



Competitive aptasensor with gold nanoparticle dimers and magnetite nanoparticles for SERS-based determination of thrombin

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Abstract

It is known that the intensity of surface-enhanced Raman scattering (SERS) of monomeric gold nanoparticles (GNPs) is insufficient for ultrasensitive analysis. The authors describe dimeric GNPs for use in a competitive SERS and aptamer based assay for thrombin. The reagent 1,2-bis(4-pyridyl) ethylene serves as both the coupling agent and the Raman reporter on the GNP dimers. In the presence of thrombin, the hybridization of two aptamers, one attached to the GNP dimers, the other to magnetic nanoparticles, is competitively prevented. This method takes advantage of the unique “hot spots” of the GNP dimers to amplify the Raman signal. This results in an ultra-sensitive thrombin assay when compared to assays using GNP monomers. The limit of detection is as low as 1 fM of thrombin. The Raman intensity, best measured at 1612 cm⁻¹, increases linearly in the 1 fM to 10 nM thrombin concentration range. The method was applied to the determination of thrombin in spiked simulated body fluid and human serum.

Keywords Gold nanoparticle dimers · Magnetic nanoparticles · Aptasensor · Competitive · Thrombin

Introduction

Thrombin plays a crucial role in the blood clotting, thrombosis and other coagulation-related reactions [1, 2]. Thrombin is not present in the blood of healthy people. The existence of thrombin in the blood without trauma would cause a series of pathophysiological changes, including coagulation abnormalities and Alzheimer’s disease [3–5]. Recently, the development of new method to detect thrombin has been a hot topic, such as electrochemiluminescence, fluorescence, colorimetry, and so on [6–8]. Among these methods, thrombin binding aptamer (TBA) was served as a recognition element to construct

specific sensors. These strategies provided affinitive and sensitive approach to monitor thrombin. However, there are some deficiencies such as labor-intensive, time-consuming and tedious pretreatment among those approaches. Therefore, it is highly significant to construct a new method to detect thrombin with high sensitivity, rapidity and specificity.

With the development of nanotechnology, various immunoassays for thrombin detection based on silver and magnetic nanoparticles have been achieved success [9, 10]. And surface-enhanced Raman scattering also emerged as a powerful technology, which has been applied widely in life science. However, the enhancement of GNP-monomers is insufficient for the ultra-sensitive analysis in biological system. Recently, Wustholz’s group reported the creation of “hot spots” in subnanometer proximity of dimers and trimers, achieving strongly SERS enhancements [11]. Simonsen proposed a new approach to quantify and sort of gold nanoparticle dimers from complex mixtures [12]. These researches proved that gold dimer can serve as an excellent SERS substrate to apply in the ultra-sensitive detection. Moreover, the target recognition elements (antibody or aptamer) can be attached onto the surface of metal nanoparticles to form SERS probes, which endows the excellent biocompatibility and high specificity to the SERS tags.

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In the fabrication of SERS nanoprobe, the functional modification is crucial for specific detection. Conventional modification commonly employed antibody as the core of the recognition mechanism [13, 14]. However, the impurities, temperature, unfreeze in experiments usually decreased the activation of the antibody-modified nanoprobe, limited its application in practical analysis. There is still an urgent requirement to design a more stable SERS probe. Aptamer is serial of artificial oligonucleotide chain with predictable and tailorable structure, permitting it to bind to target with high affinity and specificity [15], which usually appears in the form of DNA or RNA. Compared with antibody, aptamers possess variously excellent characteristics, such as small size, target binding affinity, lack of immunogenicity and specificity [16]. Importantly, it is also relatively cheap and can store stably, allowing a probable application in biochemical detection [17]. Therefore, numerous methods have been designed for immunoassay [18–20]. All the researches above illustrated that the aptamer is an excellent candidate in the construction of SERS nanoprobe.

For sake of the detection of thrombin accurately and ultrasensitively, we developed a competitive detective platform based on the GNP-dimers with the aids of magnetic separation. This method took advantage of GNP-dimers to amplify Raman signal, and used serials of competing reactions between specific aptamer and target protein. We innovatively integrated dimer nanosubstrate and magnetic separation into the competing system, greatly improved the sensitivity of thrombin detection. The strengths of the method include: (i) a remarkably amplified Raman signal that is due to the unique “hot spots” of the GNP dimers; (ii) the use of BPE as both the coupling agent and the Raman reporter in the fabrication of GNP dimers which avoids the need for additional modification steps. (iii) the competing reactions between specific aptamer and target protein multiply ensure the specificity of the whole system. (iv) Fast reaction kinetics of magnetic separation dramatically shorten the reaction time of immunoassay. Importantly, we proved that this method can detect thrombin in human serum sample with the recoveries between 99.48% and 100.47%, which demonstrates that this strategy can be served as an excellent technique to apply in biomedicine diagnosis.

Experiment section

Materials and reagents

1,2-Bis(4-pyridyl)ethylene (BPE) and trisodium citrate were acquired from Sigma-Aldrich, USA (www.sigmaaldrich.com). Chloroauric acid (HAuCl_4) was received from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) (www.reagent.com.cn). Sodium chloride (NaCl), disodium hydrogen phosphate (Na_2HPO_4), potassium dihydrogen

phosphate (KH_2PO_4), N-Hydroxysuccinimide (NHS), N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), carboxyl-functionalized magnetic beads (average diameter 300 nm), potassium chloride (KCl), magnesium chloride (MgCl_2) and silver nitrate (AgNO_3) were purchased from Aladdin-Reagent Co., Ltd. (Shanghai, China) (www.aladdin-e.com). Thrombin, Lysozyme and Hemoglobin were purchased from Sangon Biotechnology Co., Ltd. (Shanghai, China) (www.sangon.com). Human serum samples were purchased from Amyjet Scientific Inc. (www.amyjet.com) complying the rules of the local ethical committee.

The washing buffer of phosphate buffered saline (PBS, 10 mM, pH 7.4) was prepared from NaCl, Na_2HPO_4 , KH_2PO_4 , KCl and 0.05% Tween-20. The Tris-HCl buffer (pH 7.4) was prepared from 0.01 M Tris, 5 mM KCl, 5 mM MgCl_2 and 50 mM NaCl. Millipore water purification system (ELGA, London) was utilized for obtaining ultrapure water.

The sequences of all DNA strands were synthesized by Sangon Biotechnology Co., Ltd. (Shanghai, China, www.sangon.com) and listed as follows:

Thrombin binding aptamer (TBA):

5'-TGTCAGTGGGGTTGGACGGGATGGTGCCTGACTCTC-(CH_2)₇-NH₂-3'.

Aptamer-complementary: 5'-GGCACCATCCCGTCAACCCCA-(CH_2)₆-SH-3'.

Spectral measurement

The Raman spectra were acquired from a microscopic Raman spectrometer (Nippon Optical System Co., Tokyo). The system equipped with a He-Ne laser (Melles Griot, Carlsbad, CA) of 633 nm and with the energy about 1 mW. Considering the magnification of collection objectives, the sizes of collection zones were 1 μm . All spectra were calibrated with the peak intensity of silicon spotted at the 520 cm^{-1} . The typical accumulation time for the sample was 30 s. The Raman spectrum was collected by WinSpec/32 software and can be converted into the digital data. Those data were processed by the use of Origin software. Thereafter, a baseline calibration on the spectra was performed to remove the fluorescence background. The spectra acquired from the UV-vis spectrometer (Perkin-Elmer, Waltham, MA) were utilized for determining the diameter of gold nanoparticles. Fourier transform infrared (FT-IR) were used for auxiliary judging the results of the initial synthesis.

Assembly of GNP-dimers

The 30 nm AuNP was prepared according to our previous work [21]. And the detail process shows in ESM. The Raman reporter molecule of BPE has bifunctional groups that can link two AuNP to form the dimer. In the process of synthesis, BPE is quenched by silver nitrate before the gold

colloids' color changes, avoiding the endless connection of AuNP. For the assembly of the GNP-dimers, the pH value of the AuNP should be adjusted to 7. Firstly, the freshly AuNP was centrifuged at 8000 rpm for 10 min and resuspended with ultrapure water (pH 7). 15 μL volume of BPE (0.05 mM) was then added to the gold nanoparticle solutions in a few minutes, followed by the addition of 8.5 μL AgNO_3 in water (0.04 mM) to terminate the reaction. The mixture was centrifuged at 4000 rpm for 20 min with two times, and then the precipitates were finally resuspended in water. The obtained GNP-dimers were stored at 4 $^\circ\text{C}$ for further use.

Synthesis of capture probe

According to previous work [21], the synthesis of the capture probe is shown in Fig. 1a. Firstly, 100 μL of 5 mg/mL carboxyl-functionalized MNP were washed with two times by PBS (pH 7.4) buffer to eliminate the matrix effects, and we added the freshly 30 μL EDC (0.05 mg/mL) and NHS (0.05 mg/mL) solutions to activate the $-\text{COOH}$ of MNP with 2 h. After that, 100 μL amino modified TBA (100 μM) solution was added into the solution to incubate 3 h with shaking gently. The obtained capture probe was purified three times by the assistance of magnetic separation and resuspended in 1 mL PBS buffer.

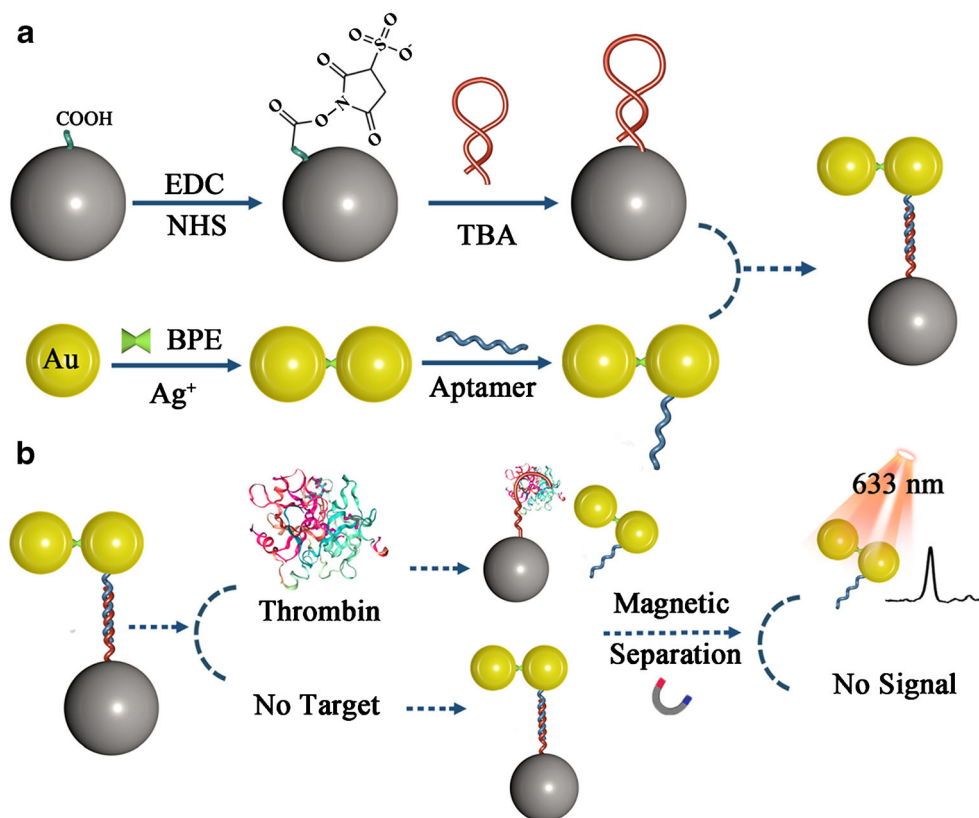
Synthesis of SERS nanoprobe

The preparation of SERS nanoprobe is following the method reported by Sun et al. [22]. The beforehand GNP-dimers were used as the enhanced substrate with a slight modification. Firstly, 100 μL of 10 μM aptamer-complementary was activated by adding the freshly 3 μL 10 mM TCEP with 1 h. After activation, the aptamer-complementary was added to the solution of the dimers at room temperature with gently shaking. After 12 h, the salting was initiated slowly with 1.0 M NaCl by steps in 24 h until the desired salt concentration (0.1 M) was obtained. The final solution was centrifuged for 20 min at 4000 rpm with two times to remove the free aptamer. Following the purification of the supernatant, the precipitate was re-distributed in 1 mL Tris-HCl (pH 7.4) buffer. At last, the obtained SERS nanoprobe can be stored steadily at least 1 month at 4 $^\circ\text{C}$. Figure 3d shows the TEM image of the SERS nanoprobe.

Assay procedure

100 μL of capture probe and 80 μL SERS nanoprobe were mixed for 2 h with gently shaking. After the formation of the assembled structure, the solution was washed two times with the assistance of magnetic separation approach and the precipitate was resuspended in Tris-HCl buffer. In a typical test, the

Fig. 1 Schematic presentation of SERS methods based on gold nanospheres dimer: **a** the formation of magnetic gold dimer sensor. **b** the competitive detection of thrombin



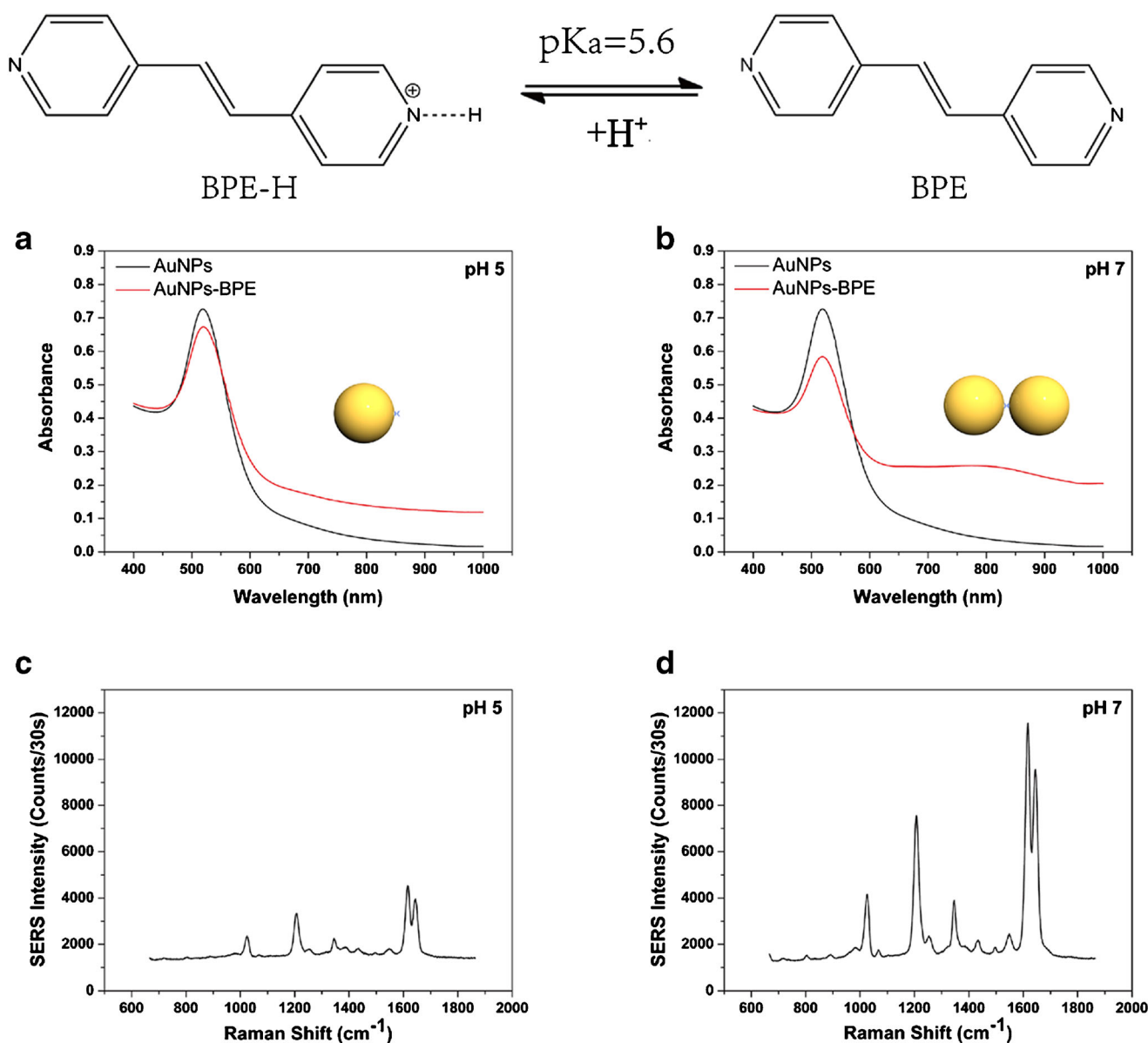


Fig. 2 Extinction spectra recorded for AuNP before (black line) and following the addition of BPE (red line) at pH 5 (a) and pH 7 (b). SERS spectra recorded with 633 nm laser excitation for AuNP after addition of BPE at pH 5 (c) and pH 7 (d)

solution was incubated with 50 μ L various concentrations of thrombin which were prepared in 0.1 M Tris-HCl buffer (pH 7.4) for 3 h at room temperature. Finally, we obtained the SERS signal from the supernatant by the approach of magnetic separation. The increasing concentration of the target would raise the Raman signal of the suspension solution (Fig. 4).

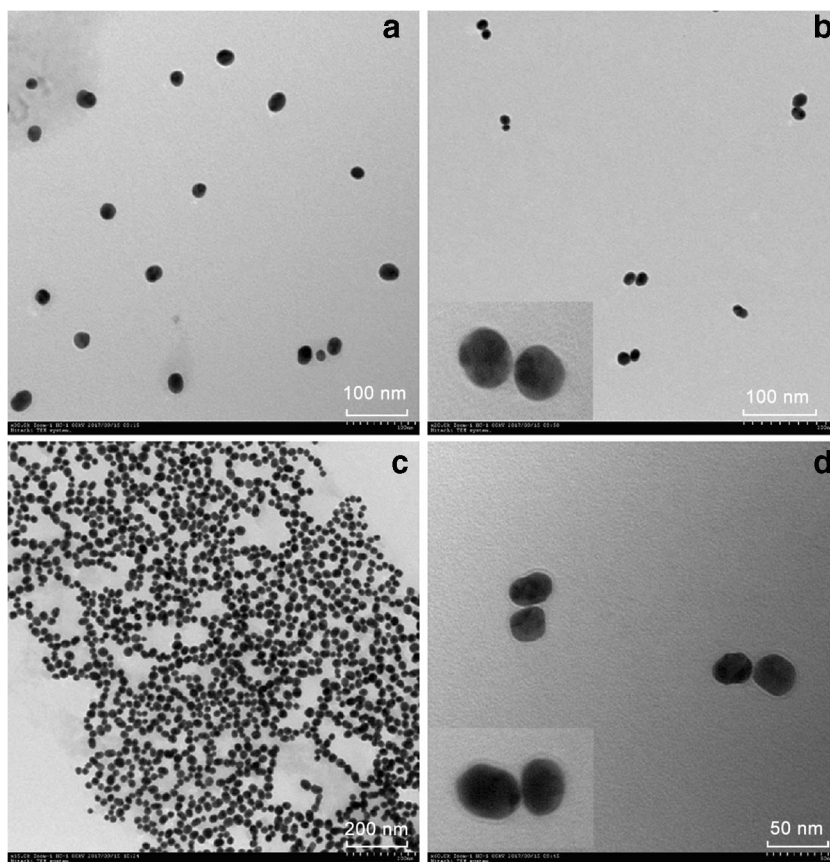
Results and discussion

Synthesis and characterization of GNP-dimers

The schematically process of GNP-dimers fabrication is described in Fig. 1a. There are two typical procedures in this

synthetic process, including the assembly of GNP-dimers with BPE and the termination reaction of $AgNO_3$. In theory the formation of gold dimers was determined by pH value. This particular property is due to the molecular structure of BPE. The pyridine groups attach to gold through the N end of BPE. If the N end has been protonated, it is difficult for BPE to attach to the Au surface [23]. According to the kinetic parameters of BPE (Fig. 2), we obtained the relationship between the molecular structure of BPE and pH value. Two characteristic conformation of BPE impacted the combination of AuNP at pH 5 and pH 7. Then we test the UV-Vis spectrum and TEM image, both results prove the formation of dimers. As the Fig. 2a shows, the UV-vis spectra are unchanged after the addition of the BPE, displaying the single-terminal combination

Fig. 3 TEM images of AuNP (a), dimers with AgNO₃ quenched (b), dimers without AgNO₃ (c) and the aptamer-modified dimers (d)

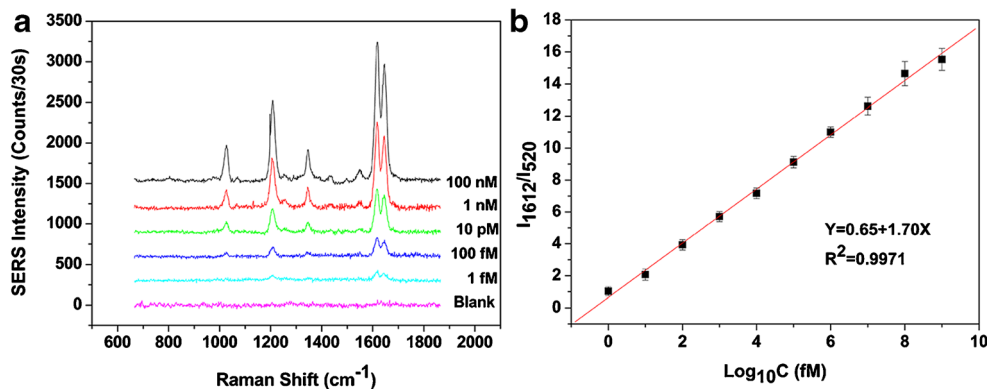


between the BPE and AuNP at the pH 5. Correspondingly, the SERS spectra measured at pH 5 exhibited weak signals of BPE (Fig. 2c). In contrast, Fig. 2b and d show that the two nonphotic pyridyl moieties of BPE can bond with AuNP to form dimers when the pH of AuNP was adjusted to 7. In this synthesis process, we used AgNO₃ to terminate the reaction between BPE and the gold dimers. AgNO₃ will coordinate with one end of the BPE [24], which will prevent the remaining BPE from attaching to the gold surface and the aggregation will be quenched. The effect of AgNO₃ can be shown in Fig. 3b and c.

Mechanism of the strategy

We fabricated the SERS nanoprobe based on the above GNP-dimers, which used as the enhanced substrate with aptamer modification. The aptamer would attach onto the surface of GNP-dimers by stepwise salt deposition. To investigate the deposition of aptamer, we examined the TEM of GNP-dimers after the modification of aptamers. From the TEM images of the SERS nanoprobe (Fig. 2d), we can see that the indication of the successful modification of aptamer surrounding the GNP-dimers. We also can find in Fig. S4 that there is

Fig. 4 The sensitivity of the competitive strategy for thrombin detection: a The Raman signals with different thrombin concentrations added. b The relationship between the values of I₁₆₁₂/I₅₂₀ and the concentrations of thrombin



no new peak emerged when the aptamer deposited into the GNP-dimers. The results indicate that the addition of the aptamer would not change the peak position of the Raman spectrum.

The schematic illustration of TBA-MNP/aptamer-dimers competitive strategy for thrombin detection is shown in Fig. 1b. At first, the hairpin TBA would be formed by the treatment of annealing. Amino-modified hairpin TBA was immobilized on the functionalized-MNP via amino-carboxyl interaction (capture probe), and thiol-modified aptamer-complementary was immobilized on the GNP-dimers via Au-S bonding (SERS nanoprobe). In the immersion of Tris-HCl, the capture probe was combined with SERS nanoprobe through the hybridization of DNA. Following the addition of the thrombin, the TBA structure was changed to connect with the target and make nanoprobe free from the MNP structure. With the increasing concentrations of the thrombin, the more TBA-MNP-thrombin complexes were formed, resulting in the increasing of Raman signal in the supernate (from nanoprobe). This method has numerous strengths compared to the traditional methods: a) the GNP-dimers improve the Raman signal significantly, comparing to the insufficient enhancement of monomer nanoparticles; b) the detected platform we constructed is highly sensitive, excellent selective and accurate quantitative detection of thrombin.

Determination of thrombin

Before the detection of thrombin, we optimized the relative parameters as shown in Fig. S5. On the basis of the optimal experiments, the quantitative application and the sensitivity of the strategy were investigated. A series of thrombin solutions with different concentrations were tested in the process of detection. We collected the unconnected gold dimers from the mixture solution with aid of magnetic separation. And the increasing target would raise the Raman signal of the suspension up. The using excitation wavelength was 632.8 nm and the energy is ca. 1 mW at sample location. The Raman peak of BPE at 1612 cm^{-1} served as the characteristic peak in this procedure. We perform nine repeated experiments at the

Table 1 Some conventional methods compared with our work. For comparison, the detection limit of this strategy was lower than the previous conventional works

Method	Target	Materials	LOD	Reference
SPR	Thrombin	AuNPs	0.1 nM	Bai et al. [26]
Fluorescence	Thrombin	Graphene	31.3 pM	Li et al. [28]
Electrochemical	Thrombin	rGO-AuNPs	0.3 nM	Wang et al. [29]
Colorimetric	Thrombin	TBA-hemin	20 nM	Li et al. [28]
SERS	Thrombin	AuNPs	0.27 pM	Yoon et al. [30]
SERS	Thrombin	GNP-dimers	1 fM	this work

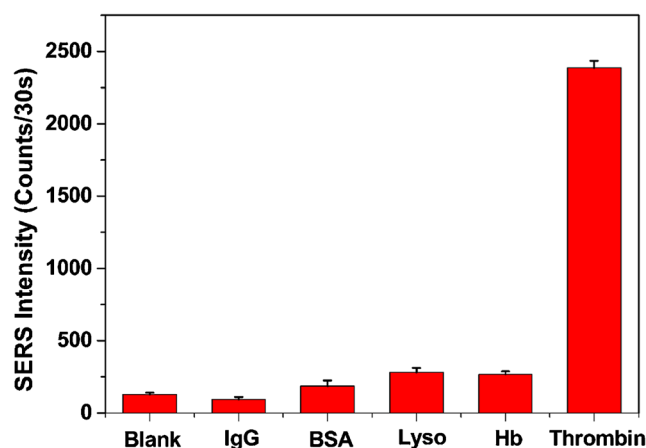


Fig. 5 Specificity of the detection of the competitive strategy for thrombin detection

same concentration of thrombin to obtain the error bar, showing the standard deviation of nine experiments. Figure 4a indicated that the intensities of Raman signals increased with the increasing concentrations (1 fM to 10 nM) of thrombin. In order to eliminate the instrument error, the ratio of Raman intensity at 1612 cm^{-1} of BPE and silicon 520 cm^{-1} of silicon (I_{1612}/I_{520}) are used for the quantitative measurements. The corresponding linear curve was determined as shown in Fig. 4b. The regression equation is $Y = 0.65 + 1.70 \log C$ (Y is the Raman peak intensity at 1612 cm^{-1} and C is the concentration of thrombin) with a relatively excellent linear relationship (correlation coefficient square of 0.9971). The control experiment (I_{1612}/I_{520}) is the highest, ca. 1.13 ± 0.178 , so the detection threshold value should be set as 1.664 (mean of the control value plus 3 times standard deviation) [25]. And the LOD of thrombin by this method was as low as 1 fM. As the Table 1 shows, compared with other methods based on SPR, fluorescence, electrochemical or colorimetric which focused on the detection of thrombin, the method indicated a higher sensitivity [26–28]. By the using of the GNP dimer, the LOD of thrombin this method is lower than the SERS immunoassay based on conventional single gold nanoparticle [29].

Specificity of the method

In order to investigate the specificity of this method, the different possible interference existed in thrombin detection were analyzed simultaneously. Herein, 100 pM IgG, BSA,

Table 2 Levels of apparent recovery with thrombin-spiked serum sample

Samples	Thrombin added (fM)	Apparent recovery ratio (%)	RSD (%)
1	5	99.48	12.62
2	50	100.47	12.26
3	500	100.18	13.81

Lysozyme (Lyso) and Hemoglobin (Hb) were separately detected under the identical experiment conditions. As shown in Fig. 5, the corresponding Raman intensities of the above added interference were similar to the blank sample. In contrast, the Raman signal dramatically increased with the present of target thrombin (100 pM). All these results demonstrated that this strategy possessed remarkable specificity for the detection of thrombin.

Application of the strategy to detect thrombin in real sample

We further examined the practicability and reliability of this method in real sample. The detections of thrombin were performed in preprocessed human serum sample. In detail, the human serum was diluted by Tris-HCl (pH 7.9) buffer with 20 times for alleviating the matrix effects. Afterwards, the different concentration gradients of standard thrombin solution (10 μ L of 1 fM \sim 10 nM) were sequentially added to 1 mL of the above serum samples. We measured the ultra-water and the human serum using this strategy. As shown in Fig. S6, the Raman signal intensities of the human serum are similar to those of the ultra-water. As the results, we determined that there was no thrombin existed in the human serum. On the basis of this result, we prepared a series of standard thrombin samples by the use of the human serum and collected the Raman intensities, respectively. The corresponding recoveries are obtained from 99.48% to 100.47% as shown in Table 2. We also proved that the strategy can apply in simulated body fluid as show in Fig. S7, which confirmed its potentiality for thrombin detection in the presence of the admixture at high excess, even in the field of clinical medical application.

Conclusion

The GNP-dimers with a competitive reaction was proposed for ultra-sensitive detection of thrombin. The GNP-dimers self-assemble by 1,2-bis(4-pyridyl)ethylene (BPE), which not only serve as the Raman reporter but also as the coupling agent between two AuNP. Compared to the GNP-monomers, the powerful SERS enhancement can be attributed to gaps in GNP-dimers which are well known as “hot spots”. The synthetic GNP-dimers were functionalized with the aptamer (the complementary) and hybridized with the TBA-modified MNP to form an entire sensing system. The formed double strand between the GNP-dimers and MNP would be unfolded with the thrombin presented. With the aid of magnetic separation, the Raman signals from the supernate increased with the target continually added. And the strengths of our novel strategy lie in: Firstly, the GNP-dimers has solved the extant deficiencies of conventional SERS substrates such as the insufficient enhancement of monomer metal nanoparticle. In addition, it

doesn't require complex processing procedure based on the solid substrate, shortening the assay time. Moreover, the whole of the probes contained SERS nanoprobe and capture probe can be stored at 4 °C to sustain activities for further usage. In terms of these advantages, the strategy achieved an extremely low LOD of 1 fM, providing a vast promise for applying in clinical medicine. However, the stability of gold-dimer in purity blood is a big problem in this strategy, and it is expected to be solved with biological coating, which is what our lab will test in the future.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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