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Polyethylenimine derivate conjugated with RGD-TAT-NLS as a novel gene vector

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Abstract. To solve the contradiction between the cell toxicity and transfection efficiency of polyethylenimine (PEI) derivate in non-viral gene therapy, a novel gene vector, P123- PEI-R18 was synthesized by using biodegradable PEI derivate conjugated with trifunctional peptide RGD-TAT-NLS. The particle size of P123- PEI-R18/DNA was around 100-250nm. The gene vector could condense DNA at the weight ratio of 2 and protect plasmid DNA from being dissolved in the blood circulation. Importantly, the complexes exhibited lower cell toxicity and higher transfection efficiency contrasted with PEI 25 kDa in vitro. P123-PEI-R18 holds high potential as a safe and efficient gene vector.

Keywords: Non-viral gene vector, polyethylenimine, P123, nuclear localization signal, αvβ3

1. Introduction

Non-viral gene vector was considered to be a promising strategy on account of non-immunogenicity, noninfection, easier preparation, and the capability of carrying large amounts of genetic materials [1–3]. Polyethylenimine (PEI) was a well-known gene transfection reagent due to the proton sponge effect [3,4]. It has been documented that the high molecular weight (HMW) PEI had high gene transfection efficiency with high cytotoxicity; Low molecular weight (LMW) PEI exhibited low cytotoxicity but low transfection efficiency [5,6]. Researches suggested that PEI derivative achieved by PEI with degradable materials show remarkable transfection efficiency and low cell toxicity [7,8].

Pluronic P123 with formula EO_{20}-PO_{70}-EO_{20} has proper Hydrophile Lipophilic Balance value [8] that can be used for polycation gene delivery. In this study, a PEI derivate PEI-P123 was synthesized by cross-linking LMW PEI with P123, which could be degraded after cell uptake so as to decrease cytotoxicity [4].The efficient transfection of plasmid DNA involves cell selection, internalization and nucleus delivery. The αvβ3 receptors exhibited high expression on tumor cells and tumor vessels, but could not be detected on quiescent blood vessels. It has been reported that Arginine-glycine-aspartate (RGD) peptide could target to αvβ3 receptors specifically [9]. Cell-penetrating peptides (CPPs) can translocate macromolecules across the plasma membrane [10]. The typical CPPs were known as TAT [11] (amino acids 49-57 of the HIV TAT protein [10]) with the sequence RKKRRQRRR. The nuclear localization signal [12] (NLS, with the sequence KKKRK) is a necessary signal sequence that

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mediates the proteins entering the cell nucleus through the nuclear pore complex [13]. Therefore, a novel peptide RGD-TAT-NLS (named R18) was synthesized by conjuncting RGD with TAT and NLS.

In the study, a polymer matrix was synthesized by cross-linking PEI with P123 and trifunctional R18 to form a new polymeric gene vector (P123-PEI-R18).

2. Materials and methods

2.1. Reagents and plasmid DNA

PEI 2KDa, N-succinimidyl-4-(N-maleimido-methyl) cyclohexane-1-carboxylate (SMCC) and 3-(4, 5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich. P123 was provided by Second Military Medical University. RPMI 1640 culture medium and fetal bovine serum (FBS) were purchased from GIBCO. GL Biochem synthesize the peptide Arg-Gly-Asp-Lys-Lys-Lys-Arg-Lys-Cys-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg (R18, MW 2439.99). The plasmids of Escherichia coli DH5α were augmented by the Tiangen EndoFree Mexi Plasmid Kit. Benzene, triphosgene, dichloromethane, N-hydroxysuccinimide, triethylamine, ethyl acetate, and absolute ethyl alcohol were bought from Sinopharm Chemical Reagent Co., Ltd.

2.2. Synthesis and characterizations of P123-PEI-R18

P123-PEI (MW 26216) was obtained by adding activated P123 and PEI 2 kDa into dichloromethane with constant stirring [11]. The SMCC solution (using dimethyl sulfoxide as solvent to 3.33 g/L) was added to P123-PEI solution (using 0.1M PBS as solvent to10 g/L) dropwise at 2:1 molar ratio with half an hour stirring at room temperature. The excess non-conjugated SMCC was removed by gel chromatography. Then R18 (using 0.1M PBS as solvent to10 g/L) was mixed into pretreated P123-PEI with stirring at 4°C overnight in the dark. The solution was lyophilized after ultrafiltration.

The ^{1}H-NMR spectra analysis was carried out at room temperature after P123-PEI and P123-PEI-R18 were dissolved in deuterium oxide.

The P123-PEI-R18/DNA complexes were prepared at different weight ratios. Then particle sizes were measured using electrophoretic light-scattering spectrophotometer. All the samples were conducted in triplicate.

2.3. DNA binding and release behavior of P123-PEI-R18/ DNA polymer

Agarose gel retardation assay was performed to investigate DNA binding ability of P123-PEI-R18. Different weight ratios of polymer/DNA complexes were prepared. Samples were analyzed by electrophoresis with the condition of 120 V and 30 min on a 1% (w/v) agarose gel in loading buffer. The gel was stained with 0.5 g/mL ethidium bromide for 15min and was illuminated by a UV illuminator to show the location of DNA.

P123-PEI-R18/DNA complexes were prepared at the weight ratio (w/w) of 20:1. Different volumes of DNase I were mixed with complex solution (10 μL) in Eppendorf tubes respectively and incubated at 37°C for 30 min. After that, all samples were treated with 2 μL of 250 mM EDTA solution, and incubated for 10 min at room temperature to passivate DNase I. Thereafter, sodium heparin (2 g/L, 10

μL) was mixed in and kept for 2 hours at room temperature. At last, the gel electrophoresis was carried out to estimate the resistant capacity of P123-PEI-R18 to DNase I.

10 μL of complex solution with the weight ratio of 20:1 for P123-PEI-R18/DNA was put into tubes. Different amounts of FBS and 2 μL varying concentrations of sodium heparin were mixed into tubes respectively and kept with 37°C for 60 min and 30 min. Next, the gel electrophoresis was conducted to evaluate the stability of complex to serum and sodium heparin.

2.4. Cell viability assay

The cytotoxicity of P123-PEI-R18 polymer was measured by MTT assay. B16 cells were distributed to each well of 96-well plate for 48h. Previous medium was abandoned. Serum-free mediums with concentrations of P123-PEI-R18 solution at 0 μg/mL, 4 μg/mL, 8 μg/mL, 16 μg/mL, 24 μg/mL and 32 μg/mL were added respectively. After the plates had been incubated for 4 h under 5% CO_2 at 37°C, fresh growth medium was replaced and kept for 72 h. Thereafter, the wells were replaced with 20 μL of 5mg/mL sterilized MTT solution and 180 μL of fresh growth medium and kept at 37°C for 4 h. Subsequently, the MTT/growth medium was replaced by 150 μL DMSO and kept for 10 min with gentle vortex at room temperature. The absorbance value at 570 nm was read by an ELISA plate reader with background subtraction. Cell viability was calculated using the following equation: cell viability (%) = (Absorbance of cells treated with nanoparticles – Absorbance of free medium alone)/ (Absorbance of control untreated cells −Absorbance of free medium alone) × 100 [14].

2.5. Gene transfection in vitro

Plasmid pGL3-Control was used to examining the transfection efficiency of P123-PEI-R18/DNA complexes. B16 cells were seeded in 24-well plates with 500 μL of growth medium added to every well and then cultured to 80% confluence. The medium was substituted by different weight ratios of complex solution (100 μL) and RPMI 1640 medium (400 μL). Cells were incubated under the condition of 5% CO_2 and 37°C for 4 hours. 500 μL of medium with 15% FBS replaced the previous one and then incubated for extra 48 hours. Then the luciferase assay was conducted according to the manufacturer's specifications. The protein contents were measured by bicinchoninic acid (BCA) protein assay kit. Transfection efficiency was expressed as the relative light units (RLUs) [11].

3. Results and discussion

3.1. Synthesis and characterization of P123-PEI-R18

The succinimidyl carbonate was used to activate the free hydroxyl groups of P123, and then these free hydroxyl groups were grafted to amino radicals of PEI to acquire P123-PEI. P123-PEI was linked with R18 by SMCC. ^{1}H-NMR spectroscopy (Figure 1) was carried out to confirm the formations of P123-PEI and P123-PEI-R18. Figure 1A exhibited the ^{1}H-NMR spectra of P123-PEI. -CH_2CH_2O- and -CH_2CH_2NH- proton peaks appear at δ3.55 ppm and δ2.51–2.7 ppm, respectively. Figure 1B exhibited ^{1}H-NMR spectra of P123-PEI-R18, where the proton peaks of P123-PEI-R18 move towards the lower magnet field compared with Figure 1A. Meanwhile -CH_2CH_2NH- proton peaks at δ2.51–2.7 ppm disappeared. These changes confirmed that P123-PEI-R18 had been synthesized successfully.

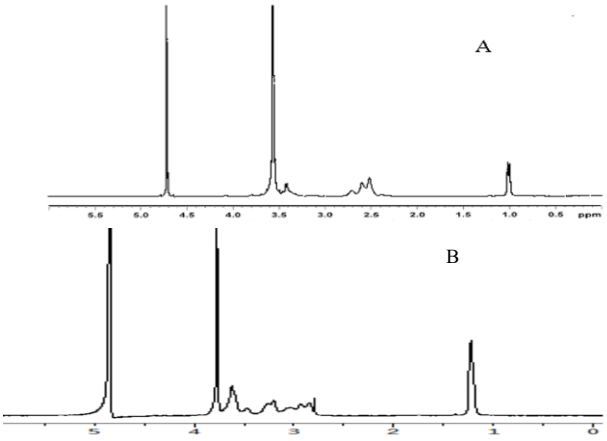

Fig. 1. ^{1}H- NMR spectra of P123-PEI (A) and P123-PEI-R18 (B).

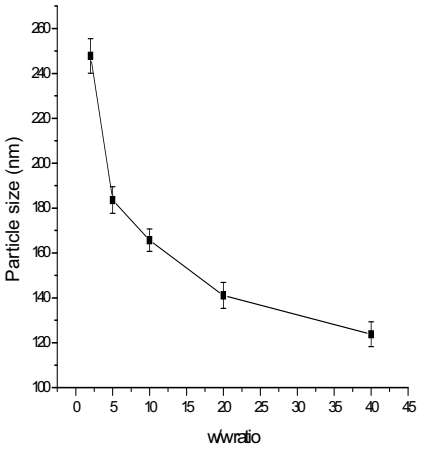

Fig. 2. Particle sizes of 123–PEI-R18/DNA polymer at w/w ratios of 2, 5, 10, 20 and 40.

3.2. Physical characteristic of P123-PEI-R18

Figure 2 showed the particle size decreased with the rise of P123-PEI-R18/DNA weight ratios. The complexes particle sizes ranged from 100 nm to 250 nm, which were suitable for gene delivery [11].

Gel retardation experiments could measure the DNA binding ability of P123-PEI-R18 (Figure 3A). The polymers could neutralize the negative charge of DNA and prevent DNA from moving to the anode [11]. DNA was condensed effectively by P123-PEI-R18 when the w/w ratio was 2.

DNA was protected by P123-PEI-R18 from the digestion of DNase I, and was released entirely under the effect of sodium heparin in the end [3]. Figure 3B showed that DNA was protected by P123-PEI-R18 until the concentration of DNase I reached 6 U DNase I/μg DNA. The stability of the P123-PEI-R18/DNA was evaluated by serum and sodium heparin. Figure 3C showed that the complex could not be dissociated even in 50% concentration of Serum. Sodium heparin was used to simulate negative charges carried by macromolecules in blood circulation [3]. P123-PEI-R18/DNA complexes were dissociated by sodium heparin when concentration was 200 μg/mL. Sodium heparin dissociated the complexes completely when concentration exceed 700 μg/mL (Figure 3D).These results indicated that the P123-PEI-R18/DNA had advisable stability in the blood.

3.3. Cytotoxicity assay

The HMW PEI was limited in transfection due to its high cytotoxicity [3]. The cell viability was determined with MTT assay at different concentrations contrast to PEI 25 kDa. P123-PEI-R18 showed lower cell toxicity than PEI 25 kDa at any ratios ($P< 0.01$) (Figure 4). P123-PEI-R18 exhibited low cytotoxicity at any concentrations, showing that the polymer was appropriate as gene vector. The lower cytotoxicity might be due to the lower amino group density compared with HMW PEI.

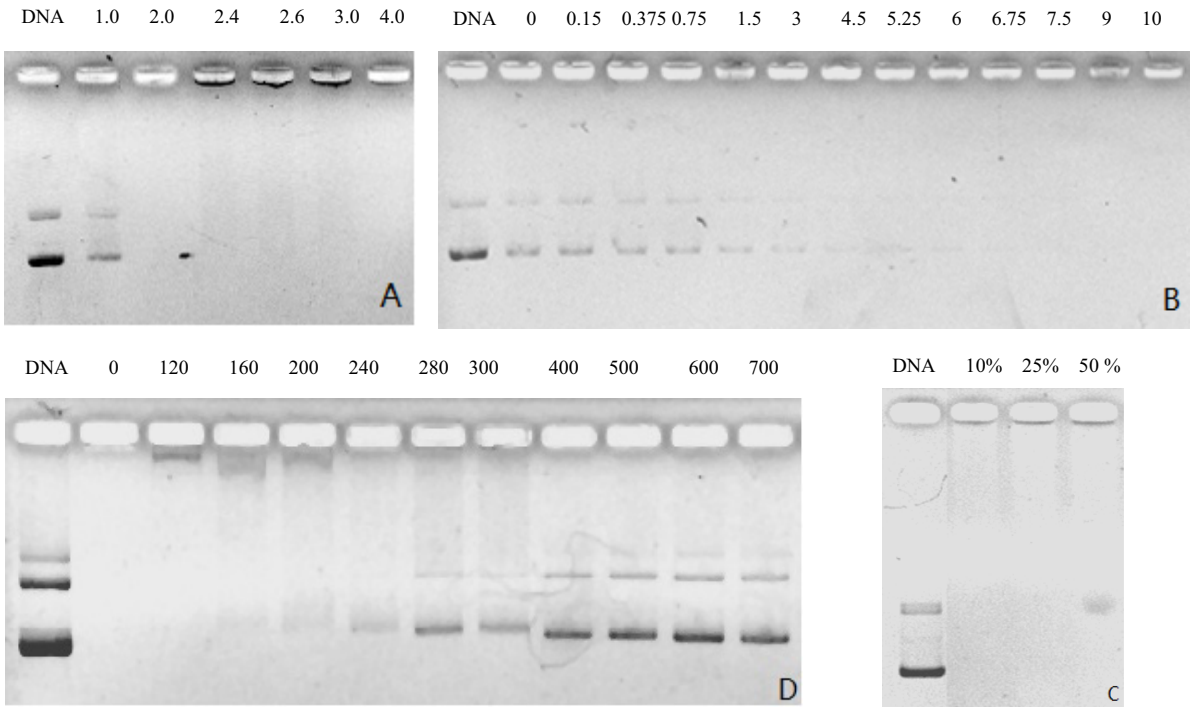

Fig. 3. Condensation and protection of plasmid DNA. (A) Agarose gel electrophoresis at varying w/w ratios; (B) Resistance dissociation by DNase I with different concentrations (DNase I/μg DNA); (C) Resistance dissociation by serum and (D) Resistance degratation by sodium heparin with different concentrations (μg/mL).

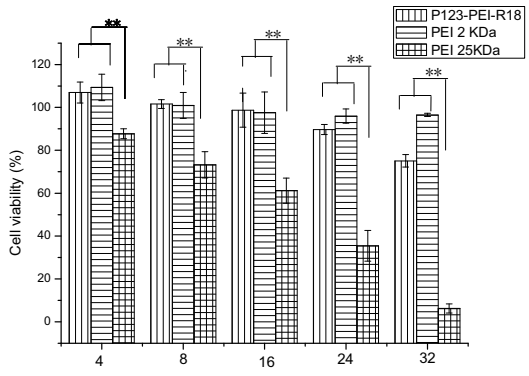

Fig. 4. Cell totoxicity of P123-PEI-R18 at varying concentrations using MTT assay(n=5,**p < 0.01).

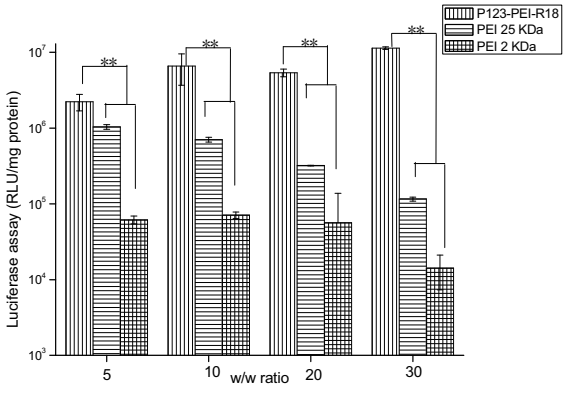

Fig. 5 Transfection efficiency of various polymer/ DNA complexes at B16 cell (n=5,**p < 0.01).

3.4. Transfection efficiency in vitro

Figure 5 showed the transfection efficiency of P123-PEI-R18/DNA complexes compared with PEI 25 kDa and PEI 2 kDa ($P < 0.01$). P123-PEI-R18 compounds exhibited higher ability of gene transfer contrast with PEI 2 kDa and PEI 25 kDa at any ratios. The P123-PEI-R18 complexes showed the optimal transfection efficiency with the weight ratio of 30. These results indicated that the P123-PEI-R18 complexes may serve as an efficient vector for non-viral gene vector in vitro.

4. Conclusion

A novel gene vector, P123-PEI-R18, was developed by cross-linking PEI with P123 and coupling a trifunctional peptide R18. It was evaluated by chemical structure, physical parameters and the gene transfection efficiency in vitro. The particle sizes of P123-PEI-R18/DNA complexes were range from 100 nm to 250 nm, which were proper for gene delivery. P123-PEI-R18 polymer could condense DNA entirely at the weight ratio of 2 and avoid digestion from DNase I when the concentration was 6 U DNase I/μg DNA. Meanwhile, P123-PEI-R18 polymer could resist dissociation induced by 700 μg/mL sodium heparin and 50% FBS. These results indicated that P123-PEI-R18 had suitable physicochemical characters as gene vector. Moreover, the polymer exhibited lower cytotoxicity and more remarkable transfection efficiency contrast to PEI 25 kDa. This novel polymer would probably become a promising reagent for gene therapy.

Acknowledgement

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