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Highly Sensitive Two-Photon Sensing of Thrombin in Serum Using Aptamers and Silver Nanoparticles

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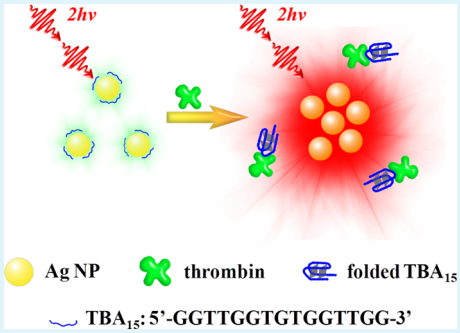
Supporting Information

ABSTRACT: Thrombin plays an important role in pathological conditions. It is important, however challenging, to detect thrombin in complex biological media for clinical practice and diagnostic applications. Here we demonstrate a label-free, fast, highly sensitive and selective two-photon sensing scheme for detection of thrombin on the picomolar level. The assay is based on interactions between thrombin and a DNA aptamer, which induce aggregation of silver nanoparticles to display significantly enhanced two-photon photoluminescence. The limit of detection (LOD) of this two-photon sensing assay is as low as 3.1 pM in the buffer solution, more than 400 times lower than that of the extinction method (1.3 nM). The dynamic range of this method covers more than 4 orders of magnitude. Furthermore, this two-photon sensing assay can be applied to detection of thrombin in 100% fetal bovine serum with LOD of 1.8 nM. In addition to the unique advantages of two-photon sensing such as deep penetration and localized detection, this method could be potentially integrated with two-photon microscopy to offer additional advantages of 3D detection and mapping for potential in vivo applications.



KEYWORDS: *two-photon photoluminescence, biosensing, thrombin, silver nanoparticles, aptamers, plasmon coupling*

INTRODUCTION

Thrombin is a multifunctional protein that produces insoluble fibrin through proteolytic cleavage of soluble fibrinogen.[1] Thrombin plays an important role in pathological conditions including the central nerve system injury, thromboembolic disease, and Alzheimer's disease.[2,3] It is important to develop simple, highly sensitive, and selective methods to detect thrombin in the blood serum for both clinical practice and diagnostic applications. Various methods, including colorimetric,[4,5] electrochemical,[6,7] fluorescence,[1,8,9] magnetic,[10] and surface enhanced Raman scattering (SERS) methods,[11] have been developed to detect thrombin. Most of these methods are based on the conformational change from a single-stranded aptamer to a quadruplex structure induced by thrombin. Sandwich binding of two affinity aptamers has also been proposed to detect thrombin for increased specificity.[12,13] It is very important to detect thrombin in the blood serum or even in vivo as thrombin generally exists in blood, which is a more complicated media than the buffer solution. Conventional methods are generally difficult to be extended to the in vivo applications, where deep penetration into biological tissues is required. Utilizing near-infrared light as the excitation source, two-photon excitation has become popular in biology and medicine because of its distinct advantages over its conventional one-photon counterparts.[14−18] These advantages include increased penetration depth, intrinsically localized excitation, less tissue autofluorescence, reduced photodamage and photobleaching. Two-photon excitation based sensing has recently been actively developed due to their potential applications for in vivo detection and 3D mapping in the biological environment.[19−24]

Noble metal nanoparticles, such as Au and Ag, have been widely employed as probes for a variety of biosensing platforms owing to their unique optical properties.[25−28] One major advantage of noble metal nanoparticles based assays is that molecular recognition events can result in color changes that can be directly visualized by naked eyes.[29−31] A number of noble metal nanoparticles based colorimetric sensors have been developed for detection of proteins,[8,32,33] peptides,[34] small molecules,[35] metal ions[36,37] and cancer cells.[27] However, a major disadvantage of the colorimetric methods is that they are difficult to be extended to the complex biological environments or in vivo applications, where high tissue penetration is required. Our recent work demonstrated that aggregation of Au and Ag nanoparticles (NPs) could result in significantly enhanced two-photon photoluminescence (TPPL) by up to 100-fold in solution and 5 orders of magnitude on the single particle level.[38−41] The observed TPPL enhancement was ascribed to enhanced two-photon excitation efficiency owing to formation of a new plasmon mode for resonance enhancement and enhanced local field at the excitation wavelength.[42] Plasmon coupling enhanced TPPL of Ag and Au NPs have been further utilized to develop two-photon sensing platforms for detection of mercury,[43] cysteine, and glutathione.[44]

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Here we demonstrate a highly sensitive TPPL based assay for detection of thrombin in the biological environment-blood serum. This assay is based on selective interactions between thrombin and a thrombin-binding aptamer (TBA_{15}), which induce aggregation of Ag nanoparticles and display significantly enhanced TPPL. The limit of detection (LOD) of our TPPL based assay is 3.1 pM in the buffer solution, >400 times better than that based on change in the extinction spectra. This assay has also been evaluated in fetal bovine serum and LOD of 1.8 nM was achieved in 100% serum. Considering the additional advantages of two-photon excitation, this TPPL based sensing assay could be readily applicable to detection of other proteins in complex media.

EXPERIMENTAL SECTION

Materials. 15-mer thrombin-binding aptamer (TBA_{15}, 5′-GGTTGGTGTGGTTGG-3′), silver nitrate ($AgNO_3$), sodium borohydride ($NaBH_4$), hydrogen tetrachloroaurate (III) hydrate ($HAuCl_4{\cdot}3H_2O$) and fetal bovine serum were purchased from Sigma-Aldrich. Human alpha-thrombin was purchased from Haematologic Technologies Inc. Trisodium citrate was purchased from BDH Chemical Ltd. L-Ascorbic acid was purchased from Alfa Aesar. Bovine serum albumin (BSA) was purchased from Sinopharm Chemical Reagent Co., Ltd. All solvents are of analytical grade and used as received without further purification. Milli-Q water (18 MΩ) was used to prepare all solutions and buffer solutions.

Preparation of Ag NPs. Ag NPs were prepared by using a seed mediated approach adapted from a previous report.[45,46] Citrate-stabilized seed solution was prepared by dissolving 1.47 mg of sodium citrate and 1.97 mg of $HAuCl_4{\cdot}3H_2O$ in 20 mL of water; 0.2 mL of 0.1 M ice cold $NaBH_4$ solution was then added under vigorous stirring. The solution changed color from orange-yellow to brownish red, indicating formation of Au nanoparticle seeds. The seed solution (3 mL) was quickly added into a mixture of $AgNO_3$ (10 mg) and sodium citrate (30 mg) in 50 mL of water. Ascorbic acid solution (30 mg in 10 mL water) was subsequently added dropwise for ∼10 min and the stirring continued for another 1 h. A brownish yellow Ag NP solution was obtained. TEM images showed that the average particle diameter was ∼15 nm.

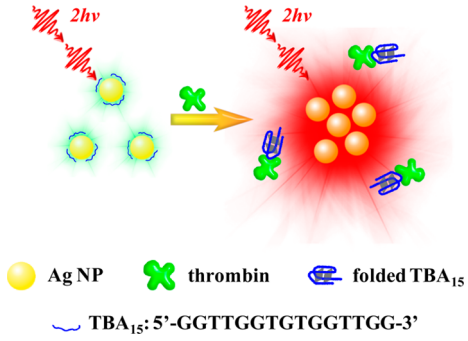
Detection of Thrombin. Two-hundred microliters of the prepared Ag NP solution was first mixed with 30 μL of 4.6 μM TBA_{15}. The mixture was kept for 5 min followed by addition of 90 μL of 20 mM Tris·HCl buffer solution (pH 7.4). Different concentrations of thrombin were subsequently added. The experiments were also performed by using serum as the media, in which different amounts of fetal bovine serum (volume ratio of 10%, 20%, 40%, 80% and 100%) were added to the above solution. All the measurements were taken after incubation for 10 min.

Instrumentations and Characterizations. The photos of the samples were taken by using a Kodak EasyShare C140 digital camera. Transmission electron microscopic (TEM) images of the nanoparticles were recorded by using a JEOL 2010 microscope. Ultraviolet−visible (UV−vis) extinction spectra were measured by using a Shimadzu UV-2550 spectrophotometer. TPPL measurements were performed by using an Avesta TiF-100 M femtosecond Ti:sapphire oscillator as the excitation source, which gives output laser pulses with central wavelength at 810 nm, pulse duration of 80 fs and a repetition rate of 84.5 MHz. The laser beam was focused onto the samples that were contained in a cuvette. The emission from the samples was collected at the direction perpendicular to the excitation beam by a pair of lenses and an optical fiber that was connected to a monochromator (Acton, Spectra Pro 2300i) coupled CCD system (Princeton Instruments, Pixis 100B). A short pass filter with a cutoff wavelength of 750 nm was placed before the spectrometer to minimize the scattering from the excitation beam. TPPL spectra of TBA_{15} protected Ag NPs in the presence of different concentration of thrombin were measured 10 min after addition of thrombin.

RESULTS AND DISCUSSION

The working principle of our two-photon sensing strategy for thrombin detection is shown in Scheme 1. The assay is based on specific interactions between thrombin and a DNA aptamer, TBA_{15}, which induce aggregation of Ag NPs and result in significantly enhanced TPPL. TBA_{15} is the first DNA aptamer isolated from in vivo selection with a binding dissociation constant of ∼100 nM with thrombin.[47] It has been previously reported that there is strong coordination interactions between the nitrogen atoms of the unfolded ssDNA and the Ag NPs.[29,48] The unfolded TBA_{15} (ssDNA) would be adsorbed onto the surface of Ag NPs, which helps to stabilize Ag NPs against aggregation.[4,49] Ag NPs with surface-adsorbed TBA_{15} can be well dispersed in the solution. Upon addition of thrombin, TBA_{15} interacts with thrombin and folds into a G-quadruplex structure. The relatively rigid structure of folded TBA_{15} molecules prevents the exposure of the DNA bases to Ag NPs, which detaches TBA_{15} from the surface of Ag NPs. Ag NPs consequently become deprotected to form aggregates.[4,29,48,50] Aggregated noble metal nanoparticles have been known to display significantly enhanced TPPL compared to unaggregated ones.[38−40,43,44] Thrombin induced aggregation of Ag NPs would thus result in significantly enhanced TPPL of Ag NPs, which could be utilized to develop a two-photon sensing scheme for selective detection of thrombin.

Scheme 1. Schematic Description of Two-Photon Sensing of Thrombin Using Ag NPs and TBA_{15}



Ag NPs were prepared by using a seed mediated approach as described in the Experimental Section. The as-prepared Ag NPs have an average diameter of 15 nm (see Figure S1 in the Supporting Information) and its extinction spectra in H_2O display a localized surface plasmon resonance (LSPR) band peaking at 395 nm (Figure 1). Upon addition of a proper amount of thrombin, the Ag NP solution rapidly changed the color from yellow to brown (Figure 1a), indicating formation of Ag NP aggregates. The obvious color change from yellow to brown can be directly visualized with the naked eyes upon addition of >20 nM of thrombin. The formation of aggregates can be verified by monitoring the changes in their UV−vis extinction spectra (Figure 1b) and TEM images (see Figure S1 in the Supporting Information). Upon addition of thrombin, the extinction of the original LSPR band at 395 nm steadily decreased, accompanied with appearance of a new LSPR band in the longer wavelength region. This new band arises from the longitudinal LSPR mode of the newly formed anisotropic Ag NPs aggregates. TEM images show that Ag NPs were assembled into chainlike structures consisting of several nanoparticles upon addition of thrombin (see Figure S1 in

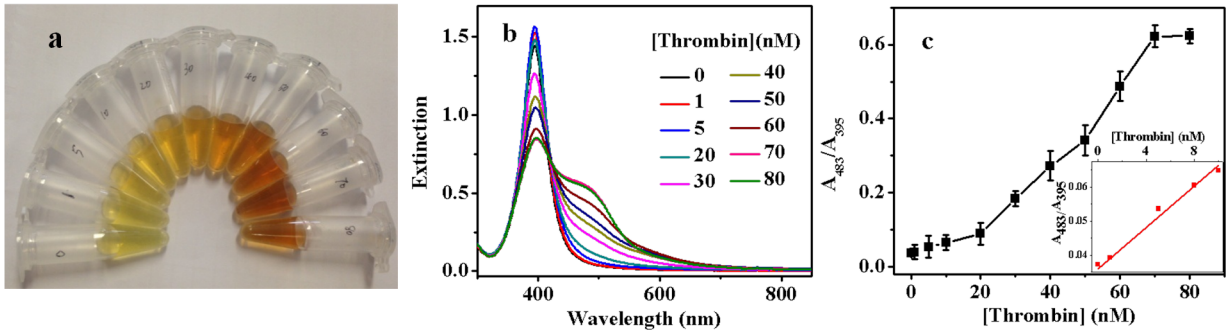


Figure 1. (a) Photographs and (b) extinction spectra of Ag NPs + TBA_{15} in the presence of different concentrations of thrombin; (c) extinction ratio $A_{483\ nm}/A_{395\ nm}$ versus [thrombin]. The inset shows that $A_{483\ nm}/A_{395\ nm}$ is linearly proportional to [thrombin] in the low concentration range. Error bars represent standard deviations obtained from three independent measurements.

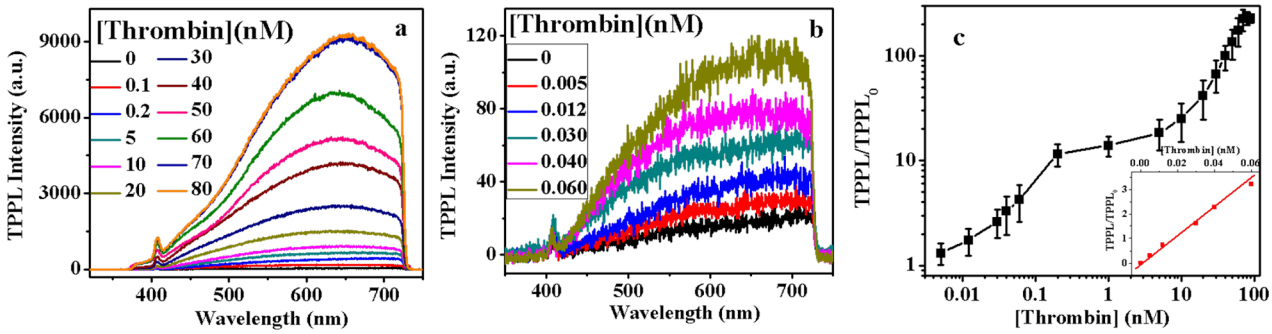


Figure 2. (a, b) TPPL spectra of Ag NPs + TBA_{15} solution upon addition of different concentration of thrombin; (c) plot of TPPL enhancement factor versus [thrombin]. The inset shows the linear plot in the low concentration range. Error bars represent standard deviations obtained from three independent measurements.

the Supporting Information). The extinction ratio between 483 and 395 nm, $A_{483\ nm}/A_{395\ nm}$, can be utilized to quantitatively determine the concentration of thrombin (Figure 1c). The LOD was determined to be ~1.3 nM based on the change in extinction spectra, which is comparable to that previously reported by Dong et al. (LOD of 0.83 nM)[4] using Au NPs.

Aggregated metal nanoparticles have been known to display significantly enhanced TPPL than the unaggregated metal nanoparticles.[38,41] Plasmon coupling in aggregated metal NPs results in formation of a strong plasmon band that is resonant with the excitation wavelength. This new plasmon band provides resonant intermediate states that greatly promote two-photon excitation processes.[42] In addition, enhanced local electric field at the excitation wavelength due to plasmon coupling also contributes to enhanced TPPL.[40,42] Furthermore, TPPL of metal nanoparticles depend on the particle size and formation of larger-sized aggregates also enhances TPPL.[39] Thrombin mediated aggregation of Ag NPs are expected to result in significantly enhanced TPPL, which could be utilized to develop a two-photon sensing platform for potentially in vivo detection of thrombin to take the unique advantages of two-photon excitation.

TPPL spectra of TBA_{15}-protected Ag NPs in the presence of different concentrations of thrombin were measured by using femtosecond laser pulses at 810 nm as the excitation source. TBA_{15}-protected Ag NPs in the absence of thrombin displayed very weak TPPL emission due to small two-photon excitation efficiency of isolated Ag NPs (Figure 2a). Upon addition of thrombin, a broad TPPL band appeared in the spectra range of 450−750 nm and gradually increased with the increasing concentration of thrombin. The integrated TPPL intensity enhancement factor (I_{TPPL}/I_{TPPL0}, where I_{TPPL} and I_{TPPL0} are the integrated TPPL intensities of the Ag NPs in the presence and absence of thrombin, respectively.) was plotted as a function of thrombin concentration. As the concentration of thrombin increases, the I_{TPPL}/I_{TPPL0} value steadily increased until a plateau reached (Figure 2c). The highest enhancement factor of 221 fold was achieved when 70 nM of thrombin was added. The TPPL intensity steadily increased when [thrombin] increases from 0.005 to 70 nM, covering a dynamic range of more than 4 orders of magnitude. Figure 2c inset shows that the TPPL enhancement factor is linearly proportional to the thrombin concentration in the low concentration range. As [thrombin] further increases, the increase of TPPL enhancement factor slows down and an inflection point was observed at ~0.2 nM. This could be understood that anisotropic aggregates form first at low concentration of thrombin, which lead to a rapid increase in TPPL intensity. At higher concentrations of thrombin, more isotropic aggregates will form (the aggregates grow in all directions). It has been previously shown that formation of anisotropic aggregates display larger TPPL enhancement compared to the isotropic aggregates.[40] Formation of isotropic aggregates will slow down the increase in TPPL enhancement. At even higher concentrations of thrombin, larger aggregates or even precipitation will form, which will cause saturation or even decrease of TPPL intensity. The LOD was estimated to be 3.1 pM from the data in Figure 2c. This sensitivity is much better compared to the LOD obtained from the change in the extinction spectra (~1.3 nM), significantly better than that of a previously reported fluorescence based method (LOD of 12 nM)[51] and comparable to that of electrochemical techniques (LOD of 1 pM).[7]

The selectivity of this two-photon sensing scheme has been evaluated against BSA as shown in Figure 3. The TPPL signal

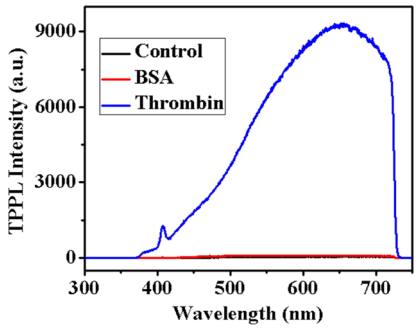


Figure 3. TPPL spectra of Ag NPs + TBA_{15} solution before (the control sample) and after addition of 70 nM thrombin or BSA.

of Ag NPs in the presence of 70 nM thrombin is much higher than that in the presence of 70 nM BSA. The observed high selectivity can be attributed to the highly specific interactions between TBA_{15} and thrombin.

It is very challenging and important to detect thrombin in the blood serum as thrombin generally exists in blood, which is a more complicated media than the buffer solution. Many reported schemes for thrombin detection were conducted in diluted serum media.[1,10,11,13,33] The TPPL scheme was tested in the presence of fetal bovine serum of different volume ratios. The TPPL signals in the complex media were found to steadily increase with increasing concentration of thrombin (see Figure S2 in the Supporting Information). However, the maximum TPPL enhancement factor strongly depends on the volume ratio of serum and rapidly decreases when serum was added. The TPPL enhancement factor decreases from 221-fold for the buffer solution (0% serum) to 4.3-fold in 100% serum. Two reasons are responsible for the observed decreasing TPPL enhancement factor. First, the serum itself displays some two-photon excitation fluorescence signal, which acts as the background noise and causes reduced enhancement factor and loss of sensitivity (see Figure S3 in the Supporting Information). On the other hand, fetal bovine serum is a complicated biological fluid containing a large number of proteins and other materials that may disrupt the aggregation of Ag NPs, resulting in a reduced enhancement factor and lower sensitivity, compared to that in the buffer solution.[52]

The TPPL results in 100% serum are shown in Figure 4. The enhancement factor is linearly proportional to the concentration of thrombin in the low concentration range (Figure 4c inset). The LOD of this TPPL method was estimated to be ~1.8 nM. The highly specific and high-affinity interactions between thrombin and the thrombin-binding aptamer allows us to discriminate against nonspecific binding and thus readily detect thrombin even in complex, contaminant−ridden samples such as blood serum. The extinction method was conducted under the same experimental condition for direct comparison, which gave an LOD of ~20 nM (see Figure S4 in the Supporting Information). The LOD of the TPPL method is over 10 times better than the extinction method. In addition, this TPPL-based detection of thrombin in the complex media is also highly selective against BSA (see Figure S5 in the Supporting Information). If the measurements were done in serum with volume ratio of 20%, the LOD could be improved to 0.1 nM for TPPL method versus 12.2 nM for the extinction method (see Figures S6 and S7 in the Supporting Information).

CONCLUSIONS

We have demonstrated a label-free, fast, highly sensitive, and selective two-photon sensing scheme for detection of thrombin on the picomolar level. The assay is based on specific interactions between thrombin and a DNA aptamer, TBA_{15}, which induce aggregation of Ag NPs and result in significantly enhanced TPPL. The LOD of this TPPL assay is as low as 3.1 pM in the buffer solution, more than 400 times lower than that of the extinction method (1.3 nM). The dynamic range of this method covers more than 4 orders of magnitude. Most importantly, this TPPL assay can be applied to detection of thrombin in 100% fetal bovine serum with LOD of 1.8 nM. Furthermore, this method could be potentially combined with two-photon microscopy to offer additional advantages of 3D detection and mapping for potential in vivo sensing applications.

ASSOCIATED CONTENT

Supporting Information

TEM images of Ag NPs in the absence and presence of thrombin, effect of serum content on TPPL enhancement, influence of two-photon excitation fluorescence of serum, UV−vis extinction method and selectivity of TPPL method in 100% serum, TPPL and UV−vis extinction methods in 20% serum. This material is available free of charge via the Internet at http://pubs.acs.org.

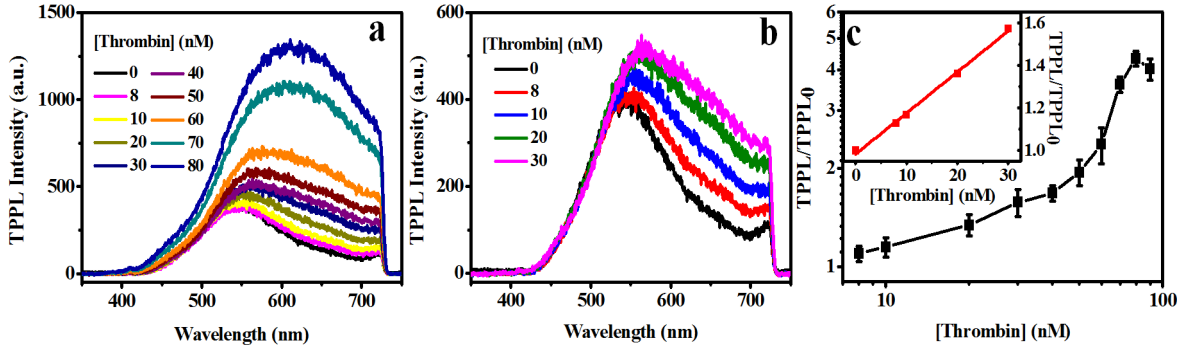


Figure 4. (a, b) TPPL spectra of Ag NPs + TBA_{15} solution upon addition of different concentration of thrombin in the presence of 100% fetal bovine serum; (c) plot of TPPL enhancement factor versus [thrombin]. The inset shows the linear plot in the low concentration range. Error bars represent standard deviations obtained from three independent measurements.

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Notes

The authors declare no competing financial interest.

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