DNA Detection

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Pyrene-Excimer Probes Based on the Hybridization Chain Reaction for the Detection of Nucleic Acids in Complex Biological Fluids**

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The sensitive and selective detection of nucleic acids is important in biological studies, clinical diagnostics, and biodefense applications. Since the DNA sequences of interest may be present in very small amounts, it is necessary to develop amplification techniques that enable the detection of trace levels of a specific sequence. Existing DNA-amplification techniques can be divided into two broad categories: thermal cycling and isothermal processing.

The polymerase chain reaction (PCR) is the most widely used thermal-cycling protocol for DNA amplification.[1] The reaction proceeds exponentially, so that trace amounts of DNA can be amplified to detectable levels. Another thermal-cycling method is the ligase chain reaction (LCR).[2] Because a thermostable ligase retains activity after multiple thermal cycles, the ligation products from one round can become the targets for the next round of ligation. In this way, the amount of product can increase in an exponential way by repeated thermal cycling. However, thermal-cycling methods are time-consuming, sometimes nonspecific, and limited to a thermostable enzyme and a laboratory setting.

In the case of isothermal amplification, a strand-displacement polymerase is often used for the continuous replication of one strand of a DNA duplex. For example, in rolling circle amplification (RCA), a circular oligonucleotide sequence serves as a template for the creation of a complementary single-stranded DNA chain containing periodic repeats of the sequence coded by the circular oligonucleotide.[3] Another technique, strand-displacement amplification (SDA), which is based on polymerization or scission,[4,5] is utilized in several sensitive DNA-detection methods.[6–8] A further development in DNA amplification is the hybridization chain reaction (HCR), in which two stable species of DNA hairpins coexist in solution until an initiator strand is introduced. The initiator triggers a cascade of hybridization events to yield nicked double helices analogous to alternating copolymers.[9–11]

The quantitation of nucleic acids in complex biological fluids is another challenge for biomedical applications, essentially because of the background signals observed for both the probe and biological fluids. However, by the introduction of pyrene moieties, the problem can be addressed effectively.[12–14] Recently, pyrene has been used as a fluorescent dye to signal the presence of ions,[15] small molecules,[16,17] nucleic acids,[12,14] or proteins.[13,18] Pyrene acts as a spatially sensitive fluorescent dye. An excimer can form when an excited-state molecule is brought into close proximity with a pyrene moiety in the ground state. The formation of the excimer results in a shift of the emission (from 375 and 398 nm for the monomer) to a longer wavelength (485 nm). The excimer also has a longer lifetime at the wavelength of 485 nm (up to 100 ns) than chromophores in biological fluids (less than 10 ns).[12–14] The long lifetime of the excimer provides an opportunity for the use of this probe for target detection in biological media, as time-resolved fluorescence measurements should enable the removal of the strong cellular background signal for efficient analysis.

Herein we describe a DNA-detection system which combines the amplification capability of HCR with the spatially sensitive fluorescence signal of pyrene molecules conjugated to hairpin probes (Figure 1). The DNA hairpins H1* and H2* are dual-labeled with pyrene moieties through a six-carbon-atom spacer at each end. Each hairpin has a stem of 18 base pairs enclosing a 6 nucleotide (nt) loop. Each also has an additional 6 nt sticky end at the 5′ end of H1* (complementary to the loop of H2*) and at the 3′ end of H2* (complementary to the loop of H1*). In the absence of target DNA, both probes (H1* and H2*) are in the closed

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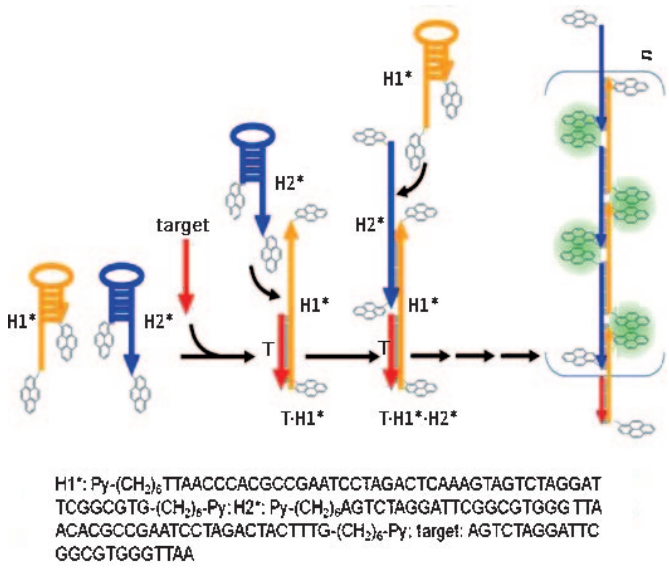

Figure 1. Working principle behind the detection of DNA on the basis of HCR amplification and the formation of pyrene excimers. Py = pyrene.

form, and the two pyrene moieties are spatially separated by the extra length of the sticky end. In this state, only the monomer emission peaks (at 375 and 398 nm) are observed. However, when the target is present in the solution, it pairs with the sticky end of H1*, which undergoes an unbiased strand-displacement interaction to open the hairpin. The newly exposed sticky end of H1* nucleates at the sticky end of H2* and opens the hairpin to expose a sticky end on H2*. This sticky end is identical in sequence to the target. In this way, each copy of the target can propagate a chain reaction of hybridization events between alternating H1* and H2* hairpins to form a nicked double-helix. In this state, a pyrene moiety on one probe is brought into close proximity to a pyrene moiety on the neighboring probe. Thus, numerous pyrene excimers are formed, each of which emits at approximately 485 nm. By observation of the emission intensities of the pyrene monomer and the excimer, the target DNA can be detected with high sensitivity.

The formation of excimers of aromatic hydrocarbons is restricted to a parallel, cofacial configuration with an interplanar distance of 3–4 Å.[19] To find the best pyrene-excimer signal, we designed and synthesized several probes with the pyrene moieties at different label positions (see Table S1 in the Supporting Information) and then compared them by the measurement of steady-state fluorescence (see Figure S2 in the Supporting Information). On the basis of the results, H1* and H2* were chosen as the HCR probes for subsequent studies.

As described above, each target DNA molecule can trigger a chain reaction of hybridization events between H1* and H2* to form a long nicked DNA polymer, with amplification of the fluorescence signal. Electrophoresis showed the products synthesized by HCR at different concentrations of target T and confirmed the proposed mechanism (see Figure S3 in the Supporting Information). Polymers of various lengths were synthesized during the HCR, and higher concentrations of the target resulted in increased consumption of H1* and H2*. To confirm the switching of the emission wavelength of pyrene moieties in this method, we visualized the solutions with UV light (see Figure S4 in the Supporting Information). A clear green color was observed by the naked eye when target DNA (1 nM) was added to the solution containing the HCR probes H1* and H2* (500 nM each). Also, real-time monitoring of the hybridization chain reaction showed that the final fluorescence intensity depends on the concentration of the target DNA (see Figure S5 in the Supporting Information).

We measured the fluorescence emission spectra of HCR probes at an excitation wavelength of 340 nm in a buffer solution containing the target DNA at varying concentrations (Figure 2). When no target DNA was present, monomer emission peaks were observed, but no observable excimer emission. The HCR probes assumed the hairpin structure, which spatially separates the pyrene moieties at the 3′ and 5′ ends and thus prevents them from forming an excimer because of the extra length of the 6 nt sticky end. However, upon the addition of target DNA to the solution, DNA polymers were formed, whereby the 3′ end sequence of one HCR probe was placed close to the 5′ end sequence of another HCR probe. After a period of 1 h, intense excimer emission was observed at 485 nm. The intensity of the emission increased in proportion to the amount of target DNA in the solution. HCR triggered by target DNA resulted in three emission peaks: two monomer peaks at 375 and 398 nm, and an excimer peak at 485 nm (Figure 2).

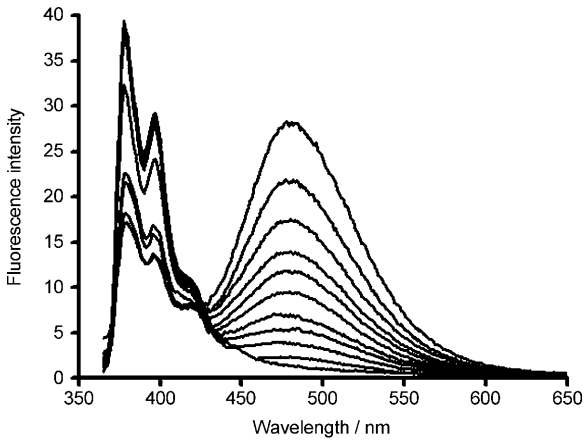

Figure 2. Response of the HCR probes (H1* and H2*) to different concentrations of the target DNA after incubation for 1 h. Concentration of the target DNA (from the top to the bottom curve at 485 nm): 5×10^{-7}, 1×10^{-7}, 2×10^{-8}, 4×10^{-9}, 8×10^{-10}, 1.6×10^{-10}, 3.2×10^{-11}, 6.4×10^{-12}, 1.28×10^{-12}, 2.56×10^{-13}, and 0 M; [H1*] = [H2*] = 500 nM; λ_{ex} = 340 nm.

On the basis of the intensity ratio of the excimer peak to either one of the monomer peaks, signal fluctuations can be cancelled, and the impact of environmental quenching can be minimized. The excimer/monomer emission ratio of the HCR probes responded proportionally to different concentrations

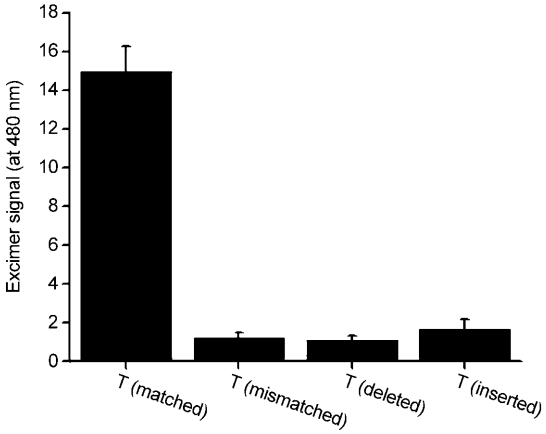

Figure 3. Response of the HCR probes (500 nM) to a matched target, a mismatched target, a target with a deleted nucleotide, and a target with an inserted nucleotide (all 1 nM). Target sequence: AGTCTAG-GATTCXGCGTGGGTTAA; T (matched): X = G; T (mismatched): X = A; T (deleted): no X; T (inserted): X = TG.

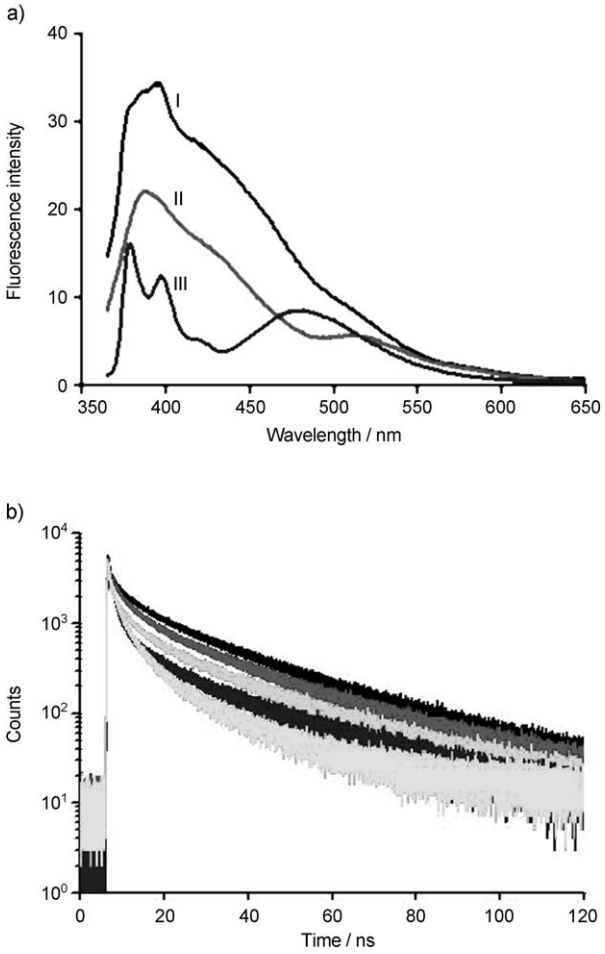

Figure 4. DNA detection in cell media. a) Steady-state fluorescence spectra of I) the HCR probes (500 nM) and the target (1 nM) in cell medium, II) cell medium only, and III) the HCR probes (500 nM) and the target (1 nM) in a buffer. b) Time-resolved fluorescence decay (at 485 nm) of the HCR probes (500 nM) in cell medium with the target DNA at various concentrations. Curves from top to bottom: with 100, 50, 10, and 0 nM of the target DNA, and the cell medium only.

of the target DNA (see Figure S6 in the Supporting Information). The limit of detection for DNA in the buffer was in the femtomolar range.

To test the selectivity of the HCR probes, we used various oligonucleotides as matched, mismatched, deleted, and inserted targets (Figure 3). Only the matched DNA triggered the reaction. Thus, the HCR probes are highly selective for completely complementary DNA. The results also reveal one important advantage of the method: detection without separation. Because only target-triggered HCR probes give excimer emission, the untriggered probes do not have to be separated from the solution prior to target detection.

Our results in the buffer system, which is relatively pure in comparison with real physiological media, demonstrate that the HCR probes have excellent selectivity and high sensitivity. We used a cell medium mixed with fetal bovine serum to investigate the feasibility of using the probes in biological samples. We measured the fluorescence spectra of the HCR probes in a sodium phosphate–sodium chloride buffer solution (SPSC; 1M NaCl, 50 mM Na_2HPO_4; pH 7.5) and in a cell medium (Figure 4a). In the buffer, the probes functioned well, and strong excimer emission was observed when the target DNA was added. Unfortunately, intense background fluorescence of the cell medium buried the signal response from the probes and made the probe signal indistinguishable from the indigenous background fluorescence. This result indicates that the measurement of steady-state fluorescence is not feasible for the direct detection of DNA in such a complex biological sample.

Most of the background fluorescence has a lifetime of less than 5 ns. By contrast, the monomer and excimer emissions of pyrene have much longer lifetimes. This large difference enables the temporal separation of the probe fluorescence signal from the intense background signal by time-resolved fluorescence spectroscopy. A short excitation pulse of 1 ns excites all chromophores in the solution that absorb at this excitation wavelength, including pyrene and the fluorogenic molecules of the cell medium. By employing a time-resolved detection technique, the decay of the fluorescence signal at a certain wavelength can be recorded. Figure 4b shows the decay of the signal at 485 nm in the cell medium containing the target DNA at various concentrations. The background signal decayed rapidly to a minimum, whereas the excimer signal remained high. The signals of the cell medium only and the HCR probes without the target decayed within the first few nanoseconds after the pulsed excitation. However, the signal of the HCR probes with the target DNA remained much longer, and its intensity was proportional to the concentration of the target. We further used the HCR probes to detect target DNA in human serum (see Figures S7 and S8 in the Supporting Information). The probes functioned as well as in the cell medium with the time-resolved detection technique. Thus, our results show that the HCR probes can be used to detect DNA in complex biological fluids by time-resolved excimer measurements.

In summary, we have developed a DNA-amplified detection method that combines the amplifying functionality

of HCR and the emission-switching property of pyrene. At least five advantages of our method have been demonstrated: 1) HCR is an excellent isothermal signal-amplification technique which does not require an enzyme; 2) the reaction takes place within about 1 h with a probe concentration of 500 nM and does not require separation; thus, the method could potentially be used for rapid detection and real-time monitoring; 3) the method is very sensitive and selective; 4) pyrene-excimer formation can be measured ratiometrically to minimize environmental effects and enable more precise detection; 5) the long lifetime and large Stokes shift of the pyrene excimer favor time-resolved analysis, which readily discriminates between the stem-open and stem-closed conformations of the HCR probes in cell media and human serum.

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