Electronic Supplementary Material (ESI) for RSC Advances.

Carbon nanotube-mediated delivery of Budesonide to macrophages

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CHEMISTRY

BIOLOGY

1. General

Multiwalled carbon nanotubes (Nanocyl 3150, 95%) were purchased from Nanocyl (Sambreville, Belgium). Chemicals and solvents were obtained from Sigma Aldrich and used without purification. ^{1}H spectra were recorded using a Bruker AVANCE DPX 400 MHz spectrometer and the residual solvent peaks were used as reference. Coupling constants (*J*) are reported in Hertz (Hz), and splitting patterns are designated as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and br (broad). ESI-Mass spectra were recorded on Waters Micromass ZQ system. Sonication was performed using a Branson Sonifier 450 ultrasonicating probe. Electron microscopy observations were carried out on a Philips CM12 microscope. XPS measurements were recorded on a VG ESCALAB 210 spectrometer.

2. CNT-BUD Nanohydrid preparation

2.1. Shortening of carbon nanotubes

CNTs (80 mg) were ultrasonicated in toluene (30 mL) for 4 days (25 W output power). A cooling system was installed to avoid evaporation of the solvent. The suspension was then centrifuged for 30 min at 10000 rpm, and the supernatant was discarded. The precipitate was taken back in H_2O (30 mL) and centrifuged again. This operation was repeated two more times with acetone (30 mL). Shortened CNTs were finally dried under vacuum (75 mg).

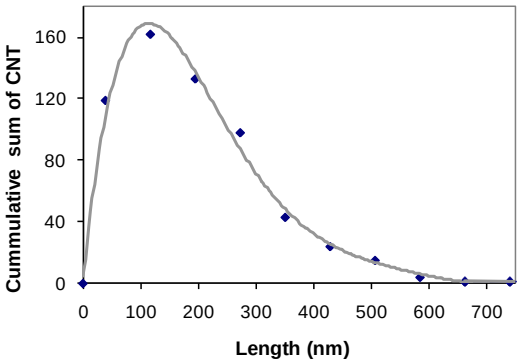


Figure S1: Size distribution of the shortened CNTs.

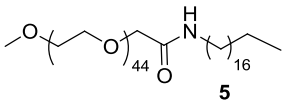
*2.2. Assembly of **1** on CNTs*

Short CNTs (10 mg) were dispersed in H_2O (4.5 mL) for 10 min using the ultrasonic probe (10 W output power). MeO-PEG_{2000}-CO-Glu(BUD)-NH-$C_{18}H_{35}$ (30 mg) dissolved in H_2O (500 µL) was then added and the mixture, cooled at 0°C, and further sonicated for 10 min. The dispersion was centrifuged (5 min, 1000 rpm). The black supernatant was collected and centrifuged (60 min, 11000 rpm). The black pellet was dispersed under sonication (10 min, 10 W output power) in H_2O (5 mL) cooled at 0°C. The dark supernatant corresponding to the MeO-PEG_{2000}-CO-Glu(BUD)-NH-$C_{18}H_{35}$/CNT nanohybrid was collected and lyophilized (6 mg).

3. Control samples preparation

3.1. CNT-CTRL (without budesonide)

3.1.1. Structure of amphiphile **5**



5

3.1.2. Assembly of amphiphile **5** on CNT

Short CNTs (10 mg) were dispersed in H_2O (4.5 mL) using the ultrasonic probe (10 W output power, 10 min). The above prepared amphiphile **5** (40 mg) dissolved in H_2O (500 µL) was then added and the mixture was further sonicated for 10 min. The dispersion was centrifuged for 5 min at 1000 rpm. The black supernatant was collected and centrifuged for 60 min at 11000 rpm. The black pellet was collected and redispersed under sonication (10 min, 10 W output power) in H_2O (5 mL). The black supernatant of MeO-PEG_{2000}-CO-NH-$C_{18}H_{35}$/CNTs was collected and lyophilized (5 mg).

3.2. Mic-BUD (with budesonide)

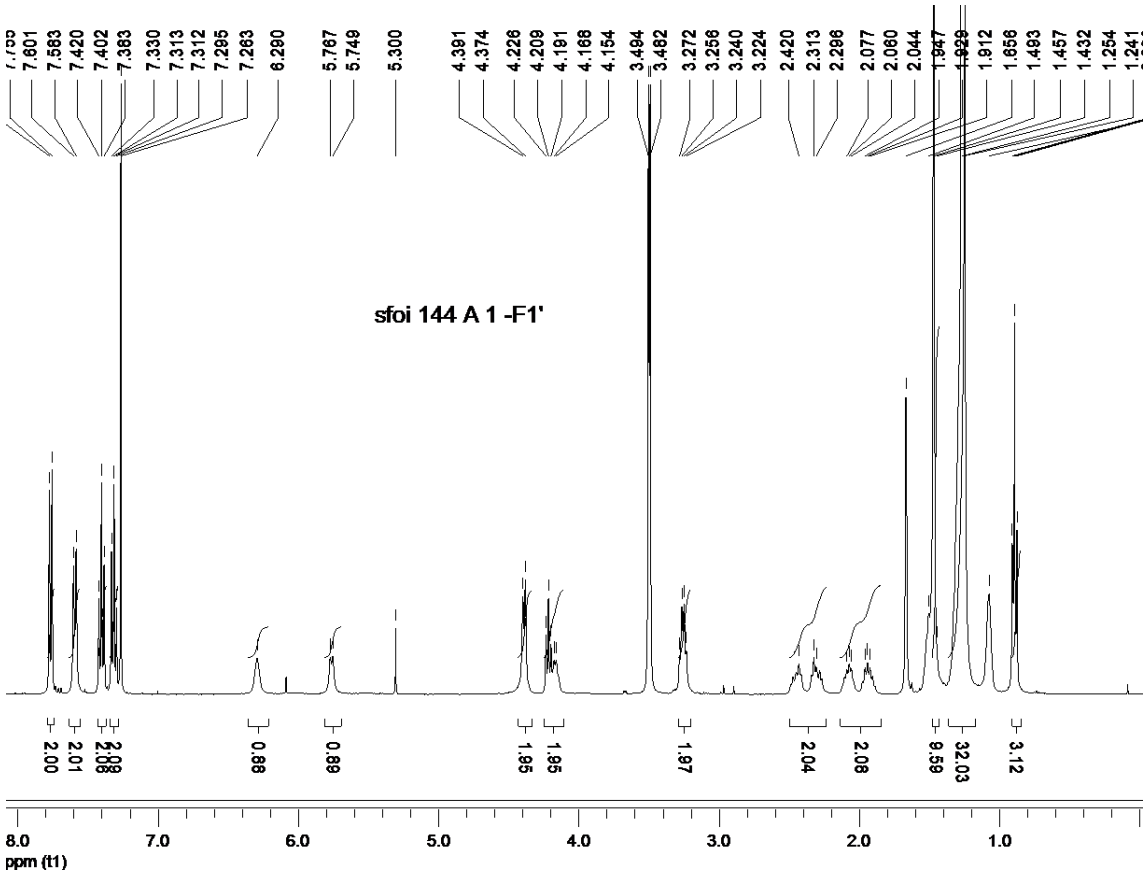
Micelles incorporating budesonide were prepared by simple self-assembly of the amphiphile **1** in water. Briefly, 1 mg of amphiphile **1** was sonicated in H_2O (1 mL) to provide a stock solution of Mic-BUD that was further diluted in complete culture medium as needed.

3.3. Mic-CTRL (without budesonide)

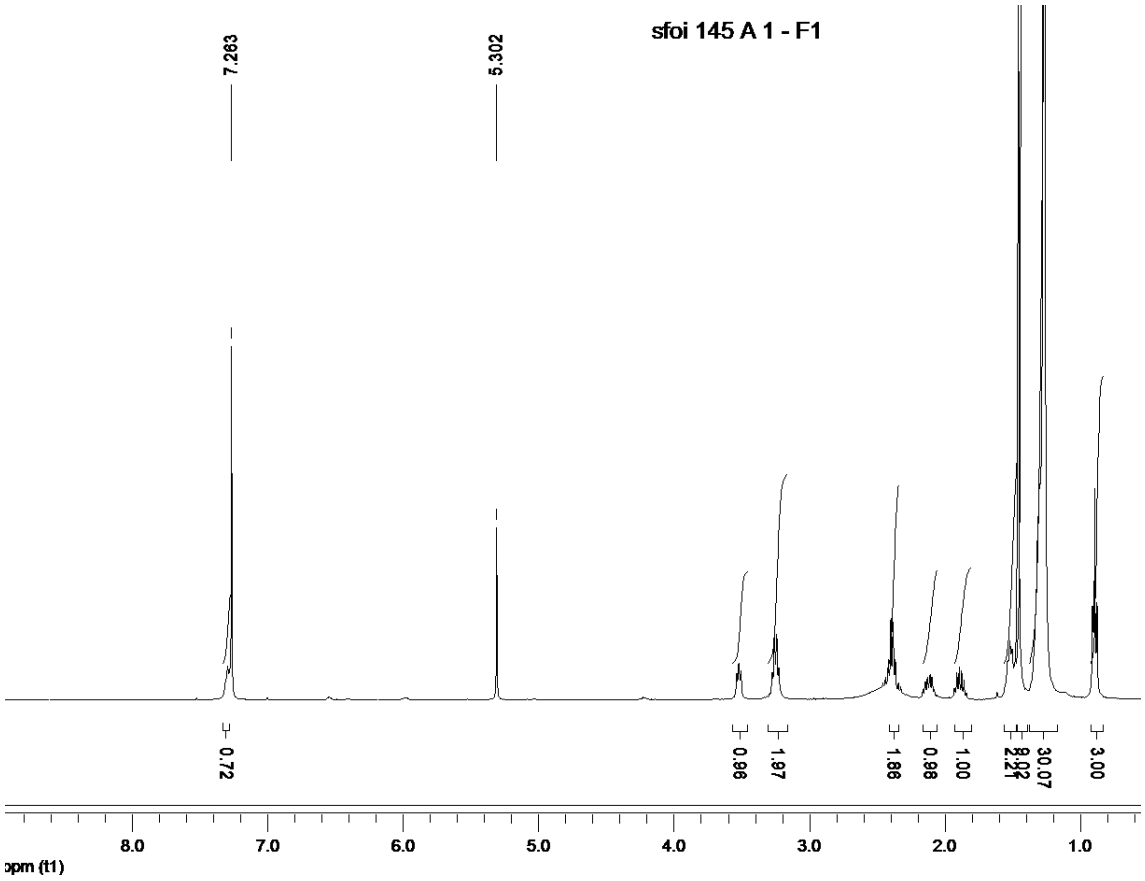
Micelles incorporating no budesonide were prepared by simple self-assembly of the amphiphile **5** in water. Briefly, 1 mg of amphiphile **5** was sonicated in H_2O (1 mL) to provide a stock solution of Mic-CTRL that was further diluted in complete culture medium as needed.

4. Copies of ^{1}H-NMR spectra

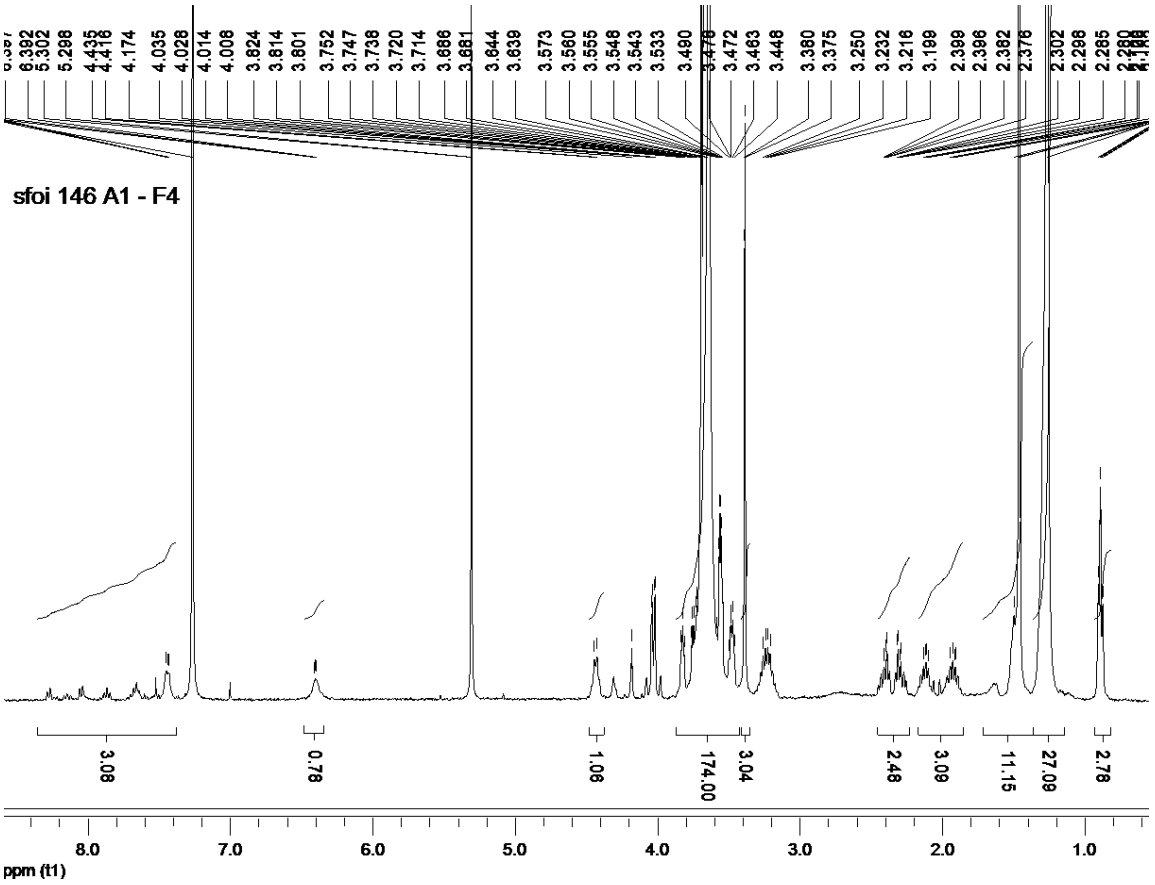
4.1. ^{1}H NMR of compound **3** (400 MHz, $CDCl_3$)



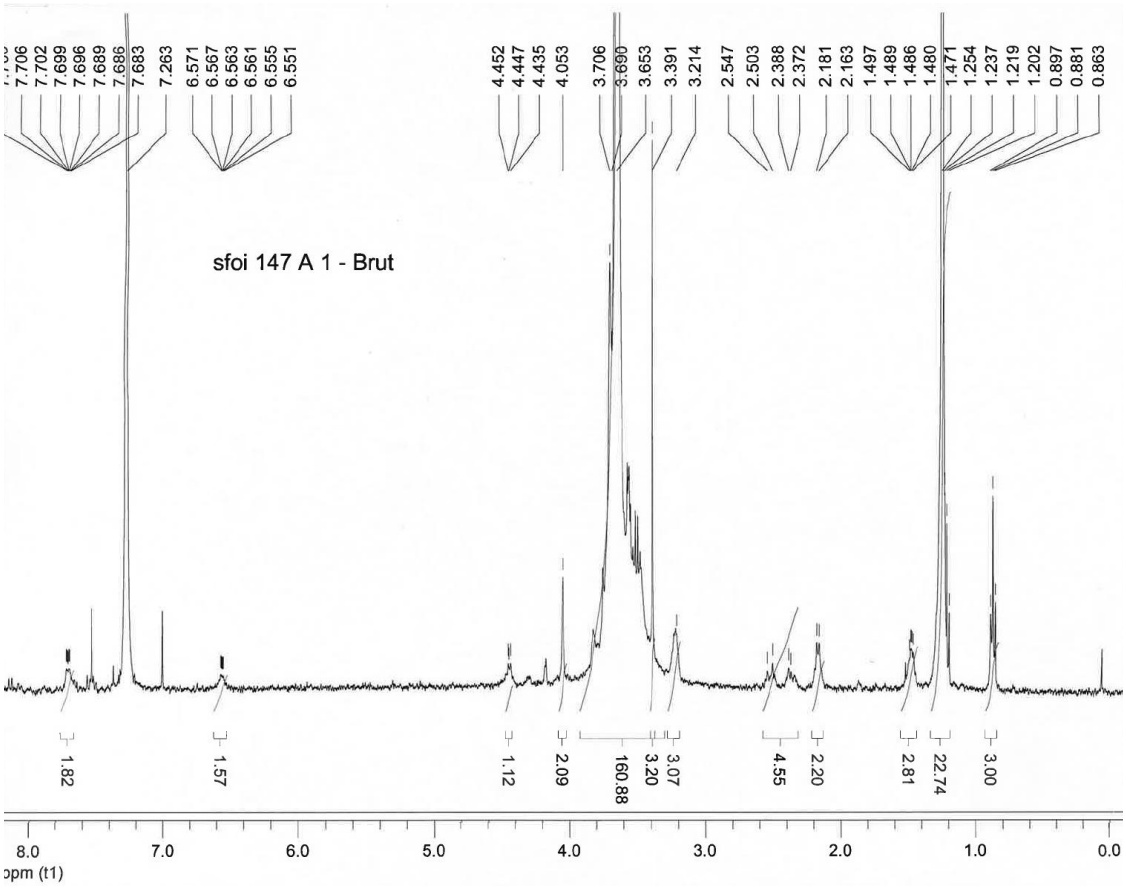
4.2. ^{1}H NMR of compound **3'** (400 MHz, $CDCl_3$)



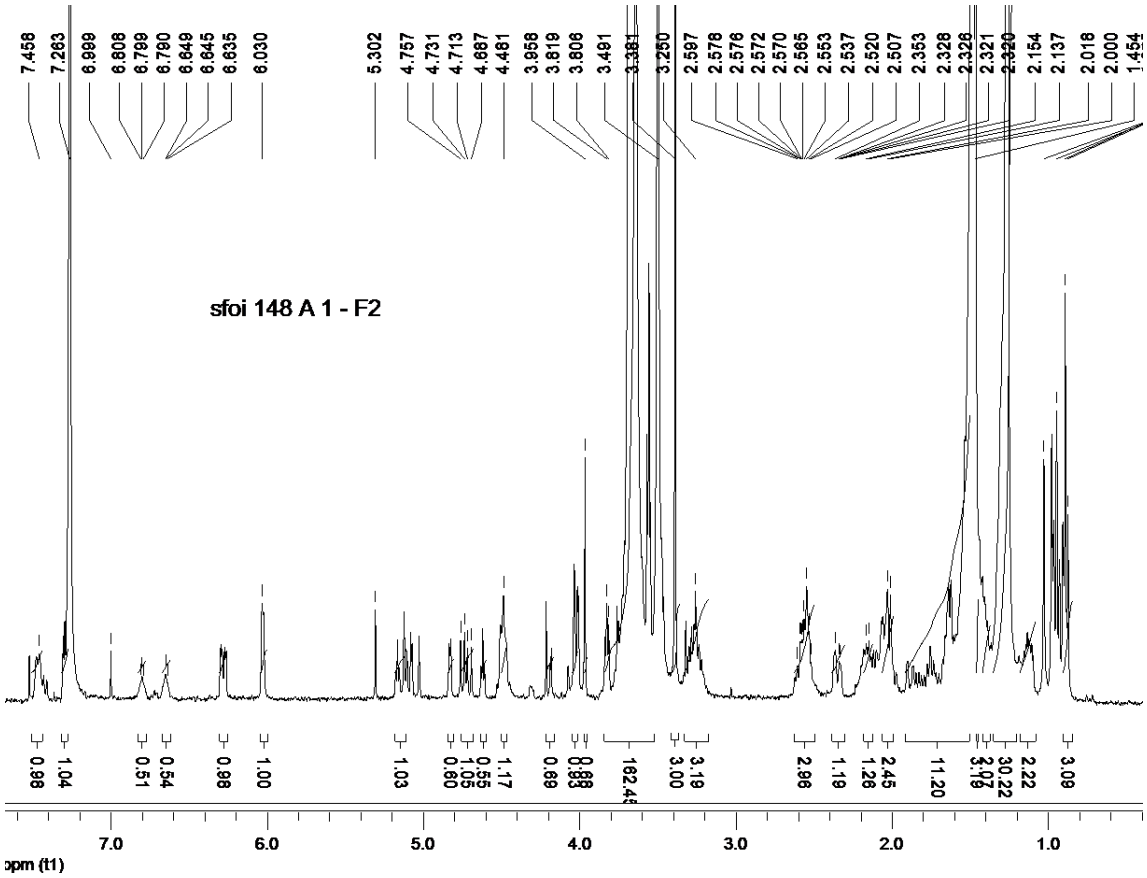
4.3. ^{1}H NMR of compound **4** (400 MHz, $CDCl_3$)



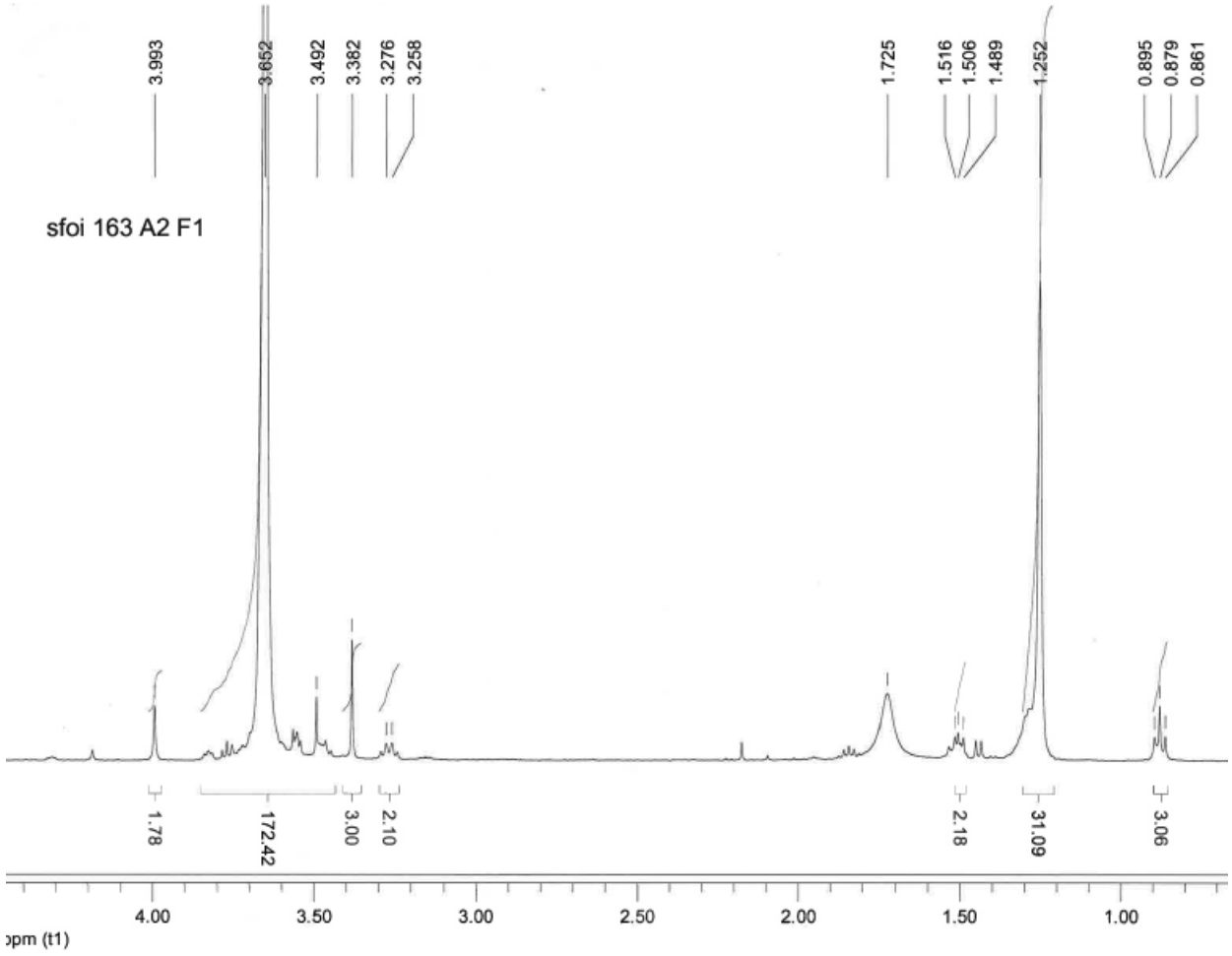
4.4. ^{1}H NMR of compound **4'** (400 MHz, $CDCl_3$)



4.5. ^{1}H NMR of compound **1** (400 MHz, $CDCl_3$)



4.6. ^{1}H NMR of compound **5** (400 MHz, $CDCl_3$)



BIOLOGY

1. Macrophages and cell culture

Murine primary macrophages were isolated from healthy untreated BALB/c mice (purchased from Charles River). Mice were maintained in our animal facility (approved by French Veterinary Services, #F67-482-2); experiments were carried out in conformity with the 2010/63/UE European animal bioethics legislation. Successive washes of the peritoneal cavity were performed with phosphate buffered saline (PBS) and harvested cells were then washed in complete culture medium (RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum, 10 μg/mL gentamycin, 50 μM β-mercaptoethanol, 10 mM HEPES; Lonza). The RAW 264.7 cell line is a BALB/c-derived transformed macrophage cell line and was kept in culture in complete RPMI medium (37°C, 5% CO_2).

Regarding incubation with nanotubes and micelles, macrophages were seeded in 96-well plates (2.10^5 cells/well; optical microscopy and ELISA test) or 24-well plates (2.10^6 cells/well; TEM) and allowed to adhere overnight before further incubation for 20-24h (37°C, 5% CO_2). In some experiments, macrophages were activated with 20 ng/mL lipopolysaccharides from *Escherichia coli* strain 0111.B4 (Sigma-Aldrich).

2. Sample preparation for cell incubation

CNT-CTRL and CNT-BUD were suspended in purified water at 1 mg/mL concentration and sonicated before being diluted (100 μg/mL and further) in complete culture medium. Mic-CTRL and Mic-BUD were formed in purified water (1 mg/mL) before further dilution in complete culture medium. BUD was first dissolved in purified water containing 5% DMSO, heated and sonicated, before being further diluted in complete culture medium (500 μM/0.25% DMSO, and further).

3. Optical microscopy

Upon 24h of incubation with CNT-CTRL and CNT-BUD (34 μg/mL or 3.4 μg/mL corresponding to 2 μM and 0.2 μM of BUD, respectively), RAW 264.7 and peritoneal macrophages were observed using an inverted Zeiss Axiovert-40CFL microscope equipped with an AxioCam ICc 1 camera.

4. TEM analysis of intracellular distribution

For TEM observation, intraperitoneal mouse macrophages were cultured on glass coverslips in 24-well plates (2.10^6 cells per well) and were allowed to adhere overnight prior to treatment with LPS (20 ng/mL) and exposure to CNT-CTRL or CNT-BUD (both 50 μg/mL) for 24 h. (37°C, 5% CO_2) At the end of the incubation time, cells were washed with Tris buffered saline (TBS) and fixed overnight at 4 °C with 2.5% glutaraldehyde. Next steps were as previously described (A. Battigelli, J. Russier, E. Venturelli, C. Fabbro, V. Petronilli, P. Bernardi, T. Da Ros, M. Prato, A. Bianco. *Nanoscale,* **2013**, *5*, 9110-9117).

5. Measurements of IL-6 secretion

Primary peritoneal mouse macrophages were either i) left untreated, ii) stimulated with LPS (20 ng/mL), iii) incubated with CNT-CTRL or CNT-BUD (50 µg/mL), iv) stimulated with LPS and incubated with either BUD or CNT-CTRL or CNT-BUD or Mic-CTRL or Mic-BUD (0.2 µM/0.02 µM and 3.4 µg/mL/0.34 µg/mL for BUD and carrier concentrations respectively). 20h to 24h later, cell culture supernatants were harvested and levels of the pro-inflammatory cytokine IL-6 were measured using a double-sandwich ELISA according to the protocol previously described (H. Dumortier, S. Lacotte, G. Pastorin, R. Marega, W. Wu, D. Bonifazi, J.P. Briand, M. Prato, S. Muller, A. Bianco. *Nano Lett.* **2006**, *6*, 1522–1528).