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A Novel SWCNT Platform bearing DOTA and β-Cyclodextrin Units. "One Shot" Multidecoration under Microwave Irradiation

E. Calcio Gaudino,[a] S. Tagliapietra,[a] K. Martina,[a] A. Barge,[a] M. Lolli,[a] E. Terreno, [b] D. Lembo, [c] and G. Cravotto.[a]*

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The functionalization of single-walled carbon nanotubes (SWCNTs) via microwave-assisted grafting reactions enables efficient multidecoration in a single step. A novel water-soluble SWCNT platform was prepared via the simple 1,3-dipolar cycloaddition of azomethine ylides under dielectric heating. Thanks to a single grafting reaction the CNT surface binds in 1:1 ratio, an aminoacidic β-cyclodextrin (β-CD) derivative and the DOTAMA moiety (1,4,7,10-tetraazacyclododecane-*N*,*N'*,*N''*,*N'''*-tetraacetic acid monoamide). This novel "one shot" synthesis compared with multistep funtionalizations, preserves the SWCNTs structural integrity (TEM images). Besides thermogravimetric analyses, the determination of β-CD and DOTA moiety grafting onto the SWCNTs surface was calculated on the basis of phenophtalein and gadolinium complexation, respectively.

Introduction

The use of carbon nanotubes (CNTs) as multi-functional nanomaterials has attracted much attention from both academics and industry thanks to their intriguing electronic,[1] optical[2] and mechanical properties.[3] Their exclusive morphology and structure make them suitable candidates for numerous applications ranging from nanoelectronics and sensorics[4] to biomedical applications,[5] first of all drug delivery[6] and imaging.[7] The main drawback of CNTs is their insolubility in aqueous media and organic solvents, resulting in their aggregation into micrometer-sized bundles. This is caused by the presence of van der Waals inter-tube forces.[8] Therefore, significant efforts have been made, in the last few years, to find appropriate methodologies that solubilise pristine CNTs via chemical functionalization[9] making them biocompatible and possibly biodegradable.[10]

In general, CNT functionalization can be achieved using two main approaches; non-covalently via supramolecular adsorption or by wrapping different functional molecules onto the tube, or otherwise using covalent functionalization onto the conjugated skeleton via various reactions.[11] As CNTs have strong aromatic character, it is possible to adsorb other aromatic molecules onto the sidewalls using stacking interactions. Non-covalent derivatization is potentially reversible and fully preserves the unique electronic properties of the material and avoids sidewall damage. While being counted among suitable procedures for covalent bond formation, cycloaddition reactions also represent an effective method for preparing functionalized CNT based materials. They generally occur between two unsaturated entities and give a cyclic product in an atom-economic manner. They do not require any extra reagents and catalysts, which often make these processes expensive and complicated.[12] They include a wide range of reactions, such as the 1,3-dipolar cycloaddition of azomethine ylides,[13] CuI-catalyzed azide-alkyne cycloaddition reactions,[14] [4+2] Diels-Alder reactions,[15] [2+1] cycloaddition reactions,[16] and other types of cycloadditions.[17]

Besides their strength and toughness, CNTs have shown excellent electrical and thermal conductivity.[18] Extensive investigations into CNT behaviour under microwave (MW) irradiation have paved the way for important applications in aircraft stealth technology and other military equipment. Dielectric heating takes advantage of the strong MW absorption exhibited by CNTs,[19] and has been extensively applied in their functionalization via cycloaddition reactions. This enabling technology ensures efficient heat transfer through the reaction mixture enhancing functionalization yields and rates.[20] An example of direct CNT MW functionalization using epoxide groups via 1,3-dipolar cycloaddition has previously been reported by our group.[21] This functionalization gave stable CNT dispersions in a range of polar solvents, including water and ethanol. As has already been mentioned, soluble or dispersible CNTs are currently being explored for use in drug delivery, therapeutic and diagnostic applications. Native and functionalized cyclodextrins (CD) have recently emerged in this research field as multifunctional supramolecular agents capable of dispersing CNTs via covalent or non-covalent interactions. However, there are many disadvantages associated to these methods, such as time consuming synthetic procedures, poor reproducibility low immobilization mass of the CDs, structural damage of the CNTs. These disadvantages can lead to the loss of their electronic conductivity and corrosion resistance.[22]

The advantage of tying a CD structure to a CNT surface can be found in the fact that its cavity remains available for the formation of host-guest complexes with organic molecules in the aqueous solution. CD-CNT adducts have been investigated for

drug delivery, biosensoring and analytical applications involving electrochemistry.[23] Moreover, single-walled carbon nanotube (SWCNT) gadolinium complexes, called Gadonanotubes, have been used as magnetic labels for cellular MRI.[24]

In this article, we introduce, a new hybrid SWCNT platform that binds β-CD units as well as DOTAMA (1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic acid monoamide). The latter, a well known macrocycle ligand, enables the formation of a stable Gd(III) complex which is used in MRI applications. This work aims to design a new multifunctional SWCNT platform by exploiting the excellent hosting properties of β-CDs and the complexation ability of DOTAMA derivatives.

We have developed a fast methodology which is based on a MW-assisted 1,3-dipolar cycloaddition used for covalent SWCNT functionalization to achieve SWCNT multimodality. This "one shot" procedure enables the SWCNT surface to be controllably functionalized with two different molecules of interest: an aminoacidic β-CD derivative and a DOTAMA-aldehyde derivative.[25] The MW-assisted synthetic route of β-CD-DOTAMA-SWCNT nanocomposites is depicted in Scheme 2. The SWCNT hybrid structure's capacity for suspension in polar solvents, including water, is dramatically increased by its high level of surface functionalization. This platform is thus a promising candidate for use as a drug carrier, MRI contrast agent[26] and theranostic system for cancer imaging and therapy.[27] The toxicity of the above mentioned modified SWCNTs was evaluated against five human cell lines and it was shown that they do not affect cell viability, even at high doses.

Results and discussion

CNT properties can be modulated by surface functionalization, depending on the nature and spatial location of functional groups. The main requirements for CNT-based platforms use as a molecular carrier are solubility, binding versatility and low toxicity. Covalent functionalization, as opposed to non-covalent, ensures higher CNT-conjugate stability and better drug release control. So far, covalent CNT functionalization has been introduced via sequential or orthogonal synthetic protocols. Triple functionalization has also been reported in the literature.[28] Multi-functionalization generally involves the use of protective groups whose sequential removal may induce alterations on the CNT surface. A fundamental requirement for the rational application of CNT derivatives in biomedical investigations and nanomedicine is to precisely know the overall loading of organic moieties bound on the surface and their relative ratio. This is particularly true in the case of orthogonal functionalization. These two parameters are a fundamental feature of CNT use as a diagnostic probe and drug carrier.

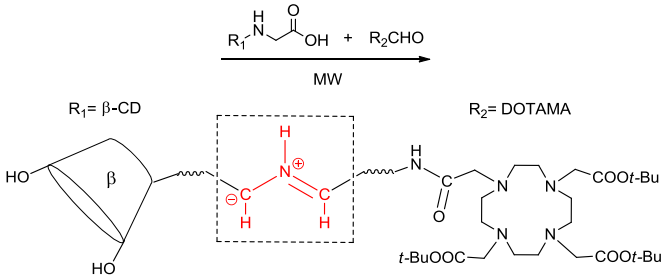


Fig. 1 Azomethine ylides generated in situ via MW-assisted decarboxylation.[29]

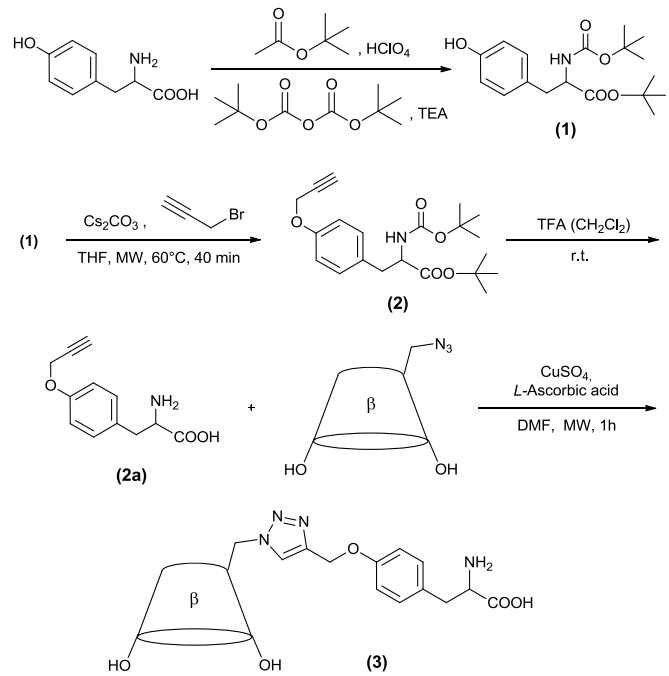
We therefore introduce a "one shot" double orthogonal covalent functionalization of SWCNT, promoted by MW irradiation.

Our synthetic strategy is based on the 1,3-dipolar cycloaddition of *in situ* generated azomethine ylides which include both a β-CD unit and a DOTAMA *tris*(*t*-butyl ester) moiety (Fig. 1).

The first step on the synthetic pathway involves the double protection of the tyrosine amino and carboxylic groups as *t*-butyl ester and *t*-butyl carbamate (Scheme 1). Protected derivative **1** was *O*-alkylated with propargyl bromide in the presence of Cs_2CO_3. The propargylated phenol derivative **2** was then deprotected under acidic conditions to give the tyrosine derivative **2a**. A MW-assisted copper-catalyzed azide-alkyne cycloaddition (CuAAC),[30] was used to obtain product **3**, in good yield (80%, Scheme 1), from the reaction of alkyne **2a** with 6-monoazido-6-deoxy-β-CD (β-CD-N_3), which was prepared according to the literature.[31] DOTAMA aldehyde derivative **4** was obtained via a Swern oxidation, following the reported procedure.[25]

Solutions of the β-CD adduct **3** and the DOTAMA derivative **4** (molar ratio = 1:2.5) were added to a suspension of SWCNTs in DMF. The mixture was sonicated for a few minutes in a cleaning bath to ensure good CNT dispersion. The mixture was irradiated with MW at 100°C (Scheme 2) for 2 h and the cooled suspension was then centrifuged.

The solid residue, consisting of the functionalized SWCNT **5**, was washed with THF and acetone, and dried under vacuum.



Scheme 1 Synthesis of the aminoacidic β-CD derivative (3) by MW-assisted CuAAC.

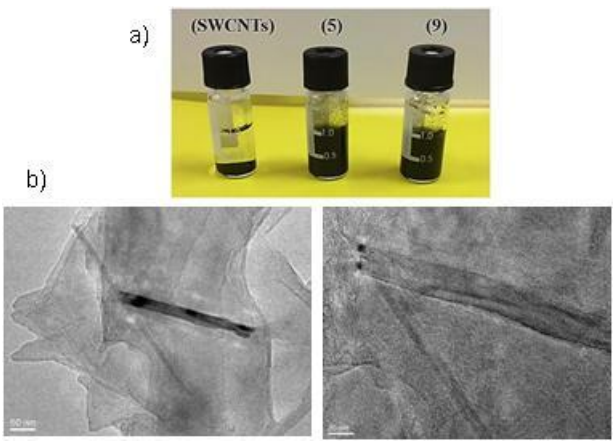


Fig. 2 a) Stability after 15-days at room temp. of a 5 mg/ml water dispersion (2 min sonication) of adducts 5 and 9. b) TEM images of functionalized SWCNTs **5.**

The ability of **5** to form a suspension is dramatically improved compared to pristine SWCNTs. Its suspension in DMF, MeOH or water is stable for several days (Fig. 2a). The morphology of nanocomposite SWCNT adduct **5** was analyzed by TEM and the images show that functionalization does not alter the SWCNT structure (Fig. 2b).

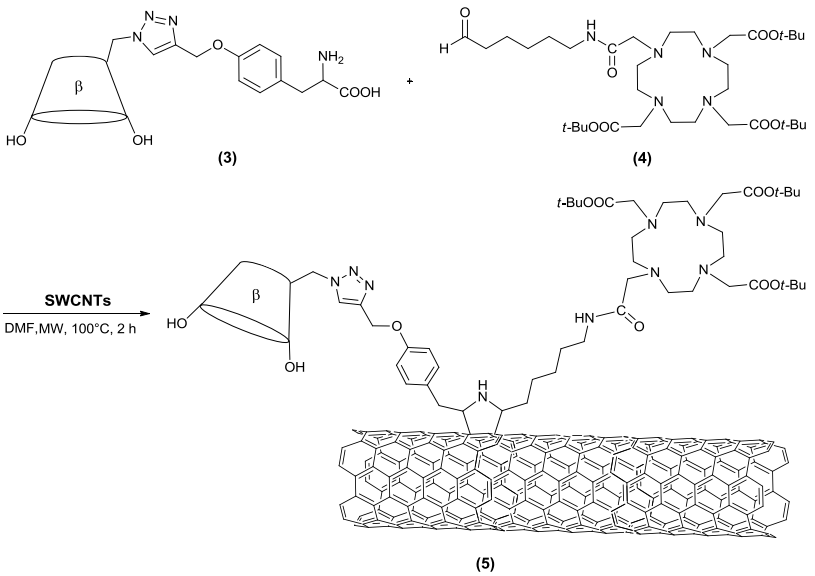
The functionalization degree of SWCNTs was first evaluated by TGA measurements, via comparison with pristine SWCNTs (Fig. 4). Heating was carried out under air. TGA analysis can provide semi-quantitative information only when it is carried out under a nitrogen atmosphere. However, the differences in combustion profiles recorded under air for pristine and modified nanotubes **5** allow the qualitative evaluation of the SWCNT surface functionalization to be carried out. Therefore, the TGA of adduct **5** showed a weight loss of about 40% at 350 °C, which could correspond to one functional group per 250 SWCNT carbon atoms.

CNT surface functionalization degree can be accurately elucidated via the quantitative determination of the β-CD and DOTAMA moieties grafted onto the carbon nanotube. In order to accurately determine the content of β-CD in the SWCNT adduct **5**, the complexation of phenolphthalein (Php), by β-CDs inclusion, was measured by means of UV-vis spectroscopy. At pH > 8.4, Php is in a conjugated quinoid form, is of a deeper colour (intense purple) than the benzenoid form and shows strong absorption at a wavelength of 553 nm. β-CD can alter this coloration because Php delocalization is disturbed by the lactonization of the ionized form upon complexation. As a consequence, absorbance changes and the pink colour disappears (scheme 3).[32] The formation of colourless 1:1 complexes in alkaline aqueous solution can thus be used for the quantitative determination of β-CD-content in polymeric materials.[33] In fact, a change in absorbance can be quantified by UV-vis spectroscopy, where the characteristic Php peak, close to 553 nm, decreases with additional amounts of β-CD.

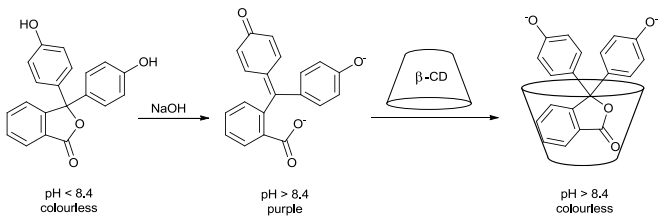
Adduct **5** was dispersed in a Php solution at pH 10.5, with the aim of quantifying the amount β-CD grafted onto the SWCNTs, and the change in Php absorbance was recorded as UV spectra. As depicted in Fig. 3, Php solution absorbance at 553 nm decreased significantly when in the presence of derivative 5. By monitoring the absorbance after 2, 5, 10, 15, 30 min and 1 h, we observed the saturation point approximately after 15 min. β-CDs grafted on SWCNTs have been quantified, and the results were compared with the calibration curve obtained with free β-CD. The absorbance of the calibration solutions of β-CD were measured at a wavelength of 553 nm at room temperature (Fig. 3). CD content was 95.7 μmol/g of SWCNT derivative **5**, which corresponds to a functionalization of about 16% by weight (referred to the whole molecule grafted to the surface). The inclusion capacity of SWCNT bound β-CD was roughly the same as native β-CD.[33a]

The amount of DOTAMA derivative bound to the SWCNT surface was determined by Gd(III) complexation followed by acid mineralization and ICP-MS analysis. To this purpose, adducts **5** were treated with trifluoroacetic acid for ester cleavage and the Gd(III) complex was then synthesized via the addition of a $GdCl_3$ water solution (pH=5.5) to the deprotected solid **5**. The suspension was shaken for 12 h then the solution was separated from solid. The solid was washed three times with water and three times with an EDTA solution to remove any unreacted Gd(III) ions, and then again with water.

The dried Gd-SWCNTs were mineralized in nitric acid at 120 °C and Gd content was determined by ICP-MS measurements. The ICP-MS data indicate a functionalization degree attributable to a weight increment of about 13% (referred to the whole molecule grafted) which is comparable to that obtained by β-CD determination. The ratio between DOTAMA and β-CD is determined by the reaction mechanism and is 1:1.



Scheme 2 "One shot" double covalent functionalization of SWCNT, promoted by MW irradiation.



Scheme 3 Php complexation by β-CDs inclusion.

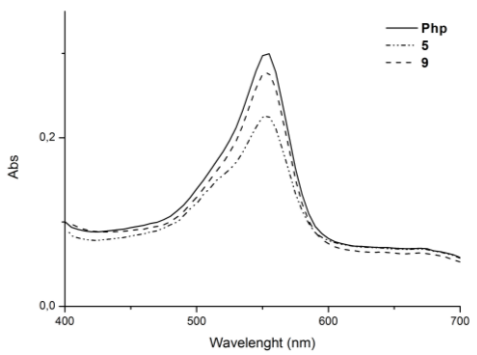


Fig. 3 β-CDs quantitative detrmination by **P**henolphthalein (Php) inclusion, measured by UV-vis spectroscopy at 553 nm.

The two approaches used to estimate Gd and β-CD content gave very similar results indicating that both these methods can be used to evaluate surface functionalization.

With the aim of demonstrating that the "one shot" procedure was the method of choice in terms of yield and functionalization degree, we compared this protocol with the sequential synthetic strategy depicted in scheme 4. SWCNTs were functionalized via a MW assisted 1,3-dipolar cycloaddition using tyrosine and octanal in DMF to obtain adduct **6** (Scheme 4), followed by the propargylation of the phenolic -OH group in the presence of Cs_2CO_3. Product **7** was analyzed by TGA which showed a covalently bound organic moiety loading of 1/110 SWCNT carbon atoms. The sequential click reaction strategy via CuAAC with the 6-monoazido-β-CD gave adduct 8 and a second coupling with the azido-DOTAMA *tris*(*t*-butyl ester) derivative[34] gave the final product **9**. The TGA profile of the latter showed a lower weight loss at 350 °C (20%) than that of the "one shot" obtained compound **5** (Fig. 4). These first qualitative data indicate the lower surface functionalization degree (about one functional group per 600 carbon atoms of the SWCNT) achieved by this second synthetic procedure, which causes the lower combustion ability of **9** compared to **5**. With the aim of quantifying the amount of molecules grafted onto **9**, the previously described quantification methods of β-CD and DOTAMA derivatives were repeated. The analysis showed slightly lower grafted β-CD content (12%, referred to the whole portion grafted) compared to the "one shot" method. The total amount of the second step grafted DOTAMA derivative was even worse (only 0.8%, referred to the whole portion grafted), with a β-CD/DOTA ratio of about 15/1.

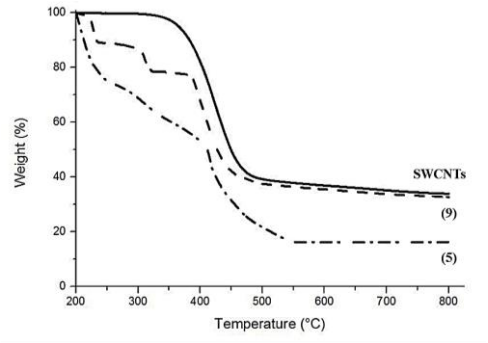
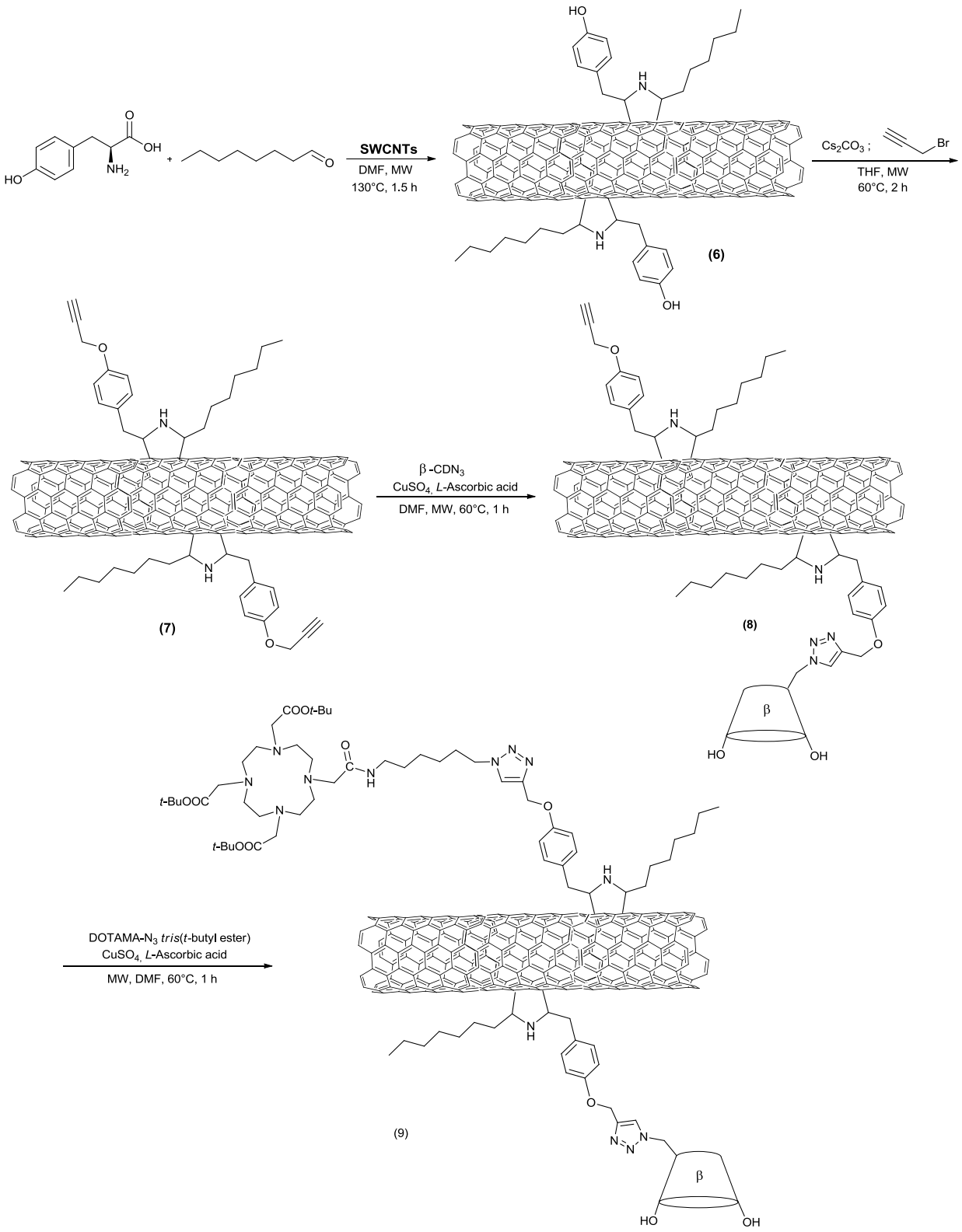


Fig. 4. Heating rate 10 °C/min from room temperature to 800 °C..

Despite the fact that MW irradiation allows high levels of covalent SWCNT functionalization to occur, prolonged thermal treatment may affect the CNT surface and damage its structure due to MW superheating effects on SWCNTs.[35] TEM analysis of adduct **9** (Fig. 5) highlights the rupture of a SWCNT tip. This should be compared to analysis of derivative **5** which was obtained using the "one shot" procedure (Fig. 2).



Scheme 4 Sequential SWCNTs functionalization via MW assisted 1,3-dipolar cycloaddition

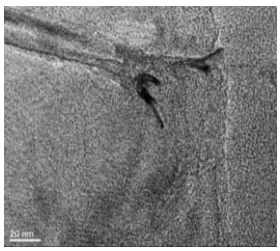


Fig. 5 TEM image of functionalized SWCNT 9.

Assessment of cell viability

The effect of SWCNT adducts **5** and **9** on cell viability was evaluated on five human cell lines of different histological origin, namely A549, Huh7, Hep2, Helf and HeLa.

The cells were treated with increasing concentrations of SWCNT adducts ranging from 0.45 µg/mL to 1000 µg/mL and cell viability was monitored after 24 hours. The results shown in Fig. 6 indicate that the two SWCNT adducts did not affect cell viability at concentrations up to 333 µg/mL in all cell lines tested. A moderate reduction in cell viability was caused by SWCNT adducts **5** and **9** in Hep2 and by SWCNT adduct **5** in Helf at the highest concentration tested.

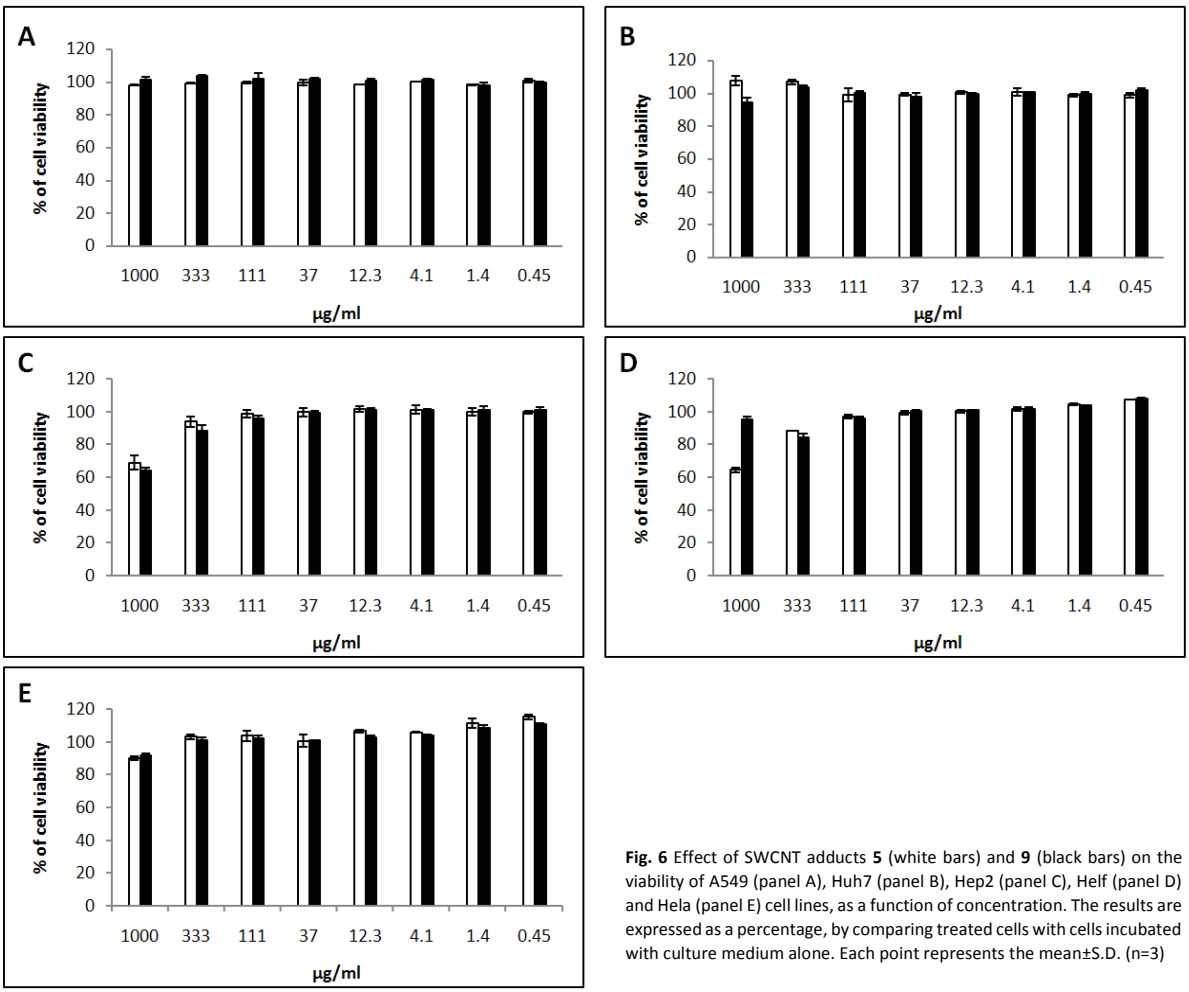


Fig. 6 Effect of SWCNT adducts **5** (white bars) and **9** (black bars) on the viability of A549 (panel A), Huh7 (panel B), Hep2 (panel C), Helf (panel D) and Hela (panel E) cell lines, as a function of concentration. The results are expressed as a percentage, by comparing treated cells with cells incubated with culture medium alone. Each point represents the mean±S.D. (n=3)

Conclusion

We have herein described a "one shot" synthetic protocol which covalently functionalizes SWCNTs with numerous active moieties via the MW-assisted 1,3-dipolar cycloaddition of azomethine ylides. Besides thermogravimetric analyses, the determination of the amount of β-CD and DOTA moieties grafted onto the SWCNT surface was calculated on the basis of the respective complexation of phenophtalein and gadolinium. The new polyvalent non-toxic SWCNTs platform will be investigated as a multi-carrier for potential diagnostic and theranostic applications.

Experimental Section

Materials and methods

All chemicals were purchased from Alfa-Aesar Italy and used without further purification. β-CD was kindly provided by Wacker Chemie (Germany).
DOTAMA-aldehyde (**4**) and DOTAMA-azido *tris*(*t*-butyl ester) derivatives were prepared following a previously reported procedure.[25,34]
MW-assisted reactions were carried out in a SynthWAVE reactor (MLS Gmbh, Milestone Srl).
Reactions were monitored by TLC on Merck 60 F254 (0.25 mm) plates, which were visualized by UV inspection and/or by heating after spraying with 5% H_2SO4 in ethanol or phosphomolybdic acid. Product purification was performed by flash-chromatography (CombiFlash RfsTeledyne ISCO) on appropriate cartridges (silica or RP18).
NMR spectra were recorded on a Bruker Avance 300 (operating at a magnetic field strength of 7T) at 25 °C; chemical shifts were calibrated to the residual proton and carbon resonance of deuterated solvents: $CDCl_3$ (1H = 7.26 ppm, 13C = 77.16 ppm) or DMSO-*d6* (1H = 2.54 ppm, 13C = 40.45 ppm).
Ultrapure water, prepared by Milli-Q Century System (Millipore, USA), was used throughout the entire study.
UV absorption measurements were performed in a UV-Vis dual-beam spectrophotometer (Agilent Technologies Cary 60, G6860AA) equipped with 1 cm path length quartz cuvette.
Non-complexed Php was separated from the functionalized SWCNT derivatives by filtration over a cellulose acetate membrane (0.2 μm) syring filter (CPS Analitica, Italy).
Thermogravimetric analyses were performed in a thermogravimetric Analyzer TGA 4000 (Perkin Elmer) at 10 °C/min operating under air with alumina crucibles containing 10–20 mg of SWCNT derivatives. Total mass loss was attributed to functional groups which were covalently attached to the sidewalls. The number of functional groups was calculated considering a fixed thermogram temperature, the residual weight of functionalized SWCNTs (W_R), its mass loss (W_L) and the molecular weight of the organic fragment (M_F) on its sidewalls = $(\%W_R/12) / (\%W_L/M_F)$.
"CellTiter 96 Proliferation Assay" (Promega, Madison, WI, USA) kit for cell viability assays was used according to the manufacturer's instructions. Absorbances were measured at 490 nm using a Microplate Reader (Model 680, BIORAD).

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Notes and references

[a] Dipartimento di Scienza e Tecnologia del Farmaco, and Interdepartmental Centre "Nanostructured interfaces and surfaces"- NIS, University of Turin, Via P. Giuria 9, 10125 Torino, Italy.
[b] Dipartimento di Biotecnologie Molecolari e Scienze per la salute, University of Turin, Via Nizza 52, 10125 Torino, Italy.
[c] Dipartimento di Scienze cliniche e Biologiche, University of Turin, Regione Gonzole 10, 10043, Orbassano (TO), Italy.
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