Research paper

**In vivo** behaviour of glyco-NaI@SWCNT ‘nanobottles’

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1. Introduction

The inner cavities of carbon nanotubes (CNTs) can accommodate a wide range of guest species [1–4]. Unprecedented structures and properties compared to those of the same material in the bulk can be observed when they are confined [5–11]. In the biomedical field, contrast agents and therapeutic compounds can be either attached to the external CNT walls or confined within the cavities of the CNTs [12–18]. The latter is attractive because CNTs can offer striking properties compared to those of the same material in the bulk can be observed when they are confined [5–11]. In the biomedical field, contrast agents and therapeutic compounds can be either attached to the external CNT walls or confined within the cavities of the CNTs [12–18]. The latter is attractive because CNTs can offer striking protection to chosen payloads, avoiding their interaction with the biological milieu [19].

Several strategies have been developed for the encapsulation of materials inside carbon nanotubes. Once filled, unless there is a strong interaction between the host nanotubes and the guest species, the ends of the CNTs need to be sealed/closed to allow selective purification of the melted salts inside the nanotubes with the spontaneous closure of the extremities during the cooling process [20,21]. The salts remain stably confined in the form of ‘nanocrystals’ inside the nanotubes while leaving the outer surface essentially unaffected, and so ready to be modified by organic molecules.

As-produced, CNTs are insoluble in almost any aqueous solution and organic solvent, and have been suggested to be toxic to mammalian cells [22], thereby presenting perceived limitations to their biological applications [23–27]. Functionalization of CNT side-walls with biologically- and biotechnologically-relevant molecules (including polymers [28], peptides [29,30], nucleic acids [31] and carbohydrates [32,33]) allows the generation of potentially stable and biocompatible dispersions. For example, non-covalent binding of aromatic molecules by π–π stacking onto the surface of the nanotubes [34] or covalent modification of their polyaromatic surface [35] allow loading of multiple molecules along the length of the nanotubes.

We have previously shown that encapsulation of radionuclide into the inner space of glycan-functionalized single-walled carbon nanotubes (glyco-X@SWCNT) may be achieved by molten filling and then covalent modification, allowing in vivo redirection of the distribution of the associated radioactivity from the thyroid to the lungs [33]. Here, we...
use steam-purified and shortened single-walled carbon nanotubes (SWCNTs) [36] filled with both ‘cold’ (NaI) and ‘hot’ (NaI$_{253}$) cargoes and subsequent functionalization with different carbohydrates to explore the basis and role of glycán in this redistribution.

2. Experimental

2.1. Purification of SWCNTs

Chemical vapour deposition (CVD) grown SWCNTs, were provided by Thomas Swan & Co. Ltd (Elicarb®). Steam purification was carried out in order to remove the amorphous carbon and graphitic shells formed during the synthesis [36]. Steam treatment was simultaneously out in order to remove the amorphous carbon and graphitic shells. Steam purification was carried out to remove the amorphous carbon and graphitic shells.

2.2. Filling of SWCNTs with NaI by molten phase capillary wetting

Purified and open-ended SWCNTs (100 mg) and NaI (1 g) were ground together and loaded into a silica ampoule. The system was evacuated and the ampoule was sealed under vacuum. The sample was subsequently annealed at 900 °C and slowly cooled down to favour the crystallisation of NaI within the hosting SWCNTs. Afterwards, the system was air opened and the sample was ground with an agate mortar and pestle. The sample was next washed in water, to remove the non-encapsulated NaI, and collected by filtration on top of a polycarbonate membrane (0.2 μm Whatman).

2.3. Synthesis of f-NaI@SWCNTs

4-(2-(bis(2-tert-butoxycarbonylamino)ethyl)amino)ethylamino)-4-oxo-butanolic acid: To a solution of tris(2-aminoethyl)amine (6 mL, 41 mmol, 5 eq.) in MeCN (200 mL) at 0 °C was added, dropwise, a solution of succinic anhydride (822 mg, 8.2 mmol, 1 eq.) in MeCN (100 mL). The reaction mixture was stirred at r.t. for 2 h, before the supernatant was decanted off and the residue redissolved in MeOH, and finally dried under vacuum to afford 4-(2-(bis(2-aminoethyl)amino)ethylamino)-4-oxo-butanolic acid as a yellow oil (2.0 g, 8 mmol, 98%).

To a solution of 4-(2-(bis(2-aminoethyl)amino)ethylamino)oxo-butanolic acid (343 mg, 1.39 mmol, 1 eq.) in dioxane (15 mL) was added di-tert-butyl dicarbonate (611 mg, 2.8 mmol, 2 eq.). The reaction mixture was stirred for 4 h at r.t., the solvent removed and the residue purified by flash column chromatography over silica (DCM:MeOH, 9:1 to 0:1) to afford the title compound as a colourless oil (246.8 mg, 0.55 mmol, 40%).

Benzyl (2-(2-(2-aminoethoxy)ethoxy)ethyl)glycinate: To a solution of benzyl (2-(2-(N-tert-butoxycarbonylamino)ethoxy)ethoxy)ethylglycinate [38] (1.1056 g, 2.79 mmol, 1 eq.) in DCM (5.6 mL) was added trifluoroacetic acid (TFA, 2.4 mL). The reaction mixture was stirred at r.t. for 1 h, before removing the solvent under vacuum to afford the title compound as a white solid (246.8 mg, 0.55 mmol, 40%).

Linker Unit 1.0

Step 1) To a solution of 4-(2-(bis(2-tert-butoxycarbonylamino)amino)ethylamino)-4-oxo-butanolic acid (1.230 g, 2.75 mmol, 1 eq.) in DMF (55 mL), were added hexfluorophosphate azabenzo triazole tetramethyl uronium (HATU, 2.091 g, 5.5 mmol, 2 eq.), hydroxybenzotriazolone (HOBt, 743 mg, 5.5 mmol, 2 eq.) and di-isopropylethylamine (DPEA, 960 μL, 5.5 mmol, 2 eq.). Finally, benzyl (2-(2-aminooethoxy)ethoxy)ethyl)glycinate (978.8 mg, 3.3 mmol, 1.2 eq.) was added and the mixture stirred at r.t. for 6 h. The reaction was evaporated to a small volume, recovered with DCM (150 mL) and washed with H$_2$O (200 mL) and brine (200 mL). The organic layer was separated, dried over MgSO$_4$, filtered and the filtrate evaporated under vacuum, to afford a yellow oil (1.846 g, 2.55 mmol, 92%).

Step 2) To Pd/C (10 mg, 10 wt% loading) under hydrogen, was added a solution of the product from step 1 (386 mg, 0.53 mmol, 1 eq.) in MeOH (dry, 3 mL). The reaction mixture was stirred at r.t. for 5 h, filtered through celite and solvent removed under vacuum. The crude product was purified by flash column chromatography on silica (DCM:MeOH, 9:1 to 0:1) to afford the title compound as a colourless oil (220.4 mg, 0.35 mmol, 66%).

A suspension of NaI@SWCNTs (6 mg) in DMF (dry, 3 mL) was sonicated for 2 min, before a solution of Linker Unit 1.0 (12.7 mg, 0.02 mmol, 1 eq.) and 2,3,5-triiodobenzaldehyde [33] (9.6 mg, 0.02 mmol, 1 eq.) in DMF (dry, 1 mL) was added. The reaction was refluxed at 130 °C for 4 days, then cooled to r.t. and filtered. The residue was washed with DMF, MeOH and dried under vacuum to afford di-Boc-f-NaI@SWCNTs as a black solid (6.8 mg).

A suspension of di-Boc-f-NaI@SWCNTs (12.2 mg) in DCM (dry, 5 mL) was sonicated for 2 min, before TFA (2.5 mL) was added. The reaction mixture was stirred at r.t. for 24 h, then evaporated, recovered with MeOH, filtered and washed with MeOH. The solid obtained was dried under vacuum to afford the title compound as a black solid (9.46 mg).

2.4. Fmoc numbering f-NaI@SWCNTs

A solution of Fmoc chloride (1 mg, 3.86 μmol, 1 eq.) in DCM (0.5 mL) was added dropwise to a suspension of f-NaI@SWCNTs (1 mg) in DCM (0.5 mL) at 0 °C. DIPEA (1.5 μL, 8.61 μmol, 2.2 eq.) was added and the reaction was stirred at r.t. for 16 h. The reaction mixture was centrifuged, the supernatant discarded and the solid recovered with MeOH then filtered and washed with MeOH. The solid obtained was dried under vacuum to afford the title compound as a black solid (9.46 mg).

2.5. Synthesis of Glycosylated-NaI@SWCNTs

2-acetamido-1-thio-(S-2-imido-2-metho xethy1)-2-deoxy-β-D-glucopyranoside 1.1: To a solution of 2-N-acetamido-3,4,6-tri-O-acetyl-2-deoxy-1-thio-(S-cyanomethyl)-β-D-glucopyranoside [39] (20 mg, 0.05 mmol, 1 eq.) in MeOH (dry, 1 mL) was added NaOMe (25% in MeOH, 12 μL, 0.05 mmol, 1 eq.). The reaction was stirred at r.t. for 16 h before being neutralised with Dowex-H$^+$. The reaction mixture was filtered and evaporated without heating. The product was obtained as a mixture of deprotected cyanomethyl (R-SCM) and ‘activated’ sugars (R-IME, 1.1)) in ratio 1:0.5 (determined by 1H NMR (CD$_3$OD)), and used in the next step without further purification.

2,3,6-O-tri-O-acetyl-4-O-[2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl]-1-thio-(S-cyanomethyl)-β-D-glucopyranoside To lactose (10 g, 29.2 mmol, 1 eq.) were added AzCl (155 mg, 1636 mmol, 56 eq.) and NaOMe (10 g, 122.6 mmol, 4.2 eq.). The reaction was stirred at 140 °C for 5 h, recovered with H$_2$O (200 mL) and extracted with DCM (3 × 200 mL). The organic layer was washed with NaHCO$_3$ satd. solution (200 mL) and brine (200 mL), then dried over MgSO$_4$, filtered and the filtrate evaporated and co-evaporated with toluene, to afford 1,2,3,6-tetra-O-acetyl-4-O-[2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl]-β-D-glucopyranoside [40] as a light yellow foam (19.9 g, 29.2 mmol, quant.).
To 1,2,3,6-tetra-O-acetyl-4-O-[2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl]-β-D-glucopyranoside (14.15 g, 20.85 mmol, 1 eq.) in an ice bath, were added HBr (33% in AcOH, 23 mL, 129.27 mmol, 6.2 eq.) and Ac₂O (4.2 mL, 43.785 mmol, 2.1 eq.). The reaction was stirred for 3 h, recovered with DCM (200 mL) and poured into H₂O (200 mL). The mixture was stirred with NaHCO₃ satd. solution (300 mL), the organic layer separated and washed with NaHCO₃ satd. solution (3 × 200 mL) and brine (200 mL). The organic layer was dried over MgSO₄, filtered and the filtrate evaporated to afford 2,3,6-tri-O-acetyl-4-O-[2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl]-1-bromo-α-D-glucopyranoside [41] as a white foam (13.27 g, 19 mmol, 91%).

2,3,6-tri-O-acetyl-4-O-[2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl]-1-bromo-α-D-glucopyranoside (14.58 g, 20.85 mmol, 1 eq.) was mixed with thiourea (3.5 g, 45.87 mmol, 2.2 eq.) and dissolved in acetone (50 mL). The reaction was stirred for 3 h at 80 °C, then evaporated. The solid was recovered with acetone (60 mL), and Na₂S₂O₅ (10.3 g, 54.21 mmol, 2.6 eq.), K₂CO₃ (4 g, 29.19 mmol, 1.4 eq.) and chloroacetonitrile (20 mL, 316.92 mmol, 15.2 eq.) were added. The reaction mixture was stirred at r.t. for 3 h, evaporated and purified by flash column chromatography on silica (EtOAc:PE, 1:1) to afford the title compound as a white foam (8.7621 g, 12.66 mmol, 63%).

4-O-[β-D-galactopyranosyl]-1-thio-(S-2-imido-2-methoxyethyl)-β-D-glucopyranoside 1.2: To a solution of 2,3,6-tri-O-acetyl-4-O-[2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl]-1-thio-(S-cyanomethyl)-β-D-glucopyranoside (100 mg, 0.14 mmol, 1 eq.) in MeOH (dry, 1 mL) was added NaOMe (25% in MeOH, 33 μL, 0.14 mmol, 1 eq.). The reaction was stirred at r.t. for 16 h before being neutralised with DowexH⁺. The reaction mixture was filtered and evaporated without heating. The product was obtained as a mixture of the deprotected cyanomethyl (R-SCM) and ‘activated’ sugars (R-IME, 1.2) in ratio 1:0.7 (determined by ¹H NMR (CD₃OD), and used in the next step without further purification.

GlNac-Nal@SWCNTs: A solution of crude 2-acetamido-1-thio-(S-2-imido-2-methoxyethyl)-2-deoxy-β-D-glucopyranosyl 1.1 (6.5 mg, 20 μmol, 1 eq.) in MeOH:DMSO (2:1, 1.5 mL) was added to a suspension of Na125I@SWCNTs (0.3 mg, 3 MBq) in DCM (0.5 mL). The reaction was stirred at r.t. for 30 min. The reaction mixture was centrifuged, the supernatant discarded and the solid recovered with MeOH, filtered and washed with MeOH. The solid obtained was dried under vacuum to afford the title compound as a black solid (0.3 mg, 3 MBq).

Lac-Na125I@SWCNTs: A solution of crude 4-O-[β-D-galactopyranosyl]-1-thio-(S-2-imido-2-methoxyethyl)-β-D-glucopyranosyl 1.2 (6.0 mg, 20 μmol, 1 eq.) in MeOH:DMSO (2:1, 1.5 mL) was added to a suspension of Na125I@SWCNTs (0.3 mg, 3 MBq) in DCM (0.5 mL). The reaction was stirred at r.t. for 30 min. The reaction mixture was centrifuged, the supernatant discarded and the solid recovered with MeOH, filtered and washed with MeOH. The solid obtained was dried under vacuum to afford the title compound as a black solid (0.3 mg, 3 MBq).

For additional schemes, structures and characterization details, namely, TGA, HREMT, STEM, TLC, NMR mass spectrometry and infrared spectroscopy see the Supporting Information.

3. Results and discussion

3.1. Preparation of ‘cold’ glyco-Nal@SWCNTs

To prepare the nanotubes for filling they were first treated with steam, followed by an HCl (aq) wash, in order to remove graphitic nanoparticles, amorphous carbon and metal catalysts that remain as impurities from their generation [36]. This method also results in simultaneous shortening of the nanotubes via a process believed to involve oxidation and decarboxylation of more reactive carbon sites present at their tips [36]. TEM images of both as-received and steam-purified SWCNTs are shown in Fig. S1; these revealed some residual iron-derived nanoparticles (from the preparative catalyst) after steam treatment. Sodium iodide (hot or cold) was filled into these carbon nanotubes to generate Na@SWCNTs by adaptation of the protocol developed by Green et al. for the creation of KI ‘nanocrystals’ in SWCNTs [5]. Thus, a mixture of steam purified SWCNTs and the metal halide was annealed above the melting point of the inorganic salt (m,p-Nal = 661 °C) inside an evacuated silica ampoule. Heating at 900 °C not only drove encapsulation of salt inside the nanotubes, but also induced the closing of their tips [20]. In this way, internal NaI crystals were isolated from the outer environment by the formation of carbon ‘nanocapsules’ [42] (or ‘nanobottles’ [30]. As a result, any residual, external NaI present after synthesis was easily removed simply by washing the sample in refluxing water.

Characterization by high-angle annular dark-field scanning transmission electron microscopy (HAADF-STEM) allowed encapsulated salt to be clearly discerned from the walls of the nanotubes (Fig. 1). Visual inspection allowed filled SWCNTs containing heavy atoms (Fig. S2, white arrowed) to be readily distinguished from empty (red arrowed). This also confirmed that washing with water after filling successfully removed all residual salt from the outer surfaces of the CNTs in the sample. Successful encapsulation of NaI was also confirmed using high-resolution transmission electron microscopy (HRTEM, Fig. 1b), allowing observation of even the crystalline lattice of encapsulated NaI. Finally, analysis of the sample with energy dispersive X-ray spectroscopy (EDX, Fig. 1c further confirmed the presence of Na and I within the encapsulated cargo.

Following successful filling to form Nal@SWCNTs, covalent
functionalization of the sidewalls was achieved under mild conditions via 1,3-dipolar cycloaddition using appropriately functionalized azo-methine ylids \[14,43,44\] (Scheme 1). The choice of ylid also simultaneously allowed introduction of a 2,3,5-triiodophenyl motif as a ‘marker’ or ‘tagging’ motif,\[45\] suitable for the ready detection of successful functionalization using electron microscopy. To allow more ready diversification of glycan, a prior strategy devised by Hong et al.\[33\] was altered through first introducing a Boc-protected bis-amine branched linker moiety 1.0 to the CNT (Scheme 1), followed by de-protection using trifluoroacetic acid (TFA) to form a functionalized SWCNT as a divergent intermediate, f-NaI@SWCNT. Fmoc-numbering \[33\] was employed to quantify the loading of the primary amine groups thus introduced; the degree of functionalization at 0.34 mmol/g.

Intermediate f-NaI@SWCNT was then subsequently glycoconjugated with appropriate amine-reactive glycan-IME imidate reagents \[46,47\], GlcNAc-IME 1.1 or Lac-IME 1.2 to introduce GlcNAc or lactose (Galβ1,4–Glc) to the linkers attached to surface of the NaI@SWCNT, respectively, forming [GlcNAc]$_2$-NaI@SWCNT or [Lac]$_2$-NaI@SWCNT (Scheme 1).

HAADF-STEM imaging of the samples after GlcNAc functionalization Fig. 2 revealed a higher intensity in the bundle area when compared to the as-filled NaI@SWCNTs, due to the presence of the 2,3,5-triiodophenyl ‘marker’ combined into the linker moiety, thereby confirming successful covalent functionalization. The introduction of the linker to the surface of NaI@SWCNTs in f-NaI@SWCNTs, [GlcNAc]$_2$-NaI@SWCNT and [Lac]$_2$-NaI@SWCNT was also confirmed by TGA (Fig. 3). Unlike the NaI@SWCNTs whose combustion starts ∼400 °C, weight loss at lower temperatures even down to ∼200 °C was observed that can be attributed to the combustion of the organic fraction attached to the nanotubes. GlcNAc and Lac functionalized SWCNTs showed higher thermal stabilities than the linker-functionalized SWCNT f-NaI@SWCNTs. During functionalization inorganic materials are employed (e.g. MgSO$_4$) that appear to remain in the functionalized samples and that contribute to an unexpected increase in the amount of inorganic residue collected after the TGA for those samples that have been through these processes (SI Fig. S4). Successful glycoconjugation

**Scheme 1.** Functionalization of NaI@SWCNTs with biantennary linker reagent 1.0 and subsequent glycosylation. Reagents and conditions: a) DMF, 130 °C, 4 days; b) DCM, TFA, r.t., 24 h; c) 1.1 or 1.2, MeOH:DMSO (2:1), r.t., 30 min.

**Fig. 1.** NaI-filled SWCNTs (NaI@SWCNTs). (a) HAADF-STEM; since the intensity of the signal scales up to ca the square of the atomic number, the heavier atoms of the guest (Na and I) appear as brighter lines along the inner cavities of the nanotubes compared to C from their walls; (b) HRTEM image; (c) EDX spectroscopy confirmed the presence of Na and I. Fe and Cu signals correspond to those from SWCNT-generation catalyst residuals and the copper grid used for supporting the sample, respectively. Si signal arises from the EDX detector.
was further confirmed by elemental combustion analyses (see Supplementary Information).

3.2. Preparation of ‘hot’ glyco-Na\textsubscript{125}I@SWCNTs.

One mode in which glycosylated ‘nanocapsules/bottle’ might be exploited is in the highly controlled and localized delivery of radioactivity. This also potentially allows quantification of biodistribution and even direct imaging of this delivery. To test the potential of our glycosylated nanotubes \textit{in vivo} we created ‘hot nanobottle’ isotopologue variants in which we replaced ‘cold’ NaI with ‘hot’, radioactive Na\textsubscript{125}I. Gamma emission from 125I allows sensitive quantification and hence whole body distribution of constructs through ‘gamma counting’ of samples. Empty SWCNTs underwent an adapted molten phase filling process: SWCNTs and an aqueous solution of the radioactive salt were placed in a silica ampoule and heated to remove water. The ampoule was then sealed under vacuum and annealed following the protocol employed for ‘cold’ NaI. The functionalization and glycoconjugation process was carried out in an essentially analogous manner to that used for non-radioactive samples to generate ‘hot’ f-Na\textsubscript{125}I@SWCNTs (0.3 mg, 3 MBq), ‘hot’ [GlcNAc\textsubscript{2}-Na\textsubscript{125}I]@SWCNTs (0.3 mg, 3 MBq) and ‘hot’ [Lac\textsubscript{2}-Na\textsubscript{125}I]@SWCNTs (0.3 mg, 3 MBq).

3.3. Biodistribution analyses of ‘hot’ and ‘cold’ glyco-NaI@SWCNTs.

Previous studies performed on the robustness of radionuclide-filled nanotubes in biological environments have shown a strong correlation between radioactivity and nanotube distribution, suggesting negligible leakage of radionuclide salts [33,48]. This is particularly the case when using radio-iodide as ‘cargo’; free iodide is readily taken up by the thyroid leading to sensitive, easily detected observation – no such free iodide was detected in these prior studies. We used this correlation to quantify the distribution of nanotubes via gamma emission from the encapsulated Na\textsubscript{125}I cargo. ‘Hot’ glyco-Na\textsubscript{125}I@SWCNTs were injected in CD-1 male mice (0.1 mg/100 µL) and biodistribution after 1 h determined from gamma radiation levels in different organs (lungs, liver, spleen, brain, gut, kidneys, muscle and heart) \textit{ex vivo} (Fig. 4).

Two variants of mammalian glycans were chosen to test influence upon biodistribution. Both have putative endogenous receptors in...
mammals with different in vivo distribution. GlcNAc was used in our prior study in which functionalized NaI@SWCNTs containing radioactive iodide was successfully delivered into the lungs [33] and was again used here in [GlcNAc]2-Na125I@SWCNT. Galactosyl-terminated, Lac, is sometimes considered to be a ‘liver-targeting’ agent due to its interaction as a ligand with the asialoglycoprotein receptors expressed on hepatocytes [49] and was selected in this study as the second glycan from animals treated with glyco-NaI@SWCNT at levels of up to 126 ng/g.tissue; iodide was also successfully observed in samples of ∼25 ng/g.tissue; iodide was also successfully observed in samples in the tissues was determined by γ-counting and expressed as percentage of the injected dose per g of tissue (%ID/g).

Fig. 4. Biodistribution of amine-functionalized and glycosylated Na125I@SWCNTs. Organs of CD-1 mice were harvested 1 h after tail-vein injection of amine-functionalized (f-Na125I@SWCNTs) and glycosylated nanotubes (GlcNAc-Na125I@SWCNTs; Lac-Na125I@SWCNTs). The distribution of the samples in the tissues was determined by γ-counting and expressed as percentage of the injected dose per g of tissue (%ID/g).

Next, in order to test whether biodistribution could be attributed to dominant, inherent physiological properties of the nanotubes, we attempted to assess the effect of an alternative injection site via intraaortic injection that necessitated preferential use of ‘cold’ glyco-SWCNTs and quantification instead by ICP-MS analysis of tissues appears to lack the sensitivity required for unambiguous biodistribution analysis. These ‘nanocapsules/bottles’ may represent a useful, sealed ‘source of radiation’. If this could be combined with the benefits of a delivery system, then this could create a potentially suitable form of ‘nano-brachytherapy’. Given the seemingly dominant control of physical properties rather than biochemical properties upon distribution observed here, we speculate that future manipulation of not only surface functionalization (e.g. at higher levels or with different types) but even via changes to carbon nanostructure at a SWCNT level might usefully influence their in vivo capabilities.

Acknowledgements

This work was financially supported by EU FP7-ITN Marie-Curie Network programme RADDEL [grant number 290023] and the EU FP7-Integrated Infrastructure Initiative–I3 programme ESTEEM2 [grant number 312483]. We also acknowledge financial support from Spanish Ministry of Economy and Competitiveness through the “Severo Ochoa” Programme for Centres of Excellence in R&D [grant numbers SEV-2015-0496, ICMAB; SEV-2017-0706, ICN2]. The ICN2 is funded by the CERCA programme. We would like to thank Thomas Swan & Co. Ltd. for supplying Elicarb® SWNT.

Finally and above all, this work on probing the potential of such ‘nanobottles’ in physiology has been inspired by many stimulating and fruitful conversations with Prof Malcolm Green – his insight and vision, as on many other occasions, has proven invaluable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ica.2019.05.032.

References