A label-free “lock-key” fluorescence aptasensing based on triplex-helix DNA and G-quadruplex for CA15-3 detection

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Abstract

Herein, we designed a label-free fluorescent aptasensor based on triple-helix DNA and G-quadruplex for carbohydrate antigen (CA15-3) detection. Triplex-helix structure can be formed with Insert G-rich DNA (IG) and Aptamer DNA (Apt), which likes a “lock” and the G-rich sequences are locked. The CA15-3 was the “key”, which specifically combined with aptamer sequences of Apt, causing IG liberated from the triplex-helix “lock”. Hereafter, the G-rich sequences of IG were formed into G-quadruplex and specifically interacted with N-methylmesoporphyrin IX (NMM), which greatly enhanced fluorescence of the solution. However, when the “key” was not existing, the “lock” would be fastened and fluorescence intensity didn’t change. According to this proposal, the concentration of CA15-3 can be effectively detected from 0.01 U mL$^{-1}$ to 5 U mL$^{-1}$ with a detection limit (LOD) of 0.01 U mL$^{-1}$. Furthermore, this proposal can be applied to spiked human serum with great precision and reproducibility.

Keywords Triplex-helix DNA; Label-free; CA15-3; G-quadruplex; Fluorescent aptasensor
Introduction

Breast cancer and ovarian cancer are main killers threatening women's health. To improve the accuracy of early diagnosis and sensitivity of breast cancer detection, some serum biomarkers were excavated and detected for many years, like UDP-galactosyl transferase, heat shock protein hsp27, mammaglobin, cytokeratin fragment 21.1 (CYFRA 21.1), nuclear matrix proteins (NMPs), Human kallikrein 5, CA 15-3, sHER2 and CA 125, 8-Iso-prostaglandin F2alpha (8-iso-PGF2alpha), miRNA, and so on. The CA15-3 was the most useful biomarker of breast cancer among them, and its concentration was under 30 U mL⁻¹ in healthy women. Also, a great quantity of CA15-3 assay methods was proposed. For instance, Sattar Akbari Nakhjavani et al. built an electrochemical biosensor to sensitively and reliably detect of CA15-3 in patient plasma with labeled with magnetic beads. Soodabeh Hassanpour et al. used nano-ink technology to propose an immunoassay of CA 15-3 by silver nanoparticles-reduced graphene oxide. Saadati Arezoo et al. designed a novel nano-bioink immunosensing to assay CA 15-3. Chi Zhang et al. constructed an impedimetric immunosensor to detect CA 15-3 based on cascade catalysis. Terävä, Joonas et al. constructed lectin nanoparticle assays for detecting CA15-3. Ai-Jun Wang et al. successfully detected CA15-3 concentration by a label-free electrochemical immunoassay. Xin Xiong et al. carried out a one-step electrochemiluminescence immunoassay to assay CA 15-3 and obtained a low detection. Undeniably, these methods were accuracy and sensitivity for CA15-3 detection. However, all these strategies realized relying on antigen-antibody binding, which is expensive, complex operation and time-consuming. In addition, these strategies aren’t conducive to portable and wearable design applications.
Due to simple, timeless, easy operation, fast response and costless, fluorescence aptamer biosensor is widely used for detection of nucleic acid, protein and exosome etc. Fluorescence signal is always produced by labeled fluorophore or compound interaction. Since fluorescent labeling of aptamers is relatively expensive, and fluorescent labeling may have a negative impact on the affinity between the target and the aptamer, non-labeled fluorescence aptamer biosensor technology has been developed. Among the label-free designs, G-quadruplex is extensively used in fluorescence aptamer biosensors relying on its outstanding property, which can specifically bind with Thioflavin T (ThT), N-methylmesoporphyrin IX (NMM), 3-(2-(4-vinlypyridine))-6-(2-((1-(4-sulfobutyl))-3,3-dimethyl-2-vinlybenz[e]indole)-9-ethyl-carbazole (9E PBIC) and carbazole derivative (CZ-BT), and so on.

Hence, we designed a label-free “lock-key” fluorescence biosensor based on triplex-helix DNA and G-quadruplex for CA15-3 detection. The CA15-3 was the key, which specifically combined with aptamer sequences of Apt, causing IG liberated from the triplex-helix “lock”. Hereafter, the G-rich sequences of IG were constructed into G-quadruplex and specifically interacted with N-methylmesoporphyrin IX (NMM), which greatly enhanced fluorescence of the solution. However, when the “key” was not existing, the “lock” would be fastened and there would not have fluorescence change. Moreover, this strategy was applied to real biological sample, it might provide potential application for breast early diagnosis and prognosis.

**Experimental**

**Reagents and chemicals**

All oligonucleotides were synthesized by Shanghai Sangong Biotech Co.
(Shanghai, China) and were purified by ULTRAPAGE (Table 1). The phosphate buffered solution, healthy human serum and streptavidin (SA) were obtained from solarbio (Beijing, China, http://www.solarbio.com). The bovine serum albumin (BSA), lysozyme and thrombin were obtained from Sigma (St. Louis, MO, USA). N-methylmesoporphyrin IX (NMM) was obtained from J&K Scientific Ltd. (Beijing, China). All of the reagents were diluted to the required concentration with distilled water before use.

**Procedure**

First of all, 200nM Apt and 100nM IG were added into phosphate buffered solution (0.01M, 2.5Mm MgCl₂, pH=5.78) for 1 hour at 30°C. And then, different concentrations of CA15-3 were added into above solution and reacted for 30min. Next, 20 mM K⁺ and 1 μM NMM were added into the reaction systems and incubated for 25min. Finally, the fluorescence spectra were obtained from the emission wavelength range of 580-650 nm at the 399 nm excitation wavelength, and the excitation and emission slits were both set at 1 nm at room temperature. In the biology sample detection, we just replaced PBS buffer with diluted human serum.

**Results and Discussion**

**The sensing principle of CA15-3 assay Feasibility of CA15-3 detection**

The principle of the “lock-key” fluorescence biosensing is based on triplex-helix DNA and G-quadruplex for CA15-3 detection (Fig.1). As a result of Watson-Crick base pairing principle and Hydrogen bond interaction, triple-helix structure could be established with Insert G-rich DNA (IG) and stems of Aptamer DNA (Apt), which was
the “lock”. The G-rich sequences were fastened with the IG. The CA15-3 was the “key”, which can specifically combine with aptamer sequences that were loop of Apt, causing IG liberated from the “lock”. Hereafter, the G-rich sequences of IG were constructed into G-quadruplex by potassium ion and specifically interacted with N-methylmesoporphyrin IX (NMM), which greatly enhanced fluorescence of the solution. However, when the “key” was not to exist, the “lock” would be fastened and there would not have fluorescence change. Only the “key” was inserted into the “lock”, the “lock” was unfastened, and the fluorescence was received.

**Feasibility of CA15-3 detection**

To confirm the feasibility of this proposal, the fluorescence spectra of experiments were carried out for CA15-3 assay. As shown in Fig.2, in the absence of CA15-3 (black line), the fluorescence spectra were low, indicating that G-rich sequences were fastened in the “lock”. Upon the addition of 5 U mL⁻¹ CA15-3 (red line), the fluorescence was significantly increased, signifying that the CA15-3 was the “key” and opened the triplex-helix “lock”.

**Optimization of experimental conditions**

To improve good property of this proposal, some experimental conditions were optimized, such as different triplex lengths, the ratio of Apt/IG, pH value, the concentration of K⁺ and the temperature of incubation. F and F₀ were the fluorescence intensity of solution with and without CA15-3, respectively. To form triple-helix structure, the acidity of solution is a critical parameter. So, the pH value was considered at first. Shown at Fig.3a, the F/F₀ ratio reached the best when pH value was 5.78. As shown in Fig.3b, when the triplex lengths of triplex-helix structure were 10 bp, the value of F/F₀ was higher than 8bp and 9bp. So, we chose the 10 bp triplex length for the subsequent experiments. Fig3c shown that the fluorescence changed with the ratio of
Apt/IG, the F/F0 ratio was highest when the ratio of Apt/IG was 2. Thus, this ratio was employed for the next experiments. To get at high sensitivity of this proposal, the concentrations of K⁺ were investigated at Fig.3d, and 20nM K⁺ was selected. As shown in Fig.3e, the maximum F/F0 signal were observed at 30°C. We found that as the reaction temperature increases, the more triplex-helix DNA forms, the background fluorescence gradually decreases, and the fluorescence ratio increases. When the temperature exceeded 30°C, it did not be conducive to form triplex-helix DNA structure and the background fluorescence increased.

**Sensitivity of assay method**

Upon the above optimization experiments, the sensitivity of “lock-key” triplex-helix DNA fluorescence biosensor for CA15-3 detection can be tested. As shown in Fig.4a, the fluorescence intensity of fluorescence aptasensor incubated with different concentrations of CA15-3 were obtained. The fluorescence intensities were gradually increased with different concentrations of CA15-3 addition. The inset in Fig.4b illustrated the linear relationship between the fluorescence intensity and the concentration of CA15-3 from 0.01 U mL⁻¹ to 5 U mL⁻¹. The regression equation was y=93.70x +873.01 with a regression coefficient of 0.98, and the detection limit was 0.01 U mL⁻¹(3σ /S, σ is the standard deviation for the blank solution and S is the slope of the regression equation). Moreover, this strategy has relatively good sensibility and linear range among the published detection methods (Table 3).

**Specificity of the proposed fluorescent assay**

In addition, the specificity of the fluorescence aptasensor was also experimented with different proteins replacement. As shown in Fig.5, an increasing fluorescence was displayed in the presence of CA15-3, demonstrating that this proposal has good specificity for CA15-3 assay. Comparatively, when the other proteins were added into
the same solution, the fluorescence intensities were lower as the blank control group.

Real samples detection

For the purpose of applying to biological samples, spiked human serum was used as a complex matrix. As shown in Table 2, the recoveries for human serum were in the range of 99.08-102.32%, and the RSD were 0.88%, 3.82% and 2.49%. A great precision and reproducibility of this “lock-key” fluorescence biosensor for CA15-3 was established.

Conclusions (optional)

As mentioned above, this label-free and simple proposal showed sensitivity and specificity for CA15-3 detection. We can obtain high sensitivity in the range of 0.01-5U mL\(^{-1}\) and credibly precision, reproducibility in spiked human serum. This proposal showed several merits in that: (1) this strategy avoided fluorophore labeling, (2) it didn’t need complicated operation technic, (3) it also provided good sensitivity and specificity for CA15-3 analysis. Therefore, this method may provide a certain reference for breast cancer early diagnosis and prognosis. And we expected that the “key-lock” strategy based on triplex-helix DNA and G-quadruplex will hold great promise applications by replacing aptamer sequences and target.

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Table 1 Oligonucleotides used in this proposal.

| Name | Base sequences |
|------|----------------|
| Apt  | 5’-CTTCCTCCGTAAGTGAAATATGACAGATCACAACCTCCCTCCTTC-3’ |
| Apt9 | 5’-CTTCCTCCGGAAAGTGAATATGACAGATCACAACCTCCCTCCTTC-3’ |
| Apt8 | 5’-CTTCCTCCGAAGTGAATATGACAGATCACAACCTCCCTCCTTC-3’ |
| IG  | 5’-GGGTTTTGGGTAGGAAGGGAGGAAGTCCTTGGGTTTTGGG-3’ |
| IG9 | 5’-GGGTTTTGGGTAGGAGGAGGAAGTCCTTGGGTTTTGGG-3’ |
| IG8 | 5’-GGGTTTTGGGTAGGAGGAGGAAGTCCTTGGGTTTTGGG-3’ |

Table 2 Recovery of CA15-3 spiked in human serum samples

| Serum sample | Added (U mL⁻¹) | Founded (U mL⁻¹) | Recovery (%) | RSD (N=3, %) |
|--------------|----------------|------------------|--------------|--------------|
| 1            | 2.5            | 2.48             | 99.08        | 0.88         |
| 2            | 5              | 5.11             | 102.32       | 3.82         |
| 3            | 10             | 10.05            | 100.48       | 2.49         |

Table 3 Different methods for CA15-3 detection.

| Detection method | Detection limit (U/mL) | Linear range (U/mL) | Reference |
|------------------|------------------------|---------------------|-----------|
| Electrochemical  | 0.3                    | 1-100               | 30        |
| Electrochemical  | 0.033                  | 0.1-120             | 31        |
| Microarray system| 17                     | 0-1280              | 32        |
| Electrochemical  | 0.5                    | 2.5-240             | 33        |
| Electrochemical  | 0.012                  | 0.1-20              | 34        |
| Type           | Value       | Units | Note     |
|---------------|-------------|-------|----------|
| Surface Plasma| 0.2         | 2.5-20| 35       |
| Resonance     | 0.0039      | 0.01-0.1| 36     |
| Fluorescence  | 0.01        | 0.01-5| Present work |
**Figure Captions**

Fig. 1 Schematic diagram of label-free “lock-key” fluorescence detection of CA 15-3 based on tiplex-helix and G-quadruplex.

Fig. 2 Fluorescence spectra of solution with and without CA15-3.
Fig. 3 The effect of pH value(a), different triplex lengths(b), the ratio of Apt/IG(c), the concentration of K⁺(d) and the temperature of incubation(e) on the fluorescence response of this system.
Fig. 4 (a) Fluorescence spectra of NMM towards different concentrations (0, 0.01, 0.05, 0.1, 1, 5, 100, 250, 500, 750 and 1000 U mL⁻¹) of CA15-3 in PBS buffer; (b) The relationship between the fluorescence intensity of NMM and different concentrations of CA15-3 in PBS buffer. The concentrations of Apt, IG, K⁺ and NMM were 200nM, 100nM, 20mM and 1μM, respectively.
Fig. 5 Fluorescence intensity (at the emission wavelength of 608 nm) of the sensor in the presence of blank, thrombin (10 μ g mL⁻¹), lysozyme (10 μ g mL⁻¹), streptavidin (10 μ g mL⁻¹), BSA (10 μ g mL⁻¹) and CA15-3 (0.1 μ g mL⁻¹), respectively. Error bars: SD, n = 3.