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PAPER

Polyamine functionalized carbon nanotubes: synthesis, characterization, cytotoxicity and siRNA binding†

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In this work we have synthesized a new series of cationic carbon nanotubes (CNTs) for siRNA binding. Both single- and multi-walled CNTs have been modified by addition or amidation reaction with short linear polyamine chains including putrescine, spermidine and spermine. All the new conjugates have been characterized with several spectroscopic and microscopic techniques. Their cytotoxic effects have been assessed on human lung carcinoma cell line. Finally, we have analyzed their capacity to bind siRNA towards the development of new carriers for gene silencing applications. The dispersibility properties and the capacity to complex siRNA of the different conjugates were found to be strongly dependent on the position of the functional groups on CNTs (*i.e.* mainly at the tips following the amidation reaction or on the sidewalls by direct C–C addition).

Introduction

Single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs) are an important class of nanomaterials,[1,2] and recent studies in the field of nanotechnology and nanomedicine have reported that CNTs could find a wide range of biological applications such as drug and gene delivery, therapeutics and diagnostics.

However, to fully exploit the properties of CNTs in nanomedicine, we need to overcome their extremely poor aqueous dispersion and their tendency to aggregate in order to obtain homogeneous dispersions in aqueous media. In comparison to other nanomaterials, functionalized CNTs (*f*-CNTs) have shown higher biocompatibility and the capacity to cross cell membranes easily.[3–5] The chemical modification of the CNT surface through covalent or non-covalent functionalization increases their solubility in both organic and aqueous solvents.[6–8] Two main approaches have been developed for the covalent functionalization of carbon nanotubes: (i) the amidation and the esterification of oxidized CNTs; and (ii) the addition chemistry to the surface of CNTs.[6,7] The first approach includes treatment of the pristine material under strong acidic and oxidative conditions, such as sonication in a mixture of concentrated nitric and sulfuric acid, or heating in a mixture of sulfuric acid and hydrogen peroxide, which results in the formation of short open tubes with oxygenated functions (mainly carboxylic groups). This approach represents a popular pathway for further modification of the nanotubes, since the carboxylic functions can react with alcohols or amines to give ester or amide derivatives. In the second approach, more elaborate methods have been developed to attach organic moieties directly onto the nanotube sidewalls (*e.g.* cycloadditions, electrophilic, nucleophilic or radical additions).[6]

In our search for new cationic carbon nanotubes towards the development of advanced systems for the delivery of nucleic acids, we have recently designed simple ammonium-functionalized CNTs and more sophisticated dendron-based nanotubes.[9,10] The different conjugates were used for gene silencing experiments *in vitro* and *in vivo* demonstrating the capacity of such systems to deliver and eventually knock-down different genes efficiently.[10] In this paper we have extended the synthesis of cationic nanotubes for gene silencing through reaction with a series of linear polyamines. Polyamines, such as putrescine, spermidine and spermine, are polycationic straight-chain aliphatic compounds, ubiquitously present in all prokaryotic and eukaryotic cells,[11] and are required in important biological processes such as cell proliferation and differentiation.[12–16] Spermidine and spermine primarily exist in aqueous solution at pH 7.4 as fully protonated polycations.[17] Therefore, they can bind to the negatively charged nucleic acid either by electrostatic interactions and/or by hydrogen bonding. They have been shown to condense DNA to stabilize its conformation or to prevent its degradation,[18] and to induce both B-to-Z and B-to-A transitions in certain DNA sequences.[19] They also modulate the structure of transfer RNA

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and other RNAs.[20] These types of polycationic molecules have been also proposed for plasmid DNA delivery and siRNA silencing.[21]

For this purpose, we have synthesized 4-aminobenzoyl derivatives of putrescine, spermidine and spermine and used them for conjugation to CNTs. We carried out addition and amidation reactions for the covalent functionalization of both SWCNTs and MWCNTs. The cationic conjugates were fully characterized by thermogravimetric analysis (TGA), transmission electron microscopy (TEM) and Raman spectroscopy. We also performed preliminary studies to analyze the impact of these polyamine-functionalized CNTs on mammalian cell cultures in terms of cytotoxic effects. Aiming to use them in order to condense and deliver siRNA, we have also studied the siRNA binding capacity of some of the new conjugates using gel electrophoresis.

Experimental section

Materials

HiPCO SWCNTs were purchased from Carbon Nanotechnologies Inc. (Lot no. R0496). MWCNTs, produced by the catalytic carbon vapour deposition (CCVD) process, were purchased as purified from Nanocyl (Thin MWCNT 95+% C purity, Nanocyl 3100®, Batch no. 071005, average diameter and length: 9.5 nm and 1.5 microns, respectively). Reagents and solvents were purchased from Fluka, Acros, Aldrich and used without further purification unless otherwise stated. Moisture-sensitive reactions were performed under Ar or N_2 atmosphere. CH_2Cl_2 was freshly distilled from CaH_2, THF from Na/benzophenone, and DMF dried over 4 Å molecular sieves. Chromatographic purification was done with silica gel Merck (Kieselgel 60, 40–60 μm, 230–400 mesh ASTM) in standard columns. TLC was performed on aluminium sheets coated with silica gel 60 F254 (Merck, Darmstadt). ^1H and ^{13}C NMR spectra were recorded on Bruker DPX 300 (operating at 300 and 75 MHz, respectively). The peak values were obtained as ppm (δ), and referenced to the solvent. The protected polyamines **13**, **19** and **25** were synthesized through stepwise elongation of the backbone and fully characterized (see ESI†). Protected polyamines **13**, **19** and **25** were further modified using already reported methods to give *N*-(4-aminobenzoyl)polyamine derivatives **15**, **21** and **27**,[22] respectively as described in ESI†. Human lung carcinoma (A549) cell line was purchased from American Type Culture Collection (ATCC) (USA) (ATCC# CCL-185). Non-coding negative siRNA (siNEG) was purchased from Eurogentec (UK). Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, glutamine, and phosphate buffered saline (PBS) and electrophoresis grade Agarose were from Gibco, Invitrogen (UK). Tris/borate/EDTA (TBE) buffer and ethidium bromide were obtained from Sigma-Aldrich (USA). The orange loading dye solution was purchased from Fermentas (UK). LDH release was assessed using Promega Cytotox 96® Non-radioactive cytotoxicity assay (Promega UK Ltd).

Preparation of modified polyamine functionalized CNTs through addition reaction

SWCNTs and MWCNTs were covalently functionalized with modified polyamines (putrescine, spermidine or spermine) using addition reactions. The diazotization-coupling procedure between CNTs and *N*-(4-aminobenzoyl)polyamine in *o*-dichlorobenzene/CH_3CN results in effective functionalization of the exohedral surface of CNTs *via in situ* generation of diazonium salts.[23]

Preparation of modified putrescine functionalized SWCNTs (1b)

General procedure: pristine HiPCO SWCNTs (30 mg, 2.5 mmol) were homogeneously dispersed in 30 mL of *o*-dichlorobenzene under Ar atmosphere through bath sonication for 2 h. To this suspension was added **15** (0.77 g, 2.5 mmol) previously dissolved in 10 mL of CH_3CN. The suspension was further sonicated for 10 min. After bubbling with Ar for 5 min, isoamyl nitrite (1.34 mL, 10.0 mmol) was quickly added and suspension was further stirred for 36 h at 60 °C under Ar atmosphere. The resulting nanotubes were re-precipitated several times from DMSO/diethyl ether, DMF/diethyl ether and methanol/diethyl ether by successive bath sonication and centrifugation (4500 rpm, 5 min, Eppendorf Centrifuge 5804R) procedures until the filtrate became colorless. Sonication and redispersion was repeated with a mixture of DMF/diethyl ether and then in diethyl ether. The product was dried under vacuum at room temperature for 2 h to give polyamine functionalized SWCNTs **1a** as a black powder. Twenty mg of Boc-protected polyamine–CNTs **1a** were suspended in 8 mL of 4 M HCl solution in dioxane and stirred at room temperature for 12 h under Ar atmosphere to cleave the Boc group. Then, solvent was evaporated under vacuum. The resulting nanotubes were washed with a mixture of CH_2Cl_2/CH_3OH several times and finally with diethyl ether and dried under vacuum for 1 h to give cationic functionalized CNTs **1b** as a black powder. The nanotubes were characterized by TEM, Raman spectroscopy and TGA. Raman D/G ratio: 0.171; TGA mass loss: 20.8% (at 600 °C); TGA analysis afforded one functional group every 73 carbon atoms, amount of functional groups: 0.91 mmol g^{-1}.[24]

Preparation of modified spermidine functionalized SWCNTs (3b). The addition reaction was performed as reported for putrescine above. The product was dried in vacuum at room temperature for 1 h to give cationic polyamine–CNTs **3b** as a black powder. Raman D/G ratio: 0.133; TGA mass loss: 15.4% (at 600 °C); TGA analysis afforded one functional group every 147 carbon atoms, amount of functional groups: 0.48 mmol g^{-1}.

Preparation of modified spermine functionalized SWCNTs (5b). The addition reaction was performed as reported for putrescine above. The product was dried in vacuum at room temperature for 1 h to give cationic polyamine–CNTs **5b** as a black powder. Raman D/G ratio: 0.185; TGA mass loss: 14.1% (at 600 °C); TGA analysis afforded one functional group every 211 carbon atoms, amount of functional groups: 0.34 mmol g^{-1}.

Preparation of modified putrescine functionalized MWCNTs (2b). The addition reaction was performed on purified MWCNTs (30 mg) as reported for the preparation of **1b**. The product was dried in vacuum at room temperature for 1 h to give cationic polyamine–CNTs **2b** as a black powder. TGA mass loss: 7.80% (at 600 °C); TGA analysis afforded one functional group every 225 carbon atoms, amount of functional groups: 0.34 mmol g^{-1}.

Preparation of modified spermidine functionalized MWCNTs (4b). The addition reaction was performed as reported for putrescine above. The product was dried in vacuum at room temperature for 1 h to give cationic polyamine–CNTs **4b** as a black powder. TGA mass loss: 8.50% (at 600 °C); TGA analysis afforded one functional group every 288 carbon atoms, amount of functional groups: 0.26 mmol g^{-1}.

Preparation of modified spermine functionalized MWCNTs (6b). The addition reaction was performed as reported for putrescine above. The product was dried in vacuum at room temperature for 1 h to give cationic polyamine–CNTs **6b** as a black powder. TGA mass loss: 9.00% (at 600 °C); TGA analysis afforded one functional group every 349 carbon atoms, amount of functional groups: 0.22 mmol g^{-1}.

Preparation of modified polyamine functionalized CNTs through amidation reaction

SWCNTs and MWCNTs were also covalently functionalized with modified polyamine (putrescine, spermidine and spermine) mainly at their tips through amide bond formation. The carboxylic acid functional groups were introduced at the tips and sidewall of CNTs using modified oxidative methods.[25] The carboxylic groups were subsequently converted into acid chloride, followed by addition of modified polyamine which results in covalent attachment of polyamines at the tips of CNTs.

Preparation of modified putrescine functionalized SWCNTs (7b)

General procedure: 100 mg of pristine HiPCO SWCNTs were suspended in 75 mL of a 3 M HNO_3 by bath sonication. The mixture was refluxed for about 48 h, sonicated for 1 h, and refluxed again for another 48 h. Then, 25 mL of 3 M HNO_3 was added and after bath sonication for 2 h, the mixture was again refluxed for 12 h. The obtained suspension was then diluted by deionized water and filtered through a polycarbonate filter (Isopore, pore size 100 nm), rinsed thoroughly with deionized water several times until the pH value was ~7. The resulting SWCNTs were resuspended in deionized water and sonicated for 5 min. The suspension was then again filtered. The black product obtained was dried and characterized by TEM, AFM and TGA.[26] A suspension of 10 mg of oxidized SWCNTs in 4 mL of neat oxalyl chloride was stirred at 62 °C for 24 h under Ar atmosphere. The excess of oxalyl chloride was evaporated under vacuum, obtaining SWCNT-C(O)Cl. The resulting nanotubes were suspended in a solution of **15** (74 mg, 0.24 mmol) in 15 mL of distilled THF or DMF followed by addition of diisopropylethylamine (DIEA) (42 μL). The resulting suspension was heated under reflux for 48 h. After cooling to room temperature and removing excess of **15** by washing several times with DMSO/diethyl ether, DMF/diethyl ether and methanol/diethyl ether by successive bath sonication and centrifugation (4500 rpm, 5 min) procedures and finally with diethyl ether. The resulting Boc-protected SWCNTs **7a** were dried at room temperature under vacuum. Eight mg of Boc-protected polyamine–CNTs **7a** were suspended in 4 mL of 4 M HCl solution in dioxane and stirred at room temperature for 12 h under Ar atmosphere to cleave the Boc group. Then solvent was evaporated under vacuum. The resulting nanotubes were washed with a mixture of dichloromethane and methanol several times and finally with diethyl ether and dried under vacuum for 1 h to give cationic functionalized CNTs **7b** as a black powder. The nanotubes were characterized by TEM, Raman spectroscopy and TGA. Raman D/G ratio: 0.117; TGA mass loss: 21.5% (at 600 °C); TGA analysis afforded one functional group every 83 carbon atoms, amount of functional groups: 0.79 mmol g^{-1}.

Preparation of modified spermidine functionalized SWCNTs (9b). The amidation reaction was performed as reported for putrescine above. The product was dried in vacuum at room temperature for 1 h to give cationic polyamine–CNTs **9b** as a black powder. Raman D/G ratio: 0.113; TGA mass loss: 18.7% (at 600 °C); TGA analysis afforded one functional group every 132 carbon atoms, amount of functional groups: 0.51 mmol g^{-1}.

Preparation of modified spermine functionalized SWCNTs (11b). The amidation reaction was performed as reported for putrescine above. The product was dried in vacuum at room temperature for 1 h to give cationic polyamine–CNTs **11b** as a black powder. Raman D/G ratio: 0.219; TGA mass loss: 17.5% (at 600 °C); TGA analysis estimates one functional group every 180 carbon atoms, amount of functional groups: 0.38 mmol g^{-1}.

Preparation of modified putrescine functionalized MWCNTs (8b)

One hundred mg of purified MWCNTs were sonicated in a water bath (20 W, 40 kHz) for 24 h in 15 mL of sulfuric acid/nitric acid (3 : 1 v/v, 98% and 65%, respectively) at room temperature.[25,27] Deionized water was then carefully added and the MWCNTs were filtered (Omnipore® membrane filtration, 0.45 μm), resuspended in water, filtered again until pH became neutral and dried. A suspension of 10 mg of *ox*-MWCNTs in 4 mL of neat oxalyl chloride was stirred at 62 °C for 24 h under Ar atmosphere. After evaporation in vacuum the resulting nanotubes were dispersed in a solution of **15** (74 mg, 0.24 mmol) in 15 mL of distilled THF or DMF followed by addition of DIEA (42 μL). The resulting suspension was heated under reflux for 48 h. After cooling to room temperature and removing excess of **15** by washing several times with DMSO/diethyl ether, DMF/diethyl ether and methanol/diethyl ether by successive bath sonication and centrifugation (4500 rpm, 5 min) procedures and finally with diethyl ether. The resulting Boc-protected MWCNTs **8a** were dried at room temperature under vacuum. The Boc protecting group was removed using 4 M HCl solution in dioxane. Then, solvent was evaporated under vacuum. The resulting nanotubes were washed with a mixture of dichloromethane and methanol several times and finally with diethyl ether and dried under vacuum for 1 h to give cationic polyamine–CNTs **8b** as a black powder. TGA mass loss: 25.5% (at 600 °C); TGA analysis estimates one functional group every 66 carbon atoms, amount of functional groups: 0.94 mmol g^{-1}.

Preparation of modified spermidine functionalized MWCNTs (10b). The amidation reaction was performed on MWCNTs as reported for putrescine above. The product was dried in vacuum at room temperature for 1 h to give cationic polyamine–CNTs

10b as a black powder. TGA mass loss: 23.2% (at 600 °C); TGA analysis estimates one functional group every 100 carbon atoms, amount of functional groups: 0.63 mmol g^{-1}.

Preparation of modified spermine functionalized MWCNTs (12b). The amidation reaction was performed on MWCNTs as reported for putrescine above. The product was dried in vacuum at room temperature for 1 h to give cationic polyamine–CNTs **12b** as a black powder. TGA mass loss: 19.1% (at 600 °C); TGA analysis estimates one functional group every 161 carbon atoms, amount of functional groups: 0.42 mmol g^{-1}.

Characterization

The thermogravimetric analyses were performed using a TGA Q500 TA instrument with a ramp of 10 °C min^{-1} under N_2 from 100 °C to 800 °C. Transmission electron microscopy (TEM) was performed on a Hitachi H600 microscope and a Philips 208 working at different accelerating voltage and at different magnification. 0.1 microgram of the sample was dispersed in 0.1 mL of methanol/water (1 : 1) by ultrasonication for 5 min and kept for 10–12 h. The solution was again ultrasonicated for 5 min before depositing and 10 μL was deposited onto a carbon coated TEM grid and dried. The images are typical and representative of the samples under observation. Raman spectra were acquired with a Renishaw instrument, model Invia reflex equipped with 532 nm, 632.8 nm and 785 nm lasers. The spectra were registered with the laser at 532 nm. After acquisition the spectra were normalized with respect to the G band and then the amplitude of the D peak was calculated.

Cell viability assay

A549 cells were maintained and passaged in DMEM media, supplemented with 10% fetal bovine serum (FBS), 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 1% L-glutamine and 1% non-essential amino acids, at 37 °C in 5% CO_2. A549 cells were incubated with polyamine–CNTs in complete media for 24 or 72 h. Toxicity was assessed by the modified LDH assay described previously using the Promega Cytotox 96® Non-radioactive cytotoxicity assay (Promega UK Ltd) according to the manufacturer instructions.[28] The assay was modified to avoid interference between *f*-MWCNTs and the LDH in the survived cells. Briefly, the LDH was quantified in the cells that survived the treatment after being artificially lysed. A549 cells were seeded at 15 000 cells (24 h) and 8000 cells (72 h) in a 96 well plate and left to attach overnight. Cells were then treated with polyamine–CNTs at 1, 10 and 100 μg mL^{-1} concentrations. In addition, 10% DMSO or 1 mM H_2O_2 were used as positive controls to induce cytotoxicity. After 24 h or 72 h treatment, cells were lysed with 10 μL of lysis buffer per 100 μL serum free media and left for 45–60 min at 37 °C. Fifty microlitres of cell lysate after centrifugation (13 000 rpm, 5 min) were mixed with 50 μL substrate mixture in a new microtiter plate and incubated for 15 minutes at room temperature. Absorbance at 490 nm was read in an ELISA plate reader. A centrifugation step was included to remove any nanotubes from the assay mixture which could interfere with the accuracy of the assay. The amount of LDH released was an indication of the number of cells that survived the treatment and expressed as cell viability. Hence, the percentage cell survival is expressed as [LDH released from tested cells − blank (media alone)/LDH released from control untreated cells] × 100.

Electrophoretic motility shift assay

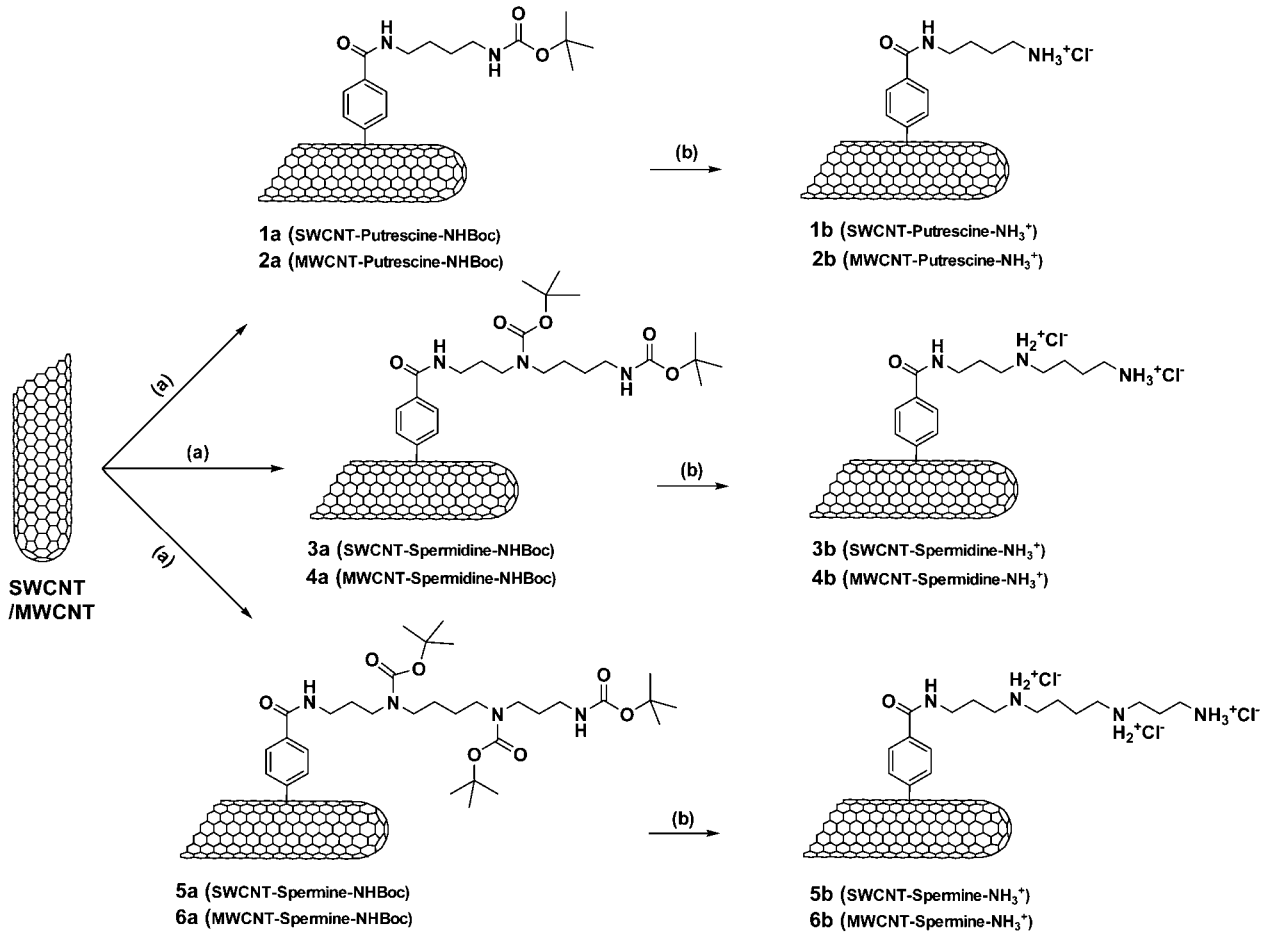
siRNA–nanotube complexes were prepared by mixing 0.5 μg of siRNA in 30 μL of 5% dextrose with polyamine–CNTs at different charge ratios (1 : 0 to 1 : 18 −/+). 0.5 μg of free siRNA was used as a control. The pH was monitored during the binding to siRNA and no variation was observed before and after the complexation or among the various CNT constructs. The pH of the dispersions and the complexes was around 6.0–7.5. At this pH all amino groups of the different constructs are more likely to be protonated. Complexes were incubated for 30 min at room temperature to allow complete complexation to occur. siRNA complexes or siRNA alone were mixed with orange dye solution (1 : 10 dilution) and loaded onto 1% (w/v) agarose/TBE gel containing ethidium bromide (0.5 μg mL^{-1}). The gel was run for 45 min at 70 mV (BioRad, UK). The gel was then photographed under UV light using GeneGenius system (PerkinElmer Life and Analytical Sciences, USA).

Results and discussion

Design, synthesis and characterization

To explore the ability of polyamine-modified carbon nanotubes to complex and eventually deliver siRNA for gene silencing, we have designed and prepared two families of CNTs functionalized by amidation reaction of the carboxylic groups and by addition of *in situ* generated diazonium derivatives to CNT aromatic surface. For this purpose, we have used both single- and multi-walled carbon nanotubes and combined them with polyamines like putrescine (1,4-diaminobutane), spermidine (1,8-diamino-4-azaoctane) and spermine (1,12-diamino-4,9-diazadodecane). Oxidized SWCNTs (*ox*-SWCNTs) and MWCNTs (*ox*-MWCNTs) were obtained after modification of earlier reported procedures.[25,29] The strong acid treatment generates defects on the sidewalls and formation of open ends that are both terminated by carboxylic acid groups. The Boc protected polyamines spermidine and spermine were synthesized in turn through stepwise elongation of 1,4-diaminobutane backbone before reaction with acid chlorides. This allows obtaining an elongated aliphatic chain of the polyamine backbone thus introducing an increasing number of amino groups per functional group. The solution phase synthesis of these orthogonally protected asymmetrical or symmetrical polyamines can be carried out using the combination of Boc, phthalimide and benzyl protecting groups (see ESI†).

For carrying out the addition reaction, we prepared 4-nitrobenzoyl derivatives of polyamines, which were eventually reduced to obtain the corresponding 4-aminobenzoyl derivatives (**15**, **21** and **27**) of each polyamine in good yields. Pristine SWCNTs and purified MWCNTs were reacted with 4-aminobenzoyl derivatives of polyamines in *o*-dichlorobenzene using the diazotization coupling procedure to produce polyamine-functionalized SWCNTs (*f*-SWCNTs) (**1a**, **3a** and **5a**) and MWCNTs (*f*-MWCNTs) (**2a**, **4a** and **6a**) (Scheme 1). This *in situ*



Scheme 1 Addition reactions to SWCNTs and MWCNTs of 4-aminobenzoyl derivatives of polyamines (putrescine, spermidine and spermine). (a) Isoamyl nitrite, *o*-dichlorobenzene : CH_3CN (3 : 1), 4-aminobenzoyl derivatives (**15**, **21** and **27**, respectively), 60 °C, 36 h; (b) 4 M HCl solution in dioxane, rt, 12 h.

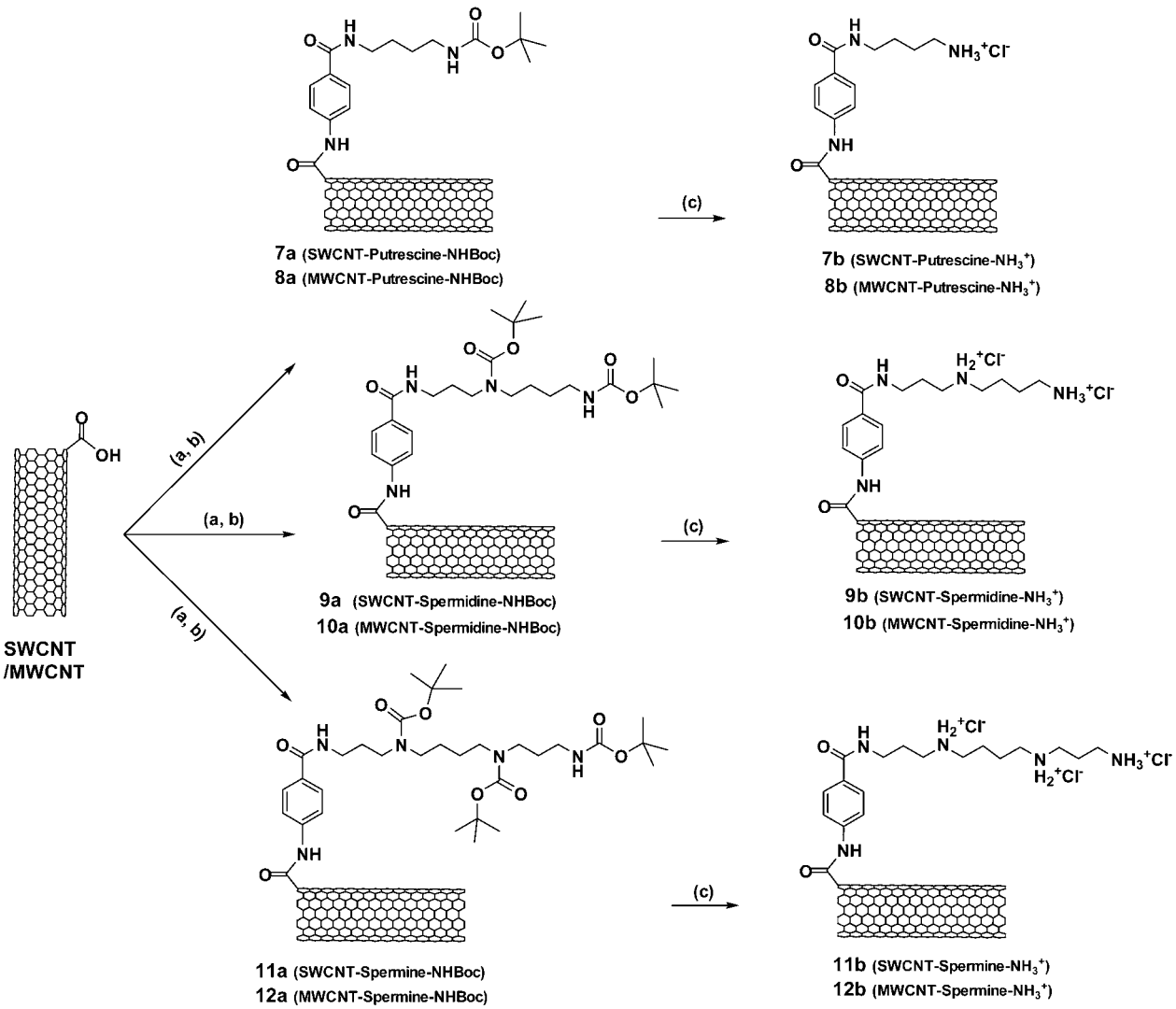
diazotization coupling to CNTs allows effective functionalization without the use of unstable aryl diazonium salts.

Alternatively, using the amidation approach, *f*-SWCNTs (**7a**, **9a** and **11a**) and *f*-MWCNTs (**8a**, **10a** and **12a**) were synthesized following the acyl chloride route,[28] allowing the oxidized CNTs to react with oxalyl chloride under argon at refluxing conditions. The resulting acid chloride derivatives were then heated under reflux with 4-aminobenzoyl derivatives of polyamines **15**, **21** and **27**, respectively (Scheme 2).

All functionalized CNTs were then treated with a solution of HCl in dioxane to remove the protecting groups affording the different cationic conjugates (**1b**–**12b**).

The degree of functionalization for the two families of polyamine-based CNTs (**1b**–**12b**) has been evaluated by TGA under N_2 atmosphere (Fig. 1). Pristine SWCNTs and purified MWCNTs lost only 1.1 and 1.2% of mass up to 700 °C, while TGA curves of the functionalized nanotubes **1b**–**12b** display a considerable decrease of weight in the range of 200–600 °C corresponding to the decomposition of the attached 4-aminobenzoyl polyamine moiety. Fig. 1(a) and (b) put in evidence the TGA differences between SWCNTs and MWCNTs, respectively, functionalized with polyamines through the addition reaction. We can derive that the exohedral surface of SWCNTs has a higher functionalization degree than MWCNTs. This is most likely because the content of carbon on MWCNTs is higher than on SWCNTs due to the presence of the additional concentric internal layers. Among the different polyamines used, we observed higher weight loss for putrescine derivative followed by spermidine and spermine owing to the presence of the increasing number of the bulky Boc protecting groups that might reduce the reactive available area, probably inducing steric hindrance that prevents further additions. The TGA curves of *f*-SWCNTs **1b**, **3b** and **5b** showed a gradual weight loss of about 20.8, 15.4 and 14.1% at 600 °C, respectively, as compared to 7.8, 8.5 and 9.0% for *f*-MWCNTs **2b**, **4b** and **6b**, respectively. On the basis of TGA weight loss, we estimated that the amount of functional groups per gram (f_w, mmol g^{-1}) of *f*-SWCNTs **1b**, **3b** and **5b** is 0.91, 0.48 and 0.34, respectively, corresponding to a degree of functionalization of one polyamine group every 73, 147 and 211 carbon atoms, respectively. In the case of MWCNTs, the number of functional groups per gram of *f*-MWCNTs **2b**, **4b** and **6b** is 0.34, 0.26 and 0.22, respectively, corresponding to a degree of functionalization of one functional group every 225, 288 and 349 carbon atoms, respectively.

Similarly, Fig. 1(c) and (d) shows the TGA curves of SWCNTs and MWCNTs functionalized with polyamines through



Scheme 2 Amidation reactions on *ox*-SWCNTs and *ox*-MWCNTs with 4-aminobenzoyl derivatives of polyamines (putrescine, spermidine and spermine). (a) Neat $(COCl)_2$; (b) 4-aminobenzoyl derivatives (**15**, **21** and **27** respectively), 60 °C, 48 h, THF (dry); (c) 4 M HCl solution in dioxane, rt, 12 h.

amidation reaction, respectively. The TGA curves of *f*-SWCNTs **7b**, **9b** and **11b** showed a gradual weight loss of about 21.5, 18.7 and 17.5% at 600 °C, respectively, as compared to 16.0% for the *ox*-SWCNTs whereas, TGA curves of *f*-MWCNTs **8b**, **10b** and **12b** showed a gradual weight loss of about 25.5, 23.2 and 19.1% at 600 °C, respectively, as compared to 13.1% for the *ox*-MWCNTs. From the TGA weight loss, we calculated 0.79, 0.51 and 0.38 mmol of functional groups per gram of *f*-SWCNTs **7b**, **9b** and **11b**, that is, one group every 83, 132 and 180 SWCNT carbon atoms. Similarly, 0.94, 0.63 and 0.42 mmol functional groups per gram of *f*-MWCNTs **8b**, **10b** and **12b** was calculated which corresponds to one group every 66, 100 and 161 MWCNT carbon atoms. In the case of the amidation reaction MWCNTs seem to give a better functionalization yield in comparison to SWCNTs, while the opposite situation was found for the direct addition to the tubes.

The polyamine–CNT hybrids were also characterized by transmission electron microscopy (TEM). The TEM images of polyamine–CNTs **1b**–**6b** synthesized by the addition reaction are shown in Fig. 2. We have observed that a high degree of de-bundling occurred in the case of polyamine-functionalized SWCNTs as compared to pristine SWCNTs, which is consistent with the degree of functionalization assessed by TGA results. Fig. 2(a)–(f) displays small bundles of polyamine–CNTs deposited from a methanol/H_2O (1 : 1) solution onto a carbon coated TEM grid. Comparing the TEM images of different polyamine–CNTs, it can be evaluated that polyamine–CNTs **3b**, **4b** (Fig. 2(b) and (e)) and polyamine–CNTs **5b**, **6b** (Fig. 2(c) and (f)) show more debundling both in the case of SWCNTs and MWCNTs in comparison to polyamine–CNTs **1b**, **2b** (Fig. 2(a) and (d)) under the same conditions. This behaviour can be attributed to the presence of the longer chains in the case of spermidine and spermine and the higher number of positive charges that can produce higher electrostatic repulsions.

Fig. 3 displays TEM images of polyamine–CNTs **7b**–**12b** synthesized by amidation reaction deposited again from a methanol/H_2O (1 : 1) solution onto a carbon coated TEM grid. In comparison to oxidized CNTs[26] bundles of polyamine–CNTs

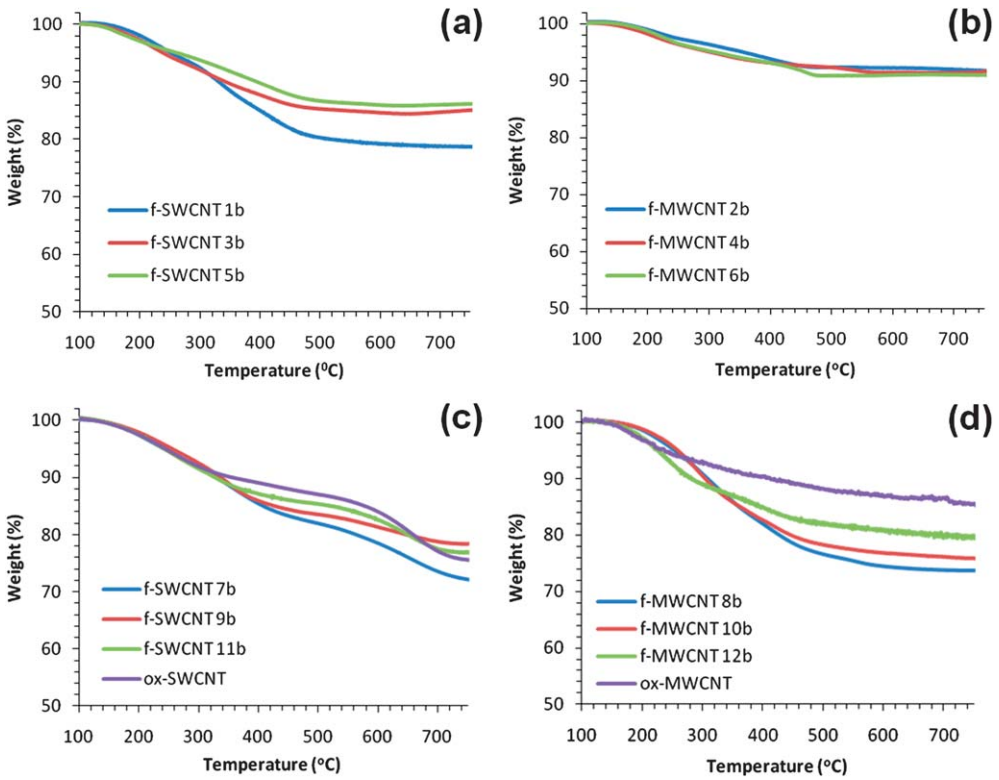


Fig. 1 Thermogravimetric analysis for *ox*-CNTs and *f*-CNTs **1b**–**12b**. (a) Thermogravimetric analysis of *f*-SWCNTs **1b**, **3b**, and **5b**; (b) Thermogravimetric analysis of *f*-MWCNTs **2b**, **4b**, and **6b**; (c) Thermogravimetric analysis of *f*-SWCNTs **7b**, **9b**, and **11b** and *ox*-SWCNTs; (d) Thermogravimetric analysis of *f*-MWCNTs **8b**, **10b**, and **12b** and *ox*-MWCNTs, in N_2 atmosphere with a ramp of 10 °C min^{-1}.

7b–**12b** are more dissociated and less tightly bound to each other, which further confirmed the functionalization with different polyamines and its role in modulating the dispersibility properties of carbon nanotubes. The nanotubes are very short in accordance with the long oxidation process. However, as can be seen from the images, nanotubes remained largely intact after functionalization.

Additional characterization was performed using Raman spectroscopy. This technique allows the evaluation of the SWCNT properties after functionalization. Although the information obtained from Raman spectra of CNTs should be considered merely qualitative, we have investigated whether the structural properties of CNTs are preserved after functionalization. The D/G ratio provides information about the quality of

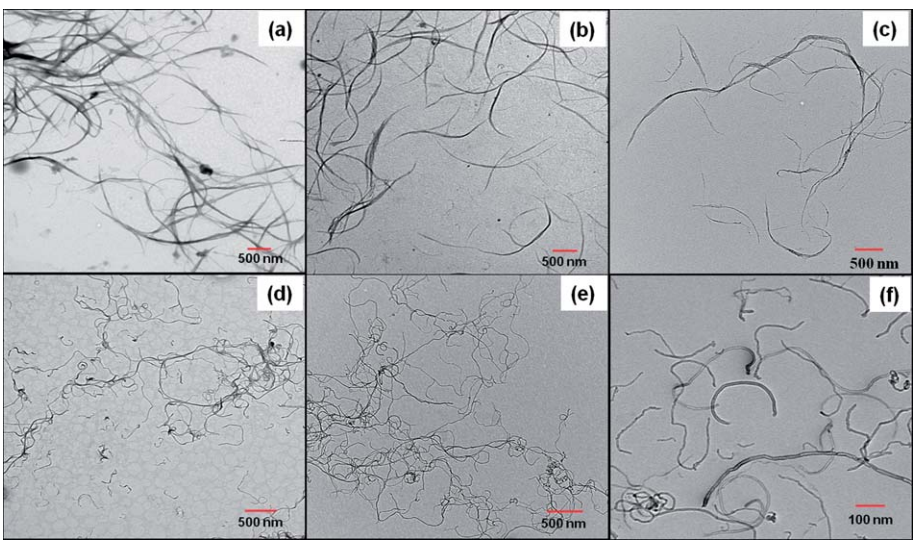


Fig. 2 TEM images of polyamine–CNTs prepared using addition reaction; TEM images of putrescine-functionalized SWCNTs **1b** (a) and MWCNTs **2b** (d), TEM images of spermidine-functionalized SWCNTs **3b** (b) and MWCNTs **4b** (e) and TEM images of spermine-functionalized SWCNTs **5b** (c) and MWCNTs **6b** (f).

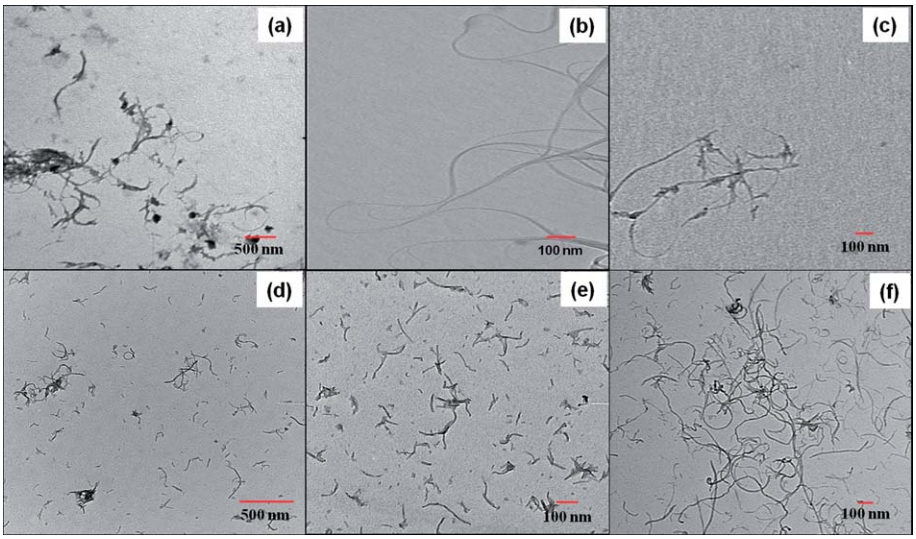


Fig. 3 TEM images of polyamine–CNT hybrids prepared using amidation reaction; TEM images of putrescine-functionalized SWCNTs **7b** (a) and MWCNTs **8b** (d), TEM images of spermidine-functionalized SWCNTs **9b** (b) and MWCNTs **10b** (e) and TEM images of spermine-functionalized SWCNTs **11b** (c) and MWCNTs **12b** (f).

these materials. The relative amplitude of D and G peaks was accurately measured for all the spectra, where the G band was much higher than the D band in all derivatives (Fig. 4). This shows that polyamine–CNTs bear a relatively low quantity of defects mainly preserving their characteristic properties, irrespective of the different types of functionalization employed. Furthermore, from a comparison between the D and G ratio on the one hand, and the radial breathing mode features on the other (RBM, 150–300 cm^{-1}) we can derive information about the degree of functionalization and, more precisely, whether *f*-SWCNTs have maintained their structures after functionalization or not. On average the D/G ratio ranges between 0.133 and 0.185 for SWCNTs **1b**, **3b** and **5b** while the value in the pristine material was 0.126, indicating that surface functionalization occurred without significantly affecting the structure of the nanotubes. In the case of *ox*-SWCNTs **7b**, **9b** and **11b** we analyzed more carefully the RBM region as the values of the D/G ratio (0.113–0.219) were thought to be due to the functional groups mainly at the tips. The RBM bands after the amidation reaction had high intensity meaning that the structure of the *ox*-SWCNTs was not disrupted. This was also considered as evidence that the oxidation and consequently the amidation reactions were more selective to the CNT tips with respect to the *in situ* formation of the diazonium salt reaction that was likely occurring across the CNT backbone surface.

Cytotoxicity and siRNA binding

With the intention of using these new families of polyamine-functionalized carbon nanotubes for binding and delivering siRNA, we analyzed the impact of their exposure to cells in terms of cytotoxic adverse effects. Human lung epithelial cells (A549) were incubated with polyamine–CNTs **1b**–**12b** at different concentrations (1–100 μg mL^{-1}) for 24 or 72 h (to assess prolonged cytotoxicity). Cytotoxicity was evaluated by the modified lactate dehydrogenase (LDH) assay (Fig. 5).

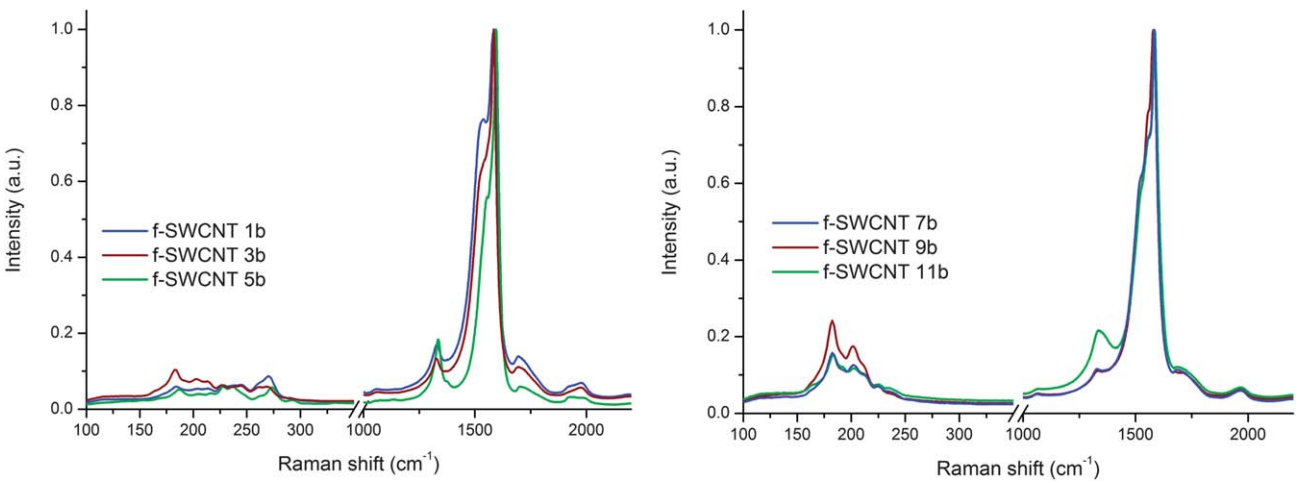


Fig. 4 Raman spectra of *f*-SWCNTs **1b**, **3b**, and **5b** (left), and of *f*-SWCNTs **7b**, **9b**, and **11b** (right).

The LDH assay measures membrane integrity as a function of the amount of cytoplasmic LDH released into the medium. LDH assay can also be used to express the cell viability by measuring the total cytoplasmic LDH remaining in the viable cells. This assay was modified to avoid any interference from the CNTs during assessment.[28] A549 cells were not affected by the different concentrations of *f*-SWCNTs both at 24 and 72 h (Fig. 5 and SI-1†). However, in the case of the *f*-MWCNTs, we observed dose-dependent cytotoxicity for the conjugates **2b** and **4b**. Interestingly, cytotoxicity decreased as the length of the polyamine increased with almost no observed cell death at 1 and 10 μg mL^{-1} of the spermine-containing nanotubes **6b** after 24 h of incubation (Fig. 5A). It seems that the length of the polyamine chain influences the cell viability particularly in the case of these *f*-MWCNTs. Similarly, the impact on cell viability was evaluated for the family of conjugates modified by the amidation reaction. Results in Fig. 5B indicate that no cytotoxicity was observed up to 100 μg mL^{-1} for 24 h exposure to the *f*-SWCNTs and *f*-MWCNTs conjugates modified by the amidation reaction (**7b**–**12b**). However, a certain degree of cytotoxicity was observed after prolonged exposure (72 h) only at the highest concentration tested (100 μg mL^{-1}) which was more significant for *f*-SWCNTs than *f*-MWCNTs (see ESI, Fig. SI-2†). Overall the cytotoxic effects are significant after 24 h only in the case of MWCNTs functionalized by addition reaction. This could be related to the dispersibility properties of the different cationic CNT conjugates. In particular, the MWCNTs (**2b**, **4b** and **6b**) modified by the addition reaction were difficult to disperse. The increased number of protonated amines in spermidine- and spermine-functionalized MWCNTs does not really facilitate the dispersion of the CNTs in the cell culture medium by comparison with putrescine-functionalized MWCNTs. The poor dispersibility likely affects cell viability.

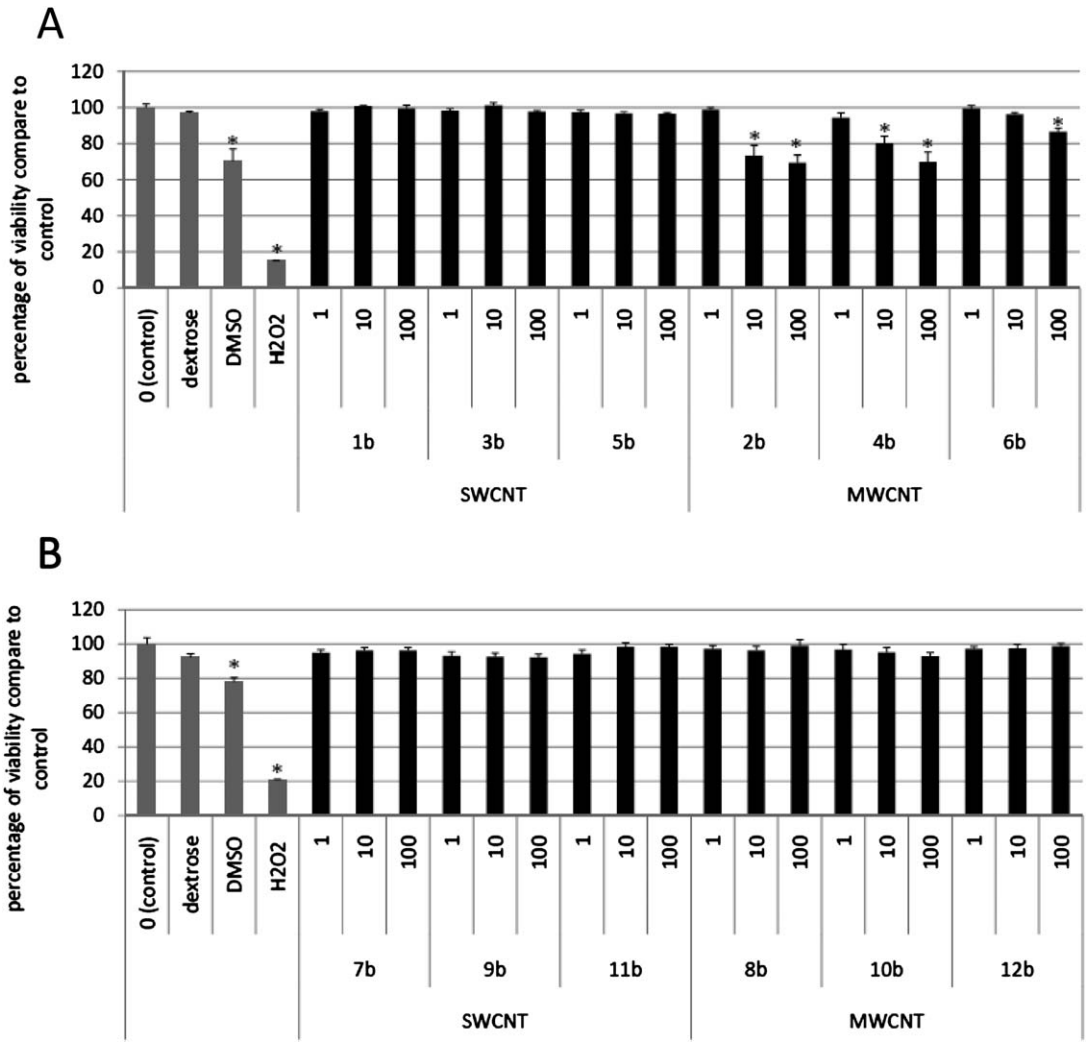


Fig. 5 Cell viability of A549 cells assessed by the modified LDH assay after 24 h exposure to *f*-CNTs (1–100 μg mL^{-1}). (A) LDH assay for *f*-SWCNTs **1b**, **3b** and **5b**, and *f*-MWCNTs **2b**, **4b** and **6b**. Cell viability was not affected by exposure to *f*-SWCNTs but was decreased by the *f*-MWCNT exposure at 10–100 μg mL^{-1} concentrations. (B) LDH assay of A549 cells treated with *f*-SWCNTs **7b**, **9b**, and **11b**, and *f*-MWCNTs **8b**, **10b**, and **12b**. Cell viability was not affected by 24 h exposure to none of these *f*-CNTs. Ten percent DMSO and 1 mM H_2O_2 in complete cell culture medium were used as positive controls. The vehicle (5% dextrose) was used as a negative control. Percentage viability was expressed as percentage of control untreated cells (*, $p \leq 0.05$).

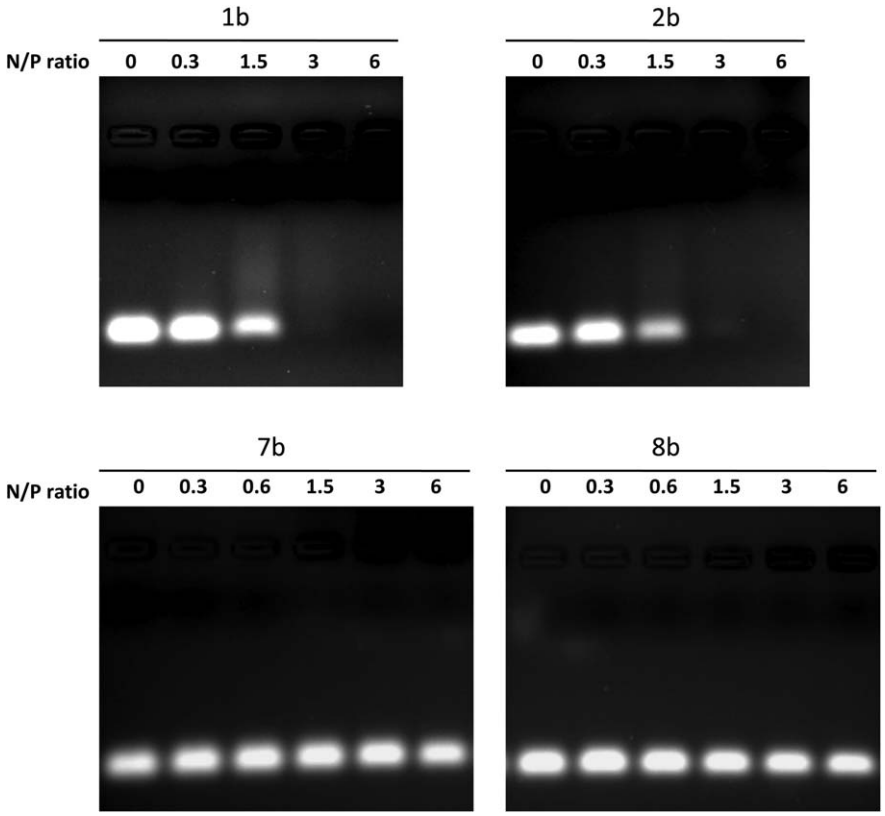


Fig. 6 Electrophoretic motility of siRNA complexed with polyamine–CNTs **1b** (top left), **2b** (top right), **7b** (bottom left) and **8b** (bottom right) (reconstituted at 1 mg mL^{-1} in 5% dextrose). Various N/P (+/−) ratios of the four conjugates were complexed to a fixed 0.5 μg of siRNA.

We previously demonstrated that cationic carbon nanotubes were able to condense nucleic acids efficiently and deliver them into mammalian cells.[9,10,30] Similarly, we have performed preliminary experiments on the complexation between polyamine–CNT conjugates and siRNA using agarose gel electrophoresis (Fig. 6). Among the three polyamine-functionalized CNT families, we tested single- and multi-walled CNTs modified with putrescine by both the addition reaction (**1b** and **2b**) and amidation (**7b** and **8b**) mainly because these nanotubes were the most dispersed in water, so we could achieve highest +/− (N/P) charge ratios at complexation with the nucleic acids. The amount of uncomplexed (free) siRNA migrating into the gel was reduced by increasing the polyamine–CNT : siRNA ratio as evidenced by a reduction in ethidium bromide fluorescence intensity signals at 3 : 1 (+/−) charge ratio, only in the case of the nanotubes functionalized by the addition reaction (**1b** and **2b**). Unfortunately, no complexation was achieved with amidated nanotubes up to 18 : 1 (+/−) charge ratio (Fig. 6 and SI-4†). We can conclude from the data obtained with putrescine-functionalized SWCNTs and MWCNTs that: (i) there was no difference in siRNA binding capacity between the *f*-SWCNTs and *f*-MWCNTs; and (ii) more efficient complexation was achieved by the nanotubes modified by the addition than amidation reaction (**1b** and **2b** *vs.* **7b** and **8b**), likely due to a more homogeneous distribution of the functional groups on the sidewall of CNTs in the case of the addition reaction. Furthermore, increasing the number of amino groups in the side chain (*i.e.* from putrescine to spermidine) in the case of amidated CNTs did not seem to improve the siRNA binding (see Fig. SI-3†), probably due to the localization of the functional groups mainly at the tips. Minor complexation of siRNA occurred at 18 : 1 (+/−) charge ratio in the case of *f*-MWCNTs **10b**.

In conclusion, we have synthesized and characterized twelve different polyamine-modified single- and multi-walled carbon nanotubes. Most of them displayed reduced cytotoxicity on A549 cells. Aqueous dispersibility was significantly improved for the conjugates modified by the amidation reaction of oxidized CNTs. Putrescine-modified CNTs were able to efficiently complex siRNA as assessed by gel electrophoresis experiments. In addition, we have also shown that these conjugates interact and can be internalized by the A549 cells (see Fig. SI-4†) using light microscopy. This finding opens the possibility to further develop some of these cationic conjugates for gene delivery and silencing. Experiments testing gene silencing using these new conjugates are in due course.

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