Synthesis and Characterization of Degradable Polycationic Polymers as Gene Delivery Carriers

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Biodegradable cationic poly(ester-amide) polymers were synthesized by double-monomer method, that showed excellent solubility in many organic solvents and water. Different degradation patterns were obtained by the regulation of monomer ratios and overall long period of time of DNA protection up to 12 days was shown by PicoGreen reagent assay. Good transfection profiles in the presence of serum and very low toxicity on mammalian cells may allow these polymers to become suitable for long-term gene delivery systems and therapeutic applications.

Key Words : Gene therapy, Gene delivery, Biodegradable polymer

Introduction

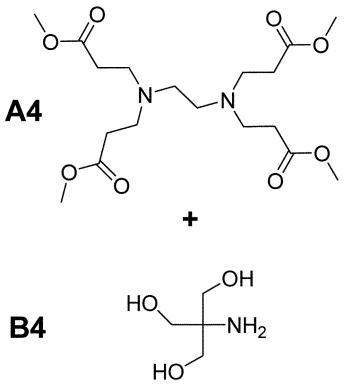
The challenging researches in polymeric vectors for gene therapy have been developed to improve the treatment of several diseases for many years. One of the effective transfection vector systems is either viral vectors or virus-associated sequences, that display very efficient target cell-specific trasfection results.[1] However, immunogenic problems and unexpected stimulation of protooncogenes caused by using viral vectors have been seriously issued so far.[2,3] Among non-viral vectors, polycationic polymers are intensively studied for the self-assembling ability with DNA, the formation of biocompatible nano-size particles, and the enhanced transport of DNA into the cell through the membrane.[4] The polyplex particles formed by cationic polymers have been characterized by charge density, size, morphology, degradation and transfection efficiency. The non-degradable polymers with high charge densities, and high molecular weight like PEI showed significant cellular toxicities which revealed the need for low-toxic, and degradable polymers possessing effective gene transfection efficacy. Many degradable polymers have been reported, but most of them degrade relatively too fast to attain long-circulating or effective controlled release of the complexed therapeutic genes.[5-9]

Materials and Methods

Materials. Ethylene diamine, Fmoc-ε-Ahx-OH, ethidium bromide, methyl acrylate, tris(2-hydroxyethyl)amine, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), N,N-Diisopropylethylamine (DIPEA), piperidine (Hexahydropyridine), ethanolamine, PEI (average molecular weight 25 kDa), and trizma base (2-Amino-2-(hydroxymethyl)-1,3-propanediol), diethyl ether were purchased from Sigma-Aldrich (St. Louis, MO). Fmoc-6-aminohexanoic acid (Fmoc-eAhx) was purchased from Novabiochem (Laufelfingen, Switzerland). N,N,N',N'-tetramethyl-O-(1H-benzotriazole-1-yl)uranium hexafluorephosphate (HBTU), and 1-hydroxybenzotriazole (HOBt) were purchased from Anaspec, Inc. (San Jose, CA). Reporter Lysis Buffer, Luciferase 1000 Assay system was a product of Promega (Madison, WI). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from GIBCO (Gaithersburg, MD). Micro BCA protein assay kit was purchased from PIERCE (Rockford, Il). PicoGreen was purchased from Molecular Probes (Eugene, OR).

Synthesis of monomer A4. Ethylenediamine methanol solution (20 mmol) was added drop wise to a stirred solution of methyl acrylate (1.5 mol) at room temperature for 48 h. Excess methyl acrylate including methanol were evaporated and final monomer A4 was obtained (99%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.37 (t, 4H, -CH_2CH_2COO-), 2.38 (s, 4H, -CH_2N-), 2.65 (t, 4H, -CH_2CH_2COO-), 3.56 (s, 12H, -CH_3).

Synthesis of A4B4 polymers and polymer surface modification. Four polymers were separately synthesized by double monomer method, as placing A4 monomer with increasing molar ratios of B4 (trizma base) monomer from 1.0 to 2.5 as Figure 1 in a glass vial at a temperature of 180 °C in a silicon bath. After 24 h, the polymers were gained and kept in a dry condition at −60 °C deep freezer. Surface hydroxyl group was reacted with Fmoc-ε-Ahx-OH to introduce primary amine. All four polymers (50 mg), Fmoc-ε-Ahx-OH (0.25 g, 0.70 mmol), 1-hydroxybenzotriazole (HOBt, 0.95 g, 0.70 mmol), N,N,N',N'-tetramethyl-O-(1H-benzotriazole-1-yl)uranium hexafluorephosphate (HBTU, 0.27 g, 0.70 mmol), and N,N-Diisopropylethylamine (DIPEA, 245 μL, 1.41 mmol) were dissolved in DMF at room temperature. After 12 h, the solutions were three times precipitated in diethyl ether for purification. The Fmoc group was removed by vortexing the polymer in 15% piperidine in DMF (v/v) solution for 5 min, followed by purification in diethyl ether precipitation, leaving the final



Polymer	Reaction Ratio A4	B4
A4B4 1.0	1	1
A4B4 1.5	1	1.5
A4B4 2.0	1	2
A4B4 2.5	1	2.5

Figure 1. The monomer structures and their reaction ratios for the polymers.

products of A4B4 1.0, A4b4 1.5, A4B4 2.0, and A4B4 2.5. NMR (300 MHz, D_2O) δ1.39 (br, $NH_2CH_2CH_2CH_2CH_2CH_2$-), 1.75 (br, $NH_2CH_2CH_2CH_2CH_2CH_2$-), 2.41 (br, NH_2CH_2-$CH_2CH_2CH_2CH_2$-), 2.42 (br, -CH_2CH_2COO-), 2.81 (br, $NH_2CH_2CH_2CH_2CH_2CH_2$-), 2.48 (br, -CH_2N-), 2.98 (t, 4H, -CH_2CH_2COO-), 4.11 (br, -$COOCH_2$-).

Primary amine quantification. Standard curve was obtained by ethanolamine solutions at increasing concentrations from 5 to 35 μg/mL. Samples were put on a test tubes in borate buffer (50 mM borate, pH 9.1), and 200 μL fluorescamine solution (1 mg/mL fluorescamine in DMF) was added for 12 min. Then 1.5 mL stop solution (DMF : water = 1 : 1) was added for measurement. The excitation and emission wavelengths were set to 390 and 475 nm, respectively, by a SFM 25 spectrofluorometer (Jasco Instruments).

PicoGreen assay. Sample polymer in HBS buffer solutions (25 mM HEPES, 150 mM NaCl, pH 7.4) was mixed with 1.0 μg pCN Luci and incubated at room temperature. After 30 min, 200 μL working solution (1 μL of PicoGreen reagent diluted in 199 μL TE buffer (10 mM Tris, 1 mM EDTA, pH 7.4)) was added for 2 min and diluted with 1600 μL TE buffer for measurement. SFM 25 spectrofluorometer (Jasco instruments) was used with the wavelength fixed at 480 nm for excitation (λ_{ex}) and 520 nm for emission (λ_{em}).

Multi Angle Laser Light Scattering (MALLS). Size-exclusion chromatography was run on a chromatography system (Dionex) equipped of Waters Styragel HR3 and Styragel HR5E columns and DMF was used as a mobile phase at a flow rate of 0.5 mL/min. MALLS detector (mini DAWN DSP, Wyatt Technology, Santa Barbara, CA) was connected to SEC system. The refractive index increment (d*n*/dc) was measured by Optilab DSP (Wyatt Technology, Santa Barbara, CA).

Degradation studies. All polymers were dissolved in PBS and separated into two groups; polyplex-solution group and polymer-solution group, both kept in a 37 °C incubator. Polyplex-solution group was incubated after complex formation with DNA (polymer/DNA, at weight ratio 30), and polymer-solution group was incubated DNA free. Polymer-solution group was mixed with 1 μg DNA for 30 min just before PicoGreen analysis until the polymer lost its self-complexing ability. PicoGreen measurement was used as above description.

Cell viability measurement. HepG2 (human hepatocellular carcinoma) cells were grown and seeded in 96-well plate at a density of 10000 cells per well in 10% FBS (fetal bovine serum) containing MEM culture medium. Cells were grown for 24 h and indicated concentrations of polymer were added and left for 48 h exposure. Then the cells were washed and 26 μL of MTT working solution (2 mg/mL MTT in PBS, filtered through 0.2 μm syringe filter) was added to each well. After 4 h incubation, the medium was removed and 150 μL DMSO was added and mixed. Percentage intensity was measured by a microplate reader at 570 nm (Molecular Devices).

Atomic Force Microscopy (AFM). The AFM images of polyplex were obtained at the weight ratio of 1 : 30 (DNA : polymer) by A4B4 2.0 at terms of time. A4B4 2.0 polymer solution was kept at 37 °C until measurement and 0.1 μg of DNA was added to solutions then incubated for 30 min to form self-assembled particles. 1 μL of the polyplex solution was dropped on the middle of a clean mica surface and left for 1 min to adsorb. The remaining solution was removed and air dried. The images were obtained on tapping mode at a scanning speed of 5 Hz with a Nanoscope a instrument equipped with an E scanner (Digital Instruments, Santa Barbara, CA).

Transfection assay. HepG2 were grown and seeded in a 24-well plate at a density of 50000 in MEM containing 10% FBS at 37 °C incubator of 5% CO_2/95% humidified air to reach 70% confluence. The polyplexes were added in 10% FBS containing condition and incubated for more 48 h then removed, washed with PBS, and followed by lysis. 120 μL Reporter lysis buffer (Promega, Madison, WI) was added and centrifuged to obtain clear lysate. Luciferase activity was measured by automatic injection of 50 μL Luciferase Assay Reagent (Promega, Madison, WI) for 10 s with a 2s delay for 10 μL lysate. Micro BCA reagent (Pierce, Rockford, IL) was used to determine protein concentration.

Results and Discussion

We designed an amide ester polymer containing random amide bonds in the ester backbone structure of the cross-linked polymer by multiple reactive moieties from two monomers, described as double-monomer methodology, Ax + By.[10] The polycondensation of monomers A4 and B4 at four different ratios resulted in four types of polymers including biodegradable backbones constructed by ester and amide bonds (Figure 1). The four polymer derivatives were classified and named as A4B4 1.0, 1.5, 2.0 and 2.5, which

means the mole ratio of B4 over A4. Usually, in many cases, the polycondensation of monomers with multiple reactive functional moieties ended up with the formation of gel-like polymers displaying poor solubility in various solvents,[11] but the four A4B4 polymers synthesized by double monomer method showed excellent solubility in water, even in many organic solvents. The condensation of two monomers formed ester bonds in its polymer structure for biodegradation, and the degradability of the polymer backbone structure was designed to be regulated with primary amine in B4 monomer by increasing B4 amount, since the primary amine in B4 will form amide bonds instead of ester bonds, which affect to slower degradation profiles when more amide bonds were present in the structures.

Synthetic large cross-linked polymers have no reference substances and their mass was not obtained by gel permeation chromatography. So, the size data was obtained by MALLS (multi-angle laser light scattering), combined with SEC without standards, in a well-established, non-invasive method for estimating an absolute molar mass (M_w) of polymer. Absolute mass (M_w) and polydispersity (M_w/M_n) at the concentration of 20 mg/mL were calculated with Astra 4.81.07 software (Wyatt Technology Corp.). As the B4 ratio increased, the molecular weight of the synthesized polymer increased (Table 1). The surface primary amines were conjugated to the hydroxyl groups by coupling Fmoc-ε-Ahx-OH to the polymers. After Fmoc group deprotection, the quantity of the primary amines resulted 2.6, 3.0, 3.8, 3.6 μmol/mg for A4B4 1.0, A4B4 1.5, A4B4 2.0, and A4B4 2.5, respectively.

The positively charged polymer self-assembled with DNA to form polymer/DNA polyplexes and the assembly degree was measured by PicoGreen reagent. PicoGreen is a very sensitive fluorescent dye which intercalates into double stranded DNA.[12] Therefore, lower PicoGreen signals from polyplex indicate compact assembly and good DNA condensation. The polymers reached significantly low intensity at weight ratio 5 by all polymers, lower fluorescence at higher weight ratios, especially with A4B4 1.5, 2.0, and 2.5, that have higher primary amine concentrations (Figure 2).

The morphology of DNA/polymer polyplex was observed by AFM, in terms of time (day) for its degradation images, as shown in Figure 3. The polymers condensed with DNA forming polyplexes had regular, globular shapes whereas polymer itself had random structures. Since the in vivo and in vitro conditions usually involve DNA/polymer polyplexes, the release of DNA from polyplex due to the degradation of polymer is considered to be important to follow up. The control DNA image was obtained by adding polymer of 1 : 0.5 (DNA : polymer) weight ratio where polymer was used for enhanced DNA binding instead of divalent metal cations. A4B4 2.0 was complexed with DNA at a weight ratio of 1 : 30 (DNA : polymer). The particles were spherically shaped without uncomplexed DNA at 0 h. After 24 h, the polyplex held its globular shape but swelling was observed at the surface of the polyplex by polymer degradation. Also, after 48 h of incubation, polyplexes showed similar images but the slow degradation kept the polyplexes in almost the same sizes as 24 h and still no protruding DNA

Table 1. Polydispersity and averaged molecular weight (g/mol) by MALLS at 690 nm laser wavelength collected at three angles, and extrapolated in a Zimm plot. *polydispersity

	M_n	M_w	M_z	M_w/M_n*
A4B4 1.0	2.82×10^2	1.55×10^3	7.67×10^3	5.50
A4B4 1.5	7.62×10^3	1.35×10^4	2.33×10^4	1.77
A4B4 2.0	1.57×10^3	3.74×10^3	2.17×10^5	2.37
A4B4 2.5	2.36×10^3	1.11×10^4	2.93×10^4	4.69

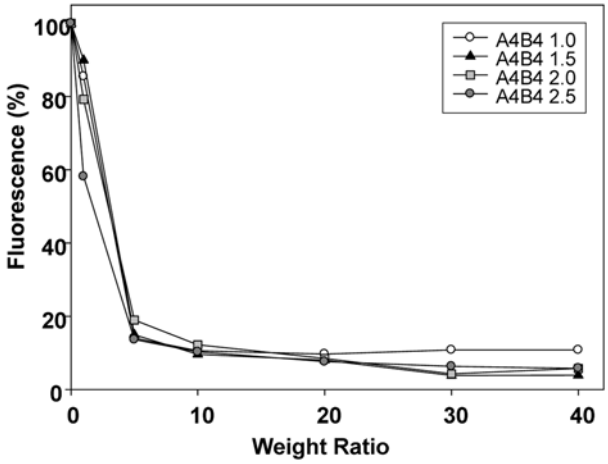


Figure 2. Polymer self-assembly assay by PicoGreen reagent.

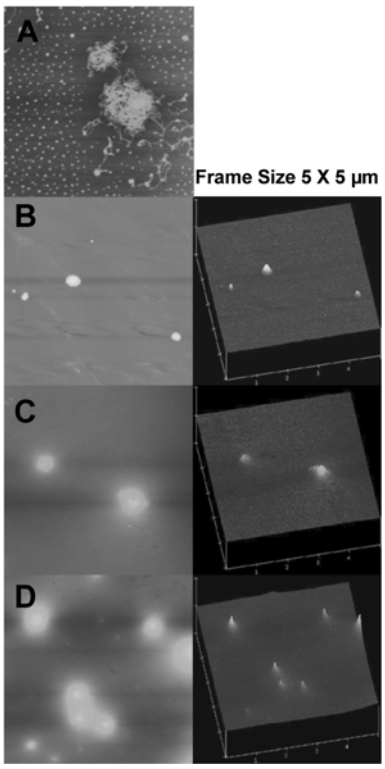


Figure 3. AFM images of A4B4 2.0 at weight ratio 1 : 30 (DNA : polymer) and its three-dimensional images. A) control DNA, B) at 0 h, C) 24 h, and D) 48 h.

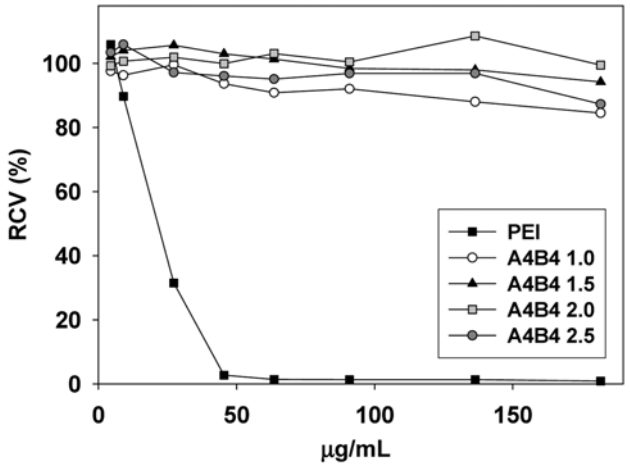


Figure 4. Cytotoxicity assay in HepG2 cells by MTT method. The increasing amounts of polymer were treated to cells for 48 h.

was observed.

Cytotoxicity was conducted by MTT assay method, where PEI was used as control (Figure 4). Cells were grown for 24 h and indicated concentrations of polymer were added and left for 48 h exposure. All A4B4 polymers showed over 85% cell viability, especially A4B4 1.5 and A4B4 2.0 showed almost no toxicity even at a very high concentration as 200 μg/mL.

Transfection efficiencies of all A4B4 polymers were investigated using luciferase reporter gene assay by luciferase activity with total proteins, in the presence or absence of serum, using PEI (1 : 1 = polymer/DNA) as a control (Figure 5). HepG2 were grown in MEM containing 10% FBS in a 5% CO_2 incubator at 37 °C and seeded in a 24-well plate at a density of 5×10^4 cells/well. The polyplexes were added to a 10% FBS containing condition and incubated for 48 h for (+) serum condition. The polyplexes were treated to cells without serum for 4 h, then washed and replaced with fresh, 10% FBS containing medium for further 44 h for (–) serum condition. Then all media was removed, washed with PBS, and followed by lysis. Increasing polymer weight ratios up to 30 showed increasing transfection efficiency, where a plateau was reached. A4B4 1.5 had good transfection efficiencies at low ratios, resulting from higher M_w and size regularity compared to other polymers. The usual protocol for transfection excludes FBS for easier cell uptake and also to avoid polymer aggregate formations, but A4B4 polymers showed good transfection efficiencies in the presence of FBS, especially better transfection effects were observed at low weight ratios as 10 and 20 by A4B4 2.5.

The polyplexes had regular, globular shapes whereas polymer itself had a random structure. To follow the degradation profiles in detail, two groups of samples were studied for the extended time. One group was the polymer group and the other group was polyplex (polymer/DNA, weight ratio of 30) group. They were both incubated under same conditions and taken at indicated time intervals, measured with Pico-Green reagent (Figure 6). As the B4 ratio increased from 1.0 to 2.5, the increased amine concentration provided by B4

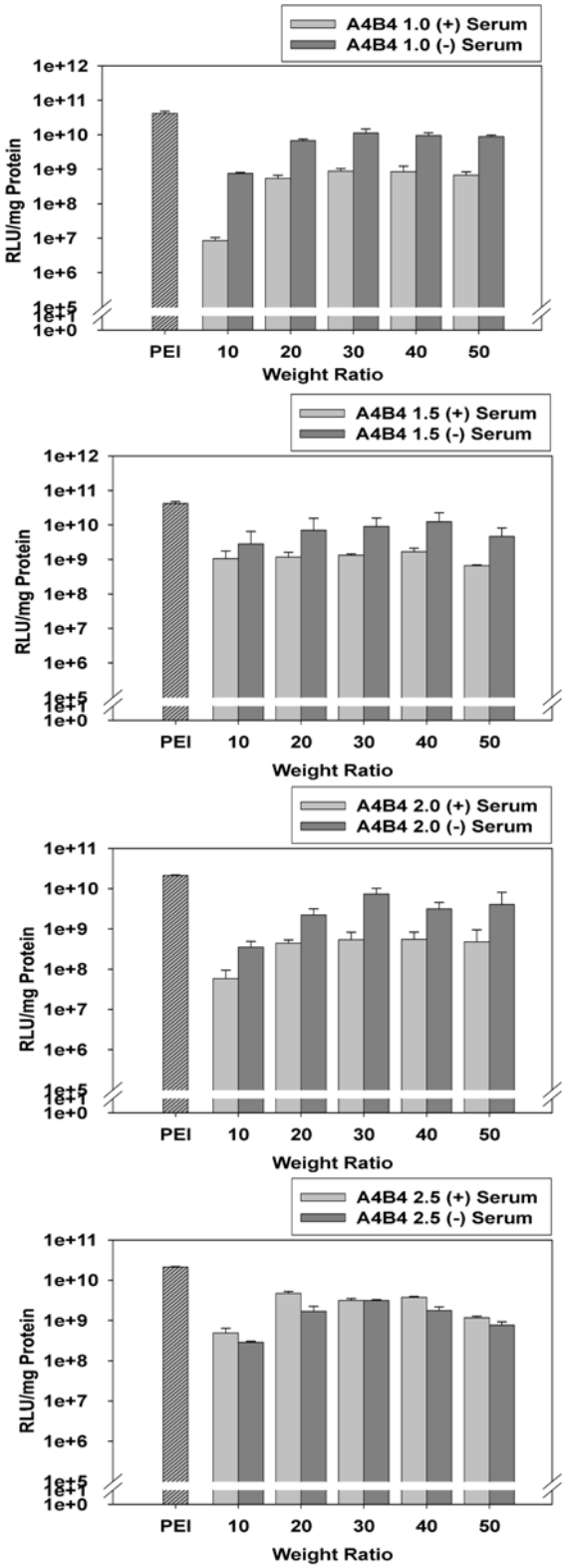


Figure 5. Transfection efficiencies of A4B4 polymers at increasing weight ratios (polymer/DNA) in HepG2 cells.

monomer will result in the formation of more amide bonds in the polymer backbone. Increased amide bonds in the polymer core structure will slow down the degradation rate than other polymers constructed at lower amine concentrations,

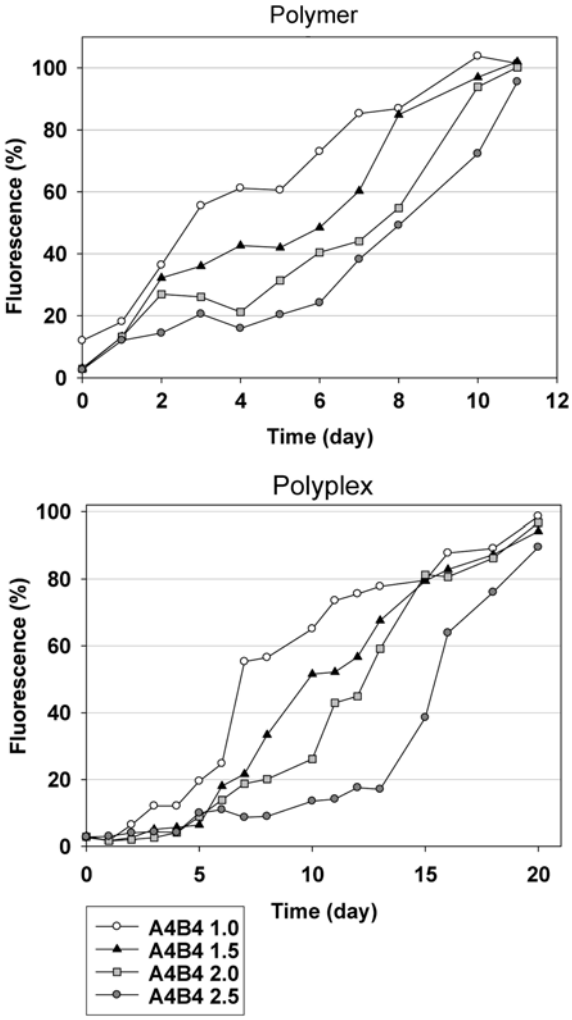


Figure 6. Degradation assay in terms of time (day) in PBS buffer conditions using PicoGreen reagent.

since labile ester bonds degraded fast under aqueous conditions especially in the presence of primary amines. In average, the free polymer showed faster degradations with simple and gradually increasing patterns, while all the polyplexed forms stably protected DNA for several days under 20% fluorescence before fast release was observed. Among the four polymers, as expected, A4B4 1.0 had the fastest degradation tendency and the slowest degradation rate was obtained by A4B4 2.5. A4B4 1.0 polymer completely lost its DNA condensing capability after 10 days but the fluorescence was still under 70% at day 10 when it was kept in the polyplexed form. A4B4 2.5 could retain DNA safely for more than 12 days under 20% fluorescence in the polyplexed form, even about 5 days in its free polymer form. Different B4 monomer ratio resulted in different bond compositions of polymer structure which resulted in different degradation rates, which could be manipulated to control the release time of complexed DNA using the mixture of different polyplex solutions of A4B4 polymers. For example, mixing A4B4 1.0 and A4B4 2.5 polyplexes, two step release of DNA will be obtained, as fast initial burst for 5 days and slow enduring release for over 20 days.

In summary, four degradable A4B4 polymers were synthesized by double-monomer method with excellent solubility. After the modification of the surface functional groups with primary amines, the polymers self-assembled with plasmid DNA in a spherical shape showing good transfection efficiencies even in the presence of serum. The polymers had different degradation tendencies, since the degradation was regulated by monomer B4 concentration at the synthesis step. Their low to no toxicity and slow degradation rates would contribute to the long-term in vivo gene delivery, and even therapeutic treatment applications.

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