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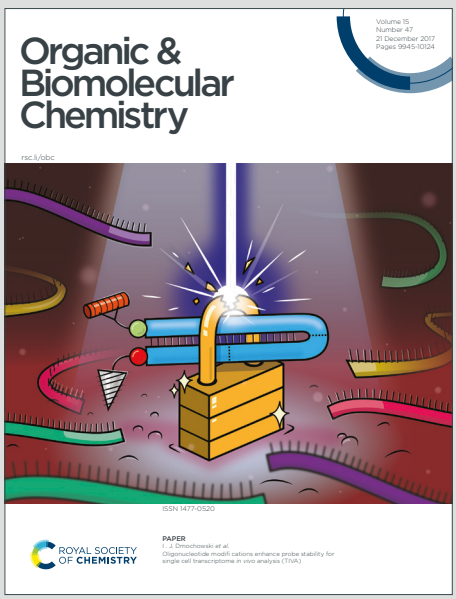

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COMMUNICATION

Self-Reporting Heavy Atom-Free Photodynamic Therapy Agents

Gulsen Turkoglu,[a,b] Gozde Kayadibi Koygun, [c] Mediha Nur Zafer Yurt[a], Naime Demirok[d] and Sundus Erbas-Cakmak [a,b]*

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Two novel, self-reporting distyryl BODIPY-based photodynamic therapy agents functionalized with singlet oxygen responsive imidazole and tertiary amine moieties are developed. Heavy atom-free photosensitizers are demonstrated to have efficient photodynamic action in MCF7 cells. Fluorescence intensity of the photosensitizers are shown to be reduced as a result of $^1O_2$ generation without any significant change in photodynamic activity.

Cancer is one of the leading causes of death and number of annual deaths worldwide approaches to 10 million people.[1] Photodynamic therapy (PDT) provides a non-invasive alternative for the treatment of certain cancers.[2] This therapy requires a photosensitizer (PS) that produce efficient singlet oxygen upon excitation. Local light exposure enables side specific activation of photosensitizer providing a remote spatiotemporal control over the therapy. Singlet oxygen generation is mediated by photoexcitation, triplet transition and energy transfer to molecular oxygen. So, unless singlet oxygen is trapped in the form of endoperoxide or similar structures, oxygen, light, and photosensitizer are necessary elements of this therapy.[3-5]

Even though therapeutics are designed to act on target cells, they may be cleared out quickly or may not be active enough to induce the desired biological outcome. On the other hand, excessive light exposure or PS load would give damage to healthy cells.[6] Therefore, it is necessary to follow photodynamic action, cellular response of the therapy, or drug accumulation. In recent years, activity monitoring of the therapeutics has provided a new approach to follow the outcome of the therapy.[7] In PDT, common cellular response of PS is oxidative damage and apoptosis. Hence, activity reporting is achieved by following apoptosis or a spectroscopic change upon singlet oxygen dependent reactions. Tang et al. reported the migration of cationic PSs from mitochondria to nucleus upon apoptosis-induced change in mitochondrial membrane potential, thereby apoptotic cells can easily be distinguished by using the location of fluorecence.[8] Akkaya group explored a similar strategy and emission is shown to be enhanced when PSs interacts with phosphotidyl serine lipids exposed upon PDT induced apoptosis.[9] In a study, metal-organic frameworks are decorated with PSs and fluorescent dyes, and caspase 3 activation by apoptosis is shown to release the emissive fluorophore.[10] Intrinsic chemiluminescence of $^1O_2$ is also used as a direct measure of PDT action.[11] Wang group reported a self-reporting therapeutic nanoplatform using $^1O_2$ mediated oxidative cleavage of the fluorophore.[12] We have reported a similar activity monitoring using cleavage of the $^1O_2$ labile linker (Z)-1,2-bis(alkylthio)ethane by means of $^1O_2$ which separates energy transfer donor acceptor units and enables detection of $^1O_2$ produced by PDT.[13,14] In a study reported by our group, singlet oxygen-initiated cascade reactions are shown to convert the photosensitizer and lead to a decrease the activity through a feedback reactions series meanwhile increasing the emission.[15]

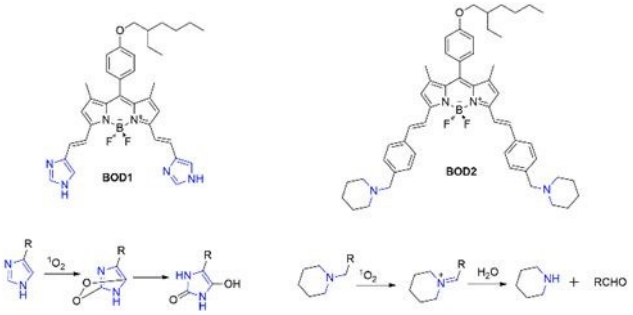

Figure 1. Structure of heavy-atom-free, activity reporting photosensitizers **BOD1** and **BOD2** (top). Reaction of imidazole ring and amines with $^1O_2$ (bottom).

[a] *Research and Development Center for Diagnostic Kits (KIT-ARGEM) Konya Food and Agriculture University, 42080, Konya, Turkey.*
[b] *Konya Food and Agriculture University, Department of Molecular Biology and Genetics, 42080, Konya, Turkey.*
[c] *Selçuk University, Department of Nanotechnology and Advanced Materials, 42250, Konya, Turkey.*
[d] *Üsküdar University, Department of Molecular Biology and Genetics,34662, İstanbul, Turkey.*

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In this work, we designed and synthesised a unimolecular activity reporting, Near-IR photosensitizer (Figure 1). In order to generate $^1O_2$, most photosensitizers rely on heavy atoms to facilitate intersystem crossing to triplet state apart from a few examples having special geometry or charge-transfer nature.[15-18] Due to potential dark toxicity of heavy atom, we developed halogen-free PSs using distyryl 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene dyes (BODIPY) **BOD1** and **BOD2** functionalized with imidazole or tertiary amines, respectively. These functional groups are attached with additional intension to have singlet oxygen responsiveness since both groups are known to be oxidized by $^1O_2$.[19-21] We propose that oxidation would change the molecules emission due to distortion of conjugation and/or $^1O_2$ mediated chemical conversion of the structure. İmidazole and histidine amino acid with an imidazole side chain are reported to produce trans-anular peroxide species as it reacts with $^1O_2$.[19,22] With this in mind, we synthesised **BOD1**. Upon generation of oxidized imidazole photophysical properties of the photosensitizer is expected to change (Figure 1). **BOD2** bears tertiary amines in the structures. Amines are also proved to be oxidized by $^1O_2$ leading to formation of imine. We proposed that, in aqueous solution imine can readily be hydrolysed and piperidine can be released generating an aldehyde on the parent PS. Being an electron withdrawing group, newly generated aldehyde is expected to change fluorescence properties of the distyryl BODIPY.

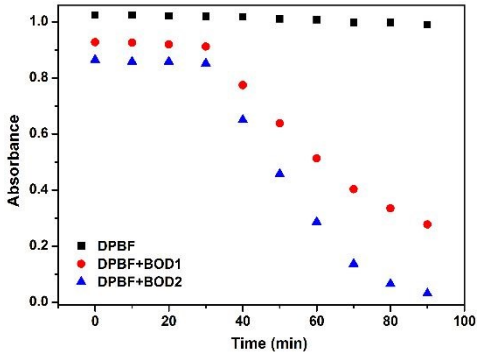

Figure 2. Change in the absorption of DPBF (50 μM) at 411 nm upon $^1O_2$ generation by photosensitizers **BOD1** (2μM) and **BOD2** (2 μM) in 1% H_2O in acetonitrile. Samples are kept in dark for 30 min and later irradiated with 637 nm LED light.

BODIPY compounds have easily accessible chemistry and they are widely used for photodynamic therapy, molecular logic gates, molecular sensors and various other applications.[23-30] Because of their chemical stability, high quantum yields and versatile chemistry we used BODIPY dye as a core photosensitizer. We prepared both PSs in 3 steps (Scheme S1). Alkyl moiety at the meso position of the BODIPY is attached to enhance lipid solubility of the molecule which would assist membrane permeability. $^1O_2$ -reactive imidazole is attached to the BODIPY core by means of Knoevenagel condensation reaction using 4-imidazole aldehyde. Tertiary amine bearing **BOD2** is synthesised from compound 3 and 4-bromomethyl benzaldehyde. Both Knoevenagel condensation and substitution with benzyl bromide take place in one pot in good yields.

For any type of photo-therapy, including photodynamic therapy, light penetration depth is a concern. Tissues are composed of light absorbing materials and to have sufficient tissue penetration photo-therapeutics needs to be activated at therapeutic window of the body, which lies at Near-IR region of the spectrum.[31] Spectral properties of photosensitizers **BOD1** and **BOD2** meet this requirement since they have absorption at 627/625 nm and emissions at 640/635 nm respectively (Figure S1).

To demonstrate singlet oxygen generation by halogen-free **BOD1** and **BOD2**, diphenyl 1,3-isobenzofuran (DPBF) $^1O_2$ trap is used.[32] Absorbance of DPBF at 411 nm decreases as the molecule reacts with generated $^1O_2$. **BOD1** and **BOD2** is either kept in dark (first 30 min) or irradiated with 637 nm light and absorbance of the trap is followed in 10 min intervals. With both molecules at 2 μM concentrations, a significant decrease in absorption is observed upon irradiation compared to PS-free DPBF solution (Figure 2, S2-4). These results clearly demonstrate the singlet oxygen generation by the two molecules and their potential to be used in photodynamic therapy although they lack heavy atoms to facilitate intersystem crossing.

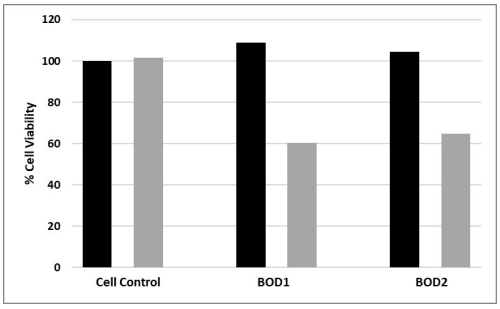

Figure 3. Cell viability assay. MCF7 cells are incubated alone or with **BOD1** (5 μM) and **BOD2** (5 μM) for 24h in dark (black), or irradiated with 637 nm light for 4h and kept in dark for additional 20h (gray).

Photodynamic action of both PSs is analysed in Michigan Cancer Foundation 7 (MCF7) breast cancer cell culture. When cells are kept in dark, no significant change in cell viability is detected when 5 μM of each PSs is used demonstrating the absence of dark toxicity (Figure 3). Upon irradiation with 637 nm Light Emitting Diode (LED) for 4h, cell viability decreases by almost 50% for both photosensitizers proving effective photodynamic action. The half maximal inhibitory concentration (IC50) of **BOD1** and **BOD2** for MCF7 cells is determined to be 197 μM and 178 μM, respectively.

Molecules are proposed to be responsive to $^1O_2$ and display a change in emission properties in the presence of $^1O_2$. Since $^1O_2$ is generated by the photosensitizer itself, chemical responsiveness of the PSs is expected to be used as an activity report. To show this, molecules are irradiated with 637 nm light

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to generate $^1O_2$ and their emissions are followed. Fluorescence intensity of **BOD1** decreased significantly while **BOD2** displayed little drop in emission intensity (Figure S5). To show that decrease in emission is due to $^1O_2$ generation but not 637 nm light, another iodine-functionalized photosensitizer (**PS-510**) having absorption at 530 is used (Figure S6). Both PSs display significant decrease in emission when samples are irradiated with 505 nm light to excite **PS-530** (Figure 4). When **PS-530** is not added to the solution, emission intensity does not change since 505 nm light cannot excite **BOD1** or **BOD2**. **BOD-1** seems to have a better $^1O_2$ responsiveness as demonstrated by the degree of change in emission even less **PS-530** is used. To further demonstrate singlet oxygen dependent chemical conversion of the PSs, ^{1}H Nuclear Magnetic Resonance (NMR) analysis of **BOD2** is done following irradiation. After 3h of irradiation, peaks resonating at 10.02 ppm corresponding to newly generated aldehyde moiety appear in **BOD2** spectra consistent with the proposed mechanism shown in Figure 1 (Figure S7). High Resolution ESI-MS spectra of the sample obtained after irradiation further supports formation of **formyl-BOD2** (Figure S10). When peak intensities are compared with unshifted pyrrolic peaks of photosensitizer 32% conversion is estimated. As opposed to **BOD2**, irradiation of **BOD1** does not lead to a change in ^{1}H NMR spectrum even the emission is quenched (Figure S8, S9). This result indicates that imidazole ring is not reactive enough towards cycloaddition with $^1O_2$ when it is conjugated to BODIPY core. Hence, decrease in **BOD1** emission is attributed to collisional quenching rather than $^1O_2$ dependent oxidation of imidazole ring.

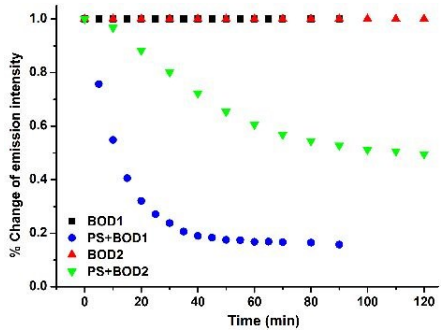

Figure 4. Change in emission intensity of **BOD1** (2 μM) and **BOD2** (2μM) upon exposure to in situ generated $^1O_2$ in 1% water in acetonitrile. $^1O_2$ is generated by irradiation of the sample with 505 nm lamp in the presence of 1 μM (for **BOD1**) or 5 μM (for **BOD2**) **PS-530**. In the absence of **PS-530**, the emission does not change (black, red).

To monitor fluorescence change of PSs upon photodynamic action MCF7 cells are incubated with PSs (5μM) and exposed to light for 4h. As it is clearly seen in Figure 5, emission decreases significantly in 637 nm light-exposed cells compared to control group which are kept in dark for the same period of time.

Photosensitizers are shown to have photodynamic action and a change in fluorescence intensity by the PDT action. An experiment is done to understand if the singlet oxygen generation capability of the PSs is preserved upon chemical conversion of the PSs by $^1O_2$. Photosensitizers are pre-irradiated with light for 1.5h and their singlet oxygen generations are analysed with the use of DPBF as described above. Results shown in Figure 6 indicate that PSs can still have efficient PDT action. Rates of decay of $^1O_2$ trap absorption does not alter significantly in pre-light treated samples compared to the results shown in Figure 2, indicating that fluorescence quenching upon exposure to light is not due to enhancement of triplet transition. Other deactivating pathways would be the major cause of fluorescence quenching.

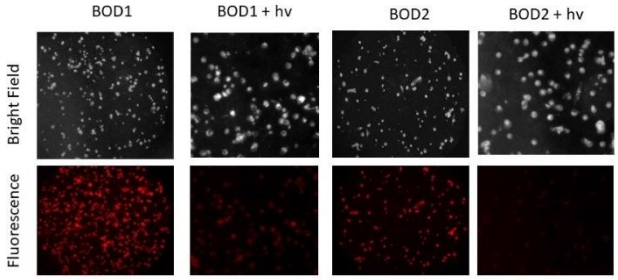

Figure 5. Photodynamic activity-reporting by **BOD1** and **BOD2** in MCF7 cell culture. 5 μM of the PSs are introduced to the cell. Cells are irradiated with 637 nm light for 4h. Singlet oxygen generation resulted in significant decrease in red emission.

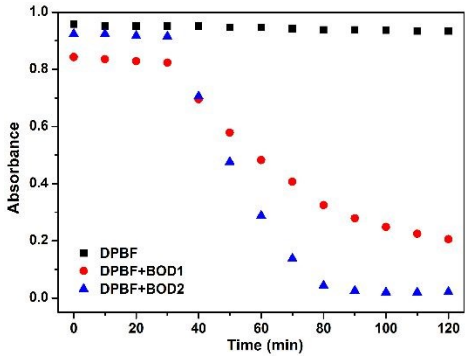

Figure 6. Change in the absorption of DPBF (50 μM) at 411 nm upon $^1O_2$ generation by pre-irradiated photosensitizers **BOD1** (2μM) and **BOD2** (2 μM) in 1% H_2O in acetonitrile. Samples are irradiated with 637 nm light for 1.5h from a 5 cm distance prior to experiment. Then, samples are kept in dark for 30 min and later irradiated with 637 nm LED light.

In this paper two different Near-IR absorbing halogen-free distyryl BODIPY photosensitizers with activity-reporting properties are presented. At micromolar concentrations PSs are shown to produce singlet oxygen efficiently and induce cell death in MCF7 cells. Absence of cytotoxic halogens is of great challenge in PDT agents, since it requires additional design criteria.[17, 18, 33] We have shown that functionalized BODIPYs can serve for this function with in-built $^1O_2$ responsive groups. Heavy atom-free photosensitizers are previously shown to have efficient PDT action when they have similar charge transfer nature or special geometry.[18] Singlet oxygen-mediated

conversion of tertiary amine moieties result in a decrease of emission in the same cell enabling visualization of photodynamic activity. Since the activity reporting depends on $^1O_2$ dependent reactions or interactions, this method provides a direct measure for the PS photodynamic activity. Both PSs are shown to preserve $^1O_2$ generation ability even after 1.5h of irradiation which would enable extension of PDT duration to longer hours if necessary. Self-activity reporting photosensitizers, like the ones reported in this research, would provide a smarter therapeutic approach, and enable better adjustment of PSs dose or light to avoid over damage to healthy cells.

Conflicts of interest

There are no conflicts to declare.

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Notes and references

‡ Author contributions: G.T., M.N.Z.Y., N.D. and S.E.C. synthesised the compounds, G.T. performed the spectroscopic analysis, G.K.K. performed the cell culture experiments, S.E.C. designed the experiments and coordinated the research.

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