Biologicals xxx (2016) 1–7

Contents lists available at ScienceDirect

Biologicals

journal homepage: www.elsevier.com/locate/biologicals

Gene delivery to neuroblastoma cells by poly (L-lysine)-grafted low molecular weight polyethylenimine copolymers

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ARTICLE INFO

Article history:
Received 27 July 2015
Received in revised form
25 December 2015
Accepted 27 March 2016
Available online xxx

Keywords:
Cytotoxicity
Gene delivery
Nanoparticles
Non-viral vector
Poly (L-lysine)
Polyethylenimine

ABSTRACT

Polyethylenimine (PEI) and poly (L-lysine) (PLL) are among the most investigated non-viral gene carriers. However, both polymers contain deficiencies that restrict their applications. In the present study, we synthesized PLL-alkyl-PEI conjugates via 6-carbon alkyl linker and investigated their possible advantages in gene delivery. Four PLL copolymers were synthesized with different molecular weights and ratios of PEI. The physiochemical properties of synthesized conjugates such as size, zeta potential, DNA condensation ability, buffering capacity and cytotoxicity were investigated. Renilla luciferase assay was employed to evaluate the gene transfection efficiency of pDNA-polymer to Neuro2A cell line. DNA condensation and particle size measurements showed that new PLL-PEI conjugates could form polyplexes in nano-scale size in the range of 99–122 nm and were able to condense DNA at low concentration. While cytotoxicity reduced in some groups, the transfection efficiency increased about 2.8 and 4 fold as compared to the unmodified PEI 1.8 kDa and 10 kDa, respectively. The results of the present study showed that the chemical modifications of PEI with PLL could significantly improve transfection efficiency and PLP10-10% shows the most promise as a new gene carrier.

1. Introduction

Most of the gene therapy clinical trials have been performed in cancers, inherited monogenic and cardio vascular diseases. Two main challenges in gene therapy include the lack of efficient gene delivery system or/and appropriate in vivo selection guidelines [1]. Various delivery strategies and gene carriers have been employed in gene therapy studies which are categorized into viral and non-viral systems. Viral vectors have been utilized in a variety of clinical trials such as retrovirals in severe combined immuno deficiency (SCID) [2], lentivirals in X-linked adreno leukodystrophy (X-ALD) [3] or recombinant adeno-associated virals (rAAV) in Leber's congenital amaurosis (LCA) diseases [4]. Although viral vectors exhibit high and rapid gene delivery efficiency, their applications have declined in recent years because (1) they have small capacity and are unable to carry large genes which are necessary for clinical applications, (2) they may activate proto-oncogenes due to random insertion of their genome into chromosomes [5], (3) they induce strong immune responses limited multiple injections and (4) there are difficulties in large scale production. Safety-concerns about the viral vectors have promoted the development of non-viral carriers. Although the transfection efficiencies of non-viral vectors are low in short-term delivery as compared with viral vectors, they have advantages including less infection risk, low cost of production, unlimited plasmid size and low immunogenicity [6]. Because of the flexibility in structures and designs of non-viral carriers, there have been many attempts to improve transfection

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http://dx.doi.org/10.1016/j.biologicals.2016.03.007
1045-1056/

efficiency and overcome their deficiencies. Liposomes and polymers are the most investigated non-viral vectors [7]. Enormous libraries of polymeric nanoparticles have been examined for gene delivery such as polyethylenimine (PEI) [8], dendritic poly (amidoamine) (PAMAM) [9], poly (β-amino ester) [10], polypropylenimine (PPI) [11] and poly (L-lysine) (PLL) [12]. Cationic polymers especially polyethylenimine and poly (L-lysine) are popular because they are positively charged and contain intrinsic affinity for negatively charged oligonucleotides and cell surfaces [13]. Both polymers can effectively condense pDNA and protect it from enzymatic degradation. PEI reveals excellent buffering capacity which can facilitate rupture lysosomes and release their cargo into cytoplasm, whereas PLL exhibits weak [0]Please provide the grant number for 'Mashhad University of Medical Sciences, Iran National Science Foundation (INSF), Iran Nanotechnology Initiative' if any. PLL/DNA polyplexes from endosomes [14]. Unlike PLL, PEI is not biodegradable and its cytotoxicity depends on its molecular weight and the type of cell tested.

In order to increase the transfection efficiency of PLL and to reduce the cytotoxicity of PEI, we synthesized copolymers by conjugating low molecular weight PEI (1.8 and 10 kDa) to a PLL core to take advantage of the properties of both PLL and PEI.

2. Materials and methods

2.1. Materials

Branched polyethylenimine with an average molecular weight of 10,000 Da (PEI 10 kDa) and 1800 Da (PEI 1.8 kDa), poly (L-lysine) (PLL; Mw ~15–30 kDa) were purchased from Polyscience, Inc. (Warrington,PA, USA). The pRL-CMV and pEGFPN1 plasmids were obtained from Promega (Madison, WI, USA). Fetal bovine serum (FBS) and Roswell Park Memorial Institute medium (RPMI) were purchased from Gibco (Gaithersburg, MD). Methylthiazoletetrazolium 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; tissue culture grade), 6-bromohexanoic acid, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were purchased from Sigma–Aldrich (Munich, Germany). Ethidium bromide was purchased from Cinnagen (Tehran, Iran). Spectra/Por dialysis membranes (Spectrum Laboratories, Houston, TX, USA) were utilized for dialysis.

2.2. Synthesis of nanoparticles

The branched polyethylenimine (1.8 kDa and 10 kDa) were dissolved in double distilled water (ddH_2O), then 14 mg (excess amount) of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was separately dissolved in ddH_2O and added to PEI while the mixture was stirred vigorously. Poly (L-lysine) was dissolved in dimethylsulphoxide (DMSO) and 6-bromohexanoic acid was added dropwise over a period of 2 h to a vigorously stirred solution. The reaction was allowed to proceed at room temperature for another 24 h and then diluted with ddH_2O. The resulted solutions were dialyzed using 8000 cut-off membrane (Spectra/Pormembrane) against 150 mM NaCl for 1 day and then ddH_2O for 2 days to remove unreacted alkylcarboxylates. The PLL-alkylcarboxylate solution was added dropwise over a period of 2 h to vigorously stirred PEI solution and the reaction was proceeded for overnight. For purification, the mixture containing PLL-PEI conjugates was transferred to a dialysis membrane with 10,000 and 25,000 cut-off for 1.8 kDa and 10 kDa PEI solutions, respectively (Spectra/Por membrane). Dialysis was performed against 150 mM NaCl saline solution for 1 day and then ddH_2O for 2 days to remove unreacted polymers. After the dialysis, the remained solution was lyophilized to yield a dry powder. Stock solutions (1 mg/ml) were prepared for further application.

2.3. Size distribution and zeta-potential measurements

The particle size and surface charge were measured for vector/pDNA polyplexes by dynamic light scattering (DLS) and laser doppler velocimeter (LDV) using a Malvern Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). The desired amount of conjugated polymers was diluted in 150 μl filtered doubly-distilled water in physiological pH (pH = 7.4) and mixed with 150 μl pDNA solution (containing desired amount of plasmid) and left for 20 min at room temperature to form the polyplexes. Then, 700 μl water was added and the solution was utilized for size and zeta potential measurements. The final polymers concentration was at cationic/plasmid (w/w) ratio at C/P = 4. Values were presented as mean ± SDfor three measurements.

2.4. Determination of buffering capacity

Synthesized conjugates with concentration of 0.4 mg/ml were dissolved in 1 ml of ddH_2O and the pH was adjusted to 12 using 1 M NaOH. The pH was measured using a pH meter (Mettler Todelo, Greifensee, Switzerland) at room temperature (about 25 °C) after addition of each 5 μL of 0.1 M HCl until pH of 2.5 was obtained. The buffering capacity of polymers was estimated from the slope of pH plot versus the amount of HCl added.

2.5. Ethidium bromide (EtBr) exclusion assay

DNA condensation ability of nanopolymers was evaluated by ethidium bromide (EtBr) exclusion assay. The fluorescence intensity of 400 ng/ml ethidium bromide solution was measured at λ_{ex} 510 nm and λ_{em} 590 nm using a Jasco FP-6200 spectrofluorimeter (Jasco Global, FP-6200, Japan). Upon addition of pDNA (10 μg/ml) to ethidium bromide solution, the fluorescence intensity increased which was regarded as 100%. The decrease of EtBr fluorescence intensity was measured following addition of predetermined amounts (2.5 μl of 1 mg/ml solution) of PLL-PEI conjugates to the pDNA-ethidium bromide solution. The experiments were performed in triplicate and results were reported by plotting the relative fluorescence intensity (%) against the carrier/plasmid ratio (w/w).

2.6. Measurement of primary amine content

The primary amines of PLL after grafting with 6-bromohexanoic acid was determined by 2,4,6-trinitrobenzenesulfonic acid (TNBS) assay using the standard method [15]. The difference in the amounts of primary amines on unmodified PLL and conjugated PLL, revealed the degrees of alkylcarboxylate grafting to PEI.

2.7. Preparation of plasmid DNA

The pRL-CMV plasmid (Promega, Madison, WI) coding Renilla luciferase reporter gene was transformed into *Escherichia coli* bacterial strain DH5α. The transformed bacteria was cultured in selective Luria–Bertani (LB) medium and plasmid was extracted from the cell pellets using Qiagen endotoxin-free Mega Plasmid kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The concentration and purity of the plasmid were estimated using the A260 and A260/A280, respectively, using a UV–Visible spectrophotometer (Pharmaspec, UV-1700; Specto, Shimadzu, Japan). The highest purity and mostly supercoiled plasmids were selected after agarose gel electrophoresis.

Please cite this article in press as: Askarian S, et al., Gene delivery to neuroblastoma cells by poly (L-lysine)-grafted low molecular weight polyethylenimine copolymers, Biologicals (2016), http://dx.doi.org/10.1016/j.biologicals.2016.03.007