行政院國家科學委員會專題研究計畫　成果報告

含胺基之新生物可分解性聚酯類高分子:合成、鑑定及基因傳遞之研究

計畫類別： 個別型計畫
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執行單位： 嘉南藥理科技大學醫藥化學系

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1. 摘要

合成可水解陽離子型新型聚酯 DMAE-PE，並且用於基因釋放的非病毒性的基因載體。實驗結果發現,DMAE-PE 能與 DNA 自主裝成奈米顆粒複合體。在核酸酵素作用下，DMAE-PE 能保護 DNA 不受此核酸酵素作用。DMAE-PE 與 PEI 比較，其細胞毒性也遠低於 PEI。

關鍵詞：生物分解、聚酯、細胞毒性

Abstract

A new cationic polycation,2-dimethylaminoethylamine-polyester (DMAE-PE) was synthesized and used as a non-viral vector for gene delivery. The DMAE-PE condensed plasmid DNA into nano-complexes. DMAE-PE provides a significant protection to plasmid DNA against restriction endonuclease. The DMAE-PE was lower cell toxicity than polyethylenimine (PEI).

Keywords: Biodegradable; Polyester; Cytotoxicity

2. Introduction

The cationic polymers that can self-assemble with DNA have been extensively studied for transfecting exogenous genes into mammalian cells [1]. A various of polycations such as poly(L-lysine) (PLL) [2-3], polyamidoamine [4], polyethylenimine (PEI) [5-6], and poly(2-(dimethylamino)ethyl methacrylate) (PDMAEMA) [7] have been proposed for the delivery of DNA into mammalian cells. These polycations not only condense DNA into structures small enough to enter cells through endocytosis, and then afford protection from nuclease degradation. Among them, Poly(L-lysine) (PLL), was the first polycation used to mediate the transfection of cells, and polyethylenimine (PEI), is of new “proton sponge” category and is hypothesized to mediate escape of plasmid DNA from the endosomal pathway [5, 8], are the standard polymer-based gene delivery systems against which new delivery systems must be compared. However, these polycations have demonstrated the tendency of polycations to mediate transfection, they are associated with considerable degree of cytotoxicity [5, 7, 9]. As a result, the design of polycation for a gene vector application generally requires a balance between transfection efficiency and short-term or long-term cytotoxicity. Thus far, there have been considerable efforts to design biodegradable polycations that are appropriate for the advancement of non-viral gene carriers. The new biodegradable and biocompatible cationic polyester (DMAE-PE) with tertiary amine in its backbone and side chains was successfully synthesized. To explore its qualification as a gene vector, its ability an DNA condensation, endonuclease enzyme and albumin protection, buffering capacity, cytotoxicity and transfection efficiency were investigated in order to assess possibilities in gene delivery.

3. Methods

3.1 *Amplification and purification of pCMV-βgal*

The plasmid pCMV-LacZ (pCMV-βgal), which contained a CMV

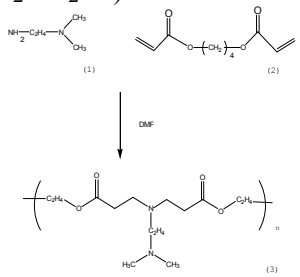
promoter to drive the β-galactosidase (LacZ) gene expression, was used [7]. The plasmid DNA was amplified and purified as reported before [7].

3.2 *Cell culture*

The cell line COS-7 (SV 40 Virus transformed African green monkey cell line, ATCC CRL-1651) was cultured in the Dulbecco's modified Eagle's medium (DMEM, GibcoBRL Co., Ltd.) with 4 mM L-glutamine adjusted to 10% heat-inactived horse serum, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose at 37°C in a humidified atmosphere of 5% CO_2.

3.3 *Polymer synthesis*

In a typical experiment, 1,4-butanediol diacrylate (1) (1.0 g, 5.04 mmol) and 2-dimethylaminoethylamine (DMAE) (2) (5.04 mmol) were weighed into separate vials and dissolved in DMF. The solution containing the 2-dimethylaminoethylamine (DMAE) (2) was slowly added to 1,4-butanediol diacrylate (1), and the polymerization of these monomers proceeded in DMF at 50℃ to yield the corresponding DMAE-PE in up to 85% yields. After 48 h, the reaction was dripped into vigorously stirring hexanes. DMAE-PE (3) was collected and dried under vacuum prior to analysis. DMAE-PE (3): ^{1}H-NMR(400MHz, DMSO-d_6, ppm) δ: 1.60 (2H, -OCH$_2$C<u>H$_2$</u>-), 2.08 (5H, -NCH$_2$CH$_2$NC$_2$<u>H$_5$</u>) , 2.41 (2H, -NCH$_2$C<u>H$_2$</u>NC$_2$H$_5$), 2.49 (2H, -OOCC<u>H$_2$</u>CH$_2$N-), 2.66 (2H, -NC<u>H$_2$</u>CH$_2$NC$_2$H$_5$), 2.69 (2H, -OOCCH$_2$C<u>H$_2$</u>N-), 3.99 (2H, -OC<u>H$_2$</u>CH$_2$-); ^{13}C-NMR (400MHz, DMSO-d_6, ppm) δ: 25.1 (-OCH$_2$<u>C</u>H$_2$-), 32.1 (-OOC<u>C</u>H$_2$CH$_2$N-), 45.6 (-NCH$_2$CH$_2$N<u>C</u>$_2$H$_5$), 51.4 (-OOCCH$_2$<u>C</u>H$_2$N-), 63.8 (-O<u>C</u>H$_2$CH$_2$-), 172.1 (-OO<u>C</u>CH$_2$CH$_2$N-).



Scheme 1. Synthesis of DMAE-PE

3.4 *Acid-Base Titration*

The ability of the polycation vectors to protonate and obtain a positive charge over the pH range 12 to 2 was determined by acid-base titration. Briefly, 10 mg of DMAE-PE, PLL and PEI were dissolved in 10 mL of 150 mM NaCl, respectively. And then 100uL of 1 N NaOH was added, and the pH of the polymer solution was recorded. The solution was titrated with increasing volumes of 0.1 N HCl and the pH was measured with a pH meter.

3.5 *Preparation and characterizations of polymer/plasmid polyplexes*

3.5.1 *Formation of polymer/plasmid polyplexes*

10 mg/mL of DMAE-PE was dissolved in the 20mM HEPES buffer (pH7.4) and its serial dilutions were made. The DMAE-PE serial dilutions were added rapidly into the DNA solutions to obtain DMAE-PE/DNA polyplexes, in which the mass ratio of DMAE-PE/DNA (w/w) was 1/2 to 300/1. After that, the polyplexes were allowed to self-assemble in HEPES buffer and incubated at room temperature for 30 minutes before measurements.

3.5.2 *DNA gel retardation and restriction endonuclease protection assay*

The DMAE-PE/DNA polyplexes were loaded into a 0.7 % agarose gel containing ethidium bromide (0.3 μg/mL) in a tris-acetate-EDTA (TAE) buffer and performed at 100 V for 45 min. After electrophoresis, the DNA bands were visualized by UV-irradiation. The DMAE-PE/DNA polyplexes at ratios of 1/1 、 5/1、 50/1 (w/w) were incubated with *Kpn I* at a concentration of 10 U/μL at 37℃ for 90 min in the provided reaction buffer. After the restriction endonuclease digesting, samples were analyzed by 0.7% agarose gel electrophoresis in the same manner as described above.

3.5.3 *Stability of the polymer/DNA polyplexes in the presence of bovine albumin*

The stability studies of the polyplexes were measured by agarose gel retardation. The polyplexes were prepared at a mass ratio

of 50/1 for 30 min, and bovine albumin was added into polyplexes solution to make final albumin concentration of 5, 25, and 50 mg/mL. The DNA release from polyplexes was determined after 6 and 12 hours at 37°C.

3.6 *Determination of the cytotoxic effect of polymers and polymer/DNA polyplexes*

Cytotoxicity of DMAE-PE in comparison with a common used gene carrier, PEI, were evaluated using the XTT assay [10].

4. Results

4.1 *Structural Characterizations of DMAE-PE (3)*

The 2-dimethylaminoethylamine-PE (DMAE-PE) (3) bearing tertiary amines in the backbone and side chain was prepared from 1,4-butanediol diacrylate (1) and 2-dimethylaminoethylamine (DMAE) (2) as scheme 1. The reaction temperature was maintained at 50 °C with yield over 90%.

The chemical structure of DMAE-PE (3) was confirmed by FT-IR, ^{1}H-NMR, and ^{13}C-NMR. Figure 1 showed the FT-IR spectra of the 1,4-butanediol diacrylate (1) and DMAE-PE (3). The peaks at 1735 cm^{-1} (C=O stretching, ester) and 1188 cm^{-1} (C-O stretching, ester) represent the absorptions of ester links in the 1,4-butanediol diacrylate (1) and DMAE-PE (3). The DMAE-PE (3) wasn't observed the peak at C=C stretching (1636 cm^{-1}, alkene) and the band absorptions was represent in 1,4-butanediol diacrylate (1). In the ^{13}C-NMR spectra, there was also accompanied with a disappearance of chemical shift at 172.1 ppm, which represented the ester carbon of the ester group in the DMAE-PE. FT-IR, ^{1}H-NMR and ^{13}C-NMR characterizations of the synthesized polymers provided clear evidence that the DMAE-PE (3) had been successfully synthesized through the reaction of the 1,4-butanediol diacrylate (1) and DMAE-PE (3). The GPC data of the DMAE-PE (3) showed that the weight-averaged molecular weight was 15,000 and with a polydispersity of 1.8, relative to polystyrene standards in THF.

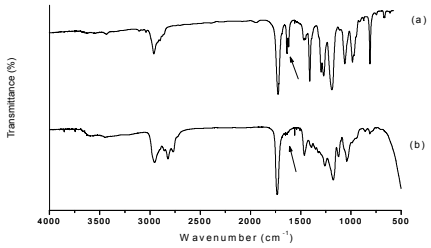


Figure 1. FT-IR spectra of 1,4-Butanediol diacrylate (a) and DMAE-PE (b).

4.2 *Buffering capacity of the various polymers*

The acid-base titration profile was obtained for PLL, PEI and DMAE-PE resulting in a proton buffering effect within the endosomal/lysosomal compartments of the cell (Figure 2). All of the polymer solutions had 11.5 to 11.7 with 1.0 N NaOH. The initial high pH protonation of the ε-amine groups of PLL can be seen as a horizontal trend above pH 8. The titration curve trend turns nearly vertical below pH 8 suggesting little buffering capacity of PLL, as all the amine groups have already been protonated. The result shown that most conformation flexible backbone and side chain (tertiary amines) of DMAE-PE was forming polyplexes with negatively charged DNA at physiological pH by electrostatic interactions.

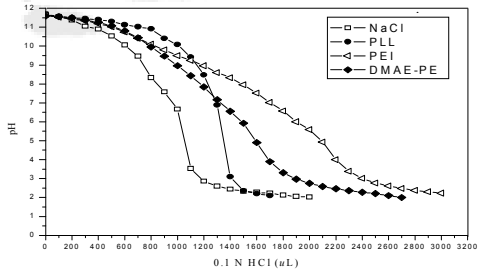


Figure 2. Acid-base titration profile of various polymers with 0.1 N HCl solution.

4.3 *Gel retardation and restriction endonuclease protection assay of DMAE-PE/DNA polyplexes*

Figure 3 illustrated the electrophoretic mobility behaviors of free DNA, DMAE-PE/DNA polyplexes on agarose gel electrophoresis. Plasmid DNA (400 ng) with increasing amounts of DMAE-PE led to the neutralization of DNA negative charges as

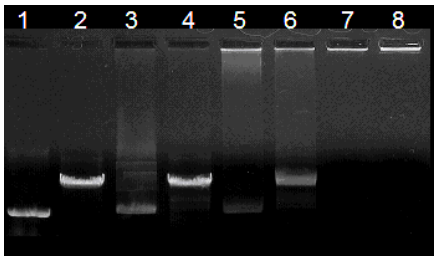


Figure 3. DNA gel retardation and restriction shown by gel retardation (Figure 3). The endonuclease protection assay of DMAE-PE. Lanes: (1) 400 ng pCMV-βgal; (2) 400 ng pCMV-βgal treated with *Kpn* I ; (3) DMAE-PE/ pCMV-βgal (w/w):1/1; (4) DMAE-PE / pCMV-βgal (w/w):1/1 treated with *Kpn* I ; (5) DMAE-PE /pCMV-βgal (w/w):5/1; (6) DMAE-PE / pCMV-βgal (w/w):5/1 treated with *Kpn* I (7) DMAE-PE /pCMV-βgal (w/w):50/1; (8) DMAE-PE / pCMV-βgal (w/w):50/1 treated with *Kpn* I .

DNA mobility on agarose gel was influenced by the presence of DMAE-PE. Plasmid DNA was partially retained by the presence of DMAE-PE at a mass ratio of 1/1 and 5/1 (lane 3 and 5) and totally retained at a mass ratio of 50/1 (lane 7). This suggests that DNA was fully complexated with DMAE-PE to form polyplexes, as complete retardation was observed at a mass ratio of above 50/1. These results showed that introduction tertiary amines of into the backbone and side chain of DMAE-PE carrying positive charges could interact with the negatively charged phosphate groups on DNA for the polyplexes formation.

4.4 *Cytotoxicity of polymers*

PEI, a well-known gene carrier, is the standard polymer-based gene delivery systems against which new delivery systems must be compared. To determine the cytotoxicity of DMAE-PE in comparison with PEI (Mw=25000) we performed a XTT assay using the COS-7 cell line. Cells were incubated with increasing amounts of DMAE-PE (ranging from 5 μg/mL to 250 μg/mL) or PEI (ranging from 5 μg/mL to 20 μg/mL). The results showed that DMAE-PE exhibited much lower toxicity on COS-7 cells than PEI in Figure 4.

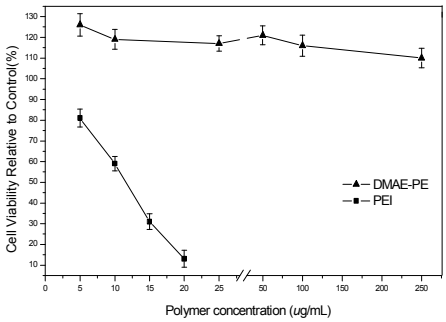


Figure 4. Toxicity of DMAE-PE and PEI in COS-7 cells. Results are presented as mean ± SD (n=3).

5. Conclusions

A novel polycation, DMAE-PE, was synthesized and characterized. Meanwhile, it shows a property of very low cytotoxicity. In addition, DMAE-PE provides a significant protection to plasmid DNA against restriction endonuclease when at mass ratios above 50/1, Herein based on these results reported, the DMAE-PE is a promising candidate for gene delivery in vivo.

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