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執行單位：嘉南醫藥科技大學醫藥化學系

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Abstract

A new cationic polymer, amine-containing polyester, bearing tertiary amines in the backbone and primary amines in the side chains was synthesized as a non-viral vector for gene delivery. The melt polycondensation reaction of the prepolymer prepared from N-Cbz-L-aspartic anhydride and N-methyldiethanolamine using titanium isopropoxide as a catalyst produced the new biodegradable amine-containing polyester. The synthesized monomer and polymer were characterized by FTIR and ^{1}H-NMR. The condensation behavior of plasmid DNA with the polymer to form self-assembly polyester/ pCMV-βgal complexes was characterized by dynamic light scattering, zeta potential, atomic force microscopy (AFM). These results suggest that the new polyester of this general structure hold promise as synthetic vectors for gene delivery and are interesting candidates for further study.

Introduction

The safe and effective delivery of therapeutic genes remains a major challenge in the field of gene therapy. So far, the use of modified viruses as gene transfer vectors has generally represented the most clinically successful approach to gene therapy. However, the limitations of viral vectors, specifically their immunogenicity and small capacity for therapeutic DNA, have led to the development of synthetic nonviral vectors.

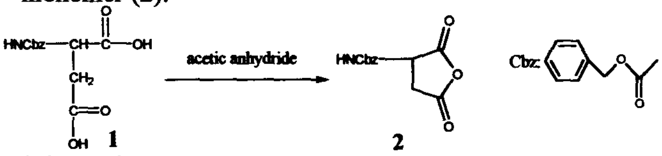
Among nonviral vectors, cationic polymer-based gene delivery systems have gained increasing attention recently. A number of polycations have been reported to effect gene transfection, including poly-L-lysine(PLL)[1], poly-(amidoamine) dendrimers[2], poly(2-(dimethylamino)ethyl methacrylate) (PDMAEMA)[3], and polyethylenimine (PEI)[4]. These polycations have been widely used as transfection vectors due to the facility with which they condense and protect DNA. While complexation of DNA with polycations is required to compact and protect DNA during early events in the transfection process, DNA and polycations must ultimately decomplex to allow efficient transcription. In view of this, degradable polycations could play an important role in the nucleus. Furthermore, these polycations can be significantly cytotoxic[5]. Therefore, to develop safe and effective transfection vectors, we have pursued the synthesis of biodegradable polycations.

To date, most polycaitons synthesized and investigated for transfection efficiency have contained pendent amines in the polymer side chain[1,6]. Herein, we report a strategy for the preparation polyester containing tertiary amines in their backbones based on polycondensation reaction of N-methyldiethanolamine to N-Cbz-L-aspartic anhydride monomer. After deprotection reaction, the polyester has contained primary amines in the polymer side chain.

Experimental part

Preparation of protected N-Cbz-L-aspartic anhydride monomer (2)

Ten g of N-Cbz-L-aspartic acid (1) was dissolved in 50 ml of acetic anhydride, and the reaction mixture was stirred for 6 hours at 35℃. The excess acetic anhydride and acetic acid were removed under reduced pressure, and diethyl ether was added to wash out the remaining acetic acid and anhydride. The solid was placed in vacuum oven for 24 hours. Scheme 1 illustrates the synthesis of the protected N-Cbz-L-aspartic anhydride monomer (2).



Scheme 1.

Preparation of prepolymers

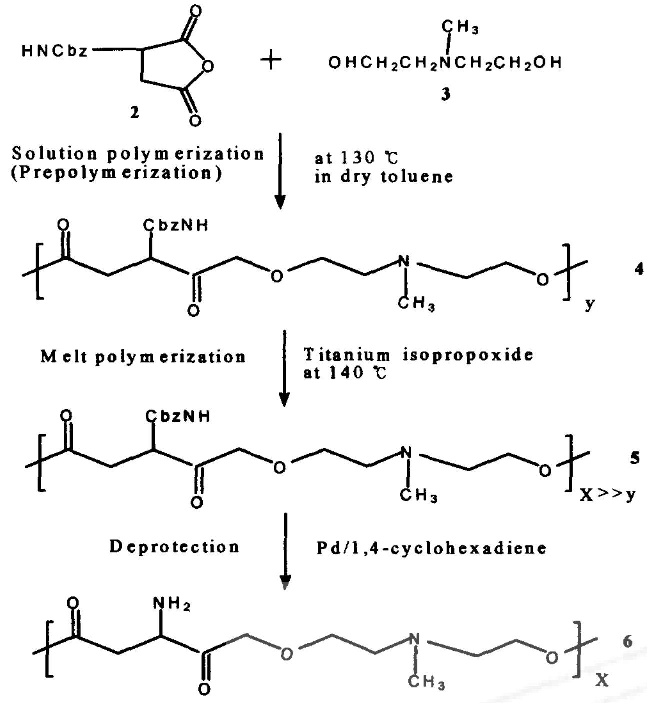
Two g (8 mmol) of N-Cbz-L-aspartic anhydride monomer(2), 0.957 g (8 mmol) of N-methyldiethanolamine (3), 3 mg (0.0016 mmol) of p-toluenesulfonic acid and 25 ml dried toluene were placed into a 50-ml round-bottomed flask which was equipped with a Deans-Stark trap. The reaction mixture was refluxed for 24 hours under nitrogen atmosphere. After evaporation of the solvent, a sticky brown prepolymer product was obtained for subsequent melt polycondensation as illustrated in Scheme 2.

Melt polycondensation of prepolymers

Five grams of the prepolymer(4) and a predetermined amount titanium isopropoxide were placed into a three-necked flask equipped with a mechanical stirrer and thermometer. Subsequently, the pressure of the reaction system was gradually decreased, and condensation polymerization was continued at 140℃ under a final reduced pressure lower than 0.5 mmHg. After reaction, the polymers were dissolved in chloroform and the insoluble materials like the residual catalyst were filtered out. Finally, the polymers were poured into excess diethyl ether. The precipitated polymers were dried in a vacuum oven at 40℃ for 24 hours.

Deprotection of amino protecting group of the new polymer

Briefly, 0.2 gram of the protected polymer (5) was dissolved in 10 ml DMF, and 10 wt% palladium-on-charcoal (1.00g) was added as a catalyst. With vigorous stirring, 1,4-cyclohexadiene was slowly added to the mixture. The reaction was continued at room temperature for 72 hours. After the deprotection reaction, the catalyst was removed by filtration. The concentrated solution was precipitated into a large excess of diethyl ether, resulting in a powder of deprotected polymer (6).



Scheme 2. Synthesis of the prepolymer and its melt polycondensation and deprotection.

Amplification and purification of plasmid DNA

Plasmid pCMV-βgal(7740 base pairs) encoding β-galactosidase was a circular DNA containing a CMV promoter and ampicillin resistant gene. The plasmid was amplified in *Escherichia coli* (DH5α strain) and purified by column chromatography (Qiagen® Plasmid Mega kit, Germany). The purity of the collected plasmid was determined by the ratio of UV absorbance at 260nm/280nm in Tris-EDTA buffer (pH 8.0). The ratio of plasmid used in the studies was between 1.8 and 2.0. Plasmid concentration was determined using the equation: 1.0 OD_{260}=50 μg/ml of plasmid DNA. (OD, optical density)

Formation of Polyester/ pCMV-βgal complexes

Stock solution of polyester (5.0 mg/ml) and dilutions were made in 20mM Hepes buffer (pH7.4). Complexes were made by adding polyester solution to pCMV-βgal solution (20 μg/ml) rapidly to obtain polyester/ pCMV-βgal (w/w) ratios from 1 to 50. The concentrations of the pCMV-βgal complexes used were all in a range where visible aggregation was not observed. The complexes were allowed to self-assemble in Hepes buffer and incubated at room temperature for 30 minutes before measurements.

Atomic force microscopy (AFM)

Polyester/ pCMV-βgal complexes were prepared in 20mM Hepes buffer (pH7.4) as described above. The sample was prepared by placing 2 μl solution on a freshly-cleaved ruby mica surface. After 3 min of contact, the sample was then rinsed with 1-2 ml distilled water. Excess liquid was taken off with filter paper (Whatman). The ruby mica was then dried with compressed air. The image was obtained on a nanoscope II (Digital Instruments, Santa Barbara, CA) equipped with D scanner. The image mode (5μm in square) scan range was set and tapping mode with silicon nitride cantilever probe was used.

Structural Characterization

The FT-IR spectrum of the N-Z-L-aspartic anhydride (2)(Fig.1A) shows the characteristic absorption peaks at 3320(NH), 1860, 1802(C=O), and 1538cm^{-1}(CN of urethane). The spectra of the protected polyester (5) (Fig.1B) and deprotected polyester (6) (Fig.1C) exhibit strong ester carbonyl bands at 1725 and 1718 cm^{-1}, respectively. The obvious feature of the deprotected polyester (6)(Fig.1C) was the absence of aromatic C-H (out of plane bending) absorptions at 694 and 750 cm^{-1} from the Cbz protecting group. This indicates the successful removal of the Cbz group and the formation of the free pendent amine groups.

The ^{1}H-NMR spectra of 2, 5, and 6 were shown in Fig.2 and consistent with the FT-IR spectrum. The disappearance of the peaks at both 7.3ppm (due to the protons of aromatic rings of the Cbz protecting group) and 5.1ppm (due to benzyl methylene proton[7]) in the deprotected polyester is a clear indication of the successful deprotection reaction.

Results and discussion

We adopted procedures of Lutz et al.[8] for the preparation of N-Z-L-aspartic anhydride. Anhydride formation was easily carried out with a high yield (90%) by cyclization of N-Cbz-L-aspartic acid in the presence of excess acetic anhydride. For the synthesis of prepolymers, N-Z-L-aspartic anhydride (2) and N-methylehtanolamine (3) reacted to form the monoester first, which subsequently underwent solution polycondensation to prepare a low molecular weight prepolymer. In addition, the use of N-Z-L-aspartic anhydride monomer instead of N-Z-L-aspartic acid for the preparation of the prepolymer (4) was desirable because the anhydride monomer dissolved in toluene for the azeotropic removal of water byproduct from the reaction mixture in order to minimize hydrolysis of the monoester and to shift the equilibrium of the reaction toward the polycondensation[9] and N-Z-L-aspartic acid did not dissolve in toluene.

The prepolymer (4) was postpolymerized by the melt polycondensation reaction to increase the molecular weight. The melt polycondensation was originally carried out at 140℃ for 3 hours under reduced pressure (lower than 0.5mmHg), and the effect of the amount of catalyst(titanium isopropoxide) on the Mw of the melt polymerized polymer using the prepolymer of Mw =1250 is shown in Fig.3. Without the catalyst, the Mw of the polymer was increased from 1250 to 2240. However, the Mw of the polymer reached its maximum (Mw=9260) in the presence of 0.25wt% titanium isopropoxide. A higher amount of titanium isopropoxide led to a decrease of molecular weight due to the decomposition of the polymer by the catalyst[10].

Because of the ester-based backbone of the polymer, removal of the protecting group required mild conditions to maximize the molecular weight of the final polymer. The Cbz group may be easily removed either by catalytic hydrogenation or by solvolysis with HBr/acetic acid[11]. To prevent the possible breakdown of the polyester backbone, we selected the catalytic hydrogenation method instead of the treatment with strong acid. 1,4-Cyclohexadiene was used as an effective hydrogen donor under mild conditions[12].

Self-Assembly of polymers with Plasmid DNA (pCMV-βgal)

The tendency of polycations to interact electrostatistically with the polyanionic backbone of DNA in aqueous solution is well-known. The ability of polyester to complex with and condense plasmid DNA is illustrated in Fig.4. Complexes reach a maximum diameter as charge neutrality is achieved and aggregation occurs. With increasing amount of polyester, the complexes size decrease sharply until the mass ratio of 20/1 was reached. A further increase in the amount of polyester does not affect the size of complexes. As shown in Fig.5, the Zeta-potential of Polyester/ pCMV-βgal complexes formed were negative at low polymer concentrations and charge neutrality was achieved near the mass ratio of 15/1, resulting in extensive aggregation. The Zeta-potential of the complexes approached a limiting value ranging from +7 to +14 mV at Polyester/ pCMV-βgal ratios above 20/1. The self-assembling complex was imaged by atomic force microscopy (AFM). The complexes discretely compact images viewed by AFM (Fig.6). The compact appearance of the complex formed with polyester verified the impression of very efficient DNA condensation measured in particle sizes. The transfection activity of the polymer/DNA complexes is highly dependent on the DNA condensation that will facilitate its uptake into the cells.

Conclusions

A new cationic polymer, amine-containing polyester, bearing tertiary amines in the backbone and primary amines in the side chains was successful synthesized. The results revealed that polyester could form a strong complex with palsmid DNA. Finally, we hypothesized that polyester of this general structure, and the L-aspartic acid derivatives into which they would presumably degradable, would be significantly less toxic than poly-L-lysine and PEI. Furthermore, a complete investigation of the biocompatibility (e.g. cytotoxicity) and transfection efficiency of the polyester is currently in progress and will be reported in a subsequent paper.

References:

1. Wagner E, Ogris M, Zauner W. Adv. Drug Deliv. Rev 1998; 30: 97–113.
2. Kukowska-Latallo JF, Bielinska AU, Johnson J, Tomalia DA, Baker JR. Proc. Nalt. Acad. Sci. USA. 1996;93:4879-4902.
3. Van de Wetering P, Cherng JY, Talsma H, Hennink WE. J. Control. Release 1997;49:59-69.
4. Boussif O, Lezoualch F, Zanta MA, Mergny MD, Scherman D, Demeneix B, Behr JP. Proc. Natl. Acad. Sci. USA 1995; 92:7297–7301.
5. Choksakulnimitr S, Masuda S, Tokuda H, takakura Y, Hashida M. J. Controlled Release. 1995;34: 233-241.
6. Van de Wetering P, Moret EE, van Steenbergen MJ, Hennink WE. Bioconjugate Chem. 1999; 10: 589-597.
7. Yokoyama M, Inoue S, Kataoka K, Yui N, Okano T, Sakurai Y. Macromol. Chem. 1989; 190: 2041.
8. Lutz WB, Ressler C, Nettleton DE. J. Am. Chem. Soc. 1959; 81: 167.
9. Won CY, Chu CC, Lee JD. J. Polymer Science. 1998; 36: 2949-2959.
10. Ynag J, Zhang S, Liu X, Cao A. Polymer Degradation and Stability. 2003; 81: 1-7
11. Anderson GW, Blodinger J, Welcher AD. J. Am. Chem. Soc. 1952; 74: 5309.
12. Felix AM, Heimer EP, Lambros TJ, Tzougraki C. J. Org. Chem. 1978; 43: 4194.
13. Vijayanathan V, Thomas T, Thomas TJ. Biochemistry 2002; 41:14085-14094.

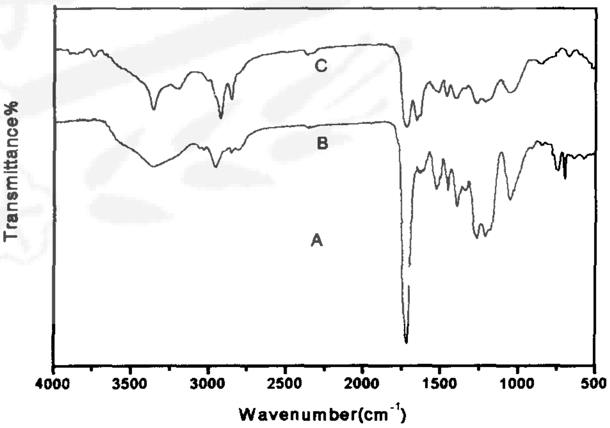


Fig.1.FTIR spectra of (A) N-Cbz-L-aspartic anhydride monomer (2), (B) N-Cbz-polyester (5), (C) deprotected polyester (6).

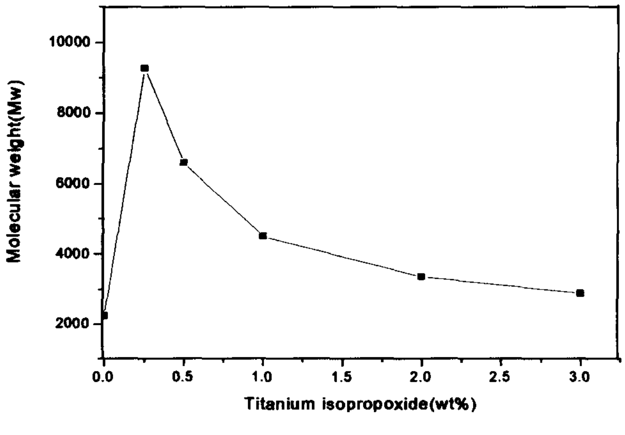


Fig.3. Effect of the amount of titanium isopropoxide catalyst on molecular weight of the melt-polymerized polymer using the prepoplymer (Mw=1250).

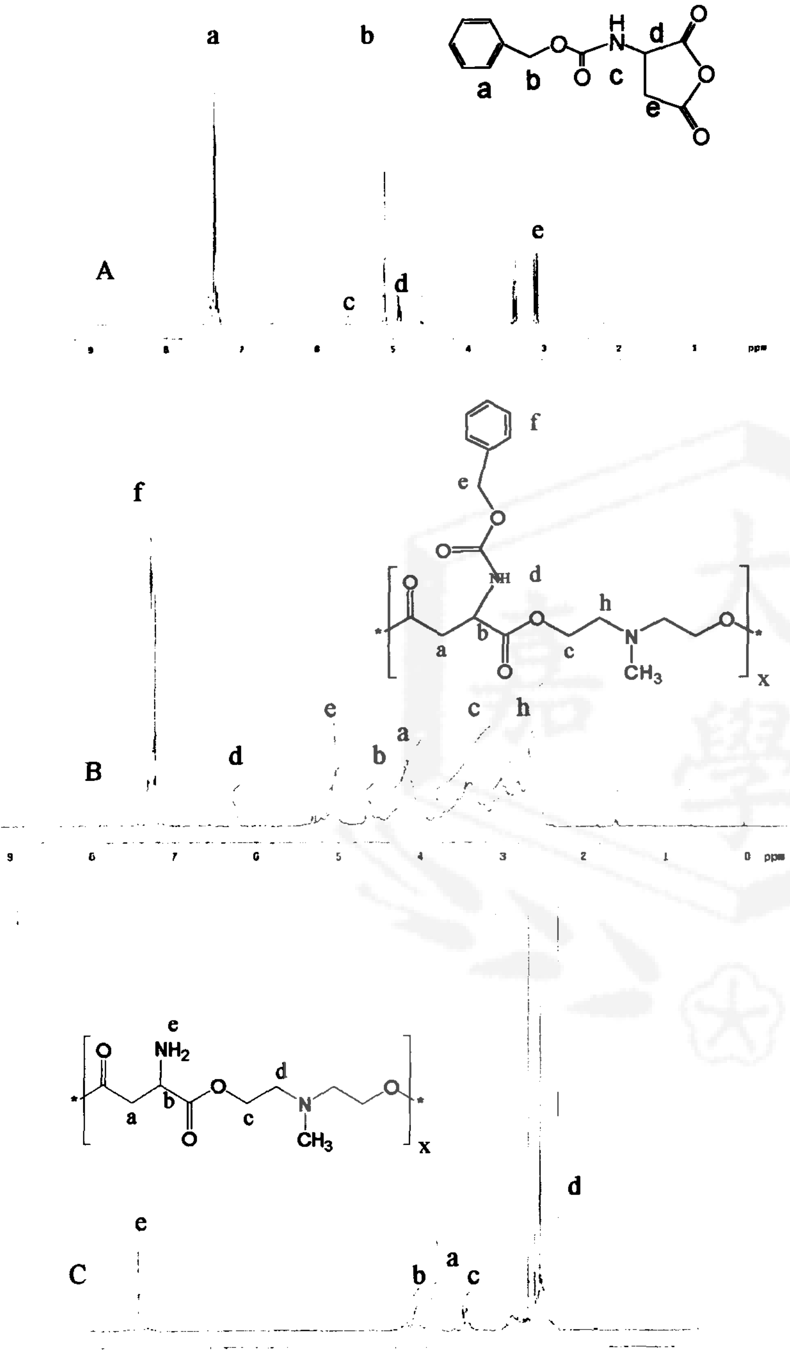


Fig.2. ^{1}H-NMR spectra of (A) N-Cbz-L-aspartic anhydride monomer (2) (B) N-Cbz-polyester (5)(C) deprotected polyester (6)

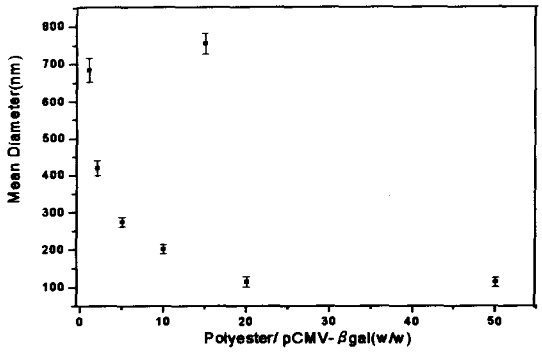


Fig.4. Mean diameters of polyester/ pCMV-βgal complexes as a function of polymer concentrations.

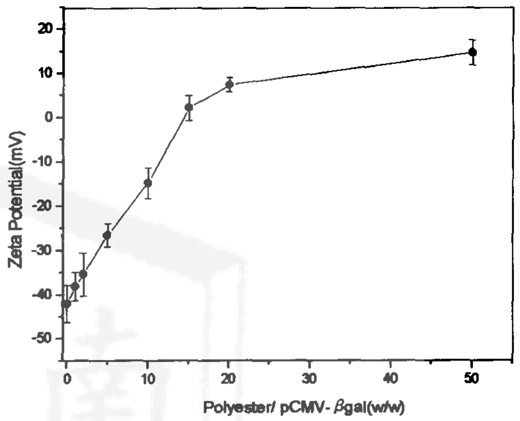


Fig.5. Zeta-potential of polyester/ pCMV-βgal complexes as a function of polymer concentrations.

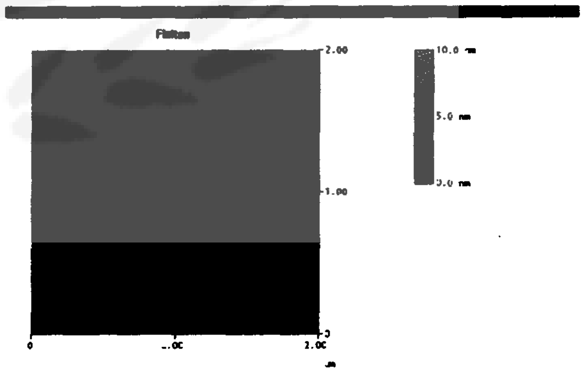


Fig.6. The AFM image of polyester/ pCMV-βgal:20/1 complex. The height of the AFM image is represented by a graded black-white scale, with the white color indicating a height more than 10nm above the ruby mica. The x and y dimensions are scaled as shown.