Aza-BODIPY Nanomicelles as Versatile Agents for *In Vitro* and *In Vivo* Singlet Oxygen Triggered Apoptosis of Human Breast Cancer

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Experimental Section

General Techniques: The general techniques and spectroscopic equipments employed were described elsewhere. The biological properties of the nanomicelles have been investigated using MCF 7, HeLa and MDA MB 231 cancer cell lines. The photoactivation was done using VINVISH PDT laser (600-720 nm, 200 J/cm^2, 50 mW/cm^2). Flow cytometric analysis was carried out in a BD FACS Aria 2 machine. Gel electrophoresis was carried out using a BIO-RAD Gel DOC XR.

Materials and Methods. Tetramethylrhodaminemethyl ester, hoechst, propidium iodide, FITC-Annexin V, 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Rose Bengal (**RB**) were purchased from S.D. Fine chemicals, India; Sigma-Aldrich, U.S.A; Merck Chemicals, Germany. DSPE was purchased from Avanti polar lipid, USA.CM-H_2DCFDA was purchased from Invitrogen. The aza-BODIPY derivatives, **1-4** were synthesized as reported earlier and characterized using different spectroscopic and analytical techniques. All the solvents used were purified and distilled before use by standard methods.

Preparation of DSPE-BODIPY nanomicelles. The **DSPE-BODIPY** nanomicelles were prepared using the lipid-hydration method. The aza-BODIPY dyes and DSPE-PEG2000-MeO were thoroughly mixed in chloroform in 5 mL round bottom flask and the solvent was removed by vacuum drying. The as formed lipid film were hydrated with phosphate buffer of pH 7.4, and vortexed at room temperature for about 15 minutes.

Transmission Electron Microscopy (TEM). TEM analysis was performed on JEOL 100 kV high resolution transmission electron microscope. The **DSPE-BODIPY** nanoconjugates (3:1, 200 μM) in PBS buffer were drop casted on the top of carbon-coated Cu grid. The samples were dried by a vacuum pump under reduced pressure for 1 h at room temperature. The accelerating

voltage of the transmission electron microscope was 100 kV and the beam current was 65 A. Samples were imaged using a Hamamatsu ORCA CCD camera.

Dynamic Light Scattering (DLS) Analysis. The DLS studies were carried out on a Nano Zeta Sizer, Malvern instruments. The samples were prepared in water/PBS buffer at required concentrations. The light scattering experiments were performed under low polydispersity index by using glass cuvettes. The hydrodynamic diameters and polydisperse indices of the samples were determined using a Malvern Zeta Nano-ZS system.

Determination of Cytotoxicity (IC_{50} value). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is a standard colorimetric assay for measuring cellular proliferation (cell growth). The cancer cells (5 x 10^3 cells per well) were added to wells of two 96 well microliter plate. One for dark cytotoxicity and another for light cytotoxicity with 150 µL Dulbecco's Modified Eagle Medium (DMEM) with 10% serum and incubated for 24 h. Then added 0.625 to 10 µM of the various **DSPE-BODIPY** conjugates in serial dilution (stock 100 mM diluted using DMEM for test) for control and incubated for 24 h and irradiated in one plate using VINVISH PDT lamp (70 W for 15 min) while the other plate was kept in dark. After 24 h of incubation, the plates were removed from the incubator and added 10 µL of MTT (5 mg/mL stock) to each well. After 4 h, the supernatant was removed taking care that the formazan crystals formed were not being removed and added 100 µL of isopropyl alcohol to each well. The plates were covered with aluminium foil and kept on a shaker until crystals were dissolved. The absorbance at 570 nm was monitored and the percentage growth inhibition was calculated using the equation 1,

$$\% \text{ Growth inhibition} = (\text{control-test})/\text{control}) \times 100 \qquad \text{……..(1)}$$

Flow Cytometric Analysis with Annexin V-FITC/PI Assay. After appropriate treatments, the cells were harvested by gentle scraping followed by their resuspension in PBS. Annexin V-FITC/PI staining was done using Invitrogen (USA) kit, following manufacturer's protocol. Annexin V-FITC is used together with propidium iodide (PI), which is one of the commonly used fluorescent probe to distinguish viable cells from dead cells. The former can penetrate through the intact and viable cells, whereas the propidium iodide can only pass through the dead cells. We studied the cell death mechanism induced by PDT using **DSPE-1** by examining the dual fluorescence of Annexin V-FITC/PI using flow cytometry. The cell populations at different phases of cell death, namely, viable (AnnexinV-FITC (negative)/PI(negative)), early apoptotic (Annexin V-FITC(+ve)/PI(-ve)) and necrotic or late-stage apoptotic (Annexin V-FITC(+ve) / PI(+ve)) were examined at different drug doses. The lower left quadrant (Q3) of each panel shows the viable cells, negative for both Annexin V-FITC and PI whereas the lower right quadrants (Q4) represent the apoptotic cells (Annexin V-FITC+/PI-). We observed that most of the cells were negative for Annexin V-FITC and PI after PDT treatment with **DSPE-1** (2 μM) in the absence of light, indicates their negligible dark toxicity toward MDA-MB-231 cells. After PDT, with the **DSPE-1** nanomicelles (2 μM), a significant number of cells were stained positive by Annexin V-FITC, indicating apoptotic mediated cell death.

Tetramethylrhodamine Methyl Ester (TMRM) Assay. Tetramethylrhodamine methyl ester (TMRM) is a cell-permeate cationic, red-orange fluorescent dye that is readily sequestered by active mitochondria. For this assay, we seeded MDA MB 231 cells in a 75 cm^2 flask and incubated for 24 h. Stock solution of the **DSPE-1** nanomicelles was prepared and it is further diluted to two different concentrations such as 1μM and 2μM in order to carry out the

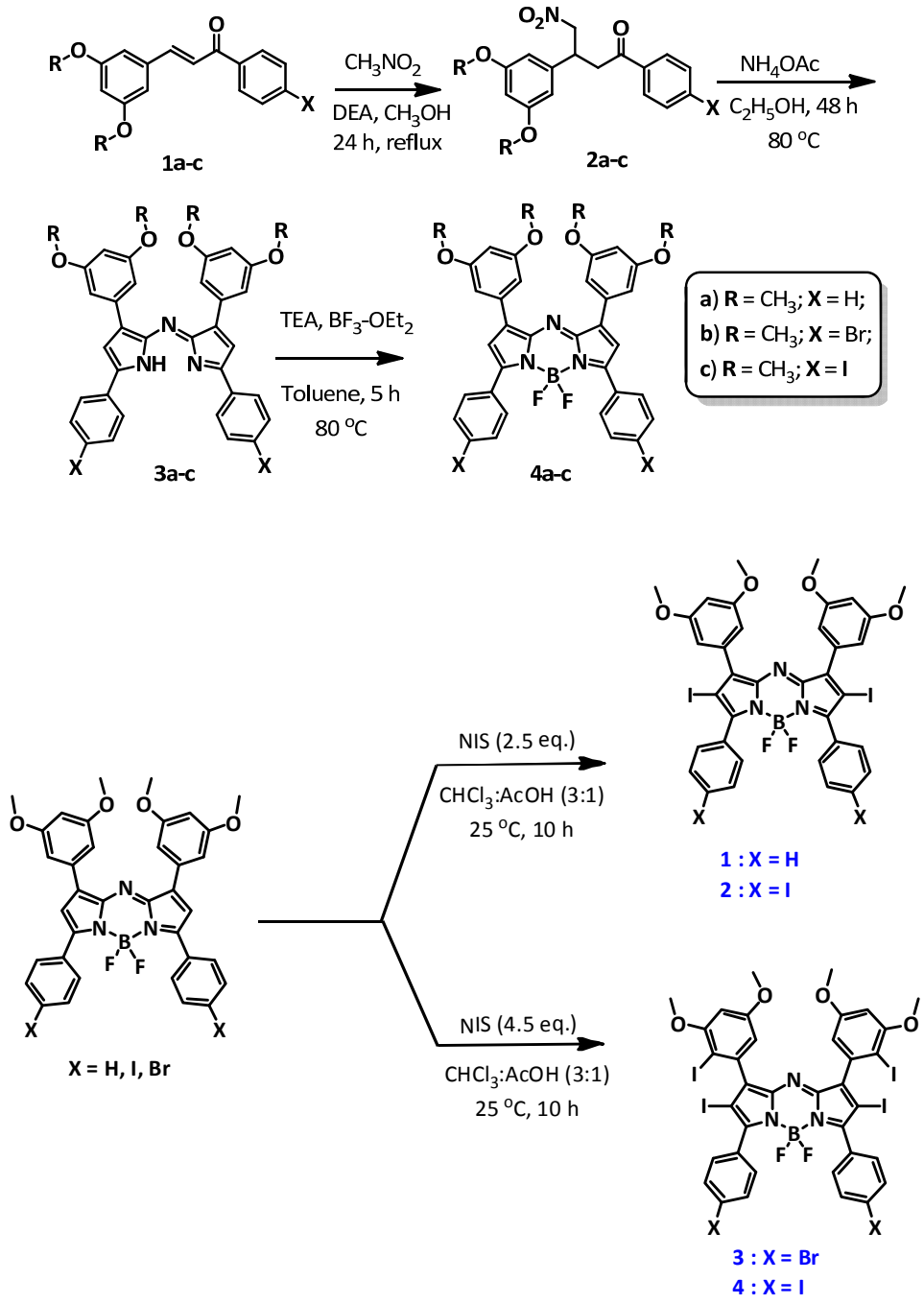
concentration dependent analysis. The nanoconjugate was then injected to the MDA MB 231 cancerous cell lines at two different concentrations and was analyzed using fluorescent microscopy.

Chromatin Condensation Assay by Hoechst Staining. To study chromatin condensation, approximately 10^5 MDA MB 231 cells were seeded in 96 well culture dishes and incubated for 24 h. Then, cells were incubated with 1 μM and 2 μM **DSPE-1** for 24 h followed by photoirradiation using VINVISH PDT lamp. Light and Dark control were taken as previously described. After 24 h of treatment MDA MB 231 cells were rinsed twice with PBS and stained with 5 μg/ml Hoechst dye for 15 min at room temperature. Cells were then washed twice with PBS and visualized under an inverted fluorescence microscope.

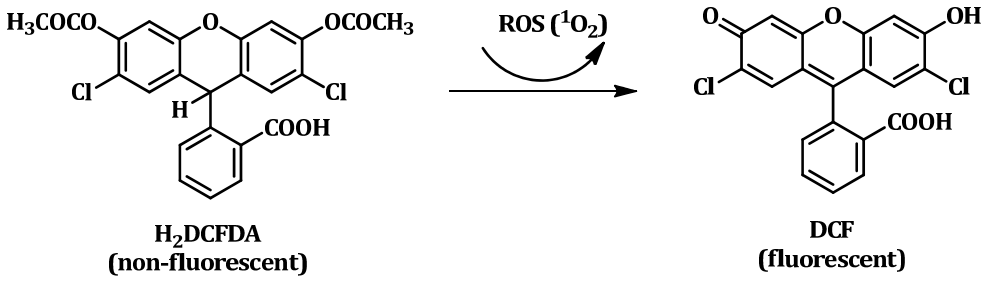
Detection of Reactive Oxygen Species. For the detection of the reactive oxygen species (ROS) produced, approximately 10^6 MDA MB 231 cells were plated in 60 mm and 96 well (BD falcon) plates with serum containing media. After 24 h, the cells were treated with 2 μM of **DSPE-1** for 24 h and irradiation was carried out for 30 min. To one of the plates, **DSPE-1** was added and kept in dark as the dark control. Immediately after PDT, cellular ROS content was determined using CM-H_2DCFDA probe according to the manufacturer's instructions (Invitrogen). Confocal images and differential interference contrast (DIC) images were acquired using Nikon A1R microscope system. Images were merged and processed using Nikon Imaging Software (NIS-Elements AR).

Animal Xenograft Studies. We evaluated the optimum dose of **DSPE-1** using NOD/SCID mice bearing breast tumor xenografts. Female NOD/SCID mice were injected with MDA MB 231 human breast cancer cells in the flank region bilaterally to establish xenograft tumors. After tumors had become palpable, their size was measured by caliper and the animals

were randomized into the following treatment groups (three animals per treatment group): animals were separated into treatment groups of (1) Non-treated-control (2) **DSPE-1**-1 mg/Kg (3) **DSPE-1**- 2 mg/Kg (4) **DSPE-1**- 4 mg/Kg. Animals were injected with the **DSPE-1** in alternative days. For evaluating the PDT using **DSPE-1** conjugate, we adapted the same model, and we used four treatment groups with necessary controls (four animals per group); (1) non-treated control (2) **DSPE** alone - 6 mg/Kg (3) **1** alone - 2 mg/Kg and (4) **DSPE-1**- 2 mg/Kg. After 24 h of injection, the tumor was irradiated with a laser light source with a wavelength of 630 nm. A light dosage of 90 J/cm^2 and fluence rate of 500 mW/cm^2 for 3 min was used for the *in vivo* PDT treatment. The animals were anesthetized with isoflurane during PDT. The **DSPE-1** was injected intraperitoneally to the animals and PDT was done after 24 h. PDT treatment was conducted twice a week. Tumors were allowed to grow to sizes of 5-6 mm in diameter before PDT treatment was carried out and were measured in alternate days for a 33-day period. Animals were sacrificed at day 33. All animal procedures were approved by the Rajiv Gandhi Center for Biotechnology Ethical Committee (IAEC/276/ASN/2015).



Scheme S1. Synthetic strategy adopted for the synthesis of aza-BODIPY derivatives, **1-4**.



H$_2$DCFDA (non-fluorescent) **DCF (fluorescent)**

Scheme S2. Mechanism of CM-H$_2$DCFDA assay: ROS mediated conversion of non fluorescent H$_2$DCFDA to strongly fluorescent (green) dichlorofluorescien (DCF).

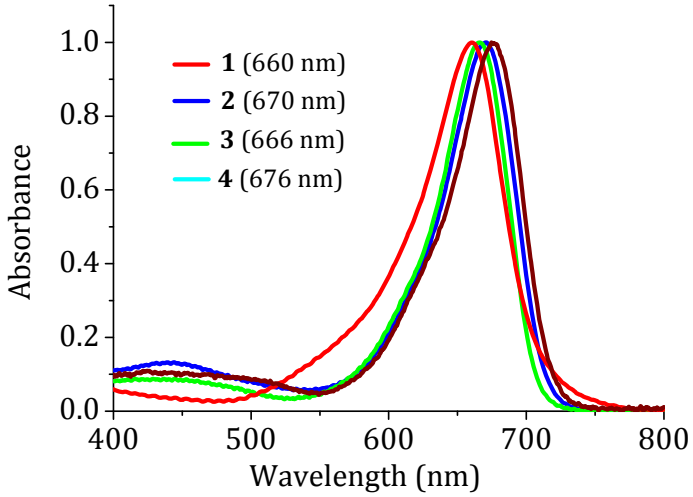


Figure S1. Normalized absorption spectra of aza-BODIPY derivatives **1-4** (2 μM each) in DMSO.

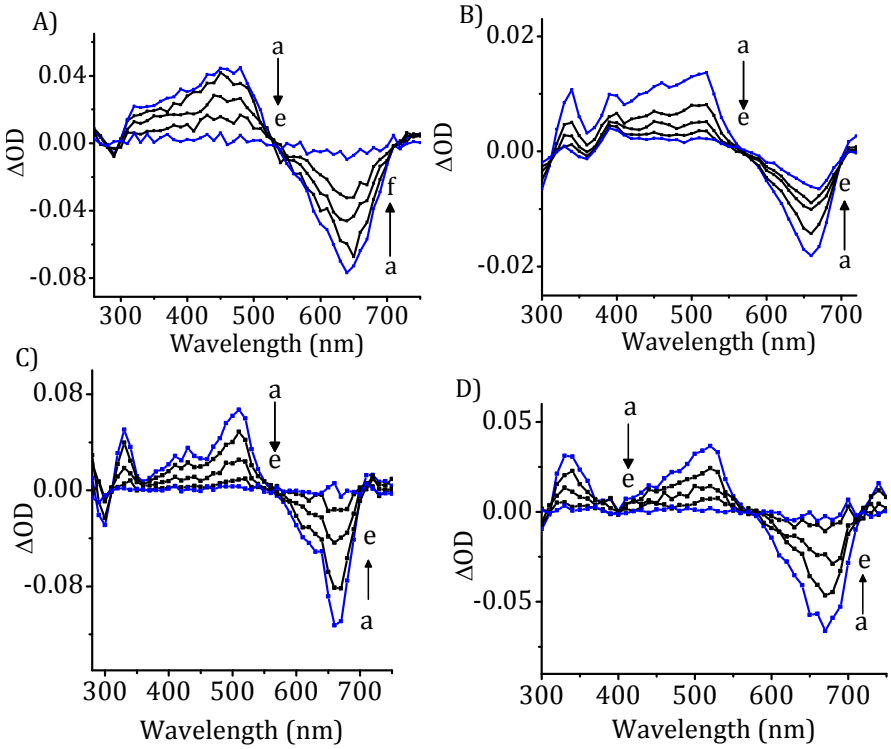


Figure S2. Transient absorption spectra of A) **1** B) **2** C) **3** and D) **4** (10 μM each) following 355 nm laser pulse excitation; time-resolved absorption spectra recorded between 0.1 and 10 μs.

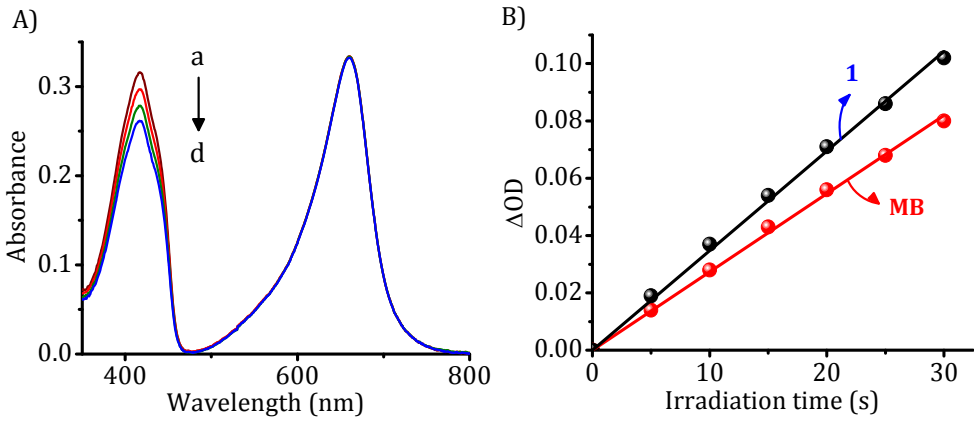


Figure S3. Representative spectra of determination of singlet oxygen through DPBF trap degradation method. A) Absorption spectra of DPBF upon irradiation in the presence of **1** (4 μM) for 15 s, (a) 0 s to (d) 15 s (recorded at 5 s interval). B) Plot of change in absorbance of DPBF at 418 nm *vs* irradiation time (λ_{irr} = 630 nm) in the presence of **1** (4 μM) against methylene blue (**MB**) (3 μM) as the standard in DMSO.

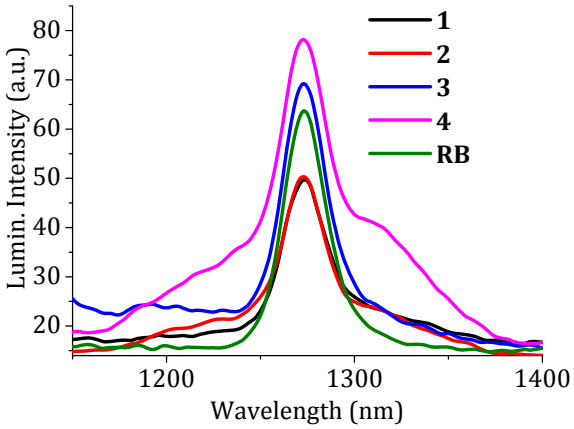


Figure S4. Singlet oxygen luminescence spectra of the aza-BODIPY dyes **1**-**4** (10 μM) and **RB** (10 μM) in acetonitrile. λ_{ex} = 630 nm.

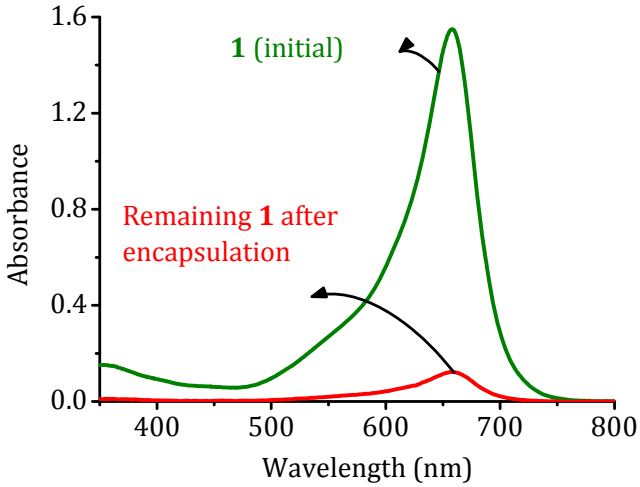


Figure S5. Determination of amount of encapsulation of **1** in DSPE micelles: Changes in the absorption of **1** (1 mM) in chloroform before and after encapsulation.

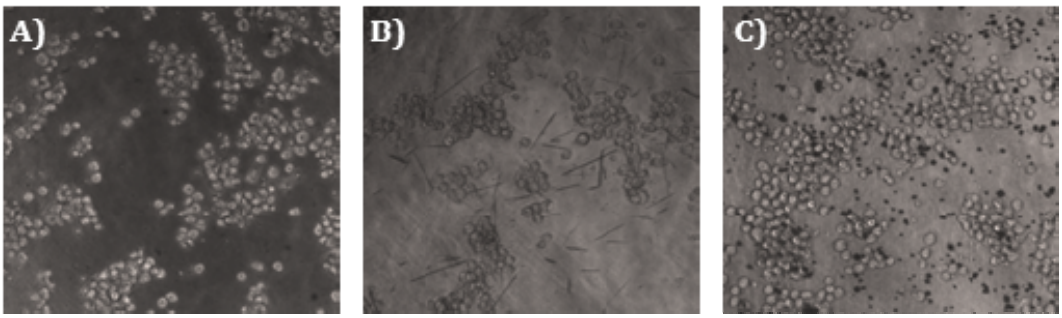


Figure S6. Dark field microscopy images of **DSPE-BODIPY** nanomicells. Panel A corresponds to **DSPE-1**, where the efficient encapsulation happens (ca. 95%), panels B and C corresponds to **DSPE-2** and **DSPE-4**, where partial precipitation/crystallization of the dye occurs.

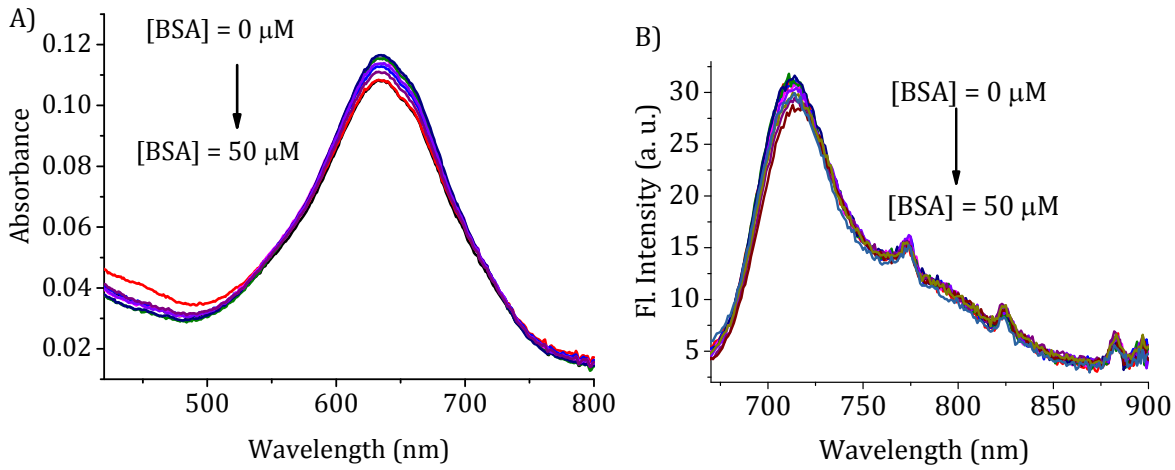


Figure S7. Stability studies of **DSPE-BODIPY** nanomicelles: Changes in the A) absorption and B) fluorescence spectra of **DSPE-1** with increase in concentration of BSA. The negligible changes in the spectra showed that there is no leakage of aza-BODIPY dyes from the DSPE micelles in biological medium.

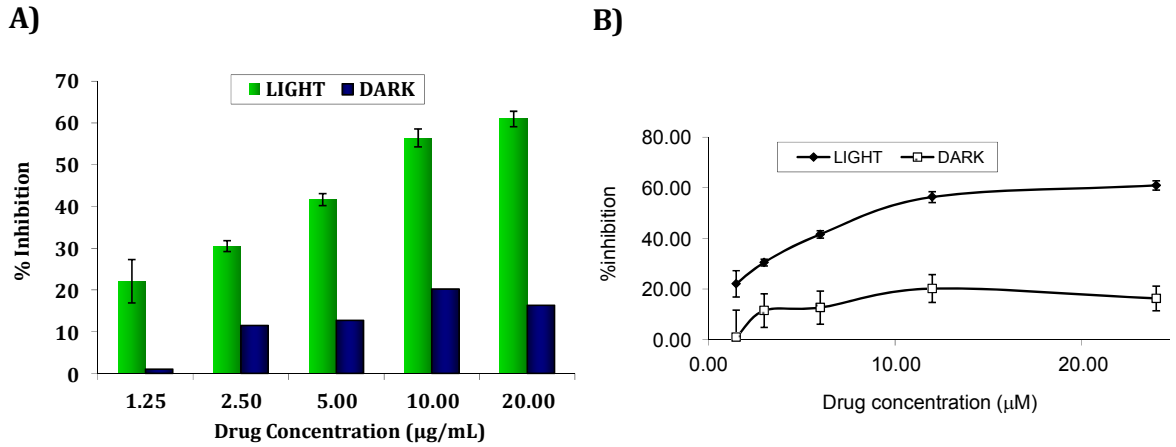


Figure S8. Histogram depicting the cytotoxicity of **DSPE-2** in light (green) and dark (blue) in MDA-MB-231 cells. B) Plot showing the percentage of growth inhibition in presence of **DSPE-2** in MDA-MB-231 cells upon irradiation using VINVISH PDT laser (600-720 nm, 50 mJ/cm^2, 1 h).

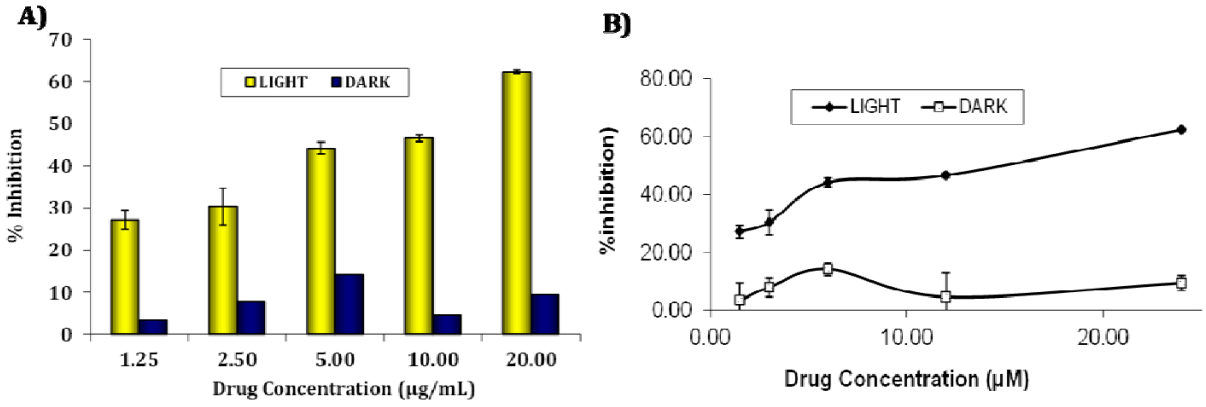


Figure S9. A) Histogram depicting the cytotoxicity of **DSPE-3** in light (yellow) and dark (blue) in MDA-MB-231 cells. B) Plot showing the percentage of growth inhibition in presence of **DSPE-3** in MDA-MB-231 cells upon irradiation using VINVISH PDT laser (600-720 nm, 50 mJ/cm^2, 1 h).

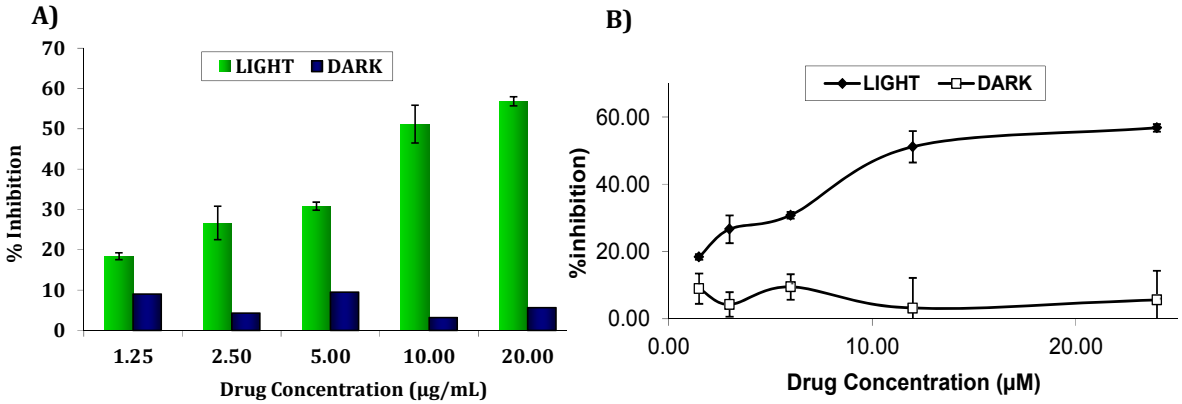


Figure S10. A) Histogram depicting the cytotoxicity of **DSPE-4** in light (green) and dark (blue) in MDA-MB-231 cells. B) Plot showing the percentage of growth inhibition in presence of **DSPE-4** in MDA-MB-231 cells upon irradiation using VINVISH PDT laser (600-720 nm, 50 mJ/cm^2, 1 h).

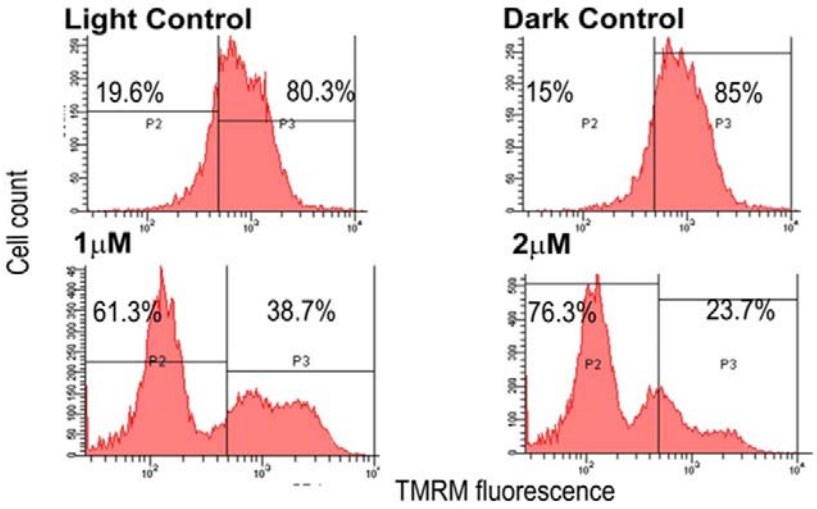


Figure S11. Quantification of TMRM assay with **DSPE- 1** through FACS analysis.

Table S1. Summary of the singlet oxygen generation efficiencies of the aza-BODIPY dyes (**1-4)** through DPBF trap method as well as 1270 nm luminescence.[a]

Compound	λ_{abs}, nm	Φ_Δ[b]	Φ_Δ[c]
1	660	0.65 ± 0.02	0.70 ± 0.04
2	670	0.68 ± 0.03	0.70 ± 0.02
3	666	0.70 ± 0.03	0.80 ± 0.03
4	676	0.80 ± 0.02	0.85 ± 0.04

[a]Average of more than three experiments, [b]singlet oxygen quantum yields through DPBF-trap degradation method with respect to methylene blue (Φ_Δ = 0.52), [c]yields calculated through 1270 nm luminescence with respect to rose bengal (Φ_Δ = 0.79).