Journal of Fluorescence, Vol. 12, No. 2, June 2002 (© 2002)

Homogeneous Phosphodiesterase and Hybridization Assays Using Europium Cryptate: Oligonucleotide Conjugates

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Received October 5, 2002

Upon conjugation to single-stranded oligonucleotides, a europium cryptate ($Eu^{3+} \subset$ tris-bipyridine) showed a marked increase in its fluorescence lifetime and was much less sensitive to fluorescence quenching by uric acid. This behavior was shown to be moderately dependent on the length and sequence of the oligonucleotide and all the single-stranded oligonucleotides studied displayed similar behavior. In contrast, a cryptate moiety attached to a double-stranded oligonucleotide did not display such an increase in its fluorescence lifetime and was quenched in presence of uric acid. Taking advantage of this unique behavior characterizing single-stranded K-ODN conjugates, a new concept of dosage based on the modulation of the cryptate fluorescence by a quencher was set up. This fluorescence quenching assay involving a single fluorescent label was applied to the monitoring of hybridization reactions and detection of a phosphodiesterase activity.

KEY WORDS: Oligonucleotide; europium cryptate; homogeneous assays; fluorescence quenching.

INTRODUCTION

As a result of the development of high-throughput screening to support drug discovery, there is an increasing demand for homogeneous assays based on simple a "mix and measure" format involving preferably fluorescent tracers. Time-resolved fluorescence techniques, characterised by an efficient rejection of the short-lived fluorescence background, allow higher a sensitivity level compared to conventional steady-state fluorescence. In this respect, lanthanide cryptates [1], because of their unique long-lived fluorescence decay and high chemical stability, are very attractive. In the cryptate-oligodeoxynucleotide conjugates (K-ODN) described herein, a europium ion is entrapped in a tris-bipyridine structure (Fig. 1). As an alternative to a FRET modulation process used in the TRACE®/HTRF® assays [2],[4] we wish to report an assay based on the selective fluorescence quenching of europium cryptate.

METHODS

The synthesis and purification of $Eu^{3+} \subset$ TBP-oligodeoxynucleotide conjugates (K-ODN) **2** to **10** (see Fig. 1) were performed essentially as previously described [3].

Fluorescence Spectra and Lifetime Measurement

The reference cryptate **1** and the K-ODN conjugates **2**–**10** were diluted to 5 μM in PBS (0.1 M phosphate,

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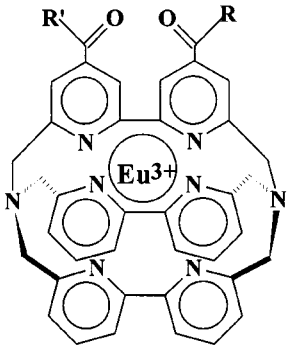
ODN	Sequence	τ (ms)
2	5'd (**K** . AAA . AAT . TTT . TTT . TTT)	1.15
3	5'd (**K** . CCC . CCT . TTT . TTT . TTT)	1.28
4	5'd (**K** . GGG . GGT . TTT . TTT . TTT)	1.21
5	5'd (**K** . TTT . TTT . TTT . TTT . TTT)	1.19
6	5'd (T . TTT . TTT . TTT . **K**)	1.32
7	5'd (TTT. TTT . TTT . TTT . TTT . **K**)	1.27
8	5'd (TT . TTT . TTT . TTT . TTT . TTT . TTT. **K**)	1.21
9	5'd (T . TTT .TTT . TTT . GGG . G**K**G)	1.07
10	5'd (**K** . AC . GCC . ACT . AGC . TCC)	1.28
11	5'd (GGA . GCT . AGT . GGC . GT)	-



1 : **R = R' = NH(CH2)2NH2**

2 to **10** : **R = NH(CH2)6 -ODN , R' = OH**

Fig. 1. $Eu^{3+} \subset$ TBP cryptate structure, fluorescence lifetime (τ in ms), and structure of the oligonucleotides (ODN) conjugates, K indicate the cryptate labeling position.

0.15 M NaCl) containing 0.1% BSA. The spectra and lifetimes measurement ($r^2 \geq 0.9994$) were recorded in time-resolved mode on an LS50 luminescence spectrometer (Perkin-Elmer) using the following settings: λ_{exc} = 305 nm/10 nm, λ_{em} = 620 nm/5 nm, Td = 0.1 ms, Tg = 0.4 ms. The lifetime values for K-ODN **2**–**10** are summarized in Fig. 1.

Microtiter Plate Measurement of Fluorescence Quenching

The reference cryptate **1** and the K-ODN conjugates **2**–**10** were diluted to 40 n*M* in PBS containing 0.1% BSA and 50 μL of the solutions were transferred to a black 96-well microtiter plate (Packard). Uric acid solutions (50 μL) prepared by serial dilution ($\frac{1}{2}$—$\frac{1}{64}$) of a fresh mother solution (0.2 g/L in 10 m*M* phosphate pH 7) were added to the wells, followed by 100 μL of PBS (0.1% BSA). An "unquenched standard" was made adding 50 μL buffer instead of uric acid. The fluorescence readings were performed on a Discovery® (Packard) time-resolved fluorimeter using a 50–400 μs window for the 620-nm channel. The quenching of fluorescence intensity (%) for a sample was expressed as the ratio of its fluorescence intensity to the unquenched standard intensity.

Monitoring of Phosphodiesterase Digestion by a Fluorescence Quenching Assay

Alkaline phosphatase (Boehringer) 5 μL (125 U) and snake venom phosphodiesterase (Pharmacia) 10 μL (0.2 u) were diluted in 500 μL of 0.1-M tris-HCl buffer, pH 9, containing 0.1 M NaCl, 15 m*M* $MgCl_2$, and 0.1% BSA. Uric acid solutions (50 μL) prepared by serial dilution (as above) were added to a microtiter plate. The K-ODN conjugate **9** (60 μL, 10 n*M* in tris buffer) was transferred in the wells, followed by 10 μL of the enzymes mix. An "unquenched standard" was made as above. The plate was incubated at 37°C and was read (620 n*M*/50–400μs) on a Discovery® fluorimeter immediately (to) and after 2 h, 3.5 h, 5.5 h, and 16 h, and the quenching (%) was computed as in above section.

RESULTS

Photophysical Properties of K-ODN Conjugates: Comparison to Reference Cryptate [3]

The absorption spectrum of K-ODN **2**–**10** displayed a maximum around 260 nm and a shoulder at 305 nm, characteristic for $Eu^{3+} \subset$ TBP (ε_{305} = 30,000 M^{-1}/cm^{-1} and ε_{260} = 17,000 M^{-1}/cm^{-1}), the 260/305 ratios were consistent with the expected value, taking account of the hypochromicity [4]. In contrast with the reference cryptate **1**, which displayed a predominant line at 620 nm, all the K-ODNs displayed a distinctive emission spectrum: in addition to the weak 578–nm transition, the 588- and 620-nm transitions, the spectrum displayed two stronger lines at 600 and 610 nm. The changes in the emission lines repartition can be explained by a modification of the symmetry around the europium ion [5]. Furthermore, in contrast with the reference **1** (lifetime τ = 0.6 ms in phosphate buffer), the single-stranded K-ODNs **2**–**10** displayed higher lifetimes (1.07–1.28 ms in phosphate buffer) irrespective of the length, sequence, or label-

ing position of the ODN (see Fig. 1). The fluorescence quenching was conveniently monitored by a decrease of the fluorescence intensity (620-nm channel) in time-resolved mode using microtiter plates. Quenching profiles (Fig. 2) were obtained by plotting the quenching as a function of quencher concentration. The reference cryptate **1** was quenched (90% quenching) in 20 mg/L uric acid (nevertheless, fluorescence can be restored by addition of fluorides [6]). In contrast, the K-ODNs **2**–**10** are less susceptible to fluorescence quenching; this susceptibility decreased slightly with the ODN length and was independent of the labeling position (data not shown). A series of K-ODNs **2**–**5** having the same length and differing only by the nature of the bases close to the cryptate moiety showed that the proximity of guanine residues in K-ODN **4** or **9** induced a better resistance to quenching (c.a. 20% quenching in 20 mg/L uric acid) compared to **1** (90% quenching).

Monitoring of DNA Hybridization by Fluorescence Quenching Assay

The single-stranded K-ODN **10** displayed a marked increase in its fluorescence lifetime (τ = 1.28 ms) and better resistance toward fluorescence quenching compared to **1** (see Fig. 2). Upon hybridization with its fully complementary sequence **11**, the lifetime of the duplex (**10** + **11**) was reduced to τ = 0.56 ms, and this duplex **10** + **11**) showed a poor resistance toward quenching (see Fig. 2). Furthermore, by warming the samples at 60°C for 15 min, the samples containing initially the K-ODN duplex (**10** + **11**) showed the same quenching profile as K-ODN **10** alone (data not shown). This finding can be used to monitor the hybridization or melting of a DNA duplex in the 20 nm; this assay is much more sensitive than the traditional A_{260} melting profile requiring a micromolar concentration range.

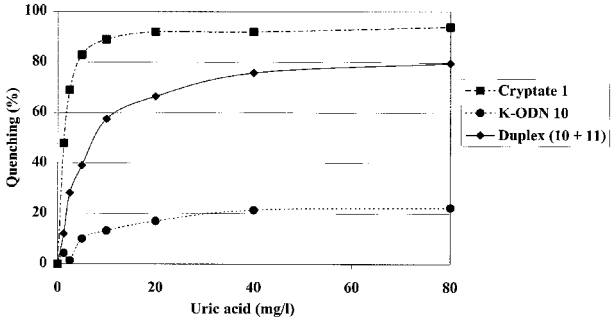


Fig. 2. Quenching profiles of single-stranded (**10**) and doubled-stranded (**10** + **11**) K-ODNs compared to reference cryptate (**1**).

Monitoring of Phosphodiesterase Digestion by Fluorescence Quenching Assay

Snake venom phosphodiesterase degrades single-stranded ODNs with natural 3′,5′-phosphodiester linkages from the 3′-end and liberating 5′-nucleotides. In this assay an excess of alkaline phosphatase was added to convert the 5′-nucleotides to nucleosides. As substrate we used the K-ODN **9**, which bears a cryptate moiety (attached to an aminohexyl-dT residue) close to the 3′-end. The phosphodiesterase activity combined with the phosphatase activity is expected to liberate a cryptate deoxynucleoside conjugate (K-dT residue). The uric acid quenching profile was monitored as a function of time. At initial time (see Fig. 3) the K-ODN **9** in pH 9 buffer display the same resistance toward quenching as observed for the series of K-ODN in pH-7 phosphate buffer. As the reaction time increases, the quenching profile is modified, and after 16 h, the quenching profile is comparable to the situation observed for cryptate **1** (see Fig. 3). A plot of the percentage of quenching as a function of time for a given uric acid concentration can be used to monitor the advancement of the enzymatic reaction. The modulation of the cryptate emission is obtained by the differential quenching affecting the cryptate, depending on whether this moiety is linked to an ODN (unquenched) or is linked to a nucleoside (quenched). This fluorescence quenching assay is a new homogeneous single-label format suitable to monitor modifications of a K-ODN substrate in the course of a reaction.

CONCLUSION

The photophysical properties of cryptate oligodeoxynucleotides conjugates (K-ODN) were shown to be very different from the parent cryptate in its non-conjugated

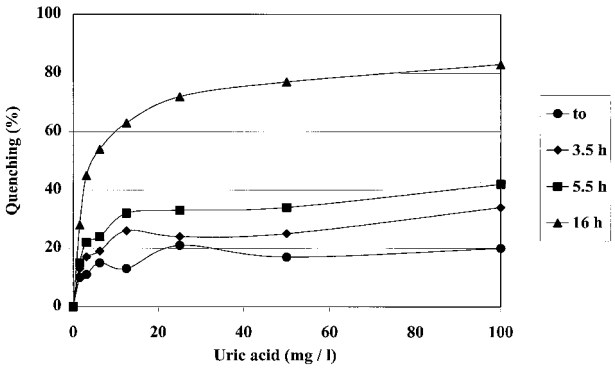


Fig. 3. Quenching profile as a function of time in the enzymatic digestion of K-ODN (**9**).

form. The fluorescence lifetime is largely increased in the K-ODN compared with a non-conjugated cryptate. A very interesting result lies in the fact that upon conjugation to single-stranded oligonucleotides, a europium cryptate was almost unsensitive to fluorescence quenching by uric acid. This behavior was shown to be moderately dependent on the length and sequence of the ODN, so far all the single-stranded K-ODNs we used displayed similar behavior. In contrast, a cryptate moiety attached to a double-stranded ODN did not display such an increase in its fluorescent lifetime and was quenched in presence of uric acid. This suggests an interaction between the cryptate moiety and a flexible single-stranded ODN, which does not exist in the case of a double-stranded ODN. Taking advantage of this unique behavior characterizing single-stranded K-ODN conjugates, a new concept of dosage based on the modulation of the cryptate fluorescence by a quencher was set up. This fluorescent quenching assay involving a single fluorescent label was applied to the monitoring of hybridization reactions and detection of a phosphodiesterase activity.

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