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A Study on Liposomal-Encapsulation of New Bodipy Sensitizers for Photodynamic Therapy

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KEYWORDS: Bodipy, photodynamic therapy, liposomes, photosensitizer, photocytotoxicity, cancers

ABSTRACT: We report a series of four efficient photosensitizers (PS) based on Bodipy core for photodynamic therapy (PDT). In the absence of hydrophilic functional groups, these PS's have been encapsulated in liposomes and examined for photocytotoxicity against human ovarian carcinoma cell line (SK-OV-3). The IC_{50} values obtained are as low as 0.350 μM, which compete with the classical photosensitizer Chlorine E6 (IC_{50}= 0.39 μM) under similar experimental conditions.

Photodynamic therapy (PDT) represents a minimally invasive therapeutic approach and emerged as a better clinical modality for treatment of various neoplastic and non-neoplastic diseases.[1-3] PDT requires a photosensitizer (PS), light source of a specific wavelength suitable for the absorption characteristics of the PS and molecular oxygen. This combination leads to the formation of highly cytotoxic singlet oxygen accompanied by the generation of reactive oxygen species (ROS), which cause tumor cell death. The most studied photosensitizers for photodynamic therapy include tetrapyrrole-based compounds (porphyrins, chlorins, and bacteriochlorins) such as porfimer sodium (Photofrin), protoporphyrin IX, and temoporfin etc.[4-6] In general, majority of PS's possess limited solubility in water; hence require various formulations that enhance their bioavailability. These formulations generally include micelles, liposomes, dendrimers and nanoparticles etc.[7-10] Among various drug delivery formulations tested, liposomes were studied widely and several clinical trials using drug-loaded liposomes are in progress.[11,12]

Liposomes are artificially constructed membrane vesicles, composed of phospholipids and other amphiphilic components. They are widely used as vehicles for drug delivery as they can encapsulate hydrophilic drugs within the aqueous regions, and also lipophilic molecules within their lipid bilayers.[13,14] Though, porphyrin based PS's are well known for PDT, there are few drawbacks, which include low molar extinction coefficients (especially in the range of 650–900 nm), involve difficult synthetic routes and their scale up is tedious. Therefore, design and developing PS's with non-porphyrin scaffolds has become important.[15] In this regard, 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (Bodipy) derivatives have attractive photophysical properties along with the ability of generating singlet oxygen with efficient triplet excited life times.[16] Bodipy based photosensitizers for PDT have been reported earlier.[17-23] However, majority of them were linked with polar functional groups or (and) oligoethylene glycol chains to enhance the hydrophilicity and cellular uptake.[17-28] In brief, dominant sector of these Bodipy photosensitizers were designed on the perception of hydrophilicity. On the other hand, photosensitizers with hydrophilic functional groups such as carboxylic acid, sulfonic acid, or sodium sulfonate result in significant reduction of the photocytotoxic activity.[29] Until the present, potent hydrophobic Bodipy based photosensitizers were not explored for PDT. This manuscript describes the liposomal encapsulation of Bodipy analogues and assessing their efficacy in PDT mediated cell death. To the best of our knowledge, this is the first study of Bodipy sensitizers for PDT *via* liposomal encapsulation.

Sensitizers for PDT are required to be designed in such a way that they acquire moderate to high triplet quantum yields in order to obtain long-lived triplet states. High fluorescence quantum yields are actually unwanted for PDT sensitizers as the fluorescence occurs *via* relaxation from singlet excited state, which means the energy absorbed on excitation does not cross to triplet states.[30] Nagano's group investigated that a 2,6-diiodo-analog of Bodipy resulted in enhanced singlet oxygen generation in comparison to the unsubstituted one. The halogen atoms (preferably I or Br) improve singlet-to-triplet intersystem crossing by heavy atom effect.[31] They also remarked that high oxidation potential would be advantageous for the PS to avoid self-oxidation.

Considering all these factors, we investigated four 2,6-diiodo 3,5-symmetric distyryl based Bodipy PS, synthesized from *meso*-*p*-nitrophenyl Bodipy (1) and *meso*-phenyl Bodipy (1a) dyes (Supporting information). These photosensitizers were named as NBDP-SAL, NBDP-NVER, NBDP-VER and PBDP-VER (Figure 1). *meso*-*p*-nitrophenyl Bodipy (1) and *meso*-phenyl Bodipy (1a) dyes were prepared using the classic one pot procedure with 2,4-dimethylpyrrole followed by the oxidation with DDQ to form dipyrrin, which in turn complexed with $BF_3.Et_2O$ in presence of base (Scheme S1, Supporting information).[32] To facilitate the intersystem crossing, 2 and 6 positions of Bodipy core were iodinated using N-Iodosuccinimide. A 2-fold Knoevenagel reaction of 3 and 5 methyl substituents of the Bodipy core[33] with aryl carboxaldehyde using catalytic amount of piperidine and AcOH in refluxing dry benzene resulted corresponding symmetrical derivatives in 35-50% yield (Scheme S2 and S3, Supporting information). All the compounds were characterized by NMR (^1H, ^{13}C) analysis and MALDI-TOF. The complete synthetic procedures along with characterization spectra are included in supporting information.

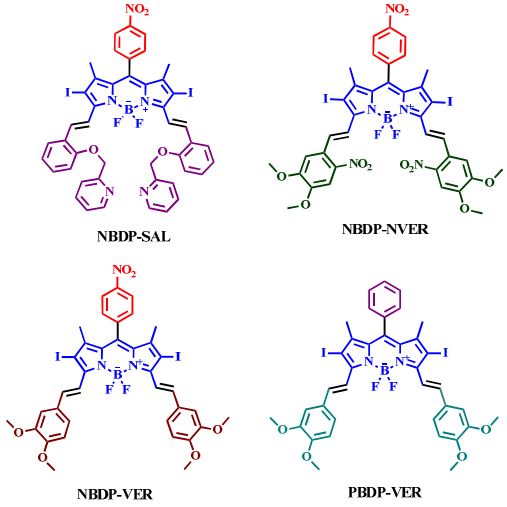

Figure 1. Molecular structures of Bodipy sensitizers for PDT

Electronic absorbance and fluorescence emission spectra of the Bodipy PS's were recorded in dimethyl sulfoxide (DMSO) (Figure 2). All of them exhibit moderate Stokes shifts ranging from 31-55 nm and possess higher extinction coefficients (Table 1). The photostability of Bodipy PS's was tested by continuous irradiation of light (200W xenon lamp) for 2 hours. The absorbance spectra measured before and after irradiation confirm that these PS are highly photostable (Figure S1, Supporting information).

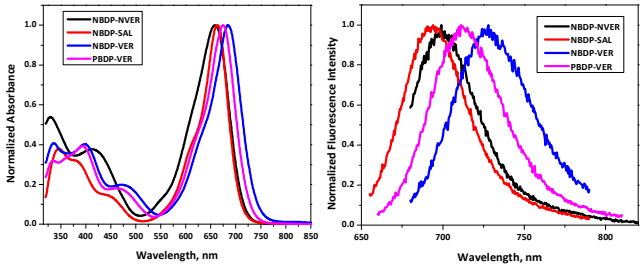

Figure 2. Normalized absorbance and emission spectra of Bodipy Bodipy PS's in DMSO

Since the core iodinated Bodipy sensitizers are expected to present good triplet quantum yields owing to heavy atom effect, nanosecond laser flash photolysis studies were carried out to understand the transient intermediates involved under laser irradiation. All the samples showed good triplet absorption, under 355 nm upon laser excitation (Figure S2, Supporting information). Figure 3 shows the transient absorption spectrum of NBDP-NVER in DMSO, which shows maxima at 380, 480 and 760 nm with bleach around 660 nm corresponding to ground state absorption. The transient intermediate from the sample, NBDP-NVER decayed by a first-order process with a lifetime of 0.76 μs, shown in inset of Figure 3. The triplet excited state quantum yields (Φ_T) of the compounds were determined by an earlier reported procedure of energy transfer to β-carotene, using $Ru(bpy)_3^{2+}$ as the reference molecule.[34] The data are summarized in Table 1 (A detailed description is given in supporting information). Excellent triplet state quantum yield of 0.90±0.03 for NBDP-NVER and moderate yields for other Bodipy PS's were obtained.

Figure 3. Transient absorption spectra of NBDP-NVER, excited at 355 nm LASER. Triplet decay at 480 nm is shown in inset.

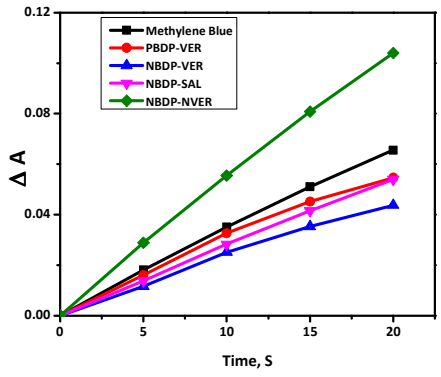

Figure 4a. Plot of ΔA of DPBF at 410 nm vs. irradiation time with Bodipy PS's along with methylene blue as the standard.

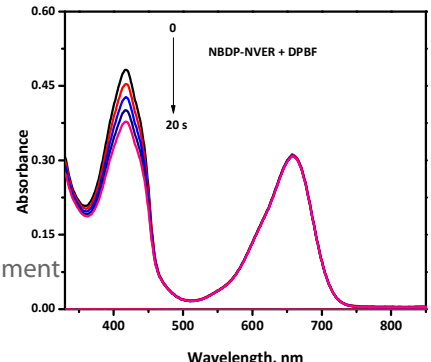

Figure 4b. Decrease in absorption of DPBF in presence of NBDP-NVER by photo-irradiation

The ability of these compounds in generating singlet oxygen can be determined by the rate of decay of 1,3-diphenylisobenzofuran (DPBF), which is a singlet-oxygen quencher. The singlet oxygen generation quantum yields (Φ $^1O_2$) were measured in DMSO using methylene blue as standard (Figure 4a). All the Bodipy PS's demonstrated good singlet oxygen generation efficiencies, with the highest of 0.80 obtained for NBDP-NVER (Figure 4b & Table 1). The generation of singlet oxygen is further confirmed from the emission of samples at 1273 nm, which is characteristic luminescence of singlet oxygen[35] (Figure S3, Supporting information). All samples exhibited considerable singlet oxygen luminescence by exciting at 600 nm.

Table 1. The photophysical properties of Bodipy PS's in DMSO

PS	Λ_{max} Abs[a] nm	Λ_{max} Ems[b] nm	Stokes shift[c]	ε[d] ($M^{-1}\cdot cm^{-1}$)	Singlet Oxygen Q.Y[e]	Triplet Quantum Yield[f]	Triplet Lifetime μs[g]
PBDP-VER	675	730	55	119197.21	0.46	0.490	1.07
NBDP-VER	684	725	41	120917.41	0.37	0.39	0.80
NBDP-NVER	657	700	43	92822.28	0.80	0.903	0.76
NBDP-SAL	664	695	31	82364.86	0.40	0.411	1.20

a) Absorbance maxima b) Emission maxima c) Difference between absorbance and emission maxima d) Molar extinction coefficients at the absorbance maxima e) Singlet oxygen quantum yields were measured using methylene blue standard f) Triplet excited state yields were determined by energy transfer to β-carotene using $Ru(bpy)_3^{2+}$ as the reference molecule g) Triplet Lifetime of the photosensitizers calculated using $Ru(bpy)_3^{2+}$ standard (0.61 μs)

Liposomes were prepared by reconstituting chloroform stocks of egg L-α-phosphatidylcholine (EPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and with PS at a mole ratio of 70:25:5 (Detailed procedure included in Supporting information). To estimate the liposome associated PS, the PS loaded liposomes were dissolved in DMSO. The observed absorbance, determined for each PS, was used to calculate the PS concentration from the standard graphs constructed with each pure PS in DMSO.

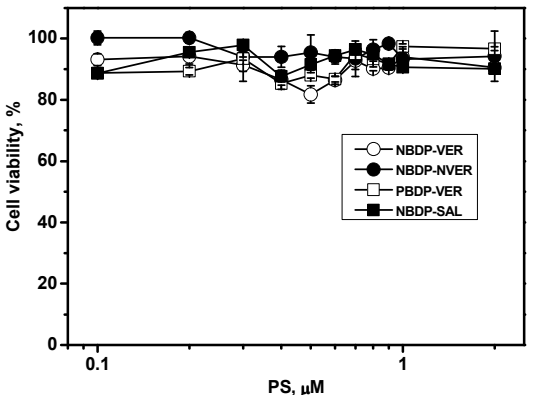

Figure 5. Cell viability of SK-OV-3 cells in the presence of free PS and light treatment. Cell viability was estimated by MTT assay using the dark plate as control plate. Cell death in the presence of PS and absence of light was negligible.

Initially, we have conducted an experiment with variation of light dosages and their effect in the presence of PS. Based on these experiments 20 Jcm^{-2} was selected as the optimal dose for PDT treatment. Light-mediated cell death was investigated with these Bodipy PS's on SK-OV-3 cells. In the PDT studies, each of the light treatments was performed in triplicate for all the four Bodipy photosensitizers in three sets. First set was subjected to light exposure which was treated with free PS (without using liposomal encapsulation) (Figure 5), where as second set was treated with liposome encapsulated PS under light exposure (Figure 6) and the third set was with liposomal encapsulated PS protected even from stray light and was treated as a dark control (Figure 7).

Light mediated cell death in the presence of sensitizers was insignificant for the first set *i.e.,* with free PS (Figure 5). The IC_{50} value (IC_{50} is concentration of PS required for killing 50% of cancerous cells) could not be determined for free PS in the tested concentration range. Whereas the cell death was very pronounced for the second set, in which cells were treated with liposome-encapsulated PS (Figure 6). It can also be seen that all of them are essentially non-cytotoxic in the absence of light (Figure 7). The IC_{50} of these photosensitizers in liposomal formulations (shown in inset of Figure 6) were comparable to that of the classical PS Chlorine E6 (IC_{50}= 0.39 μM) under the same experimental conditions. Of all the Bodipy PS's in the series, NBDP-VER exhibits the lowest IC_{50} of 0.35 μM.

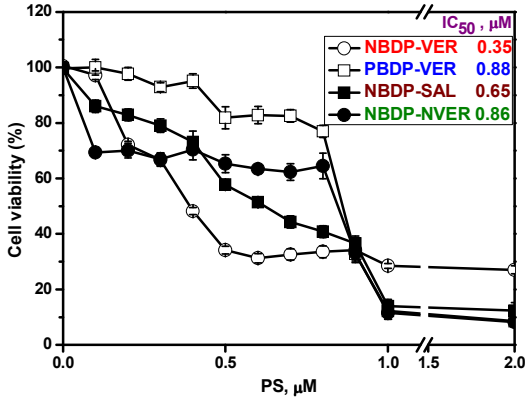

Figure 6. Cell viability of SK-OV-3 cells in the presence of various PS in liposomal formulations under light exposure

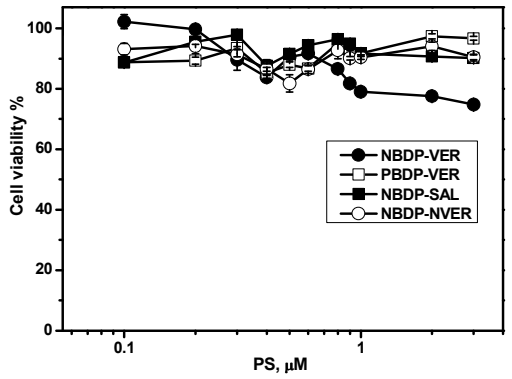

Figure 7. Cell viability of SK-OV-3 cells in the presence of various PS in liposomal formulations and without light treatment.

In conclusion, we have developed a new series of Bodipy based PS and evaluated their *in vitro* photodynamic activity using liposomal encapsulation. PDT efficacy and photophysical characteristics of these compounds noticeably depend on substituent present on them. Nitro group substitution on the 3,5 styryl moieties resulted in higher singlet oxygen and triplet quantum yields. The photosensitizers reported herein obtained competing IC_{50} values to those of classic PS, endorsing a scope to access advancements in Bodipy modification, without the limitations of hydrophilicity as much as it was focused earlier. This research paves the way for designing and developing potent formulations of Bodipy sensitizers, circumventing the need to incorporate hydrophilic functional groups.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Experimental procedures, characterization for the synthesis of compounds mentioned in Figure 1, liposomal preparation, photobleaching experiments, determination of triplet and singlet oxygen quantum yields and Figures S1−S3 (PDF)

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References

1 Dougherty, T. J.; Gomer, C. J.; Henderson, B. W.; Jori, G.; Kessel, D.; Korbelik, M.; Moan, J.; Peng, Q. Photodynamic therapy. *J. Natl. Cancer Inst.* **1998**, *90*, 889-905.

2 Hopper, C.; Photodynamic therapy: a clinical reality in the treatment of cancer. *The Lancet Oncol*. **2000**, *1*, 212-219.

3 Biel, M. A. Photodynamic therapy in head and neck cancer. *Curr. Oncol Rep.* **2002**, *4*, 87-96.

4 Bonnett, R. Photosensitizers of the porphyrin and phthalocyanine series for photodynamic therapy. *Chem. Soc. Rev.* **1995**, *24*, 19-33.

5 Ethirajan, M.; Chen, Y.; Joshi, P.; Pandey. R. K. The role of porphyrin chemistry in tumor imaging and photodynamic therapy. *Chem. Soc. Rev.* **2011**, *40*, 340-362.

6 Nyman, E. S.; Hynninen, P. H. Research advances in the use of tetrapyrrolic photosensitizers for photodynamic therapy. *J. Photochem. Photobiol. B* **2004**, *73*, 1-28.

7 Li, G.; Pandey, S. K.; Graham, A.; Dobhal, M. P.; Mehta, R.; Chen, Y.; Gryshuk, A.; Olson, K. R.; Oseroff, A.; Pandey, R. K. Functionalization of OEP-based benzochlorins to develop carbohydrate-conjugated photosensitizers. Attempt to target beta-galactoside-recognized proteins. *J. Org. Chem.*, **2004**, *69*, 158-172.

8 Derycke, A. S. L.; De Witte, P. A. M. Liposomes for photodynamic therapy. *Adv. Drug Del. Rev.* **2004**, *56*, 17-30.

9 Chen, B.; Pogue, B. W.; Hoopes, P. J.; Hasan, T. Vascular and cellular targeting for photodynamic therapy. *Crit. Rev. Eukaryot Gene Expr*. **2006**, *16*, 279-305.

10 Bai, D.; Xia, X.; Yow, C. M.; Chu, E. S.; Xu, C. Hypocrellin B-encapsulated nanoparticle-mediated rev-caspase-3 gene transfection and photodynamic therapy on tumor cells. *Eur J Pharmacol*., **2011**, *650*, 496-500.

11 https://clinicaltrials.gov/ct2/results?cond=&term=liposomes&cntry1=&state1=&recrs=ab1

12 Dragicevic-Curic, N.; Fahr, A. Liposomes in topical photodynamic therapy. *Expert Opin. Drug Deliv*. **2012**, 9, 1015-1032.

13 Choi, M. J.; Maibach, H. I. Liposomes and niosomes as topical drug delivery systems. *Skin Pharmacol Physiol*. **2005**, *18*, 209-219.

14 El Maghraby, G. M.; Williams, A. C. Vesicular systems for delivering conventional small organic molecules and larger macromolecules to and through human skin. *Expert Opin. Drug Deliv*., **2009**, *6*, 149-163.

15 Wainwright, M. Non-porphyrin photosensitizers in biomedicine. *Chem. Soc. Rev.* **1996**, *25*, 351-359.

16 Awuah, S. G.; You. Y. Boron dipyrromethene (BODIPY)-based photosensitizers for photodynamic therapy, *RSC Adv.,* **2012**, *2*, 11169-11183.

17 Chen, Y.; Zhao, J.; Xie, L.; Guo, H.; Li, Q. Thienyl-substituted BODIPYs with strong visible light-absorption and longlived triplet excited states as organic triplet sensitizers for triplet–triplet annihilation upconversion. *RSC Adv.* **2012**, *2*, 3942-3953.

18 Ozlem, S.; Akkaya, E. U. Thinking outside the silicon box: molecular and logic as an additional layer of selectivity in singlet oxygen generation for photodynamic therapy. *J. Am. Chem. Soc*., **2008**, *131*, 48-49.

19 Lim, S. H.; Thivierge, C.; Sliwinska, P. N.; Han, J.; Bergh, H. V. D.; Wagnieres, G.; Burgess, K.; Lee, H. B. In vitro and in vivo photocytotoxicity of boron dipyrromethene derivatives for photodynamic therapy. *J. Med. Chem.,* **2010**, *53*, 2865-2874.

20 Wu, W.; Guo, H.; Wu, W.; Ji, S.; Zhao, J. Organic triplet sensitizer library derived from a single chromophore (bodipy) with long-lived triplet excited state for triplet–triplet annihilation based upconversion. *J. Org. Chem.* **2011**, *76,* 7056-7064.

21 Umezawa, K.; Matsui, A.; Nakamura, Y.; Citterio, D.; Suzuki, K. Bright, Color-tunable fluorescent dyes in the Vis/NIR region: Establishment of new "tailor-made" multicolor fluorophores based on borondipyrromethene. *Chem. Eur. J*. **2009**, *15*, 1096-1106.

22 Umezawa, K.; Nakamura, Y.; Makino, H.; Citterio, D.; Suzuki, Bright, K. Color-tunable fluorescent dyes in the visible–near-infrared region. *J. Am. Chem. Soc.* **2008**, *130*, 1550-1551.

23 Yang, Y.; Guo, Q.; Chen, H.; Zhou, Z.; Guo, Z.; Shen, Z. Thienopyrrole-expanded BODIPY as a potential NIR photosen-

sitizer for photodynamic therapy. *Chem. Commun.* **2013**, *49*, 3940-3942.

24 Gallagher, W. M.; Allen, L. T.; O'Shea, C.; Kenna, T.; Hall, M.; Gorman, A.; Killoran, J.; O'Shea, D. F. A potent nonporphyrin class of photodynamic therapeutic agent: cellular localisation, cytotoxic potential and influence of hypoxia. *Br. J. Cancer* **2005**, *92*, 1702-1710.

25 He, H.; Lo, P. C.; Yeung, S. L.; Fong, W. P.; Ng, D. K. P. Preparation of unsymmetrical distyryl BODIPY derivatives and effects of the styryl substituents on their *in vitro* photodynamic properties. *Chem. Commun.* **2011**, *47*, 4748-4750.

26 Lai, Y. C.; Su, S. Y.; Chang, C. C. Special reactive oxygen species generation by a highly photostable BODIPY-based photosensitizer for selective photodynamic therapy. *ACS Appl. Mater. Interfaces,* **2013**, *5*, 12935-12943.

27 He, H.; Lo, P. C.; Yeung, S. L.; Fong, W. P.; Ng, D. K. P. Syntesis and in vitro photodynamic activities of pegylated distyryl boron dipyrromethene derivatives. *Med. Chem*. **2011**, *54*, 3097-3102.

28 Atilgan, S.; Ekmekci, Z.; Dogan, A. L.; Guc, D.; Akkaya, E. U. Water soluble distyryl-boradiazaindacenes as efficient photosensitizers for photodynamic therapy. *Chem. Commun.* **2006**, 4398-4400.

29 Lim, S. H.; Thivierge, C.; Sliwinska, P. N.; Han, J.; Bergh, H.; Wagnieres, G.; Burgess, K. H.; Lee, B. In vitro and in vivo photocytotoxicity of boron dipyrromethene derivatives for photodynamic therapy. *J. Med. Chem*. **2010**, *53*, 2865-2874.

30 Kamkaew, A.; Lim, S. H.; Lee, H. B.; Kiew, L. V.; Chung, L. Y.; Burgess, K. BODIPY dyes in photodynamic therapy. *Chem. Soc. Rev*. **2013**, *42*, 77-88.

31 Yogo, T.; Urano, Y.; Ishitsuka, Y.; Maniwa, F.; Nagano, T. Highly efficient and photostable photosensitizer based on BODIPY chromophore, *J. Am. Chem. Soc.,* **2005**, *127*, 12162-12163.

32 Wang, M.; Zhang, Y.; Wang, T.; Wang, C.; Xue, D.; Xiao, J. Story of an Age-Old Reagent: An electrophilic chlorination of arenes and heterocycles by 1-chloro-1,2-benziodoxol-3-one. *Org. Lett.*, **2016**, *18,* 1976.

33 Dost, Z.; Atilgan, S.; Akkaya, E. U. Distyryl-boradiazaindacenes: facile synthesis of novel near IR emitting fluorophores. *Tetrahedron* **2006**, ***62***, 8484-8488.

34 Marydasan, B.; Nair, A. K.; Ramaiah, D. Optimization of triplet excited state and singlet oxygen quantum yields of picolylamine–porphyrin conjugates through zinc insertion. *J. Phys. Chem. B* **2013**, *117*, 13515-13522.

35 Wozniak, M.; Tanfani, F.; Bertoli, E.; Zolese, G.; Antosiewicz, J. A new fluorescence method to detect singlet oxygen inside phospholipid model membranes. *Biochim. Biophys. Acta.* **1991**, *1*, 94-100.

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