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Optimising DNA binding to carbon nanotubes by non-covalent methods

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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 solubilize the nanotubes and cationic polymers as a bridge between nanotubes and DNA.

1. Introduction

In recent years increased attention has been paid to nanostructured 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,

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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 crucial 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-NH₂) from Avanti Polar Lipids 880128P; 1-stearoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (Lyso-PC) from 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 from 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 (four 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 13,200 rpm for 10 min and the supernatant was pipetted off. 500 µL of f-CNTs were placed in Microcon centrifugal devices, regenerated cellulose filter 100 kDa, and centrifuged at 13,200 rpm for 5 min, 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

Eighty microlitres of the f-CNTs prepared as described above at different concentrations were mixed with 2 µL of plasmid DNA of 340 µg mL⁻¹. Complexes were allowed to form for 30 min at room temperature.

2.5. Gel electrophoresis

Agarose gel electrophoresis (0.8%) 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. Sucrose (40%) 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) 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 Fig. 1): benzalkonium chloride, polyethyleneimine (PEI), 1-pyrenemethylamide hydrochloride (PMA), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[amino(polyethylene glycol)2000 (PL-PEG-NH₂), 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.

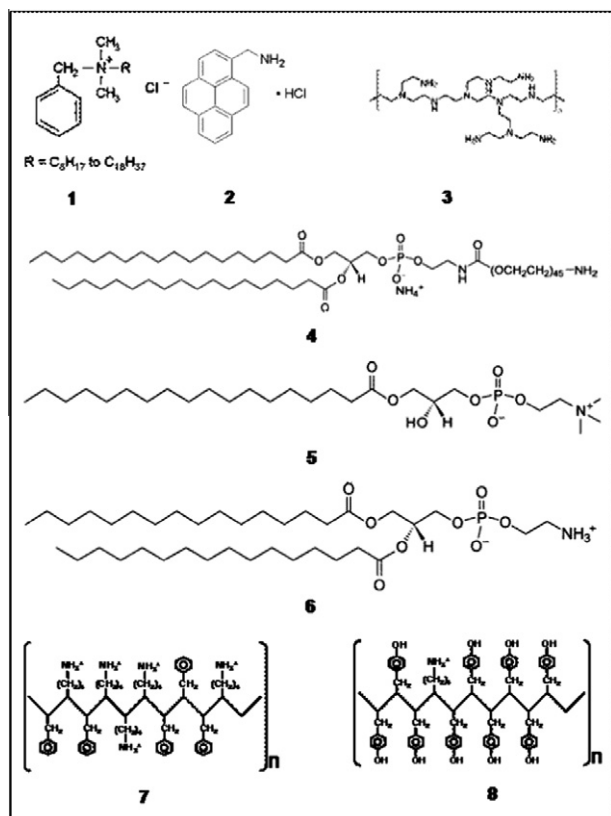


Fig. 1 – Surfactant structures: (1) benzalkonium chloride, (2) pyrenemethylamine (PMA), (3) polyethyleneimine (PEI), (4) 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[amino(polyethylene glycol)2000] (PL-PEG-NH₂), (5) 1-stearoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (Lyso PC), (6) 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (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 Figs. S1a-c). Besides, the efficiency of solubilization 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, Figs. S2-S5). In Fig. 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.

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 Fig. 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 solubilizing 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.

It was clear that with each of the surfactants tested, there was an optimum ratio of surfactant to CNTs for maximum solubilization as can be seen in Table 1. It was generally observed that the optimal mass of CNTs solubilized 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.

Fig. 4 compares this efficiency when solubilization has been optimised for each surfactant. It can be observed that the efficiency of solubilization of three types of nanotubes was in the following type order of nanotubes MWCNTs > SWCNTs > DWCNTs for benzalkonium, PEI, PL-PEG-NH₂ and poly(Lys:Tyr, 1:9), MWCNTs > DWCNTs > SWCNTs for Lyso PC,

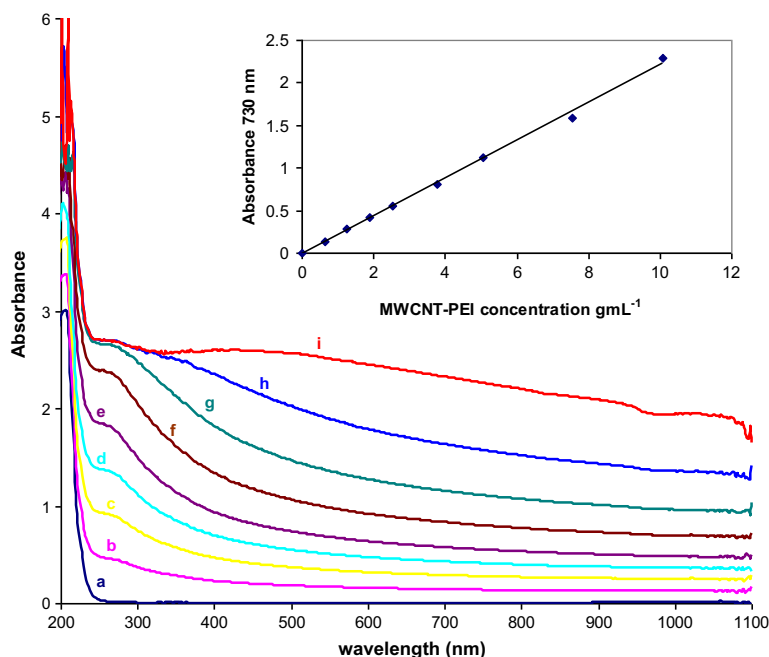


Fig. 2 – Set of spectra at increasing concentrations of dispersed MWCNT-PEI. PEI concentration was kept constant at 0.3 mg mL^{-1} and different volumes of functionalized MWCNTs were added. In the insert the absorption value at 730 nm as a function of the concentration of dispersed MWCNT-PEI is presented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

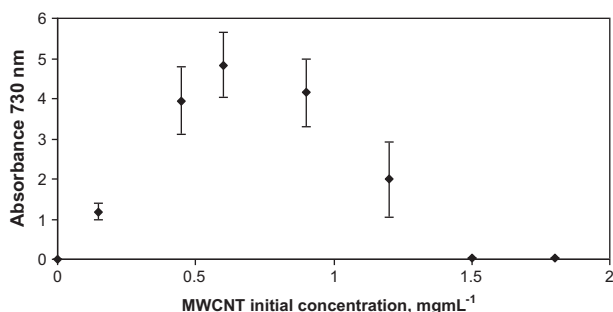


Fig. 3 – These optimisation curves were performed with all the surfactants tested and here as an example the solubilization curve for MWCNTs with LysoPC at a concentration of 0.3 mg mL^{-1} is shown. Data obtained from triplicates at each MWCNT initial concentration. A proper dilution was made to obtain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

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 solubilized better than low molecular weight surfactants (benzalkonium and PMA). When the excess surfactant was removed in the case of benzalkonium and PMA, the CNTs become not dispersed, indicating that solubilization with these surfactants requires free surfactant in equilibrium with the f-CNTs. PL-PEG-NH₂ is significantly more efficient than DPPE, which differs primarily in the absence of a PEG group, suggesting that the PEG part of PL-PEG-NH₂ 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 solubilization efficiency.

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

| 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 |

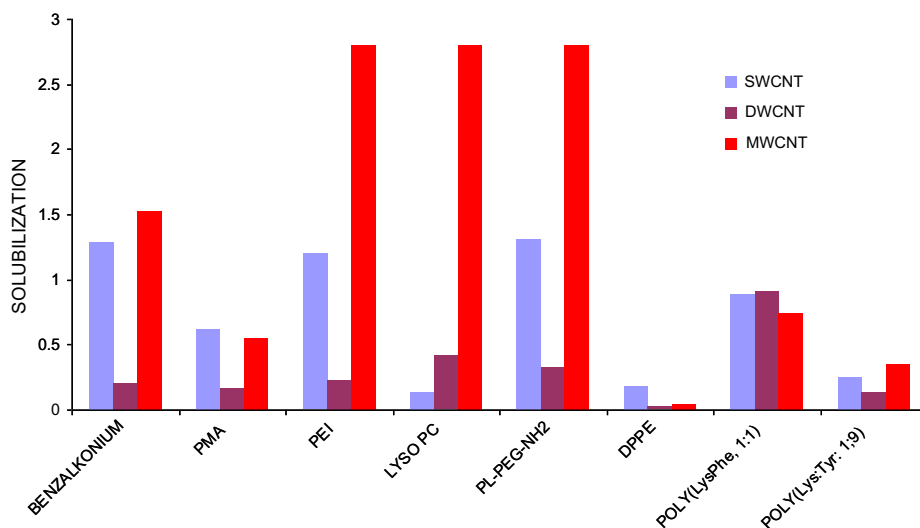


Fig. 4 – Solubilization (expressed as the absorbance at 730 nm of the suspension) as a function of the surfactant used for the different kinds of CNTs: SWCNTs, DWCNTs and MWCNTs, in the optimal conditions found for solubilization (these optimal conditions refer to the optimum found when getting the solubilization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

3.2. Optimisation 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 Fig. 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 (Fig. 5A lanes 5–8). The dispersed CNTs that most effectively bound the DNA were the PL-PEG-NH₂, poly(Lys:Phe, 1:1), and PEI, whereas the other kind of

dispersed CNTs did not show any DNA binding (see Supplementary material, Fig. 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-NH₂, 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 of 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 cationic surfactants to CNTs a good method for the condensation and binding of DNA onto CNTs.

The DNA binding capacity of each form of dispersed CNTs can be estimated from Fig. 5 by reference to the lowest concentration of nanotubes that demonstrates detectable DNA binding (for instance, lane 5 in Fig. 5A). By normalizing 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

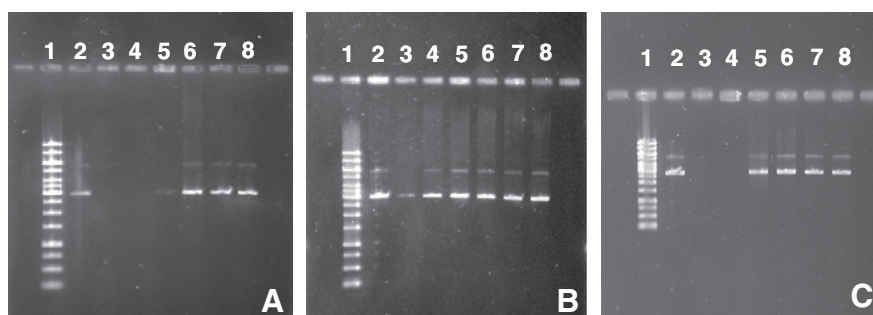


Fig. 5 – Agarose gel electrophoresis for the f-SWCNTs that effectively bind plasmid DNA: (A) PEI, (B) PL-PEG-NH₂ and (C) poly(Lys:Phe, 1:1). Lane 1: ladder, lane 2: pGL3 plasmid alone 6.8 ng μL^{-1} , lanes 3–8: f-SWCNT:plasmid DNA complexes with plasmid 6.8 ng μL^{-1} and different dilutions of f-SWCNTs from 1/1 to 1/10⁵ (1/1 refers to the best conditions found for solubilization of SWCNTs: 51 $\mu\text{g mL}^{-1}$ for PEI, 56 $\mu\text{g mL}^{-1}$ for PL-PEG-NH₂ and 37 $\mu\text{g mL}^{-1}$ for poly(Lys:Phe, 1:1).

Table 2 – Properties of the f-CNTs. The relative solubilization yield were normalized to those obtained which the highest solubilization yield (PL-PEG-NH₂ for SWCNTs and LysoPC for DWCNTs and MWCNTs).

| 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 | – |

has 10 times more binding yield compared to poly(Lys:Phe, 1:1) and 100 times more than PL-PEG-NH₂. The other f-CNTs showed negligible DNA binding.

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 solubilizing CNTs because the RNA gives high solubilization 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 (Fig. 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.

The effect of concentration of cationic polymer on DNA solubilization was investigated by agarose gel electrophoresis (Fig. 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 Fig. 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, Fig. 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

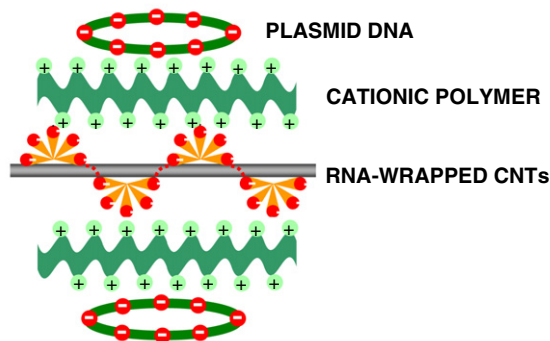


Fig. 6 – Bilayer approach with RNA-wrapped CNTs for plasmid DNA binding.

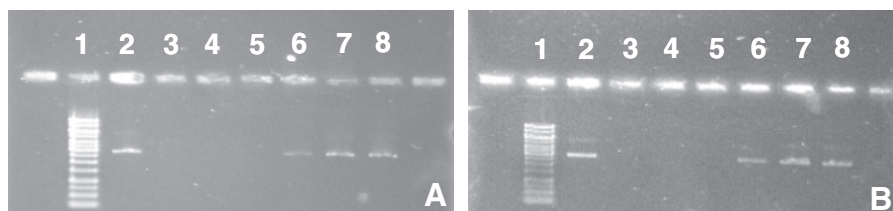


Fig. 7 – Agarose gel electrophoresis for f-SWCNTs with the bilayer approach with RNA-wrapped CNTs. (A) Effect of polylysine concentration in plasmid DNA binding: lane 1: ladder, lane 2: pGL3 plasmid 1.8 ng μL⁻¹, lanes 3–8 RNA-wrapped CNTs (34 μg mL⁻¹) with different concentrations of polylysine from 1.5 mg mL⁻¹ to 0.015 μg mL⁻¹. (B) Lane 1: ladder, lane 2: pGL3 plasmid 1.8 ng μL⁻¹, lanes 3–8: RNA-wrapped CNTs–polylysine complexes at different dilutions from 1/1 to 1/10⁵ starting in the same conditions as lane 5 in gel A.

enter the agarose). In Fig. 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.

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-NH₂ 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 solubilization and DNA binding can be achieved by this method. Overall, this study is important as good optimisation strategies for CNT functionalization for gene delivery are crucial if CNT are to be used in a health-care scenario.

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