Poly(4-hydroxy-L-proline ester): Low-Temperature Polycondensation and Plasmid DNA Complexation

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ABSTRACT: The cytotoxicity of a polycationic gene delivery system is critical for overall gene transfer efficency. Cells targeted for transfection must be able to support transcription and translation following gene delivery if treatment is to be successful. Cumulative cellular exposure time is one factor that mediates the cytotoxicity of polycations. Therefore, we hypothesized that hydrolytically degradable polycations, such as polyesters based on amino acids, could serve to reduce the cytotoxicity of polycationic gene delivery systems. In this paper, optimization of the low-temperature dicyclohexylcarbodiimide/(dimethylamino)-pyridine (DCC/DMAP)-activated polycondensation of *N*-carbobenzyloxy-4-hydroxy-L-proline (CBZ-4-hydroxy-L-proline) to ultimately yield poly(4-hydroxy-L-proline ester) and the resulting polymer's interaction with plasmid DNA is reported. The optimized polycondensation of CBZ-4-hydroxy-L-proline resulted in a polymer with $M_w = 7880$. Deprotection of the CBZ group was afforded by palladium on activated carbon-catalyzed hydrogenolysis with little cleavage of the polyester backbone. The poly(4-hydroxy-L-proline ester) was able to electrostatically complex with plasmid DNA as determined by agarose gel retardation with complete retardation occurring at the DNA:polymer ratio of 1:1 (w:w). The poly(4-hydroxy-L-proline ester) also condensed plasmid DNA into nanostructures less than 150 nm at the DNA:polymer ratio 1:3 (w:w). The in vitro cytotoxicity of the polymer was compared to that of polylysine and polyethylenimine. The minimum viability of cells incubated with poly(4-hydroxy-L-proline ester) was 85%, which is excellent when compared to the cases of polylysine (20%) and polyethylenimine (2%).

Introduction

One element critical for the overall transfection efficacy of a gene delivery system is cytotoxicity. Cell damage resulting from a cytotoxic delivery system is deleterious because following gene delivery the cell must be capable of supporting translation and transcription. Polycations such as polylysine and polyethylenimine (PEI) are the standard polymer-based gene delivery systems against which new delivery systems must be compared. Polylysine was the first polymer used to mediate the transfection of cells,[1] and PEI is of the new "proton sponge" category and is hypothesized to mediate escape of plasmid DNA from the endosomal pathway.[2,3] However, while these polymers have demonstrated the aptitude of polycations to mediate transfection, they are associated with a considerable degree of cytotoxicity.[4−6]

The goal of this work was to design and synthesize a polycation with low cytotoxicity that maintained the capability to electrostatically complex with and condense plasmid DNA into nanoscale structures. Cumulative cellular exposure time plays a primary role in the cytotoxicity of nondegradable or slowly degradable polycations.[4] Therefore, we hypothesized that if a polycation could degrade relatively rapidly, its cytotoxicity could potentially be minimized. To evaluate this hypothesis, we formulated four design criteria (hydrolytic degradation, nontoxic monomers, cationic moieties, inexpensive materials) to direct the synthesis of a polycation with minimal cytotoxicity. The key component of the design criteria is hydrolytic degradation of the polycation. Hydrolytic degradation would allow the relatively rapid degradation of the polycation, and if the working hypothesis were valid, the cumulative cytotoxic effects of a polycation should be minimized.

Using the four design criteria, we concentrated on the synthesis of amino acid-based polyesters. Four candidate amino acids that had potential for the desired polymers were 4-hydroxyproline, serine, threonine, and tyrosine. Of these amino acids, we focused on the polycondensation of 4-hydroxy-L-proline because the ester backbone of the polyester could be susceptible to nucleophilic attack by primary amines. The secondary amine of 4-hydroxyproline could provide some steric hindrance to reduce its nucleophilicity but still retain the ability to complex with plasmid DNA.

In this paper we report optimization of the low-temperature activated polycondensation of *N*-α-carbobenzyloxy-4-hydroxy-L-proline via DCC/DMAP-activated polycondensation, deprotection of the N-protecting group to produce poly(4-hydroxy-L-proline ester), determination of the effect of steric hindrance at the N-position upon the DCC/DMAP polymerization, evaluation of poly(4-hydroxy-L-proline ester)'s capability to complex with and condense plasmid DNA, and comparison of the preliminary cytotoxic screening profile of poly(4-hydroxy-L-proline ester) to both polylysine and PEI.

Experimental Procedures

Materials. Palladium on activated carbon (10%, Pd/C), 1,3-dimethyl-2-imidazolidone (DMI), 1,3-dicyclohexylcarbodiimide (DCC), 4-(dimethylamino)pyridine (DMAP), and polyethylenimine (M_w 25 000) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Boc-4-hydroxy-L-proline, CBZ-4-hydroxy-L-proline, poly-L-lysine (M_w 30 000−70 000), and (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO), and 9-fluorenylmethoxycarbonyl-4-hydroxy-L-proline (Fmoc-4-hydroxy-L-proline) was purchased from NovaBiochem (San Diego,

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Table 1. Optimization Matrix for the Polycondensation of CBZ-4-hydroxyproline; Reactions 1−10 Were Conducted at 0 °C, and Reactions 11−20 Were Conducted at 25 °C

reaction no.	temp (°C)	molar CBZ-4-hydroxy-proline concn (DMI vol, mL)	DCC equiv (DCC wt, g)	DMAP equiv (DMAP wt, mg)
1, 11	0, 25	0.5 (7.54)	2 (1.55)	0.1 (46)
2, 12	0, 25	1 (3.77)	2 (1.55)	0.1 (46)
3, 13	0, 25	2 (1.88)	2 (1.55)	0.1 (46)
4, 14	0, 25	1 (3.77)	0.5 (0.388)	0.1 (46)
5, 15	0, 25	1 (3.77)	1 (0.777)	0.1 (46)
6, 16	0, 25	1 (3.77)	2 (1.55)	0.1 (46)
7, 17	0, 25	1 (3.77)	2 (1.55)	0 (0)
8, 18	0, 25	1 (3.77)	2 (1.55)	0.1 (46)
9, 19	0, 25	1 (3.77)	2 (1.55)	0.2 (92)
10, 20	0, 25	1 (3.77)	2 (1.55)	0.5 (230)

CA). ^{1}H NMR spectra were recorded on a Bruker instrument (400 MHz, Avance DPX 400). Organic phase GPC was conducted in chloroform (1 mL/min) using two PL-Gel mixed-D columns in series (5 μm, 300 × 7.5 mm, Polymer Laboratories, Amherst, MA) with polystyrene standards on a Perkin-Elmer LC-250 with RI detection. Water-phase GPC was conducted using an Ultrahydrogel 1000 (10 μm, 7.8 × 300 mm, Waters, Milford MA) in a 0.7 M $NaNO_3$, 0.1 M Tris, pH = 7.2 mobile phase (1 mL/min) with poly(ethylene glycol) standards on a Perkin-Elmer LC-250 system with RI detection. Plasmid DNA (CMV-βGal) was produced in *E. coli* (DH5α, a kind gift from the laboratory of Dr. Richard Mulligan, Harvard Medical School, Boston, MA), isolated with a Qiagen kit and purified by ethanol precipitation. The purity of the plasmid was determined by the ratio of UV absorbance 260 nm/280 nm in double distilled water. Plasmids were ethanol precipitated until the 260 nm/280 nm ratio was 1.7 or higher. Immortalized African green monkey kidney fibroblasts (COS-7) were purchased from American Type Culture Collection (Manassas, VA) and grown at 37° C, 5% CO_2, in Dulbecco's modified Eagle's medium 90%; fetal bovine serum, 10%, penicillin 100 units/mL, streptomycin 100 μg/mL.

Methods. *DCC/DMAP Activated Polycondensation General Procedure*. The variables evaluated for the polycondensation of CBZ-4-hydroxy-L-proline are tabulated in Table 1. The polymerizations were conducted as follows: under an argon atmosphere was dissolved CBZ-4-hydroxy-L-proline in DMI. After dissolution, DMAP was added and dissolved and then DCC was added. The reaction was conducted with continuous stirring. At specified time points, the reaction was diluted with 2 mL of chloroform and filtered to remove DCU. The vial was washed out with 1 mL of chloroform and filtered into the filtrate. The filtrate was added dropwise into 100 mL of anhydrous diethyl ether and stirred for 5 min. The polymer congealed on the bottom of the flask and was isolated by decanting the ether. The polymer was quickly dried with a stream of argon, dissolved in 3 mL of chloroform, and then added dropwise into 150 mL of anhydrous diethyl ether. The polymer was isolated by filtration as a fine white powder and dried thoroughly under vacuum. Analysis of polymers from optimized reaction: Poly(*N*-CBZ-4-hydroxy-L-proline ester): ^{1}H NMR (DMSO-d_6, ppm) 2.25 (br m, 2H, CH_2), 3.57 (m, 2H, CH_2), 4.35 (m, 1H, CH), 5.03 (m, 2H, CH_2), 5.21 (m, 1H, CH), 7.25 (br s, 5H, CH_5). Poly(4-hydroxy-L-proline ester): ^{1}H NMR (D_2O, ppm) 2.68 (br m, 2H, CH_2), 3.63 (m, 2H, CH_2), 4.71 (m, 1H, CH), 5.52 (br s, 1H, CH).

DCC/DMAP Activated Polycondensation of Boc-4-hydroxy-L-proline. Under an argon atmosphere and at 4 °C, Boc-4-hydroxy-L-proline (1 g, 0.004 32 mol) was dissolved in 3.7 mL of DMI. After dissolution, DMAP (52.7 mg, 0.432 mmol) was added, dissolved, and then followed by DCC (1.78 g, 0.008 65 mol). The reaction was conducted at 4 °C with stirring for 48 h, after which the reaction was diluted with 5 mL of methanol, the DCU filtered off, and the polymer isolated by dropwise precipitation into water. Results: yield, 0.683 mg (68%), M_w = 3500, M_n = 2500.

DCC/DMAP Activated Polycondensation of Fmoc-4-hydroxy-L-proline. The polymerization of Fmoc-4-hydroxy-L-proline was conducted in the idential manner as for poly(Boc-4-hydoxyproline ester). Fmoc-4-hydroxyproline: 1 g, 0.002 82 mol. DCC: 1.16 g, 0.005 65 mol. DMAP: 34.56 mg, 0.282 mmol. DMI: 2.82 mL. Results: yield, 0.654 mg (65%), M_w = 3600, M_n = 2600.

Deprotection with Formic Acid and Pd/C. Removal of the CBZ group was accomplished using formic acid and Pd/C according to the method described in ref 11. In a 100 mL round-bottom flask the polymer (300 mg) was dissolved in 4 mL of DMF. To this solution under a nitrogen atmosphere was added 1 g of Pd/C (10%). With vigorous stirring formic acid (14 mL) was added dropwise over 15 min. The reaction was stirred at room temperature for 14 h and filtered through 3 cm of Celite; the Celite washed with 20 mL 1 N HCl and filtered through new Celite into the original filtrate. The combined filtrates were rotoevaporated using a warm (45 °C) water bath to a volume of 5 mL (white precipitate formed) whereupon 10 mL of 1 N HCl was added (precipitate dissolved). The solution was rotoevaporated as above to a volume of ~1 mL and then added dropwise to 125 mL of acetone with stirring. The polymer was isolated by filtration and dried thoroughly under vacuum. Yield: 130 mg (73%). M_w = 1500, M_n = 1200.

Gel Retardation Assay. DNA:poly(4-hydroxyproline ester) complexes were formed by mixing 50 μL of plasmid DNA stock solution (CMV-βGal, 2.225 μg/50 μL in water) with appropriate volumes of a polymer stock solution (25 μg/50 μL in water) and water added for a total volume of 100 μL. The complexes were allowed to form for 15 min at room temparature, after which 20 μL was run on a 1% agarose gel (90 V, 45 min) and the bands were visualized by ethidium bromide staining.

Quasi-Elastic Laser Light Scattering (QELS). DNA:poly(4-hydroxy-L-proline ester) complexes were formed as described above for the gel retardation assay. The formed complexes were further diluted in 900 μL of water and the complex sizes determined by QELS (Brookhaven Instruments Co. model 95 ion laser, 8 W argon laser, incident beam = 480 nm).

Cytotoxicity Assay. Immortalized African green monkey kidney fibroblasts (COS-7) cells were grown in 96 well plates at an initial cell density of 10 000 cells/well in 0.2 mL growth medium (Dulbecco's modified Eagle's medium 90%; fetal bovine serum, 10%, penicillin 100 units/mL, streptomycin 100 μg/mL). The cells were grown for 48 h, after which the growth medium was removed and replaced with growth medium containing polymer. After a 48 h incubation, the metabolic activity of each well was assayed using the thiazolyl blue ((3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, MTT) assay.[12] Briefly, to each well was added 25 μL of a 5 mg/mL MTT stock solution in sterile PBS. After 2 h incubation at 37 °C, 100 μL of extraction buffer (20% w/v SDS in DMF:water (1:1), pH = 4.7) was added to each well and the plate incubated at 37 °C overnight. The optical densities were measured at 560 nm with a microplate reader (Dynatech Laboratories MR5000, Chantilly, VA) and expressed as a percent relative to control (no polymer) cells.

Results and Discussion

The polycondensation of N-protected-4-hydroxyproline was first reported using melt transesterification.[13,14] While this method resulted in polymers with high molecular weight, it required the synthesis of methyl ester monomer precursors, the presence of a metal catalyst, and, most importantly, high temperatures (~180 °C). The amine groups in these polymers were acylated with long chain fatty acids which could not be removed to yield a polycation. Removable N-protecting groups, such as *N*-*tert*-butoxycarbonyl (BOC), carbobenzyloxy (CBZ), and *N*-(9-fluorenylmethoxycarbonyl (Fmoc), can be susceptible to thermal decomposition.[15−17] Therefore, we explored the potential of low-temperature polycondensation to produce these N-protected 4-hy-

Table 2. Weight- and Number-Average Molecular Weights of Polymers Resulting from the Reactions Listed in Table 1

reaction no.	M_w	M_n	reaction no.	M_w	M_n
1	2200	1900	11	2800	2300
2	2700	2200	12	4200	2800
3	3400	2500	13	5000	3200
4	1100	1100	14	1200	1100
5	2700	2200	15	3400	2400
6	2900	2500	16	4100	2700
7	1600	1400	17	1600	1500
8	2900	2500	18	4000	2700
9	2900	2500	19	4400	2900
10	2900	2300	20	4800	3100

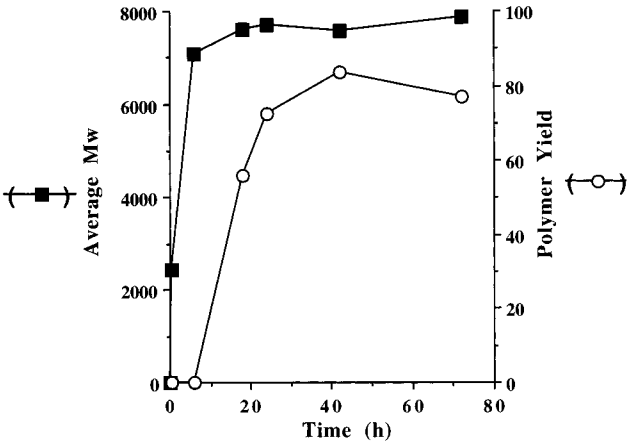

Figure 1. Kinetics of polymerization and yield of poly(CBZ-4-hydroxyproline ester) synthesized using the optimized variables ($N = 3$, standard deviations are present but are hidden under the data point markers).

droxy-L-proline derivatives. To identify a low-temperature method for the polycondensation of 4-hydroxy-L-proline, we evaluated four potential polymerization methods: (1) triphenylphosphine dichloride, (2) thionyl chloride, (3) hydroxybenzotriazole, and (4) dicyclohexylcarbodiimide/(dimethylamino)pyridine (DCC/DMAP). Using CBZ-4-hydroxy-L-proline as the monomer, only the DCC/DMAP method yielded a polymer.

To optimize the DCC/DMAP method for the polycondensation of 4-hydroxy-L-proline, we varied the monomer concentration, DCC mole equivalent, DMAP mole equivalent (with respect to the monomer mole content), and temperature according to the matrix in Table 1. The solvent used for the polymerizations (DMI) was chosen to maximize the concentration of the components in solution. The molecular weights resulting from the optimization matrix experiments are shown in Table 2. From the series of optimization reactions, the optimized reaction variables were determined: monomer concentration, 2 M; DCC mole equivalent, 2; DMAP mole equivalent, 0.5; temperature, 0 °C. The monomer concentration and DCC equivalent could not be increased further due to solubility limits, and an increase in the DMAP equivalent did not lead to a further increase in molecular weight (data not shown).

The polycondensation kinetics and yield of polymer at specific time points using the optimized parameters are shown in Figure 1. From this experiment we obtained the maximum average M_w ($M_w = 7800$, $M_n = 4400$) at 72 h and the maximum polymer yield at 40 h (83%).

The effect of steric hindrance at the N-position upon the DCC/DMAP-catalyzed polycondensation was determined by varying the size of the protecting group (BOC, 57 g/mol; CBZ, 91 g/mol; Fmoc, 179 g/mol). The reaction conditions used were as for reaction 12 in Table 1. The resulting M_n for each reaction were as follows: Boc-4-hydroxy-L-proline (2500), CBZ-4-hydroxy-L-proline (2800), and Fmoc-4-hydroxy-L-proline (2600). These molecular weights correspond to number-average degrees of polymerization of 11 (BOC), 11 (CBZ), and 7 (Fmoc). Therefore, N-substitution with moieties greater than approximately 100 g/mol may influence the DCC/DMAP-activated polycondensation of 4-hydroxy-L-proline and should be taken into account when using this method.

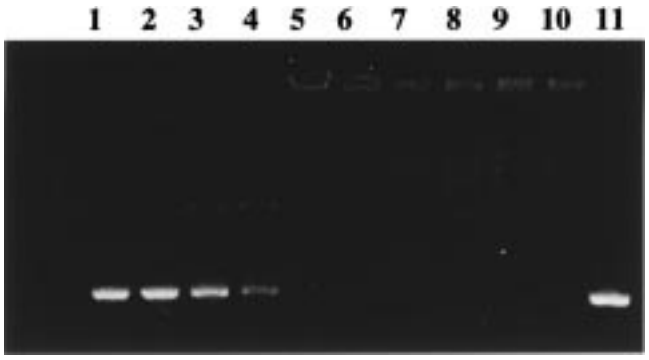

Figure 2. Electrophoretic retardation of CMV-βGal by poly(4-hydroxy-L-proline ester). Lane numbers correspond to the complex numbers in Figure 3 with the exception of 11, which is uncomplexed plasmid (DNA:polymer, 1:0, w:w).

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. Both the BOC and Fmoc protecting groups are removed under conditions known to hydrolyze ester bonds (BOC is removed by trifluoroacetic acid and Fmoc by piperidine) whereas CBZ is removable by catalytic hydrogenization. Therefore, we focused on the polycondensation of CBZ-4-hydroxy-L-proline with subsequent removal of the CBZ group. CBZ removal was accomplished by hydrogenolysis with Pd/C catalysis using formic acid as the hydrogen donor.[11] CBZ deprotection was also attempted with HBr/acetic acid.[10] The HBr/acetic acid method, with this polymer, resulted in cleavage of the ester backbone to the extent that no remaining polymer could be detected by gel permeation chromatography, but deprotection of the polymer resulting from the optimized polycondensation reaction using the formic acid−Pd/C method resulted in a deprotected polymer with $M_w = 1500$ and $M_n = 1200$. Comparison of the degrees of polymerization between the CBZ-protected polymer (number-average degree of polymerization = 18) and the deprotected polymer (number-average degree of polymerization = 8) shows that approximately one ester bond is cleaved in the polymer backbone using the formic acid deprotection method. The completeness of deprotection was assessed via ^{1}H NMR by observing the complete absence of the characteristic benzyl group peak at 7.25 ppm.

The ability of poly(4-hydroxy-L-proline) to complex with and condense plasmid DNA is illustrated in Figures 2 and 3**.** The plasmid, CMV-βGal, was incubated with increasing polymer concentration followed by 1% agarose gel electrophoresis. Agarose gel electrophoresis separates macromolecules based not only on size but also by charge. Complete gel retardation of the plasmid is evidence for DNA charge neutralization. With increased polymer content the gel retardation becomes

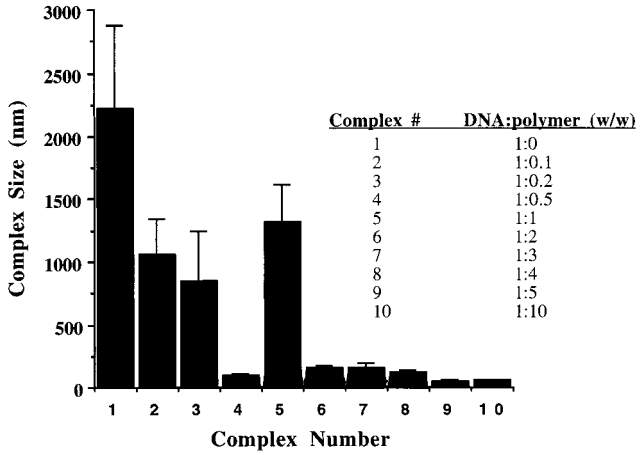

Figure 3. Sizes of DNA:polymer complexes as a function of polymer concentration.

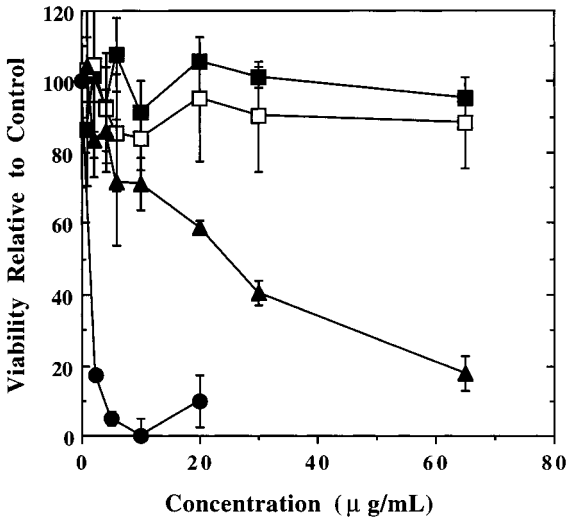

Figure 4. Cytotoxicity of 4-hydroxy-L-proline (closed square), poly(4-hydroxy-L-proline ester) (open square), polylysine (closed triangle), and polyethylenimine (closed circle) in vitro.

more prominent with complete retardation occurring at a DNA:polymer ratio (w:w) of 1:1.

The size of the polymer:DNA complexes as a function of increasing polymer content is shown in Figure 3. The complex sizes correlate well to the gel retardation experiment. With increasing polymer content, the size of the DNA:polymer complexes decreases until charge neutralization occurs at a DNA:polymer ratio of 1:1 (w:w) where large aggregates form. This aggregation pattern is well documented in the literature for DNA:polycation complexes and is a result of the elimination of charge repulsion among the complexes.[18] With increasing polymer content, the complex sizes continually decrease until the DNA:polymer ratio 1:5 is reached where a further increase in polymer content does not result in a reduction in complex size. For receptor-mediated endocytosis of the complexes, their sizes should be on the order of <150 nm.[19] Complexes in this size range for the DNA:poly(4-hydroxy-L-proline ester) occur at 1:3 (w:w) and above.

As an initial indication of the cytotoxicity of poly(4-hydroxy-L-proline ester) the polymer's effect on cell growth and metabolism of COS-7 cells in vitro was determined as a function of polymer concentration and compared to both polylysine and PEI (Figure 4).

The COS-7 cell line is a first level transfection efficiency model and was therefore used as our first level cytotoxicity model. Over the concentration range tested, the cells incubated with poly(4-hydroxy-L-proline ester) retained above 85% viablity relative to untreated control cells. Cells incubated with polylysine and PEI over the same concentration range had minimum cell viabilities of 20% and 2%, respectively. It should be stressed that this in vitro assay is a preliminary screening of the polymer's biocompatibility. A complete investigation of the biocompatibility and transfection efficiency of the polymer is currently in progress and will be reported in a subsequent paper.

In summary, the optimization of the low-temperature polycondensation of *N*-α-carbobenzyloxy-4-hydroxy-L-proline via a DCC/DMAP method is reported. The optimized polymerization parameters resulted in a polymer with $M_w = 7880$. Deprotection of the CBZ group was afforded by Pd/C−formic acid-catalyzed hydrogenolysis to yield poly(4-hydroxy-L-proline ester). The interaction of the polymer with plasmid DNA was characterized by electrophoretic mobility retardation and DNA:polymer complex sizes as a function of polymer concentration. Charge neutralization of the DNA occurred at a DNA:polymer ratio of 1:1 (w:w), and the complex sizes fell below 150 nm at the DNA:polymer ratio 1:3 (w:w). The cytotoxic profile of poly(4-hydroxy-L-proline ester) in vitro was excellent (greater than 85% cell viability) relative to polylysine and PEI. From the results reported herein we concluded that poly(4-hydroxy-L-proline ester) has potential for use in gene delivery, and we are currently investigating the polymer's biocompatibility and transfection efficiency both in vitro and in vivo.

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