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Research Paper

Antioxidant multi-walled carbon nanotubes by free radical grafting of gallic acid: new materials for biomedical applications

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Abstract

Objectives To prove the possibility of covalently functionalizing multi-walled carbon nanotubes (CNTs) by free radical grafting of gallic acid on their surface with the subsequent synthesis of materials with improved biological properties evaluated by specific in-vitro assays.

Methods Antioxidant CNTs were synthesized by radical grafting of gallic acid onto pristine CNTs. The synthesis of carbon nanotubes was carried out in a fixed-bed reactor and, after the removal of the amorphous carbon, the grafting process was performed. The obtained materials were characterized by fluorescence and Fourier transform infrared spectroscopy (FT-IR) analyses. After assessment of the biocompatibility and determination of the disposable phenolic group content, the antioxidant properties were evaluated in terms of total antioxidant activity and scavenger ability against 2,2′-diphenyl-1-picrylhydrazyl (DPPH), hydroxyl and peroxyl radicals. Finally the inhibition activity on acetylcholinesterase was evaluated.

Key findings The covalent fuctionalization of CNTs with gallic acid was confirmed and the amount of gallic acid bound per g of CNTs was found to be 2.1 ± 0.2 mg. Good antioxidant and scavenging properties were recorded in the functionalized CNTs, which were found to be able to inhibit the acetylcholinesterase with potential improved activity for biomedical and pharmaceutical applications.

Conclusions For the first time, a free radical grafting procedure was proposed as a synthetic approach for the covalent functionalization of CNTs with an antioxidant polyphenol.

Keywords antioxidant materials; carbon nanotubes; gallic acid; radical grafting

Introduction

Over recent decades, nanotechnology has received great attention concerning the development, production and use of nanoparticles for a wide range of industrial and scientific fields, such as medicine, pharmacy, agriculture, textile, food, chemical and packaging industries. Within the realm of biotechnology particular attention was focused on carbon nanotubes (CNTs), a major class of carbon-based tubular nanostructures.[1]

CNTs can be imaginatively produced by rolling up a single layer of graphene sheet (single-walled CNTs; SWCNTs),[2] or by rolling up many layers to form concentric cylinders (multi-walled CNTs; MWCNTs)[3] and closing the ends in both cases with half spheres of fullerenes. The walls of these tubes are made up of a hexagonal lattice of carbon atoms analogous to the atomic planes of graphite. Such MWCNTs can reach diameters of up to 100 nm.[4]

The unique structural, mechanical and electronic properties of CNTs were initially exploited in the field of materials science. CNTs are considered ideal materials for several applications, ranging from ultrastrong fibres to field emission displays.[5] Recently, CNTs have generated great interest in biology and pharmaceutics,[6] where suitably modified CNTs have been utilized as platforms for ultrasensitive recognition of antibodies,[7] as nucleic acids sequencers,[8] and as bioseperators,[9] biocatalysts,[10] and ion channel blockers[11] for

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facilitating biochemical reactions and biological processes.[12] In the emerging field of utilizing nanomaterials for novel and alternative diagnostics and therapeutics,[13] CNTs have been used as scaffolds for neuronal and ligamentous tissue growth for regenerative interventions of the central nervous system and orthopaedic sites,[14] substrates for detecting antibodies associated with human autoimmune diseases with high specificity[15] or multifunctional containers when being filled with tailored materials.[16] When coated with nucleic acids (DNA or RNA), vaccines and proteins, CNTs have been shown to be effective substrates for gene sequencing and as gene and drug delivery vectors to challenge conventional viral and particulate delivery systems.[17] In particular, one of the key advantages that CNTs offer in the delivery of therapeutically active molecules is the possibility of effectively crossing biological barriers. Several studies, indeed, show that CNTs interact with mammalian cells, leading to their cytoplasmic translocation.[18]

Despite this great potential, the toxicological impact and safety profile of carbon nanomaterials with regard to biological systems is not yet well investigated and is subject to an increasing number of reports. Pristine CNTs are completely insoluble in all solvents, and this has generated some health concerns; consequently, the manufacture and extensive use of nanotubes generates concern about their safe use and possible effect on human health.[19]

It should be noted that the CNTs' outstanding reactivity due to their enormous surface area, achieved by the infinitesimal size, serves as their best merit on the one hand but also their worst attribute on the other, especially when they enter human and others living creatures' bodies.[20] According to the WHO (World Health Organisation) definition, CNTs possess a fibre-like structure and the morphological similarity with asbestos fibres is the major concern in public health.[21] In a recent work, it was proved that exposing the mesothelial lining of the body cavity of mice (a surrogate for the mesothelial lining of the chest cavity) to long MWCNTs, an asbestos-like, length-dependent, pathogenic process is observed. This process includes inflammation and the formation of lesions known as granulomas.[22] In addition, in 2008, scientists reported that CNTs possess carcinogenic properties, causing mesothelioma at a high rate in intact male rats.[20]

On the basis of these considerations, several studies have hence been performed aiming to prepare derivatives of highly functionalized CNTs with reduced toxic effects.

In this study, for the first time, we have performed a covalent functionalization of CNTs with a molecule of biological interest (i.e. gallic acid) by a free radical-induced reaction. This functionalization approach exhibits two key advantages: first, materials with high biocompatibility are prepared and, second, it is possible to impart the biological properties of free gallic acid to the final nanostructures. In particular, gallic acid functionalized CNTs could be very useful because of the possible reduction of oxidative stress in biological environments. In general, oxidative stress results in oxidative alteration of biological macromolecules such as lipids, proteins and nucleic acids. It is considered to play a pivotal role in the pathogenesis of aging and degenerative diseases[23]. To cope with an excess of free radicals produced upon oxidative stress, human bodies have developed sophisticated mechanisms for maintaining redox homoeostasis.[24] These protective mechanisms include scavenging or detoxification of reactive oxygen species (ROS), blocking ROS production and sequestration of transition metals, as well as enzymatic and nonenzymatic antioxidant defences produced in the body (i.e. endogenous) and others supplied with the diet (i.e. exogenous).[25] Polyphenols, and gallic acid (Figure 1) in particular, have been widely studied for their strong antioxidant capacity and other properties by which cell functions are regulated.[26] Gallic acid is an important natural antioxidant found in several different vegetable sources widely used in the food and pharmaceutical industries. Since gallic acid is regarded as an antioxidant for preventing rancidity and spoilage in fats and oils, it has been used as an additive in cosmetics and foods such as baked goods, confectionary and chewing gum. There are many reports that gallic acid possesses free radical scavenging, anti-allergic, anti-inflammatory, anti-mutagenic and anti-carcinogenic activity.[27–30] Also, some gallic acid esters are widely used as food additives to prevent food oxidation. Gallic acid derivatives, have also been found in many phytomedicines with various biological and pharmaceutical actions, including free radical scavenging effects, induction of apoptosis of cancer cells and protection of cells from damage induced by UV light or irradiation.[31]

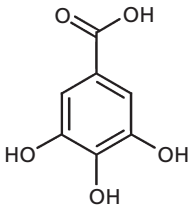

Figure 1 Chemical structure of gallic acid.

Based on these considerations, our approach was the covalent linkage of gallic acid to CNTs by means of a one-step free radical grafting reaction. The synthesis of CNTs was carried out in a so-called fixed-bed reactor and, after the synthesis, the functionalization was directly performed on pristine CNTs. We mention that we did not apply any preliminary treatment, which is commonly reported in CNT functionalization procedures. The grafting of gallic acid onto CNTs consists of a radical insertion of gallic acid on the CNT's shell performed by employing hydrogen peroxide–ascorbic acid as a biocompatible redox initiator. Using this approach, an effective, simple, single-step functionalization procedure was carried out without any kind of time-expensive derivatization process. The covalent insertion of gallic acid on CNTs was confirmed by specific analyses and in-vitro tests to confirm the effective covalent conjugation of gallic acid to CNTs. The retention of the antioxidant properties of free gallic acid in the final materials proves that, by the novel synthetic strategy proposed in this study, an effective antioxidant carbon nanostructure was prepared.

Materials and Methods

Reagents and standards

3,4,5-Trihydroxybenzoic acid (gallic acid), hydrogen peroxide (H_2O_2), ascorbic acid, 2,2′-diphenyl-1-picrylhydrazyl

radical (DPPH), Folin-Ciocalteu reagent, sodium carbonate, sulfuric acid (96% w/w), trisodium phosphate, ammonium molybdate, β-carotene, linoleic acid, Tween 20, deoxyribose, acetylcholinesterase (AchE) from *Electrophorus electricus* (AChE Type-VI-S, EC 3.1.1.7), acetylthiocholine iodide (ATCI), physostigmine, 5,5′-dithiobis (2-nitrobenzoic-acid) (DTNB), $FeCl_3$, ethylenediaminetetraacetic acid disodium salt (EDTA), dipotassium hydrogen phosphate, potassium dihydrogen phosphate, thiobarbituric acid (TBA), trichloroacetic acid (TCA), hydrochloric acid (37% w/w), acetic acid and sodium hydroxide were obtained from Sigma-Aldrich (Sigma Chemical Co., St Louis, MO, USA).

Ethanol and chloroform, were HPLC-grade and provided by Fluka Chemika-Biochemika (Buchs, Switzerland).

Instrumentation

The liquid chromatography apparatus consisted of an Jasco BIP-I (Jasco Co., Tokyo, Japan) pump and Jasco UVDEC-100-V (Jasco Co., Tokyo, Japan) detector set at 254 nm. A 250 mm × 4 mm C-18 Hibar column, particle size 5 μm (Merck, Darmstadt, Germany) was employed. As reported in literature,[32] the mobile phase was methanol–water–orthophosphoric acid (20 : 79.9 : 0.1), eluted isocratically at a flow rate of 1.0 ml/min at room temperature.

IR spectra were recorded as KBr pellets on a Jasco FT-IR 4200 (Jasco Co., Tokyo, Japan). UV–Vis absorption spectra were obtained with a Jasco V-530 UV/Vis spectrometer (Jasco Co., Tokyo, Japan).

Scanning electron microscopy (SEM) photographs were obtained with a Jeol JSMT300 A (Jeol GmbH, Eching, Germany).

The corrected emission spectra, all confirmed by excitation ones, were recorded with a Perkin Elmer LS-55 Luminescence spectrometer (Perkin Elmer Inc., Waltham, MA, USA) equipped with Hamamatsu R928 photomultiplier tube (Hamamatsu Photonics K.K., Hamamatsu, Japan). The fluorescence spectra in the solid state were recorded by using the front face accessory. The solid sample were placed between quartz plates (200 mm^2) on the sample holder.

Synthesis of carbon nanotubes

The synthesis of MWCNTs was performed in a so-called fixed-bed reactor as described previously.[33] Briefly, a quartz boat containing the prepared catalyst material was placed in the hot zone of the horizontal reactor tube. At first, the reactor was exposed to a flow of Ar (~250 standard cubic centimetres per minute (sccm)) to remove the oxidizing atmosphere; afterwards, the catalyst reduction was performed at 650°C for 30 min in a hydrogen medium (~150 sccm). The temperature was increased by 6°C/min, up to the desired growth temperature of 950–1100°C, while CH_4 was injected into the reactor. The CH_4 flow was stopped after the temperature had been maintained for 10 min. Finally, the furnace was cooled to 300°C in a flow of hydrogen, and further cooling to room temperature was carried out under a flow of Ar. The as-grown material was subsequently purified to eliminate the amorphous carbon and the catalyst particles by using a two-step method, including a thermal treatment at 450°C in air for 1 h and an acid treatment using hydrochloric acid.

Grafting of gallic acid onto purified carbon nanotubes

The grafting of gallic acid onto synthesized and purified CNTs by ascorbic acid–hydrogen peroxide redox initiator was carried out as follows: in a 25 ml glass tube, 0.25 g of gallic acid and 0.25 g of CNTs were dissolved in 25 ml of H_2O containing 10 mM ascorbic acid and 7 mM hydrogen peroxide. The mixture was maintained at 25°C for 3 h under atmospheric air.

The obtained suspension of gallic acid–CNTs (GA-CNTs) was introduced into dialysis tubes and dipped into a glass vessel containing distilled water at 20°C for 48 h with eight changes of water. Purified gallic acid–CNTs were filtered and then dried at 110°C overnight. GA-CNTs were checked to be free of unreacted gallic acid by HPLC analysis of the washing media according to the method validated in literature.[28]

Two different blank CNTs, acting as controls, were prepared. In a first experiment, the grafting reaction was carried out in the absence of gallic acid (CNTs-1), while in the second the reaction was performed in the absence of the redox initiator pair (CNTs-2).

Ocular tolerance test (HET-CAM test)

Fertilized hen's eggs were obtained from a poultry farm. Three eggs for each formulation, weighing 50–60 g, were selected and candled to discard the defective ones. The eggs were placed in a humidified incubator at a temperature of 37 ± 0.5°C for three days. The trays containing eggs were rotated manually in a gentle manner after every 12 h. On the third day, 3 ml of egg albumin was removed, using a sterile technique, from the pointed end of the egg. The hole was immediately sealed by 70% alcohol-sterilized Parafilm with the help of a heated spatula. The eggs were kept in the equatorial position for the development of chorioallantoic membrane (CAM) away from the shell. The eggs were candled on the fifth day of incubation and nonviable embryos were removed every day thereafter. On the tenth day, a window (2 × 2 cm) was made on the equator of the eggs through which formulations (0.5 ml of a 1.0 mg/ml GA-CNT suspension in water) were instilled directly onto the CAM surface and left in contact for 5 min. The membrane was examined for vascular damage and the time taken for injury to occur was recorded. To afford a semi-quantitative evaluation of the reaction, preliminary tests with reference substances were performed applying three different concentrations of Texapon ASV, sodium hydroxide and acetic acid (0.3 ml; 0.1, 1.0 and 5.0 mM) onto two CAMs, respectively, to distinguish between weak, moderate and severe effects.[34] The scores were recorded according to the scoring schemes as shown in Table 1.

Evaluation of disposable phenolic groups by Folin-Ciocalteu procedure

The amount of total phenolic groups was determined using Folin-Ciocalteu reagent procedure, according to published methods[35] with some modifications.

GA-CNTs (20 mg) were dispersed in distilled water (6 ml) in a volumetric flask. Folin-Ciocalteu reagent (1 ml) was added and the contents of flask were mixed thoroughly. After

Table 1 Scoring chart for hen's egg test–chorioallantoic membrane (HET-CAM) test

Effect	Score	Inference
No visible haemorrhage	0	Non irritant
Just visible membrane discoloration	1	Mild irritant
Structures are covered partially due to membrane discoloration or haemorrhage	2	Moderately irritant
Structures are covered totally due to membrane discoloration or haemorrhage	3	Severe irritant

3 min, 3 ml of Na_2CO_3 (7.5%) were added, and then the mixture was allowed to stand for 2 h with intermittent shaking. The absorbance of filtered mixture was measured at 760 nm against a control prepared using the blank CNTs-1 under the same reaction conditions. The same experiment was performed by using CNTs-2.

The amount of total phenolic groups in CNTs was expressed as gallic acid equivalent concentration by using an equation obtained from a gallic acid calibration curve. This was recorded by employing five different gallic acid standard solutions in water. A 0.5-ml volume of each solution was added to the Folin-Ciocalteu system to produce a final concentration of 8.0, 16.0, 24.0, 32.0 and 40.0 μm, respectively. After 2 h, the absorbance of each solution was measured to construct the calibration curve and determine the correlation coefficient (R^2).

Determination of total antioxidant activity

The total antioxidant activity of the CNT materials was evaluated according to a previously reported method.[36] Briefly, 20 mg of GA-CNTs were mixed with 1.2 ml of reagent solution (0.6 M sulfuric acid, 28 M sodium phosphate and 4 M ammonium molybdate) and 0.3 ml of distilled water. Subsequently, the reaction mixture was incubated at 95°C for 150 min. After cooling to room temperature, the absorbance of the filtered mixture was measured at 695 nm against a control prepared using blank CNTs in the same reaction. The same experiment was performed using CNTs-2.

The total antioxidant activity of the CNT materials was expressed as gallic acid equivalent concentration. By using five different gallic acid standard solutions in water, a calibration curve was constructed. A 0.3-ml volume of each solution was mixed with 1.2 ml of reagent solution to obtain a final concentration of 6.0, 12.0, 24.0, 36.0 and 40.0 μm, respectively. After 150 min incubation, the solutions were analysed by UV–Vis spectrophotometer and the correlation coefficient (R^2), the slope and the intercept of the regression equation obtained by the method of least square were calculated.

Determination of scavenging effect on DPPH radicals

Synthesized GA-CNTs were allowed to react with a stable free radical, 2,2′-diphenyl-1- picrylhydrazyl radical (DPPH), with the aim of evaluating the free radical scavenging properties of the studied materials.[37] In a volumetric flask (25 ml), 50 mg of GA-CNTs were dispersed in an ethanol solution of DPPH (100 μm) and the contents of the flask were mixed thoroughly. The sample was incubated in a water bath at 25°C and, after 60 min, the absorbance of the remaining DPPH was determined colorimetrically at 517 nm after filtration of the GA-CNTs. The same reaction conditions were applied for CNTs-1 and CNTs-2.

The scavenging activity of the tested CNT materials was measured as the decrease in absorbance of the DPPH and it was expressed as percent inhibition of DPPH radicals calculated according to Equation 1:

$$Inhibition\% = \frac{A_0 - A_1}{A_0} \times 100 \quad (1)$$

where A_0 is the absorbance of a standard that was prepared under the same conditions, but without any CNTs, and A_1 is the absorbance of CNT samples.

Scavenging activity on hydroxyl radicals

The scavenging activity on the hydroxyl radical was evaluated according to methods reported in the literature.[38] Briefly, 20 mg of GA-CNTs were dispersed in 0.5 ml of 95% ethanol and incubated with 0.5 ml deoxyribose (3.75 mM), 0.5 ml H_2O_2 (1 mM), 0.5 ml $FeCl_3$(100 mM), 0.5 ml EDTA (100 mM) and 0.5 ml ascorbic acid (100 mM) in 2.0 ml potassium phosphate buffer (20 mM, pH7.4) for 60 min at 37°C.[35] Then samples were filtered and to 1 ml filtrate, 1 ml of TBA (1% w/v) and 1 ml of TCA (2% w/v) were added and the tubes were heated in a boiling water bath for 15 min. The content was cooled and the absorbance of the mixture was read at 535 nm against reagent blank without extract.

The same reaction conditions were applied for CNTs-1 and CNTs-2.

The antioxidant activity was expressed as a percentage of scavenging activity on hydroxyl radical according to Equation 1.

β-Carotene bleaching test

The antioxidant property of synthesized GA-CNTs was evaluated through measurement of the inhibition of peroxidation in linoleic acid by using the β-carotene bleaching test.[39] Briefly, 1 ml of β-carotene solution (0.2 mg/ml in chloroform) was added to 0.02 ml of linoleic acid and 0.2 ml of Tween 20. The mixture was then evaporated at 40°C for 10 min in a rotary evaporator to remove chloroform. After evaporation, the mixture was immediately diluted with 100 ml of distilled water. The water was added slowly to the mixture and agitated vigorously to form an emulsion. The emulsion (5 ml) was transferred to different test tubes containing 50 mg of GA-CNTs dispersed in 0.2 ml of 70% ethanol, and 0.2 ml of 70% ethanol in 5 ml of the above emulsion was used as a control. The tubes were then gently shaken and placed in a water bath at 45°C for 60 min. The absorbance of the filtered samples and of the control was measured at a wavelength of 470 nm against a blank, consisting of an emulsion without β-carotene. The measurement was carried out at the initial time ($t = 0$) and successively at $t = 60$ min. The same reaction conditions were applied for CNTs-1 and CNTs-2.

The antioxidant activity ($A_{ox}A$) was measured in terms of successful bleaching of β-carotene using Equation 2:

$$A_{ox}A = \left(1 - \frac{A_0 - A_{60}}{A_0^o - A_{60}^o}\right) \quad (2)$$

where A_0 and A_0° is the absorbance measured at the initial incubation time for sample and the control, respectively, while A_{60} and A_{60}° is the absorbance measured in the samples and the control, respectively, at $t = 60$ min.

Cholinesterase inhibition assay

Inhibition of AChE was assessed by a modified colorimetric Ellman's method,[40] which is based on the reaction of released thiocoline yielding a coloured product with a chromogenic reagent. *Torpedo californica* (electric eel) AChE (Type-VI-S, EC 3.1.1.7; Sigma) was used, while ATCI was taken as the substrate of the reaction. A 40-μl volume of AChE (0.36 U/ml in buffer, pH 8) and 10 mg of GA-CNTs was added to 2 ml of buffer pH 8 (0.1 mM) and pre-incubated in an ice bath at 4°C for 30 min. Duplicate tubes were treated in the same way with 20 μl of physostigmine (0.1×10^{-3} mol/l) to assess possible interferences of the test substances utilized in the assay and to control any hydrolysis of acetylcholine that might appear not due to enzyme activity. The reaction was started by adding DTNB solution (20 μl of 0.05×10^{-3} mol/l in buffer, pH 7) and ATCI (20 μl 0.018×10^{-3} mol/l in buffer, pH 7) and tubes were placed in a water bath for 20 min at 37°C. The reaction was halted by placing the assay solution tubes in an ice bath and adding physostigmine (20 μl 0.018×10^{-3} mol/l in buffer, pH 7). The hydrolysis of acetylthiocholine was monitored by the formation of the yellow 5-thio-2-nitrobenzoate, recorded by means of a spectrophotometer at 405 nm. The percentage inhibition (%) was calculated by:

$$Inhibition\% = \frac{(A_b - A_{bc}) - (A_s - A_{cs})}{(A_b - A_{bc})} \times 100 \quad (3)$$

where A_b and A_{bc} is the absorbance of blank and blank positive control, respectively, while A_s and A_{sc} are the absorbance of sample and sample positive control, respectively.

The same reaction conditions were applied on CNTs-1 and CNTs-2 and gallic acid standard solutions.

Statistical analyses

For antioxidant and AChE inhibition measurements, five independent experiments were performed and the data were expressed as means (±SD), and analysed using one-way analysis of variance. For the inhibitory experiment, the IC50 value was determined as the concentration of an antioxidant that provides 50% inhibition

Results and Discussion

Synthesis of antioxidant carbon nanotubes

Functionalized CNTs were synthesized by radical grafting of pristine CNTs. The synthesis of CNTs was carried out in a fixed-bed reactor, and after the removal of the amorphous carbon, the grafting process was performed to obtain CNTs with antioxidant properties. As reported in previous work,[41] before the functionalization process, the CNTs were deeply investigated in terms of size and transition metal content. By checking the SEM and TEM analyses, the size of tubes were determined: the length of CNTs varied in the range of 10–30 μm, and they consist of 20–30 graphene walls with outer diameters of 10–70 nm and inner diameters of 5–25 nm. By ICP-MS and ICP-OES, in the as-grown state the Fe-content was found to be 2–7 wt %.

To confer antioxidant properties to the synthesized CNTs, gallic acid was chosen as functional molecule.

As reported, the functionalization process consists of a grafting procedure based on free radical reaction. In literature, the functionalization of CNTs by free radical-induced reactions is reported for the preparation of perfluorooctyl-derivatized CNTs by the addition of perfluorinated alkyl radicals to CNTs.[42] Furthermore, polymer-grafted CNTs have been synthesized through various types of polymerization methods such as atom transfer radical polymerization, reversible addition-fragmentation chain transfer polymerization, radiation-induced polymerization and many others.[43] The reaction mechanism involves disruption of the graphene *p*-bonded electronic structure of the side walls, with the introduction of a radical species.[44]

Phenolic group compatibility with this kind of reaction was also proven: monomers with active functional groups (phenolic groups) as side substituents were used for the preparation of chelating or grafted polymeric systems using free radical initiators. On the other hand, phenolic groups could be directly involved in the polymerization process, as it is reported that the phenolic radical undergoes a dimerization process through reaction between the hydroxyl radical and the aromatic ring.[45]

In our study, the ascorbic acid–H_2O_2 redox pair was employed as an initiator system to avoid the generation of any kind of toxic reaction products. Conventional initiators, including azo compounds and peroxides, require a relatively high reaction temperature to ensure the rapid decomposition of the initiator. In contrast, by employing a redox initiation system it is possible to perform the reactions at a lower temperature, reducing the risk of antioxidant degradation.[46]

After synthesis, GA-CNTs were purified by dialysis to remove the gallic acid that was non-covalently attached to the CNTs.

Two blank batches of CNTs, acting as controls, were prepared. In a first experiment, the grafting reaction was carried out in the absence of gallic acid (CNTs-1) to evaluate the effects of CNTs on antioxidant protocols. The second experiment (CNTs-2) was performed to prove that no gallic acid is absorbed in absence of the redox initiator pair.

Characterization of functionalized carbon nanotubes

GA-CNTs were characterized by SEM, FT-IR and fluorescence analyses to determine the tubes' morphology together with the effective formation of a covalent bond between gallic acid and CNTs. The effective presence of gallic acid was also confirmed by the Folin-Ciocalteu assay, while the biocompatibility of the proposed nanostructure was investigated by HET-CAM test.

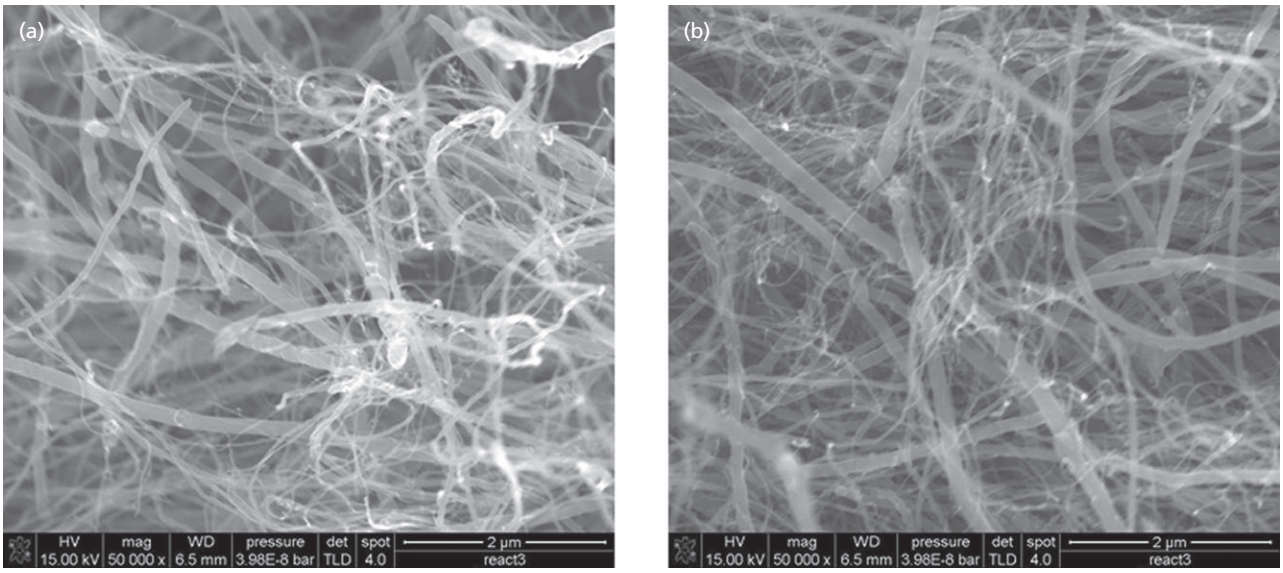

Figure 2 Scanning electron microscopy images showing the morphology of pristine carbon nanotubes (a) and gallic acid–carbon nanotubes (b).

The morphology of the gallic acid-functionalized CNTs was investigated by means of SEM imaging and no differences in the morphology of the nanotubes were observed compared with the pristine CNTs after the gallic acid insertion (Figure 2).

The covalent attachment of gallic acid to the CNTs was confirmed by FT-IR analysis. As can be seen in Figure 3, additional peaks were observed after the grafting of gallic acid on CNTs (trace b). The additional peaks appear at 1614/cm and 1327/cm, respectively, and can be well attributed to C = O stretching C-O stretching modes, thereby confirming the presence of the gallic acid carboxylic functionality on the CNTs' surfaces. In addition, the data exhibit the typical absorption peaks of the aromatic ring at 1546/cm and at 741/cm, which are associated with C = C stretching and C-H bending modes, respectively. Finally, additional peaks in the region of 3284–3502/cm indicate O-H stretching and C-H stretching modes within the aromatic rings. In contrast, no difference was observed by comparing the FT-IR spectra of CNTs-2 and pristine CNTs, showing the absence of gallic acid absorbed on the CNTs-2 surface. Note, that this observation confirms the efficiency of the employed purification procedure.

A further confirmation of the covalent functionalization of CNTs with gallic acid was obtained by fluorescence spectroscopy. Figure 4 displays the emission spectra of the free antioxidant in the solid state as well as of the gallic acid bound to CNTs. The data show a clear bathochromic shift of the emission peak from 384 nm in pure gallic acid to 445 nm in the GA-CNT conjugate. In contrast, no intensity was detected in the latter wavelength range for blank CNTs (data not shown). The red shift implies a change in the electron distribution in the aromatic ring only in the GA-CNTs and hence confirms the covalent linkage of gallic acid to the CNTs in this material.

The amount of disposable phenol group was calculated by the Folin-Ciocalteu assay. This assay is based on a complex redox reaction between phenolic compounds and phosphotungstic and phosphomolybdic acids present in the Folin-Ciocalteu reactant, which is associated with colour development. To be specific, disposable phenolic groups in the samples expressed as mg equivalent of gallic acid amounted to 2.1 ± 0.2 mg/g of dry CNTs, showing the effective functionalization of CNTs with the phenol antioxidant.

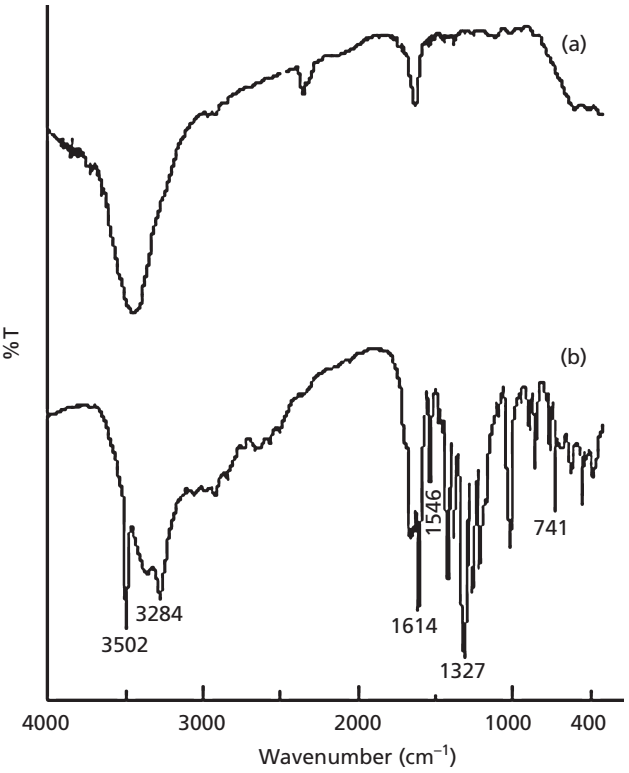

Figure 3 FT-IR spectra of the pristine carbon nanotubes (a) and gallic acid–carbon nanotubes (b).

The same experiment was performed with CNTs-2. In this case, no phenolic equivalent was detected in the sample,

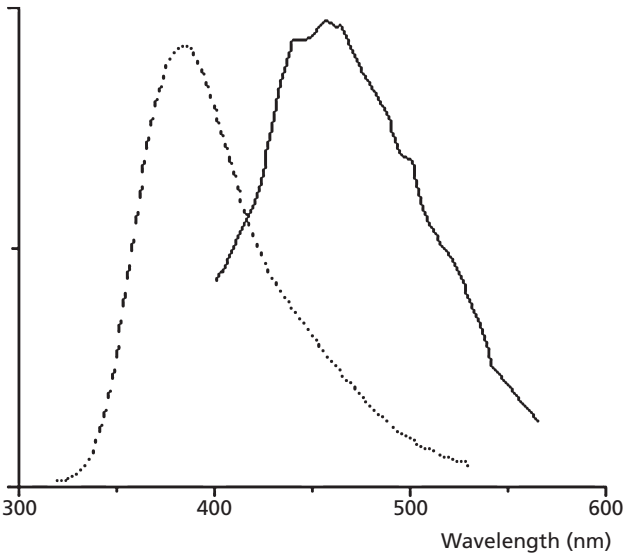

Figure 4 Bathochromic shift of the fluorescence emission spectra of gallic acid from free form (- - -) to gallic acid– conjugate (—).

showing again that no gallic acid was absorbed at the CNTs' surfaces after the dialysis process.

A further characterization was performed to determine the biocompatibility of the sample. As reported by several papers, the main goal of toxicological scientific endeavour is to safeguard human beings against the possible adverse effects of diverse types of chemicals, including pharmaceuticals, cosmetics, household products, industrial chemicals and agrochemicals.[47] No other field in in-vitro toxicology testing has driven academic, industrial and government resources to develop cell modelling systems as much as the need for alternatives to local toxicity testing.[48,49] Among the different in-vitro tests, HET-CAM has emerged as an alternative in-vivo approach for the testing of biomaterials.[50] In this method, the evaluation of biomaterials is performed by using the chorioallantoic membrane (CAM) of a developing chicken embryo that allows continuous visualization of the implant site while providing a rapid, simple and low-cost screening of tissue reactions to biomaterials. The HET-CAM, thus, is a suitable alternative to animal testing (Draize test) and it is based on the direct application of the sample onto the chorioallantoic membrane and reactions, such as haemorrhage, intravasal coagulation or lysis of blood vessels, are assessed on a time-course basis. These irritant effects may occur within 5 min of mucosal administration of sample. The CAM is a non-innervated complete tissue containing arteries, veins and capillaries, and it is technically easy to study. It responds to injury with an inflammatory process similar that observed in the conjunctival tissue of a rabbit's eye. Its well-developed vascularization provides an ideal model for ocular irritation studies.[51]

Regarding the accuracy of the method, in a recent study, a retrospective analysis revealed that the HET-CAM's overall accuracy was 65% and the overall rate of false negatives (FN) and false positives (FP) was 50% and 33%, respectively. The HET-CAM was sufficiently specific (few FPs) for water soluble compounds, but failed to identify nearly all severe irritants within this group. In contrast, it was highly sensitive

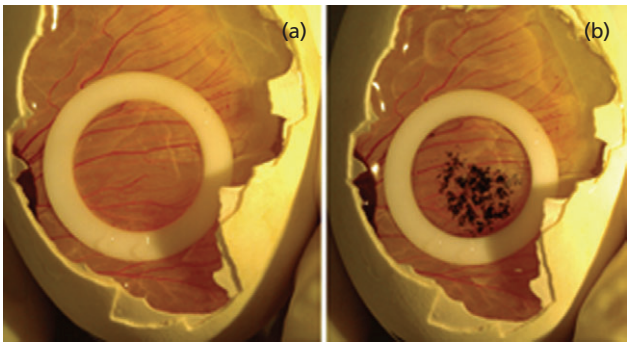

Figure 5 Photo documentation of the gallic acid–carbon nanotube's biocompatibility by hen's egg test–chorioallantoic membrane (HET-CAM) test. Untreated CAM (a) and CAM after the application of gallic acid–carbon nanotubes for 5 min (b).

(no FNs) for non- and oil-soluble substances.[52] Based on these considerations, the biocompatibility of several pharmaceutical nanostructures was assessed by this assay.[53–55]

In a model protocol, to objectify response evaluation, Texapone ASV, sodium hydrochloride and acetic acid at different concentrations were tested in parallel for reference, which enabled differentiation between none, weak, moderate and severe reactions of the possible endpoints haemorrhage, coagulation and vessel lysis. The CAM-morphology is shown in Figure 5a, while Figure 5b presents the CAM-morphology after 5 min treatment with GA-CNTs. By washing with NaCl solution (0.9% w/w), no signs of ocular irritancy have been detected; the resulting CAM morphology is, indeed, the same as that shown in Figure 5a. As a consequence, an irritancy index of zero was assigned to the synthesized CNT conjugate, confirming that the proposed CNT is nonirritant and well tolerated. The test was repeated in triplicate and no difference in the scoring charts were detected.

Determination of total antioxidant activity

This assay is based on a redox reaction between the phenol antioxidant and a molybdate reactive. In particular, there is a reduction of Mo(VI) to Mo(V) by gallic acid and the subsequent formation of a green phosphate/Mo(V) complex at acid pH. The total antioxidant activity was measured and compared with that of gallic acid and of the control, which contained no antioxidant component. We found a higher absorbance in GA-CNTs, while CNTs-1 and CNTs-2 caused no colour development. The high absorbance values indicated that the sample possesses significant antioxidant activity. According to the results, the synthesized material had significant antioxidant activity, and gallic acid equivalent amount of 3.9 ± 0.3 mg/g dry CNTs (Table 2).

The same experiment was performed by using CNTs-2, and no activity was detected.

Determination of the scavenging activity on DPPH radical

The DPPH radical is a stable organic free radical with an absorption maximum band around 515–528 nm and thus, it is a useful reagent for evaluation of the antioxidant activity of compounds. In the DPPH test, the antioxidants reduce the

Table 2 Activity of gallic acid–carbon nanotubes

	Total antioxidant activity (mg/g CNTs)	Inhibition (%)			
		DPPH radicals	Hydroxyl radicals	Peroxyl radicals	Acetycholinesterase
CNTs-1	–	13 ± 3%	18 ± 2%	4 ± 1%	7 ± 1%
CNTs-2	–	7 ± 1%	5 ± 2%	2 ± 1%	9 ± 1%
Gallic acid–carbon nanotubes	3.9 ± 0.3	92 ± 2%	63 ± 1%	98 ± 1%	57 ± 2%

DPPH radical to a yellow-coloured compound, diphenylpicrylhydrazine, and the extent of the reaction depends on the hydrogen-donating ability of the antioxidant. It has been documented that cysteine, glutathione, ascorbic acid, tocopherol and polyhydroxy aromatic compounds (e.g. hydroquinone and in particular gallic acid) reduce and decolorize DPPH by their hydrogen-donating capabilities.

CNT's scavenger ability was evaluated in terms of DPPH reducing power and expressed as inhibition (%). GA-CNTs showed high scavenging activity and blank CNTs (both CNTs-1 and CNTs-2) did not significantly interfere with the scavenger process. The inhibition (%) values, indeed, were lower than 15% in both cases (Table 2).

Determination of the scavenging activity on hydroxyl radical

Hydroxyl radicals exhibit a very high reactivity and tend to react with a wide range of molecules found in living cells. Due to their high reactivity, the radicals have a very short biological half-life. Thus, an effective scavenger must be present at a very high concentration or must possess very high reactivity towards these radicals. Although hydroxyl radical formation can occur in several ways, by far the most important mechanism *in vivo* is the Fenton reaction, where a transition metal is involved as a prooxidant in the catalysed decomposition of superoxide and hydrogen peroxide. These radicals are intermediary products of cellular respiration, phagocytic outburst and purine metabolism. Hydroxyl radicals can be generated *in situ* by decomposition of hydrogen peroxide by the high redox potential EDTA–Fe^{2+} complex. In the presence of deoxyribose substrate it forms TBARS, which can be quantitatively determined. Antioxidant activity is associated with decrease in the formation of TBARS, which can arise from donation of hydrogen or electrons from the antioxidant to the radical or by direct reaction with it. Consequently, the ability of GA-CNTs to scavenge hydroxyl radicals was evaluated by the Fenton-mediated deoxyribose assay. The obtained results reported in Table 2 imply good antioxidant activity, which shows the efficiency of the proposed CNT system to protect against the radical damage induced by hydroxyl radical. Both CNTs-1 and CNTs-2 showed no relevant scavenging activity, with inhibition (%) values lower than 20%.

Determination of the inhibition of lipid peroxidation

Linoleic acid, an unsaturated fatty acid, is usually used as a model compound in lipid oxidation and antioxidation-related assays in which carbon-centred peroxyl radicals and hydroperoxides, etc., are involved in the oxidation process. During the linoleic acid oxidation, peroxides are formed. Peroxidation of fatty acids can cause deleterious effects in foods by forming a complex mixture of secondary breakdown products of lipid peroxides. Further intake of these foods can cause a number of adverse effects including toxicity to mammalian cells. Lipid peroxidation is thought to proceed via radical-mediated abstraction of hydrogen atoms from methylene carbons in polyunsaturated fatty acids.[56]

The ability of synthesized antioxidant CNTs to inhibit lipid peroxidation was evaluated by means of the β-carotene bleaching test. Inhibition of the breakdown of lipid hydroperoxides to unwanted volatile products enables the determination of secondary antioxidants in related mechanisms. In the absence of antioxidants, oxidation products of linoleic acid simultaneously attack β-carotene, resulting in bleaching of its characteristic yellow colour in ethanolic solution. In the presence of the total extracts, oxidation products were scavenged and bleaching was prevented. The obtained results are reported in Table 2, and the good inhibition values clearly show the protection by GA-CNTs against lipid peroxidation.

Blank CNTs (both CNTs-1 and CNTs-2) did not significantly interfere with the scavenger process, the inhibition (%) values are, indeed, lower than 5%.

Cholinesterase inhibition assay

In the past few years, there has been a renewed interest in studying and quantifying the antioxidant constituents of fruits and vegetables for their potential health functionality against various diseases such as diabetes, cancer, cardiovascular disorders and neurodegenerative diseases such as Alzheimer's disease. In all these cases, the inhibition of specific enzymatic pathways by plants polyphenols is involved.[57] For this reason, specific enzymatic tests were performed on the GA-CNT conjugate to prove the beneficial effects in some neurological pathologies such as Alzheimer's disease. AChE plays an important role in the central nervous system. It catalyzes the cleavage of acetylcholine in the synaptic cleft after depolarization. Inhibitors of AChE, such as galanthamine, are used frequently in the pharmacotherapy of Alzheimer's disease: AChE is, indeed, dramatically down-regulated in the brains of patients suffering from this disease. Since there is a large amount of evidence demonstrating that oxidative stress is intimately involved in age-related neurodegenerative diseases, there have been a great number of studies that have examined the positive benefits of antioxidants to reduce or to block neuronal death occurring in the pathophysiology of these disorders.[58] The GA-CNT conjugate was tested as an AchE-inhibiting agent and good activity was found, with an inhibition value of around 60% (Table 2). The IC50 value was found to be 2.2 ± 0.9 mg/ml (4.6 μg equivalents of gallic acid considering the results of the Folin-Ciocalteu experiment),

while in control experiments the IC50 value of free gallic acid was found to be 3.1 ± 0.7 μg/ml. By comparing these data, it is clear that the enzymatic activity of gallic acid was not significantly reduced after the conjugation with the CNTs.

Uncojugated CNTs (CNTs-1 and CNTs-2) were found to have little activity on AChE (inhibition values lower than 10%) confirming that the inhibition is related to the gallic acid moiety.

These results clearly prove that a nanomaterial could have beneficial effects in patients affected by neurological pathologies such as Alzheimer's disease; this is of particular interest because one of the key application field of CNTs is their use as scaffolds for regenerative interventions of the central nervous system sites as previously mentioned.[14]

Conclusions

In this paper, for the first time, a free radical grafting procedure was proposed as a synthetic approach for the covalent functionalization of CNTs with a common antioxidant polyphenol (gallic acid). The effective covalent functionalization of CNTs was proved by instrumental analyses, while specific tests were performed to prove that the GA-CNT conjugate retains the biological properties of the polyphenol. In particular, antioxidant and enzymatic tests (DPPH and hydroxyl radical scavenging activity, inhibition of linoleic acid peroxidation, total antioxidant activity, AChE inhibition) imply the reducing power, the scavenging effect and the AChE inhibitory activity of the conjugate. Importantly, the HET-CAM test to evaluate biocompatibility indicated that GA-CNTs are nonirritant and well tolerated within the biological environment. The results clearly demonstrate the in-vitro properties of GA-CNTs in selected tests that are consistent with biocompatibility, which enhances its applicability in biomedical and pharmaceutical fields.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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