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A BODIPY-luminol chemiluminescent resonance energy-transfer (CRET) cassette for imaging of cellular superoxide

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S. Bag,[a] J.-C. Tseng[b,c]* and J. Rochford[a]*

Spectroscopic and in cellulo studies are here reported on the very first BODIPY-luminol chemiluminescent resonance energy-transfer (CRET) cassette where the luminol CL agent is covalently linked to the BODIPY energy-transfer acceptor in a molecular dyad. The efficiency of intramolecular CRET investigated for the BODIPY-luminol dyad was found to be 64% resulting in a dual emissinve response. Successful in cellulo biochemiluminescence via CRET was achieved in PMA activated splenocytes.

Introduction

Chemiluminescent (CL) imaging of reactive oxygen species (ROS) endogenously produced during tissue inflammation, provide a means for indirectly informing on biochemical processes related to wound healing,[1] microbial infection,[2] diabetes,[3] cancer,[4] cardiovascular,[5] neurodegenerative,[6] and autoimmune diseases.[7] Cell permeability, chemiluminescent quantum yield (Φ_{CL}) and commercial availability of 5'-amino-2,3-dihydro-1,4-phthalazinedione (aka luminol, Φ_{CL} = 0.25 – 2.4% dependent upon oxidant and pH)[8] has led to this system being the most widely studied for in vivo detection of ROS such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($HO^{\bullet}$). In fact, systemic delivery of luminol has recently been demonstrated as an effective means of distinguishing between acute and chronic inflammation in vivo in combination with lucigenin. In particular, luminol's chemiluminscence signal is triggered by myeloperoxidase (MPO) derived from tissue-infiltrating neutrophils during the acute phase of inflammation.[9] Unfortunately, the high energy CL photons produced from luminol (λ_{max} = 455 nm) suffer from absorption and scatter by endogenous haemoglobin and tissue, respectively, hindering its true practical potential for deep tissue imaging. One strategy to address this issue is to employ energy-transfer.[10] In fact, this very strategy is used in "glow-stick" devices through combination of (i) H_2O_2 mediated tichlorophenyloxalate (TCPO) oxidation (ii) decomposition of the high-energy 1,2-dioxetanedione product to CO_2 and UV emission (iii) intermolecular energy transfer to an appropriate fluorescent dye. Inspired by this strategy, Tseng et al. reported in vivo imaging of ROS by *chemically initiated electron-exchange luminescence* (CIEEL) where the cypridina luciferin analog 2-methyl-6-[4-methoxyphenyl]-3,7-dihydroimidazo[1,2-a]pyrazin-3-one hydrochloride (MCLA) was used in place of TCPO.[11] Intermolecular energy transfer was effected with C5 or C7 diethylthiacarbocyanine emitters, thus achieving a far visible to near infrared spectral response. Analogous reports utilizing molecular based fluorophores with the luminol CL agent are rare however. Mahammed and Gross reported also on the intermolecular CL enhancement of luminol via energy transfer to Al^{III} (λ_{fl} = 615 nm) and Ga^{III} (λ_{fl} = 625 nm) corroles.[12] In an earlier study, Burgess and co-workers reported on the only efficient intramolecular energy transfer system to date for ethynyl-luminol derivatives with π-conjugated fluorescein (λ_{fl} = 524 nm) and nile red (λ_{fl} = 634 nm) based emitters.[13] While this study held great potential due to the impressive stoke shifts reported their application in vitro/in vivo was severely limited due to poor solubility in aqueous solution. In a related study utilizing the alternative luciferin CL agent Sekiya et al. investigated the first CL energy transfer cassette employing a BODIPY fluorophore (λ_{fl} = 545 nm) including in vitro studies on HL-60 cells.[14] Herein we report preliminary data on the very first BODIPY-luminol chemiluminescent energy-transfer cassette where the luminol CL agent is covalently linked to the BODIPY energy-transfer acceptor in a molecular dyad (Fig. 1).

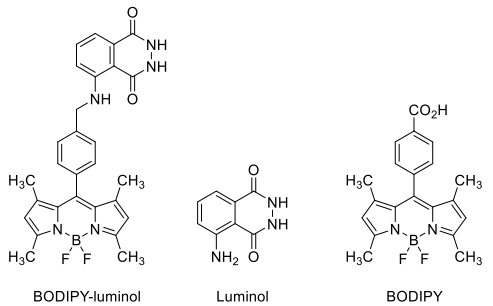


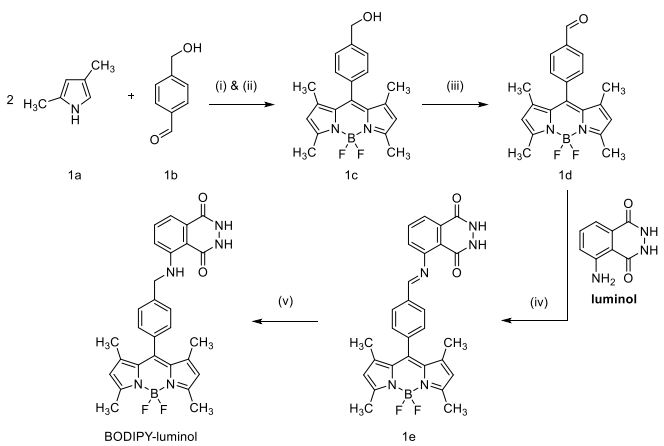
Figure 1. Molecular structure of the BODIPY-luminol conjugate alongside the luminol and *meso*-(4-carboxyphenyl)BODIPY reference systems.

In this, our first report of BODIPY-luminol conjugated chemiluminescent resonance energy-transfer (CRET) cassettes for imaging of cellular superoxide, π-mediated electronic communication between the luminol and BODIPY components is insulated through the use of a benzyl spacer. This design is by choice in order to retain the structural and electronic properties of both the independent luminol and *meso*-(4-carboxyphenyl)BODIPY monomer systems (Fig. ESI-3).

Results and discussion

Synthesis

The benzyl linked BODIPY-luminol conjugate was synthesized in a linear fashion as illustrated in Scheme 1. The *meso*-(4-benzaldehyde)-BODIPY precursor **1d** used for direct coupling with luminol was prepared via the *meso*-benzyl alcohol BODIPY intermediate **1c** from 2,4-dimethylpyrrole **1a** and 4-formylbenzyl alcohol **1b** according to a previously reported procedure.[15] Many attempts to isolate and characterize the pure imine conjugated intermediate **1e** were in vain due to its very poor solubility. Therefore coupling of **1d** with luminol, followed by subsequent reduction of the crude imine **1e**, was conducted by a one-pot method. A comprehensive description of the experimental procedure employed and spectral characterization of BODIPY-luminol can be found in the experimental section below.



Scheme 1. Synthesis of the BODIPY-luminol conjugate energy transfer cassette (i) CH_2Cl_2/TFA/Ar/r.t. (ii) DDQ/Et_3N/$BF_3.OEt_2$ (iii) CH_2Cl_2/Dess-Martin (iv) Δ/MeOH:CH_2Cl_2 (v) $NaBH_4$/MeOH.

UV/Vis absorption and fluorescence emission spectroscopy

As pointed out above, π-conjugation has been avoided through the incorporation of a benzyl spacer thus preventing electronic communication between both luminol and BODIPY components. This approach was by design to directly probe the CRET efficiency for electronically independent BODIPY and luminol components in a covalently linked dyad by comparison to their monomeric components (Fig. 1). While π-conjugation may alter electronic communication between BODIPY and luminol it is also likely to perturb frontier orbitals of both systems rendering the luminol emission and BODIPY absorption unpredictable, perhaps even hindering the efficiency of CRET due to an enhanced spectral mismatch. In fact, it is well known that the *meso*-phenyl ring on BODIPY is sufficient itself to preclude such electronic communication due to its orthogonality with respect to the BODIPY π-system.[16, 17] Considering this, the major advantage of using the benzyl linker here in addition to preserving BODIPY and luminol spectroscopic properties is the improved solubility of the BODIPY-luminol dyad, perhaps via reduced π-staking and aggregation. For example, the imine conjugated intermediate **1e** (Scheme 1) proved too insoluble to purify and prior ethynyl conjugated luminol derivatives reported by Burgess et al. also proved only soluble in DMSO precluding studies in aqueous media.[13] Negligible electronic interaction between the BODIPY and luminol subunits in the dyad system was confirmed by UV/Vis electronic absorption and emission spectroscopies (Table 1, Figs. 2, ESI-3, ESI-4). Further confirmation was also found through computational analysis for the high-energy, ROS derived, dicarboxy intermediate responsible for luminol based emission (Fig. ESI-6).

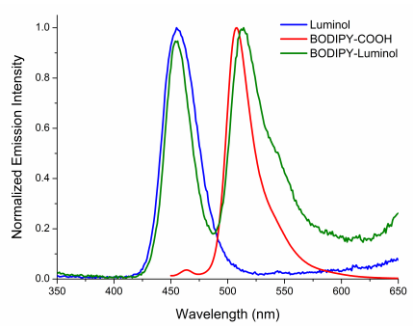


Figure 2. Normalized chemiluminescence spectra of luminol and the BODIPY-luminol cassette recorded in pH 10 Na_2CO_3:$NaHCO_3$ buffer overlayed with the photoluminescence fluorescence spectrum of *meso*-(4-carboxyphenyl)BODIPY (λ_{exc} = 400 nm).

The conjugate dyad displays an absorption spectrum consistent with the sum of both the luminol and BODIPY independent subunits recorded in an aqueous pH 10 Na_2CO_3:$NaHCO_3$ buffer

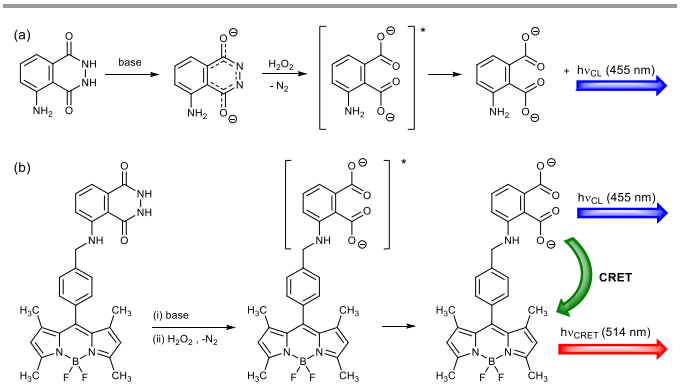
(Table 1, Fig. ESI-3). This is confirmed also by the BODIPY-luminol photoluminescence spectrum where, upon direct photoexcitation of the BODIPY $S_0 \rightarrow S_1$ electronic transition (λ_{exc} = 490 nm), the fluorescence spectrum is almost identical to that of the *meso*-(4-carboxyphenyl)BODIPY analogue (Fig. 2). A slight 6 nm red shift in the emission maximum of the dyad relative to the monomer is easily attributed to the weak inductive withdrawing effect of the carboxy substituent of the *meso*-(4-carboxyphenyl)BODIPY monomer. The latter was used in preference to the isoelectronic *meso*-(tolyl)BODIPY reference fluorophore due to the solubilizing properties of the carboxy functional group in the aqueous pH 10 buffer employed.

Table 1. UV/Vis, fluorescence emission (λ_{fl}) and chemiluminescence (λ_{CL}) data recorded in pH 10 aqueous buffer.

Compound	UV/Vis absorption λ_{max} (nm); (ε x10^4 M^{-1} cm^{-1})	λ_{fl} (nm)	λ_{CL} (nm)
Luminol	301 (0.67), 348 (0.75)	~	455
BODIPY	310 (0.79), 359 (0.52), 495 (8.10)	508	~
BODIPY-luminol	309 (1.51), 350 (1.41), 499 (8.08)	514	456, 514

Chemiluminescence

The parent luminol CL agent emits blue light upon H_2O_2/$CuSO_4$ activation (λ_{max} = 455 nm) in a pH 10 Na_2CO_3:$NaHCO_3$ aqueous buffer. While the BODIPY-luminol dyad is not optimized for quantitative CL resonance energy-transfer (CRET), due to the slight mismatch of luminol CL (λ_{CL} = 455 nm; fwhm = 1 719 cm^{-1}) and BODIPY $S_0 \rightarrow S_1$ absorption (λ_{abs} = 499 nm; fwhm = 807 cm^{-1}), overlap exists nonetheless allowing for the first proof of concept for CRET involving a luminol CL and BODIPY fluorophore energy transfer cassette. Integration of normalized luminol chemiluminescence and BODIPY-luminol absorption spectra indicates a 23% CRET compatibility (Fig. ESI-5) such that dual emission is anticipated upon exposure to ROS (Scheme 2).



Scheme 2. (a) Mechanism of oxidative chemiluminescence from luminol via a high-energy dicarboxy intermediate induced by H_2O_2/$CuSO_4$ in pH 10 aqueous buffer solution. (b) Corresponding mechanism for dual emission from the BODIPY-luminol conjugate under identical conditions.

Upon exposure of BODIPY-luminol (3.33×10^{-7} M) to a solution of H_2O_2 (1.33×10^{-3} M) with $CuSO_4$ (1.00×10^{-3} M) in pH 10 aqueous buffer dual chemiluminescence is observed, as anticipated, due to direct luminol CL in addition to BODIPY emission as a result of CRET (Fig. 3).

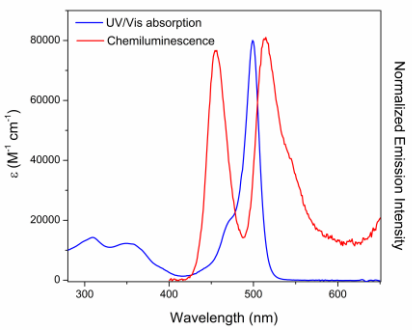


Figure 3. Overlay of electronic absorption and chemiluminescence spectra of the BODIPY-luminol conjugate recorded in pH 10 aqueous Na_2CO_3:$NaHCO_3$ buffer.

To our surprise however, upon deconvolution and integration, 64% of the CL response is attributed to the BODIPY $S_1 \rightarrow S_0$ fluorescence decay while the remaining 36% is due to direct CL from the luminol moiety of the dyad. No doubt this is a result of the close proximity of the luminol and BODIPY components within the cassette. A control experiment using equimolar concentrations of luminol and *meso*-(4-carboxyphenyl)-BODIPY subunits under identical conditions yielded a CRET efficiency of < 10%. The relative CL yield of the BODIPY-

luminol cassette is ~70% that of the parent luminol system which is unsurprising considering the flexible nature of the benzyl linkage (Φ_{CL} = 1.7% assuming Φ_{CL} = 2.4% for luminol).

In cellulo imaging studies

Noninvasive CL imaging has the potential to inform on the various roles of neutrophils and macrophages in a variety of pathological conditions. Acute tissue inflammation is largely mediated by neutrophils through chemotaxis, which rapidly passivate invading bacteria by phagocytosis ultimately forming phagosomes, within which the cells produce high levels of intragranular superoxide. Phagosomal superoxide is the primary source of many downstream ROS such as hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl).[18-21] At the late stage of inflammation, neutrophils are replaced by macrophages[1] to promote tissue repair by production of extracellular ROS.[19] While their mode of action differs, neutrophils and macrophages rely on phagocyte NADPH oxidase (Phox) as their primary source of superoxide production.[19] Together with myeloperoxidase (MPO), Phox catalyzes rapid ROS production with consumption of cellular oxygen visually evident as tissue inflammation.[21] Taking advantage of luminol's ability to penetrate both the plasma membrane and membranes of subcellular organelles, imaging of MPO activity in vivo has previously been achieved.[22, 23 24]

In cellulo tests of the BODIPY-luminol energy-transfer cassette were here performed using splenocytes activated by phorbol myristate acetate (PMA). While there is precedence, albeit ex vitro, for the self-promotion of chemiluminescence by luminol radical cations (via reaction with superoxide) PMA activation is required to induce cellular superoxide production and generate a chemiluminescence response from luminol or BODIPY-luminol. Thus PMA activation allows luminol and BODIPY-luminol to here specifically report on Phox activity for ROS production in cellulo. To emphasize the specificity of this reponse, we recently demonstrated the absence of any luminol derived chemiluminescence in cellulo/in vivo when Phox activity (and thus superoxide production) is prohibited using the Phox inhibitor apocynin.[9] Furthermore, chemiluminescence is also found absent for Phox-null animals, in vivo.[9] Figure 3 illustrates qualitative and quantitative analysis for the CL and CRET response of PMA activated splenocytes incubated with luminol and BODIPY-luminol, respectively.

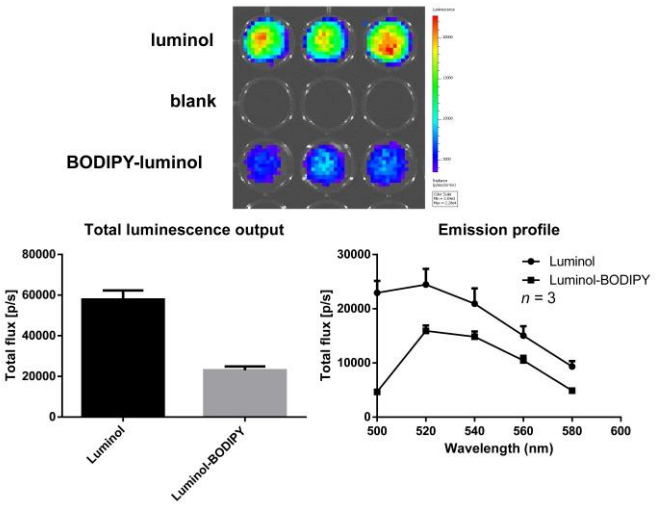


Figure 3. (Top) Biochemiluminescence of luminol, blank and BODIPY-luminol in PMA-activated splenocytes. (Bottom) In cellulo biochemiluminescence output and emission profiles of BODIPY-luminol CRET vs the parent luminol CL agent. Each well contains 2.4 x10^7 splenocytes harvested from nu/nu mice. Endogenous superoxide production was stimulated by 50 μg/ml of PMA prior to incubation with CL agents (Luminol: 66 μg/ml; BODIPY-luminol: 200 μg/ml).

The parent luminol CL agent, displays a higher total chemiluminescence output, however, this is unsurprising considering its greater Φ_{CL} yield. Although less efficient than the parent luminol system BODIPY-luminol displays an in cellulo emission profile which is dominated by the BODIPY centred CRET emission at ~ 520 nm.

Conclusions

Chemiluminescence resonance energy transfer (CRET) was successfully demonstrated for a benzyl linked BODIPY-luminol conjugate ex vitro and in cellulo upon splenocyte stimulation. To the best of our knowledge this represents the first example of CRET for any BODIPY-luminol conjugate, ex vitro or in vitro/in cellulo. Although low energy emission is dominant, the weak biochemiluminescence intensity observed in cellulo relative to luminol may be due to poor membrane permeability into intracellular phagosomes. Future studies will improve upon the CRET efficiency and cell permeability.

Experimental

Materials and methods

Sodium carbonate, sodium bicarbonate, hydrogen peroxide (10%), anhydrous copper(II) sulphate, trifluoroacetic acid, *p*-toluenesulfonic acid monohydrate, DDQ (2,3-dichloro-5,6-dicyanoquinone), triethylamine, boron triflouride etherate, tetrahydrofuran (THF), sodium borohydride, Dess-Martin periodinane [1,1,1-tris(acetyloxy)-1,1-dihydro-1,2-benziodoxol-3-(1H)-one], luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), phorbol-12-myristate-13-acetate (PMA) were all used as received from Sigma Aldrich. Toluene (ACS reagent grade) and dichloromethane (ACS reagent grade) were purchased from Pharmco-Aaper and dried over 4 Å molecular sieves prior to use. All spectra were recorded in pH 10 Na_2CO_3:$NaHCO_3$ (4:1) buffer solution. NMR spectra were recorded on a Varian spectrometer operating at 300.13 MHz for ^1H and 75.03 MHz for ^{13}C nuclei. Deuterated d_6-dimethylsulphoxide was purchased from Sigma Aldrich and the residual proton signals were used as an internal reference point for reporting the chemical shift (δ (^1H) = 2.50 ppm, δ (^{13}C) = 39.52 ppm).[25] UV/Vis absorption spectra were recorded on an Agilent 8453A diode array spectrophotometer and emission/excitation spectra recorded on a PTI Quantamaster 25 spectrometer.

Synthesis

The BODIPY-luminol conjugate was synthesized in a linear fashion as described in Scheme 1. The *meso*-(4-benzaldehyde)-BODIPY was prepared via the meso-benzyl alcohol BODIPY intermediate from 2,4-dimethylpyrrole and 4-formylbenzyl alcohol according to a reported method.[5] Subsequent coupling to luminol was conducted in a one pot method as follows. A 50 ml flask was charged with 20 ml of MeOH:CH_2Cl_2 (8:2) and to the flask was added 0.14 mmol (49 mg) of *meso*-(4-benzaldehyde)-BODIPY. Subsequently 0.14 mmol (25 mg) of luminol was added and the reaction mixture was refluxed for 1 hour. Upon cooling to room temperature one equivalent of $NaBH_4$ (0.14 mmol, 5 mg) was added and the reaction stirred for a further 2 hours. The solvents were then evaporated under reduced pressure. The crude solid was dissolved in dichloromethane, washed with dil. HCl, deionized water, brine and dried over $MgSO_4$. The pure BODIPY-luminol was finally isolated by chromatography on silica gel with an ethyl acetate:hexane (2:1) mobile phase as the second burgundy colored band. Recrystallization from dichloromethane and hexane produced analytically pure product in 40% yield. ^1H NMR δ(d_6-DMSO): 1.33 (s, 6H), 2.42 (s, 6H), 4.30 (d, 2H, J = 6.0 Hz), 6.15 (s, 2H), 6.86 (d, 1H, J = 8.40 Hz), 6.91 (d, 1H, J = 8.40 Hz), 7.21 (d, 2H, J = 8.40 Hz), 7.26 – 7.37 (m, 3H), 7.43 (dd, 1H, J = 8.40, 8.40 Hz), 11.20 (s, 2H) ppm. ^{13}C NMR δ(d_6-DMSO): 14.27, 14.34, 49.05, 109.73, 110.64, 116.65, 121.34, 126.82, 127.63, 129.92, 131.08, 134.15, 138.73, 142.83, 150.83, 151.83, 154.78, 161.55 ppm.MS (MALDI-TOF) $[M+1]^+$ m/z: calc. 514.2226; obs. 514. 2203.

In cellulo biochemiluminescence imaging

Splenocytes were harvested from 8-week old nu/nu mice (Charles River Laboratories, Wilmington MA). The cells were washed and resuspended in phosphate buffer saline (PBS) prior to loading onto a 96-well plate. Each well contains 2.4 x 10^7 cells in a total volume of 300 µl of PBS. To stimulate endogenous superoxide production, cells were incubated with 50 µg/ml of PMA. To generate biochemiluminescence, luminol or BODIPY-luminol was added into wells to reach a final concentration of 66 or 200 µg/ml respectively). Biochemiluminescence was measured using the IVIS Spectrum imaging system (PerkinElmer Inc., Hopkinton MA). A measurement of total luminescence output was first performed without any emission filter in place (open filter). Then sequential acquisitions were performed ranging from 500 to 640 nm in 20 nm intervals.

Notes and references

[a] Department of Chemistry, University of Massachusetts Boston, 100 Morrissey Boulevard, Boston, Massachusetts 02125, U.S.A.
[b] Lurie Family Imaging Center, Dana-Farber Cancer Institute, Boston, Massachusetts 02215, U.S.A.
[c] Present address: PerkinElmer Inc., 68 Elm Street, Hopkinton, Massachusetts 01748, U.S.A.

Electronic Supplementary Information (ESI) available: Experimental details, computational analysis, spectra. See DOI: 10.1039/c000000x/

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Table of contents graphic and text

Chemiluminescent resonance energy transfer is investigated for a BODIPY-luminol dyad demonstrating in-cellulo biochemiluminescence with reactive oxygen species in activated splenocytes.