Chloroquine-enhanced gene delivery mediated by carbon nanotubes

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A B S T R A C T

Polyethyleneimine-coated double-walled carbon nanotubes (DWCNTs) were used for dual gene and drug delivery, after loading the DWCNTs with the drug chloroquine, a lysosomotropic compound that is able to promote escape from the lysosomal compartment. Different forms of functionalization of the DWCNTs were examined in order to optimize this system. They included the testing of different treatments on DWCNTs to optimize the loading and delivery of chloroquine and the selection of a cationic polymer for coating the DWCNTs for optimum DNA binding and delivery. An acid oxidation treatment of DWCNTs was selected for optimum chloroquine loading together with polyethyleneimine as optimum cationic coating agent for plasmid DNA binding. Optimization of the conditions for choroquine-enhanced gene delivery were developed using luciferase expression as a model system. We have demonstrated that chloroquine-loading increases the ability of polyethyleneimine-coated DWCNTs to deliver functional nucleic acid to human cells. Cell viability tests have shown no cytotoxicity of the functionalized DWCNTs at the concentrations needed for optimum gene delivery. These results support the potential applications of this methodology in gene therapy.

1. Introduction

Carbon nanotubes (CNTs) emerged as a new tool in nanobiotechnology and nanomedicine, particularly since new functionalization methods were carried out to enhance their biocompatibility and develop novel medical therapeutics [1,2]. One of the most promising applications of CNTs in the biomedical field is in gene therapy. CNTs have been shown to be efficiently internalized by mammalian cells [3] and able to deliver gene cargoes [4]. However, transfection efficiencies obtained in cells and small animal models are not yet comparable to those reached with other delivery systems, such as viral vectors [5]. It is likely that the relatively low efficiencies are due to inefficient penetration of barriers at systemic tissue and cellular levels that the vector has to be able to overcome in order to reach its target and deliver its cargo [6]. A key barrier to overcome in order to obtain efficient gene expression is intracellular trafficking of the internalized vector [7]. Nucleic acid degradation during passage through the cellular internalization pathways is likely to play a key role in the ability of any vector to deliver a functional nucleic acid to its target in the nucleus (for DNA) or to the gene expression machinery (for RNA) in the cytoplasm. However, most nonviral vectors, including CNTs, have shown to be internalized

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by endocytosis [8] which leads to entrapment and degradation of the genetic material in lysosomes. Escape of delivered cargo from the lysosomal degradative pathway is likely to be crucial to development of therapeutic application of CNT delivery [9]. Several strategies have been developed to overcome the intracellular DNA degradative barrier for other nucleic acid delivery systems based on the destabilization of endosomal and lysosomal membranes [10]. For example, addition of lysosomotropic compounds in the cultured media has been reported to greatly enhance the transfection efficiency [11]. These compounds are weak bases that become protonated at the acidic pH inside the lysosomes and, in consequence, cause a swelling and rupture of the endocytic vesicle, releasing delivered genetic material into the cytoplasm. Carbon nanotubes can be exohedrically functionalized by attaching different functional groups or cargoes to the external walls [12,13], but can also be filled with different compounds [14,15], although optimization of filling and release remains problematic [16]. In this context, double-walled carbon nanotubes (DWCNTs) are a versatile material for a carrier design as the outside can be functionalized without modifying the integrity of the inner tube, leaving it intact for filling. The toxicity of the DWCNTs used in this work has been investigated extensively in earlier work [3,17,18], as well as their potential environmental impact [19,20]. Theoretical studies have investigated the filling and loading on the external walls of CNTs with different anticancer drugs [13,21] and subsequent release for therapeutic purposes [22]. However, the loading of CNTs with a compound to be co-delivered with plasmid DNA to improve gene delivery has not so far been investigated. We report for the first time the loading of DWCNTs with the lysosomotropic anti-malarial drug chloroquine and demonstrate its ability to enhance the cell transfection efficiency.

2. Experimental

2.1. Materials

Chloroquine diphosphate salt, poly(Lys:Phe) 1:1 hydrobromide (PLP), polyethylenimine (PEI), RNA from baker's yeast were purchased from Sigma. DSPE-PEG(2000)-amine (DSPEA-PEG) was purchased from Avanti Polar Lipids. pGL3-control vector and Bright-GloTM luciferase assay system were purchased from Promega. Qiaprep Spin MiniPrep Kit was purchased from Qiagen. DC Protein Assay was purchased from Bio-Rad. Opti-MEM I Reduced-Serum Medium (1x) and MEM medium were purchased from Invitrogen.

2.2. Synthesis of DWCNTs

DWCNTs were produced by CCVD decomposition of CH_4 over Mg(1-x)CoxO solid solution, containing small addition of molybdenum, as described earlier [23]. After the CCVD synthesis, the remaining oxide material, as well as the unprotected metal (Co, Mo) particles, were removed by treatment of the sample with concentrated aqueous HCl solution. The acidic suspension was filtered on 0.45 μm pore size cellulose nitrate membrane (Whatman) and washed with deionized water until neutrality. The resulting DWCNTs, denoted extracted DWCNTs (raw DWCNTs), were then dried overnight in an oven, at 80 °C [24]. High-resolution transmission electron microscopy showed that a typical sample consists of ca. 80% DWCNTs, the rest being SWCNTs (ca. 15%) and a few triple-walled carbon nanotubes (ca. 5%). The diameter distribution of the DWCNTs ranged from 0.5 to 2.5 nm for inner tubes and from 1.2 to 3.2 nm for outer tubes. The length of individual DWCNTs usually ranges between 1 and 10 μm, although bundles may be much longer (up to 100 μm at least) [23].

Two different treatments of the raw DWCNTs were performed (a) an oxidation treatment and, (b) an opening treatment. These treatments were used to optimize the chloroquine loading on DWCNTs and therefore, the loading yield was compared for raw, oxidized and opened DWCNTs. (a) Oxidation treatment: the walls of the DWCNTs were not expected to be functionalized during the synthesis and catalyst-elimination conditions [25]; so an additional post-synthesis treatment was required, in order to obtain oxygen-containing functional groups on the outer wall of the raw DWCNTs. This was achieved with nitric acid treatment which produces mainly carboxylic groups [26]. A sample of oxidized DWCNT was thus prepared by refluxing raw DWCNTs (30 mg) in nitric acid solution (30 mL, 3 M), at 130 °C. After 24 h, the obtained suspension was filtered and the DWCNTs were washed with deionized water until a neutral pH was obtained. A ten-minute tip-sonication step in deionized water was used to disperse the oxidized DWCNTs. After re-filtration, the so-called oxidized DWCNTs were dried overnight in an oven at 80 °C. (b) Opening treatment: raw DWCNTs were also opened by treatment with solid NaOH [27]. Briefly, raw DWCNTs (30 mg) were intimately mixed in glove-box conditions with NaOH (ca. 122.3 mg), with a purity of 98.5+% (Aldrich), in an approximate molar ratio of 1:1.3 [28]. The grey mixture-powder was then transferred into a quartz ampoule and sealed under vacuum. It was finally placed in a tubular furnace and heated at 3 °C min^{-1} to 418 °C (100 °C above the melting point of NaOH, 318 °C). The sample was kept for 240 min at this temperature and it was then slowly cooled down to room temperature at 1 °C min^{-1}. A black agglomerate with metallic reflects was obtained, which was again ground in a mortar, until a powder was obtained. In order to eliminate the excess of NaOH (at the surface of the tubes), the resulting powder was then transferred into a small quantity of deionized water, sonicated for 1 min in a sonication bath, filtered and then washed with deionized water. A reflux step, under continuous stirring in deionized water at 80 °C was used to eliminate the remaining excess of NaOH. After 24 h, the obtained grey suspension was filtered and the DWCNTs were washed with deionized water, until the colour of the filtrate could no longer be observed. The so-called opened DWCNTs were obtained from the filtration-membrane and dried overnight in an oven, at 80 °C.

2.3. Chloroquine loading of DWCNTs

About 15 mg of each non-loaded raw, oxidized and opened DWCNTs were loaded in solution with an excess of chloroquine diphosphate salt [29,30]. This anti-malarial drug is very soluble in water (100 mg mL^{-1}) and extremely sensitive to sunlight. For

the loading of each type of DWCNTs, we followed the same procedure. In a first step, three identical solutions were prepared: chloroquine diphosphate salt (4750 mg) was quickly dissolved in deionized water (50 mL) (30 s of bath sonication). Then, DWCNTs (15 mg of raw or oxidized or opened) were added to the corresponding solution and stirred 24 h at room temperature. The flasks were all the time protected from sunlight with aluminium foil. Free chloroquine was removed by filtration and subsequent washing steps. After 24 h, the three solutions were filtered to obtain chloroquine-loaded raw, oxidized and opened DWCNTs. The tubes were washed with few drops of deionized water and then ethanol, followed by drying under vacuum at room temperature.

2.4. Coating of DWCNTs with cationic polymers

Raw DWCNTs, oxidized DWCNTs (with HNO_3, 3 M) and opened DWCNTs (with NaOH) were coated with different cationic polymers: polyethyleneimine (PEI), poly(Lys:Phe, 1:1) (PLP) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol) 2000 (DSPE-PEG-NH_2). Each kind of non-coated DWCNTs (1 mg) was respectively mixed with the cationic polymer solution in deionized water (1 mL of 2 mg mL^{-1} for PEI and 1 mL of 0.5 mg mL^{-1} of PLP and DSPE-PEG-NH_2). The mixtures were sonicated in a tip sonicator (Soniprep 100 W of output power, set at 20% of maximum amplitude) for 100 s in 10 cycles of 10 s on 10 s off. The suspensions were centrifuged at 5000 rpm for 3 min and the supernatant was filtered in ultrafiltration tubes of 0.1 μm from Millipore. The filtrates were washed 4 times with deionized water (50 μL), resuspended in deionized water (0.5 mL) and ultrasonicated for 20 s (2 cycles of 10 s on 10 s off).

2.5. Coating of DWCNTs with RNA

Raw DWCNTs, oxidized DWCNTs and opened DWCNTs were treated with RNA. Each kind of DWCNTs (2 mg) were mixed with RNA solution in deionized water (1 mL of 4 mg mL^{-1}) and ultrasonicated (Soniprep 100 W of output power, set at 20% of maximum amplitude) for 100 s in 10 cycles of 10 s on 10 s off. Then, deionized water (1 mL) was added and the mixtures were sonicated in a 100 W water bath for 1 h. The suspensions were filtered on ultrafiltration tubes of 0.1 μm from Millipore. Each filtrate was washed 3 times with deionized water (50 μL) and resuspended in deionized water (1 mL).

2.6. Agarose gel electrophoresis for quantification of the chloroquine-loading of DWCNTs

0.8% Agarose gel electrophoresis in TAE buffer (mixture of tris base 40 mM, acetic acid 40 mM and EDTA 1 mM) was used to characterize the chloroquine loading of the DWCNTs and the release of the drug from the carbon nanotubes. Gel electrophoresis was run for 30 min at 90 V with 40% sucrose solution in deionised water as loading buffer (the final concentration of sucrose in the loaded samples was 6.7%). Chloroquine on its own was loaded in the gel as control to a concentration in the range of (5–75) μM. Chloroquine-loaded raw, oxidized and opened DWCNTs were also coated with RNA prior to electrophoresis. For this step, each kind of chloroquine-loaded DWCNTs (2 mg) were mixed with RNA solution (1 mL of 4 mg mL^{-1}) and ultrasonicated for 100 s (Soniprep 100 W of output power, set at 20% of maximum amplitude, 10 cycles of 10 on and 10 s off). RNA-coated carbon nanotubes were well dispersed and no centrifugation was carried out so that the amount of carbon nanotubes loaded onto the gel was accurately known. The suspensions were filtered on 0.1 μm hydrophilic PVDF filtration membranes and the solid was washed with deionized water. Each solid was resuspended in deionized water (1 mL). For the quantification of the chloroquine-loading, each sample of RNA-coated DWCNTs (20 μL of 0.24 mg mL^{-1}) dispersed in deionized water and in 0.3% hydrochloric acid were mixed with the loading buffer (4 μL) and loaded into each well.

2.7. Kinetics of chloroquine release from DWCNTs

Gel electrophoresis, as described above, was run for 30 min at 90 V with 40% sucrose as loading buffer. Filled DWCNTs were functionalized by wrapping with the amphiphilic polypeptide poly(Lys:Phe, 1:1). Each kind of chloroquine-loaded DWCNTs (1 mg) was respectively mixed with poly(Lys:Phe, 1:1) (1 mL of 2 mg mL^{-1}). The mixtures were ultrasonicated for 100 s (Soniprep 100 W of output power, set at 20% of maximum amplitude, 10 cycles of 10 s on, 10 s off) and centrifuged at 100 rpm for 5 min. The supernatants were filtered and washed with deionized water on 0.1 μm filtration membranes. Each solid was resuspended in deionized water (0.75 mL). The poly(Lys:Phe, 1:1)-coated chloroquine-loaded DWCNTs (300 μL of 60 μg mL^{-1}) were mixed with universal buffer 0.1 M pH 4.8 or 7.4 (50 μL). Universal buffer was prepared from an equimolar mixture of phosphoric acid, boric acid and acetic acid and adjusting the pH with NaOH 1 M or HCl 1 M. The mixtures of poly(Lys:Phe, 1:1)-coated chloroquine-loaded DWCNTs were incubated in a water bath at 37 °C for 24 h. Aliquots (50 μL) were removed after universal buffer addition ($t = 0$), and after 30 min, 60 min, 90 min, 2 h, 3 h, 24 h. The aliquots were filtered on 0.1 μm filtration membranes and the filtrate kept at −4 °C before electrophoresis. The gel was run for 30 min at 90 V.

2.8. Delivery of luciferase gene into HeLa cells

For the preparation of the transfection mixtures, the appropriate amount of PEI-coated DWCNTs (loaded or non-loaded with chloroquine) was mixed with the solution of pGL3 plasmid and incubated for 30 min prior transfection. These DWCNTs-PEI-pGL3 complex solutions (250 μL) were mixed with Opti-MEM Reduced Serum Media (Invitrogen) (250 μL) and supplemented with 1% antibiotics (penicillin/streptomycin). HeLa cells were cultured in 24-well plates in D-MEM (Invitrogen) without antibiotics until they reached 75% confluence. The cells were then washed with PBS and incubated with the transfection mixtures as described above for 4 h at 37 °C. After 4 h, 1 mL of MEM supplemented with 10% fetal bovine serum, 1% non essential amino acids and 1% antibiotics (penicillin/streptomycin) was added without removing the transfection mixture and the cells were incubated overnight in this medium. The medium was then replaced by fresh media and the cells were incubated for 48 h to express the luciferase gene.

2.9. *Luciferase assay*

Cells were washed twice with PBS and incubated for 15 min with lysis buffer (100 μL). After scraping the cells off the dish, the lysate was centrifuged for 15 s at 12,000*g* at room temperature and the supernatant was transferred into a new tube and kept on ice to perform the luciferase assay. Luciferase substrates (Bright-Glo™ Luciferase Assay System from Promega) (50 μL) was mixed with the lysate (50 μL) and luminescence signal was recorded in a well plate reader and averaged over 5 min. Total protein was determined in the cell lysate by the Bio-Rad DC Protein Assay which is a colorimetric method based on the Lowry assay [31].

2.10. *MTT cell viability assay*

Cells were seeded in a 96-well plate at 5000–10000 cells per well in 200 μL of media and incubated overnight. One hundred microliter of media ± RNA-coated oxidized or open non-loaded and loaded DWCNTs was added to each well and cells incubated for three days. MTT was added to each well (10 μL) and incubated at 37 °C for 3 h. The media was removed and 200 μL of DMSO was added per well to dissolve the formazan crystals. Finally, the absorbance values at 570 nm (working wavelength) and 630 nm (reference wavelength) were used as a measurement of cell viability.

2.11. *Characterization of DWCNTs*

Raman spectroscopy: Raman spectra of the non-loaded DWCNTs were obtained at 633 nm, with a LabRAM HR 800 (Jobin and Yvon) spectrometer. Chemical analysis: Elemental analysis was performed by atomic emission spectroscopy (AES). HR-TEM: The HR-TEM was carried out using a high resolution transmission electron microscope JEOL-JEM-2100F (operated at 200 kV). For sample preparation, chloroquine-loaded DWCNTs were briefly dispersed in ethanol. A few drops of each suspension were then deposited onto a 300 mesh Lacey copper TEM grid placed on absorbing paper.

3. Results and discussion

3.1. *Synthesis and characterization of the chloroquine-loaded DWCNTs*

The purpose of this work is to synthesize a gene delivery vector able to carry and co-deliver a lysosomotropic drug in order to enhance the transfection efficiency. For this purpose, DWCNTs were selected because of their versatility, as both their external wall and inner tube can be functionalized for loading with different compounds, and because of their no toxicity at concentrations potentially useful for biomedical applications. Compared to common sources, they present the advantage of being perfectly characterized in terms of morphology and purity, as well as toxicity thanks to earlier studies [3,17–20]. They also offer a better chemical and mechanical stability as compared to single-walled carbon nanotubes (SWCNTs) and thus represent the optimal choice in terms of drug/container weight ratio. DWCNTs were coated with a cationic polymer in order to bind plasmid DNA through ionic interactions, and loaded with a lysosomotropic drug to obtain an efficient gene delivery vector. The selected lysosomotropic drug was chloroquine, that promotes a lysosomal escape of the delivery vector avoiding the DNA degradation in the cellular compartment. In this section, DWCNTs were tested first for their ability to be loaded with chloroquine. Raw DWCNTs were synthesized as previously described [23] and were subjected to two different types of treatments: (a) an oxidation treatment with 3 M nitric acid which generates carboxylic acid groups on the CNT walls but also opens the tubes and, (b) a treatment with NaOH which opens the tubes but does not functionalize them. The three types of DWCNTs (raw, oxidized and opened) were compared in their ability to be loaded with chloroquine in order to select the optimum starting DWCNT material for drug loading and subsequent release. For simplicity, the different types and combinations of functionalized DWCNTs that will be used throughout the manuscript are described in Table 1. According to this table, the three types of DWCNTs that will be compared for chloroquine loading are the ones denoted as ((1), (2) and (3)).

As prepared (1)–(3) DWCNTs were first characterized by Raman Spectroscopy. As it can be seen in Fig. 1, differences in the Raman D and G bands, which are an indicator of the level of defects of the sample [31], are observed. In this way, an increasing ID/IG intensity ratio corresponds to a higher level of defects and a decreasing ID/IG ratio corresponds to a higher level of structural quality. The oxidation treatment or raw DWCNT produces an increase in the ID/IG ratio (ID/IG ratio increases from 0.18 for (1) to 0.44 for (2)). Oxidation with nitric acid is known to remove impurities from CNTs (such as amorphous carbon and metals), and therefore this increase in ID/IG ratio is indicating an increase in the defects in the carbon material which is also consistent with previous studies that have demonstrated that this treatment increases defects in carbon compounds [32]. Besides, NaOH treatment of raw DWCNTs produces a decrease in the ID/IG ratio from 0.18 for (1) to 0.10 for (3). This is consistent with previous studies that have demonstrated that treatment with molten NaOH is a recognized single step process for the simultaneous purification and opening of raw carbon nanotubes [33].

Chloroquine diphosphate salt ($C_{18}H_{26}ClN_3.2H_3PO_4$) was used for the loading of DWCNTs samples (1)–(3). Chloroquine is a hydrophobic weak base with a quinoline aromatic ring that can interact with CNT walls, both the external and the inner walls, through hydrophobic interactions (π–π stacking interactions). The DWCNTs used for this study have a wide diameter (diameter distribution of the DWCNTs ranged from 0.5 to 2.5 nm for inner tubes and from 1.2 to 3.2 nm for outer tubes) in order to increase the surface area and, therefore the ability to load the nanotubes with the drug. Loading of the drug was attempted by incubation of DWCNTs samples (1)–(3) in chloroquine diphosphate salt aqueous solution for 24 h at room temperature. Washing by filtration on a polypropylene membrane (0.45 μm) was used to remove free chloroquine. In this way, chloroquine-loaded DWCNTs samples (4)–(6) were obtained. After recovery of the DWCNTs, chemical analysis of the proportion of chlorine in the CNTs was used to quantify the chloroquine content of each preparation. As it can be seen in Table 2, both samples (5) and (6) have a similar and higher chloroquine content than sample (4). Energy-dis-

Table 1 – Different types of DWCNTs used in this work.

Construct number	Chloroquine loading procedure	Chloroquine loading	DWCNT coating	pGL3 plasmid loading
(1)	Raw DWCNT	No	No	No
(2)	Oxidized DWCNT	No	No	No
(3)	Opened DWCNT	No	No	No
(4)	Raw DWCNT	Yes	No	No
(5)	Oxidized DWCNT	Yes	No	No
(6)	Opened DWCNT	Yes	No	No
(7)	Raw DWCNT	No	RNA	No
(8)	Oxidized DWCNT	No	RNA	No
(9)	Opened DWCNT	No	RNA	No
(10)	Raw DWCNT	Yes	RNA	No
(11)	Oxidized DWCNT	Yes	RNA	No
(12)	Opened DWCNT	Yes	RNA	No
(13)	Oxidized DWCNT	Yes	PLP	No
(14)	Opened DWCNT	Yes	PLP	No
(15)	Oxidized DWCNT	No	DSPEA-PEG	Yes
(16)	Oxidized DWCNT	Yes	DSPEA-PEG	Yes
(17)	Oxidized DWCNT	No	PLP	Yes
(18)	Oxidized DWCNT	Yes	PLP	Yes
(19)	Oxidized DWCNT	No	PEI	Yes
(20)	Oxidized DWCNT	Yes	PEI	Yes
(21)	Opened DWCNT	No	PEI	Yes
(22)	Opened DWCNT	Yes	PEI	Yes
(23)	Raw DWCNT	No	PEI	Yes
(24)	Raw DWCNT	Yes	PEI	Yes

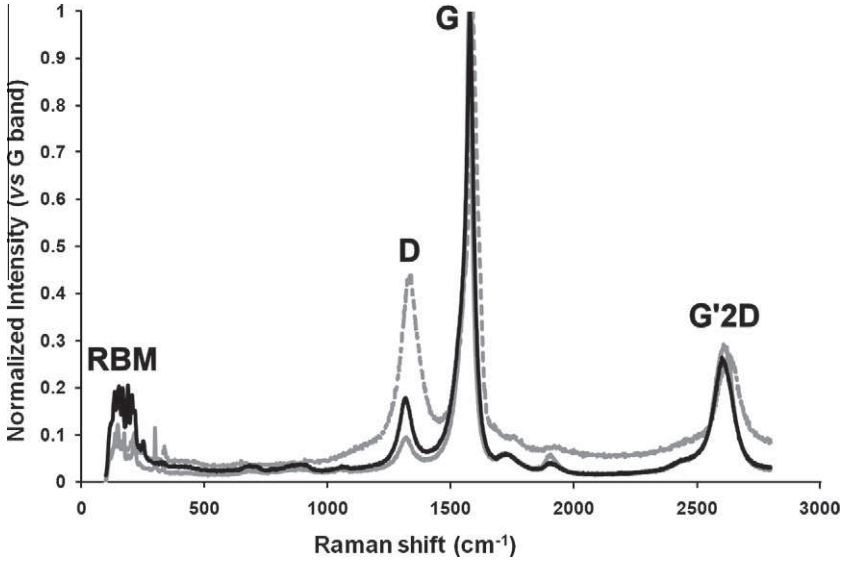

Fig. 1 – Raman spectra of the non-loaded DWCNTs. Comparison between raw (—) and oxidized (- - -) DWCNTs.

persive X-ray spectroscopy (EDX) confirmed also the presence of traces of chlorine, nitrogen, phosphorus and oxygen in chloroquine-loaded DWCNTs which is in agreement with the presence of chloroquine diphosphate on the CNTs (Supplementary material Figs. S1 and S2). Additionally, high-resolution transmission electron microscopy (HR-TEM) was used to study the structure of chloroquine-loaded DWCNTs. Fig. 2 shows a typical HR-TEM image of sample (5). As it can be seen, apparently poorly organized material mainly around individual tubes is apparent. Most of the DWCNTs are covered with some amorphous coating, which could correspond to oxidation debris created by post-synthesis treatment with HNO_3, but the presence of adsorbed chloroquine or even chloroquine filling inside the inner tube cannot be ruled out from these images given the high loading of chloroquine on the samples (see Table 2). In fact, chloroquine cannot only be adsorbed on the DWCNTs but is likely to be also located within the triangular channels formed between the nanotubes in a compact bundle. These channels are actually open and therefore, can be filled with a potential facile release. Consequently, it can be concluded that most of the chloroquine is loaded on the external walls of the DWCNTs both in debun-

Table 2 – Quantification of chloroquine of DWCNTs samples by elemental analysis[a] (before polymer coating) and electrophoresis[b] (after polymer coating of DWCNTs).

DWCNT sample	Chloroquine (wt.%)[a]	DWCNT sample	Chloroquine (wt.%)[b]
(7)	53.8	(10)	2.0
(8)	59.0	(11)	10.5
(9)	73.2	(12)	12.9

[a] Quantification of chloroquine of DWCNTs samples by elemental analysis (before polymer coating).
[b] Electrophoresis (after polymer coating of DWCNTs).

dled and bundled nanotubes. In contrast, Fig. 2b shows a HR-TEM image of sample (6). Both bundles and individual tubes with undamaged outer wall are clearly seen. Here again, the possible presence of chloroquine adsorbed on the DWCNTs outer wall is proposed, especially because NaOH treated samples are not coated by amorphous carbon. Here again, some filling inside the DWCNT cannot be ruled out.

Chloroquine-loaded DWCNTs need to be coated with a polymer in order to be dispersed in aqueous media and to be able to bind DNA for the gene delivery purposes of this work. As chloroquine seems to be mainly adsorbed on the DWCNTs walls, an important aspect to be studied is the effect of this polymer coating on the retention of the chloroquine loading of the DWCNTs. This coating has an effect on the displacement of chloroquine adsorbed on the external wall of the DWCNTs by the coating polymer. However, chloroquine that may be loaded within the triangular lattice of compact bundles should not be affected. Additionally, the polymer coating procedure includes further washing steps that can reduce the total chloroquine loading on DWCNTs. Consequently, the effect of the coating procedure on the retention of the chloroquine loading has to be evaluated. RNA was used as coating agent for the dispersion with high yield and standardization of DWCNTs samples (see Supplementary material Fig. S3) [34].

DWCNT preparations, (4)–(6) were coated with RNA (obtaining samples (10)–(12)) alongside control samples of DWCNTs non-loaded with chloroquine (1)–(3) (obtaining samples (7)–(9)). Gel electrophoresis was used to quantify the chloroquine content of the DWCNT preparations (see Supplementary material Fig. S4). Positively charged chloroquine migrates to the cathode and can be directly detected in the gel thanks to its intrinsic fluorescence, both qualitatively from its R_f (retention factor) and quantitative from its band intensity [35]. A known amount of RNA-coated DWCNTs can be loaded on the gel so that the released chloroquine can be accurately detected and quantified on the gel as the intensity of the chloroquine band is proportional to the concentration of the drug. No chloroquine band was detected for non-loaded DWCNTs samples ((7)–(9)) being only detected for chloroquine-loaded DWCNTs samples ((10)–(12)), as expected. Release of chloroquine from loaded DWCNTs was clearly promoted in acidic conditions (Fig. 3 lane c) compared to neutral pH (Fig. 3 lane b). This can be accounted for tendency of the hydrophobic nanotubes to repel the protonated nitrogen of the quinoline ring of chloroquine at acidic pH leading to the drug's rapid release from the tubes and a consequent sharp band on electrophoresis. In contrast, the samples loaded at neutral pH generated a long tail of chloroquine through the gel which is consistent with greater retention of the drug on the nanotubes and slower release throughout the electrophoresis procedure. The quantification of the loading of chloroquine was performed using the chloroquine band obtained at acidic pH as it corresponds to a complete chloroquine release from the loaded DWCNTs. This quantification is shown in Table 2. As it can be seen, the chloroquine loading on DWCNTs decreases after the polymer coating as expected. In addition, DWCNTs preparations (11) and (12) retained the loaded chloroquine on the nanotubes after polymer coating with higher yield. Therefore, DWCNTs (5) and (6) are the best starting material for obtaining optimum chloroquine loading after polymer coating. Cell viability assays were performed with samples (7)–(12) in order to investigate their cytotoxicity and select the best material for the biological applications of this work (see Supplementary material Section S4). Sample (5) was identified as the one that showed the lowest cytotoxicity. Therefore, sample (5), which corresponds to chloroquine-loaded oxidized DWCNTs, was selected as optimum for the main purpose of this work and will be used in the following studies.

3.2. *Evaluation of chloroquine-loaded DWCNTs as drug delivery system*

Chloroquine-loaded DWCNTs were evaluated as drug delivery system. For these purpose, we investigated cytotoxicity of the chloroquine-loaded DWCNTs using the HeLa human cell line as target and the MTT cell viability assay in which the MTT substrate (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) is reduced to purple formazan in the mitochondria of living cells. RNA-coated DWCNTs both chloroquine-loaded (sample (11)) and non-loaded as a control (sample (8)) were used in these studies. RNA gives a high dispersion yield (between 1 and 2 orders of magnitude higher than with the other coatings) and allowed us the testing of cytotoxicity for a wider range of DWCNTs concentrations. As it can be seen in Fig. 4a, non-loaded DWCNTs showed no cytotoxicity in the range of concentrations studied. However, chloroquine-loaded DWCNTs presented a higher cytotoxicity (LD_{50} value of 190 $\mu g\ mL^{-1}$). This higher cytotoxicity of chloroquine-loaded tubes compared to non-loaded ones was presumably due to the release of the drug, which is cytotoxic on its own (Fig. 4b). Note however that free chloroquine is cytotoxic at concentrations much higher than the total chloroquine concentration in the DWCNTs suspensions. The total dose of chloroquine carried by the DWCNTs was calculated (see Table 2) and depicted in Fig. 4b, showing a shift of one order of magnitude towards lower concentrations compared to

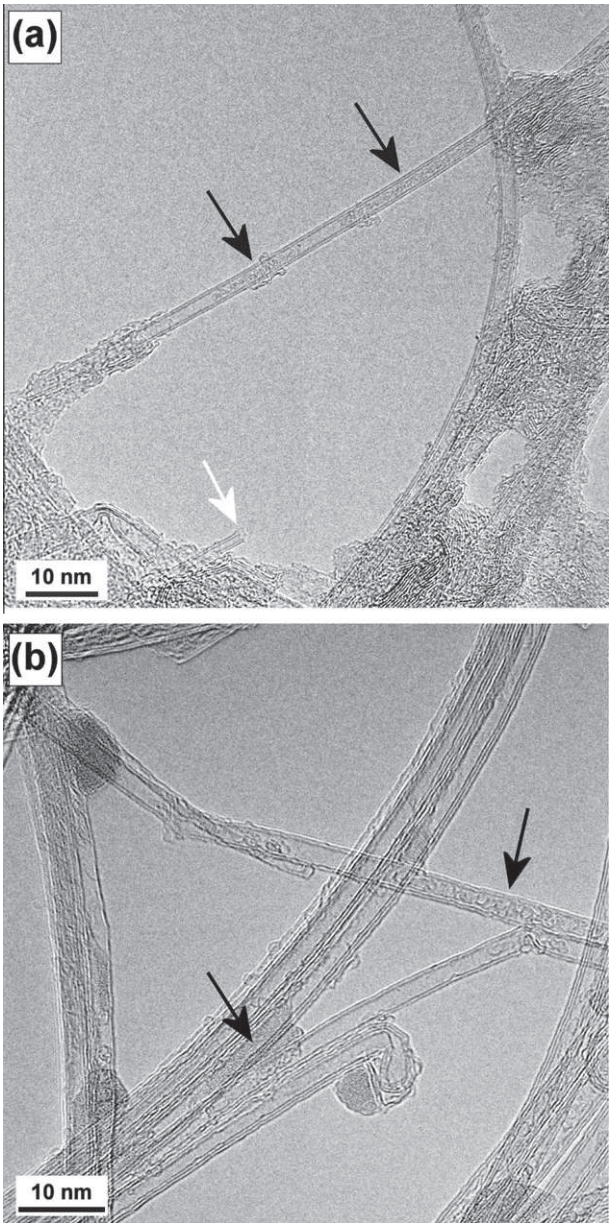

Fig. 2 – HR-TEM images of chloroquine loading within: (a) oxidized DWCNT (sample (5)). The black arrows indicate discontinuous filling with apparently poorly organized material on/in individual DWCNTs; some amorphous coatings on the outer walls are visible, as well as a destroyed tip of an oxidized DWCNT (white arrow); (b) opened DWCNTs (sample (6)): the black arrows indicate the probable presence of poorly organized chloroquine diphosphate salt around the DWCNTs or even possibly inside.

free drug. As it can be seen, DWCNTs are able to deliver the drug into the cells with higher efficiency than free drug (LD_{50} free chloroquine 46 μg mL^{-1}, LD_{50} chloroquine loaded on DWCNTs 16 μg mL^{-1}), proving the efficiency of DWCNTs as drug delivery system.

We next investigated the mechanism of drug release from the DWCNTs. The mechanism of entry of the DWCNTs in

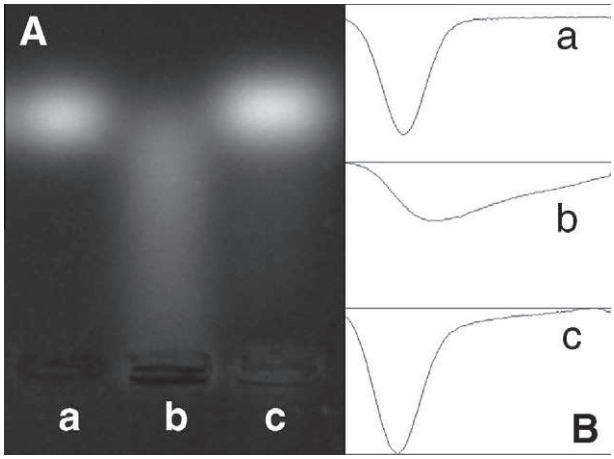

Fig. 3 – Agarose gel electrophoresis pattern of chloroquine-loaded DWCNTs. (A) agarose gel: lane a: free chloroquine 75 μM, lane b: RNA-coated chloroquine-loaded DWCNTs 0.52 mg mL^{-1} dispersed in deionized water, lane c: RNA-coated chloroquine-loaded DWCNTs 0.52 mg mL^{-1} dispersed in hydrochloric acid 0.3%. (B) Profile of the chloroquine bands in the different lanes (a–c). Sample (11) is shown in this figure as a model.

HeLa cells was studied showing internalization by an endocytic pathway (see Supplementarymaterial Section S5), which is in agreement with previous studies [8]. When the DWCNTs are internalized by this mechanism, they are brought into the cytoplasm (pH 7.4) inside an endosome that finally matures into a lysosome, an acidic cellular compartment (pH 4.8). As it was shown in Fig. 3, chloroquine release was promoted at acidic pH, the drug being retained on the loaded DWCNTs at neutral pH. The kinetics of the release of chloroquine from DWCNTs was therefore investigated at neutral and acidic pH. Chloroquine-loaded DWCNTs coated with a cationic polymer (to simulate the future gene delivery conditions) were used for this study (sample (13)). The chloroquine-loaded DWCNTs were incubated at 37 °C (physiological temperature) at pH 4.8, corresponding to the typical pH for the lysosomal compartment, and pH 7.4, corresponding to cytoplasmic pH before electrophoresis. Aliquots of these incubation mixtures were taken at different times, treated as described in the experimental section, and run in agarose gel electrophoresis (pH 4.8 Fig. 5a; pH 7.4 Fig. 5b). The intensity of the chloroquine bands was quantified by pixel intensity/counting using ImageJ imaging software (Fig. 5c). As it can be seen, the kinetics of chloroquine release from the DWCNTs was clearly accelerated at lysosomal pH (a mathematical model was developed for the kinetic description of chloroquine release from the DWCNTs that fitted the experimental kinetic curves (Supplementary material Section S6)). This fact confirms the triggered release of chloroquine from DWCNTs by a pH decrease. In this way, the results obtained in Fig. 4b are confirmed by this mechanism. The pH-triggered drug delivery from by DWCNTs in the lysosomes produces higher levels of the drug inside the cell, reaching cytotoxic values at drug concentrations lower than for free drug. The enhanced release of the drug at acidic pH from DWCNTs is clearly an advantage for gene therapy. Chloroquine remains on the DWCNTs at

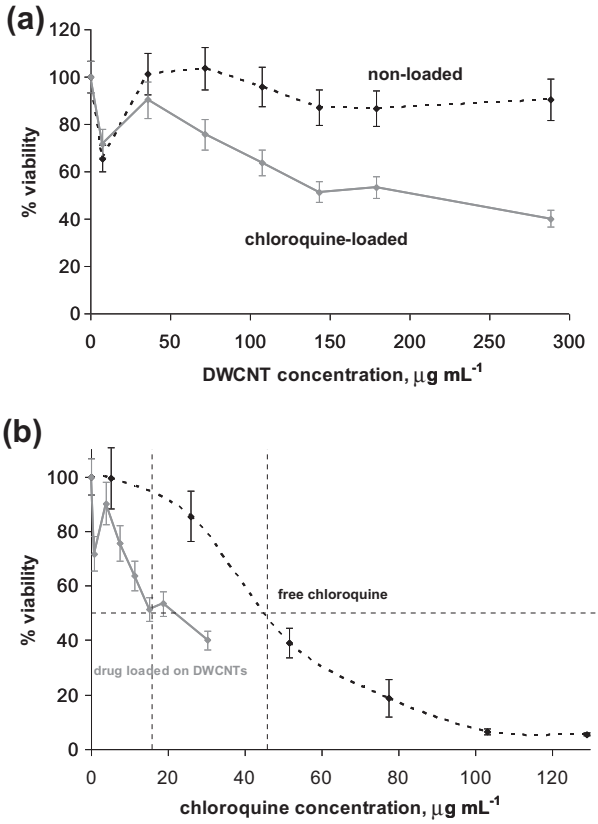

Fig. 4 – MTT cell viability tests with of HeLa cells treated with non-loaded oxidized DWCNTs (sample (8)) ((a) dotted lines) and chloroquine-loaded oxidized DWCNTs (sample (11)) ((a) black solid lines). All DWCNTs samples were coated with RNA prior to the assay. The effect of free chloroquine is shown in ((b) black line) compared to chloroquine loaded on oxidized DWCNTs ((b) grey line).

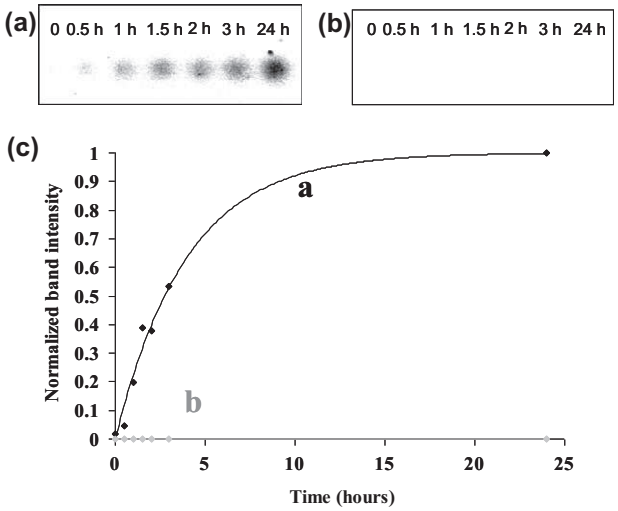

Fig. 5 – Kinetics of chloroquine release from chloroquine-loaded DWCNTs vs. pH. (a: pH 4.8, b: pH 7.4) in universal buffer 0.1 M and at 37 °C (PLP-coated oxidized DWCNT concentration 1.14 mg mL^{-1}, Sample (13) was used in this study; PLP was selected as cationic polymer as it gave the best dispersion conditions of oxidized DWCNTs and therefore, the best accuracy and sensitivity for the chloroquine detection. Furthermore, the cationic polymer simulates the in vitro drug release conditions that will be tested. In all samples, sucrose at 6.67% was added as loading solution and the gel was run at 90 V for 30 min. Agarose gel electrophoresis of the permeates of the aliquots taken and filtered at 0 min, 30 min, 60 min, 90 min, 2 h, 3 h and 24 h. The quantification of the chloroquine bands of (a) and (b) is shown in (c) at pH 4.8 (curve a, corresponding to Fig. 5a) and pH 7.4 (curve b, corresponding to Fig. 5b); the intensity band was normalized by the maximum intensity band value. Data were fitted using a mathematical model (Supplementary material).

physiological conditions and the internalization of the drug-loaded DWCNTs inside the acidic lysosomes of the cells triggers the release of the drug into the cells. Therefore, chloroquine is only released from DWCNTs in the targeted cellular compartment, the lysosome. The released drug being co-delivered with a gene would lead to enhanced gene transfection efficiencies given its lysosomotropic properties. These properties were therefore further explored in the following section.

3.3. *Evaluation of chloroquine-loaded DWCNTs as gene delivery system*

We next investigated the ability of chloroquine-loaded DWCNTs to deliver a functional gene encoding the enzyme luciferase. To interact with cells and deliver genes the nanotubes needed to be funtionalized to be able to bind DNA. In this way, DWCNTs were coated with different cationic polymers in order to disperse them in aqueous media, to test their ability in binding negatively charged plasmid DNA (pGL3 plasmid encoding luciferase gene), and in evaluating these complexes (DWCNT-cationic polymer-pGL3, both chloroquine-loaded and non-loaded) in transfecting HeLa cells. Coating of DWCNTs with the cationic polymers 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)2000] (DSPEA-PEG), (Poly(Lys:Phe, 1:1) (PLP) and polyethyleneimine (PEI) was tested (samples (15)-(20)). Good dispersion of DWCNTs was obtained with these coatings being the complexes able to bind the plasmid DNA with high efficiency (see Supplementary material Section S3). These complexes were tested to study their efficiency in delivering pGL3 plasmid and the effect of their chloroquine loading on the transfection efficiency.

As it can be seen in Fig. 6, choroquine-loaded DWCNTs coated with PEI showed the highest transfection efficiencies when compared with their controls (non-loaded DWCNTs, naked plasmid and free PEI, with free chloroquine and no free chloroquine addition). Furthermore, the ability of the coating polymer to bind plasmid DNA has an effect on the transfection efficiency. The transfection efficiencies for PEI-coated (sample (20)), PLP-coated (sample (18)) and DSPEA-PEG-coated DWCNTs (sample (16)) were in the relative efficiencies 1:0.2:0.07 at the optimum conditions, which is related to the relative DNA binding properties of DWCNTs coated with the different cationic polymers (1.0:0.16:0.037 for PEI-coated, PLP-coated and DSPEA-PEG-coated DWCNTs, respectively (see Supplementary

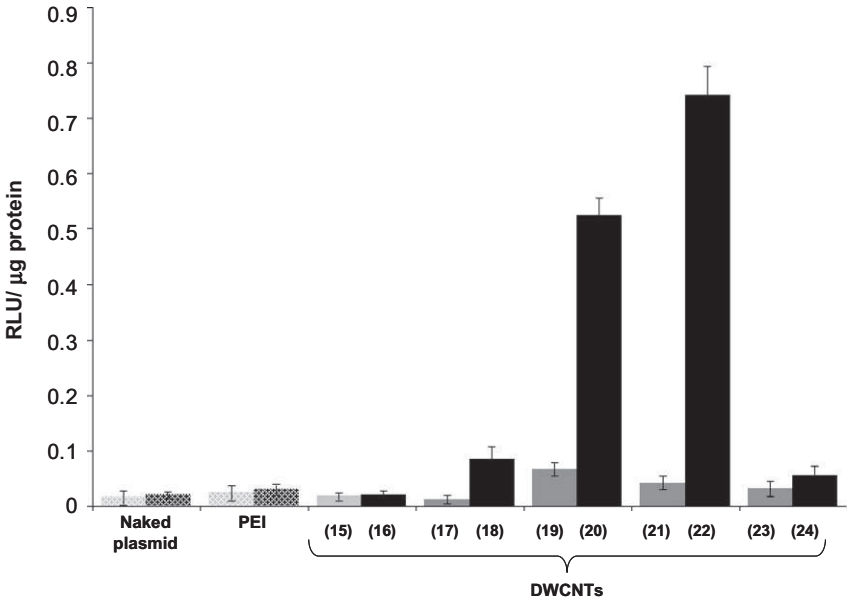

Fig. 6 – Luciferase expression (measured as relative luminescence units (RLU) per total μg of protein) from pGL3 plasmid transfected by DWCNTs without (grey bars) and with (black bars) chloroquine loading. Naked pGL3 plasmid and PEI-pGL3 complexes (PEI 0.001% (w/w) without (dotted grey bars) and with 75 μg mL^{-1} chloroquine (dotted black bars) in the transfection media are shown as controls. 75 μg mL^{-1} of free chloroquine was the concentration for optimum transfection in HeLa cells; in addition nor luciferase expression was obtained for lower free chloroquine concentrations neither for other PEI concentrations (0.01% (w/w) and lower). For transfection with DWCNTs, non-loaded oxidized DWCNTs coated with different cationic polymers and complexed with pGL3 plasmid (samples (15), (17) and (19)) in the optimum conditions (For PEI-coated DWCNTs 6 μg mL^{-1} of DWCNTs were used; for PLP-coated and DSPEA-PEG-coated DWCNTs 12 μg mL^{-1} of DWCNTs were used) were used as controls (grey bars). Transfection efficiencies of the respective chloroquine-loaded oxidized DWCNTs coated with the different cationic polymers and complexed with pGL3 plasmid (samples (16), (18) and (20)) are depicted in black bars. Transfection efficiencies of chloroquine-loaded and non-loaded raw and opened DWCNTs coated with PEI and complexed with pGL3 plasmid (samples (21)–(24)) are also depicted (DWCNTs concentration 6 μg mL^{-1}). pGL3 plasmid concentration in the transfection mixtures was 20 ng μL^{-1}. Lipofectamine was used as positive control to assess the effectiveness of the gene delivery vector based on DWCNTs; the system based on DWCNTs reached in the optimum conditions around 10% of the transfection efficiency of lipofectamine (data not shown).

material). There was a clear enhancement of transfection efficiency by using chloroquine-loaded DWCNTs, of about two orders of magnitude when compared with controls. Note that this result was obtained at a chloroquine concentration in the entire transfection mixture lower than 1 μM (estimated from the DWCNT concentration in the transfection mixture and loading yield of the nanotubes with chloroquine) that was much lower than the concentration normally added to culture media as free drug for optimum transfection (of 25–100 μM) [36–38]; and so was highly unlikely to be due to leeching out of the chloroquine prior to entry of CNTs inside cells. The other forms of DWCNTs assayed for optimizing chloroquine loading were also tested to investigate the effect of chloroquine loading on DWCNTs on the transfection efficiency. As it can be seen, as the chloroquine loading increases the transfection efficiency increases (chloroquine loading are in the order (24) < (20) < (22) corresponding to opened < oxidized < raw DWCNTs). However, as explained in Section 3.5, sample (20) showed the lowest cytotoxicity and therefore, it was selected as optimum. In addition, these DWCNT showed no cytotoxicity at the concentration levels needed for optimum gene delivery (see Supplementary material Section S4). In conclusion, chloroquine-loaded oxidized DWCNTs coated with PEI (sample (20) were selected as optimum gene delivery system. The overall approach of this methodology is illustrated in Fig. 7. It is therefore highly likely that the enhancement of transfection efficiency was due to the release of chloroquine from the loaded tubes on their capture into lysosomes and subsequent chloroquine-mediated promotion of lysosomal escape for the DNA cargo. It seems likely that the released PEI-plasmid complexes are then able to cross the nuclear envelope [39] leading to efficient plasmid transcription and subsequent translation.

4. Summary

These studies have demonstrated a new methodology for improved gene delivery based on the loading of DWCNTs with the lysosomotropic compound chloroquine capable of promoting escape of the DNA cargo from lysosomes. Chloroquine is an established anti-malarial drug that is administered to thousands of patients each year, so its toxicological properties are already well characterized. Acid treated (oxidized) DWCNTs coated with PEI and loaded with chloroquine showed the best results for gene delivery. Cell viability tests showed no cytotoxicity of the functionalized DWCNTs at concentrations needed for optimum gene delivery. The triggered

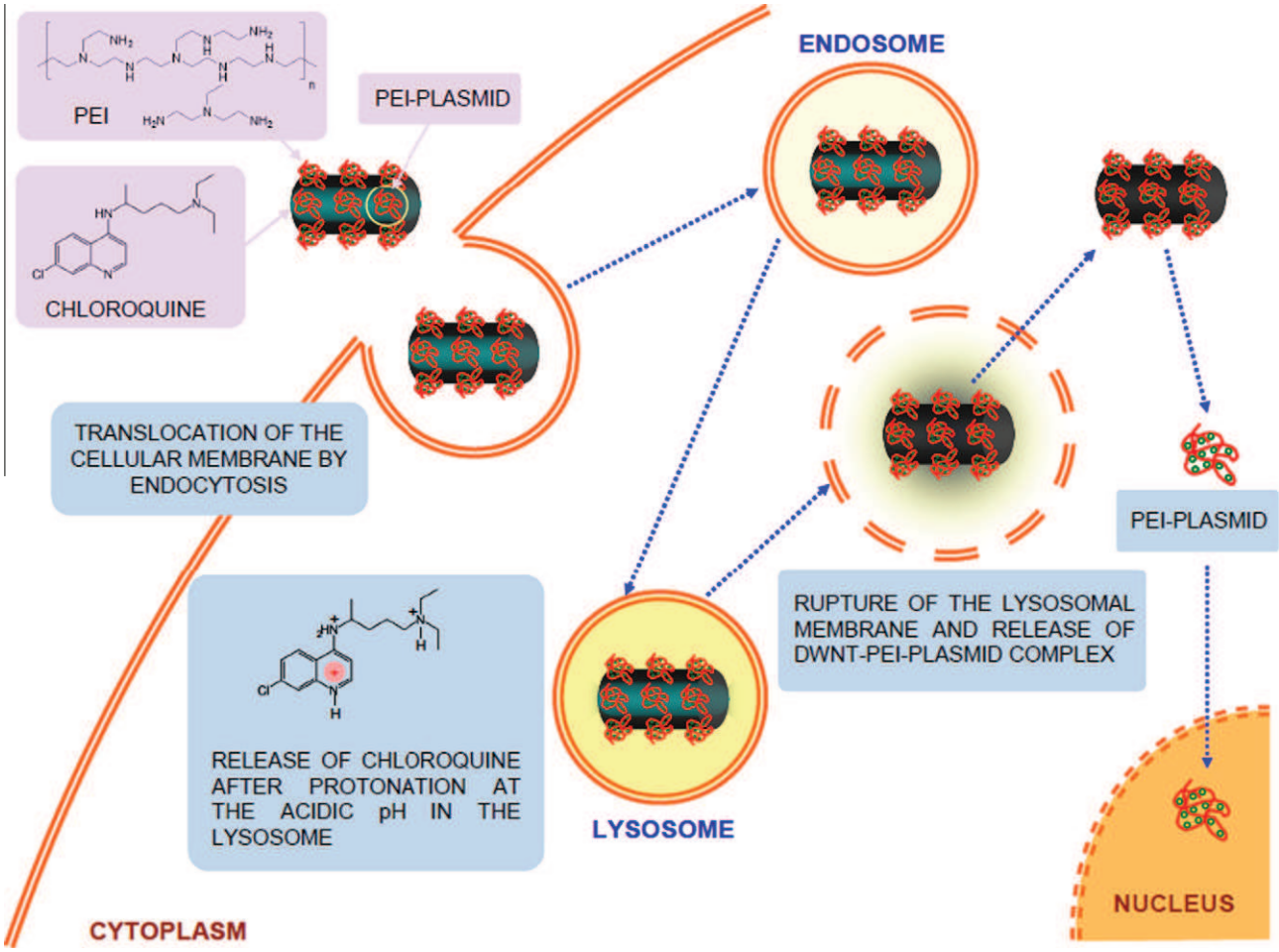

Fig. 7 – Schematic illustrating use of DWCNTs loaded with chloroquine and coated with PEI for gene delivery. The functionalized DWCNTs are internalized in the cell by endocytosis and delivered to the endosome which matures to a lysosome. Acidification causes the protonation of chloroquine and triggers its release from the DWCNT. The accumulation of protonated chloroquine in the lysosome causes swelling and the rupture of the vesicle releasing the DWCNT-PEI-plasmid complex into the cytoplasm. The plasmid is then able to diffuse through the cytoplasm and enter into the nucleus, where it is transcribed and the mRNA subsequently translated (in the cytoplasm) to yield luciferase enzyme.

release of chloroquine from the DWCNTs in the lysosomes was also demonstrated, together with the use of the DWCNTs as a dual drug and gene delivery system. These results support the potential applications of chloroquine-loaded CNTs for gene therapy.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carbon.2011.08.001.

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