Chapter 2
The Use of Single Wall Carbon Nanotubes as a Delivery System for siRNA

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Abstract RNA interfering (RNAi) is a biological process that operates in most eukaryotic cells in which small interfering RNA (siRNA) molecules inhibit gene expression. Recently, RNAi has been recognized as a therapeutic option to treat several diseases. However, the use of this gene silence technology has been limited, mainly because of the low efficiency of the different vectors in delivering significant amounts of siRNA to cells. In this context, CNTs have emerged as a novel vector to deliver siRNA for post transcriptional gene silencing purposes in vitro and in vivo due to their unique chemical and physical properties. This chapter is focused on the various strategies available and recent developments on the applications of Single Wall Carbon Nanotubes (SWCNTs) as a nanovector for siRNA delivery.

2.1 Introduction

2.1.1 CNTs as Molecular Transporters

Carbon nanotubes (CNTs) are fullerene-related structures consisting of hexagonal arrangement of sp^2 hybridized carbon atoms. Nanotubes can have a single layer of

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graphene sheets (single-walled CNTs) or can be made of multiple layers (multi-walled CNTs), and both have been explored for biological utility. When a nanotube contains only two layers, it is referenced as double-walled carbon nanotube (DWCNT).

As a molecular transporter, CNTs can shuttle various types of molecules into cells, among them proteins [1, 2], DNA [3], RNA (for references see bellow), and commonly used drugs [4–6]. In this regard, previous work from several laboratories involving different cell types have demonstrated the uptake of various types of functionalized CNTs, with most studies supporting the fact that CNTs enter cells via endocytosis [7, 8]. The endocytosis pathway is an energy-dependent process in which cells can engulf different molecules. In addition, it has been proposed that CNTs can penetrate the cells through passive-diffusion pathway in a needle-like manner [3]. However, this endocytosis-independent cell entry mechanism is still controversial, and more studies need to be performed in order to fully understand the mechanisms involved in CNT uptake by the cells.

2.1.2 Application of CNTs as a Delivery System for siRNA

Gene therapy has been recognized as a potential tool to treat several disorders. Among the various types of gene therapy available, RNA interfering (RNAi) represents a novel therapeutic approach for treating severe and chronic diseases due to its high specificity [9]. RNAi, also called post transcriptional gene silencing (PTGS), is a naturally occurring process in which a target gene is silenced. For successful knocking down of genes by RNAi, an efficient delivery system is required to allow proper membrane penetration, low immunogenicity, and protection from degradation [10]. In this regard, a variety of delivery agents have been applied to overcome these problems, but with limited degree of success [10]. In the last years, CNTs have emerged as a novel and alternative delivery agent for different biomolecules, including small interfering RNA (siRNA). In fact, CNTs not only carry the attached molecules into cells, but also present low cytotoxic effects into different types of cells [11]. In addition, CNTs provide protection to the cargo from degradation while in the circulation [11]. However, an important disadvantage related to the CNTs is its lack of solubility, and this drawback is mostly solved by incorporation of different pendant units to CNTs. Thus, functionalization of CNTs is very important for most of their use in biological applications, providing several advantages, including enhanced solubility in water, increased dispersion, and a lower tendency to form agglomerates [12]. In addition, functionalization increases the SWCNT half-life in the plasma, as demonstrated by Kirkpatrick et al. who showed that polyethylene glycol (PEG)-lipid solution in the formulation of the SWCNT complex prolonged its blood circulation time from minutes to hours [11]. In viewpoint of functionalization type, many kinds of strategies have been explored for siRNA delivery, and among these we can cite: (i) noncovalent functionalization which include the use of many biomolecules, polymers, and surfactants, and (ii) covalent functionalization that include chemical modifications [12].

This chapter summarizes the various strategies available for CNT functionalization and recent developments on the applications of Single Wall Carbon Nanotubes (SWCNTs) as a nanovector for siRNA delivery in vitro (Sect. 2.2) and in vivo (Sect. 2.3), with special focus on the hard-to-transfect cells, such as cardiomyocytes (Sect. 2.4). Section 2.5 gives a brief summary and discusses the perspectives for the use of CNTs for siRNA delivery.

2.2 Strategies Available for SWCNT Functionalization and siRNA Delivery In Vitro

Several functionalization strategies that include covalent modification or noncovalent approaches are currently available to enable biological application of CNTs. Polymers such as polyethylene glycol (PEG) and PEGylated phospholipids are known for their high biocompatibility and dispersibility, thus making them efficient surface enhancers for CNTs. Kam et al. [13] were one of the first to report the conjugation of SWCNTs to siRNA, and their use as nanocarriers. Their strategy involved the use of phospholipids functionalized SWCNTs through cleavable disulfide linkage to enable controlled siRNA release from the nanotube surface. This approach demonstrated to be highly efficient in delivering siRNA to HeLa cells and more potent than a commercially available transfection agent. The superior efficiency of these functionalized nanotubes in gene silencing was attributed to efficient siRNA cargo loading, and to the intracellular cleavable disulfide links that facilitates the endosome/lysosome escape of siRNA to the cytosol, protecting it from degradation. Extending these findings, Liu et al. [14] functionalized SWCNTs by adsorption of phospholipids grafted onto amino-terminated PEG. The complex siRNA:SWCNTs was then attached by disulfide bonds, as previously described by Kam et al. [13] and applied to human T cells and primary cells. By using this technique the authors once again demonstrated that SWCNTs can be effectively used as transporters for siRNA to silence the targeting gene on different cell types with superior results over conventional nonviral approaches, such as liposomes.

By using a different strategy, Yang and coworkers [15] investigated the ability of phagocytic cells to engulf siRNA complexed with positively charged SWCNTs (SWCNT+). Consistent with the idea that endocytosis and phagocytosis are important mechanisms of penetration of SWCNTs in the cells, siRNA:SWCNT+ complex was efficiently taken up by phagocytic dendritic cells. In addition, SWCNTs carrying CD80 targeted siRNA when incubated with dendritic cells led to significant reduction in CD80 expression in these cells, without affecting transcript expression of a housekeeping gene.

The use of siRNA has been shown efficient in the treatment of many diseases including various cancers [10]. Zhang et al. [16] explored the use of positively charged functionalized SWCNTs that were complexed to siRNA targeted to telomerase reverse transcriptase (TERT) and incubated with tumor cells. Telomerase is a cellular ribonucleoprotein found in eukariotic cells that maintains the tandem

telomeric TTAGGG repeats at chromosome ends [17], and its activation is critical for immortalization. TERT is its proteinaceous catalytic subunit which plays a critical role in tumor development and growth through the maintenance of telomere structure. Corroborating previous findings, the complex TERT siRNA:SWCNTs+ was efficient in delivering siRNA and knockdown the expression of TERT in a variety of tumor cells. In addition, TERT siRNA:SWCNT+ complex significantly suppressed the growth of LLC, TC-1, and 1H8 tumor cells and reduced cell number after 6 days of incubation with the cells [16]. Another attempt to investigate the potential of siRNA:SWCNT complex to prevent tumor progression was performed by Wang et al. [18], who employed ammonium-functionalized SWCNTs electrostatically bound to siRNA targeting cyclin A2. Cyclin A2 is a key regulator of cell cycle, and its overexpression has been detected and related to many types of cancers. The authors successfully reported the suppression of cyclin A2 expression in a tumour cell line (human erythroleukemic cell line, K526) by using the complex cyclin A2 siRNA: CNT. Moreover, it was observed growth inhibition and apoptosis of the cells incubated with the complex. Further in 2012, Chen et al. [19] also demonstrated that chemically functionalized SWCNTs can delivery siRNA to cancer cells. Accordingly, the authors used siRNA targeting MDM2 (murine double Minute clone 2) which was linked to functionalized SWCNTs by using DSPE-PEG2000-Amine, and the complex applied to breast cancer cells (carcinoma B-Cap-37). MDM2 is known to inactivate the tumor suppressor protein p53. The results indicated that siRNA-MDM2-SWCNTs complex was efficiently taken up by the cells leading to proliferation inhibition and apoptosis of the cells. Taking together, these studies confirm the efficiency of SWCNTs as nanovectors for siRNA delivery in a variety of tumor cells supporting its potential use for in vivo therapies.

The time dependence of CNTs as nanocarriers was evaluated by our group in 2010 using SKHep1 cells [20]. SKHep1 cells are a liver-derived epithelial cell line, capable of proliferating, that are not polarized. In this work, we used short

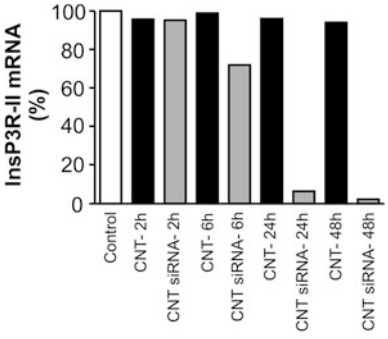

Fig. 2.1 Time dependence of SWCNTs as siRNA carriers in SKHep1 cells. Cells were incubated with the complex InsP3R-II siRNA:CNT for 2, 6, 24 and 48 h, after which media was replaced by regular culture media. Control group was exposed to a suspension of carboxylated SWCNTs alone for the time period indicated in the graph. InsP3R-II mRNA levels were examined 48 h after incubation began. Significant InsP3R-II gene knockdown was observed in cells incubated with the complex for 24 and 48 h. Reproduced from Ladeira *et al.* with permission from Nanotechnology [20]

single-wall (approximate length 200 nm) carboxylic-functionalized CNTs, and siRNA targeted to InsP3R-II, an intracellular Ca^{2+} channel present in the endoplasmic reticulum of SKHep1 cells. In order to determine the optimal time that provides maximum gene knockdown, we exposed cells to InsP3R-II siRNA: SWCNT complex for 2, 6, 24 and 48 h, and we performed quantitative real-time PCR experiments to assess levels of InsP3R-II mRNA. As shown in Fig. 2.1, a reduction in InsP3R-II mRNA levels was observed 6 h after the incubation with CNT-siRNA complex. However, higher InsP3R-II gene knockdown was achieved when cells were exposed to the complex for longer periods of time (24 and 48 h). In addition, cells incubated with CNTs alone did not show any alteration in InsP3R-II mRNA levels (Fig. 2.1). Likewise, Neagoe et al. in 2012 [21] used carboxylated

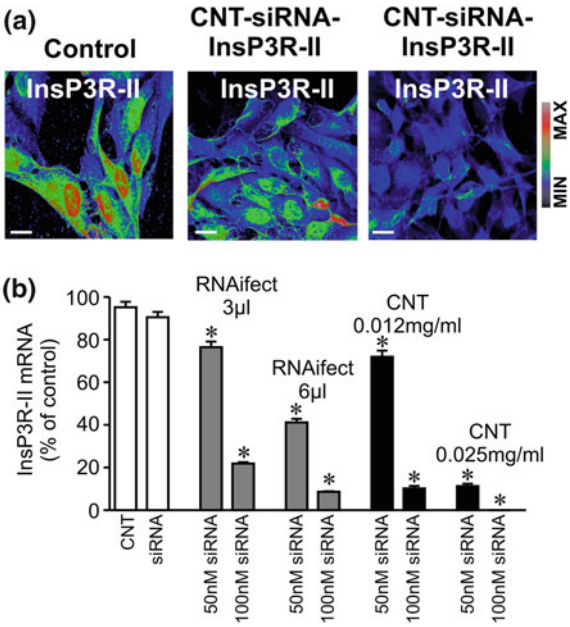

Fig. 2.2 InsP3R-II siRNA:SWCNT complex reduces InsP3R-II expression levels in SKHep1 cells. **a** Representative confocal images showing InsP3R-II labeled cells. InsP3R-II staining was significantly reduced in SKHep1 cells transfected with InsP3R-II siRNA:CNT (*middle* and *right panels*) when compared to control cells (*left panel*). Confocal images were collected 48 h after transfection began. Final concentrations of 50 nM *(middle panel)* or 100 nM siRNA *(right panel)* were efficiently delivered by SWCNTs in SKHep 1 cells. Images were pseudocolored according to the gray scale (color online). Scale bar = 10 µm. **b** Real-time PCR comparing type II InsP3R mRNA expression levels following siRNA delivery using SWCNTs or a lipid-based gene transfer system as carriers. siRNA:RNAifect reagent combination was evaluated at ratios of 50 nM:3 µL, 100 nM:3 µL, 50 nM:6 µL and 100 nM:6 µL. For siRNA:SWCNT complex formation different concentrations of SWCNTs (0.012 or 0.025 mg/mL) were added to the diluted siRNA (50 or 100 nM final concentration). Both delivery agents were efficient in suppressing InsP3R-II gene expression in SKHep1 cells. * = $p < 0.05$ when compared with control group. Reproduced from Ladeira et al. with permission from Nanotechnology [20]

SWCNTs as nanocarriers of siRNA, and by using this strategy these authors obtained efficient siRNA delivery and potent gene silencing in Hep2G cells.

In addition, we observed a concentration dependence on silencing efficiency, since a higher SWCNT to siRNA ratio produced more efficient InsP3-RII knocking down, as shown in Fig. 2.2. This finding is consistent with the idea proposed by Cherukuri et al. [22] that CNT uptake into the cells increases as a function of CNT concentration in the medium. Like previous studies, SWCNTs demonstrated superior silencing efficiency over a conventional lipid based-transfection agent (Fig. 2.2b).

We also investigated the effects of SWCNTs on SKHep1 viability by performing FACS (Fluorescence Activated Cell Sorting) in Anexin-V–FITC and propidium iodide (PI)-stained cells exposed to the same concentration of SWCNTs used in the siRNA experiments. As shown in Fig. 2.3, no effect of SWCNTs on SKHep 1 cell viability was observed indicating that under the conditions used for RNAi, CNTs do not interfere with SKHep1 functionality. These data correlated with findings from a previous study [23] showing that the uptake of SWCNTs did not adversely affect HL60 cells at similar CNT concentration.

Thus based on the literature, SWCNTs are considered to be effective carriers of siRNA in a variety of cells, regardless of the amount and type of functionalization used in the SWCNT surface.

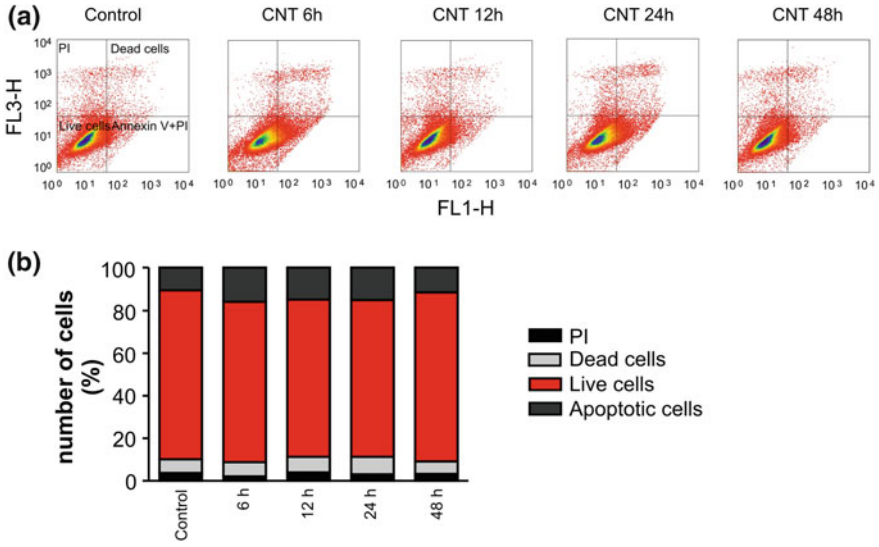

Fig. 2.3 CNTs do not affect SKHep 1 cell viability. Cellular viability was assessed by FACS in cells incubated with SWCNTs (0.025 mg/mL) for 6, 12, 24 and 48 h. **a** Representative FACS analyses shown in the histogram format. **b** *Bar graph* showing that SKHep 1 cell viability is not altered by CNT exposure at different time points. Reproduced from Ladeira et al. with permission from Nanotechnology [20]

2.3 The Use of SWCNTs for siRNA Delivery In Vivo

The potential of different functionalized SWCNTs to act as efficient siRNA delivery agents in vivo has been demonstrated in several studies and most of these studies are focused on the use of SWCNTs as siRNA platform to suppress tumor growth. Yang et al., [15] demonstrated that intravenous injection of the complex SOCS1siRNA:positively charged SWCNTs (SWCNTs +) reduced SOCS1 (suppressor of cytokine signaling type 1) expression and retarded the growth of established B16 tumor in mice, suggesting that SWCNTs-based siRNA transfer system could be used in vivo for gene therapy. Following a similar strategy, Zhang et al. [16] targeted telomerase reverse transcriptase (TERT) and efficiently suppressed tumor growth and induced the senescence of tumor cells in animal studies corroborating the idea that functionalized SWCNTs enter cancer cells providing potent RNAi in vivo.

By using a very simple approach of noncovalently complexing siRNA to pristine SWCNTs Bartholomeusz et al. [24] achieved an efficient delivery of siRNA into cancer cells. In this work, the complex SWCNT:siRNA targeted to hypoxia-inducible factor 1 alpha (HIF-1α) was added to cancer cells and also administered in vivo to mice bearing MiaPaCa-2/HRE tumor. In both cases, it was observed a specific inhibition of HIF-1α activity, demonstrating the potential for using siRNA:SWCNTs complex in vivo as a therapeutic approach for cancer.

For the use of SWCNTs in biomedical application, the study of pharmacokinetics of CNTs is very important. Cherukuri et al. [25] performed an evaluation of pharmacokinetics of SWCNTs in rabbits intravenously administered with pristine SWCNTs dispersed in a solution of surfactant Pluronic. Blood sera were analyzed by near-infrared (IR) fluorescence spectroscopy and SWCNT blood elimination kinetics was measured. The authors observed that the nanotube concentration in the blood serum decreased exponentially with a half-life of 1.0 ± 0.1 h. Although this short half-life is not ideal for biomedical applications, there are many other parameters that regulate and improve the pharmacokinetics of CNTs, and among these we can cite the surface chemistry [26]. The pharmacokinetics of SWCNTs can be significantly altered by the type of suspending agent and functionalization method, with PEGylation being the most efficient method in improving the pharmacokinetics profile of CNTs. For instance, PEGylated-SWCNTs exhibit relatively long blood circulation times and high tumour accumulation [27].

By performing in vivo studies Wang et al. [28] have shown that SWCNTs can effectively delivery siRNA into tumor cells. The authors used polyethylenimine (PEI)-functionalized SWCNTs bound by DSPE-PEG2000-Maleimide conjugation with the asparagine-glycine-arginine (NGR) peptide. The NGR peptide is widely used to target tumor environments. Then, the resulting complex was used to delivery hTERT siRNA into PC-3 cells. The results showed that SWCNTs-PEI:siRNA-NGR complex induced severe apoptosis and suppressed proliferation of PC-3 cells. In animal studies, this complex exhibited higher antitumor activity, that was enhanced by the use of near-infrared (NIR) photothermal therapy. The combination of

nanomaterials with thermal therapy may represent a promising strategy in the field of cancer treatment. Huang et al. [29] also used PEI functionalized-CNTs for the delivery of siRNAs directed against glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Corroborating previous studies, PEI-NH-SWCNTs successfully delivered GAPDH siRNA into HeLa-S3 cells resulting in suppression of GAPDH mRNA. By using this method the authors achieved silencing efficiency comparable to a commonly commercial reagent.

The in vivo delivery of siRNA to therapeutically reduce cholesterol levels was accomplished by McCarroll et al. [30] who employed as nanocarriers SWCNTs functionalized with lipids and natural aminoacids-based dendrimers. Functionalized SWCNTs were conjugated with siRNA targeting apoliprotein B (ApoB), a protein involved in cholesterol metabolism, and the efficiency of this strategy was evaluated. The complex ApoB siRNA:SWCNTs was injected in mice via tail vein and led to approximately 50 % silencing of ApoB in liver followed by a decrease in ApoB plasma levels. Importantly, neither toxic effects nor activation of the immune system was observed in mice treated with the complex.

In conclusion, the last two decades have provided remarkable work in the field of nanomedicine and the biomedical applications of SWCNTs. Thus, these data highlight the fact that functionalized SWCNTs represent effective platforms for systemic in vivo siRNA delivery.

2.4 The Application of CNTs to Hard-to-Transfect-Cells

Cardiomyocytes and other primary cells [31] are usually considered hard to transfect cells, and unfortunately, efficient gene transfer in these cells is still limited. A very useful property of CNTs is their capability to penetrate hard-to-transfect cells, such as cardiac cells, as first demonstrated by Krajcik et al. [32]. In this work, the authors used SWCNTs functionalized with hexamethylenediamine (HMDA) and poly(diallyldimethylammonium)chloride (PDDA). These functionalized $_{f}$SWCNTs were then complexed non-covalently with negatively charged siRNA directed to ERK (extracellular signal regulated kinase) and used in a primary culture of cardiomyocytes. ERK is a serine/threonine kinase that is a member of the extracellular signal-regulated kinase family of proteins, which is activated in response to numerous stimuli such as growth factors, hormones among others and is involved in the regulation of multiple signaling pathways in cardiac cells [33]. As shown by the authors, the siRNA:PDDA-HMDA-SWCNTs construct entered cardiac cells and resulted in efficient ERK knockdown. This study showed for the first time the potential of SWCNTs to carry siRNA into cardiac cells. The use of SWCNTs as a tool for siRNA delivery in knocking down experiments in muscle cells was also assessed by Lanner et al., who showed potent RNAi of TRPC3 channels in adult skeletal muscle fibers exposed to TRPC3 siRNA:CNT complex [34].

Our group also demonstrated the ability of SWCNTs to efficiently enter and carry siRNA to primary culture of cardiomyocytes [20]. In this work, short single-wall carboxylic-functionalized CNTs were incubated with cardiomyocytes for 48 h and then examined by Raman spectroscopy. Figure 2.4 shows the presence of CNTs inside neonatal cardiac cells, further confirming the ability of these nanostructures to enter these cells.

The ability of carboxylated SWCNTs complexed to siRNA targeted to InsP3R-II to efficiently knockdown InsP3R was assessed by immunofluorescence experiments (Fig. 2.5). Primary culture of cardiac cells was exposed to the InsP3R-II-siRNA: CNT complex (CNT 0.0250 mg/mL and 100 nM of siRNA) and evaluated by immunofluorescence using specific anti-InsP3R-II antibody. In muscle cardiac cells, InsP3R-II channel is found in the cytosol and nuclear envelope, as shown in Fig. 2.5. In cells treated with the InsP3RII-siRNA:CNT complex, InsP3R-II labeling was significantly reduced when compared to control cells, confirming the fact that CNTs can act as efficient nanocarriers for siRNA to cardiac cells. We also tested whether InsP3R-II-siRNA:CNT complex would affect the expression levels of another intracellular Ca^{2+} channel in the cell, the Ryanodine Receptor (RyR).

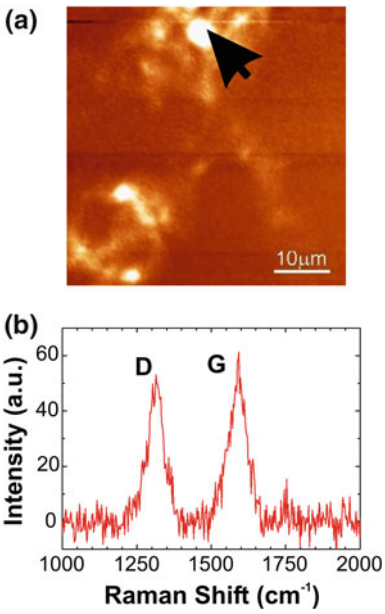

Fig. 2.4 CNTs can be uptaken by neonatal cardiomyocytes. The G band Raman peak at 1590 cm^{-1} was used for CNT detection inside the cell. **a** The plot of the carbon nanotube G band Raman intensity (see arrow) gives the CNT concentration inside the neonatal cardiomyocyte. **b** Representative Raman spectrum of neonatal cardiomyocytes exposed to CNTs (0.025 mg/mL) for 48 h taken from the area marked by the *black arrow*. The G and D carbon nanotube Raman peaks are highlighted. Reproduced from Ladeira et al. with permission from Nanotechnology [20]

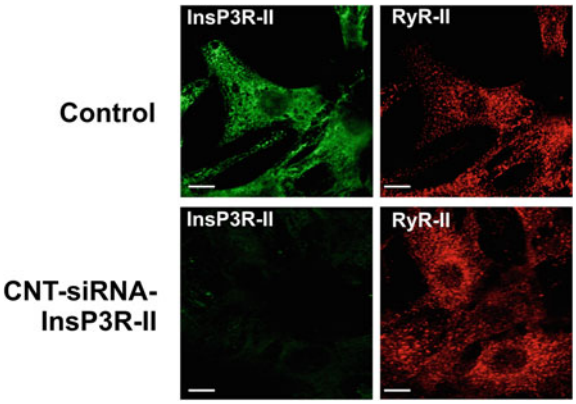

Fig. 2.5 Efficient reduction of InsP3R-II expression in neonatal cardiomyocytes exposed to InsP3R-II-siRNA:CNT complex. Representative immunofluorescence of neonatal cardiomyocytes double-labeled with anti-InsP3R-II (*left panels*), and anti-RyR-II antibodies (*right panels*). Neonatal cardiomyocytes were incubated with InsP3R-II siRNA:CNT complex (*bottom*) or with a suspension of CNTs *(top)* for 48 h, and double-labeled with anti-InsP3R-II and anti-RyR-II antibodies. SWCNTs efficiently delivered InsP3R-II siRNA (100 nM) into cardiac cells and reduced InsP3R-II expression in these cells. RyR-II immunolocalization was not altered in cells exposed to InsP3R-II siRNA:CNT complex. *Scale bar* 10 μm. Reproduced from Ladeira et al. with permission from Nanotechnology [20]

As shown in Fig. 2.5, RyR II staining and distribution throught the cells was not altered in cardiomyocytes exposed to InsP3R-II siRNA:CNT complex.

In gene knocking down experiments, CNT toxicity is a prime concern. In order to assess whether CNTs could alter cardiomyocyte viability in vitro we used a fluorescence-based live/dead assay that indicates the proportion of live and dead cells. In this experiment, cardiac cells were exposed to a solution containing CNTs for 48 h (final concentration 0.050 mg/mL). Figure 2.6 shows that incubation of neonatal cardiomyocytes with CNTs in a concentration superior to that used for siRNA experiments (0.050 mg/mL) does not alter the proportion of live/dead cells when compared with untreated cells. These findings indicate that carboxylated CNTs do not confer apparent toxicity to cardiomyocytes even when a concentration of CNTs superior to that used for the gene knocking down experiments was tested. Similar findings were obtained in a study performed by Garibaldi et al. [35] who demonstrated the biocompatibility of SWCNTs with cardiac muscle cells from a rat heart cell line H9c2. Experiments performed by Krajcik et al. [32] also confirmed the fact that functionalized cationic SWCNTs present no cytotoxic effects to cardiac cells. Taken together findings support the idea that functionalized SWCNTs confers low or no toxicity to cells in vitro [13, 22, 36–38].

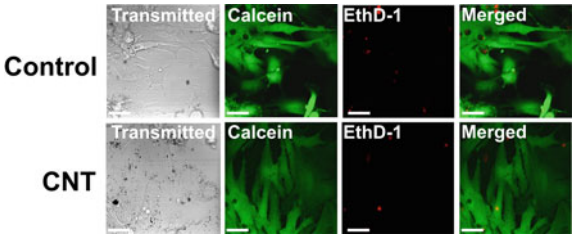

Fig. 2.6 CNTs do not alter cardiomyocyte viability. Cardiomyocytes were submitted to fluorescence-based live/dead viability assay. Dead cells were labeled with ethidium homodimer-1 (EthD-1) and living cells were labeled with calcein AM dye. Images of neonatal cardiomyocytes exposed or not (untreated control) to CNTs (0.05 mg/mL) for 48 h were collected in a confocal microscope. Reproduced from Ladeira et al. with permission from Nanotechnology [20]

2.5 Summary and Perspectives

In conclusion, based on available data in the literature, it is well established the fact that SWCNTs represent a very efficient system for siRNA delivery in a variety of cells, including cells that are hard to transfect, such as cardiomyocytes. In vivo the use of this nano-platform for siRNA based therapeutics offers great potential due to its ability to protect the siRNA from enzymatic degradation, efficient membrane penetration, and apparent low toxicity. However, regardless of the knowledge gained in recent years in biomedical applications of SWCNTs as nanovectors for siRNA, the number of studies is still limited, indicating that systemically targeting CNTs for therapeutic use remains challenging. Further work is warranted in order to optimize the siRNA:CNT formulation, the siRNA release and to achieve proper targeting of the complex. New studies should be considered in order to improve the pharmacokinetic profile of siRNA:CNT complex. In addition special attention should be taken in order to develop more efficacious route of delivery in vivo while maintaining controlled gene expression.

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