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Copper(II)-Quenched Oligonucleotide Probes for Fluorescent DNA Sensing

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Many fluorescent molecular sensors for metal ions are efficiently quenched by paramagnetic Cu^{2+} ions.[1] The quenching process is induced by coordination of Cu^{2+} either directly to donor atoms of the chromophore or to chelating groups covalently attached to the latter.[2] Cu^{2+} affinity of the fluorophore is determined by its complexing properties and ranges from weak monodentate coordination to an effective binding constant log $K = 12$ at pH 7 for calcein, a chelator modified fluorescein.[3] "Chemosensing ensembles" of Cu coordinated fluorophores have been applied to the sensing of competing binders such as amino acids.[4]

Fluorophore−quencher interactions are becoming increasingly important in bioanalytical applications that rely on the influence of analyte binding on the spatial arrangement of dye and quencher attached to the sensing molecule.[5] A prominent example is "molecular beacons",[6] single stranded DNA probes with hybridization dependent fluorescence, which are now routinely used for DNA sequence detection in real time monitoring of PCR products. These self-complementary oligo-DNAs possess a stem and loop structure and are modified at the 3′ and the 5′ end with a reporter and a quencher dye, respectively. The stem keeps these two moieties in close proximity, causing the fluorescence of the fluorophore to be quenched, while hybridization with a complementary nucleic acid target forces them apart, leading to restoration of fluorescence. Advances in genetic analysis and the limitations of molecular beacons in the detection of nucleic acids in cells or tissues[7] have stimulated a search for novel fluorophore−quencher pairs with improved signal-to-noise performance and target selectivity.[8]

Here we introduce Cu^{2+} complexes as a new type of intramolecular quencher in oligonucleotide probes. Cu^{2+} complex functionalized oligo-DNAs or DNA analogues have been studied as sequence specific RNA or DNA cleavers,[9] for metal-modulated sequence recognition,[10] in DNA-templated reactions[11] but not yet in the context of fluorimetric sequence detection.

Following the design concept of molecular beacons, the intramolecular interaction of fluorescein and Cu^{2+} complex was supported by programming **1** (Figure 1) as a self-complementary hairpin structure with stem and loop areas to bring fluorophore and Cu^{2+} quencher, attached to the 3′ and 5′ ends, in close proximity (Scheme 1).

Modified DNA oligonucleotide **1** was prepared starting from a commercially available, 3′-fluorescein labeled and 5′-aminolink 25mer DNA attached to CPG solid phase. Carboxy-functionalized 5-(2-pyridinyl)pyrazole (pypz) was attached to the 5′-amino group by standard amide coupling reaction.[11] The conjugate was released from the solid support by ammonia treatment and purified by HPLC, yielding **1** in a purity >90%. **1** was characterized by MALDI-TOF MS (*m/z* 8639) and quantified by photometry at 260 nm.

On addition of excess Cu^{2+} ions to a 0.1 μM solution of **1** at pH 7, fluorescence is efficiently quenched (Figure 2), down to 15% at $[Cu^{2+}] = 5$ μM and 5.2% of the initial value at $[Cu^{2+}] = 50$ μM. A Cu^{2+} binding constant log $K = 6.3$ (0.4) of **1** was determined fluorimetrically (Supporting Information). In preliminary investigations, significant quenching (40%) is achieved even at equimolar (i.e. 0.1 μM) Cu concentration when an analogue of **1** with the stronger chelator 2,2′-bipyridine is used.

Fluorescence is restored (Figure 2, left) on addition of fully complementary 25mer single stranded DNA **1** (0.5 μM) since intramolecular interaction of fluorescein with the Cu^{2+} complex becomes sterically impossible in the rigid, linear DNA duplex. With

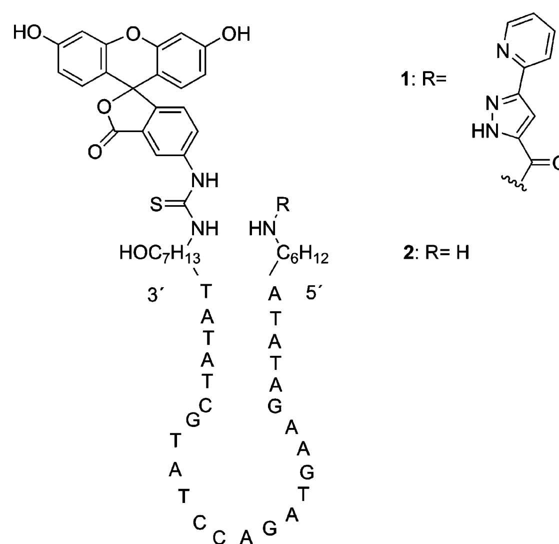


Figure 1. Synthesized DNA conjugates: oligo **1**, oligo **2**, oligo **3**; target DNAs used for hybridization experiments: **DNA 1**, **DNA 2**, **DNA 3**.

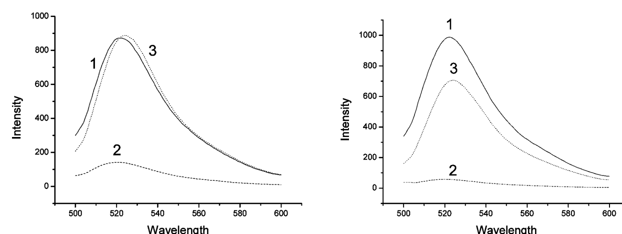
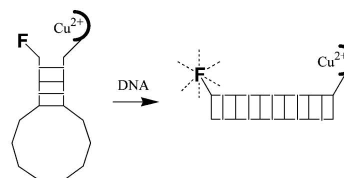


Figure 2. Relative fluorescence intensities: left, 0.1 μM oligo **1** (1), addition of 5 μM Cu^{2+} (2), addition of 0.5 μM **DNA 1** (3); right, 0.1 μM oligo **1** (1), addition of 50 μM Cu^{2+} (2), addition of 0.5 μM **DNA 1** (3); 10 mM (*N*-morpholino)propanesulfonic acid pH 7, 1 M NaCl, $T = 25$ °C.

Scheme 1. Fluorescence Recovery on Addition of Complementary DNA



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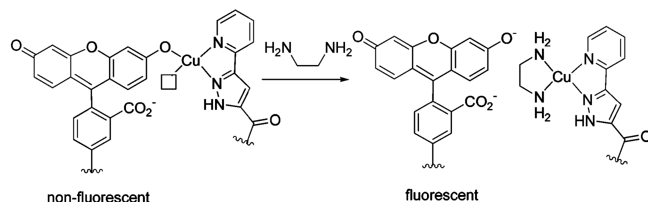
Scheme 2. Fluorescence Quenching by Intramolecular Fluorescein Coordination at Cu^{2+} (**1**) and Recovery on Addition of 1,2-Diaminoethane



a 15-fold increase of fluorescence after addition of 0.5 μM **DNA 1** to oligo **1** and 50 μM Cu^{2+} (Figure 2, right), the metal complex quencher compares in signal-to-noise performance to the best of a number of organic quencher−fluorescein pairs.[12]

In a control experiment, the fluorescein labeled precursor DNA without any further modification (oligo **2**) displayed 5% fluorescence decrease at 5 μM Cu^{2+} and 50% reduction at 50 μM Cu^{2+}, but in the latter case fluorescence increases only by a factor of 1.4 on addition of complementary DNA. Quenching of oligo **2** cannot be assigned to interaction of free Cu^{2+} ions with fluorescein since the fluorescence of unmodified fluorescein is not affected by 50 μM Cu^{2+}. Rather, the nucleobases of DNA may provide additional, low-affinity binding sites[13] and expose DNA-bound copper to intramolecular interaction with fluorescein in **2**, or in hybridized **1** where only 70% of the original fluorescence is restored (Figure 2 right, curve 3).

We suggest that the quenching process is triggered by intramolecular coordination of a phenolate donor of fluorescein, which is more basic and therefore a better donor than carboxylate,[14] to a free coordination site of the (pypz) Cu^{2+} moiety of **1**. Such a direct interaction is indicated by ~10% decrease of optical fluorescein absorbance of **1** (λ_{max} = 494 nm) upon binding of Cu^{2+}, which is also observed for calcein (λ_{max} = 492 nm), a commercially available fluorescein derivative in which coordination of Cu^{2+} to the phenolate oxygen atom is supported by chelating ($-CH_2N(CH_2CO_2^-)_2$) substituents (Supporting Information). Fluorescence of Cu^{2+}-quenched **1** is recovered on addition of competing ligands such as 1,2-diaminoethane (Scheme 2 and Supporting Information), which is assumed to block free in-plane sites of the Cu^{2+} ion in Cu^{2+} (**1**) and suppresses intramolecular fluorescein coordination. Also, an analogue of **1** in which fluorescein is replaced by pyrene (Supporting Information), a fluorophore which lacks any coordination sites for copper, is not quenched significantly at 50 μM Cu.

When in the hybridization experiments a shorter target sequence **DNA 2** (Figure 1) complementary to the loop only is used, relative fluorescence intensity of the hybrid is 13% larger than in the case of **DNA 1**. This is related to some fluorescence quenching by double-stranded DNA,[15] which is more effective than in the **DNA 1**/oligo **1** duplex due to the presence of a 6-base single-stranded spacer between dye and duplex in the latter case.

Excellent single mismatch discrimination was observed using **DNA 3** (Figure 1) in which an internal guanine nucleobase of **DNA 2** is replaced by thymine. In the presence of **DNA 3** (3 in Figure 3) practically no increase of fluorescence was observed, while on addition of complementary **DNA 2** (4 in Figure 3) fluorescence was restored. The increase of fluorescence is 20 times larger than in case of **DNA 3**.

In conclusion, Cu complexes with free coordination sites efficiently quench fluorophores with ligating properties in molecular beacon oligonucleotide probes. An advantage of this over existing probe types, which display either through space or weak contact interaction between fluorophore and quencher, is the possibility of tuning and optimizing the strength of this interaction, using for example chelating fluorophores. This should facilitate the design of superior probes with very high signal-to-noise performance in which nonspecific interactions with bioanalytical samples (leading to false positive signals) are blocked.

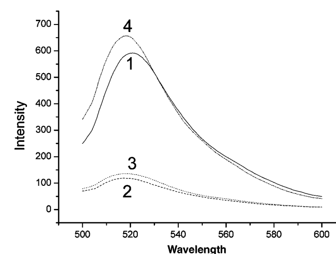


Figure 3. Relative fluorescence intensities: 0.1 μM oligo **1** (1), addition of 5 μM Cu(II) (2), addition of 0.5 μM **DNA 3** (3), addition of 0.5 μM **DNA 2** (4); 10 mM (*N*-morpholino)propanesulfonic acid pH 7, 0.1 M NaCl, T = 25 °C.

Supporting Information Available: Synthetic schemes of oligo **1**−**4**, MALDI-TOF spectra of **DNA 1**−**4**, fluorimetric titrations of oligo **1**−**4**, fluorescence recovery of **1**, fluorescence of oligo **2**, and comparison of optical and fluorescence spectra of oligo **1** and of calcein. This material is available free of charge via the Internet at http://pubs.acs.org.

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