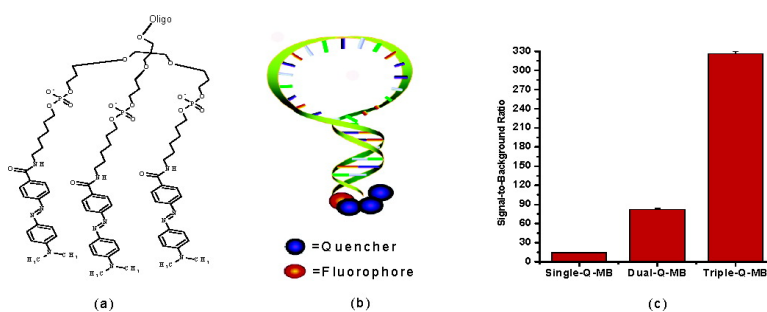Communication

Molecular Assembly of Superquenchers in Signaling Molecular Interactions

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(a)   (b)   (c)

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Molecular Assembly of Superquenchers in Signaling Molecular Interactions

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We have designed a molecular assembly of Superquenchers (SQs) for molecular interaction studies and for ultrasensitive bioanalysis. Signaling biomolecular interactions, such as DNA/RNA hybridization and protein interactions, is critically important in areas such as medical diagnosis, disease prevention, and drug discovery. While selectivity in biomolecular recognition can be achieved by capitalizing on highly selective molecular interactions, such as antibody–antigen binding and DNA base paring, the effectiveness of a molecular probe is highly dependent on the scheme and the ability to transduce a target recognition event into a measurable signal. Of many approaches, fluorescence is one of the most effective ways to signal molecular interaction and recognition. For instance, fluorogenic probes, such as Taqman,[1] molecular beacons (MB),[2,3] and protease probes[4] have been widely used for biotechnological research and development. The interaction of these molecular probes with target molecules renders unquenching of the fluorophores, yielding detectable fluorescence signal change. While exploited in broad areas, such as real-time PCR monitoring, DNA assays, and protein studies, these probes have limited increment of signal change upon interacting with their targets, mainly due to an unquenched high background signal from the probe itself. Strategies for improving the signal-to-background ratio of molecular probes promise higher assay sensitivity as well as better reproducibility. There has been encouraging progress in attempts at introducing novel signaling schemes,[5,6] exploring nanocomposites,[7,8] as well as making better quenchers using rational molecular design coupled with sophisticated synthesis.[9–11]

Herein, we have molecularly assembled an array of quencher molecules to produce SQs for use in engineering fluorogenic molecular probes with high sensitivity and specificity. We believe that using multiple quenchers to pair with one fluorophore provides better quenching efficiency because of the improved absorption efficiency and the increased probability of dipole–dipole coupling between the quenchers and the fluorophore as a result of a collective quenching effect. MB was used as a model here to test the performance of the SQs. An excellent example of fluorogenic probe, an MB,[2,3,12] is a hairpin-shaped DNA with a self-complementary stem that brings a terminal-labeled fluorophore and a quencher into close proximity. This self-quenched probe does not fluoresce until hybridized to its target because of spatial separation of the quencher and the fluorophore. In our preparation, SQs were synthesized by assembling different numbers and different types of quencher molecules using dendrimeric linkers.[13–17] The SQs were then used to label MBs. Three fluorescein (FAM)-labeled MBs with the same sequence (quencher-CC TAG CTC TAA ATC ACT ATG GTC GCG CTA GG-FAM) were prepared. A control MB, single-Q-MB, was designed in a conventional way with one DABCYL (4-(4-(dimethylamino)phenylazo)benzoic acid) quencher molecule. With a doubler linker, two DABCYLs were assembled together to the 5′ end of the Dual-Q-MB. In the Triple-Q-MB, an SQ assembled from three DABCYLs was used. These MBs were synthesized and HPLC-purified. Mass spectroscopy results confirmed the success
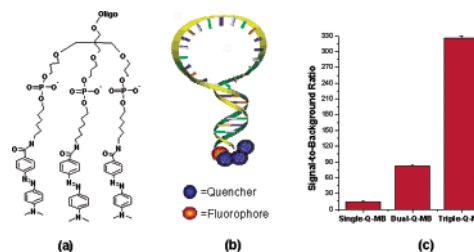

Figure 1. (a) Structure of a superquencher consisting of three DABCYLs. (b) Schematic of a molecular beacon with a superquencher. (c) Signal-to-background ratio of molecular beacons with different quenchers.

of synthesis. The structure of the SQ and the schematic of the MB with SQ are shown in Figure 1a and b, respectively.

The MBs with SQ are highly efficient in signaling the hybridization of MBs with target nucleic acids. The performance of the SQs was evaluated by comparing their quenching efficiency, which was defined as $Q\% = 100 \times (1 - (F_{MB} - F_{buffer})/(F_{hybrid} - F_{buffer}))$,[7] where F_{MB}, F_{buffer}, and F_{hybrid} were the fluorescence intensities of the MB without target, buffer, and MB–target hybrid, respectively. In the absence target, background fluorescent intensities from MBs (F_{MB}) were compared. With an increased number of DABCYL in the molecular assembly, the background fluorescence intensity decreased dramatically. On the other hand, when hybridized to target DNA (GCG ACC ATA GTG ATT TAG A), comparable fluorescence intensities were observed from all three MBs. With one DABCYL in the MB, a quenching efficiency of about 92.9% was observed, while the two DABCYL assembly quenched FAM with a quenching efficiency of about 98.75%. The SQ with three DABCYLs performed the best and had a quenching efficiency around 99.7%. Although the differences in quenching efficiency may seem small, the signal-to-background ratios[18] (S/B = $(F_{hybrid} - F_{buffer})/(F_{MB} - F_{buffer})$) of these SQ MB probes improved dramatically. It is common to use S/B in bioanalysis to determine the sensitivity of the molecular probes. The SQ MB had an S/B as high as 320-fold when hybridized to its target, which is a significant improvement compared with the 14-fold of S/B observed from the control MB (Figure 1c). This comparison clearly demonstrated that the SQ assembly greatly improves the S/B ratios and, thus, the analytical sensitivity of the fluorogenic molecular probes.

When applied to MB probes, these multiple quencher assemblies provide several favorable effects for fluorescence signaling. First, placing an SQ to an adjacent fluorophore in an MB will greatly increase the overall quenching ability in its closed conformation. This high quenching efficiency is a consequence of improved absorption extinction coefficients of the SQs and increased dipole–dipole interactions between the fluorophore and quencher molecules. Second, the SQ in the MB helps improve the probe purity. The hydrophobicity of the quencher molecules greatly increases the retention time of MB in the reverse phase HPLC, which significantly improves the separation efficiency between labeled and unlabeled DNA probes. Third, the SQ helps stabilize the hairpin
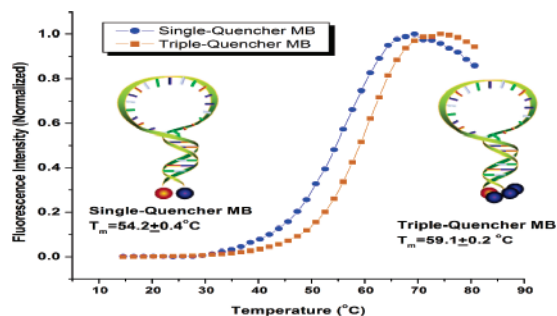
Figure 2. Melting temperature curves of different molecular beacons.

structure of the MB, as shown by the melting temperatures (T_m) of MBs (Figure 2). The T_m of the SQ MB was about 4.9 °C higher than that of the regular MB. This stabilization is believed to be a result of the hydrophobic interaction between the fluorophore and quenchers. Because of this increase in thermostability, we observed a slight improvement in the selectivity of the MB for single base mismatched DNA target. These advantages are a direct result of the SQ functionality, and there was no adverse effect on the binding kinetics of MB to its targets. Control experiments showed that the reaction kinetics of the SQ MB was similar to that of the regular MB.

The SQs can quench different fluorophores with excellent quenching efficiency. We prepared SQ MBs with fluorophores, such as FAM, TMR, and Cy3. Excellent S/B ratios were observed for all of these dyes. For instance, the SQ MB with Cy3 showed a >250-fold increase of signal change upon target hybridization.

The excellent performance of the SQ was further evidenced in MBs with different sequences. Several reported[7,18,19] MB sequences were synthesized with the SQs. Up to 300-fold increases of S/B were observed for the MBs synthesized with SQs (see Supporting Information). This was about a 20-fold improvement compared to the reported value of the regular MB.

The efficiency of SQ was also compared with that of gold nanoparticles. With their exceptional quenching capability, gold nanoparticles have been successfully used to construct fluorescent probes.[7,8] When used as the quencher in an MB, the average quenching efficiency of a gold nanoparticle to FAM has been shown to be as high as 98.68%, which is equivalent to an S/B ratio of about 76.[7] The SQ showed a higher quenching efficiency (99.5%) than that of the gold particles. Compared to the gold nanoparticle, there are a few significant advantages for using SQ for MB and other fluorogenic probe constructs: easier to prepare and manipulate, better sensitivity, and more stable. In addition, the SQ approach will leave no concerns one might have with nanoparticles in bioanalysis and bioimaging, especially for *in vivo* studies.

The approach of a chemically assembled macromolecule from individual quenchers to yield a better quencher moiety can be extended to other quencher molecules. For example, an SQ consisting of three Eclipse quenchers quenched 99.6% of fluorescence from TMR in an MB. This shows that we have developed a new strategy in using molecular assembly as molecular moieties, which can be widely useful in molecular recognition, interaction, and signaling.

The SQs may also be used in other molecular probes. Paired with FAM in an aptamer probe that selectively binds cancer marker protein platelet derived growth factor (PDGF),[20] an SQ-labeled aptamer has rendered about a 49 000-fold signal change when the probe was bound to PDGF, compared to a less than 20-fold signal change when a regular quencher was used.[20]

In conclusion, we have designed a novel molecular assembly of quencher molecules to form superquenchers with excellent quenching efficiency. The superquencher can be engineered as desired by assembling different types and different numbers of quencher molecules. By labeling a superquencher to an MB, a 320-fold enhancement of fluorescent signal was achieved, compared to about 14-fold from an MB prepared with the same monomer quencher. The molecular assembly approach has three distinguished features. (1) The assembled superquencher shows unique properties for use in engineering molecular probes. Compared with a regular MB, a SQ-constructed MB has better sensitivity, better purity, higher thermal stability, and slightly improved specificity. (2) The assembly scheme can be widely useful for different types of quencher molecules. (3) The SQ can be used for different fluorophores and in different probes. The SQs used for signaling aptamer−protein interactions have generated more than a 49 000-fold signal change when a PDGF aptamer was bound to PDGF. No detrimental effect of these SQs on the performance of probes was observed. The SQ MB showed hybridization kinetic properties similar to that of the regular MB. Our molecular assembly approach can effectively improve the sensitivity of a variety of fluorescent assays and may be widely useful for molecular recognition and interaction studies.[2,12]

Note Added after ASAP Publication. The authors' complete address was given, and Supporting Information Figure S5a on page S5 was corrected August 26, 2005.

Supporting Information Available: Preparation and characterization of superquencher MBs with different dyes and sequences. This material is available free of charge via the Internet at http://pubs.acs.org.

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