Generation of Triplet Excited States via Photoinduced Electron Transfer in meso-anthra-BODIPY: Fluorogenic Response Towards Singlet Oxygen in Solution and in Vitro

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Supporting Information Placeholder

ABSTRACT: Heavy atom-free BODIPY-anthracene dyads (BADs) upon photoexcitation generate locally excited triplet excited states by way of photoinduced electron transfer (PeT), followed by recombination of the resulting charge-separated states (CSS). Subsequent quenching of the triplet states by molecular oxygen produces singlet oxygen (\(^{1}\)O\(_2\)), which reacts with the anthracene moiety yielding highly fluorescent species. The steric demand of the alkyl substituents in the BODIPY subunit defines the site of \(^{1}\)O\(_2\) addition. Novel bis- and tetraepoxides along with bicyclic acetal products arising from a chain of rearrangements of anthracene endoperoxides were isolated and characterized. \(^{1}\)O\(_2\) generation by BADs in living cells provides fluorescent visualization of the dyads distribution promising new imaging applications.

Optical probes based on photoinduced electron transfer (PeT) in donor-acceptor dyads have found broad use in diagnostics, particularly for the detection of biomolecules, metal ions, reactive oxygen species (ROS) and measurement of intracellular pH. The PeT process leads to formation of non-emissive charge-separated states (CSS) which decay back to the ground state via different pathways. Among those is recombination of CSS, which may lead to locally excited triplet states in the molecule. Recently this process has attracted attention as a method to increase intersystem crossing without directly relying to the heavy atom effect.

The possibility for singlet oxygen (\(^{1}\)O\(_2\)) generation by donor-acceptor dyads mediated by PeT has not been realized so far in practical sense. It could be expected that \(^{1}\)O\(_2\) generation by PeT-based optical probes in biological environments would affect their optical response and simultaneously induce cytotoxicity. This is of special concern in the case of ROS detection, where sensitization of \(^{1}\)O\(_2\) by the probe itself may lead to false positives and incorrect interpretations. On the other hand, PeT-mediated \(^{1}\)O\(_2\) generation could provide a new tool for theranostic applications, since the process of charge separation can be turned on/off by various stimuli. Herein we report readily accessible heavy atom-free BODIPY-anthracene dyads (BADs) that can act as efficient triplet sensitizers, providing fluorescent response towards generated \(^{1}\)O\(_2\).

While a number of triplet sensitizers based on halogenated BODIPYs have been reported during the last decade, observations of triplet excited state formation in heavy atom-free BODIPYs are rare. In our search into efficient donor-acceptor PS, we have focused on BADs 1 and 2 (Scheme 1). BODIPYs are known to be efficient energy and electron acceptors when combined with anthracene. Although compound BAD1 has been reported to exhibit PeT, no triplet excited states formation has been noted.

\[ \text{Scheme 1. Photoinduced transformations of BADs.} \]

Upon broad-band visible light irradiation of air-saturated solutions of BADs in a range of polar solvents we observed, to our surprise, completely selective formation of BAD1-BE, which could be isolated in 5% yield along with recovered unreacted starting material (Scheme 1). In contrast, irradiation of BAD2 under the same conditions resulted in complete conversion of the substrate with formation of two products, bicyclic acetal derivative (BAD2-BA) and tetraepoxide (BAD2-TE), which were isolated in 80% and 10% yields, respectively. The structures of the products were confirmed by NMR spectroscopy and X-ray crystallography (for details see Supporting
Information (SI). Unlike BADs 1 and 2, isolated compounds exhibit bright fluorescence independent of the solvent polarity. For instance, the emission quantum yields of BADs BE in CH$_2$Cl$_2$ and hexane were determined to be 0.91 and 0.89, respectively.

The formation of products appears to be due to the sensitization of oxygen and subsequent [4+2] cycloaddition of the resulting O$_2$, which is typical for anthracene derivatives. Singlet oxygen quantum yields of BADs were measured using 1,3-diphenylisobenzofuran as an O$_2$ trap, giving values of 0.67 and 0.38 in ethanol, for BAD1 and BAD2, respectively. In order to understand the mechanism of O$_2$ formation we studied the excited state dynamics of the dyad BAD1 by broadband VIS-NIR sub-pico- to microsecond transient absorption (TA) pump-probe spectroscopy.

Figure 1. a) ps-ns Transient absorption spectra of BAD1 in dimethylformamide upon excitation at 355 nm with 35 fs pulses at delay times of 600 fs (black line), 1 ps (red line), 100 ps (green line), and 5 ns (blue line). The inset shows the blue shift of the TA spectra to 570 nm. b) Kinetics monitored at 380 nm (black line), 400 nm (red line), 425 nm (green line), 505 nm (violet line), 570 nm (cyan line) and 680 nm (magenta line) as indicated by the vertical colored bars. c) ns-µs Transient absorption spectra of degassed BADs solutions following excitation at 355 nm by 700 ps laser pulses. The spectra were integrated from 3-5 ns (black line), 10-100 ns (red line), 0.1-1 µs (green line), 1-5 µs (blue line), and 10-100 µs (cyan line). d) Kinetics observed for the bands at 570 nm and 680 nm, assigned to the BODIPY triplet state and anthracene radical-cation, respectively, in the absence and presence of oxygen (solid and dotted lines, respectively).

Immediately after photoexcitation with fs pulses at 355 nm a broad band around 360 nm due to the anthracene's singlet excited state (S) absorption, which partially overlaps with the ground state photo bleach (PB), was observed (Figure 1a). The concomitant decay of this band and simultaneous rise of the bleach at 505 nm indicate ultrafast energy transfer (EnT) from anthracene to BODIPY subunit, populating the singlet state of the latter, S$^{\mathrm{BADP}}$. Furthermore, another absorption band grows at 580 nm, and it was assigned to the BODIPY radical-anion (BDP$^-$), forming due to the PeT process. This band rises during the first 100 ps, simultaneously shifting to 570 nm, indicating a transition of the radical-anion to another excited state (see inset of Figure 1a). Synchronously with the rise of the BDP absorption (580 nm), yet another absorption band, centered at 680 nm, rises, presumably due to the anthracene radical-cation (Ant$^+$), in line with previous reports. Global fitting of the PB decays at 380 nm and 400 nm and the rise of BDP$^-$ and Ant$^+$ bands (Figure 1b) yields time constants of 1.15 ps and 0.54 ps for the EnT and PeT processes, respectively.

In the ns-µs TA experiments, a rise of an absorption band at 570 nm over 1 µs was observed, indicating formation of long-lived states (Figure 1c). Previous reports on the TA spectra of BODIPY support the assignment of this band to the BODIPY triplet state (T$^{\mathrm{BADP}}$) absorption. The band at 570 nm was quenched and decayed faster in the presence of oxygen (Figure 1d). In contrast, the anthracene radical cation- absorption band at 680 nm was impacted by oxygen significantly less. The T$^{\mathrm{BADP}}$ lifetime in the absence of O$_2$ was determined to be 41 µs. The observed transition from the bands originating in CSS to the absorption by the triplet suggests that the formation of CSS is a prerequisite for populating of T$^{\mathrm{BADP}}$.

![Figure 2](image-url)

**Figure 2.** a) Frontier molecular orbitals and their energies (in a.u.) for BADs 1 and 2. b) Diagram demonstrating transitions between excited states in BADs.

The frontier molecular orbitals diagram (Figure 2a) shows that the two highest occupied orbitals $\pi$$_{\text{ant}}$ and $\pi$$_{\text{BADP}}$ located on the anthracene and BODIPY subunits, respectively, are nearly degenerate. Density functional theory (DFT) calculations on these molecules (see SI for computational details) confirm that in BADs PeT could take place from $\pi$$_{\text{ant}}$ to singly occupied $\pi$$_{\text{BADP}}$ thus leading to singlet charge transfer state S$^{\text{CSS}}$ that is 0.4 eV more stable than the S$^{\text{BADP}}$ excited state. Unlike the valence excited states, CSS has a very low ferromagnetic exchange coupling integral due to negligible overlap of singly occupied orbitals $\pi$$_{\text{ant}}$ and $\pi$$_{\text{BADP}}$ located in mutually orthogonal molecular moieties thus leading to a very small singlet-triplet energy gap (S-T gap). Two pathways for triplet state generation from CSS may yield the lowest local T$^{\text{BADP}}$ state (Figure 2b): spin-orbit charge transfer intersystem crossing (SOCT-ISC) and radical pair intersystem crossing (RP-ISC), followed by triplet charge recombination. As has been shown in extensive works of Wasielewski and co-workers, SOCT-ISC prevails for systems with strong electronic couplings, requiring short distances between the subunits (4.3 Å in BADs). On the other hand, due to the small S-T gap in the RP state, mixing of S$^{\text{CSS}}$ and T$^{\text{CSS}}$ states is possible due to e.g. electron-nuclear hyperfine coupling. More detailed studies will be necessary to distinguish between mechanisms governing spin interconversion in BADs.

The observed PeT process is clearly manifest in the spectroscopic properties of BADs. The fluorescence of the BODIPY is quenched in polar solvents as evidenced by the negligible values of $\Phi$$_{\text{f}}$ observed, compared to the strong emission in non-polar solvents (Table S3). A broad emission band at 610 nm was observed in polar solvents. Such red-shifted broad emission bands arising from charge transfer excited states
were reported for various donor-acceptor systems. DFT calculations in vacuo show that S<sup>SS</sup> state is approximately 0.2 eV higher in energy than the valence S<sup>BDP</sup> state. The dipole moment for the S<sup>SS</sup> state was computed to be μ = 19 D in vacuo, which is much higher than that for the valence S<sup>BDP</sup> state (5 D). Interactions of CSS with polar solvent result in a decrease of the S<sup>SS</sup> state dipole moment to 1.1 D and change the relative energy ordering of the S<sup>BDP</sup> and S<sup>SS</sup> states, making PeT process favourable.

Figure 3. a) Absorption and emission spectra of BAD<sub>1</sub>. b) Changes of the emission intensity upon irradiation of BADs and reference compound solutions in CH<sub>2</sub>Cl<sub>2</sub> (5 × 10<sup>-5</sup> M) with broadband visible light. c) Change of BAD<sub>2</sub> emission upon irradiation with 532 nm laser (10 mWcm<sup>-2</sup>). d) Photo of BAD<sub>2</sub> solution before and after 5 min of irradiation, taken under excitation with 365 nm light.

When irradiated with monochromatic or broadband visible light, air-saturated solutions of BADs in polar solvents showed a gradual increase in the fluorescence (Figure 3b). For instance, irradiation of BAD<sub>2</sub> solution results in up to 100-fold increase of the fluorescence intensity due to formation of compounds BAD<sub>2</sub>-BA and BAD<sub>2</sub>-TE. No change in the emission was observed upon irradiation of the solutions in hexane even for longer periods of time (Fig. S6), confirming that the dyads do not generate O<sub>2</sub>, in the absence of the PeT process.

Scheme 2. Tentative mechanism of the formation of fluorescent products.

The formation of bicyclic acetal and tetraepoxide products from BAD<sub>2</sub> is likely to take place via an 9,10-endoperoxide intermediate (Scheme 2). The rearrangement of endoperoxides into bisepoxides can be induced either thermally or photochemically. The process is caused by the homolytic cleavage of the peroxide O-O bond, followed by rearrangement to more stable bisepoxides. Commonly such bisepoxides, containing a cyclohexadiene ring, could not be isolated, but only trapped with dienophiles. Indeed, we found no traces of this intermediate in the reaction mixture. According to previous reports, the formation of a bicyclic acetal from bisepoxide may take place via heterolytic cleavage of the epoxide C-C bond, leading to an ylide-type bipolar intermediate. This is then followed by C-O bond rupture of a second epoxide fragment, leading to rearrangement of the lateral ring and formation of the acetal bridge. The rearrangement competes with addition of O<sub>2</sub> molecule to the diene moiety leading to BAD<sub>2</sub>-TE.

In contrast, the bisepoxide BAD<sub>1</sub>-BE is stable and showed no formation of the rearrangement products. Its formation likely proceeds via the mechanism discussed above, involving O-O homolytic cleavage and further isomerization. The addition of O<sub>2</sub> to the outer ring in this case is surprising, as the central 9,10-site is the most reactive, based on frontier molecular orbital analysis. The influence of steric factors on the regioselectivity of endoperoxide formation has previously been reported for acenes with bulky substituents at the ortho-positions of the aryl groups. Comparison with BAD<sub>2</sub> shows that the unusual reactivity of BAD<sub>1</sub> can be attributed to the effect of methyl substituents in position 4 of the BODIPY core. This can be seen in the XRD data where C-4 methyl substituents in BAD<sub>1</sub> are forming a steric like shield of the C-9 position of the anthracene unit. Introduction of methyl groups into the BODIPY pyrrole rings shields the inner ring of the orthogonal anthracene residue, making the approach of O<sub>2</sub> molecule difficult. Different reactivity of BADs towards O<sub>2</sub> accounts for the variations in their fluorescence response (Figure 3b) due to the cycloaddition to the anthracene moiety, which takes place considerably faster for BAD<sub>2</sub>.

The rise of BAD<sub>2</sub> fluorescence due to cycloaddition reaction is manifested even at 1 μM concentration, and it reaches the intensities comparable to those of the emission of a strongly fluorescent reference BODIPY compound (Fig. S7). It was of special interest for us to investigate whether the sensitization process can be reproduced in live cells. For this purpose we generated appropriate water-soluble derivatives. Substitution of fluorine atoms with N,N-dimethylaminopropylene-1-residues gave corresponding BADs 3 and 4. Quaternization of the dimethylamino group with 1,3-propanesultone then gave BADs 5 and 6, bearing zwitterionic fragments (betaine) which imparted the desired aqueous solubility.

Scheme 3. Synthesis of water-soluble BADs derivatives.

To examine the fluorescence response of BADs 5 and 6 towards self-sensitized O<sub>2</sub> in cells, human breast cancer (MDA-MB-468) cells were incubated with BADs 5 and 6 (1 μM) followed by irradiation with broadband visible light (400 – 700 nm, 23.8 mWcm<sup>-2</sup>). Cells were irradiated for 0, 2.5 and 5 minutes and were visualised by confocal fluorescence microscopy. Over the time course of irradiation an increase in the fluorescence intensity was observed for BAD6 (Figure 4) indi-
cating firstly, that the chromophore had entered the cells, rather than simply associating with the external cell membrane; and secondly, that the fluorescence increased in a similar way to that observed for BAD2 in homogeneous solution. However, this behavior was not replicated in the case of BAD5, which showed no observable fluorescence on this timescale, even when irradiated with the higher light doses. Lower fluorogenicity of BAD5 is in accord with the behaviour of parent BAD1, which was shown to react with 'O₂ considerably slower than BAD2.

Figure 4. Confocal microscopy images of cells incubated with 1 µM of BAD6 after the irradiation with broadband visible light (400 – 700 nm, 23.8 mWcm⁻²) for (a) 2.5 min and (b) 5 min.

At higher concentrations of BADs evidence of morphological changes to the cells upon irradiation, most noticeably “blebbing” of the cell membrane, was observed (Fig. S2z), indicating apoptotic behaviour. Cells viability after incubation with a range of concentrations (1 – 50 µM) of BADs 5 and 6, followed by light treatment (23.8 mWcm⁻²), were assayed by MTT protocol. The results obtained indicate that both water-soluble BADs induce a significant cytotoxic effect on the cells, whereas negligible cytotoxic effects were observed in the control group under otherwise identical conditions, but without irradiation (Fig. Syz). Median lethal doses (LD₅₀) of BADs were found to be 4 µM, thus the lower dose of 1 µM was selected for imaging experiments.

In conclusion, we have demonstrated that heavy atom-free-donor-acceptor dyads can be used as 'O₂ sensitizers, whereby the triplet excited states playing the key role in oxygen sensitization form by way of photoinduced electron transfer. Moreover, the described dyads are capable of forming strongly fluorescent species with self-sensitized 'O₂ in biological media. The fluorescent response allows visualization of 'O₂ formation within the cells and, consequently, fine-tuning of the photon doses required to cause oxidative stress. These sensitizers may give rise to a promising new class of materials for photonic applications which depend on triplet excited states generation. Studies to extend the scope such systems are underway.

ASSOCIATED CONTENT

Supporting Information
Synthetic procedures, NMR, optical spectra, computational details, X-ray crystallographic data for BAD1, BAD-BE, BAD2-BA, BAD2-TE in CIF format, fluorescence microscopy images and cytotoxicity assay protocols. This material is available free of charge via the Internet at http://pubs.acs.org.

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