



Carbon nanotubes as vectors for gene therapy: Past achievements, present challenges and future goals[☆]

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ABSTRACT

Promising therapeutic and prophylactic effects have been achieved following advances in the gene therapy research arena, giving birth to the new generation of disease-modifying therapeutics. The greatest challenge that gene therapy vectors still face is the ability to deliver sufficient genetic payloads in order to enable efficient gene transfer into target cells. A wide variety of viral and non-viral gene therapy vectors have been developed and explored over the past 10 years, including carbon nanotubes. In this review we will address the application of carbon nanotubes as non-viral vectors in gene therapy with the aim to give a perspective on the past achievements, present challenges and future goals. A series of important topics concerning carbon nanotubes as gene therapy vectors will be addressed, including the benefits that carbon nanotubes offer over other non-viral delivery systems. Furthermore, a perspective is given on what the ideal genetic cargo to deliver using carbon nanotubes is and finally the gene-pharmacological impact of carbon nanotube-mediated gene therapy is discussed.

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1. Introduction

Over the past few decades, recent advances in molecular biology combined with the completion of the Human Genome Project have greatly improved our understanding of the genes involved in cellular processes and disease pathogenesis. Furthermore, both small molecular assays and high-throughput screening techniques have aided the

identification of countless genomic targets of various genetic and acquired disorders. Tremendous interest has been directed into treating diseases by introducing nucleic acids to regulate, repair, replace, add or delete a particular genetic target responsible for the manifestation of a disease. The therapeutic and prophylactic effects accomplished by successful gene therapy have given rise to the next generation of disease-modifying medical interventions, whereby a wide range of therapeutically active nucleic acids including small-interfering ribonucleic acid (siRNA), micro-ribonucleic acid (miRNA), antisense oligonucleotides (ODNs), short hair-pin ribonucleic acid (shRNA), plasmid DNA (pDNA) and RNA/DNA aptamers, have been used to manipulate gene expression at the post-transcriptional or translational level.

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As alluring as the concept of gene therapy is, not only the most important but also the most difficult challenge is the issue of gene delivery. An important prerequisite for gene therapy vectors is the ability to overcome extracellular barriers, including *in vivo* clearance mechanisms and protection of the nucleic acid cargo from degradation, while achieving specific targeting of cells or tissues. Subsequent surmountable cellular barriers include cellular uptake mechanisms, endosomal escape, nuclear entry and nucleic acid release. An ideal gene delivery vehicle should be both efficient and safe, although other characteristics are also essential including target cell specificity, efficacy and (depending on the disease indication) ability to induce sufficiently-lasting effects.

Gene therapy can be achieved by employing either viral or non-viral vectors for nucleic acid delivery. Viral vectors can achieve high transfection efficiencies and efficacy. However, their application in the clinical setting is hindered due to immunogenicity and oncogenicity concerns, poor capability to target specific cell populations and limited capacity of genetic payload [1]. Alternatively, non-viral delivery vectors exhibit particular advantages over viral vectors in terms of relative safety, the ability to deliver genes without any size limitation and the potentially facile upscale for pharmaceutical production. However, non-viral gene delivery methods have not been as successful clinically compared to their viral counterparts, due to various limitations including low transfection efficiencies and poor transgene expression [2]. The past two decades have witnessed dramatic developments in the application of nanoscience in gene therapy research, whereby various vectors have been employed in order to improve gene transfer efficacy. A plethora of nanovectors have been explored as gene therapy vehicles, including but not limited to: cationic liposomes, polymers, dendrimers, nanoparticles, peptides and carbon nanotubes (CNTs). This review will focus on the contribution of carbon nanotube-based vector technology in gene therapy, its past achievements and present challenges, with a view to offer a perspective on the direction and goals that may allow further clinical translation of this alternative technology.

2. Benefits that carbon nanotubes offer over other non-viral delivery systems

Therapeutic delivery of nucleic acids *in vivo* is challenging for a number of reasons, including lack of stability against endogenous enzymes, poor pharmacokinetic profile, and inherent incapacity to transverse cellular membranes. Carbon nanotubes (CNTs) have been utilized for various applications, including the delivery of nucleic acids for the purpose of gene therapy. CNTs consist exclusively of carbon atoms arranged in condensed atomic rings which in turn are organized in one (single-walled carbon nanotubes; SWNTs) or more (multi-walled carbon nanotubes; MWNTs) concentric sheets rolled up into cylinders. For the various applications of CNTs it is necessary to chemically tailor the outer surfaces of the CNTs in order to maximize on their unique properties [3,4]. The unusual properties of CNTs, in particular their distinctive length-to-diameter ratio, propensity to act as a template for chemical functionalization strategies and biocompatibility, make them promising candidates as molecular transporter systems. Pristine CNTs are notoriously difficult to disperse, especially in aqueous media and so various types of surface functionalizations (both covalent and non-covalent in nature) not only act to increase the solubility but also improve the biocompatibility and the propensity to deliver nucleic acids both *in vitro* and *in vivo* [4]. Fig. 1 depicts the versatility of CNTs as gene therapy vectors; pristine single-walled or multi-walled carbon nanotubes are the structural carcass upon which surface modifications can be performed to generate either chemically functionalized CNTs (for example carboxylated or aminated functionalities) or coated CNTs whereby physical adsorption of molecules is performed (for example the addition of proton rich polymers polyethylenimine (PEI) and poly(amidoamine) dendrimers (PAMAM)). The vast numbers of possibilities for CNT surface modifications make

them ideal for delivering a whole host of nucleic acids, most commonly plasmid DNA, siRNA, ODNs and aptamers.

2.1. Length-to-diameter ratio

It has been suggested that the overall size and length-to-diameter ratio of CNTs is important in determining their biocompatibility and consequently their viability as gene therapy vectors. CNT diameter varies from 0.4 to 2 nm for SWNTs and from 1.4 to 100 nm for MWNTs, while the length can reach several micrometers for both types. Furthermore, transmission electron microscopy (TEM) imaging of aqueous dispersions of SWNTs shows that they form bundles held together due to strong van der Waals interactions, whereas dispersions of MWNTs can result in better quality, individualized nanotube populations [4]. Both single-walled and multi-walled carbon nanotubes have been used as nucleic acid delivery vectors, however the most notable advancements have been achieved with multi-walled carbon nanotubes. A key question that needs to be addressed in order to further our knowledge and determine the selection criteria regarding the appropriateness of different types of carbon nanotubes as gene therapy vectors, is whether length-to-diameter ratio affects gene transfer capabilities (*i.e.* transfection efficiency). The little that is known about the importance of length-to-diameter ratio on gene transfer efficiency is that nanotube surface area, among other factors including charge density, is a critical parameter that determines the complexation of nucleic acids with CNTs [5]. This therefore raises the further question over how different types of surface modification may affect the transfection efficiency of these vector systems.

2.2. Surface modification

Even though some success in gene transfer has been reported with pristine carbon nanotubes [6], they are not restricted to their pristine (as-prepared) form and have been surface modified in multiple ways in order to overcome the challenge of aqueous dispersibility and at the same time improve their transfection efficiency, as illustrated for simplicity in Fig. 1. Multiple studies have explored different avenues of modifying the surface of carbon nanotubes for the improved delivery of nucleic acids including plasmid DNA [5,7–17], siRNA [6,18–32], miRNA [33], ODNs [34–36] and aptamers [37] into mammalian cells. Delivery of nucleic acids into cells must first cross the plasma cell membrane, and in the case of DNA must also translocate more intracellular barriers. The first *in vitro* account of nucleic acid delivery utilized positively charged, covalently amino-functionalized single- and multi-walled carbon nanotubes that effectively delivered negatively charged plasmid DNA intracellularly [7]. This concept has since been adopted in many different studies exploring the transfection capabilities of CNTs via delivery of nucleic acids both *in vitro* and *in vivo* [5,8,18–20]. Along with amino-functionalized carbon nanotubes, carboxylated carbon nanotubes have also been reported for gene transfer [21,34,37]. Due to electrostatic repulsion forces between the nucleic acid cargo and these carboxyl-coated CNTs, nucleic acids must first be amino-modified and then covalently coupled to the carboxylated CNTs for cellular delivery [34,37]. Using an alternative approach, Liu et al. were the first to demonstrate that CNTs can be chemically functionalized with other molecules used as gene transfection agents. More specifically, PEI functionalized MWNTs were used to deliver plasmid DNA to a panel of different mammalian cells (COS7, HepG2, 293 cells) [9]. Rich in amine groups, the PEI polymer itself is a versatile non-viral vector owing to its 'proton sponge effect' properties [38]. Hence, cationic polymer PEI grafted MWNTs (gMWNTs) were able to securely immobilize negatively charged pDNA onto the surface of CNTs and prevent lysosomal degradation, with transfection efficiencies similar to or even several times higher than that of PEI alone, and several orders of magnitude higher than that of naked pDNA [39]. Following this, enhanced transfection efficiencies of carboxylated MWNTs grafted

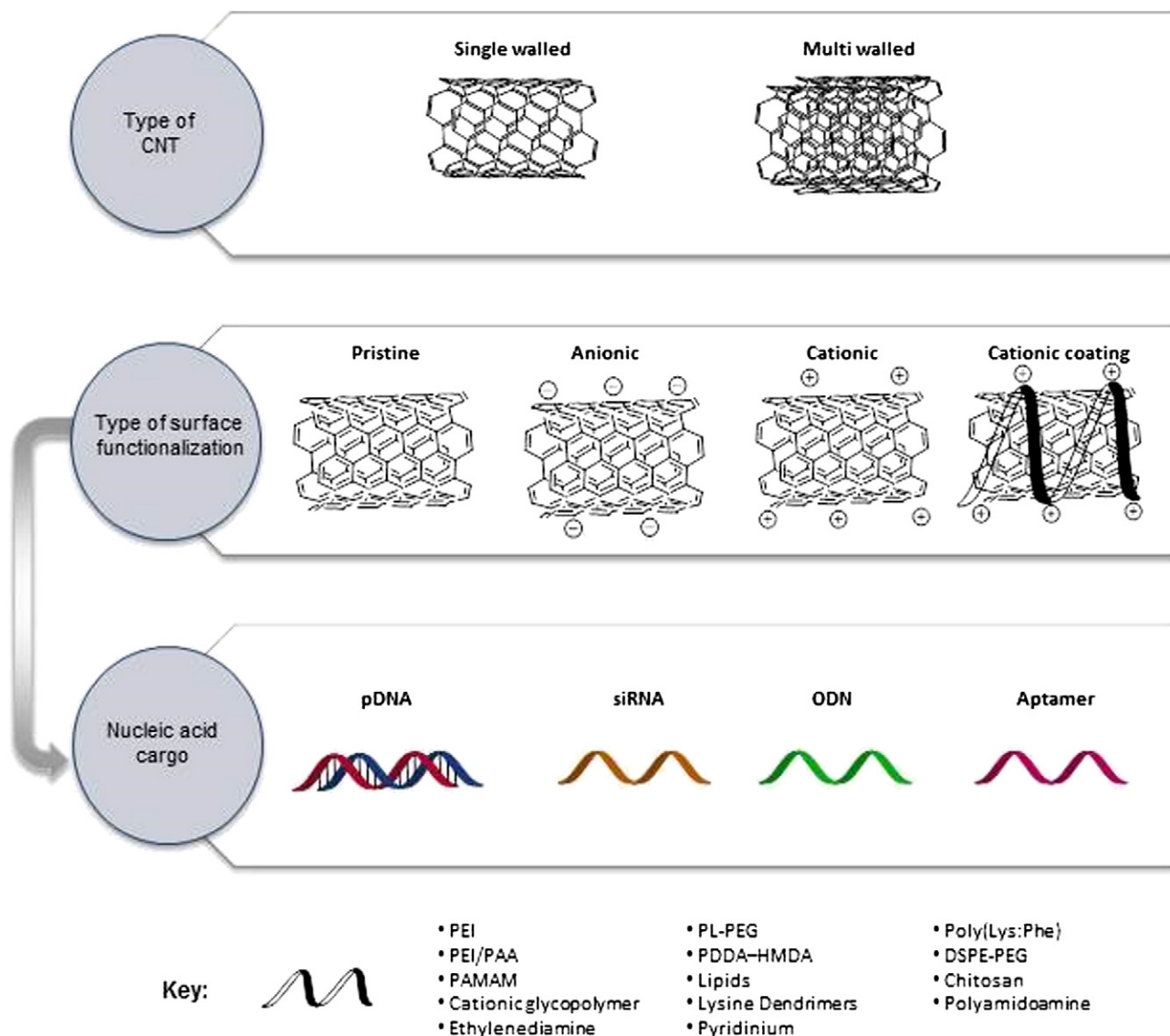


Fig. 1. Possible combinations of carbon-mediated nucleic acid delivery; types of CNT, surface modifications and nucleic acid cargos. Single-walled or multi-walled carbon nanotubes are the structural backbone upon which numerous surface modifications can be performed in order to develop efficient gene therapy vectors. CNTs may be used in their pristine form (non-modified) or surface modifications may be performed, including chemically modified functional groups (generating either cationic or anionic surface charges) or non-covalent cationic surface coatings. The extensive number of possible CNT surface modifications makes them ideal for delivering a whole host of nucleic acids, most commonly plasmid DNA (double stranded red and blue structure), siRNA (single-stranded orange structure), ODNs (single-stranded green structure) and aptamers (single-stranded pink structure). The key details are the different types of cationic coatings that have been adsorbed onto the surface of CNTs.

with PEI, PAA (polyacrylic acid) or PEI/PAA were further demonstrated whereby the delivery of pDNA mediated by PEI-MWNTs and PEI-PAA-gMWNTs showed significant improvements in gene transfection and consequent gene expression compared to both naked pDNA and PEI/PAA polymers alone [10]. A series of further studies using PEI functionalized carbon nanotubes have reported successful nucleic acid delivery [11,22,23,33,35] and it may well be developed into the next generation of gene transfection agent for *in vitro* use. Furthermore, in support of CNT functionalizations for improved nucleic acid delivery, a comparison study illustrated that a cationic dendrimer, PAMAM, can also be considered a beneficial functionalization for CNTs. Similar to PEI, PAMAM also possesses the 'proton sponge effect' [40]. Although the transfection efficiencies of MWNT-PAMAM with pDNA constructs are still lower than Lipofectamine 2000, the cytotoxicity of the hybrid decreased to 91% when compared with commercially available Lipofectamine 2000 [12,35]. Other cationic surface functionalizations include cationic glycopolymers [13], organic compounds with amine

functional groups such as ethylenediamine [14] and poly(diallyldimethylammonium)chloride-hexamethylenediamine (PDDA-HMDA) [24], phospholipid-PEG conjugates [25,26], lipids and lysine dendrimers [27] including 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)] (DSPE-PEG-amine) [28], polyamino-acid sequences such as Poly(Lys:Phe) [29], chitosan [35] and polyamidoamine dendrimers [36]. All of these surface functionalizations act to increase the surface charge density of the carbon nanotubes thus increasing the propensity to complex with nucleic acids and improve gene transfer efficiency *in vitro*. However, their translation *in vivo* is considered problematic, mainly due to the high electrostatic surface charges necessary for complexation.

3. The ideal genetic cargo to deliver using carbon nanotubes

After more than two decades of preclinical and clinical experience, some desired properties that effective non-viral gene therapy vectors

should possess are largely accepted upon. These must fit into the demanding criteria for all gene therapy vectors, including sufficient genetic cargo loading, cellular translocation, intracellular off-loading of cargo by maintaining biological activity, controllable gene expression and little/no immunogenicity/toxicity. However, less attention has been given to the correlation between vector characteristics and the choice of genetic cargo to be delivered into target cells. There is a whole plethora of possible genetic cargos to deliver, including siRNA, shRNA, miRNA, pDNA, ODNs and DNA/RNA aptamers. A key question therefore is what will be the most appropriate genetic cargo to deliver using carbon nanotubes that will match their inherent structural characteristics and interactions with cells. Table 1 gives an exhaustive overview of the carbon nanotube-mediated nucleic acid delivery studies both *in vitro* and *in vivo*.

3.1. plasmid DNA

As mentioned above, the first type of nucleic acid to be transfected successfully by carbon nanotubes *in vitro* was plasmid DNA by Pantarotto et al. [7]. Further than that, various types of surface modified carbon nanotubes (both single-walled and multi-walled) have been used to deliver plasmid DNA, chemically functionalized with amine groups [5,8], cationic glycopolymers [13], ethylenediamine [14], PEI [9–11] and PAMAM hybrids [12] (Table 1, plasmid DNA subsection). Notably, the scope of genes transfected is rather limited, with the majority of studies transfecting either green fluorescent protein (GFP) [8,11,12,15,16], luciferase or beta-galactosidase (β -gal) [7,10] marker genes, chosen for the ease and simplicity of their biological read-outs. Interestingly, although there are multiple studies reporting successful transfection of pDNA *in vitro*, there is very limited evidence to date of *in vivo* translation of these findings.

In choosing a genetic cargo to deliver, the mechanism of action must be carefully considered. It has become evident that carbon nanotubes carrying pDNA are endocytosed, followed by transgression of the endosomal membrane, DNA off-loading, nuclear translocation, DNA transcription and subsequent protein translation [41]. Since successful transfer of pDNA requires achievement of all the above challenges, it may explain the limited success seen with carbon nanotube mediated delivery of pDNA *in vivo*. Carbon nanotube mediated delivery of pDNA, in the form of nanoparticle–carbon nanotube (NP–CNT) hybrids has recently been reported in an *in vivo* canine model of restenosis [17]. The NP–CNT hybrids consist of PAA coated carboxylated single-walled carbon nanotubes complexed *via* electrostatic interactions with cationic nanoparticles containing pDNA encoding for angiopoietin 1 (Ang1) and vascular endothelial growth factor (VEGF), all of which were embedded in an implantable hydrogel thus creating a bioactive hydrogel stent, as shown in Fig. 2. In a canine femoral artery balloon-injury model, the intravenous implantation of these bioactive hydrogel stents significantly enhanced re-endothelialization of injured arteries [17].
















3.2. siRNA and mRNA

RNA interference (RNAi) is a post-transcriptional gene silencing process induced by small interfering RNAs (siRNAs) and micro-RNAs (miRNAs) [42]. Research into the therapeutic application of RNAi gained momentum after the seminal discovery in 2001 by Tuschl's group that synthetic 21-nucleotide siRNAs could trigger RNAi in mammalian cells [43]. The mechanism of RNAi is relatively simple, where initially small RNA molecules are introduced into the cytoplasm that then interact with the effector complex RNA-induced silencing complex (RISC). The interaction between the siRNA and the RISC complex leads to siRNA unwinding and consequently single-stranded RNA hybridization with the messenger RNA (mRNA) target. The hybridization causes nucleolytic degradation of the target mRNA by the RNase H enzyme Argonaute 2, ultimately leading to gene silencing. Rapid advances have been made in the development of siRNA therapeutics since the

first clinical trial of an siRNA-based drug began in 2004, while several others are currently under clinical evaluation [44]. siRNA constructs possess many valuable characteristics as gene therapy candidates, however they also have demonstrable weaknesses. Intravenous administration of unmodified siRNAs is problematic since they are unable to circulate sufficiently in the bloodstream ($t_{1/2} = \sim 15$ min) [45]. Furthermore, siRNAs may trigger a deleterious interferon response caused by innate immune system activation [46]. In addition, the delivery of siRNAs to target cells is difficult while ensuring that induction of unwanted off-target effects does not occur [47–49]. A proof-of-concept study by Kam et al. reported promising CNT-mediated RNAi data *in vitro* [25]. These investigations were performed using coated single-walled nanotubes (SWNTs) by adsorption of PEG-phospholipids (DSPE-PEG) with terminal amine or maleimide groups, which were then linked with siRNA by disulphide bonds [25]. Intracellular uptake of the nanotube conjugates saw the disulphide bonds cleaved and the siRNA cargo released, achieving more potent RNAi effects than the commercial transfection agent Lipofectamine. Since that study, considerable evidence has been obtained indicating that CNTs are viable platforms for delivering biologically active siRNA into cells both *in vitro* and *in vivo* [6,18–24,26–32]. Much of the earlier research using carbon nanotube-mediated siRNA delivery focused on silencing genes that suppressed the growth and proliferation of cancer cells. Pristine SWNTs delivering siRNA sequences (siTOX; a commercially available toxic siRNA sequence that induces cell death and siHif-1 α ; an siRNA encoding for hypoxia-inducible factor 1- α) with the aim to reduce cell viability and induce cell death in a range of *in vitro* cancer cell lines reported biological activity and a high degree of specificity [6]. Delivery of siRNA *in vitro* by surface-modified SWNTs into breast cancer cells achieved high transfection efficiencies and aided the inhibition of cancer cell proliferation and promoted apoptosis [28]. Similar results have been reported from various other cancer cell lines with knockdown of mRNA reaching around 40% and protein levels reduced by 70% [18,22,23,29,32]. Biological activity that translated to therapy was reported in the first proof-of-concept study using amino-functionalized MWNT:siRNA constructs in an *in vivo* human lung cancer xenograft model. Intratumoral administration of the MWNT:siRNA complexes resulted in biologically active siTOX, a cytotoxicity-inducing siRNA sequence, leading to delayed tumor growth and increased survival of the tumor-bearing animals [19]. Although much progress has been made using nanotube siRNA delivery for the treatment of cancer, many other therapeutic applications are emerging (Table 1, siRNA and miRNA subsections). Carbon nanotubes have been shown to transfect notoriously difficult cell types, including primary cardiomyocytes [24], skeletal muscle cells [31] and T cells [26]. PDDA–HMMA functionalized SWNTs effectively transfected primary cardiomyocyte cells with an siRNA encoding for extracellular signal-regulated kinase (siERK), achieving $\sim 75\%$ knock-down of extracellular signal-regulated kinase (ERK) target proteins and expanding the therapeutic potential of CNT-mediated RNAi applications for the treatment of heart conditions [24]. Furthermore, confirmation of successful knock-down of target mRNAs *in vitro* using functionalized carbon nanotubes has also been observed in skeletal muscle ($\sim 40\%$ reduction in the level of transient receptor potential cation channel (subfamily C, member 3) (TRPC3) protein level following transfection) [31], with the potential for the treatment of insulin-resistant conditions. Moreover, efficient RNAi of two different target mRNAs, chemokine receptor type 4 (CXCR4) and the glycoprotein, cluster of differentiation 4 (CD4), expressed in peripheral blood mononuclear cells (PBMCs) and human T cells respectively, were knocked down by approximately 60% compared to control levels. This proved that SWNTs functionalized with phospholipid-PEG (PL-PEG₂₀₀₀-NH₂⁺) have superior delivery capabilities over several existing non-viral transfection agents [26]. Further work systematically developing chemically functionalized CNTs has shown that amino-functionalized CNTs successfully condense negatively charged siRNA without the need for linker molecules, and efficiently

Table 1

Carbon nanotube-mediated nucleic acid transport and their therapeutic applications. In chronological order, studies are grouped by the type of nucleic acids delivered by CNTs: plasmid DNA, siRNA and miRNA, ODNs and aptamers. The type of CNT functionalization, the disease model (*in vitro/in vivo*) and main outcomes are summarized.

Category	Disease	Carbon nanotube (CNT)	CNT functionalization	Nucleic acid	<i>In vitro/in vivo</i>	Results	Ref.	
Plasmid DNA 	Cancer		<i>f</i> -SWNTs & <i>f</i> -MWNTs (NH ₂ -SWNTs, NH ₂ -MWNTs)	Plasmid DNA (p β -gal)	<i>In vitro</i> : •HeLa cells (cervical cancer) •CHO cells	•First example of CNT-mediated gene delivery into mammalian cells. • <i>f</i> -SWNTs facilitated higher pDNA uptake and gene expression <i>in vitro</i> than pDNA alone.	[7]	
	Cancer		•SWNT-NH ₃ ⁺ •MWNT-NH ₃ ⁺ •SWNTLys-NH ₃ ⁺	Plasmid DNA (pCMV- β gal)	<i>In vitro</i> : •A549 cells	•All three <i>f</i> -CNTs exhibited up-regulation of marker gene expression over naked DNA.	[5]	
	Varied application		<i>f</i> -MWNTs (PEI-g-MWNTs)	Plasmid DNA (pCMV-Luc gene report)	<i>In vitro</i> : •COS7 •HepG2 •293 cells	•PEI-g-MWNTs showed high transfection efficiencies: more than 3 × higher than PEI alone and 4 orders of magnitude higher than naked pDNA.	[9]	
	Cancer		<i>f</i> -MWNT (NH ₂ -MWNTs)	Plasmid DNA (pEGFPN1)	<i>In vitro</i> : •HUVECs •A375 (human melanoma)	•NH ₂ -MWNTs interact with plasmid DNA and deliver the GFP gene in cultured cells. Although transfection efficiency was low, ~5%.	[8]	
	Proof-of-concept		Vertically aligned CNTs by PECVD	Plasmid DNA (pEGFP)	<i>Ex vivo</i> (primary cell culture): •Bal17 (B-lymphoma) <i>Ex vivo</i> splenic B cells •Primary cortical neurons	•Highly efficient delivery technique, named “nanotube spearing”. •Unprecedented high transduction efficiency in difficult to transfect cells.	[15]	
	Proof-of-concept		Nanotube loaded electrodes (CNT/PEI-COOH-SAM, CNT/NH ₂ -SAM)	Plasmid DNA (pEGFP-C1)	<i>In vitro</i> : •HEK293 cells (adherent human embryonic kidney cells)	•CNT-loaded electrodes successfully transfected adsorbed pDNA into adherent cells.	[11]	
	Cancer			<i>f</i> -SWNTs (cationic glycopolymer)	Plasmid DNA (pEGFP)	<i>In vitro</i> : •HeLa cells (cervical cancer)	•Copolymer <i>f</i> -SWNTs are found to be biocompatible and exhibit transfection efficiencies comparable to Lipofectamine 2000.	[13]
	Cancer			<i>f</i> -MWNTs (PEI-g-MWNT and/or PEI/PAA-g-MWNT)	Plasmid DNA (pCMV- β -gal)	<i>In vitro</i> : •A549 cells (lung carcinoma)	•PEI-g-MWNT and/or PEI/PAA-g-MWNT showed higher transfection efficiency than either naked pDNA or PEI alone.	[10]
	Cancer			<i>f</i> -MWNTs (PAMAM hybrids)	Plasmid DNA (pEGFP-N1)	<i>In vitro</i> : •HeLa cells (cervical cancer)	•MWNT-PAMAM hybrids increased GFP transfection efficiency, whilst reducing cytotoxicity.	[12]
	Cancer			<i>f</i> -SWNTs (ethylenediamine)	plasmid DNA (p53 tagged with GFP)	<i>In vitro</i> : •MCF-7 (human breast cancer)	•Strong expression of p53 led to 40% apoptosis after 72 h exposure.	[14]
Restenosis			CNTs incorporated into nanobiohybrid hydrogel (NP-CNT)	Plasmid DNA (GFP, Ang1 & VEGF)	<i>In vitro</i> : •HUVECs <i>In vivo</i> : •Intravenous implantation	•CNTs tune the bioactivity of the stents. • <i>In vivo</i> experiments in balloon injured canine femoral artery demonstrated enhanced re-endothelialization and attenuated stenosis.	[17]	
siRNA 	Cancer		<i>f</i> -SWNTs (SWNT-PL-PEG-SS-RNA)	siRNA (lamin A/C)	<i>In vitro</i> : •HeLa cells (cervical cancer)	•Enzymatic cleavage of disulphide bonds releases the genetic cargo from SWNTs with no ill-effect on cell viability and proliferation.	[25]	
	Cancer		<i>f</i> -SWNTs (-CONH-(CH ₂) ₆ -NH ₃ ⁺ Cl ⁻)	siRNA (TERT)	<i>In vitro</i> : •TC-1 cells (cervical carcinoma) •1H8 cells (ovarian carcinoma) •LLC cells (lung carcinoma) <i>In vivo</i> : •Intratatumoral (intralesional injection of mouse bearing Lewis lung carcinoma or HeLa cell xenografts)	• <i>In vitro</i> : <i>f</i> -SWNTs suppressed mTERT expression and produced growth arrest. • <i>In vivo</i> : Injection of mTERTsiRNA:SWNT ⁺ complexes into s.c. Lewis lung tumors reduced tumor growth.	[30]	
	HIV		<i>f</i> -SWNTs (PL-PEG ₂₀₀₀ -NH ₂)	siRNA (CXCR4 and CD4 receptors)	<i>In vitro</i> : •T-cell line •MAGI cell line •Human peripheral blood mononuclear cells (PBMCs)	•Knock-down levels of CXCR4 and CD4 with <i>f</i> -SWNTs far exceed those of other non viral transfection agents.	[26]	
			<i>f</i> -SWNTs (PDDA-HMDA-SWNTs)	siRNA (ERK1 and ERK2)	<i>In vitro</i> : •Primary rat cardiomyocytes	•PDDA-HMDA-SWNTs loaded with extracellular signal-regulated kinase (ERK) siRNA suppressed ERK protein levels by about 75%.	[24]	
	Myelogenous leukemia		<i>f</i> -SWNTs (SWNT-NH ₂)	siRNA (cyclin A ₂)	<i>In vitro</i> : •K562 cells (human erythroleukemic cell line)	•The depletion of cyclin A ₂ inhibits cell proliferation and promotes apoptosis of chronic myelogenous leukemia K562 cells.	[18]	
	Insulin resistance		<i>f</i> -SWNTs	siRNA (TRPC3)	<i>In vitro</i> : •Adult mouse skeletal muscle cells	•Knockdown of TRPC3, resulted in pronounced (~70%) decrease in OAG-induced Ca ²⁺ influx and insulin-mediated glucose uptake.	[31]	

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Table 1 (continued)














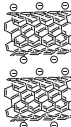
Category	Disease	Carbon nanotube (CNT)	CNT functionalization	Nucleic acid	In vitro/in vivo	Results	Ref.
	Cancer		Pristine SWNTs	siRNA (HIF-1 α , Kif11, PLK1, Tox)	<i>In vitro</i> : •MiaPaCa-2/HRE cells (pancreatic cancer) •MCF-7 cells (breast cancer) •MDA-MB-231 cells (breast cancer) •RGM1 cells <i>In vivo</i> : •Intratumoral injection (mice bearing MiaPaCa-2/HRE tumors)	• <i>In vitro</i> : SWNT:siRNA constructs are highly specific with minimal toxicity. • <i>In vivo</i> : Intratumoral injection of SWNT:siRNA complexes significantly inhibited Hif-1 α expression, but tumour growth suppression was short-lived.	[6]
	Cancer		f-MWNTs (MWNT-NH $_3^+$)	siRNA (TOX, PLK1)	<i>In vitro</i> : •Calu6 (human lung carcinoma) •SVEC 4–10 & 2F2B (murine vascular endothelial cells) •DU145 & C-33A (human prostate cancer) •A549 (human lung carcinoma) •MCF7 (human breast cancer) •HeLa (cervical cancer cells) •HEK293 (human embryonic kidney cells) •B16F10 (murine melanoma) •NIH 3T3 (fibroblast cells) <i>In vivo</i> : •Intratumoral injection (mice bearing Calu6 xenograft tumors)	• <i>In vivo</i> : MWNT-NH $_3^+$:siRNA complexes administered intratumorally can elicit delayed tumour growth and increased survival of xenograft-bearing animals. •Carbon nanotubes outperformed liposomes as intracellular delivery agents <i>in vivo</i> .	[19]
	Vascular disease		f-SWNTs (lipids and lysine dendrimers (TOT))	siRNA (ApoB)	<i>In vitro</i> : •FL83B cells (mouse hepatocytes) <i>In vivo</i> : •Intravenous (lateral tail vein)	• <i>In vivo</i> : I.V. administration silenced ApoB in the liver, plasma levels of ApoB decreased, and total plasma cholesterol decreased. Treatment was nontoxic and did not induce an immune response.	[27]
	Varied application		f-SWNTs (COOH-SWNTs)	siRNA (type I and II InsP3R)	<i>In vitro</i> : •SKHep1 (non-metastatic human hepatocellular carcinoma) •Neonatal cardiomyocytes •Rat dorsal root ganglia neurons	•f-CNT:siRNA constructs achieved transfection efficiencies greater than 95%, even in hard-to-transfect cells.	[21]
	Cancer		f-MWNTs (polycationic dendron-MWNT)	siRNA (GFP, TOX)	<i>In vitro</i> : •A549 cells (lung carcinoma) •HeLa cells (cervical cancer)	•Polycationic dendron-MWNTs significantly improved siRNA delivery with low cytotoxicity.	[32]
	Cancer		f-MWNTs (-PEI and -pyridinium)	siRNA (anti-luciferase)	<i>In vitro</i> : •H1299 cell line (luciferase expressing human lung cancer)	•Both f-CNTs:siRNA complexes showed 10–30% silencing activity and a cytotoxicity of 10–60%.	[22]
	Ischemic stroke		f-MWNTs (MWNT-NH $_3^+$)	siRNA (caspase-3)	<i>In vitro</i> : •N2a cells (mouse neuroblastoma) •Primary cortical neurons <i>In vivo</i> : •Intracerebral injection (peri-lesional stereotactic administration)	• <i>In vivo</i> : In an endothelin-1 induced stroke model, peri-lesional stereotactic administration of MWNT:siRNA complexes reduced neurodegeneration and promoted functional preservation.	[20]
	Cancer		f-DWNTs (oxidized and coated with Poly(Lys: Phe))	siRNA (survivin, GFP)	<i>In vitro</i> : •PC3 cells (human prostate cancer) •HeLa cells (cervical cancer)	•f-DWNTs:siRNA constructs achieved significant gene silencing of GFP and an apoptotic effect following survivin knock-down.	[29]
	Cancer		f-SWNTs (DSPE-PEG-amine)	siRNA (MDM2)	<i>In vitro</i> : •B-Cap-37 cells (breast carcinoma)	•f-SWNT:siRNA complexes inhibited the proliferation of B-Cap-37 cells by 44.53% at 72 h, and the apoptosis ratio was measured as 30.45%.	[28]
	Cancer		f-SWNTs (PEI)	siRNA (hTERT)	<i>In vitro</i> : •PC-3 cells <i>In vivo</i> : •Intravenous injection (tail vein injections into PC3 tumor bearing mice)	• <i>In vivo</i> : High antitumor activity due to more accumulation in tumor, without obvious toxicity in main organs. •Near-infrared (NIR) photothermal therapy combined with SWNT:siRNA treatment significantly enhanced the therapeutic efficacy.	[23]
miRNA	Cancer		f-MWNTs (PEI-g-GNR)	miRNA	<i>In vitro</i> : •HeLa cells (cervical cancer)	•PEI-g-GNR could be used as a probe carrier for more efficient transfection of cells.	[33]
ODNs	Cancer		f-MWNTs (-COOH, -PEI, -PDDA, -PAMAM, -chitosan)	ODNs (antisense)	<i>In vitro</i> : •HeLa cells (cervical cancer)	•PEI-MWNTs demonstrated efficient intracellular transporting, strong cell nucleus localization and high delivery efficiency of ASODNs.	[35]
	Cancer		f-MWNTs (polyamidoamine dendrimer modified)	ODNs (c-myc)	<i>In vitro</i> : •MCF7 & MDA-MB-435 cells (breast cancer) •HepG2 cells (liver cancer)	•These composites inhibited the cell growth in time- and dose-dependent means, and down-regulated the expression of the c-myc gene and C-Myc protein.	[36]

Table 1 (continued)

Category	Disease	Carbon nanotube (CNT)	CNT functionalization	Nucleic acid	<i>In vitro/in vivo</i>	Results	Ref.
Aptamer	Varied application		<i>f</i> -SWNTs (-COOH)	ODNs (NF- κ B)	<i>In vitro</i> : •HeLa cells (cervical cancer) •Mononuclear cells (MCs)	•SWCNT:NF- κ B decoy complexes demonstrated both efficacy and specificity compared to controls.	[34]
	Cancer		<i>f</i> -MWNTs (-COOH)	Aptamers (MUC-1)	<i>In vitro</i> : •MCF7 (breast cancer cells)	•MWNT-Apt conjugates were internalized without affecting cell viability.	[37]

deliver siRNA intracellularly, ensuing significant gene silencing [19,30]. More recently, siRNA sequences designed for caspase-3 knock-down have been effectively delivered *via* chemically functionalized CNTs by stereotactic injection to targeted brain loci, and was found to reduce neurodegeneration and promoted functional recovery of the rodent motor cortex in an endothelin-1 induced stroke model [20]. That was the first time that siRNA delivered into the central nervous system (CNS) *via* CNTs was able to produce biological and functional (motor rehabilitation) effects in an induced stroke animal model. Based on this work, CNTs can be envisioned as a delivery platform for siRNA that can be utilized for

the treatment of a variety of neurological disorders in localized brain regions and afford both therapeutic and functional recovery. Overall, our work and that of others have shown that carbon nanotube-mediated delivery of siRNA can be efficacious for a range of therapeutic applications. miRNA constructs, which are genomically encoded non-coding RNAs that help regulate gene expression, represent an alternative gene knock-down approach. However, currently there is little knowledge about the ability of carbon nanotubes to deliver miRNA sequences intracellularly. There is only one report to date [33] that describes the use of “unzipped” multi-walled carbon nanotubes as miRNA delivery vectors.

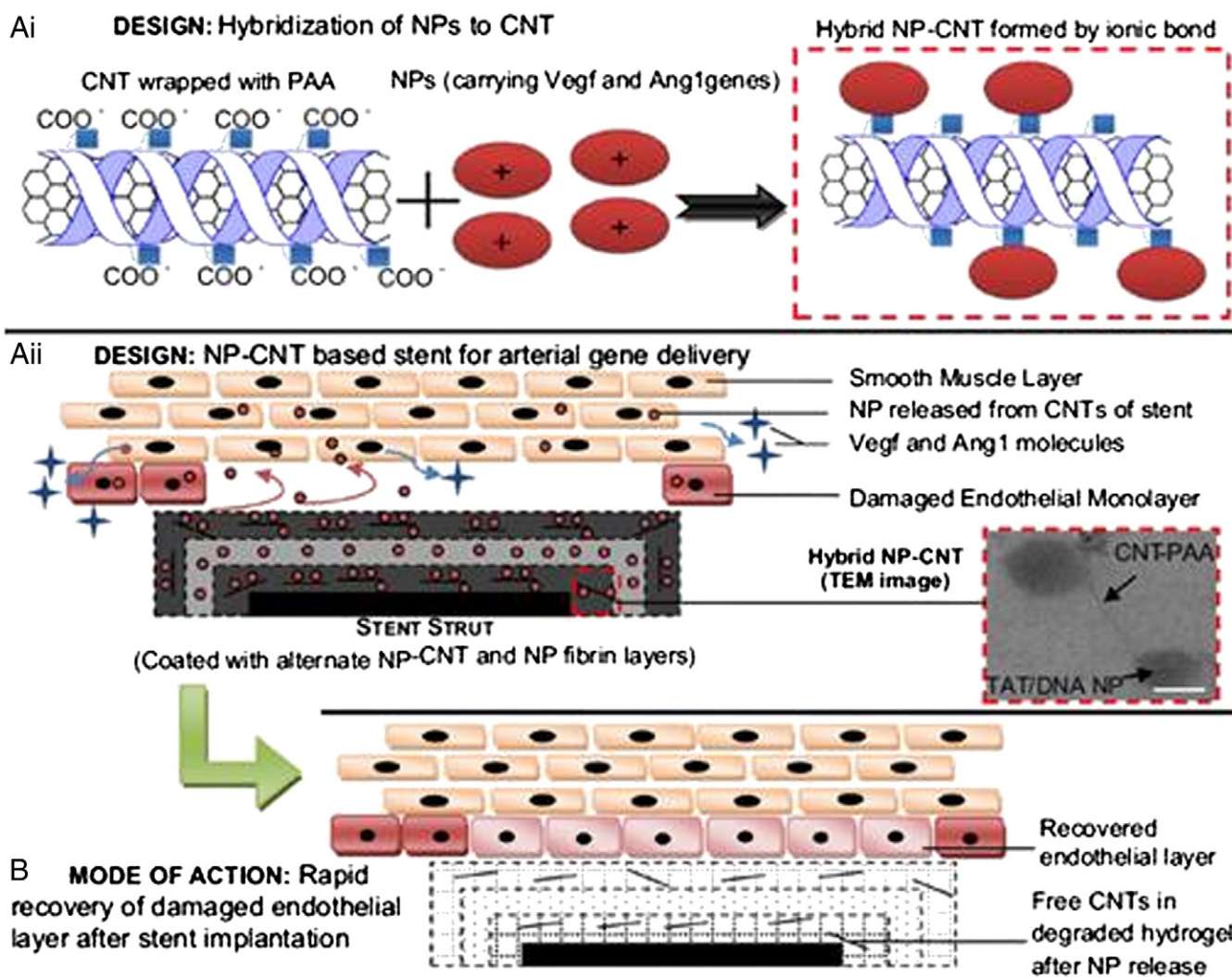


Fig. 2. Schematic representation of design and mode of action of new nanobiohybrid hydrogel based therapeutic stent. Ai) NP/CNT nanocomplex (red dotted box) formed by electrostatic bond between anionic CNT and cationic NPs; Aii) stent fabrication by L-b-L assembly of NP/CNT and NPs on stent surface using fibrin matrix, and how NPs get released from hydrogel platform to locally deliver transgene to the damaged artery for endothelial recovery (scale bar = 100 nm). B) Intima layer integration by regenerated local endothelial monolayer cells reduces the risk of restenosis. After delivery of NP payload, due to its biodegradable nature, the hydrogel will be bioresorbed and bare stent will eventually work as normal stent in the already recovered vascular segment. (Abbreviations: PAA, polyacrylic acid; NP, Tat/DNA nanoparticles; CNT, single-walled carbon nanotube; LbL, layer-by-layer; TEM, transmission electron microscope).

Image reprinted with permission from Ref. [17].

Pristine multi-walled carbon nanotubes were subjected to a series of strong acid treatments that caused longitudinal unzipping of the nanotube structure to generate flat structures, also known as graphene nanoribbons (GNRs). These GNRs were first functionalized with anionic groups by the $\text{H}_2\text{SO}_4/\text{HNO}_3$ oxidation and then coated with the cationic polymer PEI to optimally complex and transfect miRNA sequences *in vitro*. Furthermore, these carbon-based nanovectors offered protection to the nucleic acid cargo from nuclease digestion and single-strand binding protein interactions, thus enabling more efficient transfection of cells *in vitro* [33]. The lack of more published studies of carbon nanotube-mediated miRNA delivery for gene therapy applications is not surprising, especially given the incomplete understanding of the molecular mechanisms by which miRNAs regulate gene expression or the complete gene repertoire each miRNA regulates, although recent progress towards clinical applications is promising [50].

3.3. Oligonucleotides (ODNs)

Some early studies looked at the ability of both chemically functionalized (namely carboxylated) [34,35] CNTs and non-covalently modified CNTs (largely surface coatings of various cationic polymers (-PEI, -PDDA, -PAMAM) and chitosan [35] and polyamidoamine dendrimers [36]) to deliver ODNs (Table 1, ODNs subsection). ODNs have the potential as a genetic cargo to achieve therapeutic efficacy, and due to their mechanism of action have been considered decoy molecules. ODNs may be biologically active *via* different mechanisms, controlling gene

expression at either the transcriptional or translational level. However, it is widely accepted that this antisense strategy of genetic “modification” starts in the cytoplasm at the beginning of mRNA translation by binding to target transcription factors, thus blocking the translation of target RNA into target protein and modifying gene expression. Both functionalized single-walled and multi-walled carbon nanotubes have been used to successfully deliver antisense ODNs demonstrating efficacy and specificity [34–36]. Pan et al. demonstrated efficient delivery of antisense myc which led to a strong inhibition on the proliferation rate of human leukemia HL60 cells *in vitro* [36]. Furthermore, this decoy method induced apoptosis and down-regulated the expression of c-myc gene at both the RNA and protein levels, proposed as an anticancer agent [36]. Moreover, novel double functionalized MWNTs with antisense ODNs as ODNs (with cytotoxic activity) and quantum dots (for imaging), were endocytosed by human cervical cancer (HeLa) cells, achieving a high delivery efficiency of ODNs with a strong nuclear localization and consequential extensive apoptosis [35]. Although promising data using asODNs as anticancer agents has been reported, their progression towards clinical development is hindered by their inherent lack of specificity, thus making siRNA approaches that allow more specificity more attractive alternatives.

3.4. Aptamer DNA/RNA

Another relatively unexplored option is the delivery of DNA/RNA aptamers by carbon nanotubes (Table 1, aptamers subsection). Aptamers

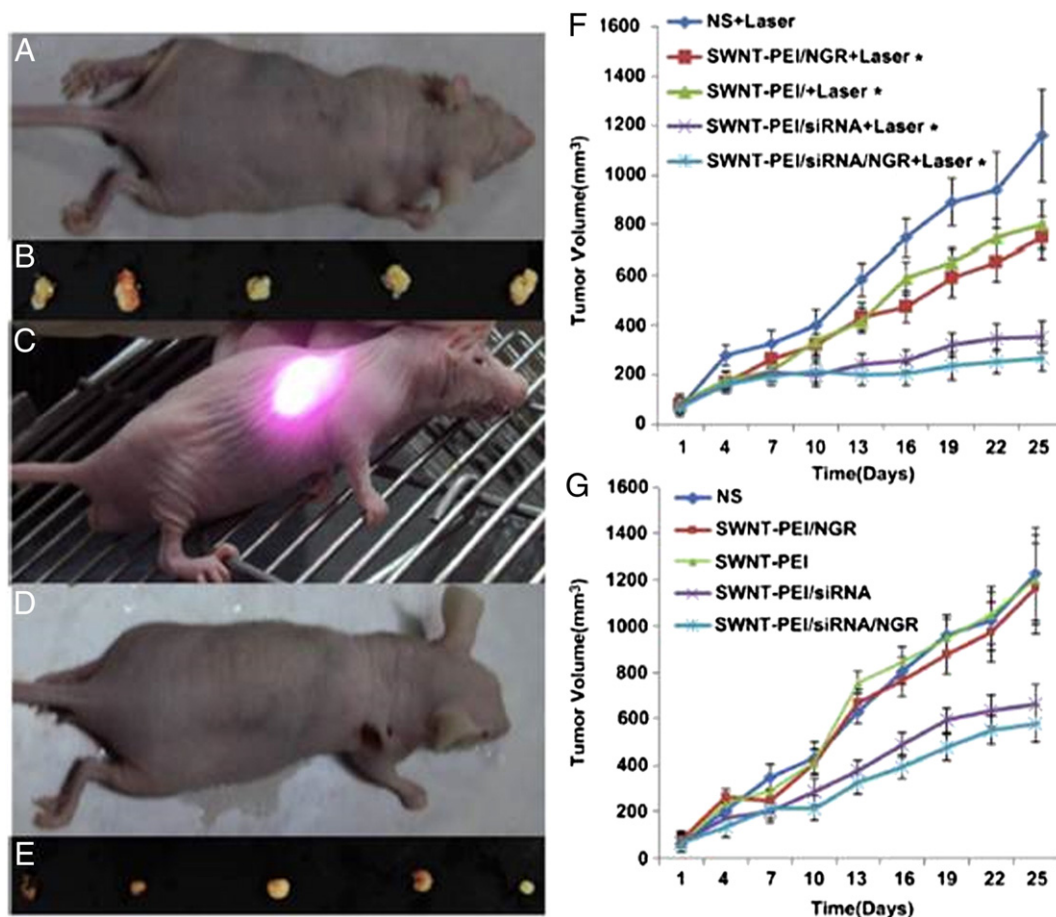


Fig. 3. Tumor-bearing nude mice model under SWNT-PEI/siRNA/NGR treatment. A) After siRNA treatment without laser irradiation; B) image of dissected tumors after siRNA therapy; C) in the process of treatment; D) after treatment; E) image of dissected tumors in the group of SWNT-PEI/siRNA/NGR + 808 nm NIR laser. Average tumor size in a PC-3 nude mouse model under treatment without (F), or with (G) laser *in vivo*. The SWNT-PEI/siRNA/NGR + laser group shows significant ($P < 0.05$) suppression of tumor growth compared to the other experimental groups with laser irradiation, and each SWNT-PEI laser group shows significant ($P < 0.05$) suppression of tumor growth compared with SWNT-PEI group, respectively. Image reprinted with permission from Ref. [23].

are short single-stranded oligonucleotides, either RNA or DNA based, that are able to recognize their intracellular targets on the basis of shape complementarity. Due to their ability to disrupt protein–protein interactions their use as inhibitors for particular intracellular pathways bestows them with vast therapeutic potential. However, the delivery of aptamers intracellularly is problematic and therefore transfection vectors have been necessary. Our laboratory has shown that MWNT–aptamer constructs can be transported intracellularly for the delivery of biologically active aptamers [13]. That approach offers the opportunity for the development of novel aptamer-based therapeutics for previously difficult to access intracellular targets due to limited intracellular translocation [37]. Despite the endless therapeutic potential that aptamers may possess as anti-cancer agents and as anti-virals against HIV and hepatitis C, there is very limited data detailing carbon nanotubes as delivery vectors for biologically active aptamer sequences [51].

4. The geno-pharmacology of carbon nanotube-mediated gene therapeutics

The main objective for the translation of gene therapy into humans is the ability to modify specific gene expression at particular cell (diseased) populations *in vivo* for an adequate amount of time, and at sufficient levels to elicit a therapeutic effect. Various challenges are presented in the journey to achieving this objective, including the formulation of genetic cargo, *in vivo* delivery, penetration of biological barriers, intracellular off-loading of genetic cargo, controlled gene expression or modification and moderation of toxicity. It is necessary to overcome these obstacles for clinically practical and efficient gene therapy, and it is possible that carbon nanotubes may be a viable gene therapy vector that can overcome some of these geno-pharmacological barriers.

Gene therapies can be divided into three main categories based on the mode of delivery to the target tissue. First of all, *ex vivo* delivery, involves the removal of target cells, transfecting them with gene therapy vectors and then returning the modified cells back to the host. This approach is limited to those cells that can be easily obtained and cultured. There is little evidence showing the use of carbon nanotubes for *ex vivo* gene transfer, but it has been reported that exogenous gene transfer has been achieved in both *ex vivo* splenic B cells and cortical neurons [15]. Both types of primary cells were cultured in conditions that favor the phenomenon of nanotube spearing; a highly efficient delivery technique that is based on the penetration of carbon nanotube–plasmid DNA complexes into cell membranes, driven by magnetic forces. Nanotube spearing using plasmid DNA containing enhanced green fluorescent protein (EGFP) immobilized onto CNTs achieved almost 100% transduction of EGFP in primary B cells 24 h after incubation and approximately 80% of primary cortical neurons expressed EGFP at 48 h as determined by fluorescent microscopy [15]. Further progress was reported in a study describing human pancreatic carcinoma cells (MiaPaCa-HRE cells) transfected with SWNT:siRNA (Hif-1 α) complexes after subcutaneous injection into mice [6]. Prior experiments by Bartholomeusz et al. saw a reduction in Hif-1 α activity in mice treated with SWNT:siRNA (encoding for Hif-1 α) complexes compared to control groups, however no suppression of tumor volumes was observed, thus suggesting an incomplete inhibition of Hif-1 α activity. As a result, MiaPaCa-HRE cells were then transfected *in vitro* with SWNT:siRNA (encoding for Hif-1 α) complexes and cultured for 24 h prior to subcutaneous implantation. An initial period of growth inhibition was observed, however after 7 days no difference was seen between treatment group and control group. It is clear that *ex vivo* gene transfer using carbon nanotube vectors has not shown great efficacy, and more work is needed to allow the possibility of clinical translation.

The second type of gene therapy approaches involves direct application of the genetic cargo to the target tissue. The majority of the work conducted *in vivo* using carbon nanotubes as gene delivery vectors for

therapeutic use are administered locally, such as intratumorally for cancer therapy or intracranially for various neurological applications.

Various *in vivo* cancer models have been established to explore the intratumoral administration of siRNA by carbon nanotubes, both pristine and surface-modified, with specificity, efficacy and therapeutic effects reported. Localized intratumoral injection of pristine single-walled nanotubes complexed with siRNA targeting Hif-1 α , effectively reduced gene activity although no suppression of tumor volume was observed, that was attributed to incomplete inhibition of Hif-1 α [6]. Podesta et al. reported therapeutic efficacy following CNT-mediated delivery of siTOX (a potent cytotoxic siRNA sequence) directly into human cancer xenografts (Calu6 cells) and reported tumor volume suppression, followed by a concomitant prolongation of tumor-bearing mice survival [19]. Importantly, it was also discovered that MWNT-NH $_3^+$ allowed efficient delivery of low doses of siRNA, thus reducing the risk of problematic off-target effects. Zhang et al. reported that intraslesional injection of mouse telomerase reverse transcriptase (mTERT) siRNA:SWNT complexes was able to suppress two types of subcutaneous tumor growth *in vivo*; murine Lewis lung carcinoma and human HeLa xenografts [30], with multiple injections of carbon nanotube–siRNA constructs being more effective.

Although the majority of research involving localized administration of siRNA:CNT constructs is centered around cancer research, other potential applications are emerging. MWNT-NH $_3^+$:siCaspase-3 constructs have been injected stereotactically into the rodent motor cortex following focal ischemic damage induced by the endothelin-1 toxin. It was observed that peri-lesional stereotactic administration of the CNT:siRNA constructs was able to not only reduce neurodegeneration but also promote functional preservation of motor capabilities in diseased animals [20].

The third type of gene therapy involves *in vivo* delivery via the blood stream, which is the most versatile delivery approach, but is very much dependent on the complex pharmacological profile of different carbon nanotubes. Multiple studies have attempted the delivery of nucleic acids *in vivo* via systemic administration carrying different siRNA molecules for the treatment of cancer [23], albeit with limited success. Not surprisingly, combinatory therapeutic approaches may be the way forward for intravenously injected carbon nanotube vectors such as the approach proposed by Wang et al. who reported the combination of RNAi and near-infrared (NIR) photothermal therapy able to significantly enhance therapeutic efficacy in tumor-bearing mice, as shown in Fig. 3 [23]. The significant anti-tumor activity reported was thought to be due to higher accumulation of the therapeutic complex (SWNT-PEI:siRNA:NGR; whereby the therapeutic siRNA sequence targets hTERT, and NGR an Asn-Gly-Arg (NGR) peptide motif improves specific tumor cell targeting) within the tumor mass, concluding that SWNT-PEI:siRNA:NGR vectors could offer a promising bimodal anti-cancer therapeutic approach by combining targeted gene therapy further enhanced by exploiting the photothermal properties of CNTs [23]. Despite these advances, there are limited studies using intravenously administered CNT-based vectors thus indicating the unmet challenge of targeting specific cells and tissues. Overall, systemically targeting CNTs for *in vivo* therapeutic use remains challenging, with most studies reporting some degree of efficacy using solid tumor models [52].

The relationship between the amount of genetic material delivered into cells (dose to host) and the therapeutic response often receives little attention in gene therapy studies. Among different models of cancer, parameters such as normal tumor vascularization, cellular and extracellular heterogeneity, lymph drainage and tumor growth rates differ so greatly, that explain why efficacy of carbon nanotube-mediated gene therapy is particularly difficult to achieve. A number of studies have revealed interesting insights into the relationship between gene dose and activity when delivered by carbon nanotubes, particularly for siRNA delivery both *in vitro* and *in vivo* [6,19,20,21,23,25,28,30,32], although, information regarding the dose of pDNA, ODNs, miRNA and aptamers and their activity is meager. Also, the way which carbon nanotubes are administered *in vivo* for gene therapy applications is becoming

more complex, beyond simple aqueous dispersions, *i.e.* in saline or dextrose solutions. More sophisticated ways to incorporate carbon nanotubes into gene therapies *per se* have evolved including CNT-loaded electrodes [11] or CNT biosensors [23]. Inoue et al. reported that CNT-loaded electrodes improved plasmid DNA transfection into adherent cells *in vitro* [11]. Furthermore, it has been reported that carbon nanotubes can be incorporated into scaffolds or other matrices for various biological applications [53,54]. In terms of gene therapy, carbon nanotubes non-covalently linked with plasmid DNA (encoding for VEGF and Ang1) have been incorporated into hydrogels with the aim to create implantable bioactive stents for the prevention therapy of stenosis which has far-reaching clinical applications for arterial narrowing associated with atherosclerosis and vascular damage following surgery and angioplasty. Furthermore, a more novel use for carbon nanotubes in gene therapy is evolving in the field of electrochemical biosensors. Functional composites of multi-walled carbon nanotubes doped with nylon 6 (PA6) have been shown to act as supporting scaffolds for single stranded DNA sequence immobilization, whereby these composites dramatically increase the amount of DNA attachment and hybridization sensitivity. Although this study does not focus on the delivery aspect of therapeutically active oligonucleotides, the importance of this novel biosensor was shown *in vitro* where high sensitivity and specificity was crucial in detecting and discriminating between the wild-type p53 gene and the mutant-type p53 gene which is involved in oncogenesis. The significance of this study lies in the ability for early diagnosis of cancer development and highly accurate monitoring of patient therapy, and so the evolution of carbon nanotube applications in the wider context of gene therapy in the form of biosensors holds great promise for both the diagnosis and monitoring of various diseases [55].

5. Conclusion

Carbon nanotubes possess an assortment of attractive characteristics that enhance their potential as gene therapy vectors. They have been shown to be able to complex and transport a range of different nucleic acids both *in vitro* and *in vivo*. Although promising, a range of challenges need to be addressed including the formulation of genetic cargo (dose to host relationship), the most efficacious route of delivery *in vivo*, achieving and maintaining controlled gene expression or modification and the moderation of toxicity. Moreover, and outside the scope of this review, there is the need to modulate inherent reactions from the host's immune system, inflammatory potential and toxicity concerns.

The need for standardization of the structural and surface characteristics of carbon nanotubes that are thought to be the most important for successful delivery of nucleic acids, would allow for more laboratories to contribute and offer greater transparency and understanding of findings between gene therapy studies and therefore accelerate developments in the exploitation of carbon nanotube-mediated gene transfer towards the clinic [17,19]. The most pertinent conclusion that can be drawn following ten years of nanotube-mediated transport of nucleic acids is that biological efficacy has mostly been demonstrated *in vitro*. The exception of CNT-mediated siRNA delivery leading to significant therapeutic efficacy has been reported *in vivo*, to afford the prediction that this is where the future developments of CNT-mediated gene therapy will lie. In terms of CNT-mediated siRNA delivery, preclinical advances have been achieved for the treatment of various cancers both *in vitro* and *in vivo*. It is also interesting to see the application of such vector systems against more challenging and complex pathologies, such as stroke. The future success of carbon nanotubes as vectors for siRNA *in vivo* will depend very much upon achievement of efficacious therapy with minimal adverse reactivity and deposition of the material in the tissue. Most of the studies to date that report therapeutic efficacy of carbon nanotube-siRNA constructs *in vivo* are administered locally, either intratumorally as anti-cancer agents [6,19,30] or *via* stereotactic injection into the brain for the localized targeting of specific loci [20].

It remains to be seen whether such strategies and routes of administration will be clinically realistic and efficacious by minimizing adverse reactions.

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