Design of double-walled carbon nanotubes for biomedical applications

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Abstract. Double wall carbon nanotubes (DWNTs) prepared by catalytic chemical vapour deposition (CCVD) were functionalized such that they were optimally designed as a nanovector for the delivery of small interfering RNA (siRNA), which is of great interest for biomedical research and drug development. DWNTs were initially oxidized and coated with a polypeptide (Poly(Lys:Phe)) which was then conjugated to thiol-modified siRNA using a heterobifunctional crosslinker. The obtained oxDWNT-siRNA was characterized by Raman spectroscopy inside and outside a biological environment (mammalian cells). Uptake of the
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custom-designed nanotubes was not associated with detectable biochemical perturbations in cultured cells, but transfection of cells with DWNTs loaded with siRNA targeting the green fluorescent protein (GFP) gene, serving as a model system, as well as with therapeutic siRNA targeting the survivin gene, lead to a significant gene silencing effect, and in the latter case a resulting apoptotic effect in cancer cells.

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1. Introduction:

In recent years, efforts have been dedicated to explore the potential biological applications of carbon nanotubes (CNTs), motivated by their size, shape and structures, as well as their unique physical properties [1-3]. Double-walled carbon nanotubes (DWNTs) bridge the gap between single-walled carbon nanotubes (SWNTs) and multi-walled carbon nanotubes (MWNTs). They are comparable to SWNTs with respect to their small diameter, but their mechanical stability is much higher than that of SWNTs. In addition, the outer wall can be functionalized without changing the mechanical and electronic properties of the inner tube [4]. These characteristics make them attractive for biomedical applications, although there has also been concern about potential harmful effects on living cells, which seem to depend on a variety of physicochemical properties (e.g. length and rigidity [5], aggregation behaviour [6], and surface functionalization [7]) and should be monitored closely. This study will tackle the challenges set for the field of nanomedicine to create a more efficient and transparent path for nano-product development, including standardization, safety, and the ability to carry and deliver a therapeutic payload [8].

Within the wide range of biomedical applications, CNTs have recently been developed as gene therapy vectors as a consequence of their ability to efficiently enter mammalian cells. Gene therapy can be achieved through the insertion of a gene (via plasmid DNA) into the host genome or through
molecules that silence the expression of a pathogenic gene, such as silencing/small interfering RNA (siRNA). siRNA consists of short double-stranded RNA segments with typically 21 to 23 bases that are complementary to the mRNA sequence of the protein whose transcription is to be silencedblocked [9]. Since RNA does not integrate into the genome, it offers greater safety than plasmid molecules. Furthermore, siRNA does not have to transfer through the nuclear membrane for its activity, but exhibits its action in the cytoplasm and therefore requires less sophisticated delivery systems than plasmid DNA, promising faster development and higher efficiencies [10]. The exact intracellular route for siRNA delivered by a suitable carrier, such as DWNTs, is shown in figure 1: After uptake, DWNT-siRNA complexes are trapped inside endosomes, which subsequently mature into acidic lysosomes, favouring the dissociation of the siRNA from the polypeptide and its consequent release into the cytoplasm. There, siRNA acts as an effector molecule for specific cleavage of the target mRNA by the RNA-induced silencing complex (RISC), leading to suppression of protein synthesis.

**Figure 1:** Schematic representation of uptake and release of siRNA inside the cell for gene silencing. After internalization, DWNT-siRNA complexes are trapped inside endosomes, which maturate into lysosomes, whose acidic environment favours the dissociation of siRNA from the DWNTs. The
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released siRNA is then free to act as an effector molecule for specific cleavage of target mRNA by RISC. The cleavage of the mRNA prevents the production of a protein. Herein, siRNA is used to prevent expression of *survivin*, an anti-apoptotic gene that is over-expressed in cancer cells.

In this study, mRNA of the *survivin* gene was employed as a siRNA target. *Survivin* is part of the protein family “inhibitors of apoptosis” (IAPs): it is highly expressed in a number of human tumours and is also involved in tumour cell resistance to anticancer drugs and ionising radiation. Evasion of apoptosis and the ability to proliferate uncontrollably are two molecular traits found in all human cancers [11]; therefore, anti-apoptotic proteins involved in signalling through specific apoptosis pathways provide attractive targets for possible drug discovery and new anticancer interventions; particularly those based on gene silencing. In this case, elimination of the *survivin* pathway leads to the lowering of the anti-apoptotic threshold in cancer cells and thus directly causes apoptosis [12], i.e. the suicide of unhealthy, cancerous cells.

Carbon nanotubes have been successfully employed as a delivery system for a variety of genes and other nucleic acids. In one of the pioneer studies, Kam *et al.* reported potent gene silencing by means of siRNA delivery using CNTs. They observed a silencing effect of over 2-fold using SWNTs in comparison with Lipofectamine®, a common transfection agent used in molecular biology [13]. A follow-up study achieved silencing of a therapeutic target, i.e. the expression of a HIV-specific cell surface co-receptor to block HIV virus entry [14]. In both cases, the applied functionalization scheme was based on cleavable disulfide bonds, which we have adapted for the study presented here. One of the first *in vivo* studies using siRNA delivered by carbon nanotubes as a therapeutic agent has been carried out by Podesta et al., who used amino-functionalized MWNTs to deliver a proprietary, toxic siRNA sequence to a human lung tumor xenograft model after intratumoral application, which resulted in tumor growth inhibition and prolonged survival of tumor-bearing animals [15]. Overall, this demonstrates that carbon nanotubes present a versatile and promising method for siRNA delivery that can potentially be applied for the treatment of a wide range of illnesses. The aim of our study is to investigate the feasibility of using double wall carbon nanotubes (DWNTs) as a new delivery vector
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for “anti-cancer” siRNA targeting the gene survivin, a major factor in contributing to the resistance of cancer cells to apoptosis.

2. Experimental:

2.1. Synthesis of DWNTs:

DWNTs were produced by catalytic chemical vapour deposition (CCVD) decomposition of CH₄ over Mg₁₋ₓCoₓO solid solution containing small quantities of molybdenum in the reactor [4]. For purification purposes and to achieve solubility in aqueous media and physiological environments, DWNTs were subjected to a mixture of nitric and sulphuric acid as described in previous studies [16, 17] with subsequent removal of agglomerates and bundles by ultracentrifugation. After oxidation, nanotubes were sterilized by autoclaving at 121 °C for 1 hour and maintained under sterile conditions for the duration of the experiment. The final product consists of single or small bundles of shorter nanotubes and will henceforth be called “oxDWNTs”

2.2. Wrapping of oxidized CNTs:

oxDWNTs at a concentration of 100 µg/mL were mixed with 50 µg/mL of Poly (Lys:Phe) in phosphate buffer (20 mM, pH 7.5) by continuous stirring for 2 hours at room temperature (± 21 °C). To remove unbound polypeptide molecules, the solution was filtered using 100 kDa filter devices (Amicon Ultra-4 centrifugal filter devices from Millipore). Next, Poly(Lys:Phe)-wrapped nanotubes were resuspended in DNase/RNase free water (Sigma Aldrich, Poole, UK) to a final DWNT concentration of 100 µg/mL.

2.3. Conjugation with siRNA:

oxDWNTs where wrapped with Poly(Lys:Phe) and subsequently mixed with non-specific RNA for Raman experiments (henceforth called “oxDWNT-siRNA”) or siRNA targeting the GFP gene
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(Silencer® GFP (eGFP) siRNA and negative control, purchased from Ambion® Life Technologies, UK) for proof-of-principle silencing experiments (henceforth called “oxDWNT-siRNA
gFP”), which allowed for siRNA binding by electrostatic interactions. Excess siRNA was removed by filtration with 100 kDa filter device (Amicon Ultra-4 centrifugal filter devices from Millipore) and the obtained oxDWNT-siRNA resuspended in DNase/RNase free water (Sigma Aldrich, Poole, UK) at a final DWNT concentration of 100 µg/mL.

For experiments with therapeutic siRNA targeting the gene survivin (siRNA
survivin) we decided to pursue another approach, which allows for intracellular siRNA detachment in a more controlled way, i.e. by cleavage of the disulfide bond by thiol-reducing enzymes aided by the acidic pH in lysosomes (based on a previous study by Kam et al. [13]). Here, oxDWNTs were first functionalized with the cationic polypeptide Poly(Lys:Phe) and then conjugated with a heterobifunctional cross-linker containing a disulfide bond (Succinimidyl 6-[3-(2-pyridyldithio)-propionamido] hexanoate, henceforth called “Sulfo-LC-SPDP”, from Pierce/Thermo Fisher Scientific, UK), which is designed for amine-to-sulfhydryl conjugation and readily reacts with thiol-modified siRNA
survivin (figure 2).

![Figure 2: Attachment of thiol-modified siRNA to Poly(Lys:Phe)-wrapped oxDWNTs using the heterobifunctional crosslinker “Sulfo-LC-SPDP”](image-url)
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25 μL of Sulfo-LC-SPDP (20 mM) were combined with prepared oxDWNT-Poly(Lys:Phe) diluted 1:1 in PBS (20 mM, pH 7.5) and incubated for 1 hour at room temperature (± 21 °C) under continuous stirring. Excess cross-linker was removed by filtration using 100 kDa filter devices (Amicon Ultra-4 centrifugal filter device from Millipore). Next, thiol-modified siRNA with the sequence 5′-Th CCACUGAGAAGACGAGCCAGAUU (J-003459-11) to target survivin was mixed with oxDWNT-Poly(Lys:Phe)-cross linker and allowed to react for 1 hour at room temperature under continuous stirring. The solutions were again filtered using a 100 kDa filter device (Amicon Ultra-4 centrifugal filter devices from Millipore) to remove excess siRNA and then resuspended in DNase/RNase free water (Sigma Aldrich, Poole, UK) at a final DWNT concentration of 100 μg/mL to obtain oxDWNT-siRNA_{survivin}.

2.4. Cell culture:

Human prostate cancer cells PC3 (ECACC, Porton Down, Salisbury, UK) were cultured in RPMI-1640 medium supplemented with 10 % (v/v) fetal bovine serum (FBS), 2mM Glutamax™ and 1 % (v/v) penicillin-streptomycin (all obtained from Invitrogen, Paisley, UK). Cells were cultured in 35 mm sterile petri dishes (Nunc, Thermo scientific) containing glass coverslips.

2.5. Raman mapping of single cells:

In order to investigate cellular uptake and toxicity, human prostate cancer cells PC3 were incubated with oxDWNT-RNA (30 μg/mL diluted in Opti-MEM serum-free cell culture medium) for up to 3 hours, after which they were washed twice with ice-cold, sterile PBS and monitored for another 21 hours. Assessment of DWNT uptake was performed at different time points ranging from 0.5 to 24 hours. After 3 hours of incubation with, the cells were washed twice with ice-cold, sterile PBS, to remove oxDWNT-RNA from the medium and fresh medium containing serum and antibiotics was added. At the end of each time point (0, 0.5, 3, 6, 9, 12, 18 and 24 hours) the medium was removed and the cells washed several times.
with PBS. Cells were subsequently fixed with 4% paraformaldehyde solution (Sigma Aldrich, Poole, UK) to prevent morphological and chemical changes during data acquisition. Finally, the samples were again washed with PBS and slides mounted and hermetically sealed using nail lacquer. Raman mapping was performed using a Renishaw InVia Raman microscope, \( E_{\text{laser}} = 1.59 \) eV (785 nm excitation wavelength). The radial breathing modes (RBMs) that are characteristic for carbon nanotubes were used to map the intensity of oxDWNT-RNA in cells and to extrapolate the nanotube diameters present in the mixture using the equation \( \omega_{\text{RBM}} = \frac{248}{d_t} \), with \( \omega_{\text{RBM}} \) being the RBM frequency in \( \text{cm}^{-1} \) and \( d_t \) the tube diameter in nm [18].

2.6. Raman spectroscopy of whole cell lysates:

While the distribution of DWNTs inside cells was monitored by Raman spectroscopy of whole cells, biochemical changes of cell constituents within this time frame was monitored by Raman spectroscopy of whole cell lysates. For that, PC3 cells were grown in medium as described above and cultured in 25 cm\(^2\) tissue culture flasks until 80% confluence was reached. At this point, cells were incubated with oxDWNT-RNA (30 µg/mL final DWNT concentration after dilution in serum-free Opti-MEM medium) for various times ranging from 0.5 to 24 hours. After 3 hours incubation, the medium containing oxDWNT-RNA was replaced with fresh medium containing serum and antibiotics after two washing cycles with PBS. At each time point, the medium was removed from the cells, followed by several washes with PBS. The cells were subsequently trypsinized, washed with ice-cold PBS, and ruptured via hypotonic shock using a lysis buffer (Tris buffer containing 50 mM Tris HCl and 150 mM NaCl at pH 7.5 and additionally 1 % NP-40, 0.2 % SDS, 1 mM phenylmethanesulfonyl fluoride (PMSF), 10 µg/mL aprotinin, 10 µg/mL leupeptin, 1 mM sodium orthovanadate (\( \text{Na}_3\text{VO}_4 \)) as cellular protease inhibitors). After lysis, suspensions were spun down for 10 min at 500 g and 4 °C (Eppendorf 5415R, Hamburg, Germany) to remove nuclei and unbroken cells, and supernatants were used to evaluate the DWNT content. For
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Raman measurements, a droplet of cell lysate (4 μL) was allowed to dry on a glass slide. Spectra of samples were recorded using a NT-MDT NTEGRA Spectra Probe NanoLaboratory inverted configuration microscope ($E_{\text{laser}} = 2.64 \text{ eV or } 473 \text{ nm}$).

2.7. GFP Knockdown in HeLa and PC3 cells:

Stable cell lines expressing green fluorescent protein (GFP) were produced via transfection with pAcGFP-N1 plasmid (Clonetech, Saint-Germain-en-Laye, France) using Lipofectamine® according to instructions in the manual. GFP expressing cells were selected using 600 μg/mL of Geneticin® (Gibco/Invitrogen, Parsley, UK). After 4 weeks of selection cells were sorted by fluorescence-activated cell sorting (FACS), whereby 80-90 % GFP-expressing cells were obtained. These cells were allowed to recover for 8 weeks and re-sorted once more for further purification. The expression of GFP allows for visualization by confocal microscopy (excitation at 488 nm and 400 mW power emission using an argon laser; detection using a 535 nm ± 15 nm band pass filter).

For oxDWNT-siRNA<sub>GFP</sub> transfection, the previously prepared complexes were diluted 1:2 in OptiMEM® medium (Invitrogen, Paisley, UK). For comparison with a commercially available transfection agent serving as the positive control, Lipofectamine® RNAiMAX, siRNA<sub>GFP</sub> complexes were prepared according to the manufacturer’s instructions (1 or 6 μl Lipofectamine® RNAiMAX, for use in 24-well or 35 mm plates, respectively, were combined with 0.2 to 1 μM siRNA<sub>GFP</sub> and added to each corresponding well). After 4 hours the medium was replaced with fresh complete medium containing serum. The cells were then incubated for further 48 hours at 37 °C in a CO<sub>2</sub> incubator.

To evaluate gene knockdown, cells were fixed with 4 % paraformaldehyde (Sigma Aldrich, Poole, UK) in PBS for 1 hour at room temperature, followed by washing with sterile PBS and treatment with 0.1% Triton X100 (Sigma Aldrich, Poole, UK) in PBS for 15 minutes to permeabilize cell membranes. Cells were then washed 3-times with PBS and TO-PRO®-3 Iodide (Molecular Probes®, 0.1 μM in PBS), a monomeric cyanine nucleic acid stain, was utilized to stain the nuclei in all samples. Images were captured at 63x magnification using a
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Zeiss LSM 510 inverted confocal microscope and analysed using LSM 510 META software. To determine the percentage of GFP knockdown after siRNA\textsubscript{GFP} delivery, cells were counted in 5 different fields. GFP expressing cells appear green (GFP, emission 505 nm) with the nucleus coloured in blue (TO-PRO®-3, emission 661 nm), while non-expressing cells only exhibit nuclear staining.

2.8. Survivin knockdown in PC3 cells:

To achieve knockdown of the survivin gene in PC3 cells, cells were incubated with oxDWN'T-siRNA\textsubscript{survivin} for 4 hours and maintained for 3-7 days to allow for gene silencing to occur. PC3 cells were initially seeded 24 hours prior to transfection PC3 cells to obtain 50 % confluency at the time of transfection in 35 mm sterile petri dishes. oxDWN'T-siRNA\textsubscript{survivin} prepared as described in the previous section was diluted 1:2 in Opti-MEM® medium (Invitrogen, Paisley, UK). Again, a commercial transfection agent, DharmaFECT®, was used to serve as the positive control. DharmaFECT-siRNA\textsubscript{survivin} complexes were prepared according to the manufacturer’s instructions: 3 \( \mu \)L DharmaFECT® reagent was combined with 400 \( \mu \)L of serum free culture medium and incubated for 5 minutes at room temperature. Next, siRNA\textsubscript{survivin} was diluted in 400 \( \mu \)L of media to a final concentration of 0.8 \( \mu \)M per 35 mm plate. The solutions were then combined and incubated at room temperature for a further 20 minutes, before being added to the corresponding wells. After 4 hours the medium was replaced with fresh complete medium (containing 10% serum) for both samples. The cells were then incubated for a further 72 hours at 37 °C in a CO\textsubscript{2} atmosphere in order to achieve a silencing effect. After 72 hours incubation cells were trypsinized and split for further incubation up to 120 hours. Cells were then stained with Trypan Blue and counted using a haemocytometer.

The level of apoptosis induced by silencing of the survivin gene was evaluated by means of the Annexin V Biotin Apoptosis Detection Kit (CalBioChem®) and subsequent analysis by flow cytometry, which permits determination of the percentage of viable cells, cells in early apoptotic stage, late apoptotic stage, and dead cells. Untreated cells, as well as cells treated with transfection agent but without siRNA\textsubscript{survivin} served as negative controls. For these
experiments, cells floating in the medium, as well as cells growing in a monolayer were recovered and combined for an apoptosis assay using the previously described kit. Combined cells were spun down and resuspended in 500 µL of medium. Next, 10 µL medium binding reagent and 2 µL Annexin V-FITC were added and the mixture incubated for 30 minutes at room temperature in the dark. Following incubation, cells were spun down and resuspended in 500 µL of a cold one-time binding buffer. After that, 10 µL propidium iodide was added and samples were placed on ice and away from light, before analysis by flow cytometry. Note that this assay can only be performed on live cells and it is unsuitable for fixed material. Measurements were obtained using the Beckman Coulter Epics XL flow cytometer analyser. Data analysis was performed utilising FlowJo software.

3. Results:

oxDWNTs were found to consist of single or small bundles of shorter nanotubes (200-2000 nm length, 296 nm ± 123 nm on average as determined by AFM and phase-analysis light scattering (PALS) [17]). TEM analysis (figure S1 in the supporting information) confirmed the Poly(Lys:Phe) coating of Poly(Lys:Phe)-wrapped oxDWNTs. Analysis by Raman spectroscopy revealed distinct peaks characteristic of DWNTs (figure 3a): the radial breathing modes (RBMs) at 150, 200, 230 and 260 cm⁻¹ (correlating with diameters of 0.9, 1.1, 1.2 and 1.58 nm [18]), as well as the D-band (1350 cm⁻¹), G-band (1590 cm⁻¹), and the 2D-band (2600 cm⁻¹), which can be clearly observed in the spectrum. Figure 3b shows a Raman map of the RBM in a mammalian cell, which has previously been exposed to oxDWNT-RNA. A strong, locally confined Raman signal was detected within the whole cell, confirming successful DWNT uptake and possible localization within endosomes, as also reported in previous work by our group, which has looked at the intracellular distribution of functionalized DWNTs in greater detail [19, 20].
An important factor for the design of DWNTs as a nano-vector is that they do not significantly modify the cell environment, so that perturbations due to the payload can be detected. Raman spectroscopy can serve as a powerful tool to assay biochemical changes in cells [19, 21]. Indeed, Raman spectra of oxDWNT-RNA inside cells (obtained by analysing whole cell lysates) exhibit several peaks in addition to those observed for DWNTs alone, of which some can be contributed to cell constituents. Figure 4 plots three of those peaks and their variation over time (24 h-time frame): DNA backbone stretching at 1090 cm$^{-1}$ [22], the CH bending mode of proteins and lipids at 1450 cm$^{-1}$ [23], and amide I $\alpha$–helix vibrations of proteins at 1660 cm$^{-1}$ [24]. The DNA backbone stretching (O-P-O) Raman peak has been described as suitable for monitoring the process of cell death due to its integral association with DNA disintegration [22]. When a comparison was made between cells exposed to
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DWNTs and unexposed controls, it was found that identical forms and patterns were maintained.

Figure 4: Time-dependent analysis of cell constituents by Raman spectroscopy after exposure to oxDWNT-RNA: DNA, protein/lipids and protein structure (Amide I α-helix). Cell constituents were not disturbed through incubation with oxDWNT-siRNA in comparison with control cells.

Next, we investigated the ability of oxDWNTs to bind and deliver siRNA into cells. siRNA has a vast potential as a therapeutic agent for various disorders, but cannot cross the cell membrane on its own due to its negative charge, and is prone to degradation by nucleases inside the cell. In this case, we used oxDWNTs to deliver two types of siRNA: first, siRNA to silence the GFP gene as a proof of principle (siRNA\textsubscript{GFP}), and secondly, siRNA to silence the gene \textit{survivin}, which has therapeutic value for cancer therapy (siRNA\textsubscript{survivin}).

Figure 5 shows fluorescent micrographs of GFP-expressing cells treated with different concentrations of siRNA\textsubscript{GFP}.
Figure 5: Percentage of cells expressing GFP in HeLa and PC3 cells treated with siRNA_{GFP}. GFP stable cell lines were produced via a protocol of 4-week selection to obtain 80-90% GFP-expressing cells, using fluorescence-activated cell sorting (FACS). GFP knockdown was achieved using either oxDWNTs or a common transfection reagent (Lipofectamine®) serving as the positive control, and was determined by confocal microscopy by counting GFP-expressing cells (green cytoplasm plus blue nucleus) against non-expressing cells (blue nucleus only). siRNA_{GFP} delivery by lipofectamine produced around 89% knockdown of GFP in Hela cells and 69% in PC3 cells for the 100 nM sample, while oxDWNTs-siRNA_{GFP} at 100 nM siRNA_{GFP} concentration induced about 40% knockdown in Hela cells and 29% knockdown in PC3 cells in comparison with the negative control. Results are presented as mean ± SEM (n=5).

Under the employed conditions, both lipofectamine® and oxDWNT showed negligible toxicity. Control cells and cells treated with non-specific siRNA (siRNA_{neg}) displayed around 80% cells
expressing GFP. The treatment with 50 nM of bound siRNA\textsubscript{GFP} using lipofectamine\textsuperscript{®} as delivery agent produced a pronounced effect in GFP expression in both cell lines, which decreased to a level of about 20\% in Hela cells and 45\% in PC3 cells. Treatment with 100 nM bound siRNA\textsubscript{GFP} delivered by lipofectamine\textsuperscript{®} decreased GFP expression to a level of about 8\% and 21\%, respectively. The knockdown effect using oxDWNT-siRNA\textsubscript{GFP} was lower than for the positive control lipofectamine, but nevertheless a significant decrease in GFP expression to a level of about 50\% was observed after only 48 hours.

We next investigated the delivery of therapeutic siRNA targeting the gene \textit{survivin} by the developed oxDWNT vectors (siRNA\textsubscript{survivin}). Figure 6 reveals the effect of siRNA\textsubscript{survivin} delivery using oxDWNTs, again in comparison with a common transfection reagent (DharmaFECT\textsuperscript{®}) and untreated cells (PC3 cells only and cells treated with DharmaFECT\textsuperscript{®} only). The results showed similar levels of apoptosis for cells treated with siRNA\textsubscript{survivin} delivered by oxDWNTs (35.89\%) and by DharmaFECT\textsuperscript{®} transfection agent (44.92\%), which are both significantly higher than the two controls; cells only (5.22\%) and cells treated with DharmaFECT\textsuperscript{®} only (18.64\%).

**Figure 6:** Flow cytometry analysis of apoptosis in cells treated with siRNA\textsubscript{survivin}, showing percentages of cells at different apoptotic stages for each treatment. PC3 cells only and PC3 cells treated with DharmaFECT\textsuperscript{®} only served as the negative controls and siRNA\textsubscript{survivin} transfected using
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the commercial transfection agent DharmaFECT® (DharmaFECT-siRNA) as the positive control. A similar apoptotic effect was obtained for oxDWNT-siRNA\textsubscript{survivin} than for the positive control DharmaFECT-siRNA\textsubscript{survivin}.

4. Discussion:

The selective and robust effect of RNA interference on gene expression, mediated by siRNA, makes it not only a valuable research tool, but also a promising therapeutic platform for the treatment of viral infections, dominant disorders, cancer, and neurological disorders [25]. In this article, we focused on the delivery of siRNA targeting the gene \textit{survivin} as a potential treatment for a range of cancers, as the survivin protein regulates apoptosis and controls cell proliferation and is expressed highly in most human tumours and fetal tissue, but is completely absent in terminally differentiated cells. The successful delivery of siRNA to target specific genes in mammalian model organisms has been achieved using a variety of methods, including liposomes, cationic lipids, polymers and nanoparticles. However, systemic toxicity remains a great concern with most of these delivery options. Thus, there is an ever-growing need to enhance the available tools that can lead to effective and safe gene delivery. The functionalized oxDWNTs developed in this work present an alternative with reasonable biocompatibility, which can simultaneously serve as an imaging agent due to their unique Raman signature within biological environments (figure 3). DWNTs have an important advantage over SWNTs, for which any structural damage disrupts their mapping and progression over time. Furthermore, as we have shown in previous studies, they are released from the cells within 24 hours after having fulfilled their task and do not accumulate within cells [20]. In this study we investigated whether oxDWNT-RNA causes any changes in the cell biochemistry within this time frame, which they did not (figure 4). This is in good agreement with a previous study of our group, which has shown that oxDWNT-RNA did not cause phosphorylation of mitogen-activate protein kinases (MAPKs), which are markers for induced cellular stress [19]. In addition, we have also previously investigated the effect of oxDWNTs on cell viability after 96 h incubation by a MTT cytotoxicity assay and found no dose-dependent toxicity, although an overall drop of cell viability of about 5-15%
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was observed, possibly due to inhibition of cell proliferation or decreased cell adhesion [17]. Overall, this indicates that oxDWNT-RNA does not have a deleterious effect on cells during their passage, although long-term effects remain to be investigated.

In terms of therapeutic efficacy, the achieved silencing effect (“gene knockdown”) was either lower than commercial transfection agents (GFP-siRNA/lipofectamine®, figure 5) or at a comparable level (survivin siRNA/DharmaFECT®, figure 6). Considering that most commercially-available \textit{in vitro} transfection agents are unsuitable for therapeutic applications due to their systemic toxicity, this indicates that DWNTs may be successfully employed to deliver therapeutic moieties, such as siRNA, to cells for the treatment of cancerous disorders, as demonstrated in this study. Future work will need to be done to study the \textit{in vivo} performance of our system, which will show whether oxDWNTs do have an advantage over cationic lipid-based transfection agents (e.g. lipofectamine® and DharmaFECT®) due to improved biocompatibility, and will allow for a comparison of the \textit{in vivo} performance of oxDWNTs with other carbon nanotube-based delivery systems in the literature.

5. Conclusions:

In summary, we show that short oxDWNTs produced by CCVD and subsequent acid treatment and functionalization are suitable for the delivery of therapeutic moieties and do not exhibit the toxic effects that have been attributed to longer CNTs [5]. Their unique Raman spectral features, such as the RBM, allow for their detection inside cells by Raman spectroscopy, while simultaneously enabling the detection of changes in cell biochemistry following oxDWNT uptake. It is demonstrated that during a period of 24 hours RNA-wrapped oxDWNTs did not cause detectable changes in the cells’ DNA, protein or lipid content. Our final goal, however, was to use oxDWNTs as a nano-vector for therapeutic payloads to achieve a clinically relevant effect. After loading oxDWNTs with siRNA to block the expression of the anti-apoptotic protein survivin, which is over-expressed in most human tumours, we successfully demonstrated apoptosis of cells after transfection with oxDWNT-siRNA\textsubscript{survivin} complexes to a similar level as obtained with siRNA\textsubscript{survivin} delivered by a standard siRNA transfection agent. It should be noted that although transfection agents are able to efficiently transport siRNA into cultured cells, they are unsuitable for therapeutic applications due to their systemic...
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toxicity. The study thereby advances the goal of developing nano-vectors for safe and efficient
delivery of therapeutic payloads.

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References:


