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Drug Delivery |*Hot Paper*|

Controlled Chemical Derivatisation of Carbon Nanotubes with Imaging, Targeting, and Therapeutic Capabilities

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Abstract: In drug delivery, carbon nanotubes (CNTs) hold a great potential as carriers because of their ability to easily cross biological barriers and be internalised into cells. Their high aspect ratio allows multi-functionalisation and their development as a multimodal platform for targeted therapy. In this article, we report the controlled covalent derivatisation of triple-functionalised CNTs with the anticancer drug gemcitabine, folic acid as a targeting ligand and fluorescein as a probe. The anticancer activity of gemcitabine was maintained after covalent grafting onto the CNTs. The functionalised nanotubes were internalised into both folate-positive and negative cells, suggesting the passive diffusion of CNTs. Overall, our approach is versatile and offers a precise chemical control of the sidewall functionalisation of CNTs and the possibility to manoeuvre the types of functionalities required on the nanotubes for a multimodal therapeutic strategy.

Introduction

Carbon nanotubes (CNTs) constitute one of the most outstanding discoveries in the quest for new materials in the last decades.[1] More than ten years ago CNTs were proposed as a new drug delivery system.[2] Their tubular shape and mechanical flexibility allow them to easily cross biological barriers and be internalised into cells independently of the cell type and the functional groups at their surface.[3] Additionally, their high specific surface area per unit weight offers great advantages over existing carriers because it provides not only multiple attachment sites for drug targeting, but also the possibility to complex nucleic acids for gene silencing and transfer.[4] The pioneering biomedical studies on CNTs by our groups opened infinite opportunities for applications in nanomedicine.[2a,5]

Since then, CNTs have attracted considerable interest in this regard and offer a promising tool for a variety of clinical applications.[6] For instance, CNTs possess intrinsic electrical and optical properties that can be exploited for biosensing and for different imaging modalities,[7] including NIR fluorescence[8] and Raman imaging,[9] photoacoustic tomography,[10] and echography.[11] Multimodal drug delivery systems hold great promise for improving cancer therapy outcomes.[12] Nonetheless, imparting cellular specificity to the carriers and improving their stability remain critical challenges. In this context, CNTs have been widely used to develop innovative cancer treatments based on chemotherapy,[6a] gene delivery,[4b,c] and/or photothermal therapy.[13]

Chemotherapy is still a gold standard in current clinical studies; however, patients continue to suffer from the lack of its selectivity, leading to severe side effects and importantly low therapeutic efficacy of the treatment. This issue can be overcome by the conjugation of a targeting ligand to the carriers specifically directed to cancer cells only.[14] One advantage of CNTs is their high aspect ratio that allows multi-functionalisation with different molecules that have specific properties or functionalities.[15] In this context, a few years ago we developed an original one-pot process for the triple functionalisation of pristine and oxidised single- and multi-walled CNTs (SWCNTs and MWCNTs).[16] This straightforward approach was based on the simultaneous reaction of three different diazonium salts generated in situ from the corresponding anilines. Therefore, in a single step it was possible to prepare CNTs functionalised with three different amines blocked by three different quasi-orthogonal protecting groups: *tert*-butylcarbamate (Boc), phthalimide (Pht) and benzylcarbamate (Z). Each of the protecting groups can be subsequently removed using specific conditions without inducing the cleavage of the other groups.

This strategy was applied for the controlled covalent derivatisation of triple-functionalised nanotubes with the FDA-approved anticancer drug gemcitabine (Gem), folic acid (FA) as

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targeting ligand and fluorescein isothiocyanate (FITC) as probe for tracking the CNT conjugates. Folic acid receptors are overexpressed on the cell surface of many types of human cancers.[17] FA has been extensively used as ligand to specifically target cancer cells.[18] Gemcitabine, as is the case with many anticancer drugs, has severe side effects, such as bone marrow depression and gastrointestinal disturbances, poor diffusion through biological barriers, low selectivity towards tumour tissues and rapid metabolism in less than 2 h. Therefore, there is a need to develop carriers for the delivery of Gem combined with a targeting ligand.[19]

The triple-functionalised CNTs described in this work were characterised by transmission electron microscopy (TEM), thermogravimetric analysis (TGA), X-ray photoelectron spectroscopy (XPS) and surface plasmon resonance (SPR). The therapeutic efficiency of the triple-functionalised CNTs was assessed in vitro on folate-positive and folate-negative cancer cells. Interestingly, we have maintained the anticancer activity of Gem after covalent grafting onto CNTs. Our approach offers precise chemical control of the nanotube surface functionalisation, allowing the decoration of their external wall with appropriate molecules. This offers also the possibility to manoeuvre the types of functionalities required on CNTs for a multimodal therapeutic strategy.

Results and Discussion

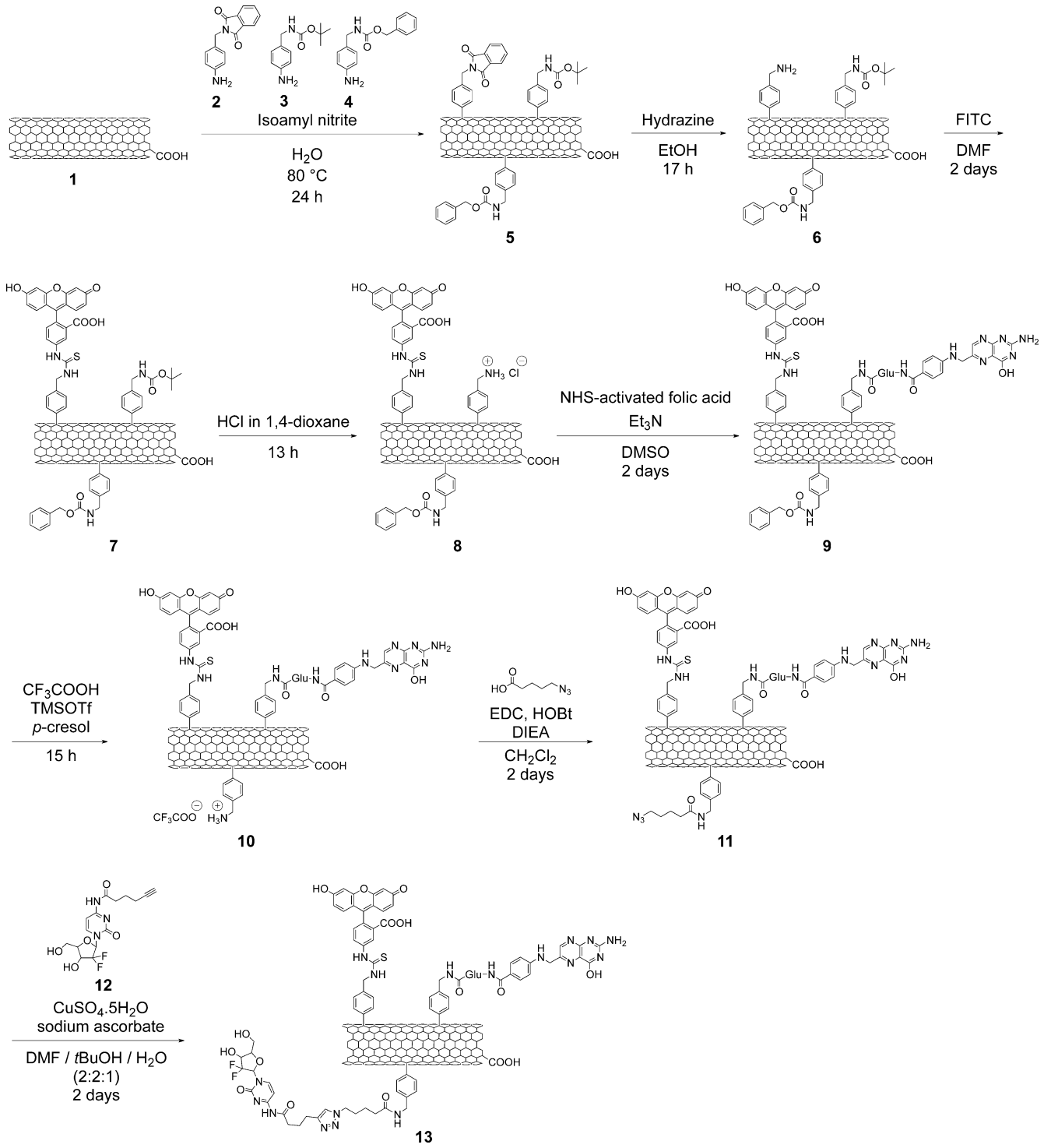
Pristine purified MWCNTs with an average diameter of 9.5 nm were provided by Nanocyl (Belgium). The MWCNTs were first oxidised by a mixture of sulfuric and nitric acid under sonication.[20] This treatment was used to shorten the CNTs from 1.5 μm to approximately 370 nm in average. Concomitantly, oxygenated functions, mainly carboxylic acids, were generated, leading to an enhanced aqueous dispersibility. The triple-functionalised MWCNTs (*f*-MWCNTs) **5** bearing protected amines were prepared according to our previous strategy (Scheme 1).[16]

The arylation reaction was performed by using the three aniline derivatives **2**–**4** bearing a primary amine function blocked by Pht, Boc and Z, respectively. The reaction was carried out in water at 80 °C to reach high levels of functionalisation.[16] The Pht protecting group was first removed using hydrazine. The Kaiser test was used to assess the amine loading, which was 410 μmol of amino groups per gram of CNTs **6** (Table 1).[16] FITC reacted spontaneously with the amine functions on the surface of *f*-MWCNTs **6** in 89% yield, according to the Kaiser test. Indeed, after the reaction, the amount of free amines drastically decreased (45 $\mu mol\,g^{-1}$), resulting in a loading of FITC corresponding to 365 μmol per gram of *f*-MWCNTs **7**. The remaining amines are not accessible for derivatisation and therefore should not interfere with the subsequent derivatisation steps because of their low reactivity. The Boc protecting group was then cleaved using HCl in 1,4-dioxane. The Kaiser test indicated that the amount of ammonium groups was 320 $\mu mol\,g^{-1}$. In parallel, we verified that fluorescein was not degraded in these acidic conditions. For this purpose, we prepared a model compound **S1** by treating benzylamine with FITC (see the Supporting Information, Scheme S1). The FITC derivative was treated in the conditions used to remove the Boc group. NMR spectroscopy confirmed that no degradation of FITC occurred (data not shown).

To endow CNTs with tumour-targeting capabilities, the ammonium moieties on the sidewall of *f*-MWCNTs **8** were derivatised with FA through its activation with *N*-hydroxysuccinimide (NHS). Again, the Kaiser test confirmed that the ammonium functions were efficiently derivatised (63% yield). The estimated amount of FA onto *f*-MWCNTs **9** was 200 $\mu mol\,g^{-1}$. The last protecting group (Z) on *f*-MWCNTs **9** was removed using a mixture of trifluoroacetic acid (TFA), trimethylsilyl trifluoromethanesulfonate (TMSOTf) and *p*-cresol.[16,21] The amount of ammonium groups assessed by the Kaiser test was 275 $\mu mol\,g^{-1}$. At this stage, we checked that both FITC and FA were stable in the conditions used to cleave the Z group. We synthesised the model compound **S2** by derivatising benzylamine with FA pre-activated by NHS (see the Supporting Information, Scheme S2). Both model compounds **S1** and **S2** were not degraded in the conditions used to remove the Z group according to NMR analysis.

The last step was the grafting of Gem onto *f*-MWCNTs **10**. This molecule has three reactive functions corresponding to an aromatic amine, a primary alcohol and a secondary alcohol (Scheme 2). We decided to link Gem to the CNTs through derivatisation of the amine function as it is known that the drug is metabolised in vivo by deamination, leading to its inactivation.[22] Thereby, this strategy should increase the stability of the drug. We investigated different approaches for the selective derivatisation of the amine moiety of Gem. We attempted to selectively derivatise the amine with 5-hexynoic acid. For this purpose, we tried to protect both hydroxyl groups with di-*tert*-butyl dicarbonate under controlled conditions by following a protocol reported in the literature (see the Supporting Information, Scheme S3).[23] Then, amidation of the amine function of Gem with 5-hexynoic acid and subsequent deprotection of the hydroxyl groups would have led to compound **12**. However, this selective protection/deprotection strategy was not successful as a mixture of monoprotected and diprotected **S3** Gem derivatives with a small quantity of triprotected molecule was obtained. The purification of this mixture was difficult, and consequently we changed the strategy. As Gem is a nucleoside analogue, we used a method developed for *N*-acylation of adenosine and cytidine nucleosides through transient protection of the hydroxyl functions with trimethylsilyl chloride (TMSCl).[22a,24] For this purpose, Gem was treated with excess TMSCl at 0 °C to protect the hydroxyl groups as *O*-silyl ethers, followed by treatment with 5-hexynoic acid pre-activated by *N*,*N*′-dicyclohexylcarbodiimide (DCC), at 60 °C for two days (Scheme 2). The hydroxyl groups were subsequently deprotected by treatment in ethanol at 45 °C to give *N*-acylated Gem **12** in 47% yield.

The triple bond of Gem derivative **12** allowed the selective ligation to CNTs by Cu^{I}-catalysed azide–alkyne Huisgen 1,3-dipolar cycloaddition (CuAAC), one of the most popular reactions in the "click chemistry" approach.[25] This reaction is highly selective, efficient and is usually performed in mild con-

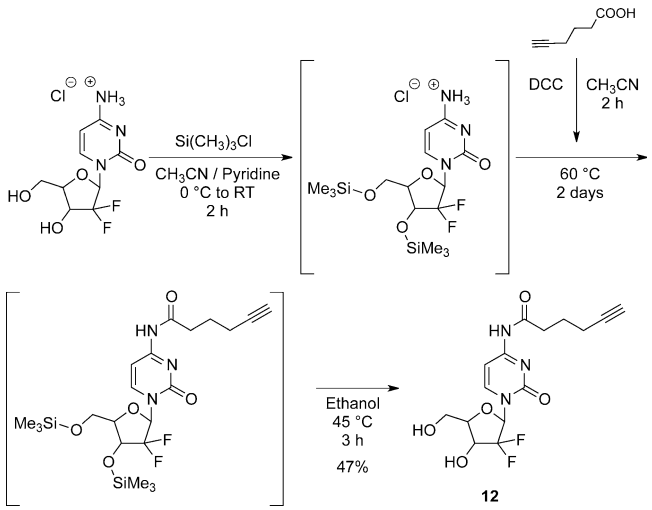


Scheme 1. Triple derivatisation of MWCNTs with FITC, FA and Gem. The remaining free amino groups are omitted in the structure of conjugates **7**, **9**, **11** and **13** for clarity reasons. They did not react most likely because of steric hindrance. EDC = *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide; HOBt = 1-hydroxybenzotriazole; DIEA = *N*,*N*-diisopropylethylamine.

ditions.[26] We optimised the conditions for the CuAAC "click" reaction on the model compound **S4** prepared by amidation between benzylamine and 5-azidopentanoic acid (see the Supporting Information, Scheme S4). The expected 1,2,3-triazole **S5** was obtained in 47% yield by using a catalytic amount of copper(II) sulfate and sodium ascorbate. Before performing the "click" reaction on the nanotubes, the amino groups of *f*-MWCNTs **10** were coupled with 5-azidopentanoic acid in the presence of *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) and 1-hydroxybenzotriazole (HOBt) as activating reagents (Scheme 1). The Kaiser test confirmed the efficiency of the reaction as 80% of the amine functions were

Table 1. Yields and loading of amine functions and molecules of interest for the different *f*-MWCNTs. These values are based on the Kaiser test.

f-MWCNTs	Amine loading [μmol g^{-1}]	Yield [% of derivatised amines]	Loading [μmol g^{-1}]
6	410	–	–
7	45	89	FITC: 365
8	320	–	–
9	120	63	FA: 200
10	275	–	–
11	55	80	–
13	–	–	Gem: 220

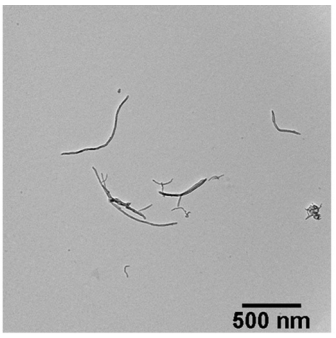


Scheme 2. Derivatisation of Gem with 5-hexynoic acid by transient protection of the hydroxyl functions with TMSCl.

converted to amide groups. Finally, the CuAAC reaction between Gem derivative **12** and azido-CNTs **11** was performed in the presence of copper(II) sulfate and sodium ascorbate leading to an amount of 220 μmol g^{-1} based on a quantitative "click" reaction. This value was in good agreement with the loading assessed from TGA (i.e., 188 μmol/g) based on the weight loss difference between *f*-MWCNTs **11** and **13** (i.e., 6.7% at 500 °C). Therefore, in eight steps we were able to obtain *f*-MWCNTs **13** bearing fluorescein, FA and Gem, named CNT-FITC/FA/Gem **13**.

The different *f*-MWCNTs were characterised by TEM, XPS and TGA in an inert atmosphere (Figure 1 and Figure 2). The observation of final CNT-FITC/FA/Gem **13** by TEM indicated that the morphology of the nanotubes was not affected by the different chemical treatments in comparison with the oxidised MWCNTs **1** and the triple-functionalised MWCNTs **5** (Figure 1 top, and see the Supporting Information, Figure S1).

XPS of *f*-MWCNTs **5** confirmed the presence of nitrogen, with the appearance of the N (1s) peak at approximately 400 eV in addition to the C (1s) and O (1s) peaks at 286 and 533 eV, respectively, in comparison with the XPS of oxidised MWCNTs **1** (Figure 1, bottom). The XPS of *f*-MWCNTs **13** showed the introduction of the F (1s) peak at 690 eV, due to the conjugation of Gem that bears fluorine atoms.



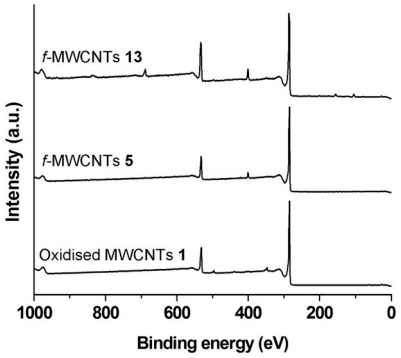


Figure 1. TEM of *f*-MWCNTs **13** (top) and XPS of oxidised MWCNTs **1**, *f*-MWCNTs **5** and *f*-MWCNTs **13** (bottom).

TGA was used to demonstrate that there was an increased weight loss after each step (Figure 2). As expected, the pristine MWCNTs were stable in N_2 up to high temperatures, whereas oxidised MWCNTs **1** and triple-functionalised MWCNTs **5** displayed a relative higher weight loss. After the grafting of FITC, FA and Gem, the weight loss progressively increased, which confirmed the sequential attachment of each of the molecules of interest onto *f*-MWCNTs **8**, **11** and **13**, respectively. Although the TGA profiles of *f*-MWCNTs **8** and **11** are similar below 500 °C, there is significant weight loss difference above 500 °C, which is consistent with an increased loading after the reaction.

To evaluate the targeting capacity of FA once bound to the CNTs, we assessed the recognition capability and the affinity of

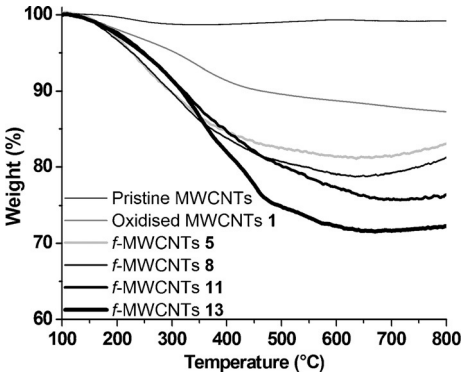


Figure 2. TGA of pristine MWCNTs, oxidised MWCNTs **1** and *f*-MWCNTs **5**, **8**, **11** and **13** performed in N_2.

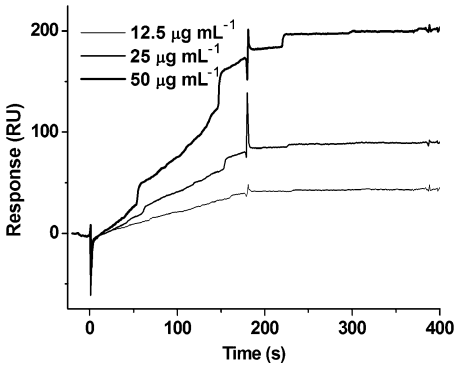


Figure 3. Sensorgrams showing the binding of *f*-MWCNTs **13** to the folate receptor at different concentrations of CNTs.

f-MWCNTs **13** towards the folate binding protein using SPR (Figure 3). The conjugate was able to bind to the folate protein on the sensor chip in a dose-dependent manner. It was not possible to obtain quantitative binding information from the SPR as a precise molecular weight for CNTs cannot be determined, due to the heterogeneity of nanotubes. This result provided evidence that the covalent attachment of FA onto CNTs maintained its recognition capacity. The *f*-MWCNTs **7** were used as control and no specific binding was observed (data not shown).

Following the study of the physicochemical characteristics of the different CNT conjugates, we then performed in vitro experiments to assess both their therapeutic and targeting capacity. The three constructs **S6**, **S7** and **S8** (see the Supporting Information, Schemes S5–S7) were further prepared as controls. The CNT-NH_3^+ **S6** sample was obtained by concomitant Boc and Z removal of *f*-MWCNTs **6** using TFA, TMSOTf and *p*-cresol, followed by counterion exchange in diluted HCl solution (see the Supporting Information, Scheme S5). MWCNTs functionalised with FITC and ammonium moieties (CNT-FITC/NH_3^+ **S7**) were synthesised by cleavage of the Z group of *f*-MWCNTs **8** (see the Supporting Information, Scheme S6). Finally, the CNT-FITC/FA/NH_3^+ **S8** was prepared from *f*-MWCNTs **10** by TFA counterion exchange with chlorine (see the Supporting Information, Scheme S7). The dispersibility of the different samples in 5% dextrose aqueous solution was satisfactory (see the Supporting Information, Figure S2).

The cytotoxicity of the different MWCNT conjugates (*f*-MWCNTs **13**, **S6**, **S7**, **S8**) was assessed using human carcinoma cells (breast MCF7 and cervical HeLa) that overexpress the folate receptors,[27] and a negative control cell line (lung A549) with very low expression levels of folate receptors.[28] After 24–72 h incubation with the different conjugates, a modified lactate dehydrogenase (LDH) assay[29] was performed to assess the biological activity of Gem once conjugated to the nanotubes. Interestingly, we observed that the anticancer activity of Gem was preserved after grafting onto CNTs as the triple-functionalised CNT-FITC/FA/Gem **13** showed the same level of cytotoxicity as Gem alone (Figure 4). In addition, there was a time-dependent cytotoxic effect with CNT-FITC/FA/Gem on all different cell lines. As expected, the control conjugates (*f*-MWCNTs

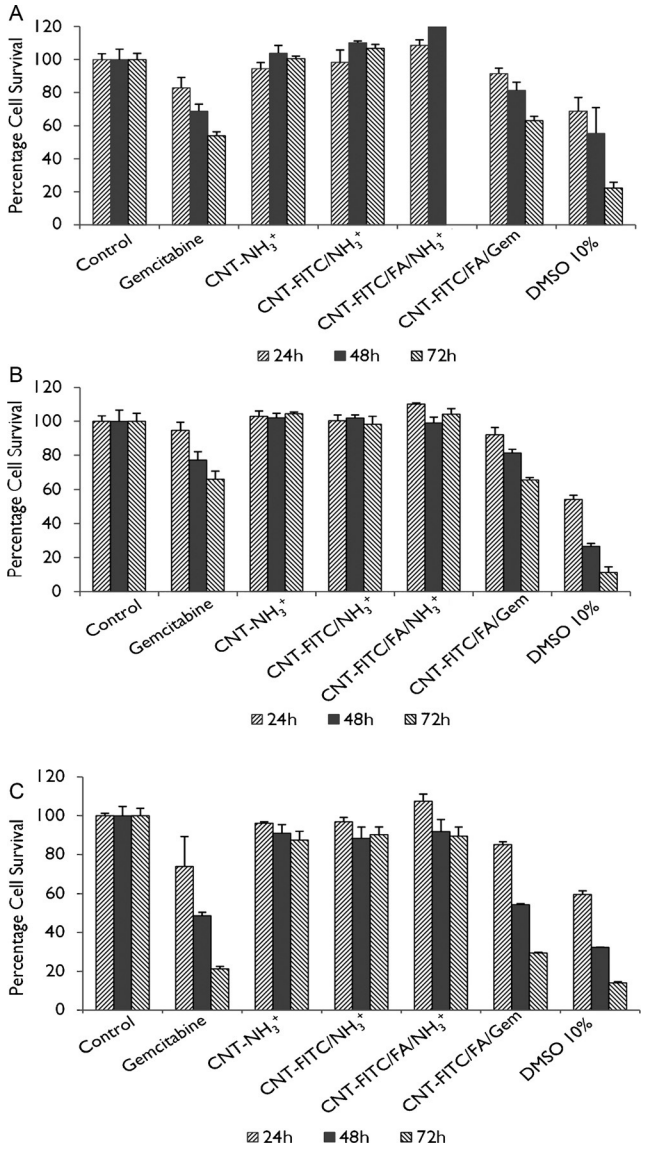


Figure 4. Cellular proliferation of folate-positive (A, B) and negative (C) cell lines after treatment with the different CNT conjugates at 20 μg mL^{-1} for 24, 48 and 72 h: A) breast cancer MCF7 cells; B) cervical cancer HeLa cells; C) lung carcinoma A549 cells. DMSO (10%) was used as a positive control.

S6–**S8**) did not show any reduction in cell viability over the 72 h incubation period, which suggested that the biological activity observed with CNT-FITC/FA/Gem was mainly due to the effective delivery of the drug molecule by the CNT carrier.

However, the data obtained in Figure 4 did not provide unequivocal evidence of cell specificity as the cytotoxic activity was not higher for MCF7 and HeLa cells (folate receptor-positive cell lines). One reason for this could be that A549 cells were shown to be very sensitive to Gem treatment (compared to MCF7 and HeLa) as evidenced by just approximately 20–40% cell viability after 72 and 48 h, respectively (Figure 4C) in comparison to approximately 60–80% with the positive cell lines (Figure 4A and B). On the other hand, light microscopy of A549 cultures treated with CNT-FITC/FA/Gem **13** for 48 h showed very limited interactions and binding of CNTs with

these FA-negative cells as compared to HeLa cells (see the Supporting Information, Figure S3 A-vi and B-vi). It is now well established that CNTs can exhibit a certain degree of cellular internalisation independent of their surface functional groups[3] by mechanisms that allow cell membrane translocation in an endocytotic-independent manner.[30] Such cellular internalisation of the constructs synthesised in this study, although limited, in combination with A549 sensitivity to Gem, could explain the non-selective cytotoxic activity obtained for all three cell lines used with the triple-functionalised MWCNTs.

Although other groups have reported the targeting efficiency of CNT-FA conjugates,[31] in most cases the conjugates are based on noncovalent surface modifications of the nanotube surface with lipids or polymers linked to FA, which makes the comparison difficult with our strategy. Only a few studies have reported the use of CNTs as carriers of Gem. In these cases, the drug was adsorbed (i.e., noncovalently linked) onto the nanotube surface.[32] Singh et al. have used the MTT assay to assess the cell viability of Gem immobilised noncovalently onto FA-MWCNTs,[32b] although it is well-documented that CNTs interfere strongly with this assay, thus producing unreliable results.[29,33] Similar to our data, the Gem/FA-MWCNTs reported by Singh et al. showed comparable cellular uptake in MCF7 cells. The constructs designed and synthesised in the present study are meant to offer higher stability due to covalent conjugation of the active molecules onto the nanotube sidewall that would eventually avoid the undesired release of the drug before reaching its target site.

Conclusion

We have developed an efficient method to covalently functionalise CNTs with a fluorescent probe, a targeting ligand and an anticancer drug. The triple functionalisation of CNTs was carefully controlled to avoid the degradation of the grafted molecules by testing the conditions on model derivatives. Gemcitabine was covalently linked to the nanotube sidewall using a "click" reaction by controlling the derivatisation of the amine function of the drug. This strategy is versatile as other probes and bioactive molecules could be covalently attached onto the CNTs. Characterisation of the CNT conjugates by TGA evidenced the successful attachment of the three molecules. SPR indicated that the affinity of FA for the folate binding protein was not affected after conjugation onto the nanotubes. Viability assays showed that the functionalised CNTs devoid of Gem were not toxic to the cells, whereas the anticancer activity of the drug was preserved after grafting onto the nanotubes, resulting in significant cytotoxicity. The functionalised CNTs were biologically active towards both folate-positive and negative cell lines, suggesting non-selective internalisation. We will further explore controlled functionalisation strategies onto CNTs with the aim of modulating their surface properties to obtain a better targeting efficiency. The methodology developed here for the triple functionalisation of CNTs is versatile and could be used to design advanced functional nanomaterials under controlled conditions.

Experimental Section

The protocols to prepare functionalised CNTs and Gem derivative **12**, as well as the cell culture and the cytotoxicity tests are detailed in the Supporting Information.

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Keywords: cancer · carbon nanotubes · drug delivery · fluorescent probes · functionalization

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