NIR fluorescent organic nanoparticles for photoinduced Nitric oxide delivery with self monitoring and real time reporting ability

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Supporting Information

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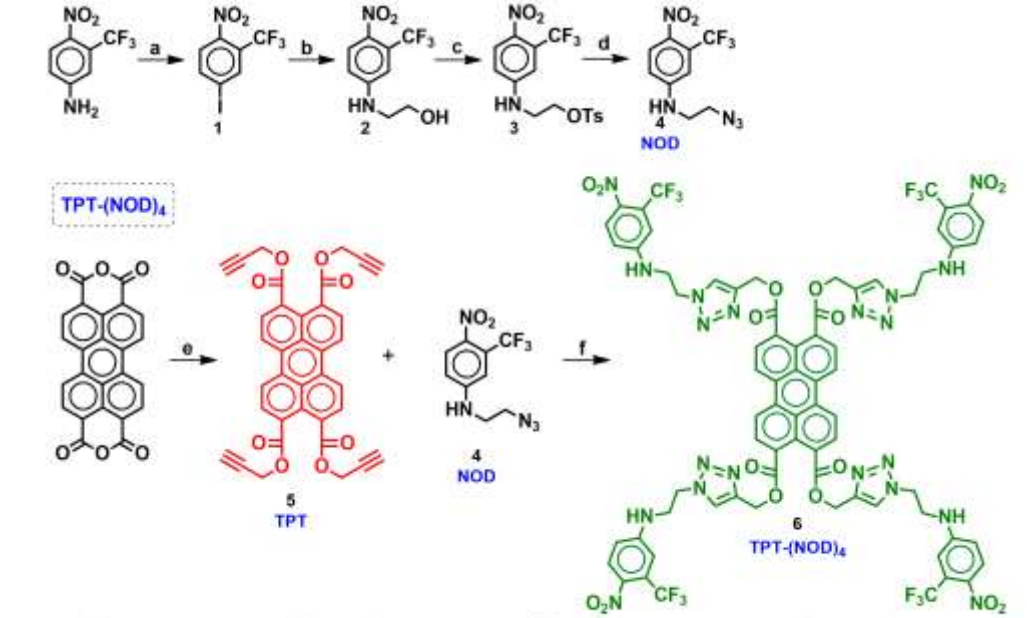
Materials and Methods

All reagents were purchased from Sigma Aldrich and used without further purification. For reaction monitoring, precoated silica gel 60 F254 TLC sheets (Merck) was used. Chromatographic purification was done with 60-120 mesh silica gel (Merck). ^{1}H NMR spectra were recorded on a BRUKER-AC 200, 400 and 600 MHz spectrometer. Chemical shifts are reported in ppm from tetramethylsilane with the solvent resonance as the internal standard (deuterochloroform: 7.26 ppm and deuterated dimethylsulphoxide: 2.5 ppm). Data are reported as follows: chemical shifts, multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet), coupling constant (Hz). ^{13}C NMR (50, 100 and 150 MHz) spectra were recorded on a BRUKER-AC 200, 400 and 600 MHz Spectrometer with complete proton decoupling. Chemical shifts are reported in ppm from tetramethylsilane with the solvent resonance as the internal standard (deuterochloroform: 77.23 ppm and deuterated DMSO: 39.52 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. Imaging was done using a fluorescence microscope (IX 51, Olympus) high-performance CCD camera with the appropriate filter using Image-Pro discovery 5.1 software.

Photochemical Experiments: Photolysis was carried out using 125 W medium pressure mercury lamp, supplied by SAIC (India) using 1M $NaNO_2$ solution as cut-off filter for transmitting light of wavelength ≥ 410nm i.e. visible light

Scheme for Synthesis of TPT(NOD)$_4$



Reaction Conditions: (a.) 3N H_2SO_4, $NaNO_2$, KI, Urea, 4 h, 0 °C - r.t (b.) ethanolamine, K_2CO_3, cat. CuI, 1 h, reflux (c.) TsCl, pyridine, DCM, 3 h, r.t (d.) NaN_3, DMF, over night, 80 °C (e.) Propargyl bromide, propargyl alcohol, DBU, DMF, 4 h, 60 °C (f.) Sodium Ascorbate, $CuSO_4.5H_2O$, (1:1:2) H_2O/EtOH/$CHCl_3$, 1 h, 60 °C.

Scheme S1: Synthesis of TPT(NOD)$_4$

Experimental procedure and Spectroscopic data

4-iodo-1-nitro-2-(trifluoromethyl)benzene (**1**).[1]

A solution of $NaNO_2$ (21.44 mmol) in water (2 mL) was added to a suspension of 4-nitro-3-(trifluoromethyl)aniline (21.44 mmol) in 3 N H_2SO_4 (22 mL) at 0 °C over a period of 1 h. Later on, KI (21.44 mmol) and urea (4.289 mmol) in water (2 mL) were added and stirred for 1 h at 0 °C, followed by stirring at ambient temperature over 2 h. The reaction mixture was then quenched by adding EtOAc. Further, the organic layer was washed with saturated $NaHCO_3$ solution (3x50mL), brine (2x50 mL), and dried over Na_2SO_4. The organic layer was then concentrated and the resultant mixture was purified by column chromatography using PET ether to obtain compound **1**, 70%, yield. ^{1}H NMR (200 MHz, $CDCl_3$) δ 8.16 (s, 1H), 8.12 – 8.06 (m, 1H), 7.62 (d, *J* = 8.4 Hz, 1H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 148.0, 142.4, 137.3, 137.1, 137.0, 136.9, 126.4, 98.9, 77.8, 77.2, 76.6.

2-((4-nitro-3-(trifluoromethyl)phenyl)amino)ethanol (**2**).[1]

To an oven dried 2 neck R.B flask equipped with a stir bar 1mmol of 4-iodo-1-nitro-2-(trifluoromethyl)benzene (**1**), 0.1 mmol of CuI, 1 mmol ethanolamine, 3 mmol of K_2CO_3 and 5 mL DMF were added. Then the R.B flask was evacuated refilled with N_2 gas (2 times) and is

heated on an oil bath at 90 °C for 1 h. Later on, reaction was quenched with water, extracted with EtOAc (2x50 mL). The organic layer was dried over Na_2SO_4 and concentrated to obtain compound **2**, which was further purified by using 40% EtOAc in pet ether, 52 % yield. ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ 8.05 (s, 1H), 7.62 (t, *J* = 4.7 Hz, 1H), 7.12 (s, 1H), 6.85 (d, *J* = 9.2 Hz, 1H), 4.87 (t, *J* = 5.3 Hz, 1H), 3.58 (q, *J* = 5.4 Hz, 2H), 3.27 (q, *J* = 5.5 Hz, 2H). ^{13}C NMR (50 MHz, DMSO-*d*$_6$) δ 153.4, 133.3, 129.6 125.8, 125.2, 124.5, 123.9, 120.9, 119.8, 118.3, 111.8, 111.0, 59.2

2-((4-nitro-3-(trifluoromethyl)phenyl)amino)ethyl4-methylbenzenesulfonate (**3**).[1]

p-Toluenesulfonyl chloride (1.5 mmol) was added in small portions to an ice cold solution of 2-((4-nitro-3-(trifluoromethyl)phenyl)amino)ethanol (**2**) (1 mmol) in pyridine (5 mL). After stirring at 0° C for 15 min, ice bath was removed and the reaction was left to stirr at room temperature for 2.5 h. The reaction mixture was then poured into stirred ice/H_2O (400 mL) extracted with EtOAc (2x50 mL), and washed successively with H_2O, aq. citric acid, and again H_2O. The organic layer was dried over Na_2SO_4 and the solvent was evaporated. The compound **3** was used without further purification, 90 % yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.92 (d, *J* = 8.9 Hz, 2H), 7.74 (d, *J* = 7.7 Hz, 2H), 7.30 (d, *J* = 7.8 Hz, 2H), 6.80 (s, 1H), 6.60 (d, *J* = 8.9 Hz, 1H), 5.15 (s, 1H), 4.23 (t, *J* = 4.7 Hz, 2H), 3.57 – 3.50 (m, 2H), 2.41 (s, 3H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 151.1, 145.7, 137.2, 132.3, 130.2, 129.1, 128.0, 127.6, 126.9, 126.3, 125.6, 125.1, 125.02, 119.6, 114.2, 113.0, 111.7, 111.6, 111.5, 111.3, 67.6, 42.3, 29.8, 21.7.

N-(2-azidoethyl)-4-nitro-3-(trifluoromethyl)aniline (**NOD, 4**).[1]

2-((4-nitro-3-(trifluoromethyl)phenyl)amino)ethyl 4-methylbenzenesulfonate (**3**) (1 mmol) and sodium azide (2 mmol) in DMF (5 mL) were stirred at 65° C for 2.25 h. After cooling, the reaction was diluted with cold H_2O and extracted with Et_2O (3×50 mL). The combined organic extracts were washed with H_2O (3×50 mL), dried over Na_2SO_4 and then the solvent was evaporated. The residue was dissolved in a small amount of Et_2O and filtered through a short plug of silica gel. Evaporation of the filtrate afforded the compound **NOD**, **4** as yellow gelatinous solid, 82 % yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.00 (d, *J* = 9.1 Hz, 1H), 7.03 – 6.85 (m, 1H), 6.72 (dd, *J* = 9.1, 2.4 Hz, 1H), 5.14 (s, 1H), 3.60 (t, *J* = 5.6 Hz, 2H), 3.44 (q, *J* = 5.5 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 151.76, 136.97, 129.42, 113.02, 111.86, 111.79, 111.73, 111.67, 50.27, 42.60, 29.90.

Tetra(prop-2-yn-1-yl) perylene-3,4,9,10-tetracarboxylate (TPT, 5).

To a solution of Perylene tetracarboxylic dianhydride (100 mg, 0.25 mmol) in DMF at 60 ˚C, were added 1,8-Diazabicyclo[5,4,0]undec-7-ene(142 µL, 0.95 mmol) and propargyl alcohol (110 µL, 1.9 mmol) and this resulting mixture was stirred for 30 min. Further, propargyl bromide (80% in toluene, 143 µL, 1.9 mmol) in DMF (0.5mL) was added dropwise to this mixture and was left for stirring for 3 h at the same temperature. The crude product was precipitated by adding water (50 ml) upon completion of the reaction and the solid was filtered out. The workup of the obtained solid was carried out by redissolving it in CH_2Cl_2 (30 mL) and washing with water as well as brine. The organic layer was dried over Na_2SO_4 and concentrated in vacuo. Thus obtained residue was purified by column chromatography with CH_2Cl_2 to afford **TPT**, compound **5** as a orange solid (68 mg, 0.12 mmol, 46 %). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.73 (d, 4H), 8.10 (d, 4H), 4.95 (s, 8H), 3.65 (s, 4H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 167.16, 133.21, 131.35, 129.41, 128.48, 123.19, 78.77, 78.50, 53.52. MALDI-TOF: [M^+] calcd for $C_{36}H_{20}O_8$: 580.116; found: 580.874.

Tetrakis((1-(2-((4-nitro-3-(trifluoromethyl) phenyl) amino)ethyl)-1H-1,2,3-triazol-4-yl)methyl) perylene-3,4,9,10-tetracarboxylate (TPT-(NOD)$_4$, 6).

Compound 5 (50 mg, 0.09 mmol), together with 4 (134 mg, 0.68mmol), $Cu_2SO_4.5H_2O$ (17 mg, 0.07 mmol) and sodium ascorbate (7.4mg, 0.04 mmol) were taken into an RB flask and a mixture of solvents i.e., $CHCl_3$ (2 mL)/EtOH (1 mL)/H_2O (1 mL) were added to the RB flask. The resultant mixture was refluxed at 65˚C for 1 h. The reaction was then cooled to room temperature and the solvent was filtered to collect the residue. The residue was washed twice with DCM and the obtained red solid compound **TPT-(NOD)$_4$,** compound **6** (68 mg, 0.12 mmol, 46 %) was sufficiently pure to carry out photophysical and photochemical studies. ^{1}H NMR (600 MHz, $CDCl_3$): d = 8.59 (d, *J* = 7.8 Hz, 4H), 8.32 (s, 4H), 8.00-7.97 (m, *J* = 7.8 Hz, 9.6 Hz, 8H), 7.62-7.60 (t, *J* = 6 Hz, 4 H), 7.00 (s, 4H), 6.83 (dd, *J* = 2.4 Hz, 9 Hz, 4H,), 5.39 (s, 8H), 4.58 (t, *J* = 6 Hz, 8H), 3.75 (dd, J = 5.4 Hz, 10.8 Hz, 8H). MALDI-TOF: [M^+] calcd for $C_{72}H_{52}F_{12}N_{20}O_{16}$: 1680.367; found: 1680.395.

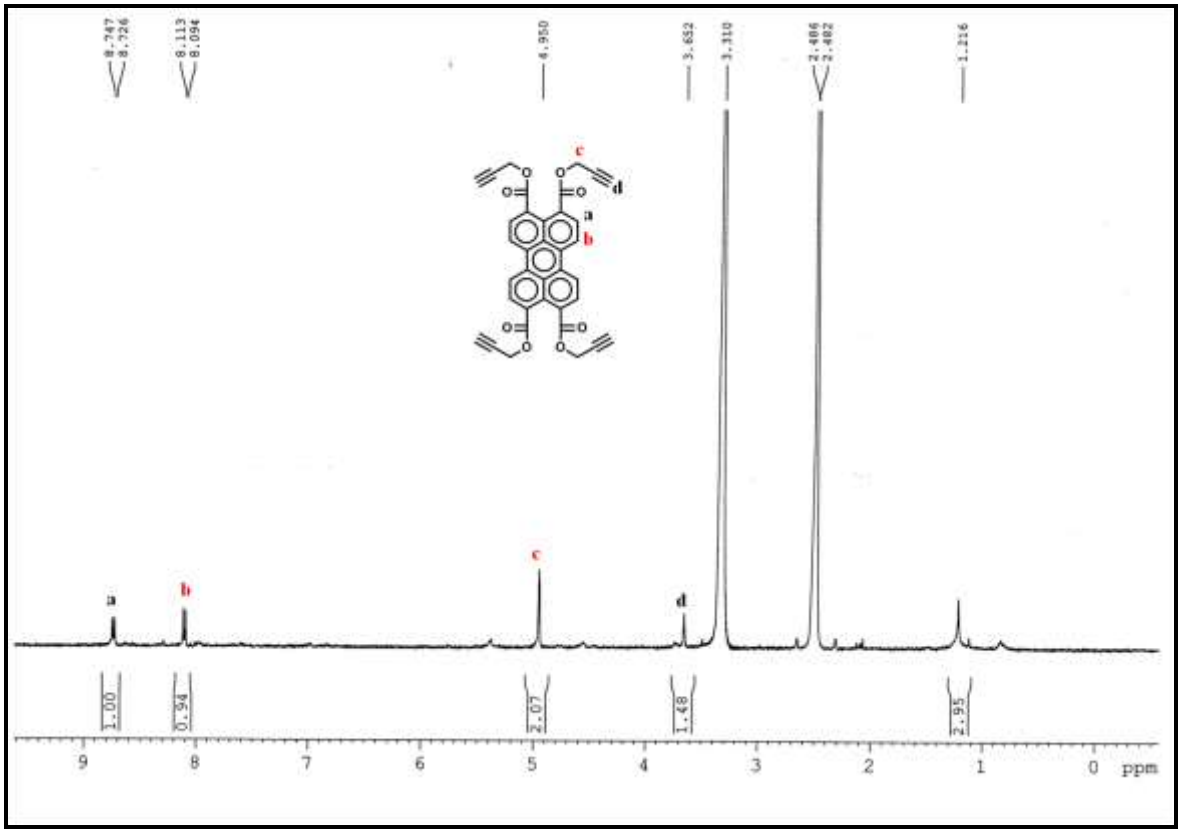


Figure S1. [1]H Spectrum of TPT-5

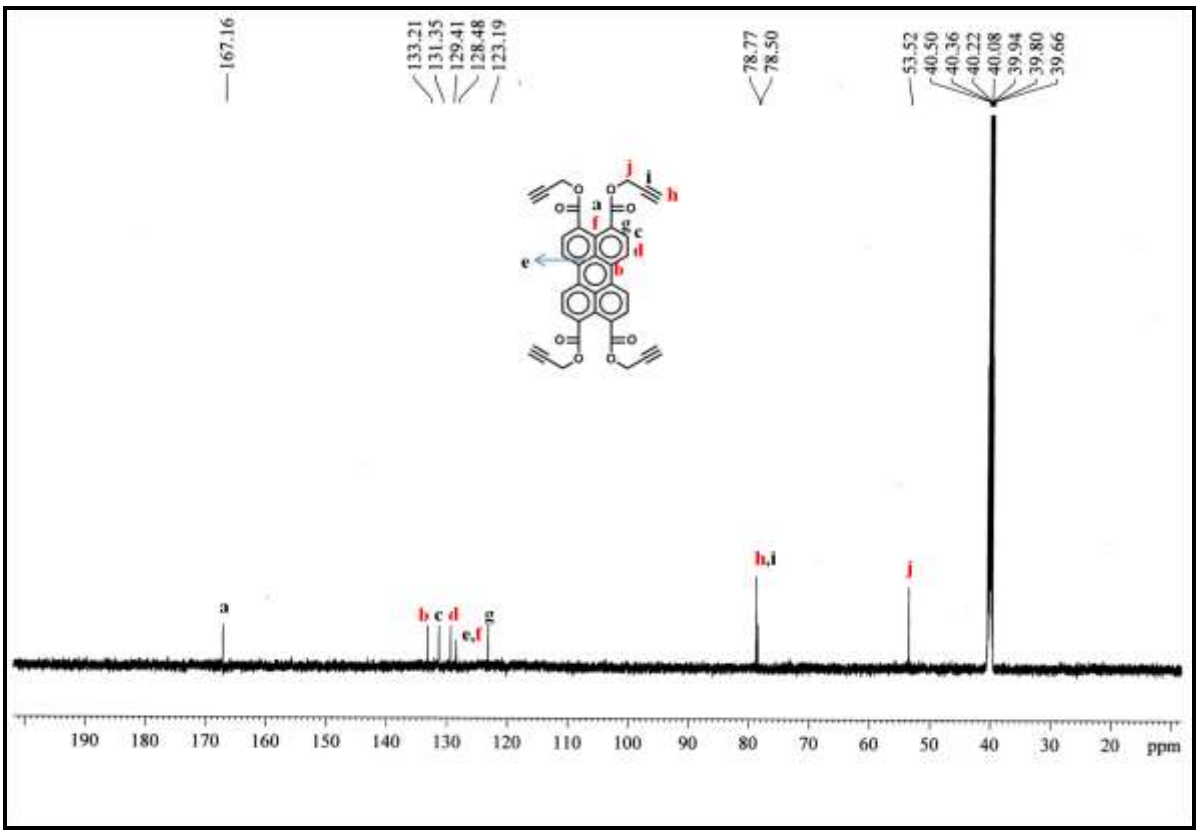


Figure S2. [13] C Spectrum of TPT-5

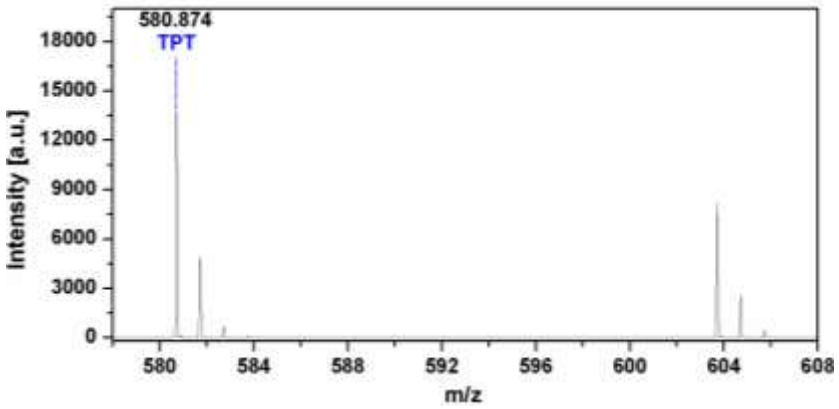


Figure S3. MALDI-TOF Spectrum of TPT-5

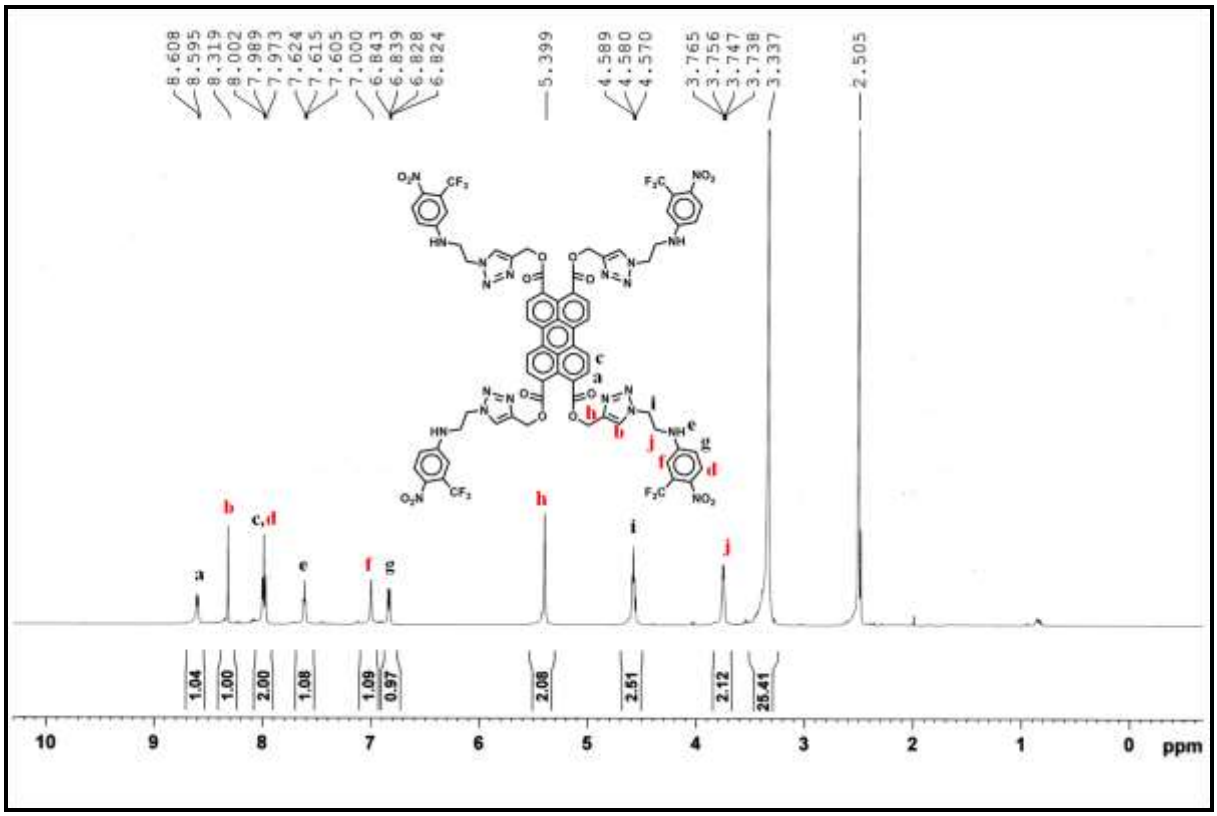


Figure S4. ^{1}H Spectrum of TPT-$(NOD)_4$

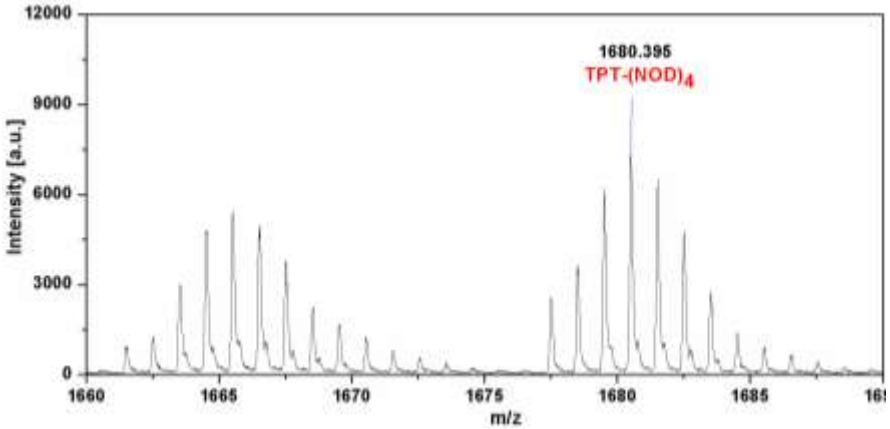


Figure S5. MALDI-TOF Spectrum of TPT-$(NOD)_4$

Preparation of NPs and Characterization Studies

The preparation of Photoresponsive TPT(NOD)$_4$ NPs was carried out using reprecipitation technique. A 30 µL of TPT(NOD)$_4$ conjugate (1 mM, 3 mL) solution in DMSO was added slowly to 3 mL of water with constant sonication. The synthesized TPT(NOD)$_4$ NPs size was determined by transmission electron microscopy (TEM) and dynamic light scattering (DLS). The photophysical properties like absorption and emission of the synthesised TPT(NOD)$_4$ NPs were studied.

Zeta Potential of TPT(NOD)$_4$ NPs

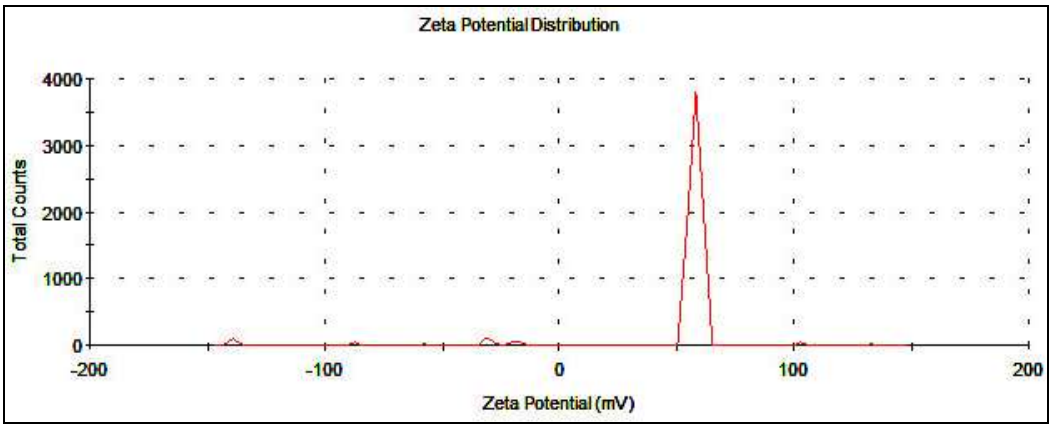


Figure S6. Zeta potential of TPT-(NOD)$_4$: + 58 mV

Hydrolytic Stability of TPT(NOD)$_4$ NPs at Different pH

To check the stability of TPT(NOD)$_4$ NPs at different pHs under dark conditions, a stock of TPT(NOD)$_4$ particles were prepared using reprecipitation technique by adding 100 µL of 1 mM TPT(NOD)$_4$ conjugate into 10 mL of water. For studying pH stability, 20 µL of TPT(NOD)$_4$ NPs from stock were suspended into 1 mL of phosphate-buffered saline (PBS) buffers of 3 different pHs (5.6, 7.4, and 8.0) each. In the same way to analyse the stability in biological environment equal amount of TPT(NOD)$_4$ NPs were dispersed into 1 mL of PBS with 10% bovine culture medium. These 4 samples were stored in dark conditions for 6 days, at 25 ± 2 °C. These samples were then extracted with DCM and were studied by ^{1}H NMR to know the remaining percentage of TPT(NOD)$_4$. We have used mesitylene as an external standard in carrying out Hydrolytic stability experiment using ^{1}H NMR.[2]

Table S1. Hydrolytic Stability study of CPy-NO NPs at Different pH and Biological conditions

Different pH and biological conditions	pH 5.6	pH 7.4	pH 8.8	Bovine culture medium
% TPT-$(NOD)_4$ NPs left after 6days[a]	98	96	98	97

[a] Analysed from ^{1}H NMR

Photolysis of TPT$(NOD)_4$ NPs and MALDI-TOF analysis

A stock of TPT$(NOD)_4$ NPs of 10 μM concentration was prepare and photolysis experiment was carried out by exposing 3 ml of this sample to 125 W medium pressure Hg lamp (≥ 410nm) and 1M $NaNO_2$ solution as cut-off filter for different time intervals over a period of 60 min. Then the emission spectra of these samples were recorded. Further the photolysed sample was subjected to MALDI-TOF analysis in order to study whether the mechanism of the photolysis involves sequential or simultaneous release of nitric oxide.

Detection and Estimation of Photoreleased Nitric Oxide

NO detection and quantification has been performed using Griess assay. Griess reagent is prepared by mixing equal volumes of 1% sulphanilamide in 5% orthophosphoric acid and 0.1% naphthylethylenediamine dihydrochloride (NED) in distilled water. Photolysis of 10 μM TPT$(NOD)_4$ NPs was carried out using 125 W medium pressure Hg lamp (≥ 410nm) and 1 M $NaNO_2$ solution as cut-off filter, for a period of 30 min after being treated with Griess reagent. At regular intervals of time aliquots of photolysed sample was collected and centrifuged. The agglomerated TPT$(NOD)_4$ NPs were filtered out and UV absorbance of the griess reagent treated resultant photolysed sample was recorded. Detection and estimation of photogenerated NO or nitrite (NO_2^-) ion was achieved by recording the increase in absorbance at ~545 nm that corresponds to the formation of azo dye.

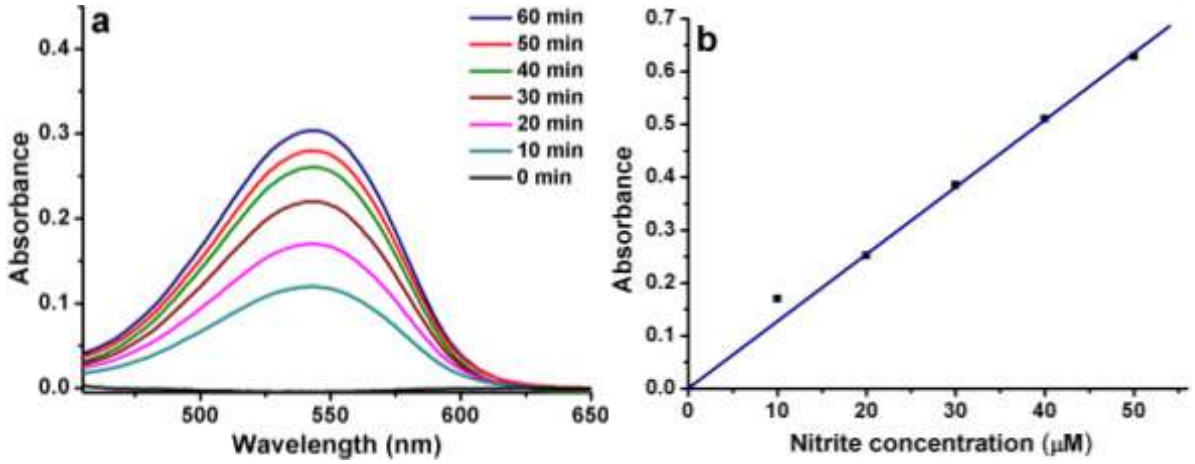


Figure S7. (a) Absorption recorded at regular intervals during photolysis of TPT-$(NOD)_4$ NPs with visible light (b) Nitrite concentration standard curve

Graph indicating precise control of light over the photolytic release of nitric oxide (NO)

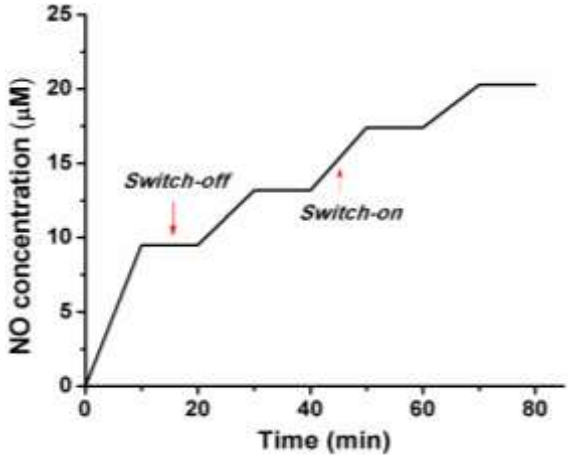


Figure S8**.** Release of nitric oxide from TPT$(NOD)_4$ NPs under bright (visible light) and dark conditions, "Switch On" and "Switch Off" implies the switching on and off of the light source, respectively.

TEM images of TPT(NOD)$_4$ NPs before and after photolysis

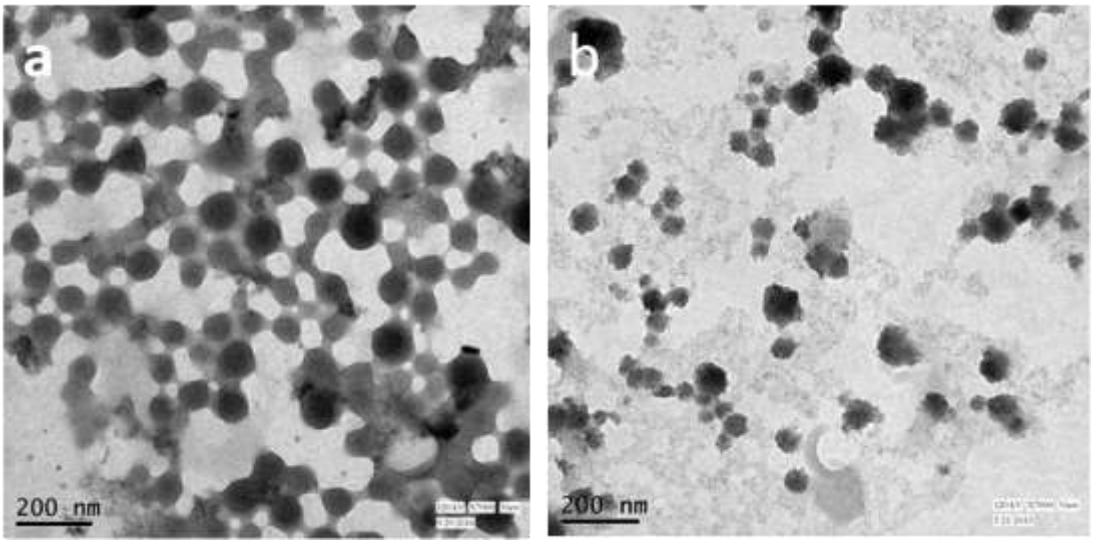


Figure S9: TEM image of TPT(NOD)4 NPs before (0 min) and after photolysis (30 min)

Time dependent cell imaging using TPT(NOD)$_4$ NPs on U87MG cell line[3]

U87MG cells were grown in monolayer on cover slips for 24 h followed by overnight serum starvation. They were then treated with TPT-(NOD)$_4$ NPs in serum free medium for 45 min, washed, media replaced and exposed to visible light source (using 125 W medium pressure Hg lamp as irradiation source (≥ 410 nm) and 1M $NaNO_2$ solution as cut-off filter) for 30 min. To study morphological changes, the cell bearing coverslips were treated and stained with Hoechst 33342. Fluorescent images were captured under Zeiss Observer Z1 microscope (Carl Zeiss, Germany) at 20x magnification. Further for studying intracellular photoinduced fluorescent changes due to NO release excitations used for collecting confocal images in Fig 7 are Blue - excitation/emission (nm) 358/461, Green 495/525, Red 596/615.

Dose dependent cytotoxicity studies of TPT(NOD)$_4$ NPs on U87MG cell line[3]

i. Before irradiation. The *in vitro* cytotoxicity of TPT(NOD)$_4$ NPs was measured using the MTT (3–(4,5–dimethylthiazol–2–yl)–2,5–diphenyltetrazolium bromide, a yellow tetrazole) assay on U87MG cell line. Briefly, cells growing in log phase were seeded into 96–well cell–culture plate at 5×10^3 cells/well. Different concentration of TPT(NOD)$_4$ NPs (0- 10 μM) were added in the wells with an equal volume of PBS in

the control wells. The cells were then incubated for 24 h at 37 °C in 5% CO_2. Thereafter, fresh media containing 50 µL MTT (1 mg/mL) were added to the 95 well plates and incubated for 4 h at 37 °C in 5% CO_2. Formazan crystals thus formed were dissolved in DMSO after decanting the earlier media and absorbance recorded at 595 nm.

ii. After irradiation. U87MG cells maintained in minimum essential medium (in 96-well cell-culture plate at concentration of 1×10^4 cells/mL) containing 10 % fetal bovine serum (FBS) and different concentration (0-10 µM) of $TPT(NOD)_4$ NPs was incubated for 4 h at 37 °C and 5 % CO_2. Then the cells were irradiated using visible light, for three different time intervals 30 min, 45 min, 60 min separately. After irradiation the cells were again incubated for 24 h. Then cytotoxicity was measured using the MTT assay as described earlier

References

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