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A novel exonuclease III-aided amplification assay for lysozyme based on graphene oxide platform

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ABSTRACT

Based on exonuclease III (Exo III) aided amplification and graphene oxide (GO) platform for fluorescence quenching, a novel, turn-on fluorescent aptasensor for lysozyme (Lys) protein was constructed. The system contains a hairpin probe (HP) and a signal probe (SP) labeled with carboxyfluorescein (FAM) at its 5′ end. HP, which consists of the aptamer sequence of Lys, is partially complementary to SP. Lys could bind with the aptamer region of the HP and facilitate the opening of the hairpin structure of HP, exposing a single-stranded sequence to hybridize with SP. This triggered the Exo III aided amplification and caused the degradation of SP, which liberated the free fluorophore labels. Upon the addition of GO, the released fluorophore could not be adsorbed and no fluorescence quenching occured, while the intact SPs could be adsorbed on GO surface with the fluorescence substantially quenched. The results revealed that the proposed method displayed fluorescence responses in a linear correlation to the concentrations of Lys within the range from 0.125 μg/ml to 1 μg/ml and the detection limit is 0.08 μg/ml. Besides such sensitivity, the proposed strategy is also low-cost and simple due to its homogeneous and fluorescence-based detection format.

1. Introduction

Nowadays, the analysis of disease-marker proteins, especially those associated with cancer diseases has been the subject of molecular biology reseach. As body's own antibiotic, lysozyme (Lys) is distributed in body tissues and secretions [1]. Lys is a single chain polypeptide that contains 129 amino acids. In vivo, it can hydrolyze linkages between N-acetylglucosamine and N-acetylmuramic acid leading to the degradation of peptidylglycan in the cell wall of Gram-positive bacteria, thus serving as a natural "drug" [2]. Many diseases such as renal diseases, monocytic leukemia and meningitis are relevant to the abnormal concentration of Lys [3–5]. Hence, the detection and quantification of Lys are of great importance. The conventional assay utilized different approaches, such as, the lytic activity determination of Lys [6], the comparison of light transmissions of crystalline Lys dilutions [7], and the immunoassay using anti-Lys polyclonal antibodies [8]. However, the applications of these methods are limited by the insufficent accuracy or the use of sophisticated instrumentation.

Aptamers are DNA or RNA oligonucleotides selected by systematic evolution of ligands by exponential enrichment in vitro, which can bind to target specifically [9,10]. Compared with antibodies, aptamers can be inexpensive, easily synthesized by chemical method. Taking use of these advantages, electrochemical, fluometric, and colorimetry aptameric sensors have been developed for Lys detection [11–14]. Although these methods can be used to determinate Lys effectively, their practical applications are limited by complicated electrode modification, relatively narrow determination range or difficulty in material synthesis. For these reasons, it is necessary to develop a sensitive, specific, cost-efficient and easily automated method for the detection of Lys.

As a DNA-modifying enzyme, exonuclease III (Exo III) possesses a double-stand specific and nonprocessive 3′–5′ exo-deoxyribonuclease activity. However, it loses activity on single-stranded DNA and 3′-protruding termini of double-stranded DNA [15–17]. Further more, Exo III digestion does not require a specific sequence for target DNA, while cyclic enzymatic digestion of DNA can be achieved. In view of these advantages, Exo III-aided amplification has been employed in various sensors [18–20].

In this work, we construct a novel fluorescent aptasensor for Lys detection by means of Exo III-aided amplification and graphene oxide (GO) based fluorescence quenching. GO is a kind of graphene derivatives; one of its important properties is that single-stranded DNA could be adsorbed on the surface of GO due to the hydrophobic and π-stacking interactions between the nucleobases and GO. As the nanoquencher, GO could quench the emission of fluorophore efficiently. In this strategy, the hairpin probe (HP) containing the Lys aptamer sequence exhibits

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a conformational change when binding to the target protein. The conformational change exposes a single-stranded tail sequence, facilitating the Exo III aided amplification and the cycling of degradation of multiple signal probes (SP), liberating the fluorophores. Without the target, no single-stranded tail sequence is exposed in the HP, and the SP cannot hybridize with the HP, avoiding the digestion of the SP by Exo III. Upon addition of GO, the fluorescence of fluorophores conjugated to single-stranded SP are greatly quenched. Whereas, fluorescence quenching does not occur to liberated fluorophores, which attributes to the weak affinity between the digested short DNA fragments and GO. To the best of our knowledge, our strategy for the first time combines the use of GO and Exo III for the construction of protein aptasensors. Further more, the strategy offers the merits of being simple, sensitive and low cost.

2. Experimental

2.1. Materials and chemicals

Human serum albumin (HSA), human immunoglobulin G (human IgG), bovine serum albumin (BSA) were obtained from Dingguo Biochemical Reagents Company (China). Recombinant human IL-6 was supplied by Pepro Tech, INC. (USA). Exonuclease III was purchased from Promega Biotech Co., Ltd. (USA). Lysozyme was obtained from Sigma-Aldrich (USA). Graphene oxide was synthesized by a modified Hummers' method [21,22]. All other chemicals were of analytical grade. Deionized and sterilized water (resistance $> 18\ M\Omega\ cm$) was used throughout.

All DNA oligonucleotides used in this work were synthesized by Sangon Biotechnology Co. Ltd (Shanghai, China). Sequences of the oligonucleotides are listed in Table 1 and the abbreviations for the work are listed in Table 2. Fluorescence measurements were carried out on a Hitachi F-7000 fluorescence spectrometer (Hitachi Ltd., Japan) at room temperature. The emission spectra were recorded from 504 to 600 nm upon excitation at 494 nm. The fluorescence intensity at 516 nm was used to evaluate the performances of the proposed assay. Both of excitation and emission slits were set as 5 nm.

2.2. Fluorescence detection of lysozyme

The reaction was performed at 37 °C for 1 h in 20 μL reaction buffer (66 mM Tris, 0.66 mM $MgCl_2$, pH 8.0) containing 30 nM HP, 100 nM SP, 40 U/mL Exo III, and the Lys of a given concentration.

And then, 8 μL 100 μg/mL GO, 72 μL deionized and sterilized water were added to obtain final volume of 100 μL. After 10 min, the fluorescence was measured and recorded at room temperture.

2.3. Gel electrophoresis analysis

The electrophoresis experiment was performed to verify the feasibility of this strategy. The samples with Exo III in the absence and presence of Lys were prepared according to the fluorescence detection procedures. The HP, the mixture of HP and SP without enzymatic digestion were also prepared. Analysis by electrophoresis was carried out on 5% agarose gel dissolved in $0.5\times$ TBE buffer (45 mM Tris, 45 mM boric acid, 1.25 mM EDTA, pH 7.9) at room temperature. The gel was stained by Gold View and run at a constant potential of 110 V for 90 min with loading of 10 μL of each sample into the lanes. The resulting gel was excited using a WD-9403 F UV device and imaged with a Canon digital camera.

3. Results and discussions

3.1. Experiment principle

As illustrated in scheme 1, a homogeneous assay format, Exo III aided amplification, aptameric target recognition, GO-based fluorescence quenching, and hairpin DNA probe were intergrated in the design of our aptasensor. The designed HP is composed of two regions, a long region that consists of the Lys aptamer sequence, whereas the other region is partially complementary to SP (cSP) [23]. Furthermore, the cSP was designed partially complementary to Lys aptamer sequence, so the HP could self-hybridize into a stable stem-loop structure, blocking the hybridization between cSP and SP. It was reported that 3′ overhang of four bases or more could be protected from digestion by Exo III [15,24]. For this reason, 10 nucleotides 3′ overhang of the HP was specially designed to avoid the digestion by Exo III in its free state. The aptasensor exhibited a signal-on architecture in response to the target. Upon the addition of target protein, the aptamer interacted with protein to form a complex of HP and target protein with the hairpin structure opened. In this case, the HP exposed a single-stranded cSP region that hybridized with SP to form a DNA duplex, which showed a blunt 3′-terminus of SP. Therefore, the Exo III could digest the SP, liberating the fluorophore and releasing the single-stranded cSP region in the complex of HP and target protein. This facilitated the Exo III aided signal amplification via cycling of the hybridization of SP with HP and the degradation of SP. After such an amplification process, multiple SPs were degraded. On the other hand, in the absence of Lys, the hybridization of SP to cSP was blocked, and the enzymatic degradation of the SPs could not take place. When GO was finally added, the intact SPs could be absorbed by GO and the fluorescence would be quenched due to the hydrophobic and π-stacking interactions between the nucleobases and GO [19,25].

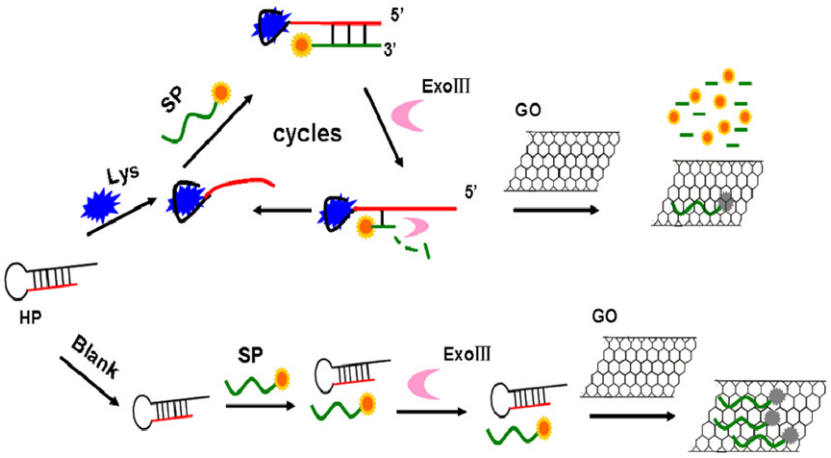
Table 2
A list of abbreviations for the work.

original words	Abbreviations
Exonuclase III	Exo III
Grapheme oxide	GO
Hairpin probe	HP
Signal probe	SP
The region of HP which is complementary to SP	cSP
Carboxylfluorescein	FAM
lysozyme	Lys
Human serum albumin	HSA
Human immunoglobulin G	IgG
Bovine serum albumin	BSA
Recombinant human IL-6	IL-6

Table 1
Synthesized oligonucleotides (5′→3′) used in the experiment.

HP:	5′-TGCACTCTTTAGTTTTT***ATCTACGAATTCAGGGCTAAAGAGTGCAGAGTTACTAG***-3′
SP:	5′- FAM- CTAAAGAGTGCA-3′

Italic bold letters of HP represent the aptamer sequence of Lys [23].



Scheme 1. Schematic representation of Exo III aided amplification assay for Lys detection.

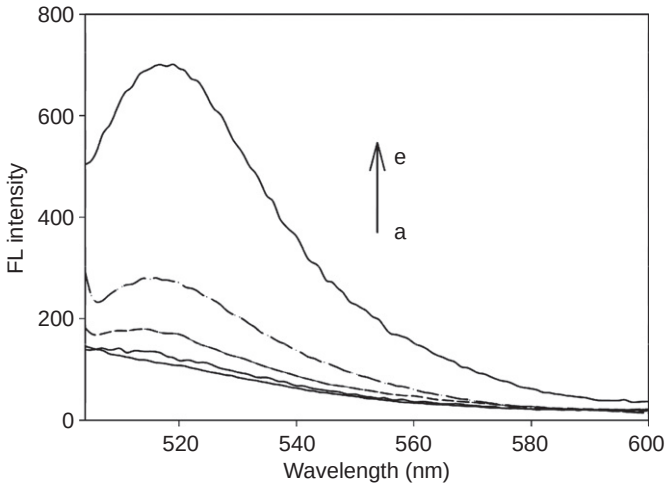


Fig. 1. The feasibility of Exo III-aided amplification assay (a) SP+GO; (b) HP+SP+GO; (c) HP+SP+Exo III+GO; (d) HP+SP+Lys+GO and (e) HP+SP+Lys+Exo III+GO. The concentration of Lys is 5 μg/mL.

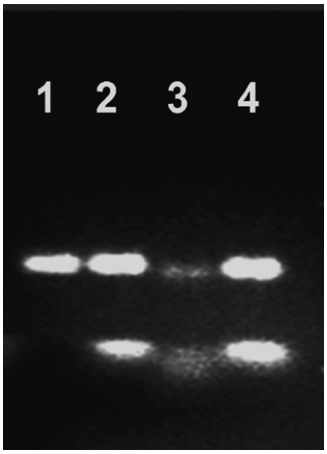


Fig. 2. Gel electrophoresis image for Exo III-aided signal amplification strategy. Lane 1, HP; lane 2, HP/SP; lane 3, Lys/HP/SP digested by Exo III; lane 4, HP/SP digested by Exo III.

By contrast, the liberated fluorophores were not absorbed by GO because of their weak interaction with GO. Hence, the fluorescent signal generated gave a measure for the amount of Lys.

Fig. 1 clearly reveals that the Exo III-aided signal amplification was achieved in this work. Even the Exo III also digested some SPs in the absence of target, which caused the small increase of background fluorescence compared with that without the Exo III, Fortunately, in the presence of target, one observed a 300% signal increase as Exo III catalyzed the exposed cSP recycling and signal amplification. In contrast, only 101% signal increase was observed without the Exo III. So the relative fluorescence with the amplification of Exo III increased almost twice as compared to that without enzyme (the relative fluorescence is defined as $(F_t - F_0)/F_0 \times 100$. F_t and F_0 are the fluorescence intensities in the presence and absence of Lys under identical conditions, respectively).

3.2. Electrophoresis characterization

The feasibility of Exo III-aided amplification strategy can be verified using agarose gel electrophoresis. As shown in Fig. 2, two bright bands were observed on the lane 4, where the target was not added into the mixture. Upon the addition of Lys, HP interacted with Lys, and the SPs which could hybridize with HP were cleaved by Exo III. The SPs were degraded by Exo III into single-stranded DNA residuals and nucleotides, and would migrate out of the gel [26]. Hence, two less bright bands were observed in lane 3. This disclosed that the addition of target can trigger the enzymatic amplification.

3.3. Optimization of assay conditions

Fluorescence responses of the Lys assay were greatly dependent on assay conditions such as the HP concentration. Fig. 3 shows that the relative fluorescence changes after incubation with a varying amount of HP. The influence of the concentration of HP was investigated from 10 nM to 60 nM. The optimum concentration of HP was observed to be 30 nM. Before 30 nM, the relative fluorescence increased with increasing concentration of HP. Nevertheless, when the concentration of HP was higher than 30 nM, the signal to background ratio gradually decreased. The relative fluorescence decreased due to the elevation of fluorescence intensity of blank. We speculated that the increase of fluorescence background was due to the fact that HPs also could be adsorbed by the GO, competing with SPs and affecting the complete quenching of fluorescence background. The effect of Exo III concentration was also investigated in the work. The relative fluorescence increased with increasing Exo III concentration until the concentration reached 40 U/mL. However,

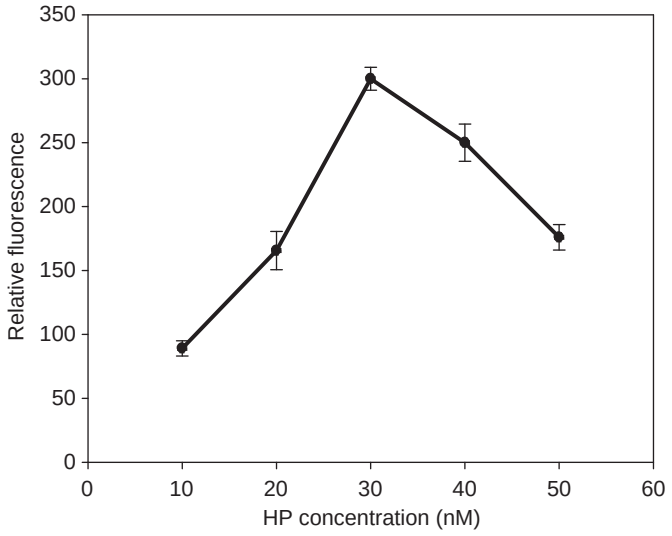


Fig. 3. Optimization of the HP concentration. The concentrations of SP, Lys, Exo III and GO were 100 nM, 5 μg/mL, 40 unit/mL, 8 μg/mL, respectively.

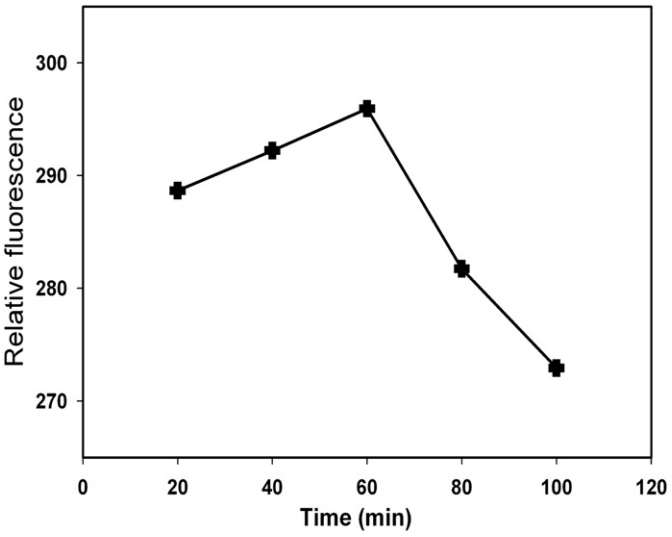


Fig. 4. Optimization of the incubiation time. The concentrations of SP, HP, Lys, Exo III and GO were 100 nM, 30 nM, 5 μg/mL, 40 unit/mL and 8 μg/mL, respectively.

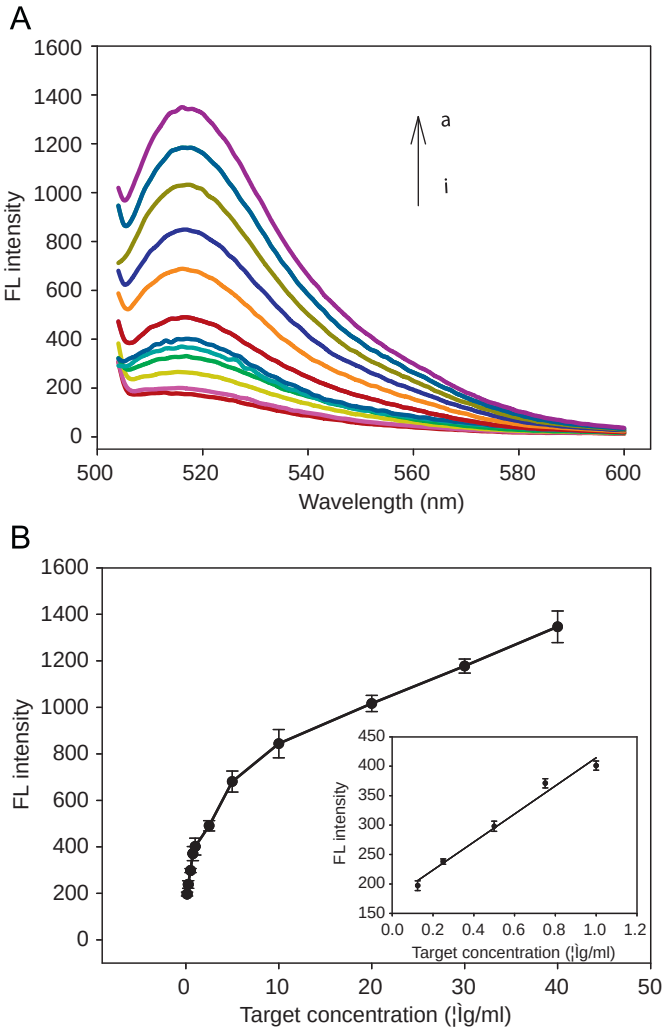


Fig. 5. (A) fluorescence spectral response of Exo III-aided amplification assay for Lys of varying concentrations. (i to a: 0.125, 0.25, 0.5, 0.75, 1, 2.5, 5, 10, 20, 30, 40 μg/mL) (B) fluorescence spectral response at 516 nm of Exo III-aided amplification assay versus Lys concentration. Inset: dependence of fluorescence intensity on the Lys concentration from 0.125 μg/mL to 1 μg/mL. The error bars represented the standard deviation of repeated measurements.

it decreased after 40 U/mL. For this reason, 40 U/mL Exo III was used in the final solution.

The influence of the incubation time was further investigated in Fig. 4. The intensity of relative fluorescence increased along with increase in the incubation time and reached its maximum at 60 min, then decreased. When the incubation time is too long, the single-stranded DNA was also digested by the Exo III [27,28]. So the SP could be digested by the Exo III, if the incubation time increased. Therefore, 60 min was chosen as the optimal incubation time.

3.4. Analytical performance

Fig. 5 depicts typical fluorescence responses of Exo III-aided amplification assay to Lys of varying concentrations. We can observe dynamically increased fluorescence peaks with increasing concentration of target Lys ranging from 0.125 to 40 μg/mL, indicating a preferable signal-on sensing mechanism. Linear regression analysis of detection data yielded the following equation: $Y=175.8+238.5X$, where Y and X denoted the fluorescence peak intensity and target concentration, respectively. The peak intensity showed a linear correlation to the concentration of target Lys in the range of 0.125 to 1 μg/mL, and the detection limit is estimated to be 0.08 μg/mL according to the 3σ rule, which provides 4 to 28 fold improvement compared with other electrochemical, ultraviolet, or colorimetry assays for Lys [11,13,14,29].

3.5. Selectivity of the aptasensor

The specificity of an assay is another critical factor besides detection sensitivity. The control experiments were also performed to evaluate the detection specificity. IgG, BSA, IL-6, HSA were employed to replace the same concentration of Lys (5 μg/mL) under the same experimental conditions for the Lys assay. A mixture of HSA and Lys, each at the concentration of 5 μg/mL, was also investigated. Fig. 6 displays the direct comparison between the fluorescent response intensities of target Lys and nontarget proteins. The measured data indicated a good selectivity of the proposed aptasensor to Lys, which was attributed to the intrinsic properties of aptamer that can specifically recognize its target molecule [30,31].

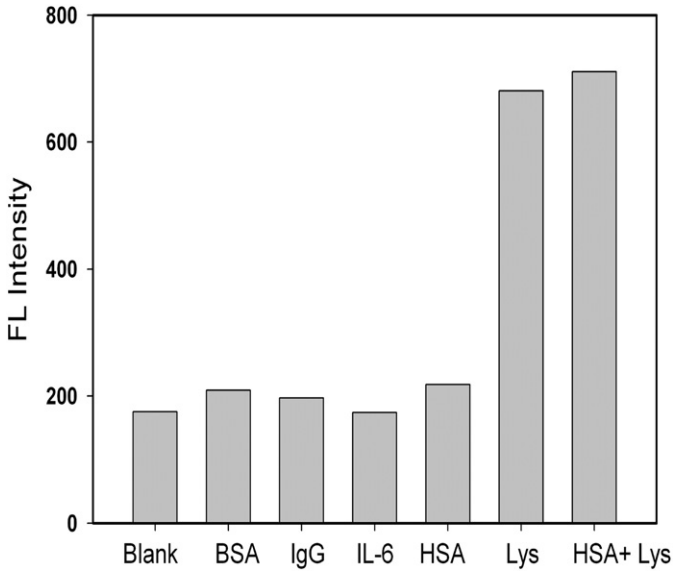


Fig. 6. The detection specificity of the assay, over BSA, IgG, IL-6 and HSA (all at a concentration of 5.0 μg/ml).

4. Conclusion

In summary, we described a novel, simple and reliable aptasensor for Lys detection by using Exo III-aided signal amplification and GO-based fluorescence quenching. This sensor was demonstrated to display good selectivity with a detection limit as low as 0.125 μg/mL. By combining Exo III-aided signal amplification and GO-based fluorescence quenching, this approach did not require specific recognition sequence for enzymatic cleavage and labeling of fluorophore/quencher pairs, which reduced the cost, lowered the background, and improved its generality for other aptasensors. This method could be widely applied to detect small molecules, other proteins and DNAs with specific designed oligonucleotides because of its excellent sequence-independent property.

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