Efficient receptor-independent intracellular translocation of aptamers mediated by conjugation to carbon nanotubes†

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Received 25th June 2010, Accepted 3rd August 2010
DOI: 10.1039/c0cc02092c

We have covalently grafted aptamers onto carboxylated carbon nanotubes to design a novel vector system that can easily translocate into the cytosol of different cell types independent of receptor-mediated uptake. We propose the use of carbon nanotubes for the efficient intracellular delivery of biologically active aptamers for potential therapeutic applications.

The use of nanostructured materials for therapeutic purposes is currently the goal of many laboratories. Carbon nanotubes (CNTs) have become increasingly important within the biomedical field as they can be easily modified and exhibit a large surface area and high *in vivo* stability.[1] Depending on whether they exist as a single cylindrical graphene layer or multiple concentric sheets they are typically referred to as single-walled (SWNTs) or multi-walled carbon nanotubes (MWNTs).[2] It has been shown that the functionalisation of these materials with peptides, small targeting ligands (such as folic acid) and monoclonal antibodies can result in active binding to specific cell receptors.[3] In addition, functionalised MWNTs have been described to have the capacity to directly translocate cellular membranes resulting in cytoplasmic delivery of their cargo.[4] This particular phenomenon is considered of importance towards the design of drug delivery systems that can achieve facile transport of biologically active agents directly into the cytoplasm.[4] Recently, our laboratories have utilised this capability of MWNTs to intracellularly deliver plasmid DNA, siRNA, fluorescent probes and anti-cancer agents.[1,5]

MWNTs have also been investigated for the construction of biosensors using both covalent and non-covalent modifications with aptamers.[6] Aptamers represent a new class of DNA or RNA oligonucleotides that adopt well defined conformations. They can be selected to recognize a wide variety of targets such as proteins, lipids, nucleic acids and sugars with high affinity and selectivity, using the SELEX methodology (systematic evolution of ligands by exponential enrichment). In addition, as small nucleic acid oligomers, they can be easily synthesized on large scale and modified with a variety of chemical agents. These properties have led to aptamers that can be considered promising candidates in cancer therapy as biomarkers (recognition of tumor antigens) or therapeutics (cytotoxic activity).[7]

In this communication, we describe a MWNT–aptamer (MWNT–Apt) hybrid prepared by coupling an anti MUC1–aptamer (sequence GGCGTACGGTAGGCGGGG-TCAACTG), modified at the 5′-end with a fluorescent probe (Cy3) and at the 3′-end with a primary amine, to the carboxylated MWNTs. MUC-1 is a membrane-associated glycoform of the mucin family and is commonly overexpressed on a broad range of epithelial cancer cell lines (breast, ovary, colon, pancreas, lung and prostate).[8,9] We investigated the physicochemical properties of the synthesized MWNT–Apt construct using transmission electron microscopy (TEM), atomic force microscopy (AFM), and thermogravimetric analysis (TGA). Furthermore, we studied the intracellular translocation and trafficking of these conjugates using laser scanning confocal microscopy.

Fig. 1A shows the synthetic pathway to prepare the MWNT–aptamer conjugates. Pristine MWNTs **1** were oxidised for 4 h with a HNO_3/H_2SO_4 mixture using a procedure described previously.[10] The resulting carboxylated MWNTs were conjugated to the aptamers using 1-[3′-(dimethylamino)-propyl]-3-ethylcarbodiimide/*N*-hydroxysuccinimide (EDCI/NHS) and isolated using filtration and thorough washings (Fig. 1A). The final MWNT–Apt construct was easily dispersed in 5% dextrose in water at 1 mg ml^{-1}.

The physicochemical characterisation of the MWNT–Apt conjugates using TEM, AFM, polyacrylamide gel electrophoresis (PAGE) and TGA was carried out (Fig. 1B–E). TEM indicated that both the oxidised MWNT (ox-MWNTs) precursors and the MWNT–Apt consist of individualised nanotubes, free from any major impurities (measured from a 0.1 mg ml^{-1} sample) (Fig. 1B and ESI†, Fig. S1), also showing that the surface of MWNT–Apt was coated (Fig. 1B, bottom panel). The constructs were then further studied by AFM indicating sharp differences in the surface morphology between the oxidised MWNTs and the MWNT–Apt (Fig. 1C and ESI†, Fig. S2).

To confirm the covalent binding of the aptamer molecules to the MWNTs (as aptamers have been known to self-assemble with MWNTs),[11] we conducted a PAGE gel and observed the aptamer bands using ethidium bromide (Fig. 1D). Lanes 1, 2 and 3 show the naked aptamer, a mix control of the aptamer with the oxidised MWNTs and the MWNT–Apt conjugate, respectively. The gel confirmed that covalent linking between

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† Electronic supplementary information (ESI) available: Cell viability, pre-incubation experiments, cellular uptake of scrambled MWNT-Apt. See DOI: 10.1039/c0cc02092c

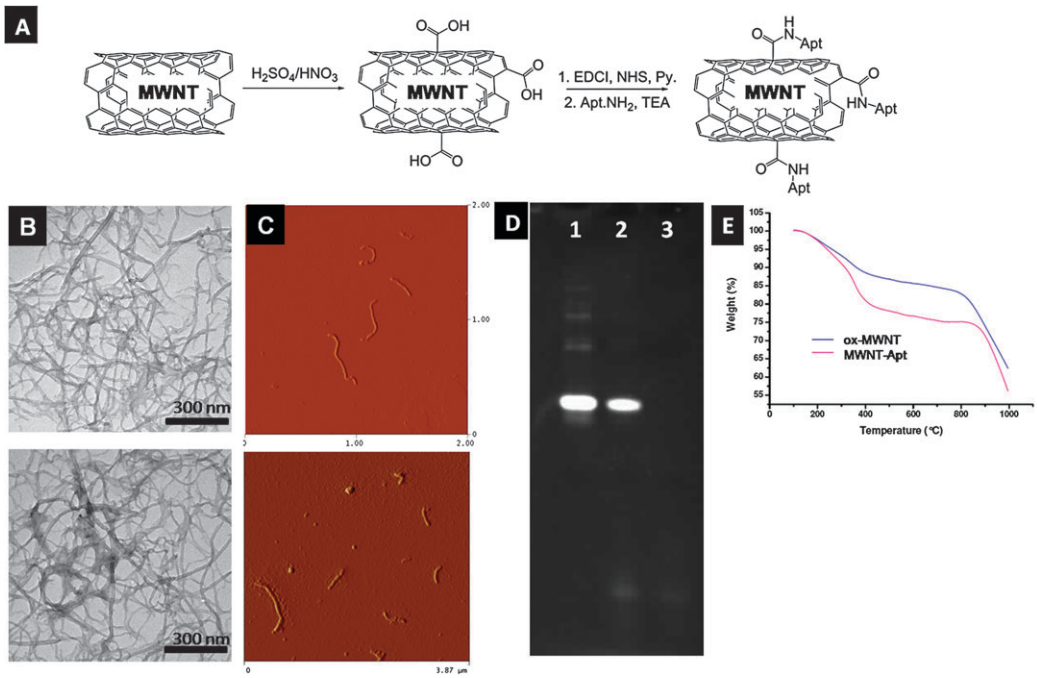

Fig. 1 Synthesis and physicochemical characterisation to MWNT–Apt constructs. (A) Synthetic route of MWNT–Apt; (B) TEM images of ox-MWNTs (top) and MWNT–Apt construct (bottom); (C) AFM images of the ox-MWNTs (top) and MWNT–Apt (bottom) in vacuum; (D) gel electrophoresis (PAGE) of aptamer alone (lane 1), aptamer mixed with ox-MWNTs (lane 2), MWNT–Apt conjugate (lane 3); (E) TGA of ox-MWNTs (blue) and MWNT–Apt (red). The sequence of the aptamer was GGCGTACGGTAGGCGGGGTCAACTG.

the aptamer and the MWNTs was taking place, since no free aptamer band could be detected only in the case of lane 3. No signal was observed from the wells in the gels, attributed to the interference of EtBr intercalation by the high MWNT concentrations as previously described for agarose gel assays in different studies.[12] In contrast, for the mixed (aptamer and oxidised MWNTs) control migration of free aptamer was consistently observed. Thermogravimetric analysis indicated further that at 500 °C weight loss for the oxidised MWNTs was significantly less than the MWNT–Apt conjugate (Fig. 1E). Taken together, these data were considered as evidence of covalent functionalisation of the MWNTs with the aptamer.

Next, we studied the cellular binding, internalisation and trafficking of the MWNT–Apt conjugates using confocal laser scanning microscopy (CLSM) in comparison to the aptamer alone (both using a concentration of 100 pmol aptamer per ml of media). The aptamer molecules chosen had been previously shown to bind to MUC-1 receptors.[9] This study was conducted using a MUC-1 positive (human breast carcinoma MCF-7) and a MUC-1 negative (human lung carcinoma Calu-6) cell line. The MWNT–Apt conjugate and the aptamer alone were incubated with the cells for 3 h in serum-free media, after which they were washed and incubated in complete media up to 24 h. At this point, the cells were washed and fixed with paraformaldehyde and the nucleus of the cells was stained with

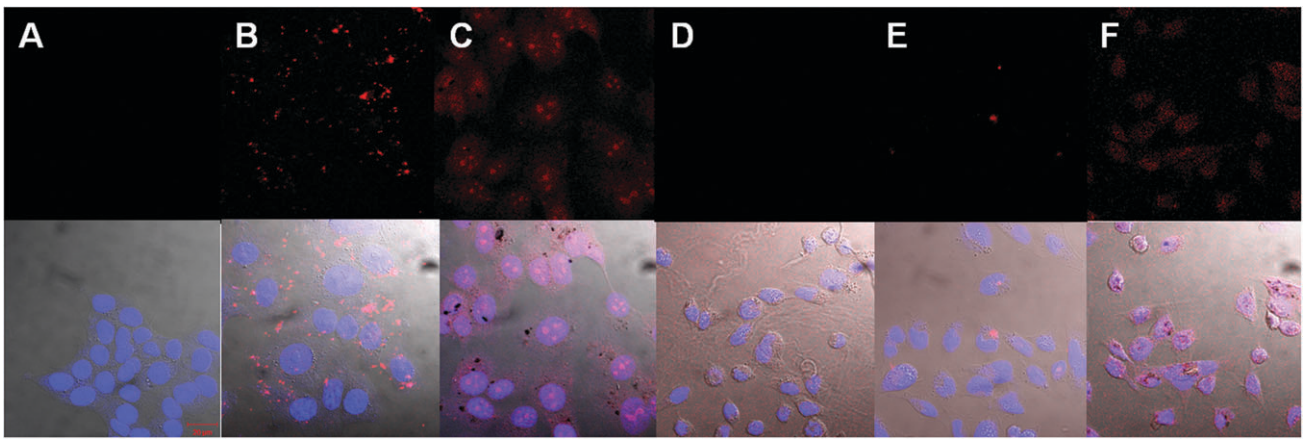

Fig. 2 Cellular binding, internalisation and trafficking of MWNT–Apt in MUC-1 positive (MCF-7) and negative (Calu-6) cells. (A) MCF-7 untreated cells; (B) MCF-7 cells treated with Cy3-labelled aptamer alone; (C) MCF-7 cells treated with MWNT–Apt; (D) Calu-6 untreated cells; (E) Calu-6 cells treated with Cy3-labelled aptamer alone; (F) Calu-6 cells treated with MWNT–Apt. Top panels show Cy3 (red) channel, bottom panels show merged DIC, TOPRO-3, and Cy3 (white, blue, red) channels.

TOPRO-3. The punctate pattern of the fluorescent intracellular signals in MCF-7 cultures indicated that the aptamers were taken up *via* an endocytotic pathway as described before (Fig. 2B).[9] When the MUC-1 negative Calu-6 cell line was used, very little uptake of the free aptamer was observed (Fig. 2E). Fig. 2C and F show that when the cells were incubated with MWNT–Apt conjugates the fluorescence signal was diffused throughout the whole cell volume. This was irrespective of MUC-1 expression on the cell surface.

In order to block the receptors on the cell surface of MCF-7 cells we also studied the effect of pre-incubation with a free anti-MUC-1 monoclonal antibody (mAb) for 1 h prior to incubation with the MWNT–Apt (see ESI†, Fig. S3). Pre-incubation with the mAb did not block the internalisation of the MWNT–Apt, even though it reduced the binding of the aptamer alone. However, no intracellular localisation could be distinguished using fluorescence microscopy, in contrast to CLSM imaging obtained at the equatorial section of the cells[13] (see ESI†, Fig. S3C & F) that confirmed intracellular trafficking of the MWNT–Apt for both cell lines. Further experiments were conducted using a MWNT–Apt construct with a scrambled aptamer sequence at the same concentration as that used in Fig. 2 (see ESI†, Fig. S4). Cytoplasmic internalisation of the MWNT–Apt conjugate was also observed throughout the cell despite the aptamer sequence being scrambled for both MUC-1 positive and negative cell lines. Lastly, an LDH cytotoxicity assay confirmed that internalisation of the MWNT–Apt did not lead to cell death (see ESI†, Fig. S5) within the 24 h period of investigation.

This study indicated that coupling of aptamers to carbon nanotubes can result in constructs that are able to translocate into the cell cytoplasm in a receptor-independent manner. Therapeutic aptamers currently suffer from poor membrane-penetration capacity and inefficient uptake by cells in the absence of extracellular recognition factors.[14] Hence, most of the therapeutic aptamers that have been described today target a variety of extracellular targets, such as ghrelin, nucleolin, and platelet-derived growth factor.[7] For the CNT–Apt presented here the issue of targeting specificity will need to be addressed with further modification of the construct if that would be desirable. The advantage such constructs offer is the possibility for direct intracellular delivery of biologically active molecules that has not been previously shown.

The present data also suggest that ligation of low molecular weight molecules onto carbon nanotubes may result in a similar non-specific internalisation of the conjugates due to the native capacity of nanotubes to cross cellular membranes.[4] Further investigation is warranted to determine the exact mechanism of such membrane translocations and cell internalisation of such constructs and any unwanted toxicological effects from these internalising nanotubes. It has been shown that chemically functionalized nanotubes typically exhibit better renal and hepatic toxicological profiles than pristine materials,[1] which is important for their success in biomedical applications.

Based on this study, we propose that carbon nanotube–aptamer constructs can be used for the purpose of facile intracellular delivery of biologically-active aptamers. In addition, MWNT–Apt conjugates can offer opportunities for the development of novel aptamer molecules towards specific intracellular targets that were previously difficult to access due to limited intracellular translocation.

This work was supported partially by the European Union FP7 ANTICARB (HEALTH-2007-201587) program, by the CNRS, the University of Trieste, the Italian Ministry of Education MIUR (Firb RBIN04HC3S) and the School of Pharmacy, University of London. The authors would also like to thank Dr Sotiris Missailidis, Open University, UK, for fruitful discussions regarding the aptamer molecules used at the beginning of this project.

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