Plasmid DNA delivery into hepatocytes using a multifunctional nanocarrier based on sugar-conjugated polyethylenimine

Research Article

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Summary

In the present study, the impact of concomitant alkylcarboxylation and sugar conjugation of high toxicity 25 kDa polyethylenimine (PEI) on the plasmid DNA delivery effectiveness into HepG2 cell line has been investigated. The abundance of asialoglycoprotein receptors in liver parenchymal cells facilitates the feasibility of polyplex delivery to the liver; therefore liver is one of the most important organs for gene therapy. This multifunctional gene carrier is composed of sugar-conjugated alkylcarboxylate PEI in which PEI interact electrostatically with plasmid DNA and condense it. Alkylcarboxylate chains improve the hydrophilic-hydrophobic balance of the polycation and galactose residues serve as a cell binding ligand for hepatocytes. PEI was alkylcarboxylated by reaction with either 2-bromoacetic or 6-bromohexanoic acids followed by grafting to dextran or galactose. Plasmid DNA condensation results showed that the galactosylated conjugates were able to condense plasmid DNA more efficiently than the dextran-grafted polymers. Conjugation of galactose and dextran onto alkylcarboxylated PEI resulted in nanoparticles smaller than 200 nm with lower zeta potential than the parent polymer. In HepG2 cells, the highest transfection efficiency was obtained with polypelexes formed with the galactose-conjugated hexylcarboxylate PEI which induced up to a 4.8 enhancement in transfection efficiency at the C/P ratio of 6 compared to unmodified PEI. Therefore, it is expected that galactose conjugation combined with alkylcarboxylation of PEI yield a safe and efficient hepatocyte targeting gene carrier.
I. Introduction

The cell membranes are not permeable to several macromolecules such as nucleic acids, therefore carriers are needed to transfer genetic materials across the membranes. Delivery of nucleic acids to cells can be achieved using different carriers including viral or non-viral vectors (El-Aneed, 2004; Hatefi and Canine, 2009). A potent non-viral gene carrier which exhibits high transfection efficiency is polyethylenimine (PEI) (Abdallah et al., 1996; Boussif et al., 1995). However, cytotoxic effects and lower nucleic acid transfer ability of PEI compared to viral systems still hamper its widespread applications in vitro and in vivo. (Boussif et al., 1995; Godbey et al., 1999a; Moghimi et al., 2005; Parhamifar et al., 2010). Both transfection efficiency and cytotoxicity seem to depend on physicochemical properties such as molecular weight, amine content and positive charge density of the polymer (Demeneix and Behr, 2005; Godbey et al., 1999b; Merlin et al., 2002; Zintchenko et al., 2008). High positive charge density on the polymer leads to electrostatic interaction of PEI with negatively charged components of cell membrane and causes membrane disruption and damage (Hunter, 2006). Different strategies have been employed to reduce PEI cytotoxicity such as PEGylation (Kichter, 2004), ligand conjugation (Ogris et al., 2003), hydrophobic modification (Liu et al., 2010) and introduction of negatively charged groups into the polymer backbone (Zintchenko et al., 2008). Hydrophobic modifications and grafting of negatively charged moieties result not only in less PEI cytotoxicity but also more efficient gene carriers by improving the hydrophilic-hydrophobic balance of the polymer (Forrest et al., 2004; Gabrielson and Daniel, 2006; Nimesh et al., 2007; Oskuee et al., 2009; Thomas and Klibanov, 2002). These modifications alter the interactions of the polyplexes with cell membranes as well as the interaction between polymer and nucleic acid (Forrest et al., 2004; Gabrielson and Daniel, 2006; Liu et al., 2010; Nimesh et al., 2007; Oskuee et al., 2009; Thomas and Klibanov, 2002). On the other hand, several different approaches have been tested in order to alter the non-specific electrostatic polyplex–cell surface interaction with the specific mechanism of receptor-mediated cellular uptake by incorporating cell-binding ligands into the polymer structure. It has been shown that these “shielded” polyplexes remained soluble and small in size, and their susceptibility to salt induced aggregation was drastically reduced (Cristiano and Roth, 1996; Ferkol et al., 1995; Sosnowski et al., 1996; Wagner et al., 1994). In the present investigation, we studied the effect of hydrophobic modification, introduction of negatively charged groups and grafting of sugar moieties in gene transfection efficiency of PEI concomitantly. The approach used was to react PEI with either 2-bromoamoeic or 6-bromohexanoic acids to impart hydrophobic nature to the polymer as well as introduction of negatively charged carboxylate groups into the polyion. Following the alkylcarboxylation, sugar moieties, dextran or galactose, were grafted onto the alkylcarboxylate derivatives of PEI to improve the biophysical properties of polyplexes and direct them into a hepatic cell line, respectively. To address the effectiveness of these new conjugates as hepatocyte gene delivery vector, we studied the condensation ability, particle size, zeta potential and cytotoxicity, as well as their transfection efficiencies in HepG2 cell line expressing asialoglycoprotein receptor (ASGP-R) and on Neuro2a cells lacking it.

II. Materials and Methods

A. Materials

Branched polyethylenimine (PEI; average MW 25 kDa), bromoacetic acid, 6-bromohexanoic acid, lactose, sodium cyanoborohydrate, N-[2-hydroxyethyl] piperazine-N’-[2-ethanesulfonic acid] (HEPES) and 2,4,6-trinitrobenzenesulfonic acid (TNBS) were purchased from Sigma-Aldrich (Munich, Germany). Dextran (MW50000) was purchased from Fluka (Munich, Germany). Plasmid pRL-CMV-luc (Renilla luciferase under control of the cytomegalovirus (CMV) enhancer/promoter), luciferase assay kit and CellTiter 96® AQueous non-radioactive cell proliferation assay (MTS) were purchased from Promega (Madison, WI, USA). Ethidium bromide was purchased from Cinnagen (Tehran, Iran). All solvents and chemicals were purchased from Sigma-Aldrich (Munich, Germany) and were of the highest purity available. Dialyses were carried out using Spectra/Por dialysis membranes (Spectrum Laboratories, Houston, TX USA).

B. Synthesis of modified PEI

The approach used to modify 25 kDa PEI was to alkylate PEI by reacting it with o-bromoalkylcarboxylic acids including 1-bromoamoeic and 6-bromohexanoic acids followed by grafting of either dextran or galactose onto PEI amine groups. Synthesis of alkylcarboxylic derivatives of PEI was carried out as described previously (Oskuee et al., 2009). Briefly, to achieve 10% of PEI amine substitution which showed the highest efficiency in the previous study, the o-bromoalkylcarboxylic acids (1-bromoamoeic acid and 6-bromohexanoic acid) were dissolved in dimethyformamide, and added dropwise over a period of 2 h to a vigorously stirred solution of PEI in the same solvent. The reactions were allowed to proceed at room temperature for 24 h. The resulting solutions were poured into a dialysis membrane (15000 cut-off, Spectra/Por membrane) and dialyzed against 150 mM NaCl for 1 day and then doubly distilled water (DDW) for 2 days to remove unreacted alkylcarboxylates. After the dialysis, the aqueous solution was lyophilized to yield fluffy powders that were characterized by 1H-NMR (DZO) spectroscopy using a Bruker Avance DRX-500 MHz NMR spectrometer (Bruker, Ettlingen, Germany).

Galactose and dextran grafting onto modified PEI amine groups were carried out as described elsewhere (Kunath et al., 2003; Zanta et al., 1997). The initial feed of sugar was set to result in 10 mole% of amine coupling in the final product. The desired amount of lactose or dextran was mixed with alkylcarboxylate derivatives of PEI plus 3 fold molar excess of sodium cyanoborohydrate based on lactose or dextran in 10 ml of borate buffer (100 mM, pH 8.0). The reaction mixture was incubated at 45 °C for 48 h with constant stirring. The sugar-grafted PEIs were lyophilized after the removal of sodium cyanoborohydrate and unreacted lactose or dextran by dialysis against 0.1 M borate pH 7.5/1.0 M sodium chloride using Spectra/Por membrane (MWCO=15000). Finally, buffer was exchanged with distilled water and the final products were characterized by 1H-NMR (DZO) spectroscopy.
C. Polymer/plasmid DNA complex formation

In all experiments, the composition of polymer/pDNA complexes was characterized by the weight/weight (C/P or w/w) ratio of the polymer to the plasmid DNA in the mixture. Different concentrations of the nucleic acid and PEI formulations were diluted at various polymer/nucleic acid mixing ratios (w/w) in separate tubes in HBG (HEPES buffered glucose solution; 20 mM HEPES, 5% glucose, pH 7). Then the solutions were mixed and incubated for 30 min at room temperature to form stable complexes.

D. Determination of the degree of grafting

Using the standard method, the amounts of accessible primary amines on alkylcarboxylate and sugar-grafted alkylcarboxylate PEIs were determined by 2,4,6-trinitrobenzenesulfonic acid (TNBS) (Snyder and Sobocinski, 1975). The degrees of grafting were calculated by the differences in the amounts of primary amines on the unmodified PEI and those on the modified-PEI. A resorcinol sulfonic acid micro-method was used to determine the amount of galactose and dextran bound to each derivative of PEI (Monsigny et al., 1988). Briefly, resorcinol solution (20 µl of 6.0 mg/ml in distilled water) and sulfuric acid (100 µl of 75% (w/w)) were added to the wells of a 96-well tray containing 20 µl of sugar-conjugated PEI dissolved in water at 10 mg/ml. Then, 50 µl of 2,6,10,14-tetramethylpentadecane was added to each well and incubation was carried out for 30 min at 90 °C, followed by 30 min incubation at room temperature in the dark. The UV absorbance of the samples was recorded at 410 nm and carbohydrate concentrations were calculated from calibration curves using several standard sucrose concentrations assayed simultaneously. Wells without PEI were used to measure the background absorbance.

E. Measurements of the particle size and zeta-potential of the polymer/pDNA complexes

The mean hydrodynamic particle size and charge measurements for polymer/pDNA complexes were performed using Dynamic Light Scattering (DLS) and Laser Doppler Velocimetry (LDV), respectively, using Malvern Nano ZS (Malvern Instruments, UK) in a salt-free buffer (20 mM HEPES, 5% glucose, pH 7.2). Various amounts of cationic polymers were diluted in 125 µl of buffer and mixed with an equal volume of the same buffer containing DNA. The measurements were carried out in automatic mode and the results are presented as mean ± SEM, n=3. Each mean represents the average value of 30 measurements.

F. Measurement of the DNA condensation by the ethidium bromide (EtBr) exclusion assay and gel retardation assay.

The ability of the modified polymers to condense DNA was studied using an ethidium bromide (EtBr) assay. Intercalation of ethidium bromide between DNA base pairs was determined by fluorescence spectroscopy (excitation: 510 nm, and emission: 590 nm) measured in a Jasco FP-6200 spectrofluorimeter (Tokyo, Japan). A solution of PEI or derivative in HBG buffer was added stepwise to a solution of the plasmid (20 µg/ml) and EtBr (0.4 µg/ml) in HBG buffer, and fluorescence intensity was measured. The fluorescence intensity of the EtBr solution in the presence of free plasmid corresponds to 0% condensation, and the fluorescence intensity without plasmid corresponds to 100% condensation. The decrease of EtBr fluorescence intensity following addition of PEI or a derivative indicates its interaction with the plasmid. All measurements were performed in triplicate and a graph was constructed by plotting the relative fluorescence intensity (%) against the polymer/plasmid DNA ratio (w/w).

In order to carry out the gel retardation assay, polyplexes were prepared as described above. Ten microliter of each polyplex mixed with loading buffer and loaded onto an ethidium bromide (EtBr) containing 1% agarose gel in TBE buffer. The gel was run at 80V for 1h and then photographed under UV light.

G. Cell culture and transfection procedure

Neuro2A murine neuroblastoma cells (ATCC CCL-131, Manassas, VA, USA) and Hep G2 (NCBI C158, Tehran, Iran) were grown in DMEM (1 g/l glucose, 2 mM glutamine) supplemented with 10% FBS, streptomycin at 100 µg/ml and penicillin at 100 U/ml. Cells were seeded at a density of 1 x 10⁴ cells/well in 96-well plates 1 day prior to transfection experiments, and grown in the appropriate medium with 10% fetal bovine serum. Different polymer/plasmid DNA weight ratios (C/P or w/w), were used to prepare the polycation/plasmid complexes (i.e. polyplexes). Polyplexes were prepared by adding 50 µl of a solution of polycation at varying concentrations in HBG to 50 µl of a solution of plasmid DNA (40 µg/ml) in HBG. Transfection was performed by adding 10 µl (equivalent of 200ng pDNA) of polyplex solution to the wells of 96-well plates containing 60–90% confluent cultures of cells in complete medium containing 10% FBS. After 4 h, the medium was replaced with a fresh complete medium and gene expression was assayed 24 h later.

H. Luciferase activity assay

Twenty four hours post transfection, the medium was removed and cells were lysed by adding 50 µl of cell lysis buffer (Promega, Madison, WI). Luciferase activity was measured using the Promega Renilla Luciferase Assay kit and protocol (Madison, WI) and a luminometer (Berthold Detection Systems, Pforzheim, Germany). The results are presented as relative light units (RLU) per number of seeded cells, mean ± s.d., n=3.

I. Cell viability assay

The cell toxicity of modified PEI derivatives complexed with plasmid DNA was evaluated using CellTiter 96® AQueous non-radioactive cell proliferation assay (MTS) (Promega, Madison, WI). Neuro2A and HepG2 cell lines were cultured in 96-well plates at 1 x 10⁴ cells per well for 24 h then treated with the same amounts of polypelexes used for transfection experiments. After 4 h, the medium was removed and replaced with fresh complete growth media and cultured for additional 24 h. MTS reagent (20 µl) was added to each well and incubated for 1.5 h at 37 °C. The absorbance of the media was measured with an ELISA plate reader at 490 nm. The cell viability (%) relative to control wells not treated with polypelexes was calculated by [A] test/ [A] control × 100.
J. Statistical analysis

Values are represented as the means ± standard deviation (SD). Results were compared using the Student's t-test. p value < 0.05 were considered to be statistically significance.

III. Results

A. Synthesis of modified PEIs

To prepare sugar-conjugated alkylcarboxylate PEI, branched 25 kDa PEI primary amines were modified with either 1-bromoacetic or 6-bromohexanoic acids to achieve 10% of substitution with respect to the primary amine content of PEI. Following the alkylcarboxylation, two sugar groups including galactose and dextran, were grafted onto the remaining PEI amines (Scheme 1). The modified polymers were labeled as PEI–X–Y%–Z, in which X represents the number of carbons in the alkyl chain, Y% is the percentage of primary amines substituted with alkylcarboxylate chains and Z represents the sugar (G=Galactose, D=Dextran) coupled to the modified PEI structure. The TNBS assay was used to determine the extent of PEI primary amine group substitution by alkylcarboxylate and sugar moieties. The degrees of substitution obtained by this method showed that the modification degree was lower than the calculated values based on the initial feed of o-bromoalkylcarboxylic acids or sugar groups to PEI. The TNBS assay demonstrated that the substitution degrees of 8% and 7% were achieved for the attachment of 1-bromoacetic acid and 6-bromohexanoic acid, respectively which were lower than the feeds. Based on the quantification of the grafting degrees made by TNBS assay and resorcinol sulfuric acid micro-method, the sugar substitution degrees were found to be approximately 4% (Table 1). Finally, the structure of sugar-grafted alkylcarboxylate derivatives of branched PEI 25 kDa were confirmed by 1H-NMR. The peaks at 1.3-2.1 ppm and 3.6-4.4 ppm were assigned to the protons of alkylcarboxylate chain and galactose respectively, while the PEI protons appeared between 2.5 and 3.2 ppm.

Scheme 1: Synthesis of galactosylated alkylcarboxylate derivatives of PEI.
Table 1:
Degree of primary amine substitution in 25 kDa PEI estimated by TNBS assay and resorcinol sulfuric acid micro-method and size and zeta potential of the plasmid/DNA complexes. Polyplexes formed at the C/P ratio of 6 in HBG buffer.

*The modified polymers were labeled as PEI–X–Y%–Z, in which X represents the number of carbons in the alkyl chain, Y% is the percentage of primary amines substituted with alkylcarboxylate chains and Z represents the sugar (G=Galactose, D=Dextran) coupled to the modified PEI structure.

<table>
<thead>
<tr>
<th>PEI Derivative*</th>
<th>Observed % of primary amines substituted by alkylcarboxylates</th>
<th>Observed % of primary amines substituted by galactose/dextran</th>
<th>Size of plasmid/polymer complex in nm (+SD)</th>
<th>Zeta potential of plasmid/polymer complex in mV (+SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEI-2-8%</td>
<td>8</td>
<td>0</td>
<td>80 (11)</td>
<td>18 (1.2)</td>
</tr>
<tr>
<td>PEI-2-8%-G</td>
<td>8</td>
<td>3.9</td>
<td>94 (8)</td>
<td>15 (0.7)</td>
</tr>
<tr>
<td>PEI-2-8%-D</td>
<td>8</td>
<td>3.8</td>
<td>113 (5)</td>
<td>13.3 (0.3)</td>
</tr>
<tr>
<td>PEI-6-7%</td>
<td>7</td>
<td>0</td>
<td>146 (4)</td>
<td>19.3 (0.6)</td>
</tr>
<tr>
<td>PEI-6-7%-G</td>
<td>7</td>
<td>3.6</td>
<td>168 (9)</td>
<td>17.3 (0.3)</td>
</tr>
<tr>
<td>PEI-6-7%-D</td>
<td>7</td>
<td>3.6</td>
<td>170 (12)</td>
<td>16.7 (0.5)</td>
</tr>
<tr>
<td>25 kDa PEI</td>
<td>0</td>
<td>0</td>
<td>78 (9)</td>
<td>33 (1.1)</td>
</tr>
</tbody>
</table>

B. Particle size and zeta potential measurements
The particle size measured by dynamic light scattering and the zeta potential measurement results are presented in Table 1 for various polyplexes formed at the C/P ratio of 6 which resulted in the highest transfection efficiency in HBG buffer. The results showed that all modified polymers are able to form nanoparticles smaller than 200 nm. All the hexanoate (C6) derivatives of 25 kDa PEI yielded complexes significantly larger than those formed with unmodified PEI \((p<0.05)\). However, the size differences between galactose/dextran-grafted conjugates and the parent alkylcarboxylated derivative were not significant. On the other word, conjugation of 4% of PEI amines by hydrophilic moieties such as dextran or galactose did not lead to larger complexes in HBG buffer. Also, it was found that the zeta potential decreases following conjugation of galactose/dextran to alkylcarboxylate PEI. There was no significant difference in zeta potential between the derivatives with two or six alkyl chain lengths. Addition of alkylcarboxylate chains and sugar moieties to PEI resulted in a decrease in the zeta potential of all conjugates ranging from 33 mV in the case of unmodified 25 kDa PEI to 15 mV and 17.3 mV for PEI-6-7%-G and PEI-2-8%-G, respectively.

C. Plasmid DNA condensation ability of modified polymers
To investigate the ability of modified polymers to condense plasmid DNA into nanosized particles, the ethidium bromide (EtBr) exclusion assay was carried out. As shown in Figure 1A, all modified polymers decreased ethidium bromide fluorescence intensity by approximately 85%. At low carrier to plasmid ratios (e.g., 0.25 or 0.5), the ability of PEI derivatives to condense DNA decreased. By adding more polymer, sufficient positive charge was provided to condense DNA comparable to that of unmodified PEI. Although unmodified 25 kDa PEI was able to fully condense the plasmid at the lowest C/P ratio tested but all the modified polymers could interact efficiently with the plasmid DNA and condense it at C/P ratio of 2 comparable with underivatized PEI. At this carrier to plasmid ratio, no significant difference in the condensation ability of modified polymers and unmodified PEI was observed. In general, the galactosylated conjugates were able to condense plasmid DNA more efficiently than the dextran-grafted polymers. The DNA binding affinity results indicated that complete condensation of plasmid by the polymers containing longer alkyl chains (PEI-6-7%-G and PEI-6-7%-D) required higher concentrations of polymer (i.e. higher w/w ratios). Gel retardation assay confirmed the condensation ability of the modified conjugates at the C/P ratios used in the transfection experiments (Figure 1B). As shown in this figure, PEI-6-7%-G which showed the highest transfection efficiency was able to retard plasmid DNA at the C/P ratios ≥ 2 comparable with unmodified 25 kDa PEI.
Figure 1: Plasmid DNA condensation by 25 kDa PEI and dextran/galactose grafted alkylcarboxylated derivatives measured by (A) ethidium bromide exclusion assay and (B) gel retardation assay. (1) DNA ladder, (2) Naked plasmid DNA, (3) 25 kDa PEI at C/P =0.8, (4) 25 kDa PEI at C/P =2, (5-8) PEI-6-7%-G at C/P ratios of 2, 4, 6 and 8, respectively.
D. In vitro Transfection

The transfection efficiency of sugar-conjugated alkylcarboxylate derivatives of PEI was investigated on HepG2 cell line expressing asialoglycoprotein receptor and on Neuro2a lacking it. The cell lines were transfected with 200 ng of plasmid complexed with varying amount of modified polymer to form different polycation/plasmid (C/P) ratios (w/w) ranging from 4/1, 6/1 and 8/1. Transfection efficiency was measured as luciferase enzyme activity, normalized to 4000 cells (RLU/4000 cells). Branched 25 kDa PEI was used as a positive control at a C/P ratio of 0.8 (N/P=6) which was found to be its optimal gene delivery ability to cytotoxicity ratio (data not shown). Transfection experiments without carrier (plasmid DNA only) resulted in undetectable gene expression in both Neuro2a and HepG2 cell lines. In both cell lines, the efficiency of dextran-grafted polymers were lower than galactosylated ones (Figure 2A and 2B). In the Neuro2a cell line lacking asialoglycoprotein receptor, PEI derivatives did not increased transfection efficiency in any C/P ratios tested (Figure 2A). Galactosylation of alkylcarboxylate derivatives of PEI was found to increase the transfection efficiency in HepG2 cell line significantly (p<0.05). Acetyl-grafted PEI enhanced the transgene expression levels. Nevertheless, the highest transfection efficiency was obtained with polyplexes formed with the hexylcarboxylate-grafted PEI. In the other word, in HepG2 cell line, PEI-2-8%-G and PEI-6-7%-G induced up to a 3.3 and 4.8 enhancement in transfection efficiency at the C/P ratio of 6, respectively, compared to unmodified PEI at the N/P ratio of 6 (p<0.05) (Figure 2B).

E. Cell toxicity assays

The cell viability of modified polymers against Neuro2a and HepG2 cells was carried out using the commercial MTS colorimetric assay. The polyplexes were prepared at the same C/P ratio used in transfection experiments in the presence of serum. Similar to the transfection tests, branched 25 kDa PEI was used as a positive control at a C/P ratio of 0.8 (N/P=6).
Both dextran- and galactose-conjugated polymers did not show increased cytotoxicity on Neuro2a cells at polymer/plasmid ratios of 4, 6 and 8 ($p>0.05$) (Figure 2C). Furthermore, no significant difference was observed in polyplex induced cytotoxicity by the galactose- or dextran-grafted polymers. On HepG2 cell line, all the galactose- and dextran-grafted conjugates did not exhibit increased cytotoxicity as compared with unmodified PEI and the cell viability was around 90% ($p>0.05$). The difference of cell viability between the modified polymers and unmodified 25 kDa PEI was approximately 35% (Figure 2C and 2D). The galactose- conjugated polymers induced more toxicity on Neuro2a cells rather than HepG2 cell line (Figure 2C and D). Overall, the sugar-conjugated PEI derivatives induced less toxicity compared to unmodified PEI on the both cell lines.

IV. Discussion

Receptor mediated gene delivery is a promising approach to transfer genetic material into specific cell types (Hashida et al., 2005). The asialoglycoprotein receptors on the surface of hepatocytes are able to recognize the galactose or N-acetylgalactosamine residues of desialylated glycoproteins (Kunath et al., 2003; Nie et al., 2011; Qin et al., 2011; Zanta et al., 1997). Since the reductive amination provides a remarkably simple route for conjugation of galactose to PEI and remains the unmodified galactose unit following the reaction for further interaction with asialoglycoprotein receptors, we decided to choose this method for creating the targeting vector for hepatocytes. This multifunctional gene carrier is composed of sugar-conjugated alkylcarboxylate PEI in which PEI interact electrostatically with plasmid DNA and condense it, alkylcarboxylate chains improve the hydrophilic-hydrophobic balance of the polyplexation and galactose residues serve as a cell binding ligand for hepatocytes.

To act as efficient gene carrier, polycations must be able to form polyplexes with DNA within the optimal size range and retain enough positive charge on the polyplex surface. Although the modified polymers formed larger polyplexes, this size range allows the nanoparticles to enter cells through endocytosis. Previously, it has been proved that average particle size depends on both the carbon chain length and degree of substitution (Nimesh et al., 2007). It was shown previously that alkylcarboxylation of PEI reduced zeta potential of polyplexes significantly ($p<0.05$) (Oskuee et al., 2009). The decrease in zeta potential is consistent with the hypothesis of charge shielding during complex formation (Kunath et al., 2003). According to this hypothesis, uncharged, hydrophilic galactose or dextran residues will most probably orientate towards the surface of the modified polymers leading to a shielding of positive charges (Kunath et al., 2003).

Conjugation of amines by negatively charged alkylcarboxylate chains may be another reason for reduced zeta potential. Following these modifications, the net charge of the polyplexes is still positive and the remaining positive charge can: (i) facilitate association of polyplexes with the negatively charged cell membranes and (ii) repulse electrostatically the cationic complexes and prevent subsequent aggregation.

Condensation of DNA and nanoparticle formation play a key role in efficiency of polymers to act as gene delivery vectors. In the all modified polymers, the conversion of primary to secondary amines was combined with the introduction of the negatively-charged alkylcarboxylate residues and addition of the hydrophilic dextran/galactose groups which would be expected to reduce the binding affinity of the modified polymers to DNA. The weakest binding affinities were associated with the dextran-grafted polymers. Dextran is a bigger pendant group than galactose. Therefore, weaker binding affinity would be expected for the dextran-conjugated polymers. Furthermore, all the polyplexes were prepared on a weight to weight ratio (C/P) rather than on a nitrogen to phosphorus molar ratio (N/P), thus, a higher molecular weight of the dextran-grafted polymers in comparison with the galactose-grafted ones could be the reason for less complete condensation at lower C/P ratios (i.e. C/P ratio of 0.25-1.5). The weaker DNA binding affinity of the polymers containing longer alkyl chains (PEI-6-7%-G and PEI-6-7%-D) could be explained by this fact that the longer alkyl chains may interfere with the interaction of the positive charged core of the modified PEIs with negative backbone of the plasmid DNA. Previously, it has been reported that acetylation of PEI led to looser or less tightly condensed polyplexes than those formed with unmodified PEI at low w/w ratios (Forrest et al., 2004; Gabrielson and Daniel, 2006). Forrest et al. reported that weaker electrostatic binding between PEI and DNA provide more efficient separation of plasmid DNA and polymer inside cells which in turn may lead to higher transfection efficiency (Forrest et al., 2004). Although there are some investigations which have been shown a correlation between weaker polymer/DNA binding and enhanced gene expression (Erbacher et al., 1997; Fischer et al., 1999; Forrest et al., 2004), but our observation suggest that facilitating dissociation of polymer/DNA complexes will not necessarily improve transfection if it is not the rate limiting step in this process.

The efficiency of sugar-conjugated alkylcarboxylate PEI in plasmid DNA delivery was investigated on HepG2 cell line expressing high levels of ASGP-Rs and on Neuro2A cell line lacking it. As no receptor-mediated uptake is expected for Neuro2A cells, the non-specific electrostatic interaction of polyplexes with cell membranes seems to be the major mechanism in the uptake of polyplexes in this cell line. On the other hand,
substitution of PEI primary amines with sugar moieties and introduction of negatively charged alkylcarboxylate groups reduced the surface charge of the polyplexes. This reduction weakens the electrostatic interaction between the polyplexes and negatively-charged cell surface components (Tseng et al., 2004) which in turn led to reduced transfection efficiency in all conjugates. Previously, it was shown by Tseng et al. that grafting of dextran onto PEI inhibited the entry of plasmid across the cell membrane (Tseng et al., 2004). Similar to the dextran-grafted polymers, the galactosylated PEI derivatives did not increase the transfection efficiency in Neuro2a cell line. The same results have been reported previously which shows a decrease in gene transfer ability of galactose- and maltose- grafted PEI in NIH-3T3 and BNL CL-2 cell lines, respectively (Erbacher et al., 1999; Kunath et al., 2003). In the all transfection experiments, the plasmid transfer ability of dextran-grafted conjugates was lower than those of galactosylated ones. This result could be explained by the fact that the polyplexes were prepared on a C/P ratio basis instead of N/P ratio, thus higher molecular weight of the conjugates containing dextran (MW=5000) than those containing lactose (MW=342) was considered to be the reason for this reduced activity. Previous investigations on dextran- and cycloextrin-grafted polymers also showed that alteration of the microenvironment around the amine groups can decrease the protonation capability of amines responsible for proton sponge effect. According to these studies, as the size of dextran and the conjugation degree increased, the transfection efficiency reduced and became undetectable (Suh et al., 1997; Tseng and Jong, 2003). Also, it was shown that a hair-like structure which was formed by DNA and dextran-grafted PEI could be responsible for the reduced gene expression at high degrees of dextran conjugation. This structure might prevent vector unpackaging or escape of polyplexes from endosomes (Tseng and Jong, 2003). The decreased transfection efficiency of dextran-grafted PEI in the present study could be explained in a similar manner. To investigate the plasmid transfer ability of sugar-conjugated PEI into hepatocytes, the transfection experiments were carried out on HepG2 cell line which expresses ASGP-Rs. These receptors are able to bind and internalize terminal galactose bearing asialoglycoproteins (Zanta et al., 1997). The galactose-conjugated alkylcarboxylate PEI demonstrated the highest transfection efficiency in HepG2 cell line. In the galactosylated form of the acetylate-grafted PEI with no significant hydrophobic shell, the cytotoxicity was reduced by the carboxylate outer layer (Figure 2D). Increase in transfection efficiency following the reduction of the net surface positive charge by adding negatively-charged carboxylate groups in the present study is consistent with the previous studies (Erbacher et al., 1997; Gabrielson and Daniel, 2006; Tseng et al., 2004). Although reducing the surface charge of a polycation vector may reduce its cytotoxicity, the least toxic polycation is not necessarily the most efficient carrier. In the PEI-6-7%G conjugates, the reduction of cytotoxicity by the carboxylate shell combined with the optimal hydrophobic-hydrophilic balance and targeting of the modified polymers by a specific ligand could be the probable reasons for increased transfection efficiency (Oskuee et al., 2009; Thomas and Klibanov, 2002; Zanta et al., 1997).

The results of viability test showed that the cytotoxicity of modified polymers has reduced significantly. A successful strategy which has been widely used to decrease PEI cytotoxicity was to reduce the polymer surface charge (Gabrielson and Daniel, 2006; Rhaese et al., 2003; Zintchenko et al., 2008). Positive-charge shielding reduces electrostatic interaction between the positive charge on the polycation and the anionic cell surfaces and thereby decreases the PEI membrane disruption effect. In the present investigation, galactose and dextran conjugation is supposed to cause positive charge shielding on PEI and decreases membrane damaging effect on both cell lines. The less toxic effect of galactose-conjugated PEI on HepG2 cell line seems to be caused by an additional specific mechanism. As HepG2 cells take up the galactose-grafted PEI by a receptor-mediated endocytosis mechanism, the concentration of modified polymers reduced in the medium by HepG2 cells whereas Neuro2a cells with no ASGP-R do not uptake the galactosylated forms through a similar process causing accumulation of more polycation in the medium and resulted in more toxic effect on Neuro2a cell line.

In conclusion, we investigated the effect of galactose/dextran conjugation and alkylcarboxylation of high toxicity 25 kDa PEI on the plasmid DNA delivery effectiveness into HepG2 cell line. The results showed that the highest transfection efficiency was obtained with the polyplexes formed with the galactosylated form of the hexylcarboxylate PEI. The most probable reasons for increased transfection efficiency of galactosylated derivatives of alkylcarboxylate PEI include targeting hepatocytes through a specific ligand, more favorable hydrophobic–hydrophilic balance and reduced cytotoxicity. Therefore, alkylcarboxylation and galactosylation of PEI can be considered as a useful strategy for developing a hepatocyte-targeting gene carrier.
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References


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