$20H_2O$, $M_r = 1973.24$, trigonal, $R\bar{3}m$, $a = b = 33.2194(3)$, $c = 38.9594(4)$ Å, $V = 37232.8(6)$ Å^3, $Z = 18$, $\rho_{calcd} = 1.584$ g cm^{-3}, $T = 188$ K, Siemens SMART CCD diffractometer, $Mo_{K\alpha}$ radiation ($\lambda = 0.71073$ Å), $\mu = 3.84$ cm^{-1}. The structure was solved by Patterson methods (SHELXS-86). Of the three nitrate ions, only 1.5 were found because of disorder. Solvent molecules were also badly disordered. All non-hydrogen atoms were refined anisotropically (SHELXL-93). Final block-diagonal matrix least-squares refinement on F^2 with all 6888 reflections and 653 variables converged to $R1$ ($I > 2\sigma(I)$) = 0.129, $wR2$ (all data) = 0.418, and GOF = 1.77. Crystallographic data (excluding structure factors) for the structures reported in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication no. CCDC-147742. Copies of the data can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge CB21EZ, UK (fax: (+44) 1223-336-033; e-mail: deposit@ccdc.cam.ac.uk).

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Design of a Molecular Beacon DNA Probe with Two Fluorophores**

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DNA/RNA analysis is of great importance in molecular biology, genetics, and molecular medicine. DNA hybridization is a major tool for the diagnosis of genetic disease, of which the clinical symptoms are linked to alterations in DNA. Identifying the mutations in the human genome has become the focus of many research efforts. One recent development is that of a novel class of oligonucleotide probe: molecular beacons (MBs).[1] Molecular beacons are single-stranded oligonucleotide probes with a hairpin structure. A fluorophore and a quencher are linked to the two ends of the strand. The five to seven bases at both ends of the beacon are complementary to each other, forming the stem, which keeps the fluorophore and the quencher in proximity to each other. The fluorescence of the fluorophore is thus quenched by the quencher through energy transfer. The loop portion of the molecule beacon is so designed that it is complementary to a target oligonucleotide of interest.[1–8] When the probe encounters the target DNA molecule it forms a hybrid and forces the stem apart. Such a conformational change moves the fluorophore and the quencher away from each other, which leads to the restoration of fluorescence. Therefore, molecular beacons emit an intense fluorescent signal at room temperature only when hybridized to their target molecules. Molecular beacons have been shown to have very high selectivity in regards to the identification of a single base-pair mismatch.[1, 2] Also, since the signal transduction mechanism is built within the MB molecules, no DNA intercalation reagent or labeling of the target molecule is needed. There have been a number of different applications of molecular beacons, such as real-time monitoring of polymerase chain reactions,[1, 2] detection of DNA/RNA hybridization in living cells,[3] DNA/RNA biosensors,[4] bacterial detection,[5] monitoring of enzymatic cleavage,[6] investigation of the progression of HIV-1 disease,[7, 8] and the study of DNA–protein interactions.[9] Molecular beacons hold great promise in studying genetics and disease mechanisms, in disease diagnostics, and in new drug development.

While MBs have been used successfully in DNA assays, their utility for quantification is limited. Currently, MBs use a

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fluorophore and a quencher attached to both ends of the stem. The sensitivity and dynamic range of MBs as probes are determined mainly by two parameters: the residual fluorescence intensity when the MBs are in the stem-closed form (incomplete quenching); and the fluorescence intensity when they are in the stem-open form. In principle, the fluorophore should be quenched completely by the quencher in the stem-closed form. In reality, however, the residual fluorescence varies greatly, because of factors such as the selection of the fluorophore/quencher pair, the synthesis of MBs, and the way the fluorophore/quencher groups are attached to the oligonucleotide. Such residual fluorescence greatly limits the detection sensitivity of MBs. In addition, MBs of this fluorophore/quencher type can rarely be used to detect the target quantitatively.

To address these problems we have developed a new strategy to design MBs with two fluorophores instead of one fluorophore and one quencher. We attach two different fluorophores (F_1 and F_2) to the two ends of the stem (Figure 1). F_1 and F_2 are chosen such that fluorescence

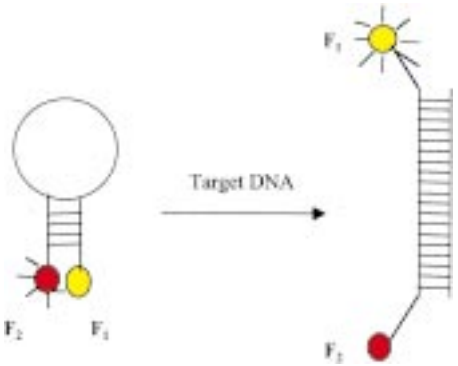

Figure 1. A schematic representation of the MB with two fluorophores. The molecular beacon was designed as a loop sequence flanked with shorter complementary sequences on the 5′- and 3′-ends. F_1 and F_2 represent the two fluorophores.

resonance energy transfer (FRET) will occur when they are in proximity. When the MB is in the stem-closed form and excited at the absorption band of F_1, the fluorescence of F_1 is quenched by F_2, while that of F_2 is observed (so-called sensitizing F_2).[10] When the MB forms a hybrid with the target, FRET is reduced or eliminated and the fluorescence of F_1 will rise while that of F_2 will diminish or disappear. Thus, the change of two fluorescence intensities can be monitored, instead of only one as in the case of a MB with a quencher and a fluorophore.

The utilization of the ratio of the intensities of the fluorescence of F_1 and F_2 (I_{F1}/I_{F2}) should provide significant advantages over measurement of I_{F1} only. First, the MBs with two fluorophores are more sensitive to the concentration of the target DNA than the current MBs. For the fluorophore/quencher MBs, the enhancement factor of the fluorophore intensity f upon hybridization with the target is defined as $I_{F1,o}/I_{F1,c}$, where subscripts o and c denote the stem-open and stem-closed forms of MB, respectively. However, the enhancement factor for the MBs with two fluorophores is defined as the change of the ratio I_{F1}/I_{F2}. This ratio is equal to $(I_{F1,o}/I_{F2,o})/(I_{F1,c}/I_{F2,c})$, which can be rewritten as $(I_{F1,o}/I_{F1,c}) \times (I_{F2,c}/I_{F2,o})$. According to the aforementioned mechanism, the fluorescence intensity of F_1 will increase upon hybridization, while that for F_2 will decrease. $I_{F2,c}/I_{F2,o}$ will always be larger than one, and, therefore, the change of I_{F1}/I_{F2} will always be larger than $I_{F1,o}/I_{F1,c}$. This means an improvement in the fluorescence detection sensitivity. Second, the MBs with two dyes will have the advantages which a ratiometric intracellular dye would have, such as better quantitation, less dependence on optical geometry, and less influence from photobleaching as well as other environmental factors that affect optical measurements.[10]

We have applied this strategy by synthesizing a two-fluorophore MB with a coumarin group (excitation 350 nm/emission 447 nm) on one end of the stem as a donor and a 6-carboxyfluorescein group (6-FAM, excitation 492 nm/emission 518 nm) as an acceptor on the other. The sequences of the MBs and different target DNAs used in this work are summarized in Table 1. All MBs and target DNA 1 were synthesized by Trilink BioTechnologies, Inc. (San Diego, CA), and DNA 2 was purchased from Integrated DNA Technologies, Inc. (Coralville, IA). All MBs were synthesized using standard phosphoramidite coupling procedures. The 5′-termini of the MBs were modified with 6-FAM and 4-(4-dimethylaminophenylazo)benzoyl (DABCYL) phosphoramidites. Each sample was deprotected with concentrated ammonium hydroxide at room temperature for 30 hours, and then purified on a polyacrylamide gel. After recovery of the full-length product, the amine-modified oligonucleotides were treated with the succinimidyl ester of 6-((7-amino-4-methylcoumarin-3-acetyl)amino)hexanoic acid (succinimidyl ester of AMCA-X), and the coumarin-labeled oligonucleotide was isolated on reversed-phase HPLC.

In the absence of a target sequence MB1 adopts a hairpin structure containing a stem of 6 base pairs (Table 1 underlined segment) and a loop of 16 bases. The stem keeps the two fluorophores in proximity, which causes a quenching of the coumarin fluorescence and an emission of FAM fluorescence as a result of energy transfer. A conformational change that opens the stem leads to the separation of the two fluorophores, and therefore restores the fluorescence of coumarin while depressing that of FAM. Figure 2 shows fluorescence spectra of MB 1 before (a) and after (b) hybridization with its target DNA 1. Fluorescence spectra were collected on a

Table 1. MB and target DNA sequences.

MB 1	5′-(6-FAM)-GCTCGTCCATGCCCAGGAAGGAACGAGC-(C6NH)-(coumarin)-3′
MB 2	5′-(DABCYL)-GCTCGTCCATGCCCAGGAAGGAACGAGC-(C6NH)-(coumarin)-3′
MB 3	5′-(6-FAM)-GCTCGT**GG**CCATGCCCAGGAAGGA**TT**ACGAGC-(C6NH)-(coumarin)-3′
DNA 1	5′-TCCTTCCTGGGCATGG-3′
DNA 2	5′-**AA**TCCTTCCTGGGCATGG**CC**-3′

The underlined bases indicates the stem sequence of the MB. Bold bases indicate the mismatched bases.

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Perkin–Elmer luminescence spectrometer (Model LS50B) with excitation at 350 nm. All fluorescence experiments were performed at room temperature. The buffer solution consists of 5 mM Mg^{2+} ions, 50 mM KCl, 20 mM tris(hydroxymethyl)-aminomethane-HCl (Tris-HCl; pH 8.0). As expected, the emission of coumarin is significantly quenched in the stem-closed form while the FAM fluorescence is strong (a), which indicates energy transfer is quite efficient for the coumarin/FAM pair. Once MB 1 hybridizes with the target DNA 1, the two fluorophores are pulled apart, and the coumarin fluorescence is largely restored (b).

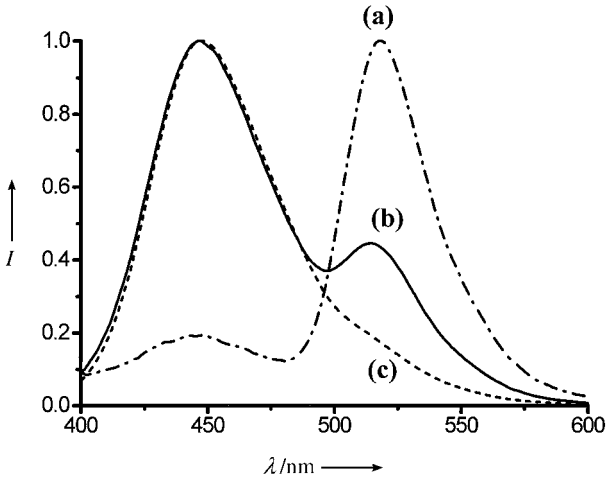

Figure 2. Normalized fluorescence spectra of MB 1 with excitation at 350 nm: a: MB 1 alone (1×10^{-7} M); b: MB 1 (1×10^{-7} M) and complementary DNA 1 (5×10^{-7} M); c: solution (b) digested by deoxyribonuclease I into mononucleotides and shorter oligonucleotide fragments. F_1 and F_2 are completely disassociated after enzymatic digestion.

We have determined the sensitivity of the MB by using different concentrations of target DNA 1. The ratio of $I_{coumarin}/I_{FAM}$ is used to report the amount of DNA 1 present. The ratio increases linearly with target oligonucleotide concentration (Figure 3 top). The excellent linear relationship indicates that a MB of this design can be used to detect the target quantitatively over a large dynamic range (1 nM to 1 μM). For a comparison, we synthesized MB 2, a conventional fluorophore/quencher (coumarin/DABCYL) molecular beacon with the same stem and loop sequence. The response of MB 2 towards different concentrations of the same target DNA 1 is shown in Figure 3 bottom, where $I_{coumarin}$ is used to report the amount of DNA 1 present. Although $I_{coumarin}$ can report DNA 1 concentration linearly over the range of 1 to 10 nM, it is clear that the increase of $I_{coumarin}$ is far from linear against the increase of DNA 1 concentration above 10 nM.

The MBs with two fluorophores are also more resistant to fluctuations caused by the experimental environment and allow quantitative determination of the target. The $I_{coumarin}/I_{FAM}$ measurement is a fluorescent ratiometric measurement with self-calibrating characteristics.[10] Since the fluctuation in fluorescence is a major source of noise in the signals, especially when the target concentration is low, the $I_{coumarin}/I_{FAM}$ measurement will not only increase the sensitivity but also make it more reliable for measurements. We carried out a statistical determination of the detection limit of DNA 1 by MB 1. The ratio of $I_{coumarin}/I_{FAM}$ for MB 1 is 0.164 ± 0.002. Based on a signal/noise ratio of 3/1, the detection limit for DNA 1 was determined to be 0.17 nM, which is one order of magnitude lower than that for many other DNA probes.[11, 12] It is worth noting that this detection limit is achieved with a conventional spectrometer with a mercury lamp and can be further improved with a more efficient optical detection

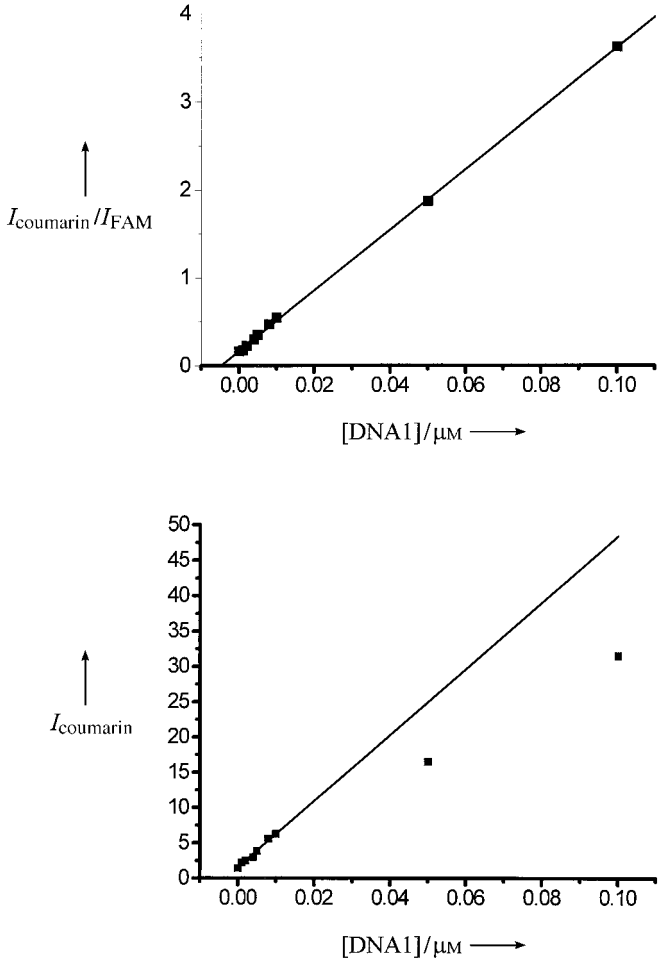

Figure 3. Top: $I_{coumarin}/I_{FAM}$ ratio for MB 1 versus [DNA 1]; bottom: $I_{coumarin}$ for MB 1 versus [DNA 1] (the intensity is given in arbitrary units). Both were excited at 350 nm. [MB 1] = 1×10^{-7} M.

system and a better optical excitation by using lasers. Moreover, the loop and the stem of the MB can be optimized to achieve higher fluorescent enhancement, thus higher sensitivity, in DNA/RNA studies.[2]

It is worthwhile pointing out the fact that the fluorescence intensity of coumarin increased further upon addition of deoxyribonuclease I to the hybrids of MB and complementary DNA. This indicated that the emission of the donor is not completely restored even in the open form of MB. A similar

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phenomenon was also observed for a MB with a fluorophore/quencher design. While having not been reported previously, this observation can be understood considering that the distance between donor and acceptor when MB is in the open form (usually 20–30 mer in length) is not significantly larger than the Förster distance (around 65 Å in this case). Thus there is considerable FRET between the donor and acceptor for MBs in the open form. This process is also manifested by the appearance of a fluorescence spectrum of MB in the open form (Figure 2b), where the FAM emission is still significant. The design of the MB with two fluorophores also provides an easy and effective way of measuring the Förster energy transfer distance for a given donor/acceptor pair. The efficiency of energy transfer can be calculated from the quenching of the donor emission in the presence of the acceptor. By designing two-fluorophore MBs with different lengths (number of bases as shown in MB1 and MB3 in Table 1), Förster energy transfer distance for a given donor/acceptor pair can thus be determined using the FRET efficiency with these different MBs.

In conclusion, we have proposed a new strategy of designing MBs which uses two fluorophores instead of one fluorophore and one quencher as the donor and acceptor. Such MBs display high sensitivity and a large dynamic range. Molecular beacons of this new design with coumarin and 6-FAM as the fluorophores can quantitatively detect the target DNA up to 1×10^{-7} M with a detection limit of 1.7×10^{-10} M with a basic spectrometer. It could also be very useful for studying protein–DNA/RNA interactions,[8] where the fluorophores are as likely to be pulled apart as to be squeezed closer. We were also able to determine for the first time the Förster distance for the coumarin/FAM pair, which was estimated to be 65 ± 1 Å. We expect MBs of this design to be effective for applications in both DNA/RNA and protein–DNA/RNA interaction studies as well as in genetic analysis based on better linearity, larger dynamic range, higher sensitivity, and less dependence on optical geometry and photobleaching.

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An Exceptionally Stable Ti Superoxide Radical Ion: A Novel Heterogeneous Catalyst for the Direct Conversion of Aromatic Primary Amines to Nitro Compounds**

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Superoxide ion ($O_2^{\bullet -}$), an active oxygen species, plays important roles in various diseases caused by oxygen toxicity such as ischemia, carcinogenesis, inflammation, diabetes, and aging.[1] Superoxide dismutases act as a defense system against oxygen toxicity in living cells by catalyzing the dismutation of $O_2^{\bullet -}$ into H_2O_2 and O_2.[2] Although it is normally reduced to H_2O, O_2 may diffuse out of the electron-transport enzyme system and interact with important biological molecules.[3] In spite of much research, neither the mode nor the site of damage due to $O_2^{\bullet -}$ in biological systems is known. Investigations on the organic chemistry of $O_2^{\bullet -}$ with simple model substances have shown that $O_2^{\bullet -}$ is a versatile species[4] and can behave as a base, as a nucleophile, and as an oxidizing or reducing agent,[5] but none of this provides an explanation for its toxicity. While the debate regarding the importance of $O_2^{\bullet -}$ in vivo continues, the chemistry of the radical remains to be completely characterized. Consequently, it is of interest to study the reactions of $O_2^{\bullet -}$ with simple organic molecules such as amines to build up a picture of its possible biological effects.

A literature search[2a, 4], [6] reveals that the generation of $O_2^{\bullet -}$ is achieved by electrolytic reduction of O_2 in DMF or by enzymes such as xanthine-xanthine oxidase. Recently, the generation of $O_2^{\bullet -}$ was described on the lattice of metal oxides such as MgO/CaO, ZnO, ZrO_2, and TiO_2 by using a photo-induced electron-transfer process.[7] However, these methods

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