Amplified detection of nucleic acid by G-quadruplex based hybridization chain reaction

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A protein-free, isothermal, self-amplified nucleic acid sensing system which was a G-quadruplex integrated hybridization chain reaction (GQ-HCR) system was developed. The G-quadruplex was closed two-thirds in the loop and one-third in the stem of one of the GQ-HCR hairpin probes. In the absence of the target molecule, the GQ-HCR probes stayed as inactive meta-stable hairpin structures and the G-quadruplex was inert. Reversely, the GQ-HCR probes could be cross-opened to start a hybridization chain reaction and the closed G-quadruplex could be released to be free when the GQ-HCR probes came across the target molecule. The GQ-HCR nucleic acid sensing system could detect as low as 7.5 nM ssDNA or RNA by the colorimetric method and 4 nM ssDNA by the fluorometric method. Less than 10 copies of dsDNA template could also be detected when PCR was combined with the GQ-HCR system (PCR + GQ-HCR). Because of these advantages, the GQ-HCR system was also studied for application in visual chip detection to obtain a satisfactory repeatable and specific result.

1. Introduction

Molecular amplification methods of nucleic acids including DNA and RNA are of vital importance to clinical diagnosis, mutational analysis, and gene therapy. They fall into two classes, enabling either template or signal amplification. Template amplification methods include polymerase chain reaction (PCR; Mullis and Faloona, 1987), ligase chain reaction (LCR; Barany, 1991), nucleic acid sequence based amplification (NASBA; Compton, 1991), rolling-circle amplification (RCA; Liu et al., 1996), loop-mediated isothermal amplification (LAMP; Notomi et al., 2000), and strand displacement amplification (SDA; Walker et al., 1992), which are very sensitive and compatible with fluorescence, colorimetry, or chemiluminescence. However, these methods are based on template replication, which increase the risk of cross-contamination from amplicons, so false-positive results frequently occur (Zou et al., 2011). To date, there have been several strategies based on signal amplification to selectively detect nucleic acids, including branched DNA (bDNA; Collins et al., 1997), invasive signal amplification, nicking endonuclease signal amplification (NESA; Kiesling et al., 2007), and hybridization chain reaction (HCR; Dirks and Pierce, 2004). Initial work by Dirks and Pierce has demonstrated a hybridization chain reaction, in which hairpins with overlapping partial complementarities were used to construct a reaction cascade that resulted in the formation of double-stranded DNA polymers up to thousands of base pairs long. HCR is a real protein-free isothermal amplification technique that relies on the self-assembly of two hairpin probes. A lot of effort based on chemical modification has been put into optimization of HCR. Most of these works concentrated on the signal reporters, to make HCR a more applicable amplification method: e.g. nano-gold particle (Pierce et al., 2006), pyrene molecule (Huang et al., 2011), fluorophore/quencher (FAM/Dabsyl; Chemeris et al., 2008), and biotin (Niu et al., 2010) modified reporter probes.

Lately, Itamar Willner’s group was the first to combine DNAzyme with HCR and achieved high detection sensitivity (Wang et al., 2011). They divided the Mg2+-dependent RNA phosphoesterase (E6; Breaker and Joyce, 1995) into two subunits which were tethered onto two DNA hairpin probes. In the presence of a target, both probes could open successively, and separated DNAzyme subunits could be reunited in the final DNA polymer. Upon binding to the fluorophore/quencher modified substrate, the reunited DNAzyme could cleave the RNA phosphoester bond of the substrate to produce fluorescence. This amplification method leads to the analysis of the target DNA with a sensitivity corresponding to 10−14 M. Earlier this year, they published another work which reported a combination of HCR probes and the divided peroxidase-like G-quadruplex DNAzyme based on a similar strategy (Shimron et al., 2012).

Unlike the splitting-DNAzyme strategy developed by Willner’s group, a new protein-free, isothermal and self-amplified
sensing system for nucleic acid detection that is based on locking the intact G-quadruplex sequence into hairpin probes of hybridization chain reaction is proposed here (GQ-HCR system, Scheme 1). This approach is another innovative trial to combine HCR with functional G-quadruplexes, which are G-rich nucleic acid sequences and are capable of forming a four-stranded structure. A special DNAzyme adopting a G-quadruplex structure utilized as the signal reporter of our system has been found to exhibit peroxidase-like activity in the presence of hemin (Li and Sen, 1996, 1998; Travascio et al., 2001). This DNAzyme has been explored for the design of various colorimetric or chemiluminescent assays in recent years (Kosman and Juskowiak, 2011). Besides their catalytic ability, some G-quadruplex sequences could bind with porphyrin molecules, such as NMM (Zhao et al., 2011) or ZnPPIX (Li et al., 2009; Li et al., 2010) to greatly enhance the fluorescence intensities of those molecules.

2. Experimental

2.1. Materials

All DNA sequences (Table S1, Supporting information) were synthesized and PAGE purified by Shanghai Sangon Biological Engineering Technology & Services (Shanghai, China), and dissolved in sterile ultrapure water to give stock solutions. Hemin was purchased from Alfa Aesar. Taq DNA polymerase, Taq buffer and dNTP were purchased from Beijing TransGen Biotech Co., Ltd. (Beijing, China). Lambda exonuclease was bought from Fermentas. ABTS was purchased from Wolsen (Xi’an, China). H2O2 was purchased from Bodi Chemical Holding Co., Ltd. (Tianjin, China). ZnPPIX was purchased from Strem Chemicals and Tyramine-HCl was purchased from Astatech (Chengdu, China). Hemin and ZnPPIX stock solutions of 5 mM were prepared with DMSO and ABTS (3.8 mM) and H2O2 (1.5 mM) were added to the GQ-HCR product and mixed completely. The color produced by DNAzyme catalyzed oxidation of ABTS could be observed afterwards. It should be mentioned that the RNA detection experiments were performed in a separate RNA-work area. And a set of reagents and consumables were prepared exclusively for RNA work. Pipettes, tubes and other plastic-ware were twice-autoclaved. Water was treated with 0.1% DEPC overnight and autoclaved twice. 5 × Tris/HCl–Na buffer (Tris 100 mM, NaCl 2 M, pH 7.2) separately, and then heated to 95 °C for 2 min and cooled down slowly to room temperature for at least 1 h before use. 20 μl GQ-HCR reaction mixtures with different amounts of the target molecule T or Tr and the pretreated GA1 and GA2 were prepared. The final concentration of each general component in the reaction solution is Tris 20 mM, pH 7.2, NaCl 400 mM, GA1 0.5 μM and GA2 0.5 μM. The reaction was performed for 1 h at 37 °C. After the GQ-HCR reaction, KCl (30 mM), hemin (1.2 μM), triton X-100 (0.002%), ABTS (3.8 mM) and H2O2 (1.5 mM) were added to the GQ-HCR product and mixed completely. The color produced by DNAzyme catalyzed oxidation of ABTS could be observed afterwards.

2.2. Instruments

Fluorescence emission measurements with ZnPPIX as the fluorophore were carried out on a Cary Eclipse Device (Varian Inc.). ZnPPIX was excited at 420 nm. The emission spectra were recorded from 550 to 670 nm (smoothing type: Savitzky-Golay, filter size 9). Real-time detection of the fluorescence produced by oxidized Tyramine-HCl in the PCR+GQ-HCR system was performed with a Varioskan Flash (Thermo Scientific). The excitation wavelength was 320 nm and emission wavelength was 410 nm. About 20 readings with a 2 min interval were recorded. PCR was performed on a C1000 thermal cycler (Bio-Rad).

2.3. Colorimetric detection of ssDNA or RNA

Probes GA1 and GA2 were mixed with 5 × Tris/HCl–Na buffer (Tris 100 mM, NaCl 2 M, pH 7.2) separately, and then heated to 95 °C for 2 min and cooled down slowly to room temperature for at least 1 h before use. 20 μl GQ-HCR reaction mixtures with different amounts of the target molecule T or Tr and the pretreated GA1 and GA2 were prepared. The final concentration of each general component in the reaction solution is Tris 20 mM, pH 7.2, NaCl 400 mM, GA1 0.5 μM and GA2 0.5 μM. The reaction was performed for 1 h at 37 °C. After the GQ-HCR reaction, KCl (30 mM), hemin (1.2 μM), triton X-100 (0.002%), ABTS (3.8 mM) and H2O2 (1.5 mM) were added to the GQ-HCR product and mixed completely. The color produced by DNAzyme catalyzed oxidation of ABTS could be observed afterwards.

It should be mentioned that the DNA detection experiments were performed in a separate RNA-work area. And a set of reagents and consumables were prepared exclusively for RNA work. Pipettes, tubes and other plastic-ware were twice-autoclaved. Water was treated with 0.1% DEPC overnight and autoclaved twice. 5 × Tris/HCl–Na buffer was prepared with sterile, DEPC treated water and re-autoclaved twice before use. RNA targets were dissolved in sterile, DEPC treated water. All solutions were subdivided into several smaller aliquotes and stored at

Scheme 1. GQ-HCR system. (A) The structure of the hairpin probes. The target could be single stranded DNA or RNA. Sequences marked with the same letter with or without “*” are complementary to each other. The solid circles “· · ·” stand for mismatches. The red line in GA1 (g1 and g2) is the closed G-quadruplex (two-thirds in the loop and one-third in the stem of probe 1). (B) The detailed process of GQ-HCR amplification. In the absence of target, two probes GA1 and GA2 keep stable hairpin structures and G-quadruplex is locked. Once the target is present, probes GA1 and GA2 are opened successively and hybridization chain reaction starts to yield DNA polymers bearing a lot of G-quadruplexes in between. (For interpretation of the references to color in this scheme, the reader is referred to the web version of this article.)
—80 °C. Each aliquot of solution was used only once. In the whole RNA detection process, contamination from environment and experimenter was prevented with the utmost effort.

2.4. Fluorometric detection of ssDNA using ZnPPIX as fluorophore

Pretreated GA1 and GA2 hairpin probes were prepared as above. 80 μl QG-HCR reactions of pretreated hairpin probes GA1 (0.5 μM) and GA2 (0.5 μM) and different concentrations of target T were performed with Tris/HCl buffer (20 mM, pH 7.2) and NaCl (1 M) for 1 h at 37 °C. After that, KCl (50 mM) and ZnPPIX (1 μM) were added into the reaction mixture. Then, the final mixed solution was excited at 420 nm and the fluorescence spectrum was recorded from 550 to 670 nm (smoothing type: Savitzky-Golay, filter size 9) on a Cary Eclipse Device (Varian Inc.).

2.5. Fluorometric detection of dsDNA with tyramine

At first, the analyse TM which contains the target sequence T was amplified by PCR. The PCR was carried out in the presence of different amounts of templates, primer1 (0.5 μM), primer2 (5'-phosphorylated, 0.5 μM), dNTP (200 μM), Taq DNA polymerase (2.5 units) in Taq buffer (20 mM Tris–HCl (pH 8.4), 20 mM KCl, 10 mM (NH₄)₂SO₄ and 2 mM MgSO₄), sterile water was added to make a final volume of 50 μl. The PCR procedure was as follows: 95 °C for 30 s; 95 °C 30 s, 50 °C 30 s, 72 °C 30 s, 40 cycles; and 72 °C 5 min. Then, each 50 μl PCR product was digested by 10 units of Lambda exonuclease for 1 h at 37 °C. After the treatment of Lambda exonuclease, pretreated probes GA1 and GA2 (0.5 μM) with NaCl (400 mM), (NH₄)₂SO₄ (90 mM), hemin (0.2 μM), Tyramine-HCl (0.8 μM), and H₂O₂ (0.8 μM) were added into the 50 μl digested PCR mixture. The real-time fluorometric detection was carried out in a Varioskan Flash (Thermo Scientific) machine (30 °C) and around 20 readings with a 2 min interval were recorded. The excitation wavelength was 320 nm and emission wavelength was 410 nm.

2.6. Visual-chip based assay of dsDNA

Visual-chip preparation: the carrier was made by a 1 mm thick transparent plastic sheet. The plastic sheet was washed thoroughly with detergent first and rinsed extensively with water, then immersed in H₂O₂ (3%) for 10 min and extensively rinsed with sterile ultra-pure water again and finally dried prior to use. 10 μl of PCR product for 1 h at 37 °C. After chain reaction, hemin (1.2 μM), ABTS (9 mM) and H₂O₂ (3 mM) were added in the drops and color differences were observed.

3. Results and discussion

3.1. Principle of GQ-HCR system

The principle of the GQ-HCR amplification is illustrated in Scheme 1. Two hairpin probes (GA1 and GA2) are designed according to the target sequence T (Scheme 1A), both with an additional 6 nucleotide (nt) sticky end. Complementary sequences are marked by the same letter with or without asterisk, such as a*-b* of probe GA1 is complementary to a-b in target T. A G-quadruplex sequence CatG4 (Kong et al., 2009; red color, Scheme 1A) is caged in the hairpin probe GA1. Two-thirds of the CatG4 sequence (g2, 16nt) is locked in the loop and one-third (g1, 6nt) is concealed in the stem which is connected to the end of the target recognition region with a mismatch base pair in between. The structures of the two probes were designed with the aid of NUPACK software (Zadeh et al., 2011). The CatG4 sequence in the closed hairpin probe GA1 cannot form a G-quadruplex structure in the absence of a target molecule. However, when the target is present, the probe GA1 could be opened instantly. The g1* of GA1 is too short to prohibit the G-quadruplex sequence in the probe to form the intra-molecular quadruplex structure, which is consistent with the structure-switching mechanism (Nutiu and Li, 2003). So probes GA2 and GA1 are opened successively and the hybridization chain reaction could proceed to obtain DNA polymers bearing a lot of G-quadruplexes in between (Scheme 1B).

3.2. Colorimetric detection of ssDNA or RNA

Initially, we investigated the self-assembled complex formation of the target T and probes GA1 and GA2. Agarose gel demonstrates that a set of long chain polymers with different molecular weights are formed when single-stranded DNA target T was introduced to trigger the chain reaction (Fig. 1). The average molecular weight of resulting polymers is inversely related to the initiator concentration, which is consistent with that of the original hybridization chain reaction (Dirks and Pierce, 2004). To prove the formation of G-quadruplex as the hybridization chain reaction proceeds, hemin, H₂O₂, and ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)) were added into the mixture at 37 °C for 30 min. After chain reaction, hemin (1.2 μM), ABTS (9 mM) and H₂O₂ (3 mM) were added in the drops and color differences were observed.
after hybridization chain reaction for 1 h at 37°C (Fig. 2A). ABTS could only be oxidized into a green colored product when target T was added into the GQ–HCR system. The intensity of the color is proportional to the concentration of the ssDNA target (Fig. S5, Supporting information).

But in living organisms except for certain viral genomes and replication intermediates, nearly all the DNA occurs as complete duplexes, which could not initiate the HCR reaction at all. On the other hand, most RNA in organisms is single stranded. Thus, we investigated the applicability of our GQ-HCR system on RNA targets. Here, a RNA target Tr with the same sequence as that of DNA target T was used and the result confirmed our anticipation.

With increasing concentration of RNA targets Tr, the color intensity of the reaction solutions presented a trend from almost colorless to light green to dark green (Fig. 2B). As little as 7.5 nM target RNA molecules could be detected by naked eyes in a 20 μl reaction mixture. The endpoint colorimetric detection is a very convenient way to realize the spot test for single stranded DNA or RNA target to get qualitative results; and the endpoint UV absorption detection at 414 nm makes the quantitative analysis of target nucleic acid possible.

3.3. Fluorometric detection of ssDNA using ZnPPIX as fluorophore

One shortcoming of the colorimetric detection is that the colorful oxidized products of ABTS fade away quickly because of photo-bleaching. Therefore, to achieve more accurate quantitative detection of the single-stranded nucleic acid targets, fluorometric assay of GQ–HCR was adopted by using ZnPPIX as the fluorophore. In the absence of G-quadruplex, ZnPPIX has very low intensity of fluorescence at 590 nm (excited at 420 nm). Contrarily, ZnPPIX upon binding to G-quadruplex dramatically enhances the fluorescence signal that can be easily monitored by a fluorometer (Fig. 3A, B). The inset is a calibration curve with fluorescence intensity data at 590 nm obtained from the spectrum, corresponding to different target concentrations (experiments were conducted in triplicate and the average values were used for plotting). The calibration curve indicates that there is a good linear relationship between fluorescence intensity and the concentration of target molecules, especially in the range 20–120 nM ($R^2 = 0.9937$). The detection limit is about 4 nM ($3\sigma/S$, in which $\sigma$ is the standard deviation for the blank solution, $n=3$, and $S$ is the slope of the calibration curve).

3.4. Principle of PCR+GQ-HCR system

In consideration of only single stranded nucleic acid molecules which could be detected and the relatively unsatisfactory sensitivity reached with the GQ-HCR system, we attempted to combine polymerase chain reaction (PCR) with the GQ-HCR system to expand its scope of application to double stranded DNA targets and make GQ-HCR more applicable in genetic detection (Scheme 2). PCR is an ideal way to amplify trace amounts of single or double stranded nucleic acids. The length and quantity of PCR product could be controlled precisely through sophisticated design of two primers and a number of thermal cycles. However, the PCR product is also double stranded DNA, which cannot trigger the cascade of hybridization chain reaction.

So one of the PCR primers was 5’-phosphorylated to obtain the duplex product in which all antisense sequences of target DNA

![Fig. 2.](image)

Colorimetric detection of nucleic acid. (A) Schematic presentation of the colorimetric detection of GQ-HCR product. Hemin, ABTS and H2O2 were added after the GQ-HCR reaction. After binding with hemin, G-quadruplex catalyzes the oxidation of ABTS by H2O2 to a green product which is observable to the naked eye. (B) Photograph of the colorimetric detection of different amounts of RNA target Tr (UCUCCACACACGACACGGCUAGAACCACU): Tube 1, H2O; Tube 2, negative control (no target); Tubes 3–8 containing 7.5 nM, 15 nM, 30 nM, 50 nM, 75 nM, and 100 nM of target RNA molecules, successively.

![Fig. 3.](image)

Fluorometric detection of ssDNA. (A) Schematic presentation of the fluorometric detection of GQ-HCR product. After binding with G-quadruplex, the fluorescence of ZnPPIX could significantly increase ($\lambda_{em} = 420$ nm, $\lambda_{em} = 590$ nm). (B) Fluorescence spectra (excited at 420 nm) of GQ-HCR reactions in the presence of different concentrations of target T. The GQ-HCR reaction was carried out for 1 h at 37°C before adding 1 μM ZnPPIX and 50 mM KCl for fluorescence detection. The inset was a calibrated curve of the average fluorescence intensity at 590 nm obtained from the spectrum data and the error bars were determined by standard deviation (SD) of the triplicate data.
would contain phosphate groups at the 5’-ends. Lambda exonuclease is a highly processive 5’-3’ exodeoxyribonuclease that can selectively digest the 5’-phosphorylated strand of duplex DNA (Avci-Adali et al., 2010). After digestion of the undesired strand, the PCR product becomes single stranded which could initiate the GQ-HCR system and realize the detection of double stranded DNA target (Scheme 2).

### 3.5. Fluorometric detection of dsDNA with tyramine

When we employed the PCR+GQ-HCR system to detect dsDNA, we found that both Lambda exonuclease digestion reaction and GQ-HCR were compatible with the PCR buffer, but the fluorophore ZnPPIX could not be applied in this method because materials like detergents in the storage and reaction buffer of Taq DNA polymerase and Lambda exonuclease seriously affect the sensitivity and resolution of fluorometric assay based on ZnPPIX. Therefore, the GQ-HCR system is an ideal platform to develop visual-chip based nucleic acid detection.

3.6. Visual-chip based assay of nucleic acids

One major merit of GQ-HCR amplification is the protein-free property. Just two unmodified DNA probes are enough to realize the signal amplification and signal report. Unlike protein enzymes which usually need to be kept in appropriate buffer at low temperature to maintain activity, DNA molecule is readily synthesized by chemical methods with excellent accuracy and reproducibility, and is very stable to denaturation (induced by temperature, for example). Therefore, the GQ-HCR system is an ideal platform to develop visual-chip based nucleic acid detection.

- Fig. 4B all the circles on the chip have been printed with the probes (GA1 and GA2) on the visual chip for the colorimetric reaction. Three samples on each column are the same. NC: no template in the PCR reaction; nucleobases in Tm1–3 which differ from those in T sequence are marked in red. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)
visual-chip based assay (Fig. 4B). Obviously, the colors of three replicates of the same sample present are quite similar to each other, and the color of TM which contains the T sequence is much deeper than that of the three other mutated samples Tm1–3 even if just one base is mutated in one of them. These results indicate high repeatability and specificity of the visual-chip based PCR+QHCR detection and also reveal its potential application in single-nucleotide polymorphism (SNP) detection. This visual-chip based colorimetric detection method is fast, easy-to-use and cost-effective compared to most fluorescent gene-chip techniques which are based on expensive equipment to read out results, and usually incorporate complicated multi-step manipulations, including DNA hybridization, signaling molecule conjugation, and repetitive washing, etc. These cumbersome procedures are not only time-consuming but often cause crucial problems that diminish the overall reliability of the assay (Wang, 2000).

4. Conclusions

In conclusion, we have developed a protein-free, isothermal, self-amplified nucleic acid sensing system GQ-HCR via the integration of HCR and G-quadruplex. The G-quadruplex sequence is locked in the loop and partially in the stem of one probe in the absence of target molecules. It would be released when the target molecule is added to initiate the hybridization chain reaction, which could achieve signal amplification and guarantee high specificity at the same time. G-quadruplex is a versatile reporter that could be an aptamer to produce fluorescence by binding with ZnPPIX, or could be a DNAzyme to oxidize different reagents to obtain colorimetric or fluorescent signals with the aid of hemin. In order to detect double stranded DNA and get much better detection sensitivity, we improved the detection system further through the combination of PCR with GQ-HCR. The PCR+GQ-HCR system not only shows very high sensitivity by using real time fluorometric assay, but also could become a convenient and portable visual-chip based detection method by printing the probes of GQ-HCR on plastic sheets. Even though the present GQ-HCR system possesses many advantages the probes are not universal enough, so both probes have to be redesigned when a new target molecule is to be detected. An extensive effort is underway in our laboratory to make GQ-HCR system more universal and sensitive.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bios.2012.05.042.

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