Functionalized Nanotubes

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Targeted Delivery of Amphotericin B to Cells by Using Functionalized Carbon Nanotubes**

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Functionalized carbon nanotubes (f-CNTs) are attracting increasing attention as new vectors for the delivery of therapeutic molecules.[1–5] In fact, carbon nanotubes (CNTs) have been shown to cross cell membranes easily and to deliver peptides, proteins, and nucleic acids into cells.[6–14] These innovative carriers present a lower toxicity, a fact that boosts their potential for biomedical applications.[1–3,15]

The use of f-CNTs for drug delivery of small molecules (e.g. anticancer, antibacterial, or antiviral agents) is still unexplored. The development of nanovectors able to carry one or more therapeutic agents with recognition capacity, optical signals for imaging, and/or specific targeting is of fundamental advantage, for example, in the treatment of cancer and/or different types of infections.[16] Theoretically, the use of f-CNTs in this approach would require the introduction of different functionalities on the external surface of the CNTs. Multiple functionalization of the tips of CNTs has been reported recently.[17] Although the method is original and interesting, it does not exploit the full surface available on the CNTs for the linking of different molecules, nor has an application for drug delivery been envisaged. We decided to explore an alternative strategy for the introduction of two different and orthogonal functionalizations to CNTs. The orthogonal methodology, which is widely used in organic synthesis, would allow the selection and control of the attachment of active molecules to the sidewalls and tips of the CNTs. This approach enabled us to simultaneously link fluorescent probes to the CNTs for tracking the uptake of material as well as an antibiotic moiety as the active molecule. For this purpose we chose fluorescein and amphotericin B (AmB), respectively. AmB is considered to be the most effective antibiotic in the treatment of chronic fungal infections.[18–20] However, the drug is highly toxic to mammalian cells;[18] one reason for this toxicity can be attributed to the formation of aggregates as a result of the lower solubility of AmB in water.[19] Conjugation of this drug to CNTs could have several advantages: 1) increased solubility of the molecule; 2) decrease in the aggregation phenomena; 3) improved efficacy owing to the internalization capacity of the CNTs; and 4) modulation of the antibiotic activity against different types of cells (mammalian, bacterial, and fungal). Herein we present the first case of AmB covalently linked to a polymeric carrier. Previously, the drug was encapsulated into colloidal or lipid systems owing to the need for slow release;[21] however, high doses are required to elicit the same efficacy as that of AmB alone. The incorporation of CNTs could allow a reduction in the amount of AmB administered. It is easy to control their dimensions and the degree of functionalization. CNTs are particularly promising delivery systems as they are non-immunogenic. [3,10]

The aim of the work described herein was first to explore a new strategy for the double functionalization of CNTs, second, to assess the characteristics of toxicity and uptake of CNTs functionalized with AmB and fluorescein towards mammalian cells, and thirdly, to evaluate the antifungal activity of CNT–AmB conjugates.

It is known that the exposure of CNTs to oxidative conditions (for example, sonication in a mixture of sulfuric and nitric acids) not only cuts the tubes and generates surface defects but can also provide abundant carboxylated sites along their sidewalls.[22,23] To establish the effect of acidic

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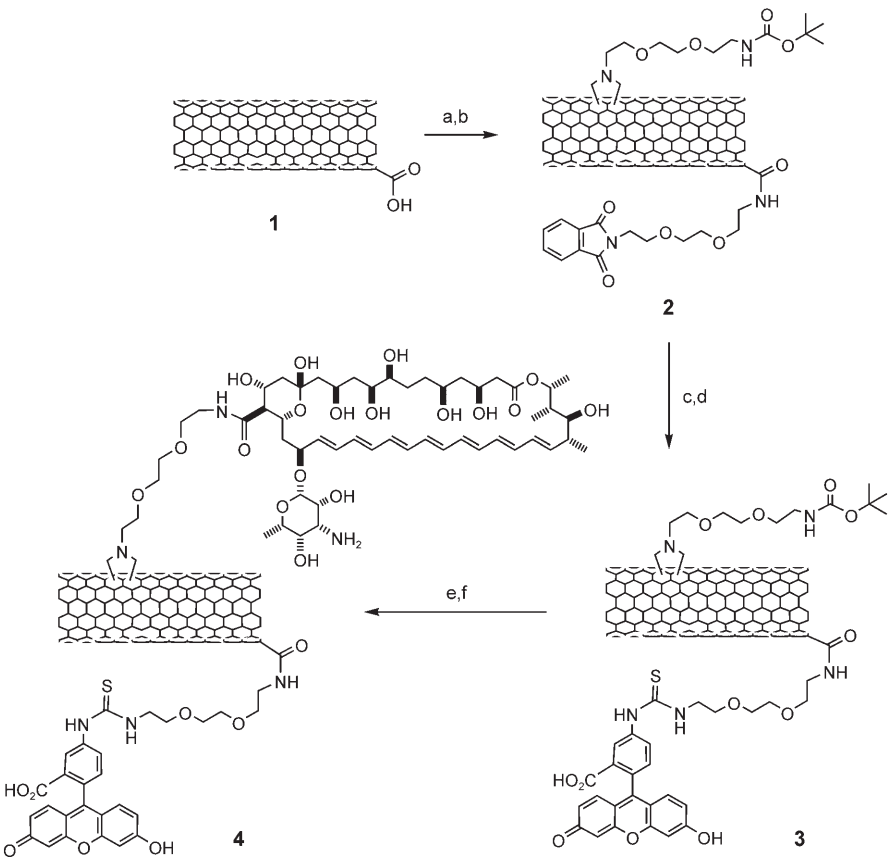
treatment on both the length of the CNTs and the number of carboxylic groups present, we prepared a series of oxidized multiwalled carbon nanotubes (MWNTs) by applying different acid conditions (Supporting Information).[24,25] The CNT-length distribution was assessed by TEM (Table 1), whereas the loadings were calculated after derivatization of the carboxylic groups (activated as acid chlorides) with Boc mono-protected diaminotriethylene glycol. After removal of the Boc group, the number of free amino groups was measured with a quantitative Kaiser test (Table 1).[9]

Evidently, the length and the loading of the MWNTs strongly depend on the duration of the acid treatment. As expected, the tube length decreases and loading increases with an increase in the duration of oxidation. For our purpose, we selected MWNTs treated for 8 h as they exhibited the most convenient length and loading.

To effect a double, orthogonal functionalization of the CNTs, the oxidized MWNTs **1** were again activated as the acid chlorides and treated with diaminotriethylene glycol (mono protected as phthalimide) (Scheme 1). This protecting group is particularly useful as it is highly stable to harsh acidic conditions and orthogonal to the Boc group, which can subsequently be introduced through 1,3-dipolar cycloaddition to the sidewalls of the MWNTs.[26,27]

MWNTs **2** were structurally characterized by ^{1}H NMR spectroscopy and TEM (Figure 1a). The ^{1}H NMR spectrum of **2** in $CDCl_3$ showed the presence of the Boc group ($\delta = 1.4$ ppm), whereas the phthalimide group gave rise to the signals at $\delta = 7.7$ and 7.8 ppm (Supporting Information). TEM analysis indicated that the lengths of individual MWNTs **2** are consistent with the data reported in Table 1. The stepwise cleavage of phthalimide, followed by removal of the Boc group gave water-soluble MWNTs, whose quantitative Kaiser test showed amino levels of 0.25 and 0.46 mmol per gram of material, respectively. Having ascertained the selective removal of the two protecting groups, we were ready to insert the desired functionalities. We initially removed the phthalimide group in MWNTs **2** with a solution of hydrazine in ethanol, and the free amino group was then coupled with FITC. The Boc group was then cleaved through the use of HCl (4 M) in dioxane, and AmB (activated at the carboxylic functional group by using HOBT and carbodiimide) was covalently linked to the amino group (Scheme 1). To avoid possible side reactions during the coupling, the NH_2 group of the mycosamine moiety of AmB was protected with Fmoc, which was eventually removed with piperidine in DMF (25%) to afford MWNTs **4**. TEM analysis of the conjugate **4** showed a morphology very similar to that of MWNTs **2** (Figure 1b).

The UV/Vis spectrum of MWNTs **4** in DMF exhibit the typical absorption bands of AmB in the range 340–420 nm



Scheme 1. a) Neat $(COCl)_2$; Pht-N$(CH_2CH_2O)_2$-CH_2CH_2-NH_2, dry THF, reflux; b) Boc-NH-$(CH_2CH_2O)_2$-CH_2CH_2-$NHCH_2COOH/(CH_2O)_n$, DMF, 125 °C; c) Hydrated NH_2-NH_2, EtOH, reflux; d) FITC, DMF; e) HCl 4 M in dioxane; f) Fmoc-AmB, HOBt/EDC × HCl/DIPEA, DMF; 25% piperidine in DMF. BOC = *tert*-butyloxycarbonyl; DIPEA = diisopropylethylamine; DMF = dimethyl formamide; EDC = N-Ethyl-N′-(3-dimethylaminopropyl)-carbodiimide; FMOC = fluorenylmethyloxycarbonyl; FITC = fluorescein isothiocyanate; HOBt = 1-hydroxybenzotriazole; Pht = phthalimide group.

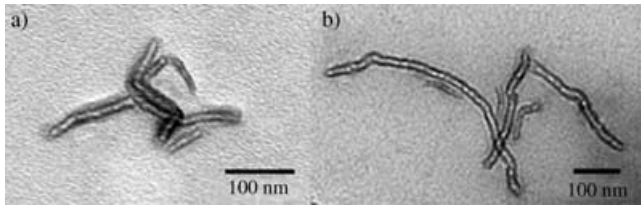


Figure 1. TEM images of MWNTs **2** (a) and **4** (b).

Table 1: Chemico physical properties of MWNTs after treatment with strong acid and derivatization of the carboxylic acid functions.

t [h]	Length [nm]	Loading [mmol g^{-1}]
1	1500–4000	0.06
3	1000–2000	0.14
5	200–1000	0.16
8	180–940	0.22
24	160–600	0.26
48	140–500	0.34

and those of FITC at 517 nm (Figure 2). Upon subtraction of the contribution of CNTs to the UV/Vis spectrum of **4**, the ratio between AmB and FITC attached to the tubes was 1.5:1. A similar result was found for the conjugate dissolved in methanol (Supporting Information).

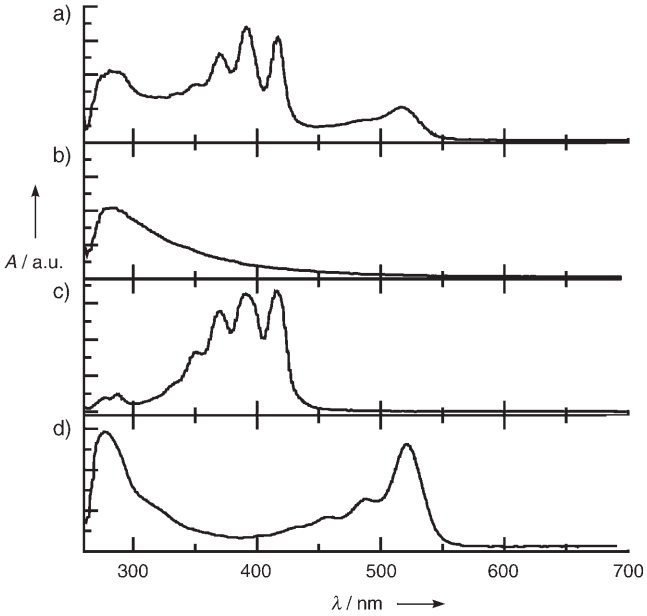


Figure 2. UV/Vis spectra of a) MWNTs **4**, b) **2**, c) AmB, d) FITC in DMF.

To assess the biological properties of the novel, doubly functionalized CNTs, we initially studied the toxicity effects of MWNTs **4** on mammalian cells, tracing its capacity to cross the cell membrane. Human Jurkat lymphoma T cells were incubated with either MWNTs **4** or AmB as the control. The cells were grown at 37 °C in RPMI medium and treated for 1 h with doses of MWNTs **4** increasing from 1 to 40 $\mu g\,mL^{-1}$. Cell viability was analyzed by flow cytometry by staining early and late apoptosis and necrosis with annexin V and propidium iodide (Figure 3). As a positive control, the cells were treated with AmB (10 $\mu g\,mL^{-1}$) alone. The dose of AmB used for the control experiment corresponded to the amount of drug covalently bound to 40 $\mu g\,mL^{-1}$ of MWNTs **4**. The conjugation of AmB to CNTs clearly reduces the toxic effects of the antibiotic on mammalian cells. At the highest doses, more than 40 % of the cells died in the presence of AmB, whereas all the cells remained alive upon treatment with MWNTs **4**. We subsequently verified that longer incubation times do not increase the percentage of dead cells. Indeed, all the Jurkat cells remained alive after treatment with MWNTs **4** for 4 and 16 h (Supporting Information). Furthermore, cell uptake of MWNTs **4** was very fast as maximum fluorescence was observed after only 1 h of incubation. This result is in accordance with the behavior of single-walled CNTs previously used as transporters for peptides and proteins.[6–8]

Attachment to CNTs modified the internalization properties of AmB. Jurkat cells incubated with MWNTs **4** at different doses and time points were analyzed by using epifluorescence and confocal microscopy. We found that the

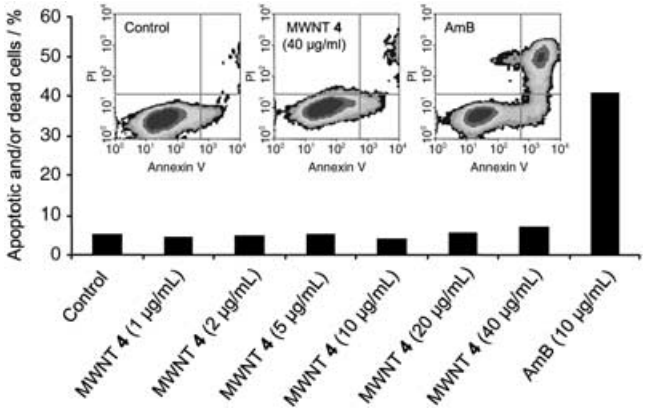


Figure 3. Percentage of early-apotopic, late-apoptotic, and/or dead Jurkat cells after treatment with MWNTs **4** at different concentrations for 1 h. After incubation and washings, the cells were stained with annexin V and propidium iodide and analyzed by flow cytometry (inset).

internalization of AmB linked to the nanotubes was dose-dependent (Supporting Information). Figure 4 clearly shows that the conjugates pass into the cell cytoplasm. Notably, the MWNTs **4** is mainly localized around the nuclear membrane, but does not cross this barrier.

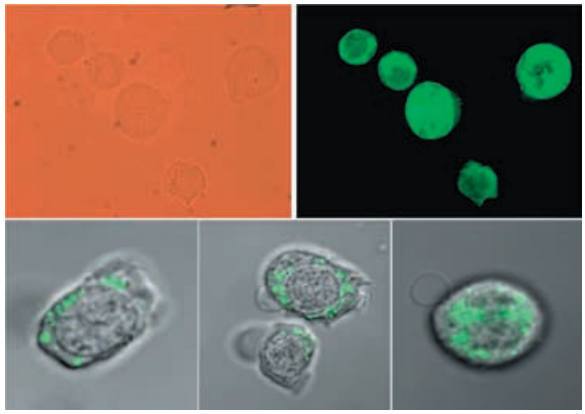


Figure 4. Epifluorescence (top) and confocal (bottom) microscopy images of Jurkat cells incubated for 1 h at 37 °C with 10 and 40 $\mu g\,mL^{-1}$ of MWNTs **4**, respectively. Jurkat cells have an average diameter of 10 μm.

The mechanism of penetration is not mediated by endocytosis because incubation in the presence of NaN_3 at 4 °C does not remarkably influence the penetration capacity of MWNTs **4** observed at 37 °C (Supporting Information).[7] We demonstrated that f-CNTs are able to enter the cell by a spontaneous mechanism: they behave like nanoneedles and pass through the cell membrane without causing cell death.[11] This mechanism was recently confirmed by Cai et al., who showed that the nanopenetration of cell membranes seems to be a unique feature of CNTs.[28] Concerning the toxic effects of AmB on mammalian cells, it is thought that this antibiotic destabilizes the cell membrane in a manner similar to the cases of fungi and yeasts.[20] Therefore, the ability of CNTs to internalize AmB rapidly into the cytoplasm of Jurkat cells

remarkably reduces the possibility of disruption of the membrane core. The covalent attachment of AmB to the nanotubes has another clear advantage: it prevents the aggregation phenomena that the drug typically displays in solution. AmB strongly self-associates in aqueous solution and increases the possibility of toxic effects to the cells. In UV/Vis spectra of MWNTs **4** in water and RPMI, we found that the aggregation is lower than that of AmB alone (Supporting Information).[19,29]

Finally, we evaluated the antifungal activity of CNTs, functionalized with AmB, against three species of fungi that are either pathogenic or may opportunistically infect humans. These included collection strains (*Candida parapsilosis* ATCC 90118 and *Cryptococcus neoformans* ATCC 90112) and clinical isolates (*Candida albicans*). In these experiments we used AmB that was covalently linked to ammonium-functionalized multi- and single-walled carbon nanotubes (MWNT-AmB **18** and SWNT-AmB **19**) (Supporting Information). We explicitly chose these types of conjugates to compare the antifungal activity of AmB bound to CNTs directly with that of native AmB, in the absence of the fluorescent probe. The minimum inhibitory concentration (MIC) values were determined after 48 h of incubation with different doses of free AmB, unconjugated CNTs, or CNT–AmB conjugates. As shown in Table 2, ammonium-functionalized CNTs, free of AmB, were inactive up to the maximal concentration (80 $\mu g\,mL^{-1}$) used against all the microorganisms tested. Conversely, MWNT– and SWNT–AmB were highly effective, indicating that the activity of the drug was not prevented by its covalent binding to both single- and multiwalled CNTs. Interestingly, when equal amounts of free and bound drug are considered, (the actual amount of AmB bound to CNTs is approximately one third by weight), conjugated AmB is definitely more potent than the free drug, particularly against the *Candida* spp. The reason for this increased activity is at present unclear, although an increased solubility of the drug by conjugation to CNTs might explain, at least in part, this finding. Alternatively, or supplementary to an increased solubility, binding to CNTs and the presence of multiple copies of AmB per CNT molecule might favor the interaction of the drug with its target, the fungal membrane. Although further investigations are necessary in this respect, the in vitro results are very promising as they indicate that appropriate conjugation can increase the effectiveness of AmB while decreasing its toxicity, as shown by the flow cytometric experiments with Jurkat cells (Figure 3). Such a development would increase the clinical use of AmB, which is, at present, limited by its narrow therapeutic index.[30]

Table 2: Antifungal activity of CNT–AmB conjugates.

CNT	Minimum inhibitory concentration (MIC)[a] [$\mu g\,mL^{-1}$]		
	C. parapsilosis ATCC 90118	*C. albicans* (c.i.)[b]	*C. neoformans* ATCC 90112
AmB	20	>80	5
SWNT–NH_3^+	>80	>80	>80
MWNT–AmB[c] **18**	1.6	6.4	0.8
SWNT–AmB[c] **19**	1.6	13.8	0.8

[a] The MIC corresponds to the lowest concentration of compound that inhibited visible growth of the organism. Results given are mean values of two independent determinations performed in duplicate. [b] c.i., clinical isolate. [c] In this table, the MIC values for MWNT–AmB and SWNT–AmB refer to the amount of AmB in the conjugates (approximately one third by weight).

In summary, we were successful in preparing CNTs containing both fluorescein and amphotericin B. Our studies revealed that AmB covalently linked to CNTs is taken up by mammalian cells without presenting any specific toxic effect. Furthermore, AmB bound to CNTs preserves its high antifungal activity. Therefore, multifunctionalized CNTs can be envisaged for the delivery of antibiotics to different types of cells by selective transport through the membrane. Finally, the covalent linkage of different drugs to CNTs is an approach that may be used to modulate the therapeutic action of the agent, thus obtaining new conjugates with interesting properties.

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