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Investigating the influence of polyplex size on toxicity properties of polyethylenimine mediated gene delivery

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ABSTRACT

Aims: Gene therapy is a promising strategy for the treatment of various diseases. Polyethylenimine (PEI) has received considerable attention for gene delivery applications due to their appropriate properties. However, their toxicity has raised concerns which cause to be used with cautious. This study aimed to prepare different complexes of PEI/DNA and evaluate their parameters affecting in vitro cytotoxicity. Also, apoptosis rate was measured to determine the mechanism of cell toxicity.
Materials and methods: The complexes were prepared through conjugation and characterized using dynamic light scattering, MTT and flow cytometry techniques.
Key findings: The particles' size was from 81 nm to 2785 nm and was increased in the HBS buffer compared to HBG buffer. In the case of branched PEIs, the size of particles was inversely associated with molecular weight. The cytotoxicity results showed that linear 250 KDa PEI was non-toxic whereas branched PEIs with lower molecular weights showed toxicity effects in a concentration dependent manner. Also, the cytotoxicity effects of branched PEIs were proportional with carrier/plasmid (C/P) ratio and were more for the polyplexes prepared in HBG buffer compared to HBS buffer after 24 h incubation. Flow cytometry results confirmed that apoptosis is the main mechanism of cell toxicity produced by polyplexes.
Significance: The results showed the effect of PEI size on its cytotoxicity. Also, the toxicity effects of PEI-derived polyplexes in vivo environment was evaluated.

1. Introduction

Gene therapy is considered as a promising treatment for not only hereditary diseases, such as cystic fibrosis and heamophilia, but also acquired diseases, such as AIDS and cancer [1]. Although it is an appropriate approach to deliver therapeutic gene materials into the cell nucleus [2], the complexity of biological systems has been an obstacle for successful gene delivery [1]. There are two major types of vehicles used in gene delivery including viral and non-viral vectors [1–3].

Though viral vectors are beneficial due to their transfection efficiency [1], their drawbacks, such as the risk of immune response [4], oncogenic effects, broad tropism [5], and difficulties regarded to their large scale production [2,6], have been limited their use for gene therapy purposes.

Recently, cationic polymers as non-viral vectors have received remarkable attention due to their low cost and simplicity of production [2,6], capability of delivering vast genetic payloads [5], and diminished immunogenicity [2]. Among all non-viral polymeric carriers, since 1995, the popularity of Polyethylenimine (PEI) as a prominent gene transfection system has increased [4,7].

There are two available forms of PEI: branched and linear. It has different molecular weights (MW) varying from a few hundred to 1500 KDa [4,8]. The 25 KDa branched form of PEI is considered as the most profitable polycation since it has noticeable transfection capability [8].

The strong electrostatic interactions between Polyethylenimine (PEIs) and DNA make the DNA condense and generate nano-sized complexes named polyplexes [1,4,9]. These positively charged

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polyplexes conserve DNA from degradation and expedite its cellular entry [4,6]. After vesicular internalization, the buffering capacity of PEI in the acidic pH of endosomes, resulting from its amine groups, leads to endosome bursting and releasing the complexes into cytoplasm. This phenomenon named “proton-sponge effect” is a hypothesis to explain the endosomal escape of polyplexes [4,8,10].

Although PEI is a predictable secure carrier, its cytotoxicity is still an unpredictable challenge [2,8,10,11]. Recent studies demonstrated that the cytotoxicity mechanism of PEI is multifactorial and depends upon MW and branching degree of PEI [12,13], particle size, and zeta potential [13,14]. Also, it motivates cell necrotic and apoptotic pathways [10]. PEI can cause two kinds of cytotoxicity during gene delivery: immediate toxicity related to free PEI resulting in destabilizing of the cell membrane before transfection, and delayed toxicity related to cellular proceeding of PEI/DNA complexes after getting into cell [8,10,11,15].

This study was aimed to control the polyplexes sizes through changing the cells' buffer and to evaluate the effect of polyplexes sizes on their cytotoxicity and DNA damage.

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagle Medium (DMEM) and propidium iodide (PI) were purchased from Sigma (Munich, Germany). PEI in branched (B) or linear (L) chemical structure with different molecular weights(B-PEI 0.6 KDa, B-PEI 1.8 KDa, B-PEI 25 KDa and L-PEI 250 KDa) was supplied by Polyscience, Inc. (Warrington, USA). All solvents were obtained from Sigma-Aldrich/Merk (Germany) and were of the highest purity available. The pRL-CMV, pEGFPN1 plasmids and [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT) were obtained from Promega (Madison, WI, USA). N-[2-hydroxythyl] piperazine-N_-[2 ethane sulfonic acid] (HEPES) was purchased from Fluka (Germany). Glucose and NaCl were supplied by Merk (Germany). Fetal Bovine Serum (FBS), tripsin, penicillin and streptomycin were prepared from Gibco (Gaithersburg, MD). Neuro2A murine neuroblastoma cells were obtained from Pasteur Institute of Iran, Tehran. In addition, plasmid extraction kit and dimethyl sulfoxide (DMSO) were purchased from Qiagene (USA) and Significancema (Germany), respectively. All of the materials were of analytical grade. Distilled water was used throughout the study.

2.2. Preparation of plasmid DNA

Plasmid DNA (pDNA) encoding *pEGFP* (Promega, Madison, Wisconsin, USA) was transformed into *Escherichia coli* bacterial strain DH5α and purified using plasmid extraction kit according to the manufacturer's protocol. Purity and identity of the plasmid were confirmed by agarose gel electrophoresis.

2.3. Preparation of DNA/polymer complexes

PEI/plasmid complexes were prepared in both HEPES-buffered saline (HBS: 20 mM HEPES, 150 mM NaCl, pH 7.2) and HEPES-buffered glucose (HBG: 20 mM HEPES, 5.2% glucose, pH 7.2). Different weight ratios of polymer/plasmid DNA in the range of 2:1 to 20:1 (*w*/w) were used to prepare the polycation/plasmid complexes (i.e. polyplexes). For this purpose, 50 μl of polycation solution in different concentrations in HBG were added to 50 μl of HBG plasmid DNA solution (40 μg/ml), mixed and incubated for 30 min at room temperature.

2.4. Particle size measurements

The sizes of the DNA-polymer complexes were measured in HBG and HBS buffers. For this purpose, desired amounts of the plasmid solution were diluted in 125 μl of buffer and mixed with an equal volume of the same buffer containing PEI. The particle size of DNA/polymer complexes was then analyzed using a Malvern Zeta sizer nano ZS (Malvern Instruments, Malvern, UK). All measurements were performed triplicate.

2.5. Viability of polyplexes

The cytotoxicity of PEI with different molecular weights, concentrations and C/P ratios was evaluated by MTT assay. Briefly, neuro2A cells were seeded at the density of 1×10^4 cells/well in 96-well plates and incubated for 24 h. Thereafter, the cells were treated with various concentrations and C/P ratios of PEI with different molecular weights. After 24 h of incubation, 20 μl of 5 mg/ml MTT in the PBS buffer was added to each well, and the cells were further incubated for 4 h at 37 °C. The medium containing unreacted dye was discarded and 100 μl of DMSO was added to dissolve the formazan crystal formed by live cells. Optical absorbance was measured at 570 and 630 nm using a microplate reader (Stat fax–2100, Awareness Technology, USA) and cell viability was expressed as a percent relative to untreated control cells. The values of metabolic activity were presented as mean ± SD of triplicate experiments.

2.6. Apoptosis assay

DNA fragmentation can be considered as an apoptotic signal in treated cells, in which creates small fragments of DNA. When stained with a quantitative DNA-binding dye such as Propidium Iodine (PI), cells that have lost DNA will take up less stain and will appear on the left of the G1 peak. Apoptotic Neuro2A cells were stained by PI followed by flow cytometry to detect the so called sub G1 peak (ref 9 & 10).

Neuro-2a cells were seeded at a density of 1×10^5 cells/well in 24-well plates and incubated for 24 h at 37 °C. One hundred microliters of PEI solutions were added to each well and incubated for 4, 24and 48 h at 37 °C. Four hundred microliters of PI color were then added to the wells and incubated for 30 min at 37 °C. Next, the cells were separated properly and kept in refrigerator for 4, 24 and 48 h and analyzed by FACScan flow cytometer (Pertec, Germany) and WinMDI Version 2.8 software. The apoptosis rates were measured by determine the Sub-G1 peak.

2.7. Statistical analysis

The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. The $p \leq 0.05$ was considered as statistically significant.

3. Results

3.1. Cytotoxicity of free PEI and PEI/plasmid polyplexes

Cytotoxicity of a range of concentration (0 to 100 μg/ml) of various molecular weights of PEI was measured on the Neuro-2a cells using MTT assay. It was found that b-250 KDa PEI had no cytotoxicity. In other molecular weights (25, 1.8 and 0.6 KDa PEI), increasing the concentration caused to decreasing the cell viability (Fig. 1).

Also, the results of cytotoxicity obtained from the various C/P ratios of PEI 25 KDa polyplexes using MTT assay are shown in Fig. 2.

The difference in cytotoxicity between C/P 4 and C/P 8, 12, 16 and 20; and C/P 2 and C/P 8, 12, 16 and 20 were statistically significant ($^{***}p < 0.001$).

Also, the viability of polyplexes with various molecular weights at C/P 6 was measured after 4, 24 and 48 h incubation with Neuro-2a cells. The results showed that 25 KDa PEI polyplexes prepared in HBG buffer exerted the highest cytotoxicity after 4, 24 and 48 h incubation.

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