Cross-linked Polyethylenimine as Potential DNA Vector for Gene Delivery with High Efficiency and Low Cytotoxicity

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Abstract Polyethylenimine (PEI) has been known as an efficient gene carrier with the highest cationic charge potential. High transfection efficiency of PEI, along with its cytotoxicity, strongly depends on its molecular weight. To enhance its gene delivery efficiency and minimize cytotoxicity, we have synthesized small cross-linked PEI with biodegradable linkages and evaluated their transfection efficiencies in vitro. In this study, branched PEI with a molecular weight of 800 Da was cross-linked by small diacrylate [1,4-butanediol diacrylate or ethyleneglycol dimethacrylate (EGDMA)] for 2−6 h. The efficiencies of the cross-linked PEI in in vitro transfection of plasmid DNA containing enhanced green fluorescent protein (EGFP) reporter gene were assessed in melanoma B16F10 cell line and other cell lines. Flow cytometry was used to quantify the cellular entry efficiency of plasmid and the transgene expression level. The cytotoxicities of the cross-linked PEI in these cells were evaluated by MTT assay. EGDMA-PEI 800-4h, a typical cross-linked PEI reported here, mediated a more efficient expression of reporter gene than the commercially available 25-kDa branched PEI control, and resulted in a 9-fold increase in gene delivery in B16F10 cells and a 16-fold increase in 293T cells, while no cytotoxicity was found at the optimized condition for gene delivery. Furthermore, the transfection activity of polyplexes was preserved in the presence of serum proteins.

Key words polyethylenimine; gene delivery; cytotoxicity; B16F10; 293T cell line

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Compared with viral vector systems, nonviral gene delivery systems have lower immunogenicity, are relatively easier to be scaled up, and have greater flexibility with regard to vector modification and DNA incorporation. Therefore, nonviral systems based on complexes of condensed DNA with polycations have attracted more attention in recent years [1]. Amine-containing polymers such as poly(lysine) [2], polyethylenimine (PEI) [3], and poly(amidoamine) dendrimers [4] have been extensively investigated. PEI is one of the most successfully and widely used nonviral gene delivery polymers reported to date. It has been known as an efficient gene carrier with the highest cationic charge density potential [5]. Due to its relatively high gene delivery efficiency [3,6,7] and ready availability, branched 25-kDa PEI has become a benchmark with which other polymers [8], especially newly designed and synthesized materials, are often compared. However it has significant cytotoxicity issues. Additionally, the long-term biocompatibility of amine-containing polymers remains an important issue for use in therapeutic applications in vivo, since these polymers are not readily biodegradable [9,10]. Consequently, recent studies have focused on the development of biodegradable cationic polymers [11−18]. But gene delivery using these biodegradable polycations has only achieved limited success. It was reported that a degradable analogue of 25-kDa branched PEI, which was produced by conjugating amino groups of 800-Da PEI to diacrylates, showed high efficient gene delivery capability together with low...
cytotoxicity [18]. So, it will be interesting to combine the favorable low toxicity of low molecular weight PEI with the higher transfection efficiency of high molecular weight PEI. The hydrolysis of the ester bonds will occur under physiological conditions within the cell after transfection and convert the cross-linked high molecular weight PEI into low toxic low molecular weight PEI.

In this study, we coupled low molecular weight 800-Da PEI together to form conjugates of high molecular weight PEI using short diacrylate linkages (containing ester bonds). Among the degradable PEI derivatives we synthesized, one degradable polyethylenimine was selected and validated with low toxicity and highly efficient gene delivery in B16F10 and 293T cell lines. We also studied the effective condensation of DNA by gel retardation assay and dynamic light scattering.

Materials and Methods

Plasmid

Transfections were performed using 2 μg per well (in 24-well plates) of plasmids encoding the enhanced green fluorescent protein gene (pEGFP-C1) (Clontech, San Jose, USA). The plasmids were amplified and purified according to standard molecular biology protocols.

Cell culture

Murine melanoma cell line B16F10 was obtained from American Type Culture Collection (Manassas, USA). 293T (human embryonal kidney) cell line was purchased from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). 293T and B16F10 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS).

General polymer cross-linking procedure

Branched 800-Da PEI, branched 25-kDa PEI, ethyleneglycol dimethacrylate (EGDMA) and 1,4-butanediol diacrylate (BDDA) were purchased from Sigma-Aldrich (St. Louis, USA), and used without further purification unless noted otherwise. Cross-linking was performed following the procedure of Lynn et al. [19,20]. One gram of PEI (branched, 800 Da) was transferred to a 10-ml flask and dissolved in 3 ml of freshly distilled methylene chloride. An equimolar amount of diacrylate linker (EGDMA or BDDA) was added, and the flask was sealed with a solvent-resistant cap. The reaction was carried out at 45 °C, with shaking, for 2, 4, 6 and 10 h respectively. The polymer was then precipitated with ether, lyophilized (18L Freeze-Dry System; Labconco, Kansas City, USA), and stored at −70 °C.

Molecular weight determination and degradation study

Capillary viscosity measurements were carried out to determine molecular weight and study degradation profile [21]. Polymer was dissolved in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4) to concentration from 60 mg/ml to 20 mg/ml. Viscosity measurements were carried out using a capillary viscometer at 25 °C. The molecular weight of cross-linked polymers was calculated using the following equation:

\[ [\eta] = KM^\alpha \]

where \( M \) is the molecular weight and \( K \) and \( \alpha \) are Mark-Houwink parameters determined from PEI standards of known molecular weight.

For degradation study, polymers were dissolved in PBS to a concentration of 60 mg/ml and pH was adjusted to 7.4. The polymer solution in viscometer was incubated in a water bath at 37 °C. Decreasing viscosities were plotted as a function of time.

Complex formation and transfection

Polymer was dissolved in PBS as 1 mg/ml stock solution. DNA-polymer complexes (polyplexes) were prepared in PBS by addition of 50 μl of polymer to an equal volume of 2 μg DNA (each well in 24-well plate) to achieve the desired polymer:DNA ratio. Polyplexes were then incubated at 25 °C for 10–15 min. Cells were cultured in DMEM according to the American Type Culture Collection protocol and seeded in 24-well plates at 1×10⁴ cells/well 24 h prior to transfection. At the time of transfection, cells were at about 70%–80% confluency. Then 100 μl of polyplexes solution (2 μg plasmid/well) and 400 μl of culture media with 10% fetal bovine serum were added to each well containing 500 μl of complete medium drop by drop. Cells were incubated at 37 °C in a CO2 incubator for 24 h before testing for transgene expression. Enhanced green fluorescent protein (EGFP) expression was quantified by flow cytometry. All samples were measured in triplicate and then repeated two or more times independently.

Cytotoxicity determination

Evaluation of cross-linked PEI cytotoxicity was performed by MTT assay [22]. B16F10 cells or other cells were seeded at a density of 1×10⁴ cells/well in 96-well
plates and incubated for 24 h. The prepared polymers were added into cell cultures in the presence of 10% FBS. After 4 h, 20 μl of 5 mg/ml MTT in PBS buffer was added. Plates were incubated for another 4 h at 37 ºC. MTT-containing medium was removed and 100 μl of DMSO was added to dissolve the formazan crystal formed by live cells. Absorbance was measured at 570 nm. The cell viability was calculated according to the following equation (Equation 1):

\[
\text{Cell viability} = \left( \frac{A_{\text{sample}}}{A_{\text{control}}} \right) \times 100\% \]

where \( A_{\text{sample}} \) represents the reading from the wells treated with polymer and \( A_{\text{control}} \) from the wells treated with PBS only.

**Gel retardation assay**

Complexes for this assay were formed at polymer:DNA ratios (\( W:W \)) of 0:1, 0.1:1.0, 0.2:1.0, 0.3:1.0, 0.35:1.0, 0.4:1.0, 0.45:1.0. In each case, an appropriate amount of cross-linked PEI (0.1 μg/μl) in PBS was mixed with 2.5 μg of the plasmid DNA (pEGFP-C1) in ddH2O, the total volume of complex solution was 50 μl. These solutions were incubated at room temperature for 10−15 min, 10 μl aliquot of the sample was mixed with 2 μl of the loading dye (bromophenol blue and xylene cyanol) solution, and loaded onto agarose gel (0.8% agarose in Tris-borate EDTA buffer). Electrophoresis was carried out at 100 V.

**Particle size measurements**

Polyplex sizes were measured by dynamic light scattering using a Brookhaven 90PLUS particle size analyzer (Brookhaven Instruments Corporation, Holtsville, USA) at 25 ºC. Complexes were prepared as in the transfection experiments, except that the total volume was scaled up to 1 ml. The complexes were then subjected to light scattering experiments. The results were expressed as mean±SD (\( n=3 \)). Polyplexes of the 25-kDa PEI and the cross-linked PEI were compared at each polymer:DNA ratio.

## Results

**Synthesis of cross-linked polyethylenimines**

By conjugation of amino groups of 800-Da PEI to EGDMA or BDDA for 2, 4, 6 and 10 h respectively, the following polymers were obtained: EGDMA-PEI 800-2h, EGDMA-PEI 800-4h, EGDMA-PEI 800-6h, BDDA-PEI 800-2h, BDDA-PEI 800-4h, BDDA-PEI 800-6h and BDDA-PEI 800-10h. The viscosity of the reaction solution increased with the reaction time, which implied that the molecular weight of the product became higher and higher. The increased viscosity of these polymers, relative to the starting material, was directly correlated with their molecular weights. We determined the intrinsic viscosity of EGDMA-PEI 800-4h with a capillary viscometer and found that the polymer had a molecular weight of about 11 kDa. \(^1\)H-NMR (500 MHz, D2O) analysis showed that the peak of double bond H \([\text{CH}_2=\text{C(CH}_3\text{)}-\] 5.66−6.39 ppm\) in EGDMA, had completely disappeared in EGDMA-PEI 800-4h, and the cross-linking was confirmed by verifying extensive ester bond formation \([\text{H signal for next to the carbonyl group }-\text{CH}_2=\text{CH}(\text{CH}_3)\text{C}=\text{O}, 2−3 \text{ ppm}]\) in the final product using \(^1\)H-NMR.

**Degradation of polymer**

Decreasing viscosities were plotted as a function of time and then converted to corresponding molecular weights (Fig. 1). When the polymer was dissolved in PBS and incubated in a water bath at 37 ºC for 10 h, it became nontoxic low molecular weight PEI. The rapid degradations might lower the cytotoxicity to the cells.

![Degradation of EGDMA-PEI 800-4h](image)

**Cross-linked polyethylenimine complex**

To demonstrate whether the cross-linked PEI possesses PEI/DNA complex forming ability, an indicator of DNA carrier capability of cross-linked PEI, agarose gel electrophoresis was employed to analyze the complex forming properties. Parameters such as polymer size increase and charge neutralization could decrease the mobility of DNA...
in agarose gel. We mixed DNA with increasing amounts of polymer to determine the ability of EGDMA-PEI 800-4h to form polyplexes with DNA. DNA was entirely retained in the wells when the ratio of DNA:cross-linked PEI is over 3:1 (W/W) (Fig. 2). The same ratio of DNA:25-kDa PEI, 3:1 (W/W), was also found to completely retard the migration of plasmid (data not shown). Therefore, cross-linked PEI did not appear to affect the ability to condense DNA. Dynamic light scattering analysis showed that EGDMA-PEI 800-4h complexes with DNA and formed smaller particles than 25-kDa PEI/DNA in ddH₂O (data not shown), and that fetal bovine serum in the culture medium could prevent this salt-induced particle growth (Fig. 3).

Cell transfection

We investigated the efficacy of the cross-linked PEI polymers in gene delivery in the murine melanoma cell line B16F10. Cells were transfected in vitro with 2 μg of plasmid DNA complexed with various polymers. Gene transfection efficiency was measured by analyzing the expression of the EGFP by flow cytometry (FACS Calibur; Becton Dickinson, Franklin Lakes, USA) (Fig. 4). Among all the synthesized PEI, the cross-linked product of branched 800-Da PEI and EGDMA was the most efficient, indicating a better combination of monomer and cross-linking agent. It was reported that 800 Da PEI and 1,3-butanediol diacrylate/1,6-hexanediol diacrylate was also a good combination of monomer and cross-linking agent.
Similar to the case in cross-linked polymer of 800-Da PEI and 1,3-butanediol diacrylate, the transfection efficiency of the cross-linked products of 800-Da PEI and BDDA was not very high [Fig. 4(A)] indicating that the transfection efficiency is evidently dependent on the nature of the linkages and the PEI used. Although the viscosity, an indicator of molecular weight, increased with increasing polymerization time, the polymerization time had little effect on the transfection efficiency of the products in B16F10 cells (Fig. 4). The starting material, 800-Da PEI, induced no measurable gene expression over the range of polymer:DNA ratios investigated (data not shown). These data are in agreement with results that PEI with less than 2000-Da has no transfection ability in various cell lines [23,24]. Compared with 25-kDa branched PEI, EGDMA-PEI 800-4h could mediate a more efficient expression of reporter genes, 9-fold more efficient in B16F10 cells, and 16-fold more in 293T cells (Fig. 5). It can also deliver genes at lower polymer:DNA ratios. When delivering genes into B16F10 cells and 293T cells, EGDMA-PEI 800-4h showed the same transfection efficiency as Lipofectamine 2000 (Invitrogen, Carlsbad, USA) (data not shown). But EGDMA-PEI 800-4h could mediate more efficient expression of reporter genes than the Lipofectamine 2000 at its optimal transfection condition in EL-4 cell line (Fig. 6).

**Cytotoxicity of cross-linked Polyethylenimine**

The cytotoxicity of cross-linked polymer was investigated by using MTT assay. The results indicated that EGDMA-PEI 800-4h was less toxic than 25-kDa PEI in B16F10 and 293T cell lines (Fig. 7). In B16F10 cells and 293T cells, EGDMA-PEI 800-4h showed 83.8% and 92.1% viability at its optimal polymer:DNA ratio (1.5:1, W/W, polymer concentration of 10 μg/ml), while 25-kDa PEI showed 64.4% and 59.5% viability at its optimal polymer concentration (polymer:DNA=3:1, W/W, 20 μg/ml polymer concentration for B16F10; and polymer:DNA=4:1, W/W, 27 μg/ml polymer concentration for 293T). Fluorescent microscopy analyses indicated that there was no significant change in cell morphology and cell growth relative to controls when transfections were performed at
optimal polymer concentrations in B16F10 and 293T cell lines (Fig. 8).

Discussion

The facilitated transfer of nucleic acids into eukaryotic cells is one of the most valuable and frequently used techniques in modern biotechnology. It is also used in many research areas including gene therapy research, studies of gene regulation, protein structure/function analyses, as well as production of recombinant proteins. The transfection reagent with low toxicity and high gene delivery efficiency becomes more and more important. PEI is an organic...
macromolecule that possesses a high cationic charge density, sometimes known as a “proton sponge”. It condenses DNA into positively charged particles that interact with anionic cell surfaces and enters cells via endocytosis. Because of the close proximity of many linker amino groups in PEI, it retains a substantial buffering capacity at a wide range of pH. The extensive buffering not only protects DNA inside the lysosome from degradation, but also leads to lysosomal swelling and rupture. This in turn provides an escape mechanism for DNA particles to the cytoplasm. Several PEI have been shown to be effective in various in vitro and in vivo transfection applications [25–27], and have evolved as versatile vectors for DNA delivery. Many researchers are focusing on the development of PEI derivative or PEI analog [13,14,18,24,25,27]. When designing the PEI-based efficient transfecting agents, we generally keep the following rules in mind, (i) the polymer should be of high cationic charge density, (ii) the polymer’s skeleton should be easily modified in order to introduce the target molecule, (iii) the system should be of low toxicity and have minimal non-specific interactions with serum proteins, and (iv) the system can be degraded under physiological conditions. Taking all of these factors into consideration, we have developed versatile delivery agents based on various diacrylate cross-linked PEI.

As previously mentioned, the complexes of EGDMA-PEI 800-4h and plasmid in PBS could achieve transfection levels up to 60% in common cell lines such as 293T, B16F10, and CHO (data not shown), and the transfection efficiency is better than commercial Lipofectamine 2000 in EL-4 cell line (Fig. 6) indicating that the cross-linked PEI can be a good transfection reagent. Many cationic non-viral vectors are known to be sensitive to serum. In contrast to cationic liposomes such as Lipofectin and Transfectam, the transfection efficiency of EGDMA-PEI 800-4h was serum-independent, an important prerequisite for in vivo gene delivery. Furthermore the presence of fetal bovine serum in the culture medium can prevent this salt-induced particle growth, probably by adsorption of serum proteins to the complex surface preventing particle-particle interactions (Fig. 3). The ability of EGDMA-PEI 800-4h to form stable complexes with DNA in the presence of serum and the potential degradability under physiological conditions are attractive features meritng further investigations in order to develop EGDMA-PEI 800-4h as an in vivo gene delivery system. Because PEI and many diacrylates are easily available in large quantities, and the process of synthesis is also simple, the production of the cross-linked PEI is very cheap.

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