

**Electrostatic-mediated Binding of DNA to Lysozyme: Evaluation of Aptamer-based
Assays for Highly Positively Charged Targets**

Lu Shi ^{a,b}, Yan Jin ^{a*}, and Juewen Liu ^{b*}

^a Key Laboratory of Analytical Chemistry for Life Science of Shaanxi Province, Key Laboratory of Applied Surface and Colloid Chemistry, Ministry of Education, School of Chemistry and Chemical Engineering, Shaanxi Normal University, Xi'an 710119, China

^b Department of Chemistry, Waterloo Institute for Nanotechnology, University of Waterloo, Waterloo, ON N2L 3G1, Canada

* Corresponding authors:

liujw@uwaterloo.ca (Juewen Liu);

jinyan@snnu.edu.cn (Yan Jin)

Abstract

Lysozyme is a highly popular protein target for the development of aptamer-based biosensors. Because lysozyme is a polycation and DNA is a polyanion, it is essential to separate the contribution of nonspecific electrostatic interactions from specific aptamer binding. In this study, various factors affecting the binding of DNA and lysozyme, including DNA sequence, DNA length, pH, and salt concentration, were explored using fluorescence polarization. We concluded that direct fluorescence polarization and fluorescence intensity change are unlikely to be directly applicable for aptamer-based biosensors to detect lysozyme because all tested DNA sequences showed binding. These fundamental studies confirm the dominant role of electrostatic binding. We further evaluated three other methods including label-free fluorescent detection using a DNA staining dye, label-free colorimetric detection using gold nanoparticles, and a fluorescent sensor based on the strand displacement reaction. In each case, we focused on a random DNA sequence that is not expected to bind to lysozyme as an aptamer. Of all the methods, only the strand displacement strategy can be potentially used to evaluate aptamer binding, as the other methods all responded to non-aptamer sequences. This study provides valuable insights for assaying aptamer binding to cationic proteins that can exhibit nonspecific attraction to DNA.

Introduction

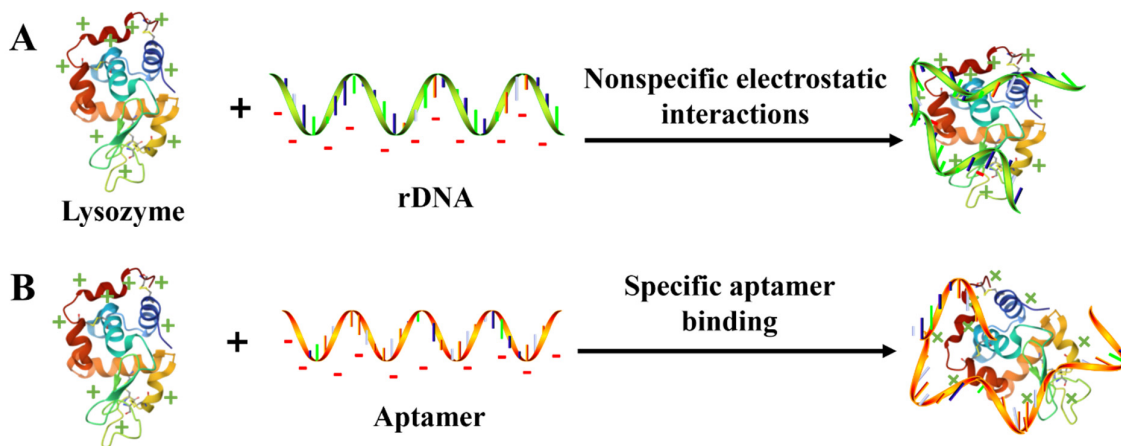
Lysozyme was discovered by Laschtschenko in 1909,¹ and later named by Fleming in 1922.² Besides its anti-inflammatory role for wound healing, hen egg white lysozyme exhibits muraminidase activity, making it a valuable natural antibacterial agent for food preservation.³ In addition, lysozyme has immunogenic properties and can cause food allergy.⁴ Therefore, efforts were made to detect lysozyme.⁵ Different from most other proteins, lysozyme is highly positively charged with an isoelectric point of 10.5-11, and each lysozyme carries eight excess positive charges at physiological pH.⁶ In addition, lysozyme has excellent heat stability and acid stability.⁷

Aptamers are single-stranded oligonucleotides (DNA or RNA) that are capable of selectively binding to target molecules.^{8,9} Among the aptamers for proteins, the most studied and used are the thrombin binding aptamers.¹⁰ The lysozyme aptamers ranked number four.¹¹ The first aptamer lysozyme was reported by the Ellington group,^{12, 13} and a dissociation constant (K_d) of 31 nM was determined. This is an RNA aptamer with a length of 80 nucleotides (80-nt). From a recent paper reviewing modification of aptamers,¹⁴ the majority of the subsequent work intended to use this aptamer used wrong sequences, such as the use of the DNA version of the aptamer, further truncation of a portion of the DNA sequence etc. without rigorous verification of binding properties.¹⁵⁻¹⁷ Many later work used a 42-nt sequence (sequence a1 in Table S1).¹⁷⁻²¹ By employing capillary electrophoresis for selection (CE-SELEX), Lammertyn's group reported a DNA aptamer for lysozyme (sequence b in Table S1).²² This aptamer was also used to develop biosensors for lysozyme.²³⁻²⁶

In 2011, Potty et al. performed a detailed study of a few aptamers for lysozyme.²⁷ They found that the binding was stronger at lower salt concentrations but much weaker with over 100 mM Na^+ . This is consistent with electrostatic-driven binding. As a cationic protein, lysozyme can easily bind to highly negatively charged DNA (Scheme 1). As early as 1976, the Record group studied the effects of ions on ligand-nucleic acid interactions.²⁸ They determined that the primary factor influencing the formation of complex structures between polyelectrolytes and nucleic acids can have significant entropic contributions from the release

of counterions. In 1999, Steinrauf et al. proposed that the interaction of lysozyme with nucleic acids fundamentally stems from electrostatic attraction as supported by gel electrophoresis data.⁶

Such nonspecific interactions can be misleading in the context of aptamer binding assays and related biosensor testing. Therefore, an interesting question is whether an assay can distinguish non-specific electrostatic interactions from specific aptamer binding. In addition, we also aim to understand the factors that can affect DNA binding to lysozyme, which may suggest an optimal condition for aptamer selection.



Scheme 1. Schematic illustration of the binding of DNA to lysozyme by two interactions: (A) nonspecific electrostatic interactions with a random DNA (rDNA); (B) specific aptamer binding. The structure of lysozyme was exported from Protein Data Bank (PDB).

Materials and Methods

Chemicals

All DNA oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA, USA). The DNA sequences used in the present study are listed Table S1. SYBR Green I (SGI), chloramphenicol (CAP), bovine serum albumin (BSA), tetrachloroauric acid (HAuCl₄) and sodium L-ascorbate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Lysozyme,

Tris(hydroxymethyl) aminomethane (Tris), and salts (NaCl and MgCl₂) were from VWR Chemicals. Milli-Q water was used to prepare all buffers and solutions. Half-area black 96-well microplates were purchased from Corning and Greiner. Quartz fluorescence cuvettes were from Agilent Technologies. Buffer 1: 50 mM Tris-HCl (pH 7.5); buffer 2: 50 mM Tris-HCl (pH 8.3); buffer 3: 50 mM KCl-NaOH (pH 12.6); buffer 4: 10 mM Tris-HCl (pH 7.5); and buffer 5: 50 mM Tris-HCl (pH 7.5, 10 mM MgCl₂).

Fluorescence Polarization Measurements with Different DNA Sequences

Fluorescence polarization measurements were performed using a microplate reader (Tecan Spark) and a 96-well black plate in buffer 1 containing 50 nM carboxyfluorescein (FAM)-labeled DNA. The DNA solutions were heated at 95°C for 1 min and then slowly cooled to room temperature prior to all experiments. The annealed sample solution (100 µL) was titrated with lysozyme to a final concentration of 2 µM. Fluorescence polarization and fluorescence intensity were monitored using a microplate reader with 478 nm excitation and 525 nm emission wavelengths.

Effect of pH

Sample solutions (100 µL) in buffers 1 (pH 7.5), 2 (pH 8.3) or 3 (pH 12.6) containing 50 nM F-rDNA (or F-a1) and different concentrations of lysozyme (0.01, 0.1, and 1 µM) were added to each well of a 96-well microplate. Fluorescence polarization and fluorescence intensity were monitored using the microplate reader with 478 nm excitation and 525 nm emission.

SGI Fluorescent Spectroscopy

Fluorescence measurements were performed using a FluoroMax-4 fluorescence spectrophotometer (Horiba Scientific, USA). Binding of rDNA to lysozyme or BSA was evaluated in buffer 1 containing 0.2 µM rDNA, 0.1× SGI and different concentrations of lysozyme. Fluorescence spectrum was collected using the fluorometer at an excitation wavelength of 495 nm.

Colorimetric Assays with AuNPs

AuNPs (13 nm in diameter) were prepared according to a previous report.²⁹ 50 μ L of 10 nM AuNPs was added in 50 μ L of the sample solution in buffer 4 containing 100 nM rDNA (or a1 DNA) and different concentrations of lysozyme (or BSA). The results were recorded using a digital camera.

Strand Displacement Reaction (SDR)

FAM-rDNA (1 μ M) and a quencher-labeled DNA (Q-DNA, 2 μ M) were first annealed in buffer 5 to generate F/Q complexes by heating for 3 min at 95°C, followed by gradual cooling to room temperature. Sample solutions containing 50 nM F/Q complexes and different concentrations of lysozyme (or BSA) were then added to each well of a 96-well microplate. After 10 min, the fluorescence intensity was monitored at 478 nm excitation and 525 nm emission.

Results and Discussion

DNA Sequences Tested. The original RNA aptamer reported by the Ellington group is an 80-nt sequence from Clone 3 (see Table S1 for the sequence).^{12, 13} However, few studies have systematically truncated this aptamer using rigorous binding assays. After removing the primer binding regions of the Clone 3 sequence, the sequence becomes Clone 3' (see Table S1). Potty tested three DNA sequences named a1, a2 and a3, all of which were of the same length.²⁷ The a1 was from a previous sensing paper, whereas a2 was related to Clone 3. Fluorescence polarization experiments indicated that they all bind to lysozyme with a similar affinity, and the authors studied a2 in their work, although a1 had a slightly higher affinity. In this work, we used the a1 sequence. It is possible that none of these DNA sequences are real aptamers for lysozyme, since they were 'in silico' derived without rigorous testing of binding side-by-side with the original RNA aptamer. In this study, we chose the a1 sequence as a starting point for comparison. In addition to the a1 sequence, we also included a few other sequences for comparison (Table S1).

Fluorescence Polarization and Quenching Assays. Fluorescence polarization (FP) is a simple, fast, and accurate assay for protein binding. Upon binding of a fluorescently labeled aptamer to its protein target, the molar volume expands, leading to decreased rotational motion and an increase in FP. FP has been used to study the binding of aptamers.^{30, 31} In this study, FAM-labeled a1 (F-a1) and a FAM-labeled random sequence (F-rDNA) were tested (Figure 1A). Interestingly, regardless of the sequence used, the FP signal increased with an increase in lysozyme concentration, while the fluorescence intensity decreased at the same time (Figure 1B-E). The a1 sequence ($K_d = 0.26 \pm 0.02 \mu\text{M}$) and the rDNA ($K_d = 0.58 \pm 0.14 \mu\text{M}$) showed comparable binding to lysozyme based on the FP.

Using fluorescence quenching, we also calculated the binding affinities of a1 and lysozyme to be $0.45 \pm 0.08 \mu\text{M}$. A similar K_d value indicated that the FP and fluorescence quenching were from the same binding reaction. F-rDNA is an arbitrary DNA sequence, and its binding to lysozyme is mainly due to electrostatic interactions rather than specific binding. Therefore, the a1 sequence does not show an advantage in binding. A more important conclusion is that, under the assay condition, FP and fluorescence quenching, as commonly used means of detecting binding of aptamers to lysozymes, may not differentiate non-specific electrostatic binding from specific aptamer binding, unless the binding from true aptamers has a much higher affinity (for example, K_d below 10 nM). Therefore, these methods are unlikely to be reliable for assaying aptamer binding to lysozymes. Nevertheless, FP appears to be a reliable binding assay, and we used it to further study nonspecific DNA binding to lysozyme.

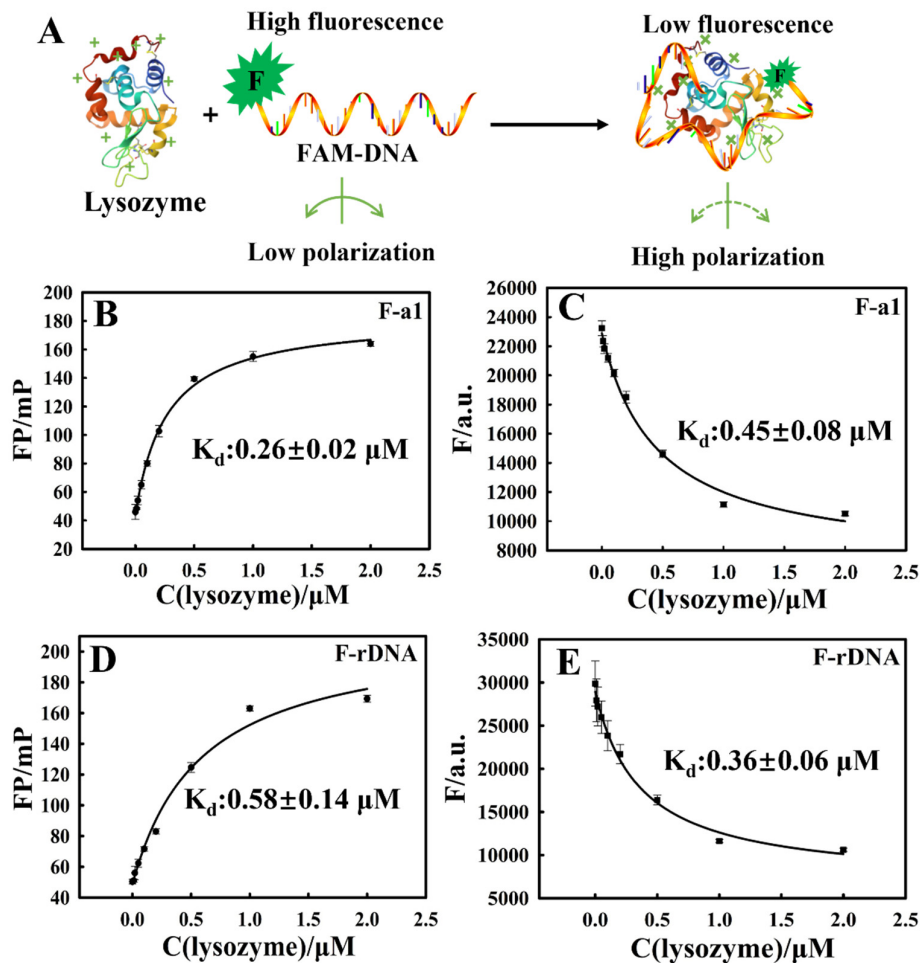


Figure 1. (A) Schematic illustration of the binding of a DNA to lysozyme by FP. FP of (B) F-a1 and (D) F-rDNA in the presence of different concentrations of lysozyme. Fluorescence intensity change of (C) F-a1 and (E) F-rDNA in the presence of different concentrations of lysozyme. The concentration of DNA was 50 nM. The error bars represent the standard deviations from three independent measurements.

Effects of DNA Sequence and Length. To further understand the binding of DNA to lysozyme, we varied the length and sequence of DNA. First, different DNA homopolymers of 15-nt, including poly-A15, poly-G15, poly-C15, and poly-T15, were tested. As shown in Figure 2A, the FP signal gradually increased as the concentration of lysozyme increased. While all FP values started from 40 mP to 60 mP, we observed the least FP change with A15, and an accurate

K_d value could not be fitted with it. For the rest three, we calculated their K_d values to be $2.7 \pm 0.8 \mu\text{M}$ (poly-G15), and $0.93 \pm 0.04 \mu\text{M}$ (poly-C15) and $2.3 \pm 0.4 \mu\text{M}$ (poly-T15), respectively. Therefore, it appeared that the binding of C15 was slightly stronger. We previously observed poly-C DNA to be the best nonspecific binder for insulin³² and also for many nanomaterials,³³ which was attributed to its lack of stable secondary structure at neutral pH allowing maximal contact with proteins and surfaces.³⁴ Compared to poly-G15, poly-C15, and poly-T15, poly-A15 has a weaker adsorption interaction with lysozyme, which might be due to its unique structural characteristics such as stronger intramolecular interactions.

Then, the effect of DNA length was studied using FAM-labeled poly-C5, poly-C15, poly-C20, and poly-C30. When examining the effect of DNA length, 5-mer is the shortest length for the company to synthesize. Typical hybridization probes are between 10 to 20-nt, and most truncated aptamers are below 50-nt. So, the range of DNA length we chose was a combination of all these factors. As shown in Figure 2B, the longer the sequence, the stronger recognition with lysozyme. The obtained K_d values were $0.93 \pm 0.04 \mu\text{M}$ (poly-C15), $0.41 \pm 0.02 \mu\text{M}$ (poly-C20), and $0.20 \pm 0.01 \mu\text{M}$ (poly-C30), respectively. Similar results were obtained for poly-A sequences of different lengths (Figure S1). Therefore, this trend was also consistent with the electrostatic binding model, and a longer DNA has stronger attraction. The results implied that the binding of DNA to lysozyme is strongly affected by the length and sequence. For a true aptamer, it needs to achieve binding beyond electrostatic interactions.

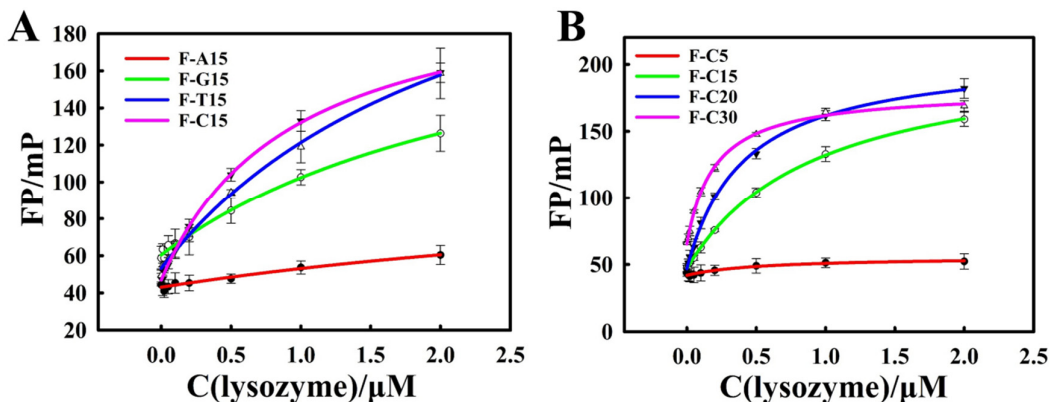


Figure 2. (A) FP of FAM-labeled 5-mer DNA sequences upon titration of lysozyme. (B) FP of FAM-labeled poly-C DNA of different lengths. The concentration of DNA was 50 nM. The error bars represented the standard deviation of three independent measurements.

Effects of pH and Salt. We further studied the effect of buffer conditions. The isoelectric point of lysozyme is 10.5-11. Thus, pH 7.5, 8.3 and 12.6 were selected to investigate the effect of pH on the interaction between DNA and lysozyme. Regardless of the DNA sequence tested, FP responded to increasing lysozyme concentration at pH values below the isoelectric point (Figure 3A and B). An increase in the attraction of DNA to lysozyme at lower pH could be attributed to the electrostatic attraction between them. When the pH is higher than the isoelectric point, lysozyme becomes negatively charged and repels the DNA.

The effects of sodium chloride (NaCl) and magnesium chloride (MgCl₂) concentrations were then investigated. For salt concentration, a good starting point is the physiological condition (e.g. 100 mM Na⁺, 2 mM Mg²⁺), and we vary our salt concentration around that. We found that the FP signal changed significantly when the concentration of Mg²⁺ was increased to 5 mM or when the Na⁺ concentration was higher than 100 mM (Figure 3C and D). The interactions between DNA and lysozyme were inhibited with increasing ionic strength, which can be attributed to charge screening by salt. At pH 8.3, similar results were obtained (Figure S2A, B), and no binding was detected at pH 12.6 (Figure S2C, D). In the presence of the DNA a1, the effect of ionic strength was similar to that of rDNA (Figure S3). The interaction between DNA and lysozyme was significantly weakened as the ionic strength increased, further confirming the critical role of electrostatic forces. In addition to charge screening, it was reported that the conformation of DNA is important for the interaction between lysozyme and nucleic acids from a co-precipitation experiment.⁶ Mg²⁺ can efficiently fold nucleic acids and increase the thermal stability of folded structures. Increased nucleic acid stability reduces DNA conformational flexibility, which could explain the decreased protein-DNA affinity with increased concentration of Mg²⁺.

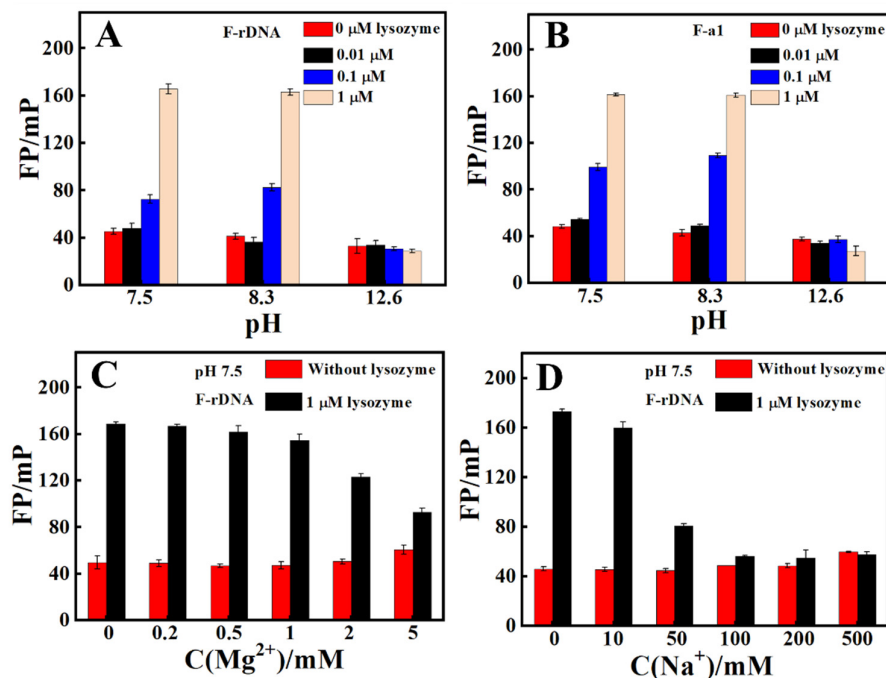


Figure 3. FP of F-rDNA (A) or F-a1 (B) at different pH (50 mM Tris-HCl, pH 7.5; 50 mM Tris-HCl, pH 8.3; 50 mM KCl-NaOH, pH 12.6) upon adding different concentrations of lysozyme. (C) FP of F-rDNA under different concentrations of Mg^{2+} . (D) FP of F-rDNA under different concentrations of Na^+ . The concentration of FAM-DNA was 50 nM. The error bars represent the standard deviation from three independent measurements.

Label-free Fluorescent Assay using Dye Staining Dyes. After understanding the electrostatic binding interactions using FP and fluorescence quenching assays, we then tested three additional commonly used aptamer sensing methods or binding assays. Nucleic-acid-binding dyes can be used as efficient indicators of aptamer-target interactions.³⁵ Different fluorescent dyes, such as SGI and thioflavin T (ThT), have been used to develop label-free biosensors and verify aptamer-target binding.³⁶⁻³⁸ It was hypothesized that the interaction between an aptamer and its target can either foster dye binding or displace the dye, leading to either an increase or decrease in fluorescence, respectively.

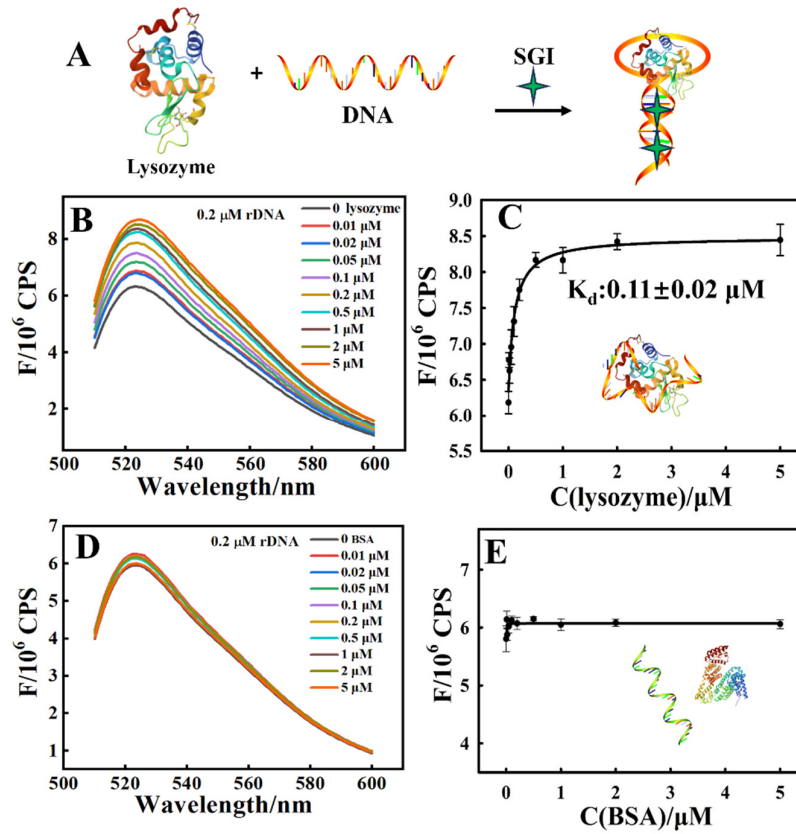


Figure 4. (A) Schematic illustration of the binding of DNA to lysozyme probed by SGI staining. Fluorescence spectra of SGI and the rDNA in the presence different concentration of (B) lysozyme or (D) BSA. Florescent intensity as a function of the concentrations of (C) lysozyme or (E) BSA. The concentration of the DNA was 200 nM and the concentration of SGI was $0.1 \times$. The error bars represented the standard deviation from three independent measurements.

In this assay, SGI was used as a signaling probe, and Figure 4A illustrates that fluorescence may increase after DNA binding to lysozyme. To confirm that the fluorescence increase was due to DNA/lysozyme binding instead of lysozyme/SIG binding, a control experiment was designed, where we titrated lysozyme into SIG and no fluorescence change was observed (Figure S4). It confirmed that the previously observed fluorescence change was due to DNA/lysozyme binding. The rDNA was used here since we aimed to examine nonspecific interactions. Figures 4B and 4C show that the fluorescence at 520 nm gradually increased as

the concentration of the lysozyme increased. According to the calculation, the K_d of the binding was $0.11 \pm 0.02 \mu\text{M}$, which is comparable to that obtained from the FP assay. This change in fluorescence can be attributed to a change in the conformation of DNA upon binding to lysozyme. BSA, which is a negatively charged protein that cannot generate electrostatic adsorption with DNA, was used as a control. Indeed, no fluorescence change was observed (Figure 4D and 4E). Since random DNA showed a selective response to lysozyme, the SGI-assisted fluorescence assay could not verify aptamer binding. At least, for such an assay, carefully designed control DNA sequences need to be tested.

Label-free Colorimetric Assays Using AuNPs. Another label-free strategy for designing aptamer-based biosensors involves citrate-capped AuNPs, taking advantage of the high extinction coefficient and distance-dependent color as well as DNA adsorption property of AuNPs.^{39, 40} A general method is to mix target molecules with an aptamer, followed by the addition of AuNPs and salt. The target is expected to bind to the aptamer and inhibit aptamer adsorption to AuNPs, leading to AuNP aggregation upon the addition of salt (Figure 5A). In the absence of the target, the aptamer is expected to adsorb onto AuNPs and increase its colloidal stability against salt. We used this method with the a1 lysozyme aptamer and the rDNA. Before adding DNA, we first tested the response of the AuNPs to lysozyme. Figure 5B shows that AuNPs aggregated when the concentration of lysozyme reached $0.02 \mu\text{M}$ even without the addition of NaCl, which can be explained by electrostatic attraction between negatively charged AuNPs and positively charged lysozyme. Interestingly, the aggregation of AuNPs decreased when the concentration of lysozyme exceeded $0.5 \mu\text{M}$, which can be explained by full capping of AuNPs by lysozyme, leading to an inversion of the surface charge of AuNPs.

We then added the a1 DNA using the method shown in Figure 5A, and a similar trend of color change still occurred, although the range of color change shifted slightly to higher lysozyme concentrations (Figure 5C). Specifically, in the absence of DNA, the AuNPs aggregated when the concentration of lysozyme reached $0.05 \mu\text{M}$. However, with DNA, the aggregation of AuNPs occurred when the concentration of lysozyme exceeded $0.1 \mu\text{M}$. In

addition, a similar trend was observed when we used the rDNA (Figure S5A and S5B). This again indicated that the color change was dominated by lysozyme interaction with AuNPs and DNA, instead of specific aptamer binding.

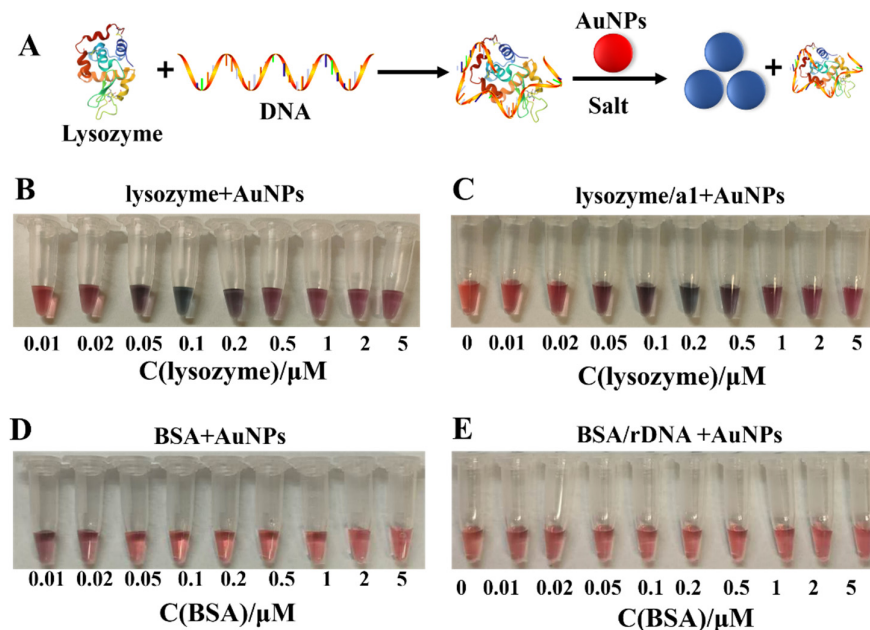


Figure 5. (A) Schematic illustration of DNA binding to lysozyme assayed using AuNPs. However, our results indicated that this method cannot be directly applied for the sensing of lysozyme due to lysozyme/AuNP interactions that was not considered in this sensing mechanism. Photographs of AuNPs in the presence of different concentration of (B) lysozyme or (D) BSA. Photographs of AuNPs after incubating different concentration of (C) lysozyme and a1; and (E) BSA and rDNA. The concentration of DNA and AuNPs were 100 nM and 5 nM, respectively.

Finally, BSA was used as a control, which showed no obvious color change regardless of the presence or absence of the control DNA (Figure 5D and E). Since BSA is negatively charged at neutral pH, the above aggregation of AuNPs upon adding lysozyme was mainly due to its positive charges. Overall, all these experiments indicated that using the label-free AuNPs is unlikely to be a reliable method for evaluating specific binding of aptamers to lysozyme, and

related sensing methods can result in false positive or false negative results, depending on the concentration of lysozyme. We previously studied many aptamers for small molecules and found that this label-free method cannot be used for their detection due to the adsorption of the target molecules to AuNPs.⁴⁰ In the current study, we extended our observation to lysozyme, a special protein.

Strand Displacement Reaction. Strand displacement reactions have been widely used in developing aptamer-based biosensors,⁴¹⁻⁴⁴ and we also tested this method for lysozyme. In this assay, a short DNA with a quencher label (Q-DNA) was hybridized to a FAM-labeled DNA aptamer, resulting in quenched fluorescence. For our purpose, we used an aptamer of chloramphenicol as a rDNA control.³⁸ We did not test any lysozyme aptamers for this reaction since there is no reliable DNA aptamer with a straightforward secondary structure available for this test. In the presence of chloramphenicol, FAM-rDNA could bind to it and release the quencher-tagged strand, leading to an increase in fluorescence (Figure 6A). Again, this aptamer is not expected to bind lysozyme and was used here to understand nonspecific interactions for this sensing method.

To achieve a low background, the ratio of F-rDNA to Q-DNA was set at 1:2. In this case, no fluorescence recovery was observed in the presence of lysozyme, and even slight fluorescence quenching occurred, which was attributed to lysozyme binding to the non-hybridized free aptamer strand (Figure 6B). For comparison, we also added chloramphenicol to this sensor, as expected, a concentration-dependent increase in fluorescence was observed (Figure 6C). The fluorescence increase from the strand displacement reaction was due to the dissociation of the two strands, and nonspecific binding by lysozyme did not promote this dissociation reaction. We reason that the strand displacement reaction might be useful to evaluate binding between lysozyme and its aptamer.

The assays tested in this work were all common sensing reactions. Other methods, such as enzyme activity assays may also be useful for evaluating the binding of specific aptamers to lysozyme.⁴⁵ Nevertheless, this work showed that one needs to be extremely careful in evaluating aptamer binding to lysozyme since DNA binds to lysozyme regardless of its

sequence and length. We recommend using mutated aptamers as the best control sequences to evaluate the binding of future aptamers. The observations made with lysozyme might also be generalizable to other polycationic targets.

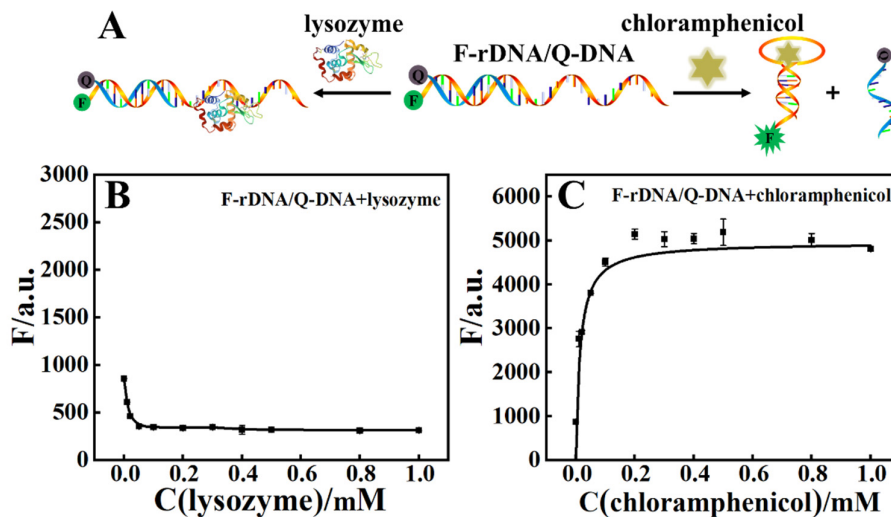


Figure 6. (A) Schematic illustration of the strand displacement assay for its specific target and nonspecific lysozyme. Fluorescent intensity of the chloramphenicol aptamer-based strand displacement sensor as a function of (B) lysozyme or (C) chloramphenicol concentration. The concentration of F-rDNA/Q-DNA was 50 nM. The error bars represented the standard deviation of three independent measurements.

Conclusions

In conclusion, the above results confirmed that lysozyme can bind to a variety of DNA sequences via electrostatic interactions. In general, the binding is stronger with longer DNA sequences, and among the different DNA sequences, poly-C DNA has the strongest binding, whereas poly-A DNA has the weakest binding. Binding is weakened by adding a high salt concentration and at high pH, especially beyond the isoelectric point of lysozyme at pH 10.5~11. These observations can be all explained by electrostatic binding. Among some common strategies for verifying aptamer binding, only the strand displacement-based assay appeared to be feasible, and this feasibility has yet to be experimentally validated by future

aptamers. This displacement strategy is less affected by the electrostatic binding between lysozyme and DNA. Other methods, such as simple FP, fluorescence quenching, DNA staining dye and AuNPs, all caused nonspecific responses, indicating that even a non-aptamer sequence produced lysozyme-concentration-dependent signal changes. This study not only provides a fundamental understanding of lysozyme binding to DNA oligonucleotides but also points out a potential method for evaluating aptamer binding and sensor design methods that can avoid false positive or false negative results. The conclusions of this study may also be applicable to other positively charged proteins.

AUTHOR INFORMATION

Corresponding Author

liujw@uwaterloo.ca

jinyan@snnu.edu.cn

Notes

The authors declare no competing financial interest.

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Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/>

Sequences of the oligonucleotides; FP changes of FAM-DNA/protein in the presence of different lengths of poly-C DNA; FP changes of F-rDNA/protein under different concentration of salt; FP changes of F-a1/protein under different concentration of salt; Fluorescence spectra

of SGI in the presence different concentrations of lysozyme; Photos of AuNPs under different conditions.

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