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SiRNA delivery with functionalized carbon nanotubes

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ABSTRACT

Carbon nanotubes (CNTs) have been studied for drug, antigen and nucleic acid delivery both in vitro and in vivo. Due to their nano-needle structure, they are supposed to cross the plasma membrane and enter directly into the cytoplasm likely upon an endocytosis-independent mechanism without inducing cell death. In this study, two cationically functionalized CNTs (CNT-PEI and CNT-pyridinium) were investigated for siRNA delivery. Both functionalized CNTs complexed siRNA and showed 10–30% silencing activity and a cytotoxicity of 10–60%. However, in terms of reduced toxicity or increased silencing activity, CNT-PEI and CNT-pyridinium did not show an added value over PEI and other standard transfection systems. Probably, the type of functionalization of carbon nanotubes might be a key parameter to obtain an efficient and non-cytotoxic CNT-based delivery system. Nevertheless, in view of the present results and importantly also of the non-degradability of CNTs, preference should currently be given to designing biodegradable carriers which mimic the needle structure of CNTs.

1. Introduction

Carbon nanotubes (CNTs) have raised great enthusiasm in the nanomedicine field as potential drug, antigen and nucleic acid delivery vehicle (Cheung et al., 2010; Patlolla et al., 2010; Bai et al., 2010). Because of their nano-needle structure (Fig. 1), they have been proposed to easily cross the plasma membrane and to translocate directly into cytoplasm of target cells, utilizing an endocytosis-independent mechanism without inducing cell death (Pantarotto et al., 2004a,b; Cai et al., 2005). Obviously, if this interesting new mechanism is applicable to a great variety of target cell types, such an endocytosis-independent cell entry mechanism of CNTs is a valuable advantage. However, there are indications for an endocytosis-dependent cell entry of CNTs (Kam et al., 2004, 2006; Heller et al., 2006; Becker et al., 2007). CNTs offer a structural advantage due to a very large surface which can be modified with functional groups and be loaded with therapeutics such as nucleic acids, drugs and proteins (Firme Iii and Bandaru, 2009; Cheung et al., 2010). Furthermore, it is expected that CNTs can be scaled-up for industrial production relatively easily (Nikolaev et al., 1999). These properties set a foundation for further exploration of CNTs for biological and therapeutic applications.

Poor solubility of CNTs in water is one of the limitations for most of their applications. Several approaches such as introducing polar or charged groups on their surface, have been investigated to facilitate their solubilisation. In addition to increasing solubility, introduction of positively charged groups at their surface also serves another purpose: such functionalized CNTs can bind molecules such as siRNA or DNA (Hu et al., 2006; Sawada et al., 2008). In comparison with chemical conjugation of DNA/siRNA to CNTs (Kam et al., 2005), nanoparticle preparation through electrostatic interactions between the negatively charged nucleic acids and the chemically functionalized cationic CNTs is much faster and convenient (Krajcik et al., 2008).

Considerable research efforts have been dedicated recently to the use of nucleic acids, especially siRNAs as novel biotherapeutics (Kim and Rossi, 2007; Pfeifer and Verma, 2001). Both DNA and siRNA molecules are rapidly degraded by nucleases present in biological fluids and not able to enter the cellular interior, with the nucleus and the cytoplasm as intracellular targets, respectively (Elbashir et al., 2001; Kurreck, 2009; Varkouhi et al., 2010a). Therefore, a key challenge to the effective and widespread use of nucleic acids is their intracellular delivery (Gilmore et al., 2004; Jeong et al., 2009; Varkouhi et al., 2010b; Keller, 2009). For this purpose, functionalized CNTs could be valuable candidates in particular in view of the reported needle-mechanism enabling direct translocation over the plasma membrane.

Several functionalized CNTs have been designed and tested for the purpose of nucleic acid delivery (Cheung et al., 2010; Al-Jamal

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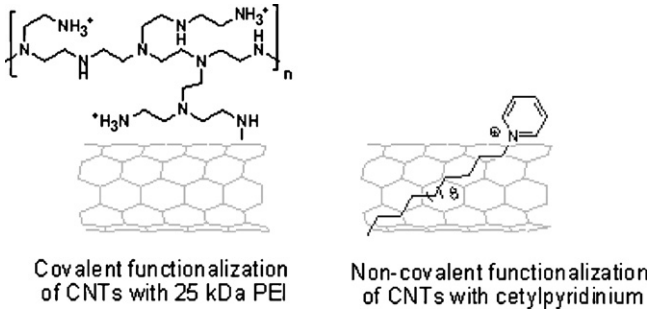

Fig. 1. Schematic representation of the two functionalized CNTs under study.

et al., 2010). Phospholipid-coated CNTs functionalized with amine-terminated polyethylene glycol (PL-PEG2000-NH_2) were shown to be efficient in siRNA and DNA delivery in human T cells and primary cells (Liu et al., 2007). Ammonium-functionalized CNTs (Wang et al., 2004) and more recently dendron-CNT (Al-Jamal et al., 2010) were reported to be efficient in siRNA delivery with low cytotoxicity. After i.v injection of PEGylated CNTs in mice, no toxicity over several months was observed (Schipper et al., 2008; Liu et al., 2008). Nevertheless, in several studies the potential toxicitiy of CNTs has been discussed and attributed to various factors such as, amongst others, length of the tubes, type of functionalization, dosage, duration of exposure, cell type, route of administration and tissue distribution. Still, most aspects regarding CNT toxicity remain uncertain (Firme Iii and Bandaru, 2009; Schipper et al., 2008; Carrero-Sanchez et al., 2006).

In this study, two newly synthesized functionalized CNTs were investigated for siRNA delivery (Fig. 1). One CNT type was functionalized covalently with the cationic polymer PEI (polyethylenimine) (CNT-PEI), which is a well-known transfection agent (Boussif et al., 1995). The silencing activity of the siRNA complexes based on CNT-PEI was compared to those based on PEI alone. The other studied CNT type was functionalized non-covalently with cationic pyridinium. The silencing activity and the cellular cytotoxicity of siRNA complexes based on these functionalized CNTs were compared with those based on the regularly used lipidic transfection agent Lipofectamine and the well-known polymeric transfectant [poly(2-dimethylaminoethyl methacrylate), pDMAEMA] (Cherng et al., 1996).

2. Materials and methods

2.1. Materials

Multi-wall Carbon nanotubes (MWCNT) (Nanocyl 3150) with a metallic catalyst content <5% and average diameter and length of 9.5 nm and 1 μm, respectively, were purchased from Nanocyl (Sambreville, Belgium). PEI 25 kDa and other chemicals were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands). The Fluc double stranded siRNA which specifically targets firefly luciferase (used against mRNA from pGL3; structure below) and anti-EGFP siRNA as non specific siRNA were obtained from Integrated DNA Technologies BVBA (Leuven, Belgium).

Sense strand: 5′-pGGUUCCUGGAACAAUUGCUUUUAca
Antisense strand: 3′-GACCAAGGACCUUGUUAACGAAAAUGU

Poly(2-(dimethylamino) ethylmethacrylate) (pDMAEMA) was synthesized and purified as described previously (Cherng et al., 1996). Lipofectamine 2000 was obtained from Invitrogen (Breda, The Netherlands). Luciferase assay reagent and reporter lysis buffer were obtained from Promega (Leiden, The Netherlands).

2.2. Shortening of carbon nanotubes

80 mg of CNTs was ultrasonicated in 30 ml of toluene for 4 days with an ultrasonicating Branson Sonifier 450 probe (25 W output power). The suspension was centrifuged for 30 min at 10,000 rpm, and the supernatant was discarded. The precipitate was taken back in 30 ml of H_2O and centrifuged again. This operation was repeated twice using 30 ml of acetone. The sample was finally dried under vacuum to yield 75 mg of shortened CNTs (Huang et al., 2002).

2.3. Preparation of CNT-PEI

CNTs (50 mg) were dispersed in 10 ml of DMF using an ultrasonic probe (15 min, 10 W output power) before 250 mg of 25 kDa branched-PEI were added. The mixture was further sonicated for 10 min and stirred for 3 days at 50 °C. The PEI-functionalized nanotubes were collected by filtration on 0.2 μm polypropylene membrane, washed with CH_2Cl_2 (15 ml), DMF (15 ml), and MeOH (3× 15 ml). Nanotubes were taken back in 10 ml HCl (pH 1), sonicated for 10 min, filtered, washed with H_2O (3× 15 ml) and MeOH (15 ml). CNT-PEI was redispersed in H_2O under sonication (10 min) and centrifuged (5 min, 2000 rpm) to remove aggregates. The black supernatant was collected and lyophilized to afford 28 mg of CNT-PEI. A control experiment was indeed carried out by mixing PEI with CNTs without heating. After the usual workup, XPS analysis showed only traces amounts of N on the nanotubes. This experiment clearly shows that there is no adsorbed PEI on the nanotube.

2.4. Preparation of CNT-pyridinium

CNTs (50 mg) were dispersed in 10 ml of H_2O for 15 min using an ultrasonic probe (25 W output power). Cetylpyridinium chloride (10 mg) was then added and the mixture was further sonicated for 10 min and centrifuged for 5 min at 2000 rpm. The supernatant was collected and centrifuged for 90 min at 11,000 rpm to remove cetylpyridinium. The precipitate was taken back in 10 ml of H_2O under sonication and centrifuged again. This operation was repeated twice. The precipitate was collected and taken back in 10 ml of H_2O under sonication (10 min, 25 W) and centrifuged for 5 min at 2000 rpm to remove aggregates. The black supernatant was collected and lyophilized to afford 20 mg of CNT-pyridinium.

2.5. Physicochemical characterization

The functionalized CNTs were dispersed in water (1 mg/ml) by bath sonication for 1 h. Electron microscopy analysis was carried out on a Philips CM12 microscope at 100 kV. XPS spectra were recorded on a VG ESCALAB 210 spectrometer. The zeta potential of the CNT dispersions in 5 mM HEPES buffer (pH 7.4) was determined at 25 °C in a DTS5001 cell using a Zetasizer 2000 unit (Malvern). The instrument was calibrated with a polystyrene dispersion with known zeta potential.

The zeta potential of the calibration standard was measured on different days and was −64 mV ± 5. This accuracy (around 95%) is reflected in the standard deviations of the zeta potentials of the CNTs.

Complexes of CNTs or PEI with siRNA were prepared by mixing 100 μl anti-luciferase siRNA (20 μg/ml: in 5 mM HEPES buffer (pH 7.4)) with different concentrations of functionalized CNTs, PEI or pDMAEMA in the same buffer. The resulting mixtures were vortexed for 10 s and then used after 30 min storage at room temperature. Complexes were prepared at different CNT, PEI or pDMAEMA to siRNA ratios (expressed as N/P ratios, where N is the number of moles of nitrogen in CNT, PEI or pDMAEMA and P is number of moles of P in siRNA). Complexes of either specific or non-specific siRNA with Lipofectamine 2000 were prepared by

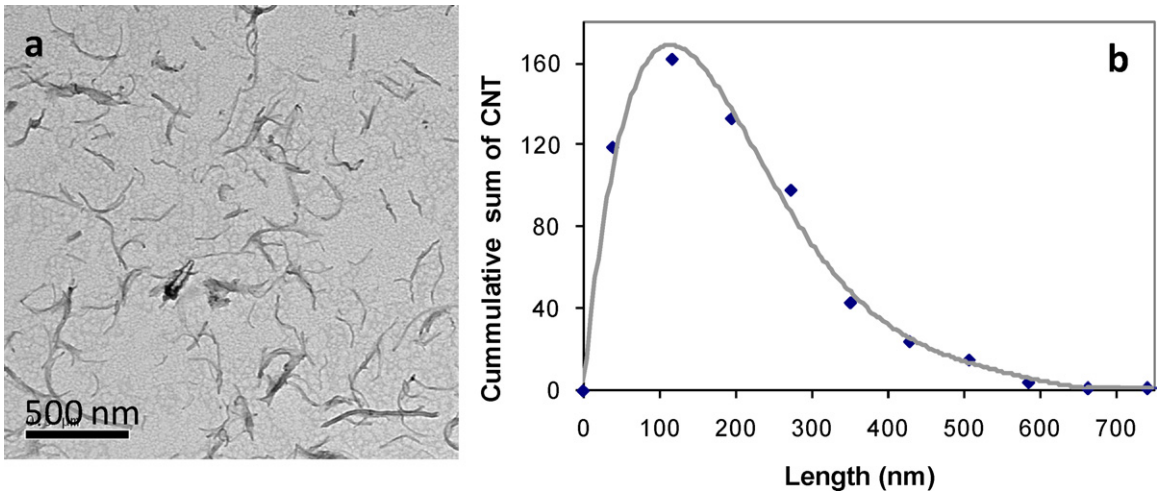

Fig. 2. TEM picture (a) and size distribution of shortened CNTs (b).

gently mixing 50 μl siRNA (20 μg/ml) in HEPES (5 mM, pH 7.4) with 3.7 μl Lipofectamine 2000 in 50 μl HEPES (5 mM, pH 7.4) followed by 30 min incubation at room temperature.

2.6. Agarose gel electrophoresis

The complexation of siRNA with the cationic vectors was investigated using agarose gel electrophoresis. The agarose gels (NuSieve® GTG® Agarose, Lonza, Rockland, ME, USA) were made at a concentration of 4% (w/v) in Tris-acetate-EDTA (TAE) running buffer and contained 0.5 μg/ml ethidium bromide. The complexes made at different N/P ratios were prepared as described above and were applied in the starting slots of the gel followed by electrophoresis at 60 V for 50 min. Naked siRNA was used as control. The siRNA bands, stained with ethidium bromide, were detected on a UV transilluminator (ImaGo compact imaging system (B&L Systems), The Netherlands).

2.7. Gene silencing and cytotoxicity

The human lung cancer cell line H1299 which expresses firefly luciferase was used to study the gene silencing activity of the different complexes. The cell line was maintained in RPMI 1640 medium with HEPES and L-glutamine (PAA laboratories GmbH, Pasching, Austria, catalog No. E15-842) completed with fetal bovine serum (FBS) (final concentration 10%, v/v) and cultured at 37 °C at 5% CO_2 humidified atmosphere. The cells (8×10^3 cells/well) were seeded into 96-well plates and cultured overnight. The anti-Luciferase siRNA complexes were added to the cells and incubated at 37 °C for 2 h. Then, the medium was removed, and fresh medium was added. Subsequently, the cells were incubated at 37 °C for 48 h, after which the luciferase protein expression was analyzed using a Luciferase reporter gene assay (Promega). The cytotoxicity of the complexes and cetylpyridinium (concentrations ranging from 1.3 to 8.6 μg/ml which correspond with its concentrations in siRNA complexes incubated with cells) was measured using the XTT colorimetric viability assay (Scudiero et al., 1988).

2.8. Determination of luciferase activity

Luciferase activity was measured after removal of the growth medium and lysis of the cells by the addition of 100 μl reporter gene lysis buffer. After a freeze/thaw cycle at −80 °C/room temperature, 50 μl of luciferase assay reagent was added to 50 μl of the cell lysate and relative light units (RLU) were measured for 10 s at room temperature using a FLUOstar OPTIMA microplate based multi-detection reader with a microinjector.

3. Results and discussion

Efficient gene silencing mediated by siRNA depends on an efficient and protected delivery of siRNA to the intra-cellular target site, the cytoplasm. CNTs offer several advantages which make them attractive carriers for nucleic acid delivery. They are thin and long, offering a large surface area to which siRNA can be bound. Remarkably, it has been observed that their nanoneedle structure facilitates their translocation over the plasma membrane into the cytoplasm via an endocytosis-independent pathway. In a study by Kostarelos et al. (2007), it was shown that CNTs were taken up by cells despite the presence of endocytosis inhibitors in the culture medium. Cell types studied included fibroblasts which are phagocytosis-deficient and prokaryotic cells (fungi, yeast and bacteria), which have no endocytosis machinery. Therefore, it was concluded that the uptake mechanism of the CNTs is passive and endocytosis-independent (Pantarotto et al., 2004a,b). Considering the endosomal localisation as one of the most problematic barriers in siRNA cytosolic delivery, this endocytosis-independent cell entry mechanism makes the CNT delivery capacity independent from endosomal escape. However, the needle mechanism is still a controversial issue and several opposite observations have been reported in the literature pointing to an endocytosis-dependent cell entry of CNTs (Kam et al., 2004, 2006; Heller et al., 2006; Becker et al., 2007).

There have been several reports on the use of functionalized CNTs for siRNA delivery. Wang et al. (2008) reported that ammonium-functionalized CNTs could electrostatically bind siRNA against cyclin A2, inducing growth inhibition and apoptosis of a human erythroleukemic cell line (K526) in vitro. Liu et al. (2007) used CNTs functionalized with amine-terminated PEG (PEG; PL-PEG2000) for siRNA delivery into human T cells and observed 60–90% knockdown of CXCR4 receptors. Ladeira et al. (2010) used siRNA coiled into carboxyl-functionalized CNT and observed >95% silencing activity in different cell lines with no cytotoxicity. Bartholomeusz et al. (2009) used siRNA complexed to pristine CNT for silencing the hypoxia-inducible factor 1 alpha (HIF-1alpha) and observed a specific inhibition of cellular HIF-1alpha activity. Moreover, intra-tumoral administration of these complexes in mice bearing MiaPaCa-2/HRE tumors significantly inhibited the activity of tumor HIF-1alpha.

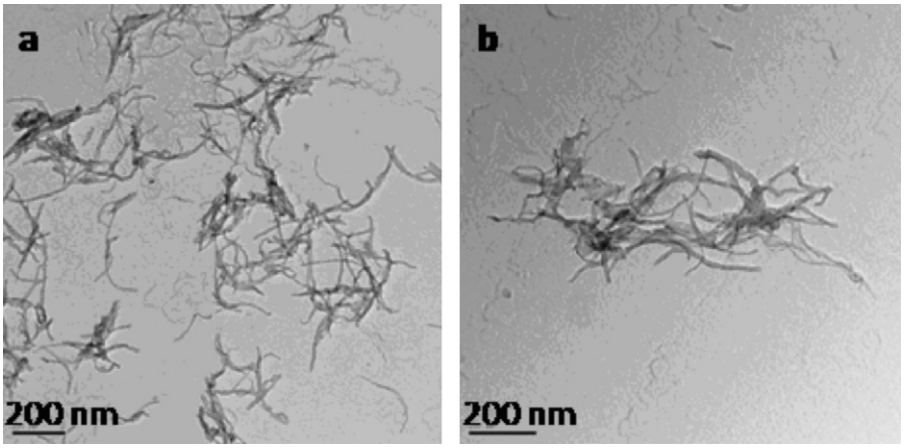

Fig. 3. TEM pictures of CNT-PEI (a) and CNT-Pyridinium (b) in dry state.

In this study, two types of functionalized CNTs (Fig. 1) were investigated for their siRNA delivery properties in vitro and compared with the polymeric transfectant pDMAEMA developed in our group (Cherng et al., 1996) and the well-known lipidic cationic transfectant Lipofectamine (Dalby et al., 2004) and PEI.

3.1. Physicochemical characterization

Full-length carbon nanotubes were first mechanically shortened by ultrasonication. Transmission electron microscopy (TEM) pictures showed that ultrasonication reduced the length of the nanotubes to a range between a few tens of nm and ~500 nm (Fig. 2a). The mean length of the nanotubes was approximately 200 nm (Fig. 2b). This value has to be compared to the initial CNTs before ultrasonication which were typically above 1 μm in length.

Cationic functionalization of the surface of CNTs was envisaged according to two different functionalization pathways, one resulting in covalently bound and the other one in non-covalently bound surface moieties. Covalent modification of the CNTs was achieved by direct amination of the graphene lattice with 25 kDa polyethyleneimine (PEI), as described by Basiuk et al. (2004). Non-covalent functionalization was done by adsorption of cetylpyridinium chloride (Py^+) onto the surface of CNTs, as reported by Mackiewicz et al. (2008) (Fig. 1). In the latter process, the hydrophobic portion of the amphiphile is adsorbed on CNT walls by van der Waals interactions while its hydrophilic head-group is oriented toward the aqueous phase. The CNT-PEI and CNT-Pyridinium samples were found to be dispersible in aqueous solutions and stable against aggregation for at least 72 h. TEM pictures (Fig. 3) show the grafted CNTs in dry state. Quantification of the degree of functionalization was achieved by X-ray photoelectron spectroscopy (XPS) which indicated the appearance of a N 1s peak at 399.9 eV (PEI) and 402.8 eV (pyridinium) which was attributed to the nitrogen atoms. From the nitrogen to carbon ratio we determined the level of functionalization of the CNTs being 28 wt% in case of 25 kDa PEI and 11 wt% in case of Pyridinium. These values indicate that the CNTs are densely covered with cationic amino groups to be used for siRNA complexation. Indeed, if we consider that all the amino groups are protonated, the cationic amino group per mg of complex is *ca.* 6 μmol for CNT-PEI and 0.3 μmol for CNT-Pyridinium.

Both types of functionalized CNT dispersions showed a positive zeta potential (10–15 mV ± 5) pointing to their ability to make complexes with negatively charged siRNA. The complex formation ability of CNT-PEI with siRNA in comparison to PEI at different N/P ratios was investigated by agarose gel electrophoresis. Fig. 4 shows that the intensity of the free siRNA bands on the gel in case of CNT-PEI reduced gradually with an increase of the N/P ratio (0.5–12) indicating complete complexation at $N/P \geq 14$. Complete binding of siRNA to PEI alone was already observed at $N/P \geq 2$.

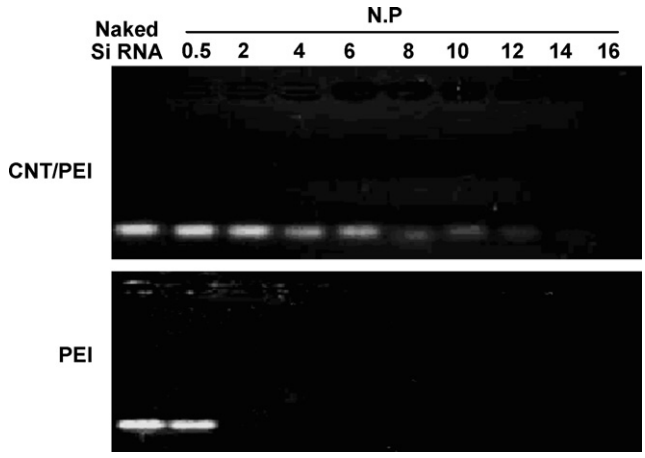

Fig. 4. Complex formation of CNT-PEI 25 kDa and PEI with siRNA as function of N/P ratio as studied by agarose gel electrophoresis.

In case of the CNT-Pyridinium, complete binding of siRNA to CNT-Pyridinium was observed at $N/P \geq 3.5$ (Fig. 5).

These results clearly show that both types of functionalized CNTs are able to complex siRNA.

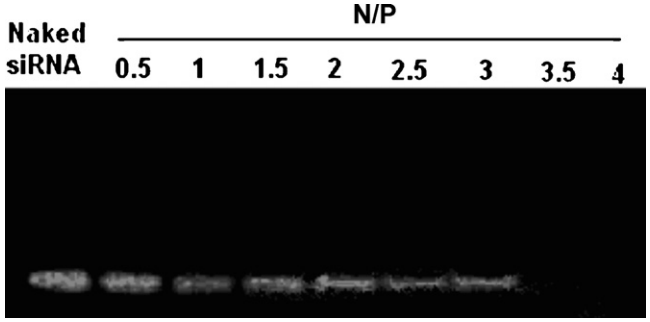

Fig. 5. Complex formation of CNT-Pyridinium with siRNA as function of N/P ratio as studied by agarose gel electrophoresis.

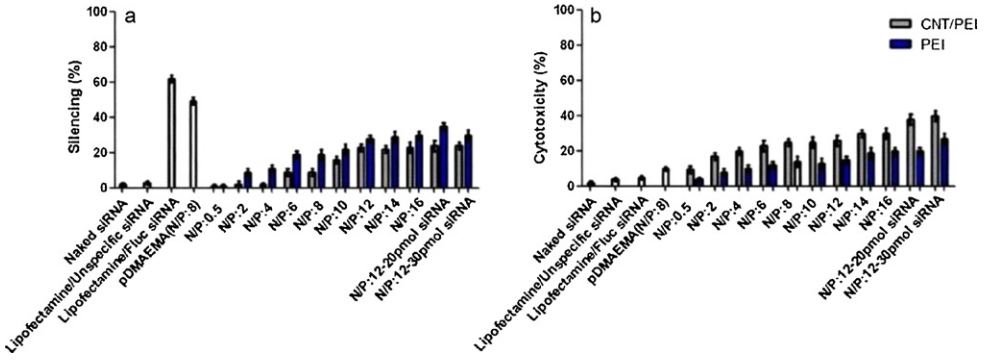

Fig. 6. a. Luciferase gene silencing after incubation of H1299 cells with siRNA complexes based on CNT-PEI and PEI alone made at different N/P ratios, in serum free medium. b. Cell viability as measured by XTT assay after incubation of H1299 cells with siRNA complexes in serum free medium (mean ± standard deviation (n = 3)).

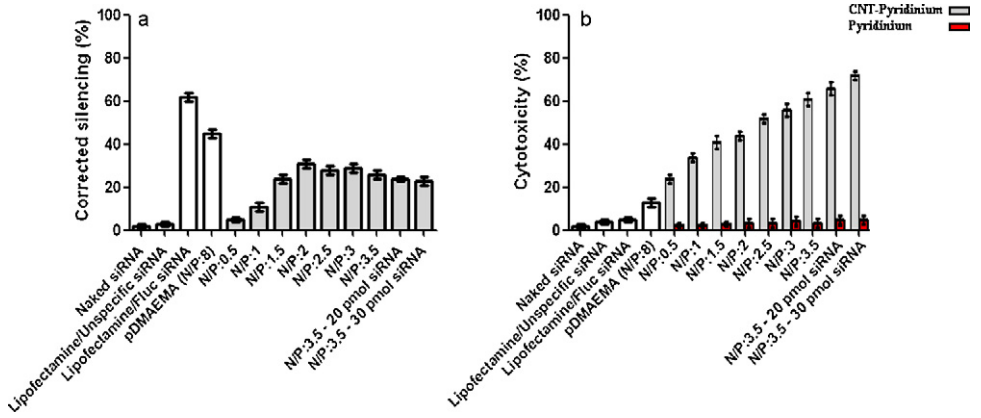

Fig. 7. a. Luciferase gene silencing after incubation of H1299 cells with siRNA complexes based on CNT-Pyridinium made at different N/P ratios, in serum free medium. b. Cell viability as measured by XTT assay after incubation of H1299 cells with siRNA complexes and Pyridinium alone in serum free medium (mean ± standard deviation (n = 3)).

3.2. Silencing activity and cell viability

Human lung cancer cells (H1299) expressing luciferase were used for the gene silencing and cell viability studies. Fig. 6a shows the gene silencing efficiency (corrected for cytotoxicity) of CNT-PEI/siRNA complexes compared to those based on PEI, pDMAEMA and Lipofectamine as reference transfectants. The silencing activity of the different formulations was corrected for cytotoxicity, so the percentage silencing reported is only related to viable cells. In the absence of serum, incubation of cells with the siRNA complexes based on CNT-PEI showed up to 20% silencing activity whereas incubation with PEI polyplexes showed 20–30% gene silencing. The results of the XTT cell viability assay showed higher cytotoxicity of CNT-PEI-based complexes than those based on PEI (Fig. 6b) which suggests that CNT enhances the cytotoxicity of PEI, while, Liu et al. showed that CNTs modified with PEI were less cytotoxic than PEI alone (Liu et al., 2005). By increasing the dose of the siRNA complexes (at N/P 12) from 10 pmol to 30 pmol per well, no significant enhancement in gene silencing activity of CNT-PEI and PEI-based complexes was observed (Fig. 6a) whereas the cytotoxicity of the CNT-PEI based complexes increased slightly up to 40% (Fig. 6b). It was observed that the gene silencing activity of the CNT-PEI based complexes was much lower than that of complexes based on Lipofectamine 2000 (60%) and pDMAEMA (50%) (Fig. 6a). In the presence of serum in the growth medium, the same results of gene silencing and cytotoxicity were observed for all siRNA formulations (data not shown). These results show that there is no added value of CNT-PEI over PEI and the reference transfectants Lipofectamine 2000 and pDMAEMA.

Fig. 7 shows the findings obtained with CNT-pyridinium/siRNA complexes. In the absence of serum, incubation of cells with the CNT-pyridinium/siRNA complexes (N/P 3.5) showed about 30% silencing activity (corrected for cytotoxicity) (Fig. 7a) and a relatively high cytotoxiciy of about 60% (Fig. 7b). By increasing the dose of the siRNA complexes (N/P 3.5) from 10 pmol/well to 30 pmol/well, no significant enhancement in the gene silencing activity of CNT-Pyridinium based complexes was observed (Fig. 7a) whereas the cytotoxicity of the complexes increased slightly up to 70% (Fig. 7b) which is probably due to the increased amount of the CNT-pyridinium in the growth medium. Cetylpyridinium in different concentrations (corresponding with its concentrations in siRNA complexes incubated with cells) showed ≤5% cytotoxicity. It is clear from the results that the cytotoxicity of the CNT-Pyridinium/siRNA complexes at the same cetylpyridinium concentrations is substantially higher related to adverse effects of the CNTs (Fig. 7b). These results reveal high cytotoxicity of the CNT-Pyridinium/siRNA complexes and limited silencing activity, with no added value of CNT-Pyridinium over Lipofectamine 2000, pDMAEMA and PEI (Fig. 7a). In several studies the potential in vitro and in vivo toxicities of CNTs have been discussed and attributed to various factors such as, amongst others, length of the tubes, type of functionalization, dosage, duration of exposure, cell type, route of administration and tissue distribution. Still, most aspects regarding CNT toxicity remain uncertain (Zhang et al., 2010; Schipper et al., 2008; Carrero-Sanchez et al., 2006; Takagi et al., 2008; Sakamoto et al., 2009; Poland et al., 2008; Muller et al., 2009; Kolosnjaj-Tabi et al., 2010; Kayat et al., 2011).

4. Conclusions

In this study, CNT-PEI and CNT-pyridinium did not show any added value over PEI, pDMAEMA, and Lipofectamine used as ref-

erence transfection agents regarding siRNA silencing activity and cytotoxicity. Despite these disappointing results obtained with the two functionalized CNT types, other literature reports encourage further nucleic acid delivery studies with other types of functionalized CNTs. Probably, the type of functionalization of carbon nanotubes might be a key parameter to obtain an efficient and non-cytotoxic CNT-based delivery system. Nevertheless, in view of the present results and importantly also of the non-degradability of CNTs, preference should currently be given to designing biodegradable carriers which mimic the needle structure of CNT*s*.

Acknowledgments

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