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# Photoluminescence and electrochemiluminescence of a Ru(II)(bpy)$_3$-quencher dual-labeled oligonucleotide probe

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**A molecular beacon oligonucleotide probe covalently labeled with Ru(II)(bpy)$_3$ and Black Hole Quencher-2 is synthesized, and hybridization assays are performed using photoluminescence and electrochemiluminescence methods of excitation.**

Oligonucleotide probes dual-labeled with reporter and quencher dyes are widely used in genetic analysis. In commercial assays, reporter fluorophores, typically based on fluorescein, rhodamine and cyanine dyes, are quenched *via* Förster Resonance Energy Transfer (FRET), where efficiency of quenching depends on $1/R^6$ and $R$ is the reporter–quencher distance. Metal-chelate complexes are a class of reporter dyes whose unique properties (*e.g*. Stokes-shifted emission, electrochemistry, long lifetime, photostability) should enable new types of genetic assays. Luminescent quenching of ruthenium complexes through FRET has been reported in an immunoassay,[1] and with dyes non-covalently bound to DNA.[2] In this study, we demonstrate that PL and ECL assays can be carried out in a homogenous fashion using a covalently attached quenching molecule, Black Hole Quencher-2™ (BHQ-2). This dark quencher, which has no native fluorescence, is attached to the 5′-end of the oligo, and the Ru(II)(bpy)$_3$ label is attached at the 3′-end. A molecular beacon (MB) stem structure[3] holds the Ru(II)(bpy)$_3$ reporter and BHQ-2 in close proximity as shown in Fig. 1. MBs are known to discriminate single base mismatches in hybridization assays.[3] To the best of our knowledge this is the first report in which ECL has been used to assay a dual-labeled fluorogeneic probe.

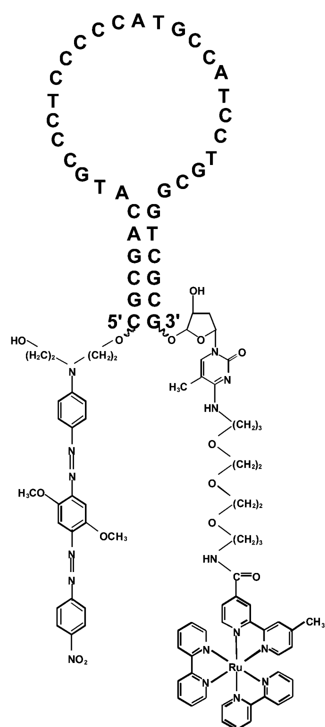

**Fig. 1** Ru(II)(bpy)$_3$/BHQ-2 MB probe.

DNA sequences were made with a Biosearch 8700 synthesizer using standard phosphoramidite reagents. The $\beta$-actin sequence with the MB stem structure is: 5′-d-*CGC-GAC*-ATG-CCC-TCC-CCC-ATG-CCA-TCC-TGC-*GGT-CGC-G*-3′. Ru(II)(bpy)$_3$-CPG solid support (sold as Pulsar-650™ by Biosearch Technologies) was used to add the 3′ label and BHQ-2 (Biosearch) was added to the 5′ end as the phosphoramidite. Complementary β-actin was made with three extra T bases at each end: 5′-d-TTT-CGC-AGG-ATG-GCA-TGG-GGG-AGG-GCA-TTT-T-3′. All synthetic DNA samples were dual-HPLC purified by anion exchange (Dionex DNA pac PA-100 column; solvent A = 0.038 M tris[hydroxymethy]aminomethane with 15% $CH_3CN$; solvent B = solvent A with 1 M NaBr) followed by reverse phase HPLC (Hamilton PRP-1 column; solvent A = 0.1 M TEAA; solvent B = $CH_3CN$). Sample purity was confirmed by analytical ion exchange and reverse-phase HPLC. ECL spectra were recorded in a fluorometer with a three-electrode arrangement (gold WE and CE, and Ag/AgCl RE) inserted into a standard cuvette; the WE faced the detector and had a diameter of 2 mm. ECL was triggered by applying a potential of 1.5 V *vs* Ag/AgCl. PL spectra were recorded in the same instrument with a 580 nm cut-on long-pass filter interposed between the cuvette and the detector; the excitation wavelength was 450 nm. Spectroelectrochemical measurements were made with a three-electrode arrangement, which included a gold mesh working-electrode (WE) in a planar glass capillary, located in the optical path of the spectrophotometer.

The mechanism of ruthenium chelate ECL with tripropylamine (TPA) as the sacrificial amine has been reported previously.[4] Electrochemical oxidation of TPA results in the formation of a $TPA^+$ radical, which spontaneously loses a proton, forming a neutral $TPA^\cdot$ radical. Electrochemically generated Ru(III)(bpy)$_3$ is reduced by $TPA^\cdot$ to electronically excited Ru(II)(bpy)$_3$, which emits light with $\lambda_{max}$ = 592 nm when it relaxes to the ground state. Deprotonation of the radical generates a high concentration of protons in the vicinity of the electrode and therefore a strong buffer is required to maintain the optimum pH of around 7.[4] Most of the buffers that operate in this pH range are electro-active at the potential required for ECL and therefore phosphate has been used. The solubility product of phosphate with divalent cations is very low, which suggested that it might be difficult to accommodate the previously reported requirement that MBs have for divalent cations,[5] but PL measurements showed that the MB performed well in the absence of $Mg^{2+}$ (Table 1). Figs. 2 and 3 show the effect of adding the complementary DNA sequence on the PL and ECL emission respectively. The signal increased to 95% of the maximum value in less than one minute, but the background signal was higher in the ECL assay. Spectroelectrochemistry suggested that this was because the BHQ-2 is bleached at potentials more anodic than +0.5 V *vs* Ag/AgCl. This is an unavoidable consequence of the Ru(bpy)$_3$ ECL reaction which is carried out at +1.5 V.

We have shown for the first time that dual labelled probes can be excited electrochemically as well as photochemically. This is

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**Table 1** PL intensities before and after hybridization. All values expressed as percentage of unquenched probe[a] in pH 8.0 Tris[a] with $MgCl_2$. Key to concentrations: Tris[a] (20 mM); Tris[b] (0.15 M); phosphate (0.15 M); TPA (0.1 M) Tween-20 (0.05%); $MgCl_2$ (10 mM)

| Buffer | Before | After |
|---|---|---|
| Tris[a], pH 8.0 | 30 | 36 |
| Tris[a], MgCl, pH 8.0 | < 1 | 26 |
| Phosphate, TPA, Tween-20, pH 7.0 | < 1 | 43 |
| Tris[b], TPA, Tween-20, $MgCl_2$, pH 7.0 | < 1 | 24 |
| Tris[b], TPA, Tween-20, $MgCl_2$, pH 8.0 | < 1 | 23 |

[a] The structure of the unquenched probe was the β-actin sequence without the MB stem structure, and without the BHQ-2.

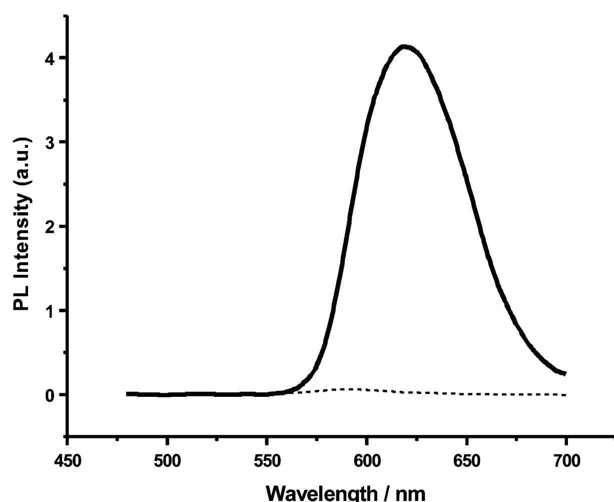


**Fig. 2** PL assay of MB (5 μM): Key: broken line before, and solid line 5 minutes after adding complement (25 μM).

an important result because the instrumentation required for electrochemical excitation and detection is considerably less expensive and more portable than that which is required for PL. Furthermore, signals derived from ECL rather than PL excitation are free from background fluorescence and scattering.[6] We are currently investigating alternative labels based on acridans.[7] These compounds have a higher quantum yield than Ru(II)(bpy)$_3$ and can be triggered under conditions that do not bleach the quenching molecule. When the reporter and quencher labels are optimized, we expect that electrochemical

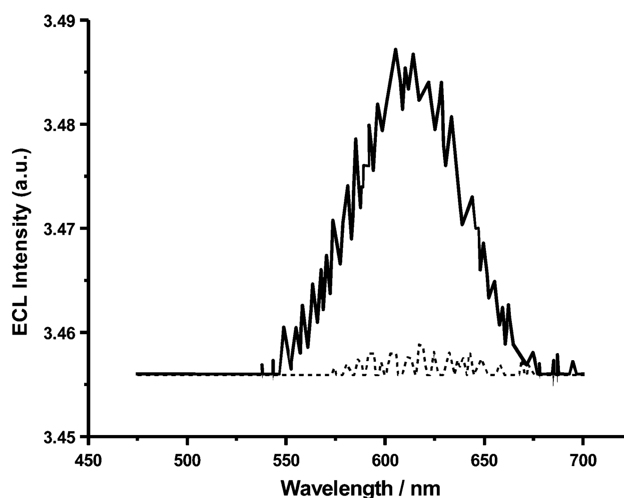


**Fig. 3** ECL assay of MB (50 μ): Key: broken line before, and solid line 5 minutes after adding complement (250 μM).

excitation of dual-labeled probes will be suitable for point-of-care and other extra-laboratory applications.

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