

Assembly of a Dual Aptamer Gold Nanoparticle Conjugate Ensemble in the Specific Detection of Thrombin when Coupled with Dynamic Light Scattering Spectroscopy

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Abstract

We demonstrate an extremely facile, rapid, specific and selective method for detecting proteins using aptamer-conjugated gold nanoparticles coupled with dynamic light scattering (DLS) at ambient conditions. Addition of proteins to aptamer-conjugated gold nanoparticles (GNPs) induced the formation of protein-aptamer-gold nanoparticle conjugate complexes. The average hydrodynamic diameter of the nanoconjugate complexes as measured by DLS, increased with the corresponding increase in protein concentration. This correlation formed the analytical basis of the assay. The nanoparticles and nanoconjugate complexes were characterized by transmission electron microscopy, ultra-violet visible spectroscopy and DLS. Various parameters affecting the assay were optimized. Using thrombin as the model analyte, we demonstrated the detection of as low as 1.41 nM (0.05 µg/mL) of the protein. A linear dynamic range of up to 300 nM (11 µg/mL) was realized. The presence of other interfering proteins such as BSA showed no effect on the assay response. The presence of other interfering proteins such as bovine serum albumin (BSA) showed no significant effect on the assay response.

Keywords: Nanotechnology; Protein detection; Thrombin; Bock; Tasset; Aptamers; Gold nanoconjugates; Dynamic light scattering; Dynamic laser light scattering

Introduction

Hybrid systems of antibody-metal nanoparticle or DNA-metal nanoparticle conjugates have proved to be effective in the development of assays for detecting various biomolecules including DNA [1-4], cancer markers and other proteins [5,6]. In particular, the use of gold nanoparticles (GNPs) conjugates in these assays has been very effective especially when the light absorption [3] and scattering [1,2,7,8] are used as detection techniques. This is because GNPs are known to have a large light absorption and scattering cross section in the surface plasmon resonance wavelength regions [9,10] that can be orders of magnitude higher than light emission from strongly fluorescing dyes. In fact, recent research demonstrated that GNPs exhibit a linear increase of scattered light intensity versus their concentration down to 0.02 pM levels [5]. This detection limit is 9 orders of magnitude lower than proteins and DNA and 4 order of magnitude lower than sensors based on light absorption [11,12]. Further, unlike fluorescent probes, GNPs are not prone to quenching and do not photo-bleach with repeated or continuous exposure to scattering light.

Recently aptamers and metal nanoparticle conjugates have been used for the detection of various chemical and biological molecules [13-17]. Aptamers are single-stranded DNA or RNA oligonucleotides that fold into well-defined 3D structures which are able to recognize with high affinity and specificity [18]. They range from 20 to 80 bases (approximately 6 to 26 kDa) and can be isolated against most targets including those that are toxic or have low immunogenicity. In comparison to antibodies, aptamers can be purified to a very high degree thus eliminating the batch-to-batch variation found in antibodies. Aptamers are also relatively more stable at ambient conditions and also under a wide range of buffer conditions. They are amenable to a various chemical modifications to facilitate their use in different scenarios. For

example, they can be modified for use in radiosopic and fluorescent applications. They can also be modified with 3' and 5' amino or biotin and thiol groups to facilitate binding to other materials. Finally they can also be modified to increase their nuclease-resistant capability. Therefore, aptamers have emerged as a novel class of ligands that rival antibodies in their therapeutic [19-21] and diagnostic [16,22-29] applications. The development of the Systematic Evolution of Ligands by Exponential Enrichment (SELEX) process has made possible the isolation of these oligonucleotide aptamer sequences with the capacity to recognize virtually any class of target molecules. Because of their small size, aptamers are useful in nanotechnological applications. They have a radius of gyration of only a few nanometers thus contributing little to the overall size on the bound nanomaterial.

As in the cases of antibody-gold nanoparticle and DNA-gold nanoparticle conjugates, aptamer-gold nanoparticle conjugates have been utilized in the detection of cancer markers [13,30], DNA, proteins [13,16,20,22,25,27-29,31,32] and small molecules [14,33] with light absorption as the preferred method of detection. Here we extend the use of aptamer-gold nanoconjugates with dynamic light scattering (DLS) as the detection technique. DLS, also known as photon correlation spectroscopy, is a technique for measuring the size of particles from

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Received June 03, 2014; Accepted July 05, 2014; Published July 10, 2014

Citation: Aaryasomayajula VSR, Severs T, Ghosh K, DeLong R, Zhang X, et al. (2014) Assembly of a Dual Aptamer Gold Nanoparticle Conjugate Ensemble in the Specific Detection of Thrombin when Coupled with Dynamic Light Scattering Spectroscopy. J Nanomed Nanotechnol 5: 210. doi: [10.4172/2157-7439.1000210](https://doi.org/10.4172/2157-7439.1000210)

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a few nanometers to a few microns. DLS spectroscopy is based on Brownian motion of particles which causes a Doppler shift of incident laser light. The diffusion constant of the particles is measured and the diffusional spherical diameter can be found from the Stokes-Einstein equation [34]. DLS has routinely been used to analyze the size and size distribution of polymer, proteins, colloids and nanoparticles.

We selected thrombin because it is a coagulation protein that has well known aptamer sequences, thus providing an excellent model system [35,36]. The binding of thrombin to these aptamers is well studied and documented, and shows much promise in nanotechnology applications. In our present study, GNPs were conjugated with two different thrombin specific thiolated aptamers namely Bock and Tasset separately; to form aptamer-conjugated GNPs. Addition of thrombin to a mixture of the two aptamer-conjugated GNPs induced the formation of nanoconjugate complexes depending on the concentration of thrombin. The average diameter of the aggregates, as measured by DLS spectroscopy, increased with the corresponding increase in the concentration of thrombin as illustrated in scheme A. This correlation formed the analytical basis of the assay.

Materials and Methods

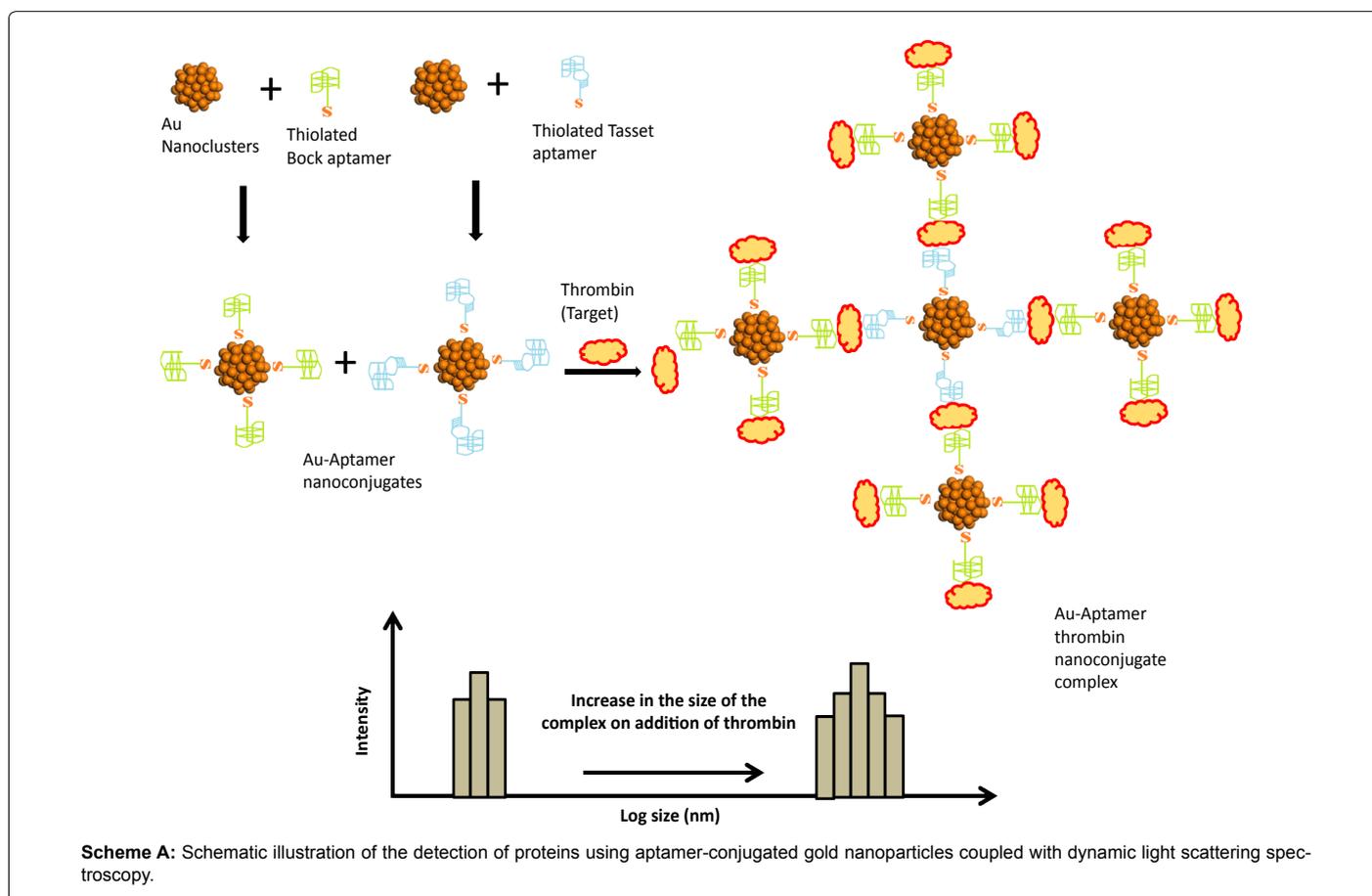
Materials and instruments

Dithiothreitol (DTT, $\geq 99.5\%$, 3483-12-3), thrombin from bovine plasma and bovine serum albumin (BSA) were purchased from Sigma Aldrich (St Louis MO). Thiolated Bock with sequence 5'-SH-(CH₂)₆-TTTTTTTTTTGGTTGGTGTGGTTGG-3' and thiolated Tasset aptamer-with sequence 5'-/5 THIO MC6-D/AGT CCG TGG TAG

GGC AGG TTG GGG TGA CT -3' were purchased from Integrated DNA Technologies (Coralville, IA). The aptamers were supplied at OD values of ≥ 20 and were sequentially diluted as appropriate. Hydrogen tetrachloroaurate (III) trihydrate ($\geq 99.9\%$ trace metals basis, CAS number 16961-25-4) was obtained from Fisher Scientific (Fair Lawn, NJ). Sodium citrate dihydrate (6132-04-3) was obtained from Spectrum Chemical Corp (New Brunswick, NJ). Gel filtration columns (NAP-5) were purchased from GE Healthcare Bio-Sciences Corp (Piscataway, NJ). All reagents and chemicals were used without further purification. DLS Spectroscopy was performed on a Malvern Nanozetasizer (ZS90). The DLS spectrometer was operated at 25°C with the detector angle at 90°, incident laser wavelength of 633 nm and 4 mW laser power. All DLS measurements were allowed a one minute equilibration time and were performed in triplicate. Measurements with poly-dispersity index (PDI) greater than 0.4 were discarded. UV-Vis spectroscopy was conducted on a Perkin Elmer Lambda 650 UV-VIS spectrometer from 200 nm to 700 nm. Transmission electron microscopy (TEM) imaging was conducted using a Hitachi 7650 transmission electron microscope.

Gold nanoparticle synthesis

GNPs were prepared by the citrate reduction of HAuCl₄·3H₂O according to a modified literature method [37]. Briefly, an aqueous solution of HAuCl₄·3H₂O (1 mM, 500 mL) was brought to reflux while stirring. 50 mL of 38.8 mM trisodium citrate solution was then added rapidly. After 15 minutes, the reaction was stopped and the mixture was allowed to cool to room temperature and subsequently filtered through a 0.45 micron filter. The concentration of the GNPs was determined by their absorbance spectra ($\lambda=523$ nm) and using appropriate extinction



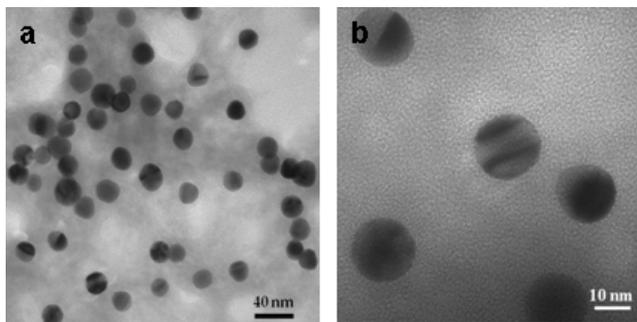


Figure 1A 1B: Transmission electron microscopic images (TEM) of gold nanoparticles (a) Lower magnification (b) Higher magnification.

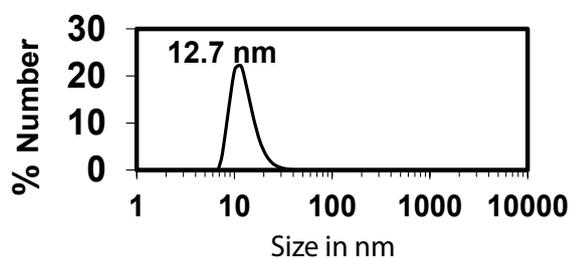


Figure 2a: DLS spectrum of the gold nanoparticles.

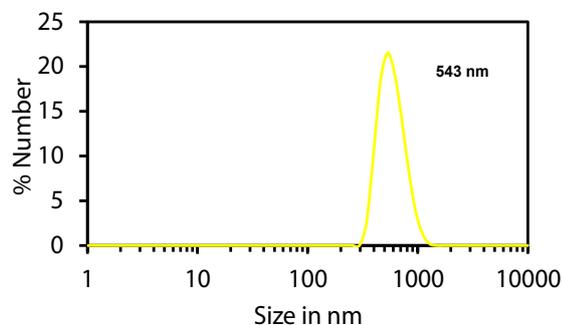


Figure 2b: DLS size distribution of gold-bock aptamer nanoconjugates.

coefficient and was found to be 0.063 μM . Upon synthesis of the citrate-stabilized GNPs, DLS spectroscopy measurements and TEM images were taken to ascertain their size before conjugation was attempted.

Conjugation of aptamers to gold nanoparticles

The GNPs were then conjugated to the thiolatedaptamer using a modified literature protocol that utilizedthiol-Au affinity [16]. The thiol modified oligonucleotides were purchased in a disulfide form which had to be cleaved using DTT. 100 μL of 1.0 OD thiol modified oligonucleotide Bock or Tassetaptamer were added to a 2 μL of 10% triethylammonium hydroxide and 7.7 mg DTT. The solution was allowed to react for one hour, and DTT was then removed via extraction with 400 μL ethyl acetate. This extraction was repeated four times to ensure complete removal of DTT. To the above solution, 1mL of previously prepared gold nanoparticles were added and incubated for 24 hours at room temperature. After the incubation 30 μL of 500 mMTris acetate (pH 8.2) buffer was added drop wise to each centrifuge

tube with gentle hand shaking. 300 μL of 1 M NaCl solution was added drop wise to each vial with gentle hand shaking. Both vials were kept in the dark for 24 hrs at ambient conditions. The conjugates were then centrifuged with a Daigger centrifuge at 6000 rpm for 30 minutes. They were washed, re-suspended and recentrifuged, and the supernatant was discarded. The precipitate was redispersed in a 1 mL solution containing 0.0076 g $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$, 0.10 g sucrose, and 2.5 μL Tween 20 [22]. The aptamer-conjugated GNPs were stored at 5°C until subsequent experiments were performed.

Thrombin detection

Bock and Tassetaptamer-conjugated gold nanoparticles were mixed together and thrombin added. The resulting thrombin-induced aggregation of the nanoconjugate complexes was monitored by DLS spectroscopy. This experiment was repeated at least four times.

Results and Discussion

Gold nanoparticle synthesis and conjugation with aptamers

Figure 1A shows a low magnification TEM image of the citrate stabilized GNPs. The GNPs were well dispersed with a very narrow size distribution. Figure 1B shows a higher magnification TEM image of the same particles. It is evident that the shape of the nanoparticles was consistently spherical with an average diameter of about 13 nm. DLS spectra were recorded to ascertain the size of these. As can be seen from Figure 2A, the average size of the GNPs was found to be 12.7 nm, which was consistent with the TEM results (Figure 1A and 1B).

Thiolatedaptamers were then conjugated to GNPs. This conjugation is based on the well-known thiol-gold affinity. Further DLS spectroscopy experiments were performed after conjugation, and the average size of the aptamer-conjugated GNPs was found to be 543 nm (for Bock aptamer-conjugated GNPs, Figure 2B) and 650 nm (for the Tassetaptamers-conjugated GNPs, Figure 2C). This change in the diameter was attributed to, the formation of the gold-aptamer nanoconjugates.

Figure 3 shows the ultraviolet-visible (UV-VIS) spectra of the GNPs and aptamer-conjugated GNPs. The surface plasmon resonance absorption of the GNPs is clearly evident at 523 nm which undergoes a slight red shift to 530 nm on conjugation of the aptamers. The intensity of the aptamer-conjugated GNPs peak at 530 nm is also reduced compared with the free GNP peak at 523 nm. The red shift and decrease in the intensity is well documented and has been attributed to the decrease in interparticle distance as a result of the binding [38] of the thiolatedaptamer onto the GNP surface. The aptamer conjugation is further confirmed by a peak at 260 nm that corresponds to the

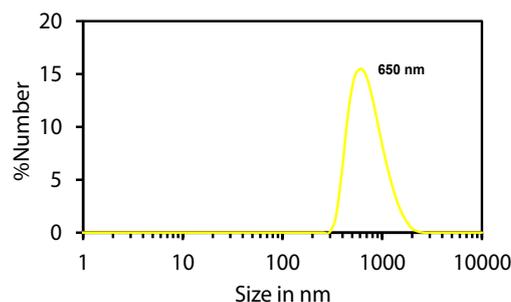


Figure 2c: DLS size distribution of gold-Tasset aptamer nanoconjugates.

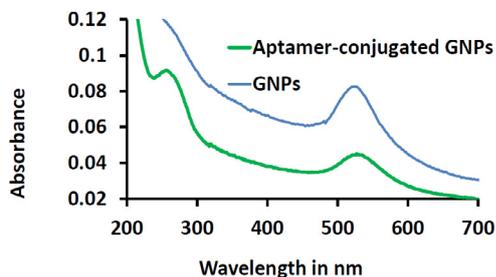


Figure 3: Absorbance spectra of gold nanoparticles and gold aptamer conjugates.

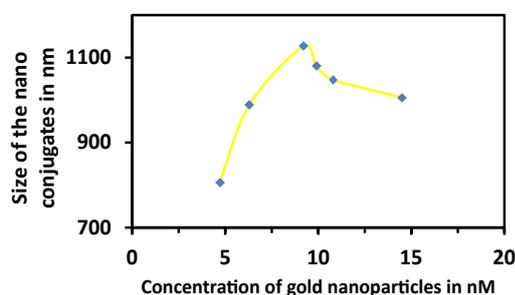


Figure 4: Size of the GNP-aptamer-thrombin nanoconjugate complex vs the GNP nanoparticle concentration used in aptamer conjugation. The amounts of Bock and Tasset aptamers were kept constant at 1 OD. The concentration of thrombin was 30 nM (1 $\mu\text{g}/\text{mL}$).

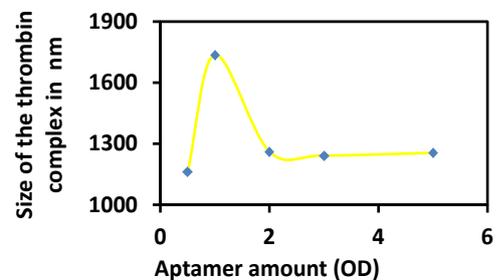


Figure 5: Effect of varying the amount of aptamers used in the conjugation reaction with GNPs nanoparticles. The GNP concentration was 9.24 nM. The concentration of thrombin was 30 nM (1 $\mu\text{g}/\text{mL}$).

absorption from the nucleic acid bases on the aptamers. The DLS and UV-VIS spectra demonstrated successful conjugation of the aptamer to the GNPs.

Thrombin detection and assay optimization

Mixing 6.25 nM of Bock and Tasset aptamer-conjugated GNPs in a 1:1 ratio by volume followed by addition of thrombin resulted in the aggregation of nanoconjugate complexes as suggested in Scheme A. The average diameter of the aggregates was estimated to be about 1000 nm. Since thrombin has binding sites to which the aptamers can bind to, the thrombin-induced aggregation of the aptamer-conjugated GNPs was expected. Based on the Scheme A, we identified two main parameters that could influence the formation of the nanoconjugate complexes. These were 1) the concentration of the gold nanoparticles and 2) the amount of the Bock and Tasset aptamers. We optimized these

two parameters as described below.

Optimization of gold nanoparticle concentration: Aptamer conjugation experiments were performed as described in section 2.3. Bock and Tasset aptamers were sequentially diluted to obtain amounts having 1.0 OD values. The aptamers were separately mixed with GNPs with concentrations from 4.70 nM to 14.45 nM. The resulting aptamer conjugated GNPs were mixed together and exposed to 30 nM thrombin as described in section 3.2. The thrombin-induced aggregation of the nanoconjugate complex was monitored by DLS spectroscopy. Figure 4 shows the plot of the size of the resulting nanoconjugate complex vs the concentration of the GNPs used in binding of the two aptamers. The maximum shift in the size of the nanoconjugate complex occurred when the concentration of the GNPs used for binding the aptamers was 9.24 nM. Therefore, for all the subsequent measurements, all the aptamer-conjugated GNPs were synthesized from 9.24 nM GNPs.

Optimization of aptamer amount: Variable amounts of aptamers ranging from 0.5 OD to 5.0 OD were separately prepared by sequential dilution of the aptamer stock solutions (OD ≥ 20) purchased from the suppliers. The aptamers were separately conjugated to the optimized concentration gold nanoparticle colloidal solution concentration of 9.24 nM. The two aptamer-conjugated GNPs were mixed together as described in 3.2 and the resulting thrombin-induced aggregation monitored by DLS spectroscopy. Figure 5 shows the resulting plot of size of the thrombin-aptamer-gold nanoparticle conjugate complex vs. the amount of the aptamers in OD units. The hydrodynamic size of the complex had a maximum at aptamer amounts of 1 OD. It is reasonable to conclude that below of 1 OD, the aptamers are the limiting factor in the formation of the complex. Above 1 OD, there was a decrease and levelling in the hydrodynamic size probably due to the nanoconjugate complex breaking down to smaller particles. For this reason 1 OD aptamer amounts were used for all further experiments.

Once the assay was optimized, it was challenged with increasing concentrations of thrombin. As expected, there was a corresponding increase in the hydrodynamic size of the nanoconjugate complexes as shown in Figure 6. As the concentration of thrombin in the parent solution was increased, there was more aggregation leading to larger hydrodynamic sizes as recorded by DLS spectroscopy. The increase in size was even observed for solutions containing as low as 1.41 nM (0.05 $\mu\text{g}/\text{mL}$) of thrombin suggesting that our assay was sensitive to low nanomolar concentrations of thrombin. The increase in the hydrodynamic size of the aggregates was found to correspond with the increase in the thrombin concentration. The detection limit of thrombin (based on the average blank signal plus 3x the standard deviation of

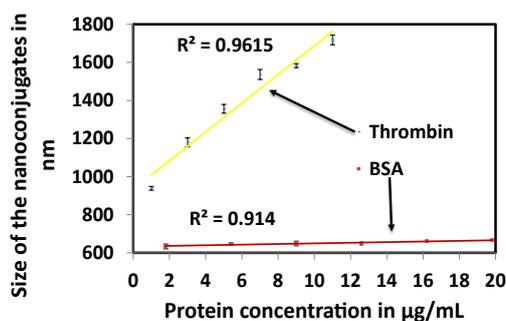


Figure 6: Increase in the average size of the GNP-aptamer-thrombin nanoconjugate complex after addition of thrombin. Aptamer amounts=1 OD. GNP concentration=9.24 nM.

Detection Mode	Detection Limit	Linear Range	Reference
Electrochemical	11 nM	0 to 50.8 nM	[39]
Surface-Enhanced Raman Spectroscopy	100 pM	-	[40]
Electrochemical	16 nM	-	[41]
Photoluminescence	1.8 nM	-	[24]
Surface-Enhanced Raman Spectroscopy	20 pM	0.1 to 10 nM	[42]
Dynamic Light Scattering Spectroscopy	1.4 nM	0 to 300 nM	This publication

Table 1: Aptamer-based thrombin biosensors.

the blank signal) was calculated to be 2.67 nM (0.1 µg/mL). Our assay was applicable to thrombin concentrations all the way up to 300 nM (11 µg/mL). To examine the selectivity of the assay, we conducted an experiment by challenging the aptamer-conjugated GNPs with bovine serum albumin (BSA). Under the exact same conditions there was no significant increase in size of the particles on adding equivalent concentrations of BSA (Figure 6). Typically, some slight increase in hydrodynamic diameter is not entirely unexpected even on addition of non-specific moieties. Table 1 shows the comparison of our technique with other aptamer-based methods. It is clear that our technique is very competitive in terms of detection limit (1.4 nM) and linear dynamic range (up to 300 nM).

Conclusions

In conclusion, we have demonstrated an extremely facile, rapid specific and selective method for detecting proteins using aptamer-conjugated GNPs coupled with dynamic light scattering at ambient conditions. A linear dynamic range of up to 300 nM (11 µg/mL) was realized using thrombin as the model analyte enabling the direct detection of as low as 1.41 nM (0.05 µg/mL) of thrombin. The presence of other interfering proteins such as BSA showed no effect on the assay response. Additional studies are underway in our laboratory to better understand the kinetics of binding to aptamer-conjugated GNPs and to test the assay on real samples. While the utility of the assay was demonstrated for protein binding/detection, the assay could easily be designed for the detection of other targets by the modification of GNPs with appropriate aptamers. Therefore, the technology has the potential to positively impact broad analytical applications in clinical, biomedical and other sectors.

Acknowledgment

We gratefully acknowledge support from NIH Area Grant 1R15CA139390-02.

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