J Mater Sci: Mater Med (2011) 22:469–479
DOI 10.1007/s10856-011-4243-5

Synthesis and characterization of ionic charged water soluble arginine-based poly(ester amide)

Jun Wu · Martha A. Mutschler · Chih-Chang Chu

Received: 5 April 2010 / Accepted: 17 January 2011 / Published online: 3 February 2011

Abstract A family of water soluble and positively charged L-arginine based poly(ester amide)s (Arg-PEAs) was synthesized and characterized. These biodegradable polymers consist of three nontoxic building blocks: L-arginine, diols, and dicarboxylic acids. The Arg-PEAs were prepared by solution polycondensation reaction of tetra-*p*-toluenesulfonic acids salts of bis-(L-arginine) α, ω-alkylene diesters and di-*p*-nitrophenyl esters of dicarboxylic acids. Optimal conditions of the monomers and polymers synthesis were investigated, and the monomers and Arg-PEAs were chemically characterized. Arg-PEAs were found to have good solubility in water and many other polar solvents. Structure–function relationship of the Arg-PEAs revealed that changing the number of methylene groups in the diol or/and diacid segment could finely tune the hydrophobic and cationic properties of the Arg-PEAs. MTT assay showed that all the prepared Arg-PEAs were non-toxic to the cell lines even at very large doses. Arg-PEAs with double bond functionality could be photo-crosslinked with polyethylene glycol diacrylate to form cationic hybrid hydrogels.

J. Wu · C.-C. Chu
Department of Biomedical Engineering, Cornell University, Ithaca, NY 14853-4401, USA

M. A. Mutschler
Department of Plant Breeding, Cornell University, Ithaca, NY 14853-4401, USA

C.-C. Chu (✉)
Department of Fiber Science and Apparel Design, Cornell University, Ithaca, NY 14853-4401, USA
e-mail: cc62@cornell.edu

1 Introduction

The rapid development of biotechnology demands new generations of water soluble, biocompatible and biodegradable synthetic polymers with various physicochemical, functional and biological properties [1–10]. For this purpose, recently, many kinds of chemical modifications have been applied to the current available synthetic biodegradable and biocompatible polymers to meet the demands in biomedical applications [1–10]. One approach is to introduce the polyether into the absorbable polymers' main or side chain, such as the PLA-*b*-PEG [11] and PLL-*b*-PEG [12, 13], which have been widely investigated in the areas of antibiofouling, self-assembly, drug/gene delivery and nanotechnology. The incorporation of polyether segment will improve the solubility, hydrophilicity of absorbable aliphatic polyesters and increase the circulation time in vivo. Another interesting approach is to introduce the poly(amino acid)s or polypeptide into the polymer backbone. The introduction of natural amino acids would bring the polymer many new properties, such as functionality, biodegradability and charge property in addition to the improvement of hydroplilicity. One example for this approach is polyester-*b*-poly(amino acid)s, such as PLA-*co*-PLL and PCL-*co*-PLL, which have been widely used in the drug delivery and tissue engineering scaffold area [4, 14, 15]. However, these modifications could hardly make the water insoluble absorbable aliphatic polyesters into water soluble polymers.

In this study, we report the design of a water soluble, biocompatible and biodegradable polymer family for biotechnology applications. This polymer family belongs to the amino acid-based poly(ester amide) (PEA, Fig. 1) category. Amino acid based-PEAs are biodegradable and biocompatible synthetic polymers having both ester and

amide linkages on their backbones, which bring good mechanical and biological properties with enzyme-catalyzed biodegradability [5, 8, 16–20]. Combining the favorable properties of polyesters, polyamides, and poly (amino acid)s, amino acid-based PEAs have shown very promising applications in the biomedical and biotechnology area [5, 8, 16–20]. The PEA backbone consists of nontoxic building blocks like α-amino acids, fatty diols and aliphatic dicarboxylic acids. The varieties of combinations of the three types of building blocks offer a variety of PEAs for different purposes. And functional groups, such as double bonds, amine or hydroxyl groups, could be incorporated into the polymer chains [8, 18]. Due to the hydrophobic amino acids used (e.g., Phe, Leu), all these amino acid-based PEAs reported so far have one common characteristic: they dissolve in organic solvents only and are not water soluble [17].

L-Arginine (Arg) is a natural amino acid present in the proteins of all life forms. It carries a positive charge at a physiological pH due to its strong basic guanidine group with an isoelectric point of 10.96 and pKa above 12.5, which is expected to have a strong capability to neutralize negatively charged polymers (proteins/nucleic acids). So L-arginine based poly(ester amide)s (Arg-PEAs) family (Fig. 2) could achieve two major goals: water solubility and cationic characteristic. The diacid and diol parts of Arg-PEAs can be utilized to not only adjust the physicochemical properties of the Arg-PEA polymers (e.g., hydrophilicity and charge density) but also to convert the resulting Arg-PEA polymers into different physical forms like hydrogels.

Our prior preliminary cell membrane penetrating and DNA transfection tests of four types Arg-PEAs had shown that the Arg-PEA/DNA complex could pass through the cell membrane and transfect rat aortic smooth muscle cells (A10) very easily with a very low level of cytotoxicity when compared with commercial transfection agents like Superfect [21], suggesting that the Arg-PEAs could have a great potential as gene delivery vector and molecular target agent. However, the details of Arg-PEA monomer and polymer synthesis and physicochemical characterizations as well as the effect of some material parameters (e.g., methylene chain length in diol and diacid segments) on

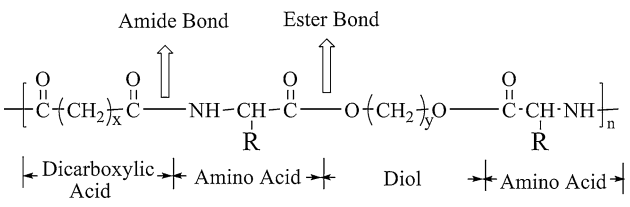

Fig. 1 Chemical structure of poly(ester amide)

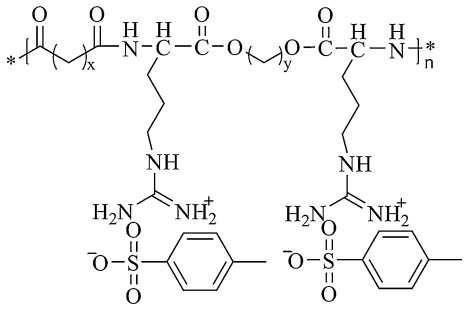

Fig. 2 Chemical structure of Arg-PEAs: *x*-A-*y*-S (S stands for toluenesulfonic acid salt), where *x* is the number of methylene groups between two closest amide groups and *y* is the number of methylene groups between two closest ester groups

Arg-PEA property were not given in the prior biologically-focused study [21]. This chemical structural modification of Arg-PEA is expected to affect its biological performance, such as the hydrophobicity of Arg-PEA could play a very important role in the DNA/Arg-PEA interaction and transfection efficiency.

In this paper, we report the details of synthesis of a Arg-PEA family. The synthesis protocols were systematically studied and optimized. The resulting cationic Arg-PEAs were characterized by standard physicochemical methods. A preliminary cytotoxicity of all the Arg-PEAs synthesized was assessed by MTT assay. These data would provide solid physicochemical support for the biological applications of Arg-PEA including delivery of small molecules/nucleic acid/protein, molecular targeting agents, and tissue engineering scaffolds.

2 Materials and methods

2.1 Materials

L-Arginine (L-Arg), *p*-toluenesulfonic acid monohydrate, fumaryl chloride, succinyl chloride, adipoyl chloride, sebacoyl chloride, ethylene glycol, 1, 4-butanediol, 1,6-hexaniol and *p*-nitrophenol were all purchased from Alfa Aesar (Ward Hill, MA) and used without further purification. Triethylamine from Fisher Scientific (Fairlawn, NJ) was dried by refluxing with calcium hydride, and then distilled before use. Superfect was purchased from Qiagen. Polyethylenimine (PEI) with a reported weight average molecular weight of 25,000, poly(L-lysine) (PLL)-hydrobromide, ethidium bromide, MTT, phosphate-buffered saline (PBS, pH 7.4), HEPES were purchased from Sigma (St. Louis, MO). Organic solvents like methanol, toluene, ethyl acetate, acetone, 2-propanol and dimethyl sulfoxide (DMSO) 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 purchased from Sigma (St. Louis, MO).

2.1.1 Synthesis of the monomers and polymers

The general scheme of the synthesis of Arg-PEAs was divided into the following three major steps: the preparation of di-*p*-nitrophenyl ester of dicarboxylic acids (I; Fig. 3a), the preparation of tetra-*p*-toluenesulfonic acid salts of bis (L-arginine), α, ω-alkylene diesters (II; Fig. 3b), and the synthesis of Arg-PEAs (III) via solution polycondensation of (I) and (II) (Fig. 3c). Monomer I has been synthesized in our prior studies [17]. Monomer II is new, and modified procedures were used to accommodate the ionic nature of the L-arginine.

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

Di-*p*-nitrophenyl esters of dicarboxylic acids were prepared by reacting dicarboxylic acyl chloride varying in methylene length with *p*-nitrophenol as previously reported [17, 18]. Three saturated and one unsaturated monomers were made; the three saturated diacid monomers were: di-*p*-nitrophenyl succate (NSu) with $x = 2$; di-*p*-nitrophenyl adipate (NA) with $x = 4$; di-*p*-nitrophenyl sebacate (NS) with $x = 8$; and one unsaturated monomer was di-*p*-nitrophenyl fumarate (NF) with $x = 2$, where x is the numbers of methylene group in the diacid. An example of the diacid monomer synthesis is given below. Di-*p*-nitrophenyl succinate (NSu) was prepared in 65% yield by the

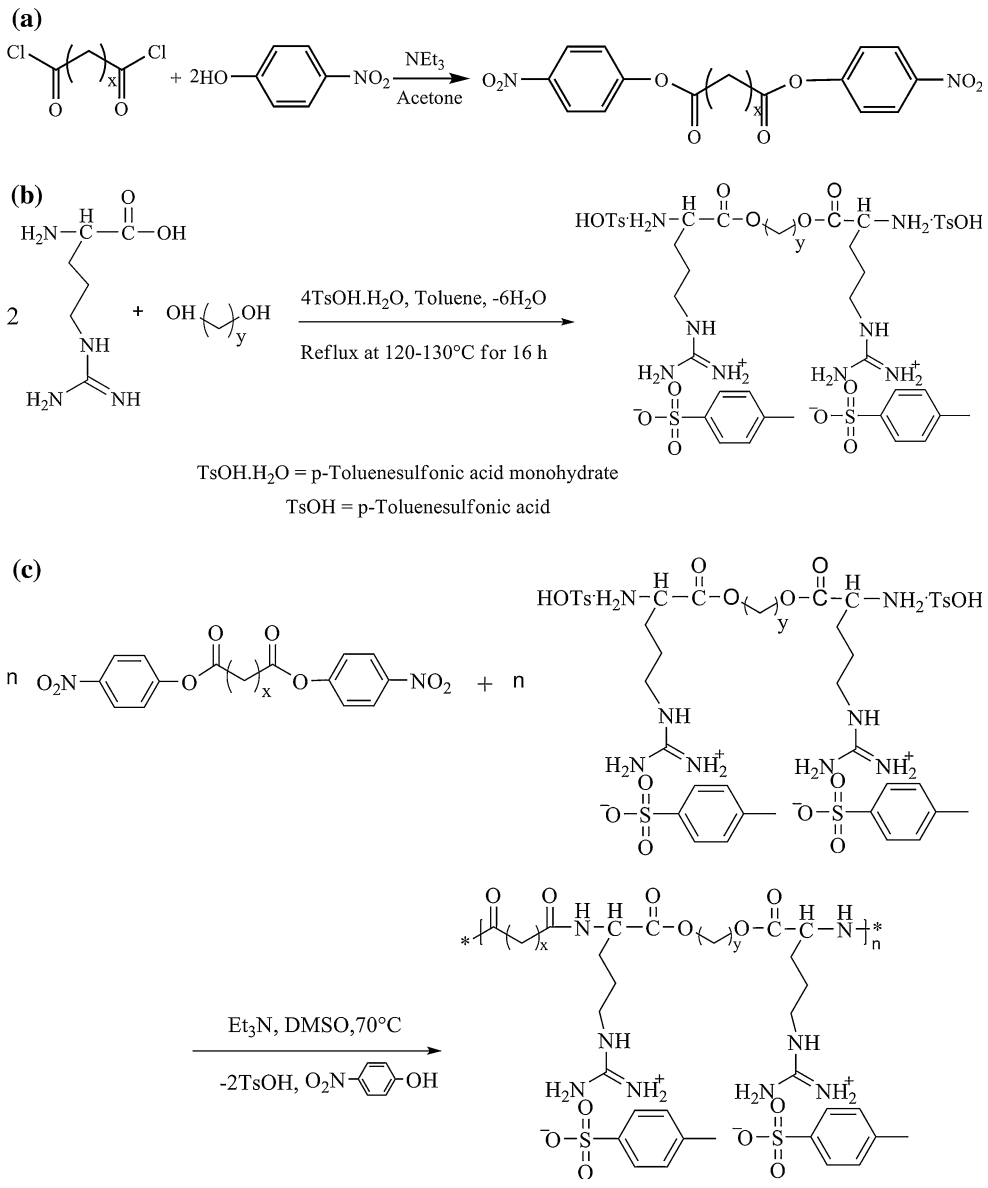

Fig. 3 **a** Synthesis of monomer I: di-*p*-nitrophenyl ester of dicarboxylic acids; **b** Synthesis of monomer II: tetra-*p*-toluenesulfonic acid salt of bis(L-arginine) alkylene diesters; **c** Synthesis of Arg-PEAs

reaction of the succinyl chloride (0.15 mol, 16 ml) with *p*-nitrophenol (0.31 mol) in acetone in the presence of triethylamine (0.32 mol). An ice/water mixture bath was used to keep the *p*-nitrophenol and triethylamine mixed acetone solution (400 ml) at 0°C. Succinyl chloride was diluted in 100 ml of cold acetone before dropped into the above chilled solution with stirring for 2 h at 0°C and overnight at room temperature. The resulting di-*p*-nitrophenyl ester of succinic acid was precipitated in distilled water, washed completely, and then dried in vacuo at room temperature before final recrystallization in ethyl acetate/DMF(4:1, v:v) for three times. The final product is needle-like, colorless crystal.

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

Because of the strong positive charge characteristic of L-arginine, the prior PEA non-charged monomer synthesis protocol (e.g., Phe, Leu) was modified. In brief, the amount of *p*-toluenesulfonic acid used for the synthesis of *p*-toluenesulfonic acid salt of L-arginine diester was doubled when compared with the prior synthesis of *p*-toluenesulfonic acid salt of non-ionic hydrophobic amino acids diesters [17, 18]. The need to double the amount of *p*-toluenesulfonic acid in the current case is because of the preferential consumption of the *p*-toluenesulfonic acid by the strong basic guanidine group on L-arginine side chain. For example, L-arginine (0.04 mol) and 1, 4-butanediol (0.02 mol) were directly mixed in a three neck round bottom flask with toluene (400 ml, b.p. 110°C) with the presence of *p*-toluenesulfonic acid monohydrate (0.082 mol). The solid–liquid reaction mixture was heated to 130°C and reflux with stirring for 24 h with 2.16 ml (0.12 mol) of water was generated. The reaction mixture (viscous solid) was then cooled to room temperature. Toluene was decanted. The resulting product was finally purified by dissolving the product in 2-propanol at 75°C with stirring and then precipitating at 4°C for three times. The ideal precipitation time is around 12 h. 2-Propanol was changed every time after precipitating and decanted afterwards, and the white sticky mass was dried in vacuo. The final product was white powder, and obtained in high yields (70 ~ 90%). Three types of monomer II were made in this study: tetra-*p*-toluenesulfonic acid salt of bis(L-arginine) ethane diesters, Arg-2-S, $y = 2$; tetra-*p*-toluenesulfonic acid salt of bis(L-arginine) butane diesters, Arg-4-S, $y = 4$; tetra-*p*-toluenesulfonic acid salt of bis (L-arginine) hexane diesters, Arg-6-S, $y = 6$. S indicated that the arg diester monomer was in the *p*-toluenesulfonic acid salt form.

2.1.4 *Synthesis of Arg-PEAs (III) by solution polycondensation of I and II monomers*

Arg-PEAs were prepared by solution polycondensation of the monomer I (Arg-2-S, Arg-4-S, Arg-6-S) and monomer II (NSu, NA, NS and NF) at a variety of combinations. Such combinations and the resulting Arg-PEAs are summarized in Table 1. The saturated Arg-PEAs are labeled as *x*-Arg-*y*-S, where *x* and *y* are the number of methylene group in diacid and diol, respectively. The unsaturated Arg-PEAs are labeled as *x*-U-Arg-*y*-S, where *x* and *y* are the number of CH and CH_2 groups in diacid and diol, respectively. U means the Arg-PEA is unsaturated. An example of the synthesis of 8-Arg-6-S via solution polycondensation is given here. Monomers NS (1.0 mmol) and Arg-6-S (1.0 mmol) in 1.5 ml of dry DMSO were mixed well by vortexing. The mixture solution was heated up to 75°C with stirring to obtain a uniformed mixture. Triethylamine (0.31 ml, 2.2 mmol) was added drop by drop to the mixture at 75°C with vigorous stirring until the complete dissolution of the monomers. The solution color turned into yellow after several minutes. The reaction vial was then kept for 48 h at 75°C in a thermostat oven without stirring. The resulting solution was precipitated in cold ethyl acetate, decanted, dried, re-dissolved in methanol and re-precipitate in cold ethyl acetate for further purification. Repeat the purification for two times before drying in vacuo at room temperature. The final Arg-PEAs are yellow or pale yellow solid powder.

2.2 Material characterization

The physicochemical properties of the prepared monomer and polymers were characterized by various standard methods. For Fourier transform infrared (FTIR) characterization, the samples were ground into powders and mixed with KBr at a sample/KBr ratio of 1:10 (w/w). FTIR spectra were then obtained with a PerkinElmer (Madison, WI) Nicolet Magana 560 FTIR spectrometer with Omnic software for data acquisition and analysis. ^{1}H NMR spectra were recorded with a Varian Unity Inova 400-MHz spectrometer (Palo Alto, CA). Deuterated water (D_2O-*d*2;

Table 1 Arg-PEAs prepared by different combination of monomers

	Arg-2-S	Arg-4-S	Arg-6-S
NSu	2-Arg-2-S	2-Arg-4-S	2-Arg-6-S
NA	4-Arg-2-S	4-Arg-4-S	4-Arg-6-S
NS	8-Arg-2-S	8-Arg-4-S	8-Arg-6-S
NF	2-U-Arg-2-S	2-U-Arg-4-S	2-U-Arg-6-S

Cambridge Isotope Laboratories, Andover, MA) with tetramethylsilane as an internal standard or deuterated dimethyl sulfoxide (DMSO-*d*6; Cambridge Isotope Laboratories) was used as the solvent. MestReNova software was used for the data analysis. Elemental analyses of the synthesized polymers were performed with a PE 2400 CHN elemental analyzer by Atlantic Microlab (Norcross, GA). The thermal properties of the synthesized Arg-PEAs were characterized with a DSC 2920 (TA Instruments, New Castle, DE). The measurements were carried out from –10 to 200°C at a scanning rate of 10°C/min and at a nitrogen gas flow rate of 25 ml/min. TA Universal Analysis software was used for thermal data analysis. X-ray diffraction data were obtained from powdered samples with a θ–θ diffractometer (Scintag, Inc., Cupertino, CA) with Cu Kα radiation (wavelength 1.5405 Å). The solubility of Arg-PEAs in common organic solvents at room temperature was assessed by using 2.0 mg/ml as a solubility standard to determine whether a Arg-PEA polymer is soluble or not in a solvent. The quantitive solubility of Arg-PEAs in distilled water at room temperature was measured by adding distilled water step by step until the clear solution was obtained. For the molecular weight measurement, Arg-PEAs were prepared at a concentration of 1 mg/ml in a 0.1% (w/v) LiCl in DMAc solution. The sample molecular weights were determined from a standard curve generated from polystyrene standards with molecular weights ranging from 841.7 to 2.93 kDa that were chromatographed under the same conditions as the samples. The standard curve was generated from a third-order polynomial fit of the polystyrene standard molecular weights.

2.3 Cell culture and cytotoxicity

Rat aortic A10 vascular smooth muscle cells (SMC)s obtained from American Tissue Culture Collection (ATCC) were provided by Dr. Bo Liu's lab at Cornell Weill Medical College. The vascular smooth muscle cell line was chosen for two reasons. (1) Vascular smooth muscle cells are the key to the formation of vascular lesions, which are major causes of stroke or infarction. (2) Vascular smooth muscle cells are very difficult to be transfected with current non-viral gene vectors. 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.

The cytotoxicity of Arg-PEAs was performed by MTT assay. Cultured SMC were seeded at an appropriate cell density concentration (3,000 cells/well) in 96-well plates and incubated overnight in a 5% CO_2 incubator at 37°C. The cells were then treated with various Arg-PEA solutions for 4 or 48 h. Cells were treated only with normal cell culture media were used as negative control (NC). PEI, PLL-HBr and Superfect treated cells were used as positive control. After 48 h incubation, 15 µl of MTT solution (5 mg/ml) was added to each well, the cell culture plate was incubated for 4 h at 37°C, 5% CO_2. After that, the cell culture medium including polymer solution was carefully removed and 150 µl of acidic isopropyl alcohol (with 0.1 M HCl) was added to dissolve the formed formazan crystal. OD was measured at 570 nm (subtract background reading at 690 nm) using a microplate reader. The cell viability (%) was calculated according to the following equation: Viability (%) = $(OD_{570\ (sample)} - OD_{690\ (sample)})/(OD_{570\ (control)} - OD_{690\ (control)}) \times 100\%$, where the $OD_{570\ (control)}$ represents the measurement from the wells treated with medium only, and the $OD_{570\ (sample)}$ from the wells treated with various polymers. Triplicates were used in each experiment.

2.4 Fabrication of Arg-PEA based hybrid hydrogel

To assess the functionality of the unsaturated Arg-PEAs, hybrid hydrogels from the unsaturated Arg-PEAs were fabricated in an aqueous medium by a photo means. PEGDA as a co-precursor (molecular weight: 4,000) was synthesized according to a modified procedure of a previously reported method [22, 23]. Several types of Arg-PEA/PEGDA hybrid hydrogel were fabricated. An example for such a fabrication is given here. An unsaturated Arg-PEA (2-U-Arg-2-S) solution of a concentration of 10.0% (w/v) was prepared by dissolving 0.5 g 2-U-Arg-2-S in 5.0 ml distilled water in a glass bottle. PEGDA (M_n = 4,000) solution (25 wt%) was then added into the prepared 2-U-Arg-2-S solution at a weight feed ratio of PEGDA to 2-U-Arg-2-S (i.e., 4.0/1.0). The photoinitiator, 4-(2-hydroxyethoxy) phenyl-(2-hydroxy-2-propyl) ketone (Irgacure 2959) was added to the precursor solution at a concentration of 0.1%(m/v). The mixed solution was then stirred for 10 min at 50°C to ensure a complete dissolution of the photoinitiator. The homogenous, transparent solution was first transferred to a custom-made 20 well Teflon mold (with 500 µm volume per well) using a micropipette, and then irradiated by a long-wavelength UV lamp (365 nm and 100 W) at room temperature for 10 min. After photo-gelation, the hydrogel samples were immersed in distilled water at room temperature for 48 h to leach out any unreacted residual chemicals. During this period, distilled water was replaced every 12 h.

3 Results

3.1 Synthesis of monomers

Four types of di-*p*-nitrophenyl esters of dicarboxylic acids (NSu, NA, NS and NF) were synthesized here as the monomer I to react with the tetra-*p*-toluenesulfonic acid

salt of bis(L-arginine) alkylene diester monomer II to provide amide linkage in Arg-PEA backbone. The synthesis and characterization of monomer I have been reported previously [17, 18]. Three types of new monomer II were prepared: Tetra-*p*-toluenesulfonic acid salt of L-arginine ethane-1,2-diester (Arg-2-S), tetra-*p*-toluenesulfonic acid salt of L-arginine butane-1,4-diester (Arg-4-S), and tetra-*p*-toluenesulfonic acid salt of L-arginine hexane-1,6-diester (Arg-6-S).

The chemical structures of these three types of Arg-based monomer II were all confirmed by FTIR and ^{1}HNMR. As shown in Fig. 4, the absorption bands of the ester group were observed in the regions $\sim$1,180 cm^{-1} (–O–) and $\sim$1,758 cm^{-1} (–CO–), and NH vibrations at 3,290 cm^{-}1. All the synthesized tetra-*p*-toluenesulfonic acid salts of bis(L-arginine) alkylene diesters are very moisture sensitive and should be stored under vacuum at room temperature.

The following are some physical and chemical details of the tetra-*p*-toluenesulfonic acid salts of bis(L-arginine) alkylene diesters monomers: *Arg-2-S*: Yield of purified product: 75%. Appearance: amorphous white powder. IR: 1,753 cm^{-1} [–CO–], 1,178 cm^{-1} [–O–]; ^{1}HNMR (DMSO-*d*6, ppm, δ): 1.63[4H,-CH_2-CH_2-CH_2-NH-], 1.82[4H,-OC (O)-CH (NH_3^+) CH_2-$(CH_2)_2$-], 2.29[6H, H_3C-Ph-SO_3-], 3.10[4H,-$(CH_2)_2$-CH_2-NH-], 4.06 [2H,^+H_3N-CH(R)-C(O)-O-], 4.39[4H, -(O)C–O-CH_2-], 7.18, 7.53[16H, Ph], 7.69 [10H, -CH_2-NH(NH_2^+)-NH_2], 8.42[6H, ^+H_3N-CH(R)-C(O)-O-]; *Arg-4-S*: Yield of purified product: 78%. Appearance: amorphous white powder. IR: 1,743 cm^{-1} [–CO–], 1,170 cm^{-1} [–O–]; ^{1}HNMR (DMSO-*d*6, ppm, δ): 1.52[4H,-OC(O)-CH(NH3 +)-CH_2-CH_2-CH_2-NH-], 1.65[4H, -(O)C–O-CH_2-CH_2-], 1.80[4H,-OC (O)-CH (NH_3^+) CH_2-$(CH_2)_2$-], 2.29[6H, H_3C-Ph-SO_3-], 3.10[4H,-$(CH_2)_2$-CH_2-NH-], 4.04 [2H,^+H_3N-CH(R)-C(O)-O-], 4.14[4H,-(O)C–O-CH_2-CH_2-], 7.18, 7.53[16H, Ph], 7.71 [10H, -CH_2-NH(NH_2^+)-NH_2], 8.40[6H, ^+H_3N-CH(R)-C(O)-O-]; *Arg-6-S*: Yield of purified product: 84%. Appearance: amorphous white powder. IR: 1,749 cm^{-1} [–CO–], 1,171 cm^{-1} [–O–]; ^{1}HNMR (DMSO-*d*6, ppm, δ): 1.34, [4H,-(O)C–O-CH_2-CH_2-CH_2], 1.61, [4H,-(O)C–O-CH_2-CH_2-CH2], 1.81, [4H,-OC (O)-CH (NH_3^+) CH_2-$(CH_2)_2$-], 2.29, [6H, H_3C-Ph-SO_3-], 3.11, [4H,-$(CH_2)_2$-CH_2-NH-], 4.03, [2H,^+H_3N-CH(R)-C(O)-O-], 4.15, [4H,-(O)C–O-CH_2-CH_2-CH_2], 7.15, 7.50 [16H, Ph], 7.73[10H, -CH_2-NH(NH_2^+)-NH_2], 8.43[6H, ^+H_3N-CH(R)-C(O)-O-].

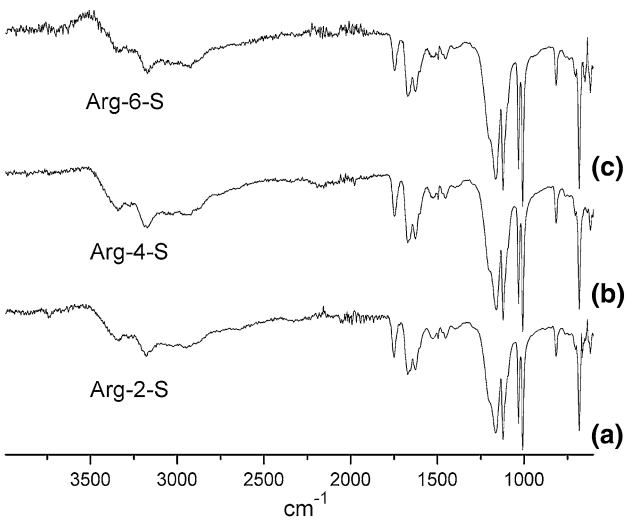

Fig. 4 FTIR spectra of the tetra-*p*-toluenesulfonic acid salts of bis(L-arginine) diesters: **a** Arg-2-S; **b** Arg-4-S; **c** Arg-6-S

3.2 Arg-PEA polymers and their property

Arg-PEAs were prepared according to the reaction scheme in Fig. 3c. The yields, glass transition temperature (T_g), molecular weight of repeating unit are given in Table 2. The reaction conditions were optimized in terms of reaction temperature and time, catalyst and its concentration, the molar ratio between two monomers, and monomer concentration.

For the chemical structure identification of all the synthesized Arg-PEAs, their structures were confirmed by both ^{1}HNMR and FTIR spectra. Figure 5 shows the FTIR spectra of three Arg-PEAs. The carbonyl bands at 1,648–1,650 cm^{-1} (amide I), 1,538–1,542 cm^{-1} (amide II), and 1,738–1,742 cm^{-1} (ester), and NH vibrations at 3,290 cm^{-1} are typical for all PEAs obtained. Figure 6 shows an example of the ^{1}HNMR spectrum of 2-Arg-2-S. The solubility of some Arg-PEAs in water and common organic solvents at room temperature is shown in Table 3. Solubility was assessed at 2.0 mg/ml at a room temperature.

For the thermal property of the Arg-PEAs, the glass transition temperature (T_g) of saturated Arg-PEAs (Table 2) ranged between 30 and 55°C. The molecular weight (MW) of 2-Arg-2-S, 4-Arg-2-S and 8-Arg-2-S (Table 4) were obtained with the help of MediVas, LLC. The MW data in Table 4 indicate that all the three Arg-PEAs had M_n between 12.5 kg/mol and 14.5 kg/mol with narrow polydispersity (PDI) of 1.07–1.10. The charge density of Arg-PEAs can be calculated by using guanidine density (mol/kg) and is shown in Table 2. Figure 7 presents a wide-angle X-ray diffraction diagram of the 3 synthesized Arg-PEAs (2-Arg-2-S, 2-Arg-4-S, and 2-Arg-6-S).

3.3 Cytotoxicity of Arg-PEAs by MTT assay

Cytotoxicity of Arg-PEAs was preliminarily evaluated by MTT assay. Poly(L-lysine) hydrobromide(PLL-HBr), poly(ethylenimine) (PEI) and Superfect were used as the

Table 2 Physical and thermal characteristics of Arg-PEAs

Polymer	Unit formula	Unit molecular weight (g/mol)	Charge density (mol/kg)	T_g (°C)	Polymer yield (%)
2-Arg-2-S	$C_{32}H_{48}N_8O_{12}S_2$	800.9	2.497	50 ± 2	80
2-Arg-4-S	$C_{34}H_{52}N_8O_{12}S_2$	828.9	2.413	46 ± 2	83
2-Arg-6-S	$C_{36}H_{56}N_8O_{12}S_2$	857.0	2.333	39 ± 2	89
4-Arg-2-S	$C_{34}H_{52}N_8O_{12}S_2$	828.9	2.413	48 ± 2	90
4-Arg-4-S	$C_{36}H_{56}N_8O_{12}S_2$	857.0	2.333	42 ± 2	88
4-Arg-6-S	$C_{38}H_{60}N_8O_{12}S_2$	885.0	2.260	39 ± 2	83
8-Arg-2-S	$C_{38}H_{60}N_8O_{12}S_2$	885.0	2.260	42 ± 2	91
8-Arg-4-S	$C_{40}H_{64}N_8O_{12}S_2$	913.1	2.190	33 ± 2	87
8-Arg-6-S	$C_{42}H_{68}N_8O_{12}S_2$	941.1	2.125	32 ± 2	92
2-U-Arg-2-S	$C_{32}H_{46}N_8O_{12}S_2$	798.9	2.503	95 ± 2	90
2-U-Arg-4-S	$C_{34}H_{50}N_8O_{12}S_2$	826.9	2.419	90 ± 2	87
2-U-Arg-6-S	$C_{36}H_{54}N_8O_{12}S_2$	855.0	2.339	81 ± 2	83

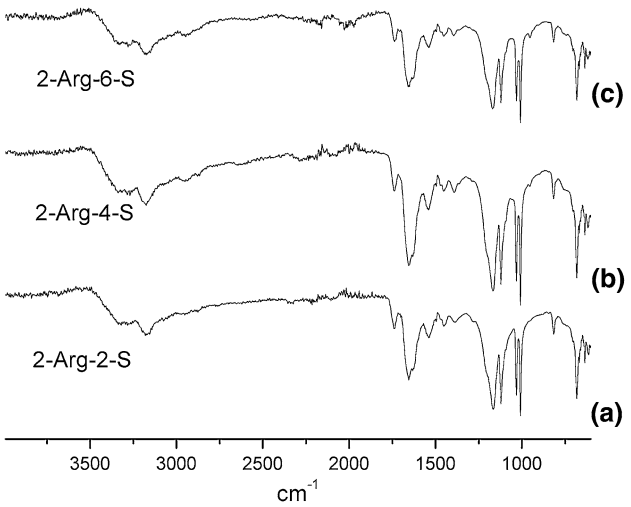

Fig. 5 FTIR spectra of Arg-PEAs: **a** 2-Arg-2-S; **b** 2-Arg-4-S; **c** 2-Arg-6-S

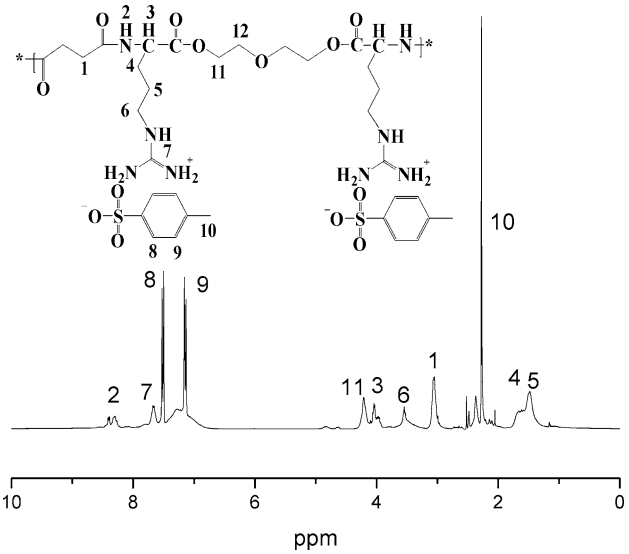

Fig. 6 HNMR spectra of 2-Arg-2-S

controls. Figure 8 showed an example of the MTT results and the data showed that all the four Arg-PEAs exhibited far better rat A10 SMC cell line viability over a wide concentration range than the three controls, PLL-HBr, PEI and Superfect, which showed significant cytotoxicity.

3.4 Fabrication of Arg-PEA/PEGDA hybrid hydrogel

Cationic hybrid hydrogels from both Arg-PEA and PEGDA precursors were fabricated in an aqueous medium by a photo means. Figure 9 showed the hydrogel image of 2-U-Arg-2-S/PEGDA (at 1.0:4.0, w/w). The left image is a completely swollen hydrogel, while the right one is a completely dried hydrogel. The presence and amounts of 2-U-Arg-2-S in the hybrid hydrogel has been confirmed by elemental analysis. The detailed property of these cationic hybrid hydrogels will be reported in a separated paper.

4 Discussion

As discussed in the introduction part, the main purpose of this paper is to report the synthesis protocols and characterization of new Arg-PEAs, so that the structure–property relationship could be well established for future designing advanced generations of Arg-PEAs.

4.1 Synthesis of monomers

The di-*p*-nitrophenyl esters of dicarboxylic acid was used to react with the tetra-*p*-toluenesulfonic acid salt of bis

Table 3 Solubility of Arg-PEAs in various solvents[a]

	2-Arg-2-S	4-Arg-4-S	8-Arg-6-S	2-U-Arg-2-S	2-U-Arg-2-S	2-U-Arg-2-S
Water	+	+	+	+	+	+
Ethanol	+	+	+	+	+	+
Methanol	+	+	+	+	+	+
2-Propanol	+	+	+	+	+	+
DMA	+	+	+	+	+	+
DMSO	+	+	+	+	+	+
THF	–	–	–	–	–	–
Acetone	–	–	–	–	–	–
Chloroform	–	–	–	–	–	–
Ethyl acetate	–	–	–	–	–	–
Hexane	–	–	–	–	–	–

[a] About 2 mg/ml was chosen as the solubility standard; + means soluble, – means insoluble

Table 4 Molecular weight information of Arg-PEAs

Polymer	M_n(kg/mol)	M_w(kg/mol)	PDI
2-Arg-2-S	12.8	13.7	1.07
4-Arg-2-S	14.4	15.9	1.10
8-Arg-2-S	13.2	14.1	1.07

(L-arginine) alkylene diester to provide amide linkage in Arg-PEA backbone. The synthesis details of the tetra-*p*-toluenesulfonic acid salts of bis(L-arginine) alkylene diesters are newly developed and reported for the first time. The amounts of *p*-toluenesulfonic acid used were the main difference of monomer synthesis between the current Arg-based monomers and other hydrophobic amino acid based monomers. According to our data, the excessive *p*-toluenesulfonic acid was needed because of the strong alkalinity of the guanidine group of Arginine. *p*-Toluenesulfonic acid preferred to react with guanidine group first to form a stable salt, then reacted with the amine group of arginine, if there were excessive amounts of *p*-toluenesulfonic acid. The only difference among the prepared three types of monomer II is the methylene chain length (y) in the diol part between the two adjacent ester groups: number of CH_2 varies from 2 to 4, and 6 (Arg-2-S to Arg-4-S, and Arg-6-S).

4.2 Synthesis of Arg-PEA polymers and their property

The reaction conditions were optimized in terms of reaction temperature and time, catalyst and its concentration, the molar ratio between two monomers, and monomer concentration. After testing, we found that the optimal polycondensation reaction conditions for the Arg-PEAs are: reaction temperature: 75°C; duration: 48 h, concentration of each monomer: 1.0–1.5 mol/l; the reaction medium: DMSO; catalyst (acid acceptor): NEt3. The molar ratio of the two monomers (I and II) should be exactly equal to 1.0: 1.0, and the molar ratio between the monomer and acid receptor is suggested to be 1.0: 1.1. The final product yields are high (>80%) under the optimized reaction conditions.

For the thermal property of the Arg-PEAs, they do not have melting points (T_m) because all the Arg-PEAs are in the amorphous state which is confirmed by the X-ray diffraction data (see Fig. 7). An examination for the effect of the number of methylene groups in the diol (y) and diacid (x) parts of the Arg-PEAs revealed that an increase in either x or y led to a lower T_g. For example, if x value was fixed at 2, the T_g decreased from 50 to 39°C when the y value was increased from 2 to 6, The same trend was observed when

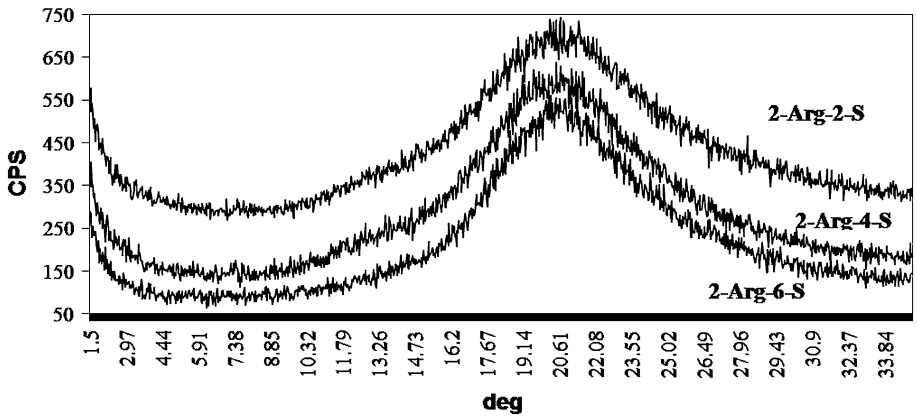

Fig. 7 X-ray diffraction diagram of Arg-PEAs

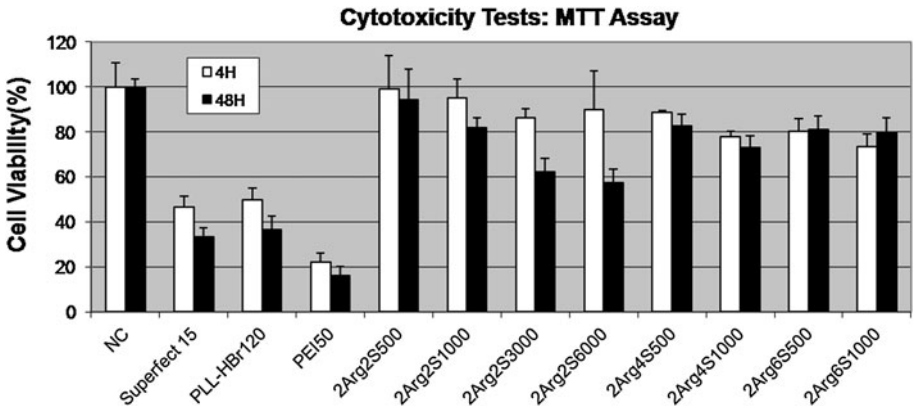

Fig. 8 Cytotoxicity tests of Arg-PEAs by MTT assay. Negative control (*NC*) is A10 SMC cell line only without any materials treatment. Various concentrations were tested on Superfect (*SF*), PLL-HBr, PEI and Arg-PEA polymers (from 2-Arg-2-S to 2-Arg-6-S). The numbers after the material name indicate the corresponding polymer concentration (ug/ml)

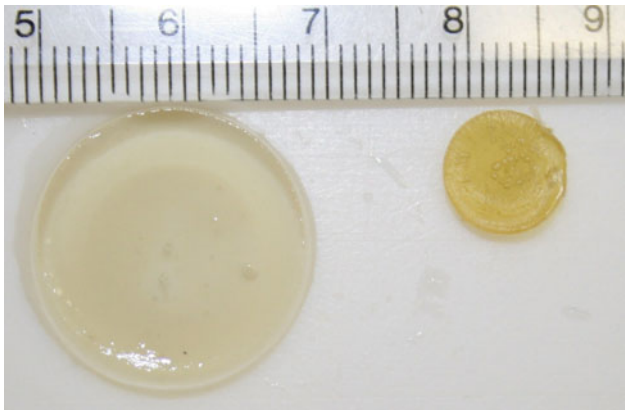

Fig. 9 Hydrogel image of 2-U-Arg-2-S/PEGDA (*left*: swollen hydrogel; *right*: dry hydrogel)

y value was fixed. This relationship is consistent with non-ionic hydrophobic amino acid-based and many other PEA systems [17], such as the Phe-PEA system, for example, if *x* value was fixed at 4, the T_g decreased from 59 to 49°C when the *y* value was increased from 4 to 6. Based on the previous reports, the Phe-based PEAs showed higher T_g than Val or Leu-based PEAs because of the stereo-hindrance effect of the aromatic groups of L-Phe, And it was observed that the T_g value of ionic Arg-PEAs is close to the corresponding non-ionic Phe-PEAs, which could be due to the stereo-hindrance effect of guanidine groups of L-arginine and the cationic property of the guanidine groups. It is not clear at this stage which is the main factor affecting the T_g of Arg-PEAs, and further studies would be focused on this area.

For the water soluble unsaturated Arg-PEAs, due to the existing of double bonds in the Arg-PEA backbone, much higher T_g values were observed when compared with the saturated Arg-PEAs. This relationship between T_g and unsaturation in PEA is also consistent with the thermal data of the water insoluble unsaturated Phe-based PEA system [18]. It is important to know that the location of the unsaturated >C=C< bonds is also critical for their effect on T_g as evident in the recent report by Pang et al. [24] in their study of Arg-based PEAs with pendant unsaturated >C=C< group. In the Pang et al. [24] study, the pendant unsaturated >C=C< group was provided by 2-allylglycine (AG). When comparing the T_g from the current unsaturated Arg-PEAs with >C=C< in the backbone versus the same Arg-PEAs (same *x* and *y*) with >C=C< in pendant location, the latter exhibited lower T_g. For example, T_g 33°C of 2-Arg-4-AG-2EG was observed versus 90°C of 2-UArg-4-S, and 27°C of 2-Arg-6-AG-2EG versus 81°C of 2-UArg-6-S. Therefore, in the event that the double bonds are located in the PEA backbone as in the current UArg-PEA case, their T_g would increase. On the other hand, if the double bonds in PEAs are located as an pendant group, their T_g would decrease instead, this suggested that the presence of the pendant double bond unit like AG could impart additional chain flexibility because of the increasing free volume from pendant double bonds which could act as internal plasticizers, lowered the intermolecular interaction between PEA chains.

For the solubility of some Arg-PEAs, due to their strong polar nature, Arg-PEAs tended to dissolve in polar solvents. All of the Arg-PEAs synthesized were soluble in polar organic solvents like DMSO, methanol or water, but did not dissolve in non-polar or weak polar organic solvents like ethyl acetate or chloroform. And the unsaturated Arg-PEAs showed no solubility difference from the saturated Arg-PEAs. The effect of *x* and *y* material parameters on Arg-PEA water solubility (Fig. 10) revealed that both *x* and *y* had a major impact on the water solubility of Arg-PEAs; and an increase in the methylene chain length in either the dicarboxylic acid part (*x*) or in the diols (*y*) part reduced the water solubility significantly due to the increasing hydrophobicity. So the water solubility of Arg-PEAs could be used as an index of polymer hydrophilicity/hydrophobicity. By adjusting the *x* or *y*, the Arg-PEA polymers' hydrophilicity/hydrophobicity could be fine tuned to meet specific needs.

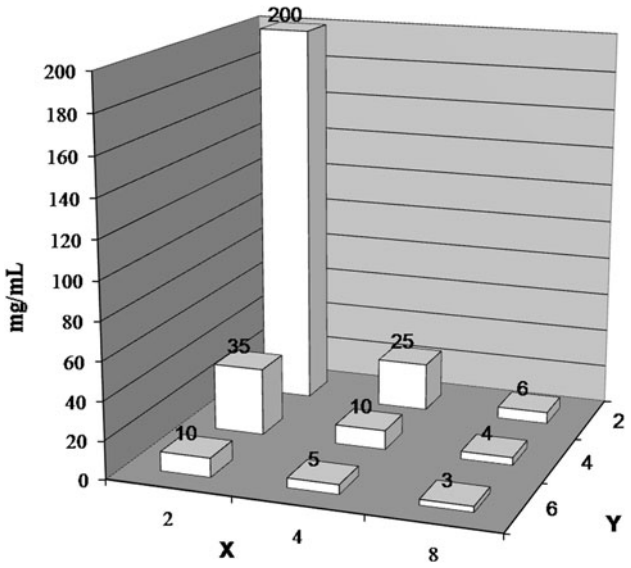

Fig. 10 Solubility of Arg-PEAs in distilled water

For the molecular weight (MW) of Arg-PEAs, the *x* or *y* values did not have any significant impact on MW and PDI of Arg-PEAs. Compared with other amino acid-based PEAs from Phe or Leu, the MW of the Arg-PEA was lower, which may be due to the more complicated and ionic chemical structure of Arg-PEAs.

The charge density of Arg-PEAs (guardinine density) was found to be controlled by both *x* and *y*; and a low *x* or *y* led to a higher charge density, and a high *x* or *y* resulted in a reduction in charge density. However, the difference in charge density of the current Arg-PEA system was not large; for example, the difference between 2-Arg-2-S (highest charge density) and 8-Arg-6-S (lowest charge density) was around 18%, so we can say all the Arg-PEAs in the present study have a similar cationic strength.

The wide-angle X-ray diffraction diagram showed some crystalline information of Arg-PEAs. Unlike some saturated hydrophobic PEAs, such as the Phe-based PEAs which are semi-crystalline polymers with melting temperatures, all the Arg-PEAs in this study are amorphous, which could be attributed to the salt form in the Arg-PEAs. The large *p*-toluenesulfonic acid counter ion adjacent to the guanidine group could prevent the tight and orderly packing of the Arg-PEA chains required for crystallization.

4.3 Cytotoxicity of Arg-PEAs

The MTT system is a simple, accurate, reproducible method of detecting living cells via mitochondrial dehydrogenase activity. An increase in cell number (cell proliferation) results in an increase in the amount of MTT formazan formed and an increase in UV absorbance. So cytotoxicity of Arg-PEAs was preliminarily evaluated by MTT assay in this study. Poly(L-lysine) hydrobromide(PLL-HBr), poly(ethylenimine) (PEI) and Superfect were used as the controls because they have been widely reported as gene carriers. The data in Fig. 8 showed that compared to Superfect, PLL-HBr and PEI, Arg-PEAs are nontoxic and very safe to the SMC cells even at a high dosage. In addition to the amino acid nature of Arg-PEAs, the relatively lower positive charge density (Table 2, guardinine density, ranging from 2.13 to 2.50 mol/kg) of Arg-PEA than the widely studied PEI and PLL-HBr which have a much higher charge density (nitrogen atoms density of 23.3 mol/kg and 4.78 mol/kg, respectively) may be responsible for the much better cell biocompatibility in Arg-PEAs. A full cellular and tissue biocompatibility of Arg-PEAs would be needed for a complete assessment of the biologically safety of Arg-PEAs for their potential biomecial applications.

4.4 Fabrication of Arg-PEA/PEGDA hybrid hydrogel

In order to obtain cationic hydrogels, hybrid hydrogels from both Arg-PEA and PEGDA precursors were fabricated in an aqueous medium by a photo means. Because Arg-PEAs have plenty of nitrogen element, while PEGDA did not have any, elemental analysis method was used to confirm the presence and amounts of 2-U-Arg-2-S in the hybrid hydrogels. According to the unpublished data, the measured amounts of 2-U-Arg-2-S is very close to the theoretical value, which confirmed that the 2-U-Arg-2-S was almost completely involved the photocrosslinking reaction. This type of cationic Arg-PEA/PEGDA hybrid hydrogel could have many potential applications in the tissue engineering scaffold and drug delivery area. A detailed physical and biological study of cationic Arg-based PEA hydrogels will be reported as a separate paper in the future.

5 Conclusions

A new family of water soluble and cationic Arg-PEAs have been successfully synthesized and characterized. Various characterization methods have been used to investigate the physicochemical properties of the Arg-PEAs. Arg-PEAs are in the amorphous salt form and proved to be water soluble. The relationship between polymer hydrophilicity and polymer structure was studied, and found that fewer CH_2 units in the Arg-PEA was, the more hydrophilic the Arg-PEA polymer became. The data from the in vitro MTT assay demonstrated that the Arg-PEA polymers are nontoxic to rat A10 SMC cells even at large dosages. The successful hydrogel fabrication from the unsaturated Arg-

PEAs in an aqueous medium offered a new type of cationic hydrogel. Based on the good biocompatibility results, positive charge property and easy hydrogel formulation, these Arg-PEAs may have the great potential applications for drug delivery vehicles and tissue engineering scaffolds, especially for the DNA/siRNA delivery, cancer therapy and wound healing applications.

Acknowledgments This study was supported by a seed grant from Cornell University Intercampus Program. We also thank Prof. Bo Liu for using her cell culture facilities.

References

1. Langer R, Tirrell DA. Designing materials for biology and medicine. Nature (London, United Kingdom). 2004;428(6982): 487–92.
2. Uhrich KE, Cannizzaro SM, Langer RS, Shakesheff KM. Polymeric systems for controlled drug release. Chem Rev (Washington, DC). 1999;99(11):3181–98.
3. Wang Y, Ameer GA, Sheppard BJ, Langer R. A tough biodegradable elastomer. Nat Biotechnol. 2002;20(6):602–6.
4. West JL. Drug delivery: pulsed polymers. Nat Mater. 2003;2(11): 709–10.
5. Chu CC. Biodegradable polymeric biomaterials: an updated overview. Biomaterials. 2007;6:6-11–6-12.
6. Langer R. Biomaterials in drug delivery and tissue engineering: one laboratory's experience. Acc Chem Res. 2000;33(2):94–101.
7. Ma PX. Biomimetic materials for tissue engineering. Adv Drug Deliv Rev. 2008;60(2):184–98.
8. Deng M, Wu J, Reinhart-King CA, Chu C-C. Synthesis and characterization of biodegradable poly(ester amide)s with pendant amine functional groups and in vitro cellular response. Biomacromolecules. 2009;10(11):3037–47.
9. Zhu A, Zhang M, Wu J, Shen J. Covalent immobilization of chitosan/heparin complex with a photosensitive hetero-bifunctional crosslinking reagent on PLA surface. Biomaterials. 2002; 23(23):4657–65.
10. Huang NF, Patel S, Thakar RG, Wu J, Hsiao BS, Chu B, et al. Myotube assembly on nanofibrous and micropatterned polymers. Nano Lett. 2006;6(3):537–42.
11. Zhu KJ, Lin X, Yang S. Preparation, characterization, and properties of polylactide (PLA)-poly(ethylene glycol) (PEG) copolymers: a potential drug carrier. J Appl Polym Sci. 1990; 39(1):1–9.
12. Katayose S, Kataoka K. Water-soluble polyion complex associates of DNA and poly(ethylene glycol)-poly(L-lysine) block copolymer. Bioconjug Chem. 1997;8(5):702–7.
13. Sawhney AS, Hubbell JA. Poly(ethylene oxide)-graft-poly(L-lysine) copolymers to enhance the biocompatibility of poly(L-lysine)-alginate microcapsule membranes. Biomaterials. 1992;13 (12):863–70.
14. Barrera DA, Zylstra E, Lansbury PT Jr, Langer R. Synthesis and RGD peptide modification of a new biodegradable copolymer: poly(lactic acid-*co*-lysine). J Am Chem Soc. 1993;115(23): 11010–1.
15. Barrera DA, Zylstra E, Lansbury PT, Langer R. Copolymerization and degradation of poly(lactic acid-*co*-lysine). Macromolecules. 1995;28(2):425–32.
16. Huang SJ, Bansleben DA, Knox JR. Biodegradable polymers: chymotrypsin degradation of a low molecular weight poly(ester-urea) containing phenylalanine. J Appl Polym Sci. 1979;23(2): 429–37.
17. Katsarava R, Beridze V, Arabuli N, Kharadze D, Chu CC, Won CY. Amino acid-based bioanalogous polymers synthesis and study of regular poly(ester amide)s based on bis(alpha -amino acid) alpha,w-alkylene diesters, and aliphatic dicarboxylic acids. J Polym Sci A Polym Chem. 1999;37(4):391–407.
18. Guo K, Chu CC, Chkhaidze E, Katsarava R. Synthesis and characterization of novel biodegradable unsaturated poly(ester amide)s. J Polym Sci A Polym Chem. 2005;43(7):1463–77.
19. Guo K, Chu CC. Synthesis, characterization, and biodegradation of copolymers of unsaturated and saturated poly(ester amide)s. J Polym Sci A Polym Chem. 2007;45(9):1595–606.
20. Guo K, Chu CC. Copolymers of unsaturated and saturated poly(ether ester amide)s: synthesis, characterization, and biodegradation. J Appl Polym Sci. 2008;110(3):1858–69.
21. Yamanouchi D, Wu J, Lazar AN, Craig Kent K, Chu CC, Liu B. Biodegradable arginine-based poly(ester-amide)s as non-viral gene delivery reagents. Biomaterials. 2008;29(22):3269–77.
22. Cruise GM, Hegre OD, Scharp DS, Hubbell JA. A sensitivity study of the key parameters in the interfacial photopolymerization of poly(ethylene glycol) diacrylate upon porcine islets. Biotechnol Bioeng. 1998;57(6):655–65.
23. Cruise GM, Scharp DS, Hubbell JA. Characterization of permeability and network structure of interfacially photopolymerized poly(ethylene glycol) diacrylate hydrogels. Biomaterials. 1998;19 (14):1287–94.
24. Pang X, Wu J, Reinhart-King C, Chu CC. Synthesis and characterization of functionalized water soluble cationic poly(ester amide)s. J Polym Sci A Polym Chem. 2010;48:3758–66.