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Technical Note

Exonuclease I-Assisted General Strategy to Convert Aptamer-Based Electrochemical Biosensors from "Signal-Off" to "Signal-On"

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Cite This: *Anal. Chem.* 2020, 92, 6229−6234

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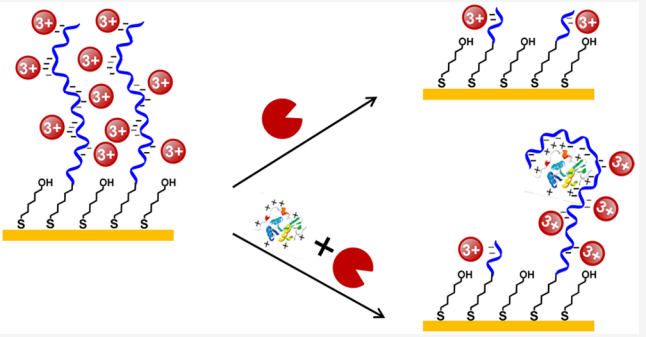
ABSTRACT: In terms of how the signal varies in response to increased concentration of an analyte, sensors can be classified as either "signal-on" or "signal-off" format. While both types hold potentials to be sensitive, selective, and reusable, in many situations "signal-on" sensors are preferred for their low background signal and better selectivity. In this study, with the detection of lysozyme using its DNA aptamer as a trial system, for the first time we demonstrated that such an aptamer-based electrochemical biosensor can be converted from intrinsically "signal-off" to "signal-on" with the aid of a DNA exonuclease. The fact that the stepwise cleavage of antilysozyme aptamer catalyzed by Exonuclease I (Exo I) is entirely inhibited upon binding lysozyme leads to the selective removal of unbound DNA probes (thiolate anti-lysozyme DNA aptamer strands immobilized on gold electrode) upon the introduction of Exo I to the sensor. With the aid of electrostatically bound redox cations ($[Ru(NH_3)_6]^{3+}$), we were able to quantitate the number of aptamer strands that are bound with lysozymes via conventional cyclic voltammetry (CV) measurements. We demonstrated that Exo I-assisted signal-on conversion protocol not only improves the sensing performance (10 times better limit of detection) but also promises a versatile strategy for DNA-based biosensor design, i.e., it can be readily adapted to other aptamer−protein binding systems (thrombin, as another example).

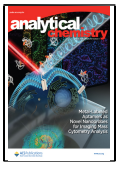


Aptamers as a major class of functional nucleic acids are conventionally selected from random-sequence libraries by systematic evolution of ligands with exponential enrichment (SELEX).[1,2] They can specifically bind to a broad range of targets, e.g., proteins, organic dyes, and drug molecules with high affinities.[3,4] Moreover, aptamers usually undergo conformational switches upon target binding, for which they have been widely adapted as the recognition elements in the design of nanoscale biosensors with various signal readout methods, such as fluorescence,[5] colorimetry,[6] chemiluminescence,[7] surface plasmon resonance (SPR),[8] and electrochemistry.[9,10]

Among above successful designs, aptamer-based electrochemical biosensors (A-EB) have attracted great interests because of their simplicity, rapid response, high sensitivity, low cost, and easy operation.[11,12] Upon binding with targets, the conformational change of immobilized aptamers results in varied distance between the covalently labeled redox species and the electrode surfaces, which strongly influences the obtained redox signals. As a result, an A-EB can have either positive (signal-on) or negative (signal-off) responses depending on the specific design and conformational switching mode.[13−17] Generally, signal-on sensors are more desirable because of their high sensitivity,[18] unlimited signal gain,[13,14] less false positives,[15] and low background signal.[19] Since most A-EB "architectures" rely on global scale conformational changes of aptamers or strand-displacement mechanisms,[15−17] the design of signal-on sensors is not always feasible or not easy to achieve.

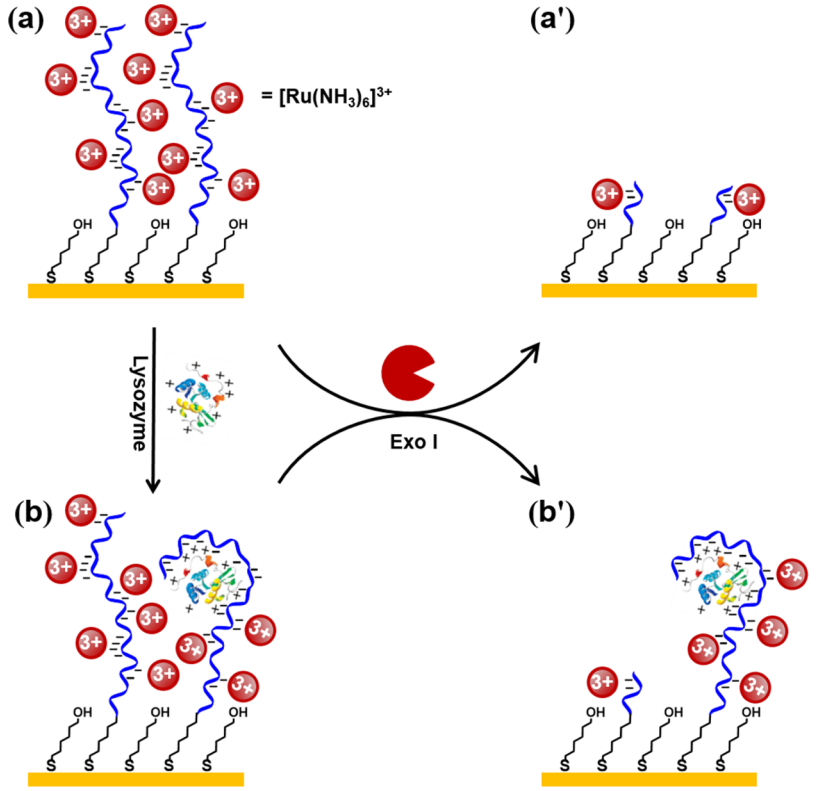
We have previously developed a label-free A-EB for lysozyme,[20] an early marker of several diseases such as AIDS, sarcoidosis, and rheumatoid arthritis.[21] In this A-EB, the interactions between positively charged lysozymes and surface-tethered aptamers lead to the partial neutralization of the negative charges on DNA phosphate backbones,[20] which inhibits the subsequent binding of multiple-charged redox cations (i.e., $[Ru(NH_3)_6]^{3+}$). As shown in Scheme 1a,b, the amount of surface-bound redox cations after lysozyme incubation decreases, which can be quantitated via conventional cyclic voltammetric (CV) measurements.[22] For extending the potential application of this A-EB, it would be more desirable if it can be "converted" from signal-off to signal-on, in order to improve its sensing performance.

Received: January 1, 2020
Accepted: April 2, 2020
Published: April 2, 2020



https://dx.doi.org/10.1021/acs.analchem.0c00005
Anal. Chem. 2020, 92, 6229−6234

Scheme 1. Schematic Illustration of Converting the A-EB for Lysozyme from "Signal-Off" (a and b) to "Signal-On" (a′ and b′) by Introducing Exo I-Hydrolysis to the Detection Procedure[a]



[a]The blue ribbons represent anti-lysozyme aptamers (HS-$(CH_2)_6$-O-5′-ATC TAC GAA TTC ATC AGG GCT AAA GAG TGC AGA GTT ACT TAG-3′ that were immobilized on gold via strong Au−S interactions. The signal readout is based on the CV measurements of electrostatically bound redox cations ($[Ru(NH)_6]^{3+}$).

It has been confirmed that Exonuclease I (Exo I, extracted from *E. coli*) is capable of catalyzing the hydrolysis of single-strand DNA (ssDNA) from its 3′-terminus but not double-stranded DNA (dsDNA).[23−27] If the hydrolysis of single-stranded aptamers by Exo I are inhibited upon forming complex with protein targets, it would be possible to convert the above A-EB from signal-off to signal-on by using Exo I to remove unbound aptamers after lysozyme incubation. As a result, only lysozyme-bound aptamers are left on the electrode to "capture" redox cations (Scheme 1a′ and b′), for which a positive sensing response can be obtained.

In this brief note, we describe our Exo I-assisted general strategy to convert aptamer-based electrochemical biosensors from "signal-off" to "signal-on". As verified by the gel electrophoresis assay, the lysozyme binding in fact protects the antilysozyme aptamer from the catalytic hydrolysis by Exo I. Upon the introduction of Exo I to the previously developed lysozyme A-EB, the sensing response was successfully converted from signal-off to signal-on. The new design shows significantly improved detection limit and lower background signal. Such an Exo I-assisted response conversion strategy was also examined with thrombin A-EB for validating its versatility.

EXPERIMENTAL SECTION

Materials and Reagents. The disulfide-modified anti-lysozyme aptamer and unmodified oligonucleotides were ordered from the Integrated DNA Technologies, Inc. (Calgary, AB). Exonuclease I (Exo I) was obtained from the Applied Biological Materials Inc. (Richmond, BC). Gold substrates (regular glass slides first coated with 5 nm Cr then 100 nm Au) were purchased from Evaporated Metal Films (EMF) Inc. (Ithaca, NY). All solutions were prepared with deionized water (≥18.2 MΩ/cm) produced from a Barnstead EASYpure UV/UF compact water system (Dubuque, IA).

DNA Purification and Immobilization. The disulfide-modified anti-lysozyme aptamer (originally selected by Kirby et al.[28]) was treated with 10 mM TCEP (reducing reagent) in 100 mM Tris buffer (pH = 7.4) for overnight to produce thiol-terminated DNA aptamer and then purified using a MicroSpin G-50 column. The gold slides were cleaned by immersion in "piranha" solution (3:1 mixture of concentrated H_2SO_4 and 30% H_2O_2) for 5 min at 90 °C, followed by rinsing thoroughly with deionized water (CAUTION: Piranha reacts violently with organic solvents, which should be handled with extreme caution). The thiolate ssDNA (freshly prepared) was immobilized on the cleaned gold substrate by spreading a 30-μL droplet of DNA solution and incubating in a humidity box for overnight. Afterward, the gold slides were rinsed with Tris buffer, then incubated in 1.0 mM MCH ($HS(CH_2)_6OH$) solution to passivate the surface. For the detection of lysozyme, a 20-μL aliquot of lysozyme solution of various concentrations was dropped on the DNA-modified gold electrode and incubated for 1 h in the humidity box.

Exo I-Catalyzed DNA Cleavage and Electrochemical Measurements. The Exo I solution of 1.25 U/μL (the minimum concentration with optimal hydrolysis efficiency toward surface immobilized ssDNA[25]) was prepared in a buffer

https://dx.doi.org/10.1021/acs.analchem.0c00005
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containing 67 mM glycine−KOH (pH 9.5), 67 mM $MgCl_2$, and 10 mM DTT to hydrolyze the surface-tethered lysozyme aptamer. For DNA hydrolysis in solution, 2.0 μL of 50 μM aptamer in the presence of different amount of lysozyme were added into 18 μL of the Exo I solution and incubated at 37 °C for 30 min. For the surface experiments, 40 μL of the Exo I solution was dropped on the DNA-modified gold electrode after lysozyme incubation and kept in the humidity box at 37 °C for 3 h (unless otherwise stated). CV measurements were performed with a CHI 600D electrochemical analyzer (Austin, TX) using a customized single-chamber, three-electrode cell. The cell has an opening at one side, where the working electrode (DNA-modified gold slide) was attached. An Ag/AgCl/3M NaCl electrode was used as the reference and a Pt wire was used as the counter electrode, respectively. All CV measurements were performed in an aqueous electrolyte containing 10 mM Tris and 5.0 μM $[Ru(NH_3)_6]Cl_3$ (pH 7.4) at room temperature.

RESULTS AND DISCUSSION

Catalytic Activity of Exo I toward the Hydrolysis of Antilysozyme DNA Aptamer upon Analyte Binding. The essential requirement for above proposed conversion of the A-EB from signal-off to signal-on (Scheme 1) is that Exo I cannot cleave the DNA aptamer upon binding lysozyme; we first performed a denaturing polyacrylamide gel electrophoresis (PAGE) assay to investigate the catalytic activity of Exo I toward anti-lysozyme DNA aptamer in the presence of different concentrations of lysozyme.

As shown in Figure 1, the clear bands observed in lane 1 and lane 2 correspond to the DNA aptamer (5.0 μM), and the

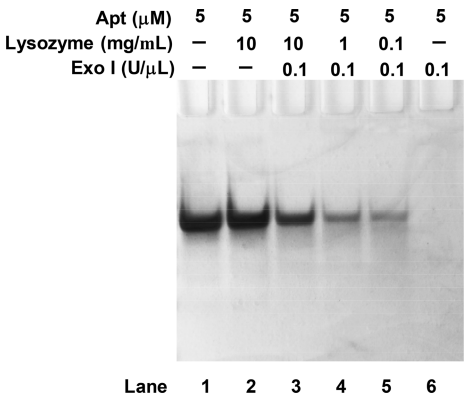

Figure 1. 10% denaturing polyacrylamide gel electrophoresis (PAGE) image of the antilysozyme aptamer (5′-ATC TAC GAA TTC ATC AGG GCT AAA GAG TGC AGA GTT ACT TAG-3′) in the presence of different concentrations of lysozyme upon incubation with Exo I (30 min at 37 °C). Listed as top insets are the concentrations of the DNA aptamer, lysozyme, and Exo I for each lane, respectively.

mixture of aptamer (5.0 μM) and lysozyme protein (10 mg/mL), respectively. The lanes 3−6 are the results of Exo I-catalyzed hydrolysis (0.1 U/μL, 30 min, 37 °C) of the mixtures containing 5.0 μM aptamer and different concentrations of lysozyme. The DNA aptamer band is still clear in lane 3, where the lysozyme concentration is high (10 mg/mL); in contrast, with decreased concentrations of lysozyme (lanes 4−6, 1.0, 0.1, and 0 mg/mL), the bands sequentially diminish, indicative of the catalytic cleavage of more and more DNA aptamer strands by Exo I. This result confirms, for the first time, that the hydrolysis of the aptamer by Exo I can be effectively inhibited by lysozyme binding, i.e., Exo I cannot cleave the aptamer−lysozyme complex. The inactivity of Exo I toward the aptamer−ligand complex may be ascribed to the steric hindrance of binding the 3′-terminus of the DNA strand by the active domain of Exo I, which has been reported previously for double-stranded DNA and other functional DNA motifs (e.g., G-quadruplex).[23,24,29]

Exo I-Assisted Conversion of Lysozyme A-EB from Signal-Off to Signal-On. Following the biochemical characterization, we introduced Exo I to the previously developed A-EB for lysozyme to explore the feasibility of converting the sensing response from "signal-off" to "signal-on". As illustrated in Scheme 1, instead of directly testing the aptamer modified electrodes (before and after lysozyme incubation) in the presence of multiple-charged redox cations ($[Ru(NH_3)_6]^{3+}$), for which a decrease in their redox response should be observed upon increasing the amount of lysozyme (positively charged) (Scheme 1a,b), the sensing interfaces (with or without the lysozyme incubation) are first treated with Exo I. According to the above gel results, in the absence of lysozyme Exo I should cleave the 42-mer aptamer strands from the 3′-end (leave only small, 11−13-mer fragments on the surface[25]), which in turn results in much less surface-bound redox cations (Scheme 1a′); more importantly, a positive sensing signal is expected as the lysozyme-bound aptamers are protected from Exo I hydrolysis and thus can capture more redox cations on surface (Scheme 1b′). The incomplete cleavage of surface tethered DNA strands has been confirmed with both electrochemical and mass spectrometry (MS) measurements previously, which was attributed to the steric hindrance of "underneath" the electrode toward Exo I/DNA interacting.[25]

Besides the semiquantitative analysis of the gel electrophoresis image shown in Figure 1 (Figure S1 in the Supporting Information), the successful conversion of the aptamer-based lysozyme sensor from "signal-off" to "signal-on" upon the introduction of Exo I hydrolysis was confirmed by cyclic voltammetric (CV) measurements in the presence of 5.0 μM $[Ru(NH_3)_6]^{3+}$.[22] This method has been confirmed to be reliable, accurate, and convenient for quantifying the amount of DNA strands (or DNA nucleotides) on the electrode surface.[22,30−33] The clear-cut single pair of CV redox peaks at −25 mV (vs Ag/AgCl) (Figure 2) represents the ideally behaved redox process of electrostatically bound $[Ru(NH_3)_6]^{3+}$. The electrostatic interaction between this multiple-charged redox cation and the negatively charged DNA backbone is strong ($K_f = (1.5 \pm 0.5) \times 10^6\ M^{-1}$),[22] for which the solution concentration to reach the saturated surface signal is low (5.0 μM) and has no interference to the observed CV peaks.[22,30] For the original A-EB, the lysozyme incubation results in a decreased CV redox peak of $[Ru(NH_3)_6]^{3+}$ (the red curve in Figure 2a), indicating less DNA strands available to bind with redox cations on the surface. By introducing Exo I, the CV peak of the blank (no lysozyme present) became much smaller (the black curve in Figure 2b) in comparison with that of the original A-EB (black curve in Figure 2a). In contrast, a pair of more pronounced CV peaks was observed after 10 μg/mL lysozyme incubation (red curve in Figure 2b). As the CV redox peak area directly reflects the amount of surface-bound redox cations, these results can be viewed as unequivocal

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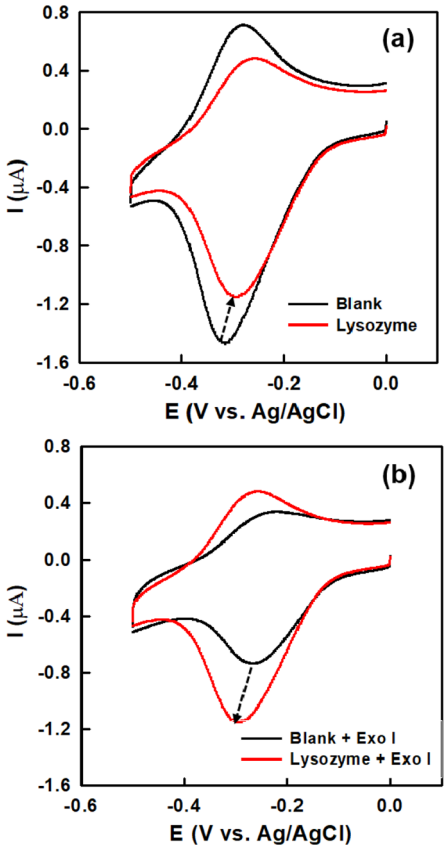

Figure 2. Representative CV responses of (a) original signal-off A-EB and (b) Exo I-assisted signal-on A-EB for lysozyme detection. The electrolyte solution was 10 mM Tris buffer containing 5.0 μM $[Ru(NH_3)_6]Cl_3$ (pH 7.4); the CV scan rate was 500 mV/s.

evidence to validate our proposed strategy to convert the E-AB from signal-off to signal-on.

Performance Comparison of the Signal-On and Signal-Off Sensors for Lysozyme Quantitation. For quantitatively evaluating the sensing performance, the total faradaic charge (Q) was obtained by integrating the CV reduction peak, which, according to Faraday's law, directly represent the amount of surface-bound $[Ru(NH_3)_6]^{3+}$. For all experiments described below, the surface density of immobilized DNA aptamer strands were kept at optimal (Γ = 1.0−3.0 × 10^{12} molecules/cm^2 as determined previously[20]) in balancing the absolute redox signal and interfacial analyte−aptamer binding efficiency.[20] We first compared the background signals (Q_0) of the original and Exo I-assisted A-EBs, which were averaged from 30 independently prepared aptamer-modified electrodes. Figure 3 displays the distribution of their background signals, which shows significant improvements upon the response conversion from signal-off to signal-on. Particularly for the original A-EB (right), its background signal has an average value of 918 nC and a standard deviation of 155 nC; in contrast, the Exo I-assisted A-EB has more than 5 times reduced background signal (158 nC) and its standard deviation is as small as 27 nC.

Based on the integrated faradic charge of the CV reduction peak (shown representatively in Figure 2), in Figure 4 we illustrate quantitatively how these two A-EBs (with and without Exo I treatment) respond to different concentrations of lysozyme. For the original A-EB, the integrated charge decreases monotonically with increased concentration of

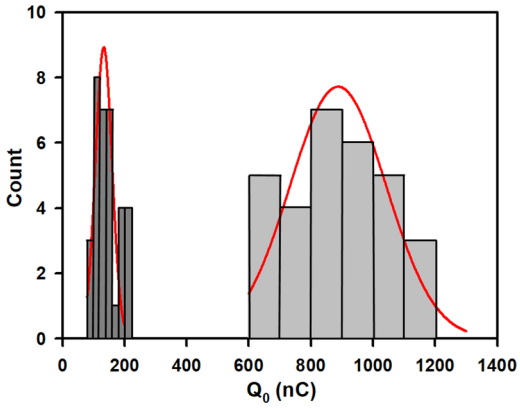

Figure 3. Distribution of the integrated charges of the blank derived from 30 individual A-EBs with original signal-off (right) and Exo I-assisted signal-on responses (left), respectively.

lysozyme (namely, signal-off, Figure 4a); while for the Exo I assisted A-EB (Figure 4a′), the sensing response becomes stronger when the concentration of analyte increases (a rising trend, i.e., signal-on). In both cases, the sensing response becomes saturated upon incubation with high concentrations of lysozyme (>50 μg/mL).

Figure 4b,b′ shows the linear responses of the signal-off A-EB and Exo I-assisted signal-on A-EB at low concentration ranges; both showed excellent linearity as indicated by the R^2 values. Based on the "$3\sigma/b$" rule (σ is the standard deviation of the intercept of the linear regression line, and b is the slope), their limits of detection (LODs) for lysozyme were determined to be 144 ± 8 ng/mL (10 ± 0.6 nM) and 10 ± 0.4 ng/mL (0.70 ± 0.02 nM), respectively. The more than 10-times improved LOD of Exo I-assisted signal-on A-EB should be mainly attributed to its significantly reduced background signal in comparison with the intrinsic signal-off sensor. More importantly, this LOD is much lower than the physiological concentration of lysozyme (0.5−50 μg/mL);[21,34] compared with the previously reported sensors for the quantitation of lysozyme,[9,12,35−38] the Exo I-assisted A-EB of lysozyme has either lower or comparable LOD (Table S1). It should be noted that the role of Exo I in improving the LOD upon converting an A-EB from signal-off to signal-on (i.e., differentiating the analyte-bound and free DNA aptamers on surface to reduce the background signal) is different from previous studies, where the exonucleases have been adapted for cyclically amplifying the sensing signal.[36,39,40]

The selectivity of our Exo I-assisted signal-on A-EB was then tested with 10 μg/mL lysozyme and 1.0 mg/mL of three other interfering proteins, i.e., cytochrome c (Cyto c), bovine serum albumin (BSA), and thrombin (Thr). As shown in Figure S6 (Supporting Information), only lysozyme yields a significant increase in the sensing response; three other interfering proteins with 100-fold higher concentration have no discernible influences in the sensing response (i.e., indistinguishable from the blank). To further evaluate the application potential of the Exo I-assisted signal-on A-EB for lysozyme detection, we have tested it in human serum using spiked samples. Briefly, 100-fold-diluted healthy human serum samples were examined by Exo I-assisted signal-on A-EB before and after adding different concentrations of lysozyme. With their sensing responses all falling into the linear range shown in Figure 4b′, we have determined the rates of recovery of the spiked lysozyme samples in serum, which showed

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Anal. Chem. 2020, 92, 6229−6234

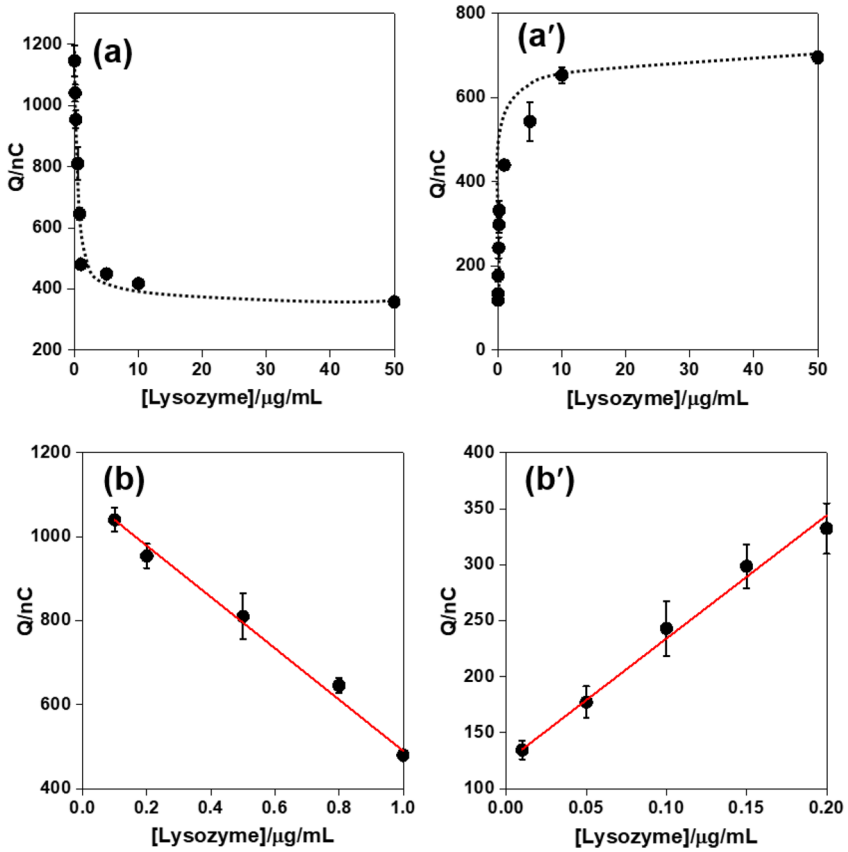

Figure 4. Sensing responses of (a) original signal-off A-EB and (a′) Exo I-assisted signal-on A-EB as a function of the lysozyme concentration; their linearized responses at low concentration ranges are shown in parts b and b′, respectively. The black dotted lines in (a) and (a′) are to guide the eyes only; the red solid lines in parts b and b′ are the best linear fits ($y = -610.9x + 1101$, $y = 1100x + 124.1$) to the experimental data (solid circles in black) with R^2 values of 0.988 and 0.993, respectively. The error bars were obtained from at least three independently prepared electrodes.

satisfactory results (between 94.4 and 108.2%, with a RSD < 6%; Table S2). The excellent selectivity against other more abundant serum proteins and near-unity recovery rates of the spiked samples augment the capability of the newly developed Exo I-assisted signal-on A-EB for quantitative analysis of real samples.

Versatility Evaluation of the Exo I-Assisted Signal-on A-EB. To confirm the versatility of such an Exo I-assisted response conversion strategy, we have explored the same testing procedure of A-EB for thrombin detection. The original A-EB for thrombin adopts an antithrombin DNA aptamer that folds to a G-quadruplex upon analyte binding.[41] Initially, we have confirmed the hydrolysis of aptamer by Exo I can be effectively inhibited by thrombin binding (Figure S7 in the Supporting Information). The corresponding Exo I assisted A-EB shows a linear response to thrombin at the nanomolar range (Figure S8); the obtained LOD (2.0 ± 0.1 nM) was improved from the more elegant design by Xiao et al. via target-induced strand displacement and methylene blue (MB) tagging (3 nM).[16] The Exo I-assisted A-EB for thrombin is also based on conventional CV measurements using electrostatically bound redox cations, for which no covalent labeling of the DNA aptamer with redox moieties is necessary.[20,22]

As demonstrated above, the strategy to build Exo I-assisted signal-on A-EB with excellent sensing performance can be extended to many other systems, thus validating its practical application potentials with other electrochemical measurements as well (Figure S9 and Figure S10). Besides protein targets, this method should be able to accommodate a wide range of nucleic acid detection since the 3′-terminus of DNA in a hybridized complex is also protected from Exo I hydrolysis.[23] Moreover, besides the sensors based on electrostatic redox labeling, the gel results show in Figure 1 (lanes 3–6) indicate that this Exo I assisted signal-on A-EB could be easily adapted for other nonelectrochemical techniques that are able to quantitatively characterize DNA on surface (e.g., fluorescence techniques based on noncovalent intercalating dyes) which, however, would be much more difficult for the original signal-off A-EB where the total amount of DNA aptamer remains unchanged before and after lysozyme binding.

CONCLUSION

In summary, we demonstrated in this work that the hydrolysis of anti-lysozyme aptamer by Exo I is inhibited upon lysozyme binding. With the aid of such a protein-binding based protection effect, we introduced the step of Exo I hydrolysis to our previously developed A-EB for lysozyme and successfully converted its sensing response from "signal-off" to "signal-on", by which both the selectivity and sensitivity of the E-AB have been significantly improved. The versatility of the Exo I-assisted response conversion strategy was also confirmed with another popularly studied A-EB for the quantitation of thrombin. We envision that the Exo I-assisted signal-on A-EBs are potentially applicable with broad analytical techniques and to many different fields, from on-site environmental monitoring to point-of-care diagnosis.

https://dx.doi.org/10.1021/acs.analchem.0c00005
Anal. Chem. 2020, 92, 6229–6234

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.0c00005.

Detailed experimental procedures and additional experimental data including the gel electrophoresis characterization and CV confirmation of the conversion of A-EBs for lysozyme and thrombin from signal-off to signal-on, LOD comparison with those reported in the literature, selectivity, and recovery test (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was jointly supported by the National Natural Science Foundation of China (NSFC, Grant 21573024), Beijing Natural Science Foundation (Grant 2182026), and Natural Sciences and Engineering Research Council (NSERC) of Canada. X.G. thanks the China Scholarship Council to support her visit in Canada.

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