Electronic Supplementary Information

Far-Red Fluorescent Carbon Nano-Onions As Biocompatible Platform For Cellular Imaging

Stefania Lettieri,[a] Adalberto Camisasca,[ab] Marta d'Amora,[c] Alberto Diaspro,[cde] Takashi Uchida,[f] Yoshikata Nakajima,[f] Keiichi Yanagisawa,[f] Toru Maekawa,[f] Silvia Giordani*[ag]

[a] *Nano Carbon Materials, Istituto Italiano di Tecnologia (IIT), via Morego 30, 16163, Genoa, Italy*

[b] *Department of Chemistry, University of Genoa, Via Dodecaneso 31, Genoa, 16145, Italy*

[c] *Optical Nanoscopy, Nanophysics, Istituto Italiano di Tecnologia (IIT), via Morego 30, 16163, Genoa, Italy*

[d] *NIC@IIT, Istituto Italiano di Tecnologia (IIT), Via Morego 30, Genoa, 16163, Italy*

[e] *Department of Physics, University of Genoa, Via Dodecaneso 33, Genoa, 16145, Italy*

[f] *Bio-Nano Electronics Research Centre, Toyo University 2100, Kujirai, Kawagoe, Saitama 350-8585, Japan*

[g] *Department of Chemistry, University of Turin, via Giuria 7, 10125, Turin, Italy*

email silvia.giordani@iit.it

Material and methods

Materials

All solvents and reagents were purchased from Sigma-Aldrich in high purity grade and were used as received. All reactions and measurements were carried out under ambient conditions, unless otherwise noted.

Instrumentation

Thermogravymetric analysis (TGA)

TGA was conducted on a TA Q500 analyser, using a Pt pan as sample holder. After equilibrating the sample at 30 °C for 5 min and then at 100 °C for additional 20 min, the measurement was performed in air using a heating rate of 10 °C/min. The sample weight was monitored until 900 °C.

Raman spectroscopy

Raman spectra were measured on a Horiba Jobin Yvon HR 800 UV LabRam Raman microscope. For the Raman measurements, the samples were deposited directly on silicon wafer and excited with a built-in 632 nm laser.

Absorption and fluorescence spectroscopy

Absorption spectra were recorded on an Agilent Cary 8454 UV–vis diode array spectrophotometer. Fluorescence spectra were taken on a Horiba Jobin Yvon Fluoromax-4 spectrofluorometer in 1.00 cm × 1.00 cm quartz glass cells. The CNO samples were dispersed in DMSO or cell medium (DMEM) to a final concentration of 1 mg mL^{-1}. The dispersion of CNO was sonicated for 15 min at 37 kHz and then diluted respectively in DMSO or cell medium (DMEM) to achieve final concentrations of 20, 10 and 5 μg mL^{-1}.

Dynamic light scattering (DLS) and zeta-potential measurements

DLS Measurements were performed on the Malvern Nano-ZS instrument operating in backscattering (173°) mode and analyzed with the software Zetasizer, with automatic selection of the optimal detector position and

number of independent measurements. The CNO samples were dispersed in water or PBS 0.01M (PBS pH 7.4, composition 0.14 M NaCl, 0.0027 M KCl, 0.010 M PO_4^{3-}) to a final concentration of 1 mg mL^{-1}. The samples were sonicated for 10 min at 37 kHz and then diluted respectively in water and PBS 0.01M to achieve final concentrations of 5, 10 and 20 μg mL^{-1}. The CNOs samples were sonicated for additional 20 min and the size of the particle was measured. Zeta-potential measurements were performed on the same apparatus using the disposable zeta-potential cuvettes.

High-resolution transmission electron microscopy (HRTEM)

HRTEM observation was carried out using a transmission electron microscope (JEM-2100, JEOL) equipped with a charge coupled device camera (ORIUS SC1000, Gatan, Inc.). The acceleration voltage was set at 80 kV. The specimens were prepared dropping a droplet of solution of CNOs dispersed in ethanol onto holey carbon films supported by copper grids.

X-ray photoelectron spectroscopy (XPS)

XPS analysis was carried out using an X-ray photoelectron spectroscopy microprobe (PHI Quantes, ULVAC-PHI) with a monochromatic Al Kα (1486.6 eV) radiation source. The specimens were prepared dropping a droplet of solution of CNOs dispersed in ethanol onto an Au coated Si substrate so that the surface charge of CNOs caused by generated photoelectrons could be dramatically reduced. Note that the binding energy was calibrated measuring that of the typical C 1s.

Confocal microscopy

Fluorescence imaging was performed with a laser scanning confocal microscope equipped with a resonant scanner (Nikon A1R) using a Plan Apo VC 60x Oil DIC N2 objective.

NMR spectroscopy

NMR spectroscopy was performed on a Bruker Avance III 400 MHz system (400.13 MHz for 1H and 100.62 MHz for 13C) in CDCl3, MeOD or DMSO-d_6 purchased from SigmaAldrich.

High-resolution mass spectrometry (HRMS)

The Accurate Mass measurements (HRMS) were performed on a Waters SYNAPT G2 High Resolution Mass Spectrometry instrument equipped with an Electrospray Ionization interface and coupled to a Waters ACQUITY UPLC. Electrospray ionization in positive mode was applied in the mass scan range 50-1200 Da. The analysis were performed on a Waters ACQUITY UPLC BEH C18 column 100 x 2.1 mm ID (particle size 1.7 µm) with an in-line filter. The mobile phase was 0.1% formic acid in H2O and 0.1% formic acid in acetonitrile.

Fluorescence quantum yield

Fluorescence quantum yields were determined by the comparative method of Williams et al.[1] Toward this, the integrated fluorescence intensities of a known dye and the tested compound were compared and the fluorescence quantum yields were calculated using the following equation: $\Phi x = (\Phi_{st})\ (Grad_x / Grad_{st})\ (\eta^2 x/\eta^2_{st})$. st and x denotes the standard and test respectively, while Φ is the fluorescence quantum yield. Grad is the gradient obtained from the plot of integrated fluorescence intensity vs. absorbance of the dye at the excitation wavelength. η represents the refractive index of the used solvents.

Biological methods

Sample preparation

The samples of **oxi-CNOs** and **fluo-CNOs** were prepared by suspending CNOs in sterile Dulbecco's modified Eagle's medium (DMEM) (Life Technologies) at final concentrations of 0.5, 1, 5, 10, 20 µg mL^{-1} followed by sonication for 10 min at 37 kHz.

Cell cultures

HeLa (derived from a human cervix carcinoma) and MCF-7 (human mammary gland adenocarcinoma) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies) supplemented with

10% fetal bovine serum (FBS) (Life Technologies), 100 IU/ml penicillin and 100 μg/ml streptomycin (Life Technologies) at 37 °C in a humidified 5% CO_2 balanced-air atmosphere. Cells were passaged at 80% confluency and split 1:10 in fresh media and discontinued after passage 15.

Cell viability

Hela and MCF7 cells were seeded in 96-well plates at the concentration of 5,000 cells/well and left in the incubator overnight to allow cells adhere to the well at 37 °C in a 5% CO_2 humidified environment. Following the incubation, the maintenance medium was removed and replaced with 500 μL of **oxi-CNOs** and **fluo-CNOs** dispersions at the final concentrations of 0.5, 1, 5, 10 and 20 μg mL^{-1}. Cell viability was determined after 12, 24, 48 and 72 hours of exposure to the dispersions of CNOs by WST-1 assay, according to the manufacturer's instructions (Roche Applied Sciences). Before the cell viability test, a volume of 10μL WST-1 reagent was added to each well, followed by 2 hours of incubation in the same environment. The cell viability was determined by optical absorption (450 nm test wavelength, 690 nm reference wavelength) in a standard plate reader. Data were expressed as the mean ± standard deviation (mean ±SD); significance was determined by one-way analysis of variance (ANOVA) in order to compare each treatment group with the negative controls. A value of $p \leq 0.05$ was considered statistically significant.

Cellular imaging

For cellular imaging, MCF7 cells were plated on chambered coverglass (Thermo Scientific Nunc Lab-Tek II) and cultured in DMEM culture growth medium at 37 °C in a 5% CO_2 humidified environment for 24 hours. Subsequently, the culture medium was removed and cells were incubated with 5, 10 and 20 μg mL^{-1} suspension of **fluo-CNOs** for another 24 hours. As a control, cells were left untreated (data not shown). After the incubation time, the plated cells were rinsed three times with phosphate-buffered saline (PBS) (0.1 M, pH 7.4) and then incubated with a solution of Hoechst 33342 (5 μg mL^{-1}) (Sigma) or LysoTracker Green (75 nm) (Life Technologies) for 15 min. The cells were rinsed three times and filled with PBS (0.1 M, pH 7.4) and observed with the confocal microscope.

Synthetic procedures

BODIPY

1. was synthetized according to a previously reported procedure[2]. 706 mg of dimethyl benzaldehyde was dissolved in a degassed solution of EtOH (20 mL) and DCM (280 mL). 0.974 mL (9.4 mmol) of 2,4-dimethylpyrrole was added and the condensation was initiated with few drops of TFA. The reaction mixture was stirred at RT for 16 hours in the dark. After 16 hours, tetrachloro-1,4-benzoquinone (1144 mg/7.85 mmol) was added, followed by stirring for 30'. The solvents were then removed under vacuum and the dark residue re-dissolved in 150 mL of DCM. DIPEA (4.9 mL) was added and after 30' BF_3OEt_2 (5.2 mL) was added. The mixture was stirred for 3 hours. The crude was eluted on a silica plug using DCM before purification by column chromatography (SiO_2, DCM: hexane/50:50, increasing amount of DCM) to obtain a red powder (850 mg, 49%). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 1.43 (s, 6H), 2.08 (s, 6H), 2.56 (s, 6H), 4.78 (s, 1H), 5.97 (s, 2H), 6.63 (s, 2H).

2. 220 mg of **1.** (0.6 mmol) and dimethylamino-benzaldehyde (1.34 g, 0.009 mol) were dissolved in 50 mL of dry toluene and deoxygenated by purging with di-nitrogen (N_2). Piperidine (2.4 mL), glacial acetic acid (2.9 mL) and a catalytic amount of $Mg(ClO_4)_2$ were added and the reaction mixture was refluxed at 150 °C for 27 hrs with a Dean-stark condenser. The crude was eluted on a silica plug using acetone before purification by chromatography (SiO_2, EtOAc: hexane/2:8, increasing amount of EtOAc). The pure fractions were distilled, and the pure compound precipitated from DCM in hexane to obtain a black powder (145 mg, 40%). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 1.50 (s, 6H), 2.14 (s, 6H), 3.06 (s, 12H), 4.78 (s, 1H), 6.61 (s, 2H), 6.66 (s, 2H), 6.75 (d, *J* = 8.4 Hz, 4H), 7.21 (d, *J* = 16.1 Hz, 2H), 7.56 (d, *J* = 8.5 Hz, 5H), 7.61 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 158.04, 152.38, 151.44, 140.38, 137.26, 136.80, 135.86, 132.44, 129.11, 124.84, 124.50, 117.67, 115.33, 113.93, 112.67, 19.84, 13.65. HRMS-ESI: m/z: calcd. for $C_{39}H_{41}N_4OBF_2$ +: 630,3341 [M+H]+, found: 630,3363.

3. 50 mg of **2.** were dissolved in 10 mL of acetone. 500 µL of CH_3I were added and the solution was stirred in a sealed screw cap vial at 70 °C for 24 hours. The reaction was filtered and the back solid washed with DCM to remove any impurities. A dark blue solid was obtained (50 mg, 96%). ^{1}H NMR (400 MHz, MeOD)

δ 8.07 – 7.87 (m, 4H), 7.80 (d, J = 16.4 Hz, 1H), 7.58 – 7.49 (m, 1H), 6.93 (s, 1H), 6.71 (d, J = 10.1 Hz, 1H), 3.74 (d, J = 7.6 Hz, 9H), 2.11 (s, 3H), 1.58 (d, J = 18.7 Hz, 3H). ^{13}C NMR (101 MHz, MeOD) δ 158.28, 151.79, 146.65, 142.59, 138.93, 136.36, 133.52, 133.12, 128.46, 124.61, 121.49, 121.46, 120.46, 117.92, 114.98, 56.41, 18.45, 12.50. HRMS-ESI: m/z: calcd. for $C_{41}H_{47}N_4OBF_2$+ : 330,1909 [M]2+, found: 330,1891.

p-CNOs

The synthesis of small pristine carbon nano-onions (**p-CNOs**) was performed by thermal annealing of detonation nanodiamonds (d-DNs) of 5 nm average particle size in a tube furnace under a positive pressure of helium at 1650°C.

oxi-CNOs

A dispersion of **p-CNOs** (50 mg) was prepared by ultrasonication (20' at 37 kHz) in 30 ml of a 3 M solution of nitric acid (HNO_3). The solution was stirred under reflux conditions for 48 h. The **oxi-CNOs** were separated from the reaction mixture by centrifugation (15 min at 1800 rpm) and filtered off on a nylon filter membrane (pore size 0.2 μm) and washed with dH_2O, DMF, methanol and acetone. After drying overnight at RT, 51.2 mg of **oxi-CNOs** were obtained as a black powder.

fluo-CNOs

A dispersion of **oxi-CNOs** (10 mg) was prepared by ultrasonication (30' at 37 kHz) in 10 mL of anhydrous DMF. To the mixture were added consecutively 9.2 mg (0.08 mmol) of NHS, 12 mg (0.01 mmol) of DMAP and 14 μL of EDC. The reaction mixture was briefly sonicated and after the addition of 4 mg (0.0044 mmol) of red-BODIPY stirred at room temperature for 20 hrs under di-nitrogen atmosphere. The **fluo-CNOs** were filtered off thought a nylon membrane (pore size 0.2 μm) and washed with fresh DMF, THF and MeOH to remove the unreacted dye and the remained reagents. 12 mg of **fluo-CNOs** were recovered as a black powder.

BODIPY characterization

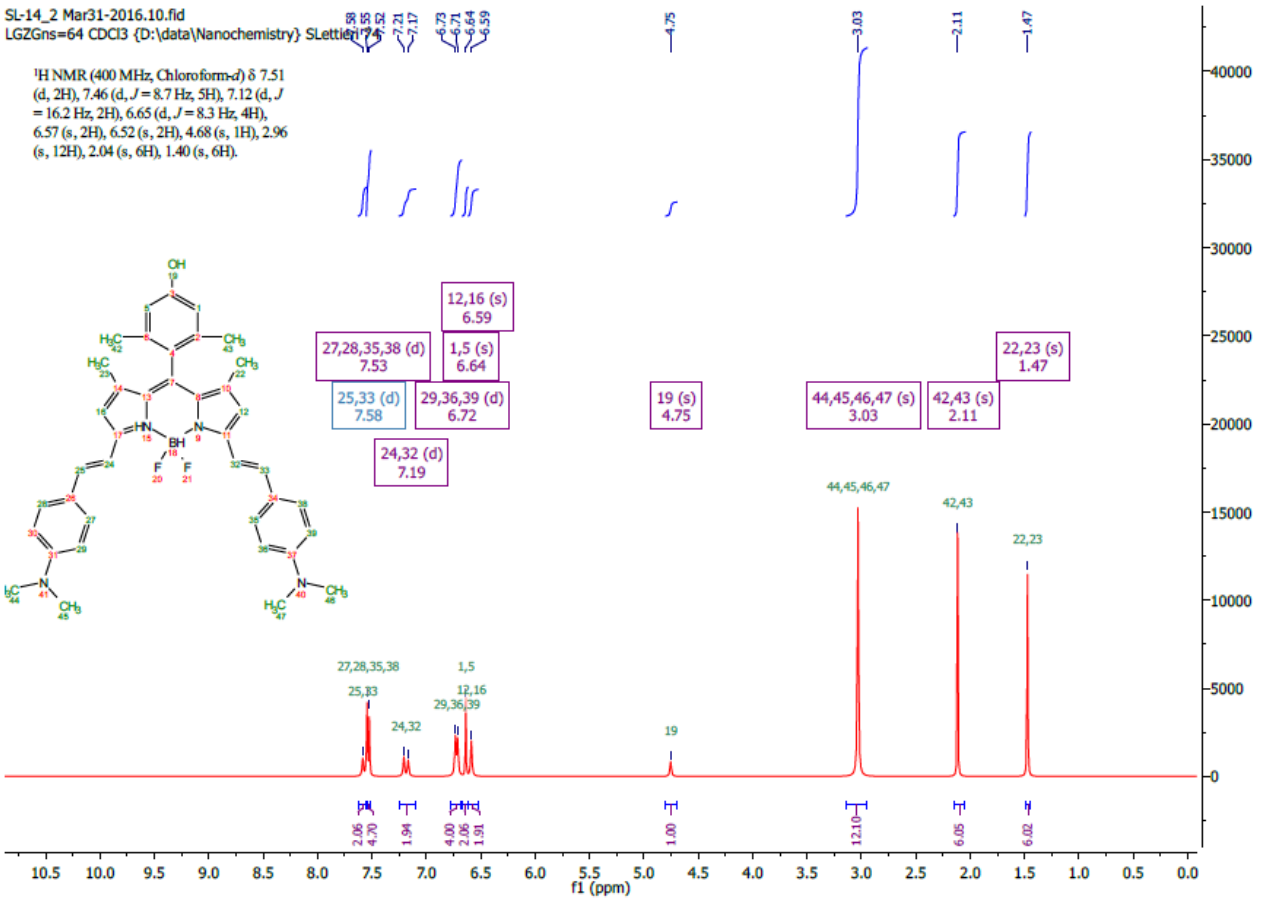

Fig. S1 ^{1}H NMR spectrum in Chloroform-*d* of **BODIPY 2.**

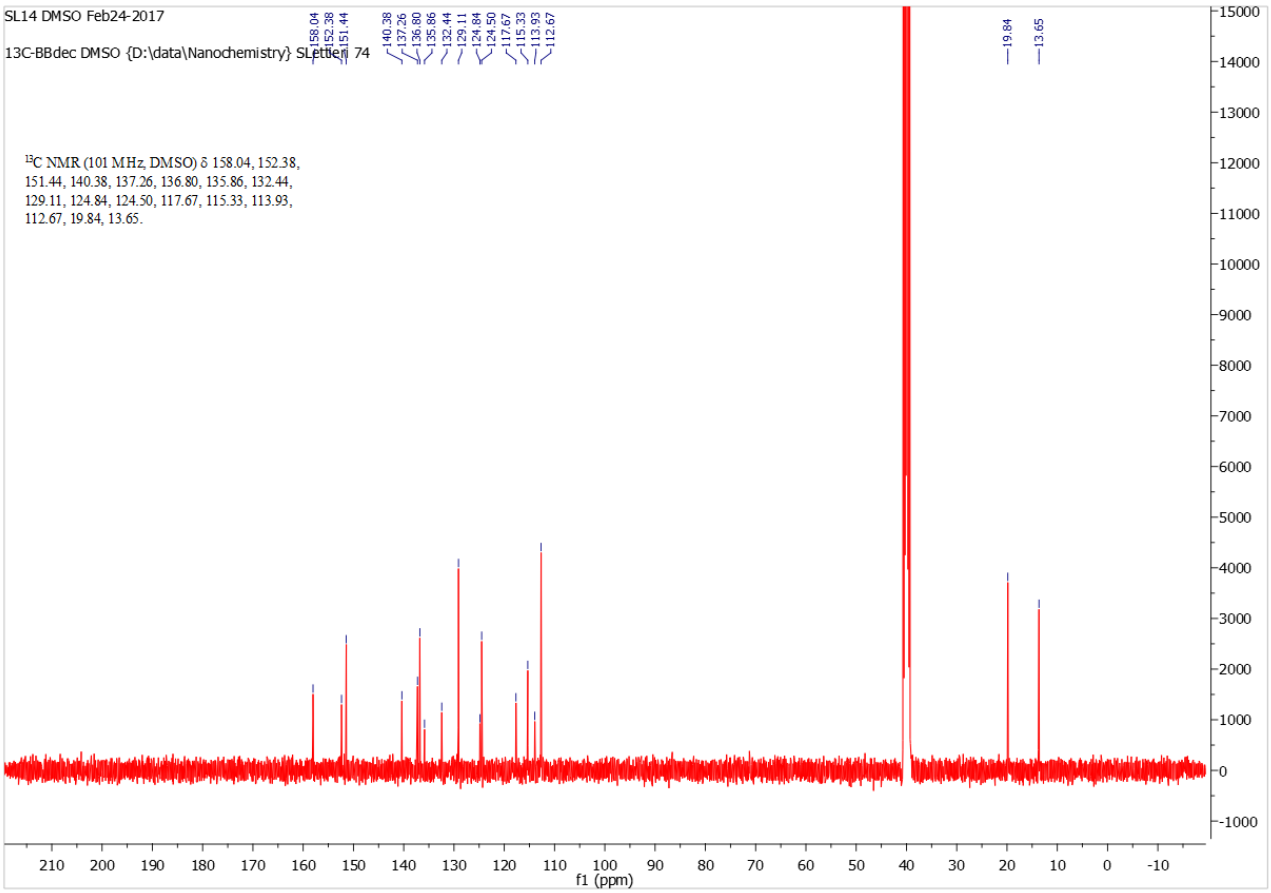

Fig. S2. ^{13}C NMR spectrum in DMSO-d_6 of **BODIPY 2**.

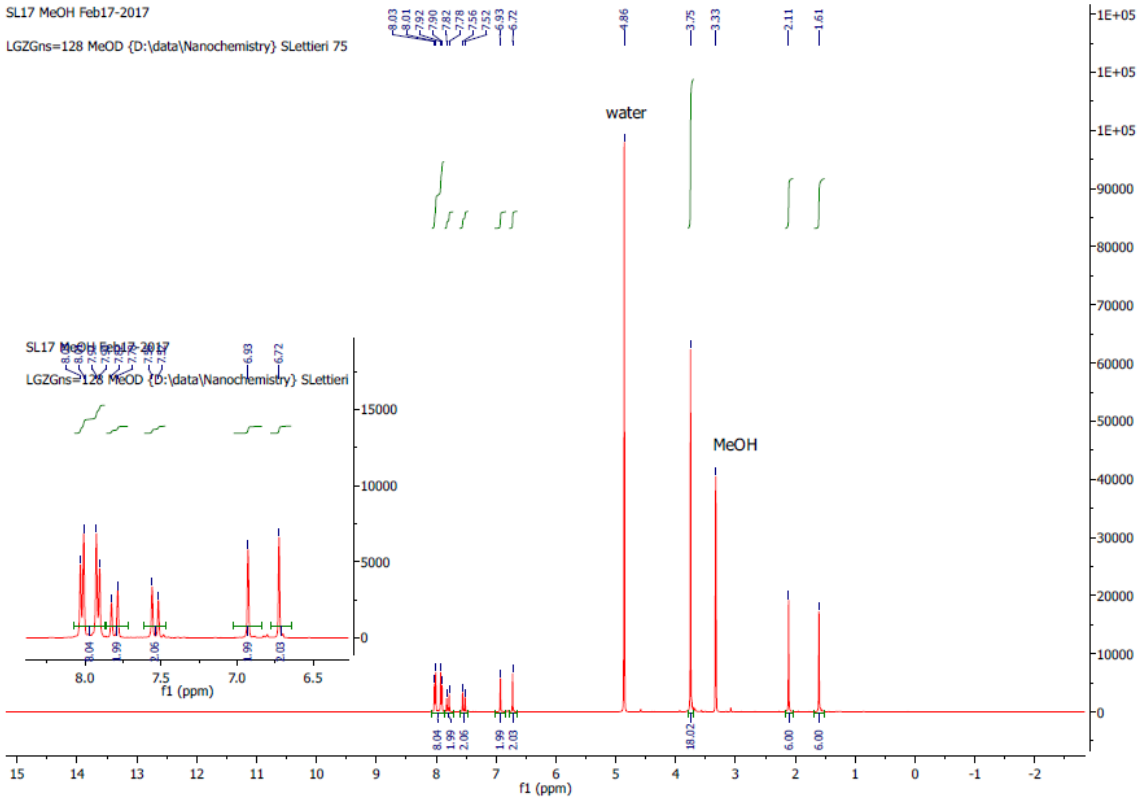

Fig. S3 ^{1}H NMR spectrum in MeOD of **BODIPY 3**.

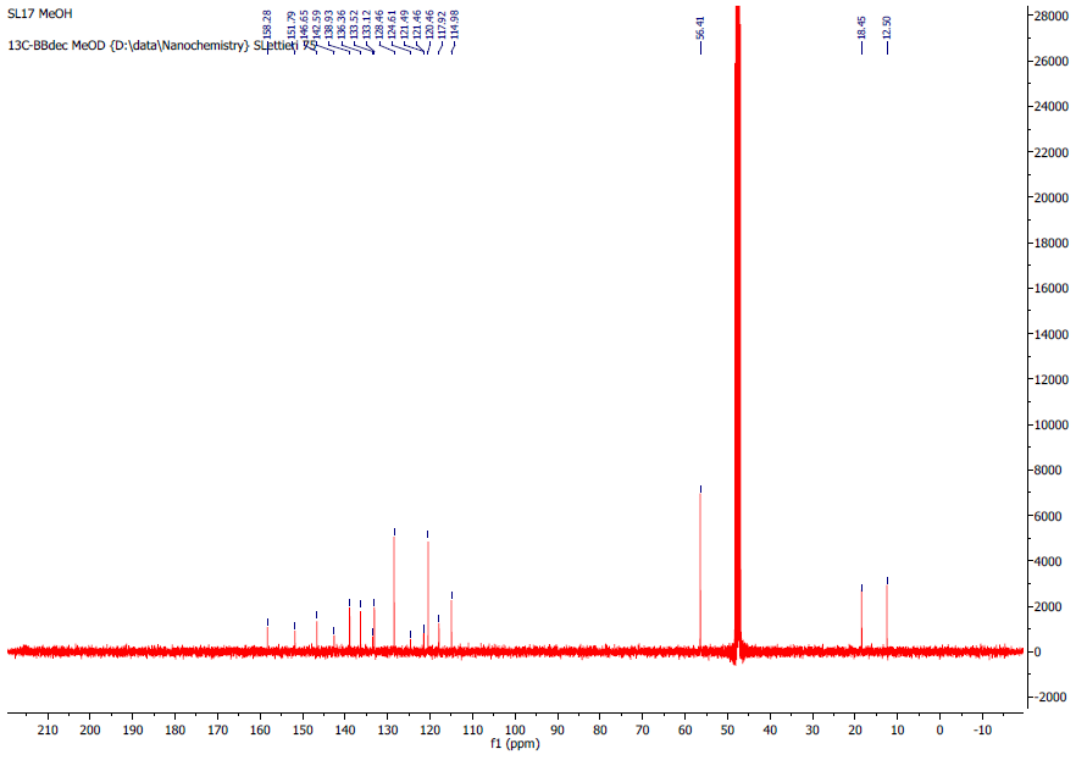

Fig. S4 ^{13}C NMR spectrum in MeOD of **BODIPY 3**.

Spectroscopic characterization

UV-Vis Absorption and Fluorescence Spectroscopy

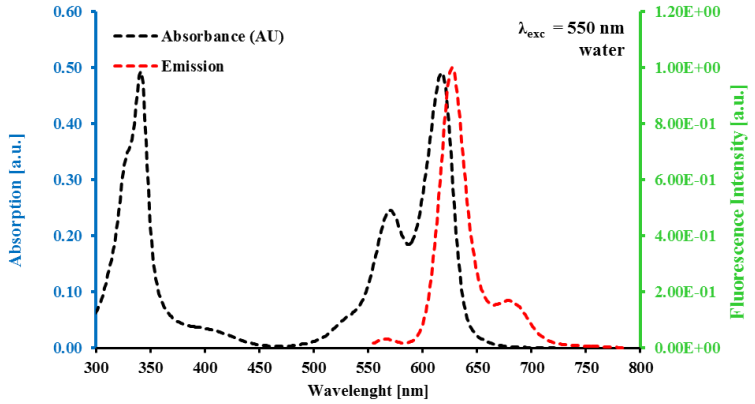

Fig. S5 Absorption (black line) and emission (red line) spectra in water of **BODIPY 3**.

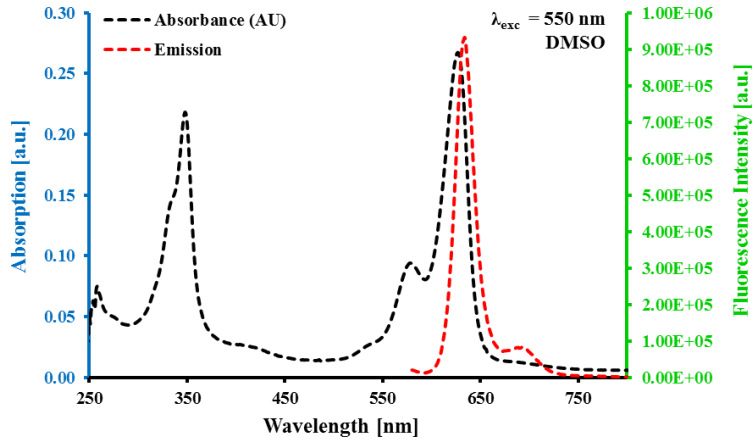

Fig. S6 Absorption (black line) and emission (red line) spectra in water of **BODIPY 3**

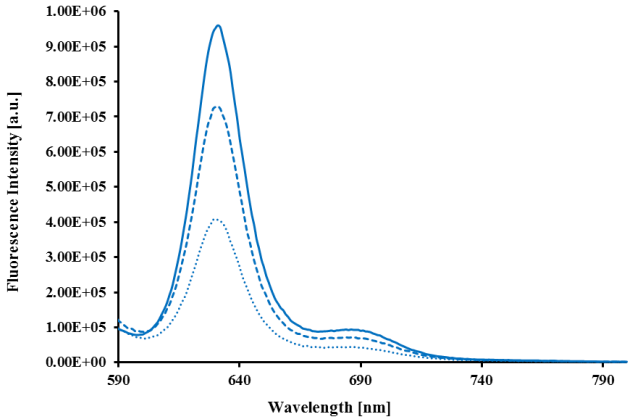

Fig S7 Emission spectra in DMEM of **fluo-CNOs** at 20 μg mL^{-1} (solid line), 10 μg mL^{-1} (dash line); 5 μg mL^{-1} (dot line). Excitation at 561nm; Emission at 632 nm.

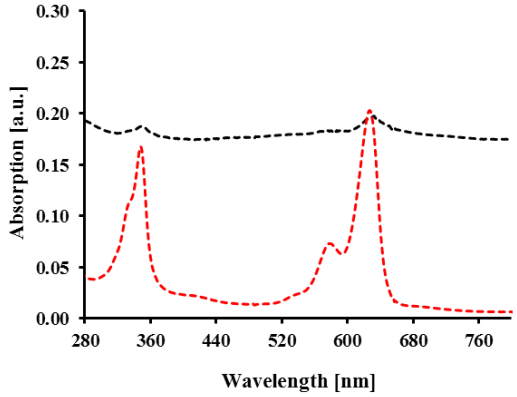

Fig. S8 Absorption spectra of **BODIPY 3** (red line) and **fluo-CNOs** (black line) (20 μg mL^{-1}).

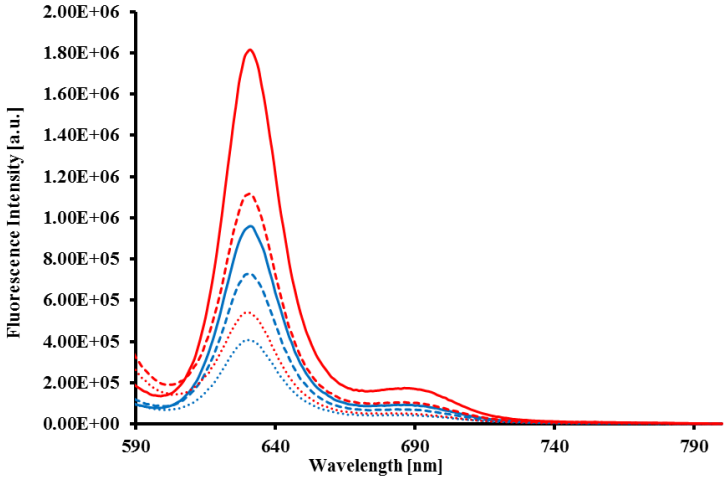

Fig. S9 Emission spectra in DMEM (blue) and 0.01% Tween20 DMEM solution (red) of **fluo-CNOs** at 20 μg mL^{-1} (solid line), 10 μg mL^{-1} (dash line); 5 μg mL^{-1} (dot line). Excitation at 561 nm; Emission at 632 nm.

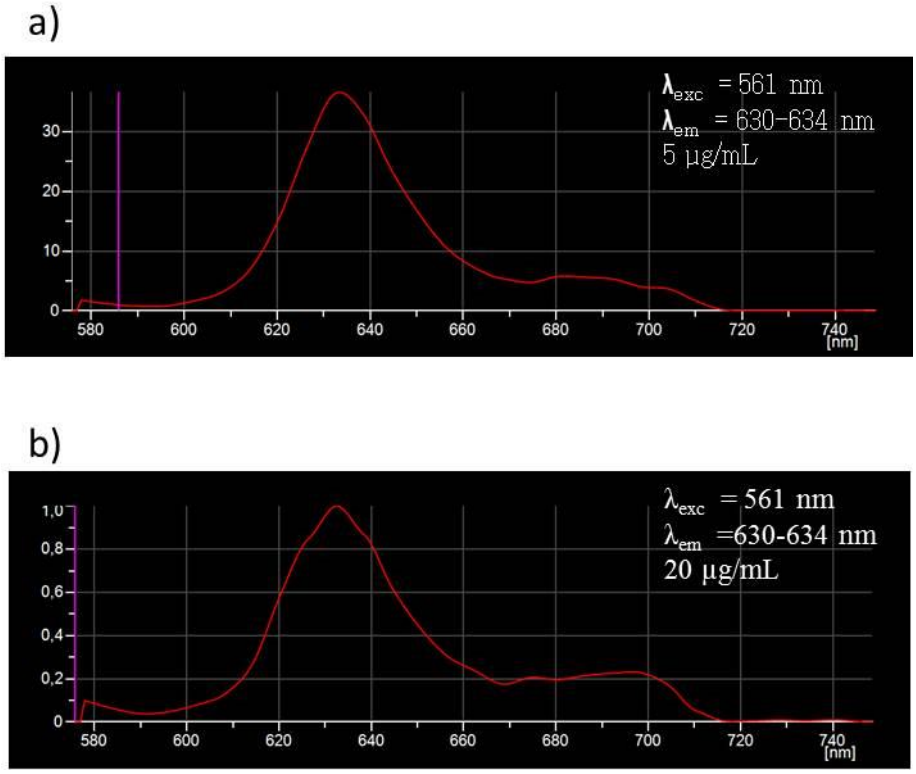

Fig. S10 Laser confocal microscopy emission spectra of **fluo-CNOs** dispersed in DMEM at 5 µg mL^{-1} (a), 20 µg mL^{-1} (b). Excitation at 561nm; Emission at 630-634 nm.

X-ray photoelectron spectroscopy

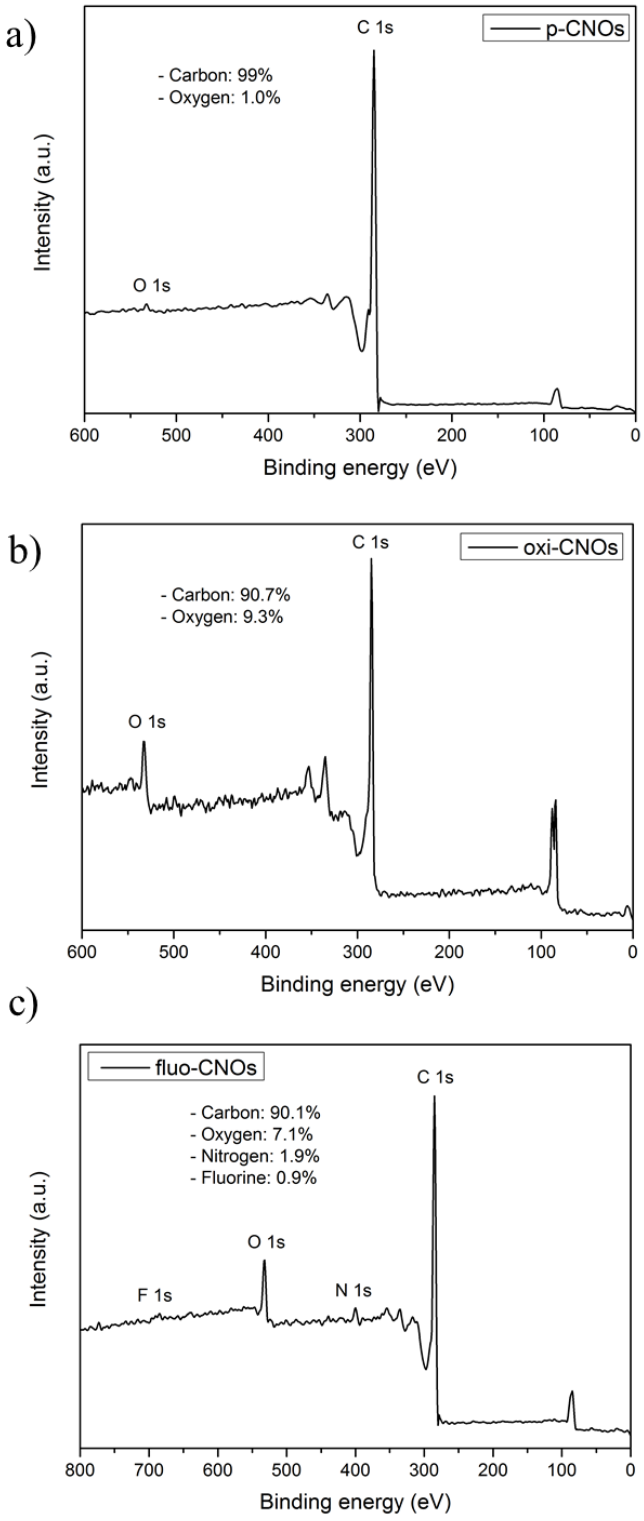

Fig. S11 XPS survey spectra of the functionalized CNOs.

Table S1 Chemical state and area (%) of the different peaks of C1s spectra in functionalized CNOs.

Sample	C-C sp^2 (%)	C-C sp^3 (%)	C-O (%)	C=O (%)	COOH (%)	π-π* (%)	sp^2/sp^3
p-CNOs	62.66	16.47	5.17	3.99	0.18	11.40	3.80
oxi-CNOs	49.03	30.25	4.43	1.95	9.93	4.40	1.62
fluo-CNOs	49.03	30.47	4.79	2.43	9.93	3.36	1.61

CNOs characterization

Dynamic light scattering (DLS)

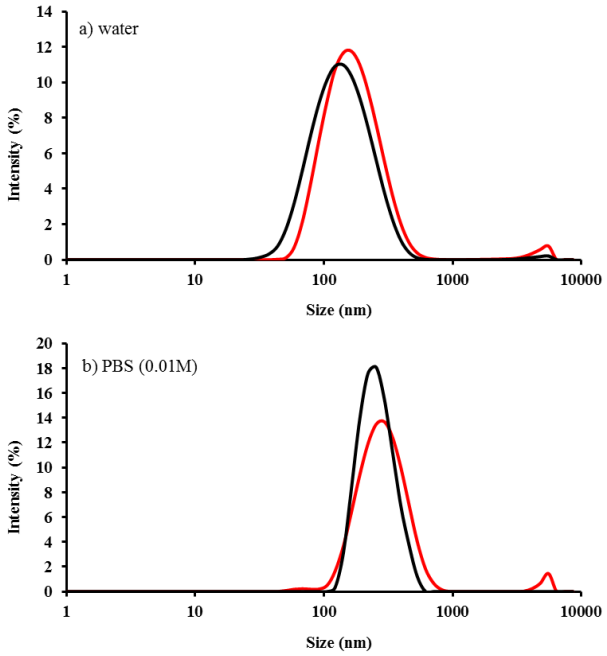

Fig. S12 Effective hydrodynamic diameter of **oxi-CNOs** (black line) and **fluo-CNOs** (red line) at a concentration of 5 µg mL^{-1} in water (a) and in PBS (b).

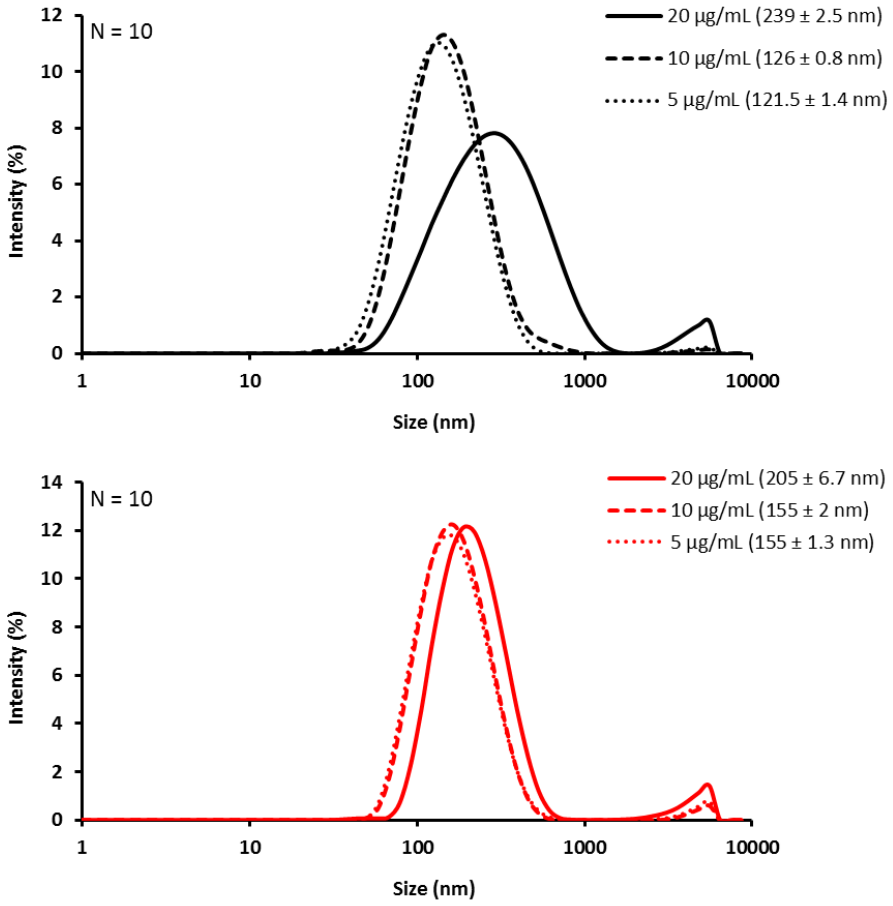

Fig. S13 Effective hydrodynamic diameter of **oxi-CNOs** (black) and **fluo-CNOs** (red) in water at three different concentrations.

Table S2 Effective hydrodynamic diameter obtained from dynamic light scattering (DLS) measurements of **oxi-CNOs** and **fluo-CNOs**.

Average size (nm) Intensity (%) In water	***oxi-CNOs***	***fluo-CNOs***
5 µg mL^{-1}	121 ± 1.4 nm	155 ± 1.3 nm
10 µg mL^{-1}	126 ± 0.8 nm	155 ± 2 nm
20 µg mL^{-1}	239 ± 2.5 nm	205 ± 6.7 nm

Biological data

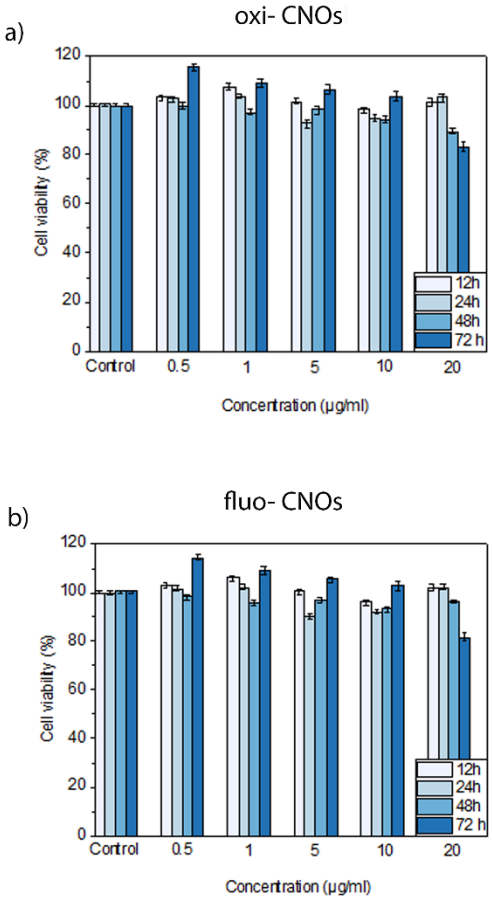

Fig. S14 Cellular viability of Hela cells treated with different concentrations (0.5; 1; 5; 10 and 20 µg mL^{-1}) of **oxi-CNOs** (a) and **fluo-CNOs** (b) for 12, 24, 28 and 72 hours, revealed by WST 1 assay. Viability (%) is evaluated for the samples of CNOs against a non-treated control (ctrl). Data are expressed as mean ± standard error as calculated from three separate experiments.

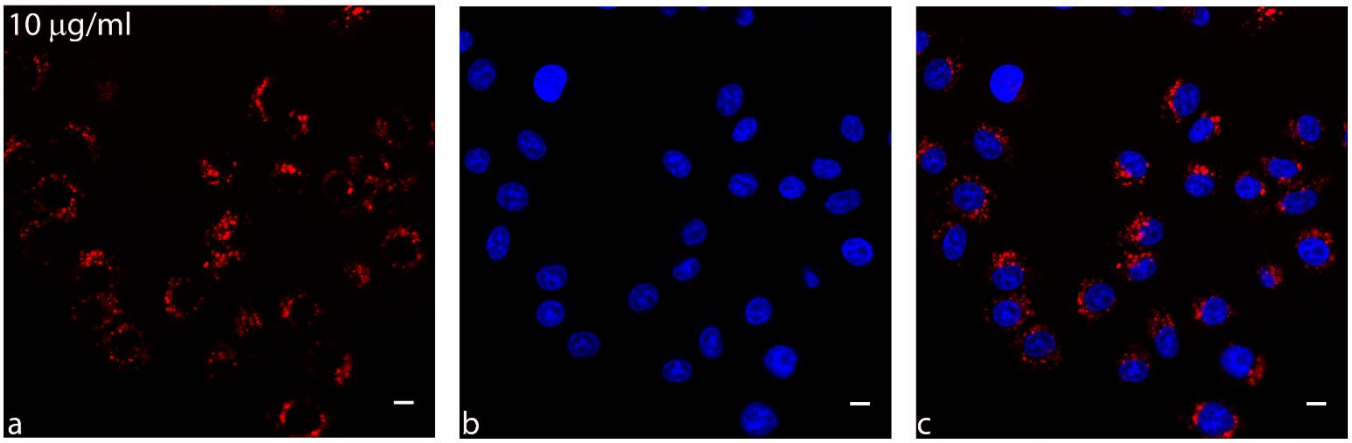

Fig. S15 Confocal images of living MCF7 cells after incubation for 24 hours with 10 µg mL^{-1} of **fluo-CNOs**. (a) **fluo-CNOs** (red); (b) nuclei stained with Hoechst 33342 (blue); (c) merged images. Scale bars=10µm.

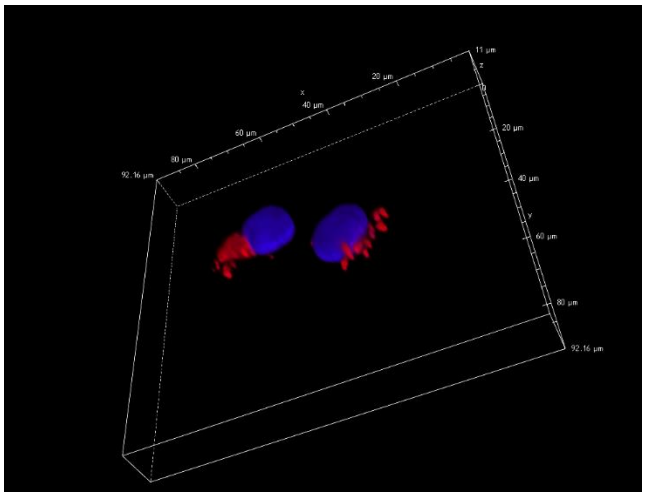

Fig. S16 MCF7 cells volume rendering of the three dimensional confocal stacks. Cells were incubated for 24 hours with 20 µg mL^{-1} of **fluo-CNOs** and stained with Hoechst 33342.

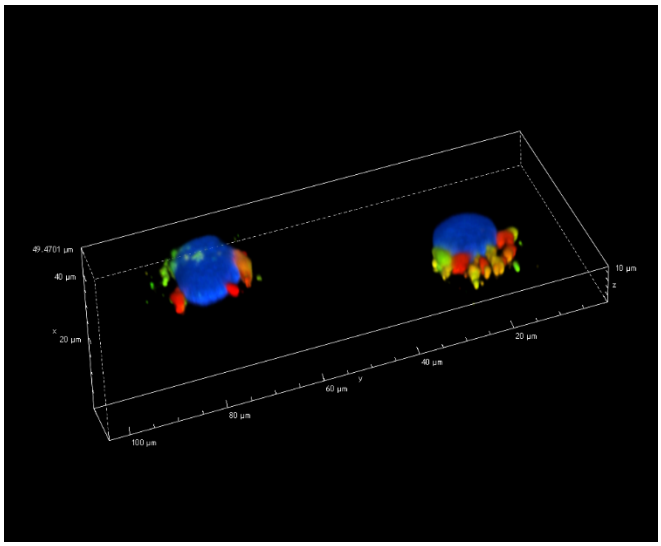

Fig. S17 MCF7 cells volume rendering of the three dimensional confocal stacks. Cells were incubated for 24 hours with 20 µg mL^{-1} of **fluo-CNOs** and stained with Hoechst 33342 and Lysotracker green.

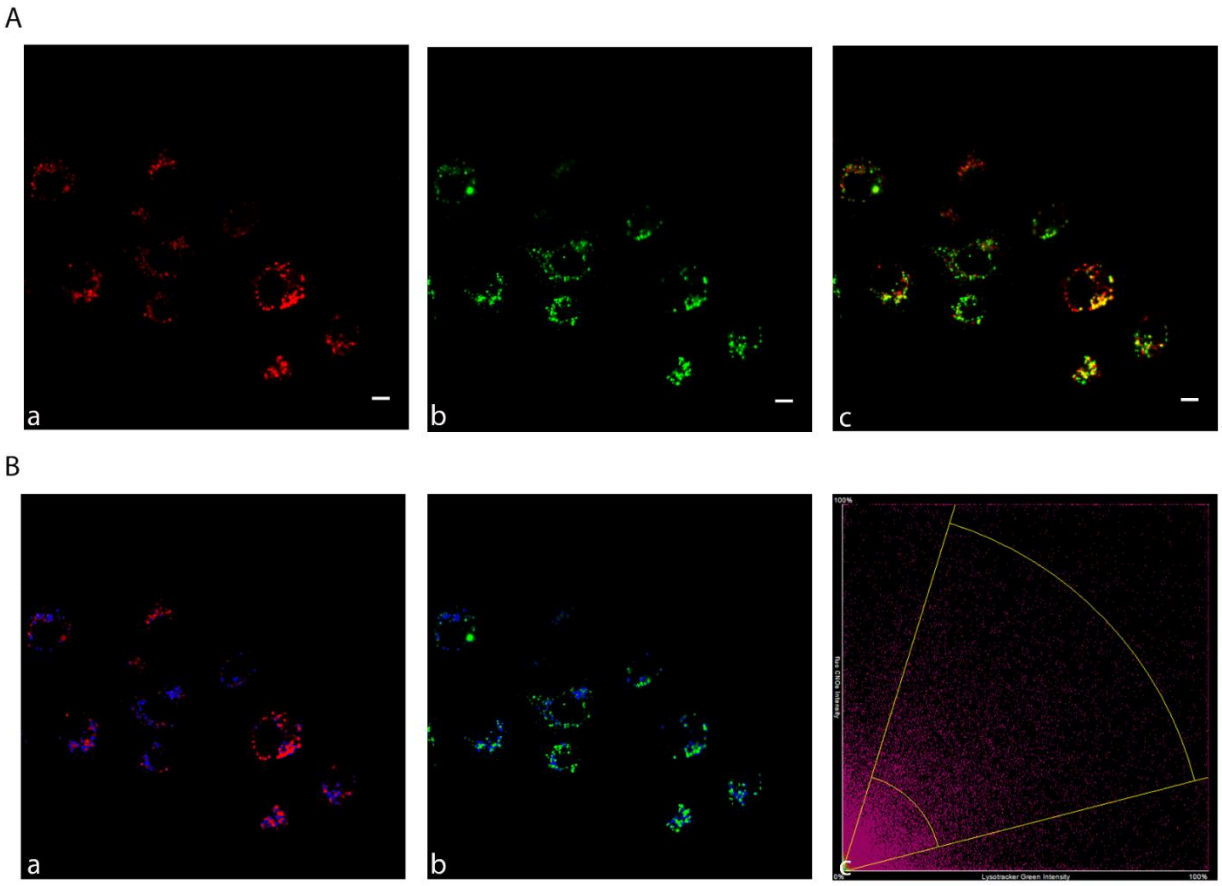

Fig. S18 (A) Confocal images of MCF7 cells after incubation for 24 hours with 20 µg mL^{-1} of **fluo-CNOs**. (a) **fluo-CNOs** (red); (b) lysosomes marked with Lysotracker green (green); (c) merged images. Scale bars = 10 µm. (B) Colocalization mask (blue) illustrating the overlap of red (a) and green (b) signals; (c) graph summarizing thresholder Pearson's correlation coefficient obtained from colocalization of **fluo-CNOs** with Lysotracker green. Pearson's correlation coefficient = 0.60987.

References

1. A.T.R. Williams, S. A. Winfield and J. N. Miller, *Analyst.*, 1983, **108**, 1067-1071.

2. J. Bartelmess, M. Baldrighi, V. Nardone, E. Parisini, D. Buck, L. Echegoyen and S Giordani, *Chem. - A Eur. J.*, 2015, **21**, 9727-9732.