A cyclic enzymatic amplification method for sensitive and selective detection of nucleic acids

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Received 7th April 2010, Accepted 7th May 2010
DOI: 10.1039/c0an00215a

Based on Exonuclease III (Exo III) and displacing probes, we have developed a Cyclic Enzymatic Amplification Method (CEAM) for sensitive and selective detection of nucleic acids. In this design, the displacing probe is non-fluorescent on its own and cannot be digested by Exo III until displacement hybridization by a target sequence, leading to release of free non-quenched fluorophore. Because a single target sequence can lead to the release and digestion of numerous fluorophore strands from the displacing probe, a remarkable signal amplification is achieved. With this method, DNA can be detected in the picomolar range with a high selectivity and within less than 20 min.

Introduction

The ability to sense and detect ultralow concentrations of specific nucleic acid sequences is important in clinical diagnostics and biodefense applications.[1] Among many methods developed for this purpose, amplification is one of the most significant ways because it offers the highest analytical sensitivity.[2] There are two types of amplification that have been widely used: target sequence amplification and signal amplification. Target sequence amplification amplifies target sequence to such a level that is detectable with traditional assays, while a signal amplification method utilizes enzymatic reaction or other signal amplification mechanism to transduce target binding events to measurable signal. Typical examples of target amplification include polymerase chain reaction (PCR),[3] rolling circle amplification (RCA)[4] and their derived techniques, such as helicase-dependent amplification (HDA)[5] *etc.* These sequence amplification techniques have the advantages of high sensitivity and selectivity (single nucleotide discrimination), but they are also limited by a series of drawbacks including complex treatment procedures, easy contamination and high costs.[6]

In contrast, signal amplification methods, such as nicking enzyme signal amplification (NESA)[6a,7] and autonomous replication of DNA/FokI cutter units,[8] have been developed for simple and convenient DNA detection. These amplification methods are simple, and also do not require expensive instruments. However, one of the common limitations of these enzyme signal amplifications is that the enzyme used requires target DNA with a specific sequence for enzyme recognition, such as "–GATCC–" for Nicking endonuclease and "–CATCC–GGATG–" for FokI Enzyme, thus set an restriction on target sequence selections.[9] Consequently, a novel platform for sensitive and selective monitoring of any nucleic acid sequence with rapid, simple manipulation is greatly desired.

Toward this end, Zuo *et al.* have developed an Exo III-aided target recycling method for sensitive and selective amplified fluorescence DNA detection.[10] The method uses Exo III for enzymatic cleavage while a molecular beacon as a signalling probe. The amplified DNA assay is designed in such a way that once a molecular beacon recognizes and hybridizes to its target sequence to form a blunt 3′ terminus, it will be immediately digested by Exo III to produce free un-quenched fluorophore and ultimately releasing the target. The released target can then hybridize with another, whence the cycle starts anew, leading to significant amplification of the signal. The approach achieves picomolar detection simply by mixing the modified molecular beacon, Exonuclease III, and complementary target DNA and incubating for 2 h at 37 °C. The method does not require a specific recognition site in the target sequence because Exo III is a sequence-independent enzyme. However, as a single strand stem-loop hairpin probe, molecular beacons are difficult to design and expensive to make.[11]

In this paper, we report an enzymatic signal amplification method based on Exo III[13] and displacing probe,[12,14] called Cyclic Enzymatic Amplification Method (CEAM) for simple, rapid, sensitive, selective and inexpensive detection of nucleic acids. Displacing probes, developed by Li *et al.* based on the principle of displacement hybridization,[12] consists of two complementary oligonucleotides of different lengths labelled with fluorophore and quencher respectively. In comparison with molecular beacons, these probes are much easier to design, synthesize, purify and thus cheaper and easier to use. In our design, the displacing probe is non-fluorescent on its own and cannot be digested by Exo III until displacement hybridization by a target sequence, leading to the release of free non-quenched fluorophore. Because a single target sequence can lead to the release and digestion of numerous fluorophore strands from the displacing probe, a remarkable signal amplification is achieved. With this method, DNA can be detected in the picomolar range with single base discrimination selectivity within less than 20 min.

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Results and discussion

Principle of CEAM

The principle of CEAM is shown in Fig. 1. This system consists of a displacing probe and Exo III. Exo III catalyzes

the stepwise removal of mononucleotides from blunt or recessed 3′-hydroxyl termini of duplex DNA, while it is less active on single-stranded DNA or 3′ protruding termini of double-stranded DNA. The displacing probe is composed of two complementary oligonucleotides of different lengths. The longer positive strand is labelled with a fluorophore at the 5′-end and the shorter negative strand is labelled with a quencher at the 3′-terminus, so that the fluorophore and the quencher groups are in close proximity in the duplex probe.[12] Thus, in the absence of target DNA, the probe is non-fluorescent due to the quenching of the fluorophore by the quencher. In the presence of target DNA, the quencher strand can be displaced and the fluorophore becomes fluorescent because the formation of a much stable and longer Fluorophore probe/target duplex. More importantly, after binding to the target, the 3′ of the fluorophore strand changes from 3′ protruding to a recessed terminus and becomes an ideal substrate for Exo III digestion. As a result, target binding to the fluorophore strand leads to digestion of the fluorophore strand and subsequently releasing the target DNA strand to bind to another 3′-protruding termini double stranded probe to initiate the next round of cleavage. This cyclic reaction will repeat again and again until all double stranded probes are consumed and all fluorophores light up, resulting in a significant fluorescent signal amplification. Ultrasensitive detection of DNA can be achieved because in principle a single copy of target DNA could lead to complete consumption of all displacing probes present in the system and complete restoration of fluorescence.

Traditional displacing probes with a blunt 3′-terminus can be nonspecifically cleaved by Exo III, causing a relatively high background signal. To solve this problem, we elongated the 3′-termini of quencher strand to form a enzymatic inactive protruding terminus. Our experiments clearly indicated that with the lengthening of the quencher probe, background digestion of the fluorophore probe significantly inhibited. However, a protruding terminus that is too long would reduce the FRET efficiency between the fluorophore and the quencher. Overall, our experimental results indicated that a displacing probe with 4 guanine bases protruding at 3′-termini of quencher probe remarkably resists Exo III cleavage, while maintaining a low fluorescence background.

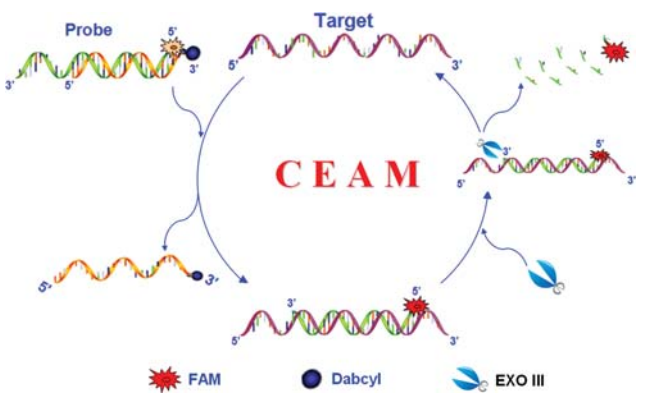

Fig. 1 Working principle of the cyclic enzymatic amplification method (CEAM).

Verification of CEAM

As previously described, the displacing probe undergoes a displacement hybridization with its target and provides an active site for Exo III. Cyclic enzymatic amplification is then triggered. As shown in Fig. 2(a), with the addition of a low concentration of target DNA (*i.e.* 5 nM), fluorescence intensity slightly increased, indicating the successful dislodgement of the quencher probe from the fluorophore probe by a longer target sequence. The increase of fluorescence intensity was very small because one target sequence could only displace one quencher strand. By contrast, a remarkable rise in fluorescence intensity was observed upon the addition of Exo III, which was likely due to the rapid cyclic displacement of quencher sequence by target sequence and subsequent cleavage of the fluorophore strand by Exo III(Fig. 2a). Further kinetic analysis of target binding reaction and Exo III digestion indicated that the latter is orders of magnitude faster than the former when target concentration is in the sub-nM range, with the toehead exchange reaction being the rate-determining step. Because a single target sequence can lead to the release and digestion of numerous fluorophore strands, a remarkable signal amplification was observed. Electrophoresis experiment was performed, which clearly revealed complete digestion of the fluorophore strand, while other sequences including the quencher strand, target strand remained intact (Fig. 2b). The results confirmed that CEAM enables a significant signal amplification.

Amplified detection of target with high sensitivity

Signal amplification nature of the CEAM enables ultrahigh sensitive DNA detection. Fig. 3(a) shows the fluorescence intensity observed upon different concentrations of targets with this method. The results showed that fluorescence intensity increased as the concentration of the targets increased. Fig. 3(b) shows the relationship between the fluorescence intensity and target concentration. As the target concentration increases, the rate of fluorescence enhancement increases. A good linear relationship between the fluorescence signal change (ΔF) and the target concentration was from 0.05 nM to 2.5 nM for sensitive quantitation with a correlation coefficient of 0.9932 for the linear

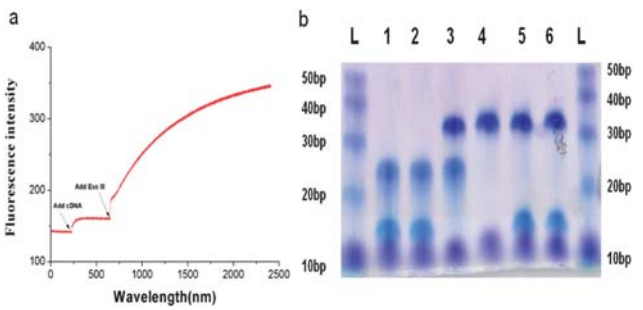

Fig. 2 (a) Time course study of CEAM at low target concentrations. (b) Denatured PAGE analysis of the products by the cyclic enzymatic amplification method. Lanes 1–6: (1): F probe + Q probe (2) F probe + Q probe + Exo III:, (3): F probe + cDNA, (4): F probe + cDNA + Exo III, (5): Q probe + cDNA, (6): Q probe + cDNA + Exo III. The fluorophore probe, quencher probe and cDNA were 5.0×10^{-8} mole respectively. All samples were incubated at RT.

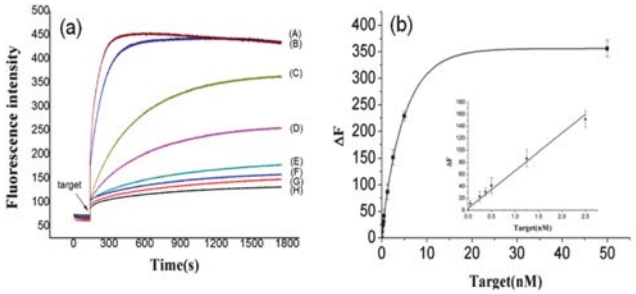

Fig. 3 (a) Detection of different concentrations of target based on CEAM. Experiments were performed in the presence of 5U Exo III with 5 $\times$ 10^{-8} M fluorophore probe, 1 $\times$ 10^{-7} M quencher probe, and different concentrations of target. The curves from top to down contain the target with 5.0 $\times$ 10^{-8}, 2.5 $\times$ 10^{-8}, 5.0 $\times$ 10^{-9}, 2.5 $\times$ 10^{-9}, 5 $\times$ 10^{-10}, 2.5 $\times$ 10^{-10}, 5 $\times$ 10^{-11} and 0 M, respectively. All samples were incubated at RT. (b) The relationship between the fluorescence enhancement and target DNA concentration. The data shown here and in the following figures are mean values and standard deviations obtained from at least three independent experiments.

calibration curve shown in the inset. The regression equation was $\Delta F = 58.98C_{target} + 7.71$. Based on 3 times standard deviation of 6 measurements of blank samples, the detection limit was about 24 pM, which is comparable, or superior to existing signal amplification technologies.[15] It is worthy pointing out that such a high sensitivity is achieved within less than 20 min. Zuo *et al.* reported a detection limit of 10 pM within 30 min using Exo III and molecular beacons,[10] which is comparable to ours. The sensitivity was reported to further improve by extending the reaction to 24 h. However, no significant improvement of detection limit was observed with our method even after extending the reaction to 24 h. Of course, such a long assay time might not be favorable for many applications where time is a critical factor.

To confirm that the high sensitivity of DNA detection is a result of the cyclic enzymatic amplification, control experiments with target at various concentrations reacting with the displacing probes in the absence of Exo III were also carried out (Fig. 4). However, the detection limit is only 1 nM, which is two orders of magnitude less sensitive than CEAM.

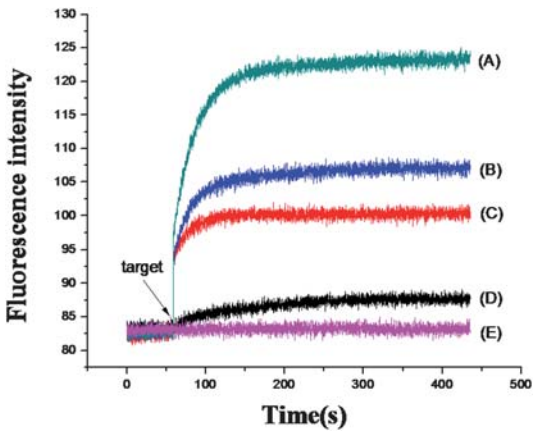

Fig. 4 Response of displacing probe to various concentrations of target. Experiments were performed in the absence of Exo III with 5 $\times$ 10^{-8} M fluorophore probe, 1 $\times$ 10^{-7} M quencher probe and different concentrations of target. The curves from (A) to (E) contain the target with 1 $\times$ 10^{-8}, 8 $\times$ 10^{-9},5 $\times$ 10^{-9}, 1 $\times$ 10^{-9} and 0 M, respectively. All samples were incubated at RT.

Selectivity of the CEAM

Our CEAM displays an excellent detection selectivity. As shown in Fig. 5, completely matched target triggered 1$\sim$2 times higher signal change than a single-base mismatched target did. The good selectivity probably comes from two reasons. On the one hand, displacing probe is considered a probe with good selectivity for single-base mismatch detection because of the different melting temperature between fully matched and mismatched target.[12] On the other hand, different enzymatic activity responding to match and mismatch target adds an extra layer of selectivity. A complete match produces a recessed fluorophore strand, while a mismatch would yield a protruding 3′-terminus, thus causing a significant difference on Exo III digestion activity. The excellent selectivity of CEAM makes it a promising technique for detection of single nucleotide polymorphisms.

CEAM for detection of HFE

Based on the principle established above, one could utilize CEAM for DNA detection in clinical diagnostics and biodefense applications, especially disease-associated single nucleotide polymorphisms (SNP). For instance, we designed a CEAM probe for human hemochromatosis (HFE) gene mutation detection. HFE gene is one of the most common single gene disorders that causes hereditary hemochromatosis in Caucasians. The difficulty in detecting this mutation with classical single stranded probes lies in the fact that the G/A is the least destabilized mismatch, and thus requires a probe with extremely high specificity. Our results showed that with a CEAM approach one can easily distinguish a mutation gene from a wild-type gene at concentrations as low as 10 pM (Fig. 6).

Conclusions

In conclusion, we have designed a Cyclic Enzymatic Amplification Method based on Exo III and displacing probes. The method offers high sensitivity and excellent selectivity for nucleic

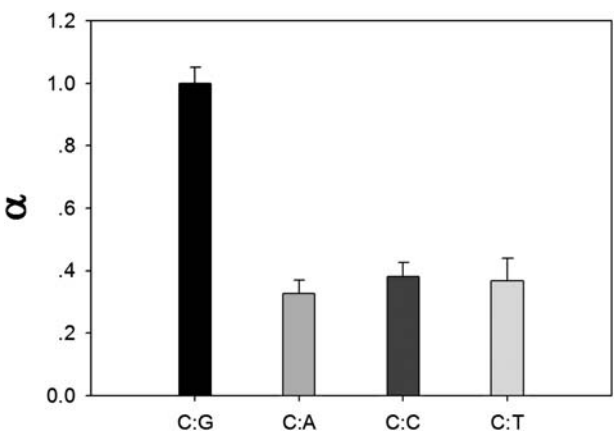

Fig. 5 Single-base mismatch detection using CEAM.

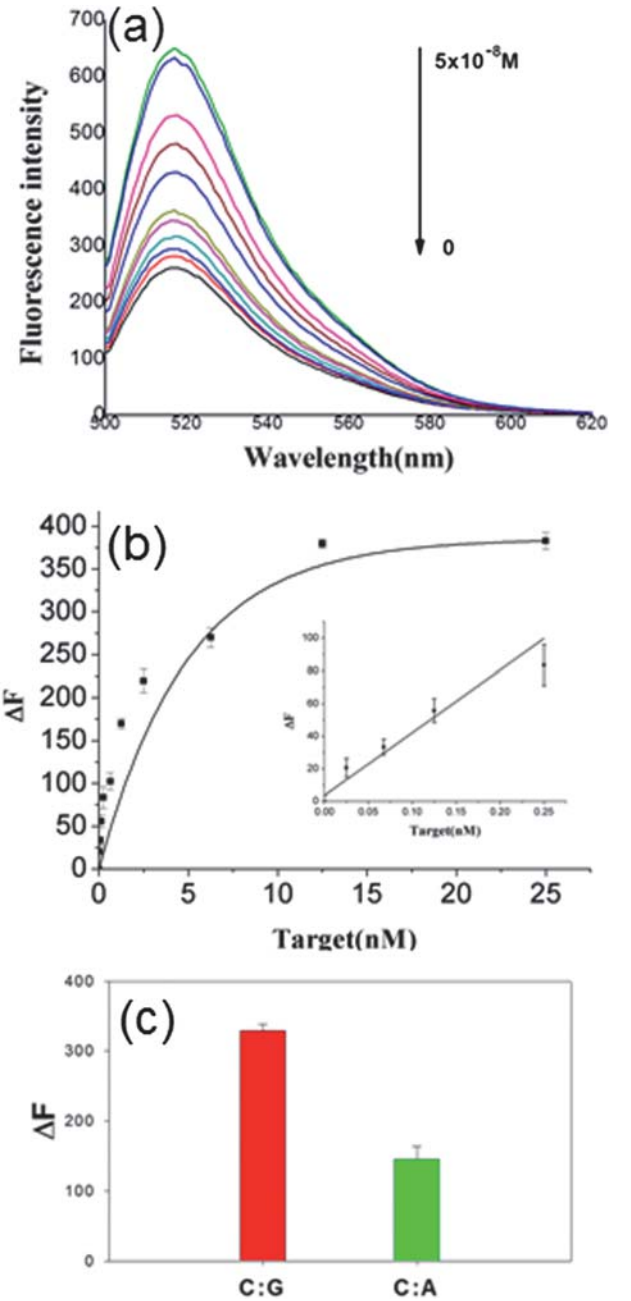

Fig. 6 Detection of different concentrations of HFE target based on CEAM. Experiments were performed in the presence of 5U Exo III with 5 × 10^{-8} M fluorescence probe, 1 × 10^{-7} M quencher probe, and different concentrations of target. (a) Fluorescence spectra of this amplified DNA detection method over a range of target DNA concentrations. The curves from top to bottom represent the target with 5 × 10^{-8}, 2.5 × 10^{-8}, 1.25 × 10^{-8}, 5 × 10^{-9}, 2.5 × 10^{-9}, 1.25 × 10^{-9}, 5 × 10^{-10}, 2.5 × 10^{-10}, 1.25 × 10^{-10}, 5 × 10^{-11} and 0 M, respectively. All samples were incubated at RT. (b) The relationship between the fluorescence enhancement value and target DNA concentration. (c) The selectivity comparison of CEAM for cDNA and single-base mismatched DNA.

acid detection with rapid, simple procedures. Such a method does not require a recognition sequence in the target DNA, thus has much better flexibility for choosing a target sequence and a wider applicability. CEAM can be carried out at a constant temperature (37 °C or Room Temperature) and just with one-step manipulation, avoiding the complex handling procedures required by other methods like PCR or RCA. Furthermore, a signal change can be achieved within 15 min, effectively decreasing the time-cost of clinical diagnostics. Also, superior to double labelling molecular beacons, displacing probes with one-dye-labeled are easy to design and inexpensive to make.[11b] Based on these advantages, CEAM should be potentially exploited in the field of study on life science and practically applied in clinical diagnostics, mutation detection, and biodefense applications.

Experimental

Materials

Exonuclease III was purchased from Takara Biotechnology Co. Ltd. (Dalian, China) and used without further purification. The probe DNA, target DNA and mismatched DNA were synthesized on a PolyGen DNA synthesizer and the reagents were purchased from Glen Research (Sterling, VA, USA). All DNA sequences are listed in Tables 1 and 2.

DNA synthesis

DNA was synthesized on a PolyGen DNA synthesizer. A DABCYL CPG column was used for the synthesis 3′-DABCYL labled quencher probe, while FAM phosphoramidite was used to couple the fluorophore to the 5′ ends of the Fluorophore probe. The complete probe sequences were then deprotected in concentrated ammonia overnight at 65 °C and purified by high-pressure liquid chromatography.[16] HPLC was performed on an Agilent 1100 series HPLC using a Promosil C18 reversed phase column (5 μm, 250 × 4.6 nm). The collected product was then vacuum-dried, desalted with a NAP-5 column, and stored at −20 °C for future use. Absorbance was measured on an Agilent 8453 UV/Vis spectrometer and used to calculate the concentration.

Fluorescence measurements

Fluorescence measurements were carried out on a RF-5301-PC Fluorescence Spectrophotometer (Shimadzu, Japan). Excitation and emission wavelengths were set at 490 and 517 nm, respectively, with 5 nm bandwidth. The emission spectra were obtained

Table 1 Sequences for oligonucleotides for CEAM[a]

Name	Sequence
Fluorophore probe	5′-FAM-AGCAACCTCAAACAGACACCATGG-3′
Quencher probe	5′ - GTTTGAGGTTGCTGGGG-Dabcyl-3′
cDNA	5′-GGATTCCATGGTGTCTGTTTGAGGTTGCTG TAGC-3′
G>A DNA	5′-GGA TTC CATGGTGTCTGTTTAAGGTTGC TGTAG C-3′
G>C DNA	5′-GGATTCCATGGTGTCTGTTTCAGGTTGCTG TAGC-3′
G>T DNA	5′-GGATTCCATGGTGTCTGTTTTAGGTTGCTG TAGC-3′

[a] Underlined letters represent the mismatched site.

Table 2 Sequences for oligonucleotides for HFE detection by CEAM[a]

Name	Sequence
Fluorophore probe	5′-FAM-CTCCACCTGGCACGTATATCT-3′
Quencher probe	5′-TA CGTGCCAGGTGGAGGGGG-Dabcyl-3′
cDNA	5′-CATAGTCCAGATATACGTGCCAGGTGG AGTA CG -3′
G>A DNA	5′-CATAGTCCAGATATACGTACCAGGTGG AGTA CG-3′

[a] Underlined letters represent the mismatched site.

by exciting the samples at 490 nm and scanning the emission from 500 to 650 nm in steps of 1 nm. All experiments were conducted in 20 mM Tris-HCl (pH 8.0) buffer containing 5 mM $MgCl_2$ and 50 mM NaCl. The amplified detection of target were performed in 400 μl solution consisting of 5.0×10^{-8} M fluorophore probe and 1×10^{-7} M quencher probe, 5 units of Exonuclease III and varying concentrations of DNA target at RT for 0.5 h.

Gel electrophoresis

A 20% denatured PAGE analysis of the products by the cyclic enzymatic amplification reaction was carried out in 1 × TBE (pH 8.3) at 1 W power for about 2 h. After Stains-All staining, gels were scanned.

Acknowledgements

This work was financially supported by the NSFC (20805038), MOE (200803841013), and National Basic Research Program of China (2007CB935603, 2010CB732402).

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