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Ultrasensitive colorimetric carcinoembryonic antigen biosensor based on hyperbranched rolling circle amplification

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In this study, a hyperbranched rolling circle amplification (HRCA)-based colorimetric biosensor for carcinoembryonic antigen (CEA) is developed with high sensitivity and specificity. A CEA aptamer can bind with its target (CEA) to form a complex due to their high affinity, and the introduced CDNA cannot hybridize with the aptamer. Thus, free CDNA can propagate the HRCA reaction to form a large number of single-stranded DNA (ss-DNA). ss-DNA can be easily adsorbed onto AuNPs and prevent salt-induced AuNPs aggregation, which causes the change in the color of the system. It is found that the absorbance intensity ratio (A_{520}/A_{660}) has a linear relationship with the concentration of the target in the range of 5 pM–0.5 nM, and the detection limit is as low as 2 pM ($S/N = 3$).

1. Introduction

Recent development in biotechnologies offers a wide variety of signal amplification tools, such as polymerase chain reaction (PCR) and rolling circle amplification (RCA). Because PCR requires complex thermal cycling steps, in addition to sophisticated and expensive equipment, it is considered to be complicated. The process of RCA is much simpler than that of PCR, which includes the following steps. First, a short DNA primer (CDNA) hybridizes with the DNA template (padlock probe) to fold into a ringlike structure in a head-to-tail manner. With the assistance of DNA ligase, the padlock probe is then circularized, and the subsequent RCA process is accomplished by Phi 29 DNA polymerase.[1] However, the sensitivities of the RCA-based biosensors are less than that of PCR.[2] To solve this problem, hyperbranched rolling circle amplification (HRCA) has been developed based on the RCA reaction,[3] which is an isothermal and exponential amplification through the turn-by-turn cascade of primer extension and strand displacement.[4,5] The products of HRCA consist of large amounts of single-stranded DNA (ss-DNA) and double-stranded DNA (ds-DNA) with various lengths. Many HRCA-based biosensors had been developed for high-sensitivity detection.[6,7] Because most of these sensors are based on fluorescence or electrochemical detection, these approaches usually need complex labelling and expensive measurement.

Au nanoparticles (AuNPs) represent a class of materials that exhibit a high extinction coefficient and broad absorption in the visible range.[8] Au atoms on the surface possess unoccupied orbitals, into which nucleophiles can donate electrons.[9,10] It has been reported that ss-DNA and ds-DNA exhibit different binding properties toward unmodified AuNPs.[11,12] Because ss-DNA is flexible and can partially uncoil its bases, it can be easily adsorbed onto AuNPs and prevent salt-induced AuNP aggregation by enhancing the electrostatic repulsion between ss-DNA-adsorbed AuNPs, which results in the color change of the solution. Many colorimetric assays have been developed based on this mechanism. For example, Chang's group pioneered a colorimetric sensing approach for the determination of adenosine triphosphate using aptamer-modified gold nanoparticles.[13] Luo *et al.* developed a novel aptamer-based colorimetric biosensor for the multiplex detection of adenosine, thrombin and cocaine.[14] These studies demonstrated that colorimetric assays exhibit characteristics of being simple, rapid as well as permitting direct visual detection without the need for complicated equipment.[15] However, the sensitivity is not good, which limits their application. It is necessary to determine methods to improve the sensitivity of the colorimetric assay.

The ultrasensitive determination of disease-marker proteins has attracted substantial research efforts due to their broad applications in the diagnosis, prevention, and treatment of diseases.[16–18] Here, a highly sensitive and label-free method for analyzing proteins on the basis of AuNPs-mediated HRCA has been developed. Carcinoembryonic antigen (CEA), one of the most studied tumor markers associated with liver, colon, breast and colorectal cancers,[19] has been chosen as an example. The assay is easy to implement for visual detection, which combines the merits of high sensitivity of HRCA and the simplicity of Au-NP-based colorimetric sensors.

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2. Experimental section

2.1 Chemicals

The sequence of the CEA aptamer[20,21] and the other DNA oligonucleotides were synthesized by Sangon Inc. (Shanghai, China). Their sequences are shown below:

CEA aptamer: 5′-ATACCAGCTTATTCAATT-3′

CDNA: 5′-AATTGAATAAGCTGGTAT-3′

Primer 1: 5′-GCA TTT CAG TTT ACG-3′

Primer 2: 5′-TTG CGA AAT GTA AAC-3′

Padlock: 5′-P-**TATTCAATT** GCA TTT CAG TTT ACG GTT TAC ATT TCG CAA **ATACG TGCT**-3′

In the padlock probe, the binding region for the HRCA primer 1 was shown as a wavy line, and the region with the same sequence as the HRCA primer 2 was shown as underlined portions. *Escherichia coli* (*E. coli*) DNA ligase set (including *Escherichia coli* DNA ligase, 10× *Escherichia coli* DNA ligase buffer, and 10× BSA (0.05%)) was obtained from Takara Biotechnology Co., Ltd. (Dalian, China). The deoxynucleotide solution mixture (dNTPs), Bst DNA polymerase large fragment, and their corresponding buffer were purchased from New England Biolabs (NEB). SYBR Green II was purchased from Xiamen Biovision Biotechnology Co. Ltd. (Xiamen, China). $HAuCl_4$ (99.999%) and sodium citrate dehydrate were purchased from Dingguo Biotech (Beijing, China). All other chemicals were of analytical reagent grade and obtained from Sigma Chemical Co., USA. In this experiment, double-distilled water (Milli-Q, Millipore, resistance 18.2 MΩ) was used throughout the experiments. DNA buffer solutions were prepared by dissolving DNA into 50 mmol L^{-1} Tris–HCl (pH 7.4).

2.2 HRCA reaction and AuNP-based colorimetric assay

Different concentrations of CEA were mixed with the aptamer probe (5 nM) in equal volume for 30 min. The same volume of CDNA (5 nM) was then introduced into above solution for incubation at 37 °C for 30 min with vibration. The ligation reaction was performed in the ligation buffer solution containing 6 U *E. coli* DNA ligase, 0.05% BSA and 0.167 mM nicotinamide adenine dinucleotide (NAD), which was incubated at 37 °C for 60 min. Then, the HRCA reaction was simultaneously carried out at 63 °C for 60 min in the aforementioned solution, which included 40 nM primer 1, 40 nM primer 2, 9.6 U Bst DNA polymerase and 0.6 mM dNTP.

AuNPs with an average diameter of 13 nm were prepared according to the literature.[22] For the colorimetric assay, 50 μL of HRCA product was added to 150 μL of the gold colloid and after mixing for some minutes, an additional 15 μL 3 M NaCl was added. The resulting solutions were observed by the naked eye or quantified by UV-vis absorption spectroscopy (Perkin Elmer Instruments, USA). Because of the difference between the aggregation degree of Au-NPs between absorbance at wavelengths of 520 nm and 660 nm, the ratio of absorbance 520/absorbance 660 (A_{520}/A_{660}) was chosen to examine the effect of proposed method.

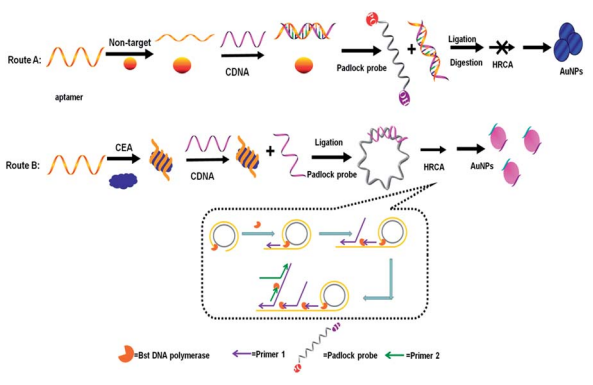
3. Results and discussion

3.1 Principle of the colorimetric biosensor

The protocol of the proposed colorimetric biosensor is shown in Scheme 1. In the absence of the target (CEA), the aptamer hybridizes with CDNA to form ds-DNA, and no CDNA hybridizes with the padlock to initiate HRCA. Only the padlock probe and HRCA primers exist in solution, the dosage of primers are not high enough to prevent salt-induced AuNP aggregation, and the color of the solution is blue (Route A). In the presence of the target CEA, the CEA binding aptamer prefers to form a CEA/aptamer complex *in lieu* of the aptamer–CDNA duplex. Thus, free CDNA hybridizes with the padlock probe, shown in bold, which can be ligated and circularized in the presence of *E. coli* DNA ligase. The obtained circular padlock probes are further purified by the digestion of the unreacted linear padlock probes and the excess oligonucleotides using exonucleases I and III. Subsequently, primer extension and strand displacement take place in the presence of circular padlock probes, two primers, and Bst DNA polymerase, producing a large number of ss-DNA fragments of variable lengths. By this method, each target can propagate an HRCA reaction to form a large number of ss-DNA. Because ss-DNA can be easily adsorbed onto AuNPs and prevent salt-induced AuNPs aggregation, the color of the solution is red (Route B). Based on this principle, a colorimetric method for CEA determination can be developed.

A simple experiment has been performed to verify our presumption. First, faradaic electrochemical impedance spectroscopy was applied to characterize the binding between the aptamer and target (CEA). As shown in Fig. 1(A), when the probe aptamer is immobilized on the gold electrode surface, the R_{ct} value is about 200.0 Ω (curve a) because the negatively charged phosphate backbone on the electrode prevents the redox of $[Fe(CN)_6]^{3-/4-}$ on the electrode. After treating with the target, the R_{ct} value increases to 544.9 Ω (curve b). The reason is because of the formation of aptamer/CEA on the electrode, which retards further interfacial electron transfer reaction of the redox probe. This result indicates that the aptamer can bind with CEA.

Because ss-DNA can combine with SYBR Green II to produce strong fluorescence signals, which is used to check whether the



Scheme 1 Principle of the proposed HRCA-based colorimetric biosensor.

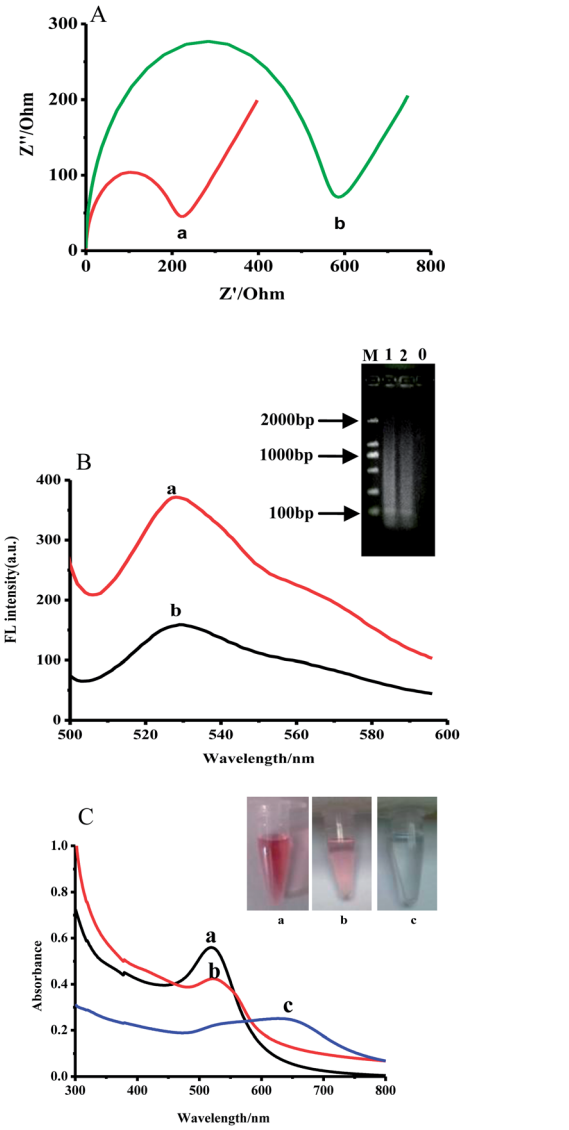


Fig. 1 (A) Nyquist plots of electrodes. (a) Probe aptamer/MCH-modified gold electrode; (b) aptamer/MCH-modified gold electrode after interaction with 8×10^{-9} mol L^{-1} CEA. (B) Fluorescence spectra of HRCA solution (a) with and (b) without CEA ([CEA] = 8×10^{-9} mol L^{-1}); inset: HRCA products are electrophoresed in 2% agarose gel. The DNA ladder is indicated in lane M. Lanes 0, 1 and 2 represent the HRCA products from [CEA] = 0, [CEA] = 8×10^{-9} mol L^{-1} and [CEA] = 8×10^{-10} mol L^{-1}, respectively. (C) UV-vis absorption of (a) citrate–AuNPs, HRCA–AuNP solution (b) with and (c) without CEA ([CEA] = 8×10^{-9} mol L^{-1}).

products of the HRCA solution contain large amount of ss-DNA. As shown in Fig. 1(B), in the presence of the target CEA, an obvious fluorescence signal can be detected (curve a), indicating that the existence of the target can trigger the HRCA reaction, which forms a large number of ss-DNA. However, in the absence of the target, the fluorescence intensity is weak (curve b). This weak signal reason may be because nothing can trigger the HRCA reaction; a few of the non-reaction primers combine with SYBR Green II to produce a weak fluorescence signal. The HRCA products at different target concentrations are analyzed by gel electrophoresis. As shown in the inset of Fig. 1(B), long ladder-type bands are observed in the presence of 8×10^{-9} mol L^{-1} CEA (lane 1) and a dark ladder-type band is observed in the presence of 8×10^{-10} mol L^{-1} CEA (lane 2). However, no bands in the absence of CEA are observed (lane 0) because no HRCA occurs. These results suggest the validity of the proposed mechanism.

Fig. 1(C) shows the UV-vis absorption spectra and the corresponding photographs of AuNP colloids at different conditions. The color of the citrate–AuNP solution is red, and the absorption band is observed at around 520 nm (tube a and curve a in Fig. 1(C)). In the presence of CEA, the HRCA reaction can be initiated, and the color of the AuNP solution containing the HRCA product after salt adjusting is red, and the absorbance does not cause significant variation (tube b and curve b in Fig. 1(C)). However, in the absence of the target (CEA), nothing can trigger the HRCA reaction. Only the HRCA primers exist in solution, and the dosage of primers are not high enough to prevent salt-induced AuNP aggregation. At this stage, the color of the AuNP solution containing HRCA primers after salt adjusting is blue. A new absorption band at around 660 nm appears, and the intensity at around 520 nm decreases (tube c and curve c in Fig. 1(C)). These results also demonstrate the feasibility of the proposed system.

3.2 Optimization of the reaction conditions

To obtain high amplification efficiency of HRCA, the concentrations of primers, dNTP substrates and Bst DNA polymerase are optimized. As shown in Fig. 2(A), the absorbance intensity ratio (A_{520}/A_{660}) increases with the increase of primer concentrations from 0.005 μM to 0.04 μM, suggesting that primer concentration leads to the improvement of HRCA efficiency. However, when the concentration of the primers exceeds 0.04 μM, the absorbance intensity ratio (A_{520}/A_{660}) decreases. This can be explained by the fact that high concentrations of exogenous primers might adversely cause primer dimerization.[23] Therefore, 0.04 μM is selected as the optimum concentration of primers in the subsequent research. In addition, with the increase of dNTP concentration, A_{520}/A_{660} enhances gradually and finally reaches a constant after 0.6 mM. Thus, the concentration of dNTP is 0.6 mM (Fig. 2(B)). For the concentration of Bst DNA polymerase, it is found that A_{520}/A_{660} increases with the increase of Bst DNA polymerase concentrations first and then reached a stabilized platform when the concentrations are greater than 9.6 U (shown in Fig. 2(C)). Hence, the concentration of Bst DNA polymerase is set at 9.6 U.

A long HRCA reaction time is expected to generate more copies of the products for better signal amplification. SYBR GREEN II-based time-dependent fluorescence has been applied to monitor the HRCA reaction. As shown in Fig. 2(D), the fluorescence intensity increases gradually at the beginning, showing that HRCA products are generated continuously, whereas the fluorescence intensity trends to a constant value at 60 min, indicating the saturation of the HRCA products. Therefore, 60 min is chosen as the optimum time for the HRCA reaction.

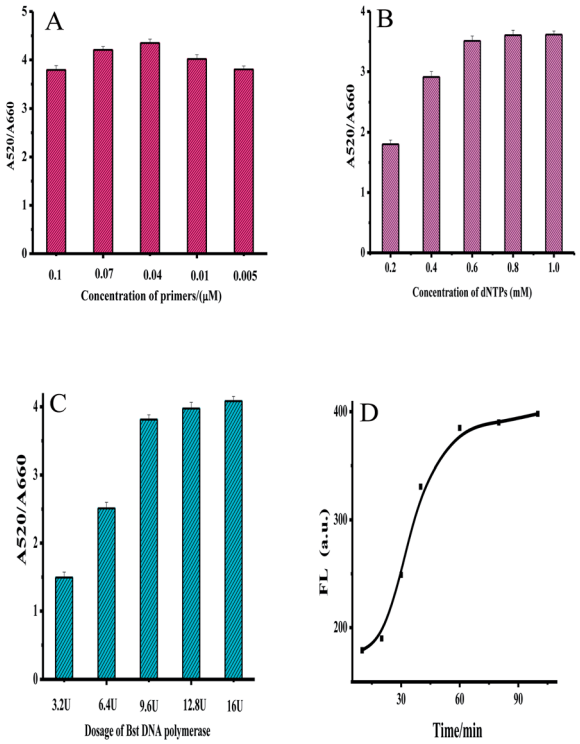


Fig. 2 Effects of the concentrations of (A) primers, (B) dNTP and (C) Bst DNA polymerase on the absorbance intensity ratio in the presence of 8×10^{-9} mol L^{-1} CEA. (D) SYBR GREEN II-based time-dependent fluorescence intensity changes in HRCA reaction ([CEA] = 8×10^{-9} mol L^{-1}).

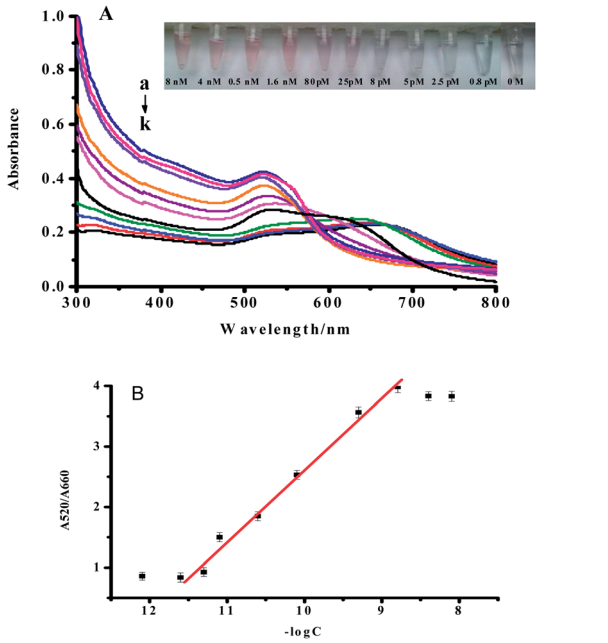


Fig. 3 (A) Color change of the solution with increasing concentrations of CEA and UV-vis absorption spectra of the system at different CEA concentrations. From a to k: 8.0×10^{-9}; 4.0×10^{-9}; 1.6×10^{-9}; 5.0×10^{-10}; 8.0×10^{-11}; 2.5×10^{-11}; 8.0×10^{-12}; 5.0×10^{-12}; 2.5×10^{-12}; 0.8×10^{-12}; and 0 mol L^{-1}. (B) Variation of the absorbance intensity ratio against the different CEA concentrations. Inset: corresponding calibration plot of the logarithm of target CEA concentrations in the range from 5.0 pM to 0.5 nM.

3.3 Quantitative analysis of CEA

To further characterize the detection range of this assay, a series of samples containing different concentrations of CEA have been tested. As shown in Fig. 3(A), the color of the solutions changes gradually from red to blue upon the decrease of the CEA concentration. Meanwhile, a dramatic red shift is observed with the decrease of the target concentration from the absorbance spectra of the detected samples. Only one peak located at 520 nm is detected in the presence of high concentrations of CEA. Along with the decrease of the CEA concentration, a new broad absorption appears, whereas the absorbance at 520 nm gradually red-shifts and decreases. Accordingly, the absorbance peak ratio at 660 and 520 nm was employed to quantitatively scale the CEA concentration. A_{520}/A_{660} has a good linear relationship with the logarithmic concentration of CEA in the range of 5.0 pM–0.5 nM (Fig. 3(B)), and the regression equation is expressed as

$$A_{520}/A_{660} = 1.19 \log C + 14.57,\ R = 0.9946,$$

where C is the CEA concentration, and R is the correlation coefficient. A detection limit of 2.0 pM ($S/N = 3$) was achieved, which is much lower than the values from previously reported fluorescence,[24] chemiluminescence[25] or electrochemical approaches,[26] which may be explained by the combination of the high efficiency of the HRCA and the high specificity of the aptamer.

Six replicated experiments were performed at a CEA concentration of 50 pM; the relative standard deviation (RSD) was 5.8%, suggesting that the proposed method has good reproducibility.

3.4 Specificity of the HRCA-based colorimetric assay

To demonstrate the specificity of the sensors for biological samples, several common proteins were used to test the selectivity of the proposed colorimetric sensor. 1 μM alpha fetoprotein (AFP), lysozyme, bovine serum albumin (BSA) and thrombin were chosen as interferents, and the concentration of CEA was set as 80 pM. From the visual observation (Fig. 4, inset), the color of the mixed solution still remains red only in the presence of CEA, resulting from the product of the HRCA reaction to prevent salt-induced AuNP aggregation. When the concentrations of interferents were more than 10 000 times higher than the CEA concentration, the color of the salt-induced AuNP solutions turns blue, almost similar to the blank. The values of the absorption ratio (A_{520}/A_{660}) for the system treated with different targets were also recorded (Fig. 4). In the presence of 80 pM CEA, the value increases significantly, compared with the blank, whereas in the presence of 1 μM of the other protein, the values are similar to or slightly higher than the value of the blank. The reason is because insufficient ss-DNA had been produced and induced the aggregation of the AuNPs. This demonstrates that the HRCA-based biosensor is highly specific

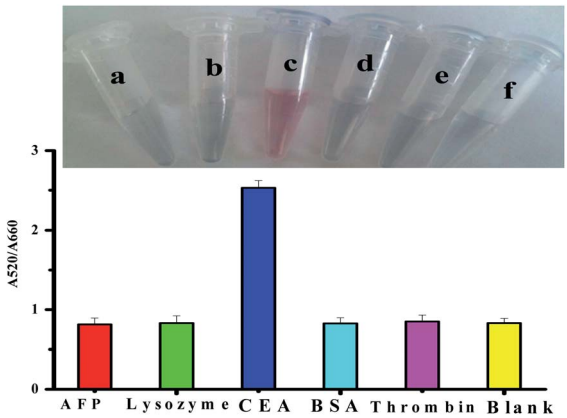


Fig. 4 Absorbance intensity ratio of the proposed sensor with different interferents and targets. The inset is the corresponding color at different interferents and targets. (a) Thrombin; (b) AFP; (c) CEA; (d) BSA; (e) lysozyme; and (f) blank. The concentration of CEA is 8×10^{-11} mol L^{-1}, while the concentrations of other interferents are 1×10^{-6} mol L^{-1}.

and has good selectivity for the discrimination of CEA from other proteins.

4. Conclusions

In summary, a new selective and sensitive colorimetric sensing system for CEA by merging the speediness and convenience of a colorimetric assay with isothermal and exponential amplification of HRCA technology has been developed. This homogeneous assay system not only eliminates thermal cycling but also achieves improved assay characteristics (*e.g.*, wide linear response range, low detection limit and high specificity). It holds promising potential for broad applications in the biodetection of diseases, biochemical study, and in environmental and clinical applications.

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