EFFECT OF POLYMER MOLECULAR WEIGHT ON THE DNA/PEI POLYPLEXES PROPERTIES

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Abstract. Cationic polymers have been used to condense DNA by electrostatic interaction into small particles (polyplexes), for protecting the DNA from degradation and enhancing its uptake *via* endocytosis. Polyethylenimine (PEI) is one of the most advanced delivery systems that can condense DNA efficiently forming PEI/DNA complexes. The effect of PEI molecular weight (2k, 5k and 25k) on the physicochemical and biological properties of the polyplexes was investigated. As the molecular weight of PEI increased, the condensation ability, surface charge increased while complexes size decreased. PEI 25k has the lowest buffer capacity compared to 2k and 5k PEI. Transfection efficiency of examined polyplexes was higher in MCF7 cells than in HeLa cells. 25k PEI formed smaller polyplexes and achieved higher transfection efficiencies (into two cell lines HeLa and MCF7) than 2k PEI and 5k PEI. Attachment of different hydrophobic amino acid residues and suitable targeting ligands onto the surface of 25k PEI will increase its transfection efficiency.

Key Words: Polyethylenimine; DNA; characterization; cell lines; zeta potential; transfection; molecular weight.

INTRODUCTION

Gene therapy has become the research focus for many laboratories in pharmacy, medicine, biochemistry and chemical engineering worldwide. However, the growing potential of gene therapy for both genetically based and infectious diseases will not achieve its goals until the issue of gene delivery will be resolved.

Gene therapy vehicles can be categorized into two groups: viral and non-viral systems. Viral carriers were naturally evolved to infect cells and transfer their genetic materials into the host cells. The acute immune response, immunogenicity,

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and insertion mutagenesis, uncovered in gene therapy clinical trials, have raised serious safety concerns about some commonly used viral vectors. The limitation in the size of the transgene that recombinant viruses can carry and issues related to the production of viral vectors present additional practical challenges [7].

Non-viral delivery systems can potentially have considerable advantage over viral counterparts because of the greater control of their molecular composition for simplified manufacturing and analysis, flexibility in the size of the transgene to be delivered, and relatively lower immunogenicity [10]. Unfortunately, the efficiency of gene delivery by the non-viral systems is less than that of the viral counterparts. In addition, physical properties such as size and zeta potential can play a critical role in their efficiency. In both delivery systems, selected modifications that can produce safe, efficient and targetable gene carriers are desirable [5].

The use of cationic polymers confers several advantages, due to the fact that they are durable, inexpensive, easy to prepare, purify and chemically modify, as well as of their enormous stability [13]. Polyplexes are formed by electrostatic interactions between negatively-charged plasmid DNA and a cationic polymer. DNA condensation by cationic polymers is an essential step for non-viral gene delivery since DNA itself is almost immobile in extracellular matrix.

Polyethylenimine (PEI) is a stable, easy to handle, inexpensive cationic polymer [8]. It has gained significant interest as non-viral gene delivery systems through condensation of DNA into compact particles, uptake into the cells, release from the endosomal compartment into the cytoplasm, and uptake of the DNA into the nucleus [11].

PEI-mediated gene delivery is based on the electrostatic interactions of the polycation with the negatively charged phosphate groups of DNA. DNA condensation is therefore a function of the cation-to-anion ratio, e.g. the PEI nitrogen-to-DNA phosphate (N/P) ratio. Condensation protects the DNA from degradation by nucleases, and the compact particles can be taken up by cells via natural processes such as adsorptive endocytosis, pinocytosis and phagocytosis [6]. The complexation and condensation behaviour is dependent on several polymer characteristics, such as molecular weight, number and the density of charges, in addition to the composition of the complexes, e.g. the ratio of polymer to DNA.

The physicochemical properties of different molecular weights of PEI, such as condensation ability, size, surface charges and finally the stability of the PEI-DNA complexes may be important factors to obtain a higher transfection efficiency of the polycation vectors. The aim of the present study was to correlate the effect of cationic polymer molecular weight on the physicochemical and biological properties of the polyplexes.

MATERIALS AND METHODS

MATERIALS

Low-molecular-weight, (2 kDa, 5 kDa) branched PEI is obtained from Lupasol G100, BASF, Ludwigshafen, (Germany). 25 kDa branched PEI, FCS were purchased from Sigma–Aldrich (Madrid, Spain). The Bradford protein assay was obtained from Coomassie Brilliant Blue R250, Sigma-Aldrich. Salmon sperm DNA was from MP Biomedicals (llc, France), RPMI 1640 medium with L-Glutamine was from Gibco, Invitrogen corporation, UK. Penicillin and streptomycin were obtained from Fluid Bioscience International, USA. Trypsin and EDTA were purchased from Biowest, South America. pSVβgal was from Promega (Madison, WI). ONPG (o-nitrophenyl-beta-D-galactopyranoside) was from Sigma-Aldrich. BSA (Bovine serum albumin) was obtained from AMRESCO chemicals, USA. All other reagents are of analytical reagent grade and the doubly distilled water is used all along.

PREPARATION OF PEI/DNA COMPLEXES

Polyplexes formation was performed according to Boussif procedure [2] as follows: DNA/PEI complexes were prepared at different N/P ratios. The N/P ratios were calculated based on PEI nitrogen per nucleic acid phosphate (1 ug of DNA is 3 nmol of phosphate, and 1 ul of PEI stock solution contains 10 nmol of amine nitrogen). Appropriate amounts of DNA and polymer solution were mixed and vortexed. The resulting polyplexes were incubated for 30 min at room temperature before use.

TRANSMISSION ELECTRON MICROSCOPE

PEI/DNA complexes at N/P 10 of different PEI molecular weights were analyzed on negative stain electron microscopy using a JEM 1230 electron microscope (Jeol LTD, Tokyo, Japan). A drop of PEI/DNA complex suspension in 0.1 N NaCl was applied to copper coated with carbon grid, the excess was drawn off with filter paper; an aqueous solution of 1% ammonium molybdate was used as a negative staining agent; wait for 2 min at room temperature; remove the excess solution with a filter paper; then examine under the electron microscope. The PEI/DNA complex size was estimated with the Gatan software provided with the instrument.

ZETA POTENTIAL MEASUREMENTS

Hundred μg DNA was complexed with the appropriate amount of polymer in 10 mL 0.15 M NaCl giving N/P = 10 polyplex. Zeta-potential measurements were carried out in the standard capillary electrophoresis cell of the Zetasizer 2000 HS (Malvern, UK) at 25ºC. Average values were calculated with the data from four runs.

DISSOCIATION OF THE COMPLEXES IN NaCl SOLUTION

Dissociation of polyplexes in NaCl solution was evaluated by measuring the concentration of DNA released from the precipitated PEI/DNA complexes using UV absorption spectroscopy [8]. Absorbance of DNA at λ = 260 nm was used for analysis. The measurement was organized as follows: (i) a set of vials with DNA (30 μg/mL) solutions with various contents of NaCl was prepared, (ii) polycation stock solution was added to each vial to prepare complexes at N/P = 1.0, (iii) the solution was allowed to equilibrate overnight, (iv) the precipitate was removed by centrifuging (at 5000 rpm for 20 min) with microcentrifuge (Thermo Scientific, IEC CL31R multispeed centrifuge, USA) and the absorbance of DNA in supernatant solution was measured. The NaCl concentration corresponding to the 50% release of DNA from complexes was chosen as a characteristic of the complex stability [17].

COAGULATION KINETICS OF COMPLEXES IN NaCl SOLUTION

The kinetics of coagulation of the PEI/DNA complexes was investigated by measuring the turbidity (λ_{310}) in 0.15 M NaCl solutions. The complexes were prepared at N/P 1.5 in water. Then the ionic strength of solution was increased up to 0.15 M NaCl by fast addition of 4 M NaCl stock solution. The measurements of the complexes solution turbidity were started immediately after the addition of NaCl [17].

DNA CONDENSATION ASSAY

The ability of the polymers to condense DNA was determined by measuring the change in Ethidium Bromide (EB)/DNA fluorescence after the addition of the polymer forming the polyplexes. Polyplexes formation is known to be accompanied by a loss of fluorescence ($\lambda_{excitation}$ = 490 nm, $\lambda_{emission}$ = 590 nm) [4]. A sample containing 5 μg/mL of DNA and 0.8 μg/mL of EB in 150 mM NaCl was used to calibrate the machine to 100% fluorescence against background fluorescence of 0.8 μg/mL of EtBr in a Perkin Elmer fluorescence luminescence spectrometer LS 55 (UK). The polymer was added in a stepwise fashion (2 μL/step) then the solution was vortexed. Then the sample was allowed to stabilize and after 2 min the relative fluorescence was measured [4].

CELL CULTURE

HeLa (human cervical carcinoma) and MCF7 (human breast cancer) cell lines were cultured according to standard protocols. The cells were seeded at a density of (1×10^5–10^6) on 6-well plates. Shortly, cells were maintained in RPMI 1640 supplemented with 10% FCS, 1% penicillin/ streptomycin at 37ºC in a

humidified incubator with 5% CO_2 atmosphere. Cells were split using Trypsin/ EDTA when confluent.

IN VITRO TRANSFECTION WITH β-GAL PLASMID

The transfection efficiencies of different molecular weight PEIs (2k, 5k and 25k) were examined by transiently transfecting HeLa and MCF-7 cell lines with pSV-β-galactosidase (β-gal) expression plasmid. The PEI transfection was performed as follows: immediately prior to transfection, the growth medium was substituted with serum free RPMI 1640 (1 mL per well). Then, PEI/DNA transfection mixtures at N/P 10 (containing 2.5 μg of β-gal plasmid) prepared as discussed above then were pipette dropwise to the cell cultures. Cells were exposed to transfection agents for six hours, after which the cells washed with saline solution then 2 mL complete medium was added to the cells. After 48 hours, the β-gal activity was determined.

β-GALACTOSIDASE ASSAY

The β-galactosidase activity was determined using ONPG (o-nitrophenyl-beta-D-galactopyranoside) solution (0.1g ONPG, 0.0585 g Na_2HPO_4 and 0.065 NaH_2PO_4 in 50 mL of distilled water adjusted to pH 7.8. Before use, β-Mercaptoethanol was added to the ONPG solution to a final concentration of 0.046 M. Cells in 6 well plates were lysed with 400 μL lysis buffer (10 mM Tris, 150 mM NaCl, 50 mM NaF, 1 mM Na_3VO_4 and 0.5% NP-40 after adjusting pH to 7.6 in 500 mL). Plates were vortexed briefly then incubated on ice for 10 minutes. The lysing vortex-incubation cycle was repeated 3 times. The lysate has transferred to microfuge tube. 30 μL of lysate was plated in 96-well plates then 180 μL of the ONPG solution was added. During the incubation, the β-galactosidase enzyme cleaves the β-bond from ONPG resulting in the formation of the yellow o-nitrophenol molecule. Plates were incubated at 37 ºC till the development of an appropriate yellow color. The intensity of the β-galactosidase activity was measured by an ELISA reader at a wavelength of 405 nm (SunRise Remote Touch Screen, TECAN, Austria). β galactosidase activity units were standardized for protein concentration determined by the Bradford protein assay using bovine serum albumin standards.

RESULTS AND DISCUSSION

ELECTRON MICROSCOPE OF PEI/DNA POLYPLEXES

In order to study the influence of the PEI molecular weight on the PEI/DNA polyplexes shape and size, electron micrographs of PEI/DNA complexes (N/P 10)

were shown in Figure 1. The three examined PEI formed complex particles appeared in the majority of micrographs as spherical structures. Figure 2 shows that PEI 25k has formed compact complex particles with DNA of about 55 nm in diameter. In contrast, PEI 2k formed relatively large complexes of about 228 nm in diameter while PEI 5k formed complexes with a mean diameter of 174 nm.

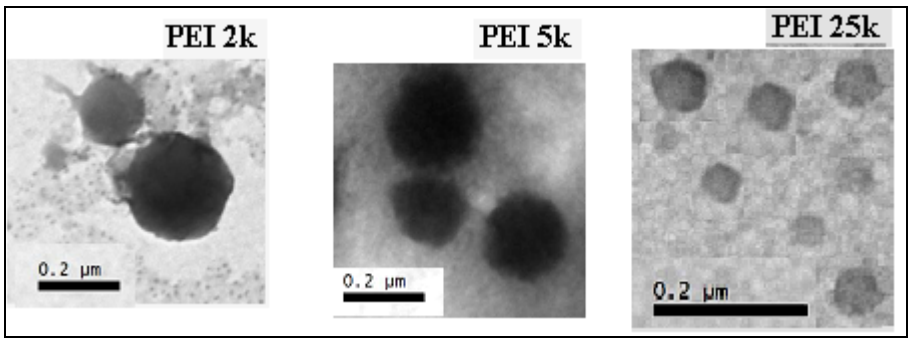

Fig. 1. Negative stain electron micrograph of PEI/DNA complexes (N/P 10) of different molecular weights.

An increase in the molecular weight of the PEI results in a decrease in complex size. This can confer protection against degradation in the extracellular environment, and improved cell uptake by electrostatic interactions of the polycation with the negatively charged cell surface. Cationic polymers can be combined with DNA to form a particulate complex, and can condense DNA molecules to a relatively small size. This can be crucial for gene transfer, as small particle size may be favourable for improving transfection efficacy, particularly *in vivo*.

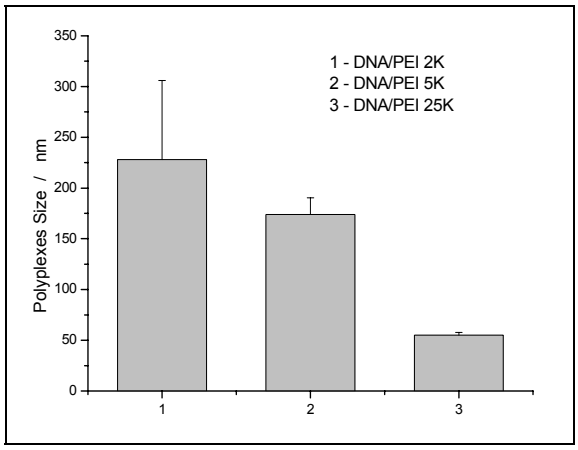

Fig. 2. Size measurements of different DNA/PEI polyplexes (n = 5).

ZETA POTENTIAL OF PEI/DNA POLYPLEXES

Transfection complex formation is based on the interaction of the positively charged polycations with the negatively charged phosphate groups of the nucleic acid. The size and surface charge density of transfection complexes can be related to the transfection efficiency of a reagent. The information of the overall charge of transfection complexes by zeta-potential measurements can be speeded up by the development of better non-viral DNA delivery vectors for gene therapy [14]. Values of the zeta-potential of cationic polymer indirectly reflect polyplex surface net charge and can therefore be used to evaluate the extent of interaction of the polymer cationic charges with the DNA.

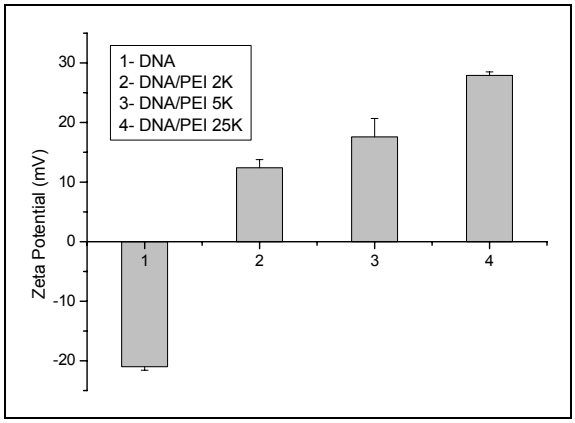

Fig. 3. Zeta potential of different DNA/PEI polyplexes (n = 5).

A comparison between the zeta potential of different molecular weight PEI formed polyplexes was presented in Figure 3. As the PEI molecular weight increased, the surface charge of the PEI/DNA complexes increased and their sizes decreased. The zeta potential of DNA was found to be around –21±0.6 mV. The zeta potentials for DNA/PEI (2k, 5k and 25k) polyplexes were 12.4±1.4 mV, 17.6±3.1 mV and 27.9±0.6 mV, respectively.

PEI 25k has the highly positively surface charged complexes with DNA. It is considered the more stable and the most probable PEI to be uptaken by the cells during the gene transfection process

DISSOCIATION OF PEI/DNA COMPLEXES IN NaCl SOLUTIONS

The stability of PEI/DNA complexes in the body fluids during transport as well as their ability to release DNA in the cytoplasm or nucleus of the target cell are prerequisites for their effective *in vivo* transfection. So, we studied the stability of the complexes in NaCl solutions.

When PEI/DNA complexes were prepared in NaCl solutions of increasing salt concentration (ionic strength), the charges on PEI and DNA macromolecules of the complexes are screened, cooperative binding becomes weaker and dissociation of the complexes occurs [16]. The DNA release was estimated spectrophotometrically using the procedure described in the methods (Fig. 4).

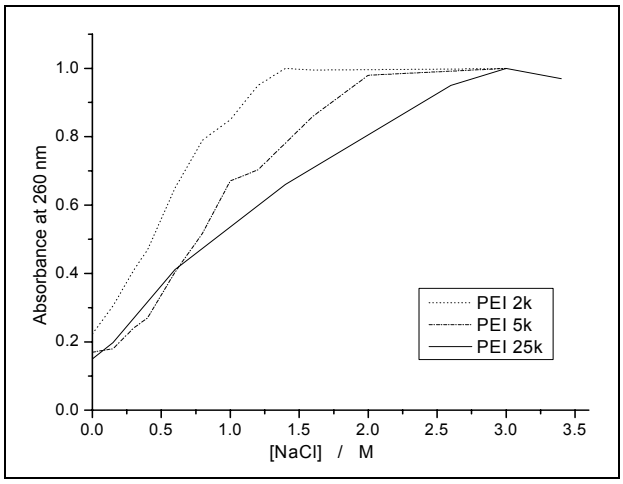

Fig. 4. Dissociation of PEI/DNA complexes in NaCl solutions.

There were two values of C^{50} and C^{100} that can be used to describe the dissociation stability characteristics of the investigated complexes. C^{100} represents NaCl concentration at which complete dissociation of the complex occurred and 100% of DNA is released. 50% of DNA is released from the complex at NaCl concentration, called C^{50} value. The results of the three examined PEIs in the preparation of PEI/DNA complexes were summarized in Table 1. It was found that the molecular weight increased, as C^{50} and C^{100} increased. So, the higher the polycation molecular weight, the higher the stability of polyplexes to dissociate in NaCl solutions.

Table 1

pKa values determined by potentiometric titration with HCl

Polymer Average M.W.	C^{50} (M)	C^{100} (M)	pKa
PEI 2k	0.441	1.3	8.6
PEI 5k	0.46	1.4	7.8
PEI 25k	0.91	1.8	7.11

The proton ability of PEI is an important property for its success as a gene delivery vehicle. A useful descriptor of PEI's protonability is its pKa, which has remained elusive because rigorous analysis of experimental data to obtain PEI's pKa would require one to include an ionization constant for every amine group [8].

Buffer capacity can be defined as "maximum amount of either strong acid or strong base that can be added before a significant change in the pH will occur" [20]. It is a measure of protection of a buffer against changes in pH. Cationic polymers like PEI are assumed to induce facilitated endosomal escape due to the uptake of protons by the basic amino groups when the pH in the endosomes decreases from 7.4 to 5.1. This buffering effect causes an increase in osmotic pressure in the endosome, leading to the disruption of the endosomal membrane to facilitate polyplex transport into the cytoplasm (proton sponge mechanism) [2]. Therefore, the buffer capacity of the polymers, i.e. the capacity of reversible binding of protons in this pH range, may be a relevant parameter for the endosomal escaping capability of their polyplexes [12].

The pKa of PEI in this pH range decreased with increasing the molecular weight: pKa ~8.6 for 2k PEI, 7.8 for branched 5k PEI, and 7.11 for 25k PEI. This large buffering capacity above pH 7 would appear to be due to the secondary amines that are present in all PEIs [12, 13]. The results indicated that PEI 25k has the lowest buffer capacity 2k and 5k PEI. Forrest *et al.* [6] also proved that proper reduction of PEI buffering capacity can effectively enhance *in vitro* gene delivery activity.

KINETICS OF COAGULATION OF PEI/DNA COMPLEXES

It is well known that DNA bases have a strong optical absorption in the ultraviolet region (~260 nm) which is related to the base of the DNA [1]. It was shown from Figure 5 that the turbidity of the PEI/DNA complexes was increased with time and the optical density of the complexes at the range > 300 nm increased with time which may be due to the increase in the scattering caused by DNA condensation and the appearance of aggregates in the solution [18]. Positively charged polyplexes showed a tendency to aggregate as a function of incubation time. Aggregation was also dependent on parameters such as surface charge and ionic strength of the medium [6].

So, a comparison between the aggregation behaviors of different PEI M.W. complexes was made by comparing their absorbance change at λ_{310nm} with time. The wavelength of 310 nm was chosen because it is beyond the absorption range of DNA and polymer [18]. Figure 5 showed the change of turbidity of the PEI/DNA complexes with time for three different PEI/DNA complexes in saline solution.

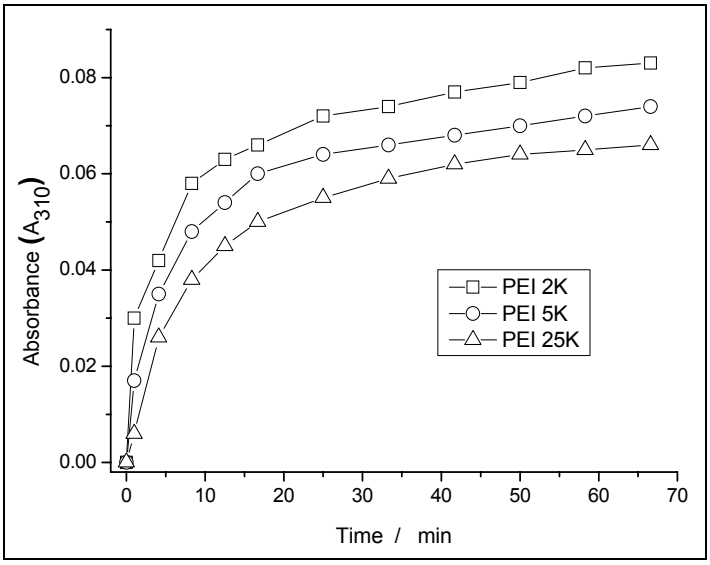

Fig. 5. The time dependence of absorbance (A_{310}) of the PEI/DNA complexes for different M.W. PEI in 0.1 M NaCl.

The kinetics of PEI/DNA complex coagulation was dependent on the molecular weight of the PEI used for complex formation, where The PEI/DNA complexes prepared from 25k PEI (the highest M.W.) showed the slowest coagulation rate. As the used M.W. PEI decreased, the turbidity of the complexes increases, so the aggregated complex amount increased. All the complexes stabilized after 50 min.

The coagulation of PEI/DNA complexes may be explained as follows: non-stoichiometric polycation/DNA complexes prepared in water with an excess of polycation (N/P 1) possess positive surface charges preventing the complexes from aggregation. After addition of NaCl to a final concentration of 0.15 mol/l, positive surface charges are successively screened, the complexes become more hydrophobic and start to coagulate due to the hydrophobic interactions as well as van der Waals forces.

DNA CONDENSATION

The ability of different molecular weight PEI to condense DNA was quantified by measuring the fluorescence intensity emitted upon addition of polymer to EB-DNA complex. The fluorescent light could be quenched by the addition of polymer which competed with EB to bind DNA [22]. This was a proof that the polymer intercalates to the base pairs of DNA.

The results were provided as relative fluorescence intensity, where a value of 100% denoted the fluorescence intensity for EB-DNA without polymer. The relative fluorescence intensity decreased rapidly with increasing N/P ratio due to the inaccessibility of DNA for EB when complexed with the PEIs (Fig. 6).

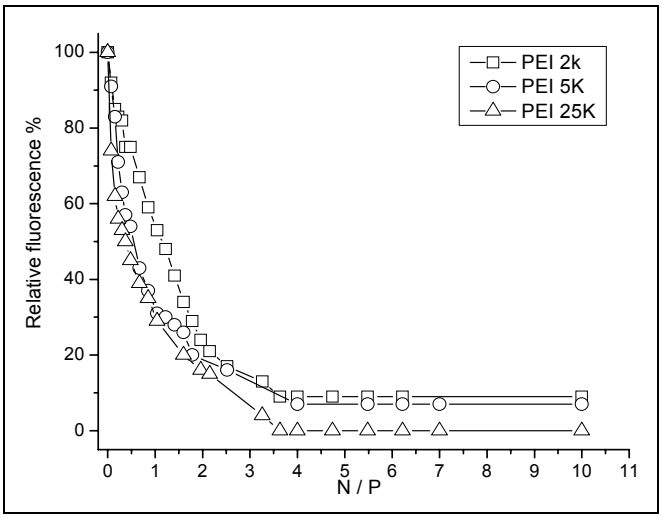

Fig. 6. The relative fluorescence intensity of the DNA-EB after the addition of the PEI polymer.

Although all the three different M.W. of PEI were able to condense the DNA as shown by the decreasing relative fluorescence intensity, the efficiency by which different M.W. PEI polymers condensed the DNA was different.

The condensation efficiency can be estimated from three parameters: (1) the amount of positive charge of polycation required to reach the IC_{50} value, which can be defined as the positive to negative ratio (+/–) of polycation to DNA at which 50% of the initial fluorescence is quenched; (2) the residual (F_{RES}) value of fluorescence indicating the extent of condensation; and (3) the charge ratio (R_{min}) at which the (F_{RES}) occurs [20]. The values of IC_{50}, F_{RES} and R_{min} measured from the relative fluorescence intensity curve (Fig. 3) for the different molecular weight PEI were shown in Table 2.

Table 2

Condensation parameters of PEI/DNA by different PEI M.W.

Polymer Average M.W.	IC_{50}	F_{RES}	R_{min}
PEI 2k	1.1	9%	3.64
PEI 5k	0.55	7%	3.4
PEI 25k	0.37	Zero	3.1

At N/P ≥ 3.64, all polymers almost completely suppressed fluorescence. However, at lower N/P ratios, 25k PEI was able to condense plasmid DNA better than 2k and 5k PEI because the IC_{50} of approximately 0.37, 0.55 and 1.1 was determined for 25 k PEI, 5k PEI and 2k PEI, respectively (Table 2). So, the most efficient PEI/DNA complex was PEI 25k, which exhibited the lowest F_{RES}, R_{min} and IC_{50} values. PEI 5k, however, was efficient for condensation, but slightly higher R_{min} and IC_{50} values than PEI 25k and lower R_{min}. 2k PEI has relatively large IC_{50} and F_{RES} values. These results suggested that reduced DNA condensation efficiency of 2k and 5k PEI, when compared with 25k PEI at low N/P ratios, seemed to be a consequence of its lower molecular weight. Polycations which condense DNA at lower charge ratios form polyplexes which are more resistant to disruption by polyanions and more stable against nuclease degradation and can be easily uptaken by cells.

TRANSFECTION EFFICIENCY

PEI/DNA complexes of 2k, 5k and 25k PEI at N/P 10 (for example) were chosen to be tested comparatively for their transfection efficiency in HeLa and MCF7 cell lines. Polyplexes were prepared with β-gal plasmid DNA. Cell extracts of transfected cells were measured for β-galactosidase activity with a spectrophotometric assay after 48 hours of the transfection experiment. The internalization of polyplexes resulting in successful transfection appears to depend on the cell type and the kind of polymer used. Branched PEI polyplexes appear to mediate transfection via both the lipid-raft-dependent and clathrin-dependent pathways. In HeLa cells, however, both the lipid-raft-dependent and clathrin-dependent pathways are involved, with the former being more pronounced [21]. Figure 7 shows the relative β-galactosidase activity of PEI/DNA complex with different PEI molecular weights. In general β galactosidase activity in MCF7 cells was higher than that in HeLa cells which suggested a higher transfection efficiency in MCF7 cells. These data verify the dependence of DNA/PEI polyplexes cellular uptake on the type of cells. The molecular weight of used PEI polymer increased, as the transfection efficiency increased in both cell lines.

A decrease in the complex size from 228 nm (PEI 2k) to 55 nm (PEI 25k) may lead to an increase in the level of β galactosidase activity from 2151 mU/mg protein (PEI 2k) to 4034 mU/mg protein (PEI 25k) with MCF7 cells because the endocytosis is more efficient with particles < 150–200 nm and the velocity of cytoplasmic movement was found to be a function of particle size [16]. Likewise, Zeta potential values of PEI/DNA complexes were 12.4, 17.6 and 27.9 mV for the 2k, 5k and 25k PEI polymer complexes showed a higher level of transfection efficiency as seen in Figure 7. A higher value of zeta potential and smaller complex size could be relevant to the increase in the transfection efficiency.

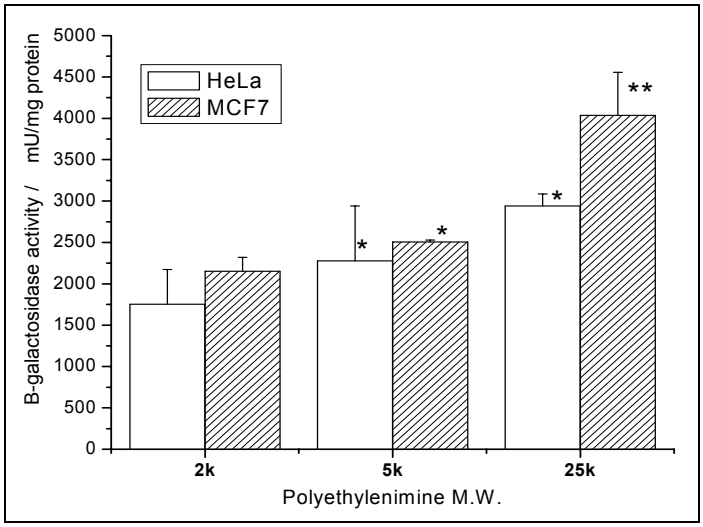

Fig. 7. β galactosidase activity of the PEI/DNA complex with HeLa and MCF7 cell lines. The β gal concentrations were normalized to protein concentration of each sample (*: $P < 0.05$ and **: $P < 0.01$ with Anova Test)

At the cell surface, the rate of entry into cells varied with cell type. After cellular uptake of the gene delivery systems by endocytosis, the endosomal release was another critical barrier which affected the efficiency of gene transfer since most DNA was retained in the endosomes and eventually degraded or inactivated by lysosomal enzymes. A number of strategies have been developed to enhance endosomal release. One involves using gene delivery systems with high buffer capacity known as "proton sponge" presumably able to reduce the acidification of the endosome resulting in swelling and membrane rupture [9].

Following the release from the endosome, the transgene traffics toward the nucleus through the cytoplasm where it could be exposed to degradation by cytosolic nucleases. The transfection efficiency of the DNA might also be reduced by the inability to dissociate from the delivery vector as only free DNA could be transcribed in the nucleus.

CONCLUSION

As the molecular weight of PEI increased the condensation ability, surface charge increased while complexes size decreased. 25k PEI formed compact, spherical, smaller and stable DNA/PEI polyplexes and achieved higher transfection efficiencies (into two cell lines HeLa and MCF7), than 2k PEI and 5k PEI.

Our objective was to provide a framework for the future design and synthesis of optimal non-viral vectors for gene therapy and disease control. A better

understanding of physicochemical mechanisms, polyplexes structure and transport across cellular membranes should facilitate advances toward the rationalization and conception of new vectors. Complete condensation of DNA protects against degradation in the extracellular environment, improves cell binding by electrostatic interactions of the polycation with the negatively charged cell surface, and improves endocytosis. Attachment of different hydrophobic amino acid residues and targeting ligands onto the surface of 25k PEI which will increase its transfection efficiency will be the subjects of our future investigations.

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