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Research paper

An efficient vector for gene delivery: α,β-poly (3-dimethylaminopropyl-D,L-aspartamide)

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Abstract

PSI, as the potential peptide-like intermediate, is subject to simple chemical modification in order to obtain good non-viral carriers for gene delivery. This paper describes the facile synthesis and preliminary evaluation of α,β-poly (3-dimethylaminopropyl-D,L-aspartamide) (PDAI) as a vector. Reaction of PSI with 3-dimethylamino-1-propylamine afforded PDAI in *N*,*N*-dimethylformamide (DMF) solution. Such biophysical properties of PDAI/DNA complexes as the particle size and the zeta potential were determined by dynamic light scattering assay. The complexes prepared at weight ratios ranging from 2 to 3 have an average size of around 200 nm and a zeta potential of around 10.0 mV. Gel electrophoresis assays confirmed that PDAI could compact DNA to form the complexes and protect DNA from enzymatic degradation by DNase I at the weight ratio above 2.0. Furthermore, PDAI was found to transfect HepG2 cells at a much higher efficiency than commercially available polyethylenimine (PEI) ($W_w = 75{,}000$ Da). MTT cytotoxicity assay demonstrated that PDAI also showed much less toxicity than did PEI, suggesting that PDAI is a new class of transfection reagent to be used as a safe vector.

Keywords: PDAI; PDAI/DNA complex; Gene delivery; Transfection efficiency

1. Introduction

Gene therapy holds a prospect for treatment and prevention of congenital and acquired diseases by introducing small DNA or RNA sequences into cells. The type of vector used is a key to success in gene delivery [1]. So far there have been two main approaches to gene delivery concerning both viral and non-viral vectors. Besides viral gene carriers, non-viral gene delivery systems such as cationic polymers are being investigated intensively to circumvent some of the problems encountered in using viral vectors [2,3]. To enhance transfection efficacy numerous cationic polymers [4,5] have been used for transfection. Though these polycations improve transfection in comparison to naked DNA, they still have relatively low transfection efficacy, or they are associated with the toxicity in vitro or in vivo, or their preparation is complicated.

Recently biodegradable polycations with hydrolysable chemical bonds are now emerging as a new generation of synthetic carriers. Peptide derivatives of poly-D,L-aspartamide (PSI) are gaining much attention. They show both flexibility and advantage in designing drug carriers [6–14] such as a large amount of drug loading capacity and the potential for low toxicity and superior biocompatibility. However, polycations[13,14] based on PSI are comparatively rare, their synthesis is also inconvenient.

We herein report our effort toward designing a new derivative of PSI, α,β-poly (3-dimethylaminopropyl-D,L-aspartamide) (PDAI), as a potential carrier for gene delivery. PDAI

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vector was conveniently synthesized from ring-opening reactions of primary amine to PSI. The polymer was characterized by NMR, elemental analysis and gel permeation chromatography (GPC). Its DNA complexation, ability to protect DNA against enzymatic degradation, and cytotoxicity were investigated. The transfection efficiency of PDAI/DNA complexes was evaluated in HepG2 cells using the reporter gene β-galactosidase. Lipofectin® and polyethylenimine were used as positive controls.

2. Materials and methods

2.1. Materials

D,L-Aspartic acid, linear poly (ethylenimine) (PEI) ($M_w = 75{,}000$ Da), *O*-nitrophenyl-β-D-galactopyranoside (ONPG), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were all purchased from Sigma Chemical Company (St. Louis, MO). Lipofectin® and RPMI Medium 1640 were obtained from Gibco, BRL (Grand Island, NY). The pSV-galactosidase control vector with SV40 early promoter, enhancer and *LacZ* gene was supplied by Promega Corporation (Madison, WI). The pSV-galactosidase plasmid was amplified in *Escherichia coli*, and then the plasmids were isolated and purified.

2.2. Preparation and characterization of PDAI

Poly-D,L-succinimide (PSI) was prepared as described in the literature [6]. Briefly, D,L-aspartic acid (10 g) was mixed with 5 g of 85% phosphoric acid in the flask. The flask was heated in an oil bath at 180 °C for 4 h. The vitreous mass obtained was dissolved into 50 ml of *N*,*N*-dimethylformamide (DMF) and the solution was poured into a beaker containing 200 ml of water. A flaky precipitation formed which was centrifuged, washed with water until neutral, and dried.

A solution of 3 g PSI in 15 ml DMF was separated and added to 10 ml *N*,*N*-dimethylamino-1-propylamine (DAP) or ethanolamine. The reaction mixture was rapidly stirred at ambient temperature for 24 h. The solution was then acidified with glacial acetic acid to pH 4. To remove excess DAP or ethanolamine, the solution was diluted with water, dialyzed against deionized water and lyophilized.

The NMR spectra of PDAI and PHEA were recorded with a Bruker DRX-500 NMR spectrometer in D_2O solution. Infrared spectra were recorded with a Nicolet 170SX FT-IR spectrometer. Samples were pressed into KBr pellets. The elemental compositions of PDAI and PHEA were determined by elemental analysis (EA-240C, Perkin-Elmer). The average molecular weight (M_w) and polydispersity (PD) of PDAI and PHEA were determined by GPC (TL-9800, Shimadzu) with a TSK-gel column (G3000SW, Tosoh Corporation, Japan). GPC was performed at a flow rate of 0.5 ml/min, the mobile phase was 1% acetic acid. The calibration curve was prepared with protein standards of different molecular weight including rabbit phosphorylase B (97,400 Da), bovine serum albumin (66,200 Da), rabbit actin (43,000 Da), bovine carbonic anhydrase (31,000 Da), trypsin inhibitor (20,100 Da) and hen egg white lysozyme (14,400 Da).

2.3. Preparation and characterization of PDAI/DNA complexes

PDAI was dissolved in 0.5% acetic acid. The pH of the solutions was adjusted to 5.0 with NaOH. The final concentration was 1 mg/ml. Then the solution was sterile filtered through a 0.22 μm filter, and diluted to 0.5 mg/ml by RPMI 1640.

The required volume of 500 μg/ml PDAI solution was added to PBS containing 50 μl of 0.2 mg/ml DNA (diluted from 1 mg/ml by RPMI 1640 by gentle pipetting to form complexes of a selected weight ratio). The mixture was vortexed rapidly for several seconds and left for 30 min to ensure the complexes would completely form at room temperature.

The diameters and zeta potentials of the complexes were determined by photon correlation spectroscopy by using a 90 Plus Particle Sizer (Brookhaven Instruments, Holtsville, NY). The physical stability of each complex was studied by agarose gel electrophoresis (0.9% agarose in TAE buffer).

2.4. Nuclease resistance of PDAI/DNA complexes

Each sample contained 2 μg DNA complexed with PDAI at different weight ratios. After incubation for 30 min at room temperature, 200 U/ml DNase I (5 units) buffer (50 mM Tris–Cl, pH 7.6, and 10 mM $MgCl_2$) was added, and each sample was incubated at 37 °C. Samples were then loaded onto a 0.8% agarose gel stained with EtBr and subjected to electrophoresis.

2.5. Cytotoxicity evaluation

Cytotoxicity of different reagents was measured using the MTT dye reduction assay. HeLa, L929 and HepG2 cells were seeded in a 96-well plate at a density of 2.0×10^4 cells/well and incubated overnight as described in the transfection session. Then the cells were incubated in 100 μl serum free medium containing selected amount (from 1 to 35 μg) of PDAI, PHEA and PEI. After 12 h, the medium was removed and the cells were rinsed twice with PBS. The wells were refilled with complete medium and cells were cultured for another 24 h. Next, 20 μl of MTT (5 mg/ml) solution was added into each well and was allowed to react for 4 h at 37 °C. A total of 200 μl of DMSO was added to each well and the plate was incubated for 30 min at room temperature. Absorbance at 490 nm was measured with an ELISA plate reader (Bio-Rad, Microplate Reader 3550).

2.6. Study of gene transfection

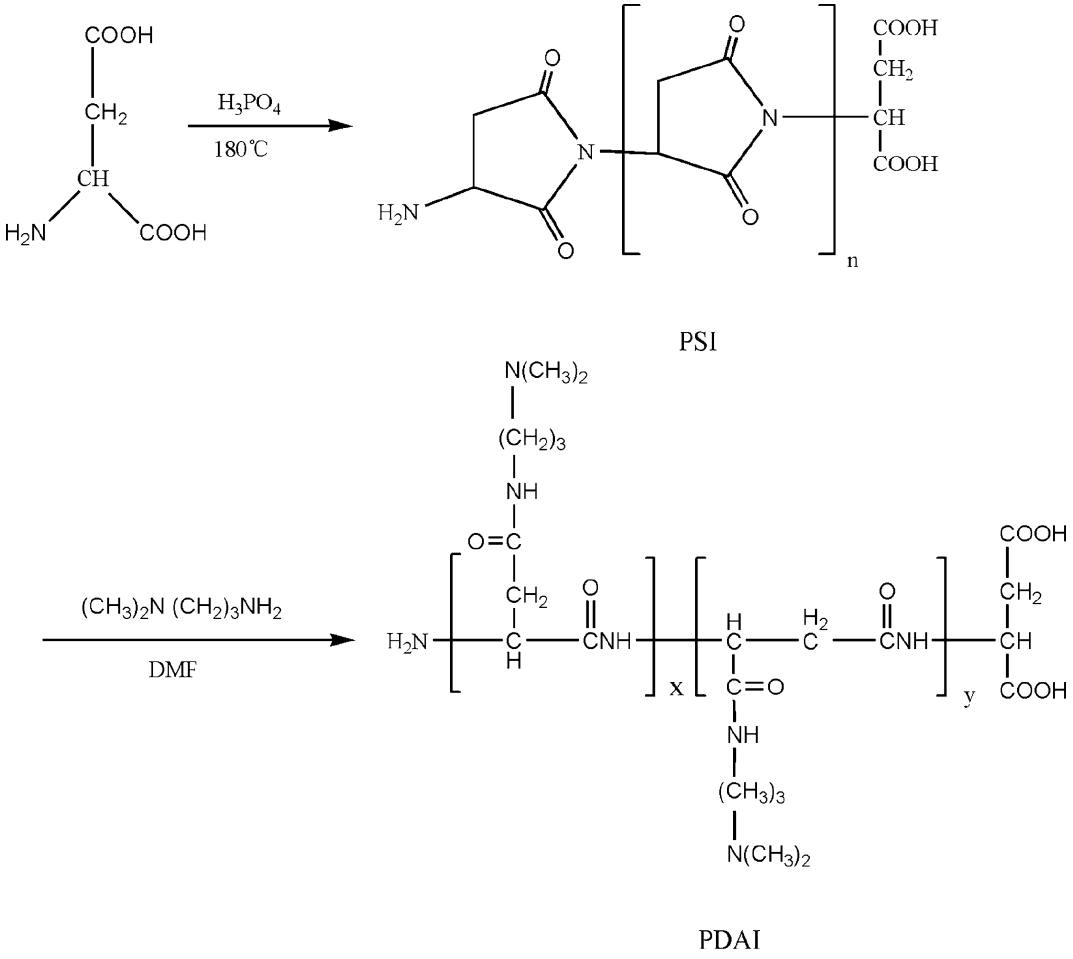
In a typical transfection experiment, HepG2 cells were seeded at a density of 5.0×10^5 cells/dish in 60 mm culture dishes with 5 ml of complete medium (RPMI 1640 containing 10% serum) and incubated for 24 h prior to transfection. Transfection was performed on cells that were approximately 70% confluent. Before transfection, the complete medium was removed and cells were rinsed once with 1× PBS. The naked DNA, PDAI/DNA complexes and PEI/DNA (containing 300 μg of DNA) were diluted in 2 ml RPMI 1640 medium, and then were used to refill the dishes. After incubation at 37 °C for 6 h, serum and RPMI 1640 medium were added to the dishes so that the final volume of medium was 5 ml and contained 10% serum. After another 18 h, the medium containing complexes was removed. The cells were rinsed twice with 1× PBS, harvested, and resuspended in 1× PBS. Transfection with lipofectin/DNA complexes was performed as a positive control. To determine the transfection efficiency, the β-galactosidase activity was measured through ONPG assay [15] using a UV spectrophotometer (Shimadzu UV-2201).

3. Results and discussion

3.1. Preparation and characterization of PDAI

PDAI was easily prepared by complete aminolysis of PSI in DMF solution at room temperature. During the reaction, a partial racemization occurred and opening of the succinimide rings proceeded at two sites, so the final product possessed D,L configuration and α,β-structure. Scheme 1 gives the synthetic route and the chemical structure of PDAI.

Spectroscopic data (FT-IR and ^{1}H NMR) about PHEA are in accordance with a previous report [16]. FT-IR spectrum of PDAI shows structural bands at: 3360 cm^{-1}(–NH– and –NH$_2$); 1720.7 cm^{-1} (amide I) and 1651 cm^{-1} (amide II) are assigned to the peptide bond. ^{13}C NMR spectrum of PDAI reveals the characteristic peaks at: δ 43.92 (–N(**C**H$_3$)$_2$), δ 170–175 (**C**=O), δ 26.87, δ 26.23 and δ 56.04 (–**C**H$_2$–**C**H$_2$–**C**H$_2$–N=). In addition, its ^{1}H NMR spectra clearly manifested the peaks at: δ 3.22 (s, 6H, –N(C**H**$_3$)$_2$), δ 2.81 (m, 2H, –CH–C**H**$_2$–CO–NH–) and 4.64 (m, 1H, –NH–C**H**–CO–CH$_2$–) belonging to the protein backbone.



Scheme 1. Synthesis of PDAI.

Table 1
Physicochemical character of PDAI

Sample	Theoretical %			Experimental %			M_w (kDa)	PD
	C	H	N	C	H	N		
PHEA	54.27	8.54	21.10	53.58	8.81	20.53	72	1.27
PDAI	45.57	6.32	17.72	45.69	6.19	17.84	65	1.43

Table 1 shows the results of the physical properties for PDAI and PHEA. The content of C, H and N elements is found to be nearly parallel to the theoretical value. Polydispersity (PD) index of polymers is around 1.0, indicating that PDAI and PHEA have comparatively homogeneous molecular weights.

3.2. Characterization of PDAI/DNA complexes

The interaction between the plasmid and the polycations at different ratios is analyzed by gel retardation electrophoresis. Fig. 1 shows that the plasmid DNA in complexes migrated in agarose gels. As the proportion of PDAI increased, there was a drop in the ethidium bromide staining intensity of DNA that entered the gel. At weight ratios of 0.4 or 0.6, a fraction of plasmid is free to migrate in the gel. Entire retardation occurred when the ratio increased to 2.0. This polymer property is important for the condensation of DNA and transfection efficiency.

Surface properties of PDAI/DNA complexes were determined by laser light scattering. The particle size and zeta potential of PDAI/DNA complexes varied with weight ratios, as shown in Fig. 2. Measurements of zeta potential also helped to confirm that the PDAI is able to bind DNA. With the weight ratio increasing, the zeta potential was observed to gradually rise (Fig. 2(A)). Neutral surface charge was observed when aggregates formed. The positive values of zeta potential indicated that the surface charge prevented aggregation of particles. The slightly positive zeta potential (around 10 mV) was reported to result in the best transfection efficiency [17,18], so PDAI/DNA complexes prepared at weight ratio varying from 2 to 3 were expected to be suitable for the gene transfer into cells. Finally, the surface zeta potential increased to approximately +28.0 mV when the ratio was above 5.0.

The size of a polymeric gene delivery complex is known to dramatically affect transfection efficiency [19,20]. Fig. 2(B) shows how particle size depends on the weight ratio. Particle size increased when the weight ratio was lower than 0.8, at which point polyplexes were observed to have the biggest size and tendency to aggregate. Although the weight ratio went up from 2.5 to 5, particle sizes remained at around 200 nm with a low standard error, indicating that the PDAI is capable of forming homogeneous nanoparticles with DNA due to its highly positively charged tertiary amine. These results also indicated that it was possible to prepare complexes of a proper particle diameter for high transfecting efficiency by adjusting the weight ratio.

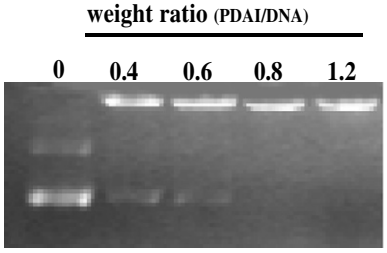


Fig. 1. Gel retardation assay of PDAI/DNA complexes.

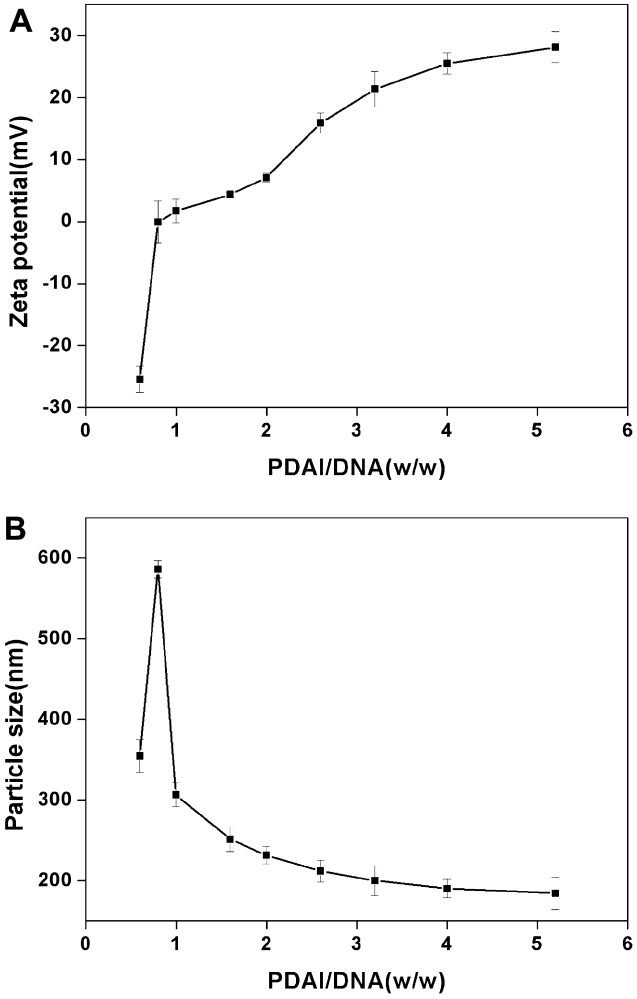


Fig. 2. (A) Zeta potential of PDAI/DNA complexes and (B) particle size of PDAI/DNA complexes.

3.3. Nuclease resistance of PDAI/DNA complexes

Protection of plasmid DNA from nucleases is one of the crucial factors for efficient gene delivery in vivo as well as in vitro [21] and the protective effect to DNA in the complexes was examined using DNase I as a model enzyme. Fig. 3 reveals that naked plasmid DNA was nearly completely degraded within 30 min, some extent of degradation could be observed at a weight ratio of 1.0, for the complex-

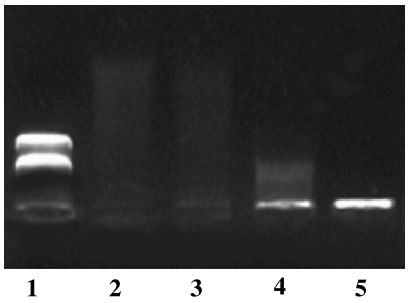


Fig. 3. Degradation effect of PDAI/DNA complexes by nuclease. From the left to the right examples in the lanes was separately: (**1**) naked DNA; (**2**) naked DNA + DNase I; (**3**) PDAI/DNA (0.25:1) + DNase I; (**4**) PDAI/DNA (1:1) + DNase I; (**5**) PDAI/DNA (2:1) + DNase I.

es having a less compact structure failed to fully protect DNA from enzymatic degradation. Whereas DNA was condensed with PDAI at the ratio of 2.0, the plasmid DNA was efficiently protected from the enzymatic hydrolysis.

3.4. Cytotoxicity evaluation

Fig. 4 shows the toxicity of PDAI to L929, HeLa and HepG2 cell lines compared to PHEA and PEI. PEI is reported to be very toxic to cell lines [22,23], our study also gave similar results, the dose-dependent cytotoxicity was observed with increasing concentrations of PEI, inducing a higher toxicity than PHEA and PDAI due to the fact the PEI has proved to trigger lysosomal disruption in cells [24,25]. In comparison, PDAI was found to be much less cytotoxic to three cell lines tested. It is also interesting to note that both HepG2 cells and HeLa cells were observed to grow well at the concentration of PDAI or PHEA even as high as 1000 μg/ml. We believe that the biodegradable peptide structure is a key factor to low cytotoxicity to cellular viability. The results further demonstrated that PDAI is a biocompatible and safe vector.

3.5. Gene transfection study

PDAI was assessed for in vitro transfection efficiency in HepG2 cells at different weight ratios ranging from 0.66 to 6.67 and compared to commercially available PEI and lipofectin available for transfection reagents. The results based on the expression of β-galactosidase reporter gene are illustrated in Fig. 5. Using the PDAI/DNA complexes, it was found that the β-galactosidase activity increased as the weight ratio increased from 0.66 to 2.67. The maximal activity of gene expression occurred at a weight ratio of 2.67. When the weight ratio exceeded 2.67, a gradual decline was observed. Similar to PDAI/DNA complexes, the transfection efficiency of PEI/DNA complexes was dependent on the weight ratio, but PDAI showed prominent improvement in the transfection ability compared to PEI, especially the optimal transfection effect reached around 75% level of galactosidase activity compared to

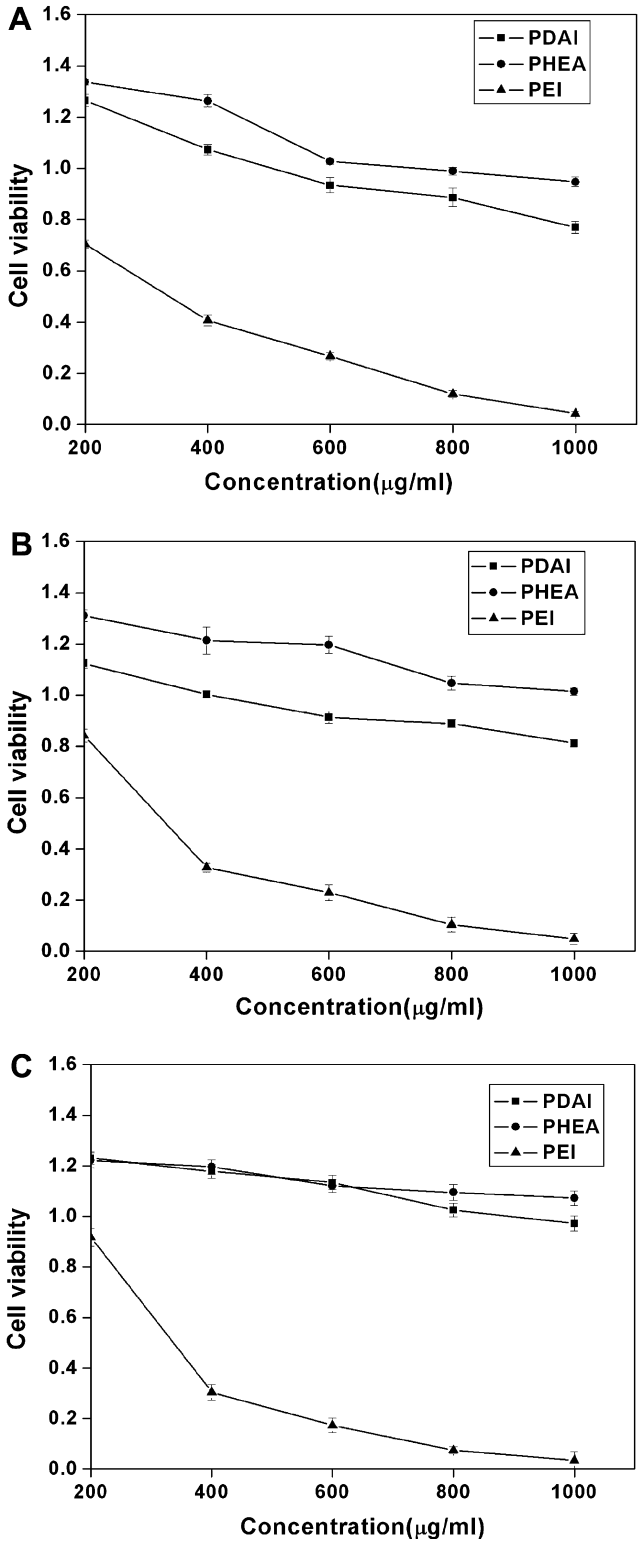


Fig. 4. Cytotoxic valuation of PDAI in (A) L929 cells, (B) HeLa cells and (C) HepG2 cells.

lipofectin. The most effective transfection of PEI/DNA was obtained at a ratio of 1.33. The different optimum ratios in the transfection of PEI/DNA and PDAI/DNA complexes may be associated with the difference in charge density or the biocompatibility of the polymers.

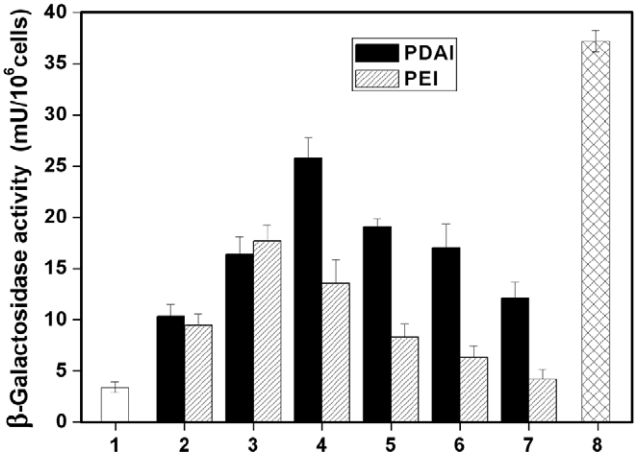


Fig. 5. Weight ratio-dependent β-galactosidase expression in HepG2 cells transfected with PDAI/DNA complexes in comparison with PEI/DNA. Results are expressed as mean values ± SD (**1**) naked plasmid; (**2**) PDAI (PEI)/DNA = 0.66; (**3**) PDAI (PEI)/DNA = 1.33; (**4**) PDAI (PEI)/DNA = 2.67; (**5**) PDAI (PEI)/DNA = 4; (**6**) PDAI (PEI)/DNA = 5.33; (**7**) PDAI (PEI)/DNA = 6.67; (**8**) Lipofectin. Results are expressed as mean values ± SD from one representative experiment ($n = 6$) of three performed ($P < 0.05$).

The surface of mammalian cells is negatively charged due to carboxylic groups of the sialic acid of glycoproteins, the sulfate groups of proteoglycans and the phosphate groups of glycerophosphates [26]. Suitable size and surface charge are necessary for the gene vector to cross cellular membranes. Highly positively-charged complexes may interact more strongly with the cellular surface so as to trigger cellular endocytosis. In addition, the bigger size and too many positive charges may activate the lysosomal system and eventually lead to complex removal [27], so transfection efficiencies are expected to drop when the weight ratio rises to a certain value. The transfection levels decreased most likely due to the increasing toxicity of the vector as can be seen in Fig. 4.

The gene-activated matrices (GAM) are a platform for gene delivery and as bioreactors for seeding cells to secrete plasmid-encoded proteins that enhance natural healing process. The encapsulated DNA content can be controlled by different ratios with polymers and the conditions in the incorporation process [28,29]. PDAI, as a polycationic biomaterial with good biodegradability, little cytotoxicity and high gene transfection efficiency, will be attempted as a GAM material in future research.

4. Conclusions

PSI in DMF solution allowing for facile modification yields PDAI. The polycation with the biodegradable peptide linkage was further characterized with DNA forming polyplexes, exhibiting the ability to form stable nanoparticles and protect DNA against enzymatic degradation. The bioconjugate proved to have little cytotoxicity in mammalian cells, and high transfection efficiency comparable to that of PEI in HepG2 cells. Therefore, PDAI has the potential to become a biocompatible and efficient gene delivery system.

Acknowledgements

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