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Cytotoxicity and *in vivo* tissue compatibility of poly(amidoamine) with pendant aminobutyl group as a gene delivery vector

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ABSTRACT

To design successful polymeric gene delivery vehicles with good biocompatibility and highly efficient gene transfer ability is one of the great scientific challenges in modern gene therapy. Poly(amidoamine) with pendant aminobutyl group (PAA-BA) has been proved to exhibit high transfection efficiency against bone marrow stromal cells (BMSCs) *in vitro*. In this work, based on previous research, PAA-BA's biocompatibility including *in vitro* cytotoxicity determined by effect on BMSCs' morphology, viability, membrane damage and apoptosis/necrosis, and *in vivo* tissue compatibility determined by muscular and hepatic tissue response were further investigated in comparison to branched polyethylenimine (PEI) 25 kDa. The results demonstrated that PAA-BA possess much better cytocompatibility than PEI, yielding slight cell morphological change, high cell viability and mild effect on cell membrane damage as well as inducing less apoptotic/necrotic cells at optimal N/P ratio. PAA-BA also exhibited better tissue compatibility, reflected by no or less inflammatory response in the site of muscle injection at the same (0.03% w/v) or higher concentration (0.1% w/v) and no hepatic tissue morphological change with normal hepatocytes. We concluded that PAA-BA was promising and safe candidate for *in vitro* BMSCs gene delivery and had potential for in future *in vivo* gene therapy.

1. Introduction

Gene therapy has attracted progressive attention for the treatment of numerous intractable diseases, however, the lack of biocompatible and efficient gene delivery systems is an obstacle to their clinical application [1]. The safety problem of virus as highly potent gene delivery vector, including severe immunological, toxicological responses and random integration into host genome, has limited its application [2]. Recently increasing interest has been paid to the design of non-toxic and highly efficient polycationic vectors for gene therapy [3]. However, for most polymeric vectors, high transfection efficiency is mostly afflicted with high toxicity [4]. Higher dosage of polycation generally increase transfection efficacy, but are associated with low cell survival rate [5] as well as tissue toxic response [6]. Therefore in practical applications, both transfection efficacy and biocompatibility should be considered to optimize the gene delivery vectors for the balance of effectiveness and safety.

The term "biocompatibility" encompasses many different properties of the gene vectors, however, two important aspects of the biomaterial screening refers to their *in vitro* cytotoxicity and *in vivo* tissue compatibility behavior [7,8]. In cell-based gene therapy, the cationic nature of the polymeric gene vectors can induce immediate or delayed cytotoxicity by mechanisms involving necrosis and apoptosis [9,10]. Polyethylenimine (PEI) and poly(L-lysine) (PLL), two kind of commonly used polycations, have demonstrated their extensive cytotoxicity by means of inducing apoptosis in a range of human cell lines [11]. When applied in *in vivo* gene delivery, positively charged polyplexes will interact with negatively charged serum proteins or red blood cells as well as accumulate in certain tissue or organ, resulting in tissue toxic response [6]. The toxicity of gene carriers may compromise gene transcription and translation processes and potentially limits protein expression [12]. Cationic carriers may not only exacerbate, attenuate or even mask the effects of delivered nucleic acids *in vitro* [9], but also hinder *in vivo* nucleic acid delivery due to the toxicity associated with their formulations [13]. Thus, it is critical to evaluate the biocompatibility issues of gene delivery vectors, both on a cellular and systemic basis, before *in vivo* application.

In our previous work, we have reported the synthesis, transfection and intracellular trafficking characteristics of poly(amidoamine)s with pendant primary amine in the delivery of plasmid DNA to bone marrow stromal cells (BMSCs) [14–16]. It was found that poly(amidoamine) with pendant aminobutyl group (PAA-BA in

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Scheme 1) demonstrated higher transfection efficiency and percentage of nuclear localization than branched PEI 25 kDa. As well as high transfection efficiency of about 29%, PAA-BA also had much lower cytotoxicity against BMSCs with IC50 of 100 μg/mL, almost one order of magnitude higher than PEI. These results motivated us to perform an additional and comprehensive evaluation of biocompatibility of PAA-BA in order to ensure its safety for future application. In this work, pluripotent BMSCs were still used as gene modification target cells to investigate PAA-BA's cytotoxicity, including MTT and lactate dehydrogenase (LDH) assay as well as apoptosis analysis. Mice intramuscular injection and intravenous administration as two major *in vivo* gene delivery routes were also carried out to evaluate the tissue compatibility.

2. Materials and methods

2.1. Materials

Branched PEI 25 kDa, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoplium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma–Aldrich (St. Louis, MO). Minimum essential medium alpha (α-MEM), penicillin/streptomycin, fetal bovine serum (FBS) and trypsinase were obtained from Gibco BRL (Grand Island, NY). The Cytotoxicity Detection Kit and In Situ Cell Death Detection Kit were purchased from Roche Diagnostics (Branchburg, NJ, USA). Annexin V-FITC Apoptosis Detection Kit was obtained from BD Biosciences (BD Pharmingen, San Diego, CA). Triton X-100 was purchased from Thermo Fisher Scientific Inc. (Rockford, IL, USA). PAA-BA with average molecular weights (*Mw*) of 1.17×10^4 (*Mw/Mn* = 2.80) was synthesized as described in our early work [14,15]. PAA-BA and PEI were separately dissolved in 150 mM NaCl as 1 mg/mL and then the solutions were filtered through a 0.22 μm Milipore filter system (Milipore, Billerica, MA) for sterilization.

pGL-3 as luciferase reporter gene was purchased from Promega (Madison, WI, USA). The plasmid was propagated in *Escherichia coli*, and was isolated and purified by PureYield™ Plasmid Midiprep System (Promega, Madison, WI, USA). The absorbance at the wavelength of 260 and 280 nm was measured by UV Spectrophotometer (UV-2401, Shimadzu, Kyoto, Japan).

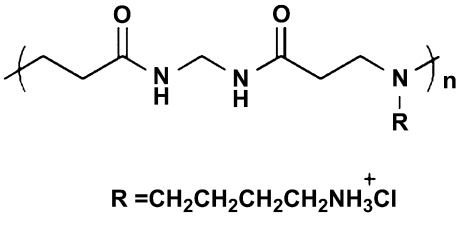
2.2. BMSCs isolation and expansion

Primary BMSCs were isolated from bilateral femurs of 9-day-old Sprague–Dawley rats as described in our previous work [16]. In brief, the femurs were cut at distal and proximal ends under sterile condition, then the bone marrow was flushed out by using a 1-mL steriled syringe with 600 μL of α-MEM containing 10% FBS and 100 U/mL penicillin/streptomycin. The marrow solution was centrifuged at 1000 rpm for 5 min, suspended in fresh complete medium and directly seeded on a single flask and incubated at 37 °C, 5% CO_2. After 72 h, the non-adherent cells were removed from the medium. The confluent cells were trypsinized on day 7. Then BMSCs were subcultured and 4-5th generations were used in this work.

2.3. Preparation of polyplexes and BMSCs transfection

PAA-BA and PEI/DNA complexes at different N/P ratio were respectively prepared by mixing polymers at desired concentrations with certain quantity of pDNA. Briefly, each polymer was diluted proportionately at appropriate concentration in 150 mM NaCl to form 100 μL of solution. 1 μg of plasmid was also diluted in 150 mM NaCl to form another 100 μL of solution. Equal volume of solutions was quickly mixed together in micro centrifuge tube and gently vortexed for 15 s. Then the mixtures were incubated at room temperature for 30 min and used for transfection immediately.

In BMSCs transfection experiment, 4-5th generation BMSCs were seeded at density of 1×10^5, 5×10^4 and 5×10^3 cells/well in 12-, 24- and 96-well plates respectively. The cells were allowed to grow until 60–70% confluence after which the complete medium was replaced with serum-free α-MEM. Certain volume of polycation/DNA complexes solution (containing 2 μg, 1 μg and 0.25 μg pDNA for 12-, 24- and 96-well plates respectively) was added into each well and incubated for 4 h at 37 °C. Then, after removing the serum-free medium, each well was supplemented with fresh complete medium, and incubated at 37 °C for 24–48 h. Morphological changes of transfected BMSCs were observed and captured by using a light microscope(Leica, Wetzlar, Germany).



Scheme 1. The chemical structure of PAA-BA.

2.4. Cell viability assay post-transfection

BMSCs were transfected by PAA-BA or PEI/pGL-3 complexes at various N/P ratios from 5 to 100 in 24-well plates. After 4 h, 24 h and 48 h of transfection, MTT assay was performed to measure cell viability post-transfection. Three wells of each group were randomly chosen and washed twice with serum-free α-MEM. 100 μL of filtered MTT solution (5 mg/mL) was added to each well. After incubation at 37 °C for 4 h, the medium in the wells was aspirated carefully and then 0.5 mL of DMSO was added to dissolve any insoluble formazan crystals. Then a total 600 μL solution of each well was transferred into three wells of 96-well plate and the absorbance was measured at 490 nm on an ELX Ultra Microplate Reader (Bio-tek Instruments Inc, USA). Untreated cells were taken as control with 100% viability. The relative cell viability was calculated as a percentage relative to untreated control cells.

2.5. LDH assay

BMSCs were transfected by PAA-BA or PEI/pGL-3 complexes at various N/P ratios from 10 to 100 in 96-well plates. After 4 h and 8 h of transfection, LDH assay was performed to measure the membrane damaging effects by using a Cytotoxicity Detection Kit according to the manufacturer's protocols. Briefly, each sample was divided into two sections: maximum LDH (considered as high control) and experimental LDH release. Wells with untransfected cells were considered as low control. Experimental LDH wells and low control wells were added with 10 μL of PBS for volume correction. High control wells were added with 10 μL of 9% Triton X-100 and incubated for 1 h at 37 °C for complete lysing. Then 50 μL of the supernatant was collected from each well through centrifugation (12000 g for 5 min) to remove cell debris and was transferred into a separate 96-well plate, followed by adding 50 μL of LDH substrate, shaking at room temperature for 30 min in the dark and reading absorbance at 490 nm with an ELX Ultra Microplate Reader. Release of total LDH for each sample was calculated according to the following formula: $\%\,\text{Total LDH release} = \frac{\text{exp. value} - \text{low control}}{\text{high control} - \text{low control}} \times 100$

2.6. Apoptosis analysis by terminal deoxynucleotidyl transferase (TdT)-mediated-UTP nick end labeling (TUNEL) staining

BMSCs were transfected by PAA-BA or PEI/pDNA complexes at N/P ratios of 5, 10, 15 and 20 in 24-well plates with cover slip on the bottom. After 24 h of transfection, BMSCs were evaluated by TUNEL assay to identify apoptotic cells by using an In Situ Cell Death Detection Kit. Briefly, BMSCs grown on cover slip were washed twice with ice-cold PBS, and fixed with 4% paraformaldehyde in PBS for 30 min at room temperature. After washed twice with PBS, the cells were treated with 0.3% H_2O_2-methanol followed by another twice PBS washing. Then the cells were treated with permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 10 min on ice. Samples were washed twice with PBS again and incubated with the TUNEL reaction mixture containing FITC-labeled dUTP and TdT for 1 h at 37 °C in a humidified atmosphere in the dark. Finally, the samples were incubated with anti-FITC-peroxidase (POD) conjugate. POD activity was shown by diaminobenzidine (DAB). At last samples were counterstained with hematoxylin. For each experimental set, untransfected BMSCs were incubated in the absence (blank control) or presence (positive control) of DNase I pretreating, in addition to negative control group prepared by omitting TdT from the reaction mixture.

2.7. Apoptosis analysis by annexin V-FITC and PI staining

BMSCs were transfected by PAA-BA or PEI/pDNA complexes at N/P ratios of 5, 10, 15 and 20 in 12-well plates. After 24 h of transfection, an Annexin V-FITC Apoptosis Detection Kit was used to detect the apoptotic rate of BMSCs post-transfection through fluorescence-activated cell sorter (FACS). The cells in each group were harvested and washed twice with cold PBS. After centrifugation, the supernatant was removed and 195 μL of binding buffer and 5 μL of annexin V-FITC were added. The cells were incubated for 15 min in the dark at room temperature, and then centrifuged again. Following supernatant removal, 190 μL of binding buffer and 10 μL of propidiumiodide (PI) were added to the cell pellet. Then, the cells were incubated for 5 min in the dark followed by adding 300 μL of PBS. Untransfected BMSCs served as four control groups, which were respectively treated as follows: without annexin V-FITC or PI staining as dual negative control, no PI but annexin V-FITC staining as annexin V positive control, no annexin V-FITC but PI staining as PI positive control, both annexin V-FITC and PI staining as dual positive control. Two color flow cytometric analyses were performed on a FACSort™ (BD Biosciences, San Jose, CA) and the signal was detected by FL1 and FL3 channels. The FL1 and FL3 discriminator was set on 10^2 in order to distinguish positive and negative cells. A gate was set on scatter plots of control groups to establish quadrant marker and the samples were analyzed