Electronic Supplementary Material (ESI) for RSC Advances

Clicking dendritic peptides onto single walled carbon nanotubes

-Supporting Information-

Materials: As prepared single walled carbon nanotubes (AP-SWCNTs, Carbon Solutions, Inc.), concentrated sulfuric acid (Merck, 98% AR grade), nitric acid (Merck, 70% AR grade), fluoropore membranes (hydrophobic) 0.22 μm (FGLP, Millipore), *N*,*N*-dimethylformamide (DMF, Aldrich 99.9%), *N*-(3-Dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDC, Fluka), *N*-hydroxybenzotriazole (HOBt, Auspep), 3-chloropropylamine hydrochloride (Aldrich, 98%), sodium azide, *N,N,N', N'', N''*-pentamethyldiethylenetriamine (PMDETA, Aldrich, 99%), copper (I) bromide (CuBr, Aldrich, 98%), trifluoroacetic acid (TFA, Aldrich, 99%), dichloromethane (DCM, Aldrich, 99%), fluorescein isothiocyanate (FITC, Aldrich, 98%), potassium bromide (KBr, Aldrich, 99%), tissue culture polystyrene (TCPS) dish and 96-well plate (Iwaki cell biology, Crown Scientific) and deuterochloroform ($CDCl_3$, Cambridge Isotope, 99% D) were used as received unless otherwise stated.

Synthesis of 3-azidopropylamine: Similar to a method previously reported,[1] 3-chloropropylamine hydrochloride (10.0 g, 77 mmol, 1.0 equiv) and sodium azide (12.5 g, 192 mmol, 2.5 equiv) were reacted in DMF (36.0 mL) at 100 °C for 48 hours. The reaction mixture was cooled to room temperature, poured into ethyl ether (250 mL), and extracted with a saturated aqueous NaCl solution (500 mL). The organic layer was separated, dried over $MgSO_4$, and filtered. The supernatant was concentrated to obtain the product as yellow oil.

Preparation of N_3-CNTs: In a 250 mL round bottom flask, EDC (46.3 mg) and HOBt (32.4 mg), were added with a stir bar, capped with a rubber septum and Cu wire then degassed for 1 hour. DMF (70 mL) was then added to the round bottom via a pre-purged syringe. The solution was then stirred over night (~ 12 hours). AP-SWCNTs were cut by ultrasonication in an acid bath of 3:1 (v/v) solution of concentrated H_2SO_4:HNO_3 at 0 °C for 8 hours at (1 mg/mL) to produce 4 wt% acid treated (or carboxylated) SWCNTs.[2] Carboxylated SWCNTs (41.1 mg) were placed in a 20 mL vial, capped with a rubber septum and Cu wire, then sonicated in DMF (10 mL) for 2 hours at room temperature. In a separate 20 mL vial, azidopropylamine (24.2 mg) and DMF (10 mL) were added and purged for 1 hour with nitrogen gas. Both the CNT-DMF solution and azidopropylamine-DMF solution were then added to the EDC/HOBt mixture via cannulas. The reaction was then stirred at room temperature for 24 hours, followed by filtration over a FGLP and rinsed with DMF, then dried under vacuum.

Preparation of K3-CNTs: N_3-CNTs (10.1 mg) were sonicated in DMF (5 mL) for 30 minutes resulting in a suspension. *N-tert*-butoxycarbonyl (Boc)-K3[3,4] (30 mg, 3.6×10^{-5} mol) was added and the suspension was degassed by bubbling nitrogen gas for 40 minutes. PMDETA (3.0 mg, 5.1μL) was dissolved in DMF (5 mL) and degassed by bubbling nitrogen gas for 30 minutes. CuBr (4.0 mg, 0.5 equiv of Boc-K3) was placed in a vial and purged with nitrogen gas for 30 minutes. PMDETA/DMF was transferred into the CuBr vial under nitrogen and mixed by bubbling nitrogen gas for 10 minutes. The mixture was the transferred under nitrogen into the DMF containing N_3-CNTs and Boc-K3, which had been degassed for 40 minutes. The mixture was stirred for 24 hours under nitrogen atmosphere. The solution was filtered over a FGLP and washed with DMF under vacuum filtration and allowed to air dry under vacuum for 12 hours. The filter was placed in DMF and sonicated to resuspend the CNT composite. The DMF was removed over two days by blowing nitrogen over the solution. Residual solvent was removed under high vacuum. The K3-CNTs were then dissolved in a solution of 30% TFA in DCM for 1 hour to remove the Boc groups. The deprotected K3-CNTs were washed over a FGLP.

Preparation of FITC-K3-CNTs: K3-CNTs (8.6 mg) were dissolved in PBS to which fluorescein isothiocyanate (FITC) (2.25 μmol, 0.875 mg) was added from a stock solution (2 mg/mL in PBS). The solution was reacted overnight at 4 °C then washed over a FGLP and isolated by sonication in EtOH to remove CNTs from the filter.

IR analysis: IR spectra were acquired using a Nicolet Avatar 370MCT from Thermo Electron Corporation equipped with a transmission accessory. The transmission spectra were obtained in the range of 650-4000 cm^{-1} at a resolution of 2 cm^{-1}. The CNTs were dispersed in dry potassium bromide (KBr) then compacted into discs for IR analysis.

Raman: Raman scattering analysis was performed on a Witec alpha300R microscope in Raman mode using a 100x objective with a numerical aperture (NA) of 0.9 and a 532 nm (E_{laser} = 2.33 eV) laser. CNTs were deposited on a glass slide from a buffer solution, dried, then rinsed 3 times with MilliQ to remove buffer salt. The sample surface was mounted perpendicular to the excitation source. Single spectra were collected using integration times of 10 seconds. Spectral analysis was conducted with Witec project v1.90 software.

Atomic Force Microscopy: AFM images were acquired in ambient conditions with a Multimode and NanoScope V controller (Digital Instruments, Bruker, Santa Barbara, USA) using NSC15 silicon probes (Mikromasch, USA) operating in tapping mode. These probes have a fundamental resonance frequency between 250 – 400 kHz, nominal spring constant of 42 N m^{-1} and nominal tip radius of 10 nm. Amplitude set-points were typically 70% to 90% of the cantilever free amplitude. Topographic height images were obtained at a scan rate of 1 Hz with feedback controls optimized for optimum image quality. All images represent flattened data using the NanoScope Analysis version 1.20 software (Digital Instruments, Bruker, USA). Dendron functionalized CNTs were deposited on a glass slide from a buffer solution, dried, then rinsed 3 times with MilliQ to remove buffer salt.

MSC culture: Female Wistar rats (~100 g, Animal Care Unit, School of Medicine, Flinders University of South Australia) were sacrificed by anesthesia under the guidelines approved by the Institutional Animal Care and Utilisation Committee. The MSCs derivation procedure has been previously published.[5] Bone marrow cells were collected from the femurs and tibias by flushing the bone cavity with Dulbecco's modified eagle medium (DMEM-low glucose containing 2 mM L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin, 0.1 mM MEM nonessential amino acid, and 10% foetal bovine serum, pH 7.4). Marrow cells were filtered through a 100 μm nylon mesh. Red blood cells were lysed using RBC lysis buffer (0.15 M ammonium chloride, 10 mM potassium bicarbonate, 0.1 mM EDTA) for 5 minutes. After centrifugation, cells were resuspended in the culture medium and then seeded in TCPS dishes. After 24 hour incubation at 37 °C and 5% CO_2, nonadherent cells were discarded by rinsing with phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4 with pH = 7.4) and the adherent cells were grown to 90% confluence. The culture medium was replaced every 2 days. Colony-forming unit fibroblasts-like cells were detached by trypsinisation and collected by centrifugation as the passage 1 cells. Passage 3 cells were used in this study.

MSC culture with CNTs: Cell density of 20,000 cells/cm^2 was seeded in 96-well TCPS microplates. After 24 hour incubation, FITC-K3-CNTs and CNTs with concentration of 0, 1, 2, 5, 10, 20, 50, and 100 μg/mL was added into medium for further 24 and 48 hours. At these two time points, cell morphology was assessed by an upright microscope (Nikon Eclipse 50i). Captured images were processed using NIS-Elements BR 3.0x software.

Cytotoxicity of CNTs: The cytotoxicity of CNTs was determined through the dye-uptake technique, which is based on the neutral red absorption by living cells.[6] After 24 hours and 48 hours incubation with CNTs, 50 μg/mL neutral red dye in medium was added to each well in the microplates and incubated for

2 hours at 37°C. Following this period of time, samples were washed with PBS three times to remove any remaining unincorporated dye. The dye was then released from the cells using 300 µL of lysis solution (1% acetic acid, 50% ethanol and 49% MilliQ water). The plate was agitated for 5 minutes before 100 µL of solution was transferred to a 96 well microplate to be read at 570 nm wavelength in the spectrometer.

Confocal microscopy: After 48 hours of incubation with 10 µg/mL FITC-K3-CNTs and CNTs, cells were gently washed with PBS three times then fixed by 4% paraformaldehyde in PBS for 20 minutes and then rinsed with PBS twice on a glass cover slide. F-actin and nuclei were stained by 500 nM Phalloidin-TRITC and 100 nM DAPI, respectively, for 1 hour. Fluorescence microscopy images were captured using a laser scanning confocal system (TCS SP5, Leica, Germany).

Supplementary figures:

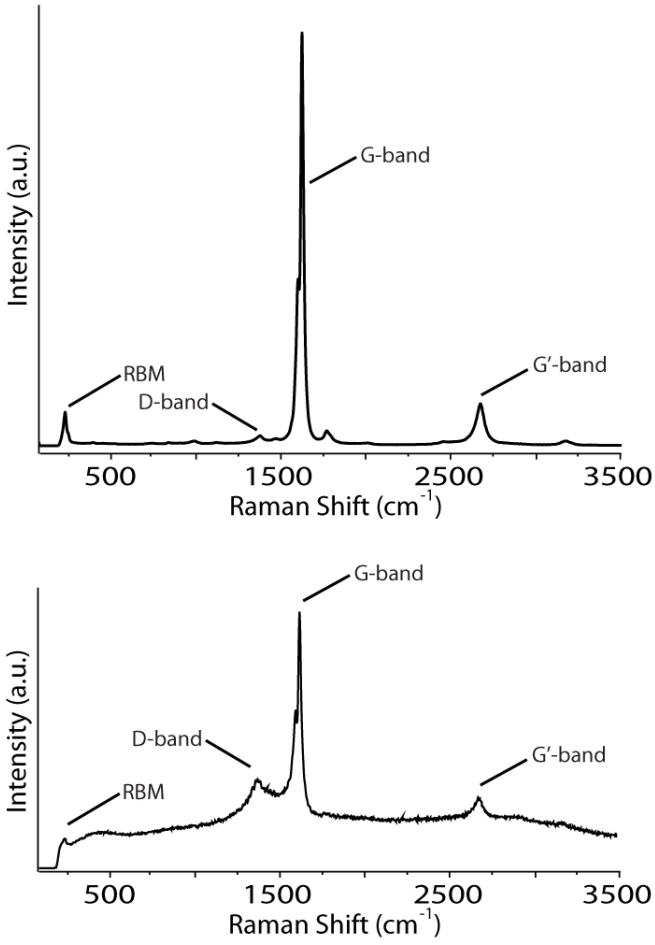

Fig. S1 Raman spectra of pristine single walled carbon nanotubes (top) and Boc-K3-CNTs (bottom).

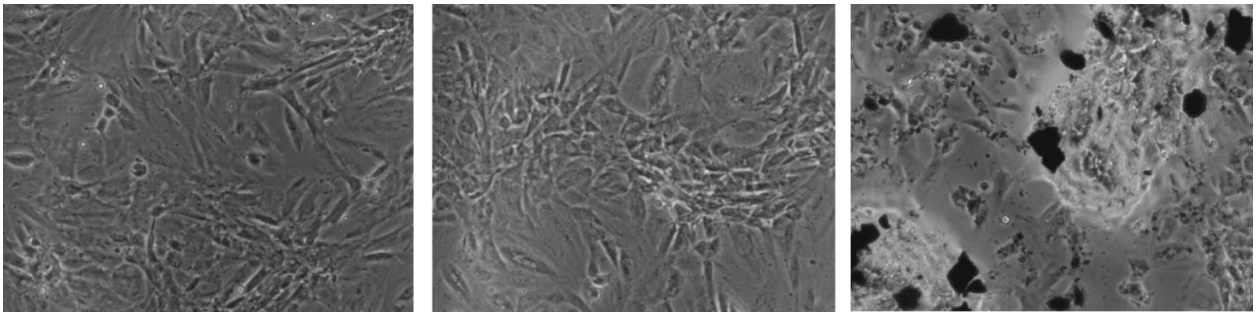

Fig. S2 Brightfield microscopy images of rMSCs after 48 hours of incubation with no CNTs (left), 50µg/mL of FITC-K3-CNTs (middle), and 50µg/mL of COO^--CNTs (right).

References:

1 Gondi, S. R., Vogt, A. P. & Sumerlin, B. S. Versatile Pathway to Functional Telechelics via RAFT Polymerization and Click Chemistry. *Macromolecules* **40**, 474-481, doi:10.1021/ma061959v (2007).

2 Marshall, M., Popanita, S. & Shapter, J. Measurement of functionalised carbon nanotube carboxylic acid groups using a simple chemical process. *Carbon* **44**, 1137-1141 (2006).

3 Haridas, V., Sharma, Y. K. & Naik, S. Multi-Tier Dendrimers with an Aromatic Core. *European Journal of Organic Chemistry* **2009**, 1570-1577, doi:10.1002/ejoc.200801150 (2009).

4 Haridas, V. *et al.* Gelation and topochemical polymerization of peptide dendrimers. *New Journal of Chemistry* **35**, 303, doi:10.1039/c0nj00544d (2011).

5 Clements, L. R. *et al.* Mesenchymal stem cell attachment to peptide density gradients on porous silicon generated by electrografting. *physica status solidi (a)* **208**, 1440-1445, doi:10.1002/pssa.201000320 (2011).

6 Low, S. P., Williams, K. A., Canham, L. T. & Voelcker, N. H. Evaluation of mammalian cell adhesion on surface-modified porous silicon. *Biomaterials* **27**, 4538-4546, doi:10.1016/j.biomaterials.2006.04.015 (2006).