Target-assembled exciplexes based on Scorpion oligonucleotides

Molecular Beacons [1], Scorpion primers [2], HyBeacons [3] and TaqMan probes [4,5] are generally single-stranded oligonucleotides that utilize a fluorophore and quencher. In the dark (quenched) form, they adopt a conformation with the quencher close to the fluorescent dye. On hybridization to the target molecule, they undergo a conformational change (Molecular Beacons and Scorpion probes), an environmental change for the fluorophore (HyBeacons) or enzymatic cleavage (TaqMan), resulting in a fluorescence change.

Scorpion uni-probes, which are extremely sensitive and sequence-specific, comprise a single-stranded fluorescent probe sequence held in a hairpin–loop by complementary stem sequences at each end (Figure 1). The probe contains a 5′ reporter dye and a nearby internal quencher directly linked to the 5′ end of a PCR primer via a blocker, which prevents Taq DNA polymerase from extending the primer. Taq DNA polymerase extends the PCR primer, making the complementary strand of the target. During the next cycle, the hairpin–loop unfolds and the loop region hybridizes intramolecularly to the newly synthesized target, giving increased fluorescence intensity [6].

A recent approach [7–10] to diminish background fluorescence uses exciplexes assembled from components that hybridize at the target sequence. This end-point exciplex method uses two components that are weakly or non-fluorescent at the detection wavelength, resulting in background fluorescence of <1%. For exciplex (or excimer) emission the exci-partners (exciplex partners) must approach closely; ~3.5 Å (1 Å = 0.1 nm) for a pyrene excimer [11,12]. Therefore the potential resolution of the exciplex is in the order of the thickness of 1 bp. FRET (fluorescence resonance energy transfer) systems work over 10–100 Å, which is ~3 bp. In the present study, we describe exciplex emission for Scorpion-based nucleic acid detectors (Figure 2), comprising a Scorpion target (34-mer), bearing one exci-partner on the 5′-terminus, plus a second probe oligonucleotide (8-mer), bearing the other exci-partner, on its 3′-terminus (Figure 2). The exci-partners (5′-pyrenyl and 3′-N′-methyl-N′-naphthalenyl) and structures are shown in Figure 2(C).

The following probes were used: Scorpion-5′-N (5′-naphthalenyl-labelled Scorpion), Exciprobe-3′-PY (3′-pyrene-labelled Exciprobe; Exciprobe is a short oligonucleotide, see Figure 2B), Scorpion-5′-PY (5′-pyrene-labelled Scorpion), Exciprobe-3′-N (3′-naphthalenyl-labelled Exciprobe), Exciprobe-3′-p (3′-phosphate-labelled Exciprobe; control).

INTRODUCTION

Molecular Beacons [1], Scorpion primers [2], HyBeacons [3] and TaqMan probes [4,5] are generally single-stranded oligonucleotides that utilize a fluorophore and quencher. In the dark (quenched) form, they adopt a conformation with the quencher close to the fluorescent dye. On hybridization to the target molecule, they undergo a conformational change (Molecular Beacons and Scorpion probes), an environmental change for the fluorophore (HyBeacons) or enzymatic cleavage (TaqMan), resulting in a fluorescence change.

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Abbreviations used: exci-partner, exciplex partner; Exciprobe-3′-N, 3′-naphthalenyl-labelled Exciprobe; Exciprobe-3′-p, 3′-phosphate-labelled Exciprobe; Exciprobe-3′-PY, 3′-pyrene-labelled Exciprobe; LES, locally excited state; Scorpion-5′-N, 5′-naphthalenyl-labelled Scorpion; Scorpion-5′-p, 5′-phosphate-labelled Scorpion; Scorpion-5′-PY, 5′-pyrene-labelled Scorpion; TFE, trifluoroethanol; Tm, ‘melting’ temperature of DNA; Tris buffer, 10 mM Tris/0.1 M NaCl (pH 8.4).

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Scorpion oligonucleotides were from Biosearch Technologies (Novato, CA, U.S.A.), DNA probes and DNA targets from Sigma-Proligo (Paris, France), and deuterium oxide from Goss Scientific Instruments. N-methyl-N-naphthalen-1-yl-ethane-1,2-diamine dihydrochloride exci-partner, 1-pyrenemethylamine and protocols for attachment to oligonucleotides and Scorpion probes have been described previously [8]. Water was distilled and purified by ion exchange and charcoal (MilliQ, Millipore). Tris buffer (10 mM Tris/0.1 M NaCl, pH 8.4) was prepared from analytical grade materials.

HPLC was performed on an Agilent 1100 Series system, with both diode array and fluorescence detection for online acquisition of spectra. The columns used were: Zorbax Eclipse X DB-C8 column (125 mm × 4.6 mm, 5 µm), or Luna C18 (2) (25 cm × 4.6 mm, 5 µm) using a 0–50 % (v/v) acetonitrile/water gradient.

NMR spectra were recorded on a Bruker 300 MHz (7.05 T) spectrometer (Avance-300) at 300 MHz for 1H NMR and 121 MHz for 31P. Chemical shifts (δ) are in p.p.m., relative to Me₄Si (0.00) for 1H spectra and the intramolecular phosphate (0.00) for 31P spectra.

UV–visible absorption spectra were measured at 20°C on a Peltier-thermostatted Cary-Varian 1E UV–visible spectrophotometer. Quantification of oligonucleotide components used millimolar absorption coefficients (εᵥ) of 79.9 for Exciprobe-3'-p, and 312.3 for Scorpion target. Molar absorption coefficients were calculated by the nearest neighbour method [13]; the contribution of the exci-partners was small and could be neglected. T_m (‘melting’ temperature of DNA) values (first derivative method), on the basis of Aᵥ260, were determined in Peltier-thermostatted quartz cuvettes using a Cary 4000 UV–visible spectrophotometer.

Fluorescence emission and excitation spectra were recorded in 2 ml four-sided quartz thermostatted cuvettes using a Peltier-controlled Cary-Eclipse or a Shimadzu RF-5301PC spectrofluorophotometer, with the temperature controlled by circulating water from a Haake GH water cooler. Spectra were recorded in Tris buffer containing various percentages of TFE (trifluoroethanol). Excitation wavelengths for both LES (locally excited state) and exciplex emission were optimized in each experiment. Spectra were corrected for any TFE, buffer or naphthalene background emission under the optimized experimental conditions as appropriate. A working solution (700 µl) was prepared, for example, by taking an aliquot (11 µl of 0.224 mM stock) of Scorpion-5'-PY (final cuvette concentration, 3.52 µM), Exciprobe-3'-N (0.7 µl, final cuvette concentration, 3.80 µM), Tris buffer (70 µl) and Milli-Q water (618.3 µl). The emission spectrum was recorded and TFE sequentially added [to give 19.6 %, 32 % and 41 % (v/v)], with emission spectra sequentially taken.
After attachment of 1-pyrenemethylamine (giving Scorpion-5′-PY), the free Scorpion oligonucleotide eluted early (26 min), followed by the 5′-pyrene oligonucleotide-conjugate (30 min) and unconjugated 1-pyrenemethylamine (33 min). Eluted peaks were detected at multiple wavelengths (260, 280, 340 and 480 nm), with a typical yield of ~23%. The 348 nm absorbance band is due to pyrene (also affects 260 nm band). The ratio of absorbance at 260 nm (A = 0.303) to 345 nm (A = 0.026) was ~11.6. Attachments of 1-pyrenemethylamine to 8-mer probe resulted in $A_{260}/A_{345}$ ratio of 3.0 [8,14]. Appropriate fractions were combined and freeze-dried, and the modified oligonucleotides characterized by $^3$P NMR spectroscopy in $^2$H$_2$O. $^3$P NMR spectroscopy of the unmodified Scorpion probe provided a control confirming the presence of the phosphoramidate bond [15].

After attachment of 1-naphthalenemethylamine to give Scorpion-5′-N, HPLC showed elution of the 3′-naphthalene oligonucleotide conjugate (29 min), and unconjugated 5′-methyl-N′-naphthalen-1-yl-ethane-1,2-diamine (34 min), with a typical yield of ~83%. UV/visible absorption spectra of unmodified Scorpion-5′-p (5′-phosphate-labelled Scorpion) and Scorpion-5′-N were similar to those described previously [8]. The shoulder at 310 nm on the 260 nm absorption band substantiated the presence of attached naphthalene. In addition to the phosphodiester signal at ~0 p.p.m., a new $^3$P NMR signal at ~10.2 p.p.m. is attributable to phosphoramidate in Scorpion-5′-N [15].

**RESULTS**

Exciplex emission of Scorpion-5′-PY plus Exciprobe-3′-N was not observed in Tris buffer (5 °C) in the absence of TFE. Sequentially adding TFE (19.6%, 32% and 41%, v/v) led to increased fluorescence intensity at 466 nm, with the largest increase at 32% (v/v) TFE (Figure 3). No exciplex signal was seen for 47–90% (v/v) TFE.

No exciplex band at 466 nm in the corresponding control system in 32% (v/v) TFE/Tris buffer formed when the naphthalenyl group on Exciprobe-3′-N was replaced by a phosphate (Exciprobe-3′-p), even after heating the system to 60 °C and re-annealing by cooling back to 5 °C.

The $T_m$ value for the Scorpion-5′-p alone in 41% (v/v) TFE/Tris buffer was 50.0 ± 1.0 °C (Figure 4), and for Exciprobe-3′-N and Scorpion-5′-PY (full system) together in 41% (v/v) TFE/Tris buffer (Figure 4), values were 16.0 ± 0.5 and 52.0 ± 0.5 °C respectively.

No exciplex signal was detected at any TFE concentration for the system composed of Scorpion-5′-N and Exciprobe-3′-PY.

**DISCUSSION**

Sigmoidal melting curves on the tandem duplex Scorpion systems provided strong evidence of duplex formation (Figure 4). The single melting transition of Scorpion-5′-p alone ($T_m$, 50.0 ± 1.0 °C) corresponds to opening the hairpin of the Scorpion (Figure 4, inset), which is consistent with previous studies [16–18]. For the full exciplex system of Exciprobe-3′-N and Scorpion-5′-PY, the two transitions (16.0 ± 0.5 and 52.0 ± 0.5 °C) probably correspond to the melting transitions of the short 8-mer Exciprobe-3′-N and Scorpion hairpin-loop respectively [19], the value of the higher $T_m$ being close to that of the parent Scorpion-5′-p under the same conditions (Figure 4).

Additional evidence of duplex formation comes from the emission spectra. The Scorpion target alone showed no exciplex peak at 466 nm in the absence of Exciprobe-3′-N. The addition of Exciprobe-3′-N to the Scorpion target caused a 2–4 nm red shift of the pyrene LES emission, accompanied by its quenching. A new structureless band appeared at ~466 nm (Figure 3), which is characteristic of a naphthalene–pyrene exciplex [7–10]. Exciplex emission is achievable due to interaction of the


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