A Label-free “Lock-key” Fluorescence Aptasensing Based on Triplex-helix DNA and G-quadruplex for CA15-3 Detection

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Herein, we designed a label-free fluorescent aptasensor based on triple-helix DNA and G-quadruplex for carbohydrate antigen (CA15-3) detection. The triplex-helix structure can be formed with inserted G-rich DNA (IG) and aptamer DNA (Apt), which like a “lock” to lock the G-rich sequences. The CA15-3 was the “key”, which specifically combined with aptamer sequences of Apt, resulting in liberating IG from the triplex-helix “lock”. Then, the G-rich sequences of IG were formed into G-quadruplex and specifically interacted with *N*-methylmesoporphyrin IX (NMM), which greatly enhanced the fluorescence of the solution. However, when the “key” did not exist, the “lock” was fastened and fluorescence intensity did not change. With this proposed method, the concentration of CA15-3 can be effectively detected from 0.01 to 5 U mL⁻¹ with a detection limit (LOD) of 0.01 U mL⁻¹. Furthermore, this proposed biosensor can be applied to spiked human serum with great precision and reproducibility.

Keywords Triplex-helix DNA, label-free, CA15-3, G-quadruplex, fluorescent aptasensor

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Introduction

Breast cancer and ovarian cancer are main killers threatening women's health. To improve accuracy of early diagnosis and sensitivity of breast cancer detection, some serum biomarkers have been extracted and detected over a course of many years. They include UDP-galactosyl transferase,¹ heat shock protein hsp27,² mammaglobin,³ cytokeratin fragment 21.1 (CYFRA 21.1),⁴ nuclear matrix proteins (NMPs),⁵ human kallikrein 5,⁶ CA 15-3, sHER2 and CA 125,⁷ 8-iso-prostaglandin F2alpha (8-iso-PGF2alpha),⁸ miRNA,⁹ and others. CA15-3 has proven to be the most useful biomarker for breast cancer, and its concentration is less than 30 U mL⁻¹ in healthy women.^{10,11} Also, a great quantity of CA15-3 assay methods has been proposed. For instance, Akbari Nakhjavani *et al.*¹² built an electrochemical biosensor to sensitively and reliably detect CA15-3 in patient plasma labeled magnetic beads. Hassanpour *et al.*¹³ used nano-ink technology to propose an immunoassay for CA 15-3 by silver nanoparticles-reduced graphene oxide. Saadati *et al.*¹⁴ designed a novel nano-bioink immunosensing to assay CA 15-3. Zhang *et al.*¹⁵ constructed an impedimetric immunosensor to detect CA 15-3 based on cascade catalysis. Terävä *et al.*¹⁶ constructed lectin nanoparticle assays for detecting CA15-3. Wang *et al.*¹⁷ successfully detected CA15-3 concentration by a label-free electrochemical immunoassay. Xiong *et al.*¹⁸ carried out a one-step electrochemiluminescence immunoassay to assay CA 15-3 and obtained a low detection

limit. Undeniably, these methods demonstrated accuracy and sensitivity for CA15-3 detection. However, all these strategies were realized by relying on antigen-antibody binding, which is expensive, involves complex operations and is time-consuming. In addition, these strategies are not conducive to portable and wearable design applications.¹⁹

Due to its simple, time-saving, easy-to-operate, rapid-response and low-cost features, the fluorescence aptamer biosensor is widely used for detection of nucleic acids,^{20,21} proteins^{22,23} and exosomes,^{24,25} *etc.* A 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 because of its outstanding properties, one of which is that it can specifically bind with Thioflavin T (ThT),²⁶ *N*-methylmesoporphyrin IX (NMM),²⁷ 3-(2-(4-vinylpyridine))-6-(2-((1-(4-sulfobutyl))-3,3-dimethyl-2-vinylbenz[e]indole)-9-ethyl-carbazole (9E PBIC),²⁸ and carbazole derivative (CZ-BT),²⁹ *etc.*

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 to become liberated from the triplex-helix “lock”. The G-rich sequences of IG were constructed into G-quadruplex and specifically interacted with *N*-methylmesoporphyrin IX (NMM), which greatly enhanced the fluorescence of the solution. However, when the “key” did not exist, the “lock” remained fastened and there was no fluorescence change. Moreover, this strategy was

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Table 1 Oligonucleotides used in this proposal

Name	Base sequences
Apt	5'-CTTCCTCCCTGAAGTGAATATGACAGATCACAACTTCCCTCCTTC-3'
Apt9	5'-CTTCCTCCCGAAGTGAATATGACAGATCACAACTCCCTCCTTC-3'
Apt8	5'-CTTCCTCCGAAGTGAATATGACAGATCACAACTCCTCCTTC-3'
IG	5'-GGGTTTGGGTAGGAAGGGAGGAAGTCCTTGGGTTTGGG-3'
IG9	5'-GGGTTTGGGTAGGAGGGAGGAAGTCCTTGGGTTTGGG-3'
IG8	5'-GGGTTTGGGTAGGAGGAGGAAGTCCTTGGGTTTGGG-3'

applied to real biological samples, and is expected to have the potential application for breast cancer 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, 200 nM Apt and 100 nM IG were added into phosphate buffered solution (0.01 M 2.5 mM MgCl₂, pH 5.78) for 1 h at 30°C. And then, different concentrations of CA15-3 were added into the above solution and reacted for 30 min. Next, 20 mM K⁺ and 1 μM NMM were added into the reaction systems and incubated for 25 min. 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 replaced PBS buffer with diluted human serum.

Results and Discussion

The sensing principle of CA15-3 assay and 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 the Watson-Crick base pairing principle and hydrogen bond interaction, a triple-helix structure could be established with inserted 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 the loop of Apt, causing IG to become liberated from the “lock”. The G-rich sequences of IG were constructed into

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

Fig. 2 Fluorescence spectra of solution with and without CA15-3.

G-quadruplex by potassium ion and specifically interacted with *N*-methylmesoporphyrin IX (NMM), which greatly enhanced the fluorescence of the solution. However, when the “key” did not exist, the “lock” remained fastened and there was no fluorescence change. Only when the “key” was inserted into the “lock”, and the “lock” was unfastened, then the fluorescence was achieved.

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 the good properties of the proposed biosensor, some experimental conditions were optimized, such as triplex length, the ratio of Apt/IG, pH value, the K⁺ concentration and the incubation temperature. *F* and *F*₀ were the fluorescence intensity of solution with and without CA15-3, respectively. To form a triple-helix structure, the acidity of the solution is a critical parameter. So, the pH value was considered first. As shown in Fig. 3a, the *F*/*F*₀ ratio reached the optimal level when pH value was 5.78. As shown in Fig. 3b, when the triplex length of the triplex-helix structure was 10 bp, the value of *F*/*F*₀ was higher than when the length was 8 or 9 bp. So, we chose the triplex length of 10 bp for the subsequent experiments. Figure 3c shows that the fluorescence changed with the ratio of

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 200 nM, 100 nM, 20 mM, and 1 μ M, respectively.

Apt/IG, and the F/F_0 ratio was the highest when the ratio of Apt/IG was 2. Thus, this ratio was employed for the next experiments. To obtain high sensitivity of the proposed biosensor, the concentrations of K⁺ were investigated at Fig. 3d, and 20 nM K⁺ was selected as optimal. As shown in Fig. 3e, the maximum F/F_0 signal was observed at 30°C. We found that as the reaction temperature increases, more triplex-helix DNA forms, the background fluorescence gradually decreases, and the fluorescence ratio increases. When the temperature exceeded 30°C, it was not conducive to forming triplex-helix DNA structure and the background fluorescence increased.

Sensitivity of assay method

With 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 the fluorescence aptasensor incubated with different concentrations of CA15-3 were obtained. The fluorescence intensities were gradually increased with the addition of different concentrations of CA15-3. The inset in Fig. 4b illustrates the linear relationship between the fluorescence intensity and the concentration of CA15-3 from 0.01 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 ($3\sigma/s$, σ is the standard deviation for the blank solution and s is the slope of the regression equation). Moreover, this strategy has relatively good sensitivity and linear range among the published detection methods (Table 2).

Table 2 Different methods for CA15-3 detection

Detection method	Detection limit/ U mL ⁻¹	Linear range/ U mL ⁻¹	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 immunosensor	0.012	0.1 – 20	34
Surface plasma resonance	0.2	2.5 – 20	35
Fluorescence	0.0039	0.01 – 0.1	36
Fluorescence	0.01	0.01 – 5	Present work

Fig. 5 Fluorescence intensity (at the emission wavelength of 608 nm) of the sensor in the presence of blank, thrombin (10 $\mu\text{g mL}^{-1}$), lysozyme (10 $\mu\text{g mL}^{-1}$), streptavidin (10 $\mu\text{g mL}^{-1}$), BSA (10 $\mu\text{g mL}^{-1}$) and CA15-3 (0.1 $\mu\text{g mL}^{-1}$), respectively. Error bars: SD, $n = 3$.

Table 3 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

Specificity of the proposed fluorescent assay

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

Real sample detection

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

Conclusions

As mentioned above, the proposed label-free and simple biosensor showed sensitivity and specificity for CA15-3 detection. We can obtain high sensitivity in the range of 0.01 – 5 U mL⁻¹ as well as credible precision and reproducibility in spiked human serum. This proposed sensor showed several merits in that: (1) this strategy avoided fluorophore labeling, (2) it did not need complicated technical operation, and (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 “lock-key” strategy based on triplex-helix DNA and G-quadruplex will hold great promise for applications by replacing aptamer sequences and the target.

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