Synthesis and Characterization of a New Family of Cationic Amino Acid-Based Poly(ester amide)s and Their Biological Properties

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ABSTRACT: A new family of positively charged and water soluble amino acid-based poly(ester amide)s (PEAs) consisting of nontoxic L-arginine, diols, and aliphatic dicarboxylic acids building blocks was synthesized and characterized. The L-arginine based PEAs (Arg-PEAs) were prepared by a solution polycondensation of two monomers: tetra-*p*-toluenesulfonic acids salts or hydrochloride acid salts of bis-(L-arginine) α, ω-alkylene diesters (monomer **II**), and di-*p*-nitrophenyl esters of saturated or unsaturated dicarboxylic acids (monomer **I**). Optimal reaction conditions were studied as functions of type of solvents and acid acceptors, concentrations of reactants. The molecular weights (M_n and M_w) of Arg-PEAs measured by GPC ranged from 20,000 to 60,000 g mol^{-1} with a rather narrow molecular weight distribution below 1.5. The chemical structures were confirmed by IR and NMR spectra. Arg-PEAs obtained were all amorphous materials with T_g from 33 to 125°C, depending on the number and the type (saturated vs. unsaturated) of methylene groups in diols or diacids, and the type of counter-ions attached to the guanidine group of the Arg-based PEAs. The Arg-PEAs had a high solubility in all polar solvents, including water. Preliminary studies of cell morphology and DNA capture capability of Arg-PEAs indicated that this new family of cationic PEAs was nontoxic and more biocompatible than a commercial transfection agent (Superfect®), and can successfully capture plasma DNA. The strong positive charge of Arg-PEAs as well as their good water solubility could provide unique characteristics for potential gene transfection or other charge preferred biomedical applications. J Appl Polym Sci 124: 3840–3853, 2012

Key words: L-arginine; positively charged poly(ester amide); water soluble; biomaterials; biodegradable; amino acids

INTRODUCTION

Aliphatic polyesters are a common class of absorbable polymers with typically poor thermal and mechanical properties. Polyamides generally have excellent thermal and mechanical properties, but slow to biodegrade due to amide linkage and stronger intermolecular interactions. In an effort to design new biodegradable polymers with both good thermal and mechanical properties, many new poly(ester amide) (PEA) polymers composed of both ester and amide blocks on their backbones have been studied widely for many years, and these PEA polymers were based on aliphatic diamines to provide the amide linkages in PEAs.[1–5]

In addition to aliphatic diamines, α-amino acids, because of their abundant availability from natural resources and potential biodegradability of their derivatives under certain enzymatic catalyzed conditions, have been chosen as the source to provide the amide linkage in the biodegradable amino acid-based poly (ester-amide)s (AA-PEAs) in many reports.[6–28] α-amino acid derived PEA polymers have three building blocks: (amino acid, diols, and diacids). Very recently, poly (ethylene glycol) has been used to replace the traditional diols for designing new generations of AA-PEA.[29,30]

AA-PEAs have a wide range of thermal, physical, biodegradation, and biological properties, and some of them have built-in functionality, depending on the type of amino acids, diols, and diacids used. For example, the unsaturated poly(ester amide)s (AA-UPEAs) have carbon-to-carbon double bonds on the AA-PEA backbones, and these double bonds are photo-reactive and were used to fabricate hybrid hydrogels.[6,10,11,15,16] Other examples of functional amino acid-based PEAs reported in the literatures include the pendant carboxylic acid in the L-lysine block,[14] pendant amine group in the L-lysine segment,[20,31] and pendant multifunctional groups like carboxylic acid, amine, and hydroxyl in the corresponding amino acid blocks.[20,32–34] These amino acid-based PEAs could be one of the most promising biodegradable biomaterial candidates for biomedical applications.

Additional Supporting Information may be found in the online version of this article.

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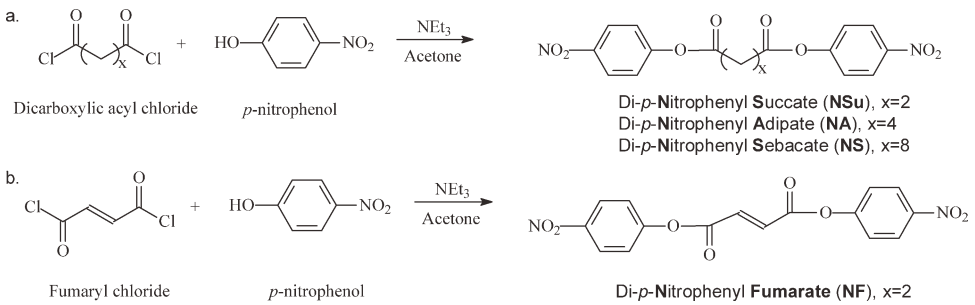


Scheme 1 Synthesis of nitrophenol-based monomer I. (a) Three saturated di-*p*-nitrophenyl esters of dicarboxylic acids, NSu, NA, and NS; (b) one unsaturated di-*p*-nitrophenyl esters of dicarboxylic acid, NF.

Out of 20 naturally occurring amino acids, only the hydrophobic amino acids, such as L-valine, L-leucine, L-isoleucine, DL-norleucine, L- and DL-phenylalanine, and DL-methionine[17] and glycine[28] and L-alanine,[23] and one hydrophilic L-lysine (Lys)[14,20,31] had been applied in all amino acids based PEAs reported so far; and most of these AA-PEAs are neutral-charged and dissolve only in organic solvents. The only reported charged AA-PEA polymers synthesized from natural amino acid was based on L-lysine.[14,20,31] These negatively[14,19] or positively charged[20,31] AA-PEAs were hetero-chain copolymers and still could not dissolve in an aqueous medium. The lack of water solubility of these reported amino acid-based PEAs limits their applications in biomedical fields that require aqueous environment for cell friendly environment in tissue engineering.

In this article, the chemical synthesis and optimization process of a new family of positively charged arginine-based PEAs that can dissolve in an aqueous medium was reported. This new family of (+) AA-PEAs were synthesized from L-arginine (Arg) with its positive charged guanidine group intact as the pendant group. Arginine, lysine, and histidine are the only three naturally occurring amino acids with positively charge in the 20 naturally occurring amino acids. Arg was adopted in this study because of its strongest basicity. The arginine side chain consists of three nonpolar methylene groups and the strongly basic δ-guanidino group. With a pKa 12, the guanidino group is ionized over the entire pH range including the physiological pH in which proteins exist naturally. This unique property of guanidino group in Arg is the basis for the aqueous solubility and positive charge of arginine-based AA-PEA (Arg-PEA) polymers. During the synthesis, the protonated guanidino group on arginine is so stable that it may eliminate the need of tedious protection and deprotection steps of functional groups as commonly encountered in the traditional polypeptide synthesis. The elimination of protection and deprotection steps could also improve yields.

In this research, the synthesis and characterization of a series of L-arginine-based poly(ester-amide)s (Arg-PEAs) by solution polycondensation are described. The effects of the reaction solvents, monomer concentration, and acid acceptors on the molecular weight of the resulting Arg-PEA polymers were investigated. The combined characteristics of aqueous solubility and positive charge in Arg-PEAs along with the reported study that Arg-based polymers could enter cells more efficiently than other polycationic homopolymers[36] could provide great potentials as transfection agents for the negatively charged nuclear acids like plasmid DNA and siRNA via electrostatic interaction. A preliminary study of using Arg-PEAs to capture plasmid DNA was conducted to demonstrate the potential of such an application. Cell morphological changes upon exposure to this new family of Arg-PEAs were also examined for a preliminary cytotoxicity evaluation.

EXPERIMENTAL

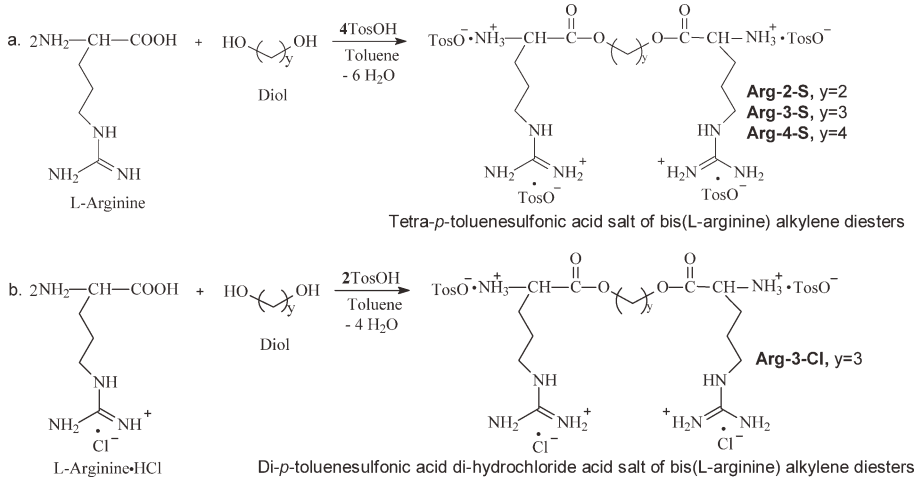
Materials and methods

Materials

L-arginine (Sigma), L-arginine hydrochloride (Sigma), *p*-toluenesulfonic acid monohydrate (TosOH) (Alfa Aesar, Ward, Hill, MA), succinyl chloride, sebacoyl chloride, adipoyl chloride, ethylene glycol, 1,3-propanediol (Avocado Research Chemicals), 1,4-butanediol (Alfa Aesar, Ward Hill, MA), and *p*-nitrophenol (J. T. Baker, Phillipsburg, NJ) were used without further purification. Triethylamine from Fisher Scientific (Fairlawn, NJ) was dried by refluxing with calcium hydride, and then distilled. Toluene, ethyl acetate, acetone, 2-propanol, and dimethyl sulfoxide (DMSO) solvents were purchased from VWR Scientific (West Chester, PA) and were purified by standard methods before use. Other chemicals and reagents if not otherwise specified were all purchased from Sigma (St. Louis, MO).

Synthesis of monomers

The general synthesis scheme of Arg-PEAs was divided into three major steps, and are shown in



Scheme 2 Synthesis of tetra-*p*-toluenesulfonic acid salt of bis(L-arginine) alkylene diester monomers **II**. (a) three di-*p*-toluenesulfonic acid salt of bis(L-arginine) alkylene diesters: Arg-2-S, Arg-3-S and Arg-4-S; b. di-*p*-toluenesulfonic acid dihydrochloride acid salt of bis(L-arginine) alkylene diesters, Arg-3-S-Cl.

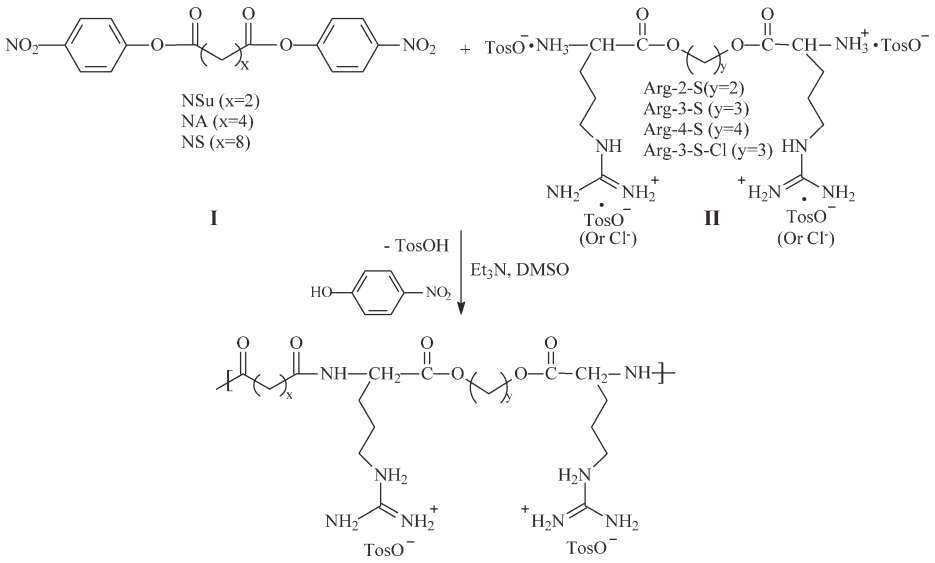
Schemes 1–3: the preparation of di-*p*-nitrophenyl ester of dicarboxylic acids (**I**), the preparation of tetra-*p*-toluenesulfonic acid salts of bis (L-arginine), α, ω-alkylene diesters (**II**), and the polycondensation of monomers (**I**) and (**II**) for the synthesis of Arg-PEAs polymers.

Synthesis of di-p-nitrophenyl ester of dicarboxylic acids (I)

Di-*p*-nitrophenyl ester of dicarboxylic acids were prepared by reacting dicarboxylic acyl chloride of varying methylene length (x) with *p*-nitrophenol as previously reported[14–17,26–30] and shown in Scheme 1. Four monomers of different methylene chain length were made: di-*p*-**N**itrophenyl **Su**ccate (**NSu**), $x = 2$; di-*p*-**N**itrophenyl **A**dipate (**NA**), $x = 4$; di-*p*-**N**itrophenyl **S**ebacate (**NS**), $x = 8$; di-*p*-**N**itrophenyl **F**umarate (**NF**), $x = 2$. Monomer NF was unsaturated (i.e., providing functional C=C double bond in the AA-PEA backbone), while NA, NS, and NSu were all saturated. The detailed synthesis procedure of monomer **I** was given in our prior publications.[10,14–17,26–30,32–34]

Synthesis of tetra-p-toluenesulfonic acid salt of bis(L-arginine) alkylene diesters (II)

This arginine-based *p*-toluenesulfonic acid salt of diester monomer was the first time reported. Arg-based monomers were synthesized by a modified synthesis procedure from the previously reported



Scheme 3 Synthesis of arginine based poly(ester-amide)s polymers from the solution polycondensation of monomers **I** (Scheme 1**)** and **II** (Scheme 2).

method.[10,14–17,26–30] In the modified procedures, the amount of *p*-toluenesulfonic acid (TosOH) used was doubled when comparing with the previously reported synthesis of *p*-toluenesulfonic acid salt of hydrophobic amino acids diesters. This modification was due to the strong basicity of the guanidino group on Arg.

Four Arg-based monomers were made, and they were different in number of methylene groups in the diols unit (*y*) as well as the type of counter-ion salt: tetra-*p*-toluenesulfonic acid salt of bis(L-arginine) **ethane** diesters **Arg-2-S** (with 2 methylene groups); tetra-*p*-toluenesulfonic acid salt of bis(L-arginine) **propane** diesters, **Arg-3-S** (with 3 methylene groups); tetra-*p*-toluenesulfonic acid salt of bis(L-arginine) **butane** diesters, **Arg-4-S** (with 4 methylene groups); di-*p*-toluenesulfonic acid di-hydrochloride acid salt of bis(L-arginine) propane diesters, **Arg-3-S-Cl** (with 3 methylene group). The two types of counter-ion salts attached onto the guanidine group of Arg in the Arg-PEA were: toluenesulfonic ion (S) and chloride ion (Cl).

The synthesis of toluenesulfonic acid salt of arginine diester monomer **II** was illustrated by a representative example, Arg-3-S. L-arginine (0.02 mol) and 1,3-propanediol (0.01 mol) were condensed under refluxed toluene (d.p. 110°C, 80 mL) with the presence of TosOH (0.04 mol). The heterogeneous solid–liquid reaction mixture was heated to 120°C and refluxed for 24 h after 1.08 mL (0.06 mol) water was generated and collected by a dean-stark apparatus. The resulting mixture was cooled down to room temperature. With toluene decanted, the dry mixture was then purified by repeated precipitation in 2-propanol for three times. The final product was white powder, and obtained in quantitative yields ($\sim$ 90%). The reaction scheme is shown in Scheme 2.

In the case of di-*p*-toluenesulfonic acid di-hydrochloride acid salt of arginine diester, the starting Arg monomer used is L-arginine hydrochloride instead of L-arginine. Because the counterion Cl^- was preattached onto the arignine monomer, only 1 to 1 ratio of *p*-toluenesulfonic acid to arginine.HCl was needed for the diester synthesis. All the remaining steps were the same as the di-*p*-toluenesulfonic acid procedures described above.

Synthesis of Arg-PEA polymers by solution polycondensation of (I) and (II)

The Arg-based PEA polymers were prepared by solution polycondensation of the above two monomers (**I** and **II**) as shown in Scheme 3. The resulting Arg-PEA polymers are listed in Table I. There were totally four unsaturated and six saturated Arg-PEAs synthesized. The saturated Arg-PEAs were labeled as *y*-Arg-*x*-S or Cl, while the unsaturated Arg-PEAs were labeled as *y*-UArg-*x*-S or Cl. *x* and *y* were the number of methylene groups in diols and in diacids segments, respectively, while S and Cl are the TosOH or chloride counter ions, respectively. U stands for unsaturation.

TABLE I
Arg-PEA Polymers Synthesized by Different Combinations of Monomers I and II

Monomers	Arg-PEAs	Type	Counter ion
NSu + Arg-2-S	2-Arg-2-S	Saturated	TosOH
NSu + Arg-3-S	2-Arg-3-S	Saturated	TosOH
NA + Arg-3-S	4-Arg-3-S	Saturated	TosOH
NA + Arg-4-S	4-Arg-4-S	Saturated	TosOH
NS + Arg-3-S	8-Arg-3-S	Saturated	TosOH
NS + Arg-4-S	8-Arg-4-S	Saturated	TosOH
NF + Arg-2-S	2U-Arg-2-S	Unsaturated	TosOH
NF + Arg-3-S	2U-Arg-3-S	Unsaturated	TosOH
NF + Arg-4-S	2U-Arg-4-S	Unsaturated	TosOH
NF + Arg-3-Cl	2U-Arg-3-S-Cl	Unsaturated	Chloride

An example of the synthesis of 8-Arg-3-S via solution polycondensation is given here. Monomers NS (1.0 mmol) and Arg-3-S (1.0 mmol) in 0.54 mL of dry DMSO were mixed well by vortexing. The solution mixture was gradually heated up to 70°C while keeping vortexing to maintain a uniform mixture. Triethylamine (0.31 mL, 2.2 mmol) was added dropwise to the mixture with a vigorous stirring until a complete dissolution of both NS and Arg-3-S monomers. The reaction vial was then kept at 70°C for 48 h in a thermostat oven without stirring. The resulting Arg-PEA polymer (8-Arg-3-S) in the solution was precipitated out in a cold ethyl acetate, decanted, and dried. The dried polymers was further purified twice by redissolving in methanol and reprecipitated in cold ethyl acetate and final drying *in vacuo* at room temperature.

Materials characterization and preliminary biological assessment

The chemical structures of the synthesized monomers and polymers were characterized with standard chemical methods.

In Fourier Transform Infrared (FTIR) characterization, the samples were ground into fine powders and mixed with KBr at a sample/KBr ratio of 1 : 10 (w/w). FTIR spectra were obtained with a PerkinElmer (Madison, WI) Nicolet Magana 560 FTIR spectrometer with Omnic software for data acquisition and analysis.

NMR spectra were recorded with a Varian (Palo Alto, CA) Unity INOVA 400-MHz spectrometer operating at 400 and 100 MHz for ^1H and ^{13}C NMR, respectively. Deuterated dimethyl sulfoxide (DMSO-d_6, Cambridge Isotope Laboratories) was used as the solvent.

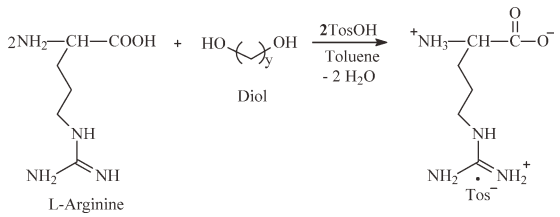
Elemental analyses of the synthesized polymers were performed with a PE 2400 CHNS elemental analyzer by Atlantic Microlab (Norcross, GA).

Thermal property of the synthesized monomers and Arg-PEA polymers were characterized with a DSC 2920 (TA Instruments, New Castle, DE). The measurements were carried out from −10 to 300°C at a heating rate of 10°C min^{-1} and at a nitrogen gas flow rate of 25 mL min^{-1}. TA Universal Analysis software was used for thermal data analysis including the determination of the glass transition temperature (T_g).

The number- and weight-average molecular weights (M_n and M_w) and molecular weight distribution (MWD) of the synthesized Arg-PEAs were determined by a model 510 gel permeation chromatograph (Waters Associates, Milford, USA) equipped with a high-pressure liquid chromatography pump, a Waters 486 UV detector, and a Waters 2410 differential refractive index detector. Tetrahydrofuran (THF) was used as the eluent (1.0 mL min^{-1}), and less than 10 μL of DMF was mixed to increase the polymer solubility in THF. The columns were calibrated with polystyrene standards with a narrow MWD.

A preliminary cell morphology study was conducted for providing an initial glimpse of the cytotoxicity of this new family of Arg-PEAs. Rat aortic A10 vascular smooth muscle cells (SMC)s were obtained from American tissue culture collection (ATCC). The SMCs were grown as recommended at 37°C in 5% CO_2 in Dulbecco's minimal essential medium (DMEM) supplemented with 10% FBS (Germini, Woodland, CA) and antibiotics. A representative Arg-PEA (2-Arg-2-S) of two concentrations (15–1500 μg/100 μL of cell medium) was added into the cell culture medium containing SMC (10,000 cells/well) in 96-well plates, and were incubated for 48 h. The cell morphology was viewed and recorded after 48-h incubation. The commercial transfection agent (Superfect®) and experimental agent (PEI) at their suggested optimal concentrations along with a blank cell culture medium served as the controls.

For a preliminary assessment of the feasibility of these new Arg-PEAs to capture plasmid DNA, a gel retardation assay was conducted to quantify the amounts of Arg-PEAs required to condense DNA to form polyplex, the first key step in nonviral gene transfection application.[35] Plasmid DNA COL(-772) was provided by Prof. Bo Liu of Weill Cornell Medical College. All plasmids were prepared using Qiagen endotoxin-free plasmid Maxi kits according to the supplier's protocol. The quantity and quality of the purified plasmid DNA was assessed by spectrophotometric analysis at 260 and 280 nm as well as by electrophoresis in 1% agarose gel. The purified plasmid DNA were resuspended in a TE buffer and frozen in aliquots. The Arg-PEA Polymer/DNA complexes formulated over a wide range of the polymer to DNA weight ratio with the above protocol were analyzed by gel electrophoresis in a 1% agarose gel stained with ethidium bromide (10 μg mL^{-1}) with TAE buffer at 100 V for 60 min. DNA migration was visualized by a UV illumination.



Scheme 4 Synthesis of *p*-toluenesulfonic acid of arginine when the molar ratio of *p*-toluene acid to arginine is one to one. No ester group was formed between arginine and diol.

RESULTS AND DISCUSSION

Synthesis of monomers

Four types of di-*p*-nitrophenyl esters of dicarboxylic acids monomers, **I** (NSu, NA, NS, and NF) were synthesized and characterized. All these four monomers **I** were obtained as white crystals with high yields (68–82%). The melting temperature of NSu, NA, NS, and NF were 185, 124, 108, and 238°C, respectively. These four monomers **I** have been reported in our previous studies,[10,14–17,20,26–30,32–34] and will not be discussed further here.

The tetra-*p*-toluenesulfonic acid salts of bis(L-arginine) alkylene diesters monomers **II**, however, are new. Because of the resonance structure, the guanidino group on the side chain of Arg exhibits a very higher basicity than that of the primary amine group on arginine backbone, the added TosOH during these monomers synthesis would prefer to interact with the guanidino group first, and then with the primary amine, if any excessive TosOH acid would be available. Hence, if the molar ratio of TosOH to Arg was one to one as in the previously reported synthesis of hydrophobic and neutral charged AA-PEAs (e.g., L-Phe or Leu-based PEAs), all the TosOH would most likely be consumed by the guanidino group in Arg with virtually no TosOH available for the primary amine in Arg. As a result, no ester bond could be formed between the —OH group on diol and the —COOH group on Arg as shown in Scheme 4. The lack of ester formation under the above reaction condition was confirmed by FTIR, NMR, and elemental analysis. The TosOH salt of Arg product in Scheme 4 was crystalline with a defined melting temperature, whereas the true Arg

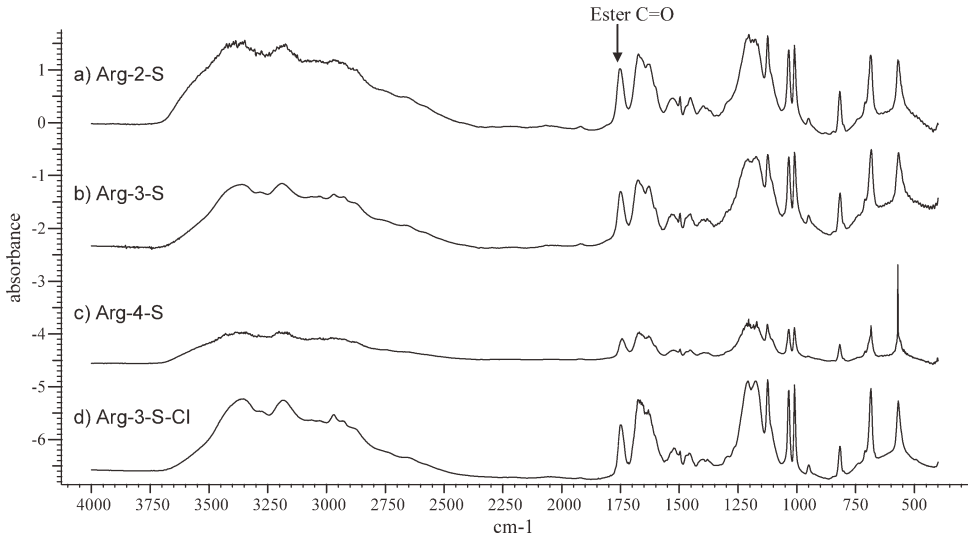


Figure 1 FTIR spectra of the tetra-*p*-toluenesulfonic acid salts of bis(L-arginine) diesters monomer **II**. (a) Arg-2-S (tetra-*p*-toluenesulfonic acid salt of bis(L-arginine) ethane-1,2-diester); (b) Arg-3-S (tetra-*p*-toluenesulfonic acid salt of bis(L-arginine) propane-1,3-diester); (c) Arg-4-S (tetra-*p*-toluenesulfonic acid salt of bis(L-arginine) butane-1,4-diester); (d) Arg-3-S-Cl (di-*p*-toluenesulfonic acid di-hydrochloride acid salt of bis(L-arginine) propane-1,3-diester.

diester monomer was actually amorphous without any melting temperature.

The need to have excessive TosOH for the successful synthesis of Arg diester monomers was also confirmed in the synthesis of di-*p*-toluenesulfonic acid di-hydrochloride acid salt of bis(L-arginine) diester. In the synthesis of this monomer, the guanidino group of the starting material, L-arginine hydrochloride acid was already preoccupied by Cl^- counter ion. As a result, only 1 : 1 molar ratio of TosOH to L-arginine hydrochloride acid was needed for the ester formation, as demonstrated in Scheme 2.

Four types of *p*-toluenesulfonic acid salt of Arg diester monomers (**II**) were synthesized: Arg-2-S, Arg-3-S, Arg-4-S, and Arg-3-S-Cl. The differences among them were the number of methylene groups in the diol (i.e., between the two ester groups in the resulting monomer) as well as the type of counter ion. The number of —CH_2— varied from 2 to 4 from Arg-2 to Arg-4. Their chemical structures were all confirmed by FTIR and NMR. The FTIR spectra are shown in Figure 1. Absorption bands of ester group were observed in the regions $\sim$ 1172 cm^{-1} (—O—) and $\sim$ 1748 cm^{-1} (—C=O). NMR spectra are shown in Figure 2. The toluenesulfonic acid salts of Arg diesters obtained were all amorphous and with no melting peak shown in the DSC scan.

Tetra-p-toluenesulfonic acid salt of L-arginine ethane-1,2-diester (Arg-2-S)

Arg-2-S was reprecipitated from 2-propanol, with 75% yield of purified amorphous white powder. IR: 1753 cm^{-1} [—C(O)—], 1178 cm^{-1} [—O—]; ^{1}H NMR (DMSO-d_6, ppm, δ): 1.63 [4H, —CH_2—**C**H_2—CH_2—NH—], 1.82 [4H, —OC(O) —CH(NH_3^+)—**C**H_2—(CH_2)$_2$—], 2.29 (6H, **H**$_3$C—Ph—SO_3—), 3.10 [4H, —(CH_2)$_2$—**C**H_2—NH—], 4.06 [2H, $^+$**H**$_3$N—**C**H(R)—C(O)—O—], 4.39 [4H, — (O)C—O—**C**H_2—], 7.18, 7.53 [16H, Ph], 7.69, bump between 6.70 and 7.53 [10H, —CH_2—N**H**(N**H**$_2^+$)—N**H**$_2$], 8.42 [6H, $^+$**H**$_3$N—CH(R)—C(O)—O—]; ^{13}C NMR (DMSO-d_6, ppm, δ): 20.84 (H$_3$**C**—Ph—SO_3—), 24.13 [—OC(O)—CH(NH_3^+)—CH_2—**C**H_2—CH_2—NH—], 27.16 [—OC(O)—CH(NH_3^+)—**C**H_2—(CH_2)$_2$—NH—], 40.15 [—(CH_2)$_2$—**C**H_2—NH—C(NH_2^+)—NH_2], 51.74 [$^+$H$_3$N—**C**H(R)—C(O)—O—], 63.35 [—OC(O)—**C**H_2—**C**H_2—], 125.47, 128.43, 138.63, 144.24 (**Ph**), 156.78 [—NH—**C**(NH_2^+)—NH_2], 169.21 [—**C**(O)—O—]. Elemental analysis: calculated % C: 47.45, H: 5.88, N: 10.54, S: 12.06; found % C: 46.54, H: 6.06, N: 9.81, S: 11.72.

Tetra-p-toluenesulfonic acid salt of L-arginine propane-1,3-diester (Arg-3-S)

Arg-3-S was re-precipitated from 2-propanol with 62% yield of purified amorphous white powder. IR: 1749 cm^{-1} [—C(O)—], 1172 cm^{-1} [—O—]; ^{1}H NMR (DMSO-d_6, ppm, δ): 1.55 [4H, —CH_2—**C**H_2—CH_2—NH—], 1.79 [4H, —OC(O) —CH(NH_3^+)—**C**H_2—(CH_2)$_2$—], 1.97 [2H, —(O)C—O—CH_2—**C**H_2—], 2.30 (6H, **H**$_3$C—Ph—SO_3—), 3.10 [4H, —(CH_2)$_2$—**C**H_2—NH—], 4.07 [2H, $^+$**H**$_3$N—**C**H(R)—C(O)—O—], 4.24 [4H, —(O)C—O—**C**H_2—CH_2—], 7.16, 7.49 [16H, Ph], 7.62, bump between 6.70 and 7.49 [10H, —CH_2—N**H**(N**H**$_2^+$)—N**H**$_2$], 8.38 [6H, $^+$**H**$_3$N—CH(R)—C(O)—O—]; ^{13}C NMR (DMSO-d_6, ppm, δ): 20.82 (H$_3$**C**—Ph—SO_3—), 24.21 [—OC(O)—CH(NH_3^+)—CH_2—**C**H_2—CH_2—NH—], 27.24 [—OC(O)—CH(NH_3^+)—**C**H_2—(CH_2)$_2$—NH—], 31.24 [—OC(O)—CH_2

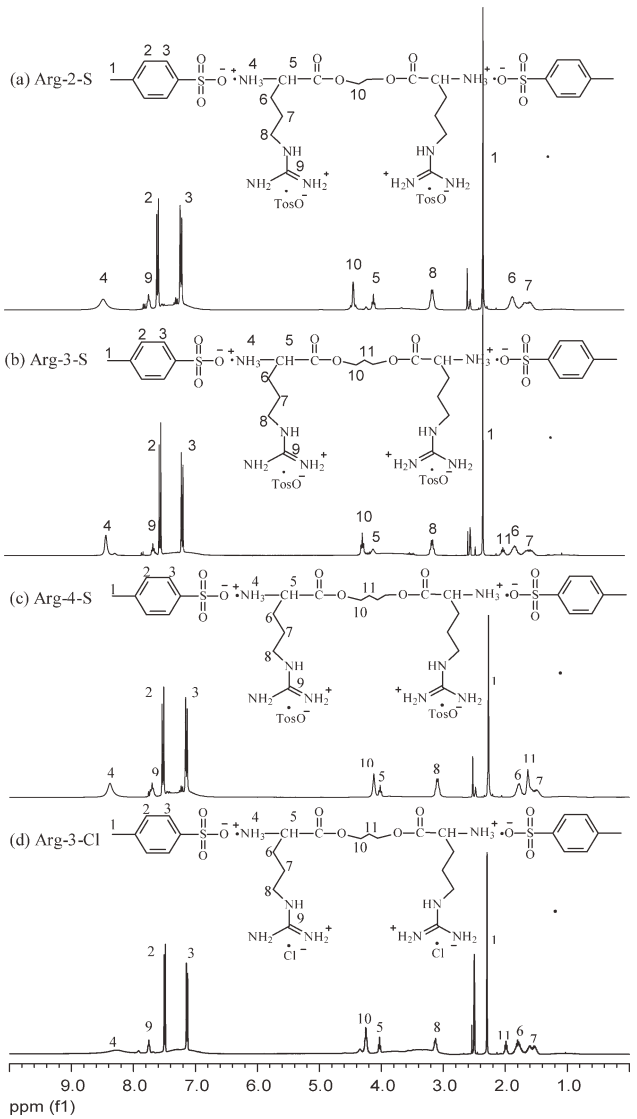


Figure 2 ^{1}H NMR spectra of four types of tetra-*p*-toluenesulfonic acid salts of bis(L-arginine) diesters monomer **II**. (a) Arg-2-S; (b) Arg-3-S; (c) Arg-4-S; (d) Arg-3-S-Cl.

CH_2CH_2—], 40.06 [—$(CH_2)_2$—**C**H_2—NH—C(NH_2^+)—NH_2], 51.73 [^+H_3N—**C**H(R)—C(O)—O—], 62.43 [—OC(O)—**C**H_2—CH_2—**C**H_2—], 125.46, 128.36, 138.47, 144.46 (**Ph**), 156.78 [—NH—**C**(NH_2^+)—NH_2], 169.22 [—**C**(O)—O—]. Elemental analysis: calculated % C: 47.94, H: 5.99, N: 10.40, S: 11.90; found % C: 47.19, H: 6.14, N: 10.05, S: 11.94.

Tetra-p-toluenesulfonic acid salt of L-arginine butane-1,4-diester (Arg-4-S)

Arg-4-S was reprecipitated from 2-propanol and acetone with 65% yield of purified amorphous white powder. IR: 1743 cm^{-1} [—C(O)—], 1170 cm^{-1} [—O—]; ^{1}H NMR (DMSO-d_6, ppm, δ): 1.52 [4H, —OC(O)—CH(NH_3^+)—CH_2—C**H**$_2$—CH_2—NH—], 1.65 [4H, —(O)C—O—CH_2—C**H**$_2$—], 1.80 [4H, —OC(O)—CH(NH_3^+)—C**H**$_2$—$(CH_2)_2$—], 2.29 (6H, **H**$_3$C—Ph—SO_3—), 3.10 [4H, —$(CH_2)_2$—C**H**$_2$—NH—], 4.04 [2H, ^+H_3N—C**H**(R)—C(O)—O—], 4.14 [4H, —(O)C—O—C**H**$_2$—CH_2—], 7.18, 7.53 [16H, Ph], 7.71, bump between 6.77 and 7.53 [10H, —CH_2—N**H**(N**H**$_2^+$)—N**H**$_2$], 8.40 [6H, $^+$**H**$_3$N—CH(R) —C(O)—O—]; ^{13}C NMR (DMSO-d_6, ppm, δ): 20.84 (H$_3$**C**—Ph—SO_3—), 24.38 [—OC(O)—CH(NH_3^+)—CH_2—**C**H_2—CH_2—NH—], 25.46 [—OC(O)—CH_2**C**H_2—], 27.23 [—OC(O)—CH(NH_3^+)—**C**H_2—$(CH_2)_2$—NH—], 40.08 [—$(CH_2)_2$—**C**H_2—NH—C(NH_2^+)—NH_2], 51.75 [^+H_3N—**C**H(R) —C(O)—O—], 65.23 [—OC(O)—**C**H_2—$(CH_2)_2$—], 125.46, 128.40, 138.56, 144.35 (**Ph**), 156.78 [—NH—**C**(NH_2^+)—NH_2], 169.32 [—**C**(O)—O—]. Elemental analysis: calculated % C: 48.43, H: 6.10, N: 10.27, S: 11.75; found % C: 47.43, H: 6.25, N: 10.12, S: 11.74.

Di-p-toluenesulfonic acid di-hydrochloride acid salt of L-arginine propane-1,3-diester (Arg-3-S-Cl)

Arg-3-S-Cl was re-precipitated from 2-propanol and acetone with 65% yield of purified amorphous white powder. IR: 1743 cm^{-1} [—C(O)—], 1170 cm^{-1} [—O—]; ^{1}H NMR (DMSO-d_6, ppm, δ): 1.59 [4H, —CH_2—C**H**$_2$—CH_2—NH—], 1.78 [4H, —OC(O)—CH(NH_3^+)—C**H**$_2$—$(CH_2)_2$—], 1.99 [2H, —(O)C—O—CH_2—C**H**$_2$—], 2.29 (6H, **H**$_3$C—Ph—SO_3—), 3.12 [4H, —$(CH_2)_2$—C**H**$_2$—NH—], 4.03 [2H, ^+H_3N—C**H**(R)—C(O)—O—], 4.25 [4H, —(O)C—O—C**H**$_2$—CH_2—], 7.14, 7.50 [16H, Ph], 7.75, bump between 6.75 and 7.50 [10H, —CH_2—N**H**(N**H**$_2^+$)—N**H**$_2$], 8.26 [6H, $^+$**H**$_3$N—CH(R)—C(O)—O—]; ^{13}C NMR (DMSO-d_6, ppm, δ): 20.84 (H$_3$**C**—Ph—SO_3—), 24.25 [—OC(O)—CH(NH_3^+)—CH_2—**C**H_2—CH_2—NH—], 27.24 [—OC(O)—CH(NH_3^+) —**C**H_2—CH_2—], 31.23 [—OC(O)—CH_2**C**H_2CH_2—], 40.26 [—CH_2—**C**H_2—NH—], 51.67 [^+H_3N—**C**H(R)—C(O)—O—], 63.20 [—OC(O)—**C**H_2—CH_2—**C**H_2—], 125.46, 128.39, 138.52, 144.40 (**Ph**), 156.97 [—NH—**C**(NH_2^+)—NH_2], 169.36 [—**C**(O)—O—].

Synthesis of Arg-PEA polymers

Side chain of Arg consists of three nonpolar methylene groups and a strong basic δ-guanidino group. With a *p*Ka value about 12, the guanidino group is ionized over the entire pH range including physiological pH. The ionized guanidino group is planar and hence the positive charge is effectively distributed over the entire group due to resonance. In the protonated form, the guanidino group is unreactive, and only very small fractions of the nonionized form are present at a physiological pH. Because of this strong resonance, basicity and unreactivity of the guanidino group, the regular protection and deprotection steps required for synthesizing amino acids-based monomers is unnecessary. The strong electrostatic interaction between the acidic TosOH and the basic Arg guanidino group would provide the

TABLE II
Effect of the Monomer Concentration on the Polycondensation of Monomer II (Arg-3-S) with Monomer I (di-*p*-Nitrophenylsebacate, NS) at Molar Ratio 1/1 in DMSO Solvent and the Presence of NEt_3 at 70°C for 48 h

Monomer concentration (mol L^{-1})	Appearance[b]	Yield (%)	η_{red}[a] dL g^{-1} (H_2O)
0.6	S	85.6	0.10
1.2	S	96.6%	0.14

[a] 12 mL of solution with DMSO as solvent, 30°C.
[b] S: homogeneous solution.

protection. On the other hand, the removal of the toluenesulfonic acid counter ion from the guanidino group would also become very difficult.

Effect of monomer concentration, solvents and acid receptor on the molecular weight of Arg-PEAs

Arg-PEAs were prepared according to the well-established solution polycondensation of monomers **I** and **II**. Molecular weights of polymers from polycondensation or step growth polymers increase at a slower rate with a lower conversion; and a very high conversion (i.e., >95%) is required to reach moderately high molecular weights, when comparing with an addition polymerization. It is crucial to optimize the polycondensation reaction conditions to achieve higher molecular weight.

The effect of reaction temperature and time on the molecular weight of AA-PEAs had been well established in previous publications.[6,10,11,16,17,20] It was found that a higher reaction temperature up to 70°C increased the polycondensation reaction rate two to three times than that at room temperature, and the reaction was completed within 50 h; but a further increase in reaction temperature would lead to a lower molecular weight AA-PEA, although at an even higher reaction rate. Therefore, 70°C reaction temperature and 48-h reaction time were adopted as part of the optimum reaction conditions for Arg-PEA polymer synthesis.

TABLE III
Effect of Reaction Solvents on the Polycondensation of the Monomer II (Arg-3-S) with Monomer I (di-*p*-Nitrophenylsebacate, NS) at Molar Ratio 1/1 and the Presence of NEt3 at 70°C and 48 h

Solvent	Appearance[b]	Yield (%)	η_{red}[a] dL g^{-1} (H_2O)
DMA	S	85.6	0.11
DMF	S	63.4	0.11
DMSO	S	83.4	0.12
Methanol	S	52.3	0.03
Ethanol	S	74.5	0.07

Concentration of each monomer 0.6 mol L^{-1}.
[a] 12 mL of solution with DMSO as solvent, 30°C.
[b] S: homogeneous solution.

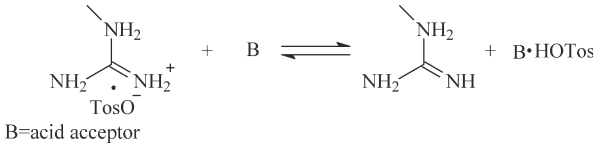
In the present work, monomers Arg-3 and NS (resulting polymer 8-Arg-3-S) were used as an example to illustrate the effects of monomer concentration, nature of solvents and type of acid acceptor on the resulting molecular weight of Arg-PEAs via solution polycondensation.

Monomer concentration effect. Both 0.6 mol L^{-1} (previously reported) and 1.2 mol L^{-1} monomer concentrations (**I** and **II** monomers) were prepared for the polymerization, and the reduced viscosity of the resulting Arg-PEA polymers was compared and shown in Table II. The monomer concentration can not be too low to have insufficient molecular interactions, or too high to have insufficient molecular collision due to viscosity. An equal molar ratio of the starting monomers **I** and **II** (Schemes 1 and 2) at a concentration of 1.2 mol L^{-1} in the solution polycondensation was found to be the best for preparing higher molecular weight Arg-PEAs.

Solvent effect. as shown in Table III, five organic solvents were tested, DMA, DMF, DMSO, methanol, and ethanol. DMSO was found to be the best for synthesizing AA-PEAs because the resulting Arg-PEAs had the highest reduced viscosity, a stable reaction condition due to the high boiling point of DMSO, and the least safety concern to biological applications.

Acid acceptor effect. The acid acceptor plays a crucial role in this Arg-PEA polycondensation reaction, because, during the polycondensation, TosOH was detached and released from the amino acid diester salt (monomer II) as a byproduct as shown in Scheme 5 below. An acid acceptor, B, could neutralize the TosOH byproduct and shift the reaction more toward the completion, i.e., better yields. B = acid acceptor.

To shift the equilibrium in Scheme 5 to the right, i.e., to increase the available free amine in the Arg diester monomer **II** for its condensation with di-*p*-nitrophenyl ester of dicarboxylic acids, monomer **I**, two conditions need to be satisfied: proper temperature and acid acceptor. A 70°C had been proved the most effective in our prior and current studies. The acid acceptor, B, also plays an indispensable role in



Scheme 5 Reaction equilibrium of toluenesulfonic acid salt of monomer **II** and acid acceptor K_2CO_3.

TABLE IV
Effect of Acid Acceptors on the Polycondensation of *p*-Toluenesulfonic Acid Salt (Arg-3-S) with di-*p*-Nitrophenylsebacate (NS) at Molar Ratio 1/1 in DMSO Solvent at 70°C and 48 h[a]

Acceptor (B)	Molar ratio B/monomer 2	Appearance[b]	Yield (%)	η_{red} dL g^{-1}
NEt_3	2.1	S	85.6	0.1
NEt_3	4.2	S	85.6	0.06
TMED	1.05	H	99.9	N/A
TMED	2.1	H	99.9	N/A
K_2CO_3 + Ceolite 4 Å	1.05	H	70.0	N/A
K_2CO_3 + Ceolite 4 Å	2.1	H	81.2	N/A

[a] Concentration of each monomer 0.6 mol L^{-1}.
[b] S: homogeneous solution, H: heterogeneous mixture-K_2CO_3 is insoluble in DMA, TosOK, and TMED 2HOTos precipitated.

the polycondensation reaction. This is because the acid acceptor leads to the generation of free amino groups from di-*p*-toluenesulfonic acid salts of bis (L-α-amino acid) α,ω-alkylene diesters, monomer **II**, by removing TosOH from the acid salts. The resulting free amino groups are required for the aminolysis of the active ester groups in monomer **I** for the formation of AA-PEAs. In the case of Arg-PEA polycondensation reaction, the TosOH or HCl was not only attached onto the primary amines, but also attached onto the guanidino group on the Arg side chain. Because of the higher basicity of the guanidino group compared with the primary amine, acid acceptor is expected to remove TosOH or HCl preferentially from the primary amine first, and then the guanidino group.

Tertiary amines, such as triethylamine (NEt_3) and *N*,*N*,*N*′,*N*′-tetramethylethane-1,2-diamine (TMED), inorganic salts like potassium carbonate or sodium carbonate were all suitable acid acceptors in this reaction (Table IV). The difference among these acid acceptors lies in their solubility of the resulting salts in the reaction medium. For example, TosOH salts of NEt3 were soluble in DMA, whereas the TosOH salts of TMED precipitated as crystals in DMA. Acid acceptor K_2CO_3 and its TosOK salt were insoluble in DMA which could facilitate the purification of AA-PEAs. This self-separation of low molecular weight byproducts from the AA-PEA polymers would be highly desirable for the purification of the resulting AA-PEAs. In addition, the precipitation of the salt byproducts would shift the equilibrium polycondensation reaction to the right, i.e., promoting polycondensation reaction. Especially, water and CO_2 will be released from the interaction of K_2CO_3 with TosOH.

Both FTIR and NMR spectra proved the efficiency of these three types of acid acceptors (NEt_3, TMED, and K_2CO_3) to promote AA-PEA formation. In this study, NEt_3 was eventually chosen due to its simplest synthesis process: overnight in 70°C oven without stirring. In the case of TMED and K_2CO_3 acid acceptors, due to the heterogeneous systems, continuous stirring was required, and the resulting salts byproducts were hard to be removed completely from the resulting polymers (filtration and dialysis against water had been tried), and hence the reduced viscosity was not available.

All the Arg-PEAs synthesized are listed in Table V. Their molecular weight varied from 20,000 to 60,000 g mol^{-1} with narrow polydispersity (M_w/M_n from 1.03 to 1.57). The solubility of Arg-PEAs in THF was low, and therefore less than 10% DMF was added to improve the solubility during GPC measurement with THF as an eluent.

Chemical structure identification

The structures of the Arg-PEAs were confirmed by both IR and NMR spectra. Figure 3 shows the FTIR spectra of some representative Arg-PEAs: 2-Arg-2-S, 4-Arg-3-S, 4-Arg-3-Cl, 2U-Arg-3-S. The carbonyl bands at 1648–1650 cm^{-1} (Amide I), 1538–1542 cm^{-1} (Amide II), 1738–1742 cm^{-1} (ester), and NH vibrations at 3290 cm^{-1} are typical for all AA-PEAs

TABLE V
Physical Property of Poly(ester amide)s Obtained by Polycondensation of Monomer I (Active Diester) with Monomer II Salt According to Scheme 3

Poly(ester amide) *x*-Arg-*y*	Empirical formula (FW)	Yield[a] (%)	T_g (°C)	M_n (kg mol^{-1})	M_w (kg mol^{-1})	M_n/M_w
2-Arg-2-S	$C_{32}H_{48}N_8O_{12}S_2$ (800.91)	86	38 ± 2	38.1	39.7	1.04
2-Arg-3-S	$C_{33}H_{50}N_8O_{12}S_2$ (814.92)	99	80 ± 2	33.8	34.8	1.03
4-Arg-3-S	$C_{35}H_{54}N_8O_{12}S_2$ (842.98)	98	56 ± 2	130.8	205.8	1.57
4-Arg-4-S	$C_{36}H_{56}N_8O_{12}S_2$ (857.00)	85	42 ± 2	24.6	25.5	1.03
8-Arg-3-S	$C_{39}H_{62}N_8O_{12}S_2$ (899.09)	96	39 ± 2	58.6	89.7	1.53
8-Arg-4-S	$C_{40}H_{64}N_8O_{12}S_2$ (913.11)	97	33 ± 2	40.2	50.3	1.25
2U-Arg-2-S	$C_{32}H_{46}N_8O_{12}S_2$ (798.88)	99	92 ± 2	N/A	N/A	N/A
2U-Arg-3-S	$C_{33}H_{48}N_8O_{12}S_2$ (812.91)	99	103 ± 2	N/A	N/A	N/A
2U-Arg-4-S	$C_{34}H_{50}N_8O_{12}S_2$ (826.94)	99	125 ± 2	N/A	N/A	N/A
2U-Arg-3-Cl	$C_{19}H_{34}N_8O_6Cl_2$ (540.53)	98	67 ± 2	36.4	44.5	1.22

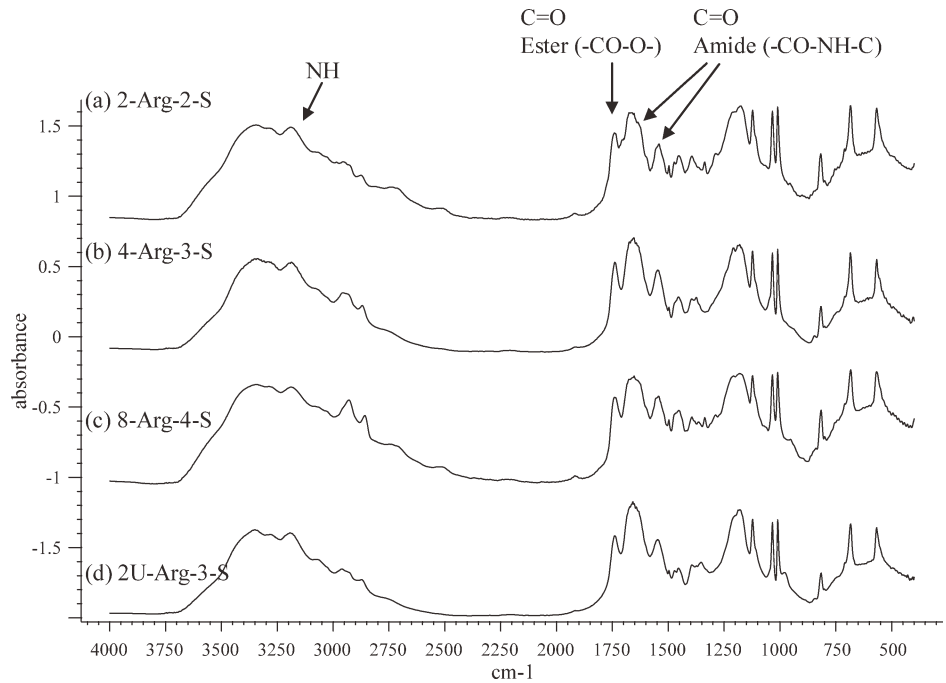


Figure 3 FTIR spectra of synthesized Arg-PEAs: (a) 2-Arg-2-S (NSu + Arg-2-S); (b) 4-Arg-3-S (NA + Arg-3-S); (c) 8-Arg-4-S (NS + Arg-4-S); (d) 2U-Arg-3-S (NF + Arg-3-S).

obtained. The corresponding ^{1}H NMR and ^{13}C NMR spectra of the four representative AA-PEAs are given in Figures 4 and 5. The ^{1}H NMR spectra confirmed the expected Arg-PEA structure with the expected NH, aromatic, and aliphatic signals. The two >C=O signals shown in the ^{13}C NMR spectra are also typical for all the AA-PEAs.

Thermal properties of Arg-PEAs

As shown in Table V, All Arg-PEAs synthesized in this study were amorphous without melting temperatures. Their glass transition temperature, T_g, ranged from 33 to 125°C, depending on the length of methylene chain in diols and diacid segments, the type of methylene chain (i.e., saturated vs. unsaturated) and the type of salt counter ions (i.e., $TosO^-$ salt vs. Cl^-).

An examination of the effect of the number of methylene groups in dicarboxylic acid (x) and in diols (y) of AA-PEAs on T_g revealed that an increase in methylene chain length would reduce T_g. For example, at a constant x (e.g., 4 or 8), an increase in y led to a lower T_g (from 56°C of 4-Arg-3-S to 42°C of 4-Arg-4-S); similar T_g pattern was also observed in the case of an increasing x at a constant y (from 80°C of 2-Arg-3-S, 56°C of 4-Arg-3-S to 39°C of 8-Arg-3-S). This observation was attributed to a higher AA-PEA chain flexibility due to a longer methylene chain length in either the diol or diacid segments, and, therefore, easier chain segmental mobility (i.e., lower T_g). This observation, however, was not found when x was very short, e.g., $x = 2$, and the opposite methylene chain effect was observed, i.e., at a smaller x, higher y resulted in a higher T_g (2-Arg-2-S vs. 2-Arg-3-S; 2U-Arg-2-S and 2U-Arg-3-S vs. 2U-Arg-4-S). This unusual methylene chain length effect on T_g in those Arg-PEAs having very short methylene chain length in both diacid (x) and diol (y) segments could be attributed to an increasing ionic charge density of the guanidine group in the Arg segment as both shorter x and y led to a shorter spacing between two adjacent guanidine groups. An increasing ionic charge density could facilitate a stronger intermolecular hydrogen bond formation. When x was short (e.g., 2), and y was longer (from 2 to 4), the guanidine groups were closer toward the amide group on the Arg-PEA backbone rather than the ester group, and probably forms a stronger intermolecular hydrogen bonding, and, therefore, a lower freedom of chain rotation (i.e., higher T_g). When x was longer, the intermolecular interaction would be offset by the higher flexibility of the longer methylene group. The effect of different x or y on T_g was more profound when x or y was short; for example, the T_g difference between 4-Arg-3-S and 4-Arg-4-S ($\Delta T_g = 14$°C) was more than double of the T_g difference between 8-Arg-3-S and 8-Arg-4-S ($\Delta T_g = 6$°C). Similar findings were also observed between 4-Arg-3-S and 8-Arg-3-S ($\Delta T_g = 17$°C) and between 4-Arg-4-S and 8-Arg-4-S ($\Delta T_g = 9$°C).

As shown in Table V, the unsaturated C=C bond in the unsaturated Arg-PEAs polymer backbone increased T_g due to the higher rigid C=C bond in the diacid segment. For example, 2U-Arg-2-S had T_g

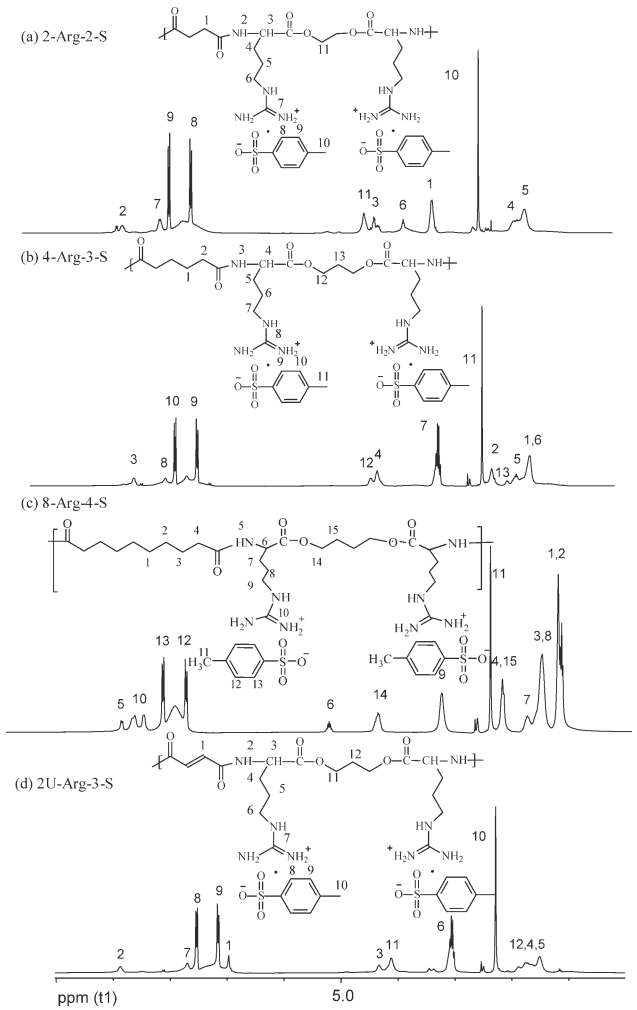


Figure 4 ^{1}H NMR spectra of synthesized Arg-PEAs in DMSO solvent: (a) 2-Arg-2-S (NSu + Arg-2-S); (b) 4-Arg-3-S (NA + Arg-3-S); (c) 8-Arg-4-S (NS + Arg-4-S); (d) 2U-Arg-3-S (NF + Arg-3-S).

more than double of the T_g of 2-Arg-2-S (92 vs.38°C). The influence of the type of counter ion salt in the Arg-PEAs on their T_g can be observed when comparing 2U-Arg-3-S (103°C) to 2U-Arg-3-Cl (67°C). Because $TosO^-$ counter ion is much bulkier than Cl^-, this bulkier counter ion could restrict the rotational freedom of the Arg-PEA backbone, and hence a higher T_g.

When the T_g of the Arg-based PEAs was compared with the T_g of nonpolar amino acids based PEAs reported in the literatures at the equivalent x and y, the T_g of the Arg-based PEAs was generally lower as shown in Table VI. This may suggest that the presence of nonionic side chain groups like isopropyl in Val and benzyl in Phe could reduce the freedom of polymer chain rotation more than ionic side chain groups like guanidine group in Arg. When x was short (e.g., 2U-Arg-4-S vs. 2U-Phe-4 in Table VI), the trend was reversed again as shorter x could result in a higher charge density in Arg-PEAs

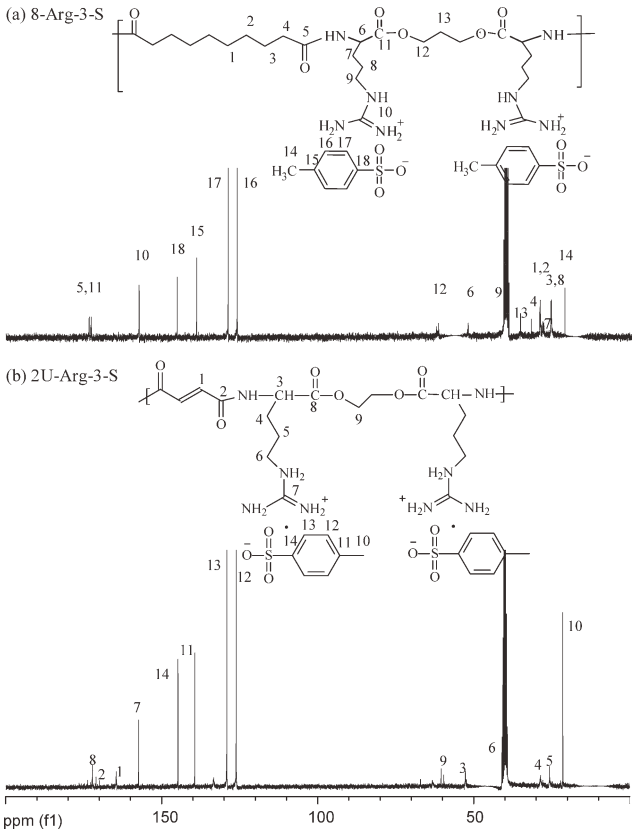


Figure 5 ^{13}C NMR spectra of synthesized Arg-PEAs. (a) 8-Arg-3-S (NS + Arg-3-S); (b) 2U-Arg-3-S (NF + Arg-3-S).

which could result in a stronger intramolecular/intermolecular interaction between guanidine group and other polar groups like amide and ester groups. Such ionic molecular interaction is lacking in the nonpolar AA-PEAs, and hence only the bulkness of the side group could affect T_g.

TABLE VI
Comparison of Glass Transition Temperature (T_g) of the Newly Synthesized Arg-Based PEAs with Prior Nonpolar Amino Acid-Based PEAs

Amino acid-based poly(ester amide)s	Glass transition temperature, T_g (°C)
4-Arg-4-S	**42 ± 2**
4-Leu-4	45
4-ILeu-4	47
4-Val-4	58
4-Phe-4	59
8-Arg-3-S	**39 ± 2**
8-Phe-3	48
8-Leu-3	48
8-Arg-4-S	**33 ± 2**
8-ILeu-4	37
8-Val-4	44
8-Leu-4	47
8-Phe-4	47
2U-Arg-4-S	**125 ± 2**
2U-Phe-4	103

The data of nonpolar amino acid-based PEAs were from Refs. 16 and 17.

TABLE VII
Solubility of Three Typical Arg-PEAs in Solvents at Room Temperature (25°C)

Poly(ester amide) *x*-Arg-*y*-S	Solvent							
	DMSO; DMA; DMF	Methanol	Ethanol	Water	THF	Ethyl acetate	Acetone	Chloroform
2-Arg-2-S	++	++	++	++	±	−	−	−
4-Arg-3-S	++	+	+	+	±	−	−	−
8-Arg-4-S	++	+	+	±	±	−	−	−

(++): Very soluble, form clear liquid phase (>0.1 g mL^{-1}) ; (+): Soluble (>0.05 g mL^{-1}, <0.1 g mL^{-1}); (±): less soluble, cloudy or turbid but without distinct phase separation (<0.5 g mL^{-1}); (−) Insoluble.

Solubility of Arg-PEAs

The solubility of Arg-PEAs (0.1 g) in organic solvents (1.0 mL) at room temperature was assessed and is summarized in Table VII. Because of the strong polarity nature, all of the Arg-PEAs synthesized were soluble in polar organic solvents like DMSO or water, but not soluble in non-polar organic solvents, such as ethyl acetate or chloroform. But, an increase in methylene chain length in dicarboxylic acid (*x*) or in diols (*y*) segments reduced solubility as expected due to an increase in hydrophobicity,

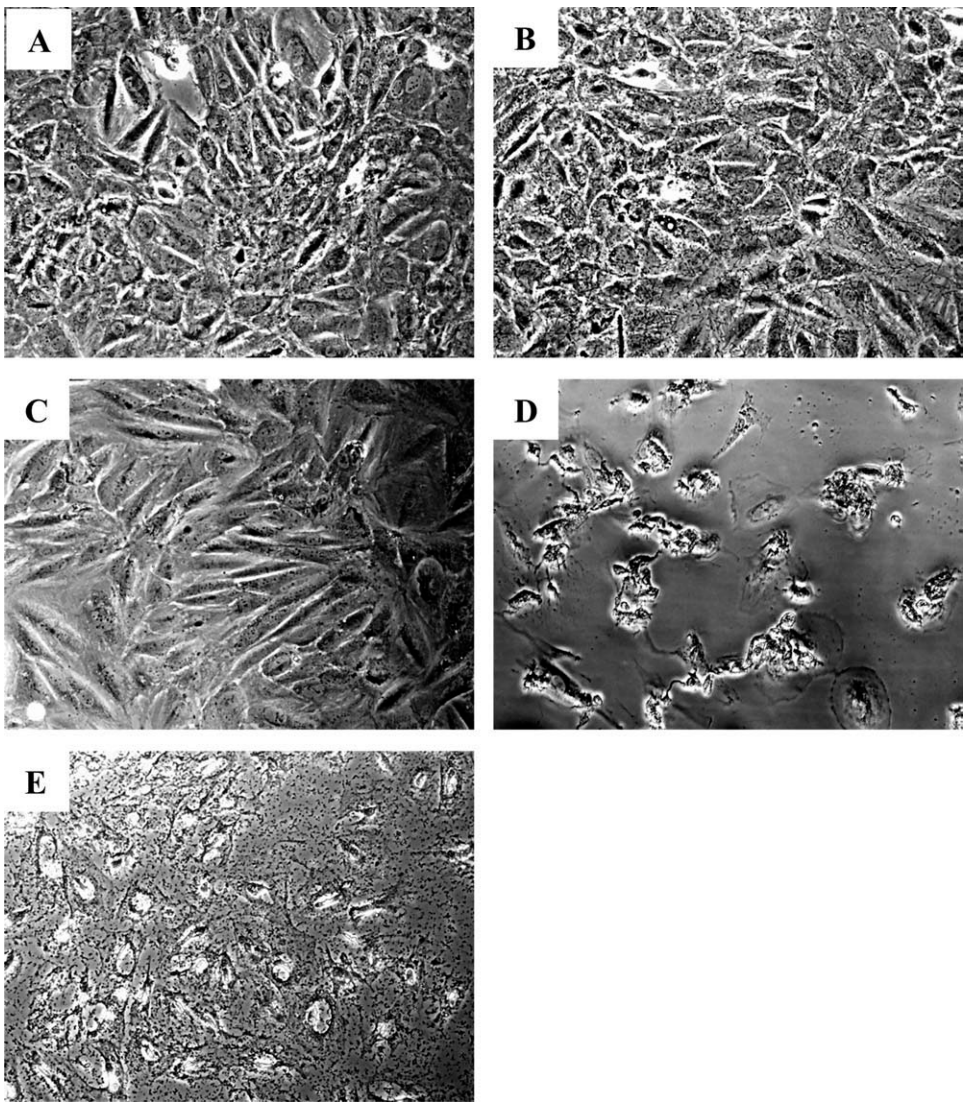


Figure 6 Rat aortic smooth muscle cell morphology upon 48-h exposure to Arg-PEAs (2-Arg-2-S) polymers at two different concentrations (15–1500 μg/100 μL of cell medium). A—blank cell medium control; B—2-Arg-2-S at 15 μg/100 μL; C—2-Arg-2-S at 1500 μg/100 μL; D—commercial transfection agent Superfact® at 15 μg/100 μL as control; E—experimental agent (PEI) at 15 μg/100 μL as control.

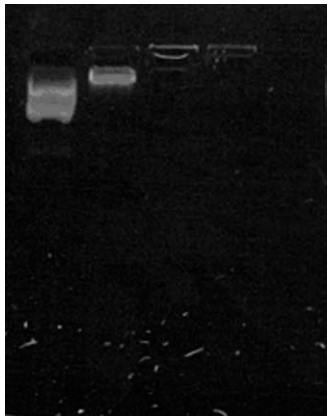


Figure 7 Agarose gel electrophoresis to examine the effect of amounts of 2-Arg-2-S needed for the condensation of plasma. Lane 1 (far left)—1.0 g COL(-772)/LUC plasmid DNA; lane 2 (second from left)—weight ratio of 2-Arg-2-S/DNA = 4/1; lane 3 (third from left)—weight ratio of 2-Arg-2-S/DNA = 17/1; lane 4 (fourth from left)—weight ratio of 2-Arg-2-S/DNA = 42; and lane 5 (far right)—weight ratio of 2-Arg-2-S/DNA = 68.

For example, 2-Arg-2-S had a higher solubility than 4-Arg-3-S, and was much more soluble in polar solvent or water than 8-Arg-4-S. On the contrary, all those reported nonpolar amino acid-based PEAs from Phe, Leu or Val dissolved in chloroform very well.[6,10,11,14–18,26–33]

Preliminary cytotoxicity of Arg-PEAs

A preliminary evaluation of the cytotoxicity of these newly synthesized Arg-PEAs was performed by visual assessment of the cell morphological assay of rat aortic smooth muscle cell line. As show in Figure 6, the Arg-PEA treated SMC [Fig. 6(B,C)] exhibited cell morphology nearly identical to the blank cell culture medium [Fig. 6(A)], while the SMC treated by commercial Superfect® [Fig. 6(D)] and experimental PEI [Fig. 6(E)] transfection agents showed very unhealthy cell morphology indicated by the clumping together of SMC into irregular shaped aggregates.

Preliminary DNA condensation capability of Arg-PEAs

The capability to condense plasma DNA was examined over a wide range of weight ratios of Arg-PEAs to DNA. An example of a typical result obtained during gel electrophoresis experiments for all Arg-PEA polymers is shown in Figure 7. In this example, the movement of the plasmid DNA in the gel was retarded as the amounts of the 2-Arg-2-S PEA polymer increased, demonstrating that the 2-Arg-2-S PEA polymer was able to bind to DNA, neutralize its charge at a concentration above certain Arg-PEA to DNA weight ratios. As the weight ratio of the Arg-PEA to DNA increased, more gel retardation occurred (from Lane 2 to 5, Fig. 7). At the weight ratio of Arg-PEA/DNA 4 : 1 (Lane 2), the complex moved slightly from its original well location toward the anode site, indicating that four times of 2-Arg-2-S was not sufficient to neutralize DNA, and the complex still possessed some negative charges. A complete neutralization of Figure 7 Agarose gel electrophoresis to examine the effect of amounts of 2-Arg-2-S needed for the condensation of plasma. Lane 1—1.0 μg COL(-772)/LUC plasmid DNA; Lane 2—weight ratio of 2-Arg-2-S/DNA = 4/1; Lane 3—weight ratio of 2-Arg-2-S/DNA = 17/1; Lane 4—weight ratio of 2-Arg-2-S/DNA = 42; Lane 5—weight ratio of 2-Arg-2-S/DNA = 68. DNA was achieved at a Arg-PEA polymer to DNA weight ratio ~ 17 (Lane 3).

These gel electrophoresis retardation results demonstrate the condensation capability of Arg PEA polymers to DNA, and provide the basic formulation information for subsequent transfection experiments. It is important to note, however, that strong binding and efficient DNA condensation do not always correlate directly with gene-delivery efficiency, probably because tight DNA binding may prevent its release and hence transcription.[35] A good polymer-based transfection agent must, therefore, balance sufficient binding strength to initially protect the plasmid DNA payload with subsequent capability to release the plasmid DNA later.[35]

CONCLUSION

A series of water soluble positively charged Arg-based poly(ester amide)s (Arg-PEA) were synthesized by solution polycondensation of tetra-*p*-toluenesulfonic acids salts (or hydrochloride acid salts) of bis-(L-arginine) α, ω-alkylene diesters (monomer **II**) and di-*p*-nitrophenyl esters of saturated or unsaturated dicarboxylic acids (monomer **I**). The unique guanidine group of Arg provides the positive charge, water solubility and some special biological functions, and most of all, provides the convenience during poly(ester amide) synthesis. The *p*-toluenesulfonic acid functioned both as a catalyst for ester formation and a protector to the guanidine group, and the amounts needed were, therefore, doubled for the synthesis when compared to non-polar amino acids based PEAs.

Both saturated and unsaturated Arg-PEAs were synthesized, and the effects of type of solvents and acid acceptors and monomer concentration on polymer synthesis were examined for achieving an optimized reaction condition. The Arg-PEA polymers obtained had good yields with DMSO as the solvent and triethylamine as the acid acceptor. The molecular weights measured by GPC ranged from 30,000 to 60,000 g mol^{-1} with rather narrow MWD of 1.03–1.6. Chemical structures of the monomers and Arg-PEAs

polymers were confirmed by FTIR and NMR. The resulting polymers were amorphous with T_g ranged from 33 to 125°C, depending on the length and type of methylene chain and the type of salt. The Arg-PEA polymers were cationic and soluble in all strong polar solvents including water, but insoluble in nonpolar solvents. A preliminary cell morphology study of Arg-PEAs indicated that this new family of cationic AA-PEAs was nontoxic and more biocompatible than commercial and popular experimental transfection agents (Superfect® and PEI). Because of these unique characteristics, Arg-PEAs could have the potential as gene carriers for transfection, drug carrier, and many other bioengineering applications.

References

1. Goodman, I.; Vachon, R. N. Eur Polym J 1984, 20, 529.
2. Goodman, I.; Vachon, R. N. Eur Polym J 1984, 20, 539.
3. Goodman, I. Eur Polym J 1984, 20, 549.
4. Goodman, I.; Valavanidis, A. Eur Polym J 1984, 20, 241.
5. He, Y.; Qian, Z. Y.; Zhang, H. L.; Liu, X. B. Colloid Polym Sci 2004, 282, 972.
6. Guo, K.; Chu, C. C. J Biomater Sci Polym Ed 2007, 18, 489.
7. Arabuli, N.; Tsitlanadze, G.; Edilashvili, L.; Kharadze, D.; Goguadze, T.; Beridze, V.; Gomurashvili, Z.; Katsarava, R. Macromol Chem Phys 1994, 195, 2279.
8. Armelin, E.; Paracuellos, N.; Rodriguez-Galan, A.; Puiggali, J. Polymer 2001, 42, 7923.
9. Asin, L.; Armelin, E.; Montane, J.; Rodriguez-Galan, A.; Puiggali, J. J Polym Sci A Polym Chem 2001, 39, 4283.
10. Guo, K.; Chu, C. C. J Polym Sci Polym Chem 2007, 45, 1595.
11. Guo, K.; Chu, C. C. Biomaterials 2007, 28, 3284.
12. Fan, Y. J.; Kobayashi, M.; Kise, H. J Polym Sci A Polym Chem 2001, 39, 1318.
13. Fan, Y. J.; Kobayashi, M.; Kise, H. J Polym Sci A Polym Chem 2002, 40, 385.
14. Jokhadze, G. M. M.; Panosyan, H.; Chu, C. C.; Katsarava, R. J Biomater Sci Polym Ed 2007, 18, 411.
15. Guo, K.; Chu, C. C. J Polym Sci A Polym Chem 2005, 43, 3932.
16. Guo, K.; Chu, C. C.; Chkhaidze, E.; Katsarava, R. J Polym Sci A Polym Chem 2005, 43, 1463.
17. Katsarava, R.; Beridze, V.; Arabuli, N.; Kharadze, D.; Chu, C. C.; Won, C. Y. J Polym Sci A Polym Chem 1999, 37, 391.
18. Katsarava, R. D.; Kharadze, D. P.; Japaridze, N. S.; Omiadze, T. N.; Avalishvili, L. M.; Zaalishvili, M. M. Makromol Chem Macromol Chem Phys 1985, 186, 939.
19. Katsarava, R. D.; Kharadze, D. P.; Toidze, P. L.; Omiadze, T. N.; Japaridze, N. N.; Pirtskhalava, M. K. Acta Polym 1991, 42, 95.
20. Deng, M.; Wu, J.; Reinhart-King, C. A.; Chu, C.-C. Biomacromolecules 2009, 10, 14.
21. Montane, J.; Armelin, E.; Asin, L.; Rodriguez-Galan, A.; Puiggali, J. J Appl Polym Sci 2002, 85, 1815.
22. Okamura, A.; Hirai, T.; Tanihara, M.; Yamaoka, T. Polymer 2002, 43, 3549.
23. Paredes, N.; Rodriguez-Galan, A.; Puiggali, J.; Peraire, C. J Appl Polym Sci 1998, 69, 1537.
24. Peredes, N.; Casas, M. T.; Puiggali, J. Macromolecules 2000, 33, 9090.
25. Rodriguez-Galan, A.; Fuentes, L.; Puiggali, J. Polymer 2000, 41, 5967.
26. Tsitlanadze, G.; Kviria, T.; Katsarava, R.; Chu, C. C. J Mater Sci Mater Med 2004, 15, 185.
27. Tsitlanadze, G.; Machaidze, M.; Kviria, T.; Djavakhishvili, N.; Chu, C. C.; Katsarava, R. J Biomater Sci Polym Ed 2004, 15, 1.
28. Paredes, N.; Rodriguez-Galan, A.; Puiggali, J. J Polym Sci A Polym Chem 1998, 36, 1271.
29. Guo, K.; Chu, C. C. Biomacromolecules 2007, 8, 2851.
30. Guo, K.; Chu, C. C. J Appl Polym Sci 2008, 110, 1858.
31. De Wit, M. A. W. Z.; Atkins, K. M.; Mequanint, K.; Gillies, E. R. J Polym Sci A Polym Chem 2008, 6, 6376.
32. Guo, K.; Chu, C. C. J Appl Polym Sci 2010, 117, 3386.
33. Pang, X.; Chu, C. C. Biomaterials 2010, 31, 3745.
34. Pang, X.; Wu, J.; Reinhart-King, C.; Chu, C. C. J Polym Sci A Polym Chem 2010, 48, 3758.
35. Pack, D. W. H. A.; Pun, S.; Stayton, P. S. Nat Rev Drug Discov 2005, 4, 581.
36. Wang, S. B.; Liu, Y. G.; Weng, L. F.; Ma, X. J. Macromol Biosci 2003, 3, 347.