treated from 0 to 100 nM were presented in Fig. S16A, and we were able to detect 8.32 pM thrombin (Fig. S16B). Other common enzymes and abundant proteins did not cause significant interference in the assay (Fig. S17). Hence, the success of both assays for trypsin and thrombin suggests the applicability of the strategy. The platform can be readily extended to detect virtually any protease. Moreover, since this approach can be used for the assay of other proteases by simply changing the substrate domain of the peptide, it may have great potential in biomedical applications in the future. Further, other SERS-active amino acids such as tyrosine and tryptophan can be considered as intrinsic Raman reporter with different spectra, which offers opportunity for high throughput detection platforms and protein-based molecular logic devices.

4. Conclusion

In summary, we have demonstrated a novel “turn-on” SERS biosensor for proteases based on enzyme assays. Our assay offers high sensitivity by integration of catalytic amplification of peptides through enzymatic reactions, and highly sensitive detection of peptide induced 4-MBA modified AuNPs aggregations. Requiring no complicated procedures and recognition units, this assay is much simpler to carry out. The strategy showed enhanced sensitivity and provided new opportunities to enzyme assays. Further, we also demonstrated the universality of this method through application in thrombin detection. The principle of this assay can be extended to other enzymes and assay kits, and high throughput platform may be developed. By using SERS-active peptides, label-free detection of proteases could be established with improved specificity, while peptides combining multiple cleavage sites of proteases could be designed for SERS-based logic gate operations of proteases and biosensors development. Potential applications include clinical diagnosis and fundamental fields such as molecular interactions and protease research.

Note

Additional experimental results of optimization of AuNPs, SEM images are presented in Supporting information.

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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.2014.10.065.

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