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Polylysine-modified polyethylenimine inducing tumor apoptosis as an efficient gene carrier

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ABSTRACT

Polyethylenimine (PEI) is receiving increasing attention as a gene carrier with high transfection efficiency. However, its high charge density and cytotoxic effects limit its application. Polylysine (PLL) is another polymeric gene carrier with good biodegradability and biocompatibility, although its lack of endosomal escape ability strongly impairs its transfection efficiency. In this study, PLL was introduced to PEI by ring-opening polymerization of ε-benzyloxycarbonyl-L-lysine *N*-carboxyanhydride, followed by deprotection of carbobenzyloxy groups. As-prepared PEI-PLL multiarm hyperbranched copolymers were characterized as gene carriers *in vitro* by measuring their particle size, zeta potential, cytotoxicity, transfection efficiency, and cell internalization. The optimum transfected efficiency of PEI-PLL was nearly seven times higher than that of PEI with a molecular weight of 25 kDa. Furthermore, pKH3-rev-casp-3 plasmid DNA was used as a gene for anti-tumor treatment in a xenograft model using nude mice. Compared with 25 kDa PEI, PEI-PLL exhibited better tumor inhibition effects in 23 days. In addition, terminal deoxynucleotidyl transferase dUTP nick end labeling, immunohistochemistry, and western blot analysis were used to determine the anti-tumor mechanism of PEI-PLL. The results showed that tumor cell apoptosis led to tumor inhibition, which could be attributed to pKH3-rev-casp-3 inducing poly(ADP-ribose) polymerase-1 cleavage. PEI-PLL is a promising gene carrier candidate for further application *in vivo*.

1. Introduction

Gene therapy has the prospect of becoming an efficient treatment option following chemical, radioactive, and surgical operation methods to treat cancers. Safety, high efficiency, and stability are three main aspects of gene therapy for tumor treatment [1–5]. Failure of gene delivery to target tumor organs and transfection-induced damage to normal tissues and organs reduce the therapeutic effect of this method. Therefore, the development of an efficient gene carrier is the key to the success of gene therapy for cancers [6–9].

Non-viral gene carriers are potential safer alternatives to viral gene carriers because they are not associated with immune safety concerns. Several polycations have been explored as non-viral gene carriers, including polyethylenimine (PEI), chitosan, poly(L-lysine), poly(β-amino ester), and polyamidoamine dendrimer, as well as some natural polycations, such as cyclodextrin and gelatin [10–14]. Hyper-branched PEI and polylysine (PLL) are two of the most widely studied gene carriers with effective gene transfection activities. However, their transfection abilities do not meet the requirements of clinical application. PEI and PLL possess different features, and these features could complement one another very well. Hyper-branched PEI with a molecular weight of 25 kDa (PEI25k) is considered the gold standard for gene transfection reagents. The "proton sponge" effect related to endosomal escape ability is regarded to account for the relatively high transfection efficiency of PEI. However, the prospects for the application of PEI are negatively affected by its non-degradability and high cytotoxicity. In order to overcome these shortages, many researchers introduce PEG on the surface of the PEI to enhance biocompatibility, reduce cytotoxicity, and protect DNA from degradation [15]. However, is modified with a high degree of PEGylation, i.e., 50 PEG units are grafted to a PEI chain, PEI will has low toxicity and high solubility, but the transfection efficiency *in vitro* is only a tenth of original level [16].

After the PLL was introduced, these problems were solved in a simple and direct manner. PLL is a synthetic polypeptide with good biodegradability and biocompatibility [17,18]. It has been well established as a gene carrier to efficiently condense DNA and protect DNA from nuclease attack [19,20]. But under the physiological conditions (pH = 7.4) the primary amine of the PLL is protonated completely with no buffer capacity, so it cannot efficiently escape the endosome without endosomolytic agents, which strongly impairs its transfection ability [21,22]. Therefore, in order to make up the defects for each other's weaknesses, the development of a PEI-PLL conjugation system is both appealing and practical [23–25].

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In this study, a series of PEI-PLL copolymers were synthesized at different ratios. Evaluations of particle size and zeta potential as well as gel retardation assay were carried out to prove the ability of PEI-PLL to condense *p*DNA. Cytotoxicity, *in vitro* transfection efficiency, and cell internalization were assessed to confirm the transfection ability of PEI-PLL. After a nude mouse model of xenograft tumor was subjected to anti-tumor therapy, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, immunohistochemistry, and western blot analysis were also used to verify the anti-tumor mechanism of PEI-PLL.

2. Materials and methods

2.1. Materials

Branched PEI25k was purchased from Aldrich. ε-Benzyloxycarbonyl-L-lysine *N*-carboxyanhydride [Lys(Z)-NCA] was prepared as described by Daly [26,27]. Trifluoroacetic acid was purchased from GL Biochem Ltd. (Shanghai, China). Hydrobromic acid in acetic acid 33% (v/v) was purchased from ACROS. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Amresco (Solon, Ohio, USA). Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco (Grand Island, USA). Cell lysate and a luciferase reporter gene assay kit were purchased from Promega (Mannheim, Germany). A BCA protein assay kit was purchased from Pierce (Rockford, IL, USA). Calf thymus DNA was purchased from Sigma (St. Louis, MO, USA). Primary anti-poly(ADP-ribose) polymerase-1 (PARP1) antibody was purchased from Ebioscience (San Diego, USA), whereas anti-cleaved PARP1 was obtained from Abcam (Cambridge, England).

Human cervical cancer HeLa cells were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences and cultured in DMEM with 10% FBS at 37 °C and 5% CO_2. The purity grades of plasmid pGL-3, plasmid pKH3, and plasmid pKH3- rev-casp-3 were measured on a UV spectrophotometer using the absorbance reading at 260 and 280 nm.

Female BALB/C nude mice (5–6 weeks old; weight, 18–22 g) were purchased from Vital River Company (Beijing, China) and kept at an SPF-level laboratory (Northeast Normal University). The mice were given water with water-soluble vitamin C and aseptic food. All experimental procedures were in accordance with the guidelines for laboratory animals established by the Animal Care and Use Committee of Northeast Normal University.

2.2. Synthesis of polymers

PEI-poly(benzyloxycarbonyl-L-lysine) [PLys(Z)] (PEI-PLL) was synthesized by ring-opening polymerization (ROP) using PEI25k as a macro-initiator and Lys(Z)-NCA as the monomer. Briefly, PEI25k and Lys(Z)-NCA were separately dissolved in dried chloroform. After complete dissolution, the Lys(Z)-NCA solution was added to the PEI solution with different feed ratio list in Table 1; the mixture was stirred for 72 h at 30 °C. Next, the solution was concentrated and precipitated with excess diethyl ether. After filtration, PEI-PLys(Z) was obtained by drying under vacuum at room temperature for 24 h (Yield: 83%, 82%, 85% and 79% respectively for copolymers with four different PEI and Lys(Z)-NCA feed ratios). PEI-PLL was obtained by removing the protected benzyloxycarbonyl group of PEI-PLys(Z). Different PEI-PLL was named as PEI-PLL1-4 according to different feed ratios list in Table 1. One gram of PEI-PLys(Z) was dissolved in 10 mL of trifluoroacetic acid at room temperature. Three milliliters of hydrobromic acid in 33% acetic acid (v/v) was then added. After 2 h, the solution was added dropwise into an excess of ethyl ether. A slightly yellow solid was collected and dried under vacuum at room temperature overnight, after which the solid was dissolved in water and dialyzed (molecular weight cut-off, 7000 Da) against water (500 mL; changed four times over 48 h). The end products were collected by lyophilization (Yield: 82%, 80%, 78% and 82% respectively).

Table 1
Molecular weight of PEI-PLL.

Polymer	Feed ratio, molar		Determined by ^{1}H NMR			Determined by GPC	
	PEI	Lys(Z)-NCA	PEI, wt%	PLL, wt%	Mn	Mn	Mw/Mn
PEI-PLL1	1	327	45.5	54.5	54.9 kDa	5856	1.40
PEI-PLL2	1	123	60.9	39.1	41.0 kDa	4200	1.41
PEI-PLL3	1	55	71.6	28.4	35.0 kDa	3900	1.23
PEI-PLL4	1	20	86.3	13.7	29.0 kDa	3301	1.27

2.3. Polymer characterization

^{1}H NMR spectra were recorded on a Bruker AV-400 spectrometer (Bruker, Ettlingen, Germany). The measurements were carried out at room temperature, and a mixed solvent of CF_3COOD and D_2O was used.

2.4. Transfection activity

The transfection efficiencies of PEI-PLL1/DNA, PEI-PLL2/DNA, PEI-PLL3/DNA, and PEI-PLL4/DNA *in vitro* were evaluated in HeLa cell lines compared with PEI25k. The cells were seeded into 96-well plates at 1.0×10^4 per well 24 h before transfection and grown in 200 μL of DMEM containing 10% FBS. The medium was replaced with 190 μL of fresh DMEM before the complex was added. The plasmid DNA was added to the carrier solution at different weight ratios (20, 10, 5, 2.5, and 1), and the solution was mixed (containing 0.2 μg of pGL3-luc). Before being added to cells, the complex solutions were incubated for 30 min at room temperature. The cells were then incubated with complexes for 48 h at 37 °C. The Promega Luciferase Assay system was used to evaluate the expression of the reporter gene. The protein content of the lysate was detected using a Micro BCA Protein Assay Kit (Pierce). The efficiency of gene transfer was reported in units of LUC per milligram of cellular protein.

2.5. Polymer/plasmid DNA complex characterization

The zeta potential and particle size of the complexes were determined by dynamic light scattering using a particle analyzer (Zeta-PALS, Brookhaven, NY). Measurements were performed with different Carrier/DNA-pGL3 (wt/wt) ratios, ranging from 1 to 40, after 20 min of incubation at room temperature.

We evaluated the ability of the complexes to bind plasmid DNA by agarose gel electrophoresis. PEI25k and PEI-PLL2 solution at weight ratios ranging from 0 to 0.6 were added to 0.5 μg of *p*DNA, and the total volume of the complex was determined to be 15 μL. Before gel electrophoresis was carried out, the complexes were incubated for 30 min at room temperature. For further verification, heparin was continuously added at different concentrations to the complex under the same experimental conditions. The results were analyzed by 1% agarose gel electrophoresis following the same steps mentioned above.

2.6. MTT assay

Through transfection *in vitro*, PEI-PLL2 was selected as the best carrier to measure cell viability using MTT assay. HeLa cells were seeded in 96-well plates with 200 μL of DMEM containing 10% FBS for 24 h before the start of the experiment. The toxicity experiment was performed using a method similar to the transfection experiment. After transfection for 48 h, 20 μL of MTT solution (5 mg/mL in PBS) was added to each well; the mixture was incubated for 4 h. Finally, the solution was removed and replaced with 200 μL of dimethyl sulfoxide. The solution