Homogeneous Detection of Nucleic Acids by Transient-State Polarized Fluorescence

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We describe a transient-state polarized fluorescence-based method for detecting nucleic acids. An active ester of the phthalocyanine dye La Jolla Blue™ was coupled to an oligonucleotide containing an amino group at its 5' end, and the conjugate was purified by HPLC chromatography. We monitored the hybridization characteristics of the conjugate with complementary oligonucleotides and RNA as targets by transient-state polarized fluorescence measurements. The method was comparable in sensitivity to isotopic and nonisotopic heterogeneous detection systems and was capable of detecting 1 fmol of a 382-base-long RNA transcript from human immunodeficiency virus type 1 (HIV-1) generated in a self-sustained sequence replication (3SR) reaction.

Indexing Terms: phthalocyanine • self-sustained sequence replication • hybridization

Nucleic acid hybridization assays are important for the clinical detection of viral and microbial pathogens and genetic disorders (1, 2). Currently, assay formats are predominantly based on heterogeneous hybridization, in which the target nucleic acid is sequenced on a solid support to allow separation of hybridized and unhybridized detection probe (3). Although these heterogeneous assays display good sensitivity, the necessity of separation and wash steps makes automation difficult. A number of homogeneous hybridization assays exploit the properties of donor and acceptor fluorophor-labeled oligonucleotides to transfer or quench fluorescence energy when hybridized to an analyte (4, 5). Arnold et al. (6) described a chemiluminescence-based detection assay that exploits the differential rates of hydrolysis of acridinium ester-labeled oligonucleotide probes. Although sensitivities in the attomole range are reported, their assay formats require the addition of reagents to trigger the chemiluminescent reactions. Homogeneous assays for which conventional fluorophors, such as fluorescein, are used are compromised by background fluorescence from biological test samples. However, these systems permit the monitoring of hybridization reactions in real time.

Here, we report the use of an oligonucleotide labeled with the phthalocyanine dye La Jolla Blue™ (Diatron Corp., San Diego, CA) (see Figure 1) to detect complementary DNA and RNA in a homogeneous format using transient-state polarized fluorescence (TSPF)³ (7, 8). This fluorophor is highly sensitive in homogeneous immunoassays (9) and has the advantages of an absorbance maximum at 685 nm, a high molar absorption coefficient, and low nonspecific binding to biological molecules by its axial polyethylene glycol (PEG) ligands (10). Furthermore, the emission wavelength maximum (705 nm) of the dye in the near infrared region is well separated from the intrinsic fluorescence of biological molecules. The use of fluorescence detection, therefore, allows excellent discrimination between the dye signal and background signals in the test sample. Our approach is based on the increase in polarized fluorescence resulting from longer rotational relaxation times (11) of duplexes of the La Jolla Blue–oligonucleotide conjugate and its complementary targets. We monitored changes in polarized fluorescence with instrumentation that generates short excitation pulses with a laser diode source, and then used a photomultiplier-based transient-state detection system to measure the fluorescence signal (12). This simple nonisotopic "mix-and-read" hybridization assay has a sensitivity equivalent to some of the more complex heterogeneous assays currently available for the detection of single-stranded DNA and RNA.

³ Nonstandard abbreviations: TSPF, transient-state polarized fluorescence; PEG, polyethylene glycol; SSP, saline–sodium phosphate; DMSO, dimethyl sulfoxide; DEPC, diethyl pyrocarbonate; 3SR, self-sustained sequence replication; HIV-1, human immunodeficiency virus type 1; and BBSH, bead-based sandwich hybridization.

Fig. 1. Structure of La Jolla Blue
Absorption maximum, 680 nm; ε = 1.7 × 10⁶ L · mol⁻¹ · cm⁻¹; quantum yield = 70%

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Materials and Methods

Materials

Phosphoramidites and Aminolink-2 reagent used in DNA synthesis were purchased from Applied Biosystems (Foster City, CA). The 20× saline–sodium phosphate (SSP) buffer contained 3.6 mol/L NaCl and 200 mmol/L NaH₂PO₄, pH 7.4. T4 kinase (EC 2.7.1.78) was obtained from New England Biolabs (Beverly, MA). Dimethyl sulfoxide (DMSO) and sorbitol (molecular biology grade) were obtained from Sigma Chemical Co., (St. Louis, MO), and dextran sulfate (sodium salt) was purchased from Pharmacia (Uppsala, Sweden). N-Methyl-2-pyrrolidone was obtained from Fluka (Buchs, Switzerland) and stored over 4A and 5A sieves.

All solutions used in handling RNA were treated with 1 mL/L diethyl pyrocarbonate (DEPC) and autoclaved to inactivate RNases. Derivatization of oligonucleotides and the hybridization reactions were performed in presiliconized microcentrifuge tubes obtained from Denve Scientific (Denville, NJ); cat. no. C19033). Radioactivity was measured by Cerenkov counting with a Beckman (Fullerton, CA) LS 7800 liquid scintillation counter. Oligonucleotides and the La Jolla Blue-conjugated derivatives were quantified by their absorbances at 260 and 680 nm with a Beckman DU-7400 or Perkin-Elmer (Norwalk, CT) Lambda 4C UV–Visible spectrophotometer. La Jolla Blue was synthesized by Diantron. All other reagents were obtained from Aldrich Chemical Corp. (Milwaukee, WI).

Procedures

Instrumentation. Fluorescence polarization measurements were performed on a Diamtron transient-state fluorometer, which has a 685-nm laser diode (Toshiba, Tokyo, Japan; Model TOLD9140) to excite the sample. The laser diode was pulsed for 2 ns at a 10-MHz repetition rate, and a thermal controller was used to maintain the diode temperature at 40 ± 0.1 °C. The output optics consisted of an optical fiber centered at 705 nm, a liquid crystal polarizer, and a photomultiplier tube (Hamamatsu, San Jose, CA; Model R2949). Emission from the sample was detected with time-correlated single-photon counting (13), with a resolution of 75 ps. Each sample was viewed for 10 s with the output polarizer oriented parallel (par.), then perpendicular (perp.), to the excitation polarization. In this way we obtained two histograms representing the parallel and perpendicular fluorescence decay profiles. The two fluorescence components, S(par.) and S(perp.), were calculated by summing the total counts over a portion of the data where the transient-state fluorescence signal has the highest signal-to-background ratio. Figure 2 shows a typical profile of the parallel and perpendicular fluorescence components vs time. The transient-state polarization is expressed as:

\[ \text{TS polarization (mP)} = \frac{1000 [S(\text{par.}) - S(\text{perp.})]}{[S(\text{par.}) + S(\text{perp.})]} \]

The transient-state instrumentation was configured to automatically read up to 12 reaction tubes with a volume of 1 mL or 100 μL. The optical system was thermally controlled at 35 ± 0.1 °C. Dedicated microprocessors were used to control mechanism movements, temperature, and transient-state data acquisition.

Oligonucleotide synthesis. We synthesized oligonucleotides on an Applied Biosystems 394 DNA/RNA synthesizer using standard phosphoramidite chemistry. The hexylamine-linked oligonucleotides were synthesized by using the AminoLink2 reagent in the last step of the automated synthesis. The tritylated and 5′-aminohexyl oligonucleotides were purified by reversed-phase HPLC as described previously (14). The purified oligonucleotides migrated as single bands on a 15% denaturing polyacrylamide gel. The sequences of the oligonucleotides are as follows:

90-422, 5′-AATTAGCCGATGATCACAATCACA-TGCT-3′; 86-272, 5′-TCTAATTACTACCTCTTTCTCTCT-GCTAGACT-3′; 92-157, 5′-AGCAGTTGAGTTGATACATC-TGAGTTCTATT-3′; 86-273, 5′-AGTCTAGAGAAGAGGTTAGAATTTAGA-3′; 89-255, 5′-TTATTTG-GCCCCGGCTGTGTTTTCGATCTCT-3′; 89-263, 5′-AATTTAATACGACTACTATAGGGATTAAAATGGT-GTTATCCATATTGTCTCCTACT-3′; 88-336, 5′-TACTCCTGGCCCCACAGGGCTACTGGGAC-3′. Underlined bases in oligonucleotide 89-263 denote the presence of a T7 promoter sequence, and the transcription initiation sequence is in boldface (15).

Synthesis of aminohexyl oligonucleotide derivative. Oligonucleotide 90-422 (10 nmol) was phosphorylated with T4 kinase and [γ-³²P]ATP by standard procedures (16). The phosphorylated oligonucleotide derivative was dissolved in 200 μL of a solution containing 0.1 mol/L 1-methylimidazole, 0.1 mol/L 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-HCl, and 0.25 mol/L ethylenediamine, pH 6.0. After 16 h at room temperature the reaction mixture was precipitated twice with 8 mol/L LiCl/absolute ethanol and redissolved in 10 mmol/L Tris-HCl and 1 mmol/L EDTA, pH 7.4.
Conjugate synthesis and purification. Oligonucleotide 90-422 (−500 nmol) was dissolved in 250 µL of 0.2 mol/L collidine, pH 7.7, in a 1.5-mL Denville centrifuge tube. After addition of 25 µL of 8 mol/L LiCl and 825 µL of absolute ethanol, the mixture was cooled in solid CO₂ and centrifuged at 8800 × g for 30 min in a microcentrifuge at 0 °C. After removal of the supernate, the pellet was redissolved in 1.3 mL of collidine buffer. A 100-µL aliquot (40 nmol) of the oligonucleotide solution was added to a dried sample (400 nmol) of the bis-N-hydroxysuccinimide ester of La Jolla Blue in a centrifuge tube. After addition of 25 µL of 8 mol/L LiCl and 50 µL of collidine buffer, the mixture was cooled to 0 °C. Finally, 75 µL of N-methylpyrrolidone was added and the reaction mixture was incubated at room temperature for 1 h and then maintained at 4 °C for a further 18 h. Absolute ethanol (750 µL) was added and the reaction products were precipitated. The pellet containing the conjugate and unreacted oligonucleotide was redissolved in 100 µL of collidine buffer. HPLC purification was performed on a C₈ reversed-phase analytical column (4.6 × 250 mm) with a gradient of acetonitrile in 0.1 mol/L triethylammonium acetate, pH 6.8. Effluent was monitored at 675 nm.

Self-Sustained Sequence Replication (3SR)™ amplification of human immunodeficiency virus type 1 (HIV-1) RNA. We used the oligonucleotide primers 89-255 and 89-263 to amplify a 382-base region of the env gene of HIV-1 RNA, as described previously (15). The target used was 0.1 amol of HIV-1 RNA extracted from infected CEM cells (17). The amplification reactions contained 100 mL/L DMSO and 150 g/L sorbitol and were carried out in a volume of 50 µL for 1 h. The concentration of 3SR (SIBIA, La Jolla, CA) product was estimated to be 1000 nmol/L, on the basis of a predetermined sandwich hybridization efficiency of 35% for this target using Trisacyrly-oligonucleotide capture beads and ³²P-labeled detection oligonucleotide.

Detection of 3SR RNA product by sandwich hybridization. Oligonucleotide 86-273 was attached to a Trisacyrly-GF-2000 support as described previously (18). Serial dilutions of the 3SR RNA product were assayed by bead-based sandwich hybridization (BBSH) (15) with Trisacyl-oligonucleotide 86-273 capture beads and 100 fmol of ³²P-labeled oligonucleotide 90-422 probe.

Fluorescence polarization assays. The desired amount of conjugate was dissolved in 5 µL of probe buffer (100 mmol/L Na₂HPO₄, pH 7.5, 175 mmol/L NaNCl, 0.5 mL/L Tween 20, 1 g/L PEG 2000), and mixed with 10 µL of the DNA or RNA analyte in DEPC-treated H₂O and 15 µL of hybridization buffer (10× SSP, 200 g/L dextran sulfate). Hybridizations were performed under the various conditions described in Results. After a brief centrifugation of the samples to remove condensation, 70 µL (or 1 mL where noted) of reading buffer (100 mmol/L Na₂HPO₄, 150 mmol/L NaCl, pH 7.5) was added, the solutions were transferred to glass cuvettes, and the transient-state fluorescence polarization values were measured.

Results

La Jolla Blue–oligonucleotide conjugate synthesis. The phthalocyanine dye La Jolla Blue (Figure 1) was activated as its bis-N-hydroxysuccinimide ester and coupled with either the aminoethyl or aminohexyl derivatives of the oligonucleotide 90-422 in aqueous collidine buffer. Following ethanol precipitation of the crude conjugates, the pellets were resuspended in the Tris-EDTA buffer at pH 8.1 and subjected to two cycles of reversed-phase HPLC purification. Comparison of the absorbances at 260 and 680 nm of the purified dye–oligonucleotide conjugates indicated an equimolar oligonucleotide-to-dye ratio (data not shown).

Optimization of hybridization conditions. Preliminary experiments indicated that the dye–oligonucleotide conjugates were nonspecifically adhering to the surface of the glass cuvettes. Initially, this problem was alleviated by using the 3SR reaction solution as the hybridization medium. This approach was effective in maintaining the conjugates in solution at subfemtomole concentrations (data not shown). The conjugates were dissolved in 0.5 mL/L Tween 20/1 g/L PEG-2000 and then hybridized in 5× SSP/100 g/L dextran sulfate. Before carrying out the studies described below, we found that the change in polarized fluorescence of the conjugates upon binding to the nucleic acid targets was dependent on the temperature of the hybridization reaction (data not shown). Temperature optima of 45 °C and 60 °C were determined for duplex formation with oligonucleotide DNA and 3SR RNA targets, respectively.

Detection of complementary DNA. We studied the time course of the hybridization reactions between the conjugate containing the 30-base hexylamine-derivatized oligonucleotide 90-422 (350 fmol) and a 25-fold molar excess of complementary and noncomplementary 30-base oligonucleotides by acquiring transient-state polarized fluorescence measurements at 49-s intervals. When oligonucleotide 92-157 was the complementary target (Figure 3), the fluorescence polarization values increased with time and reached a maximum after 5 min. In contrast, the fluorescence polarization values for the reactions with noncomplementary oligonucleotides showed no time-dependent increase from their initial values at time zero. Figure 4 shows the results of a titration experiment in which a 45-min hybridization between 5 fmol of La Jolla Blue–oligonucleotide 90-422 conjugate and increasing amounts of complementary oligonucleotide 92-157 was monitored by polarized fluorescence. The level of sensitivity was ~1 fmol. No improvement in the sensitivity of the assay was observed when an ethylamine group was substituted for the hexylamine group in the dye–oligonucleotide conjugate (data not shown).

Detection of complementary RNA. An antisense 382-base-long RNA transcript of the env gene of HIV-1 was generated by 3SR amplification and was detected with the complementary La Jolla Blue–oligonucleotide 90-422 conjugate. The rate of hybridization was slower with this more complex target (data not shown), and
hence, assays were typically conducted for 1 h at 60 °C. The dynamic range and the limit of sensitivity of the TSPF method were determined by incubating aliquots of the target RNA solution (1000 nmol/L) with two different amounts (10 and 0.2 fmol) of conjugate probe. It appears from Figure 5 that whereas the limit of sensitivity with 10 fmol of conjugate probe was ~16 fmol (0.016 μL) of the target RNA, as little as 2 fmol (0.002 μL) of the same RNA solution could be detected with 0.2 fmol of conjugate probe. At this lower probe concentration, the high intensity of the fluorescence signal (>100 000 photon counts/20 s) ensured highly reliable counting statistics.

Detection of complementary RNA by sandwich hybridization. We also assayed aliquots of the 3SR RNA solution with BBSH. A Trisacryl resin-bound 30-mer oligonucleotide, 86-273 (18, 19), complementary to the 3SR-generated RNA, was used as the affinity support, and 32P-labeled 90-422 oligonucleotide, which is complementary to another region of the 3SR product, was used as the detection probe. We used the Trisacryl-oligonucleotide beads and the detection oligonucleotide in a hybridization reaction with the 3SR RNA target at 42 °C for 90 min. Excess probe was washed away and the radioactivity associated with the solid support was used to estimate the amount of hybridized target. The limit of sensitivity with the BBSH method was ~1 fmol (0.001 μL) of the target RNA solution (Figure 6).
Discussion

The TSPF method offers a viable homogeneous nonisotopic system for detecting target nucleic acids. The sensitivity of this approach is due to a combination of factors: (a) the fluorescence characteristics of the La Jolla Blue; (b) transient-state fluorescence detection; (c) the laser light source of the instrumentation; and (d) the single-photon counting device used for detection of the emitted signals. These features contribute to a 50- to 100-fold improvement in sensitivity relative to steady-state fluorescence polarization-based detection with fluorescein-labeled nucleic acid probes (20). The polarized fluorescence method was shown to detect 100 fmol of a 3SR-generated HIV-1 env region RNA transcript with a 30-base-long fluorescein-modified oligonucleotide. The TSPF detection system is comparable in its sensitivity with $^{32}$P detection and with other heterogeneous nonisotopic methods that use lanthanide chelates for time-resolved fluorescence detection (21) and chemiluminescence-based alkaline phosphatase–oligonucleotide conjugates (14). However, unlike the lanthanide chelate-based and enzyme–oligonucleotide conjugate-based detection systems that can only use a fraction ($\leq 20\%$) of the 3SR product solution, the entire 3SR reaction mixture can be used in the TSPF assay, thereby providing a fivefold increase in sensitivity.

Quantitation of an unknown sample can be achieved with calibration curves (see Figure 5). However, a number of different probe concentrations may be necessary for a wide dynamic range. An attractive feature of this fluorescence method is its potential for continuously monitoring the kinetics of homogeneous hybridization reactions. However, the current TSPF assay is compromised by poor hybridization efficiencies; target concentrations substantially higher than the probe are required to drive the reaction to completion (Figure 5). Incorporation of the following changes in the TSPF system may improve the sensitivity of the assay: The use of shorter oligonucleotide probes should provide a larger change in fluorescence polarization and faster hybridization, and any steric hindrance due to the presence of the large axial ligand on the phthalocyanine dye may be minimized by using a smaller axial ligand than that of the La Jolla Blue. Based on the fluorescence intensities of 0.2 fmol of conjugate probe (>100 000 photon counts/20 s), it should therefore be possible to use lower concentrations of probe to detect targets in the attomole range. The ultimate limitation of the assay, however, is the dependence of the rates for hybrid formation on probe and target concentrations. At extremely low concentrations, this factor will become the critical determinant for the sensitivity of the assay.

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References