



Design and Fabrication of a Silver Nanocluster-Based Aptasensor for Lysozyme Detection

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Abstract

Lysozyme is a universal part of the innate immune system and is present in various bodily fluids. It is also vastly studied as a biomarker for a variety of diseases and is used in the food industry as a preservative. However, due to lack of a universal and precise detection method, new techniques are highly sought after for this purpose. Here, we introduce a silver nanocluster-based aptasensor to detect lysozyme with high sensitivity. The sensor consists of an RNA aptamer and a complementary DNA sequence with a silver nanocluster template. Silver nanoclusters were synthesized by reduction of silver nitrate in the presence of complementary DNA sequence and characterized by transmission electron microscopy, UV-Visible, fluorescence, and circular dichroism spectroscopy. Emission of the silver nanoclusters switched between low and high intensity based on the quenching ability of the aptamer for DNA-silver nanoclusters and the binding affinity of the aptamer for lysozyme. In the presence of the aptamer, DNA-silver nanoclusters show low fluorescence due to the hybridization of the aptamer, and hence disruption in the formation of DNA-silver nanoclusters. Upon addition of lysozyme, aptamer binding to the DNA-templated silver nanoclusters will be inhibited due to the stronger interaction between the aptamer and lysozyme. Presence of the free DNA template leads to a restored fluorescence signal of DNA-silver nanoclusters which is dependent upon the lysozyme concentration. By utilizing this strategy, the complementary DNA-templated silver nanoclusters sensor can detect concentrations of lysozyme lower than 10 nM via a signal in the visible spectral region. The proposed sensor presents valuable features such as low-cost, high sensitivity, easy fabrication, and convenient operation for detection of lysozyme.

Keywords Lysozyme · Silver nanocluster · Aptamer · Biosensor

Abbreviations

Apt	RNA aptamer
cDNA	Complementary DNA
cDNA-Apt	Complementary-aptamer
AgNCs	Silver nanoclusters

HSA	Human serum albumin
Cyt C	Cytochrome C

Introduction

Lysozyme is a ubiquitous enzyme of innate immune system present in various bodily fluids (tears, saliva, mucus, and serum) and is active in the catalytic hydrolysis of the polysaccharide wall of bacteria [1, 2].

Correlation of lysozyme with certain diseases involving the eyes [2], kidneys [3, 4], rheumatoid arthritis [5], and leukemia [6] has been reported consistently in the literature. In addition, lysozyme has been used as an anti-inflammatory drug for wound care and as a food preservative in cheese, shrimp, and sausage. However, due to allergenic features of lysozyme, detecting accurate levels of lysozyme is critical in food products [7, 8]. Therefore, determination of lysozyme concentration has attracted extensive attention by researchers in the medicine and food industry.

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To date, analysis of lysozyme has been accomplished by enzyme-linked immunosorbent assay (ELISA), nephelometric methods, lysoplate methods, and separation techniques such as polyacrylamide gel electrophoresis (PAGE) and high-performance liquid chromatography (HPLC) [1, 9].

Although these approaches have made great contributions toward the assay of lysozyme, limitations such as lengthiness, availability of commercial antibodies for immunoassay methods, interference from other proteins, and expensive procedures warrant an improved method [10]. Aptamers as the new substitute recognition elements for commercial antibodies are widely accepted among scientists in a variety of fields. Aptamers have outstanding features like more stability, high selectivity, affinity, and easy synthesis compared with antibodies that make them much more desirable [11]. A variety of sequences have been developed as anti-lysozyme aptamers in the recent years [7, 12, 13], when multiple aptamer-based analytical methods have also been established for lysozyme detection. In this regard, diverse techniques include fluorescence [1, 14–18], electrochemical [8, 19–21], surface plasma resonance (SPR) [22], colorimetric [23–25], magnetic relaxation switch [26], luminescence [27], chemiluminescence [28], surface-enhanced Raman scattering (SERS) [29], and MALDI-TOF MS [30].

For example, Marty et al. developed the most sensitive electrochemical aptasensor for detection of lysozyme. In their report, the electrode surface-linked aptamer and biotinylated antibody were used to capture lysozyme in a sandwich format. Lysozyme was determined via differential pulse voltammetry of the generated enzyme reaction product, 1-naphtol. Although this sensor achieves an impressive femtomolar detection limit, the method requires optimization of parameters such as temperature condition, pH, antibody, and enzyme concentration [8]. Interestingly, the enzymatic signal amplification is coupled to a wide variety of output signal for lysozyme detection. For example, Zhang et al. have demonstrated the nicking-polymerization reaction in order to obtain fluorescence [18] and SERS [31] signal amplification for lysozyme. Park et al. have also used amplified DNzyme signal for detection of lysozyme [25].

Although the signal amplification causes high sensitivity (in the femtomolar range), the labeling process and several binding and separation steps make the experiments complex. None of these techniques could serve as a simple, fast, low-cost method for lysozyme detection, and that is what we aim to achieve in this study.

DNA-templated silver nanoclusters (DNA-AgNCs) with a few atoms have been developed as a new class of fluorophores. These kinds of fluorophores have excellent features such as photostability, nontoxicity, low-cost, biocompatibility, and facile synthesis [32, 33]. Therefore, DNA-AgNCs have attracted a great deal of attention as a novel nanobiosensor for detection of DNA [34–36], miRNA [37–40], metal ions [41–46], and single nucleotides [47, 48]. In addition,

silver-based nanoclusters are used for protein detection [49–52], that in some cases are combined with an aptamer for target detection [53–56].

Herein, we introduce an aptamer-mediated sensor for lysozyme detection based on DNA-AgNCs. This sensor involves two oligonucleotide strands, an RNA aptamer for lysozyme detection and a partially complementary DNA (cDNA) to the anti-lysozyme aptamer. The reported DNA template of anti-lysozyme aptamers by Ellington et al. has been widely used [13]. Willson et al. characterized DNA and RNA forms of the aptamer biophysically [57]. The results showed that the RNA form of aptamer had higher affinity than the DNA analog; however, for both cases, electrostatic interactions mainly contributed to lysozyme and aptamer association. Therefore, in this report, we used the RNA form of aptamer to design the nanobiosensor.

The cDNA consists of the D'DNA sequence as a template for AgNCs synthesis introduced by Sharma et al. [58]. AgNCs nested in D'DNA sequence have high fluorescence quantum yield and stability.

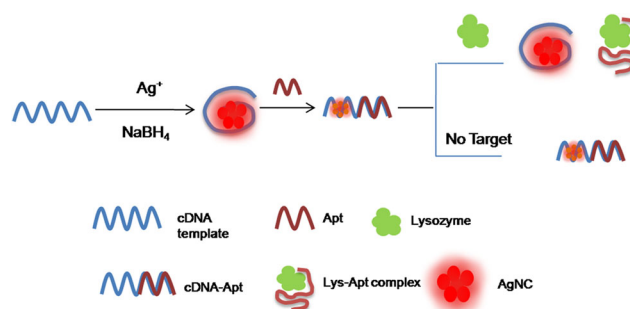
As depicted in Scheme 1, we propose a detection mechanism based on the concept that in the presence of lysozyme, the aptamer-induced fluorescence quenching of cDNA-AgNCs decreases as a result of lysozyme–aptamer complex formation.

In this study, some problems of the most fluorescence-based sensors including covalent attachment of fluorophore and immobilization of oligonucleotide have been tackled. This yields a simple, inexpensive, and fast method for detection of lysozyme. Moreover, utilization of an aptamer as a recognition element instead of an antibody, elimination of the need for using advanced blotting techniques as well as complex instruments such as SPR, HPLC, and MALDI-TOF-MS make this design more desirable for industrial applications.

Material and Methods

Materials

Silver nitrate (AgNO_3), sodium borohydride (NaBH_4), Tris base salt, acetic acid, boric acid, Xylene Cyanol FF,



Scheme 1 Principle of cDNA-templated AgNCs-based aptasensor

Lysozyme, HSA, Cyt C, and α -Amylase were purchased from Sigma-Aldrich Chemical Company. DNA SYBR™ Gold from Invitrogen and MetaPhor™ Agarose were also used for gel electrophoresis. DNA and RNA oligonucleotides were purchased from IDT, Leuven, Belgium: Apt an RNA oligonucleotide with the sequence: 5'-AUC AGG GCU A AA GAG UGC AGA GUU ACU UAG-3', and cDNA—a DNA oligonucleotide with the sequence: 5'-CTG CAC TCT TAC CCG AAC CTG GGC TAC CAC CCT TAA TCC CCT AGC CCT GAT-3'. The underlined sequence within cDNA is a silver nanocluster template termed D'DNA (5'-AAC CTG GGC TAC CAC CCT TAA TCC CC-3') which is a template for synthesis of AgNCs as reported by Sharma et al. [58]

Instruments Fluorescence spectra and UV-Visible absorption were measured by a SpectraMax M2 multi-mode plate reader (Molecular Devices Corporation, Orleans Drive Sunnyvale, California, USA) and Shimadzu UV3600 respectively. 3D fluorescence spectroscopy of samples was done by Jasco FP-6300 spectrofluorimeter. Circular dichroism measurements were done on a Jasco J-715 CD spectropolarimeter. Transmission electron microscopy was performed by Zeiss-EM10C-100 kV. Agarose gels were run by horizontal electrophoresis (Bio-RAD), and visualized with a transilluminator UV lamp at 254 nm and photographed with a digital camera. Milli-Q water with an electrical resistance of 18 M Ω cm was prepared with a Millipore Synergy water purification system and used for preparation of solutions.

Synthesis of Silver Nanoclusters

Silver nanoclusters were synthesized in the presence of Apt, cDNA, and cDNA-Apt. AgNCs were synthesized according to previous work by Sharma et al. [58]. The final ratio of DNA: AgNO₃: NaBH₄ was considered 1:6:6 and Tris-acetate (pH 6.5) was selected as reaction buffer. Finally, cDNA-Apt-AgNCs were synthesized by incubation of Apt and cDNA-AgNCs in 1:1 ratio for 1 h at room temperature.

Silver Nanocluster Characterization

The visible spectrum of silver nanoclusters was measured in 300–700 nm range. 3D fluorescence spectra were monitored in 300–650 nm range to find the best excitation wavelength. CD spectra of the free cDNA and cDNA-AgNCs were measured from 200 to 350 nm at the 1-nm interval. A quartz cuvette with 0.1 cm pathlength was used. CD spectra of free D'DNA and D'DNA-AgNCs were measured as controls. The final concentration of DNA was 30 μ M. Transmission electron microscopy (TEM) was performed on a Zeiss-EM10C at 100 kV and

was used for further characterization of cDNA-AgNCs. Formvar carbon-coated grid Cu Mesh 300 was used as the substrate and imaging was done at room temperature, in a vacuum environment. The final concentration of cDNA was adjusted to 100 μ M. TEM measurements were replicated six times on one sample, to analyze the size distribution of AgNCs.

Lysozyme Detection

AgNCs prepared with cDNA were mixed with 500 nM Apt in 200 μ l Tris-boric acid buffer. Different concentrations of lysozyme (2, 10, 25, 50, 125, 250, and 500 nM) were then added to the solution and incubated at room temperature for 4 h. This process provided sufficient lysozyme-Apt binding and maximum emission intensity for AgNCs. All experiments were repeated six times.

Results and Discussion

Principle of cDNA-Templated AgNCs-Based Aptasensor

Silver nanoclusters were synthesized in the presence of cDNA which becomes partially complementary to the aptamer (Apt). As seen in Scheme 1, in the absence of lysozyme, the fluorescence intensity is low because the interaction of Apt with partially complementary DNA (cDNA-Apt) may disrupt AgNCs synthesis. Upon addition of lysozyme, due to the stronger affinity between lysozyme and Apt, the free cDNA template allows rigid formation of cDNA-AgNCs, leading to higher fluorescence intensity.

Synthesis of Silver Nanocluster

The absorption and emission spectra of cDNA-AgNCs in the absence and presence of Apt were investigated to verify the effect of Apt on the fluorescence properties of AgNCs. The UV-Vis results show that cDNA and cDNA-Apt-AgNCs have the same strong peak at 420 nm as well as a weaker absorption peak at 530 nm due to the formation of AgNCs by D'DNA sequence, being consistent with Sharma et al.'s report [58]. The cDNA-Apt-AgNCs display lower absorbance at 530 nm, demonstrating that the Apt disrupts formation of AgNCs (Fig. 1a). The maximum emission intensity of cDNA and cDNA-Apt-AgNC were obtained by excitation in the range of 300–650 nm. As shown in Fig. 1b, the cDNA-AgNCs and cDNA-Apt-AgNCs have the highest emission intensity at 530 nm excitation.

To rule out the possible interference of the Apt in fluorescence emission, AgNCs were synthesized in the presence of Apt. The absorbance and maximum emission intensity were

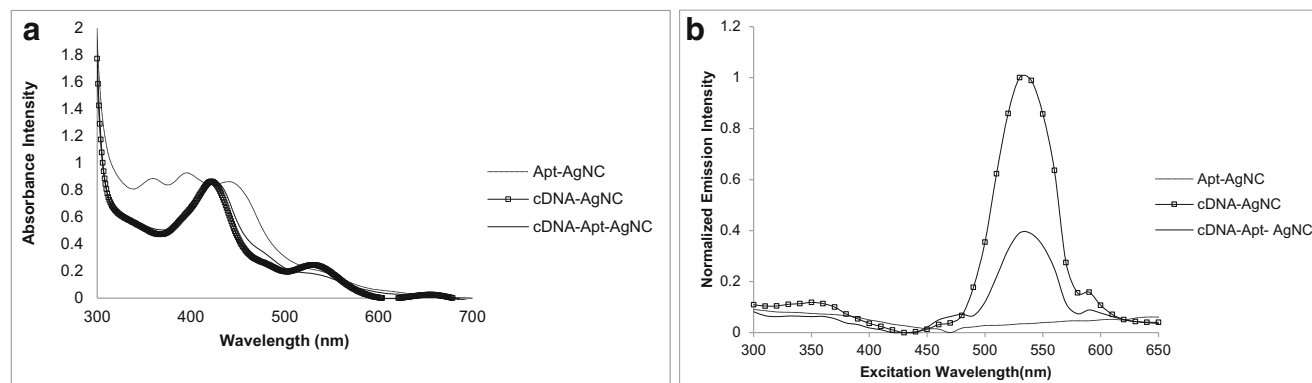


Fig. 1 **a** UV-Vis spectra of AgNCs in the presence of Apt (dotted line), cDNA (white square) and cDNA-Apt (solid line). Final concentration of nucleic acids were adjusted to 70 μ M. **b** Emission intensity as a function of excitation wavelength for AgNCs formed with Apt, cDNA, and

cDNA-Apt. The emission intensity for cDNA-AgNC was normalized to 1 at 530 nm. Tris-acetate buffer (50 mM) was selected as reaction buffer. A quartz cell with 1 cm path length was used

investigated by excitation in the range of 300–650 nm. The Apt-AgNCs do not show any absorbance at 530 nm although there are several peaks at lower wavelengths (Fig. 1a). Moreover, Apt-AgNCs do not show any emission by excitation at 300–650 nm. Therefore, there is no interference with emission of AgNCs (Fig. 1b).

Characterization of cDNA-AgNC by CD Spectroscopy and TEM

CD spectroscopy has been used for characterization of DNA-templated silver nanocluster. The CD spectrum of free cDNA has positive ellipticity at 275 nm and a negative band near 245 nm with crossover at 260 nm, showing a single-stranded B-DNA [59]. According to previous studies, DNA may display an i-motif-like structure in the presence of Ag^+ ions [60, 61]. Conversely, the CD spectrum of cDNA undergoes a little red shift of ~ 3 nm after synthesis of AgNCs (Fig. 2). This is consistent with the previous

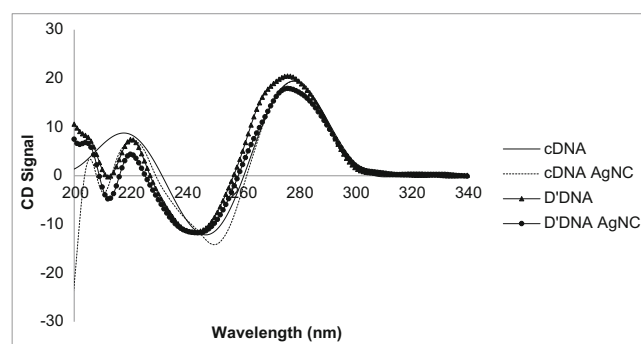


Fig. 2 Far UV CD spectra of free cDNA (solid line) and cDNA-AgNC (dotted line). D'DNA (black triangle) and D'DNA-AgNC (black circle) were used as control. Tris-acetate buffer (50 mM) was selected as reaction buffer. A quartz cuvette with 0.1 cm path length was used. Final concentration of DNAs was adjusted to 30 μ M. A reference spectrum of the buffer has been subtracted and the y-axis shows the ellipticity in mdeg

study of Sharma et al. in which D'DNA-AgNCs does not show any i-motif signatures [58].

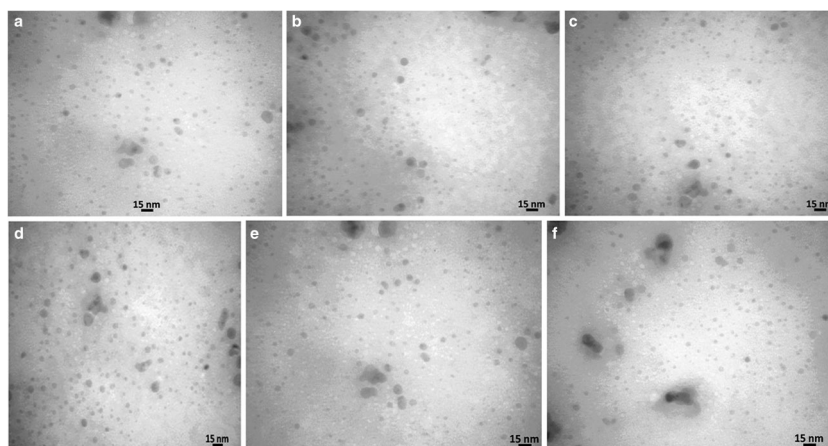
Figure 3 shows TEM images of cDNA-AgNCs. Analysis of the images showed that the average particle diameter of AgNCs is about 4.2 nm (Online Resource, Fig. S1a). There were also particles with an average size of 30 nm showing that large silver nanoparticles have been synthesized with surface plasmon peak of around 400 nm (Fig. 3). A previous study by Petty et al. also reported a strong peak around 400 nm which shows larger Ag nanoparticles may be formed beside silver nanoclusters [62]. As seen in Online Resource (Fig. S1b), there is a large particle that might be the result of AgNCs aggregation. In line with the previous study by Wilcoxon et al., beam heating or cluster melting occurs during imaging which may lead to fusion of the clusters [63].

Optimization of AgNCs Synthesis

The sensitivity of lysozyme detection greatly depends on assay conditions such as fluorescence intensity and aptamer affinity. In recent studies, Tris-buffer with MgCl_2 and high concentration of NaCl were used for selective lysozyme detection by aptasensors [13, 14, 20, 64–67]. This buffer condition can disrupt the electrostatic interaction of the positively charged lysozyme with the negative backbone aptamer, thereby inhibiting the non-specific binding of lysozyme [13].

To achieve the best reaction conditions, fluorescence of AgNCs was investigated in the presence of NaF and Mg-acetate. For this purpose, the effect of 50 mM Tris-acetate buffer with 5 mM magnesium acetate, 150 mM sodium fluoride, and 700 mM boric acid were investigated on AgNCs emission. cDNA-AgNCs solutions were excited at 530 nm and the emission was measured at 610 nm. Online Resource, Fig. S2 shows that both magnesium and sodium fluoride salts caused a decrease of cDNA-AgNC

Fig. 3 TEM images of cDNA-AgNC. Size of AgNCs yield was estimated to be 2–5 nm. Final concentration of cDNA was adjusted to 100 μ M. TEM was performed on a Zeiss-EM10C–100 kV, formvar carbon-coated grid Cu Mesh 300 was used as substrate and imaging was done at room temperature, in vacuum environment



emission while boric acid had a positive effect on AgNC emission, leading to enhancement of cDNA-AgNCs emission. To obtain the time with the highest emission, the fluorescence intensity of cDNA-AgNCs was compared in Tris-acetate and Tris-boric acid buffers during 8 h (by 2 h interval). Although AgNCs emission has an increasing trend during 8 h in Tris-acetate buffer, it reaches to maximum intensity by 4 h in Tris-boric acid. Results showed that Tris-boric acid buffer can also accelerate formation of AgNCs (Online Resource, Fig.S3).

Confirmation of Apt and cDNA-AgNCs Interaction

Fluorescence Measurements

The effect of different concentrations of Apt (2, 4, 6, 8 μ M) was investigated on cDNA-AgNCs (2 μ M). Results showed that Apt leads to decrease of cDNA-AgNCs emission. This indicates that Apt can disrupt AgNCs synthesis in an equimolar fashion (Online Resource, Fig. S4). The fluorescence intensity did not decrease by further addition of Apt. Therefore, 1:1 ratio of Apt to cDNA was selected for further experiments.

Agarose Gel Electrophoresis

Apt binding to the cDNA was further verified by agarose gel electrophoresis. Apt was mixed with free cDNA and cDNA-AgNCs and then incubated for 2 h at room temperature. Apt, free cDNA, and cDNA-AgNC were used as controls. Samples with 34 μ M final concentration were loaded on a 3.5% agarose gel and imaged under a UV lamp at 254 nm. SYBR gold was used to stain the samples. As shown in Online Resource, Fig.S5, cDNA-Apt was formed upon addition of Apt to cDNA. Since the complex is heavier than cDNA, this indicates a successful hybridization. The orange color of cDNA-Apt-AgNCs shows the inherent emission of the D'DNA-silver nanoclusters that was also observed in the presence of the hybridized strands.

Evaluation of the Aptamer-Lysozyme Interaction and the Aptamers Specificity

It has previously been reported by Girvin et al. that elimination of Mg^{+2} reduces the lysozyme -aptamer interaction by 67-fold. Therefore, we assessed the effect of Tris-magnesium acetate on lysozyme binding by comparing Tris-acetate and Tris-boric acid buffer in 0.8% agarose gel. Different concentrations of lysozyme (5, 10, 25, 50, 100, 150, and 200 μ M) were mixed with a constant concentration of Apt (5 μ M). Samples were incubated at room temperature for 1 h. Upon addition of lysozyme to Apt, the complex formed and remained in the well due to the altered charge; while the free Apt with negative charge migrated through the gel.

Online Resource, Fig. S6a shows that association of lysozyme-Apt in Tris-acetate is weaker in comparison with Mg-Tris-acetate buffer. By increasing the lysozyme concentration, more lysozyme-Apt complex will be formed and less free Apt will remain in Tris-Mg reaction buffer; although there is free Apt even at the highest concentration of lysozyme (Online Resource, Fig. S6b). While lysozyme-Apt complex has been prepared with 1:1 ratio, there is not much free Apt in 1: 5 and higher ratios in Tris-boric acid buffer. The results show that Tris-boric acid enhances lysozyme binding to Apt more than Tris-Mg (Online Resource, Fig. S6c).

Specificity of the Apt was investigated by comparing with CytC and HSA. CytC has a pI value of ~ 10 , yielding a positive charge (similar to lysozyme with pI ~ 11). HSA was selected as a negative control with pI of ~ 4.7 . CytC or HSA were mixed with Apt (5 μ M). Samples were incubated at room temperature for 2 h (in Tris-boric acid buffer). Analysis of gel electrophoresis showed that Apt bound to lysozyme and Lys-Apt complex remained in the well, whereas free Apt moved down. Apt did not bind to CytC or HSA as there was no complex in the well after 2 h of incubation (Online Resource, Fig.S6d). Therefore,

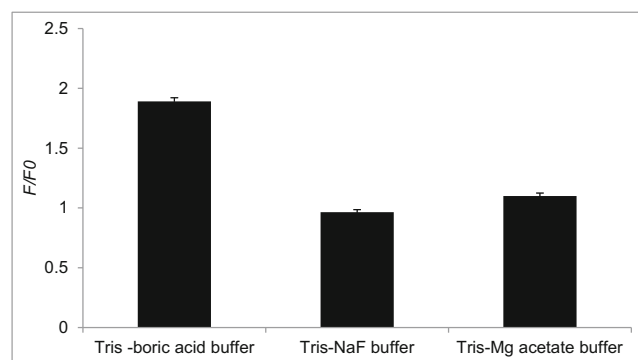


Fig. 4 Fluorescence ratio (F/F_0) of AgNCs after addition of Apt, in the presence (F) and absence (F_0) of lysozyme at 610 nm, in three different buffers. All solutions were excited at 530 nm. Standard deviations are indicated on each column

it can be assumed that Tris-boric acid buffer not only can increase the binding affinity of Apt to lysozyme (Online Resource, Fig. S6a, b) but also can block non-specific binding between Apt with CytC and HSA proteins (Online Resource, Fig. S6d).

Buffer Effect on Nanobiosensor Function

Fluorescence intensities of AgNCs were investigated after addition of Apt in the presence (F) and absence (F_0) of lysozyme at 610 nm (with Tris-Mg, Tris-NaF, and Tris-boric acid), to obtain highest fluorescence ratio (F/F_0). The 1:1 ratio of Apt to lysozyme was incubated in three different buffers separately and mixed with cDNA-AgNCs. As seen in Fig. 4, the highest F/F_0 ratio was obtained in Tris-boric acid buffer. Therefore, Tris-boric acid buffer was selected as the optimum buffer for lysozyme detection since it enhances the AgNCs emission and Apt-binding affinity as well as the F/F_0 ratio.

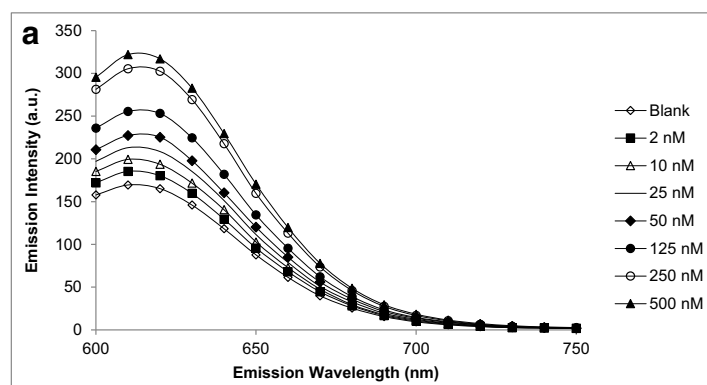


Fig. 5 Sensitivity of cDNA-AgNCs-based aptasensor for lysozyme detection; Tris-boric acid buffer was used as reaction buffer (700 mM). All solutions were excited at 530 nm. **a** Fluorescence emission of AgNCs after Apt addition with increased concentrations of target, blank (white diamond), 2 nM (black square), 10 nM (white triangle), 25 nM (solid

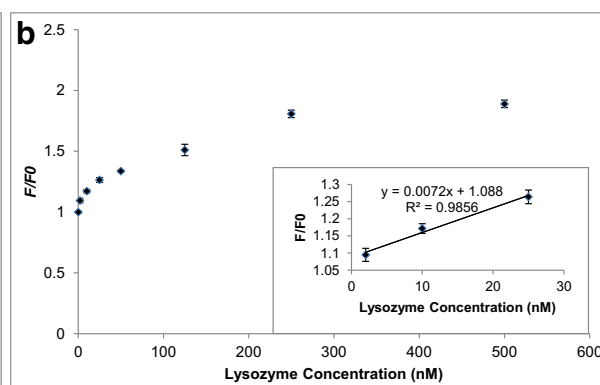
Lysozyme Detection

In the presence of Apt, the fluorescence of cDNA-AgNCs is low as expected, likely because cDNA hybridization with Apt disrupts the silver nanoclusters binding site. In the presence of lysozyme, fluorescence is restored as the binding of lysozyme to Apt decreases hybridization of Apt with cDNA. As shown in Fig. 5a, fluorescence intensity is gradually recovered by increasing lysozyme concentration from 2 to 500 nM to achieve the linear relationship between the probe fluorescence and lysozyme.

The F/F_0 ratio was plotted against lysozyme concentrations, where F and F_0 are the fluorescence intensities at 610 nm of cDNA-AgNCs after adding Apt in the presence and absence of lysozyme, respectively. The results revealed that our sensor displays fluorescence response in a linear correlation to the concentration of lysozyme within the range of 2–25 nM. The detection limit of lysozyme was calculated to be 5.6 nM, based on $3\sigma/\text{slope}$ (σ is the standard deviation of the blank) (Fig. 5b). We achieved a relative standard deviation (RSD) of 3.3% from a series of three experiments with 25 nM lysozyme concentration, indicating a good reproducibility of our findings.

As a control, the possible effect of lysozyme on fluorescence intensity of cDNA-AgNCs was investigated by adding different concentrations of protein (2–500 nM) to cDNA-AgNCs in the absence of Apt. There was a negligible effect on cDNA-AgNCs upon lysozyme addition. Therefore, the fluorescence enhancement is not the result of the interaction between cDNA and lysozyme. This has occurred due to the strong affinity of lysozyme to its Apt (Online Resource, Fig. S7).

So far, anti-lysozyme aptamers have been used as a recognition segment in various sensors. As seen in Table 1, most fluorescence aptasensors have detection limits in the nanomolar range, while we achieved a lower LOD than some reports [14, 17]. It is important to note that the approach



line), 50 nM (black diamond), 125 nM (black circle), 250 nM (white circle), 500 nM (black triangle); **b** plot of fluorescence ratio (F/F_0) at 610 nm versus lysozyme concentration. The insert shows the low concentration range

Table 1 Summary of aptasensors for lysozyme detection

Materials	Signal output	LOD	Reference
FAM-labeled aptamer/anionic polymer	FRET	53 nM	[14]
Carbon dot-labeled aptamer/graphene oxide	FRET	0.06 nM	[16]
Pyrene-conjugated cDNA	Fluorescence	0.2 nM	[1]
ExonucleaseIII/graphene oxide film/FAM-labeled signal probe	Fluorescence	5 nM	[15]
Nicking endonuclease/DNA polymerase/magnetic nanoparticles	Fluorescence	3.6 fM	[18]
Aptamer-conjugated SiNP/polyelectrolyte	Fluorescence	25 nM	[17]
Gold film/graphene oxide film	SPR	0.5 nM	[22]
DNAzyme/nicking enzyme/DNA polymerase	Colorimetric	0.1 fM	[25]
Avidin-alkaline phosphatase/antibody/amine-labeled aptamer	Electrochemical	4.3 fM	[8]
Nicking endonuclease/DNA polymerase/AuNP-functionalized Raman probes/modified gold chip/modified magnetic bead/amine-labeled aptamer	SERS	1 fM	[31]
Aptamer-conjugated iron oxide nanoparticles	Magnetic relaxation switch	0.5 nM	[26]
Carbon nanotube/europium (III)	Luminescence	0.9 nM	[27]
Au nanoparticles/magnetic nanoparticles/DNA polymerase/nicking enzyme/amine-labeled aptamer	Chemiluminescence	0.2 pM	[28]
	MALDI-TOF-MS	1 nM	[30]

presented here is comparable with the fluorescence-based method combined with exonuclease III-aided signal amplification of similar LOD [15].

Recently, Yang et al. have demonstrated a FRET-based aptasensor from carbon dots to graphene oxide [16]. Although they showed a low LOD for detection of lysozyme, the non-specific absorption of biological sample component to the graphene surface might be a cause for false positive results. The fluorescence-based aptasensors are very effective in detecting lysozyme. However, the process of oligonucleotide attachment to a fluorophore in these structures can be quite cumbersome. To tackle this issue, we used an intrinsically fluorescent transducer element through a cDNA-templated extension by adding the fluorescent D'DNA sequence for stabilizing emissive AgNCs.

Other aptamer-based methods relying on SPR [22], luminescence [27], magnetic relaxation switch [26], and MALDI-TOF-MS [30] have also been used for lysozyme detection. Although these methods show nanomolar detection limits, their application can be costly. To the best of our knowledge,

the lowest detection limit for lysozyme has been reported to be 0.1 fM, using the enzymatic signal amplification [25]. The enzymatic signal amplification is also combined with fluorescence [18], SERS [31], or colorimetric [25] detection methods to increase sensitivity. However, better sensitivity in these reports has been gained at the expense of complexity, multiple purification steps, the inconvenience associated with limited enzyme stability, and finally the cost.

Besides aptamer-based sensors, other detection methods have also been used for detection of lysozyme (Table 2). While our method is much more sensitive than HPLC (currently used in clinical and analytical laboratories), it has also achieved lower LOD relative to turbidimetry, PRLS, and imprinting techniques.

The data in Table 3 show that LOD of the presented design is comparable with other reported AgNCs-based sensors for protein detection. The most common designs currently used are structure-switching aptamer biosensors, in which, the AgNCs-template is directly linked to a location on the aptamer [53, 54]. In addition, some of them are based on signal turn-off

Table 2 Summary of other methods for lysozyme detection

Recognition element	Detection method	LOD	Reference
Antibody	ELISA	0.01 nM	[68]
Reverse phase polymeric column with phenyl functional groups	HPLC	0.034 μ M	[69]
Micrococcus lysodeikticus	Turbidimetry	0.13 μ M	[70]
Imprinting technique	fluorescence	14 nM	[71]
	PRLS	13 nM	[10]

Table 3 Summary of nanocluster-based aptasensors for protein detection

Materials	Analyte	Signal Output	LOD	Reference
DNA-AgNC	Thrombin	Fluorescence	1 nM	[54]
DNA-AgNC	Thrombin	Fluorescence	1 nM	[55]
DNA-AgNC	PDGF-BB	Fluorescence	0.37 nM	[56]
DNA-AgNC	adenosine deaminase	Fluorescence	—	[53]
DNA-AgNC/cDNA	Lysozyme	Fluorescence	5.6 nM	Our work

due to target binding, causing a conformational change and substantially fluorescence quenching [55]. Although simple, this design can affect the original affinity of aptamer and may suffer from false positive results due to the interferences in signal quenching.

To overcome these problems, we have presented a turn-on process for lysozyme sensing, relying on the D'DNA-AgNCs template fused to cDNA which serves as the competitive ligand for binding to RNA lysozyme aptamer. The oligonucleotides used are commercially available at a very competitive cost without any stability issues, even in the case of RNA sequence. Note that the sequence of the competitive aptasensor has required sensitivity for detection of lysozyme in serum (49–180 nM) [68], saliva (116 nM) [72], and tears (0.114 mM) [2]. In some other recent reports, fluorescence turn-on methods have been presented for detection of other proteins [53, 54, 56]. There is a negligible difference between the LOD of our nanobiosensor and reported detection limit for thrombin and PDGF-BB, which can be explained by greater affinity of anti-thrombin aptamer ($K_d = 0.5$ nM) [54], and anti-PDGF-BB aptamer (in picomolar range) [56].

Conclusion

Several sensors have been developed to detect lysozyme in multiple contexts. However, the simplicity in function, feasibility and stability of designed sensor are the main differentiating factors for our design with large-scale production capacity. The aptamer is much cheaper than an antibody (as the biorecognition element), and AgNCs provide high emission stability (as the transducer element). In summary, we have introduced a cheap, label-free, and on-off switching aptasensor for detection of lysozyme with the best capacity for industrial production. The innovative approach in coupling lysozyme-aptamer-binding affinity with aptamer-induced quenching of cDNA-AgNCs fluorescence makes it possible to detect lysozyme in nanomolar range. Emission of the silver nanoclusters switches between low and high intensity in the absence and presence of lysozyme, respectively. We believe that the simplicity and re-usability of this strategy can be extended to other AgNCs-based aptasensors for a more diverse range of protein detection.

Compliance with Ethical Standards

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

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