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Short communication

LSPR biomolecular assay with high sensitivity induced by aptamer–antigen–antibody sandwich complex

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ABSTRACT

Herein we demonstrate a sensitive approach for protein detection based on peak shifts of localized surface plasmon resonance (LSPR) induced by aptamer–antigen–antibody sandwich structures. The applicability of the proposed method is demonstrated using human α-thrombin as a model analyte. While the binding of thrombin to its specific receptor, thrombin binding aptamer (TBA) modified on Au nanorods (AuNRs), causes a measurable LSPR shift, a subsequent binding of an anti-thrombin antibody to the captured thrombin can exhibit a nearly 150% amplification in the LSPR response. This enhanced signal essentially leads to an improvement of limit of detection (LOD) by more than one order of magnitude. In addition, the use of TBA as thrombin recognition units makes the biosensor reusable. The feasibility of the proposed method was further exploited by the detection of thrombin in human serum, opening the possibility of a real application for diagnostics and medical investigations.

1. Introduction

In recent years, label-free biomolecular assay based on localized surface plasmon resonance (LSPR) of noble metal nanostructures have attracted increasing interest to the research community. LSPR-based biosensings rely on either colloidal aggregation (Jain and El-Sayed, 2010) or local refractive index changes (Guo et al., 2010; Hall et al., 2011a). Since most organic molecules have higher refractive indices than buffer solutions, binding organic molecules to plasmonic nanoparticles induces an increase in the local refractive index, resulting in a red-shift of the extinction and scattering spectrum. (Jeffrey et al., 2008) This LSPR shift-based sensing offers several advantages over the aggregation-based assays, including multiplexed detection, device miniaturization, accessibility to a single nanoparticle, regeneration of biological receptors, compatibility with microfluidics and a broad range of functionalization chemistries (Mayer and Hafner, 2011).

Despite the undisputed advantages of the LSPR shift-based assays, the sensitivity of this approach is orders of magnitude lower than other plasmonic biosensors, in which continuous noble metal films in the attenuated total internal reflection geometry are involved (Kabashin et al., 2009). Diverse strategies have been therefore reported to improve the sensitivity of LSPR shift-based biosensors. Many have focused on the increase of refractive index sensitivity of nanoparticles by controlling their sizes and shapes because refractive index sensitivity of metal nanoparticles is largely influenced by their structural geometry (Mayer et al., 2010; Sherry et al., 2005). Other attempts include the development of single nanoparticle plasmonic sensors that improve the absolute detection limits and hold great potential for multiplexed assays (Guo et al., 2011b; Nusz et al., 2008). Lastly, the effective changes in refractive index based on molecular binding events are also proven to be useful for the increase of LSPR sensitivity. Specifically, there have been three ways introduced for the increase in refractive index change using molecular bindings. First approach involves the increase of molecular mass bound to plasmonic particles. It is reported that LSPR shifts are roughly in proportion to the mass of the molecules bound to the surface of plasmonic structures (Whitney et al., 2005). Based on this recognition, a second antibody was used to bind to amyloid-β-derived diffusible ligands, a biomarker for Alzheimer's disease, to enhance the LSPR shift (Haes et al., 2005). Second method is to use chromophores coupled with LSPR signals of nanoparticles. The coupling between nanoparticles and resonant dyes was found to be useful for the detection of small molecules (Das et al., 2009; Zhao et al., 2007). Lastly, nanoparticle pairs have been created. Plasmonic nanoparticles exhibit a strong distance-dependent coupling with neighboring particles when an inter-particle distance is less than ~2.5 times of particle radii (Sonnichsen et al., 2005). Such coupled plasmonic nanostructures enable high-resolution monitoring of molecular conformation (Jain et al., 2007) and the amplification of LSPR shifts (Hall et al., 2011b).

Here, we demonstrate a simple approach to enhance LSPR shift by the construction of aptamer–antigen–antibody sandwich structures. The conjugation of an antibody to antigen enlarges the mass

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of the target molecule, which leads to a significant enhancement of LSPR shift. Through this strategy, the limit of detection (LOD) of thrombin was improved by more than one order of magnitude. In addition, the use of aptamer as a capture molecule provides reusable capability of the biosensor. As a result, we can construct a calibration curve and detect analytes with a same nanoparticle, as previously demonstrated by our group (Guo and Kim, 2011). The recycled, single nanoparticle ultimately eliminates plasmonic variations among different nanoparticles, and eventually improves the reproducibility and sensitivity of the sensor.

2. Experimental

2.1. Darkfield microscope system

An Olympus IX71 inverted microscope employs an oil immersion ultra-dark-field condenser (IX-ADUCD U-DCW, NA 1.2-1.4, Olympus) and a 100× Plan Semi Apochromat objective (UPLFLN100× OI, NA 1.3-0.6, Olympus) was used for plasmonic scattering detection. Detail setup of this optical system was reported previously (Guo et al., 2011a).

2.2. Substrate bioconjugation

The 5′-thiol-modified thrombin aptamer (5′-C6-S-S-$(T)_5$-GGT TGG TGT GGT TGG-3′, thrombin binding aptamer (TBA)) with a dissociation constant of 25 nM were activated and immobilized onto the Au nanorods (AuNRs) according to previous report.(Guo et al., 2011c; Liu and Lu, 2006) The deprotected thiol-TBA was then diluted to 1 μM by deionized water, and injected into the flow cell containing AuNRs modified 2D chip. The flow cell was kept in a dark drawer at room temperature over night. After aptamer modification, the chip was rinsed with water, and then 100 μM 6-mercaptohexanol was injected into the flow cell and incubated for another 3 h to ensure full-surface coverage of AuNRs so that non-specific protein adsorption would be suppressed. The aptamer-modified glass chip was rinsed thoroughly with deionized water and incubated in 1× TEA buffer at 4 °C before use.

2.3. LSPR measurements

Thrombin stock solutions were diluted with thrombin binding buffer to appropriate concentration. Before thrombin detection, binding buffer was injected into the flow cell and incubated for 15 min. Then, Thrombin solutions at specific concentration were injected into the flow cell and incubated for 30 min. The LSPR scattering spectra before and after protein binding were recorded. A peak fitting based on Lorentzian algorithm was used for data processing by using OriginPro 8.0 for accurate and efficient determination of λ_{max}, and the corresponding wavelength shift ($\Delta\lambda_{max}$) was calculated.

After thrombin binding to TBA modified AuNR, a solution containing 50 nM anti-thrombin (monoclonal Antibody, Sigma) in binding buffer was injected to the flow cell and incubated for 20 min. Antibody would bind to TBA bounded thrombin, which would generate a significant LSPR shift.

Control experiments were carried out to check the specificity of this system. A solution of anti-rat Igg2b antibody (50 nM) prepared in binding buffer was injected to an aptasensor with aptamer bounded thrombin and the LSPR spectra before and after the solution injection were tested.

In order to test the performance of the sensor in complex matrixes, standard solutions of thrombin were added to male human serum (diluted 1:1 with binding buffer). The spiked samples were tested and compared with those results from samples spiked in pure binding buffer.

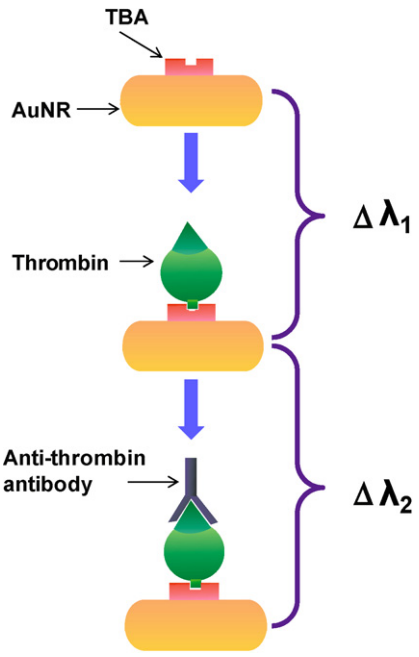

Fig. 1. Schematic diagram of antibody-enhanced LSPR shift.

2.4. Regeneration of aptamers

After each cycle of thrombin detection, the flow cell was rinsed with 2 M NaCl for 5 min, and then rinsed with copious distilled water. It is reported that the aptamer would readily unfold the three-dimensional structure of the aptamer without damaging the oligomer structure at high ionic strength. (Bini et al., 2007) Therefore, after this treatment, the bound thrombin is released from TBA. The three-dimensional-structured TBA could be easily regenerated by refilling the flow cell with binding buffer.

3. Results and discussion

3.1. Principle of the sensor

Limitations of LSPR-peak-shift assays lie in the relatively low sensitivity compared with conventional propagating SPR as well as other sensing methods, e.g. ELISA and fluorescent-based protein assays. Local refractive index changes induced by biomolecular interactions at the surface of metallic nanostructures are closely related to biomolecular density and molecular weight. In general, the higher biomolecular concentration tends to generate the denser molecular layer bound on metal surface, ultimately leading to a larger peak shift. On the other hand, comparing with small molecules, biomolecules with large molecular weight could generate high degree of LSPR shift due to large local refractive index change upon biomolecular binding. Herein we demonstrate the feasibility of using aptamer–antigen–antibody complex to enhance the LSPR shift.

Antibodies are widely used in LSPR-based immunosensors as receptors. It is reported that anti-thrombin antibody binds to specific sites of thrombin, which is different from the TBA binding sites. (Kang et al., 2008) Because TBA is used to capture thrombin molecules, it is possible to use thrombin antibody as a specific probe to enhance LSPR signals. Fig. 1 shows the mechanism of the proposed detection scheme. TBA-modified AuNRs are incubated in

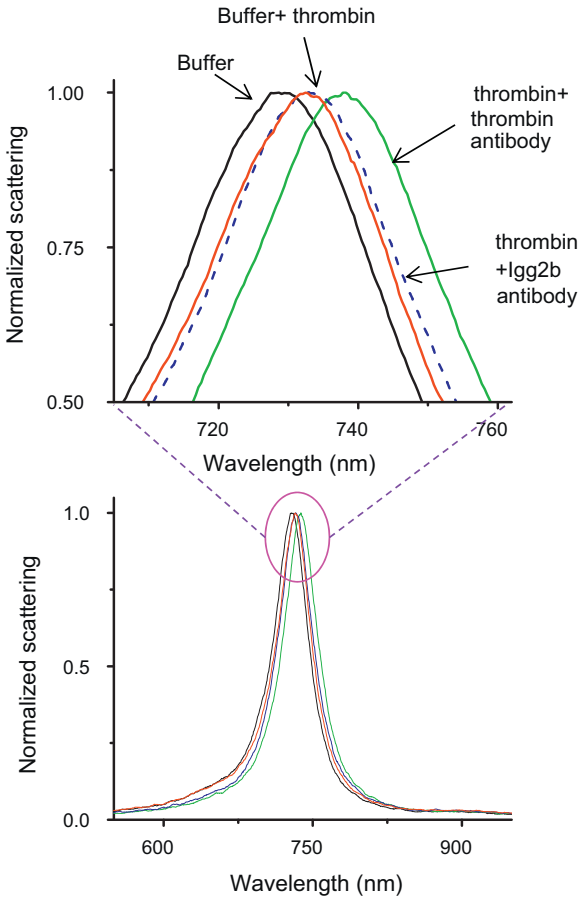

Fig. 2. Steady-state LSPR spectra of a thrombin aptamer modified sensor upon sequential incubation in binding buffer (black), 10 nM thrombin in buffer (red) and 50 nM anti-thrombin antibody (green). The dash line represents spectral of incubating a 10 nM thrombin-bounded AuNR into a solution of 50 nM anti-rat Igg2b antibody. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

a thrombin solution to trigger thrombin bind to the TBA-modified AuNRs. This thrombin binding event generates a LSPR shift ($\Delta\lambda_1$). The $\Delta\lambda_1$ is directly related to a concentration of the thrombin, thus it can be used for thrombin quantification. After a steady stage peak shift is obtained (i.e., saturated thrombin binding), the LSPR shift can be amplified by the incubation of the thrombin-bound AuNRs in a solution containing anti-thrombin antibodies. The binding of anti-thrombin to thrombin causes further local refractive index changes at the AuNR surface, generating secondary red-shift of LSPR peak ($\Delta\lambda_2$).

Fig. 2 shows the steady-state LSPR spectra of a typical TBA-modified AuNR by the sequential incubation of the TBA-modified AuNR in binding buffers, i.e., 10 nM thrombin and 50 nM anti-thrombin antibodies in binding buffer, respectively. Upon anti-thrombin antibody binding to the thrombin-bound AuNR, the LSPR shift increased from 3.52 nm ($\Delta\lambda_1$) to 8.89 nm ($\Delta\lambda_1 + \Delta\lambda_2$). The result indicates the potential of using the anti-thrombin antibody for the enhancement of LSPR signals. The specificity of this antibody-induced LSPR peak-shift was confirmed by replacing the anti-thrombin antibody with anti-rat Igg2b antibody (50 nM), and maintaining other experimental conditions unaltered. The corresponding spectrum is shown in Fig. 2 (dash curve). Minimal peak shift was observed after the incubation of the thrombin-bound AuNR in the anti-rat Igg2b solution. The result shows that the LSPR shift of the aptasensor is specific for the anti-thrombin antibody.

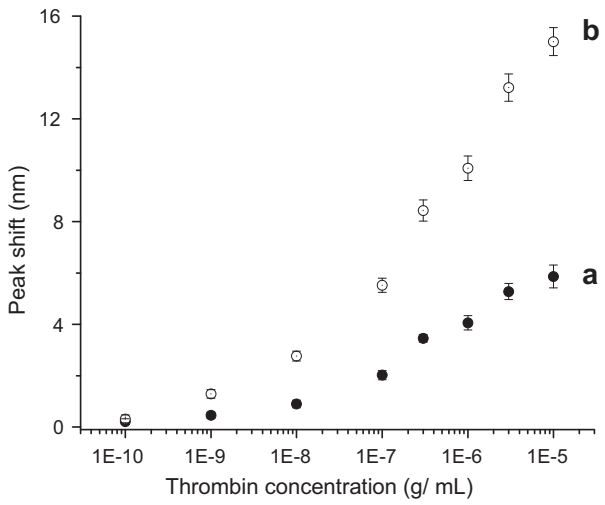

Fig. 3. Dose–response curves for thrombin detection, (a) without antibody enhancement; (b) with antibody enhancement. Error bars represent standard deviations of five replicates.

3.2. Reuse of the sensor

To generate a calibration curve and detect an analyte with a same single particle, it is essential to reuse the nanoparticle in the course of assay. Herein we use the DNA aptamer as a protein capture unit to make the sensor reusable. It is known that the addition of high concentrations of salt (e.g. NaCl or KCl) causes disruption of the hydrogen bonds and electrostatic interactions responsible for most of the aptamer-target association. (Radi et al., 2006) A routine aptamer regeneration protocol that involves the use of 2 M NaCl, was adopted to regenerate the aptasensor in the present study. The 15-mer aptamer (TBA) forming a G-quadruplex structure in the binding buffer, (Vairamani and Gross, 2003) was incubated in a thrombin solution for 30 min. Thrombins bind to the G-quadruplex-structured aptamer through protein–aptamer interaction, as discussed extensively in previous reports. (Ellington and Szostak, 1992; Joachimi et al., 2007) After the thrombin detection, the aptasensor can be readily regenerated by the incubation of the aptasensor in a solution of 2 M NaCl to release the bound thrombin. Our previous investigation revealed that the aptasensor can be reused for 80 times with the variation of relative binding efficiency less than 5% (Guo and Kim, 2011).

3.3. Analytical merits of the sensor

The proposed aptasensor was exploited for thrombin detection in the concentration range from 0.1 ng/ml to 10 μg/ml. The corresponding peak wavelength shifts before and after the addition of anti-thrombin antibody were plotted against thrombin concentration (Fig. 3). A wide dynamic range was obtained for both cases. It should be noted that both calibration curves were plotted based on a same AuNR, based on the aforementioned reusable approach. The recycles of a AuNR brought much improvement to the reproducibility of the sensor, as can be seen from Fig. 3; the relative standard deviations (RSDs) of the steady-state responses of the sensor were less than 10% ($n = 5$), while the RSDs of our previous report based on an averaged LSPR response from a large number of nanoparticles were more than 20% (Guo et al., 2011c).

The LOD of our reusable single nanoparticle aptasensor in the presence and absence of antibody enhancement was exploited. Ten randomly selected AuNRs with varied peak wavelengths were investigated in detail (see Supporting information Table S1). An averaged LOD of 1.6 pM was achieved after the antibody-induced

signal enhancement. When compared with the averaged LOD of 18.3 pM without the antibody enhancement, the LOD of the aptasensor with the antibody enhancement was improved by a factor of 11. It is worth to mention that the LOD of the proposed aptasensor is superior to most of the previous LSPR-based biosensors, including both single nanoparticle based (Guo et al., 2011c; Nusz et al., 2008) and large-ensemble based ones. (Fan et al., 2010; Wang et al., 2010) In addition, the proposed reusable single nanoparticle aptasensor is also comparable with the commercially available propagating SPR instruments (Frasconi et al., 2010; Piliarik et al., 2010) in terms of detection limit, yet with additional advantages in high spatial resolution and small sample volume.

3.4. Human serum assay

The reliability of the aptasensor to detect thrombin in complex matrixes was evaluated by the detection of samples with thrombin-spiked, undiluted human serum. Three samples with the final thrombin concentration of 0.05 nM, 5 nM and 50 nM were examined. The recoveries of these standard-addition samples are shown in Supporting information Table S2. The recoveries of standard addition were in the range of 81.8–86.0%. A weak matrix effect was observed considering the small amount of peak shift measured in serum compared with buffer (an averaged peak shift of ~17%). The origin of this signal drop in complex serum matrix could be ascribed to a reduced concentration of thrombin available for the binding caused by the interaction with other components in serum, as discussed previously. (Centi et al., 2007; Xiao et al., 2005)

4. Conclusion

In summary, we report an effective approach to enhance the sensitivity of LSPR shift-based biosensors by the formation of aptamer–antigen–antibody sandwich complex. DNA aptamers immobilized on AuNR were used as a receptor to capture a target protein in solution. High stability of aptamers makes the sensor reusable, allowing that a calibration curve generation and sample detection can be performed with a single nanoparticle. The sensitivity of the proposed sensor was improved more than one order of magnitude assisted by the antibody enhancement. Although only thrombin detection is demonstrated herein, in view of the wide applications of aptamer and antibody in protein detection, the proposed approach can be used for many other protein assays when the following two conditions are satisfied: (i) an antibody and an aptamer targeted to a same protein are available; and (ii) no obvious sharing of binding sites between an aptamer and an antibody.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2011.10.047.

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