Synthesis and Characterization of the Novel Transfection Reagent Poly(amino ester glycol urethane)

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Poly(ester urethane) (PEU) is a class of biodegradable polymer that has been applied as tissue-engineering scaffolds with minimum toxicity. Despite its unique biocompatibility, there have been no reports in modifying the PEU backbone to design a soluble, PEU-based DNA carrier. We have developed a method of incorporating tertiary amines and poly(ethylene glycol) (PEG) into PEU to synthesize a soluble poly(amino ester glycol urethane) (PaE_GU) as a novel transfection reagent. Parallel to this, we have synthesized poly(amino ester) (PaE) and poly(amino ester urethane) (PaEU) as the control polymers. The test transfection reagent PaE_GU and the control PaE were similar in their properties of being soluble and buffering pH in water and their capabilities of self-assembling with DNA and transfecting the target cells. Significantly, PaE_GU exhibited faster hydrolysis kinetics than PaE, half-lives of 19 and 36 h for PaE_GU and PaE, respectively, underlying PaE_GU's unique property of low cytotoxicity. However, in comparison to PaE_GU, the other control polymer, PaEU, was not readily dissolved in water, indicating the importance of PEG units in PaE_GU in increasing polymer hydrophilicity. This study demonstrated a useful synthesis scheme for the PEU-based transfection reagent PaE_GU. The combination of tertiary amine, ester, PEG, and urethane units in the polymer backbone constitutes a feasible approach for the future design of low-toxicity gene transfer vectors.

Introduction

Nonviral vectors are attractive alternatives to viral vectors due to their ease of production, low immunogenicity, and lack of pathogenicity.[1−4] However, nonviral vectors also suffer from relatively low levels of transgene delivery in comparison to those of viruses. Although higher vector amounts have always been perceived as alleviating this problem, the intrinsic toxicity of nonviral vectors, such as the toxicity of polymeric transfection reagents and their accumulation inside the cytoplasm, limits the vector doses. This dilemma underlines the importance of developing new classes of low-toxicity nonviral DNA carriers while maintaining their effectiveness in transfection.

Polymeric transfection reagents generally are formulated by biodegradable polymers to improve biocompatibility. These polymers are incorporated with positively charged amine groups for DNA binding and are connected through hydrolytically unstable linkages such as ester or urethane linkages to induce biodegradability and generate nontoxic degraded compounds.[5−11] Additionally, cationic polymers would provide a pH-buffering ability, allowing a "proton sponge" to be formed, which would assist in the escape of vectors from the endocytosis pathway and improve transfection efficiency.[12−15]

Among a variety of biodegradable polymers, poly(ester urethane) (PEU), has lately attracted interest as tissue-engineering scaffolds for its elastic, biodegradable, and cell-adhesive properties.[16−20] Importantly, PEU and its degradation products have shown minimum cytotoxicity in vitro and in vivo.[16−19] Despite the unique biocompatibility of PEU as a tissue-engineering scaffold, to our knowledge, it appears that no previous reports have increased the hydrophilicity of this polymer to generate a soluble PEU-based DNA carrier.

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With the goal of developing PEU as the backbone for a new transfection reagent, we investigated a two-step scheme of using poly(ethylene glycol) acrylate, 2-diethylaminoethylamine, and 1,4-diisocyanatobutane for polymerization to generate a novel reagent poly(amino ester glycol urethane) (PaE_GU). In parallel, we have synthesized poly(amino ester) (PaE) and poly(amino ester urethane) (PaEU) as control polymers. Our results showed that the hydrophilicity and fast hydrolysis kinetics (half-life of 19 h) properties of PaE_GU were different from those seen in PEU as tissue-engineering scaffolds, which would be of interest to others in the field of biodegradable polymers and gene delivery. We present and discuss the results that characterize and compare PaE_GU and the control polymers in pH-buffering capacity, degradation, polymer/DNA condensation, transgene delivery, and cytotoxicity in this report.

Experimental Procedures

Materials. 2-Diethylaminoethylamine, 2-hydroxyethyl acrylate, dibutyltin dilaurate, tetrahydrofuran (THF), and *N*,*N*-dimethylformamide (DMF) were purchased from Fluka (Buchs, Switzerland) and were dried by passing over calcium hydride and then distilled before use. Phosphate-buffered saline (PBS, pH 7.4) was purchased from Sigma Co. (St. Louis, MO). Poly(ethylene glycol) acrylate (M_w = 375) and 1,4-diisocyanatobutane were purchased from Aldrich (Milwaukee, MI). Hind III endonuclease was purchased from New England Biolabs (Ipswich, MA). 5-Bromo-4-chloro-3-indoyl-β-galacto-pyranoside (X-gal) and CellTiter 96 AQueous one solution for the MTS assay were purchased from Promega (Madison, WI).

Synthesis of the Test Transfection Reagent Poly(amino ester glycol urethane), PaE_GU. The test transfection reagent PaE_GU was synthesized following the scheme in Figure 1A. First, poly(ethylene glycol) acrylate and 2-diethylaminoethylamine with a C═C/NH_2 molar ratio of 2/1.1 were mixed in anhydrous THF solvent under liquid nitrogen purge inside a reaction flask. The mixture was heated to 50 °C

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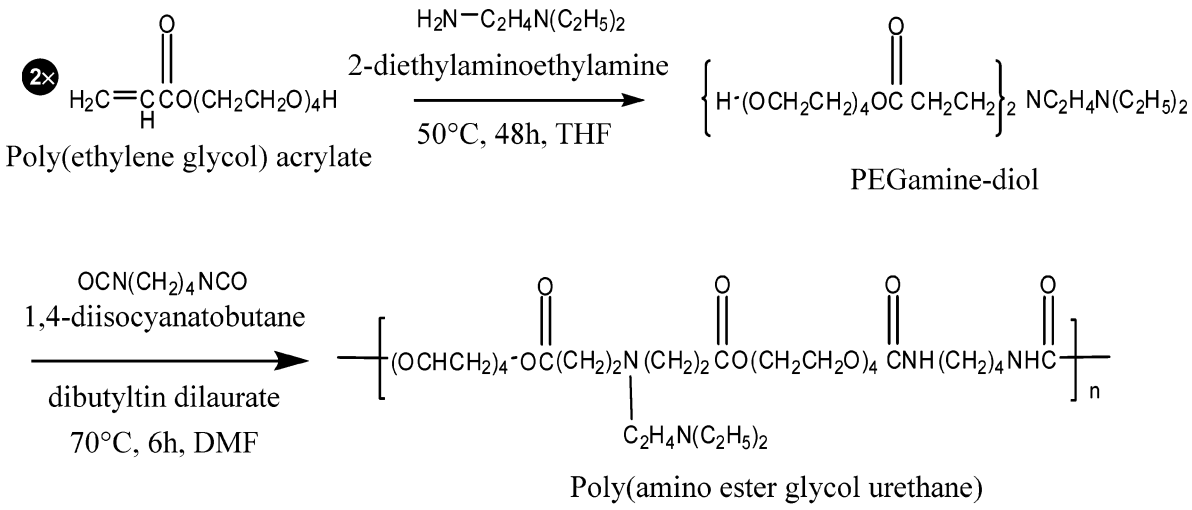
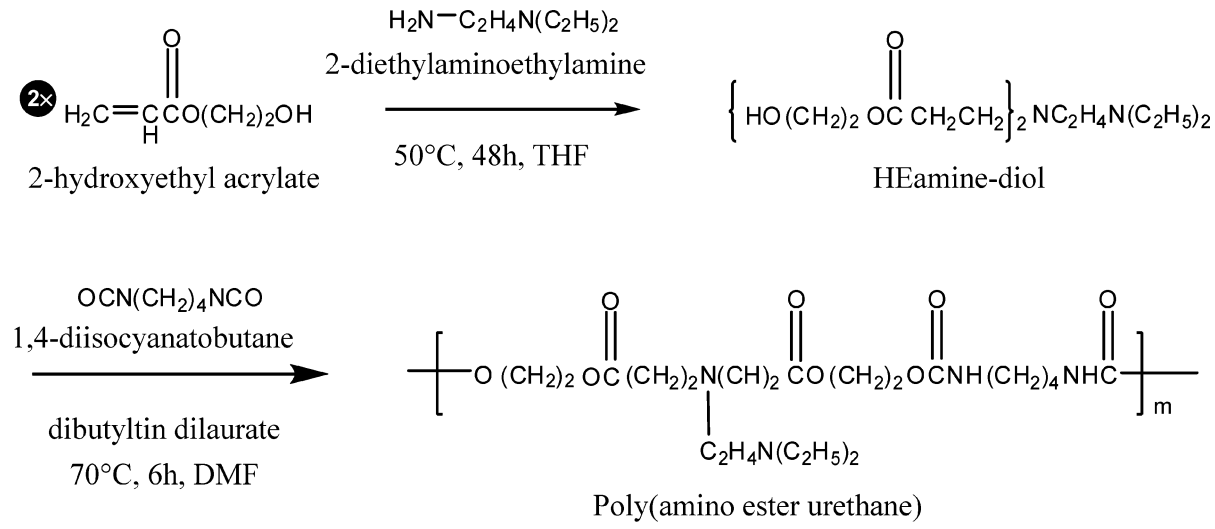
(A) Synthesis of Poly(amino ester glycol urethane), PaE_GU



(B) Synthesis of Poly(amino ester urethane), PaEU



(C) Synthesis of Poly(amino ester), PaE

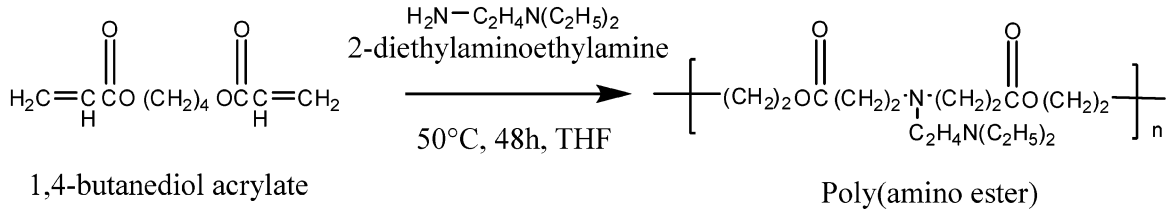


Figure 1. Synthesis schemes for the test transfection reagent (A) PaE_GU and the control polymers (B) PaEU and (C) PaE.

for reaction for 48 h to generate the monomer PEGamine-diol. Afterward, the reaction solution was added dropwise to 10 mL of stirring hexanes. Reactant 2-diethylaminoethylamine was soluble in hexanes; the undissolved product PEGamine-diol was separated from the liquid phase and then vacuum-dried at 40 °C. In the second reaction, the synthesized PEGamine-diol and 1,4-diisocyanatobutane with a OH/NCO molar ratio of 1/1.1 were mixed in anhydrous DMF solvent in a three-necked reaction flask under dry nitrogen purge. The mixture was then heated to 70 °C for reaction for 6 h with 10 wt % dibutyltin dilaurate as the catalyst. An excess amount of methanol was slowly added into the reaction mixtures until no un-reacted isocyanate groups were detected. After reaction, PaE_GU was precipitated in anhydrous ethyl ether and vacuum-dried at 40 °C.

Synthesis of the Control Polymers Poly(amino ester urethane), PaEU, and Poly(amino ester), PaE. Synthesis of PaEU (Figure 1B) followed the same scheme for PaE_GU synthesis, except that 2-hydroxyethyl acrylate and 2-diethylaminoethylamine were used to generate the monomer HEamine-diol, which was then reacted with 1,4-diisocyana-

tobutane in the second reaction to produce PaEU. Synthesis of PaE followed the scheme in Figure 1C. 1,4-Butanediol diacrylate (1.0 g, 5.04 mmol) and 2-diethylaminoethylamine (5.04 mmol) were dissolved in THF in separate vials. The 2-diethylaminoethylamine solution was then slowly added into the 1,4-butanediol diacrylate solution. Polymerization of these two monomers took place at 50 °C.[7] After 48 h, the reaction solution was dripped into vigorously stirring hexanes. PaE was collected and vacuum-dried prior to analysis.

Characterization of Synthesized Monomers and Polymers. The synthesized PEGamine-diol and HEamine-diol monomers and final polymers were first characterized by Fourier transform infrared (FT-IR, Perkin-Elmer Spectrum 824, spectra recorded as KBr pellets) spectroscopy to identify their functional groups (Figure 2). Their structures were then further examined by nuclear magnetic resonance (NMR, Varian Unity Inova 500 MHz spectrometer). Chemical shifts in ^{1}H NMR and ^{13}C NMR spectra (Supporting Information) were reported in parts per million (ppm). A 99.8% pure DMSO-d_6 was used as the solvent in characterization. The molecular weights of the polymers were determined by high-performance liquid chromatography/gel permeation chromatography analysis (HPLC/GPC, Waters model LC-2410). THF was used as the eluent, and polystyrene was used as the reference in HPLC/GPC. The sample concentration in THF was 8.0 mg/mL. The flow rate was 1.0 mL/min. The weight- and number-average molecular weights (M_w and M_n, respectively) were calibrated with standard polystyrene samples.

Acid−base titration was used to evaluate the buffering capacity of synthesized polymers. However, because PaEU was not readily dissolved in water and formed insoluble clumps in solution, only PaE_GU and PaE were included in this assay. Approximately 10 mg of test polymers was dissolved in 10 mL of 150 mM NaCl, and then 100 μL of 1 N NaOH was added to the solution to adjust the pH to the alkaline range at approximately 11.5. Approximately 0.1 N HCl was used as the titratant to lower the pH to acidic conditions at approximately 2.0. The titration increment size was 100 μL. Hydrolytic degradation of polymers over time was measured by dissolving PaE_GU and PaE in PBS solution (pH 7.4) at a concentration of 10.0 mg/mL and then incubating in a water bath at 37 °C. Hydrolytic degradation was stopped at various points in time, and samples were vacuum-dried to remove water. The residual molecular weights of the polymer were determined by HPLC/GPC.

Plasmid DNA Preparation. The plasmid pCMV-EGFP carrying an enhanced green fluorescent protein (EGFP) expression cassette was used in this research. This EGFP expression plasmid was constructed by cloning the 0.8 kb EGFP cDNA from plasmid pEGFP-N2 (Clontech, Palo Alto, CA) downstream of the CMV promoter in plasmid pAAV-MCS (Stratagene, La Jolla, CA). The finished 5.8 kb plasmid pCMV-EGFP has only one Hind III endonuclease cleavage site. In addition to pCMV-EGFP, a pCMV-lacZ plasmid carrying the reporter β-galactosidase has also been used to evaluate PaE_GU-mediated transfection. Plasmids were amplified in *Escherichia coli* (DH5α strain) and purified by the Qiagen Plasmid Purification Midi Kit (Hilden, Germany).

Preparation and Characterization of Polymer/DNA Complexes. To prepare the polymer/DNA complex, 5.0 mg of polymer was dissolved in PBS (pH 7.4) and mixed with plasmid DNA at a mass ratio of polymer/DNA ranging from 0.5/1 to 150/1 (w/w). The polymer/DNA mixture was incubated at room temperature for 30 min to allow for self-assembly of polyplexes before further characterization.

The hydrodynamic sizes of polymer/DNA complexes were determined by dynamic light scattering (Nicomp 380 system, USA) at 25 °C using a 5 mW He−Ne laser ($\lambda = 633$ nm) as the incident beam at a scattering angle of 90°. The surface charges of polymer/DNA complexes were determined by the electrophoretic mobility at 25 °C with a zeta-potential system (Nicomp Instruments, USA).

The charge interaction between polymer and DNA was evaluated by a gel retardation assay. Electrophoresis was carried out in this assay with different mass ratios of polymer/DNA complexes loaded into parallel wells of a 0.7% agarose gel containing 0.3 μg/mL of ethidium bromide in a Tris-acetate-EDTA (TAE) buffer. Polymer-free DNA was used as the control. Electrophoresis was performed at 100 V for 45 min. The mobility of DNA was analyzed by gel imaging under UV irradiation.

Polymer protection of DNA from enzyme attack was evaluated by a restriction endonuclease assay. In this assay, polymer/DNA complexes at mass ratios of 1/1, 5/1, and 50/1 (w/w) were incubated with endonuclease Hind III at a concentration of 20 U/μL at 37 °C for 60 min in the provided reaction buffer. Afterward, samples were loaded into parallel wells of agarose gel for electrophoresis analysis as described in the gel retardation assay.

Cell Culture and Transfection. Human HT-1080 fibroblasts (ATCC, Manassas, VA) were grown in Dulbecco's modified Eagle's medium (DMEM, Biosource, Rockville, MD) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. Approximately 5×10^5 cells were seeded in 40 mm dishes and fed with complete DMEM medium for 12 h prior to transfection. Polymer/DNA complexes for transfection were prepared by incubating various amounts of polymer (in PBS) with 1 μg of DNA (in DMEM), with polymer/DNA mass ratios from 10/1 to 150/1, for 30 min. DMEM was then added to the mixture to adjust the final volume to 1100 μL. Transfection was carried out by replacing the culture medium with the 1100 μL transfection cocktail. One hour later, the transfection cocktail was replaced with complete DMEM medium, and cells were incubated for an additional 48 h before reporter analysis.

Analysis of Reporter Expression. Flow cytometry analysis of EGFP-transfected cells was conducted with a FACSCalibur (Becton Dickinson) benchtop flow cytometer equipped with an argon laser at 488 nm excitation and a band-pass filter at 505−530 nm to detect EGFP. Untransfected cells were used as the control. The cells were appropriately gated by forward and side scatters, and 10 000 events per sample were collected. Additionally, transfected fibroblast monolayers were imaged by the Nikon ECLIPSE TS100-F fluorescence microscope for EGFP detection with a Nikon GFP-L filter set (EX 480/40, DM 505, BA 510). Expression of reporter β-galactosidase from PaE_GU-mediated pCMV-lacZ transfection was evaluated by X-gal staining. Transfected HT-1080 monolayers were fixed (0.25% glutaraldehyde; 5 min at 4 °C), stained with 1.25 mg/mL X-gal solution containing 1 mM $MgCl_2$, 0.5 mM $K_4Fe(CN)_6 \cdot 3H_2O$, and 0.5 mM $K_3Fe_3(CN)_6$ in sterile PBS for 24 h at 37 °C, and imaged. Positive cells were visible as blue spots. Untransfected HT-1080 monolayers were used as the control.

Evaluation of HT-1080 Cell Viability. The mitochondrial activity of polymer-treated cells was measured using the CellTiter 96 AQueous one solution cell proliferation assay system according to the manufacturer's protocol.[21] In this cell viability/proliferation assay, cells were treated with polymers at various concentrations for 1 h. Afterward, the test medium was removed, and the cells were washed with PBS and then incubated for an additional 48 h before the MTS assay. The methoxyphenyl-tetrazolium salt (MTS) compound was reduced by viable cells to form a colored formazan product, which was quantified colorimetrically at 490 nm using a spectrofluorometer (Sunrise/Tedan, Austria). Untreated HT-1080 cells were used as the control.

Results and Discussion

Characterization of Synthesized Polymers. Figure 1 schematically shows the synthesis of the test transfection reagent poly(amino ester glycol urethane), PaE_GU, and the control polymers, poly(amino ester), PaE, and poly(amino ester urethane), PaEU. Detailed procedures were described in the Experimental Procedures section. Figures 2A and 2B report the FT-IR spectra of the reactant and product following the PaE_GU synthesis scheme (Figure 1A). We characterized the intermediate PEGamine-diol after the first reaction. Figure 2A shows that

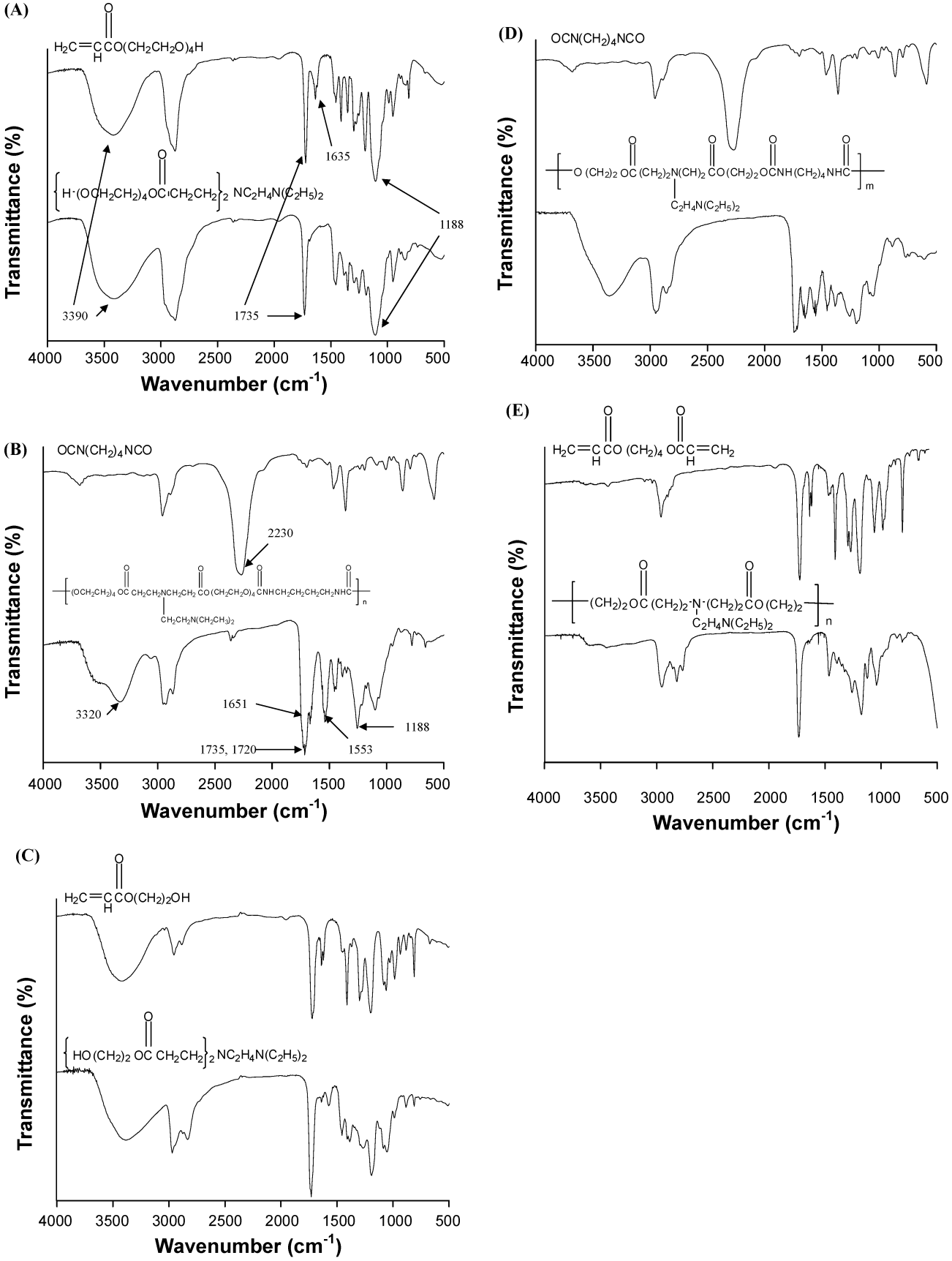


Figure 2. FT-IR spectra of (A) poly(ethylene glycol) acrylate and PEGamine-diol, (B) 1,4-diisocyanatobutane and PaE_GU, (C) 2-hydroxyethyl acrylate and HEamine-diol, (D) 1,4-diisocyanatobutane and PaEU, and (E) 1,4-butanediol diacrylate and PaE.

the adsorption peaks of the sample were at 1735 cm^{-1} (C═O stretching, ester), 1188 cm^{-1} (C−O stretching, ester), and 3390 cm^{-1} (O−H stretching, alcohol), indicating the presence of ester and alcohol links, which were derived from the poly(ethylene glycol) acrylate. Because there was no alkenes' absorption peak at 1635 cm^{-1} after the reaction, we confirmed that the alkenes

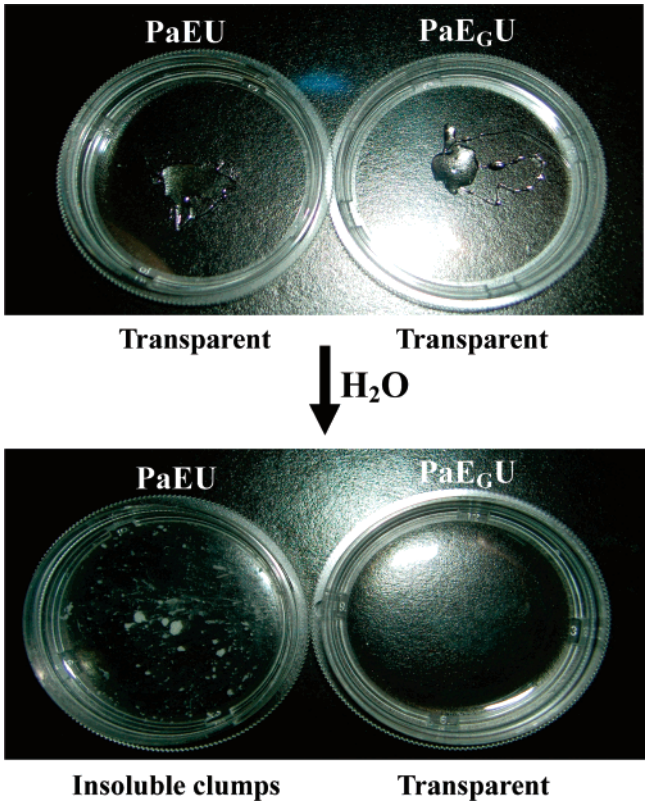


Figure 3. Gross images of the (A) pure PaEU and PaE$_G$U polymers and (B) PaEU−water and PaE$_G$U−water mixtures. PaEU was not readily dissolved in water and formed insoluble white clumps in suspension.

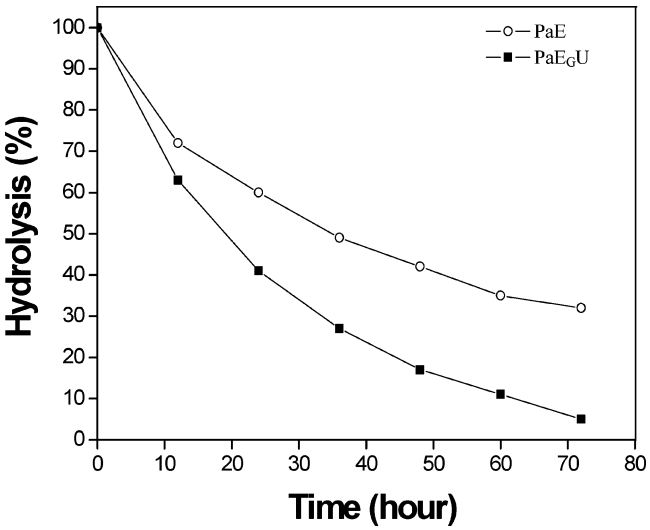


Figure 4. Hydrolytic degradation of PaE$_G$U and PaE incubated in PBS buffer at pH 7.4 and 37 °C. Degradation is expressed as percent degradation over time based on the HPLC/GPC data.

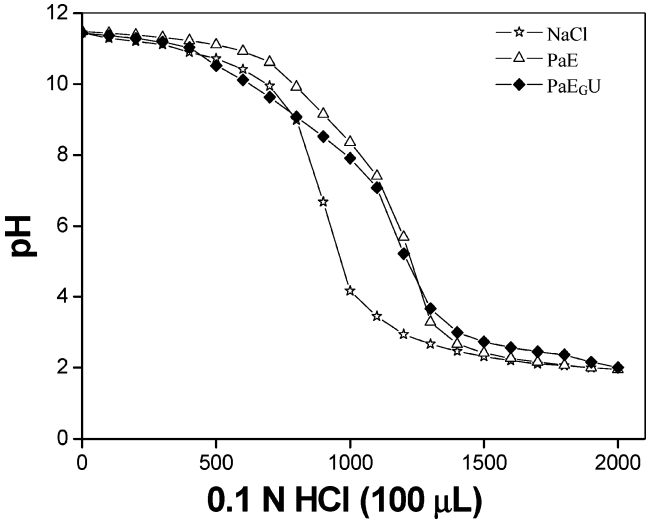


Figure 5. Acid−base titration profiles of the PaE$_G$U and PaE polymers and the NaCl control solution.

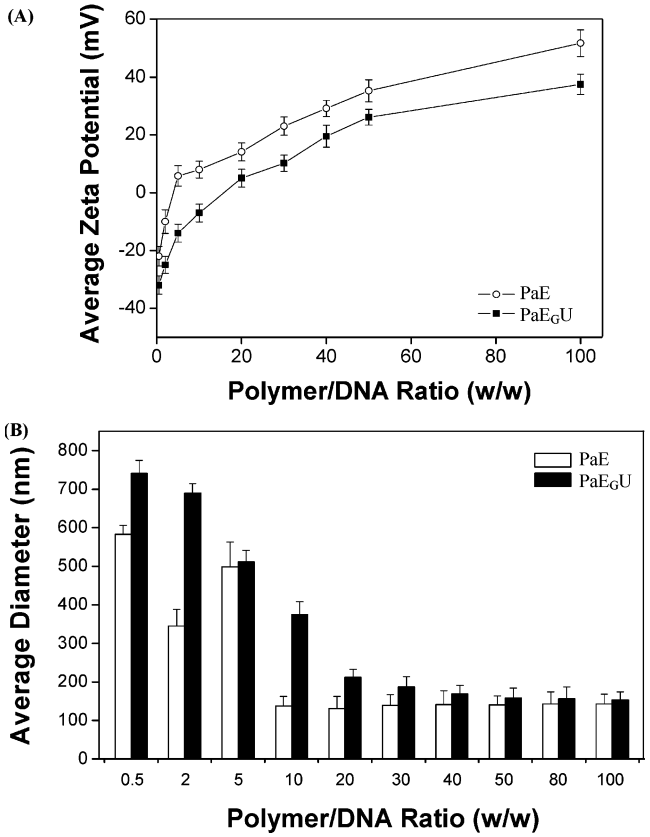


Figure 6. (A) Zeta-potential and (B) size of polymer/DNA complexes at different polymer/DNA mass ratios (w/w). Results are presented as mean ± standard deviation ($n = 3$).

in the reactant poly(ethylene glycol) acrylate were changed to the ethyl group in the PEGamine-diol.

We then characterized the product of the second reaction. Figure 2B shows that the absorption peaks of the PaE$_G$U were at 1735 cm^{-1} (C═O stretching, ester), 1188 cm^{-1} (C−O stretching, ester), 1720 cm^{-1} (C═O stretching, urethane), 3320 cm^{-1} (N−H stretching, urethane), 1651 cm^{-1} (C═O stretching, amide), and 1553 cm^{-1} (N−H bending, amide), indicating the presence of ester and urethane linkages. Additionally, because there was no isocyanates derived peak at 2230 cm^{-1}, we confirmed that after polymerization the isocyanates in the reactant 1,4-diisocyanatobutane were successfully changed to the urethane group in our final product PaE$_G$U. Similar analysis of the FT-IR adsorption peaks in Figures 2C, 2D, and 2E has been used to differentiate the reactant and the product following the synthesis schemes for the control polymers PaEU (Figure 1B) and PaE (Figure 1C).

The FT-IR analysis, along with the ^{1}H and ^{13}C NMR data (Supporting Information), confirmed the designated functional groups in the synthesized polymers and polymer structures. We further determined the molecular weight of the polymer by HPLC/GPC. The results showed that PaE$_G$U, PaEU, and PaE had weight-average molecular weights of 17 500, 18 100, and

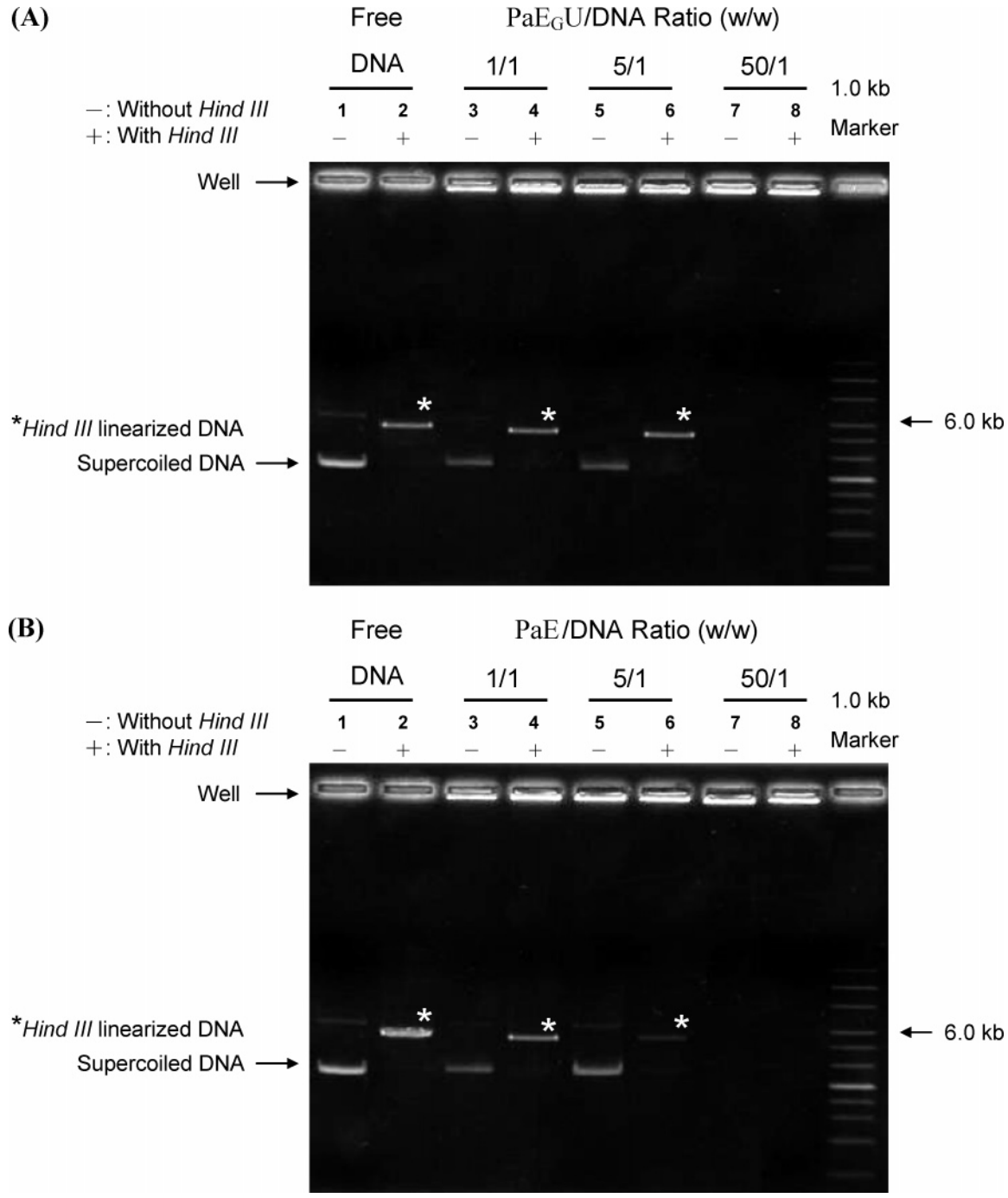


Figure 7. DNA gel retardation and restriction endonuclease protection assays of (A) PaE_GU and (B) PaE: without endonuclease Hind III (−); with endonuclease Hind III (+). The ethidium bromide staining at the loading wells indicated that DNA was retarded or retarded and protected by polymers.

15 700 as well as polydispersity indices of 1.4, 1.8, and 1.6, respectively.

Among the three synthesized polymers, the test PaE_GU and the control PaE were readily dissolved in water; however, the other control PaEU had limited solubility in water. Figure 3 shows that although the gross images of pure PaE_GU and PaEU were both transparent, after mixing with water (10 mg/mL), PaE_GU was dissolved and the solution remained transparent, yet PaEU formed insoluble white clumps. This result indicated the importance of incorporating the PEG units in the PaE_GU backbone to increase polymer hydrophilicity.

Comparison of Hydrolysis Kinetics of PaE_GU and PaE. Hydrolysis of synthesized PaE_GU and PaE was tested by dissolving polymers in PBS and then incubating at 37 °C over a period of 72 h (Figure 4). A percentage of the degradation profile was calculated from the relative change in M_w of the aliquot at a point in time to the initial M_w. In this hydrolysis test, both PaE_GU and PaE exhibited continuous degradation during the test period. Specifically, Figure 4 shows that PaE_GU and PaE had estimated half-lives of 19 and 36 h, respectively. Because both PaE_GU and PaE included ester linkages and amine groups in their polymer backbones, their continuous degradation in water can be explained by the hydrolytic ester cleavage of the polymer, catalyzed by the nucleophilic amine groups for attack of water.[22,23]

The presence of urethane linkages and PEG units in the PaE_GU backbone decreased the hydrolysis half-life to 19 h from the that of PaE of 36 h, or by 47%. This result is consistent

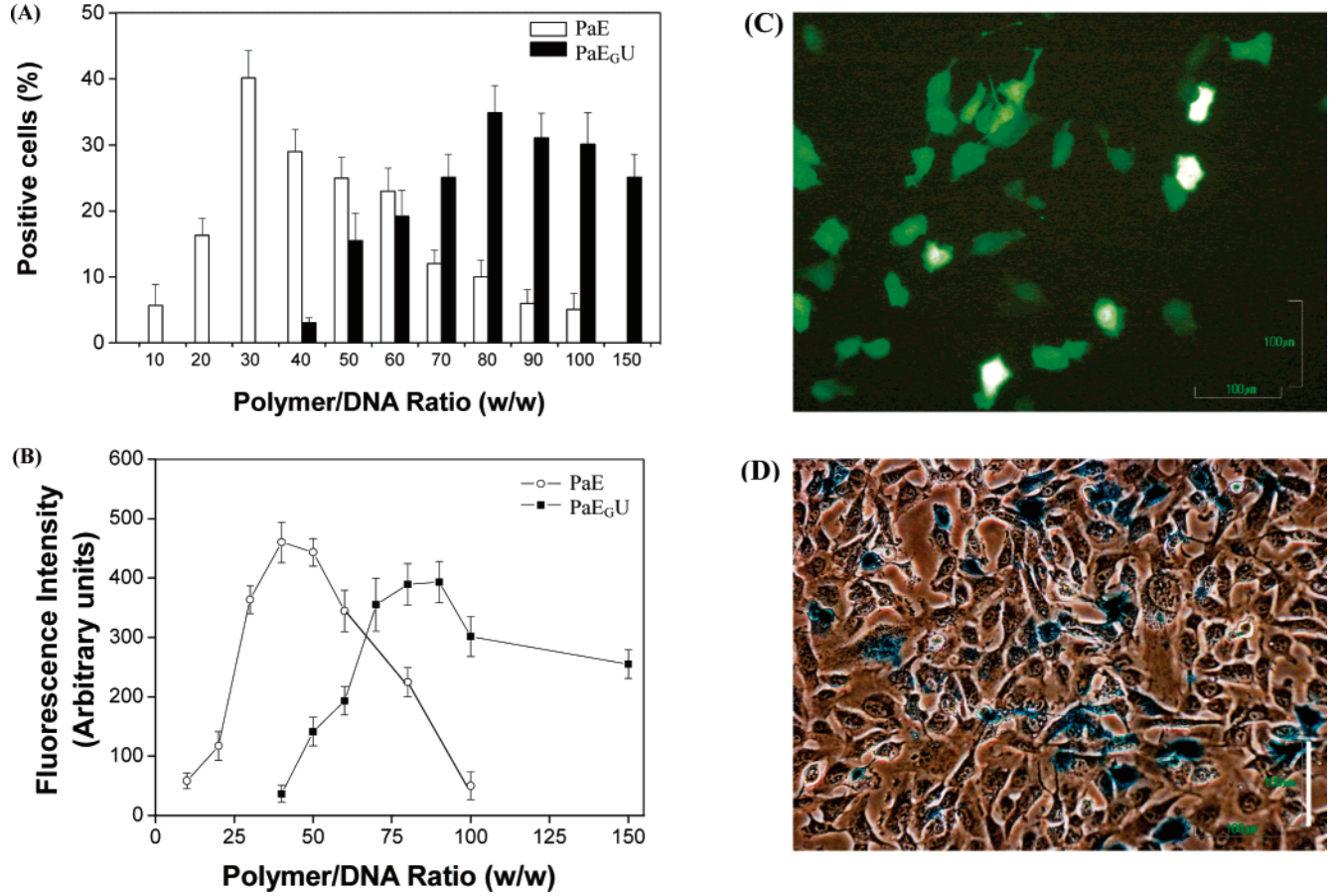


Figure 8. (A) EGFP-positive cells (%) of PaE$_G$U- and PaE-mediated transfection. The percentage of positive cells was analyzed by fluorescence-activated flow cytometry. Results are presented as mean ± standard deviation ($n = 3$). (B) Fluorescence intensities of PaE$_G$U- and PaE-mediated transfection. Results are presented as mean ± standard deviation ($n = 3$). (C) Fluorescence micrograph of PaE$_G$U/DNA (80/1, w/w) mediated transfection of HT-1080 cells using EGFP as the reporter. (D) Phase-contrast micrograph of X-gal staining of PaE$_G$U/DNA (80/1, w/w) mediated transfection of HT-1080 cells using β-galactosidase as the reporter. The bar is 100 μm in length.

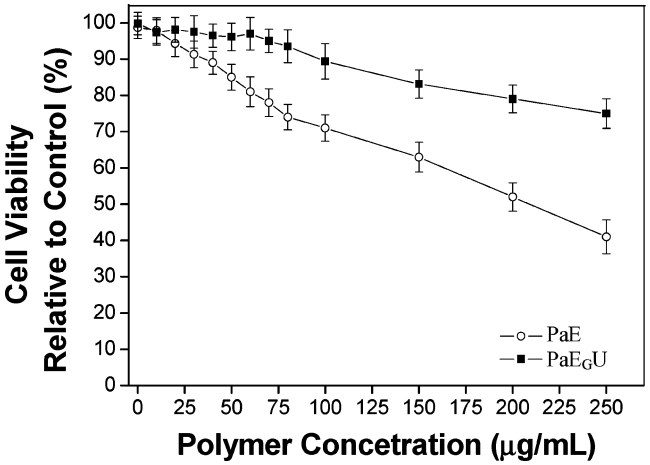


Figure 9. Assay of cell viability after treatment with PaE$_G$U or PaE. The viability of untreated HT-1080 cells was assigned a value of 100. Results are presented as mean ± standard deviation ($n = 3$).

with previous findings that the urethane group, similar to the ester group, is capable of not only elevating the hydrophilicity of the polymer but also promoting hydrolysis.[5,6] Application of hydrolytic polycations in gene transfer has been proposed with low cytotoxicity by dispersing the intrinsic high cationic charge through degradation.[23−26] Thus, the distinct hydrolytic behavior of PaE$_G$U represents a large advantage in its biocompatibility. Additionally, this property would facilitate releasing of DNA from the PaE$_G$U/DNA complex in transfection.

Comparison of pH-Buffering Capacity between PaE$_G$U and PaE. Previous reports have indicated the importance for polymeric gene transfer vectors to possess the pH-buffering "proton sponge" ability to assist the escape of vectors from the endosomal/lysosomal compartments.[12−15] In Figure 5, we examined the acid−base titration profiles of PaE$_G$U, PaE, and the NaCl control. Both polymers exhibited similar pH-buffering profiles against the increment of HCl acid, with buffering capacities between pH 11.5 and 7.0.

Zeta-Potential and Size Analysis of Polymer/DNA Complex. Figure 6A shows the zeta-potential values of PaE$_G$U/DNA and PaE/DNA complexes at various mass ratios, ranging from 0.5/1 to 100/1 (w/w). The zeta-potential steadily changed toward positive values associated with an increase in polymer/DNA mass ratio. The values became larger than zero as the mass ratios of PaE$_G$U/DNA and PaE/DNA were larger than 20/1 and 5/1, respectively. Figure 6B reports the particle sizes of PaE$_G$U/DNA and PaE/DNA complexes. It was noticeable that the average diameters of PaE$_G$U/DNA (150−160 nm) and PaE/DNA (125−140 nm) were not significantly affected by the polymer/DNA ratio when ≥50/1 and ≥10/1, respectively. Under these conditions, the particle diameters were both smaller than the general particle size required for cellular endocytosis at 200 nm,[27,28] suggesting their potential in forming condensed polymer/DNA polyplexes for transfection.

Gel Retardation Assay and Restriction Endonuclease Protection Assay of the Polymer/DNA Complex. PaE$_G$U and PaE were capable of forming complexes with plasmid DNA,

and their interactions were tested in the gel retardation assay. Figures 7A and 7B show that PaE_GU and PaE behaved similarly in interacting with DNA. Plasmid DNA was partially retarded on agarose gel electrophoresis in the presence of PaE_GU and PaE at the polymer/DNA mass ratios of 1/1 and 5/1 (lanes 3 and 5) and was totally retarded at the mass ratio of 50/1 (lane 7).

We also investigated whether the plasmid DNA was protected from endonuclease digestion in the PaE_GU/DNA and PaE/DNA complexes. The free DNA was completely linearized after 60 min of incubation with endonuclease Hind III, as demonstrated by the DNA band (5.8 kb) in lane 2 in Figures 7A and 7B. Similarly, DNA digestion was observed at PaE_GU/DNA and PaE/DNA mass ratios of 1/1 and 5/1, indicating that DNA was not fully condensed with polymer at these two doses. However, the plasmid DNA was completely protected in the PaE_GU/DNA and PaEU/DNA complexes at a mass ratio of 50/1, indicating that both polymers had sufficient electrostatic interaction with DNA at this dose against endonuclease digestion.

Transfection Activity in Culture. To compare the transfection efficiency of our test reagent PaE_GU and the control PaE, we applied EGFP as the reporter to visualize and quantify transfected cells. Human HT-1080 fibroblasts grown as monolayers were transfected with the EGFP-encoding plasmid at various polymer/DNA ratios. Twenty-four hours after transfection, a significant number of EGFP-positive cells could be identified under the fluorescence microscope. Forty-eight hours after transfection, fluorescence-activated flow cytometry was applied to quantify the percentage of EGFP-positive cells, which can be considered as the average transfection efficiency (Figure 8A).

The highest efficiency of PaE_GU-mediated transfections occurred at a polymer/DNA mass ratio of 80/1, which generated 35% of EGFP-positive cells. This was 87% of the highest efficiency of the PaE-mediated transfection (mass ratio at 30/1). Figure 8B compares the fluorescence intensities of PaE_GU- and PaE-mediated reporter expression. The highest fluorescence intensity of PaE_GU-mediated transfections occurred at the mass ratio of 90/1; this intensity was slightly smaller (by 15%) than the highest intensity generated from the PaE-mediated EGFP expression (mass ratio at 40/1).

Figure 8C shows a typical fluorescence micrograph of PaE_GU/DNA (80/1) transfected HT-1080 cells 24 h after transfection. The monolayer of cells exhibited diffuse fluorescence, indicating strong EGFP expression. A similar result of PaE_GU-mediated transfection is illustrated in Figure 8D, where β-galactosidase was applied as the reporter and its expression was revealed by X-gal staining of the transfected monolayer 48 h after transfection.

Using human HT-1080 fibroblasts as the transfection targets allows us to compare the PaE_GU transfection with our previous work of adeno-associated virus serotype 2 transduction (EGFP-positive cells was approximately 78%, flow cytometry analysis) and FUGENE6 reagent (Roche, Indianapolis, IN) transfection (EGFP-positive cells was approximately 45%).[29] In addition to HT-1080 cells, human embryonic kidney 293 (HEK293) cells, human Caco-2 enterocytes, and Chinese hamster ovary (CHO-K1) cells were also transfected by PaE_GU for transgene expression (Supporting Information).

Cytotoxicity of PaE_GU and PaE. In addition to achieving transgene delivery, a successful transfection reagent should exhibit minimum cytotoxicity. It has been reported that incorporation of hydrolytic ester linkages contributed to the low toxicity of PaE-based transfection reagent.[7−11] Indeed, Figure 9 shows that both our synthesized PaE_GU and PaE caused a decrease of less than 10% in cell viability after incubation with 25 μg/mL polymers. When the polymer concentration was increased to 100 μg/mL, the viabilities of PaE_GU- and PaE-treated cells were approximately 90% and 71%, respectively. Significantly, even when the concentration of PaE_GU was as high as 250 μg/mL, 3.4-fold higher than the concentration used in Figure 8A to achieve 35% efficiency of transfection, the cell viability was still maintained at 75%, indicating that the new transfection reagent PaE_GU exhibited little toxicity toward the target cells.

Conclusion

We have synthesized a new transfection reagent poly(amino ester glycol urethane), PaE_GU, and characterized its properties using poly(amino ester), PaE, and poly(amino ester urethane), PaEU, as the controls. Among these three polymers, PaEU had limited solubility in water and formed insoluble clumps in solution. PaE_GU and PaE were similar in their properties of being soluble and buffering pH in water and their capabilities of self-assembling with DNA and transfecting the target cells. It is significant that PaE_GU exhibited faster hydrolysis kinetics than PaE, underlying PaE_GU's unique property of low cytotoxicity. Our study thus suggests that engineering soluble cationic polymers to incorporate ester, PEG, and urethane units in the backbone constitutes a useful approach for the future design of gene transfer vectors.

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Supporting Information Available. ^1H and ^{13}C NMR data, images of PaE_GU-mediated transfection of HEK293 cells, human Caco-2 enterocytes, and CHO-K1 cells. This material is available free of charge via the Internet at http://pubs.acs.org.

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