Enhanced anticancer activity of multi-walled carbon nanotube–methotrexate conjugates using cleavable linkers†

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Methotrexate was tethered to multi-walled carbon nanotubes through different cleavable linkers exploiting the ammonium functionalities introduced by 1,3-dipolar cycloaddition reaction of azomethine ylides to the nanotubes. The new nanobio-hybrid conjugates were internalized into human breast cancer cells and it was shown that the cytotoxic activity was strongly dependent on the presence and type of linker.

Nanomaterials are currently being developed for the delivery of biologically-active cargoes into living systems for disease diagnosis and therapy.[1,2] In fact, the efficacy of many potent and promising drug molecules is limited by their low water solubility, increased drug resistance and highly cytotoxic side effects, thus necessitating an efficient way of systemic transportation. One such type of nanomaterials, carbon nanotubes (CNTs), has attracted particular attention as carriers for biologically relevant molecules, because of their unique physicochemical and biological properties.[3–6] The synthesis of novel nanobio-hybrids based on CNTs represents therefore a valuable alternative to achieve enhanced cellular uptake, increased efficacy of such therapeutic agents and, hopefully, reduced side effects. Recently, several research groups have independently developed different strategies to achieve effective drug delivery to tumours using functionalized CNTs.[7–10] Towards this goal, the triggered release of cytotoxic agents from the nanotubes intracellularly has received limited attention. Introduction of cleavable linkers to facilitate controlled release of the drug from CNTs has been suggested using disulfide bonds, sensitive to pH changes and reducing agents.[11,12] However, this strategy suffers from the *in vivo* instability, as endogenous thiols can easily cleave the disulfide bridge during blood circulation.[13,14]

Here, we conjugate multi-walled carbon nanotubes (MWNTs) with the common anticancer drug methotrexate (MTX), a folic acid antagonist extensively used for first-line therapy. However, its conjugation to a carrier system is considered critical, because of its low cellular uptake.[15] Therefore, we have covalently linked MTX to MWNTs using a variety of cleavable linkers, including groups sensitive to intracellular enzymes, and demonstrate that the cytotoxic efficacy of the conjugates is dependent on the type of cleavable linker used. Oxidized CNTs have been functionalized with amine moieties through the 1,3-dipolar cycloaddition reaction of azomethine ylides.[16,17] The amount of functional groups was assessed by thermogravimetric analysis (TGA) and the Kaiser test (see ESI†). MWNTs **2** was obtained by direct coupling of MTX activated with HATU [*O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate], a common reagent in peptide chemistry, and DIEA (diisopropylethylamine) (Scheme 1). The coupling occurred *via* both α and γ carboxylic acids of the MTX Glu moiety.[15,18] The negative Kaiser test confirmed the completeness of the reaction. MWNTs **3** and **4** were then synthesized, each containing a different type of cleavable linker between the drug and the nanotube backbone.

Such cleavable CNT–drug conjugates must be sufficiently stable to sustain physiological conditions, for extended blood circulation. They also need to extravasate through the vasculature into the diseased site and be able to translocate plasma membranes and release the drug only inside the target cells by hydrolytic or enzymatic cleavage. In this context, the rational design of the linker is a challenging and critical factor to ensure the overall efficacy of the conjugate system. We introduced two different linkers: MTX was bound to MWNTs **1** either *via* the tetrapeptide Gly-Leu-Phe-Gly (MWNTs **3**) or the 6-hydroxyhexanoic ester (MWNTs **4**) (Scheme 1). The peptide linker was chosen as it is selectively cleaved by proteases overexpressed in tumour cells.[19–21] Instead, 6-hydroxyhexanoic ester was selected as an esterase-sensitive, hydrophobic spacer that has been widely used in prodrug conjugate synthesis.[22–24] The latter has been selectively coupled in the α-position of MTX, before conjugation to the amino groups of MWNTs **1** (see ESI†). In the case of MWNTs **4**, the carboxylic functions of the oxidized tubes were also derivatized with the fluorescent marker rhodamine B to investigate the cellular uptake of the conjugates.

The characterization of MWNTs **2–4** and their precursors was performed using TGA and complementary spectroscopic and microscopic techniques including transmission electron microscopy (TEM).

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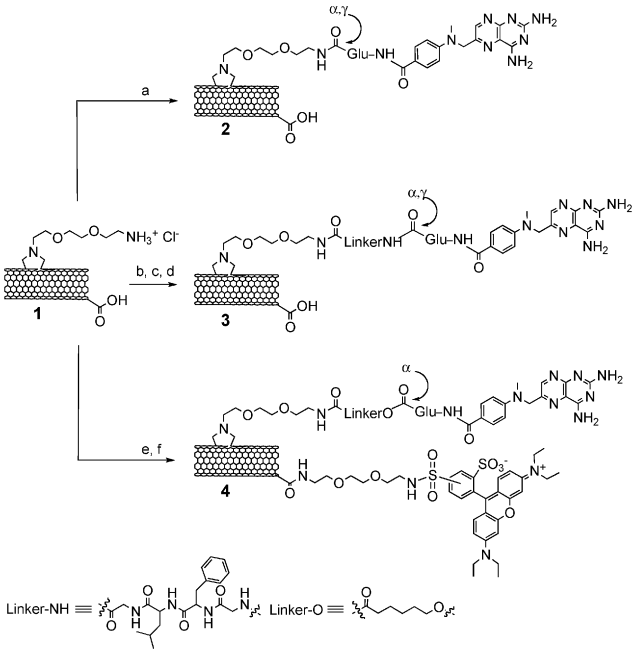

Scheme 1 Synthesis of MWNTs **2–4**. *Reagents and conditions*: (a) MTX, DIEA, HATU, DMF, 60 °C, 48 h; (b) Boc-Gly-Phe-Leu-Gly-OH (**5**), DIEA, HATU, DMF, 45 °C, 48 h; (c) HCl, 1,4-dioxane, 10 h; (d) MTX, DIEA, HATU, DMF, 60 °C, 48 h; (e) MTX-Linker-O (**6**), DIEA, DIC, DMF, 60 °C, 48 h; (f) rhodamine-NH-TEG-$NH_3^+Cl^-$ (**7**), DIEA, HATU, DMF, 60 °C, 48 h. Syntheses of products **5–7** are reported in ESI.†

The amount of functional groups in the different functionalized MWNTs was quantified by TGA. The weight loss for the highly functionalized conjugates was directly correlated to the increase of mass around the CNTs introduced at each step (Fig. 1). TEM analysis of the different conjugates showed that all derivatives were structurally similar (Fig. 2), with an average length around 300 nm and a diameter of 9.5 nm.

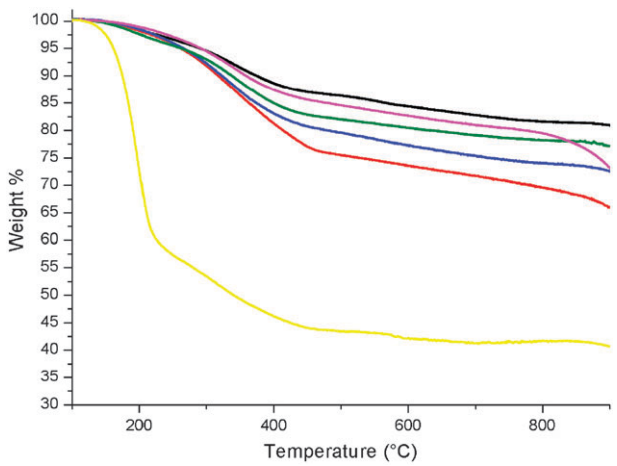

Fig. 1 TGA curves for MWNTs **1** (black line), MWNTs **2** (blue line), MWNTs **3** (red line), MWNTs **4** (yellow line), MWNTs **22** (green line), and MWNTs **23** (purple line). All of the experiments were performed under a N_2 atmosphere. The molecular structures and the syntheses of MWNTs **22** and **23** are reported in ESI.†

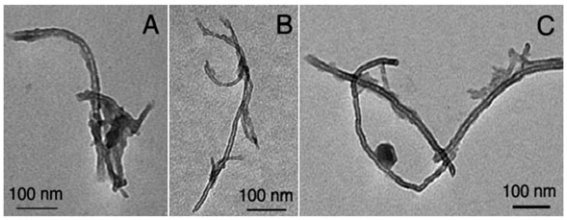

Fig. 2 TEM images of (A) MWNTs **2**, (B) MWNTs **3** and (C) MWNTs **4**.

To establish the capacity of these conjugates to translocate into mammalian cells, human breast carcinoma (MCF-7) cells were incubated with the rhodamine-labelled MWNTs **4** (Fig. 3). As a control (Fig. 3A–D) we used the cells alone and treated with MWNTs **1** devoid of the dye. The nanotubes were efficiently internalized and red fluorescent signals, due to the rhodamine B conjugated to the tubes, were observed throughout the cytosol at a concentration that did not affect cell viability. The differential interference contrast (DIC) images (Fig. 3D and F) display a viable cellular morphology of MCF-7 and the presence of few black nanotube aggregates inside the cells.

We next investigated the cytotoxic activity of MWNT conjugates **2–4** against MCF-7 using a modified version of the LDH assay (see ESI†). As shown in Fig. 4, the MWNT conjugates **3** and **4** were able to induce cell death in 90% and 20% of cell cultures, respectively after 24 h incubation in comparison to the controls MWNTs **1** and MTX alone. The remarkably high and statistically significant cytotoxic activity of MWNTs **3** compared to MWNTs **4** is most probably due to the efficient enzymatic hydrolysis of the drug tethered by the cleavable peptide inside the cells in comparison to the more stable ester bond.[25] These findings also suggest the ability of the conjugates to offer an improved mechanism for enhanced cellular uptake that the MTX molecules suffer from.[23] Several reasons may account for the higher cytotoxic activity of MWNTs **3** in comparison to MTX alone, including the ability of CNT carriers to cross the plasma membrane intact and release their cytotoxic payload by selective enzymatic cleavage.

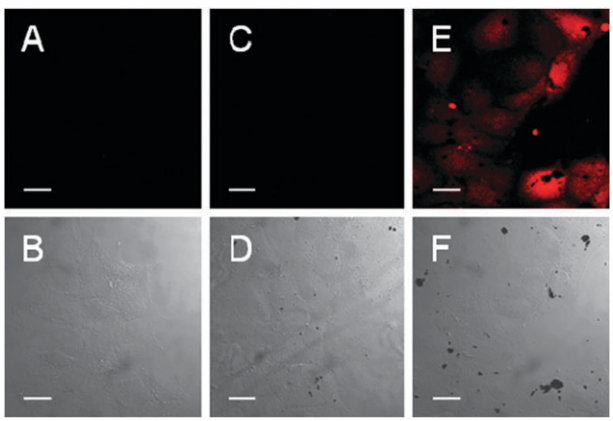

Fig. 3 Confocal microscopy images of MCF-7 cells incubated in the absence (A, B) and presence of 10 μg ml^{-1} of MWNTs **1** (C, D), and MWNTs **4** (E, F) for 24 h. Top panels depict rhodamine optics (red channel) and bottom panels show differential interference contrast (DIC) images. Scale bar is 20 μm in all images.

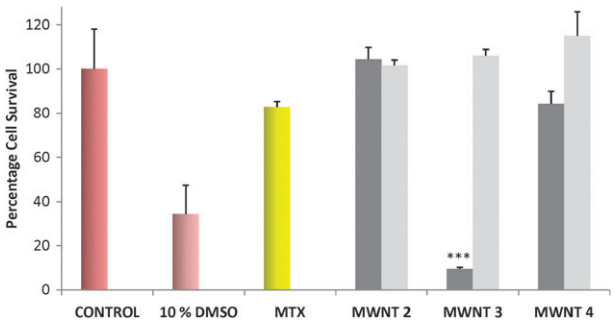

Fig. 4 Cell survival of MCF-7 cells after treatment with MWNT conjugates **2–4** for 24 h (dark grey bars). MTX concentration was kept constant at 10 μM with and without MWNTs (see Table S1†). Light grey bars correspond to the cytotoxicity effects of MWNTs **1** devoid of the drug used as control at the same dose of the related MWNT–MTX conjugate. 10% DMSO was used as a positive control for cytotoxicity. *** indicate statistical significance ($p < 0.005$) between MWNTs **3** and MTX alone.

Alternatively, the greater lipophilicity of these linkers could promote higher affinity with the tumour cell plasma membrane *via* hydrophobic interactions. More mechanistic studies are warranted to better understand the uptake and intracellular release of MTX from the MWNTs.

In summary, we have designed and synthesized a novel generation of chemically functionalized MWNT conjugates carrying an anticancer agent based on cleavable linkers. These initial investigations using such conjugates indicate that the activity of the drug strongly depends on the type of linker used. High cytotoxic activity could be observed using an enzyme-sensitive peptide linker. This approach in combination with the previously reported capacity of functionalized CNT to translocate through the plasma membrane may open promising alternatives for improved cancer therapeutics of even clinically established molecules, such as methotrexate.

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