Electronic Supplementary Material (ESI) for RSC Advances.

Comparative assessment of the *in vitro* toxicity of some functionalized carbon nanotubes and fullerenes

Carolina Canapè, Stéphanie Foillard, Roberta Bonafè, Alessandro Maiocchi, Eric Doris

CHEMISTRY

BIOLOGY

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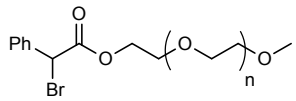
1. General

Chemicals were purchased from Aldrich and used as received. Multi-walled carbon nanotubes (Nanocyl 3150, 95+C%) were purchased from Nanocyl (Sambreville, Belgium). Methyloxy-PEG-amines were purchased from Iris Biotech (Marktredwitz, Germany). C60-fullerenes were purchased from MER Corp (Tucson, USA). ^{1}H NMR were recorded on a Bruker Avance spectrometer (400 MHz). Transmission electron microscopy images were collected on a field emission Philips CM12 microscope equipped with a CCD camera operated at 100 kV. XPS spectra were recorded on a VG ESCALAB 210 spectrometer. Quantification of the degree of functionalization was achieved by X-Ray Photoelectron Spectroscopy (XPS) (oxygen O1s peak at 533 eV and amine N1s peak at 400 eV).

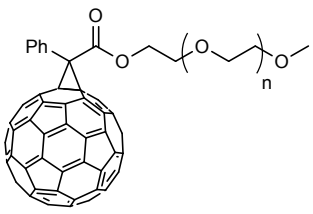
2. Preparation of functionalized fullerenes

*2.1. Synthesis of polyethylene glycol 2-Bromo-2-phenylacetate **1** (for fullerene functionalization)*



A typical procedure is given for the synthesis of PEG_{5000} derivative. PEG_{2000} and PEG_{10000} were prepared according to the same procedure. 2-Bromo phenyl acetic acid (215 mg, 10 equiv.) and PEG_{5000} monomethyl ether (1 g, 1 equiv.) in dry CH_2Cl_2 (20 mL) were cooled to 0 °C. Dicyclohexylcarbodiimide (DCC) (309 mg, 15 equiv.) and dimethylaminopyridine (DMAP) (37 mg, 3 equiv.) were added and the mixture was stirred 30 min at 0 °C and 24 h at room temperature. The precipitate was removed by filtration over celite. The filtrate was dried ($MgSO_4$) and concentrated under vacuum. The product was purified by chromatography over silica (CH_2Cl_2/MeOH 99:1 to 90:10) to give PEG_{5000} 2-bromo-2-phenylacetate **2** as a white, waxy solid and in 94 % yield. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.37 (s, 3H); 3.4-3.85 (m, H-PEG), 4.25-4.4 (m, 2H), 5.38 (s, 1H), 7.3-7.4 (m, 3H), 7.5-7.6 (d, 2H, 6.8 Hz).

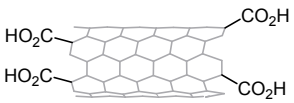
2.2. Functionalization of fullerene with Polyethylene glycol (Full-PEG_{2000}, Full-PEG_{5000}, Full-PEG_{10000})



A typical procedure is given for the synthesis of Full-PEG_{5000}. Full-PEG_{2000} and Full-PEG_{10000} were prepared according to the same procedure. A solution of C_{60} fullerene (14.6 mg, 1 equiv.) in 15 mL of dry toluene was added to PEG_{5000} 2-bromo-2-phenylacetate (200 mg, 1 equiv.) in 5 mL of dry THF. 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) (8 µL, 2.5 equiv.) was added and the mixture was stirred at room temperature for 6 h under nitrogen. The mixture was concentrated under vacuum and the residue was adsorbed over silica and purified by column chromatography over silica (CH_2Cl_2/MeOH 99:1 to 90:10) to give PEG_{5000}-fullerene in 42% yield. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.36 (s, 3H); 3.4-3.85 (m, H-PEG), 4.5-4.6 (m, 2H), 7.3-7.6 (m, 3H), 7.9-8.2 (m, 2H).

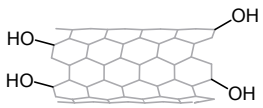
3. Preparation of functionalized carbon nanotubes

3.1. Preparation of short carboxylated carbon nanotubes (sCNT-CO_2H)



Carboxylated carbon nanotubes were prepared by chemical shortening of carbon nanotubes. 100 mg of CNTs were reacted with 16 mL of a 3/1 mixture of 98% sulfuric acid / 65% nitric acid for 3 h at 70 °C. The suspension was diluted with 60 mL of H_2O and centrifuged for 1 h at 10000 rpm. The supernatant was discarded and the precipitate was taken back in 30 mL of H_2O and centrifuged again. This operation was repeated twice using 30 mL of acetone. The precipitate was redispersed again in H_2O under sonication and filtered on a 5 µm PTFE membrane to remove nanotube aggregates. The aqueous phase was filtered on a 0.2 µm polypropylene membrane and the collected nanotubes were washed extensively with H_2O (15 mL). The nanotubes were redispersed in H_2O under sonication and lyophilized to afford 15 mg of chemically shortened CNTs. The oxidative process introduces carboxylic groups (COOH) at the surface of the nanotubes.

3.2. Preparation of short hydroxylated carbon nanotubes (sCNT-OH)



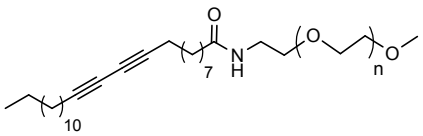
Introduction of hydroxyl groups on the surface of the carbon nanotubes was achieved by lithium aluminium hydride reduction of the carboxylated CNTs (sCNT-CO_2H). Thus, 36 mg of $LiAlH_4$ were dispersed in 1 mL of anhydrous Et_2O, before 34 mg of sCNT-CO_2H were added in one portion. The suspension was sonicated for 1 h in an ultrasonic bath and further stirred for 1 h at room temperature. 35 mg of $LiAlH_4$ in 1 mL of Et_2O were again added and the mixture was stirred for 24 hours at room temperature. The reaction was quenched by 2 mL of ethyl acetate and 2 mL of aqueous 1 N NaOH. The suspension was centrifuged for 30 min at 10000 rpm. The precipitate was redispersed in 10 mL of H_2O and centrifuged again. This operation was repeated with aqueous 1 N HCl and acetone. The precipitate was resuspended in 10 mL of H_2O under sonication and centrifuged at 2000 rpm for 5 min. The black supernatant was collected and lyophilized to afford 20 mg of hydroxylated CNTs.

3.3. Preparation of sCNTs functionalized with PEG amphiphiles (sCNT-PEG_{2000}, sCNT-PEG_{5000} and sCNT-PEG_{10000})

3.3.1. Mechanical shortening of carbon nanotubes (sCNT)

80 mg of CNTs in 30 mL of toluene were first mechanically shorted by continuous ultrasonication (probe) for 4 days (25 W output power). The suspension was centrifuged for 30 min at 10000 rpm, and the supernatant was discarded. The precipitate was taken back in 30 mL of H_2O and centrifuged again. This operation was repeated twice using 30 mL of acetone. The precipitate was finally collected and dried under vacuum to afford 75 mg of shortened sCNT.

3.3.2. Synthesis of PEG-amphiphiles **2** (PEG_{2000}, PEG_{5000}, and PEG_{10000}-amphiphiles) for the coating of CNTs

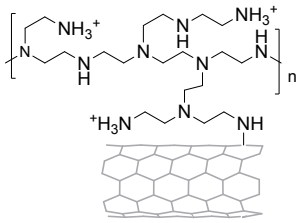


A typical procedure is given for the synthesis of PEG_{5000}-amphiphile. PEG_{2000}-amphiphile and PEG $_{10000}$-amphiphile were prepared according to the same procedure. The starting 2,5-dioxo-pyrrolidin-1-yl pentacosa-10,12-diynoate (activated lipophilic chain) was prepared according to the procedure of Ogier *et al*.[1] Under N_2, a solution of 2,5-dioxo-pyrrolidin-1-yl pentacosa-10,12-diynoate (1.2 equiv., 113 mg), diisopropylamine (1 equiv., 35 µL), and methoxy-PEG_{5000}-amine (1 equiv., 1 g) in anhydrous CH_2Cl_2 (20 mL) was stirred at room temperature for 24 h. After concentration under vacuum, the crude product was purified by chromatography over silica (CH_2Cl_2/MeOH, 95/5) to afford the PEG_{5000}-amphiphile as a waxy white solid in 90% yield. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.75-0.85 (t, 3H, J=6.8 Hz); 1.15-1.25 (m, 22H), 1.25-1.35 (m, 4H), 1.37-1.47 (m, 4H), 1.50-1.60 (m, 2H), 2.05-2.13 (t, 2H, J=7.6 Hz), 2.13-2.20 (t, 4H, J=6.8 Hz), 3.30 (s, 3H), 3.34-3.42 (m, 2H), 3.50-3.62 (m, H-PEG), 3.72-3.76 (t, 2H, J=4.8 Hz), 7.23 (s, 1H).

3.3.3. Functionalization of sCNTs with PEG-amphiphiles **2** (sCNT-PEG_{2000}, sCNT-PEG_{5000}, sCNT-PEG_{10000})

A typical procedure is given for the synthesis of sCNT-PEG_{2000}. sCNT-PEG_{5000} and sCNT-PEG_{5000} were prepared according to the same procedure. The PEG_{2000}-amphiphile was dispersed in 20 mL of H_2O using the ultrasonic probe (20 min, 10 W output power) before 20 mg of mechanically shortened nanotubes (sCNT) were added. The mixture was further sonicated for 30 min (25 W output power). The suspension was centrifuged for 5 min at 2000 rpm and the precipitate (aggregates) was discarded. The supernatant was centrifuged for 90 min at 10000 rpm and the precipitate was collected. The latter was redispersed in 20 mL of H_2O under sonication and centrifugation at 10000 rpm was repeated. This sequence was repeated twice. The precipitate was redispersed in 5 mL of H_2O and the suspension was irradiated at 254 nm for 4 hours to induce polymerization of the amphiphiles around the nanotubes. The suspension was then diluted with 10 mL of H_2O and centrifuged (5 min, 2000 rpm). The black supernatant was collected and lyophilized to afford 16 mg of PEG_{2000} coated CNTs.

3.3.4. Preparation of polyethyleneimine-functionalized carbon nanotubes (sCNT-PEI)



sCNTs (50 mg) were dispersed in 10 mL of DMF using the ultrasonic probe (15 min, 10 W output power) before 25 kDa branched-PEI (250 mg) was added. The mixture was further sonicated for 10 min and stirred for 3 days at 50 °C. The PEI-functionalized nanotubes were collected by filtration over 0.2 µm polypropylene membrane, washed with CH_2Cl_2 (15 mL), DMF (15 mL), and MeOH (3 x 15 mL). Nanotubes were taken back in 10 mL of aqueous pH 1 HCl and sonicated for 10 min. The nanotubes were filtered and washed with H_2O (3 x 15 mL) and MeOH (15 mL). PEI-CNTs were redispersed in H_2O under sonication (10 min) and centrifuged (5 min, 2000 rpm). The black supernatant was collected and lyophilized to afford 28 mg of sCNT-PEI.

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1. Chemicals

Foetal bovine serum (FBS), FGM-2 Fibroblast Basal Medium, RPMI-1640 Medium, Eagle's Minimum Essential Medium (MEM), Dulbecco's Modified Eagle Medium (DMEM), EGM-2 Clonetics Endothelial Cell Growth Medium-2, L-Glutamine, non essential AA, Sodium pyruvate, Trypsin/EDTA, Trypsin Neutralizing Solution and HEPES Buffered Saline were purchased from Lonza. Trypan blue solution, L-Glutamine, Dulbecco's Phosphate Buffered Saline (PBS), MTT (Thiazolyl Blue Tetrazolium Bromide), Physiological saline solution 0.9% and pen-strep antibiotics were purchased from Sigma-Aldrich. LDH assay mixture reagent was obtained from Roche Applied.

2. Sample particles stock solution and media preparation

For *in vitro* assays, nanomaterials were weighted and dispersed in medium containing 5 % serum in order to obtain a stock solution. The sonication method for dispersion was water bath or mild probe sonication to ensure that the energy provided is high enough to de-agglomerate the nanoparticles but sufficiently mild to prevent damaging the surface functionalization of the nanomaterials. Full-PEG_{2000} was sonicated with cycles of 5 minutes in ice cold bath and 1 minute pause until complete dispersion; sCNT-PEG_{2000} was sonicated for 2 minutes with a probe sonicator. Full-PEG_{5000}, Full-PEG_{10000}, sCNT-PEG_{5000}, sCNT-PEG_{10000}, sCNT-CO_2H, sCNT-OH, and sCNT-PEI were simply sonicated for 1 hour in a water bath.

For Full-PEG_{2000}, Full-PEG_{5000} and Full-PEG_{10000} a concentration of 1 mg/mL was selected as stock solution and 1:2 serial dilutions were prepared in order to obtain the following concentrations: 500, 250, 125, 62.5, and 31.25 µg/mL.

Because of optical interference issue for, sCNT-PEG_{2000}, sCNT-PEG_{5000}, sCNT-PEG_{10000}, sCNT-CO_2H, sCNT-OH and sCNT-PEI a concentration of 250 µg/mL was selected as stock solution and diluted to 125, 100, 75, 50, 25 µg/mL.

Hemolysis test was performed on 1 mg/mL preparations obtained by weighing dry powders and dispersing them in physiological saline 0.9%; the obtained dispersions were then sonicated for 1 hour in a water bath.

3. Cell Culture

All cells derived from cryo-cultures; all cell lines were maintained as a monolayer culture in 75 cm^2 plastic tissue culture flasks, excepted murine macrophage J774 which were grown in sterile 55 cm^2 dishes. Human hepatocellular carcinoma (HepG2) cells were seeded in Eagle's minimum essential medium (MEM) and supplemented with 2 mM L-Glutamine, 0.1 mM non-essential AA, 1 mM Sodium pyruvate, 10% fetal bovine serum (FBS) and pen-strep antibiotics (2%). Normal human dermal fibroblast (NHDF) cells were seeded in FGM-2 (Fibroblast Growth Medium-2 medium) and supplemented with pen-strep antibiotics (2%) and 5 % fetal bovine serum. Human epithelial colon cancer (CaCo2) cells were cultured in Eagle's Minimum Essential Medium (MEM) supplemented with 20% fetal bovine serum (FBS) and with pen-strep antibiotics (2%). Mouse granulocytes (U937) were cultured in RPMI 1640 medium containing 10% (v/v) fetal bovine serum (FBS) and with pen-strep antibiotics (2%). Human umbilical vein endothelial (HUVEC) cells were cultured in Clonetics Endothelial Cell Growth Medium-2 (EGM-2) supplemented with pen-strep antibiotics (2%). Murine macrophage J774 A1 cells were cultured in DMEM medium supplemented with 10% (v/v) fetal bovine serum (FBS) and with pen-strep antibiotics (2%). Cultures were maintained at 37 ºC in a humidified atmosphere containing 5% CO_2.

4. Cell viability

Cell viability was evaluated immediately before incubation with test materials using trypan blue staining. The cell suspension was mixed with an equivalent volume of 0.4% trypan blue solution, and cell counting was performed using hematocytometer with a light microscope.

4.1. Cytotoxicity assay

For cytotoxicity assays cells were seeded in 96-well microplates at a density of 100,000-150,000 cells/mL in medium containing 5% FBS and pen/strep. After 24 h of cell attachment (60-80% confluence), plates were washed with 100 µL/well phosphate buffered saline (PBS) and the cells were treated with increasing concentrations of each nanomaterial (200 µL/well) prepared in 5% FBS containing medium for 24 h. Three replicate wells were used for each control and test concentrations per microplate. Cytotoxicity was assessed using the assays as outlined below.

4.1.1. MTT assay

The MTT assay is based on the protocol described by Mossmann.[2]

Briefly, for the purposes of the experiments at the end of the 24 h incubation time, the plates were discarded from the remaining liquids and washed with 100 µL phosphate buffered saline (PBS)/well. An aliquot of 100 µL of MTT (5 mg/mL) prepared in culture medium, was added to each well and the plates were incubated for 3 h at 37 °C in a 5% CO_2 humidified incubator. After this incubation period the medium was discarded, then 150 µL of isopropanol were added to each well to extract the dye and the plates were shaken at 240 rpm for 10 min. The homogenous solutions were then transferred to 96-well filtering plates (0.22, 0.45 or 1 µm pore size), and centrifuged at 1500 rpm for 5-15 minutes to ensure elimination of the carbonaceous nanoparticles. The absorbance was measured at 570 nm on the plate reader (Perkin-Elmer). All values were corrected from background absorbance (no cells). The absorbance values of control cells was set as 100 % viability and MTT conversion of treated cells was expressed as a percentage relative to the control cells as follow:

$$\text{Viability \%} = \frac{A\ sample - A\ blank}{A\ control - A\ blank}$$

4.1.2. LDH assay

Cytotoxicity induced by nanoparticles was assessed by lactate dehydrogenase (LDH) leakage into the culture medium. Following exposure about 120 µL of all supernatants were recovered and transferred to 96-wells filtering plates with 0.22, 0.45 or 1 µm pore size, depending on compounds behavior and size and centrifuged at 1500 rpm for 5-15 min order to recover cleared supernatants. The lactate dehydrogenase (LDH) assay is a means of measuring membrane integrity as a function of the amount of cytoplasmic LDH released into the medium. The LDH assay was performed according to the instructions provided by Roche Applied Company. In each plate, 3 wells of cells were left unreacted (low control) while 3 others were treated with the lysis solution provided in the kit to induce total release of LDH (high control). LDH assay enzyme was prepared by reconstituting the lyophilisate enzyme in 1 mL double distillate water for 10 min and mixed thoroughly. Next, the LDH assay mixture was made by mixing equal amounts of the assay substrate, enzyme and dye solutions. An aliquot of 100 µL of this mixture was added to the cleared supernatants. The plates were protected from light and incubated at room temperature for 20–30 min. The absorbance was measured at wavelength of 490 nm on the plate reader (Perkin-Elmer). Percentage of viability was calculated as follows:

$$\text{Viability \%} = 100 - \frac{A\ sample - A\ low\ control}{A\ high\ control - A\ low\ control}$$

4.1.3. Hemolysis test[3]

Hemolysis tests were performed on rat and human blood. Rat blood was obtained by canulating the abdominal aorta of a male rat, and collecting about 10 mL of blood in a lithium-heparin test tube. Human blood was purchased from a local Transfusion Centre; 5 mL of blood from 4 donors were collected in lithium-heparin test tubes. All samples were maintained at 2-8 °C, until their use and just before performing the assay, a blood pool of the human blood donors was made by mixing 1 mL of each sample. Positive and negative controls were prepared by adding respectively 1 mL of isotonic and hypotonic solution (physiological saline 0.9 % and double distilled water) in test tubes. Sample solutions were prepared as described in the paragraph above (see sample particles stock solution and media preparation) and conditioned at 37 °C in a water bath. An aliquot of 100 µL of fresh blood (rat or human) was added to each test tube, gently mixed and incubated for 30 minutes in a static water bath; after incubation, all test tubes were centrifuged at 2110 *g* for 15 min. Separated plasma were transferred on a 96-well filtering plate, centrifuged for 15 min at 2110 *g* to clear them from nanoparticles, and read in a spectrophotometer at 490 nm to detect hemoglobin that is spread into the solution in case of cell disruption. The percentage of hemolysis was obtained correcting optical densities (O.D.) values from blank absorbance, and then by applying the following formula:

% Hemolysis = (O.D. Test Article – O.D. Negative Control)/(O.D. Positive Control – O.D. Negative control)

Hemolytic potential was evaluated by in accordance with the following classification: formulations with a hemolysis value of <10% to be nonhemolytic while values > 25% to be at risk for hemolysis.[4]

4.1.4. Statistical Analysis

Each experiment was repeated at least three times in triplicate and mean±SD for each value was calculated. Results are given relative to 'control cells' (100% viability). The statistical significance between groups was assessed with GraphPad Prism 5 built-in software. Results were compared by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. All results were presented as mean ± SD; *p* values of less than 0.05 were considered to be statistically significant. Data (where appropriate) were fitted to a sigmoidal curve and a four parameter logistic model used to calculate the half maximal inhibitory concentration (IC_{50}), which was the concentration of nanomaterials which caused a 50% cell growth inhibition in comparison to untreated controls. The IC_{50} values were reported ±95% Confidence Intervals (±95% CI).

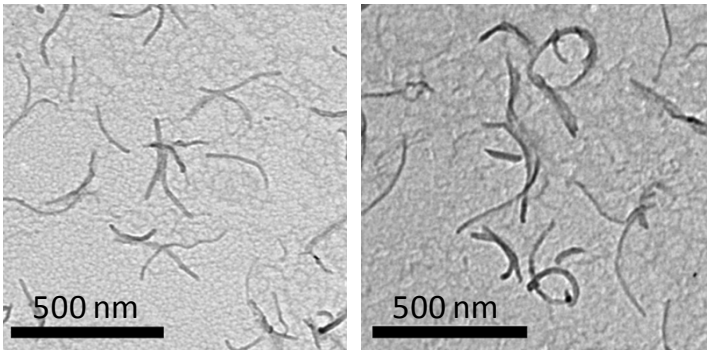


Figure S1: TEM images of mechanically shortened CNTs.

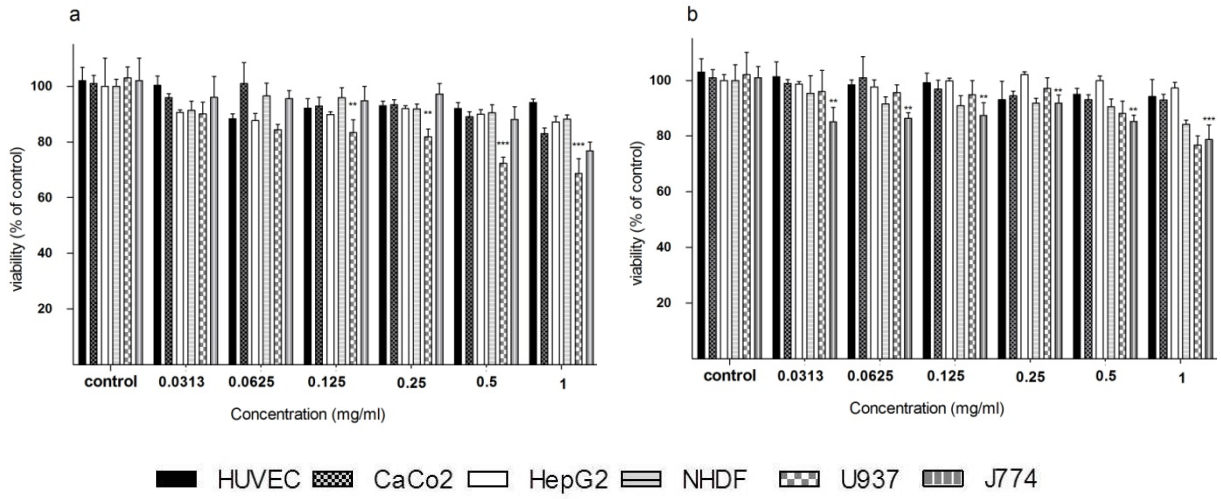


Figure S2. Viability of cells assessed with the MTT assay, after incubation for 24 h with a) Full-PEG_{5000} and b) Full-PEG_{10000}. Results are expressed as a percentage of control mean ± SD. Asterisks indicate significant difference from control insult treatment as determined by ANOVA followed by the Dunnett's Multiple Comparison Test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$).

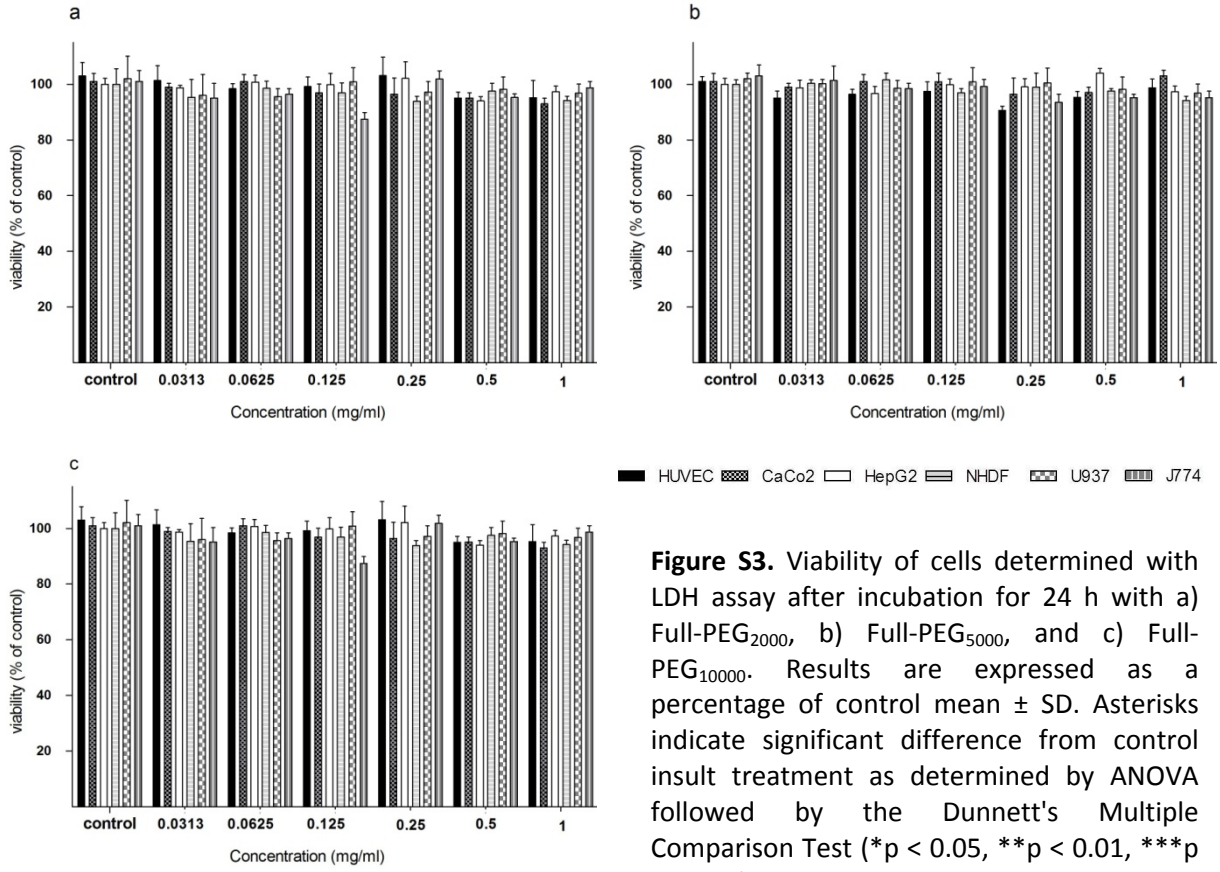


Figure S3. Viability of cells determined with LDH assay after incubation for 24 h with a) Full-PEG_{2000}, b) Full-PEG_{5000}, and c) Full-PEG_{10000}. Results are expressed as a percentage of control mean ± SD. Asterisks indicate significant difference from control insult treatment as determined by ANOVA followed by the Dunnett's Multiple Comparison Test (*p < 0.05, **p < 0.01, ***p < 0.005).

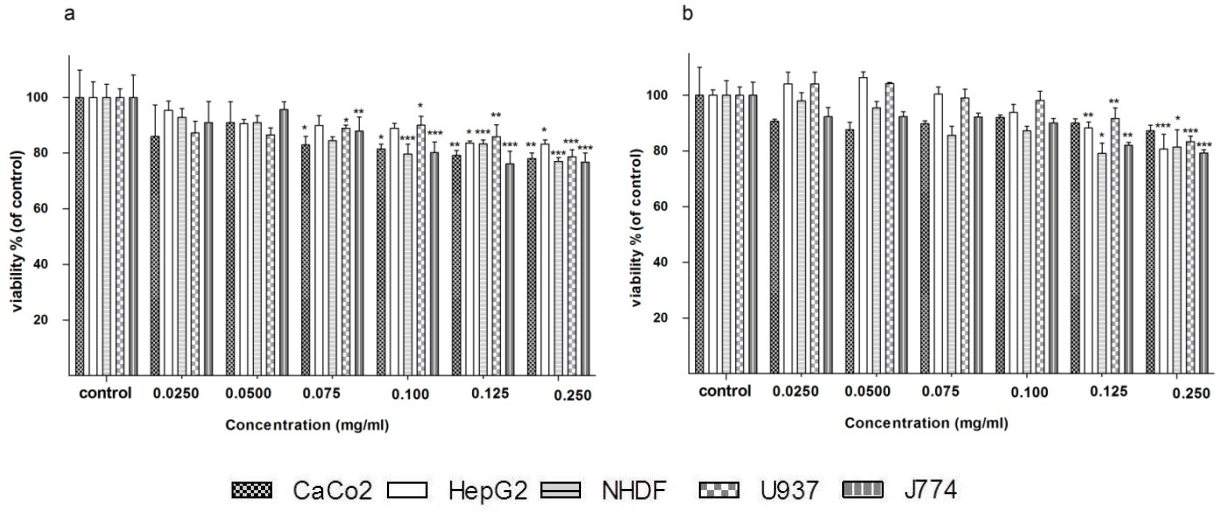


Figure S4. Viability of cells, assessed with the MTT assay after incubation for 24 h with a) sCNT-CO_2H and b) sCNT-OH. Results are expressed as a percentage of control mean ± SD. Asterisks indicate significant difference from control insult treatment as determined by ANOVA followed by the Dunnett's Multiple Comparison Test (*p < 0.05, **p < 0.01, ***p < 0.005).

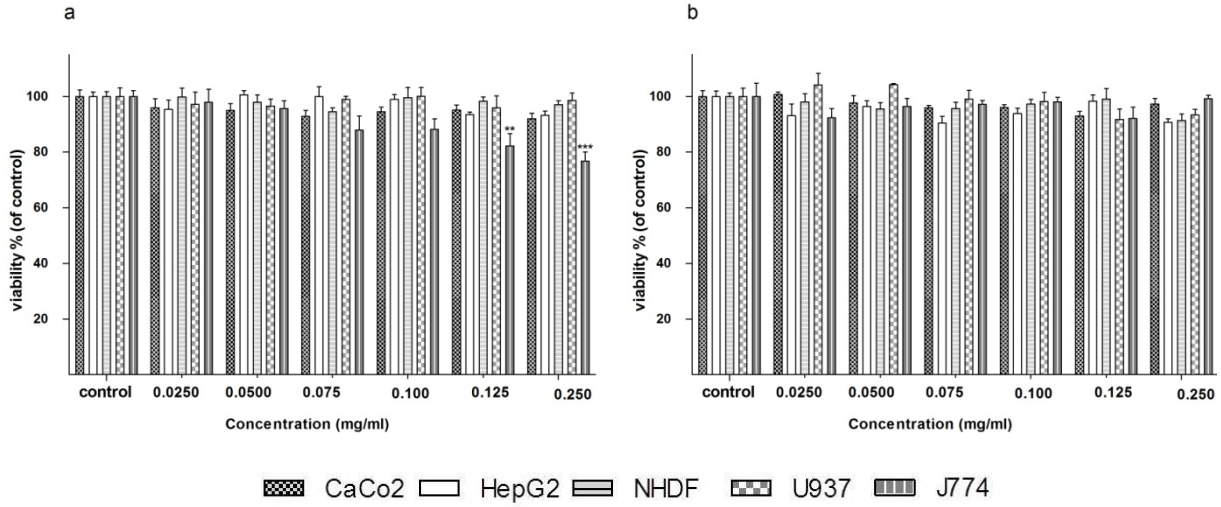
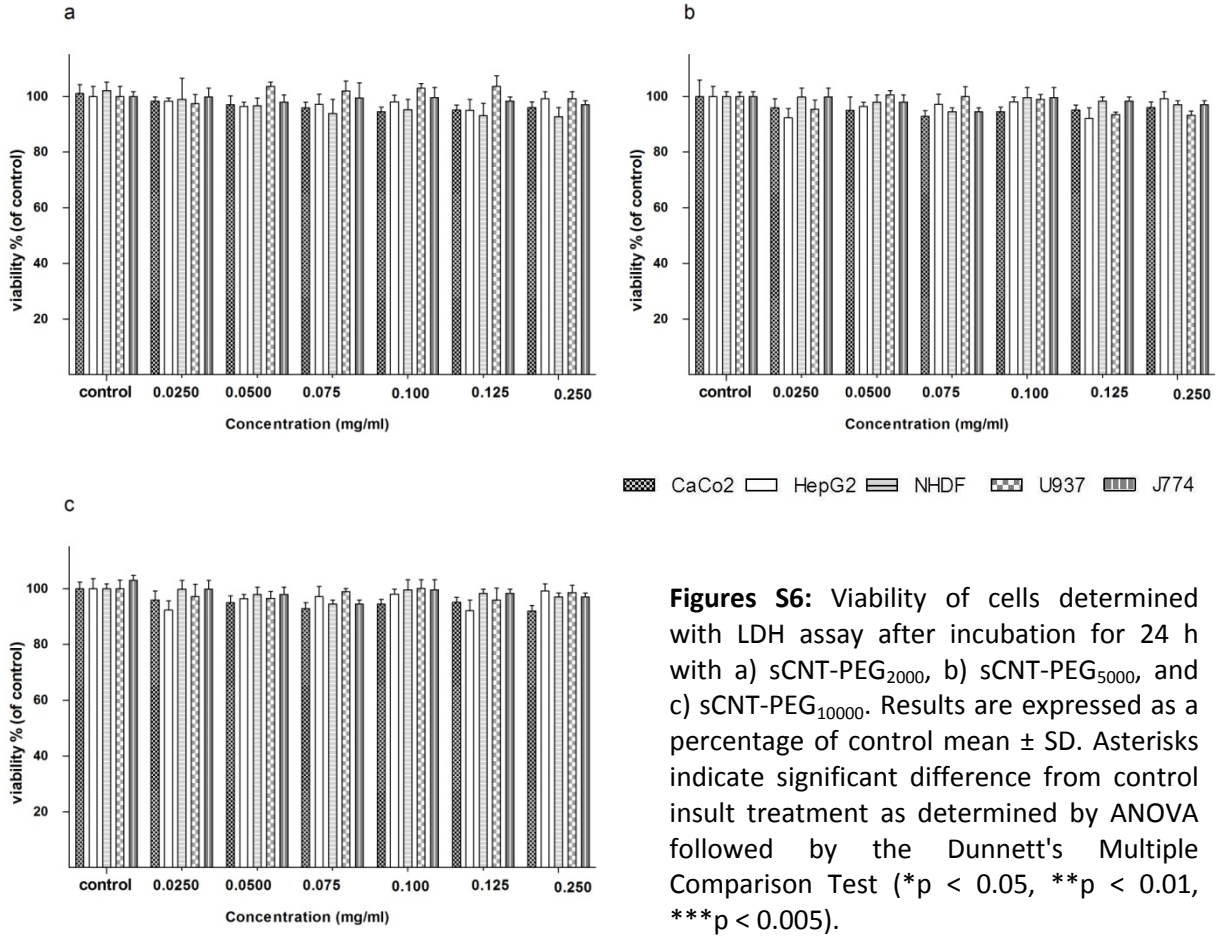


Figure S5. Viability of cells determined with LDH assay after incubation for 24 h with a) sCNT-CO_2H and b) sCNT-OH. Results are expressed as a percentage of control mean ± SD. Asterisks indicate significant difference from control insult treatment as determined by ANOVA followed by the Dunnett's Multiple Comparison Test (*p < 0.05, **p < 0.01, ***p < 0.005).



Figures S6: Viability of cells determined with LDH assay after incubation for 24 h with a) sCNT-PEG_{2000}, b) sCNT-PEG_{5000}, and c) sCNT-PEG_{10000}. Results are expressed as a percentage of control mean ± SD. Asterisks indicate significant difference from control insult treatment as determined by ANOVA followed by the Dunnett's Multiple Comparison Test (*p < 0.05, **p < 0.01, ***p < 0.005).

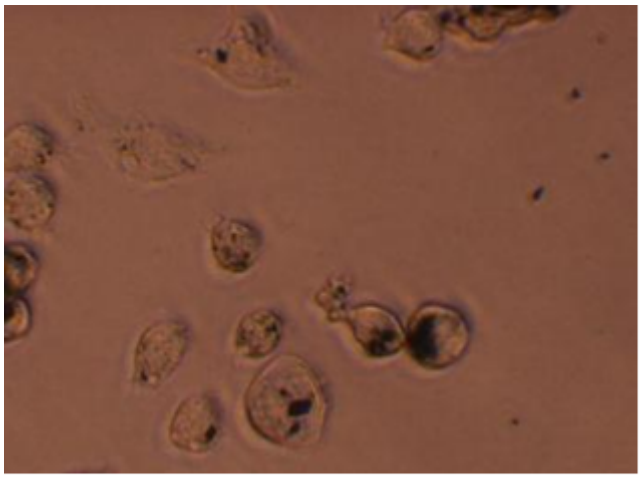


Figure S7. Optical image of J774 cells that were incubated at 37°C for 24 h with sCNT-PEG_{2000} dispersed in serum containing media.

References and Notes

1. J. Ogier *et al.*, Enhanced drug loading in polymerized micellar cargo. *Org. Biomol. Chem.* **2010**, *8*, 3902.
2. T. Mossmann *et al.*, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Meth.* **1983**, *65*, 55.
3. Procedures involving animals were conducted in accordance with Italian and international laws on the use of animals for experimental purposes (L.D. 116/92; Directives 86/609/EEC and 2010/63/EU) and AVMA guidelines for the euthanasia of animals.
4. K. Amin *et al.*, In vitro hemolysis: guidance for the pharmaceutical scientist. *J. Pharm. Sci.* **2006**, *95*, 1173.