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Aptamer-mediated turn-on fluorescence assay for prion protein based on guanine quenched fluophor

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ABSTRACT

An aptamer-participated haprin structure was designed by employing cellular prion protein (PrP^C) as a model protein, and thus an aptamer-mediated turn-on fluorescence assay for proteins was developed in this contribution. The designed aptamer-participated haprin structure consists of three segments. Namely, an aptamer sequence located in the loop, three guanine bases at 3′-terminal, and a fluophor modified at 5′-terminal. It was found that the guanine bases at the 3′-terminal could quench the fluorescence of the fluophor such as tetramethyl-6-carboxyrhodamine (TAMRA) at the 5′-terminal about 76.6% *via* electron transfer if the guanine bases are close enough to the fluophor, and the quenched fluorescence could get restored when the target protein is present since the interaction, which could be confirmed by measuring fluorescence lifetime, between TAMRA-aptamer and the target protein forces the guanines away from TAMRA so that TAMRA-modified aptamer changes into turn-on state. A linear relationship was then constructed between the turn-on fluorescence intensity and the concentration of PrP^C in the range from 1.1 to 44.7 μg/mL with a limit of detection of 0.3 μg/mL (3σ).

1. Introduction

Prions, including Creutzfeldt–Jakob disease (CJD), Gerstmann–Straussler–Scheinker disease (GSS), fatal familial in human, bovine spongiform encephalopathy (BSE) in cattle and scrapie in sheep, have the hallmark of accumulation of abnormal prion protein (PrP^{Sc}), which are converted from the host-encoded normal prion protein (PrP^C) [1]. PrP^C and PrP^{Sc} have the same amino acid sequence but are different in conformation since PrP^C has high content of alfa-helix and PrP^{Sc} is rich in beta-sheet structure [2]. PrP^C takes part in a variety of processes of cellular metabolism including copper homeostasis [3], apoptosis [4], signal transduction [5], and its translocation is very important in the conversion of PrP^{Sc} [6]. For a long time, human contaction with animals affected with BSE had been believed safe and did not show any apparent problems. The announcement of possible transmission of BSE to humans in 1996, however, created enormous concern. Therefore, rapid and sensitive diagnostic methods for prions are in pressing need to ensure consumer protection [7].

Currently, methods including conformation-dependent assay [8], protein misfolding cyclic amplification (PMCA) [9], and combination with new ligands [10,11] coupled spectroscopic techniques [12] have been established. For example, Schmerr and co-workers developed a capillary electrophoresis-based non-competitive immunoassay [13], while Craighead and co-workers developed a nanomechanical resonator assays for prion protein detection [14]. The two methods, which are based on the recognition of antibody of prion protein, are sensitive, but some complicate functionlization steps are compulsory along with indispensable expensive apparatus. In addition, the affinity of the clearly identified antibodies of prion protein in native form is too low to be applied in practical conditions [7].

Binding study in aptamer with its target is a new strategy in molecular recognition chemistry [15–17], and therefore the aptamer that can specifically bind to prion protein with high affinity may be another new ligand in the detection of PrP. Efforts have been made in the screening of prion aptamers, especially in the selection of RNA aptamers [18–23]. It is known that RNA is susceptible to nucleases and cannot be applied in body fluids unless be modified [23]. In such case, DNA aptamers with the same binding affinity and specificity as RNA aptamers have to be selected to solve this problem. To our knowledge, there are only two research groups reporting the selection of DNA aptamers against PrP up to now [24,25] and only Irudayaraj and co-workers proposed a DNA aptamers based method in prion protein detection [26]. Their coupling of aptamer-mediated magnetic and gold-coated magnetic

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nanoparticles as detection assay for PrP^C is a smart success, but at least four functionalization steps are needed and thus their method should have to further consider how to overcome much more difficulties in real routine analysis.

It has reported that fluorescent dye including rhodamine, oxazine, fluorescein, Bodipy-FL, pyrene, stilbene, and coumarin dyes can be quenched by guanine *via* electron transfer when guanine is close enough [27,28]. That occurs to us to develop an aptamer-mediated turn-on fluorescence assay for the detection of PrP by designing an aptamer with haprin structure, whose 3′-terminal was seriated by three guanine bases and 5′-terminal was functionalized with tetramethyl-6-carboxyrhodamine (TAMRA). It was found that guanine bases could efficiently quench the fluorescence of TAMRA in the absence of target protein *via* electron transfer owing to the close proximity between guanine bases and TAMRA. In the presence of target protein, however, the interaction between aptamers and its target forces the guanine bases away from TAMRA, making the TAMRA-aptamer change into turn-on state, and thus the fluorescence of TAMRA gets restored as the results. It was found that PrP^C ranging from 1.1 to 44.7 μg/mL could be detected following our present design, showing that the designed aptamer-mediated fluorescence assay has the potential to be applied in routine analysis of PrP^C with the advantages of rapidness, specificity and sensitivity and possibly extends to abnormal prion protein (PrP^{Sc}) detection if security ensured.

2. Experimental

2.1. Materials

TAMRA-aptamer was synthesized by Beijing Sunbio-technology Co. (Beijing, China) and the complementary DNA was commercially available from Invitrogen Co. (California, USA). 2-(N-morpholino)ethanesulfonic acid (MES) was from Sino-American Biotech. (USA). 0.02 M MES buffer was prepared by dissolving 0.3905 g MES in 100 mL ultra-pure water (18.2 MΩ) and adjusted pH to 6.25 with NaOH. Human immunoglobulin (H-IgG) and thrombin were purchased from Sigma–Aldrich (Missouri, USA). Lysozyme, fibrin, pepsin, chymotrypsin and human serum albumin (HSA) were purchased from Shanghai Biotech. (Shanghai, China).

Other commercial reagents such as sodium chloride and guanidine hydrochloride were analytical reagent grade without further purification. All water used was ultra-purified with LD-50G-E Ultra-Pure Water System (Lidi Modern Waters Equipments Co., Chongqing, China).

2.2. Apparatus

The fluorescence of TAMRA-aptamer was measured with a F-2500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan), and the circular dichroism (CD) spectra were obtained by a J-810 spectropolarimeter (JASCO Co., Japan), while the fluorescence lifetime was measured with FL-TCSPC fluorescence spectrophotometer (Horiba Jobin Yvon Inc., France).

2.3. Preparation of recombinant human prion protein

The plasmid of recombinant human prion protein (23-231) was constructed according to Ref. [29] and expressed in *Escherichia coli* BL21 (DE3). A fresh overnight culture was used for inoculation in 0.5 L of 2× YT medium which contains 16 g trypton, 10 g yeast extract and 5 g NaCl. Incubation was performed at 37 °C and induced by 5 mg/mL isopropyl-D-thiogalactopyranoside. After 4 h, bacteria were harvested by centrifugation and sonicated in buffer A (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole, pH 8.0). The resulting solution was washed by buffer A containing 2 M NaCl, 0.5% Triton X-100 and 2 M urea and then incubated with buffer C (6 M guanidine hydrochloride, 0.5% Triton X-100, 10 mM β-mercaptoethanol, 10 mM Tris–HCl pH 8.0, 100 mM Na_2HPO_4) overnight. Followed to centrifuge at the speed of 12,000 rpm and 4 °C for 15 min, the soluble protein fraction was purified by nickel–nitrilotriacetic acid (NTA) agarose resin (Genview). The immobilized folding protein was refolded by applying a gradient of buffer C to buffer D (10 mM Tris–HCl, 100 mM Na_2HPO_4, pH 8.0) and extensively washed with 40 mL buffer D, then eluted with buffer E (20 mM NaAc, 150 mM NaCl, pH 4.0). The purified proteins were analyzed by SDS-polyacrylamide gel electrophoresis and its conformation was confirmed by its CD spectra.

2.4. General procedure

The interaction between TAMRA-aptamer and PrP^C or other proteins was carried out in the 0.02 M MES buffer (pH 6.25) containing 0.25 M NaCl after TAMRA-aptamer was denatured by incubating in boiling water for 10 min and then chilling down in ice water for 5 min. After that, certain concentration of proteins including target protein PrP^C and control proteins were added into denatured TAMRA-aptamer and the mixture was immediately transferred for the fluorescence measurements.

For calculation of quenching efficiency, 2.0×10^{-7} M TAMRA-aptamer was incubated with 5.0×10^{-7} M complementary DNA (cp-DNA) at 37 °C for 30 min in 0.02 M MES buffer (pH 6.25) containing 0.25 M NaCl.

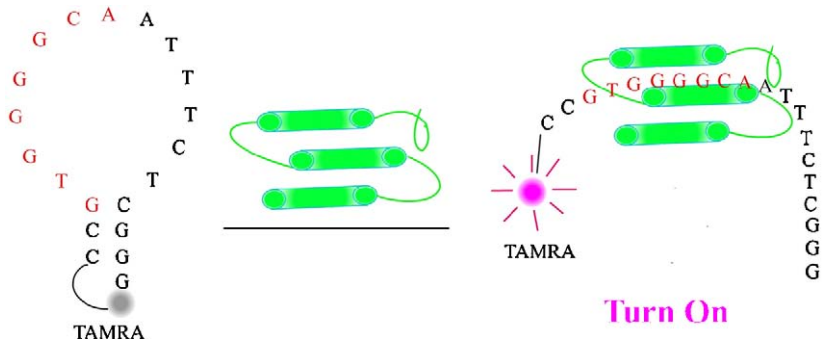
3. Results and discussion

3.1. State investigations of the designed TAMRA-aptamer

Scheme 1 displays our strategy for this contribution. As a new designed probe, the state of the designed TAMRA-aptamer including the quenching effect of guanine on the TAMRA, the conformation of the designed probe, is very important for our further applications and thus it should at first make some characteristics.

The quenching efficiency (Q) of guanine is related to the number of guanine and the distance away from fluorphor according to Ref. [28]. In our strategy, there are three guanine bases in the complementary stem of the one modified with TAMRA, thus the quenching efficiency of guanine is theoretically relative high. If we define the fluorescence of TAMRA-aptamer in haprin structure as F_0 and that in opening structure as F, then Q can be calculated as $(F - F_0)/F$. In this calculation, DNA complementary to the loop segment of TAMRA-aptamer was used to open the haprin structure of TAMRA-aptamer. The fluorescence intensity at 583 nm, which arises predominately from the TAMRA emission, was used to calculate the quenching efficiency (Fig. 1). It was found that the quenching efficiency was 76.6%, which is close to reference reports [30], suggesting that the design of our strategy is successful and might be application for the detection of PrP^C.

The CD spectrum of the designed TAMRA-aptamer was measured to confirm its conformation. It was found that the CD spectrum of TAMRA-aptamer in the absence of complementary DNA is different from that of its single-stranded complementary DNA (cp-DNA), which has a negative peak around 250 nm and a positive peak nearby 270 nm, as can be seen from Fig. 2. However, if TAMRA-aptamer hybridizes with its cp-DNA and forms a hybrid, the negative peak nearby 250 nm has an increase of the amplitude, which usually indicates an increase in helicity of DNA. The phenomenon suggests that the haprin structure of TAMRA-aptamer opened and double-stranded structure could have been formed. Moreover, it was found that the negative peak gets blue-shifted and



Scheme 1. Schematic presentation of PrP^C detection with aptamer-mediated fluorescence strategy.

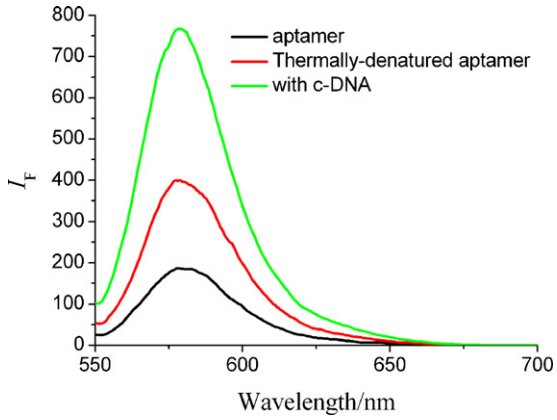


Fig. 1. Fluorescence emission of TAMRA-aptamer in different states and its hybrid with its complementary DNA (cp-DNA). TAMRA-aptamer, 2.0×10^{-7} M; DNA, 5.0×10^{-7} M; pH, 6.25; NaCl, 0.25 M.

positive peak red-shifted when TAMRA-aptamer coexists with its complementary DNA.

Since the core region of selected aptamer contains only eight nucleotides, the interaction between PrP^C and TAMRA-aptamer might be difficult to open the haprin structure. In such case, we should consider some thermal denaturation for the TAMRA-aptamer in order to promote the interaction between PrP^C and TAMRA-aptamer. Fig. 1 shows the fluorescence emission of TAMRA-aptamer in natural and thermally denatured states and the one of the hybridization with its complementary DNA in the presence of 0.25 M NaCl. It was found that the fluorescence of TAMRA-aptamer either in nature state or in thermally denatured state gets decreased in the presence of 0.25 M NaCl, indicating that NaCl can promote the formation of haprin structure. By comparing the fluorescence of TAMRA-aptamer in thermally denatured state in the presence of 0.25 M NaCl with that of the hybrid with cp-DNA and the one in nature state, we could conclude that the thermally denatured step just opens the haprin structure to some extent in the presence of 0.25 M NaCl, the reason we speculate is that there is a balance between TAMRA-aptamer anneal in the presence of 0.25 M NaCl and thermally denatured. And the coexistence of the thermally denatured TAMRA-aptamer with 0.25 M NaCl might make the interaction between PrP^C and TAMRA-aptamer easier.

3.2. *Spectral feature of the interaction between designed TAMRA-aptamer and PrP^C*

Fig. 3 shows the results by incubating denatured TAMRA-aptamer with different concentration of PrP^C. In the absence of PrP^C, the aqueous medium has very weak fluorescence emission because the fluorescence of TAMRA gets quenched resulting from the enough proximity of guanine at the 3′-terminal to the TAMRA group located at 5′-terminal of TAMRA-aptamer. With the addition

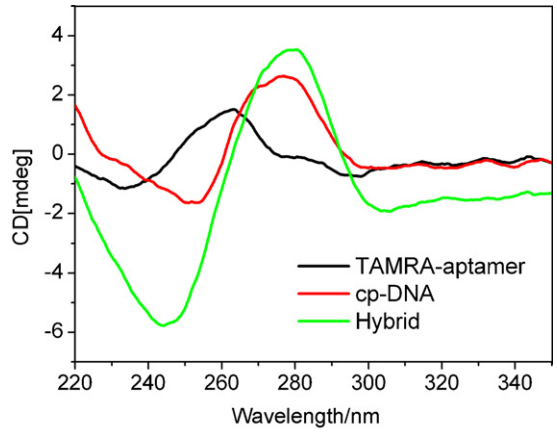


Fig. 2. CD spectra of TAMRA-aptamer (black), its complementary single-stranded sequence (cp-DNA, red) and their hybrid (green). TAMRA-aptamer, 1.0×10^{-6} M; complementary DNA, 2.0×10^{-6} M; pH, 6.25; NaCl, 0.25 M. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

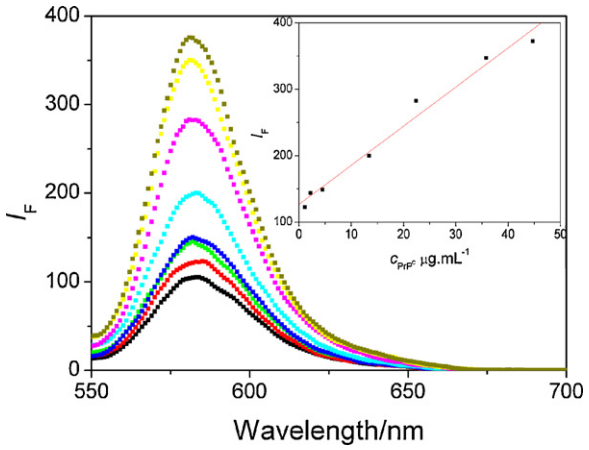


Fig. 3. The fluorescence emission of TAMRA-aptamer with increasing PrP^C. Inserted is the calibrated curve of PrP^C, which can be expressed as $I_F = 126.5 + 5.9c_{PrP}$ in the range from 1.1 to 44.7 μg/mL with the correlation of $R = 0.991$ and the limit of detection (3σ) of 0.3 μg/mL. Denatured TAMRA-aptamer, 1.0×10^{-7} M; pH, 6.25; NaCl, 0.25 M.

Table 1
Fluorescence lifetimes (τ_t) obtained with two-exponential fit of the fluorescence decay curves.

Sample and concentration of PrP^C	τ_1 ($\times 10^{-10}$ s)	τ_2 ($\times 10^{-9}$ s)	A_2/A_1 [a]
Aptamer	6.26	2.93	1.34
Aptamer plus 2.24 μg/mL PrP^C	6.89	3.04	1.35
Aptamer plus 13.41 μg/mL PrP^C	8.87	3.61	2.21

[a] A_2/A_1 represents for the ratio of the percentage of τ_2 and τ_1.

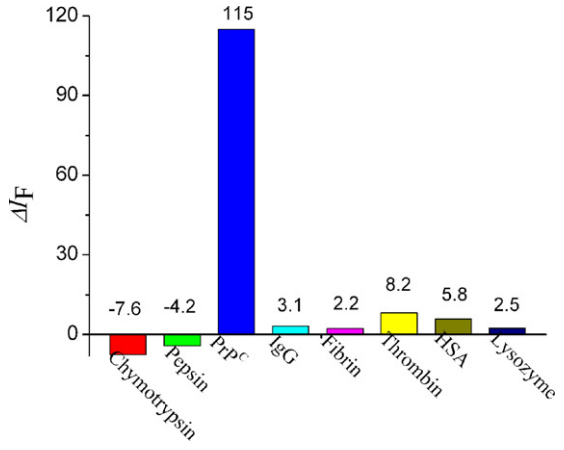


Fig. 4. Specificity of aptamer-mediated fluorescent assay. IgG, chymotrypsin, lysozyme, fibrin, HSA and pepsin were 100 μg/mL, while thrombin were 30.53 μg/mL and PrP^C 17.88 μg/mL; pH, 6.25; NaCl, 0.25 M.

of target protein PrP^C, however, the interaction between PrP^C and TAMRA-aptamer induces TAMRA-aptamer into a turn-on state and the guanine bases are forced away from the TAMRA group with the results of the fluorescence getting restored gradually. It was found that the restored fluorescence intensity follows a linear relationship, which could be expressed as $I_F = 126.5 + 5.9c_{PrP}$ in the range from 1.1 to 44.7 μg/mL with the correlation of $R = 0.991$ and the limit of detection (3σ) of 0.3 μg/mL.

3.3. Mechanism of the interaction of between TAMRA-aptamer and PrP^C

In order to confirm the mechanism involving in the interaction of TAMRA-aptamer and PrP^C, we measured the fluorescence lifetime. Table 1 shows the fluorescence lifetime obtained with two-exponential fit of the fluorescence decay curves. In the absence of PrP^C, TAMRA-aptamer has two lifetimes belonging to the turn-off (τ_1) and turn-on (τ_2) state, respectively. With the addition of PrP^C, the fluorescence lifetime gets increased gradually and τ_1 increase from 0.626 to 0.887 ns and τ_2 from 2.93 to 3.61 ns. Meanwhile, the ratio of the percentage of τ_2 and τ_1 gets increased from 1.35 to 2.21, corresponding to the increasing percentage of TAMRA-aptamer in turn-on state.

The specificity of designed TAMRA-aptamer-mediated fluorescence assay was also examined using other proteins. About 2- to 5-fold of concentration of IgG, chymotrypsin, lysozyme, fibrin, thrombin, HSA and pepsin were incubated and detected individually with the TAMRA-aptamer-mediated fluorescence assay. The peak currents were shown in Fig. 4, it can be seen that no significant cross-reactivity was detected for these proteins and expected that the developed TAMRA-aptamer-mediated fluorescence assay could exhibit a high degree of selectivity for PrP^C detection.

4. Conclusions

In summary, we proposed a cost-effective aptamer-mediated fluorescence assay for the detection of PrP^C. The fluorescence of designed aptamer was quenched by guanine in the absence of target protein PrP^C and TAMRA-aptamer is in turn-off state. In the presence of PrP^C, the interaction between PrP^C and TAMRA-aptamer forces guanine base at 3′-terminal away from TAMRA functionalized at 5′-terminal and TAMRA-aptamer then gets in turn-on state, thus the fluorescence restored. We suppose that the aptamer-mediated fluorescence assay has the potential to apply in routine analysis of PrP^C with the advantages of specificity and sensitivity, and this strategy can also be extended to the detection of PrP^{Sc} if security ensured and other proteins whose aptamers had been selected.

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