Amplifying Fluorescent Polymers

Direct Synthesis of an Oligonucleotide–Poly(phenylene ethynylene) Conjugate with a Precise One-to-One Molecular Ratio**

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Conjugated polyelectrolytes have great potential in biochemical sensor applications[1–8] because of their unique light-harvesting[9,10] and superquenching[11–14] properties. Among them, water-soluble poly(phenylene ethynylene)s (PPEs) are attractive candidates in optical biosensing because of their facile synthesis and high fluorescence quantum yields in aqueous solution.[5,6,15] Highly sensitive bioprobes can be constructed with these polymers for selective target recognition. To achieve this objective, the polymer must be conjugated with a biomolecule such as a DNA strand, a peptide, or a protein. Such conjugation can be accomplished by coupling PPEs with pendant reactive groups to biomolecules with specific reactive moieties, for example, by formation of an amide bond between a PPE functionalized with a carboxylic acid group and an amine-functionalized biomolecule. Although some progress has been made in the coupling of PPEs to biotin,[11,16] there remains a clear need to develop new strategies for coupling PPEs to oligonucleotides and proteins. Effective strategies for coupling such biomolecules to conjugated polymers would have significant implications for a variety of fields, including bioanalysis and biomedical diagnostics.[17] Unfortunately, the coupling of PPEs to large biomolecules is fraught with difficulty. First, the introduction of reactive pendant groups can be challenging and may also change the polymer properties. Second, unfavorable steric and electrostatic interactions between the polymer and the target biomolecule result in a low coupling efficiency. Moreover, the coupled product has chemical and physical properties similar to those of the free polymer, which makes it difficult to separate the conjugated product from the unreacted polymer. Finally, the degree of coupling to the polymer is difficult to control because of the nature of the polymer, the poor coupling efficiency, and the lack of effective separation methods.

Herein, we introduce a versatile and effective synthetic method for coupling oligonucleotides to conjugated polyelectrolytes. Instead of synthesizing the polymer and oligonucleotide separately before coupling, we treat the oligonucleotide as an "end-capping" monomer in the Pd-catalyzed step-growth polymerization of a PPE-based conjugated polyelectrolyte. The oligonucleotide takes part in the polymerization process and is incorporated into the PPE chain as an end-capping unit. The oligonucleotide-functionalized monomer is bound to a controlled pore glass (CPG) solid support, thereby allowing the DNA–PPE conjugate to be easily separated by centrifugation. Furthermore, this method not only allows an oligonucleotide to be conjugated to a PPE chain, but it can also be extended to other biomolecules such as biotin by using a similar approach with biotin phosphoramidite.

Scheme 1 shows the process of making a DNA–PPE conjugate. On a CPG support, an oligonucleotide with a defined sequence was synthesized from the 3′ end to the 5′ end by using standard phosphoramidite chemistry (a). 5′-Dimethoxytrityl-5-iodo-2′-deoxyuridine 3′-[(2-cyanoethyl)-(*N*,*N*-diisopropyl)]phosphoramidite (5I-dU phosphoramidite) was used to introduce a 5I-dU residue as the last base of the oligonucleotide (b), which provided the functionalization necessary to render the oligonucleotide active as a monomer for the PPE. Under Sonogashira conditions, the 5I-dU base couples to terminal alkynes with high efficiency.[18,19] Without deprotection and cleavage from the CPG solid support, the 5I-dU-functionalized oligonucleotide was added to the PPE polymerization solution as an end-capping monomer, thereby allowing the polymer chain to cross-couple with the CPG-linked oligonucleotide (c). After polymerization, the CPG was washed and centrifuged several times until no PPE was detected in the supernatant. This procedure was followed by overnight incubation in ammonia to cleave the DNA–PPE

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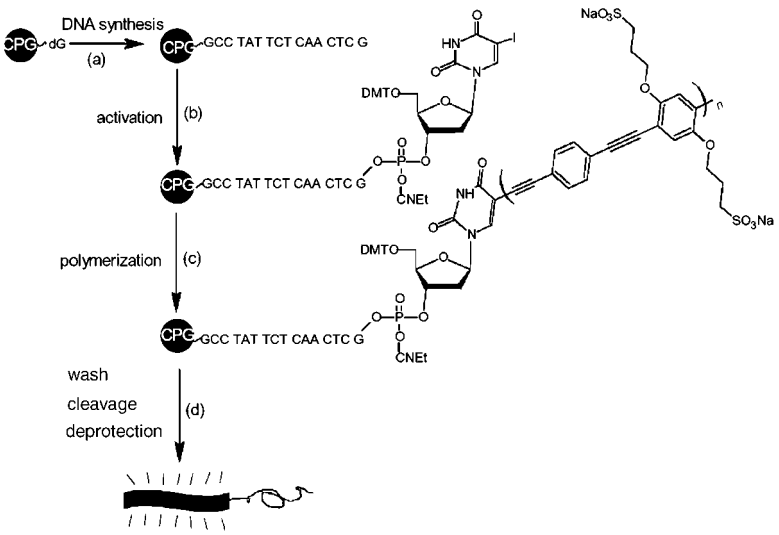
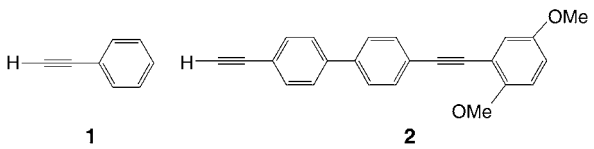


Scheme 1. Schematic representation of the solid-state synthesis of the DNA–PPE conjugate; for details see text. DMT = dimethoxytrityl.

conjugate from the solid support and to remove the protecting groups from the oligonucleotide bases. The product, DNA–PPE, was obtained after further desalting by means of ethanol precipitation (d).



Scheme 2. Model molecules used to couple to the 5I-dU-functionalized oligonucleotide.

Two small organic molecules (Scheme 2) were used to demonstrate that the 5I-dU-modified oligonucleotide is able to conjugate to the polymer monomer and oligomer present in the polymerization reaction mixture. In one model reaction, ethynylbenzene (**1**) was coupled with the CPG-linked DNA. In a second reaction, 4-[(2,5-dimethoxyphenyl)-ethynyl]-4′-ethynyl-1,1′-biphenyl (**2**) was used to mimic a small PPE oligomer. The coupling products from both model reactions were analyzed with reversed-phase gradient HPLC/ESI MS. The molecular weights observed by mass spectrometry matched those calculated from the product structures, a result indicating that the CPG-linked oligonucleotide is able to undergo cross-coupling with terminal acetylenes under Sonogashira coupling conditions.

In the synthesis of DNA–PPE, the CPG from four CPG columns (1-μmol scale) containing the 5I-dU-modified oligonucleotide was transferred to a 100-mL round-bottomed flask containing dimethylsulfoxide (DMSO, 20 mL). The PPE monomers disodium 3-[2,5-diiodo-4-(3-sulfonatopropoxy)-phenoxy]propane-1-sulfonate (690 μmol) and 1,4-diethynylbenzene (694 μmol) were then added to the solution with stirring under a gentle flow of argon. The resulting solution was deoxygenated by several cycles of vacuum–argon degassing. Another solution comprising $[Pd(PPh_3)_4]$ (20 μmol) and CuI (20 μmol) in DMSO (10 mL) was likewise deoxygenated and subsequently added dropwise to the monomer solution. The final mixture was again deoxygenated and stirred at room temperature under argon for 24 h. The resulting solution was viscous and brown-yellow in color, and it exhibited an intense blue-green fluorescence under near-UV illumination. The solution was then centrifuged and the precipitated CPG was washed several times with DMSO and water until the supernatant was clear and colorless. After washing, the CPG was still yellow-green and highly fluorescent. The CPG was then incubated in ammonia at 55 °C to cleave the oligonucleotide from the CPG and to deprotect the bases. After overnight incubation, the CPG became white whereas the liquid phase turned yellow-green and fluoresced under UV illumination, thus indicating that the PPE coupled to DNA was cleaved from the CPG as a result of the cleavage of DNA from the support. A control synthesis was carried out by following the same procedures and experimental conditions, but a 16-mer DNA without the 5I-dU base was used. After three repeated rinses prior to cleaving DNA from the solid support, the control CPG became white, thus indicating no PPE coupled to the DNA in this control experiment. The solutions that resulted after ammonia incubation of the CPG derivatized with 5I-dU oligonucleotide and of the control CPG were desalted by ethanol precipitation, dried, and redissolved in deionized water. Figure 1 compares the fluorescence emission spectra of the DNA–PPE and control solutions. The DNA–PPE solution shows an emission band with a maximum at 520 nm, which is consistent with a previous report that this PPE emits at 520 nm in water (the emission is broad because the PPE is aggregated in water).[5] Deaggregation of the PPE, induced by the addition of a nonionic surfactant, dispersion into agarose gel, or changing to methanol as the solvent,[5] shifts its emission maximum to

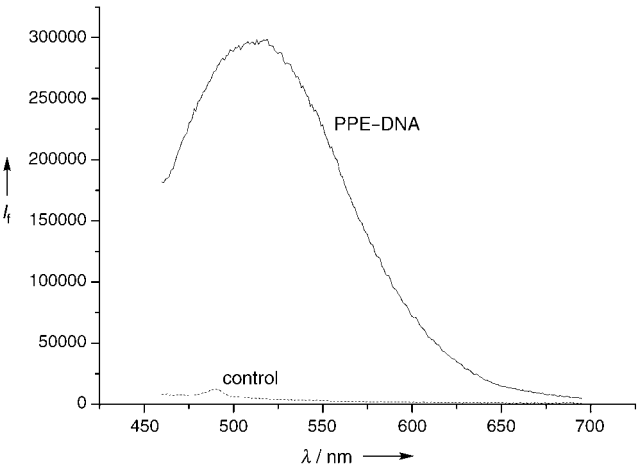


Figure 1. Fluorescence emission spectra of DNA–PPE and the control solution. I_f: fluorescence intensity.

455 nm (see the Supporting Information). The strong fluorescence from the DNA–PPE solution and the lack of fluorescence from the control solution suggest that the PPE has been successfully coupled to the DNA.

Mass spectrometry analysis indicated that the DNA structure remained intact after exposure to the polymerization conditions (see the Supporting Information). Data from DNA hybridization experiments revealed no significant change in the binding capability of these oligonucleotides (see the Supporting Information).

The fact that the PPE is coupled to the 5I-dU-modified DNA was further confirmed by gel electrophoresis, in which a 0.5% agarose gel was used to analyze the DNA–PPE conjugate, the PPE, and the DNA obtained from the control synthesis. As shown in Figure 2, in the DNA lane (3), only one band is observed, while in the PPE lane (2), no DNA band exists. By contrast, there are two bands in the DNA–PPE lane (1), which suggests that at least two types of DNA are present. One is probably the "free" DNA (this band has a migration rate similar to that in lane 3), and the second is likely to be the DNA–PPE conjugate. The DNA–PPE band migrates very little in the agarose matrix; the reasons might be the rigid rod structure[7] of the PPE and the large molecular weight of the PPE–DNA conjugate. Quantitative analysis of the DNA–PPE lane revealed that the ratio of the overall intensity for the DNA band to that for the DNA–PPE conjugate band is about 1.2:1, a result that indicates a yield higher than 45% for the coupling reaction between the DNA strand and the PPE.

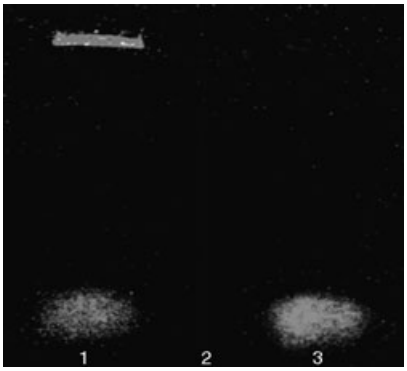
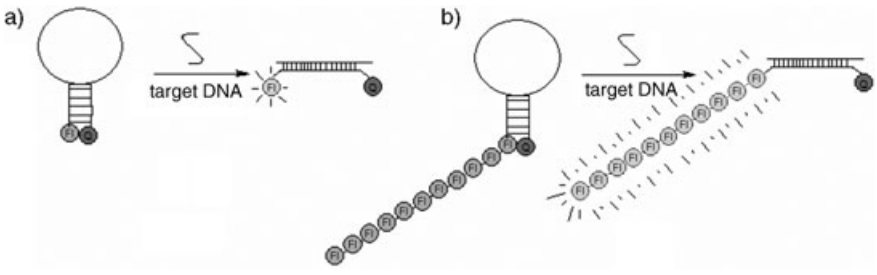


Figure 2. Gel electrophoresis of PPE–DNA (lane 1), PPE (lane 2), and DNA (lane 3) samples. Conditions: agarose (0.5%), 1× TBE buffer (0.089 M tris(hydroxymethyl)aminomethane (Tris), 0.089 M borate, and 2 mM ethylenediaminetetraacetate (EDTA), pH 8.2–8.4), 90 V for 20 min. The gel was prestained with ethidium bromide for DNA detection. Pictures were taken with a camera in fluorescence mode with a 540–640 nm band pass filter. This filter passes emission from ethidium bromide, thereby indicating the presence of oligonucleotide, while removing the emission for PPE under these conditions (PPE emits at 455 nm in agarose gel).

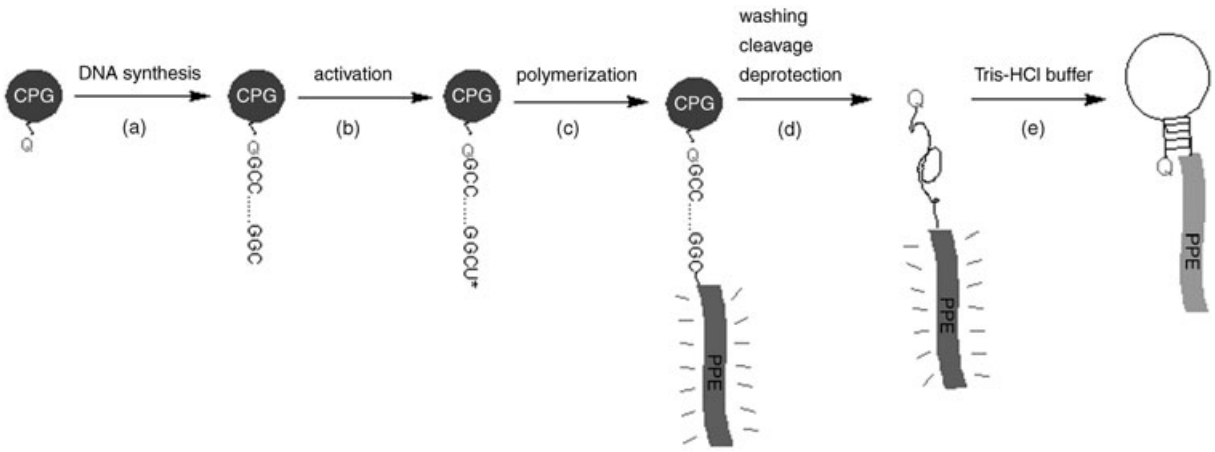
The successful establishment of this conjugation method allows us to construct a variety of biosensors with conjugated polymers, where precise control of the conjugation ratio of recognition molecule to polymer and complete separation of free conjugated polymer from biofunctionalized conjugated polymer are crucial. Our first attempt was to synthesize a molecular beacon with a conjugated polymer chain as its fluorophore. A molecular beacon[20,21] is a hairpin-shaped oligonucleotide with a fluorescent dye (Fl) at one end and a quencher (Q) at the other end. In the absence of the target DNA, the fluorescent dye and quencher molecule are brought close together by the self-complementary stem of the probe, and the fluorescence signal is suppressed. The perfectly matched DNA duplex is more stable than the single-stranded hairpin, so the molecular beacon readily hybridizes to its target sequence, thereby disrupting the stem structure, separating the fluorophore from the quencher, and restoring the fluorescence signal (Scheme 3a). Compared to a traditional molecular beacon, this new design uses a polymer chain as the fluorophore to amplify the fluorescence signal. When the molecular beacon is in its closed state, the polymer chain will be brought close to the quencher. It is expected that the fluorescence of the conjugated polymer will be strongly suppressed because of the superquenching property of the conjugated polymer. After target-DNA binding, the fluorescence of the conjugated polymer will be restored as a result of the increased separation distance between the conjugated polymer and the quencher (Scheme 3b).

The synthesis of the molecular beacon followed the same procedure as described above, except that a 3′-DABCYL-modified quencher CPG was used instead of a regular base CPG (Scheme 4; DABCYL = 4-(4-(dimethylamino)phenylazo)benzoic acid). As a universal quencher, DABCYL has been widely used as the quencher in molecular-beacon synthesis. Another reason why DABCYL was chosen was because its absorption overlaps with the PPE emission. Quenching experiments revealed that the Stern–Volmer quenching constant of DABCYL to PPE in free solution is about $4\times10^{6}\,\text{M}^{-1}$ (see the Supporting Information). The molecular-beacon sequence synthesized was 5′-PPE-CCTAGCTCTAAATCACTATGGTCGCGCTAGG-DABCYL-3′. Theoretical calculations indicated that this sequence could form a stable hairpin structure.

Figure 3 shows the response of the molecular beacon labeled with the conjugated polymer to a fivefold excess of its target DNA. When a lower concentration of the target was



Scheme 3. Working principles of a molecular beacon (a) and a conjugated-polymer-labeled molecular beacon (b). In a regular molecular beacon, one fluorophore is used to report a target-binding event, whereas a chain of fluorophores is used in the polymer-labeled molecular beacon. Q = quencher, Fl = fluorophore.



Scheme 4. Schematic representation of the solid-state synthesis of the PPE-labeled molecular beacon. Q stands for the DABCYL quencher.

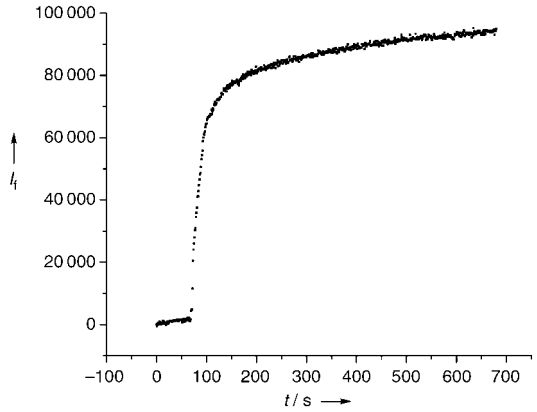


Figure 3. Response of the PPE-labeled molecular beacon synthesized according to Scheme 4 to its target DNA. Target sequence: 5′-GCG AC-CATA GTG ATT TAG A-3′. Buffer conditions: 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM $MgCl_2$, 0.1% Tween 20.

used, a slower reaction profile was observed. When a large excess of random-sequence DNA was added to the molecular-beacon solution as a negative control, no substantial change in the fluorescence intensity was observed. These hybridization results suggested that 1) the DABCYL molecule quenches the polymer chain when the molecular beacon is in the hairpin conformation and 2) the molecular beacon labeled with conjugated polymer functions as a normal molecular beacon that selectively hybridizes to its target DNA.

In conclusion, a novel synthetic method has been developed for the conjugation of a water-soluble PPE with an oligonucleotide. Coupling was achieved by carrying out the PPE polymerization reaction in the presence of a 5I-dU-terminated oligonucleotide linked to a CPG support. The DNA–PPE product can be easily separated from free PPE by centrifugation. The conjugation reaction is simple, fast, and easily controllable. The efficiency of the DNA–PPE coupling is high. Mass spectrometry analysis and the results of a DNA-hybridization study indicated that the polymerization conditions were so mild that the DNA exposed to them not only remained structurally intact but also kept its biorecognition capability. The new method has four distinct advantages: stoichiometric labeling of DNA to the polymer chain, easy separation to enable high purity of the desired final product, high yields for the DNA–PPE conjugation, and a stable product as a result of covalent conjugation between the biomolecules and the PPE. This new method makes it possible to efficiently couple a fluorescent amplifying polymer with biomolecules for bioanalysis and biosensor applications. As an example of using a conjugated polymer for DNA sensing, a molecular beacon with a conjugated polymer chain as the signaling element was prepared. Without the strategies described herein, the preparation of such a molecular beacon would be extremely difficult or even impossible. This molecular beacon gave a strong fluorescence signal specifically for the complementary sequence and showed promising results in bioanalysis. The physical, chemical, spectral, and biological properties of the DNA–PPE hybrid material are currently being investigated. The application of PPE-labeled molecular beacons to highly sensitive bioassays[22] is in progress.

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