Ammonium and Guanidinium Dendron–Carbon Nanotubes by Amidation and Click Chemistry and their Use for siRNA Delivery

Alessia Battigelli, Julie Tzu-Wen Wang, Julie Russier, Tatiana Da Ros, Kostas Kostarelos, Khuloud T. Al-Jamal, Maurizio Prato, and Alberto Bianco**

***A** series of multi-walled carbon nanotube (MWCNT) conjugates is described, functionalized with different dendrons bearing positive charges at their termini (i.e. ammonium or guanidinium groups). The dendrimeric units are anchored to the nanotube scaffolds using two orthogonal synthetic approaches, amidation and click reactions. The final nanohybrids are characterized by complementary analytical techniques, while their ability to interact with siRNA is investigated by means of agarose gel electrophoresis. The demonstration of the cell uptake capacity, the low cytotoxicity, and the ability of these cationic conjugates to silence cytotoxic genes suggests them to be promising carriers for genetic material.*

1. Introduction

Carbon nanotubes (CNTs) represent a unique material for applications in nanoelectronics, composite materials and energy storage, to mention a few.[1] In addition, during the last decade, the potential use in the biomedical field has also attracted great interest due to the CNT ability to cross cell membranes like tiny needles ("nanoneedles").[2] In particular, we demonstrated that covalently functionalized CNTs can enter inside different cell types through energy-dependent or independent methods, i.e. endocytosis or nanoneedle mechanism, respectively.[3] Moreover, when administered in vivo, organ biodistribution profile was highly dependent on the degree of chemical functionalization[4] with rapid excretion via the renal route achieved for highly chemically functionalized CNTs.[5] These properties have paved the way for applications of CNTs as drug or gene delivery systems.

Recently, the discovery of RNA interference (RNAi) process has introduced a new concept in gene delivery.[6] The possibility to silence specific genes using siRNA (small interfering RNA) has shown a great advantage in comparison to DNA, because knock-down with siRNA occurs in the cytoplasm[7] and does not need nuclear uptake, as for DNA. On the other hand, the use of naked siRNA is limited by its low cytoplasmic delivery and in vivo reduced stability. This has prompted researchers to design suitable delivery systems to protect siRNA from nuclease degradation and to facilitate its cellular uptake, like cationic polymers, liposomes, or nanoparticles.[8] In 2005 Kam et al.[9] reported an efficient use of single-walled CNTs (SWCNTs) as carriers for siRNA, covalently linked through a disulfide bond to poly(ethylene glycole) chains, adsorbed, in turn, on the surface of the nanotubes. In this way, once inside endosomes or lysosomes, the cleavage of the disulfide bridge allowed

Dr. A. Battigelli, Dr. J. Russier, Dr. A. Bianco
CNRS, Institut de Biologie Moléculaire et Cellulaire
Laboratoire d'Immunopathologie
et Chimie Thérapeutique
67000 Strasbourg, France
E-mail: a.bianco@ibmc-cnrs.unistra.fr

Dr. A. Battigelli, Dr. T. Da Ros, Prof. M. Prato
Dipartimento di Scienze Chimiche e Farmaceutiche
Università di Trieste 34127, Trieste, Italy
E-mail: prato@units.it

Prof. K. Kostarelos
Nanomedicine Laboratory
UCL School of Pharmacy
University College London
London WC1N 1AX, UK

Dr. J. T.-W. Wang, Dr. K. T. Al-Jamal
Drug Delivery Group, Institute of Pharmaceutical Science
King's College London
London SE1 9NH, UK

DOI: 10.1002/smll.201300264

small NANO MICRO

the release of the oligonucleotide sequences from the nanotubes. Cellular studies performed on HeLa cells confirmed the occurring of siRNA release and the resulting gene silencing. Since then, our and other groups[10] reported several studies on the efficacy of CNTs as transfecting agents for siRNA in vitro and in vivo, exploiting different synthetic strategies to complex the genetic material on the surface of CNTs.

One of the most promising approaches for gene delivery is represented by polycationic nanomaterials able to efficiently complex and compact genetic materials.[11] In this context, in 2009 our group reported the functionalization of MWCNTs with modified positively charged polyamidoamine (PAMAM) dendrons of first and second generation and the subsequent supramolecular complexation with siRNA.[12] The electrophoretic motility on agarose gel of these complexes showed that increasing the branching of the dendritic structure enhanced the ability of CNTs to complex siRNA, most likely due to a higher water dispersibility of the conjugates and to the higher amount of positive charges on the surface of the nanotubes. Moreover, in vitro studies[13] of these conjugates showed an effective cellular delivery of the genetic material and an efficient silencing of specific genes.

Even though nowadays various examples of conjugation of CNTs with linear[14] or branched[15] polyamines have been reported, in order to exploit them in the near future as clinical therapeutic systems, more detailed information about the structure-delivery relationship is undoubtedly needed, because of the high number of parameters that can influence their physico-chemical properties and the complex structure of such hybrid materials. For this reason, in this work we focused our attention on the design, synthesis and investigation of organically functionalized MWCNTs, bearing different types of positively charged functional groups, to understand better the complexation ability of the genetic material and to find out the best conditions for the complexation and the subsequent delivery into the cellular environment. In particular, we wanted to perform a comparative study on the ability of different kinds of MWCNTs functionalized with guanidinium groups to complex and release the genetic material and to study their capability to penetrate into the cells. The guanidinium group was selected because it possesses a higher basicity in comparison to amines (pK_a = 13.5 in water)[16] which renders complexation with siRNA more stable at physiological pH.[17] Moreover, several studies showed that the presence of guanidinium groups on dendrimers[18] or peptoids[19] facilitates the cellular uptake of the entire molecules. For these reasons a series of MWCNTs derivatives functionalized with dendrons of first (G1) and second (G2) generation was synthesized, exploiting the amidation or the click reaction to link the dendrons to CNTs. Using these two approaches, different distances between the carbon surface and the positive charges were obtained. Once assessed the ability of the obtained conjugates to complex siRNA, the cellular uptake and the gene silencing of CNT/siRNA complexes were evaluated, demonstrating the efficacy of this series of MWCNTs as transfecting agent.

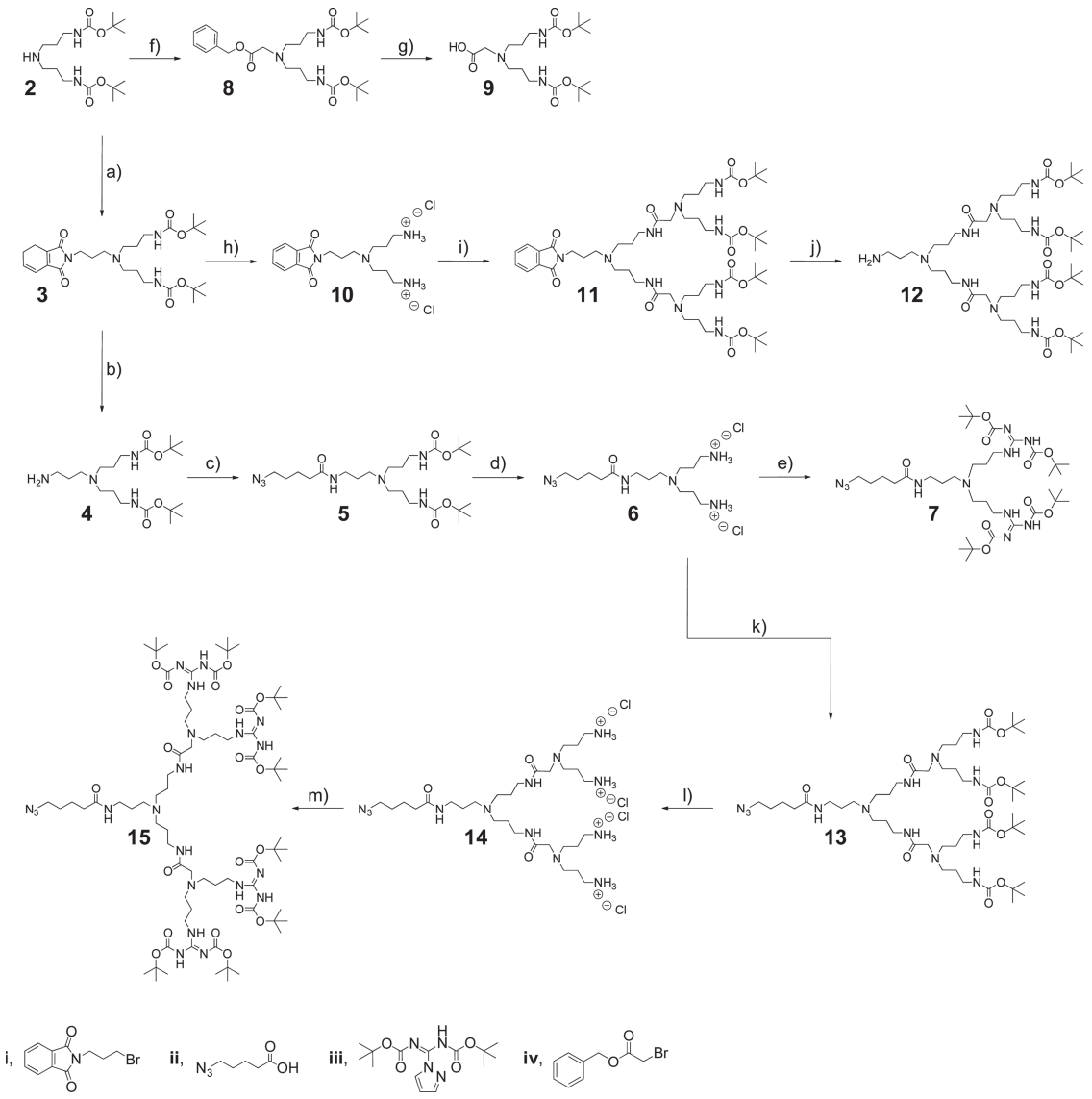
2. Result and Discussion

2.1. Synthesis of the Dendron Library

Initially, to achieve a sufficiently polar organic functionalization for the dendron–CNTs of zero generation, Boc mono-protected diaminotriethylene glycol **1** (see Supporting Information) was prepared, as already reported in the literature.[20] The synthetic pathway used for the preparation of G1 and G2 dendrons is illustrated in **Scheme 1**. The preparation of G1 dendron started from Boc di-protected compound **2** (see Supporting Information for details),[21] the addition of *N*-(3-bromopropyl)phthalimide to the central secondary amine group of **2**, in the presence of Na_2CO_3 at 75 °C, afforded the phthalimido-derivative **3**. Subsequent deprotection of the phthalimide group using hydrazine hydrate gave G1 dendron **4**, which was then used to functionalize MWCNTs via amidation reaction (see **Scheme 2**). In order to introduce an azide function, necessary for the click reaction, the free amine group of **4** was coupled with 5-azidopentanoic acid using EDC × HCl and HOBt as coupling reagents, leading to the formation of compound **5**. After the cleavage of the Boc group with HCl in dioxane, compound **6** was obtained while the guanidinium groups were introduced through the addition of *N*,*N*'-bis(*tert*-butoxycarbonyl)-1H-pyrazole-1-carboxamidine. The reaction product was purified by column chromatography, obtaining the first generation dendron **7** with an azide function at the core, ready for subsequent functionalization of MWCNTs through click reaction (see **Scheme 3**).

To perform the synthesis of G2 dendron, another branched building block was synthesized. The addition of benzyl bromoacetate to compound **2** and the subsequent cleavage of benzyl group afforded the dendritic derivative **9** (Scheme 1). The synthesis of G2 dendrons with an amino group at the focal point for the coupling with CNTs started from the cleavage of the two Boc protecting groups of compound **3**. The two free amino functions of compound **10** were coupled with two equivalents of **9**, affording compound **11**. After the deprotection of the phthalimido group via hydrazine treatment, the second generation dendron **12**, necessary for the amidation coupling to MWCNTs, was obtained in good yield.

The synthesis of the second generation dendron bearing an azide group at the focal point started from compound **6**, following the same strategy used to generate compound **12**. First, the free amine groups were coupled to the branched building block **9**, in the presence again of EDC×HCl and HOBt, affording compound **13**. The removal of the Boc protecting group led to the free amino derivative **14**, further modified introducing Boc protected guanidinium groups by addition of *N*,*N*'-bis(*tert*-butoxycarbonyl)-1H-pyrazole-1-carboxamidine. In this way the second generation dendron **15** was obtained after purification through column chromatography. The presence of the azido group at the core of the molecule was eventually used to link this dendron to CNTs via click chemistry.

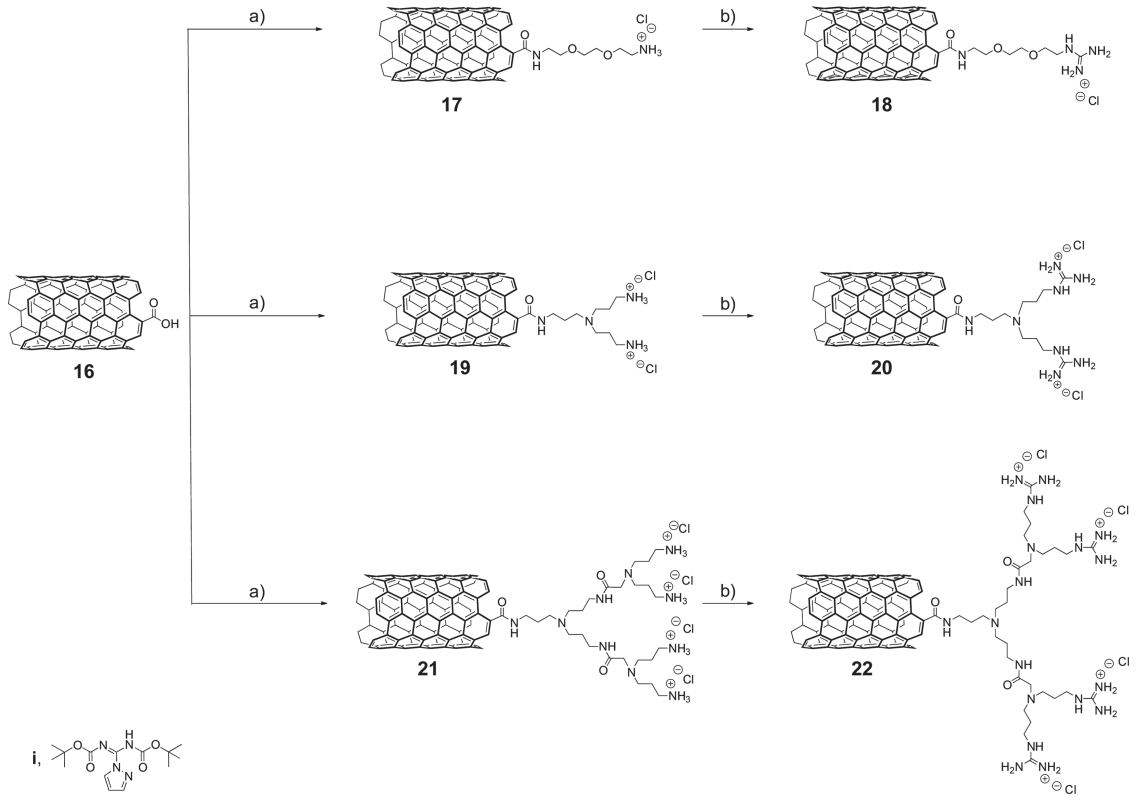


Scheme 1. Synthesis of the zero, 1st and 2nd generation dendrons. a) **i**, Na_2CO_3, CH_3CN, 75 °C, 24 h, 77%; b) Hydrazine hydrate, EtOH/Toluene, 50 °C, overnight, 92%; c) **ii**, EDC×HCl, HOBt, DIEA, CH_3CN, 48 h, 96%; d) HCl in dioxane, 2 h, quantitative; e) **iii**, DIEA, CH_3CN/THF, 48 h, 53%;f) **iv**, DIEA, CH_3CN, 48 h, 76%; g) H_2, Pd/C, MeOH, 2 h, 94%; h) HCl in dioxane, 2 h, quantitative; i) **9**, EDC×HCl, HOBt, DIEA, CH_3CN, 48 h, 71%; j) Hydrazine hydrate, EtOH/Toluene, 50 °C, overnight, 71%; k) **9**, EDC×HCl, HOBt, DIEA, CH_3CN, 72 h, 54%; l) HCl in dioxane, 2 h, quantitative; m) **iii**, DIEA, CH_3CN/THF, 48 h, 65%.

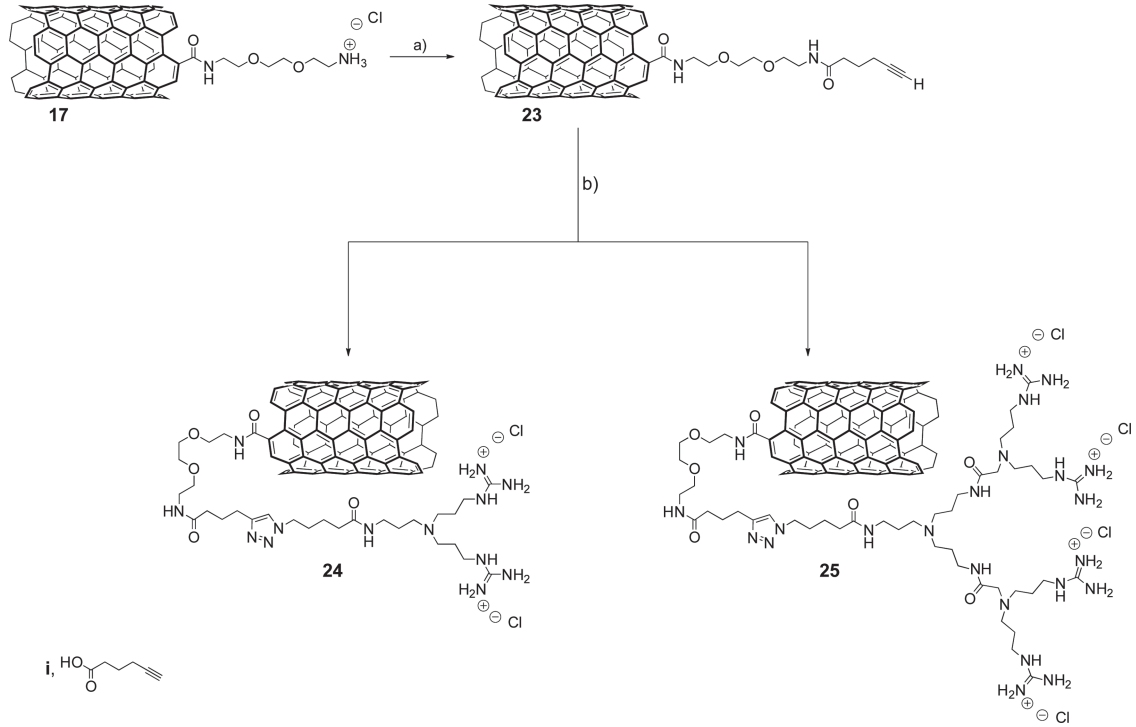
2.2. Functionalization of MWCNTs

The first step in the functionalization of CNTs was the oxidation of pristine material, using a mixture of sulfuric and nitric acid (3:1, v/v) under sonication for 24 h.[22] One of the advantages of the oxidation treatment is to increase the dispersibility of the CNTs. Moreover, the oxidation affords shortened and opened CNTs, creating defects on the sidewall of the material and introducing carboxylic functions mainly at the nanotube tips (see Supporting Information for details, and Figure S1 and S2). The presence of these groups on the surface of CNTs can be used to easily link different linear or ramified chains with a high degree of functionalization. For this purpose, these carboxylic groups were modified via the amidation reaction to obtain the conjugates dendron–CNTs of zero, first and second generation, bearing ammonium or guanidinium groups at their termini (Scheme 2). Oxidized MWCNTs were refluxed under argon in oxalyl chloride for 24 h, to generate the reactive acyl chloride derivatives. After evaporation of the excess reagent, the tubes were redispersed in dry THF. The corresponding amidated conjugates were obtained under reflux in the presence of one of the three different Boc protected compounds **1**, **4** or **12**. After the treatment with a solution of HCl in dioxane, the resulting ammonium derivatives of G0, G1 and G2 **17**, **19** and **21**, respectively, were isolated and characterized by TGA and Kaiser test (Scheme 2). To form the guanidinium dendron-MWCNTs, *N*,*N*'-bis(*tert*-butoxycarbonyl)-1H-pyrazole-1-carboxamidine was allowed

NANO MICRO small



Scheme 2. Synthetic scheme of the functionalization of CNTs with dendron of generation zero, 1st and 2nd. a) 1. **1**, **4** or **12**, ($COCl_2$), THF, 65 °C, 72 h; 2. HCl in dioxane, overnight; b) 1. **i**, DIEA, DMF, 48 h; 2. HCl in dioxane, overnight.



Scheme 3. Synthetic scheme of the functionalization of CNTs with dendron of 1st and 2nd generation through click reaction. a) **i**, EDC×HCl, HOBt, DIEA, DMF, 72 h; b) **7** or **15**, 2,6 lutidine, $Cu(MeCN)_4PF_6$, THPTA, NMP, 72 h; 2. HCl in dioxane, overnight.

to react with the amino groups of MWCNTs **17**, **19** and **21**. Following the deprotection of the Boc group, the final guanidinium derivatives of zero, first and second generation **18**, **20** and **22** were obtained (Scheme 2).

MWCNTs were then functionalized with the dendrons **7** and **15** using the click reaction. Initially, an acetylene group was introduced into MWCNTs **17**, using an excess of 5-hexynoic acid, in the presence of EDC×HCl and HOBt, affording functionalized MWCNTs **23** (Scheme 3).

Finally, "Cu catalyzed click reaction"[23] of MWCNTs **23** with the dendron of the first generation **7** and the second generation **15** in the presence of $[Cu(MeCN)_4PF_6]$, as catalyst, 2,6-lutidine, and tris-(hydroxypropyltriazolylmethyl) amine (THPTA), as stabilizing ligand,[24] afforded functionalized MWCNTs **24** and **25** (Scheme 3).

2.3. Characterization of the Nanohybrids

The dendron–MWCNT conjugates of the different generations were then characterized by thermogravimetric analysis (TGA), Kaiser test and transmission electron microscopy (TEM) to assess the level of functionalization and the morphology of the different CNTs. The degree of functionalization was evaluated by TGA under nitrogen, calculating the weight loss from the curves at 500 °C. Comparing the thermogravimetric profiles of the different intermediates, it was possible to calculate the functionalization value of each conjugate (**Figure 1**).

Quantitative Kaiser test[25] was also used to estimate the amount of free primary amino groups and the obtained values were compared with those derived from TGA, showing a fairly good agreement between the two techniques (see Supporting Information, Table S1).

Concerning the conjugates obtained through the amidation reaction, TGA measurements showed a functionalization of 120 and 145 μmol/g for G1 conjugates **19** and **20**, respectively, whereas the Kaiser test gave a functionalization of 140 and 80 μmol/g, respectively. In the case of G2 conjugates **21** and **22**, from the TGA weight loss, we calculated 80 and 60 μmol/g of functional groups, in good agreement with Kaiser test, which showed 60 and 40 μmol/g, respectively. The lower functionalization of G2 conjugates in comparison to G1 nanotubes is probably due to the higher steric hindrance of the building blocks increasing the generation of the branched molecules, which accounts for a reduced functionalization efficiency. Considering the nanomaterials achieved by click reaction, G1 and G2 conjugates showed a functionalization of 80 and 50 μmol/g, respectively, calculated only from the TGA curves at 500 °C, as the Kaiser test is not suitable to quantify the amount of guanidinium moieties.

To study the morphology of the functionalized material, dendron–CNTs were dispersed in DMF with a concentration of 0.1 mg/mL and then observed by TEM. The structure of the conjugates resulted unchanged, confirming that the chemical treatments employed to functionalize CNTs do not affect the structural features of the material (**Figure 2**).

2.4. Complexation Study with siRNA

The ability of dendron functionalized MWCNTs to complex the genetic material was determined and quantified by GelGreen displacement assay and agarose gel electrophoresis. The lower the amount of free siRNA was detected, the

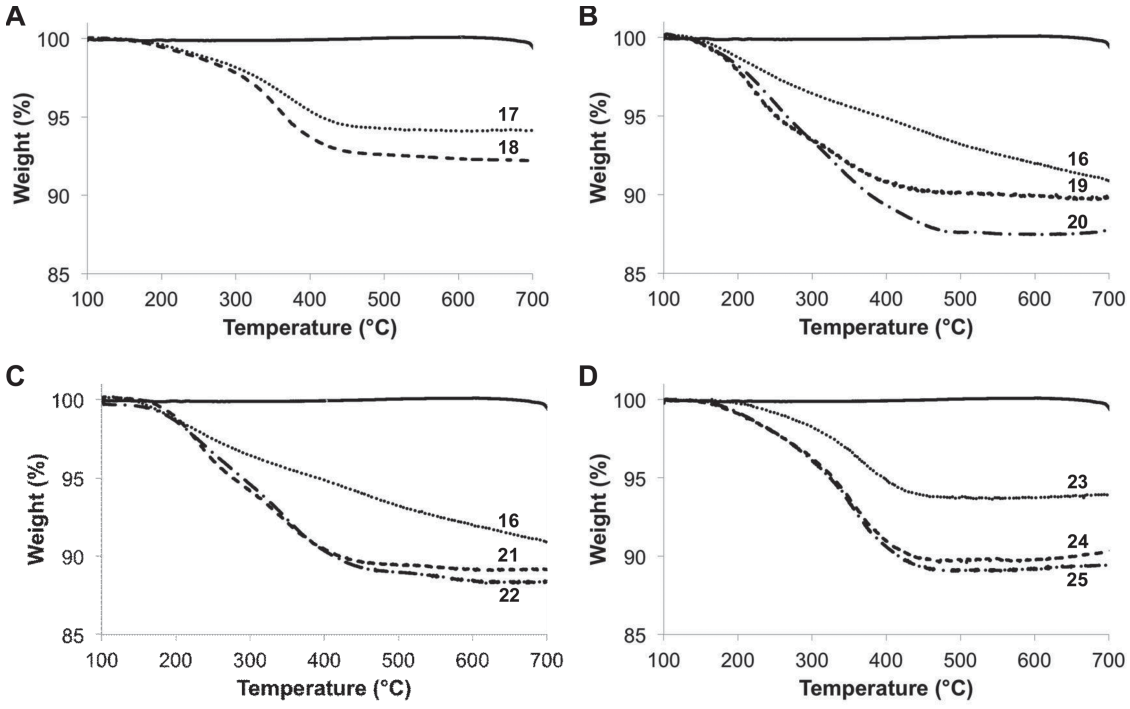


Figure 1. Thermogravimetric curves of G0 MWCNTs **17** and **18** (A), G1 MWCNTs **19** and **20** (B), G2 MWCNTs **21** and **22** (C) and click functionalized MWCNTs **24** and **25** (D) and their intermediates. Black curves correspond to MWCNTs pristine material.

NANO MICRO small

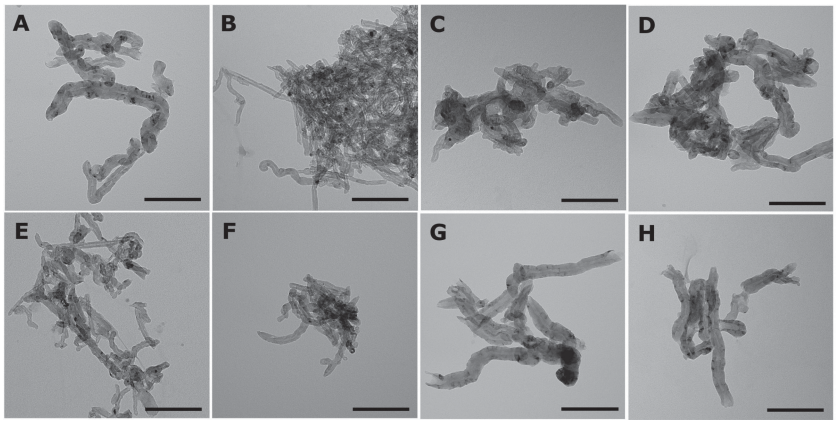


Figure 2. TEM images of ammonium G0 MWCNTs **17** (A), G1 MWCNTs **19** (B), G2 MWCNTs **21** (C) and of the corresponding guanidinium derivatives **18**, **20** and **22**, respectively (D, E, F). TEM images of click MWCNTs of 1st (**24**) and 2nd (**25**) generation (G and H, respectively). Scale bars correspond to 200 nm.

higher is the ability of the conjugates to complex genetic materials. In the cases of the guanidinium derivatives prepared through amidation (**18**, **20** and **22**), a similar behavior of siRNA complexation was observed among the different tested generations (**Figure 3**). In particular G0 guanidinium conjugate (**18**) showed the highest complexation ability of the studied conjugates with 15% of free siRNA detected at +/– charge ratio 3:1 and 5:1. Upon increasing the charge ratio (N/P, nitrogen/phosphate), the amount of free siRNA measured decreased proportionally until reaching a plateau at higher charge ratios. Unexpectedly, siRNA complexation ability was inversely proportional to the dendron generation number: the higher the generation was, the lower the siRNA complexation capability obtained.

In this study we also exploited a different approach to bind the dendrons to CNTs (i.e. the click reaction) aiming to investigate if the distance between CNTs and the terminal charged groups could modulate the interaction with siRNA. The results showed that increasing the length reduced the ability of the conjugates to complex the genetic materials. The CNTs functionalized through click reaction (**24** and **25**) displayed lower abilities to complex siRNA compared with the other conjugates. This phenomenon can be due to a non-specific interaction between the long alkyl chain and the triazole moieties present in the conjugates through the click reaction and

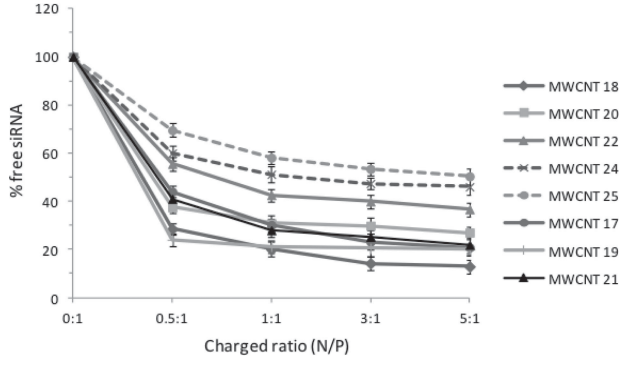


Figure 3. Percentage of free siRNA (unbound) measured by GelGreen displacement assay.

the CNT surface, thus hampering the exposition of the guanidinium groups to complex siRNA. In view of these results, the amidation approach seems to work better so that further studies were carried out to compare the guanidinium conjugates with the corresponding ammonium-based nanotubes, including the G0 precursors.

Interestingly, unlike the guanidinium derivatives, all ammonium conjugates (G0, G1 and G2) possessed similar abilities to complex siRNA independently from dendron generations, in particular at higher charge ratios. Around 20% of free siRNA was detected when complexed with G0, G1, and G2 ammonium-based CNTs (**17**, **19** and **21**) at 3:1 or 5:1 charged ratios (N/P). All ammonium conjugates displayed similar or better siRNA complexation capability in comparison to their guanidinium counterparts.

Comparing the different dendron generation effect on siRNA complexation, the results here obtained are in contrast with those previously reported.[12] Indeed, in this latter we observed that increasing the dendron generation enhanced the ability to complex siRNA. This discrepancy is probably due to the different way in which the material was functionalized. In our previous work, CNTs were firstly functionalized through 1,3-dipolar cycloaddition, which takes place mostly at the sidewalls of the material, whereas here we functionalized oxidized CNTs through amidation, which occurs preferentially at the tips of CNTs. Moreover, studies conducted by other groups in which pyrene[26] or poly(propylene imine) dendrimers[27] were functionalized with guanidinium groups showed also that these types of nanoparticles possessed better complexation profiles with genetic materials (i.e. DNA) than the corresponding ammonium conjugates.

Results similar to GelGreen displacement were obtained by agarose gel electrophoresis (Figure S3). siRNA was complexed with dendron CNTs at different N/P charge ratios (up to 10:1). Depending on the degree of complexation, the fluorescence signals of siRNA (due to intercalation with GelRed) was either detected when retained in the wells upon complexation with dendron–CNTs (quenched fluorescence) or when migrated to the front line as free siRNA. As shown in Figure S3, almost no siRNA complexation was observed from the CNTs through click reaction (**24** and **25**) within the charge ratio up to 10:1. Charge ratio dependent siRNA complexation was instead measured for the other dendron–CNT conjugates through amidation reaction as reduced siRNA migration was imaged at higher charge ratios (5:1 or 10:1). Complete restriction of siRNA migration occurred at 10:1 charge ratio in the case of complexation with ammonium G1 CNTs **19**.

2.5. Cell Uptake and Cytotoxicity Studies

To assess the suitability of our conjugates for gene delivery in vitro, dendron–CNTs complexed with siRNA were incubated

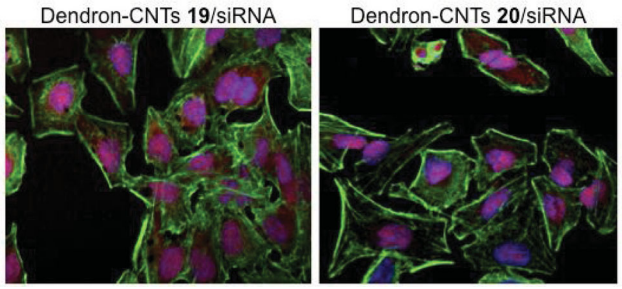


Figure 4. Confocal microscopy images of A549 cells after 1 hour of incubation with dendron–CNTs **19** or **20**/siRNA (Alexa 546 labelled) complexes 5:1 charge ratio. Complexes were prepared and incubated with cells for 1 h in serum-containing media. F-actin is stained in green, nucleus in blue. siRNA uptake is represented by the red signals.

with A549 lung cancer cells and their cellular uptake and distribution were studied. From the complexation studies, G1 CNTs resulted the most interesting, since G2 conjugates showed lower ability to complex siRNA. For this reason we decided to investigate more in detail ammonium and guanidinium derivatives, **19** and **20** respectively, and their ability to interact with cancer cells. A549 cells were treated with dendron–CNT **19** or **20** complexed with Alexa 546 labelled siRNA for 1 h and then imaged by confocal microscopy. As shown in **Figure 4**, in the cells treated with ammonium CNTs **19**/siRNA complexes, the genetic material, evidenced by red signals, was found to be homogenously present in the cell cytoplasm. In comparison, lower red fluorescence signals were detected in the cells treated with guanidinium CNT **20**/siRNA complexes. The results here are in good agreement with the gel electrophoresis study in which guanidinium CNTs exhibit lower complexation capacity of siRNA than the corresponding ammonium counterparts.

The cellular uptake of all the conjugates complexed with Alexa 546 labelled siRNA at different N/P ratios (1:1, 5:1 and 10:1) was quantified after 24 hours of incubation by measuring the fluorescence intensity in the cell lysate (**Figure 5**).

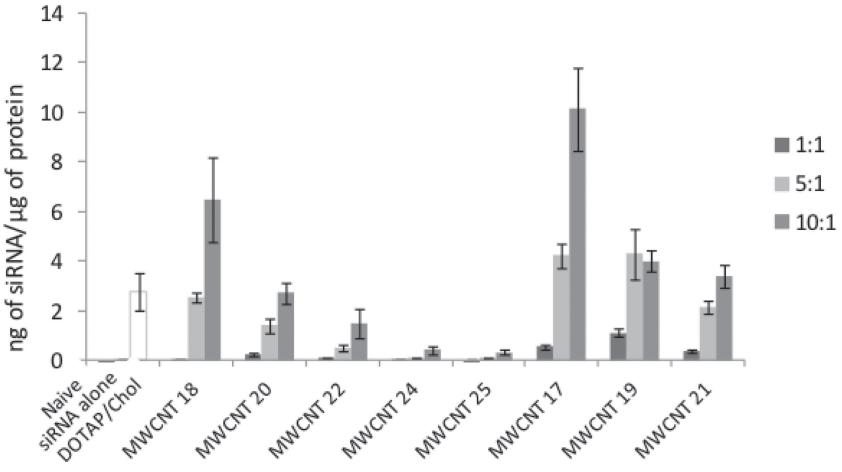


Figure 5. Cellular uptake of dendron–CNTs/siRNA (Alexa 546 labelled) complexes in A549 cells. Cells were incubated with *f*-CNTs/siRNA complexes (1:1, 5:1, and 10:1 +/– charge ratios) for 24 h. The amount of intracellular siRNA was estimated by measuring the fluorescence signals at 590 nm using fluorescent siRNA calibration curve. Total protein concentration per well was determined by BCA assay.

The results were again in agreement with the gel agarose electrophoresis results (Figure S3). CNTs functionalized through click reactions (**24** and **25**) were not able to transport the genetic material into cells as shown by the low fluorescence signals detected in cell lysates. Nevertheless, siRNA was found to be internalized into cells when complexed with the other derivatives, with higher uptake observed in case of ammonium conjugates (**17**, **19** and **21**) compared to guanidinium ones (**18**, **20** and **22**). Moreover, in both cases, higher dendron generations resulted in lower cellular uptake. This phenomenon was more remarkable for the guanidinium derivatives. Collectively, the results indicate that chemical functionalization plays an important role for the internalization of siRNA.

The effect of CNTs on cell viability is an important parameter that is necessary to investigate when the properties of CNTs are studied for biomedical purposes. Previous works have shown the potential toxicity of pristine long CNTs.[28] However, it was found that the shortening and the covalent functionalization of the nanotubes significantly decreased their cytotoxicity and alleviated the pathogenic effects in vivo.[28] In order to assess the impact of functionalization of CNTs on cell viability, cytotoxicity assay on A549 cells was performed. Cells were incubated with dendron–CNTs for 24 or 72 h and the LDH assay showed that the cell viability was not affected by the treatment with different guanidinium dendron–CNTs (Figure S4). There was no cell killing induced by all ammonium CNT conjugates at 24 h (Figure S5A). However, cell survival was reduced as a function of CNT concentrations in the cells treated with G1 and G2 ammonium dendron–CNTs at 72 hours (Figure S5B). The low cytotoxicity measured using guanidinium dendron–CNTs is very encouraging since toxicity is one of the most common drawbacks of many non-viral vectors for gene therapy.[29] However, the cellular uptake measured was not affected by the cytotoxicity of these conjugates, since this experiment was conducted after 24 h of CNT incubation where all the conjugates did not show any significant variation on cell viability. Therefore, there seems to be multiple factors involved in the toxicity induced by different dendron–CNT conjugates and further studies are needed to understand the mechanism.

2.6. In Vitro Gene Silencing

The ability of dendron–CNT conjugates **19** and **20** to deliver functional siRNA was further tested in vitro after an incubation of 24 (data not shown) or 48 h (**Figure 6**), using a concentration of CNTs which resulted non toxic from the previous cytotoxicity test. siRNA specific for PLK1 gene (siPLK1) was chosen because silencing of this gene results in cancer cell death. In agreement with siRNA complexation and cellular uptake results, conjugate **19** seemed to be more

NANO MICRO small

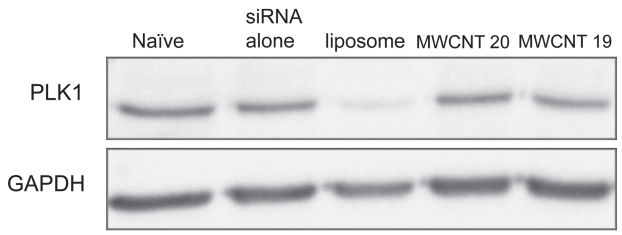


Figure 6. Evaluation through western blot of protein expression of in vitro siPLK1 silencing by cationic liposome (4.3:1 mass ratio), and MWCNTs **19** and **20** (5:1 charge ratio) in HeLa cells at 48 h.

effective than conjugate **20** in silencing PLK1 gene in HeLa cells. More than 5% but less than 10% PLK1 protein reduction was induced by dendron–CNTs **20**. On the other hand, around 25% protein reduction was achieved by dendron–CNTs **19**-mediated siPLK1 silencing at 48 hours post transfection compared with naïve cells and siRNA alone. Cationic liposomes (DOTAP/cholesterol) were also used to compare the gene silencing efficiency. Although liposomes displayed better gene silencing ability than dendron–CNTs **19** by a factor of 3, it was found that the liposome-mediated gene silencing effect remained constant at 24 and 48 h. Contrarily to liposome, lower protein expression was measured in the samples collected at 48 hours post transfection by dendron–CNTs **19** compared to the 24 h sample. The enhanced silencing effect at longer time point indicates that siRNA complexed with dendron–CNT was still stable and might possess a slow but efficient release of genetic material. Since the intracellular siRNA concentration was reduced over time as the consequence of cell proliferation, the reduction of a quarter of PLK1 protein measured at 48 h post transfection is promising. This could be also an advantage for in vivo applications as the ideal siRNA/vector complexes should be stable for several hours or few days after systemic injection until reaching the organs of interests for siRNA silencing.

3. Conclusion

A series of dendrons of first and second generation have been synthesized, possessing ammonium or guanidinium groups at their termini. The functionalization of the nanotubes was achieved using different covalent approaches in order to study the properties of the resulting conjugates in the complexation of siRNA. In particular, the presence of an amine or an azide moiety at the focal point of the dendron has been exploited to link the ramified structures to CNTs via amidation or click reactions. In this way the distance between the nanotubes and the charged groups of the dendron has been altered, introducing an additional parameter in the study of interaction with siRNA.

All the derivatives have been characterized by TGA and Kaiser test to evaluate the degree of functionalization. Their ability to interact with siRNA was investigated using GelGreen displacement and agarose gel electrophoresis. CNTs functionalized through amidation were more efficient in siRNA complexation than those functionalized through the click chemistry. Higher siRNA complexation was observed with the ammonium derivatives, compared to the guanidinium conjugates. Moreover, cellular uptake and western blotting studies confirmed the electrophoresis results that ammonium derivatives display the best siRNA uptake and gene silencing activity compared to the other conjugates studied here. Finally, the low cytotoxicity of all the derivatives revealed a promising relevance of these conjugates as gene delivery system. Silencing studies in vivo are currently under investigation.

4. Experimental Section

Material and Reagents: MWCNTs (20–30 nm) were purchased from Nanostructured and Amorphous Materials Inc (USA). Chemicals were purchased from Sigma-Aldrich, Acros and Alfa Aesar, and used as received without any further purification. All solvents used for synthesis were analytical grade. When anhydrous conditions were required, high quality commercial solvents treated with molecular sieves (porosity 4 Å) were used (DMF). THF was dehydrated by using Dry Solvent Station GTS 100. Water was purified using a Millipore filter system MilliQ.

Oxidation of MWCNTs: MWCNTs were firstly oxidized under sonication in a water bath (20 W, 40 kHz) for 24 h in a sulfuric acid/nitric acid solution (3:1 v/v, 98% and 65%, respectively) at room temperature. Deionized H_2O was then carefully added and the oxidized MWCNTs were filtered (Omnipore PTFE membrane filtration, 0.45 µm), re-suspended in water and filtered again until the pH became neutral. MWCNTs were then dialyzed against water (Spectra/Por dialysis membrane MWCO 12 000–14 000 Da) for 3 days and then lyophilized, affording *f*-MWCNTs **16** (see SI Figure S1 and S2).

General Procedure for Amidation Reaction: 16 (100 mg) was suspended in oxalyl chloride (10 mL) and sonicated in a water bath for 30 min. The reaction mixture was stirred at 60 °C for 24 h under Ar atmosphere. Oxalyl chloride was removed under reduced pressure and a solution of compound **1** (100 mg) in dry THF was added to CNTs. The reaction mixture was stirred for 48 h at 65 °C under Ar atmosphere. After cooling the suspension to room temperature, CNTs were filtered over a PTFE membrane (0.45 µm). The solid recovered on the filter was dispersed in DMF, sonicated in a water bath until thorough dispersion of the CNTs and filtered over a PTFE membrane (0.45 µm). This sequence was repeated 3 times with DMF, twice with MeOH and once with Et_2O and then CNTs were dried under vacuum. CNTs were dialyzed against water (Spectra/Por® dialysis membrane MWCO 12 000–14 000 Da) for 3 days and then lyophilized.

General Procedure for Boc Deprotection on CNTs: CNTs possessing Boc protecting groups (50 mg) were suspended in a 4N HCl solution in dioxane (50 mL) and sonicated in a water bath for 30 min. The reaction mixture was stirred overnight at room temperature. CNTs were filtered over a PTFE membrane (0.45 µm) and washed as described before.

General Procedure for Guanidilation Reaction on CNTs: CNTs **17** (10 mg) was dispersed in DMF (10 mL) and sonicated in a water bath for 30 min. DIEA (0.2 mL) and ***N,N'***-bis(*tert*-butoxycarbonyl)-1H-pyrazole-1-carboxamidine (100 mg) were added to the suspension and the reaction mixture was stirred 48 h at room temperature. CNTs were filtered over a PTFE membrane (0.45 µm) and washed as described before.

f-MWCNTs 23: CNTs **17** (50 mg) were suspended in DMF (50.0 mL) and sonicated in a water bath for 30 min. Hexinoic acid (50 μL, 0.45 mmol), DIEA (1.6 mL, 9 mmol) and HOBt (122 mg, 0.9 mmol) were added to the suspension, in an ice bath, and then EDC×HCl (259 mg, 1.35 mmol) was subsequently added. The reaction mixture was stirred for 3 days at room temperature. CNTs were filtered over a PTFE membrane (0.45 μm) and washed as described before, affording *f*-MWCNTs **23**.

General Procedure for Click Reaction: CNTs **23** (10 mg) was suspended in NMP (10 mL) and sonicated in a water bath for 30 min. Compound **7** (50 mg, 63 μmol), THPTA (5.4 mg, 13 μmol) and 2,6-lutidine (0.4 ml, 3.1 mmol) were sequentially added and the reaction mixture was degassed, bubbling Ar for 30 min. $Cu(MeCN)_4PF_6$ (23 mg, 63 μmol) was then added to the solution and the reaction mixture was stirred at room temperature for 3 days under Ar atmosphere. CNTs were filtered over a PTFE membrane (0.45 μm) and the solid recovered on the filter was dispersed in DMF, sonicated in a water bath until thorough dispersion of the CNTs and filtered over a PTFE membrane (0.45 μm). This sequence was repeated once with H_2O, once with saturated aqueous NH_4Cl solution, once with H_2O, twice with DMF, twice with MeOH and once with Et_2O and then CNTs were dried under vacuum, affording *f*-MWCNTs **24**.

Electrophoretic Mobility Shift Assay: In this experiment non-coding siRNA Alexa 546-labelled siNEG (UGC-GCU-ACG-AUC-GAC-GAU-G55; MW: 14450.3 Da) (Eurogentec, UK) were used. siRNA (0.25 μg in 30 μL of 5% dextrose) was complexed to *f*-MWCNT in 30 μL of 5% aqueous solution of dextrose at different N/P charge ratios. Free siRNA (0.25 μg) were used as a control. The suspensions were left at room temperature for 30 min to allow the formation of the complexes. After staining with 6x Orange Loading Dye (Fermentas, UK), the suspensions were added to 1% agarose gel (Life Technologies, UK) containing GelRed (Biotium, USA) stain, followed by electrophoresis in 0.5x Tris-Borate-EDTA buffer (Sigma-Aldrich, UK) at 75 mV for 45 min. The gels were then visualized under UV light using ChemiDoc MP system (BioRad, UK).

GelGreen Displacement Assay: The GelGreen Nucleic Acid Gel Stain, 10 000× in H_2O (Biotium, USA) was diluted in water to become 100× concentrated. Thirty-five microlitres of GelGreen were firstly complexed with siRNA (0.25 μg in 25 μL 5% dextrose) and allow equilibrating for 30 min (100% fluorescence intensity). The GelGreen-siRNA solutions were then mixed with equal volume (60 μL) of dendron CNT solution (complexing agent) at different concentrations to achieve the complexation ratios: 0.5:1, 1:1, 3:1, and 5:1 +/− charge ratios. After 1 h of incubation at room temperature, dispersions were centrifuged at 13 000 rpm for 30 min (to remove dendron–CNTs:siRNA complexes) and the supernatants (containing siRNA unbound to dendron–CNTs) were transferred to a black-bottom 96-well plate. Amounts of the unbound siRNA were quantified by measuring the florescence signal at 554 nm using a FLUOstar OPTIMA plate reader (BMG Labtech, UK) and presented as a percentage of total fluorescence signals at 0:1 +/− charge ratio (100% free siRNA).

Evaluation of Fluorescence siRNA Complex Uptake in A549 Cells by Laser Scanning Confocal Microscopy: Dendron–CNTs/siRNA complexes were prepared at 5:1 charge ratio and incubated with cells for 1 h in serum-containing media. Cell were rinsed, fixed, permeabilised and subsequently stained with Alexa Fluor 488 phalloidin (green colour, F-actin), TO-PRO-3 (blue colour, nucleus). Cellular uptake of siRNA Alexa 546-labelled was evidenced by the red signal. Confocal images were captured using a Nikon Eclipse Ti Inverted confocal microscope (Nikon, UK).

Quantification of Fluorescently Labelled siRNA Complex Uptake in A549 Cells: A549 cells were incubated with dendron–CNTs/siRNA complexes (1:1, 5:1 and 10:1 charge ratios) for 24 h. Cells were washed, lysed, centrifuged, and then the cell lysates were transferred to a 96-well plate followed by measuring the fluorescence signals at 590 nm in a FLUOstar OPTIMA plate reader (BMG Labtech, UK). A standard curve of fluorescent siRNA was established in order to calculate the siRNA concentration per well. Total protein concentration per well was later determined by BCA assay kits (Pierce BCA protein assay kit, Thermal Scientific, UK). Results were presented as ng of siRNA per μg of protein. Cationic liposome DOTAP/cholesterol was also used to complex siRNA at 4.3 mass ratios as a positive control.

Cell Viability Assay: A549 cells were seeded in 96-well plates and incubated with dendron CNTs (200 μL of 10, 50 and 100 μg/mL) in complete media for 24 h or 72 h. Cytotoxicity was examined by modified LDH assay[30] using CytoTox96 assay kits (Promega, UK). In brief, at the end of incubation period, media was removed and replaced with 100 μL of phenol and serum free media, containing 9% v/v Triton X-100. After an incubation of 45–60 min at 37 °C, the cell lysate was transferred into microtubes and centrifuged at 13 000*g* for 5 min. 50 μL of the supernatant (free from dendron CNT material) was then transferred to a fresh 96-well plate and mixed with 50 μL reconstituted substrate in each well. The plate was covered with foil and incubated for 15 min at room temperature. The reaction was stopped by adding 50 μL of the stop solution to each well. The absorbance was read at 490 nm in a plate reader (FLUOstart OPTIMA, BMG Labtech, UK) and the results are expressed as the percentage of cell survival (mean ± S.D.) and calculated using the below equation:% Cell survival = (A490 nm of treated cells/A490 nm of untreated control cells) x100.

Western Blotting of siPLK1 Silencing: HeLa cells were seeded onto 6-well plates with antibiotics free RPMI-1640 medium overnight. 24 h later, siPLK1 (Eurogentec, UK) was complexed with MWCNTs **19** and **20** in 5% dextrose at charge ratio 5:1 (+/−) for 20 min. Complexes solutions were diluted in serum free medium and added to cells to achieve the final concentration of siPLK1 80 nM per well. After 4 h, serum was added to each well to supplement the medium (10%). At 24 h and 48 h post transfection, cells were washed twice with ice-cold PBS and collected directly into lysis buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40 and 0.5% sodium deoxycholate). The cell lysates were left on ice for 30 min and mixed by vortex in between. Samples were then cleared by centrifugation at 13 000 rpm for 30 min at 4 °C and the supernatants containing proteins were collected. Protein concentrations were examined using BCA assay kits (Pierce BCA protein assay kit, Thermal Scientific, UK) and 15 μg of protein from each sample was resolved in 10% SDS-PAGE gels and transferred to Hybond ECL nitrocellulose membranes (GE Healthcare, UK). After blocking in 3% BSA at room temperature for 1 h, the blots were incubated with mouse monoclonal PLK1 antibody (abcam, UK) at 10 μg/mL overnight on ice. The blots were then incubated with the secondary antibody, horseradish peroxidase linked anti-mouse antibody (Cell Signalling Technology, USA) at 1:1000 dilution for 1 h at room temperature. The specific bands were detected using chemiluminescent kits (Immun-Star

Chemiluminescent Kit, BioRad, UK) and imaged and quantitatively analysed using ChemiDoc MP imaging system and Image Lab software (BioRad, UK). Cells treated with cationic liposome (DOTAP/cholesterol)/siPLK1 complexes at 4.3:1 mass ratio was used as a positive control following the same protocol. GAPDH was used as an internal reference (house-keeping) gene.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

This work was supported by CNRS (PICS 2010 Program), University of Trieste and Italian Ministry of Education MIUR (cofin Prot. 2010N3T9M4 and Firb RBAP11C58Y). Partial funding from Biotechnology and Biological Sciences Research Council (BB/J008656/1) is also acknowledged. A.B. is grateful to Università italo-francese/Université franco-italienne for supporting her international PhD (VINCI program 2008). We are also thankful to C. Fabbro for her help on TGA measurements.

[1] a) A. Jorio, G. Dresselhaus, M. S. Dresselhaus, in *Carbon Nanotubes: Advanced Topics in the Synthesis, Structure, Properties and Applications*, Springer-Verlag, Berlin, Heidelberg **2008**; b) M. Terrones, *Annu. Rev. Mater. Res.* **2003**, *33*, 419.

[2] D. Pantarotto, R. Singh, D. McCarthy, M. Erhardt, J.-P. Briand, M. Prato, K. Kostarelos, A. Bianco, *Angew. Chem. Int. Ed.* **2004**, *43*, 5242.

[3] L. Lacerda, J. Russier, G. Pastorin, M. A. Herrero, E. Venturelli, H. Dumortier, K. T. Al-Jamal, M. Prato, K. Kostarelos, A. Bianco, *Biomaterials* **2012**, *33*, 3334.

[4] K. T. Al-Jamal, A. Nunes, L. Methven, H. Ali-Boucetta, S. Li, F. M. Toma, M. A. Herrero, W. T. Al-Jamal, H. M. M. ten Eikelder, J. Foster, S. Mather, M. Prato, A. Bianco, K. Kostarelos, *Angew. Chem. Int. Ed.* **2012**, *51*, 6389.

[5] a) Z. Liu, C. Davis, W. Cai, L. He, X. Chen, H. Dai, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 1410; b) M. R. McDevitt, D. Chattopadhyay, J. S. Jaggi, R. D. Finn, P. B. Zanzonico, C. Villa, D. Rey, J. Mendenhall, C. A. Batt, J. T. Njardarson, D. A. Scheinberg, *PLoS One* **2007**, *2*, e907.

[6] a) A. Fire, S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver, C. C. Mello, *Nature* **1998**, *391*, 806; b) S. M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, T. Tuschi, *Nature* **2001**, *411*, 494.

[7] G. Meister, T. Tuschl, *Nature* **2004**, *431*, 343.

[8] a) A. Aigner, *J. Biomed. Biotechnol.* **2006**, *2006*, 71659; b) K. A. Whitehead, R. Langer, D. G. Anderson, *Nat. Rev. Drug Discov.* **2009**, *8*, 129; c) M. Morille, C. Passirani, A. Vonarbourg, A. Clavreul, J.-P. Benoit, *Biomaterials* **2008**, *29*, 3477.

[9] N. W. S. Kam, Z. Liu, H. Dai, *J. Am. Chem. Soc.* **2005**, *127*, 12492.

[10] a) J. E. Podesta, K. T. Al-Jamal, M. A. Herrero, B. Tian, H. Ali-Boucetta, V. Hedge, A. Bianco, M. Prato, K. Kostarelos, *Small* **2009**, *5*, 1176; b) W. Cheung, F. Pontoriero, O. Taratula, A. M. Chen, H. He, *Adv. Drug Deliv. Rev.* **2010**, *62*, 633.

[11] a) C. K. Goldman, L. Soroceanu, N. Smith, G. Y. Gillespie, W. Shaw, S. Burgess, G. Bilbao, D. T. Curiel, *Nat. Biotechnol.* **1997**, *15*, 462; b) D. Putman, C. A. Gentry, D. W. Pack, R. Langer, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 1200; c) Y.-Z. Xiang, Z.-H. Feng, J. Zhang, Y.-L. Liao, C.-J. Yu, W.-J. Yi, W. Zhu, X.-Q. Yu, *Org. Biomol. Chem.* **2010**, *8*, 640.

[12] M. A. Herrero, F. M. Toma, K. T. Al-Jamal, K. Kostarelos, A. Bianco, T. Da Ros, F. Bano, L. Casalis, G. Scoles, M. Prato, *J. Am. Chem. Soc.* **2009**, *131*, 9843.

[13] K. T. Al-Jamal, F. M. Toma, A. Yilmazer, H. Ali-Boucetta, A. Nunes, M.-A. Herrero, B. Tian, A. Eddaoudi, W. T. Al-Jamal, A. Bianco, M. Prato, K. Kostarelos, *FASEB J.* **2010**, *24*, 4354.

[14] P. Singh, C. Samorì, F. M. Toma, C. Bussy, A. Nunes, K. T. Al-Jamal, C. Ménard-Moyon, M. Prato, K. Kostarelos, A. Bianco, *J. Mater. Chem.* **2011**, *21*, 4850.

[15] J. McCarroll, H. Baigude, C.-S. Yang, T. M. Rana, *Bioconjugate Chem.* **2010**, *21*, 56.

[16] P. S. Pallan, K. N. Ganesh, *Biochem. Biophys. Res. Comm.* **1996**, *222*, 416.

[17] T. A. Theodossiou, A. Pantos, I. Tsogas, C. M. Paleos, *ChemMedChem* **2008**, *3*, 1635.

[18] I. Tsogas, Z. Sideratou, D. Tsiourvas, T. A. Theodossiou, C. M. Paleos, *ChemBioChem* **2007**, *8*, 1865.

[19] T. Schröder, N. Niemeier, S. Afonin, A. S. Ulrich, H. F. Krug, S. Bräse, *J. Med. Chem.* **2008**, *51*, 376.

[20] K. Kordatos, T. Da Ros, S. Bosi, E. Vázquez, M. Bergamin, C. Cusan, F. Pellarini, V. Tomberli, B. Baiti, D. Pantarotto, V. Georgakilas, G. Spalluto, M. Prato, *J. Org. Chem.* **2001**, *66*, 4915.

[21] S. P. Rannard, N. J. Davis, *Org. Lett.* **2000**, *2*, 2117.

[22] J. Liu, A. G. Rinzler, H. Dai, J. H. Hafner, R. K. Bradley, P. J. Boul, A. Lu, T. Iverson, K. Shelimov, C. B. Huffman, F. Rodriguez-Macias, Y.-S. Shon, T. R. Lee, D. T. Colbert, R. E. Smalley, *Science* **1998**, *280*, 1253.

[23] a) H. C. Kolb, M. G. Finn, K. B. Sharpless, *Angew. Chem. Int. Ed.* **2001**, *40*, 2004; b) T. Palacin, H. Le Khanh, B. Jousselme, P. Jegou, A. Filoramo, C. Ehli, D. M. Guldi, S. Campidelli, *J. Am. Chem. Soc.* **2009**, *131*, 15394; c) S. Rana, J. W. Cho, *Nanoscale* **2010**, *2*, 2550.

[24] X.-M. Liu, A. Thakur, D. Wang, *Biomacromolecules* **2007**, *8*, 2653.

[25] a) E. Kaiser, R. L. Colescott, C. D. Bossinger, P. I. Cook, *Anal. Biochem.* **1970**, *34*, 595; b) V. K. Sarin, S. B. H. Kent, J. P. Tam, R. B. Merrifield, *Anal. Biochem.* **1981**, *117*, 147.

[26] K. Ohara, M. Smietana, A. Restouin, S. Mollard, J.-P. Borg, Y. Collette, J.-J. Vasseur, *J. Med. Chem.* **2007**, *50*, 6465.

[27] L.-A. Tziveleka, A.-M. G. Psarra, D. Tsiourvas, C. M. Paleos, *J. Control. Release* **2007**, *117*, 137.

[28] a) C. A. Poland, R. Duffin, I. Kinloch, A. Maynard, W. A. H. Wallace, A. Seaton, V. Stone, S. Brown, W. MacNee, K. Donaldson, *Nat. Nanotechnol.* **2008**, *3*, 423; b) Y. Liu, Y. Zhao, B. Sun, C. Chen, *Acc. Chem. Res.* **2013**, *46*, 702; c) H. Ali-Boucetta, A. Nunes, R. Sainz, M. A. Herrero, B. Tian, M. Prato, A. Bianco, K. Kostarelos, *Angew. Chem. Int. Ed.* **2013**, *52*, 2274; d) C. Bussy, M. Pinault, J. Cambedouzou, M. J. Landry, P. Jegou, M. Mayne-L'hermite, P. Launois, J. Boczkowski, S. Lanone, *Part. Fibre Toxicol.* **2012**, *9*, 46.

[29] H. Lv, S. Zhang, B. Wang, S. Cui, J. Yan, *J. Control. Release* **2006**, *114*, 100.

[30] H. Ali-Boucetta, K. T. Al-Jamal, K. H. Müller, S. Li, A. E. Porter, A. Eddaoudi, M. Prato, A. Bianco, K. Kostarelos, *Small* **2011**, *7*, 3230.

Received: January 24, 2013
Revised: February 20, 2013
Published online: May 6, 2013

