

Carbon nanotube cell translocation and delivery of nucleic acids *in vitro* and *in vivo*

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In the last few years, the carbon nanotube (CNT) field has seen a new direction of investigation growing rapidly, along with the interest of more researchers from diverse fields of expertise interested in this new material in an attempt to exploit their properties in biomedical applications. Here we describe the most recently reported work on the application of CNT for gene encoding nucleic acid (DNA and RNA) delivery purposes by using *in vitro* and *in vivo* models. Several groups have now successfully observed the cellular internalisation of nucleic acids with the aid of CNT following very different protocols. The main processes for the internalisation pathways and intracellular release of the nucleic acids are here reviewed. Furthermore, we have just started to see some initial studies of *in vivo* work using siRNA-CNT conjugates to achieve silencing in tumour tissue. Admittedly, it is still very early days for the technology, but future studies are necessary, and will surely appear, in order to determine the feasibility of bringing the CNT closer to the clinic.

1. Introduction

Carbon nanotubes (CNT) consist exclusively of carbon atoms arranged in a series of graphene-like planar sheets rings rolled up into a tubular structure. Generally, CNT are classified in two categories based on their structure and dimensions: single-walled carbon nanotubes (SWNT), which consist of one layer of cylindrical graphene and have diameters from 0.4 to 2.0 nm and lengths

in the range of 20–1000 nm; and multi-walled carbon nanotubes (MWNT), which contain several concentric graphene sheets and greater dimensions with diameters in the range of 1.4–100 nm and lengths from 1 to a few microns. Several methods for the production of both types of tubes and the modulation of their dimensions have been described.¹ CNT have attracted the attention of various research groups as the possible applications of these tiny carbon structures

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Lara Lacerda

Ms Lara Lacerda completed her First Degree in Pharmaceutical Sciences at the University of Coimbra in 2003. Currently she is pursuing a PhD degree in carbon nanotubes and nanomedicine at the University of London under the supervision of Kostas Kostarelos. Her research focuses on the development of functionalised carbon nanotubes for biomedical applications such as drug and gene delivery. Lara is an associate member of the Royal Society of Chemistry (UK).



Alberto Bianco

during 1992 (with Professor M. Mutter), at the University of Tübingen in 1996–1997 (with Professor G. Jung, as an Alexander von Humboldt fellow) and at the University of Padova in 1997–1998 (with Professor G. Scorrano). He currently is a Research Director at CNRS in Strasbourg (France). His research interests focus on the development of carbon-based nanomaterials as therapeutic vectors; the applications of functionalized carbon nanotubes and fullerenes in nanomedicine; and the synthesis of fullerene-peptides as new ligands for immunotherapy. He is member of the American Chemical Society, the French Group of Peptides and Proteins, and the European Peptide Society.

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spread rapidly among very different fields. Their extraordinary properties, such as high electrical and thermal conductivity, great strength and rigidity, have been developed for a wealth of applications, including resilient composite materials,² field emission,³ energy storage,⁴ and molecular electronics,⁵ among many others.

Pristine (as-prepared) CNT aggregate readily in bundles and are difficult to suspend effectively as a consequence of strong van der Waals forces. Therefore, the application of CNT as novel 'dry' materials has been explored previously due to their lack of solubility in most solvents that posed an obstacle for the utilization of CNT in 'wet' applications such as in biology and medicine. However, after the discovery of functionalisation of CNT using different types of organic reactions it has been possible to individualise and disperse them in solvents. Common techniques used to functionalise CNT are: (a) defect-site, (b) covalent sidewall, (c) non-covalent exohedral and (d) endohedral functionalisation.⁶ The simplest method to achieve aqueous suspensions of CNT is by wrapping and coating using polymers, surfactants or biomolecules with the aid of sonication.⁶ An alternative approach is the introduction of chemical moieties on the nanotube surface *via* an organic reaction (covalent

functionalisation), such as the 1,3-dipolar cycloaddition reaction, which has been primarily used in our laboratories.⁷ Once the issue of CNT aqueous dispersibility was dramatically improved, several research groups began investigating the potential of CNT in the fields of biotechnology and biomedicine,^{8,9} since the development of water-soluble CNT automatically renders them usable in contact with biological fluids and environments.

A further important development was the discovery that CNT can non-covalently bind single stranded¹⁰ and double stranded^{11,12} DNA. Due to the latter, the complex formation between CNT and nucleic acids allowed the exploration of the hypothesis that CNT can be used as gene delivery systems. In the present article we highlight the current state-of-the-art in the delivery of nucleic acids by using CNT that have used both *in vitro* and *in vivo* models, and highlight the development of CNT as gene delivery vectors.

2. Delivery of nucleic acids by CNT

Exogenous gene delivery and expression in live cells is a complex biological phenomenon that has been applied for a variety of purposes, most commonly gene identification and gene therapy. The

optimum delivery of genes in diseased tissue is considered today to be a bottleneck in both viral (*i.e.* use of genetically modified viruses) and non-viral (*i.e.* use of chemical moieties) gene therapy applications. There is an ever-growing need to enhance the available tools that can lead to effective and safe gene delivery and expression. CNT are some of the most recent nanomaterial constructs to be developed as gene delivery vectors, stemming from both their attractive physicochemical and electronic features as well as the initial observations that they can be internalised in mammalian cells very efficiently.^{13,14} A summary of the different studies published today using CNT for the delivery of nucleic acids in *in vitro* and *in vivo* models is presented in Table 1.

2.1 *In vitro* models

2.1.1 DNA transfection. Our groups published the first studies^{11,12} to demonstrate CNT-mediated gene delivery and expression leading to production of marker proteins encoded in double-stranded plasmid DNA (pDNA). In these initial studies we observed that pDNA was able to associate in a condensed globular conformation through electrostatic interactions at the surface of CNT covalently functionalised with ammonium groups (Fig. 1). The delivery



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Professor Maurizio Prato obtained his Laurea degree in chemistry in 1978 from the University of Padova, Italy, where he was appointed Assistant Professor in 1983. He moved to Trieste as an Associate Professor in 1992 and was promoted to Full Professor in 2000. He spent a postdoctoral year in 1986–1987 at Yale University, was a Visiting Scientist at the University of California, Santa Barbara, in 1991–92, and Professeur Invité at the

Ecole Normale Supérieure in Paris, France, in July 2002. His research interests focus on the synthesis of novel functional materials, including functionalized fullerenes and carbon nanotubes, for applications in materials science and medicinal chemistry. His scientific contributions have been recognized by National awards, which include: Federchimica Prize (1995, Association of Italian Industries) and the National Prize for Research (2002, Italian Chemical Society).



Kostas Kostarelos

Professor Kostarelos is the Chair of Nanomedicine at The School of Pharmacy of the University of London, a Fellow of the Royal Society of Medicine (FRSM) and a Fellow of the Institute of Nanotechnology (FIoN). He obtained his Diploma in Chemical Engineering and PhD from the Department of Chemical Engineering, Imperial College London. Following his promotion to Assistant Professor of Genetic Medicine and Chemical

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Table 1 *In vitro* and *in vivo* models used to delivery nucleic acids with CNT

	Nucleic acid	CNT	Model	Ref.	
<i>In vitro</i>	DNA	pDNA (β gal)	SWNT-NH ₃ ⁺	CHO cells	11
		pDNA (β gal)	SWNT-NH ₃ ⁺ SWNT-Lys-NH ₃ ⁺ MWNT-NH ₃ ⁺	A459 cells	12
	pDNA (CMV-Luc)		MWNT	293 cells	15
			MWNT-PEI	COS7 cells	
			MWNT-ox-NH ₂ MWNT-ox-NH-PEI	HepG2 cells	
	pDNA (EGFPN1)		MWNT-ox-NH-PEI-FITC	HUVEC cells A375 cells	16
			MWNT-NH ₂		
			MWNT-COOH MWNT-OH		
	pDNA (EGFP-c1)		MWNT-CH ₂ CH ₂ CH ₃	Bal17 cells mouse splenic B cells and primary culture of mice cortical neurons	17
			CNT(Ni)-COOH CNT-COOH		
	pDNA (UC19)	FAM-dsDNA-NH ₂	MWNT-COOH	<i>E. coli</i> strain DH5 α	18
			SWNT-COOH	1H8 cells TC-1 cells LLC cells	20
	Cy3-ssDNA		SWNT	HeLa cells HL60 cells	22,23
	Cy3-ssDNA-SH		SWNT-DGPE-PEG-SH and SWNT-DGPE-PEG-maleimide	HeLa cells	24
	ssDNA		SWNT	Murine myoblast stem cells 3T3 cells	25,26
Cy3-ssDNA	Cy5-ssDNA	SWNT (long and short)	IMR 90 cells	27	
			A549 cells MC3T3-E1 cells		
RNA	PI-poly(rU) RNA siRNA-SH	SWNT SWNT-DGPE-PEG-SH and SWNT-DGPE-PEG-maleimide	A10 cells	28	
			MCF7 cells		
	siRNA (murineTERT) siRNA (humanTERT)	SWNT-CONH-(CH ₂) ₆ NH ₃ ⁺	HeLa cells	24	
			HeLa cells		
	SH-siRNA (CXCR4) SH-siRNA (CD4) SH-siRNA (Luc)	SWNT-PL-PEG ₂₀₀₀ -SH SWNT-PL-PEG ₅₄₀₀ -SH	Human T cells and peripheral blood mononuclear cells	29	
<i>In vivo</i>	DNA	FAM-dsDNA-NH ₂	SWNT-COOH	Tumour-bearing mice	20
	RNA	si-RNA (murineTERT)	SWNT-CONH-(CH ₂) ₆ NH ₃ ⁺	Tumour-bearing mice	20
		si-RNA (humanTERT)			

of pDNA and expression of β -galactosidase (marker gene) in CHO cells was

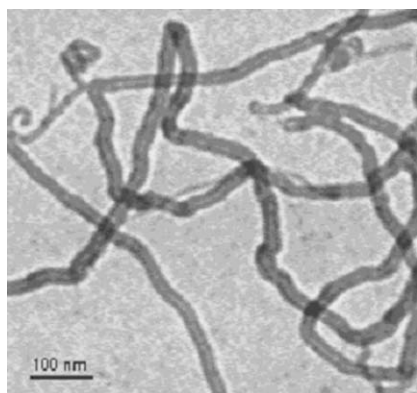


Fig. 1 Water soluble MWNT functionalised through the organic 1,3-dipolar cycloaddition reaction.

found to be approximately 10 times higher than naked pDNA alone. Furthermore, it was found that the charge ratio (+/-) CNT-NH₃⁺/pDNA was an important factor determining the level of gene expression. Very interestingly, on a more fundamental level, we also observed that CNT had the capacity to 'pierce' the plasma membranes in a manner previously unreported.¹¹ These studies initiated a number of independent investigations from various groups with the aim of CNT-mediated gene delivery.

The next study to reproduce the concept was that by Liu *et al.*¹⁵ who reported the non-covalent association of pDNA with CNT functionalised with polyethyleneimine (PEI) groups, which contain a high density of terminal amine

groups, an overall highly cationic macromolecule commonly used as a transfection agent. The CNT-PEI/pDNA complexes tested at different charge ratios in different cell lines were shown to produce much higher levels of gene expression than pDNA alone. Furthermore, it was reported that the transfection efficiency with the CNT-PEI constructs was about 3 times higher than with PEI alone, postulating a similar endosome-mediated intracellular uptake. However, the exact mechanism leading to the reported upregulation of gene expression by the CNT-PEI conjugate was not identified.

Later, work reported by Gao *et al.*¹⁶ elucidated that the surface functionalisation group of the CNT also plays a role

in the formation of the complexes between the CNT and pDNA. That team studied the gene delivery of pDNA by CNT functionalised with four different chemical groups: amino, carboxyl, hydroxyl and alkyl. They found that only the positively charged, amine-functionalised CNT were able to complex and deliver the pDNA. Similar to our own original observations, this report described that complexes between CNT and pDNA enhanced the gene delivery and expression compared to naked DNA alone, however they did not improve the transfection efficiency compared to a commercially available transfection agent (Lipofectamine 2000).

Cai *et al.*¹⁷ proposed a new strategy to introduce exogenous pDNA immobilised on CNT into mammalian cells. The CNT used in these studies contained Ni particles enclosed in their tips, which allowed a magnetic 'spearing' technique (exposure to an external magnetic field followed by centrifugation) onto the CNT–pDNA complexes. The results from these investigations reported gene expression in 80–100% of the cell population for the CNT with Ni, while CNT deprived of Ni particles did not produce any gene expression. Although, the use of such technique is limited to *in vitro* or *ex vivo* gene transfer, this was the first work to utilise the plasma membrane 'piercing' effect that we originally proposed in an alternative experimental setup and that can lead to very high gene expression.

Interestingly, CNT have been shown to be able to deliver exogenous genes not only into mammalian cells but also into bacteria. Rojas-Chapana *et al.*¹⁸ demonstrated that oxidised, water-dispersible CNT can deliver pDNA into *E. coli* (ratio of transformation efficiency/transformants of about 32) by opening up temporary nanochannels across the cell envelope. The authors described that addition of CNT in a suspension containing *E. coli* and pDNA and application of a microwave frequency¹⁹ resulted in the orientation of the CNT tips perpendicularly to the cell surface and subsequently plasmid delivery into the bacteria. In the authors' own words "CNT appear to be *needle-pricking the cells*". Again the concept of cell wall 'piercing' was proposed to explain the observations made in these studies. Zhang *et al.*²⁰ have confirmed that

CNT functionalised with short dsDNA are internalized by different murine tumour cell lines without any associated cytotoxic effects. The conjugates were tracked by fluorescence microscopy, since the dsDNA was fluorescently labeled. This work did not describe gene expression using this dsDNA. Moreover, very recently our original observation and subsequent hypothesis of CNT acting as 'nanoneedles' of the plasma membrane has been almost identically reproduced using CNT non-covalently functionalised with block copolymer molecules interacting with microglia cells.²¹ The work accumulating gradually by different groups is confirming our proposals that novel, very interesting mechanisms other than endocytosis are contributing to the high levels of cellular internalisation of CNT.

There have also been a few published studies using single-stranded DNA (ssDNA) as the CNT coating material to render the CNT water dispersible. Of course such nucleic acids do not encode for exogenous genes, therefore are not relevant to gene delivery applications, however intracellular translocation of the ssDNA is reported. Kam *et al.* have reported the translocation of ssDNA into the nuclei of HeLa cells by two different CNT structures: (a) Cy3-labeled ssDNA directly linked to CNT through a non-covalent adsorption^{22,23} and (b) Cy3-labeled ssDNA covalently linked to a polyethylene glycol chain that was part of a phospholipid molecule which was adsorbed onto the CNT.²⁴ These studies reported that ssDNA is released from the CNT after NIR excitation of the CNT²³ or after enzymatic cleavage of the disulfide bonds²⁴ and translocated into the cell nucleus. The authors postulate endocytotic uptake of the whole CNT–ssDNA constructs. Heller *et al.*^{25,26} have also reported the cellular internalisation of CNT coated by ssDNA. Although the long-term goal of this team is to develop resilient optical sensors based on CNT for *in vitro* and *in vivo* applications, they have used ssDNA to achieve the solubilisation of the CNT in aqueous solutions and demonstrated an endocytosis-dependent internalisation and perinuclear localisation without penetration into the nuclear envelope of the CNT–ssDNA complexes incubated with mammalian cells.

More recently, Becker *et al.*²⁷ have reported the importance of length on the corresponding toxicity of CNT–ssDNA complexes that are uptaken into cells (approx. 200 nm). This report showed length-dependent cellular uptake by fluorescence microscopy. Longer CNT were labelled with Cy3-ssDNA and found to be excluded from internalisation by cells, while shorter tubes labelled with Cy5-ssDNA were found in the cytoplasm. Although this study was oriented towards the length-dependent toxic effects of CNT, one can conclude that CNT–ssDNA complexes are able to internalise into a variety of cell lines. What should be highlighted however from these studies using CNT dispersions coated with macromolecules is that the surface coating on the CNT, ssDNA or PEGylated lipid in the cases above, is—as expected—critically important in the interactions between cells and CNT. The endocytotic mechanism of uptake reported is thought to be a result of the nucleic acid or lipid coat on the CNT surface recognised by the cells instead of the CNT backbone.

2.1.2 RNA transfection. CNT were first shown to transport non-functional (biologically) RNA into mammalian cells by Lu *et al.*²⁸ The authors reported the cellular uptake of CNT–RNA polymer poly(rU) hybrids formed through non-specific binding with the CNT. However, this study reported only the cellular uptake and the cytotoxicity of the CNT–RNA constructs; the propidium iodide labelled RNA was simply used to fluorescently label the CNT–RNA hybrids and follow their cellular internalisation by fluorescence microscopy. No biological function was reported in that work.

In a subsequent publication, Kam *et al.*²⁴ have studied a siRNA–CNT conjugate linked with disulfide bonds to PEGylated lipids coating the CNT surface, and have shown that the conjugates were internalised into HeLa cells and also reported siRNA-mediated gene silencing. In these studies, different siRNA constructs were successfully delivered by CNT, and two targeted genes were silenced: (a) the gene encoding lamin A/C protein (localised inside the nuclear lamina of cells) and (b) the luciferase gene. This work also compared

the gene silencing efficiency between the siRNA–CNT conjugates and the more conventional Lipofectamine–siRNA complexes to find that the CNT improved the degree of gene silencing.

The work reported by Zhang *et al.*²⁰ pushed the development of CNT as siRNA delivery systems further, as they employed ammonium-functionalised CNT to mediate the delivery of *TERT*siRNA into tumour cells (murine and human) and silence the *TERT* gene, which is critical for the development and growth of tumours. They observed that treatment of different tumour cells with the CNT–*TERT*siRNA complexes led to the suppression of the cell growth. More recently, and with an alternative therapeutic endpoint, Liu *et al.*²⁹ have shown the delivery of siRNA molecules able to silence the expression of the cell-surface receptors CD4 and co-receptors CXCR4 in human T cells and peripheral blood mononuclear cells by CNT. Because both cell-surface receptors are necessary for HIV access and infection of T cells, the authors have suggested that siRNA–CNT conjugates can potentially be used for the treatment of HIV. Furthermore, they observed that conjugates of CNT covalently linked by disulfide bonds to siRNA had greatly improved the silencing in T cells compared to Lipofectamine 2000 and other liposome-based formulations.

Fig. 2 schematically summarizes the general strategies that have been described to introduce nucleic acid–CNT conjugates into cells, regardless of the structure of the nucleic acid and the achievement of gene expression (DNA) or protein silencing (siRNA). The main internalisation pathways to accomplish cellular delivery of nucleic acids by CNT are as follows (Fig. 2): phagocytosis of nucleic acids covalently linked to CNT²⁹ (A), injection of nucleic acids through nanochannels formed by CNT¹⁸ (B), ‘penetration’ or ‘piercing’ of nucleic acids adsorbed on the surface of CNT¹⁷ (C_I) or complexed with CNT by electrostatic forces^{11,12} (C_{II}); and finally, endocytosis of nucleic acids electrostatically complexed¹⁵ (D_I), covalently linked²⁴ (D_{II}) or adsorbed^{22,23,25} (D_{III}) to CNT. The mechanisms suggested to describe intracellular release of the nucleic acid from the CNT are illustrated as well in Fig. 2 as follows: electrostatic dissociation of the complexes^{11,12,15,16} (E); enzymatic cleavage of the disulfide linkages that hold the nucleic acid onto the CNT²⁴ (F); and lastly, nucleic acid release following excitation of the CNT with NIR radiation²³ (G).

2.2 *In vivo* models

2.2.1 DNA transfection. Very interestingly, there are hardly any studies in

the literature describing the *in vivo* gene expression by CNT-mediated delivery of pDNA. Zhang *et al.*²⁰ have recently injected FAM-labeled dsDNA–CNT conjugates into rodents. The CNT–dsDNA conjugates consisted of CNT functionalised with ssDNA, which later were annealed with the complementary ssDNA labeled with FAM. These conjugates were intratumorally administered in mice bearing Lewis lung carcinoma tumours. The study has confirmed the uptake of the conjugates by observing that almost all tumour cells were fluorescent, however, the experiment was performed only for the purpose of *in vivo* imaging and tracking of the CNT–DNA. No further attempt has been reported today to deliver gene expressing DNA in live tissue.

2.2.2 RNA transfection. The *in vitro* studies described above using CNT–siRNA complexes by Zhang *et al.*¹⁹ were also extended to *in vivo* studies in the same report. The activity of CNT–*TERT*siRNA was verified after intratumoral injections in mice bearing Lewis lung carcinoma tumours or HeLa cell xenografts. Tumour growth was inhibited after treatment with CNT–*TERT*siRNA complexes and average tumour weight was significantly reduced when compared to that of tumours from untreated animals. Moreover, they reported injection of the CNT–*TERT*siRNA complexes at multiple sites in the tumour mass resulted in more effective treatment than injections at a single injection point. This work is the first and only report† currently in the literature that showed how CNT can offer a promising new technology for the development of advanced cancer treatment applications. However, much more work is definitely needed and will surely appear in order to explore and carefully define the opportunities and limitations of CNT as delivery systems of nucleic acids using *in vivo* models.

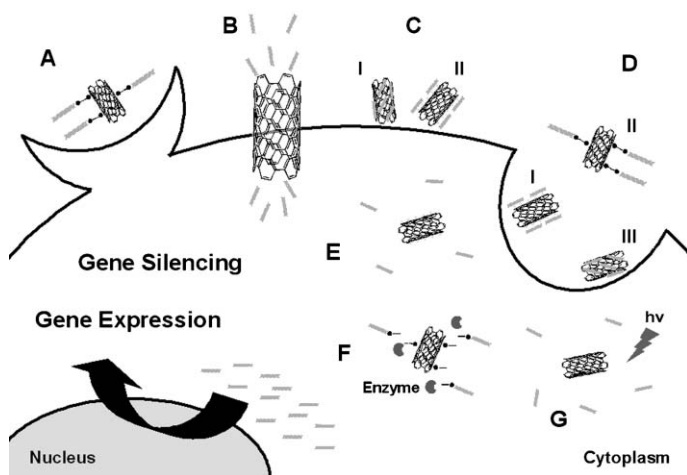


Fig. 2 Suggested strategies for cellular delivery of nucleic acids by CNT: phagocytosis of nucleic acids covalently linked to CNT²⁹ (A), injection of nucleic acids through CNT nanochannels¹⁸ (B), penetration of nucleic acids adsorbed on the surface of CNT¹⁷ (C_I) or complexed with CNT by electrostatic forces^{11,12} (C_{II}); and endocytosis of nucleic acids electrostatically complexed¹⁵ (D_I), covalently linked²⁴ (D_{II}) or adsorbed^{22,23,25} (D_{III}) to CNT. Suggested strategies for intracellular release of the nucleic acid from the CNT: electrostatic dissociation^{11,12,15,16} (E), enzymatic cleavage^{24,29} (F) and radiation²³ (G).

† The paper by Yang *et al.*, Single-walled carbon nanotubes-mediated *in vivo* and *in vitro* delivery of siRNA into antigen-presenting cells, published online on 6 July 2006 in *Gene Therapy*, was recently retracted in *Gene Therapy*, 2007, **14**, 920. doi:10.1038/sj.gt.3302962.

3. Future work and directions

Several biomedical applications of CNT have been proposed in the last few years. Concerning the utilisation of CNT in the delivery of nucleic acids, the encouraging findings described so far in the literature are highlighted herein. CNT are versatile platforms for nucleic acid delivery *in vitro* and *in vivo* because of their high surface area, facile functionalisation of their surface, and their ability to cross the cell membranes. To our knowledge, it is crucial to functionalise the surface of CNT, in order to transform non-functionalised CNT (insoluble in most solvents) into water-soluble and biocompatible CNT with an improved toxicity profile. On the other hand, with a growing number of functionalisation routes, many important questions remain unanswered. Each functionalisation method is probably producing CNT with different characteristics, which will lead to differences in the mechanism of CNT metabolism, degradation or dissolution, clearance and bioaccumulation. On the other hand, most non-viral gene delivery systems today suffer from both limited levels of gene expression and an unfavorable toxicity profile due to their highly cationic surface character. Therefore, opportunities for CNT-based gene transfer systems are still ample.

The development of CNT as delivery systems for nucleic acids is still in its nascent stages and many more studies are needed to determine the advantages and limitations offered by these novel materials. Most challenges are still ahead for the biomedical utilisation of CNT. The administration or implantation of CNT and their complexes with gene encoding or silencing constructs in an *in vivo* setting are almost completely unexplored, despite the groundbreaking work by several groups.³⁰ Moreover, the toxicological and pharmacological profiles of CNT systems used as therapeutics will have to be better elucidated prior to any clinical studies undertaken. In conclusion, we would like to stress that although we are still in very early stages

of development, nucleic acid delivery systems based on CNT may well become viable and effective, particularly in combination with their electrical or semiconducting properties and an improved toxicity profile.

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