Optimising DNA binding to carbon nanotubes by non-covalent methods

Vanesa Sanz[1,2±*], Ewa Borowiak[3], Petar Lukanov[4], Anna Marie Galibert[4], Emmanuel Flahaut[4], Helen M. Coley[1], S.Ravi P. Silva[2] and Johnjoe McFadden[1]

[1]Faculty of Health and Medical Sciences, University of Surrey

Guildford, GU2 7XH (United Kingdom)

[2]Nano-Electronics Centre, Advanced Technology Institute, University of Surrey

Guildford, GU2 7XH (United Kingdom)

[3]Szczecin University of Technology

Centre of Knowledge Based Nanomaterials

Pulaskiego St. 10

70-322 Szczecin (Poland)

[4]Universite Paul Sabatier-Inter-University Research and Engineering Centre on Materials (CIRIMAT)

118 Route de Narbonne

F-31062 Toulouse Cedex 9 (France) 353

[±]current address: Institute of Nanoscience of Aragón, University of Zaragoza, Campus Río Ebro, Mariano Esquillor s/n, 50018 Zaragoza, Spain

*Corresponding author, fax: +34 976 762776, e-mail address: vasanz@unizar.es (V. Sanz)

Abstract. The use of carbon nanotubes as a gene delivery system has been extensively studied in recent years owing to its potential advantages over viral vectors. To achieve this goal, carbon nanotubes have to be functionalized to become compatible with aqueous media and to bind the genetic material. To establish the best conditions for plasmid DNA binding, we compare the dispersion properties of single-, double- and multi-walled carbon nanotubes (SWCNTs, DWCNTs and MWCNTs respectively) functionalized with a variety of surfactants by non-covalent attachment. The DNA binding properties of the functionalized carbon nanotubes were studied and compared by electrophoresis. Furthermore, a bilayer functionalization method for DNA binding on SWCNTs was developed that utilized RNA-wrapping to solubilise the nanotubes and cationic polymers as a bridge between nanotubes and DNA.

1. Introduction

In recent years increased attention has been paid to nano-structured materials such as carbon nanotubes. Carbon nanotubes have received considerable interest in the biomedical field in areas such as drug and gene delivery, scaffolds for tissue growth, biosensing and diagnostics, because of their biocompatibility, low cytotoxicity and their ability to cross the cell membrane[1 - 4]. Although the exact mechanisms by which CNTs cross the cell membrane are under debate[5,6] much research has shown that CNTs accumulate in the cell without toxic effects[3,4]. Encouraging, SWCNT localise in tumours in mice, probably because of increased vascularisation inherent in tumours, making tumour targeting a feasible approach[7]. However, one of the still remaining problems when using carbon nanotubes for these applications is the inherent difficulty in handling them as they tend to aggregate in bundles through strong attractive interactions which are very difficult to disrupt. Therefore, the development of functionalization methods to obtain stable suspensions of carbon nanotubes is primordial. Functionalization of CNTs has been performed by covalent and non-covalent approaches[8]. Covalent modification (i.e. amidation[9], esterification[10], reduction of nitro groups[11] and cleavable disulfides[12]) changes the structural and electrical properties of CNTs whereas non-covalent approaches retain CNTs in their native state. Furthermore, non-covalent methods are usually quite simple and quick, involving steps such as ultrasonication, centrifugation and filtration. Besides, when using carbon nanotubes for biomedical applications, the functionalization method has crutial implications. For example, the retention of the native structure of the carbon nanotube can be advantageous for CNT taking-up and processing in the cell. However, the surfactant has to be carefully selected as they are known to permeabilize plasma membranes being cytotoxic on their own which could limit the possible biomedical applications of such functionalized carbon nanotubes.

One of the most promising research applications in the field of nanotechnology has been the use of carbon nanotubes (CNTs) as gene delivery systems for silencing deleterious

genes[12,13]. However, the use of carbon nanotubes as gene delivery vectors requires functionalization to disperse the nanotubes in aqueous media and to render them able to effectively bind to DNA. It has been reported that a variety of single-stranded DNAs, short double-stranded DNAs, and RNAs can disperse SWCNTs [14, 15], and that DNA is able to insert into the opened cavity of MWCNTs in a non-specific manner[16]. However, these methodologies would require high amounts of the purified genetic material in order to functionalize and use them as gene delivery systems. Different covalent methodologies have been developed based on the chemical modification of the carbon nanotube surface to introduce positively charged groups or maleimide groups for DNA binding through ionic interactions or through covalent bounds to thiol-terminated oligonucleotides, respectively. However, as it was stated above, these methods disrupt the structure of carbon nanotubes and also the functionalization procedures are usually time consuming and tedious. The use of non-covalent approaches is an alternative to these methods. The use of non-covalent approaches renders the cationic groups available for negatively charged DNA binding by ionic interactions. However, there has been no systematic investigation of the functionalization of CNTs for optimal binding of DNA, which is the subject of this study. In this paper, a comparative study on the non-covalent functionalization of CNTs for DNA binding is presented. The general approach was to use amphiphilic molecules that wrap the surface of CNTs through their hydrophobic regions leaving the hydrophilic groups exposed rendering them soluble in aqueous media. Tests were carried out with single-walled, double-walled and multi-walled carbon nanotubes (SWCNTs, DWCNTs, MWCNTs, respectively) in order to compare their dispersion properties. Cationic surfactants that can effectively bind negatively charged DNA were additionally used to bind plasmid DNA for designing functionalized CNTs for gene delivery purposes. Furthermore, the introduction of the cationic functionalities, mainly amine groups, allows further attachment of groups such as targeting moieties for targeting purposes and fluorophore markers for cell tracking. In addition, a new

functionalization method for DNA binding based on a bilayer approach with RNA-wrapped SWCNTs is also presented. The functionalization methods and conclusions described in this work for DNA binding to carbon nanotubes are not only important for gene delivery purposes but also for other applications of carbon nanotubes in the biomedical field such as biosensing.

2. Materials and methods

2.1. Materials.

Carbon nanotubes were prepared by the CVD method in our lab[17 - 19]. Benzalkonium chloride from Fluka 12060 > 95.0 %; polyethyleneimine (PEI) from Sigma P3143 50 % w/v; 1-pyrenemethylamide hydrochloride (PMA) 95 % from Aldrich 401633; 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)2000 (PL-PEG-NH_2) from Avanti Polar Lipids 880128P; 1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine (Lyso-PC) form Avanti Polar Lipids 855775P: 1,2,dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE) > 99 % from Sigma P1348; RNA from baker's yeast from Sigma R6750; Poly(Lys:Phe, 1:1) hydrobromide from Sigma P3250; Poly(Lys:Tyr, 1:9) hydrobromide form Sigma P2025; polylysine 0.1 % w/v from Sigma P8920; bovine serum albumin from Sigma A3294.

2.2. Preparation of functionalized CNTs.

The appropriate amount of CNT (0.15 mg to 2 mg) was mixed with 1 mL of cationic surfactant (0.3 $mg.mL^{-1}$ in double distilled water) and the mixture was ultrasonicated in a Soniprep for 40 s (4 cycles of 10 s on and 10 s off) and then sonicated for 2 h in water bath (3 W) at room temperature. The suspension was then centrifuged at 13200 rpm for 10 minutes and the supernatant was pippeted off. 500 μL of f-CNTs were placed in Microcon centrifugal devices, regenerated cellulose filter 100 KDa, and centrifuged at 13200 rpm for 5 minutes, the filtered was then washed three times with 50 μL of bidistilled water and finally recovered by resuspending in 500 μL of bidistilled water.

2.3. Preparation of surfactant:CNT optimisation curves.

To obtain these solubilization curves, different amounts of CNTs (0.075 mg, 0.225 mg, 0.3 mg, 0.45 mg, 0.75 mg, 0.9 mg) were mixed with 400 μL of distilled water. Then, 100 μL of surfactant solution 1.5 $mg.mL^{-1}$ were added and the samples were sonicated as described above.

2.4. Preparation of f-CNTs-DNA complexes.

80 μL of the f-CNTs prepared as described above at different concentrations were mixed with 2 μL of plasmid DNA of 340 $\mu g.mL^{-1}$. Complexes were allowed to form for 30 min at room temperature.

2.5. Gel electrophoresis.

0.8 % agarose gel electrophoresis in tris-acetate-EDTA (TAE) buffer was used to study the interaction of plasmid DNA with functionalized carbon nanotubes. The gel was run for 45 min at 90 V. 40 % sucrose was used as loading buffer for the plasmid DNA-*f*-CNTs complexes (a 10 μL sample were charged in each well prepared by mixing 8 μL of the complexes with 2 μl of loading buffer l) and ethidium bromide was used for DNA staining.

2.6. Molecular absorption spectroscopy.

Molecular absorption spectra were recorded in a Varian Cary 5000 UV-VIS-NIR spectrophotometer using a 1 cm optical pathway quartz cuvette.

3. Results and discussion

3.1. Functionalization of SWCNTs, DWCNTs and MWCNTs with cationic surfactants

SWCNTs, DWCNTs and MWCNTs were used for this study and several surfactants were tested (see figure 1): benzalkonium chloride, polyethyleneimine (PEI), 1-pyrenemethylamide hydrochloride (PMA), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)2000 (PL-PEG-NH_2), 1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine (Lyso-PC), 1,2,dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE), Poly(Lys:Phe, 1:1) hydrobromide and Poly(Lys:Tyr, 1:9) hydrobromide. The overall

objective was to functionalize CNTs for the development of methods to attach DNA to CNTs. Therefore, we selected surfactants carrying cationic groups such as amine and choline in order to bind negatively charged plasmid DNA.

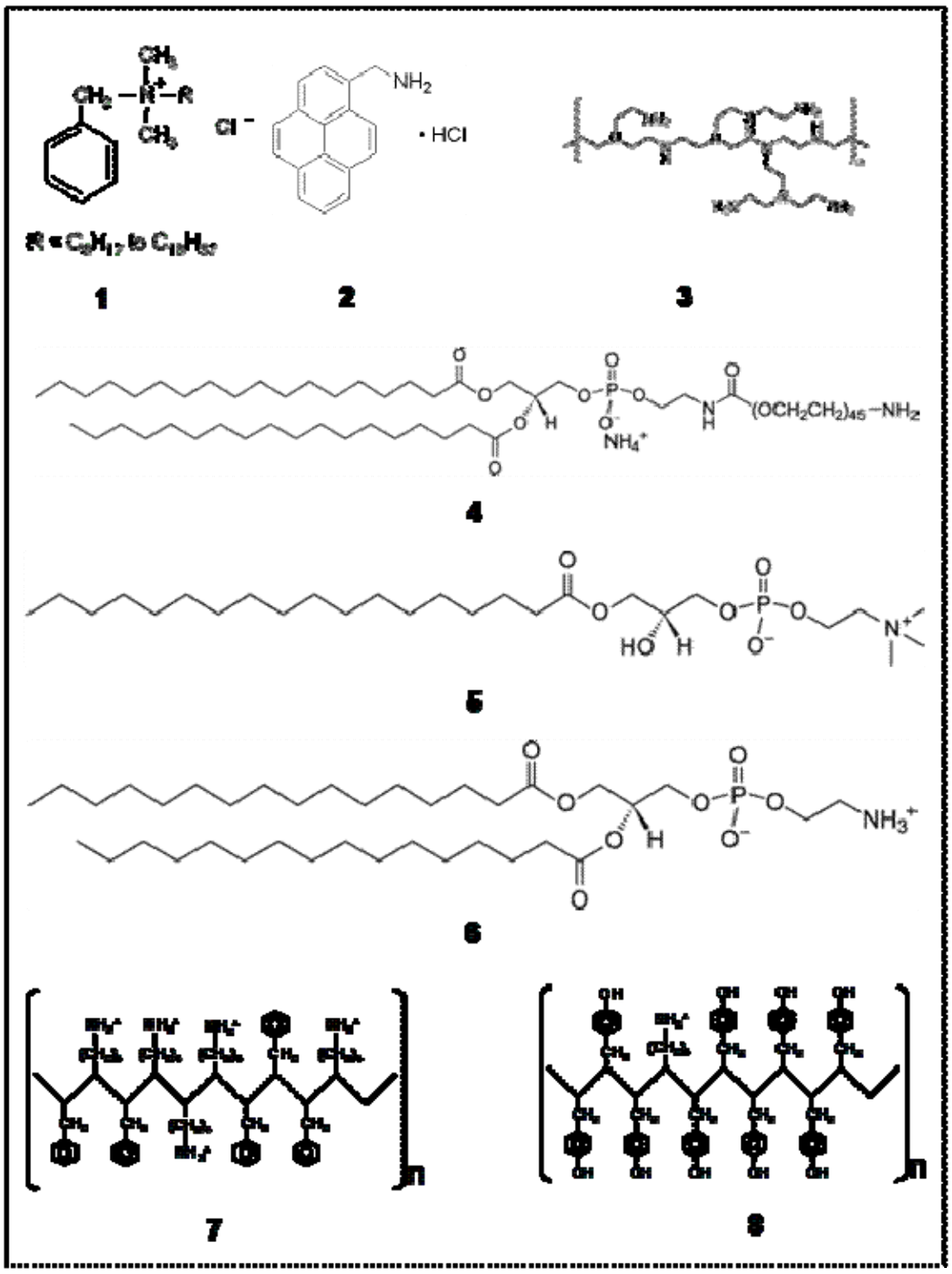

Figure 1. Surfactant structures: 1) benzalkonium chloride, 2) pyrenemethylamine (PMA), 3) polyethylenimine (PEI), 4) 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycosl)2000] (PL-PEG-NH_2), 5) 1-stearoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (Lyso PC), 6) 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE), 7) Poly(Lys:Phe, 1:1), 8) Poly(Lys:Tyr, 1:9).

Our method of dispersion of CNTs was to mix the CNTs with surfactants to promote suspension by sonication, and centrifugation in order to remove the bundles complexes. These dispersion method produce individual nanotubes which was confirmed by atomic force microscopy measurements (see supplementary material, figures S1a-c). Besides, the efficiency of solubilisation was measured by VIS-NIR spectroscopy as CNTs absorb in this optical region. A 730 nm absorption line was selected as the working wavelength to estimate the quantity of solubilized CNTs. This working wavelength was selected as suspended carbon nanotubes absorb at this wavelength which is also free of background absorption from the tested surfactants (see supplementary material, figures S2-S5). In figure 2, a set of spectra as a function of dispersed CNT concentration keeping constant the surfactant concentration is shown. As can be seen, as the concentration of dispersed CNTs increases the absorption at 730 nm linearly increases. Furthermore, the presence of the surfactant, do not contribute to the absorbance value at this wavelength (the linear calibration curve crosses at zero value at the y axis). This result shows that any free surfactant or non-covalently attached to CNTs do not interfere in the measurement of the dispersed CNT concentration which shows that this method can be used to determine the dispersion yield.

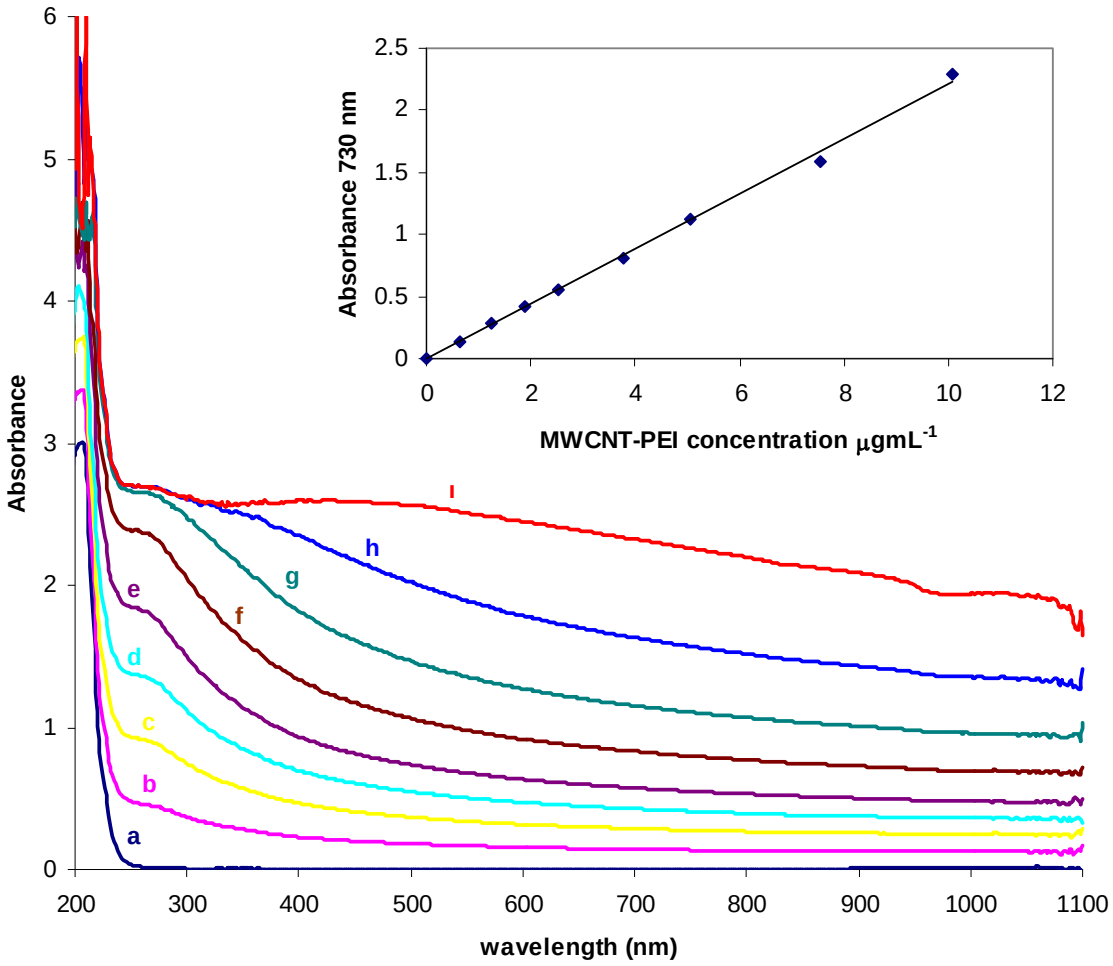

Figure 2. Set of spectra at increasing concentrations of dispersed MWCNT-PEI. PEI concentration was kept constant at 0.3 mgmL^{-1} and different volumes of functionalized MWCNTs were added. In the insert the absorption value at 730 nm as a function of the concentration of dispersed MWCNT-PEI is presented showing a linear relationship.

We found that the ratio of surfactant to CNT was crucial in order to optimise the dispersion[20]. An example of one of these dispersion curves is shown in figure 3, showing MWCNTs dispersed with Lyso PC. In this dispersion curves the concentration of surfactant was kept constant and the amount of CNTs was varied in the dispersion mixture. The yield of dispersion of CNTs was obtained by measuring the absorption value of the suspension at the selected wavelength, as it was stated above. As can be seen, as the amount of CNTs in the dispersion mixture increases the concentration of dispersed CNTs increases until a maximum

is reached where the optimum conditions for dispersion are obtained. Above this optimal concentration of nanotubes in the dispersion mixture, the yield of dispersed nanotubes decreases. This is likely to be due to limiting concentrations of surfactant being shared between large numbers of nanotubes such that insufficient active surfactant is available for solubilising each nanotube. This effect support the fact that the CNTs are actually being dispersed by the surfactant as limiting concentrations of surfactant lead to not properly or not completely dispersed CNTs.

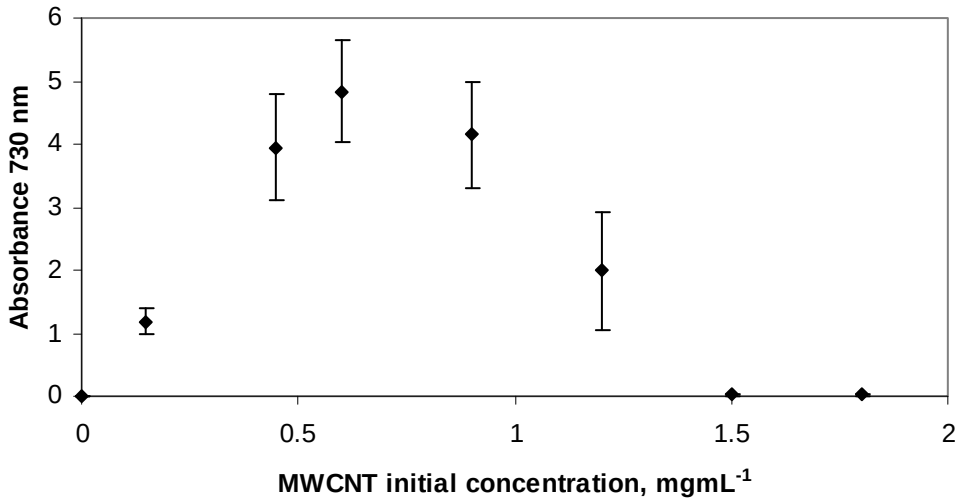

Figure 3. These optimisation curves were performed with all the surfactants tested and here as an example the solubilization curve for MWCNTs with LysoPC at a concentration of 0.3 $mg.mL^{-1}$ is shown. Data obtained from triplicates at each MWCNT initial concentration. A proper dilution was made to obtain an absorbance value in the linear range of the spectrophotometer.

It was clear that with each of the surfactants tested, there was an optimum ratio of surfactant to CNTs for maximum solubilisation as can be seen in table 1. It was generally observed that the optimal mass of CNTs solubilised with low molecular weight surfactants tended to be higher than the optimum mass obtained with higher molecular weight surfactants, attributed to the increased hydrophobicity of the low molecular weight compounds.

SURFACTANT	SWCNTs	DWCNTs	MWCNTs
BENZALKONIUM	5	1.5	5
PMA	6	5	6
PEI	5	3	6
PL-PEG-NH_2	2	0.5	3
LYSO PC	0.5	0.5	2
DPPE	0.5	5	2
POLY(Lys:Phe, 1:1)	3	6	1.5
POLY(Lys:Tyr, 1:9)	2	6	2

Table 1. Optimum CNT/surfactant ratio for the best dispersion. In this table the optimum CNT/surfactant (w/w) for each surfactant are given.

Figure 4 compares this efficiency when solubilisation has been optimised for each surfactant. It can be observed that the efficiency of solubilisation of three types of nanotubes was in the following type order of nanotubes MWCNTs > SWCNTs > DWCNTs for benzalconium, PEI, PL-PEG-NH_2 and poly(Lys:Tyr, 1:9), MWCNTs>DWCNTs>SWCNTs for Lyso PC, SWCNTs>MWCNTs>DWCNTs for PMA and DPPE, and DWCNTs>SWCNTs>MWCNTs for poly(Lys:Phe, 1:1). When comparing surfactants, the best conditions for solubilization of CNTs were obtained with phospholipids, followed by non-biological surfactants and finally polypeptides. When comparing the solubilization yield for the non-biological surfactants, PEI solubilised better than low molecular weight surfactants (benzalconium and PMA). When the excess surfactant was removed in the case of benzalkonium and PMA, the CNTs become not dispersed, indicating that solubilisation with these surfactants requires free surfactant in equilibrium with the f-CNTs. PL-PEG-NH_2 is significantly more efficient than DPPE, which differs primarily in the absence of a PEG group, suggesting that the PEG part of PL-PEG-NH_2

molecule plays an important role in the solubilization process. Conversely, the high solubilization yield for Lyso PC compared well to DPPE suggesting that increasing the number of acyl chains (in DPPE) decreases the solubilisation efficiency.

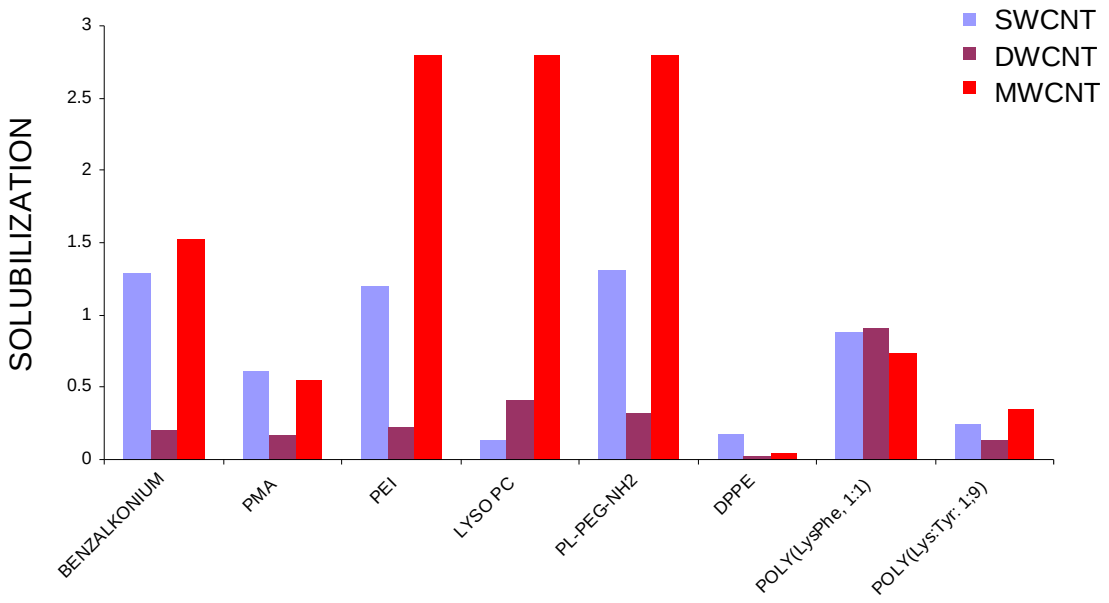

Figure 4. Solubilization (expressed as the absorbance at 730 nm of the suspension) as a function of the surfactant used for the different kinds of CNTs: SWCNTs, DWCNTs and MWCNTs, in the optimal conditions found for solubilisation (these optimal conditions refer to the optimum found when getting the solubilisation curve as shown in figure 3).

3.2. Optimization of DNA binding

To test the use of dispersed CNTs with the cationic surfactants as gene carriers, we studied the binding of plasmid DNA to these dispersed CNTs by agarose gel electrophoresis. The plasmid used for this study was the pGL3 plasmid (from Promega) that encodes the luciferase enzyme (lane 2 figure 5A). Binding of plasmid DNA to functionalized CNTs inhibits EtBr intercalation[21], as the DNA is in a condensed form. The level of binding can thereby be assessed by the measurement of the non bound DNA. The CNTs dispersed by the non

covalent attachment of cationic surfactants described above complexed with DNA (CNT:DNA) were prepared for each surfactant at various mixing ratios to determine the effectiveness of DNA binding. In this way, a constant amount of plasmid DNA was incubated with decreasing concentrations of dispersed CNTs. After running the agarose gel, the excess of plasmid DNA can be followed as a band for free plasmid DNA (figure 5A lanes 5-8). The dispersed CNTs that most effectively bound the DNA were the PL-PEG-NH_2, poly(Lys:Phe, 1:1), and PEI, whereas the other kind of dispersed CNTs did not show any DNA binding (see supplementary material, figure S6). A constant amount of plasmid DNA was also incubated with decreasing amounts of free surfactants as a control (see supplementary material, table S1). It was observed that only PL-PEG-NH_2, poly(Lys:Phe, 1:1), and PEI surfactants were able to bind plasmid DNA. It was also found that the surfactant non-covalently attached to CNTs is more efficient to bind plasmid DNA. After determining the amount to surfactant attached to CNTs (see supplementary material), it was found that surfactant bound to CNTs leads to a better condensation of DNA. This conclusion makes the non-covalent attachment of cationinc surfactants to CNTs a good method for the condensation and binding of DNA onto CNTs.

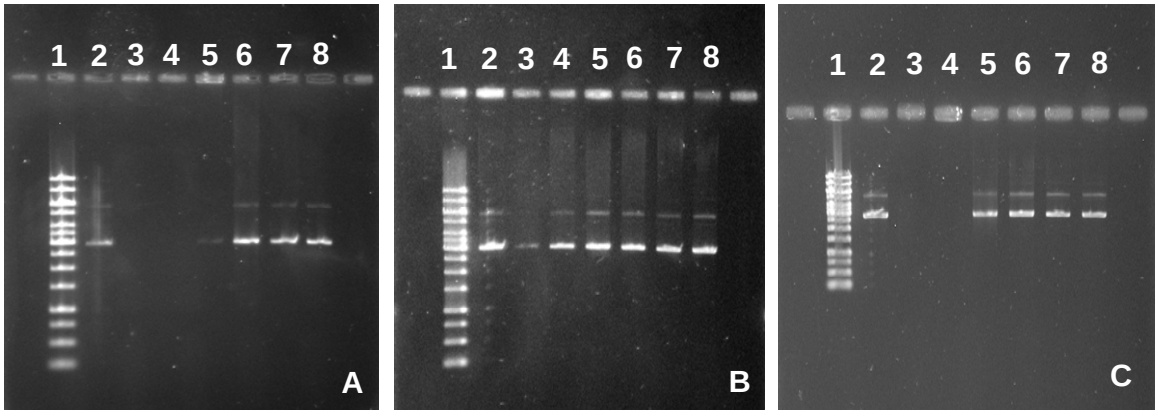

Figure 5. Agarose gel electrophoresis for the f-SWCNTs that effectively bind plasmid DNA: A) PEI, B) PL-PEG-NH_2 and C) poly(Lys:Phe, 1:1). Lane 1: ladder, lane 2: pGL3 plasmid alone 6.8 ng.μL^{-1}, lanes 3-8: f-SWCNT:plasmid DNA complexes with plasmid 6.8 ng.μL^{-1}

and different dilutions of f-SWCNTs from 1/1 to $1/10^5$ (1/1 refers to the best conditions found for solubilization of SWCNTs: 51 $\mu g.mL^{-1}$ for PEI, 56 $\mu g.mL^{-1}$ for PL-PEG-NH_2 and 37 $\mu g.mL^{-1}$ for poly(Lys:Phe, 1:1).

The DNA binding capacity of each form of dispersed CNTs can be estimated from Figure 5 by reference to the lowest concentration of nanotubes that demonstrates detectable DNA binding (for instance, lane 5 in Figure 5A). By normalising this value to the DNA concentration it is possible to obtain a DNA binding capacity of each f-CNT as shown in Table 2. It can be seen that the best results were obtained for PEI which has 10 times more binding yield compared to poly(Lys:Phe, 1:1) and 100 times more than PL-PEG-NH_2. The other f-CNTs showed negligible DNA binding.

SURFACTANT	RELATIVE SOLUBILIZATION YIELD FOR SWCNTs	RELATIVE SOLUBILIZATION YIELD FOR DWCNTs	RELATIVE SOLUBILIZATION YIELD FOR MWCNTs	WEIGHT OF BOUND DNA PER WEIGHT OF f-SWCNTs (mg DNA.mg-^{1} f-SWCNTs)
BENZALKONIUM	0.98	0.22	0.55	-
PMA	0.47	0.18	0.20	-
PEI	0.91	0.25	1.00	120
PL-PEG-NH_2	0.10	0.46	1.00	0.092
LYSO PC	1.00	0.36	1.00	-
DPPE	0.13	0.03	0.02	-
POLY(Lys:Phe, 1:1)	0.67	1.00	0.26	18.2
POLY(Lys:Tyr, 1:9)	0.19	0.14	0.12	-

Table 2. Properties of the f-CNTs. The relative solubilization yield were normalized to those obtained which the highest solubilization yield (PL-PEG-NH_2 for SWCNTs and LysoPC for DWCNTs and MWCNTs).

3.3. Functionalization of RNA-wrapped SWCNTs by a bilayer approach

We also examined functionalization of SWCNTs with biological molecules such as nucleic acids and proteins. RNA-wrapped CNTs are an attractive method of solubilising CNTs because the RNA gives high solubilisation yields and is non-cytotoxic[22]. However, RNA-wrapping confers negative charges on the carbon nanotubes which then makes them unsuitable for DNA binding. To overcome this problem we investigated the use of a cationic ion or molecule that can act as bridge between the negatively charged RNA wrapping the CNT, and the negatively charged plasmid DNA (Figure 6). The following cationic polymers were investigated: poly(Lys:Phe, 1:1), PEI and polylysine (data not shown). The best results were obtained using the cationic polymer polylysine as a bridging molecule. With poly(Lys:Phe, 1:1) and PEI it was observed a higher aggregation of the dispersed CNTs owing to the cationic molecules acting as ionic bridges between negatively charged RNA-wrapped CNTs. As this aggregation was lower for polylysine the studies with this functionalization method were carried out with this polymer. Furthermore, it was quantified the amount of plasmid DNA that polylysine on its own is able to bind as a control. This amount was determined as 1.40 mg DNA per mg of polylysine which is higher than for PEI and poly(Lys:Phe, 1:1) (see supplementary material, table S1). This property also makes polylysine a good choice for the development of this bilayer approach for DNA binding to carbon nanotubes.

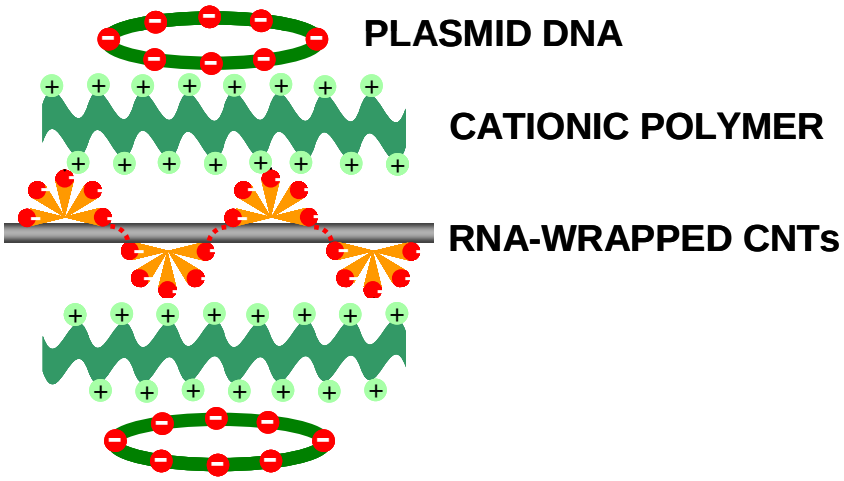

Figure 6. Bilayer approach with RNA-wrapped CNTs for plasmid DNA binding.

The effect of concentration of cationic polymer on DNA solubilisation was investigated by agarose gel electrophoresis (Figure 7). The results showed that the complex between RNA-wrapped CNTs and polylysine is positively charged when the concentration of polylysine is high which is the best condition for DNA binding (see figure 7A) we observe. As the concentration of polylysine is decreased, the binary complex becomes negatively charged because the RNA is in excess of the polylysine. There is also a RNA:polylysine ratio at which the binary complex becomes neutral. These effects on functionalized CNT surface charge can be observed during the electrophoresis process of the sample preparation (see supplementary material, figure S7), negatively charged CNTs run towards the positive electrode and *vice versa* (although this can be seen only in the well as the CNTs are too long and rigid to enter the agarose). In Figure 7B the plasmid DNA concentration is optimised. These studies show that the optimum DNA binding is 0.071 mg DNA per mg RNA-wrapped CNTs, when working with 45 μg polylysine per mg of RNA-wrapped CNTs. This data confirms that the condensation of plasmid DNA is more efficient in this bilayer approach than with polylysine on its own.

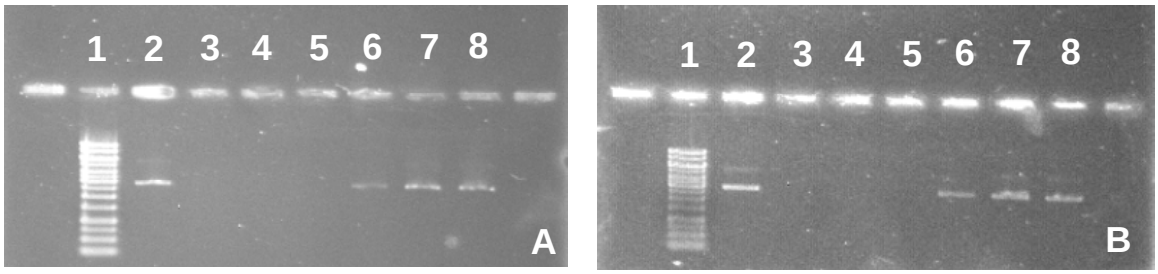

Figure 7. Agarose gel electrophoresis for f-SWCNTs with the bilayer approach with RNA-wrapped CNTs. A) effect of polylysine concentration in plasmid DNA binding: lane 1: ladder, lane 2: pGL3 plasmid 1.8 ngμL^{-1}, lanes 3-8 RNA-wrapped CNTs (34 $\mu g.mL^{-1}$) with different concentrations of polylysine from 1.5 $mg.mL^{-1}$ to 0.015 $\mu g.mL^{-1}$. B) lane 1: ladder, lane 2: pGL3 plasmid 1.8 $ng.\mu L^{-1}$, lanes 3-8: RNA-wrapped CNTs-polylysine complexes at different dilutions from 1/1 to $1/10^5$ starting in the same conditions as lane 5 in gel A.

4. Conclusions

In conclusion, we have compared the solubilization properties of SWCNTs, DWCNTs and MWCNTs with different kinds of surfactants using non-covalent functionalization. The best conditions for solubilization are with the use of phospholipids with PL-PEG-NH_2 for SWCNTs and LysoPC for DWCNTs and MWCNTs. Furthermore, the solubilization yields with the surfactants tested are in general higher for MWCNTs and SWCNTs than for DWCNTs. The solutions of f-CNTs obtained by the solubilization methods presented here are very stable (several months). The use of these functionalized CNTs for development of gene delivery systems was also studied. The best conditions for plasmid DNA binding were obtained with PEI, but, given its cytotoxicity, the best combination for solubilization and DNA binding is poly(Lys:Phe, 1:1), which is less toxic. Furthermore, a bilayer functionalization method based on RNA-wrapped CNTs and the use of cationic polymers shows that comparable solubilisation and DNA binding can be achieved by this method.

Overall, this study is important as good optimisation strategies for CNT functionalisation for gene delivery are crucial if CNT are to be used in a healthcare scenario.

Acknowledgements

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