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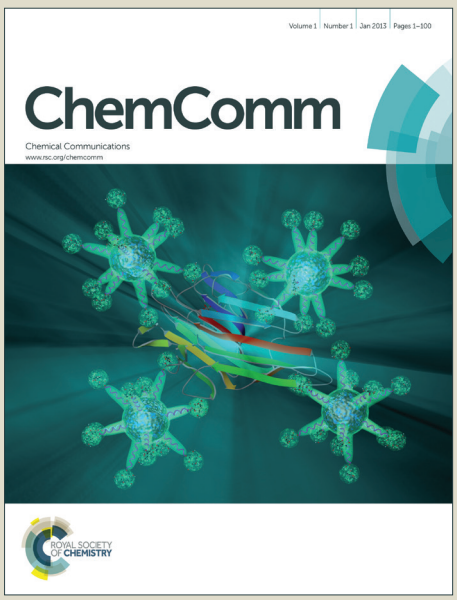

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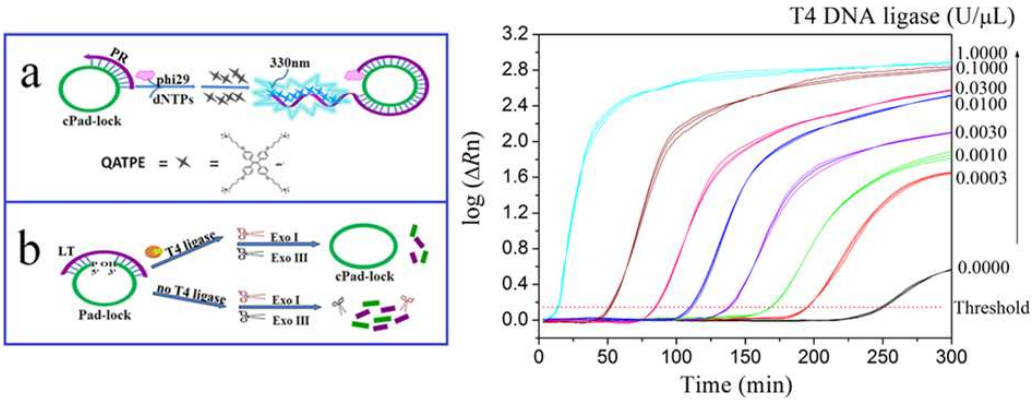

Real-time monitoring of rolling circle amplification (RCA) was achieved by the super-aggregation of a tetraphenylethene dye QAPTE along single-stranded DNA products and consequent enhanced aggregation-induced emission. QATPE is non-sequence specific, therefore can work for all RCA reactions. Real-time RCA not only makes the kinetic analysis of RCA reaction possible, but also makes RCA more suitable for biosensor design. To validate this, it was used to measure T4 DNA ligase activity.

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Real-time monitoring of rolling circle amplification using aggregation-induced emission: applications for biological detection

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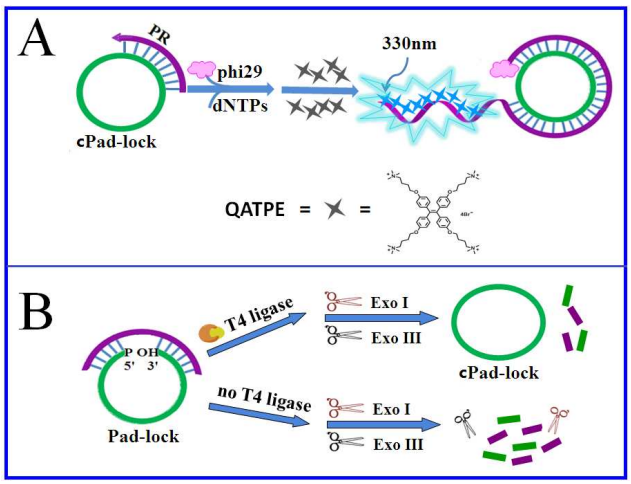
We report that QATPE, an aggregation-induced emission-active tetraphenylethene dye, can be used as a non-sequence-specific ssDNA probe for real-time monitoring of all rolling circle amplification (RCA) reactions，thus making RCA more suitable for biosensing applications.

Nucleic acid amplification has been widely used in many research fields, including medical science, molecular biology and forensic science.[1] There have been a number of approaches towards nucleic acid amplification, the most common being the polymerase chain reaction (PCR).[2] The breakthrough from qualitative to quantitative PCR analysis was achieved by real-time quantitative PCR (RT-qPCR), which was launched by ABI company in 1996. For real-time monitoring of PCR amplification, a non-sequence-specific fluorescent dye, such as SYBR Green I (SG I)[3] or a sequence-specific fluorescent-labelled DNA probe is used.[4] SG I represents the most common approach towards detection and many commercially available RT-qPCR kits are based on SG I.[5] As a non-specific minor groove binder of double-stranded DNA (dsDNA), SG I can be used to detect any PCR product, avoiding the need for specific probe design towards different targets.

The replication of DNA by rolling circle amplification (RCA)[6] is conducted at a constantly low temperature, eliminating the need for thermal cycling and high-temperature denaturation of dsDNA in PCR.[7] Isothermal nucleic acid amplification techniques are able to avoid certain limitations of PCR.[8] As we known, there are many inherent advantages in real-time methods for monitoring amplification reactions, and achieving real time monitoring of RCA reactions will certainly promote their use in biosensing.[9] However the real-time monitoring of RCA is not well established. This is partly due to the lack of a universal fluorescent dye that can bind all single-stranded DNA (ssDNA) products of RCA.

Aggregation-induced emission (AIE) has provided a new opportunity for the development of real-time quantitative RCA (RT-qRCA).[10] The principle lies behind non-emissive fluorescent dyes, which aggregate and become fluorescent in the presence of ssDNAs.[11] Tetraphenylethenes (TPE) have received much attention as AIE-active dyes because they are easy to synthesize, ready to use, photostable and yield high levels of fluorescence.[12] Here, we have shown that the AIE-active TPE dye 1,1,2,2-tetrakis{4-[(trimethylammonium) butoxy]phenyl} tetraphenylethene tetrabromide (QATPE) (Scheme 1) can be used as a non- sequence-specific ssDNA fluorescent probe for real-time monitoring of RCA. To confirm the validity of RT-qRCA in biosensing applications, we used it to measure T4 DNA ligase activity.



Scheme 1. Schematic illustration of the mechanism of the real time monitoring of RCA and its application for T4 DNA ligase detection: (a) AIE caused by the super-aggregation of QATPE molecules along single-stranded RCA product makes the real-time monitoring of RCA reaction possible. (b) T4 DNA ligase activity detection based on real-time RCA reaction.

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QATPE has four phenyl rings, linked by single bonds to a central ethylene bridge. The free rotations of the single bonds promote energy dissipation via non-radiative channels, making free QATPE almost non-emissive. However, these intramolecular rotations are restricted when the positively charged QATPE dye aggregates on the surface of certain biomolecules, such as negatively charged ssDNA and fluorescence is emitted.[13] Unlike SG I, which shows higher fluorescence response to dsDNA than to ssDNA, QATPE produces more fluorescence in response to ssDNA than to dsDNA (Figure S1), so represents a potential non-sequence-specific fluorescent dye for ssDNA detection. In this scenario, QATPE aggregates on single-stranded RCA products and produces an AIE signal. This signal would increase with the lengthening of the RCA product (Scheme 1a) and the progress of the RCA reaction would be monitored in real-time.

To confirm the ability of QATPE to detect RCA products, we first performed end-point detection reactions. As shown in Figure 1a, QATPE only produced a fluorescence signal in the presence of all RCA reaction components (circular template **cPad-lock**, primer **PR**, polymerase phi29 and dNTPs). This demonstrated that the production of a long-stranded RCA product can trigger the aggregation of QATPE, which enhances AIE fluorescence.

To confirm that the fluorescence signal was directly related to RCA product synthesis, RCA reactions were run with different **PR** concentrations before adding QATPE. The intensity of the resulting fluorescence signals was dependent on the **PR** concentration (Figure 1b). The RCA reaction products were also separated by gel electrophoresis and QATPE was used as the staining dye. The intensity of the bands increased with **PR** concentration (Figure 1c). Additionally, the migration rate of the band was slower than that of the largest marker fragment (7000 bp), demonstrating the formation of very long RCA products. The super-aggregation of QATPE along the long single-stranded RCA products results in the emission of strong AIE signal. This result also demonstrated that QATPE can be used as a staining dye to realize the visualization of ssDNA in agarose gel electrophoresis assay. Unlike ethidium bromide, which is added to the agarose gel to detect dsDNA, QATPE is conveniently added directly to the reaction solution, indicating the stability of the ssDNA/QATPE complex, which does not dissociate during electrophoresis.

Changes in the fluorescence signal were examined at different time points during the RCA reaction. To achieve this, a 3 mL RCA reaction solution was prepared and 100 µL were removed at different time points during the reaction. The fluorescence signal (475 nm) was recorded immediately after the addition of 40 µM QATPE. Almost no fluorescence change was observed for the reaction system without **PR**. However, in the presence of **PR**, an increase in fluorescence signal was observed with increasing reaction time (Figure 1d). This further demonstrates that the increase in fluorescence is directly related to the synthesis of RCA product. The fluorescence-time curves generated when QATPE was added before the reaction were comparable to those when QATPE was added afterwards (Figure 1d), thus indicating QATPE does not affect RCA reaction efficiency. In addition, QATPE give a very fast fluorescence response to the RCA product, which is important for real-time monitoring. Indeed, a stable fluorescence signal was reached in 6 s after QATPE addition (Figure S2).

The feasibility of RCA products in triggering the aggregation of QATPE was further characterized by atomic force microscopy (AFM). As shown in Figure 1e, the AFM image shows RCA product strands with lengths of several hundred nanometers and with heights of approximately 0.5 nm, consistent with the diameter of ssDNA reported in previous studies,[14] demonstrating the successful generation of long ssDNA product from a starting template of only 15 nm length. With the addition of QATPE, the size of DNA strands is obviously enlarged. Software analysis of the AFM images reveals that the heights of DNA strands increase to approximately 2 nm after mixing with QATPE (Figure 1f) demonstrating the super-aggregation of QATPE triggered by single-stranded RCA product.

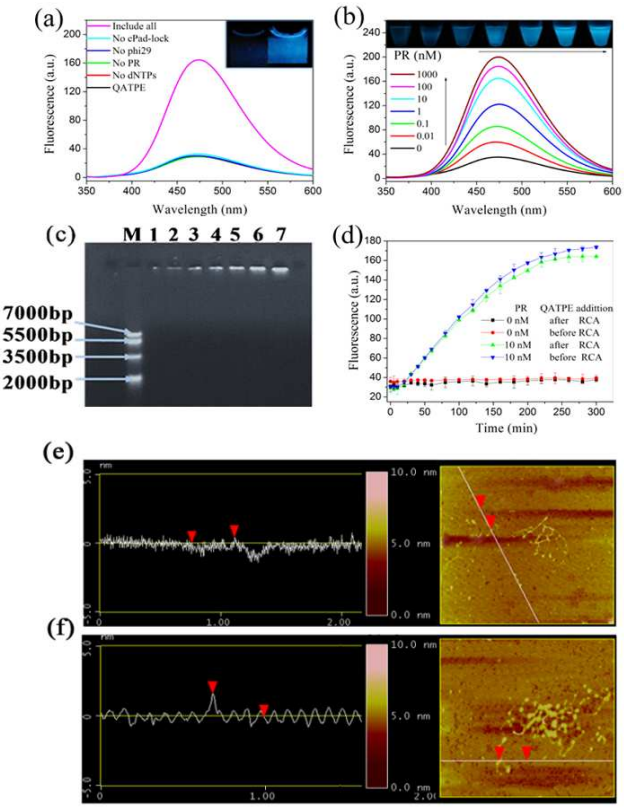

Figure 1. RCA product detection by the end-point mode. (a) Feasibility of QATPE for the detection of RCA product. Insert: fluorescent photographs of corresponding samples under UV lamp excitation (left: no **PR**; right: include all); (b) End-point fluorescence signal of the RCA reaction solutions containing different concentrations of **PR**. Inserts: fluorescent photographs of the corresponding solutions under UV lamp excitation; (c) Agarose electrophoresis analysis of above RCA solutions stained by QATPE. (Lane M) DNA marker. **PR** concentration (Lane 1→7) were 0, 0.01, 0.1, 1, 10, 100, 1000 nM respectively; (d) Time-dependent fluorescence signal change of the RCA reaction solutions containing 0 or 10 nM **PR**. Addition of QATPE before and after RCA reaction gives similar fluorescence-time curves. All experiments were performed in triplicate; AFM images and height profile of the RCA product before (e) and after (f) mixing with QATPE.

Next, we investigated whether QATPE can monitor the RCA reaction in real time. For high-throughput real-time monitoring of the RCA reaction, we used a commercial RT-qPCR instrument (StepOnePlus™ Real-Time PCR system, ABI, United State) and added QAPTE before the RCA reaction. We detected an increase in fluorescence signal that was dependent on both **PR** concentration and reaction time (Figure S3), indicating that QATPE is suitable for monitoring the RCA reaction in real-time.

The real-time monitoring of the RCA reaction may be more suitable for applications in biological detection. To test this, we developed a detection platform for T4 DNA ligase activity quantitation.[15] Many studies have shown that the activity levels of DNA ligases are closely related to the pathogenesis of cancers and the DNA ligase presents an attractive target for broad-spectrum antibacterial therapy and cancer therapy, so sensitive detection of DNA ligase activity is highly significant for early diagnosis and prognostic evaluation of cancers.[16] Herein, a linear oligonucleotide (**Pad-lock**) with a phosphorylated 5'-end was used. When hybridizing to another oligonucleotide (**LT**) at adjacent sites, it could be circularized by T4 DNA ligase (Scheme 1b) to form the circular template (**cPad-lock**) of RCA reaction. Then, T4 DNA ligase-triggered amplification of **cPad-lock** by RCA was monitored in real-time.

The resulting fluorescence–time curves were dependent on T4 DNA ligase concentration (Figure 2a). T4 DNA ligase activity can be measured from the fluorescence-time curves in two ways. One is based on the relationship between T4 DNA ligase activity and the fluorescence signal at a certain time point (e.g. 250 min, Figure 2b and Figure S4). This approach is similar to the end-point detection, but one advantage is that the time point can be selected arbitrarily from the fluorescence-time curves to obtain the best detection result.

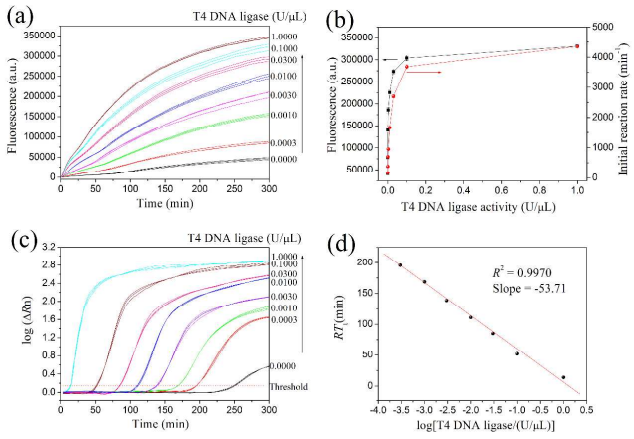

Figure 2. T4 DNA ligase activity detection using real-time RCA. (a) Fluorescence-time curves for the detection systems containing different concentrations of T4 DNA ligase; (b) fluorescence signal change at 250 min or initial reaction rate change as a function of T4 DNA ligase activity; (c) log(Δ*R*n)-time curves for the detection systems containing different concentrations of T4 DNA ligase; (d) Linear relationship between RT_t value and the logarithm of T4 DNA ligase activity. All experiments were performed in triplicate.

Another detection approach is based on the relationship between T4 DNA ligase activity and the initial reaction rate (Figure 2b), which can be measured from the initial part of each fluorescence–time curve. With these two approaches, we obtained similar measurements for T4 DNA ligase activity, although the second approach appeared to give better detection precision.

The software of the RT-qPCR instrument can produce log(Δ*R*n)-time curves (Figure 2c), where Δ*R*n is the fluorescence difference between the reporter fluorophore and the baseline set by the instrument. Log(Δ*R*n)-time curves have a sigmoidal distribution, meaning a low increase in signal at the beginning of the reaction, followed by a rapid increase and an eventual plateau. This distribution can be attributed to the shorter reaction times needed to generate a detectable signal with increasing T4 DNA ligase concentration. A linear relationship was obtained between the RT_t value (the reaction time at which the fluorescence signal reaches the set threshold) and the logarithm of T4 DNA ligase activity in the range of 0.003~1 U/μL (Figure 2d), offering a much wider linear quantitative range (over 3.5 orders of magnitude in terms of T4 DNA ligase activity) than end-point detection. This method was able to selectively detect T4 DNA ligase activity and no obvious fluorescence signal enhancement was observed in the presence of non-targeted proteins or enzymes (Figure. S5).

The StepOnePlusTM Real-Time PCR system has several fluorescence detection channels, which can detect signals from several fluorophores. For this study, we should select an optimal channel that would detect fluorescence at the closest range to the maximum emission wavelength of QATPE (475 nm). The best option was the 6-carboxyfluorescein channel. However, the maximum emission wavelength (~520 nm) of this fluorophore is still much larger than that of QATPE. The mismatching of the detection wavelength will certainly impair the sensitivity of RT-RCA-based detection. To solve this issue, one way is addition of a suitable detection channel to the instrument. The other more feasible way is to screen or design AIE-active dyes with a fluorescence emission wavelength compatible with the instrument.

In summary, real-time monitoring of the RCA reaction was achieved by utilizing the super-aggregation of QAPTE along single-stranded DNA products and consequent increased AIE. QATPE is non-sequence specific, therefore can work for all RCA reactions. Real-time RCA not only makes the kinetic analysis of RCA reaction possible, but also makes RCA more suitable for applications in biological detection. Compared to end-point detection, real-time RCA has many advantages: (1) more options can be provided for target measurement, using the fluorescence signal at designated reaction times, the initial reaction rate or the unique data processing software of the RT-qPCR instrument; (2) high throughput detection is possible (96 samples can be detected simultaneously using the RT-qPCR instrument); (3) post-reaction detection measures are not necessary, which simplifies and shortens each experiment; (4) the reaction tubes need not be opened when the reaction is finished, reducing the risk of product contamination. To validate our real-time quantitative RCA method, we used it to

design a detection platform for T4 DNA ligase activity quantitation. The data processing software of the commercial RT-qPCR instrument produced a much wider linear quantitative range compared to end-point detection. The developed real-time RCA technique can also be easily extended to design other detection platforms for DNA, microRNA, T4 polynucleotide kinase phosphatase and other biomolecules.[15a] We next goal is to find an AIE-active fluorophore, with a wavelength more compatible with the RT-qPCR instrument.

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