Facile Synthesis of Advanced Photodynamic Molecular Beacon Architectures

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Nucleic acid photodynamic molecular beacons (PMBs) are a class of activatable photosensitizers that increase singlet oxygen generation upon binding a specific target sequence. Normally, PMBs are functionalized with multiple solution-phase labeling and purification steps. Here, we make use of a flexible solid-phase approach for completely automated synthesis of PMBs. This enabled the creation of a new type of molecular beacon that uses a linear superquencher architecture. The 3′ terminus was labeled with a photosensitizer by generating pyropheophorbide-labeled solid-phase support. The 5′ terminus was labeled with up to three consecutive additions of a dark quencher phosphoramidite. These photosensitizing and quenching moieties were stable in the harsh DNA synthesis environment and their hydrophobicity facilitated PMB purification by HPLC. Linear superquenchers exhibited highly efficient quenching. This fully automated synthesis method simplifies not only the synthesis and purification of PMBs, but also the creation of new activatable photosensitizer designs.

INTRODUCTION

Activatable photosensitizers modulate singlet oxygen production in response to interactions with a variety of target biomolecules (*1*). Nucleic acid-activatable photosensitizers are based on hybridization to specific nucleic acid target sequences. Nucleic acid photodynamic molecular beacons (PMBs) synthesized with porphyrin or phthalocyanine photosensitizers have recently been developed (*2, 3*). Molecular beacons are hairpin-loop structures that hold a quencher and fluorophore together, until the loop portion hybridizes to a target sequence, causing stem separation and unquenching. PMBs are an extension of prototypical molecular beacons that offer single base mismatch sensitivity and large fluorescence signal differences between the hybridized and nonhybridized states (*4, 5*). Excluding molecular beacons, alternative strategies for DNA control of singlet oxygen have also been proposed. Single-walled nanotubes that bound and quenched a chlorin-*e*6 conjugated aptamer were displaced when the aptamer bound its thrombin target, leading to photosensitizer activation (*6*). Another approach was based on the hybridization and displacement of pyropheophorbide-labeled oligonucleotides to another quenching DNA strand (*7*). These multicomponent systems make use of separate quenching and photosensitizing molecules, which simplifies the synthesis and purification of each individual component but adds a layer of uncertainty of how the subunits behave in diverse hybridization conditions. Here, we report a new synthesis method for PMBs that facilitates their synthesis and purification and also permits the development of novel photodynamic beacon architectures.

Although production of standard molecular beacons is routine, there have been few reports of synthesis and purification of PMBs. Normally, the nucleic acid portion is produced using standard DNA synthesis methods, and then one beacon terminus is labeled with a photosensitizer and the other is labeled with a quenching (or self-quenching) moiety. There are several approaches to the conjugation of these functional groups. To simplify the labeling procedure, previous efforts resulted in the development of a porphyrin phosphoramidite (*7*). While this phosphoramidite is ideal for labeling the 5′ terminus of an oligonucleotide in the final addition cycle of a solid-phase synthesis, no subsequent additions are possible. The most common approach is the incorporation of amine-modified functional groups with different protecting groups that permit sequential deprotection and labeling of each terminus using an activated ester or acid-modified labeling moiety. In the case of self-quenched beacons, only a single type of conjugatable group is required since the same labeling reaction may be repeated at both termini. In some cases, it may be challenging to find a suitable solvent for dissolving the hydrophobic photosensitizer with the hydrophilic nucleic acid. If the oligonucleotide is conjugated at the 3′ prior to synthesis, purification problems can arise using reverse-phase HPLC, because short degradation sequences labeled with a hydrophobic photosensitizer or quencher can elute at the same time as the ion-paired full-length product. Solid-phase synthesis approaches have been described for dual terminal oligonucleotide labeling (*8*) and are applied here to automate PMB synthesis and simplify purification.

RESULTS AND DISCUSSION

To generate a complete solid-phase synthesis approach for PMBs, we first labeled controlled pore glass (CPG) with a photosensitizer. Pyropheophorbide was a suitable choice, since this fluorescent photosensitizer was stable under the harsh conditions of multiple DNA synthesis cycles and oligonucleotide deprotection. The photosensitizer was conjugated to the CPG using a simple dehydration reaction after removing the CPG FMOC group (Figure 1A). After conjugation and washing, the statically charged CPG displayed a dark green color (Figure 1B). A solid-phase packing scheme was developed using a vacuum line, in which empty columns were two-thirds filled to ensure reproducible synthesizer reagent flowthrough during the synthesis (Figure 1C). For the quenching moiety on the opposing 5′ terminus, we employed a commercially available phosphoramidite quencher, BlackBerry quencher dT (BBQ-dT). This is nonterminating and so could be used for multiple additions. As

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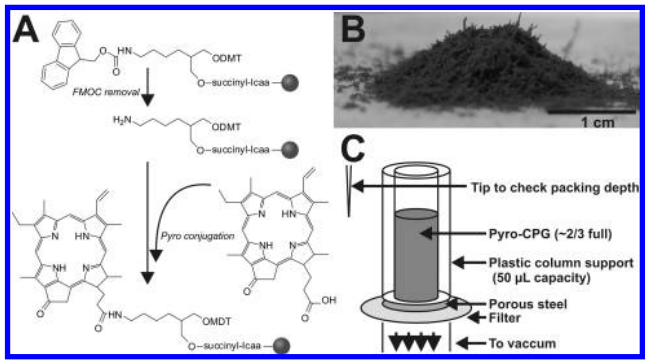

Figure 1. Solid-phase support for automated PMB synthesis. (A) Synthetic scheme for generation of pyropheophorbide solid-phase CPG support. (B) Photograph of pyropheophorbide CPG. (C) Packing method for achieving reproducible solid-phase synthesis.

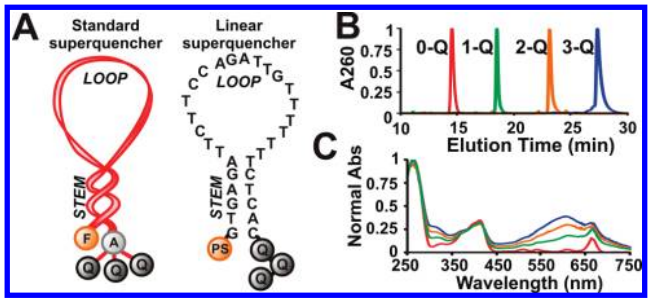

Figure 2. Solid-phase generation of new PMB architectures. (A) Standard (left) and linear (right) superquenchers. F represents the fluorophore, A represents an adapter phosphoramidite, and Q represents the quencher. The linear superquencher details the sequence used. PS represents pyropheophorbide. (B) HPLC retention times of the 0, 1, 2, or 3 quencher PMBs. (C) Absorption profiles of the molecular beacons: color scheme as in B.

an example of a useful application of this solid-phase synthesis approach, we generated a new class of PMBs that feature the first linear superquencher.

Superquenchers are assemblies of multiple quenchers that efficiently quench molecular beacon fluorescence when the beacon is in the inactive state (*9*). These have not been previously explored as singlet oxygen quenchers. Standard superquenchers use a trebler phosphoramidite that branches the oligonucleotide, permitting multiple quencher additions in the next addition cycle (Figure 2A, left). While this approach is effective, it requires the use of an additional specialty phosphoramidite (the trebler phosphoramidite, in addition to the quencher phosphoramidite). Instead, we used multiple additions of the BlackBerry quencher phosphoramidite at the 5′ terminus to achieve the first "linear superquencher" (Figure 2A, right) that has multiple quencher moieties attached sequentially (via phosphodiester bonds) in series rather than in parallel. The PMB included pyropheophorbide on the 3′ terminus, the oligonucleotide sequence indicated in the figure (which is a shared stem beacon (*10*) with one stem complementary to a portion the target sequence), and 0, 1, 2, or 3 BlackBerry quenchers linked on the 5′ terminus. After synthesis, the PMBs were deprotected with methylamine/ammonium hydroxide (1:1) for 15 min at 55 °C and were then purified by reverse-phase HPLC. This rapid protocol resulted in PMBs that could be synthesized and purified in a single day. The PMB identities were confirmed with mass spectroscopy. Figure 2B shows the reverse-phase HPLC elution profiles of the beacons. As expected, each additional quencher increased the hydrophobicity and retention times of the beacon. One advantage of the increased retention time was that the linear superquencher could be distinguished from pyropheophorbide-labeled degradation products that tend to elute near the full-length beacon with no quencher or a single quencher (note that Figure 2 shows traces for the purified beacons). The addition of 2 or 3 hydrophobic quenching moieties shifted the elution time so that HPLC purification of the full-length product was straightforward. The absorption profile of the 0, 1, 2, or 3-quencher beacons shown in Figure 2C confirmed that the oligonucleotide (260 nm) and pyropheophorbide (410 and 680 nm) ratios remained constant, while each additional quenching moiety contributed to the broad peak from 500 to 750 nm. The separate spectra of pyropheophorbide and BlackBerry quencher are shown in Supporting Information Figure 1.

The fluorescence response of the beacons was characterized to confirm the beacon activation. As shown in Figure 3A, all

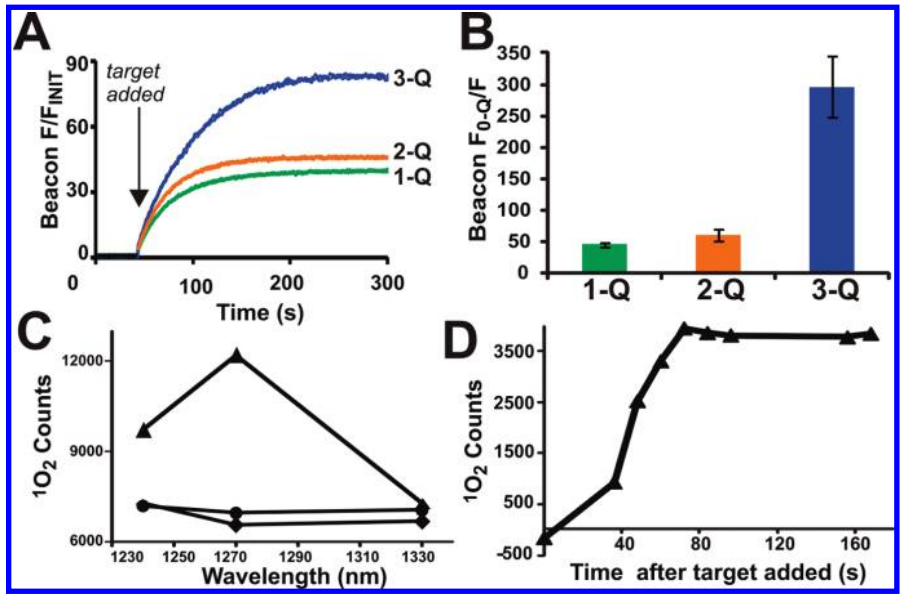

Figure 3. Characterization of linear superquencher (A) 50 nM PMB fluorescence response to addition of a 10-fold excess of target DNA in buffered saline. F is the beacon fluorescence and F_{INIT} is the beacon fluorescence at the initial time point. (B) Beacon fluorescence quenching compared to the 0-Q beacon. (C) Direct measurement of singlet oxygen luminescence for the 1-Q beacon. Triangles: beacon + target. Circles: buffer alone. Diamonds: beacon alone. (D) Singlet oxygen luminescence activation kinetics of the 1-Q beacon measured in buffered saline supplemented with 75% D_2O.

beacons rapidly hybridized to the target nucleic acid, resulting in increased fluorescence. While the single and double quencher were activated 30- and 40-fold, respectively, the triple quencher had a higher activation of approximately 90-fold. This activation is similar to the 100-fold activation observed using gold as a potent molecular beacon quencher (*11*), yet it is not as effective as the 300-fold activation observed with standard branched superquenchers (*9*). However, when the fluorescence intensity of the closed beacon was compared to the 0-Q beacon that lacked any quencher (as opposed to comparing the closed beacon to the target-activated beacon), 300-fold quenching was indeed observed (Figure 3B). This shows that the quenching of the triple quencher was extremely effective, but there was some residual intramolecular quenching in the activated state likely due to the extremely hydrophobic nature of the photosensitizer and quenchers. The singlet oxygen generation of the beacons was next examined. As shown in Figure 3C, in the closed state, the beacon with only 1 quencher generated lower singlet oxygen luminescence than that of the buffer alone. However, when the target was added, a clear singlet oxygen luminescence peak appeared at 1270 nm as expected. Maximum singlet oxygen activation occurred within 80 s of target addition (Figure 3D). Because the singlet oxygen luminescence of the closed-state beacon was below the instrument detection limits with even the 1-Q beacon, we could not accurately determine the activation of the 2-Q or 3-Q PMBs. It would be expected to be similar to the activation of fluorescence, since the degree of fluorescence quenching is highly correlated to the degree of singlet oxygen quenching (*12*).

In summary, the solid-phase approach was effective for generating PMBs that are more convenient to synthesize and purify. By using pyropheophorbide modified CPG, the entire PMB could be synthesized in one run on a standard DNA synthesizer using only one specialty quenching phosphoramidite. Purification was also facilitated by the change in HPLC retention time induced by the presence of multiple quenchers. This synthetic approach permitted the generation of a modified and powerful singlet oxygen superquencher, in which the quenchers were assembled linearly rather than in a branched configuration. The 3-Q PMB displayed favorable characteristics for purification and low-background activation. Fully automated DNA synthesis methods facilitate effective nucleic acid activatable photosensitizer design and implementation.

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Supporting Information Available: Experimental procedures and absorption spectra of BBQ and pyropheophorbide. This material is available free of charge via the Internet at http://pubs.acs.org.

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