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PAPER

Functionalization of multi-walled carbon nanotubes with coumarin derivatives and their biological evaluation†

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We report the synthesis and the characterization of different multi-walled carbon nanotubes (MWCNTs) linked to natural molecules, 5,7-coumarins and/or oleic acid, obtained from purified pristine MWCNTs by a cascade of chemical functionalization. The activities of these modified MWCNTs were investigated *in vitro* on human umbilical vein endothelial cells (HUVECs) by evaluating their ability to influence cell viability and to induce cell apoptosis. Our data showed that pristine MWCNTs are markedly cytotoxic; conversely, the carboxylated carbon nanotubes, much more readily dispersed in aqueous solutions and CNT-Link, the key intermediate designed by us for the drug anchorage, are biocompatible at the tested concentrations (1 and 10 μg ml^{-1}).

Introduction

Owing to their unique electrical, physicochemical, and structural properties carbon nanotubes (CNTs) have attracted much research interest in many fields, including the emerging one of biological nanotechnology.[1–3] For instance, CNTs have been utilized as nanoelectronic biosensors to detect various biomolecules released by cells,[4–6] as vectors to deliver drugs and vaccines into cells[7] and as nanostructured scaffolds for tissue engineering.[8] Generally, proven success in other fields may not translate to the use of CNTs in medicine for reasons including inconsistent data on cytotoxicity and limited control over functionalized-CNT behavior, both of which restrict predictability. Thus, before such materials can be used for drug delivery in human therapy, their toxicity and biocompatibility need to be thoroughly investigated, especially the concern of interfacing between CNTs and mammalian cells.

The earliest study dates from 2001[9] and research since then has been focused mainly on lung toxicity,[10–11] skin irritation[12] and cytotoxicity.[9–13,14] However, the reported cytotoxic effects of CNTs in mammalian cells are controversial, with some reports demonstrating their cytotoxic effect,[15,16] whereas others demonstrated their biocompatibility.[17,18] A variety of factors can affect the CNT cytotoxicity: type of CNTs (single wall or multi-wall), impurities, lengths of CNTs, aspect ratios, dispersion, chemical modification; furthermore, the different methodologies employed to assay CNT toxicity can also justify these controversial results.[19] Finally, the efficiency, as well as the toxicity of CNTs, are also related to the characteristics and properties of the drug used to functionalize the CNTs. In particular, although various chemically modified CNTs have been used in many fields, the degree of toxicity has not been studied in depth and there is a need for further scientific evaluation and studies. Since the incomplete characterization of the CNT materials following purification and functionalization could be responsible for the contradictory cytotoxicity results, an ideal study requires a parallel monitoring between the chemical, physical and structural properties of the nanomaterial (through SEM, TEM, FTIR analyses) and their effects on its biocompatibility. In this paper we report the synthesis and characterization of a novel drug delivery system based on multiwalled carbon nanotubes (MWCNTs) first functionalized (through a cascade of chemical functionalization) and then conjugated to the natural compound 5,7-coumarin, to produce a MWNT-link-coumarin conjugate able to improve the bioactivity of 5,7-coumarin by increasing its capability to reach the intracellular target. In fact, the 5,7-coumarin residue was chosen as model of natural molecules by virtue of its chemical and biological properties. Coumarins, a vast class of natural products widely occurring as secondary plant metabolites, exhibit a broad range of biological activities;[20] especially, 7-hydroxycoumarins show antioxidant,[21–22] antiangiogenic[23] and cytostatic[24] properties and are able to activate apoptosis pathway.[25] Furthermore, we have tried to further increase the capability of MWNT-link-coumarin to reach the cell target by esterification of MWNT-link-coumarin with oleic acid. Oleic acid influences the membrane structure and function[26–27]

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and, in particular, the changes in the membrane fluidity;[28] it is considered as a model compound since, among the other free fatty acids, it is one of the most effective penetration enhancers.[29] There is evidence that the capability of a drug to interact with and to cross phospholipidic membranes may be modulated by its esterification with opportune aliphatic side chains, such as fatty acids. Thus, in our study, different samples of multi-walled carbon nanotubes were obtained by sequential steps of carboxylation, acylation, amine modification and finally, conjugation with the natural mentioned compounds (Fig. 1). The properties of these modified MWCNTs were investigated *in vitro* in human umbilical vein endothelial cells (HUVECs) by evaluating their capability to influence cell viability and to activate cell apoptotic machinery. Apoptosis is an important process during normal development for the maintenance of tissue homeostasis and the elimination of unwanted or damaged cells from multicellular organisms. Several lines of evidence have indicated that endothelial cell apoptosis may limit angiogenesis and actively lead to vessel regression in adult neovascularization.[30]

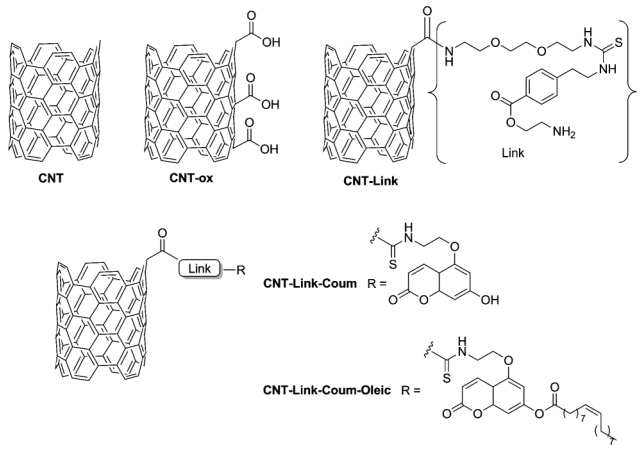

Fig. 1 Six samples of MWCNTs synthesized for biological studies.

Results and discussion

Synthesis of functionalized MWCNTs: CNT-Ox, CNT-Link, CNT-Link-Oleic, CNT-Link-Coum, CNT-Link-Coum-Oleic

We developed a method for the functionalization of carboxylated MWCNTs, which allows the anchorage of target compounds such as fatty acids and coumarins by means of a linker moiety. CNT-Ox, prepared by oxidation of purified pristine MWCNTs (CNT sample) using a mixture of sulfuric acid/nitric acid (molar ratio 1 : 3), were converted into the corresponding acyl chlorides by treatment with oxalyl chloride at reflux. The key step of the synthesis of functionalized MWCNTs is represented by the acquisition of the sample CNT-Link. The chemical derivatization, depicted in Fig. 2, requires the coupling of MWCNT-acyl chlorides with amine **1** in the presence of triethylamine and the subsequent cleavage of the Boc protecting group by treatment with HCl in dioxane to give compound **2**. The reaction coupling effectiveness was qualitatively demonstrated by EDAX analysis and quantitatively by potentiometric argentometric titration.[31]

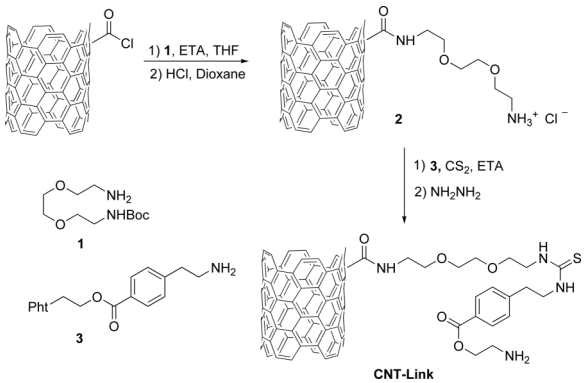

Fig. 2 Synthesis of CNT-Link.

The amine loading of compound **2** was determined also by the Kaiser test,[32] giving a value of 1.11 mmol g^{-1}, close to that obtained by argentometric titration (1.12 mmol g^{-1}).

In Fig. 3a it was reported the roughly SEM image, performed on a JEOL JSM 5600LV instrument operating at 20 kV, used in mapping mode to acquire the compositional information of sample **2**; the image in Fig. 3b shows an uniform distribution of chloride atoms on the carbon nanotubes. The EDAX spectrum in Fig. 4 confirms a remarkable presence of chlorides in the sample.

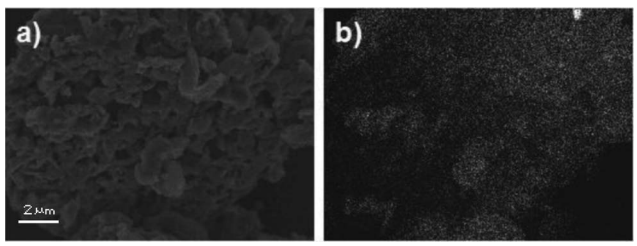

Fig. 3 (a) SEM images of **2**; (b) chloride distribution on **2**.

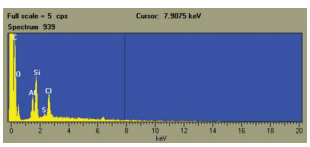

Fig. 4 EDAX analysis on **2**.

In the second step, the 2-(1,3-dioxoisoindolin-2-yl)ethyl-4-(2-aminoethyl)benzoate **3**, was *in situ* converted to the corresponding isothiocyanate by treatment with carbon disulfide in the presence of triethylamine and then coupled with **2**. The synthetic scheme towards CNT-Link was completed by the removing of the phthalimido protecting group using a solution of hydrazine in ethanol at room temperature. The quantity of the free amino groups (1.10 mmol g^{-1}) on CNT-Link was measured with a quantitative Kaiser test.[32] The CNT-Link has been further derivatized with oleic acid and coumarins in order to evaluate the impact of natural moieties on the cell viability (Fig. 5). The reaction of CNT-Link with oleyl chloride in the presence of triethylamine afforded the sample CNT-Link-Oleic; the samples CNT-Link-Coum and CNT-Link-Coum-Oleic have been obtained by coupling of coumarin isothiocyanate derivatives (see ESI†) with the free amino groups of CNT-Link. This method was chosen because

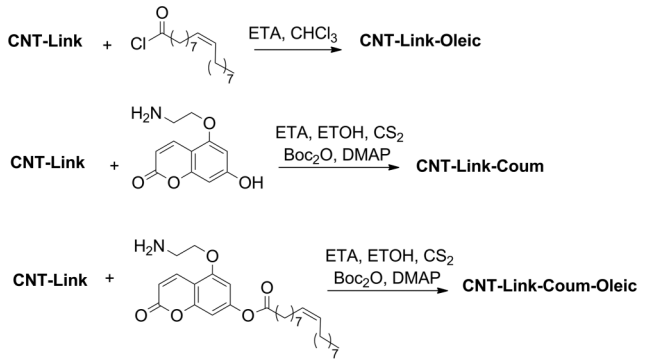

Fig. 5 Synthesis of samples CNT-Link-Oleic, CNT-Link-Coum and CNT-Link-Coum-Oleic.

allows for a rapid and clean preparation of isothiocyanate in high yields and purity without the need for subsequent work-up.[33]

Characterization of CNT, CNT-Ox, CNT-Link, CNT-Link-Coum, CNT-Link-Oleic and CNT-Link-Coum-Oleic

TGA, FT-IR, UV/Vis and HRTEM analyses were used to characterize the MWCNT samples. The degree of functionalization of the multiwalled carbon nanotubes: CNT, CNT-Ox, CNT-Link, CNT-Link-Oleic, CNT-Link-Coum and CNT-Link-Coum-Oleic was obtained by comparing the TGA data (see Table 1) obtained by thermal decomposition of the surface groups under inert atmosphere. The pristine MWCNTs (CNT sample) heated up to 900 °C did not show any significant weight loss; this is indicative of the absence of any functional group or other thermally degradable substances somehow introduced. The thermal degradation of functionalized MWCNTs occurs through a multistep process; the first stage, up to a temperature of 150 °C, corresponds to the evaporation of the adsorbed water while at 900 °C it may be ensured a complete loss of any organic functional groups.[34] The weight loss of CNT-Ox was 15% at 900 °C while it reaches, at the same temperature, the values of 29%, 36%, 39% and 36% for CNT-Link, CNT-Link-Coum-Oleic, CNT-Link-Coum and CNT-Link-Oleic respectively (after subtraction of the weight at 150 °C, which corresponds to the water loss). In the case of CNT-Link-Coum-Oleic sample, TGA data indicate a lower weight loss with respect to CNT-Link-Coum one; this value indicates that the coupling between the amino group of CNT-Link and the isothiocyanate of 5-(2-aminoethoxy)-2-oxo-2*H*-chromen-7-yl-oleate is carried out in a lower yield, maybe due to the steric hindrance of the oleyl residue. In order to exclude that the coumarins were linked to MWCNTs by π–π stacking a sample of CNT-Link was treated with 5-(2-aminoethoxy)-7-hydroxy-2*H*-chromen-2-one in ethanol for 12 h without the reagents CS_2, ETA, $(BOC)_2O$ followed by the standard washing/filtration/sonication/work-up procedures. The TGA profiles of CNT-Link sample and CNT-Link black sample were found superimposable.

Table 1 Comparative study of the functionalization of samples CNT, CNT-Ox, CNT-Link, CNT-Link-Oleic, CNT-Link-Coum and CNT-Link-Coum-Oleic

Sample code	Δm (%) 150 °C	Δm (%) 900 °C	Coum and/or FA (%)
CNT	0	2	—
CNT-Ox	3	18	—
CNT-Link	7	36	—
CNT-Link-Coum	3	42	10
CNT-Link-Coum-Oleic	4	40	7
CNT-Link-Oleic	0	36	7

Quantitative results related to the weight loss as inferred by TGA (Δm %) at 150 °C and 900 °C. At the column on the right is reported the estimated percentage of Coumarin and/or Fatty Acid (FA) loaded on MWCNTs (calculated by the weight difference with CNT-Link).

The FTIR spectra (Fig. S1 in ESI†) of pristine MWCNTs and the modified MWCNTs indicated the successful chemical modifications according to the synthetic scheme reported in Fig. 5. The presence of coumarins covalently bonded to the nanotubes was also confirmed by UV spectra (Fig. S2 in ESI†). CNT-Link-Coum-Oleic and CNT-Link-Coum samples show absorbance at 240–300 nm, while no absorbance was detected for the sample CNT-Link; a different spectrum was registered for the free coumarin derivative (compound **7**, (9*Z*)-5-(2-aminoethoxy)-2-oxo-2*H*-chromen-7-yl-octadec-9-enoate, in ESI†). Pristine and functionalized MWCNTs were observed by HRTEM and the images were shown in Fig. 6 (low magnification images on the left side, high magnification ones on the right side). It is evident that the pristine MWCNTs (CNT sample) are entangled and randomly oriented. The outer surface of MWCNTs is smooth and well graphitized. Pristine MWCNTs exhibit a clear multiwalled tube structure of 15–20 layers and the graphite layers do not stay always continuous along the growth orientation in some regions, which results from point defects and faults between graphitic carbon planes; moreover, the HRTEM analysis revealed an average length of 10–20 μm, with a diameter close to 15–20 nm. Following oxidation, MWCNTs (CNT-Ox) were noticeably dispersed and shortened: the length was reduced from micrometres to nanometres scale. The length distribution was used to quantify the extent of shortening by the oxidation process. The average length of CNT-Ox was between 200 and 1000 nm. The magnified TEM image of CNT-Ox shows for MWCNTs an erosion of external layer in many points as a result of the oxidative insertion of terminal functional groups (mainly –COOH groups).

In the typical TEM images of MWCNTs functionalized with coumarins and/or oleic acid, it can be observed that multiwalled carbon nanotubes are often covered with amorphous materials probably due to coumarins or oleoyl residue bound on MWCNTs. In addition, from the comparison between the high magnification TEM images of CNT or CNT-Ox with CNT-Link-Coum, CNT-Link-Coum-Oleic and CNT-Link-Oleic, it can be clearly seen that the surface of the multiwalled carbon nanotubes, in these latter cases, has been deeply modified: the organic molecules densely decorate the side walls of the MWCNTs, as observed by the presence of diffuse hazy contrast at the walls.

Effect of MWCNTs on HUVECs

The ability of the multiwalled carbon nanotubes under investigation to affect cell viability were studied on HUVECs by Trypan Blue exclusion assay after exposing cells to various concentrations of CNT, CNT-Ox and CNT-Link (1 and 10 μg mL^{-1}) for 24 h. This assay was preferred to other commonly used method in cytotoxicity assessment, like MTT Tetrazolium Salt Assay, because the hydrophobic CNTs, with a very high specific surface

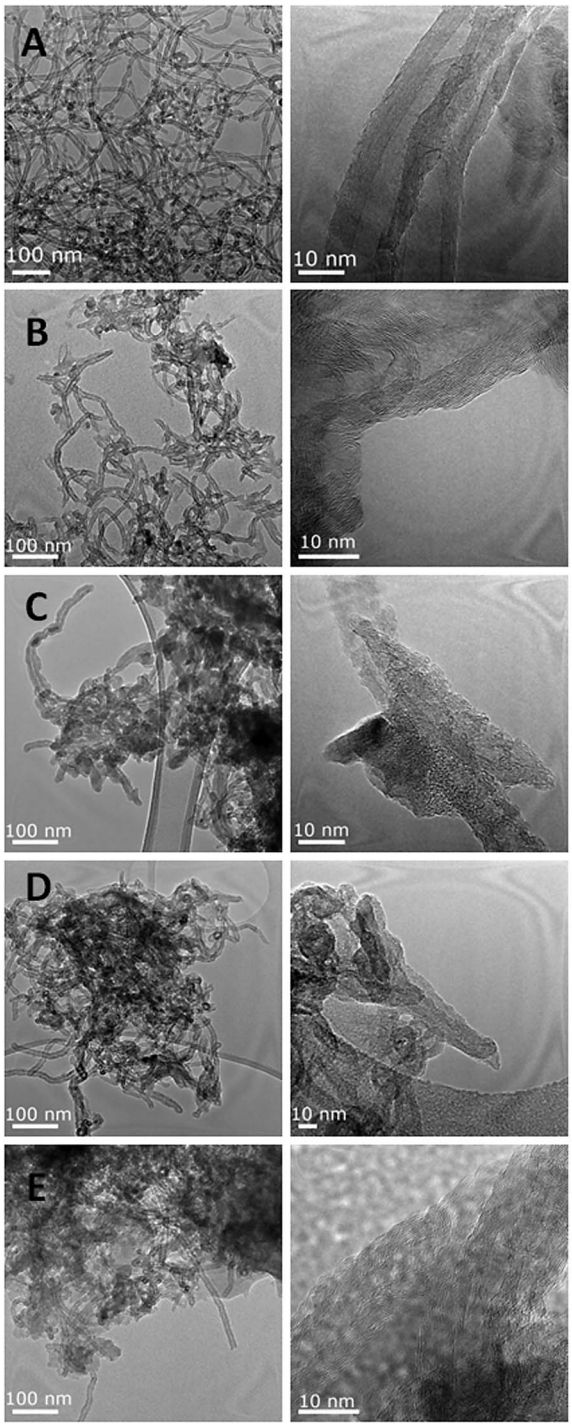

Fig. 6 Representative HRTEM images of CNT (A), CNT-Ox (B), CNT-Link-Coum (C), CNT-Link-Coum-Oleic (D) and CNT-Link-Oleic sample (E). Low magnification images are on the left side, high magnification ones are on the right side.

area and high chemical activity, might interact with the dye and affect the experimental data.[19] Endothelial cells are often used to investigate the safety of biomaterials, especially if to be used for systemic drug delivery and by injective route. The obtained data were expressed as a percentage of the values obtained from cells incubated with negative control extracts. The activation of cellular preapoptotic events was also investigated through expression of effector caspase-3 in HUVECs, exposed for 24 h to the selected CNTs at the concentrations of 1 and 10 μg mL^{-1}, by Western blot analysis using β-actin as housekeeping protein. Apoptosis requires specialized cellular machinery, including a family of cysteine proteases termed caspases.[35] Among the identified caspases, caspase-3 (CPP32)[36] is believed to serve as a general mediator of apoptosis pathway being activated early during apoptosis.

As shown in Fig. 7, a significant cell necrosis was observed for pristine MWCNTs at the highest concentrations tested (10 μg mL^{-1}). Pristine carbon nanotubes are already known to be harmful to living cells in culture due to the hydrophobicity and tendency to aggregate in large size bundles, and also to the toxic effects from impurities that can be classified as carbonaceous (amorphous carbon and graphitic nanoparticles) and metallic particles (typically transition metal catalysts). On the contrary, we observed that not only oxidized MWCNTs (CNT-Ox), much more

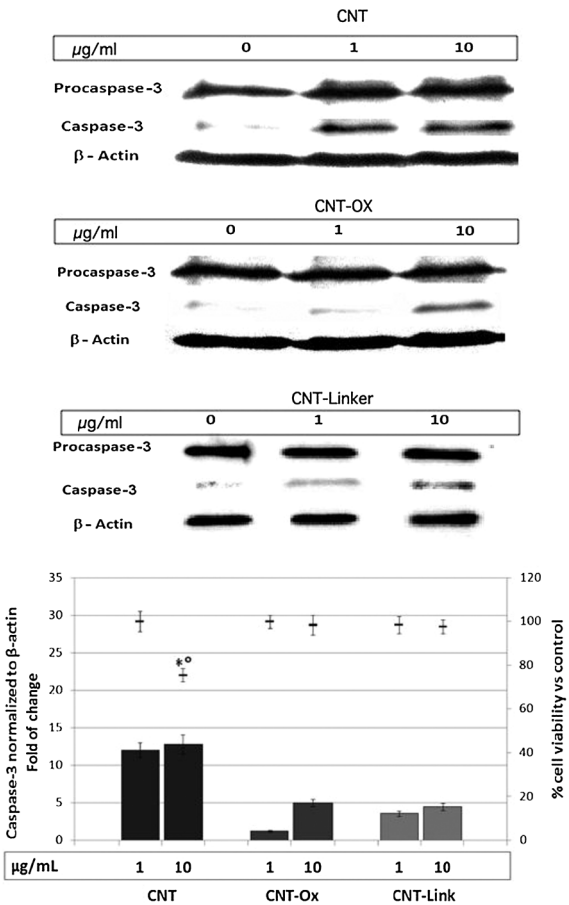

Fig. 7 Cell viability (dashes) and caspase-3 activation (columns) in HUVECs exposed for 24 h to CNT, CNT-Ox and CNT-Link (1–10 μg mL^{-1}) or to the vehicle (PBS). Cultures treated with the vehicle alone were used as controls. **Trypan blue assay**: Each point represents mean ± SD of three experiments. Data represent percentage of viable cells (mean percentage) calculated from the number of viable cells in treated samples *vs.* untreated control. * $P < 0.05$ *vs.* respective 1 μg mL^{-1} exposure. ° $P < 0.05$ *vs.* control. **Caspase-3 activation**: Representative images from three independent experiments. Results by densitometry are reported as fold change against control and expressed as mean ± SD of three experiments. Caspase-3 bands intensity values were normalized to the corresponding β-actin value.

readily dispersed in aqueous solutions, but also CNT-Link (that are functionalized so that can be linked to bioactive drugs) were not toxic in our experimental conditions. In agreement with these results, immunoblotting data have provided clear and significant evidence of apoptosis involvement only for CNTs pristine at all the concentrations tested.

We further investigated the biological activities of MWCNTs functionalized with 5,7-coumarin and/or oleic acid. Since the antiproliferative and antiangiogenic effects of coumarins are already known in literature[23,24] we evaluated caspase-3 activation in HUVECs exposed to the selected functionalized MWCNTs. Results showed that CNT-Link-Coum was able to increase caspase-3 expression in a dose-dependent way without affecting cell viability (Fig. 8). Moreover, only at concentration of 10 μg ml^{-1}, CNT-Link-Oleic slightly reduced cell viability (probably due to altered membrane permeability) and induced caspase activation. Thus, taken together, these data suggest that this effect induced by CNT-Link-Coum is specifically due to the coumarin moiety. These results support the hypothesis that oleic acid derivatization can produce a more hydrophobic structure, enhancing CNT cell uptake. However, CNT-Link-Coum-Oleic appeared less able to increase caspase-3 activation than CNT-Link-Coum (although it showed a higher capability to affect cell viability in the Trypan blue assay), so esterification with oleic acid does not appear to be useful to improve the bioproperties of CNT-Link-Coum.

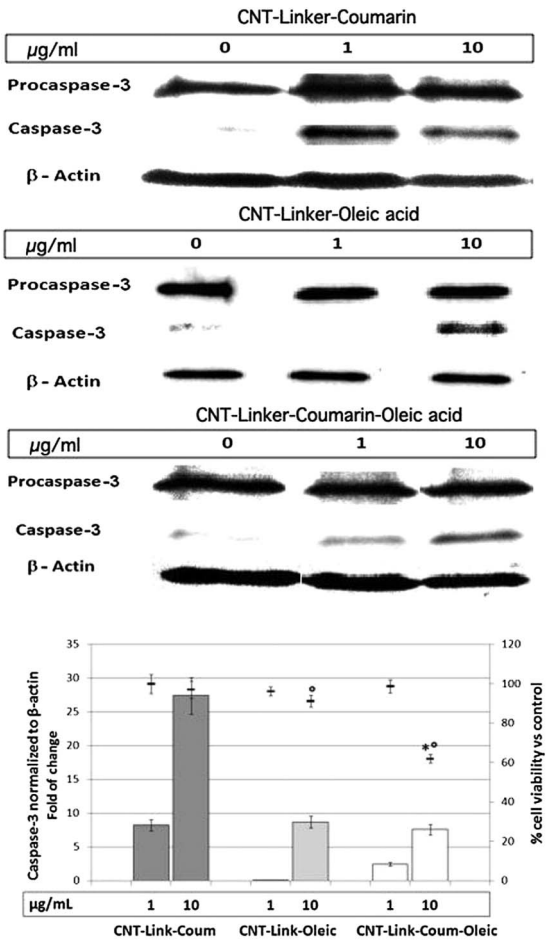

Fig. 8 Cell viability (dashes) and caspase-3 activation (columns) in HUVECs exposed for 24 h to the functionalised nanotubes CNT-Link-Coum, CNT-Link-Oleic and CNT-Link-Coum-Oleic (1–10 μg mL^{-1}) or to the vehicle (PBS). Cultures treated with the vehicle alone were used as controls. **Trypan blue assay**: Each point represents mean ± SD of three experiments. Data represent percentage of viable cells (mean percentage) calculated from the number of viable cells in treated samples *vs.* untreated control. * $P <$ 0.05 *vs.* respective 1 μg mL^{-1} exposure. ° $P < 0.05$ *vs.* control. **Caspase-3 activation**: Representative images from three independent experiments. Results by densitometry are reported as fold change against control and expressed as mean ± SD of three experiments. Caspase-3 bands intensity values were normalized to the corresponding β-actin value.

Experimental

Preparation of CNT sample

MWCNTs were produced by catalytic chemical vapor deposition (CCVD) from isobutane on Fe/Al_2O_3 catalyst; then synthesized MWCNTs were subjected to purification, as reported in previously procedure, giving the CNT sample with purity >95%.[37]

Synthesis of CNT-Ox sample

500 mg of pristine MWCNTs, sonicated in a water bath (60 W, 35 kHz), in 100 mL of sulphuric acid/nitric acid mixture (3 : 1 v/v, 98% and 69% respectively) were stirred at 60 °C for 6 h. 500 mL of deionized water was then added and the mixture was decanted for 2 h and two fractions was collected: an upper solution containing a so-called "light MWCNTs", and a bottom solution containing a so-called "heavy MWCNT" material. Then the supernatant (~300 mL) was recovered, filtered under vacuum on a 0.1 μm Millipore membrane, carefully washed with deionized water until pH became neutral and dried. Light MWCNTs were obtained in a yield of 40%. Heavy MWCNTs were recovered with a yield of 50%. Light MWCNTs were titrated to determine the concentration of acidic sites present according to literature method.[38] Briefly, MWCNTs were heated at 100 °C to remove carbon dioxide and water. Ox-MWCNTs were added into 100 mL of 0.01 N sodium hydroxide and stirred for 48h. The sample was centrifuged at 10,000 rpm for 15 min. Unreacted NaOH was titrated with 0.01 N hydrochloridric acid. The total acidity of the sample CNT-Ox, was found to be 1.8 mmol g^{-1}.

Synthesis of CNT-Link sample

Light Ox-MWCNTs (100 mg) were heated in 30 mL of neat oxalyl chloride at reflux for 48h; the excess of volatile reagent was removed to give the corresponding acetyl chlorides which, without further purification, were coupling to *tert*-butyl-2-(2-(2-aminoethoxy)ethoxy)ethylcarbamate. At the suspension of MWCNT-C(O)Cl in dry tetrahydrofuran (THF, 10 mL) 0.028 mL (0.20 mmol) of triethylamine (ETA) and 47 mg (0.19 mmol) of **1** were added. The reaction was left to stirring for 12 h and then filtered under vacuum on a 0.1 μm Millipore membrane, carefully washed with THF and methanol. The residue was treated with 5 mL of HCl 4 M in dioxane for five hours and then filtered under vacuum on a 0.1 μm Millipore membrane and carefully washed with dioxane and then with deionized water to guarantee complete elimination of excess chloride. The determination of NH_2 loading on compound **2** was calculated by potentiometric argentometric titration (1.12 mmol g^{-1}) and by Kaiser test (1.11 mmol g^{-1},

see ESI†). To a solution of 2-(1,3-dioxoisoindolin-2-yl)ethyl 4-(2-aminoethyl)benzoate **3** (0.15 mmol, 51 mg) in absolute ethanol (2 mL), CS_2 (1.5 mmol, 114 mg) and triethylamine (0.15 mmol, 0.02 mL) were added. The reaction mixture was stirred for 30 min at room temperature and then cooled on an ice bath. Di-*tert*-butyl dicarbonate (Boc_2O, 0.15 mmol, 33 mg), dissolved in absolute ethanol, was added followed by the immediate addition of a catalytic amount of dimethylaminopyridine (DMAP, 1–3 mol%) in absolute ethanol (1 mL). The reaction mixture was kept in the ice bath for 5 min and then was allowed to reach the room temperature. When evolution of gas from the reaction mixture had ceased (approximately 15 min), 100 mg of compound **2** and triethylamine (0.15 mmol, 0.02 mL) were added and the reaction mixture was stirred at room temperature for 12h. The mixture was filtered under vacuum on a 0.1 μm Millipore membrane and carefully washed with ethanol. The residue was treated with a solution of hydrazine hydrate in ethanol 25% (10 mL) at reflux for 24h. The mixture was cooled, filtered under vacuum on a 0.1 μm Millipore membrane and carefully washed with ethanol to give the sample CNT-Link. The NH_2 loading (1.1 mmol g^{-1}) was calculated by Kaiser test.[32]

Synthesis of CNT-Link-Oleic sample

100 mg of CNT-Link have been dispersed in chloroform (15 mL) and sonicated in a water bath (60 W, 35 kHz) for 30 min at room temperature. Then, triethylamine (0.12 mmol, 0.017 mL) and oleoyl chloride (0.12 mmol, 36 mg) were added and the suspension was stirred at reflux for 24 h. The mixture was cooled, filtered on a 0.1 μm Millipore membrane and washed with $CHCl_3$ and methanol until disappearance of the starting material from the solution to give sample CNT-Link-Oleic.

Synthesis of CNT-Link-Coum sample

To a solution of 5-(2-aminoethoxy)-7-hydroxy-2*H*-chromen-2-one (0.15 mmol, 30 mg) in absolute ethanol (5 mL), CS_2 (1.5 mmol, 114 mg) and triethylamine (0.15 mmol, 0.02 mL) were added. The reaction mixture was stirred for 30 min at room temperature and then cooled on an ice bath. Di-*tert*-butyl dicarbonate (Boc_2O, 0.15 mmol, 33 mg), dissolved in absolute ethanol, was added followed by the immediate addition of a catalytic amount of dimethylaminopyridine (DMAP, 1–3 mol%) in absolute ethanol (1 mL). The reaction mixture was kept in the ice bath for 5 min and then allowed to reach the room temperature. After that evolution of gas from the reaction mixture had ceased (approximately 15 min), 100 mg of CNT-Link and triethylamine (0.15 mmol, 0.02 mL) were added and the reaction mixture was stirred at room temperature for 12 h. The mixture was filtered under vacuum on a 0.1 μm Millipore membrane. The solid residue was washed for three-time with ethanol (3 × 20 mL) and each time sonicated for 10 min and separated from the supernatant by filtration. The solid residue was then dried under vacuum at 50 °C to give sample CNT-Link-Coum.

Synthesis of CNT-Link-Coum-Oleic sample

The sample CNT-Link–Coum-Oleic was synthesized starting from 5-(2-aminoethoxy)-2-oxo-2*H*-chromen-7-yl oleate (see ESI†) (0.15 mmol, 73 mg), following the procedure above reported for the synthesis of CNT-Link-Coum.

Cell culture and treatments

The Human Umbilical Vein Endothelial Cells (HUVECs) were isolated from freshly obtained human umbilical cords by collagenase digestion of the interior of the umbilical vein as described by Jaffe and coworkers[39] and were cultured in medium 199 with supplementation in gelatin pretreated flasks as described by Speciale and coworkers.[40] The MWCNT samples were diluted to a concentration of 2 mg ml^{-1} stock solution in 1× phosphate-buffered solution (PBS). The particles were vortexed for 1 min and then indirectly sonicated for 5 min at 4 °C immediately prior to preparing treatment-dilutions into serum-free medium. The dilutions were again vortexed prior to being added to the cells to insure adequate dispersion. The subconfluent cells were treated for 24 h in serum-free medium with various doses of CNT (1–10 μg mL^{-1}). Control cells were not exposed to MWCNT. Phenol (64 g L^{-1}) was used as a positive toxic control (data not shown). At the end of the exposition time, cells were immediately processed and/or preserved at –80 °C until analysis as expected for each test.

Cell viability

The capability of the different MWCNT derivatives to influence HUVEC viability was evaluated by means of Trypan blue exclusion assay. This dye does not pass through intact cell membranes so that live cells are excluded from staining. Briefly, 20 μl cell suspension was mixed with 20 μl Trypan blue isotonic solution (0.4% w/v) and loaded into a haemocytometer for both live and dead cell counting.

Western blot analysis

Caspase-3 activation was evaluated by Western blot. Briefly, cell lysates were prepared in nondenaturing buffer (10 mM Tris HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 5 mM $EDTANa_2$, 1 mM DTT, 1 μg mL^{-1} Leupeptine, 1 mM Benzamidine, 2 μg mL^{-1} Aprotinine) and protein concentration was determined using the Bradford assay.[41] For immunoblot analyses, 40 μg of protein lysates per sample were denatured in 4X SDS-PAGE sample buffer (260 mM Tris-HCl, pH 8.0, 40% v/v glycerol, 9.2% (w/v) SDS, 0.04% bromophenol blue, and β-mercaptoethanol as reducing agent) and subjected to SDS-PAGE on 16% polyacrylamide (29 : 1 Acrylamide : BisAcrylamide) gels. Separated proteins were transferred to nitrocellulose membranes (Hybond-P PVDF, Amersham Bioscience). Residual binding sites on the membrane were blocked by incubation with TBS-T (10 mM Tris, 100 mM NaCl, 0.1% Tween 20) containing 5% (w/v) nonfat milk powder (Amersham Bioscience) overnight at 4 °C. Membranes were then probed with specific primary antibody (Rabbit Anti-Caspase-3 Polyclonal Antibody or anti-β-actin monoclonal Antibody) followed by peroxidase-conjugated secondary antibody (HRP Labeled Goat Anti-Rabbit Ig) and visualized with an ECL plus detection system (Amersham Biosciences). The equivalent loading of proteins in each well was confirmed by Ponceau staining. Caspase-3 intensity values were normalized to the corresponding β-actin value.

Statistical analysis

All the experiments performed on human cells were carry out in triplicate and repeated three times. Results are expressed as means ± SD from three experiments and statistically analysed by a one-way ANOVA test, followed by Tukey's HSD, using the statistical software ezANOVA (http://www.sph.sc.edu/comd/rorden/ezanova/home.html). Differences in groups and treatments were considered significant for $P < 0.05$.

Conclusions

Functionalized multi-walled carbon nanotubes samples CNT-Ox, CNT-Link, CNT-Link-Coum, CNT-Link-Oleic and CNT-Link-Coum-Oleic, were prepared by a cascade of chemical reactions starting from purified pristine MWCNTs. The various data (TEM, TGA, FTIR, SEM, EDAX, UV-vis and titrations) proved that successful carboxylation, acylation, amine modification and, finally, coumarin conjugation have been carried out. Moreover, the characterization, performed for each synthetic step, allowed the monitoring of the main factors able to affect the sample cytotoxicity. Consistent with other previous reports, we showed that pristine MWCNTs are markedly cytotoxic. Conversely, the functionalized multi walled carbon nanotubes, CNT-Ox, much more readily dispersed in aqueous solutions and CNT-Link, key intermediate for the drug anchorage, are biocompatible since they do not significantly affect cell viability at the tested concentrations (1 and 10 μg ml^{-1}). We demonstrated that, when linked to MWCNTs (CNT-Link-Coum), the 5,7-coumarin residue can modulate cell signaling pathway, such as caspase cascade, in HUVECs, despite the very little coumarin amount loaded on and thus delivered by the functionalized carbon nanotubes (10% estimable by TGA analysis, 0.1μg of coumarin on 1μg of CNT-Link-Coum). This concentration is significantly lower than that of the free 5,7-coumarin, which is known to produce similar effects at concentration >10 μg ml^{-1} (data not shown). This effect seems to be specifically due to the coumarin moiety since no significant caspase-3 activation is observed when CNTs are derivatized with oleic acid alone or when CNT-Link-Coum is esterified with oleic acid. The findings of this preliminary study about the bioactivity of CNT-Link-Coum allow us to suggest that very low doses of CNT-Link-Coum are able to activate cell signaling pathways related to the apoptotic process without significantly influence cell viability.

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