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Original Research

Effective in vitro gene delivery to murine cancerous brain cells using carbon nanotube-polyethylenimine conjugates

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Abstract

Objective(s): Carbon nanotube (CNT) has been widely applied at molecular and cellular levels due to its exceptional properties. Studies based on conjugation of CNTs with biological molecules indicated that biological activity is preserved. Polyethylenimine (PEI) is explored in designing novel gene delivery vectors due to its ability to condense plasmid DNA through electrostatic attraction. In this study functionalization and grafting polyethylenimine onto the surface of carbon nanotube was used to improve the solubility and biocompatibility.

Materials and Methods: The effect of molecular weight of polymer on final efficacy of vectors has been investigated using three different molecular weights of polymer. In this study no linker was used and both segments (PEI and CNT) were directly attached resulted in the synthesis of three different vectors. Synthesized vectors were tested for their ability to condense plasmid DNA and cellular toxicity using ethidium bromide and MTT assays. Size and Zeta potential of nanoparticles was determined using Malvern zeta sizer. Evaluation of transfection efficiency of vectors was carried out on N2A cell line by different methods including qualitative fluorescence imaging, flow cytometry and luciferase assay.

Results: All three synthesized vectors bear positive surface charges with sizes in the range of 85-190 nm. More than 80 percent of treated cells were viable and in the case of V25 significant improvement in reducing cytotoxicity compared to unmodified polymer was observed. Obtained results indicated that vector containing PEI 1.8 kDa has the greatest improvement in terms of its transfection efficiency compared to unmodified polymer.

Conclusion: Conjugation of PEI with carbon nanotube les to new vectors with lowered cytotoxicity and higher transfection efficiency. The highest transfection efficiency was obtained with the lowest molecular weight PEI.

Keywords: Carbon nanotube, Disulfide bond, Functionalization, Gene delivery, Polyethyleneimine

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Introduction

Once carbon nanotube (CNT) was discovered by Iijima [1] numerous range of studies has focused on their unique properties. Aside from their chemical properties, their nanoscale size, extreme flexibility, high tensile strength, optical activity [2] and large current density have made them candidates for physical and chemical studies aimed at preparing nano-composites, biosensors [3] and field-effect transistors (FETs) [4]. The hydrophobic nature of SWNT renders it insoluble in aqueous media and has prompted attempts to improve its solubility by either non-covalent or covalent functionalization methods [5-7]. SWNT is able to enter cells and this is a key characteristic which different studies on designing new carriers for drug [8] and gene [9] delivery has focused on. We recently reported that nanovectors based on non-covalent functionalization of SWNT with alkylated polyethylenimines were efficient gene vectors in vitro and in a systemic gene delivery system [10]. In this study we have investigated covalent functionalization of SWNT. Among polycations available for functionalizing SWNT, PEI has the advantage of providing high densities of terminal primary amines, as well as being one of the most widely used non-viral vectors for transfection studies [11].

Attempts have been made to improve PEI transfection efficiency while lowering cytotoxicity by chemical modifications with peptides [12-15] or alkyl groups [16].

In the present study we have synthesized three DNA nanovectors by covalent conjugation of SWNT to PEI molecules of different molecular weights through amide bond formation to investigate the effect of polymer size on DNA transfection efficiency and cytotoxicity.

Materials and Methods

Materials

Single-walled carbon nanotubes, 25 kDa branched polyethylenimine, N-hydroxybenzotriazole (HBOt), and 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), solvents and all other chemicals were purchased from Sigma-Aldrich (Munich, Germany). Branched polyethylenimines (1.8 kDa and 10 kDa PEI) were purchased from Polyscience, Inc (Warrington, USA). Dialyses were carried out using Spectra/Por dialysis membranes (Spectrum Laboratories, Houston, USA).

PTFE membranes (200 nm) were obtained from Chemlab, Spain.

Carboxylate functionalization of SWNT

Carboxylation of SWNT was carried out as described in literature [17]. Briefly, SWNT (10 mg) was suspended in 30 ml of 2.5M HNO_3 with sonication for 3 minutes, heated 36 h at 75°C, sonicated an additional 30 minutes then heated at 75°C again for 36 h. The reaction mixture was filtered through a 200 nm PTFE membrane and the retentate washed until the pH of the filtrate became neutral. The retentate (SWNT-COOH) was dried at 65^{o}C for 24 h (Figure 1).

Coupling of functionalized SWNT

The procedure for conjugation of different molecular weights of PEI can be summarized as follow. Briefly, SWNT-COOH (1, 4 mg) was dispersed in 8 ml water by sonication then EDC (240 mg) added and the mixture stirred 45 min at room temperature.

PEI (0.088 mmol for PEI 1.8 kDa, 0.022 mmol for PEI 10 and 25 kDa) and HOBt (168.8 mg) dissolved in 7 ml water was added dropwise and the mixture stirred at room temperature for 5 days.

Solubilized PEI-grafted SWNT was separated from the reaction mixture by filtering through a 200 nm PTFE membrane and further purified by dialysis against 3L of distilled water three times using Spectra/Por® (3500 MWCO for PEI 1.8 kDa, 12000-14000 for PEI 10 kDa and 25000 for PEI 25 kDa) membrane to remove reactants (Figurer 1). The resulting vectors were named as V1.8, V10 and V25 in which the number shows the molecular weight of polymer used in vector structure.

The PEI-grafted SWNTs were lyophilized and used to prepare 1mg/ml stock solution.

IR spectroscopy

FTIR spectra were used to confirm the modification of SWNT surface and attachment of different groups [18]. IR spectra were recorded in KBr discs from 4000 to 500 cm^{-1}.

Thermogravimetric analysis

Degree of grafting of PEI onto the SWNT surface was determined by thermogravimetric analysis [19-21].

Thermogravimetric analysis diagrams were obtained using TGA 50 instrument (Shimadzu, Japan) by heating the sample at 10ºC/min rate to 800ºC in air.

DNA condensation analysis

Condensation ability of SWNT derivatives was analyzed ethidium bromide (EtBr) assay by fluorescence spectroscopy (Jasco FP-6200 spectrofluorimeter, Tokyo, Japan) with excitation at 510 nm and emission at 590 nm.

The fluorescence intensity of 1ml of EtBr solution (400 ng/ml in Hepes buffered Glucose (HBG)) complexed with pDNA (5µg) was set to 100%. Solution of the SWNT derivative (1 mg/ml) was added in aliquots of 2.5 µl and fluorescence intensity was recorded until it reaches a constant value.

Determination of average size of polyplexes

Size and zeta potential of SWNT-PEI conjugates and their surface charges was carried out using the Malvern Zeta Sizer in automatic mode and DTS software (Malvern Instruments, UK).

Since for all vectors plasmid DNA was completely condensed at C/P=2 (polyplexes were prepared at this ratio based on 5 µg plasmid DNA) in all cases, this ratio was used for determination of both size and zeta potentials. Results are presented as mean ± SD for three independent measurements.

Cell culture

Murine neuroblastoma (N2A) cells (ATCC CCL-131) were cultured in DMEM (1g/L glucose, 100 µg/mL streptomycin, 100 units/mL penicillin and 2 mM glutamine) supplemented with 10% fetal bovine serum (FBS), and maintained at 37ºC in 5% CO_2 atmosphere.

Cells were cultured at the density of 10^4 cells /well in of 96-well plates 24 h before treatment.

Cytotoxicity assay

The viability of cells after treatment with vectors was measured by treating N2A cells with 20 µl of polyplexes with three different C/P ratios (4, 6 and 8 based on 0.2 µg plasmid); four replicates each, for 4 h under normal culture conditions.

After replacement of medium with fresh medium with 10% FBS, cells were cultured for an additional 18 h at 37ºC. Cell viability was measured using the MTT assay procedure [21]. Resulting formazan crystals upon addition of MTT reagent were dissolved in 100 µl DMSO and the absorbance was recorded at wavelength 590 cm-1 and 630 cm^{-1} as reference. Untreated cells were used as control. Cell viabilities were analyzed using a microplate reader (Tecan, Switzerland) at absorbance.

Transfection activity

Promega Renilla Luciferase Assay kit and protocol was used in order to evaluate the transfection efficiencies of SWNT-PEI derivatives. N2A cells were treated with 20 µl of polyplexes prepared with Renilla luciferase plasmid DNA at three weight ratios (C/P: 2, 4 and 6) based on 0.2 µg plasmid; four replicates each, for 4 h under normal culture conditions. Then medium was replaced with fresh medium with 10% FBS and the cells cultured for an additional 18 h at 37ºC. Cells lysate was used to determine Renilla luciferase activity by luminometer (Berthold Detection Systems, Pforzheim, Germany) as Relative Light Unit (RLU).

Data were presented as RLU/number of cells [16]. The similar procedure was used for fluorescent imaging except for preparing C/P ratios based on 0.4µg EGFP plasmid.

Results and Discussion

Synthesis and characterization of PEI-grafted SWNTs

PEI was conjugated to SWNT through amide bond to terminal carboxylate group introduced onto the surface of SWNT by oxidation (Figurer 1).

Attachment of PEI improved SWNT solubility in aqueous media and to provide the positive charge on SWNT required for DNA condensation.

FTIR spectra of carboxylated SWNT contained a peak at 1736 cm-1 indicating the introduction of carboxyl group by the oxidation reaction (Figure 1).

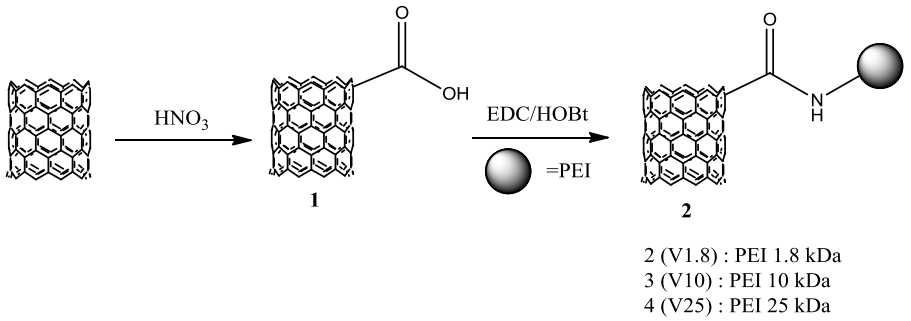


Figure 1. Schematic presentation of synthetic steps used in preparation of SWNT-PEIs conjugates.

After conjugation with amine groups on PEI, this peak was replaced by a characteristic peak at 1630 cm^{-1} confirming the formation of amide bond and methylene groups of the conjugates were observed at around 2820 and 2940 cm^{-1} while amine peaks appeared at 3430 cm^{-1} for all three products (Figurer 2). Scanning electron microscopy (SEM) was employed to study the surface topography and measure the diameters of polyplexes prepared with SWNT-PEI conjugates (Figure 3).

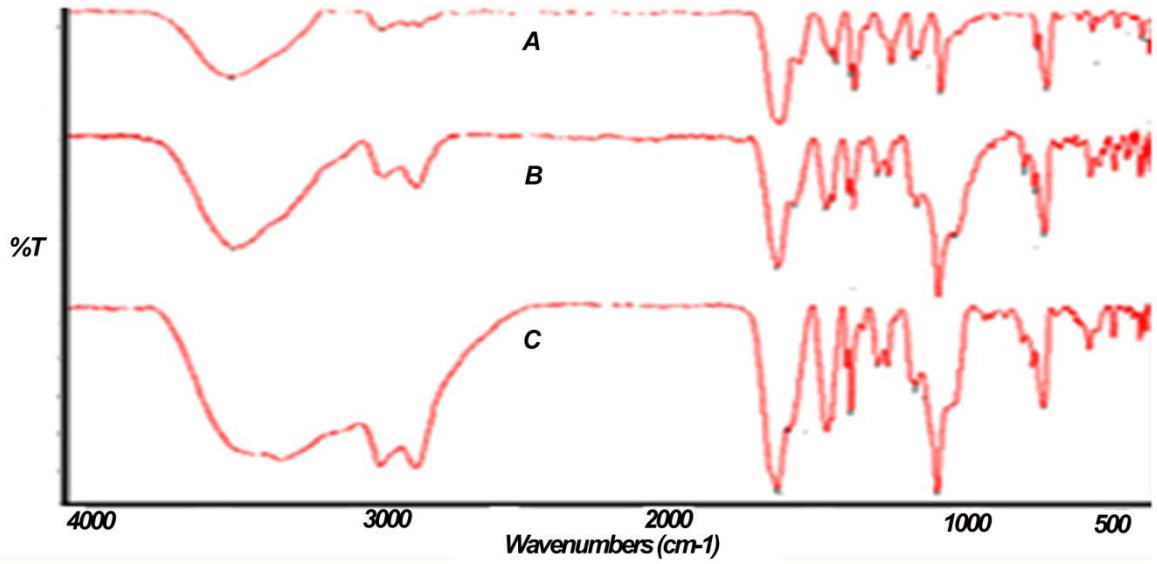


Figure 2. FTIR spectra of three SWNT-PEIs: A) V1.8 B) V10 C) V25.

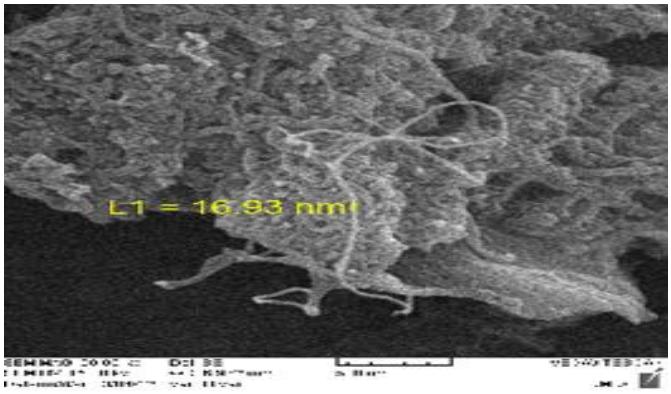


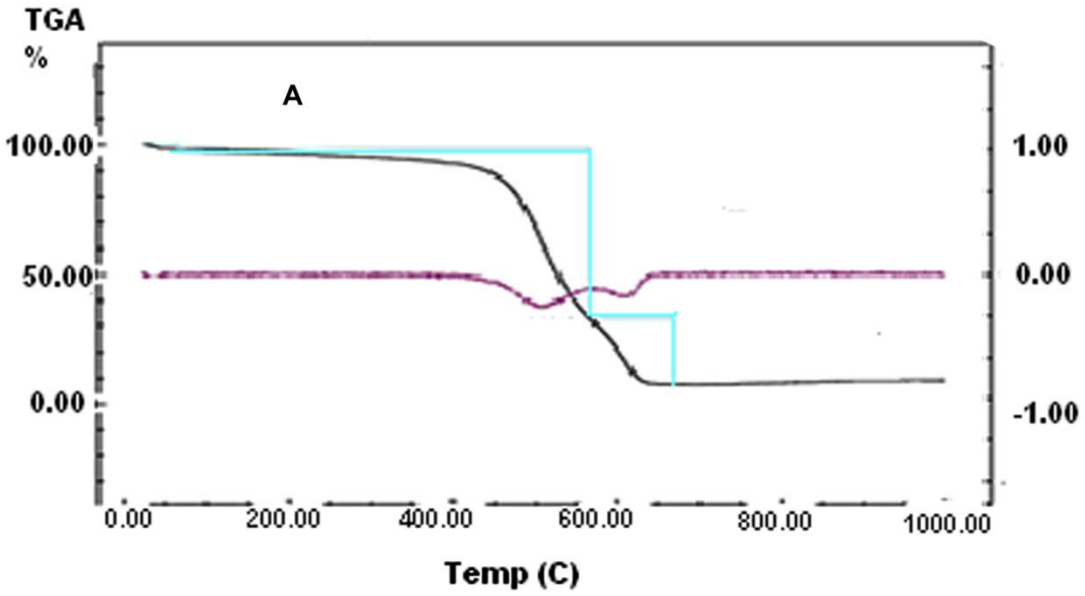
Figure 3. SEM image of SWNT-amide-PEI1.8 (V1.8).

The SEM micrographs clearly showed that SWNTs were coated with PEI along the length of the SWNTs, so that diameters increased from 1.4 nm to 16-20 nm. These topographic changes confirm the chemical functionalization of carbon nanotubes.

Thermogravimetric analysis

Content of SWNT and weight percent of grafted moieties was determined by thermogravimetric analysis (TGA) (Figure 4). As previously reported, SWNT-COOH is more thermally stable than underivatized SWNT [22]; the TGA diagram showed that carboxylated SWNT corresponded to COOH group (1mmol COOH/1mg of SWNT-COOH).

The percent of grafting onto SWNT for V1.8 was 83% corresponding to weight ratio of 2.67 µmol/mg.



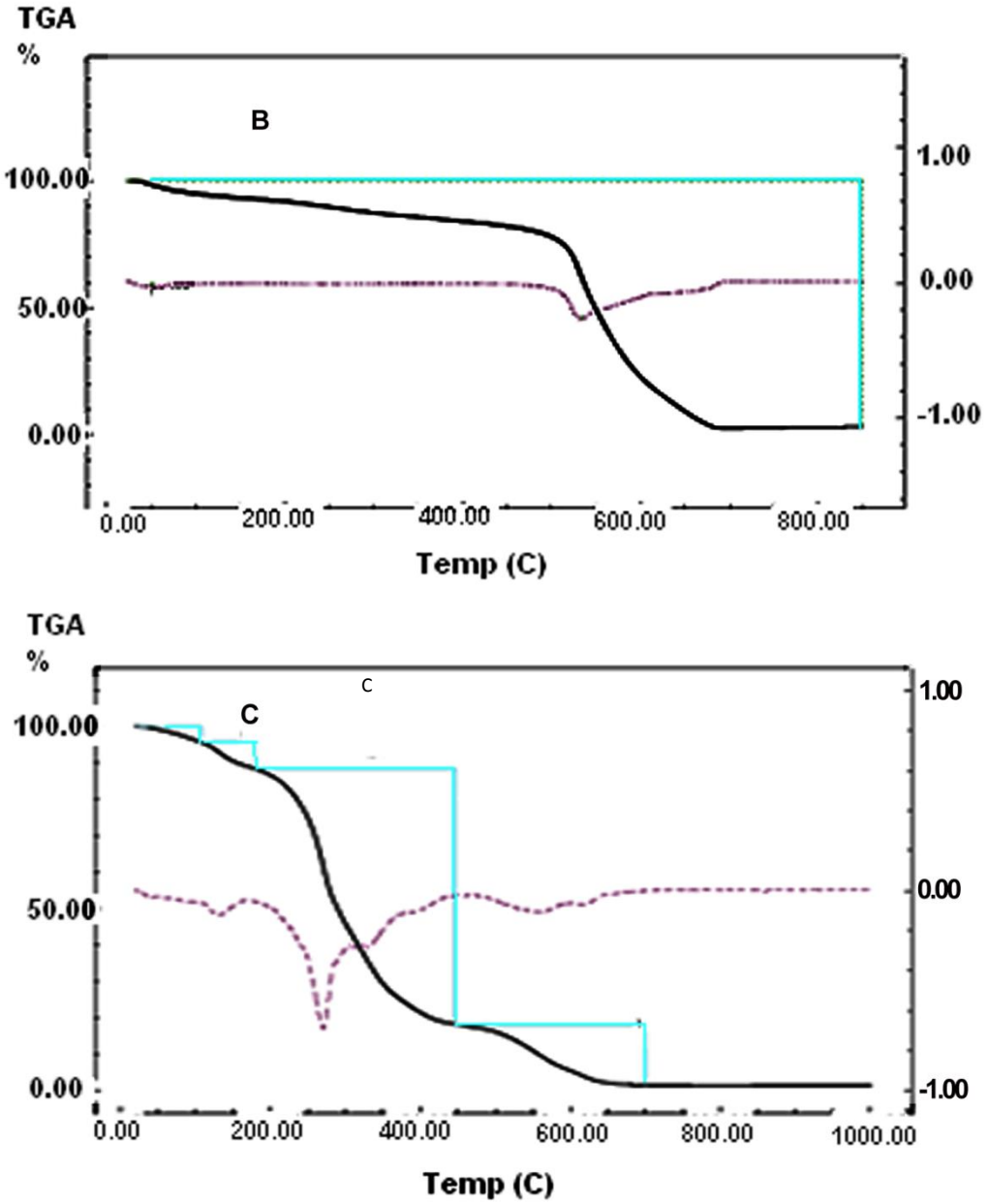


Figure 4. TGA diagrams of A) pristine SWNT B) SWNT-COOH C) SWNT-amide-PEI1.8 (V1.8).

Buffering capacity of SWNT-PEI conjugates

The buffering capacity of PEI, due to the presence of primary amines, is believed to enable endosomal escape and avoiding degradation in lysosomes through proton sponge effect [23].

Measurement of buffering capacities of vectors was carried out by adjusting pH to 12 using 0.4 µg/ml stocks, and titration by adding 5 µl aliquots of 0.1 N HCl (Figure 5).

DNA condensation by SWNT-PEI conjugates

Results of Et-Br condensation assay are shown in Figure 6. As diagram shows all three SWNT-PEI conjugates (V1.8, V10 and V25) condensed plasmid effectively.

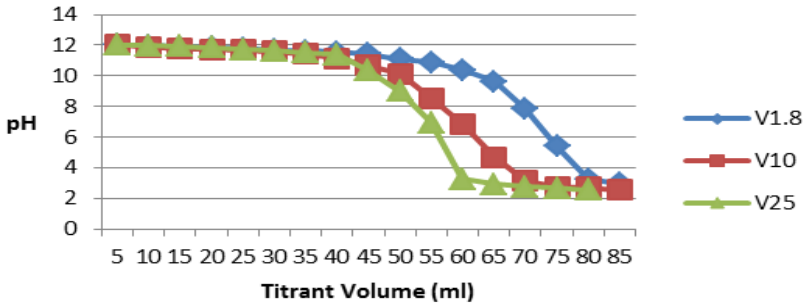


Figure 5. Buffering capacity of vectors was measured by titration with HCl 0.1 N solution.

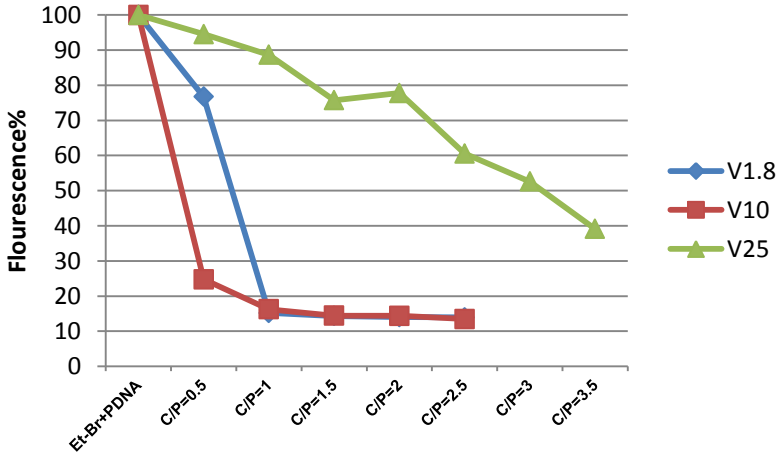


Figure 6. Ethidium bromide assay for determination of vectors ability in plasmid condensation.

Size and Zeta potential of SWNT-PEI conjugates

Size and surface charge of nanoparticles have a major impact on their transfection activity [24]. All three vectors bear positive charges in the range of and sizes up to 200 nm (Figure 7).

Cytotoxicity of polyplexes prepared with SWNT-PEI conjugates.

Cytotoxicity of vectors on N2A cells were evaluated using MTT assay and results are summarized in Figure 8. As it can be seen V1.8 and V10 polyplexes exhibited no significant cytotoxicity ($P<0.05$) (Figure 8). Control 25 kDa PEI exhibited significant cytotoxicity ($P<0.05$) under all conditions tested.

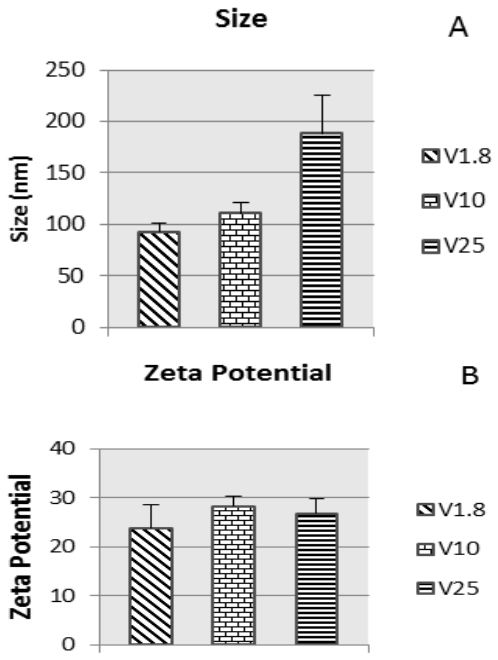


Figure7. A) Size B) Zeta potential of polyplexes at C/P=2.

Transfection activity of polyplexes prepared with SWNT-PEI conjugates

The most remarkable increase in transfection efficacy of vectors compared to unmodified polymer was observed in the case of V1.8. In accordance with previously reported data luciferase expression levels is increased as the C/P ratios is elevated [25]. For V10 polyplexes at different C/P ratios no improvement in efficacy compared to free PEI 10 kDa at similar C/P ratio was observed. This can be attributed to reduced number of surface amines of 10 kDa PEI available [26] when conjugated to SWNTs, as well as less total 10 kDa PEI when conjugated to the surface of SWNTs. Similar results have been observed in this laboratory for non-covalent attachment of alkylated PEI to SWNT surfaces [27].

In the case of V25 polyplexes only low or no significant levels of expressed luciferase was detected (Figure 9). Size and surface charge of polyplexes play an important role on transfection level [28, 29].

The size of polyplexes was in the range of 92–188.3 nm with zeta potentials of 23–28.4 eV. It seems that size of nanoplexes is the key factor for their efficacy as V25 has the lowest efficiency and has the largest size (Figure 9).

One issue that has to be considered in vectors based on PEI is that transfection ability and cytotoxicity often show the same trend. PEIs with higher molecular weight are more efficient transfection agents, but they are also more toxic [30]. The smaller PEIs are more easily eliminated via excretion pathways.

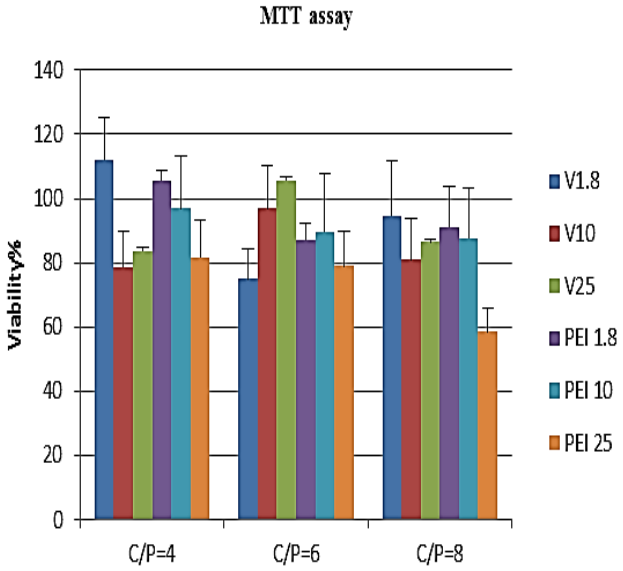


Figure 8. MTT assay of synthesized vectors compared to unmodified polymers.

Different degradable linkages such as ester, amide, imine, carbamate and ketals have been used [31].

Since PEI 1.8 kDa shows no cytotoxicity, its attachment to SWNT via degradable amide linker [32] can lead to higher transfection and yet no significant cytotoxicity.

It was previously reported that number of grafted PEI molecules to a specific area on the surface of SWNTs depends on the PEI molecular weight [30], more of PEI molecules attached for PEI 1.8 kDa and less for higher molecular weights PEIs.

Though PEI 1.8 kDa has lower number of amines but higher number of attached would lead to improved transfection compare to PEI 1.8 kDa. In the case of PEI more amine groups are shielded by SWNT surface due to larger size of polymer so two effects cancel each other and no net change is observed.

But for V25 shielding effect is prominent and reduced trans-fection efficacy is observed.

Fluorescence imaging using green fluorescence protein (EGFP) plasmid DNA

Enhanced green fluorescence protein (EGFP as a reporter gene) was used for qualitative comparison of the transfection efficiency of SWNT-PEI conjugates. Expression of EGFP plasmid DNA was detected as fluorescent images (Figure 10). Polyplex prepared with V1.8 induced EGFP expression more efficiently than unmodified 1.8 kDa PEI.

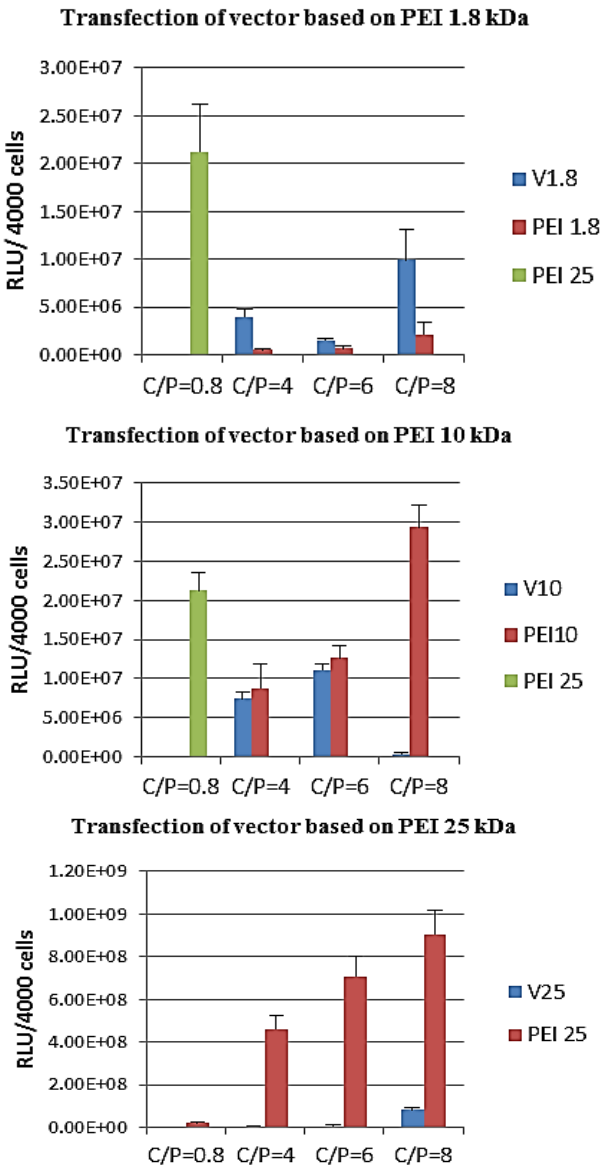


Figure 9. Transfection of vectors using Renilla luciferase assay.

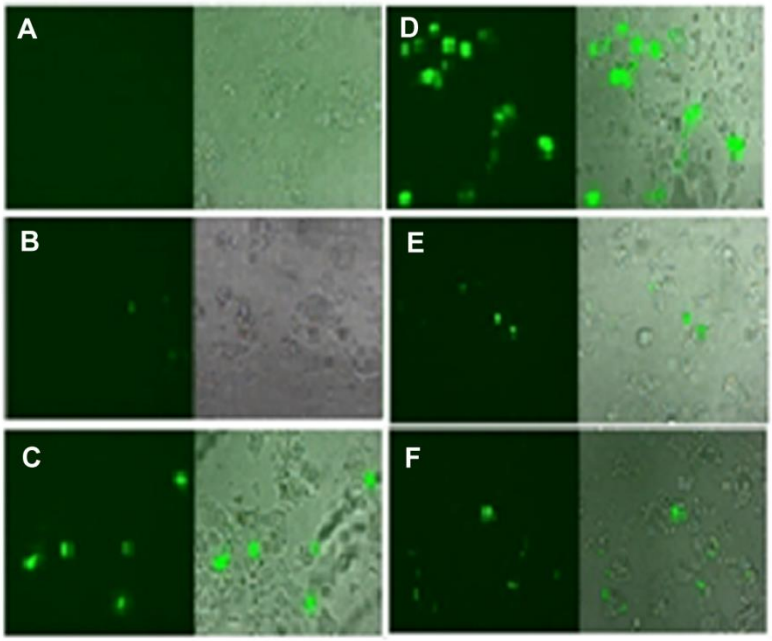


Figure 10. Fluorescent microscopy on N2A cells transfected by EGFP plasmid. A) PEI 18 kDa B) PEI 10 kDa C) PEI 25 kDa D) V1.8 E) V10 F) V25.

Conclusion

The goal of this study was to combine transfection activity of PEI with SWNT ability to penetrate into cells for designing novel DNA nanocarries. Three vectors were synthesized upon PEIs with three different molecular weights and SWNTs conjugation. The lowest molecular weight PEI used (1.8 kDa) yielded conjugates with the greatest enhancement of transfection efficiency relative to the corresponding free PEI. It has previously been reported [33] that PEI molecules of molecular weight 1.8 kDa and lower can be coupled to SWNTs in larger numbers than can higher molecular weight PEIs, for which the PEI/SWNT ratio is relatively constant. Inaccessibility of some NH2 groups on higher molecular weight PEIs conjugated to SWNT may account for their lower enhancement of transfection efficiency relative to free PEI of the same molecular weight.

Acknowledgments

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