Published in final edited form as:

J Am Chem Soc. 2005 September 21; 127(37): 12772–12773. doi:10.1021/ja053482t.

Molecular Assembly of Superquenchers in Signaling Molecular Interactions

Chaoyong James Yang, Hui Lin, and Weihong Tan *

Department of Chemistry, University of Florida, Gainesville, FL32611

Abstract

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 a molecular beacon, a 320 folds enhancement of fluorescent signal was achieved, compared to about 14-fold from a molecular beacon prepared with the same monomer quencher. Our molecular assembly approach can effectively improve the sensitivity of a variety of fluorescent assays and can be widely useful for molecular interaction studies

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 pairing, 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 probes⁴ 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 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 because of a collective quenching effect of these multiple quenchers. MB was used as a model here to test the performance of the SQs. An excellent example of fluorogenic probe, a MB^{2;3;12} is a hairpin shaped DNA with a self-complementary stem that brings terminal labeled fluorophore and 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 that had one quencher DABCYL (4-(4-(dimethylamino) phenylazo) benzoic acid) molecule. With a doubler linker, two DABCYLs were assembled together to the 5' end of the Double-Q-MB. In the Triple-Q-MB, a SQ assembled from three DABCYLs was used. These MBs were synthesized and HPLC-purified. Mass spectroscopy results confirmed the success of synthesis. The structure of the SQ and the schematic of the MB with SQ are shown in Figure 1a and 1b 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, with buffer, and with MB–target hybrid, respectively. In the absence target, background fluorescent intensities from MBs (F_{MB}) were compared. With 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 3 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 ratios¹⁸ ($S/B = (F_{Hybrid} - F_{Buffer}) / (F_{MB} - F_{Buffer})$) 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 folds when hybridized to its target, which is a significant improvement compared with 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 a SQ to an adjacent fluorophore in a 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, 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 hair-pin 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 mismatches target DNA. These advantages are a direct result of the SQ, 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/Bs were observed for all these dyes. For instance, the SQ MB with Cy3 showed more than 250 folds 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 folds of S/B were observed for the MBs synthesized with SQs (See Supporting information). This was about 20 times improvement compared to the reported value with 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 a MB, the average quenching efficiency of gold nanoparticle to FAM has been shown as high as 98.68%, which is equivalent to a S/B ratio of about 76.⁷ The SQ showed a higher quenching efficiency (99.5%) than that of the gold particles. Compared to gold nanoparticle, there are a few significant advantages to use SQ for MB and other fluorogenic probe constructs: easier to prepare and manipulate; better sensitivity; more stable. In addition, the SQ approach will leave no concerns one might have with nanoparticles in bioanalysis and bioimaging, especially in in-vivo studies.

The approach of chemically assembled macromolecule from individual quenchers to yield better quencher moiety can be extended to other quencher molecules as well. For example, a SQ consisting of three Eclipse quenchers quenched 99.6% of fluorescence from TMR in a 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 a SQ labeled aptamer has rendered about 49,000-fold of signal change when the probe bound to PDGF, compared with 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 a MB, a 320 folds enhancement of fluorescent signal was achieved, compared to about 14-fold from MB prepared with the same monomer quencher. The molecular assembly approach has three distinguished features: 1. The Superquencher assembled shows unique properties for used 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 has generated more than 49,000 folds of signal changes when PDGF aptamer bound to PDGF. No detrimental effect of these SQs on the performance of probes was observed. The SQ MB showed similar hybridization kinetic properties to that of the regular MB. Our molecular assembly approach can effectively improve the sensitivity of a variety of fluorescent assays and can be widely useful for molecular recognition and interaction studies.^{2;12}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

This work is partially supported by NIH R01 GM66137 and NIH Genomic Center grants. C.J.Y. received support as an ACS Division of Analytical Chemistry Fellow sponsored by Merck.

References

1. Holland PM, Abramson RD, Watson R, Gelfand DH. *Proc.Natl.Acad.Sci.U.S.A* 1991;88:7276–7280. [PubMed: 1871133]
2. Tan W, Wang K, Drake T. *Curr.Opin.Chem.Biol* 2004;8:547–553. [PubMed: 15450499]
3. Tyagi S, Kramer FR. *Nat.Biotechnol* 1996;14:303–308. [PubMed: 9630890]
4. Matayoshi ED, Wang GT, Krafft GA, Erickson J. *Science* 1990;247:954–958. [PubMed: 2106161]
5. Chen LH, McBranch DW, Wang HL, Helgeson R, Wudl F, Whitten DG. *Proc.Natl.Acad.Sci.U.S.A* 1999;96:12287–12292. [PubMed: 10535914]
6. Du H, Disney MD, Miller BL, Krauss TD. *J.Am.Chem.Soc* 2003;125:4012–4013. [PubMed: 12670198]
7. Dubertret B, Calame M, Libchaber AJ. *Nat.Biotechnol* 2001;19:365–370. [PubMed: 11283596]
8. Maxwell DJ, Taylor JR, Nie SM. *J.Am.Chem.Soc* 2002;124:9606–9612. [PubMed: 12167056]
9. Xia, W.; Whitten, D.; McBranch, D. U.S. Patent. 2005030579. 2005.
10. Cook, RM.; Lyttle, M.; Dick, D. U.S Patent. US15082. 2001.
11. May JP, Brown LJ, Rudloff I, Brown T. *Chem.Commun* 2003;(8):970–971.
12. Peng XH, Cao ZH, Xia JT, Carlson GW, Lewis MM, Wood Wi. C, Yang L. *Cancer Res* 2005;65(5): 1909–1917. [PubMed: 15753390]
13. Shchepinov MS, Udalova IA, Bridgman AJ, Southern EM. *Nucleic Acids Res* 1997;25:4447–4454. [PubMed: 9358151]
14. Dubber M, Frechet MJ. *Bioconjugate Chem* 2003;14:239–246.
15. Hussain M, Shchepinov MS, Sohail M, Benter IF, Hollins AJ, Southern EM, Akhtar S. *J.Controlled Release* 2004;99:139–155.
16. Li Z, Jin RC, Mirkin CA, Letsinger RL. *Nucleic Acids Res* 2002;30:1558–1562. [PubMed: 11917016]
17. Luehrsen KR, Davidson S, Lee YJ, Rouhani R, Soleimani A, Raich T, Cain CA, Collarini EJ, Yamanishi DT, Pearson J, Magee K, Madlansacay MR, Bodepudi V, Davoudzadeh D, Schueler PA, Mahoney W. J. *Histochem. Cytochem* 2000;48:133–145. [PubMed: 10653594]
18. Marras SAE, Kramer FR, Tyagi S. Genotyping SNPs with molecular beacons. *Methods Mol.Biol* 2003;212:111–128. [PubMed: 12491906]
19. Vijayanathan V, Thomas T, Sigal LH, Thomas TJ. *Antisense & Nucleic Acid Drug Development* 2002;12:225–233. [PubMed: 12238811]
20. Vicens MC, Sen A, Vanderlaan A, Drake TJ, Tan W. *ChemBioChem* 2005;6:900–907. [PubMed: 15812865]

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.

Figure 2.
Melting temperature curves of different molecular beacons.