Demonstration of G-quadruplex-assisted hybridization chain reaction for nucleic acid detection

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***Abstract*—In recent years, the hybridization chain reaction (HCR) has been proposed as an alternative to polymerase chain reaction (PCR) for diagnosis. Unfortunately, the sensitivity of the HCR methods are still far below PCR and researchers focus on ways to improve it by investigating different HCR designs. While earlier designs exploit fluorescently labelled probes for detection, here we propose an HCR system that combines G-quadruplex formation and fluorescence for detection of single stranded DNA sequences with concentrations as low as 20 pM. We show that G-quadruplex-mediated oxidation of Amplex Red results in fluorescence increase and lowers the detection limit by about 10-fold, in comparison to HCR using only fluorescently labelled HCR probes.**

Keywords— G-quadruplexes, hybridization chain reaction, nucleic acid detection, signal amplification.

I. Introduction

Hybridization chain reaction is an enzyme-free method for nucleic acid detection. In this procedure, the target DNA/RNA initiates a hybridization cascade between two or more hairpin-formed sequences through a toehold mediated strand displacement to realize signal amplification [1]. Hybridization chain reaction (HCR) provides a feasible solution for nucleic acid detection without target amplification.

HCR is based on the release of potential energy stored in the form of hairpins. The HCR system contains a number of hairpin forming DNA sequences, in which each hairpin has a self-complementary stem, a loop and a flanking toehold sequence. (Figure 1) In principle the hairpins share partially complementary sequences however, their hybridization is blocked by an already occupied stem sequence. Only upon the addition of the trigger which is a single stranded DNA/RNA initiator, the first hairpin molecule unwinds, releasing one strand of the stem. The triggers sequence achieves this due to its larger complementary sequence. The unwinded first hairpin component is now free to trigger hybridization with another component of the HCR system. This process continues until a large number of hairpin components are joined in formation of an elongated or sometimes, dendrimeric complex. [2]

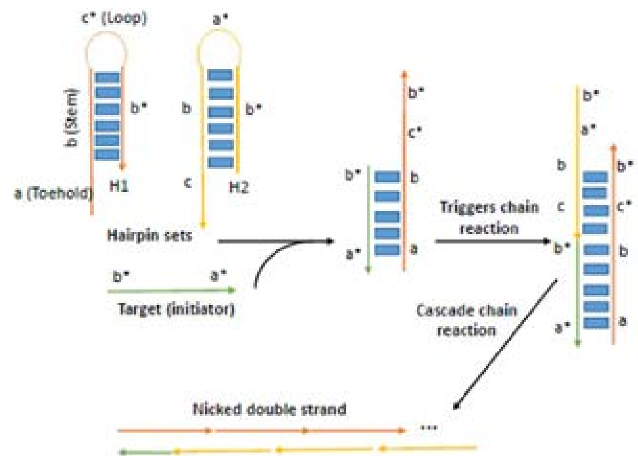

Fig. 1. An earlier HCR design by Dirks and Pierce [3]

The detection of the HCR may rely on different techniques. While fluorescence is very common, there have been several other approaches to detect the HCR product, including electrochemistry [4], chemiluminescence [3], and colorimetric methods [3].

HCR presents to be an alternative to PCR, however, the identification is provided by sequence specific hybridization of short oligonucleotides to a bigger target instead of enzymatic amplification. As in PCR, HCR provides nucleic acid detection at low concentrations, and provides some advantages in

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comparison. First of all, HCR may be performed at room temperature and does not require a thermal cycler for the reaction to complete [5]. The lack of equipment enables the HCR to be performed in the field, and removes the initial setup cost for DNA detection.

Another advantage is the lack of enzymes for HCR to happen. Without the need of enzymes, the cost of reaction is lower and cold storage of the components is unnecessary. However, claims have been made that the enzyme amplified HCR could improve the sensitivity which are shown to be comparable to those of PCR based assays. However, the vulnerability of enzymes greatly limits the applications of enzyme amplified HCR nucleic acid assays in complex biological samples. The design of the hairpins should be thermodynamically more quantitative with regard to the energy stored in the hairpins and the interaction between the hairpins and the target, since a relatively small variation may adversely affect the amplification efficiency of HCR or even fail to initiate HCR at all [5].

On the other hand, HCR has its own disadvantages. In comparison to PCR, HCR does not provide similar levels of sensitivity. To be a legitimate contender, various enzymatic amplification schemes were therefore coupled to HCR to enhance the assay sensitivity significantly [5]. However, no HCR system managed to achieve sensitivities equivalent to the PCR technique.

Here we introduce a novel HCR system that exploits G-quadruplex formation and G-quadruplex-mediated Amplex Red (™) reduction reaction for detection of the trigger sequence. It is our aim to stimulate formation of the intermolecular G-quadruplexes between HCR components in the presence of the trigger, and the G-quadruplexes were then allowed to reduce non-fluorescent Amplex Red molecules and convert them into fluorescently active resofrin. The fluorescence was detected using fluorescence spectrometry. Although we successfully detected the trigger sequence at concentrations as low as 100 pM, contrary to our expectation, the fluorescence decreased with increasing trigger sequence, instead of increasing, indicating that a more complex mechanism is undergoing.

II. HCR DESIGN

We designed four different oligonucleotide sequences which are called GH1, GH2f, GH3, GH4 and also GT as the trigger molecule. (Table 1) In the absence of the GT, trigger sequence, the GH1-4 are expected to form unimolecular hairpins. In the presence of GT, the GH1 is expected to bind HT, unwinding its stem and presenting the other half of the sequence in the solution. Next, GH2f is expected to bind the unwound half of the GH1. As GH2f is no longer in hairpin mode, the fluorescent moiety FAM is distanced from the quencher moiety, BHQ, and increases in fluorescence at 520 nm. The unwinding of GH2f, releases half of the strand, making it available for binding by GH3, and then GH4. GH4, containing the sequence identical to GT at the 5' end, continues the chain reaction by binding another GH1 in the solution. The process continues until the equilibrium is reached. the equilibrium is expected to shift exponentially in relation to the GT concentration.

TABLE 1. The DNA sequences used in this study. The bold nucleotides indicate guanines that are expected to participate in formation of G-quadruplexes.

component name	**Sequence (5'-3')**
GH1	5'AAGATGGAAGGAGGCCCAGTTTCATGGGCCTCC TTC**GGG**A -3'
GH2f	5'/FAM/TGGGCCTCCTTCCGTTTTGAAGGAGGCCC ATGAAAC/BHQ-1/-3'
GH3	5'A**GGG**TA**GGG**C**GGG**AAAACGGAAGGAGGCCCA CCATTATGGGCCTCCTTC-3'
GH4	5'TGGGCCTCCTTCCATCTTGAAGGAGGCCCATAA TGG -3'
GT	5'TGGGCCTCCTTCCATCTT -3'

Both GH1 and GH3 are designed with extra flanking sequences at 3' and 5' ends, respectively. These flanking sequences contain guanine stretches. While there is one 3nt guanine stretch on GH1, the GH3 has three. When GH1 and GH3 are bound to GH2f at the equilibrium, aforementioned stretches are expected to form intermolecular G-quadruplex structures. [6] (Fig. 2.)

After reaching the equilibrium, hemin is added to the solution to bind the newly formed G-quadruplexes. This G-quadruplex-hemin complex is known to have horseradish peroxidase-like enzymatic activity. [7] Briefly, this complex can react with Amplex Red (™) in the presence of H2O2, and produce resofrin, a fluorescent molecule that emits light at 580 nm. Our design aimed to detect GT concentration through this emission. In order to increase sensitivity, we have chosen to use förnster resonance energy transfer between FAM and resoferon to detect the signal by exciting FAM at 490 nm. This enabled the excitement of resofrin only by FAM in close proximity if the GH2f hairpin is unwound.

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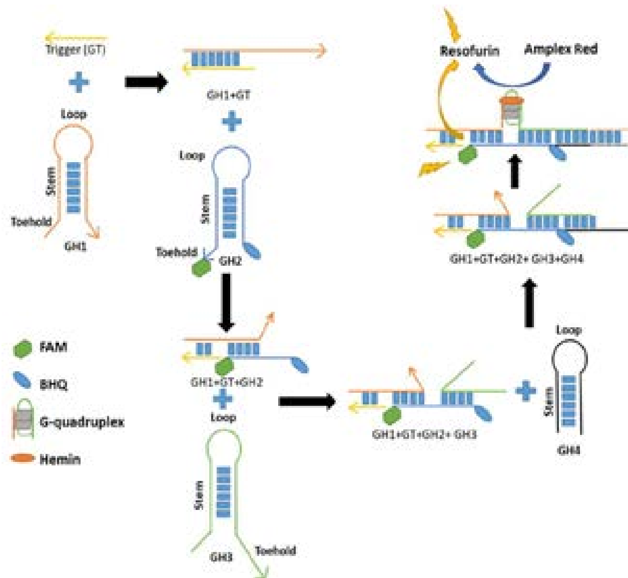

Fig. 2. G-quadruplex-assisted hybridization chain reaction designed in this study.

III. METHODS

The DNA sequences were obtained commercially and stocks were prepared at 100 µM. The GH mix is prepared by adding each GH component at 5 µM in 5X SSCT buffer containing %0.1(v/v) Tween20, 75 mM sodium citrate, 750 mM sodium chloride and 150 mM potassium chloride at pH 7. The GH mix preparation was completed with heating up to 85°C for 10 minutes, then quickly put in ice for 10 minutes. The GH mix was then incubated in the presence of varying GT concentrations overnight. After overnight incubation the GH mix was diluted further with a 5x SSCT buffer containing 50 nM hemin, 200 µM H_2O_2 and 20 µM Amplex Red. All fluorescence measurements were performed with excitation at 490nm and with bandwidths of 5nm.

IV. RESULT AND DISCUSSION

Prior to the addition of the Amplex Red, the FAM fluorescence was measured. The FAM fluorescence showed dramatic difference between in the presence of 50 nM and 200 uM trigger sequence. (Figure 3) Unfortunately no significant difference was detected between 0, 2 and 20 pM.

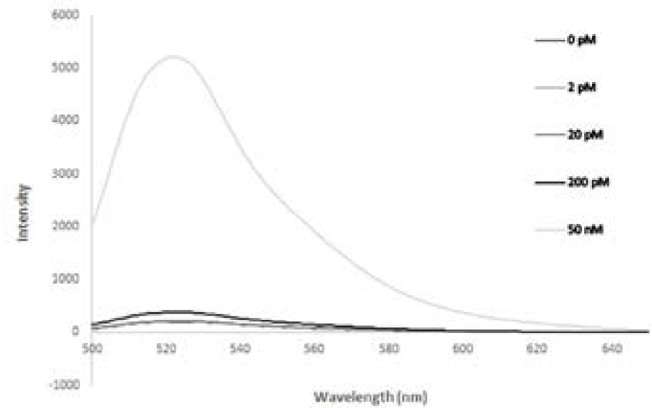

Fig. 3. Fluorescence obtained at different GT concentrations prior to addition of Amplex Red.

Although FAM fluorescence by itself was not enough to distinguish pM level GT concentrations, it became apparent upon addition of Amplex Red. After 15 min of reaction time, the FAM fluorescence decreased dramatically and a new peak appeared belonging to resorufin. (Figure 4) The produced resorufin was detectable through FRET as FAM was excited at 490 nm. While 2 pM was still not distinguishable from 0 pM, higher concentrations showed increasing fluorescence.

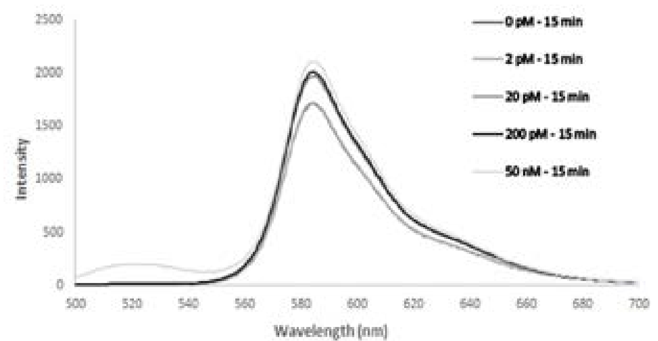

Fig. 4. Fluorescence obtained at different GT concentrations after addition of Amplex Red and allowed to react for 15 minutes.

The ability to achieve FRET indicates that the FAM has maintained close proximity to resorufin. This could be attributed to possible intercalation of resorufin into DNA due to its aromatic structure.

Unfortunately, no linear range was detected and further studies are required to investigate linear detection limits. However, as it stands, qualitative detection was achieved for concentrations as low as 20 pM.

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