Boron dipyrrromethene (BODIPY) functionalized carbon nano-onions for high resolution cellular imaging†

Juergen Bartelmess,a Elisa De Luca,b Angelo Signorelli,a Michele Baldrighi,a Michele Becce,a Rosaria Brescia,c Valentina Nardone,d,e Emilio Parisini,d Luis Echegoyen,f Pier Paolo Pompa and Silvia Giordani*a

Carbon nano-onions (CNOs) are an exciting class of carbon nanomaterials, which have recently demonstrated a facile cell-penetration capability. In the present work, highly fluorescent boron dipyrrromethene (BODIPY) dyes were covalently attached to the surface of CNOs. The introduction of this new carbon nanomaterial-based imaging platform, made of CNOs and BODIPY fluorophores, allows for the exploration of synergistic effects between the two building blocks and for the elucidation of its performance in biological applications. The high fluorescence intensity exhibited by the functionalized CNOs translates into an excellent in vitro probe for the high resolution imaging of MCF-7 human breast cancer cells. It was also found that the CNOs, internalized by the cells by endocytosis, localized in the lysosomes and did not show any cytotoxic effects. The presented results highlight CNOs as excellent platforms for biological and biomedical studies due to their low toxicity, efficient cellular uptake and low fluorescence quenching of attached probes.

Introduction

In the flourishing research field of nanomedicine, the outstanding results provided by carbon nanomaterials continuously stimulate their improvement in different areas, such as regenerative medicine, drug delivery and bioimaging.1-3 For these purposes, fullerenes,1-3 carbon nanotubes,7,8 carbon diamonds9,10 and graphene based nanostructures11,12 have recently been investigated, providing excellent results and standing results provided by carbon nanomaterials continuously stimulate their improvement in different areas, such as regenerative medicine, drug delivery and bioimaging.1-3 For these purposes, fullerenes,1-3 carbon nanotubes,7,8 carbon diamonds9,10 and graphene based nanostructures11,12 have recently been investigated, providing excellent results and showing, in some cases, the possibility of combining multiple features on a single nano-platform.3 In the search for the best (i.e. the most effective and the least toxic) nano-vector, carbon nano-onions (CNOs)13 have played, until now, only a minor role. Their possible application in nanomedicine has not been fully explored, despite extensive investigations in other fields of research,14 such as tribology,15,16 sensing,17,18 catalysis,19 and their use in supercapacitors.20-22 Their application in the context of biomedicine is limited to very few examples,17,23 in which few-layered (≈5 nm average diameter) CNOs have demonstrated very promising properties in cell penetration, along with low cytotoxicity and low inflammatory potential.23 Furthermore, following synthetic strategies developed for other carbon nanomaterials,24 pristine CNOs (p-CNOs) can be decorated with a large variety of functional groups.14,25 A common reaction for covalent CNO functionalization is the so-called Tour reaction,26 which allows for the introduction of a large variety of functional groups,25 for example benzoic acid. The fluorescent tag used in this study is a boron dipyrrromethene (BODIPY) derivative. BODIPY dyes show excellent optical properties like high molar extinction coefficients as well as high fluorescence quantum yields in combination with good stability.27,28 They are therefore widely used as imaging agents in biology.29-31 In addition, the application of BODIPY dyes in systems for solar energy conversion,32-34 light-driven hydrogen generation,35 and for photodynamic therapy of cancer37,38 has been studied. Electrogenerated chemilumines-
Results and discussion

Synthetic aspects

BODIPY functionalized CNOs (BODIPY-CNOs) and the related benzoic acid-BODIPY ester 2, used as reference compound, were synthesized by an ester condensation reaction (Scheme 1). The raw CNO material (p-CNO) was prepared by the annealing of nanodiamond particles with a diameter of approx. 5 nm, following reported procedures. Benzoic acid functionalities were introduced by reacting p-CNOs with 4-amino-nobenzoic acid and sodium nitrite in an acidified DMF/water mixture, an adaptation of the Tour reaction. Subsequently, benz-CNOs were reacted with N-hydroxysuccinimide (NHS) and the meso-phenol substituted BODIPY 1. The esterification reaction was carried out in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) or N,N’-dicyclohexylcarbodiimide – DCC, as indicated in Scheme 1 and 4-(dimethylamino)pyridine (DMAP) in dry THF. The BODIPY-CNO nanomaterial was purified by subsequent centrifugation and several re-dispersion steps in THF, while 2 was purified by column chromatography.

Reference compound 2

The reference compound 2 was synthesized to study the spectroscopic properties of this new BODIPY fluorophore without being influenced by the presence of the CNO nanomaterial. In general, a close connection between carbon nanomaterials and chromophores makes it difficult to investigate the properties of the bound chromophore in great detail. Usually, a broadening and weakening of the fluorescence signal is observed, mainly due to the size and strong intrinsic absorption of the carbon nanostructures and possible electronic interactions.

X-ray quality crystals of 2 were obtained as orange rhombic plates by re-crystallization from dichloromethane–methanol (1 : 3 v/v) at −20 °C. The diffraction derived structure of 2 is presented in Fig. 1; additional details, tables and a number-

Crystallographic data for 2: CCDC 1015701, C_{26}H_{23}N_{2}O_{2}F_{2}B, M = 444.27, orthorhombic, a = 18.4695(18), b = 43.0104, c = 10.9507(11) Å, α = β = γ = 90°, V = 8699.0(15) Å³, T = 100 K, space group Fdd2, Z = 16, 6167 reflections measured, 2757 independent (R_{int} = 0.043). The final wR_{2} was 0.154.
ing scheme for the molecule are provided in the ESI. 2 crystallizes in the orthorhombic crystal system, space group Fdd2. The boron atom is coordinated in a tetrahedral geometry by two nitrogen and two fluoride atoms. The BODIPY core is near-planar, and the dihedral angle between the two pyrrole rings is 9.58°. This conformation limits the possible resonance of the e member cycle, with the two moieties forming an angle of 79.9(6)°. The planarity of the indacene core, which forms an extended conjugated system, is an essential requirement for the optical properties of this class of compounds. The meso-phenyl group is nearly orthogonal to the indacene 12-member cycle, with two moieties forming an angle of 38.82°. This last value shows a lack of extensive π coupling between the two phenyl groups, although partial delocalization involving the carboxylate group is still possible. Indeed, the rather short carboxylic C–O distance [1.406(6) Å] indicates a partial double bond character. ATR FTIR spectroscopy reveals the presence of a carbonyl stretching band at 1738 cm−1 and no –OH functionality (Fig. S2†), corroborating the successful esterification of 1 with benzoic acid, leading to 2.

### Characterization of the CNOs

**BODIPY-CNOs** were characterized by a wide variety of analytical, spectroscopic and microscopic techniques. Full characterization of the efficiency of nanodiamond conversion and the purity of the p-CNOs was obtained using a combination of TGA, LR and HRTEM (with EELS), Raman and FTIR spectroscopy. Similar techniques were used for the characterization of benz-CNOs, verifying a successful covalent functionalization with benzoic acid by the Tour reaction.

Raman spectroscopy indicated a successful covalent functionalization of the p-CNO starting material, reflected as an increase of the D-band at 1320 cm−1, compared to the

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Toluene</td>
<td>504</td>
<td>91.8</td>
<td>517</td>
<td>13</td>
<td>0.60</td>
</tr>
<tr>
<td>DCM</td>
<td>502</td>
<td>103.6</td>
<td>516</td>
<td>14</td>
<td>0.57</td>
</tr>
<tr>
<td>DMSO</td>
<td>502</td>
<td>86.4</td>
<td>516</td>
<td>14</td>
<td>0.67</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>498</td>
<td>87.5</td>
<td>511</td>
<td>13</td>
<td>0.51</td>
</tr>
</tbody>
</table>

The spectroscopic data for compound 2 in various solvents are summarized in Table 1. Briefly, the absorption spectra of 2 in toluene show an absorption maximum at 504 nm with a molar extinction coefficient of 91.8 × 10³ M⁻¹ cm⁻¹. A minor solvent dependency is observed. In acetonitrile the absorption maximum undergoes a hypsochromic shift of 6 nm to 498 nm. The emission maximum of 2 in toluene is located at 517 nm with a fluorescence quantum yield of 0.60, while the emission maximum in acetonitrile is at 511 nm with a fluorescence quantum yield of 0.51. In comparison, 1 in toluene has an absorption maximum at 503 nm, an emission maximum at 515 nm, and a slightly higher fluorescence quantum yield (0.64). When comparing the absorption spectra of 1 and 2 in acetonitrile, an increased absorption is observed for the latter in the UV-region, with a maximum at 231 nm (Fig. 2). This can be attributed to the absorption of the phenyl group of the benzoic ester moiety. ATR FTIR spectroscopy reveals the presence of a carbonyl stretching band at 1738 cm⁻¹ and no –OH functionality (Fig. S2†), corroborating the successful esterification of 1 with benzoic acid, leading to 2.

![Fig. 1](image1.png)

**Fig. 1** Crystal structure of 2. Structure of a single BODIPY-ester molecule (top) and the remarkable high-symmetry crystal cell, containing 16 symmetry-generated molecules (bottom). Color code: pink: boron; grey: carbon; green: fluorine; light gray: hydrogen; blue: nitrogen; red: oxygen.

![Fig. 2](image2.png)

**Fig. 2** Normalized absorption (left axis) and fluorescence (right axis) spectra of 1 (green) and 2 (orange) in acetonitrile. Excitation at 485 nm.

### Table 1 Photophysical data of 2 in toluene, dichloromethane (DCM), DMSO and acetonitrile

This journal is © The Royal Society of Chemistry 2014
G-band at 1580 cm\(^{-1}\) (Fig. 3). The D/G ratio increases from 0.96 for the \(p\)-CNOs to 1.64 for the benz-CNOs and for the BODIPY-CNOs. FTIR spectroscopy proved the nature of the functional groups introduced based on their characteristic stretching vibrations. While \(p\)-CNOs showed no significant IR bands, benz-CNOs displayed some distinct IR bands in the region between 620 and 1800 cm\(^{-1}\), which changed significantly upon esterification with 1 (Fig. 4). The most distinctive feature is a carbonyl band at around 1640 cm\(^{-1}\) (Fig. 4, inset).

Thermogravimetric analysis confirms the successful functionalization of the CNOs (Fig. S3†). \(p\)-CNOs decompose at around 570 °C, without any weight loss at lower temperatures. Benz-CNOs revealed significant weight loss starting at around 150 °C and decomposed completely at around 570 °C as well. In the low temperature domain, the weight loss of BODIPY-CNOs was significantly larger due to an increased organic functionalization with BODIPY fluorophores. The degree of functionalization of the CNO nanomaterial was estimated from the weight losses, as described in the literature, assuming that one CNO consists of 6 carbon shells. The TGA of benz-CNOs and BODIPY-CNOs performed in air shows a weight loss at 400 °C of about 10% and additional 18%, respectively. We estimated about 55 benzoic acid functionalities per onion for benz-CNOs and approx. 37 BODIPY molecules per CNO for BODIPY-CNOs.

UV-vis-NIR absorption spectroscopy of BODIPY-CNOs revealed the typical absorption features of CNOs as a broad plasmonic absorption over the whole spectral range (Fig. 5) and a distinct absorption band, with a maximum at 502 nm in dimethyl sulfoxide (DMSO), which can be attributed to BODIPY. Upon photoexcitation, fluorescence emission with a maximum at 512 nm was observed. Comparison of the maximum fluorescence intensities of a BODIPY-CNO dispersion with that of a solution of 2 in DMSO allowed for an estimation of the BODIPY-CNO’s fluorescence quantum yield with a value of about 0.17 (i.e. 25% of the fluorescence quantum yield of 2). The BODIPY centered absorption at the excitation wavelength of 490 nm was similar for both samples. All spectroscopic results support the successful covalent functionalization of CNOs with the bright fluorescent BODIPY dye. Notably, while many other dye molecules covalently linked to different carbon nanostructures exhibit a strong fluorescence quenching, which limits their use in imaging applications, BODIPY-CNOs largely overcome this problem. The attachment of BODIPY to the CNOs leads in fact merely to a small reduction of the fluorescence emission. This fluorescence quenching observed for BODIPY-CNOs can be ascribed to the high absorption of the bulk CNO material and not to electron/energy transfer events, which usually result in
very pronounced fluorescence quenching. These conclusions are consistent with our recent report using NIR fluorescent BF₂-chelates of azadipyrromethene dyes in combination with CNOs, and very promising for the design and application of fluorescent labels based on CNOs for biological imaging.

DLS and Z-potential measurements were performed in order to characterize the nanoparticles’ behavior under physiological conditions (Table S2†). DLS measurements were performed in phosphate buffered saline (PBS) at pH 7.4 to mimic the conditions used in biological experiments. Initially, benz-CNOs and BODIPY-CNOs were dissolved in DMSO at a concentration of 1.0 mg mL⁻¹ and then diluted with PBS to a final concentration of 10 μg mL⁻¹. Z-potential measurements were conducted instead in a low ionic strength medium (phosphate buffer 0.01 M, pH 7.4), at a 20 μg mL⁻¹ CNO concentration and without prior dispersion in DMSO, in order not to alter the characteristics of their surface. Under these conditions, BODIPY-CNO agglomerates show a bimodal dimensional distribution, featuring averages of 110 ± 16 nm (43%) and 426 ± 93 nm (57%), which display a Z-potential of −23 mV. As expected, this value is less negative than the one found for benz-CNOs, −39.7 mV.

AFM analyses were performed to estimate the size of the nano-onions. Fig. 6 illustrates two typical topographic images of individual BODIPY-CNOs, deposited on mica. The height distribution analysis of about 100 individual CNOs is plotted in Fig. 6c and clearly reveals the predominance of CNOs with an average diameter of 5 nm and a few larger CNOs.

TEM was used to characterize the carbon nanomaterials and confirmed the initial conversion of nanodiamonds to CNOs. The presence of agglomerates of BODIPY-CNOs with a size of few hundred nm was confirmed by bright field (BF) TEM investigations (Fig. 7a). HR-TEM analysis shows that individual CNOs have an average diameter of 5–7 nm, with 6–8 concentric graphitic shells, 3.4 Å apart (Fig. 7b and S4†). A few larger particles were found, up to 13 nm and 14 concentric graphitic shells. For all the samples EEL spectra at the carbon K-edge region (270–360 eV energy loss) show the typical near-edge fine structure for CNOs (Fig. 7c and S4†), with a narrow peak corresponding to the 1s→π* transition (285 eV), indicating predominantly sp²-bonded carbons and a weaker peak at about 292 eV, corresponding to a 1s→σ* transition.

**In vitro toxicity investigation and cellular biodistribution of CNOs in MCF-7 cells**

In order to investigate the *in vitro* toxicity of different preparations of CNOs, the metabolic activity of MCF-7 cells was determined upon exposure to increasing concentrations of benz-CNOs and BODIPY-CNOs (from 0.5 to 10 μg mL⁻¹). Cell viability was measured after 24, 48, and 72 hours of incubation using the WST-8 test (Water-Soluble Tetrazolium salt) (Fig. 8). The administration of functionalized CNOs did not affect the
cellular viability as compared with the cell control (Fig. 8a), even after prolonged exposure (72 h) (Fig. 8a and b).

To visualize the intracellular distribution of CNOs, confocal live cell imaging was performed using fluorescent BODIPY-CNOs. Representative images of the subcellular localization of BODIPY-CNOs (10 \( \mu \text{g mL}^{-1} \)) in living MCF-7 cells treated for 48 hours with the nanoparticles are shown in Fig. 9. The CNOs were efficiently taken up by the cells and were found to localize predominantly in the cytoplasm and in the perinuclear region (Fig. 9a and d). To probe the intracellular fate of CNOs in greater detail, the distribution of BODIPY-CNOs into vesicular compartments was analyzed in living cells in combination with the Lysotracker probe, a specific marker of lysosomes (Fig. 9b and e). As highlighted by the yellow colocalization signals in Fig. 9c and f, BODIPY-CNOs localize in lysosomal vesicles, in line with previous reports on nanoparticles.56,57 Conversely, unconjugated BODIPY 2 showed only weak staining of cellular membranes (data not shown), as BODIPY is an intrinsically lipophilic fluorescent dye.58

The internalization of CNOs in the lysosomes was further studied by colocalization analysis. Fig. S5B† shows the white colocalization mask applied to the image of BODIPY-CNOs and lysosomes in Fig. S5A.† The white signal (Fig. S5Ba and b†) represents the overlapping regions of green CNOs and red lysosomal signals that are also represented in the middle area of the scatter plot (Fig. S5Bc†). Pearson’s correlation coefficient

**Table 8** Cellular viability of MCF-7 cells after exposure to different CNOs. Viability of MCF-7 cells exposed to CNOs was evaluated by the WST-8 assay. Viability of CNO treated cells was expressed relative to non-treated control cells (Ctrl). Viability of cells treated for 24, 48, and 72 hours (a) with 0.5, 1, and 10 \( \mu \text{g mL}^{-1} \) benz-CNOs and (b) with 0.5, 1, and 10 \( \mu \text{g mL}^{-1} \) BODIPY-CNOs.

**Table 9** Cellular uptake of BODIPY-CNO nanoparticles visualized by confocal microscopy imaging in living cells. Representative confocal images of MCF-7 cells incubated for 48 hours with 10 \( \mu \text{g mL}^{-1} \) BODIPY-CNOs. (a–c) Large field of view with several cells, and (d–f) imaging at single cell resolution. (a, d) Green fluorescent BODIPY-CNOs, (b, e) lysosomes stained with Lysotracker Red probe, (c) merged images. Hoechst 33342 was used for vital staining of the nucleus (f). Note the high level of BODIPY-CNO uptake, and the colocalization (yellow) of the BODIPY-CNO within the lysosomes.
(PCC) was used to quantify the degree of colocalization. The PCC measured was 0.7892.

In Fig. S6 some representative stacks of the optical sectioning of a cell incubated with BODIPY-CNOs (10 μg mL⁻¹) and Lysotracker are shown. The signal of the internalized CNOs matches with the signal of the lysosomes throughout the volume of the cell. The movie of the complete z-stack acquired is shown in the ESI Movie M1.†

In summary, the presented data verify that CNOs can abundantly enter cells without exerting toxic effects on the investigated MCF-7 cells (although further studies are needed to completely elucidate this point). The CNO nanomaterials are internalized by endocytosis and do not enter the nucleus of the cells.

Conclusions

Boron dipyromethene (BODIPY) functionalized CNO conjugates have been synthesized and characterized. The high fluorescence of the nanoparticles allowed high-resolution imaging in MCF-7 human breast cancer cells. The CNOs were efficiently taken up by the cells and localized in lysosomes. Cell viability measured up to 72 hours following incubation did not show significant cytotoxicity. BODIPY-CNO conjugates have the necessary characteristics for further development of theranostic nano-platforms which combine targeting, imaging and therapeutic capabilities, due to their low cytotoxicity and the low fluorescence quenching of the attached fluorescence probes. In combination with the synthetic versatility of BODIPY dyes, CNOs are very promising for the future preparation of nanomaterials with tailor-made photophysical properties for various biological and theranostic applications.

Experimental

Materials and methods

All starting materials, reagents and solvents were purchased from Sigma-Aldrich in high-purity grade and used without further purification. SPECTROPHOTOMETRIC or HPLC grade solvents were used for UV-vis and fluorescence studies. All measurements were performed at room temperature and under ambient conditions, unless otherwise noted. All instrumental details and additional procedures are summarized in the ESI.†

Synthetic procedures

1: BODIPY fluorophore 1 was synthesized following a previously published procedure.13

2: Benzoic acid (122.1 mg/1.00 mmol), NHS (115.1 mg/1.00 mmol), DCC (206.3 mg/1.00 mmol) and DMAP (122.2 mg/1.00 mmol) were dissolved in 40 mL of dry THF at 0 °C under a N₂ atmosphere. The solution was allowed to warm up to room temperature and stirred for another 5 h. Then, 1 (67 mg/0.20 mmol) dissolved in 10 mL of dry, deoxygenated THF was added and the reaction mixture was stirred at room temperature for 5 days. The solution was filtered and after evaporation of the THF, the crude product was purified by column chromatography (SiO₂, hexane–dichloromethane (DCM) 3:1 (v/v) with rising amounts of DCM). 2 was obtained as a bright orange solid in 33% yield (29 mg/0.065 mmol). X-ray quality crystals were obtained as orange rhombic plates by re-crystallizing from dichloromethane–methanol 1:3 (v/v) in the freezer (−20 °C).¹¹ H NMR (400 MHz, CDCl₃) δ 8.23 (d, 2H, J = 8.3 Hz), 7.67 (t, 1H, J = 7.5 Hz), 7.54 (t, 2H, J = 7.8 Hz), 7.37 (m, 4H), 6.01 (s, 2H), 2.57 (s, 6H), 1.48 (s, 6H).¹³ C NMR (100 MHz, CDCl₃) (14.6, 121.4, 122.7, 126.6, 128.6, 128.7, 129.3, 130.2, 131.5, 132.6, 133.9, 140.1, 143.2, 151.6, 155.8). HRMS-ESI: [M+H]⁺ calculated for C₂₆H₂₄BF₂N₂O₂: 445.1899 [M + H]⁺, found: 445.1911. 

Benz-CNO: NaNO₂ (1.47 g/21.3 mmol) was dissolved in 20 mL deionized (DI) water and cooled to 0 °C. This solution was added at once to a solution of 4-aminobenzoic acid (2.88 g/21.0 mmol) in 30 mL DMF at 0 °C. Conc. HCl (200 μL) was added and the mixture was stirred for 30 min at 0 °C. p-CNOs44–46 (31 mg) were dispersed in 20 mL DMF by ultrasonication for 20 min and the dispersion was added to the reaction mixture, which was stirred at 0 °C for 4 h and at RT for additional 3 days. Following this, the CNOs were separated from the reaction mixture by centrifugation (30 min, 2100 g) and purified by subsequent re-dispersion - centrifugation steps in DI water, DMF, and methanol. After drying at 60 °C overnight, 25 mg of benz-CNOs were recovered.

BODIPY-CNO: Benz-CNOs (10 mg) were dispersed in 20 mL dry THF and the dispersion was deoxygenated with N₂. Then NHS (9.2 mg/0.08 mmol), DMAP (12.0 mg/0.08 mmol) and EDC (12.4 mg/14 μL/0.08 mmol) were added and the mixture was heated under reflux for 1 h. Following this, 1 (13.6 mg/0.04 mmol) was added and the reaction mixture was heated for another 44 h under reflux. After cooling to room temperature, the CNOs were precipitated by centrifugation (30 min/2100g) and separated from the supernatant. Subsequently, the solid was re-dispersed by brief ultrasonication in THF and again centrifuged. This process was repeated four more times. The obtained solid was dried at 60 °C overnight. Approx. 12 mg of BODIPY-CNOs were recovered.

CNO preparation for cellular studies. Benz- and BODIPY-CNOs were dispersed in DMSO (1 mg mL⁻¹) and intensively sonicated as previously described.23 For cellular in vitro experiments, all CNOs preparations were sonicated for 15 min at 50 kHz (100% intensity), diluted to the desired concentrations in a cell culture medium (DMEM), and sonicated for 15 min at 50 kHz before adding to the cells.

Cell cultures. MCF-7 cells (human mammary gland adenocarcinoma cell line ATCC HTB-22) were cultivated in DMEM with 50 μM glutamine, supplemented with 10% FBS, 100 U mL⁻¹ penicillin and 100 mg mL⁻¹ streptomycin. Cells were incubated in a humidified and controlled atmosphere with a 95% to 5% ratio of air/CO₂, at 37 °C.

WST-8 assay. MCF-7 cells were seeded in 96 well microplates at a density of 5000 cells per well at a final volume of 100 μL and incubated for 24 h in a humidified atmosphere at 37 °C.

This journal is © The Royal Society of Chemistry 2014 Nanoscale, 2014, 6, 13761–13769 | 13767
and 5% CO₂ to obtain a subconfluent monolayer (60–70% of confluence). The culture medium was removed and replaced with 100 µL of medium containing CNOs at the final concentrations of 0.5, 1, and 10 µg mL⁻¹. The metabolic activity of all cultures was determined after 24, 48 and 72 h of exposure to CNOs, using a standard WST-8 assay (Sigma). Assays were performed following the procedure previously described. Data were expressed as mean ± SD. Differences in cell proliferation between cells treated with CNOs and the control were considered statistically significant with a p-value <0.05.

Confocal microscopy. Fluorescence imaging was performed with a SP8-STED microscope (Leica Microsystems, GmbH, Germany) using a 63× oil immersion objective (HC PL APO CS2 63×/1.40 OIL). BODIPY-CNOs were excited at 488 nm and the emission was acquired in the spectral window at 500–560 nm. Lysosomes were imaged by exciting the LysoTracker Red DND-99 with the 577 nm line of the white light laser (WLL, Leica), and acquired in the emission range of 600–680 nm. The nucleus stained with Hoechst 33342 (Sigma) was excited with the 405 nm wavelength and acquired at 415–480 nm.

CNOs incubation for cellular imaging. MCF-7 cells were seeded in 3.5 cm glass bottom dishes (World Precision Instruments, FD35-100) and incubated for 24 h in a humidified atmosphere at 37 °C and 5% CO₂ to obtain a subconfluent monolayer (60–70% of confluence). After 24 h the medium was removed, and the cells were incubated with a suspension of BODIPY-CNOs (10 µg mL⁻¹). As a control, cells were left untreated (not shown). After 48 h of incubation at 37 °C with CNOs, the cells were washed three times with PBS (pH 7.4) and incubated for 30 minutes with 75 nM LysoTracker Red DND-99 (L7528, Life Technologies). The medium was then replaced with a fresh medium and the cells were transferred to the microscope incubator (Life Imaging Services, Switzerland). The temperature was maintained at 37 °C using the Cube and Box temperature control system, and the humidified 5% CO₂ atmosphere was maintained using an automated gas mixer system (The Brick; Life Imaging Services).

Acknowledgements

We are grateful to the Istituto Italiano di Tecnologia (IIT) for funding. The authors wish to thank Dr Agustin Molina-Ontario (UTEF) for the preparation of the pristine CNOs; Dr Marco Frasconi, Dr Marco Salerno, Dr Farouk Ayadi, Sine Mandrup Bertozzi, Giammarino Pugliese (IIT Genova) for instrumental support and helpful discussions. LE wishes to thank the NSF, PREM Program (DMR-1205302) and the Robert A. Welch Foundation (Grant AH-0033) for generous support.

Notes and references


