Polyethylenimine-grafted polycarbonates as biodegradable polycations for gene delivery

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Polycations as one of non-viral vectors have gained increasing attentions. In this paper, polyethylenimine(PEI)-grafted polycarbonates (PMAC-g-PEIx) were synthesized as a kind of biodegradable polycations for gene delivery. Backbone polymer, poly(5-methyl-5-allyloxycarbonyl-trimethylene carbonate) (PMAC), was synthesized in bulk catalyzed by immobilized porcine pancreas lipase (IPPL). Then, PMAC-O, the allyl epoxidation product of PMAC, was further modified by PEI with low molecular weight (x = 423, 800 and 1800). The MWs of PMAC-g-PEIx, measured by GPC–MALLS, were 81,900, 17,9900 and 200,600 g/mol with polydispersities of 1.2, 1.4 and 1.7, respectively. PMAC-g-PEIx could form positively charged nano-sized particles (30–90 nm) with pDNA, and all the three PMAC-g-PEI/DNA polyplexes had similar buffer capabilities. In vitro experiments demonstrated that the PMAC-g-PEIx showed much low cytotoxicity and enhanced transfection efficiency could be found in comparison with PEI25K in 293T cells. Furthermore, pre-incubation of PMAC-g-PEI1800 showed a weakening binding capacity with DNA. The biodegradability of PMAC-g-PEIx can facilitate the efficient release of pDNA from polyplexes and reduce cell cytotoxicity. These results suggested that PMAC-g-PEIx would be a promising non-viral biodegradable vector for gene delivery system.

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1. Introduction

Gene therapy has been attracting more attentions for the treatment of genetic and acquired diseases that are currently incurable, such as immunodeficiency [1], cystic fibrosis [2], and Parkinson’s disease [3]. In response, two primary gene vehicle classes have been developed: recombinant viruses and non-viral vectors [4]. The former has high transfection efficiency for both DNA and RNA to numerous cell lines [5], but including toxicity, immunogenicity, combining limited molecular DNA and hard for large-scale preparation acceptable for clinical testing [6]. Alternatively, non-viral vectors, such as liposomes, cationic polymers, dendrimers, nanoparticles and so on, show many advantages including less immune reaction to tissue, control of the structure of molecule, cell/tissue targeting, fitting various plasmid sizes, low cost and easy to synthesis [7].

Among non-viral systems, polycations are proposed because it is easy to form self-assembly with DNA [7]. Polyethylenimine (PEI) has been one of the most popularly and powerfully employed cationic gene carriers. High molecular weight PEI, such as PEI25K, shows a high transfection efficiency but also a high cell cytotoxicity. Low molecular weight PEI (Mw ≤ 2000), by contrast, has demonstrated a low cytotoxicity but also cannot be used as gene vectors due to rather unsatisfactory transfection efficiency. Therefore, modifying low molecular weight PEI as gene carriers are extensively investigated now, such as Michael addition of PEI (x = 600, 1200 and 1800) with polycaprolactone diacrylate [8], cycloexodrin (CD)-modified PEI derivatives [9], dextran-hexamethyleneisocyanate grafted PEIs [10], diithiobis(succinimidylpropionate) or dimethyl-3,3’-dithiobispropionimidate modified low molecular weight PEI [11]. The transfection efficiency of these materials was comparable to that of PEI25K and the cytotoxicity was reduced to some degree [8–11]. The results showed that the charge density should not be too high which may combine membrane and destroy the cells metabolism, while the degradability of polycations would benefit to decrease the cytotoxicity. However, it remains challenging to obtain efficient gene vectors by careful molecular design, which combine the advantages of PEI25K with the low cytotoxicity of the low molecular weight PEIs.

Aliphatic polycarbonates are one of the important biodegradable polymers with good biocompatibility, favorable mechanical properties, low toxicity and biodegradability [12]. The properties of aliphatic polycarbonates could be further modified and designed by introducing pendant functional groups, which can be used to not

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only adjust the properties of polycarbonates but also facilitate further modification. Recently, aliphatic polycarbonates with pendant functional groups have been synthesized by ring-opening polymerization (ROP), such as hydroxyl [13], amino [14], allyl [15], carboxyl [16], etc. The synthesis of novel cyclic monomers containing a variety of functionalities has become an important method for the development of new biodegradable polymer materials with special properties for different applications.

Tin(II) 2-ethylhexanoate, which has been approved for surgical and pharmaceutical applications by the FDA, is generally employed as the catalyst for the synthesis of biomedical polymers. However, it has been reported that tin(II) 2-ethylhexanoate cannot be removed by a purification process such as the dissolution/precipitation method; thus, the residual Sn may be concentrated within matrix remnants after hydrolytic degradation [17]. To avoid the potential harmful effects of metallic residues in biomedical polymer materials, enzymatic polymerization is one of the powerful candidates for polymer synthesis [18]. Up to now, various kinds of biodegradable polymers have been synthesized by enzymatic ring-opening polymerization, such as polystyres and poly-carbonates [18–20]. In our previous studies, we have reported that immobilized porcine pancreas lipase (IPLL) on silica particles can effectively catalyze the ring-opening (co)polymerization of different six-membered cyclic carbonates [20,21]. The stability and recyclability of native enzyme can be improved significantly by immobilization [22].

In this paper, a biodegradable polycarbonate, PMAC-g-PEIx, is presented as a non-viral gene vector. Grafted PEI residues were introduced to form stable polyplexes with pDNA due to their strong positive charges, while backbone polycarbonates was employed to reduce the cytotoxicity by controlling the charge density and preventing from combining cell membrane with its hydrophobic property. And also the biodegradability of polycarbonates would benefit to release DNA easily. Physicochemical properties of this vector were characterized and the DNA loading was evaluated.

2. Materials and methods

2.1. Materials

PMAC was synthesized according to the literature [23,24] and recrystallized for several times before use. 3-Chloroperoxybenzoic acid (mCPBA, 70–75% water) was purchased from Sigma–Aldrich Co. and used as received. PEI (x = 423, 800 and 25K) and PEI1800 were purchased from Sigma–Aldrich Co. and Alfa Co., respectively. IPLL was prepared according to He et al. [21, 3–4-[3-Dimethylamino]propyl]-3-aminopropyltrimethoxysilane (MTT), Dubeczko’s phosphate buffered saline (PBS), 3-Dubeczko’s Modified Eagle’s Medium (DMEM), fetal bovine serum (FBS) and trypsin purchased from Invitrogen Corp. GelRed™ was purchased from Biotime (CA, USA). The human embryonic kidney transformed (293T) cells were incubated in DMEM containing 10% FBS and 1% antibiotics (penicillin–streptomycin, 10,000 U/C, and a Malls device (DMW EDS, Wyatt Technology) as the index detector. Ultrapure water (20% acetonitrile, weight ratio) was used as eluent. Flow rate was 1.0 ml/min.

2.2. Synthesis of PMAC-g-PEIx

2.2.1. Synthesis of PMAC by enzymatic ring-opening polymerization

The vessel containing MAC and IPLL (0.25 w/w) with a magnetic stirring bar was sealed and dried with anhydrous phosphorus pentoxide as desiccant in vacuum. Then the vessel was placed in an oil bath at 80 °C for 2 h. The reaction mixture was dissolved in dichloromethane and the insoluble IPLL was removed by filtration. The solvent was condensed and poured into methanol to precipitate the polymer. The resulting polymer was dried in vacuo (yield: 86%).

2.2.2. Allyl epoxidation of PMAC

PMAC (0.10 g), 4 molar excess of mCPBA (70–75% water) and 5 mL CHCl3 were charged into a flask with a magnetic stirring bar. The reaction mixture was refluxed for 12 h. Then the solution was filtered and precipitated into methanol twice. The obtained polymer (PMAC–O) was dried in vacuo (yield: 72%).

2.2.3. Synthesis of PMAC-g-PEIx

PMAC–O (0.10 g), 10 molar excess of PEIx (x = 423, 800 and 1800) and 20 mL CHCl3 with a magnetic stirring bar in a flask was placed in oil bath at reflux for 2 h. The mixture was condensed and 1 mL HCl aqueous solution was added. Then the neutralized products were dialyzed against distilled water (MWCO: 3500) at 4 °C for 24 h. Then the solution was lyophilized for 2 days to obtain the PEI-grafted polycarbonates (PMAC-g-PEIx).

2.3. Characterization

1H NMR spectra were performed on a Mercury VX-300 spectrometer using CDCl3 or D2O–DMSO as the solvent. Gel permeation chromatography (GPC) measurements were performed on Waters 2690 HPLC equipped with Ultrahydrogel 250 and 2000 columns (temperature 40 °C, and a Malls device (DMW EDS, Wyatt Technology) as the index detector. Ultrapure water (20% acetonitrile, weight ratio) was used as eluent. Flow rate was 1.0 ml/min.

2.4. MTT cytotoxicity assay in vitro

The cytotoxicity of the resulting polycations was evaluated by MTT assay in 293T cells using PEI25K as the control. Generally, the 293T cells were seeded in a 96-well tissue culture plate at a density of 6000 cells/well in 100 μL DMEM medium containing 10% FBS for 24 h, and were exposed to the polyplex solutions at a series of concentrations for another 48 h. Then, 20 μL MTT (5 mg/mL) solutions was added to each well and further incubated for 4 h. After that, the medium was exchanged by 150 μL DMSO to dissolve the formazan crystal formed by proliferating cells. The concentration of the proliferating cells in each well was confirmed by the absorbance of solvent at 570 nm using a microplate reader (Bio-Rad, Model 550, USA). The relative cell viability (%) was calculated according to the following:

\[
Cell\% = \frac{A_{sample} - A_{control}}{A_{control}} \times 100%
\]

where A_{sample} was obtained in the presence of polymers and A_{control} was obtained in the absence of polymers. The results were presented as the average values of four runs.

2.5. Agarose gel electrophoresis

pDNA condensing ability of PMAC-g-PEIx was examined by agarose gel electrophoresis. PMAC-g-PEIx/DNA polyplexes at various weight ratios ranging from 0.1 to 1.75 were prepared by adding appropriate volumes of polymer solution (2 mg/mL) to 1.3 μL of pG3-3 DNA (77 ng/μL in 40 μM Tris–HCl buffer solution). The volumes of the polyplexes were made up to a total volume of 6 μL with 150 mM NaCl solution. The polyplexes were then incubated at room temperature for 30 min, and were loaded on agarose gel (0.7%, w/v) containing GelRed and with Tris–acetate (TAE) running buffer at 80 V for 80 min. The location of pDNA bands was visualized with a UV lamp using a Vilber Lourmat imaging system (France).

2.6. Zeta potential measurements

Zeta potential measurements of polyplexes were carried out using a Nano-ZS 3600 (Malvern Instruments, USA) with a He–Ne laser beam (633 nm, fixed scattering angle of 90 °) at 25 °C. PMAC-g-PEIx/DNA polyplexes at weight ratios ranging from 2.5 to 160 were prepared by the same method with above agarose gel electrophoresis. After 30 min incubation in 100 μL ultrapure water, polyplex solutions were diluted to final volume of 1 mL before measurements.

2.7. Morphology of PMAC-g-PEIx/DNA polyplexes

PMAC-g-PEIx/DNA polyplexes were prepared at weight ratios of 10 and 60. Then the morphologies of the resulting polycations were observed by FEI10-200 transmission electron microscope (TEM). The samples were prepared by dropping materials solution onto the copper grid with Formvar film and dried at 30 °C.
adding 0.1 M NaOH, and then the solution was titrated by 0.1 M HCl. During titration, the pH value of the mixture was measured using a microprocessor pH meter (pH 211, Germany).

2.9. Transfection experiments in vitro

The pGL-3 and pEGFP-C1 plasmid DNA were both used to evaluate the transfection efficiency of PMAC-g-PEIx. For comparison, PEI25K at the N/P ratio of 10 was used as the positive control. The 293T cells were seeded at a density of 6 × 10⁴ cells/well in a 24-well plate in 1 mL DMEM medium containing 10% FBS and grown to reach 70–80% confluence prior to transfection. After incubation 30 min at room temperature, the cells were treated with polyplex solution (1 µg/pDNA) at various weight ratio from 2.5 to 160 for 4 h at 36 °C. Then, medium of each well was exchanged for fresh medium, and the cells were further incubated for another 48 h before assay.

For green fluorescent protein assay, the cells expressing green fluorescent protein were observed by the fluorescence-activated cell sorting. For luciferase assay, the growth medium was removed and the cells were washed by 200 µL PBS and then lysed using 200 µL Reporter Lysis Buffer (Piez). After two cycles of freezing and thawing, the solution was transferred into microtubes and centrifuged for 3 min. Luciferase activity of the supernatants was measured using 100 µL of luciferase assay reagent on a Lumat LB 9507 tube luminometer (Berthold) for 30 s. The Light units (LU) valued by a Protein Assay Reagent Kit (Piez) were normalized against protein concentration in the cell extracts, which were measured with chlorin-luminometer (Lumat LB9507, Egkg: Berthold, Germany). The total protein was measured according to a BCA protein assay kit (Piez) and luciferase activity was reported in terms of RLU/mg cellular protein. Data were shown as mean ± standard deviation (S.D.) based on two independent measurements.

2.10. Pre-incubation of PMAC-g-PEIx for DNA binding

The property of release DNA associated with the degradation of polycations was further investigated using PMAC-g-PEI1800 as a model. The polycations were dissolved in 150 mM NaCl solvent (2 mg/mL) and incubated at 37 °C for different periods. Then the ability of polycations to condense pGL-3 was evaluated by agarose gel electrophoresis.

3. Results and discussion

3.1. Synthesis and characterization of polymers

As summarized in Scheme 1, a multi-step reaction was performed for the synthesis of the biodegradable PMAC-g-PEIx vectors construct comprising PEI covalently linked to a PMAC backbone. As shown, polycarbonates with pendant allyloxycarbonyl group, poly(5-methyl-5-allyloxycarbonyl-trimethylenecarbonate)(PMAC), was synthesized in bulk using IPPL as the catalyst. The ring-opening polymerization was carried out at 80 °C for 2 h, while the products would be insoluble for a longer time than 2 h. It is probably due to high reactivity of the ester bond which resulted in the uncontrollable occurrence of transesterification reaction during the polymerization.

![Scheme 1. Synthesis of PMAC, PMAC–O and PMAC–g-PEIx (x = 423, 800 and 1800).](image)

![Fig. 1. 1H NMR (300 MHz) spectra of PMAC (CDCl₃), PMAC–O (CDCl₃), as well as PMAC-g-PEI1800 (D₂O–DMSO, volume ratio of 1:5).](image)
The allyl groups of PMAC were then completely epoxidized by 3-chloroperoxybenzoic acid in refluxing chloroform for 12 h to gain PMAC–O. The results could be confirmed by the 1H NMR shown in Fig. 1. For both PMAC and PMAC–O, 1.1–1.2 ppm were assigned to –CH3, 4.2–4.3 ppm were assigned to –OCOCH2C–, and 4.5–4.6 ppm were assigned to –OC2H2CH2. In the PMAC spectrum, characteristic allyl peaks (5.8–5.9 and 5.2–5.3 ppm) were clearly shown. After epoxidation reaction, the allyl proton signals disappeared and the oxirane proton characteristic signals (3.2–3.3 and 2.6–2.8 ppm) appeared. And also the signals of –OC2H2CH2 around 4.5 ppm were separated to 4.5 and 3.9 ppm. The resulting active epoxy groups of PMAC–O could provide an effective approach to further modification by its reactions with many nucleophilic reagents, such as alcohols, amines and carboxyl acids.

In designing the polycations, three different low molecular weight PEIx (x = 423, 800 and 1800) were chosen for their low toxicity. Briefly, PEI was covalently grafted onto PMAC–O via its exposed amino groups. PEI (10 molar equiv.) was employed to perform the PEIylation completely. The reaction mixture was then neutralized with 1 m HCl to form ammonium salt. In a neutral circumstance, the PMAC-g-PEIx should be more stable by avoiding the possible nuclear attack of PEI molecules due to the susceptibility of ester bonds on polycarbonates backbone. Fig. 1 showed that the oxirane proton characteristic signals were lapped over by PEI signals at 2.6–3.2 ppm. PMAC-g-PEIx was insoluble in CHCl3 but soluble in H2O. However, 1H NMR spectrum of PMAC-g-PEIx in D2O only showed PEI and D2O signals, due to the amphiphilicity of PMAC-g-PEIx polymers resulting in self-assembly potential. Therefore, 1H NMR spectrum of PMAC-g-PEI1800 in D2O–DMSO was shown in Fig. 1, that –CH2 characteristic signals of PMAC was visible.

The molecular weight of PMAC-g-PEIx was also measured by GPC–MALLS system. The results showed symmetric and narrow
molecular weight distributions. As expected, increasing the grafted-PEI molecular weight resulted the increase of polyacations molecular weight. The MWs of PMAC-g-PEx (x = 423, 800 and 1800) were 81,900, 179,900 and 200,600 g/mol and their PDI values were 1.2, 1.4 and 1.7, respectively. And also, the calculated compositions of PMAC-g-PEx from 1H NMR spectrum were close to theoretical values, that almost 100% of pendent epoxide groups of PMAC-O were modified with PEI.

3.2. Cytotoxicity assay of PMAC-g-PEx in vitro

MTT assay was performed in order to examine the cytotoxicity of polyplexes. Generally, the cytotoxicity of polyacation depends on the molecular weight, the cell binding and permeable membranes [26]. It is reported that one reason of the cytotoxicity of polyacations is resulted by aggregation and adherence on cell surface to form significant necrosis [27]. Previous works have also reported that biodegradability of polymeric gene carriers can reduce their cytotoxicity [28,29]. The cytotoxicity of PEI25K was found to be very high as well-known. In contrast, as shown in Fig. 2, PMAC-g-PEx polyplexes were observed to exhibit much lower cytotoxicity compared to PEI25K. It may be because that PMAC backbone could shield and reduce the positive charge density which resulted in partially preventing its combination with cellular surface, while biodegradability would also contribute to its lower cytotoxicity. Additionally, the cytotoxicity of PMAC-g-PEx showed a strong correlation with the PEI molecular weight, in good agreement with PEx series. On the other hand, at the concentration lower than 0.7 mg/mL, PMAC-g-PEI423 and PMAC-g-PEI800 had low cytotoxicity than PEI423 and PEI800, respectively, while the cytotoxicity of PMAC-g-PEI1800 was higher than that of PEI1800. PMAC backbone grafted by PEI with higher molecular weight showed a lower contribution to reduce the charge density. The backbone polymer contents within PMAC-g-PEI1800 was comparatively lower than that in PMAC-g-PEI800 and PMAC-g-PEI423.

3.3. Buffer capability of PMAC-g-PEx

The buffer capability will help the polyplex to escape from the endosomes and promoting transfection efficiency [30] according to the “proton sponge” hypothesis [31]. In this paper, the buffer capabilities of PMAC-g-PEx, PEx and also PEI25K were evaluated by acid-base titration assay over the pH values ranging from 10.00 to 2.00. NaCl (150 mM) solvent was employed as the control. As expected, the acid-base titration curves of PMAC-g-PEx were almost identical with that of PEI25K and PEx (shown in Fig. 3). Moreover, the buffer capabilities of PMAC-g-PEx were slightly lower than that of PExs, respectively, while PMAC-g-PEI1800 showed a high buffer capability than PMAC-g-PEI800 and than PMAC-g-PEI423. The higher the charge density was, the better the buffer capability showed. PMAC-g-PEx series showed good buffering capacity in the pH range 5–7.

3.4. Characterization of PMAC-g-PEx/DNA polyplexes

Electrostatic interactions between the negatively charged nucleic acids and the cationic PMAC-g-PEx series induced formation of PMAC-g-PEx/DNA polyplexes, which was investigated by agarose gel retardation and TEM measurements.

As shown in Fig. 4, the ability of PMAC-g-PEx to condense pGL-3 was evaluated using a DNA retardation assay by agarose gel electrophoresis with PMAC-g-PEx/DNA weight ratios ranging from 0 to 2.0:1. The resulting polyplexes comprising different molecular weight PEI showed a different DNA binding capacity. PMAC-g-PEx could completely retard DNA migration at a polymer/DNA weight ratio of 2.0, 1.0, and 0.5, respectively (Fig. 4A–C). The required polymer/DNA ratios were inversely proportional to PEI molecular weight, that PMAC-g-PEI1800 showed a higher DNA binding capacity than PMAC-g-PEI800 and than PMAC-g-PEI423. In fact, a lower charge density, as well as a lower molecular weight, can impair the condensation capability [32]. These results suggested that a high charge density would be good for DNA binding within our experiments.

The morphology of the PMAC-g-PEx/DNA polyplexes observed by TEM measurement was shown in Fig. 5. At weight ratios of 10 and 60, all of the polyplexes displayed average sizes less than 100 nm except PMAC-g-PEI423/DNA polyplexes at weight ratio 10 (Fig. 5, A1). This may be that PMAC-g-PEI423 could not combine DNA completely at weight ratio 10. In contrast, PMAC-g-PEI423/ DNA polyplexes at weight ratio 60 formed regular nanospheres with a size of about 30 nm (Fig. 5, A2). On the other hand, PMAC-g-PEI800 and PMAC-g-PEI1800 could combine pGL-3 completely at weight ratios of both 10 and 60, while smaller nano-sized particles could be observed at higher weight ratio (Fig. 5, B2 and C2) than at lower weight ratio (Fig. 5, B1 and C1). DNA polyplex with PMAC-g-PEI1800 displayed a larger size than with PMAC-g-PEI800 and PMAC-g-PEI423. Thus, on the TEM photographs, the smallest DNA polyplex about 30 nm could be found at weight ratio
60 with PMAC-\(g\)-PEI423 (Fig. 5, A2), while the largest polyplex about 90 nm was formed at weight ratio 10 with PMAC-\(g\)-PEI1800 (Fig. 5, C1). This result showed that PMAC-\(g\)-PEI could condense \(p\)DNA into nano-sized particles appropriate for gene delivery at suitable ratios. The trend of the sizes is in accordance with the results of molecular weight measurements and gel retardation experiments.

The cellular membrane was negative and zeta potential of carrier/DNA polyplexes are closely related for the complexes introduced into cells [33]. Positive charge of polyplex is thought to be helpful for its absorption to negatively charged cellular membrane, also leading to efficient intracellular trafficking [33]. In this paper, zeta potentials of PMAC-\(g\)-PEIx complexed with pGL-3 are shown in Fig. 6. At weight ratio 2.5–160, zeta potentials displayed all positive values showing complete complexation. And also, zeta potentials of PMAC-\(g\)-PEIx polyplexes increased rapidly according to the polyplex weight ratio, finally reaching the positive plateau values. All three PMAC-\(g\)-PEIx polyplexes showed very similar zeta potentials of about 35 mV and no significant differences between them. The positive charge of PMAC-\(g\)-PEIx polyplexes would be helpful for its further applications in vivo.

Fig. 5. Transmission electron microscope of (A) PMAC-\(g\)-PEI423, (B) PMAC-\(g\)-PEI800 and (C) PMAC-\(g\)-PEI1800 polyplexes with pGL-3 at weight ratios of 10 (A1, B1, C1) and 60 (A2, B2, C2).

Fig. 6. Zeta potential of the polyplexes at weight ratios ranging from 2.5 to 160.
3.5. Transfection in vitro

In vitro transfection experiments of PMAC-g-PEIx/DNA complexes were assessed in 293T cells in the absence of serum. PEI25K, the most widely used polycation gene vector and considered highly efficient, was used for comparison at N/P ratio of 10.

3.5.1. Green fluorescent protein assay

In order to directly visualize the infected cells, the green fluorescent protein assay of the samples was estimated with pEGFP-C1 used as a reporter gene. Transfection ability of the PMAC-g-PEI at different weight ratio was evaluated in the transfection of pEGFP-C1 to 293 T cells, while no significant differences could be observed. Fig. 7 showed the transfection results at a weight ratio of 80. Fig. 7(A1–D1) represent the fluorescent micrographs, and Fig. 7(A2–D2) represent the light inverted images. The transfection efficiencies green fluorescent protein assay mediated by PMAC-g-PEIx have much more bright green fluorescent spots than that by PEI25K, especially in the case of PMAC-g-PEI1800. Moreover, Fig. 2 showed that PMAC-g-PEIx presented much lower cytotoxicity than PEI25K even at a higher polymer concentration. These results suggested that PMAC-g-PEIx might be suitable for in vivo gene transfection.

3.5.2. Luciferase assay

Furthermore, the quantitative analysis of PMAC-g-PEIx as gene vectors was carried out by reporter gene pGL-3. As shown in Fig. 8, the polyplexes showed transfection efficiencies comparable to or

![Fig. 7. The transfection efficiency green fluorescent protein in 293T cells expressed by (A) PMAC-g-PEI423, (B) PMAC-g-PEI800 and (C) PMAC-g-PEI1800 at weight ratio 80, and (D) PEI25K PEI at N/P ratio 10. The fluorescence micrographs of both cells are in A1–D1, while the light inverted micrographs are in A2–D2.](image)

![Fig. 8. The transfection efficiency of PMAC-g-PEIx (x = 423, 800 and 1800) complexed with pGL-3 at weight ratios from 2.5 to 160 in 293T cells.](image)
even significantly higher than that of PEI25K. Within the range of weight ratios tested, all the three PMAC-g-PElx/pG-L3 polyplexes displayed the same change trend. The transfection efficiency increased gradually and reached a plateau along with the weight ratio. PMAC-g-PEI423 and PMAC-g-PEI800 reached their maximum efficiency plateaus at weight ratio of about 60, while PMAC-g-PEI1800 reached its maximum at a relatively lower weight ratio of 40. This result was consistent with the gel retardation result showing that PMAC-g-PEI1800 showed the highest DNA binding capacity among the three. And also, even at a rather high weight ratio of 160, the transfection efficiency was still found to be on a plateau and achieved nearly 3–10 times higher than the optimal value of PEI25K (at N/P ratio 10) [11]. It presented again that PMAC-g-PElx had apparently lower cytotoxicity and higher transfection efficiency than PEI25K, suggesting that PMAC-g-PElx could be a promising non-viral gene delivery vector.

In addition, compared with the three PMAC-g-PElx samples, PMAC-g-PEI1800 has a relatively better transfection efficiency. It was reported that the transfection efficiency depends on the particle size of polycation/DNA complexes [34], which is determined by the physical properties of the cationic polymers rather than the size of the DNA molecule [7]. The larger particle size would promote the transfection efficiency by increased sedimentation of complexes, which was favorable for cell attachment and subsequent uptaking [34]. The larger particle size would be also convenient for the escape of DNA from the complexes into nucleus. And also, it is proven that the high buffering capacity in the endosomal pH range 5–7 originating from the unique polyamine structure is very important for the high transfection efficiency of PEI which helps the polyplex to escape from the endosomes and promote transfection efficiency [30]. Therefore, the higher transfection efficiency of PMAC-g-PEI1800 might be attributed to its largest particle size shown in Fig. 5 and the best buffer capability shown in Fig. 3. The highest zeta potential of PMAC-g-PEI1800 among the three PMAC-g-PElx samples (shown in Fig. 6) also contributed to its highest transfection efficiency, which was necessary to bind the negatively charged cell via electrostatic interaction [33]. This result also supported previous findings that gene transfection efficiency of low molecular weight PEI was greatly enhanced by covalent coupling to other polymers [35].

3.6. Pre-incubation of PMAC-g-PEI1800 for DNA binding

It was reported that the potential advantage of biodegradable gene vectors as compared to their non-degradable counterparts is their reduced cytotoxicity and the avoidance of accumulation of the polymer in the cells after repeated administration [36]. Also, the degradability of the polymer can be helpful as a tool to release the plasmid DNA into the cytosol. Therefore, the influence of polycations degradation on their binding capacity with DNA was investigated in this paper. Comparative agarose gel electrophoresis experiments about PMAC-g-PEI1800 pre-incubated in 150 mM NaCl solvent at 37 °C for a different period of time were performed. As shown in Fig. 9, the binding capacity of PMAC-g-PEI1800 decreased gradually along with the pre-incubation time from 0 to 48 h. PMAC-g-PEI1800 without pre-incubation could completely retard DNA migration at a much lower weight ratio of 0.5. After pre-incubation for 24 h, PMAC-g-PEI1800 released partial DNA at weight ratios of 0.25 and 0.5, while the critical PMAC-g-PEI1800/DNA weight ratio for complete retardation of DNA was about 0.75. And also, PMAC-g-PEI1800 could form stable complexes with DNA at a weight ratio of about 1.25 after pre-incubation for 32 h. Moreover, after pre-incubation for 48 h, pDNA released from PMAC-g-PEI1800 polyplex was observed within the range of weight ratio tested. It meant that PMAC-g-PEI1800 was degraded in that condition and that it could not condense pDNA anymore because of the cleavage of backbone bonds. Thus, increasing the pre-incubation time would result in the poor binding capacity. However, the result would be not a bad thing. The lower charge density of the polycations could be profitable for decreasing their cell cytotoxicity. Considering that the fast degradation rate is believed to bring the possibility of insufficient gene delivery to cell nucleus, future reports will describe the importance of the control of polycation degradation rate, as well as their influence on gene delivery transfection efficiency in vitro and also in vivo.

4. Conclusion

In this study, PEI-grafted biodegradable PAMC-g-PElx (x = 423, 800 and 1800) polymers were synthesized for non-viral gene delivery systems. Toxicity, buffer capability, DNA binding capacity as well as transfection efficiency depended on the polymeric vectors. The required polycations/DNA ratios decreased along with increasing the PEI molecular weight, while PMAC-g-PElx could form positively charged nano-sized particles (<100 nm) with
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