

## Fluorene-Morpholine based Organic Nanoparticles: Lysosome-targeted pH-triggered Two-photon Photodynamic Therapy with Fluorescence Switch On-Off

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## 1. General information

**Materials:** All reagents were purchased from Sigma Aldrich and used without further purification. Acetonitrile and dichloromethane were distilled from CaH<sub>2</sub> before use. <sup>1</sup>H NMR spectra were recorded on a BRUKER-AC 200 MHz spectrometer. Chemical shifts are reported in ppm from tetramethylsilane with the solvent resonance as the internal standard (CDCl<sub>3</sub>: 7.26 ppm). Data are reported as follows: chemical shifts, multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet), coupling constant (Hz). <sup>13</sup>C NMR (50 MHz) spectra were recorded on a BRUKER-AC 200 MHz Spectrometer with complete proton decoupling. Chemical shifts are reported in ppm from tetramethylsilane with the solvent resonance as the internal standard (deuteriochloroform: 77.0 ppm). UV/vis absorption spectra were recorded on a Shimadzu UV-2450 UV/vis spectrophotometer, fluorescence emission spectra were recorded on a Hitachi F-7000 fluorescence spectrophotometer, FT-IR spectra were recorded on a Perkin Elmer RXI spectrometer and HRMS spectra were recorded on a JEOL-AccuTOF JMS-T100L mass spectrometer. Photolysis of Fluo-Mor NPs were carried out using 125 W medium pressure Hg lamp supplied by SAIC (India) for one photon (1PE) and 740 nm diode laser for two-photon (2PE). Chromatographic purification was done with 60–120-mesh silica gel (Merck). For reaction monitoring, precoated silica gel 60 F254 TLC sheets (Merck) were used. Cell culture media and all the other materials required for culturing were obtained from Gibco, USA.

## 2. Synthesis of Fluorene-Morpholine (Fluo-Mor) conjugates:

Fluo-Mor (**7**) was synthesized following previous reports starting from commercially available fluorene (**1**) as shown in **scheme 1**. Brief synthetic procedures have been discussed herein.

### 2.1. a) Synthesis of 2-nitrofluorene (**2**)<sup>1</sup>

Two grams (12.03 mmol) of fluorene (**1**) was dissolved in 16.68 mL of warm glacial acetic acid in a two-necked round bottomed flask. The temperature was brought to 50 °C, and 2.8 mL (45.25 mmol) of concentrated HNO<sub>3</sub> was added with stirring in the course of 15 min. During the addition, the solution became yellow, and a small amount of material precipitated. The temperature was then brought to 60–65 °C, when the precipitate dissolved and the colour of the solution deepened. Stirring was continued, and heating continued until the temperature of the mixture reaches 80 °C. After five minutes, heating was removed, and the mixture was allowed to cool to room temperature. The yellow solid product was collected on a Büchner funnel and washed with two 2-mL portions of cold glacial AcOH containing 0.5 g. of potassium acetate. It was then washed several times with water and dried. Compound **2** was obtained in 90% yield. FTIR, UV and <sup>1</sup>H, <sup>13</sup>C NMR values were matched with literature. <sup>1</sup>H NMR (200 MHz, CDCl<sub>3</sub>): 8.41 (s, 1H), 8.33-8.29 (d, 1H, J = 8 Hz), 7.90-7.86 (d, 2H, J = 8.6 Hz), 7.65-7.62 (m, 1H), 7.46-7.43 (d, 1H, J = 7.2 Hz), 4.02 (s, 1H). <sup>13</sup>C NMR (50 MHz, CDCl<sub>3</sub>): 148.08, 146.79, 144.81, 143.92, 139.47, 128.87, 127.43, 125.42, 123.12, 121.33, 120.47, 119.86, 36.96.

### b) Synthesis of 7-iodo-2-nitrofluorene (**3**)<sup>2</sup>

To 2-nitrofluorene (**2**) (2 g, 9.5 mmol) in 30 mL of glacial acetic acid was added iodine (1 g, 3.9 mmol). The solution was stirred at room temperature for 10 min, after which were added 10 mL of conc. H<sub>2</sub>SO<sub>4</sub> and NaNO<sub>2</sub> (0.67 g, 9.7 mmol). The solution was heated under reflux for 30 min and poured onto ice. Compound **3** was obtained as yellow solid in 95% yield and

collected by filtration. FTIR, UV and  $^1\text{H}$ ,  $^{13}\text{C}$  NMR values were matched with literature.  $^1\text{H}$  NMR (200 MHz,  $\text{CDCl}_3$ ): 8.43 (s, 1H), 8.36-8.32 (d, 1H,  $J = 8$  Hz), 8.023 (s, 1H), 8.00-7.809 (m, 2H), 7.66-7.62 (d, 1H,  $J = 8$  Hz), 4.05 (s, 1H).  $^{13}\text{C}$  NMR (50 MHz,  $\text{CDCl}_3$ ): 148.07, 147.13, 144.79, 143.91, 139.43, 139.00, 136.53, 129.51, 128.85, 125.4, 121.87, 94.77, 36.94.

#### **c) Synthesis of 7-cyano-2-nitrofluorene (4)<sup>3</sup>**

2-Nitro-7-iodo-fluorene (**3**) (0.500 g, 1.48 mmol) and CuCN (0.14 g, 1.56 mmol) in dry DMF (5 mL) were refluxed for 1.5 h under  $\text{N}_2$ . Upon cooling, a solution of  $\text{FeCl}_3 \cdot 6 \text{H}_2\text{O}$  (0.593 g) in 36% HCl (0.15 mL) and water (2 mL) was added and the mixture was heated at 60–70 °C for 1 h, and allowed to cool to room temperature with stirring. Compound **4** was precipitated out in ~50 % yield and collected by filtration, then washed with water and 95% ethanol. The FTIR, UV and  $^1\text{H}$ ,  $^{13}\text{C}$  NMR values were similar with that of the previous report.  $^1\text{H}$  NMR (200 MHz,  $\text{CDCl}_3$ ): 8.46 (s, 1H), 8.38-8.33 (d, 1H,  $J = 10$  Hz), 8.00-7.809 (m, 2H), 8.04 (s, 1H), 7.77-7.74 (d, 1H,  $J = 7.6$  Hz), 4.10 (s, 1H).  $^{13}\text{C}$  NMR (50 MHz,  $\text{CDCl}_3$ ): 147.93, 147.20, 145.84, 144.62, 143.69, 134.69, 131.61, 129.69, 129.05, 123.43, 121.89, 118.93, 111.94, 36.94.

#### **d) Synthesis of 7-nitro-fluorene-2-carboxylic acid (5)<sup>3</sup>**

Compound **4** (1 g, 4.22 mmol) was refluxed in an equimolar mixture of 70%  $\text{H}_2\text{SO}_4$  (4 mL) and acetic acid (4 mL) for 48 h. Upon cooling, the yellow compound **5** was precipitated and collected by filtration. Pure compound **5** was obtained after running it through flash column chromatograph (230-400 silica gel) using 20 % EtOAc-pet ether as eluent in 50% yield. The product was confirmed after matching the spectral data with literature.  $^1\text{H}$  NMR (200 MHz,  $\text{CDCl}_3$ ): 8.36 (s, 1H), 8.31-8.28 (d, 1H,  $J = 6$  Hz), 8.18 (s, 1H), 8.04-7.96 (m, 2H), 7.85-7.81 (d, 1H,  $J = 8$  Hz), 4.16 (s, 1H).  $^{13}\text{C}$  NMR (50 MHz,  $\text{CDCl}_3$ ): 170.3, 147.93, 147.20, 145.84, 144.62, 143.69, 134.69, 131.61, 129.69, 129.05, 123.43, 121.89, 118.93, 111.94, 36.94.

#### **e) Synthesis of 2-(7-nitro-fluoren-2-yl)-benzothiazole (6)<sup>3</sup>**

Compound **6** was synthesized by initially refluxing compound **5** (0.400 g, 1.54 mmol) and thionyl chloride (3 mL) for 4 h under N<sub>2</sub>. Excess thionyl chloride was distilled off, and the residue was dried *in vacuo*. The dried residue was mixed with 2-aminobenzenethiol (0.26 mL, 1.54 mmol) in NMP (2.5 mL). The mixture was then heated at 100–110 °C for 15 h. Upon cooling, 95% ethanol was added, and the precipitate was collected by filtration, washed with ethanol and hexane, and dried *in vacuo* to yield compound **6** in 70 % yield. The spectral data were matched with literature. <sup>1</sup>H NMR (200 MHz, CDCl<sub>3</sub>): 8.72 (s, 1H), 8.63(s, 1H), 8.57-8.43(m, 4H), 8.38 (d, 1H, J=8.1 Hz), 8.29 (d, 1H, J=9.0 Hz), 7.77 (t, 1H, J=8.1 Hz), 7.68 (t, 1H, J=7.2 Hz), 3.75 (s, 2H). <sup>13</sup>C NMR (50 MHz, CDCl<sub>3</sub>): 166.5, 152.21, 147.93, 147.20, 142.3, 139.40, 136.08, 134.69, 131.61, 129.69, 129.05, 128.7, 124.93, 123.45, 121.89, 115.98, 114.20, 111.94, 109.3, 36.43.

**f) Synthesis of 4,4'-((2-(benzo[d]thiazol-2-yl)-7-nitro-fluorene-9,9-diyl)bis(ethane-2,1-diyl))dimorpholine (7)<sup>4</sup>**

A mixture of compound **6** (0.050 g, 0.145 mmol), 4-(2-bromoethyl)morpholine (0.028 g, 0.145 mmol), KI (0.003 g, 0.015 mmol), and DMSO (5 mL) was stirred at room temperature, to which powdered KOH (0.034 g, 0.61 mmol) was slowly added under N<sub>2</sub>. The colour of the reaction mixture changed from bright yellow to dark green immediately after KOH addition, and, after 1h, the reaction mixture was poured into water and extracted with hexanes. The organic extract was washed with water, dried over Na<sub>2</sub>SO<sub>4</sub>, and concentrated, affording 0.040 g dark brown crude. Purification was accomplished using flash column chromatography (230-400 silica gel) with 30 % EtOAc/hexanes, providing 0.030 g of dark yellow solid (70 % yield). FTIR (KBr, cm<sup>-1</sup>): 1190, 1438, 1720, 2925. UV-vis (citrate buffer): λ<sub>max</sub> (log ε): 350 (0.55). <sup>1</sup>H NMR (200 MHz, CDCl<sub>3</sub>): 8.43 (s, 1H), 8.36-8.32 (d, 1H, J = 8.4 Hz), 8.31-8.27 (d, 1H, J = 8 Hz), 8.023 (s, 1H), 8.00-7.85 (m, 3H), 7.66-7.62 (d, 1H, J = 8 Hz), 7.42-7.030 (m, 2H), 4.05-4.00 (t, 8H, J = 5 Hz), 2.51-2.49 (t, 8H, J = 1.2 Hz), 2.21-2.18 (t, 4H, J = 2.2 Hz),

1.65-1.64 (t, 4H,  $J = 1.8$  Hz).  $^{13}\text{C}$  NMR (50 MHz,  $\text{CDCl}_3$ ): 166.5, 152.21, 147.93, 147.20, 142.3, 139.40, 136.08, 134.69, 131.61, 129.69, 129.05, 124.93, 123.45, 115.98, 114.20, 111.94, 109.3, 67.61, 63.11, 51.31, 43.37, 40.07. For  $\text{C}_{32}\text{H}_{34}\text{N}_4\text{O}_4\text{S}$   $[\text{MH}^+] = 571.2301$ , found 571.2303.

## 2.2 Characterization of Fluo-Mor Conjugate

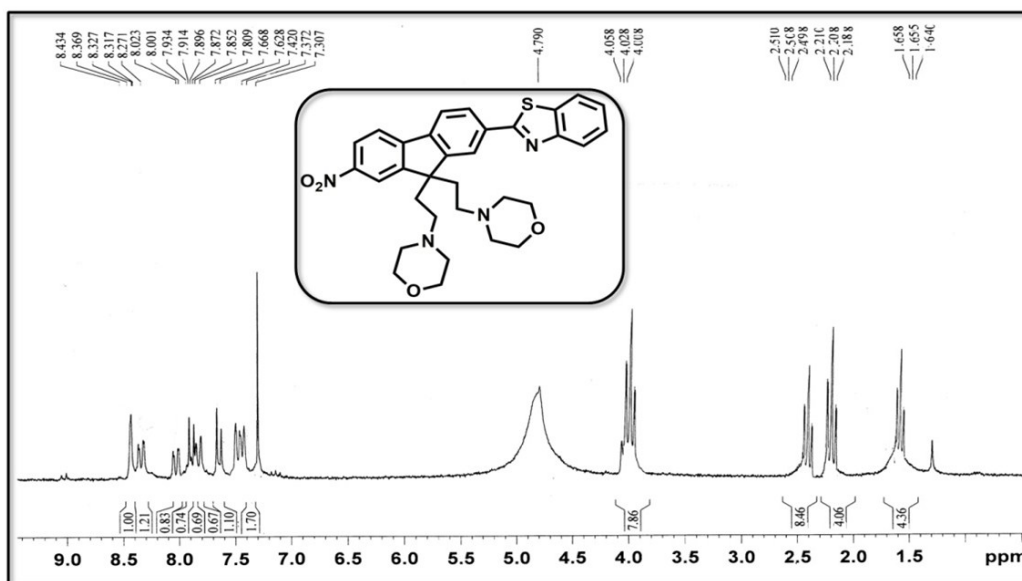


Figure S1  $^1\text{H}$  NMR of Fluo-Mor (7)

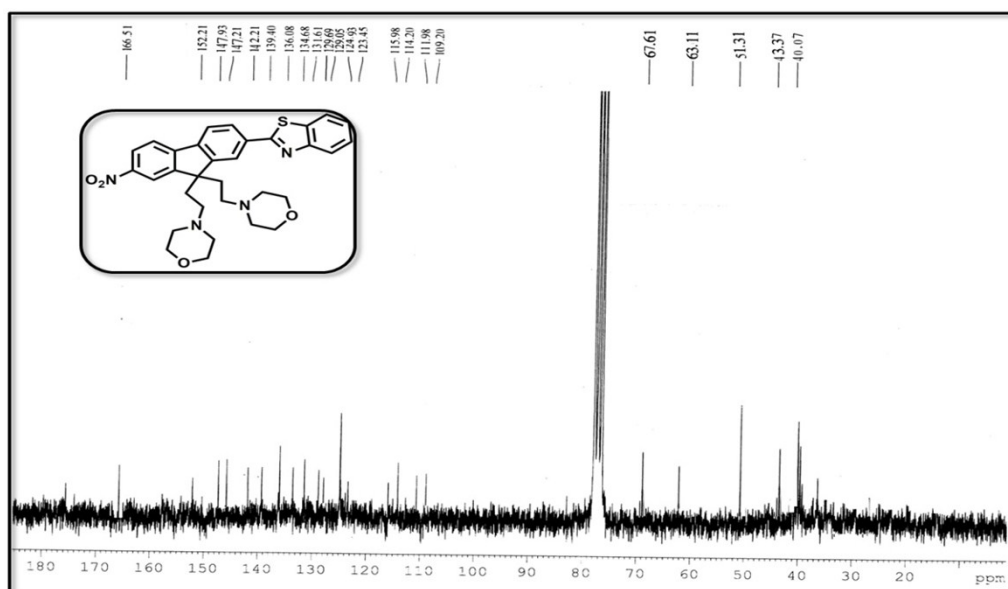
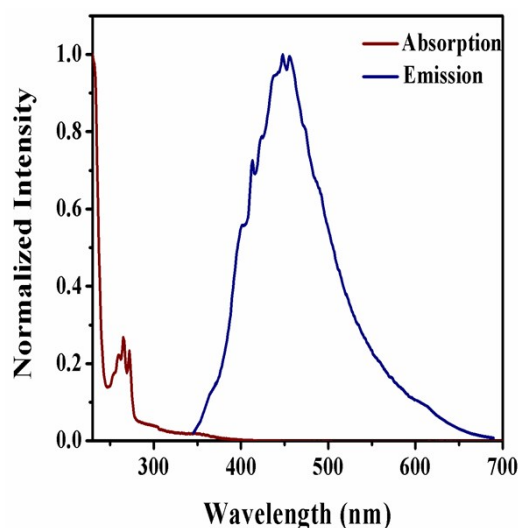


Figure S2  $^{13}\text{C}$  NMR of Fluo-Mor (7)

## 3. Synthesis of Fluo-Mor Nanoparticles (NPs)<sup>5</sup>

Reprecipitation technique was followed to synthesize Fluo-Mor NPs.<sup>5</sup> To a vial containing 20 mL Millipore water, 10  $\mu$ L of 3 mM solution of Fluo-Mor conjugate in acetone was slowly added at room temperature under controlled stirring. The size and shape of the Fluo-Mor NPs was verified by UV/vis, fluorescence, TEM, DLS and zeta potential measurements.

### 3.1 Absorption and Emission spectra of Fluo-Mor NPs



**Figure S3** Normalized absorption and emission spectra of Fluo-Mor NPs

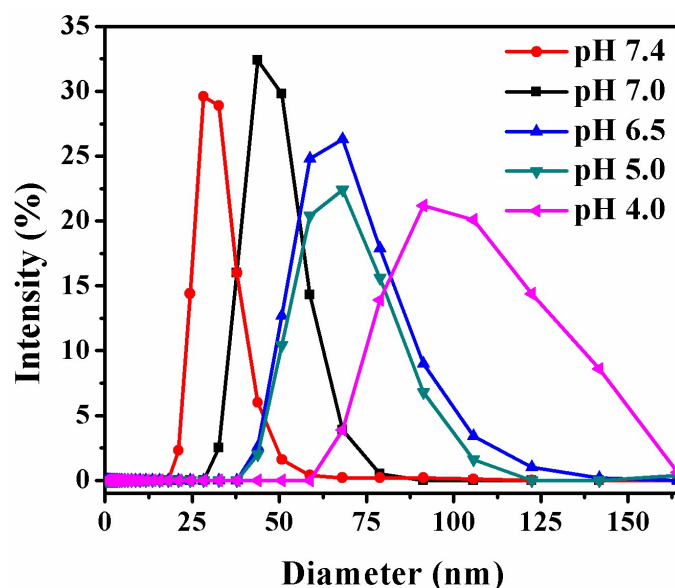
### 3.2 Hydrolytic Stability of Fluo-Mor NPs at pH 7.4:

1 mL of  $2 \times 10^{-4}$  M solution of Fluo-Mor NPs was added in PBS containing 10% fetal bovine serum with pH = 7.4. All the tubes were kept in ultrasonic for 10 min to make the solutions homogeneous and stored at 37 °C in dark condition for 96 h. Then all the solutions were analyzed by reverse phase HPLC to examine the remaining percentage of the Fluo-Mor NPs.

**Table S1** The remaining percentage of Fluo-Mor NPs in dark condition at pH 7.4

NPs	Time (day)	% of Fluo-Mor NPs depleted	
		(pH 7.4) PBS	(pH 7.4) FBS
Fluo-Mor	7	1	3

### 3.3 pH-dependent DLS study of Fluo-Mor NPs



**Figure S4** DLS spectra of Fluo-Mor NPs at different pH-values

## 4 Photophysical and Photochemical Properties of Fluo-Mor NP

### 4.1 Measurement of fluorescence quantum yields<sup>6</sup>

The quantum yield of the Fluo-Mor NPs was determined by reference point method.<sup>6</sup> Quinine sulfate in 0.1 M H<sub>2</sub>SO<sub>4</sub> (literature quantum yield: 54%) was used as a standard sample to calculate the QY of Fluo-Mor NPs. Fluo-Mor NPs were dispersed in different citrate buffer solutions having pH values in between 3-7.4. The absorbance values of the solutions at the excitation wavelength were measured with UV-Vis spectrophotometer. Photoluminescence (PL) emission spectra of all the sample solutions were recorded by Hitachi F-7000 fluorescence spectrophotometer at an excitation wavelength of 360 nm.

$$\frac{\Phi_S}{\Phi_R} = \frac{A_S (Abs)_R \eta_S^2}{A_R (Abs)_S \eta_R^2}$$

Where  $\Phi$  represents quantum yield, **Abs** represents absorbance, **A** represents area under the fluorescence curve, and  $\eta$  is refractive index of the medium. The subscripts **S** and **R** denote the corresponding parameters for the sample and reference, respectively.

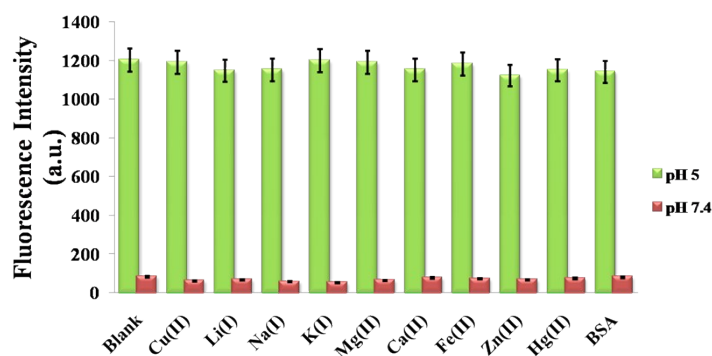


**Table S2** pH dependent fluorescent Quantum yields of Fluo-Mor NPs

Sample	Integrated emission intensity (I)	Abs. At 350 nm (A)	Refractive index of solvent ( $\eta$ )	Quantum yield at 350 nm (Q)
Quinine sulfate	55337.365	0.0717	1.33	0.54
pH=3	99712.93	0.711	1	0.098
pH=4	99995.93	0.667	1	0.104
pH=5	99500.53	0.651	1	0.107
pH=6	92600.49	0.626	1	0.103
pH=7	45604.88	0.431	1	0.074
pH=7.4	9711.762	0.167	1	0.04

Fluorescence quantum yield (excitation wavelength 350 nm, error limit within  $\pm 5\%$ ).

#### 4.2 Competitive experiments in presence of various inorganic metal ions and hydrophobic serum protein



**Figure S5** Effects of different metal ions (200  $\mu\text{M}$ ) on fluorescence intensity of 70  $\mu\text{M}$  Fluo-Mor NPs at pH 7.4 and 5.0. Blank = Fluo-Mor NPs only; all other bars represent Fluo-Mor NPs in presence of respective metal ions and BSA.

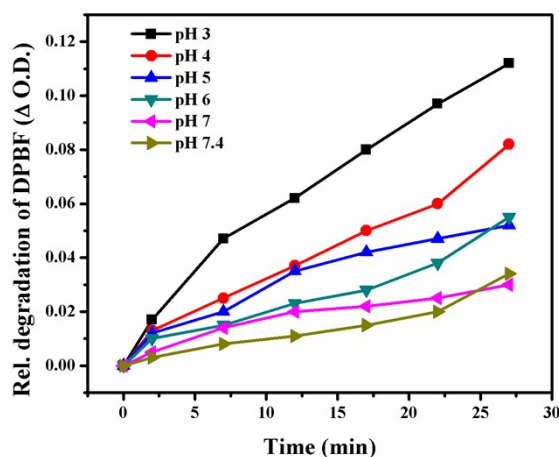
#### 4.3 Measurement of singlet oxygen quantum yields<sup>7</sup>

The singlet oxygen quantum yields ( $\Phi_{\Delta}$ ) of Fluo-Mor NPs were determined using Rose Bengal as the reference with a yield of 0.83 in Acetonitrile (ACN). An equimolar mixed

solution of Fluo-Mor NPs (34  $\mu\text{M}$ ) and DPBF (35  $\mu\text{M}$ ) were prepared in citrate buffer solutions containing 0.5% DMSO. In the solution, the concentration of the photosensitizer was adjusted to possess the same absorbance (typically 0.1) at 365 nm and the initial concentration of DPBF was  $1.26 \times 10^{-4}$  M. During the experiment, the solutions were stirred vigorously in presence of  $\text{O}_2$  gas. The solutions were further irradiated with UV/vis light of  $\geq 365$  nm for 1PE and with 740 nm diode laser ( $30 \text{ mW/cm}^2$ ) for 2PE, the bleaching of the absorption band of DPBF at 410 nm was monitored. The solution of DPBF alone was also irradiated and the obtained result was subtracted to diminish the errors originating from the photo-activity of DPBF. The  $\Phi_{\Delta}$  of each solution was calculated by the following equation,

$$\Phi_S = \frac{K_S}{K_R} \times \Phi_R$$

Where, K is the slope of the photodegradation plot of DPBF against time (s) and the subscripts S and R denote the sample and the reference respectively and  $\Phi_R$  is the singlet oxygen quantum yield of the reference (Rose Bengal).



**Figure S6** Photodegradation study DPBF at 418 nm at different pH under 2 PE irradiation (with 740 nm diode laser)

**Table S3** Singlet oxygen quantum yield of Fluo-Mor NPs at different pH values under two-photon excitation

pH (in Citrate Buffer solution containing 0.5 % DMSO)	$\Phi_{\Delta}^a$
3	0.49
4	0.29
5	0.26
6	0.12
7	0.09
7.4	0.06

<sup>a</sup>Rose Bengal as reference with known  $\Phi_{\Delta} = 0.74$  in water

## 5. *In vitro* application of Fluo-Mor NPs

### 5.1 Sub-cellular localization studies

To study the lysosome targeting of Fluo-Mor NPs, Human Colon cancer HT-29 cells ( $10^5$  cells/well) were anchored on 12-well plates and allowed to adhere for 12 h. The cells were then further incubated with Fluo-Mor NPs with different concentrations (75, 100 300  $\mu\text{g}/\text{mL}$ ) for 4 h in a humidified 5%  $\text{CO}_2$  atmosphere at 37 °C cell culture medium. Thereafter, the cells were fixed in 4 % paraformaldehyde for 20 min, after which the cells were rinsed twice with phosphate buffered saline (PBS) in five minutes interval. Then, the cells were again treated with 0.1 % Triton-X for 15 min. Following similar washing with PBS buffer (1X, pH 7.4), the colon cancer cells were stained with LysoTracker Red DND-99 (100 nM) and further incubated for 1 h at 37 °C. After washing the cells with PBS buffer, imaging was done using an Olympus FV1000 confocal microscope with the appropriate filter.<sup>8</sup>

### 5.2 Photocytotoxicity assay (MTT Assay)

Cytotoxicity of Fluo-Mor NPs with and without irradiation on human colon cancer HT29 cells was determined by conventional MTT assay. Cells in their exponential growth phase were trypsinised and seeded in 96-well flat-bottom culture plates at a density of  $10^5$  cells per well in 200 $\mu$ L of DMEM complete medium. The cells were allowed to adhere and grow for 12 hours at 37 °C in an incubator and then the medium was replaced with 200  $\mu$ L fresh incomplete medium containing phosphate buffer saline (PBS) and Fluo-Mor NPs. Fluo-Mor NPs treated cells were incubated for another 4 h. Afterwards, the cells were irradiated with UV/vis light of wavelength  $\geq 365$  nm (0-5 min) and further incubated for about 16 hours at 37 °C.<sup>9</sup> Following treatment for 16 hrs at 37 °C, MTT solution (5mg/ml in PBS) was added and incubation was prolonged for 5 h at 37 °C. Inhibition of cell proliferation was monitored by MTT (Himedia) assay.<sup>10</sup> Absorbance of the MTT-formazan product dissolved in DMSO (Himedia) was estimated at 595 nm using an ELISA plate reader (Thermo, USA). The inhibition of cell viability was calculated using the formula:

$$\% \text{ viability} = (\text{Test absorbance}/\text{Control absorbance}) \times 100$$

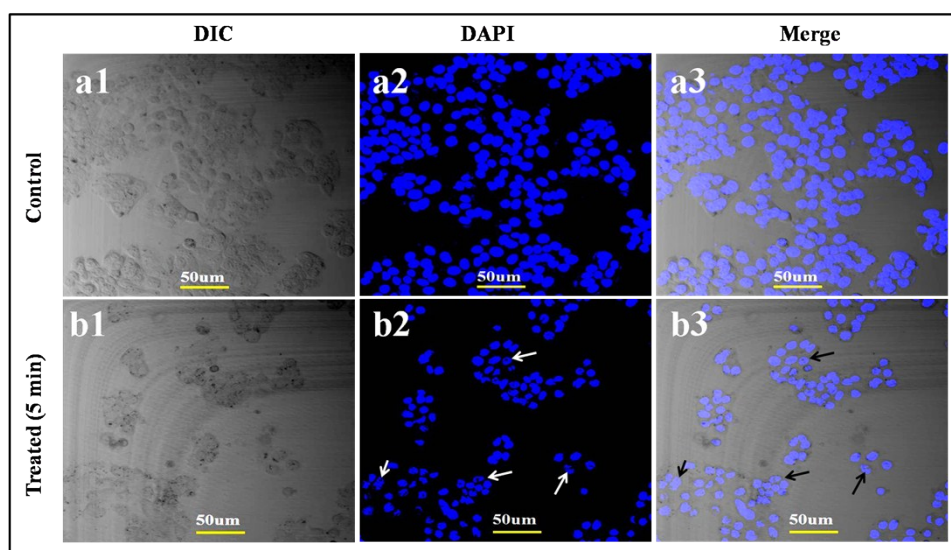
### **5.3 Live-dead Assay<sup>11</sup>**

Live-dead assay was performed by live-dead viability/cytotoxicity kit for mammalian cells (Invitrogen), as per manufacturer protocol. The drug treated (48h) HT-29 cells ( $1 \times 10^5$  cells/mL) were stained with 150 $\mu$ L live-dead assay reagent (2 $\mu$ M Calcein AM and 4  $\mu$ M EthD-1) for 30 min at 25°C. It was then washed twice in PBS (150 $\mu$ L) and visualization under confocal microscope (CLSM; Olympus FV 1000 attached with an inverted microscope 1X 81, Japan).

### **5.3 DAPI staining for nuclear morphology observation**

To determine whether Fluo-Mor NPs induced cell death is correlated with apoptosis, nuclear morphology of cells were assessed upon staining with 4', 6-diamidino-2-phenylindole (DAPI). Evaluation of normal or apoptotic cells depends on their morphological

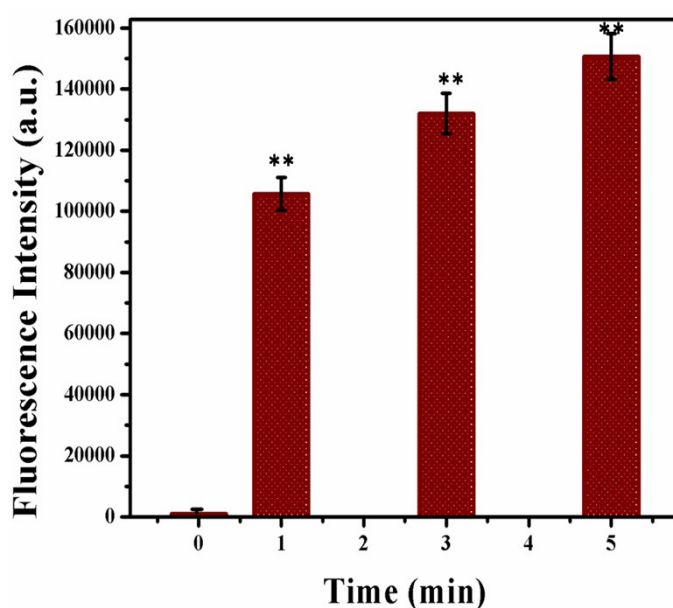
characterization. Normal nuclei (smooth nuclear) and apoptotic nuclei (condensed or fragmented chromatin) are easily distinguished. Nuclear condensation and fragmentation is one of the special features of apoptotic cell death, which was seen in HT 29 cells after 5 min treatment of cells with UV-vis light ( $\geq 365$  nm) in presence of Fluo-Mor NPs followed by 12 h of incubation. DAPI staining for nuclear morphology observations was performed as described by Mukhopadhyay et al.<sup>12</sup> HT 29 ( $10^5$  cells/well) cells were treated with Fluo-Mor NPs for 12 h after treatment to UV-vis light ( $\geq 365$  nm) for 5 min along with the control (PBS). Then, cells were rinsed with phosphate buffer saline (PBS) and fixed in pretreatment solution for 5 min. Cells were incubated for 30 min with 10  $\mu$ L of 4', 6-diamidino-2-phenylindole (DAPI, Sigma) staining solution. Morphological alterations of nucleus like condensation or fragmentation were observed by confocal microscopy (CLSM; Olympus FV 1000 attached with an inverted microscope 1X 81, Japan).



**Figure S7** Study of morphological changes of cell nucleus in presence of Fluo-Mor NPs by staining with DAPI dye; **(a)** before irradiation of light, **(b)** after 5 min of light irradiation; **(1)** bright-field images, **(2)** fluorescence image of DAPI at 410 nm **(3)** overlays of the bright-field images and the fluorescence images. Scale bar =50  $\mu$ m

## 5.5 Intracellular Singlet Oxygen Detection<sup>7</sup>

The production of ROS in cells was determined by using DCFH-DA as the probe<sup>13</sup>, which can diffuse through the cellular membrane and further react with ROS to generate fluorescent dye. Briefly, after incubated with 300  $\mu\text{M}$  Fluo-Mor NPs for 4 h in the 96-well plate, the cells were washed with PBS and then incubated with 4  $\mu\text{L}$  of 10 mM DCFH-DA stock solution in PBS solution for 30 min. Then the cells were carefully washed by PBS for two times and added with 100  $\mu\text{L}$  PBS. After irradiated with a medium pressure mercury lamp with suitable 0.1 M  $\text{CuSO}_4$  solution as filter ( $\geq 365$  nm) ( $20 \text{ mW cm}^{-2}$ ) for 5 min and further incubated for 16 h. The medium was then replaced with 200  $\mu\text{L}$  fresh incomplete medium containing phosphate buffer saline (PBS) and incubated for 10 min at 37 °C. After washing with Dulbecco's modified eagle medium (DMEM), the fluorescence signal was determined by a Olympus FV1000 confocal microscope with excitation at 488 nm and emission at 530 nm. The singlet oxygen production ability of the blank cells incubated in dark was taken as the 100% baseline.



**Figure S8** Intracellular singlet oxygen measurements using DCFH-DA assay: gradual

increase in fluorescence quantum yield of DCF dye with respect to time of irradiation ( $\geq 365$  nm), Values are presented as the mean  $\pm$  standard deviation of three different observations and the significance level was evaluated by comparing with the negative control using Student's t-test statistics: **\*\*P < 0.01**

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