Gene Technology

Functionalized Carbon Nanotubes for Plasmid DNA Gene Delivery**

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Dedicated to Professor Giorgio Modena on the occasion of his 80th birthday

Exploration of the biological and medical applications of carbon nanotubes (CNTs) is a rapidly expanding field of

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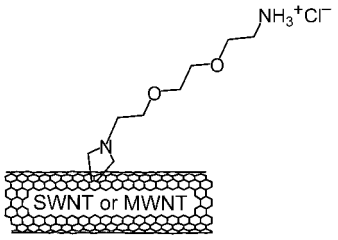
[**] This work was supported by the Centre National de la Recherche Scientifique (CNRS), Università di Trieste, and Ministero dell'Istruzione, dell' Università e della Ricerca (MIUR; cofin 2002, prot. 2002032171). Transmission electron microscopy (TEM) analysis was performed at the microscopy facility of the Institute of Biomedical Problems and was cofinanced by CNRS, Région Alsace, Louis Pasteur University, and the Association de la Recherche pour le Cancer. The authors wish to acknowledge C. D. Partidos for helpful and stimulating discussions. We thank Mr. Claudio Gamboz (Centro Servizi Polivalenti di Ateneo (CSPA), Università di Trieste) for his great help with the TEM measurements.

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

  DOI: 10.1002/anie.200460437

research.[1–11] In particular, the use of CNTs as carriers of biologically active molecules holds great promise.[6,11] Functionalized carbon nanotubes are interesting as material for engineering a novel gene delivery system.[12] Herein, we show that ammonium-functionalized CNTs (*f*-CNTs) are able to associate with plasmid DNA through electrostatic interactions. Upon interaction with mammalian cells, these *f*-CNTs penetrate the cell membranes and are taken up into the cells. The nanotubes exhibit low cytotoxicity and *f*-CNT-associated plasmid DNA is delivered to cells efficiently; gene expression levels up to 10 times higher than those achieved with DNA alone were observed. These findings reveal a novel combination of properties attributable to soluble carbon nanotubes and establish the potential of these structures as components of advanced delivery systems for a variety of therapeutics.

Carbon nanotubes were covalently modified by using a method based on the 1,3-dipolar cycloaddition of azomethine ylides.[13,14] Both single-walled and multi-walled carbon nanotubes (SWNTs and MWNTs) were functionalized with a pyrrolidine ring bearing a free amino-terminal oligoethylene glycol moiety attached to the nitrogen atom. The presence of



this functional group increases the solubility of carbon nanotubes remarkably, particularly in aqueous solutions.[14] The concentration of functional groups on the carbon nanotubes was calculated as about 0.55 and 0.90 mmol g^{-1} for *f*-SWNTs and *f*-MWNTs, respectively.[15]

The electrostatic interactions of the positively charged ammonium *f*-CNTs with the phosphate groups of plasmid DNA were studied by TEM. Figure 1 A shows a bundle of *f*-SWNTs deposited from an aqueous solution onto a carbon-coated TEM grid. Although one might expect repulsion between the positive charges of the ammonium salts, which could lead to bundle disruption, nanotube association patterns such as those shown in Figure 1 A were observed throughout. These bundles are less tightly bound than pristine CNTs, probably because of the presence of the functionalization chains. When a solution of *f*-SWNTs (720 μg mL^{-1}) in water was mixed with plasmid DNA (5 μg mL^{-1}) in a 6:1 (+/−) charge ratio, globular and supercoiled structures were observed in different regions of the nanotube surface (see black arrows in Figure 1 B and C).

Spherical, toroidal, or supercoiled structures between 15 and 300 nm in diameter are typically obtained when plasmid DNA is allowed to interact with positively charged groups or cations. Such interaction leads to varying degrees of plasmid condensation depending on the charge density, the hydrophobic character of the interaction, and the number of plasmid DNA molecules in the condensate.[16] We observed

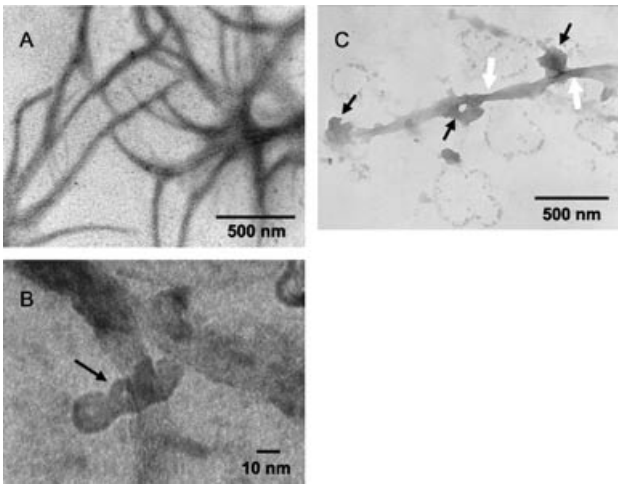


Figure 1. TEM images of *f*-SWNTs (A) and *f*-SWNT:DNA complexes (B and C).

tighter packing of the *f*-SWNTs (see white arrows in Figure 1 C) within regions where condensation of plasmids onto the carbon nanotube bundles took place.

To determine whether it is possible to use these *f*-SWNTs for intracellular delivery applications we studied their interaction with mammalian HeLa cells. We recently reported that CNTs functionalized with a fluorescent group (fluorescein isothiocyanate) and a fluorescent peptide are able to traverse cell membranes.[6] We did not necessarily expect to observe this property for the positively charged *f*-CNTs used in this study since the high number of charged ammonium groups could interfere with the mechanism of cell binding and uptake. Fluorescence detection is not possible for the ammonium-CNTs described herein because of the lack of an appropriate chromophore. The interaction of the *f*-CNTs with cells was therefore studied by TEM. HeLa cells were incubated with ammonium *f*-SWNTs and *f*-MWNTs at a concentration of 2.5 mg mL^{-1}. The nanotubes were allowed to interact with the cells for 1 h and were then embedded in an epoxy resin. Ultrathin sections of the polymer (about 90 nm thick) were cut on an ultramicrotome with a diamond knife and examined by TEM. Figure 2 shows HeLa cells incubated with *f*-MWNTs. The various cellular compartments are indicated by white arrows in Figure 2 A. Many nanotubes are clearly visible inside the cell. Subsequent magnifications (Figure 2 B and C) provide a higher-resolution view of the intracellular localization of the *f*-MWNTs. Interestingly, a degree of nuclear localization of the nanotubes was observed consistently throughout the samples. Careful analysis of the cell sections also permitted observation of nanotubes in the process of crossing the plasma membrane barrier.

Figure 2 D shows an *f*-MWNT during interaction with the cell membrane and uptake into the cell. The observed nanotube has a diameter of about 20 nm and an apparent length of around 200 nm. Although the mechanism of cellular uptake is still unclear, the semirigid and elongated form of the tube rules out an endocytosis process.[17] This deduction was confirmed by preincubation of the cells with sodium azide or 2,4-dinitrophenol, typical inhibitors of energy-dependent cell

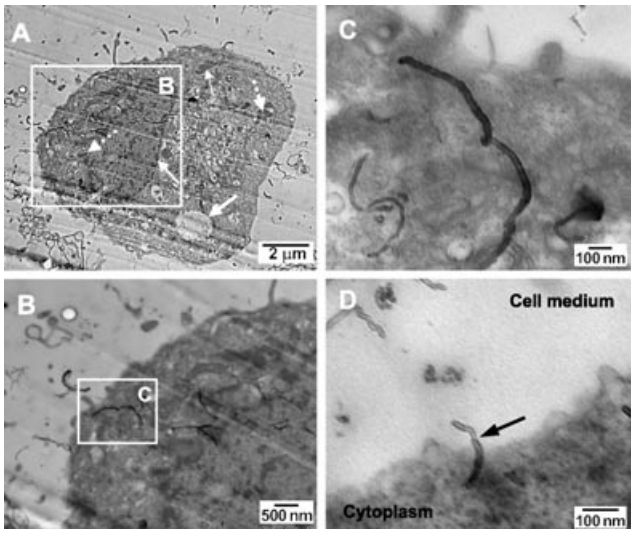


Figure 2. Ultrathin transverse section of HeLa cells treated with *f*-MWNTs. After incubation, the cells were fixed, stained, dehydrated, and embedded in Epon 812 resin. Ultrathin layers (90 nm thick) were cut with an ultramicrotome. A) The entire cell; B) and C) two subsequent magnifications. D) A multi-walled carbon nanotube crossing the cell membrane. Dotted white arrow, chromatin; dashed white arrow, a mitochondrium; thin white arrow, Golgi complex; medium white arrow, nuclear membrane; thick white arrow, a vacuolum.

processes such as endocytosis. The carbon nanotubes used in this study probably enter the cell by a spontaneous mechanism in which they behave like nanoneedles and pass through the cell membrane without causing cell death.[18] Very recently published molecular dynamics simulation data suggest that hydrophobic nanotubes with hydrophilic functional groups can spontaneously insert into a lipid bilayer.[19] Such mechanistic modeling results correlate well with our experimental observations on the interaction between *f*-CNTs and plasma membranes (Figure 2D). We believe that the cationic functional groups bind the nanotubes to the cell membrane, then a spontaneous insertion mechanism allows the nanotubes to pass through the biomembrane as predicted by theoretical studies. Subsequent translocation of the *f*-CNTs within the intracellular region could follow this non-endocytotic process.

The ability of the ammonium-functionalized carbon nanotubes to enter cells and potentially reach their nuclei was further exploited for the delivery of plasmid DNA to the cell. Figure 3 shows the levels of marker gene (β-galactosidase; β-gal) expression in CHO cells after exposure to nanotubes connected to plasmid DNA encoding the gene.

As with other nonviral gene delivery vectors,[20–22] the charge ratio between the ammonium groups at the SWNT surface and the phosphate groups of the DNA backbone seems to be a determinant factor in the level of gene expression. *f*-SWNT/DNA charge ratios between 2:1 and 6:1 (+/−) led to 5–10 times higher levels of gene expression than treatment of the cells with DNA alone. No cytotoxicity was observed in this study (the highest nanotube concentration used in our gene delivery experiments was 1.2 mg mL^{-1}),[18] even when the *f*-SWNT:DNA complexes were incubated with the CHO cells for 3 h. We observed an increase in gene expression with increasing incubation times for *f*-SWNT:DNA complexes with charge ratios that resulted in optimum gene delivery capacity (i.e. between 2:1 and 6:1); the three-hour incubation period led to peak gene delivery for these complexes. The functionalized carbon nanotubes used in this study offer considerable advantages over other nanomaterials recently explored as components of systems for the delivery of DNA to mammalian cells.[23–25] The nanotubes

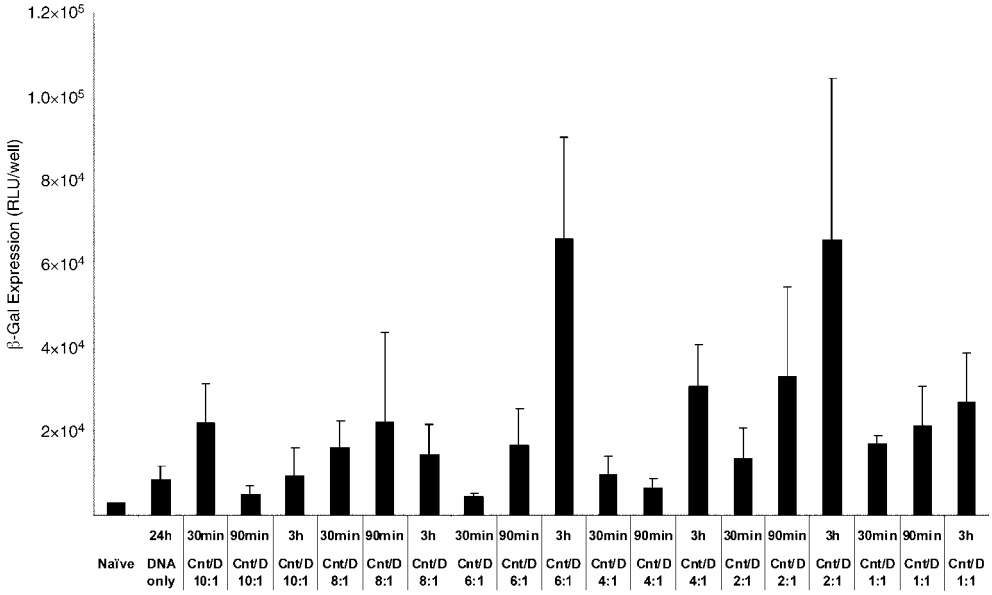


Figure 3. Delivery of plasmid DNA by *f*-SWNTs and expression in cells. Levels of marker gene (β-gal) expression in CHO cells in relative light units (RLU) per mg total protein. Various *f*-SWNT/DNA charge ratios were tested with three different incubation time periods. Toxicity manifested as cell detachment and death was not observed at any point during this study. *f*-SWNT and DNA are denoted in the figure as Cnt and D, respectively.

described herein allow relatively facile further functionalization of their surface and are therefore chemically versatile, they are capable of penetrating the cell membrane, and they have a lower cytotoxicity than other nanomaterials. Other cationic macromolecules, such as peptides, dendrimers, and liposomes generally achieve effective delivery of DNA by causing destabilization of the cell membrane, which leads to pronounced cytotoxicity.[26–29] Preliminary comparative gene expression data for lipid:DNA and *f*-CNT:DNA delivery systems show that our first generation of functionalized carbon nanotubes is less effective for transfection in vitro than lipid:DNA systems.

The study reported herein constitutes the first example of the utilization of carbon nanotubes as components for engineering a novel nanotube-based gene delivery vector system. The functionalized nanotubes formed supramolecular complexes with plasmid DNA through ionic interactions. These complexes are able to bind to, and penetrate within cells by what seems to be an endosome-independent mechanism. *f*-SWNTs complexed with plasmid DNA were able to facilitate higher DNA uptake and gene expression in vitro than could be achieved with DNA alone. In view of these interesting properties, the delivery of other types of therapeutic agents by *f*-CNTs through noncovalent interactions of the nanotubes with the agent can be envisaged.

Experimental Section

f-SWNT:DNA complexes: *f*-SWNTs were hydrated in deionized water at a concentration of 6 mg mL^{-1}. Plasmid DNA (pBgal, Clontech) was hydrated in deionized water at a concentration of 1 mg mL^{-1}. Aliquots were stored frozen at −20 °C until needed. The appropriate volume of nanotubes was diluted to a total volume of 300 μL in Optimem. pBgal (3 μg) was added to a separate sample of Optimem (300 μL). The diluted nanotubes were added dropwise to the DNA and the mixture was pipetted briefly. Complexes were allowed to form for 10 min prior to use. This process was repeated for each charge ratio tested. For the electron microscopy investigations, the nanotubes and DNA were always allowed to interact in water. An aqueous sample containing *f*-SWNT:DNA complexes was deposited onto a 300-mesh copper grid coated with a Formvar/carbon support film (Taab Labs Ltd.). Prior to preparation, the grids were "glow discharged" in an Emitech K350G system (Emitech Ltd) for 3 min at 30 mA (negative polarity). Imaging was carried out with a FEI/Philips CM120 BioTwin transmission electron microscope (Eindhoven) at an accelerating voltage of 120 KV, and with a Philips TEM 208 instrument at an accelerating voltage of 100 KV.

Preparation of cell sections for TEM analysis: SWNTs and MWNTs were purchased from Carbon Nanotechnology, Inc. and Nanostructured & Amorphous Materials, Inc., respectively, and were functionalized as described in the literature.[14] HeLa cells (1.25×10^5) were cultured in Dulbecco's minimal essential medium in a 16-well plate at 37 °C in the presence of 5% CO_2 until 75% confluency was reached. The cells were then incubated with a solution of *f*-SWNT and *f*-MWNT (2.5 mg mL^{-1} each) in phosphate-buffered saline (PBS) for 1 h, washed twice with PBS, and fixed by treatment with 2.5% glutaraldehyde in a cacodilate buffer (0.075 M sodium cacodilate, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 4.5% sucrose, pH 7.3) for 2 h at room temperature. An aliquot (10% v/v) of a saturated solution of picric acid in cacodilate buffer (1/10) was added to each well and the cells were incubated overnight at 4 °C. The specimen was washed three times with distilled water (15 min each wash) then treated with a 1% OsO_4 solution in cacodilate buffer for 2 h at room temperature. Cells were carefully rinsed with distilled water and post-fixed with a 2% solution of uranyl acetate in water overnight at 4 °C. After several washes, the cells were dried by treatment with 70% and 90% ethanol for 10 min each, and twice with absolute ethanol for 20 min. A fresh sample of Epon 812 resin was prepared as suggested by Electron Microscopy Sciences and distributed through the cells in each well. The plate was stored in an oven at 65 °C for three days. Each resin block was then removed from the plastic support and cut. A Reichert-Jung Ultracut-E ultramicrotome with a diamond knife (Ultramicrotomy 45°) was used to cut the resin containing the cells into 90-nm thick slices. Three consecutive slices were deposited on a formvar grid and observed through a Hitachi 600 electronic transmission microscope at 75 kV. Images were taken with an AMT high-sensitivity camera at various levels of magnification.

Gene delivery studies: CHO cells (ATCC) were grown to 90% confluency in F12K medium containing 10% fetal bovine serum and 1% penicillin/streptomycin (all from Gibco) in 96-well tissue culture dishes (Corning-Costar). CHO cells are one of the most popular cell lines used for gene transfer studies since they exhibit adequate levels of gene expression after treatment with various nonviral transfection agents; these cells are also commonly used for genetic screening purposes.[30] The culture medium was removed, *f*-SWNT:DNA complexes (50 μL) were added, and the cells were analyzed in triplicate under each set of test conditions. After 30, 90, or 180 min, the transfection medium was removed and replaced with fresh culture medium. As a control, three wells were transfected with DNA (0.25 μg) in Optimem (50 μL). Cells were incubated for 48 h then harvested. β-galactosidase activity was measured by using the Tropix Galactolight Plus kit and a Berthold 9507 luminometer according to the manufacturers' instructions.

Received: April 25, 2004
Revised: June 24, 2004

Keywords: carbon nanotubes · gene delivery · plasmid DNA · supramolecular chemistry

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