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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 geno-pharmacological impact of carbon nanotube-mediated gene therapy is discussed.
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Contents

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

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