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An enzyme-mediated universal fluorescent biosensor template for pathogen detection based on a three-dimensional DNA walker and catalyzed hairpin assembly†

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An enzyme-mediated universal fluorescent biosensor template for rapid detection of pathogens was developed based on the strategy of a three-dimensional (3D) DNA walker and catalyzed hairpin assembly (CHA) reaction. In the bacterial recognition step, a strand displacement reaction between bacteria and the double-stranded complex caused the release of the walker strand. The walker strand triggered the DNA walker to produce an enzyme fragment, and the DNA walker used gold nanoparticles (AuNPs) as the track to provide an excellent DNA ligand anchoring area. In the CHA step, the enzyme fragment induced the CHA cycle to yield fluorescence signals, which greatly enhanced the conversion ratio of trigger DNA and the sensitivity of the fluorescent biosensor. The effect of the distance and density of the DNA ligand was studied by adjusting the length of poly-adenine (PolyA), and was further explored by its reaction kinetics. By comparing the maximum reaction rate ($V_{\max}$), Michaelis constant (K_m) and turnover number (K_{cat}), the optimized PolyA probe was assessed and identified. In this work, the optimized PolyA-DNA probe exhibited an outstanding sensitivity in *Salmonella typhimurium* (*S. ty*) detection, which is 11.9 times and 4.6 times higher than those of the SH-DNA and the MCH treated SH-DNA. Meanwhile, a detection limit of 28.1 CFU mL⁻¹ was achieved in *Escherichia coli* (*E. coli*) detection. Furthermore, the biosensor achieved good selectivity and high repeatability with recoveries of 91%–115% for real sample detection. Considering these advantages, this template has great potential as a routine tool for pathogen detection and has wide applications in the field of global public health and food safety.

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1. Introduction

Infectious diseases caused by food-borne and water-borne pathogens pose an increasing threat to public health and the global economy.^{1,2} According to statistics from the World Health Organization (WHO), almost one in ten people around the world get sick from eating contaminated food every year. Food-borne diseases are estimated to have killed 420 000 people, out of whom 125 000 are children under the age of five. In order to prevent the spread of infectious diseases,

developing a sensitive and accurate analytical method for pathogen detection is of great significance to food safety and global public health.^{3–6}

In the past, a large number of pathogen analysis methods, such as the culture and colony counting method, the polymerase chain reaction (PCR) and the enzyme-linked immunosorbent assay (ELISA) have been developed.^{7–9} However, these traditional methods are costly and labor-intensive and require specialized instruments and operators, which are difficult to meet the requirement of bioanalysis nowadays.¹⁰ In order to overcome these defects, biosensors with high performance have been explored to use point of care testing (POCT) for pathogen detection.^{11–13} Fluorescent biosensors have attracted wide attention as a candidate POCT technique because of their excellent specificity, high speed and ease of use.^{14,15} The reported fluorescent biosensors, such as DNA walkers, have the same working principle, including target capture, displacement and detection. However, the original DNA walker showed a lack of sensitivity, which is a key factor in the construction of biosensors. To overcome this constraint, various strategies

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have been developed including increasing the anchored surface area of the target ligand^{16–18} and developing signal amplification methods.^{19–21}

Accumulating evidence has demonstrated that the anchored surface area undergoes a dimensional development. Initially, a one-dimensional (1D) DNA footprint or two-dimensional (2D) DNA origami track^{22–24} was studied and used, which can perform stepwise walking autonomously and progressively. However, as the research progressed, researchers found that these connection methods had limited travel space and few steps to walk. Nowadays, a three-dimensional (3D) steric structure has shown superior detection performance due to the effective cargo payload capacity, a large surface area for molecule immobilization and recognition, and rapid kinetics of target recycling.^{16,18,25} For example, Yang *et al.* have reported a DNA walker nanomachine that can walk randomly along a 3D track to detect specific nucleic acids. In this context, gold nanoparticles (AuNPs) as an analytical carrier to increase the anchored surface area were proposed.²⁶ To date, there are two main ways of binding the target ligand to the surface of AuNPs: Au–S connection^{27,28} and Au–N connection.^{29–31} As we know, with the Au–S connection it is difficult to control the distance of the target ligand on the surface of AuNPs and to avoid nonspecific DNA–Au binding through the flexible single stranded DNA (ssDNA) to the AuNP surface, all of which reduces the hybridization efficiency and causes insignificant sensitivity of the DNA walker.³² To precisely control the separation and conformation of the adjacent DNA ligands, poly-adenine (PolyA) binding on the AuNP surface has been studied, which has the same adsorption affinity as the Au–S connection. The surface density of the DNA ligand can be programmatically regulated by changing the PolyA length which provided opportunities for precise control of the orientation and conformation of PolyA–DNA and the nonspecific DNA–Au binding can be eliminated by the PolyA coating on the AuNP surface, thus improving the hybridization efficiency and detection sensitivity.^{30,33,34} Furthermore, the tuning of the PolyA length can regulate enzyme activity by changing the local salt concentration on the AuNP surface and eliminating steric hindrance between the enzyme and the substrate, which can optimize the reaction performance.³⁵

Apart from the anchored surface area, the signal amplification strategy is also important for the fluorescent biosensor. Recently, enzyme-free DNA recycling amplification has attracted much attention because of its advantages of simplicity and low cost.^{36,37} Catalyzed hairpin assembly (CHA) is a sensitive and enzyme-free technique, which has been widely studied for signal amplification and nucleic acid detection. In the reaction system, two hairpin structures are kinetically trapped and have a stable structure without a trigger DNA from the enzyme-catalyzed product of the DNA walker. In the presence of a trigger DNA, the DNA can perform the strand displacement reaction, leading to the opening of two hairpin structures and recycling of the DNA.³⁸ CHA achieves signal amplification by the strand displacement reaction only. With the addition of a molecular beacon (MB), CHA can generate

the fluorescent signal to achieve signal detection, thus realizing sensitive analysis of target.^{39,40}

In this work, a fluorescent biosensor template containing functional AuNP-based 3D DNA walker and CHA was proposed for pathogen detection. As a track of the 3D DNA walker, AuNPs can provide an appropriate anchoring area for the DNA ligand; the distance and conformation between the adjacent PolyA–DNA probes have been systematically studied by changing the length of PolyA with real-time fluorescence intensity monitoring. Meanwhile, the maximum reaction rate ($V_{\max}$), Michaelis constant (K_m), turnover number (K_{cat}) and K_{cat}/K_m were compared to explore the reasons for detection performance with different lengths of PolyA. In the whole system, the effective dual signal amplification involves the DNA walker and CHA reaction, which had greatly enhanced the conversion ratio of target DNA and the sensitivity for the detection of target pathogens. And the fluorescent biosensor used as a template with only the aptamer sequence for the target pathogen and a partial sequence that is complementary to the aptamer changed when changing the detection pathogen such as *S. ty* and *E. coli*. Furthermore, the proposed fluorescent biosensor template has achieved good selectivity and obtained good performance in the detection of real samples compared with quantitative real-time PCR (qPCR), it is believed that this proposed template can facilitate public health and global food safety.

2. Materials and methods

2.1 Reagents and instrumentation

Gold chloride hydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$) and 2-mercaptoethanol ($2\text{-C}_2\text{H}_6\text{OS}$) were purchased from Sigma-Aldrich (USA). Sodium citrate trihydrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 3\text{H}_2\text{O}$) was purchased from Chengdu Kelong Chemicals Co., Ltd (Chengdu, China). Nt. BbvCI was obtained from New England Biolabs (Beijing, China). Tryptone Soy Agar (TSA) was purchased from AoBoXing (Beijing, China). LB Broth (LB) was purchased from Sangon Biotech (Shanghai, China). All DNA sequences listed in Table S1† were synthesized and purified by Sangon Biotechnology Co., Ltd (Shanghai, China), all of which were kept at 95 °C for 5 min, and slowly cooled down to room temperature for at least 2 h before use.

The fluorescence spectra were obtained using a LS55 fluorescence spectrophotometer (PerkinElmer, USA). Absorption spectra were recorded using a PerkinElmer Lambda 25 UV/VIS spectrometer (PerkinElmer, USA). Optical density at 600 nm was determined using a NanoDrop One (Thermo, USA). Nanoparticles were characterized by transmission electron microscopy (TEM) (Tecnai G2 F20 S-TWIN, FEI, USA) and Dynamic light scattering (DLS) (ZEN 3690, Malvern, UK). Polyacrylamide gel electrophoresis (PAGE) images were scanned using an Azure c300 Biosystem (Azure Biosystems, USA). Quantitative real-time PCR (qPCR) was carried out using LightCycler® 96 (Roche, Switzerland).

2.2 Preparation of gold nanoparticles (AuNPs)

AuNPs were synthesized according to a previous report.⁴¹ In brief, 47 mL of ultrapure water containing 1 mL of $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ (0.01 g mL^{-1}) was heated under vigorous stirring. Then, 3 mL of 1% (v/v) $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 3\text{H}_2\text{O}$ was added into the boiling solution. After this the solution was kept boiling and stirred for 30 min, and the mixture was naturally cooled down to room temperature with stirring. The obtained AuNPs were stored at 4 °C for further use. The concentration of AuNPs was calculated using $C = A 450/\epsilon 450$.⁴²

2.3 Synthesis of PolyA-DNA-AuNPs (PDAs)

The functional processing of AuNPs using a PolyA-DNA probe is the basis of this experiment. Briefly, PolyA-DNA and prepared AuNPs were mixed in a molar ratio of 200 : 1 for 16 h at 25 °C. Buffer A (0.1 M PBS, 2 M NaCl, pH 7.4) was then slowly added to the mixture at room temperature for 40 h (the final concentration of NaCl was 0.2 M). Subsequently, the mixture was centrifuged and washed with buffer B (10 mM PBS, 0.2 M NaCl, pH 7.4) three times at 14 000g for 20 min to remove excess PolyA-DNA. The obtained PolyA-DNA-AuNPs (PDAs) were resuspended in buffer C (10 mM PBS, 0.1 M NaCl, 1 mM MgCl_2 , pH 7.4) for further use.

2.4 Preparation of SH-DNA-AuNPs (SDAs) and MCH treated SDAs (MSDAs)

Thiol-modified DNA was fixed on the AuNP surface by the salt-aging method.³ Briefly, 10 μL of SH-DNA (100 μM) were mixed with TCEP (20 mM) for 1 h to cleave the disulfide bond and then diluted to 400 μL . This activated SH-DNA solution was incubated with 625 μL AuNPs. Then, buffer A was slowly added to the mixture for salting and the final concentration of NaCl was 0.2 M. After centrifugal washing, the obtained SH-DNA-AuNPs (SDAs) were redissolved in buffer C to 540 μL and stored at 4 °C for future use. During the regulation process of 2-mercaptoethanol (MCH), 540 μL of SDAs were treated with MCH (the final concentration was 20 mM) for 1.5 h at 25 °C. The excess MCH and displaced SH-DNA were removed by centrifugation at 14 000g for 20 min and the supernatant was discarded. Then, the MCH treated AuNPs (MSDAs) were washed three times with buffer B. The particles were subsequently resuspended in buffer C and stored at 4 °C.

2.5 Procedure of pathogen detection

The procedure of pathogen detection was simple: only annealing, replacement, incubation, and fluorescence signal measurement were needed. In the typical pathogen recognition procedure, solution A (the mixed solution of 1 μL of 100 μM c-Apt, 3 μL of 100 μM Apt and 96 μL reaction buffer) was kept at 95 °C for 5 min and annealed to 25 °C to form the Apt@c-Apt duplex. Then, 20 μL of the pathogen was mixed with 16 μL of solution A and the mixture was incubated at 37 °C for 30 min to release c-Apt. After centrifugation at 6000 rpm for 5 min, the pathogen was separated and the supernatant containing c-Apt was collected. Subsequently, 16 μL

PDAs, 16 μL of 1 μM H1, 16 μL of 1 μM H2, 32 μL of 1 μM F@Q and 0.06 $\text{U } \mu\text{L}^{-1}$ Nt.BbvCI were added into the above collected solution and incubated at 37 °C for 50 min. Finally, the whole reaction solution was tested using a LS55 fluorescence spectrophotometer at room temperature.

2.6 Bacterial strain and culture

Salmonella typhimurium (ATCC 14028, *S. ty*), *Escherichia coli* (ATCC 25922, *E. coli*), *Yersinia enterocolitica* (*Y. e*), *Staphylococcus aureus* (ATCC 6538, *S. a*) and *Shigella sonnei* (*S. s*) were kindly provided by West China School of Public Health, Sichuan University. All of the bacteria were grown in Lysogeny Broth (LB) medium for 12 h at 37 °C under 200 rpm. Then, the cultured bacteria were gradient diluted to obtain different optical densities at 600 nm (OD_{600}). Meanwhile, the bacterial concentrations corresponding to each OD_{600} were quantified by the culture and colony counting method. After incubation at 37 °C for 16 h on agar medium, the colonies on the plates were counted to establish the relationship with OD_{600} . The standard linear calibration curves are shown in Fig. S1.† The bacterial concentration by OD_{600} was calculated using the standard linear calibration curves.

2.7 Polyacrylamide gel electrophoresis (PAGE) assay

PAGE was used to test and verify the behavior of DNA assembly in this work. In the gel electrophoresis assay, the assembly product of each sample (5 μL) was mixed with 6 $\times$ loading buffer (1 μL) and the mixture was loaded into 10% polyacrylamide gel. The electrophoresis was driven by 120 V for 40 min in 1 $\times$ TAE buffer, the gel was stained with 4S Red Plus nucleic acid for 15 min and imaged using a gel imaging system.

2.8 Preparation of real samples

In order to analyze the practical application capability of the biosensor system, the quantitative analyses of real samples (fresh chicken nuggets and milk) were performed, and they were purchased from a local Xinnanmen market in Chengdu. Five pieces of chicken have been inoculated with different concentrations of bacteria and minced in a sterile environment. Then, the minced chicken samples were placed in 20 mL reaction buffer and centrifuged at 8000 rpm for 20 min to remove excess precipitation, and the supernatants were collected as a real sample for analysis. Before using for testing, sterilized milk was mixed with different concentrations of bacteria, which was then detected using the fluorescent biosensor. In addition, to verify the reliability of the biosensor, qPCR was used to detect these samples for comparison.

2.9 Quantitative real-time PCR (qPCR)

To verify the reliability of the fluorescent biosensor method, qPCR was employed to detect *S. ty* and *E. coli* for comparison. Firstly, the genomic DNA of *S. ty* and *E. coli* with varying concentrations were extracted using a kit, which was used as a DNA template for qPCR detection. The reaction system of qPCR and the primer sequences of *S. ty* and *E. coli* are shown in Tables S3 and S4.† The detection conditions of qPCR were

as follows: first, annealing at 95 °C for 30 s; second, 40 rounds of cycling reaction including denaturation at 95 °C for 5 s, annealing at 55 °C for 10 s, and extension at 72 °C for 15 s; finally, the standard curves between the *S. ty* and *E. coli* concentration and the value of C_q were established as shown in Fig. S2.†

3. Results and discussion

3.1 Principle of the fluorescent biosensor template

The working principle for amplified detection of *S. ty* is shown in Fig. 1. The detection process can be divided into three steps for the whole reaction, namely: target recognition, a DNA walker and the CHA reaction.

In the first step, the complementary sequence (c-Apt) can be released from the double-stranded structure formed by the binding between the aptamer (Apt) and *S. ty*. The released c-Apt acts as the starting DNA to trigger the DNA walker reaction. In the second step, PolyA-DNA was connected to the AuNP surface by the salt-aging method. When c-Apt encountered PolyA-DNA, based on the rule of Watson–Crick base pairing, the hairpin structure of the PolyA-DNA probe opened, resulting in the formation of the double-stranded structure of c-Apt@ PolyA-DNA. Then, the double-stranded structure is cleaved by Nt.BbvCI specifically into two fragments, in which one enzyme fragment (E_f) is released. Then, c-Apt automatically binds to another PolyA-DNA strand and initiates a new cleavage process to generate large quantities of E_f in a catalytic

fashion because the number of complementary bases of c-Apt@ PolyA-DNA is decreased, which forms the basis of the CHA reaction.

In the third step, the released E_f is used as trigger DNA to initiate the reaction of CHA that promotes the generation of fluorescence signals and improves the sensitivity of the fluorescent biosensor. In detail, the existing E_f is hybridized with the H1 and the hairpin structure opens, exposing an active ssDNA region of H1. Then the hairpin H2 is hybridized with the active ssDNA region and a H1@H2@ E_f complex is formed. As the chain displacement reaction proceeds, this complex resolves into the H1@H2 dipolymer. The liberated E_f is able to trigger another CHA cycle. To achieve quantitative detection of *S. ty*, the H1@H2 dipolymer is capable of hybridizing with the fluorescence–quencher complementary structure (F@Q) to cause the separation between the fluorescent probe and quencher, causing fluorescence signal emergence. Hence, this fluorescent biosensor template has great potential for the detection of *S. ty*. By contrast, in the absence of *S. ty*, c-Apt is not released and cannot initiate the separation of the DNA walker and CHA efficiently. Therefore, the PolyA-DNA, H1, H2 and F@Q retain their original structures and no longer generate fluorescence signals.

3.2 Characterization and feasibility of the fluorescent biosensor template

In order to verify the performance of the fluorescent biosensor template, the connection between PolyA-DNA and AuNPs was

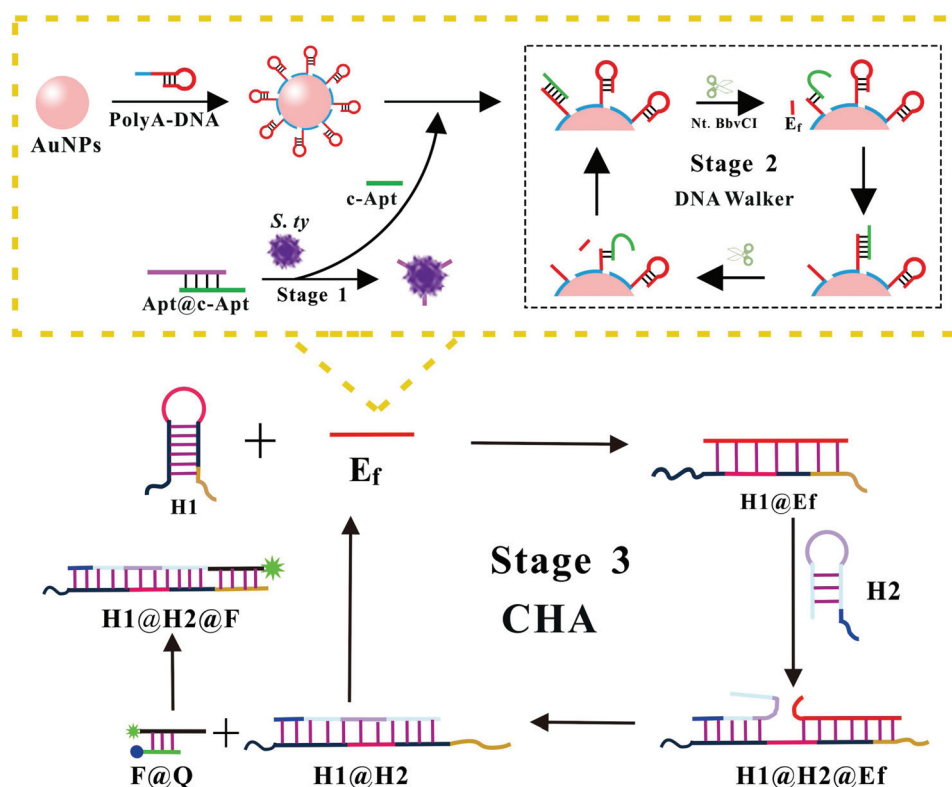


Fig. 1 Schematic illustration of the fluorescent biosensor based on a 3D DNA walker moving on the AuNP surface and the CHA reaction.

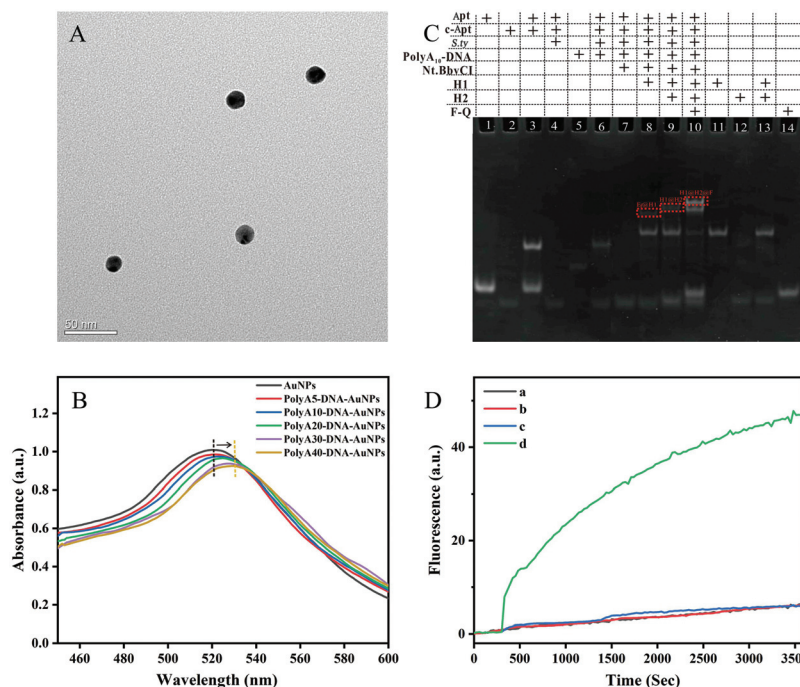


Fig. 2 Characterization of the connection between PolyA-DNA and AuNPs and verification of the feasibility. (A) The TEM image of the bare AuNPs. The scale bar is 50 nm. (B) UV-Vis spectra of AuNPs and functional PolyA-DNA. (C) PAGE image verifying the feasibility. Reaction conditions: lane 1: 16 μL (3 μM) Apt; lane 2: 16 μL (1 μM) c-Apt; lane 3: lane 1 + lane 2; lane 4: lane 3 + 20 μL *S. ty*; lane 5: 16 μL PolyA₁₀-DNA, lane 6: lane 4 + lane 5; lane 7: lane 6 + 0.06 U μL^{-1} Nt.BbvCI; lane 8: lane 7 + 16 μL H1; lane 9: lane 8 + 16 μL H2; lane 10: lane 9 + 32 μL F@Q; lane 11: 16 μL H1; lane 12: 16 μL H2; lane 13: lane 11 + lane 12; lane 14: 32 μL F@Q. (D) The real-time fluorescence measurement for verifying the performance. Reaction conditions: curve a: without Nt.BbvCI and *S. ty*; curve b: with *S. ty*; curve c: with Nt.BbvCI; curve d: with Nt.BbvCI and *S. ty*.

characterized first. As shown in Fig. 2A, the TEM result shows that the size of bare AuNPs was uniform indicating that the AuNPs were successfully synthesized. The UV-Vis absorption spectra of bare AuNPs show the characteristic peak at 518 nm (black line), as shown in Fig. 2B. After PolyA-DNA modified the bare AuNPs, the absorption peak of the functional PDAs shows an obvious red shift. The wavelength shift ranging from 518 to 527 nm was positively related to the length of PolyA, which implied that the size of assembled PDAs increases successively with the increase of the length of the PolyA tail. The direct proof was provided by the DLS results in Fig. S3.† The same tendency from 14.1 ± 0.3 to 26.5 ± 0.5 in terms of size was observed. The experimental phenomenon is due to the fact that the assembled PolyA tail exhibited electrostatic and steric repulsion to the hairpin DNA, which promotes the extension of the hairpin DNA into solution. With the increase of the length of the PolyA tail, the number of hairpin DNA attached to the surface of AuNPs decreases, thus the acting force of the PolyA tail to each hairpin DNA connected increases, so that the hairpin DNA is in a complete standing-up state and the particle size increases successively.

Subsequently, the PAGE assay was carried out to verify the feasibility of the biosensor template. The result is shown in Fig. 2C. When *S. ty* was added, the band of Apt disappeared as the specific binding between *S. ty* and the complementary sequence c-Apt stays away from Apt, as shown in lane 3 and

lane 4. When the PolyA₁₀-DNA encountered Apt@c-Apt@*S. ty*, the bright band of c-Apt@PolyA₁₀-DNA is observed (lane 6) and the band of E_f appeared (lane 7) by the specific cleavage of Nt.BbvCI. Then H1 and H2 are separately added, and the double-stranded structure of H1@H2 is observed (lane 9). Upon the introduction of the signal probe F@Q, the band of the tripolymer of H1@H2@F appeared (lane 10). These results provide strong evidence that the biosensor template is rationally designed. Meanwhile, PAGE was employed to verify the specificity between Apt and *S. ty*. As illustrated in Fig. S4,† four other kinds of bacteria show an obvious Apt@c-Apt band, implying that Apt can specifically bind to *S. ty*. In addition, the performance of the biosensor system was also investigated using the fluorescence experiment. As shown in Fig. 2D, a low fluorescence signal is obtained, when *S. ty* and Nt.BbvCI aren't in resolution (curve a), and only *S. ty* (curve b) or Nt.BbvCI (curve c) is added. In the presence of *S. ty* and Nt.BbvCI (curve d), a relatively strong fluorescence intensity is observed, which indicates that this biosensor shows effective signal amplification in response to the target bacteria.

3.3 Assessing surface coverage effects on the performance of the fluorescent biosensor template for *S. ty* detection

To achieve the optimal performance of the biosensor system, two key experimental parameters, including the molar ratio of Apt/c-Apt (Fig. S5A†) and reaction time (Fig. S5B†), were deter-

mined. The molar ratio of 3 : 1 and the reaction time of 50 min with the highest F/F_0 were selected in this work. As research progressed, we found that the change in the PolyA length affects the density and conformation of the PolyA-DNA probes on the surface of AuNPs, which implied that the change in the length of PolyA could further affect the enzyme activity of the biosensor. To confirm the supposition, the following formula (Michaelis–Menten equation) was used to evaluate the enzyme activity:

$$V = \frac{V_{\max} \times [S]}{K_m + [S]}$$

where “ V ” represents the reaction rate, “ $V_{\max}$ ” represents the maximum reaction rate, “[S]” represents the substrate concentration, and “ K_m ” represents the Michaelis constant, which means the substrate concentration at half the maximum reaction rate in the enzymatic reaction. The substrate in the enzymatic reaction is the most appropriate when K_m is at a low level. The turnover number (K_{cat}) that is positively related to the catalytic efficiency of the enzyme is calculated by using the following formula, where “[E_0]” is the total concentration of the Nt.BbvCI in each system.

$$K_{\text{cat}} = \frac{V_{\max}}{[E_0]}$$

By calculating the K_m and K_{cat} constants above, the specificity constant (K_{cat}/K_m) as an effective second-order rate constant to reflect the enzyme efficiency and function needs to be evaluated. In order to achieve this aim, the reaction kinetics of different states were analysed by monitoring the fluorescence intensity in real time, as shown in Fig. S6.† When Nt.BbvCI and 10^3 CFU mL^{−1} *S. ty* are present in the reaction solution, the lowest value of K_m is calculated to be 0.2 μM, which indicates that the most appropriate substrate is PolyA₁₀-DNA-AuNPs, as shown in Fig. 3A (red line). Similarly, the highest value of K_{cat} is calculated to be 0.04 min^{−1} in the PolyA₁₀ state (green line), indicating that the state of PolyA₁₀-DNA-AuNPs has the highest catalytic efficiency for Nt.BbvCI. Variations in K_m and K_{cat} show that the density and conformation of PolyA₁₀-DNA connected to the surface of AuNPs is the best substrate condition and provides the highest catalytic efficiency of Nt.BbvCI. Meanwhile, upon changing the density of MCH, the K_m is 1.1 times lower and the K_{cat} is 1.1 times higher than those of SDAs, which shows the importance of the density and conformation of the DNA probe.

Furthermore, the efficiency of the Nt.BbvCI (K_{cat}/K_m) in each state was compared. As shown in Fig. 3B, the value of K_{cat}/K_m is distinctly improved from 5A to 10A, and it reaches its maximum value at 10A, which indicated the cleavage efficiency of Nt.BbvCI was the best on the condition. In addition, K_{cat}/K_m of PolyA₁₀-DNA shows a higher cleavage efficiency compared with SDAs, showing that Nt.BbvCI in the PolyA₁₀-DNA state has better enzyme efficiency and function on the AuNP surface. The value of K_{cat}/K_m improved slightly using MCH to reduce nonspecific adsorption and adjust the loading density of SH-DNA, indicating that density regulation

can eliminate the steric hindrance between Nt.BbvCI and the cutting site and change the local salt concentration, thus improving the Nt.BbvCI efficiency. Interestingly, as shown in Fig. 3C, the $V_{\max}$ shows the same trend as K_{cat}/K_m . The variation of $V_{\max}$ with different PolyA lengths pointed out that the cleavage efficiency of Nt.BbvCI is affected by the change in the DNA probe density. Therefore, the PolyA tail can be used not only as an anchoring block between AuNPs and the DNA probe, but also as an adjustable fixture for adjusting the density and conformation of the DNA probe to change the efficiency of Nt.BbvCI.

To verify the importance of density and conformation, the detection limit in each system was tested, as shown in Fig. S7.† The lowest detection limit of *S. ty* is 22.9 CFU mL^{−1} in the PolyA₁₀-DNA system, which has increased 17.6 times compared to the PolyA₄₀-DNA system. We also observed that the density adjustment by MCH increases by 2.6 times the detection limit compared to SDAs as shown in Fig. 3D. The detection limit of the PolyA₁₀-DNA system for *S. ty* is one to four orders of magnitude lower than that of the previously reported methods (Table S2a†) and this PolyA₁₀-DNA detection system has the advantages of being fast and low-cost compared with the cultured bacterial sample⁴³ and laser-induced breakdown spectroscopy (LIBS).⁴⁴ In addition, there are some methods that reach a lower LOD than this work (Table S2b†). For example, the reported PCR,⁷ immunology-based assays⁴⁵ and surface-enhanced Raman scattering (SERS)⁴⁶ methods have realized the sensitive detection of *S. ty*, and their LODs have reached 7–9, 10 and 4 CFU mL^{−1}, respectively. However, the PCR method requires expensive equipment and has a longer detection time; immunology-based assays are faced with the challenge of cross-reaction with closely related antigens and antigen variation. Although SERS can achieve low-cost and ultra-sensitive bacterial detection, the reporter molecule used in the SERS method shows obvious signal attenuation during the detection process.⁴⁶ Through the comparison of Michaelis–Menten equation constants and detection limit, it was found that a system with an optimal PolyA tail has an appropriate density and conformation of the DNA probe, thus improving the collision probability and efficiency of Nt.BbvCI, leading to a low detection limit. And based on the above experimental phenomena, PolyA₁₀-DNA achieves the optimal performance for the detection of *S. ty* in this fluorescent biosensor.

3.4 Generality of the fluorescent biosensor template

To illustrate whether the fluorescent biosensor is suitable for the detection of other bacteria, *E. coli* with its DNA aptamer was tested. *E. coli* is a Gram-negative bacterial species found ubiquitously in the intestinal flora of animals, and the occurrence of pathogenic variants causes major public health problems.^{47,48} Simple and sensitive detection of *E. coli* is very important for food safety and clinical diagnostics. As shown in Fig. S8,† the tripolymer of H1@H2@F appeared (lane 11) in PAGE principle analysis, which indicates that the use of the biosensor is a rational strategy for the detection of *E. coli*.

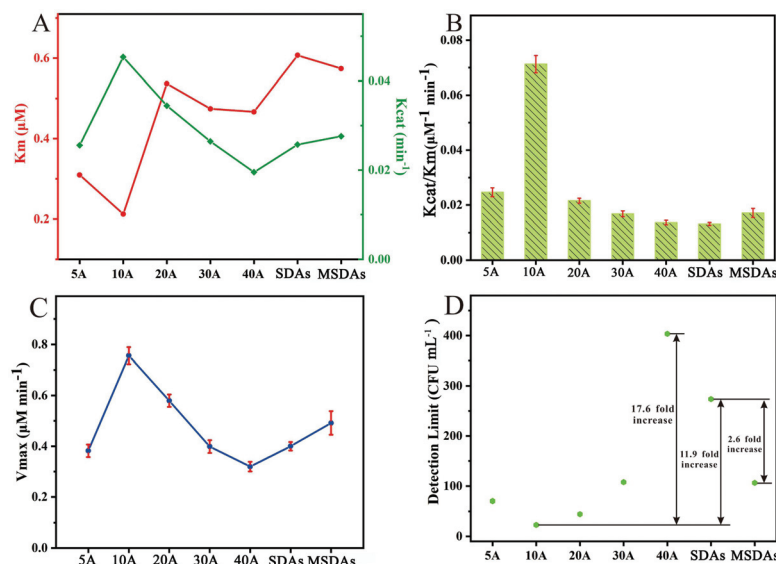


Fig. 3 Effect of surface coverage on the performance of the biosensor system. (A) Comparison of the kinetic parameters of Nt.BbvCI with different lengths of PolyA; (B) the comparison of the specificity constant of Nt.BbvCI in solution; (C) the programmable change of V_{max} with the change of the surface coverage; (D) comparison of the detection limit in different surface coverage. All experiments were repeated five times.

Furthermore, the specificity of Apt of *E. coli* was confirmed by PAGE as shown in Fig. S9.†

By real-time fluorescence intensity determination, as shown in Fig. S11,† the lowest calculated value of K_m is 0.1 μM , which indicates that the most appropriate length is PolyA₁₀ for Nt.BbvCI. Correspondingly, the highest K_{cat} is 0.04 min^{-1} in the PolyA₁₀-DNA state, which shows the length of PolyA with the highest catalytic efficiency of Nt.BbvCI is PolyA₁₀, as shown in Fig. S10A.† The density and conformation of PolyA-DNA is adjusted by changing the length of PolyA, which causes the K_m and K_{cat} to change. This phenomenon indicated that the proposed template of PolyA₁₀-DNA had good performance in *E. coli* detection. In addition, K_{cat}/K_m and V_{max} also show the same tendency as shown in Fig. S10B and C,† indicating that the cleavage efficiency of Nt.BbvCI is the best in the PolyA₁₀-DNA system. Having successfully demonstrated the performance of cleavage efficiency of the Nt.BbvCI in the *E. coli* assay, a standard linear calibration curve was obtained by comparing the fluorescence change with the concentration of *E. coli*. As shown in Fig. 4A–E, the values of the detection limit were 82.1 CFU mL^{-1} for PolyA₅-DNA, 28.1 CFU mL^{-1} for PolyA₁₀-DNA, 79.9 CFU mL^{-1} for PolyA₂₀-DNA, 391.1 CFU mL^{-1} for PolyA₃₀-DNA and 547.4 CFU mL^{-1} for PolyA₄₀-DNA, respectively. The detection limit of the PolyA₄₀-DNA system shows a 19.5 times decrease compared with the lowest detection limit of *E. coli*, as shown in Fig. 4F. At the same time, the detection limit of the PolyA₁₀-DNA system for *E. coli* was compared with previously reported methods (Table S2a and b†). From these experimental data we believe that the PolyA₁₀-DNA probe improves DNA hybridization and Nt.BbvCI digestion due to several reasons. Firstly, an appropriate DNA surface coverage resulted in a lower amount of phosphate groups with negative charge on the AuNP surface, which in turn reduced the local

salt concentration and minimized salt-induced enzyme efficiency inhibition. Secondly, suitable PolyA₁₀-DNA attached to the AuNP surface prevents the nonspecific adsorption of the DNA probe, which lifts the probe to maintain an upright orientation to ease target hybridization and increase enzyme accessibility. Finally, suitable PolyA₁₀-DNA altered the final DNA probe density, greatly reducing the spatial and electrostatic repulsion between the adjacent DNA strands. Generally speaking, the PolyA₁₀-DNA probe with a suitable length achieved excellent performance for pathogen detection by improving the hybridization ability of the probe, probe–target assembly efficiency and enzymatic activity at the AuNP surface.

3.5 Selectivity of the fluorescent biosensor template

The selectivity of the proposed fluorescent biosensor is mainly attributed to the application of the highly specific bacterial aptamer. In this study, other bacteria including *Y. e.*, *S. s* and *S. a* with concentrations of 10^2 , 10^3 , 10^4 , and 10^8 CFU mL^{-1} were tested to evaluate the selectivity of the constructed biosensor. In the detection system of *S. ty*, a high relative fluorescence enhancement is obtained when *S. ty* was added and the fluorescence signal from *S. ty* becomes increasingly higher than that of non-target bacteria as shown in Fig. 5A. 10^2 CFU mL^{-1} is the minimum concentration and the fluorescence signal from *S. ty* is three times higher than those from other bacteria, which indicates that this proposed fluorescent biosensor possesses remarkable selectivity to *S. ty* than other bacteria. Similarly, in the detection system of *E. coli*, there is no significant fluorescence intensity increase upon the addition of non-target bacteria except for *E. coli* (Fig. 5B), and the fluorescence signal from the minimum concentration of 10^2 CFU mL^{-1} *E. coli* is three times higher than those from other bacteria. According to these experimental results, the proposed

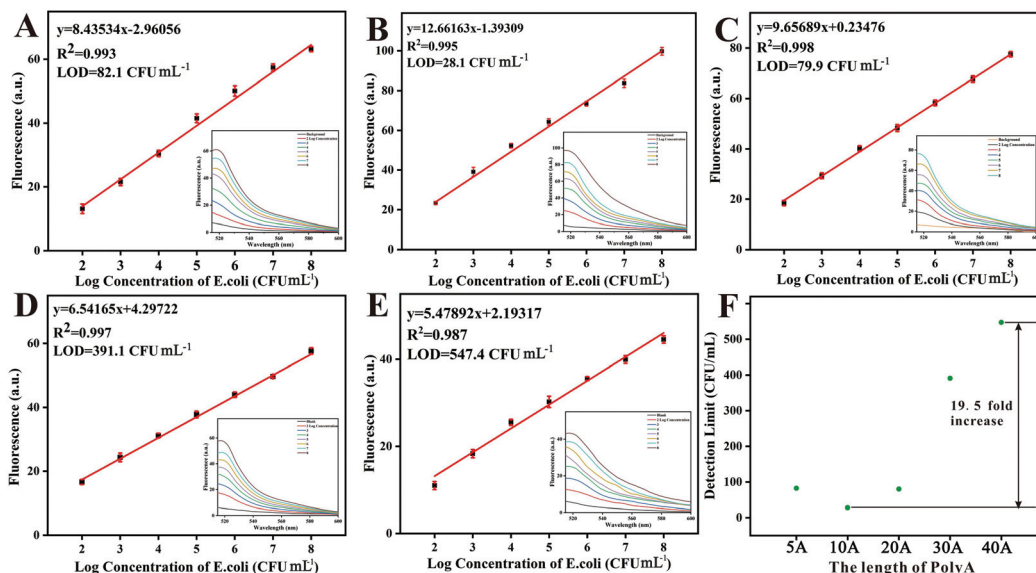


Fig. 4 The relationship between the *E. coli* concentration and the fluorescence intensity change at 520 nm. (A) PolyA₅-DNA-AuNPs; (B) PolyA₁₀-DNA-AuNPs; (C) PolyA₂₀-DNA-AuNPs; (D) PolyA₃₀-DNA-AuNPs; (E) PolyA₄₀-DNA-AuNPs. (F) Comparison of the detection limit in different surface coverages. Reaction conditions: 16 μ L (3 μ M) Apt, 16 μ L (1 μ M) c-Apt, 20 μ L *E. coli* with different concentrations, 16 μ L PolyA-DNA-AuAPs with different lengths, 16 μ L H1, 16 μ L H2, 32 μ L F@Q and 0.06 U μ L⁻¹ Nt.BbvCI.

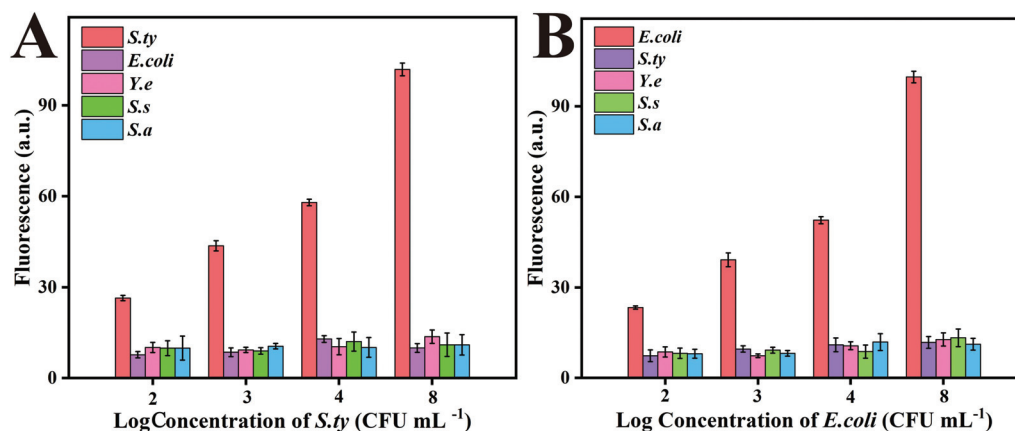


Fig. 5 Selectivity of the proposed fluorescent sensor toward the target. (A) The bacterial solutions with 10^2 , 10^3 , 10^4 , and 10^8 CFU mL⁻¹ were detected using the *S. ty* aptamer; (B) the bacterial solutions with 10^2 , 10^3 , 10^4 , and 10^8 CFU mL⁻¹ were detected using the *E. coli* aptamer. Each experiment was conducted three times.

biosensor shows good selectivity to target bacteria. Moreover, the biosensor template can be extended to selectively detect other molecules with a specific aptamer just by changing the aptamer sequence of the target and the partial complementary sequence that matches the aptamer sequence.

3.6 Real sample application in chicken and milk

Pathogens in food and water pose a serious threat to public health and financial stability; so the analysis performance of the fluorescent biosensor template was assessed by spiking bacteria of varying concentrations in chicken and milk. As demonstrated in Table 1, the spiked recovery varied within the range of 91%–115% and the relative standard deviation (RSD)

varied from 3% to 10% when the concentration of *S. ty* in the chicken and milk varied between 1.4×10^2 CFU mL⁻¹ and 1.4×10^6 CFU mL⁻¹. Compared with qPCR, the discrepancy is the highest at 14% and the lowest at 1%, indicating that our fluorescent biosensor template provides reliable test results in real sample testing. The real sample detection of *E. coli* was also completed, as shown in Table 2. The spiked recovery and the RSD varied from 96% to 108% and 6% to 8%, respectively. Similarly, the discrepancy from qPCR is 12% at the highest and 3% at the lowest. At the same time, the natural samples were detected and the results are shown in Table S5.† For natural samples, neither method could detect *S. ty* and *E. coli*, indicating that the samples were not contaminated with *S. ty*

Table 1 Application of the proposed biosensor for *S. ty* detection ($n = 3$)

Samples	<i>S. ty</i> added (CFU mL ⁻¹)	Detected by the sensor (CFU mL ⁻¹) Concentration $\pm$ SD	Detected by the qPCR (CFU mL ⁻¹) Concentration $\pm$ SD	Discrepancies between qPCR and the sensor (%)	Recovery (%)	RSD (%)
Chicken	1.4×10^2	$(1.6 \pm 0.1) \times 10^2$	$(1.5 \pm 0.1) \times 10^2$	1	112	3
	1.4×10^4	$(1.4 \pm 0.1) \times 10^4$	$(1.2 \pm 0.2) \times 10^4$	12	100	7
	1.4×10^6	$(1.3 \pm 0.1) \times 10^6$	$(1.4 \pm 0.6) \times 10^6$	7	91	10
Milk	1.4×10^2	$(1.3 \pm 0.1) \times 10^2$	$(1.2 \pm 0.1) \times 10^2$	8	95	9
	1.4×10^4	$(1.4 \pm 0.1) \times 10^4$	$(1.2 \pm 0.1) \times 10^4$	14	102	7
	1.4×10^6	$(1.6 \pm 0.1) \times 10^6$	$(1.4 \pm 0.2) \times 10^6$	11	115	6

Table 2 Application of the proposed biosensor for *E. coli* detection ($n = 3$)

Samples	<i>E. coli</i> added (CFU mL ⁻¹)	Detected by the sensor (CFU mL ⁻¹) Concentration $\pm$ SD	Detected by the qPCR (CFU mL ⁻¹) Concentration $\pm$ SD	Discrepancies between qPCR and the sensor (%)	Recovery (%)	RSD (%)
Chicken	8.4×10^2	$(8.4 \pm 0.6) \times 10^2$	$(8.9 \pm 0.6) \times 10^2$	6	100	7
	8.4×10^4	$(8.0 \pm 0.7) \times 10^4$	$(8.9 \pm 0.9) \times 10^4$	11	96	8
	8.4×10^6	$(8.4 \pm 0.5) \times 10^6$	$(8.8 \pm 0.4) \times 10^6$	6	99	6
Milk	8.4×10^2	$(9.1 \pm 0.7) \times 10^2$	$(8.6 \pm 0.9) \times 10^2$	6	108	8
	8.4×10^4	$(8.8 \pm 0.5) \times 10^4$	$(8.5 \pm 0.6) \times 10^4$	3	105	5
	8.4×10^6	$(8.3 \pm 0.5) \times 10^6$	$(9.2 \pm 0.8) \times 10^6$	12	98	6

and *E. coli*. All these results showed that target detection can be realized, and it is reasonable to anticipate that the template can find wide applications in real sample analysis.

4. Conclusion

In summary, an enzyme-mediated universal fluorescent biosensor template based on a 3D DNA walker and CHA was constructed successfully for pathogen detection. Firstly, due to the robust manipulation capacity of the DNA walker and the superior signal amplification capacity of CHA, as low as 22.9 CFU mL⁻¹ for *S. ty* and 28.1 CFU mL⁻¹ for *E. coli* can be detected within 50 min. Secondly, the specificity of this strategy was significantly enhanced because of the excellent identification ability of the pathogen aptamer and strict sequence design of the DNA walker and CHA system. Thirdly, compared with SH-DNA and SH-MCH states, our strategy fully employed the PolyA-DNA probe to ensure the stability and conformation between adjacent DNA probes on the AuNP surface. By tuning the length of PolyA, the cleavage efficiency of Nt.BbvCI was precisely regulated, which is the basis of the CHA reaction and one of the decisive factors for good performance of the fluorescent biosensor. Fourth, the fluorescent biosensor template has good universality and can be extended to detect other pathogens with a specific aptamer just by changing the aptamer sequence of the target pathogen and the partial sequence that is complementary to the aptamer sequence. We believe that the novel biosensor template can provide opportunities for better application of biomolecule detection. Our proposed template can facilitate food safety and global public health.

Conflicts of interest

The authors declare no conflicts of interest.

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