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Validity of Poly(1, 6-bis-(*p*-carboxyphenoxy hexane)-*co*-(sebacic anhydride)) Copolymer in Biomedical Application

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ABSTRACT: To further enhance the performance of biodegradable polymer-based medical devices, there is an increasing need to obtain independent control of key properties such as mechanical strength, degradation rate, and bioactivity. In this study, biodegradable copolymers of poly(1,6-bis-*p*-carboxyphenoxyhexane-*co*-sebacic anhydride) (CPH:SA) are synthesized, via melt condensation techniques, at three different molar ratios (7 : 3, 5 : 5, and 3 : 7). Tablets of the copolymers are prepared by mold casting at high temperature. Using an *in vitro* degradation test, copolymer tablets demonstrate a suitable mechanical strength, a slight decrease in pH value, and a slow degradation rate. High cell viability is observed on the surface of the copolymer tablets. The 3-(4,5dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) assay and live/dead staining demonstrate reduced toxicity and high cell survival. *In vitro* testing with C2C12 cells reveals good cellular attachment and spreading on the tablet surfaces, with the best properties displayed by the 7 : 3 molar ratio copolymer. Materials composed of CPH:SA have the potential to serve as medical implants. © 2012 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 128: 3687–3695, 2013

KEYWORDS: biodegradable; biomaterials; copolymers; biocompatibility; polycondensation

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INTRODUCTION

In modern medical practice, bone substitutes, such as bone screws and bone plates, are made primarily of metal such as titanium or 316 L.[1] However, the elastic moduli of these metals are 5–10 times higher than human cortical bone.[2,3] This modulus mismatch can lead to a stress shielding effect, resulting in decreased bone mineral mass and occasionally leading to bone fracture after removal.[4] Stress shielding is caused by the absence of load on the healing bone because the metal substitutes take over all the stress transfer.[5] Other potential problems with the use of metal substitutes are corrosion and accumulation of metallic particles in the vicinity of the implant[6] or at distant body parts, including draining lymph nodes, spleen, and liver.[7] Such corrosion and accumulation may alter osteoblast behavior even at subtoxic levels. Removal of the metal implant after bone union requires secondary surgery, which can cause further injury to the patient.

To overcome the shortcomings of metal substitutes, degradable polymer substitutes have been studied. Members of the polyester class of polymers, such poly(L-lactide) (PLLA) and polycaprolactone (PCL), have attracted wide attention due to their biodegradability and biocompatibility.[8] However, PLLA has several disadvantages, including insufficient strength and lack of desired bioactivity.[9] Moreover, implanted PLLA induces inflammation, which has been attributed to the formation of lactic acid during PLLA degradation.[10] PCL also is a biodegradable biomaterial that is biocompatible and has a very long degradation time,[11] but PCL's compressive strength, tensile strength, and elastic modulus all would have to be increased to provide sufficient biomechanical performance.[12] Therefore, inferior mechanical strength, short degradation period, and local tissue acidity limit the utility of these polyester polymer bone substitutes.

To address these concerns, our laboratory has proposed a novel class of biodegradable polyanhydrides that may be suitable for orthopedic application. This class of polymers is characterized by chemistry-dependent surface erosion,[13] a characteristic that permits maintenance of critical mechanical properties during the process of degradation.[14] Payload release, moderate pH

microenvironments, and superior protein stabilization capabilities[15] are others advantages of polyanhydrides in biomedical applications. Notably, polyanhydrides previously have been approved by the United States Food and Drug Administration for clinical use, with demonstrated degradation, metabolism, and excretion in humans.[16] Initial studies suggested that poly (sebacic anhydride) (PSA) exhibits a faster-than-desired degradation rate.[17] On the other hand, polyanhydrides of aromatic acids exhibit more appealing mechanical properties and stability,[18] along with longer release[19] and degradation times[20,21] when compared with aliphatic polyanhydrides.

In this study, PSA and poly(1,6-bis-*p*-carboxyphenoxyhexane) (CPH) were selected as components of a copolymer. As reported in this article, polyanhydride copolymers were fabricated with different molar ratios of CPH and PSA. Tablets of the resulting copolymer, formed by mold casting, exhibited high mechanical strength, slow degradation, and excellent biocompatibility, suggesting that poly(1,6-bis-*p*-carboxyphenoxyhexane-*co*-sebacic anhydride) (CPH:SA) copolymers could be used as bone substitutes in place of current metal or polymer substitutes.

MATERIALS AND METHODS

Materials

1,6-Dibromohexane was purchased from Acros. Sebacic acid and acetic anhydride were obtained from Sigma. *p*-Hydroxybenzoic acid was supplied by Lancaster. Sodium hydroxide was from J.T Baker. The live and dead cell double-staining kit was obtained from Fluka. All reagents were analytical grade.

Synthesis of 1,6-Bis(*p*-carboxyphenoxy)hexane and the Prepolymer

p-Hydroxybenzoic acid (13.8 g) and sodium hydroxide (8.0 g) were dissolved in 40 mL of DI water, and 1,6-dibromohexane (7.69 mL) was added dropwise into the mixture. The resulting solution was refluxed for 3.5 h. Sodium hydroxide (10 mL at 5*M*) was added, and the mixture was refluxed for another 2 h. After cooling, the precipitate was washed with methanol, and then dissolved in 300 mL of DI water. The resulting solution was heated to 60°C and then acidified to pH 2 by the addition of sulfuric acid. The resulting precipitate was filtered, washed with acetone, and dried under vacuum at room temperature.

CPH or sebacic acid (5 g) was refluxed with acetic anhydride (100 mL) at 150°C under nitrogen protection for 20 min. The unreacted acetic anhydride was removed under vacuum at 60°C. The crude prepolymer was recrystallized from dichloromethane and then washed with a 1 : 1 (v/v) mixture of anhydrous ethyl ether : petroleum ether. Prepolymer was dried under vacuum and stored at −20°C before use.

Synthesis of CPH:SA Copolymers

CPH and SA were combined at molar ratios of 7 : 3, 5 : 5, and 3 : 7 and polymerized by melt condensation at 180°C under vacuum for 90 min. The crude product was dissolved in dichloromethane and then purified by precipitation in ice-cold ethyl ether. The precipitate was separated by filtration and washed with ethyl ether. The copolymer was dried under vacuum at room temperature for 24 h. A generalized procedure for synthesis of these copolymers is presented in Figure 1. For subsequent descriptions, CPH:SA synthesized at molar ratios of 3 : 7, 5 : 5, and 7 : 3 are abbreviated as H3S7, H5S5, and H7S3, respectively.

Molecular Structure and Molecular Weight

^{1}H-NMR spectroscopy was performed on a 500-MHz NMR spectrometer (Varian Unityinova 500 NMR) at room temperature with $CDCl_3$ to study molecular structure. Molecular weight and molecular weight distributions were determined by gel permeation chromatography (GPC; Jasco RI-2031, PU-2080) and ^{1}H-NMR, using ratios of δ = 2.40 ppm (—CH_2—CH_2COO—) and δ = 6.94 ppm (—C=CH—C) for the calculation of molecular weight. FTIR spectra were measured using Fourier transfer infrared (Perkin-Elmer system 2000) with KBr pellets.

Thermal Properties and Crystalline Phase Analysis

Thermal properties of the copolymers (melting point, T_m, and enthalpy changes, ΔH) and degree of crystallinity were measured using a differential scanning calorimeter (DSC; Perkin Elmer, Diamond DSC). A temperature ramp of 10°C/min with a modulation of ±1°C/min was used. The materials' crystal structures were measured by multipurpose X-ray diffraction (Rigaku, Ultima IV) within 2θ = 5–50° at the scan speed 0.5°C/min. Thermogravimetric analysis was carried out on a thermogravimetric analyzer (TA instrument, SDT Q600) at a heating rate of 5°C/min from room temperature to 400°C under nitrogen gas flow.

Preparation and Morphological Characterization of Copolymer Tablets

For each tablet (diameter 8 mm, height 4 mm), the total weight of copolymer sample placed in the mold was 300 mg. The copolymer in the mold was heated to 150°C in a high-temperature furnace, and maintained at this temperature for 120 min. Any air bubbles in the polymers were removed by applying a pressure spring on the mold. To study surface morphology, the dried tablets were coated with gold before imaging by scanning electron microscopy (SEM; Hitachi S-5000).

In Vitro Degradation of Copolymer Tablet

Each of the three kinds of copolymer tablet was placed in a separate release bottle. Ten milliliters of phosphate-buffered saline (PBS, pH 7.4) was added to each bottle, and the bottles were placed in a shaking bath (37°C, 100 rpm) for up to 15 weeks.

The degradation rate was assessed as weight loss. Specifically, sample tablets were retrieved at predetermined time points (0, 1, 2, 4, 6, 9, 12, and 15 weeks), dried under vacuum at room temperature, and weighed. (To ensure that drying was complete, the cycle of drying and weighing was repeated until the tablets achieved a constant weight.) The residual weight percent was calculated as $(W_d/W_o) \times 100\%$, where W_d was residual weight at the predetermined time and W_o was the original weight of the dried copolymer tablet.

Degradation also was assessed by testing the pH of the fluid (i.e., the PBS in which the tablet was being shaken). Specifically, the pH of the fluid was measured (at each of the above time points) by using a pH meter (Shindengen).

Mechanical Testing

To measure mechanical properties of the copolymers, sample tablets were retrieved at the predetermined time points (0, 1, 2, 4, 6, 9, 12, and 15 weeks) and dried under vacuum at room temperature. Specifically, compressive strength and elastic modulus were measured for the copolymer tablets. The tablets were mounted on

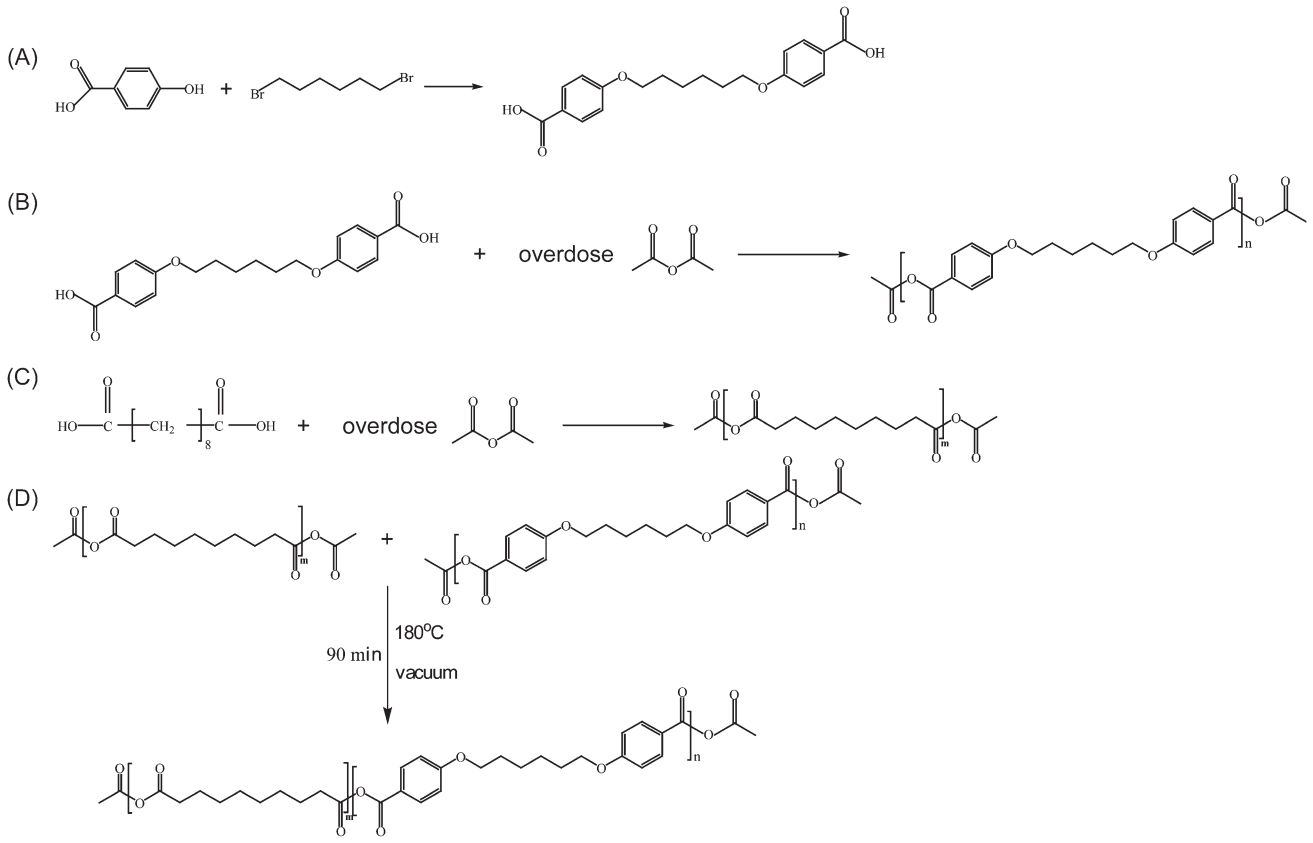

Figure 1. Synthesis scheme of (A) CPH, (B) prepolymer of CPH, (C) prepolymer of SA, and (D) CPH:SA copolymers.

a universal testing machine (Shimazu AGS-2000 G) and subjected to axial loading at a compression speed of 0.5 mm/min. The load versus displacement data were recorded.

Toxicity Testing

C2C12 is a mouse myoblast cell line; because these cells are capable of differentiation into myoblasts and osteoblasts, cytotoxicity in this cell line is functionally relevant for potential bone substitutes. Cells were cultured, using standard methods, in medium consisting of Dulbecco's modified eagle's medium supplemented with 10% fetal bovine serum. C2C12 cells were seeded at 5×10^5 cells/well in 24-well plates. After cell adhesion for 1 day, testing samples were added to the wells. Cultures then were incubated for 1 week, during which the medium was changed every 2 days. At the end of the week, cell viability was test by addition of MTT solution at 2.5 mg/mL. Cytotoxicity of the three kinds of copolymer tablets was evaluated by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method. MTT, a yellow tetrazole, is reduced to a purple formazan by the mitochondria of living cells; the number of living cells is quantified by measuring optical density at 570 nm.

Cell viability also was assessed by fluorescence, using the live and dead test in the same culture as MTT assay. The cell culture medium was withdrawn, and then the cells were washed twice with sterile PBS. Stock solutions of the green (calcein-AM) and red (ethidium homodimer) fluorescent dyes were diluted 1 : 500 and 1 : 1000 into sterile PBS, and cells were stained (30 min in the dark) with 1 mL/well of the diluted fluorescent dye. The dye solution was removed before imaging by inverted fluorescence microscopy (Zeiss Axiovert 200). Cell viability was quantified by counting the live (green-stained) and dead (red-stained) cells in a given field.

Cell Adhesion

C2C12 (5×10^5 cells/well) were seeded above each tablet, and the cells and tablet were cocultured for 1 week. The medium was changed every 2 days, as above. After 1 week, the cell-seeded copolymer tablets were washed twice with PBS and then immersed in 2.0% glutaraldehyde solution at 4°C overnight. The samples were washed three times with DI water, and then dehydrated by immersion (10 min/cycle) in a series (50, 70, 95, and 100%) of solutions of increasing ethanol concentrations. Finally, the samples were dried under vacuum for 2 h. The dried samples then were coated with gold–palladium sputtering and examined with SEM as above.

RESULTS AND DISCUSSION

Characterization of Copolymers

The molecular structures of copolymers were characterized by ^{1}H-NMR. For 1,6-bis(*p*-carboxyphenoxy) hexane anhydride, the peaks at 7.96 and 6.94 ppm correspond to the aromatic proton of anhydride bonds, and peaks at 4.10 and 1.92 ppm represent the methene protons of the CPH segments. The sharp peaks at 1.3, 1.6, and 2.4 ppm are attributed to sebacic acid (Figure 2). The molecular weights, as measured by GPC and ^{1}H-NMR, are shown in Table I. The polydispersion of the three copolymers was less than 1.50, which indicated that the molecular weight distribution was very uniform at all three molar ratios. In FTIR

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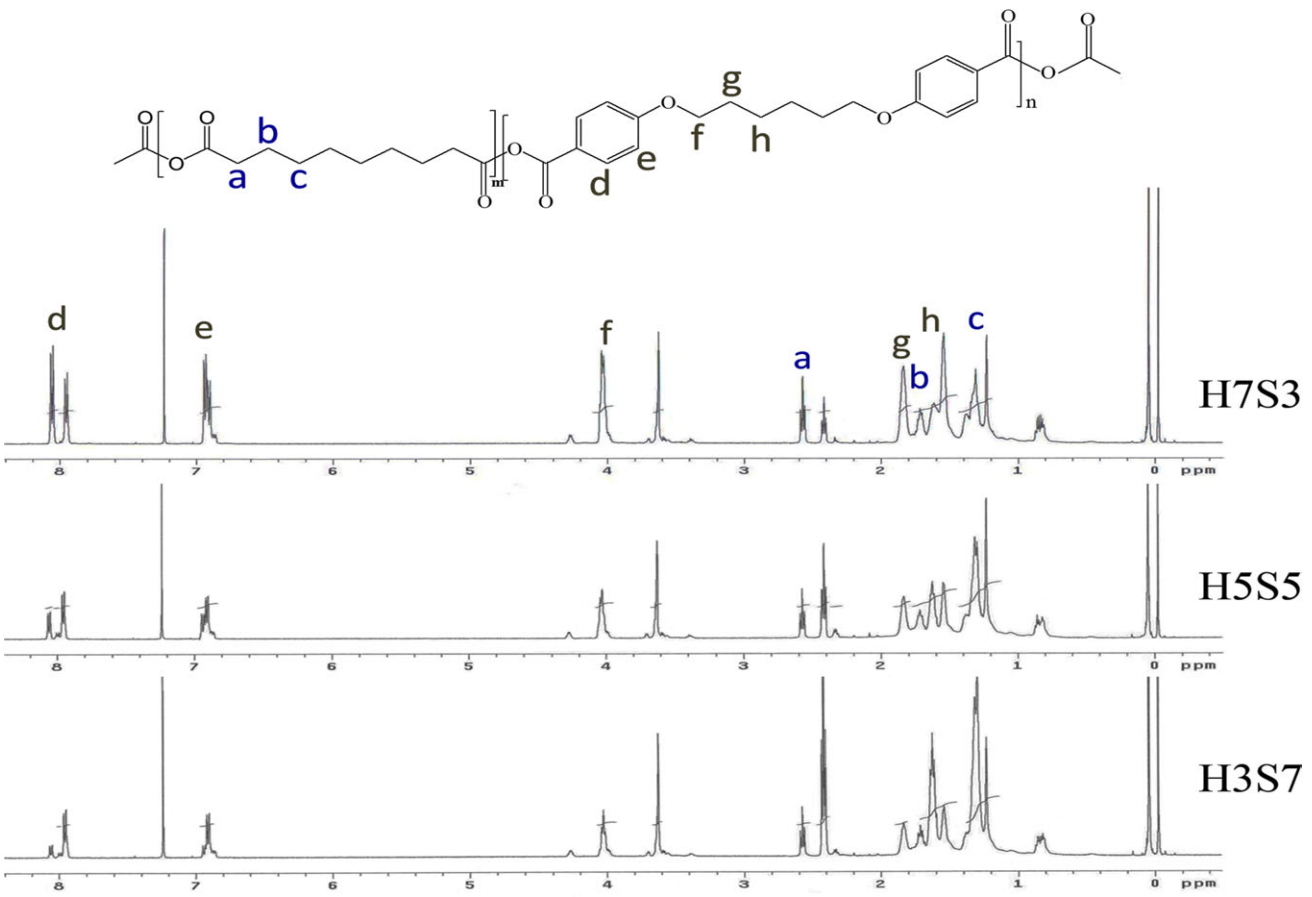

Figure 2. [1]H-NMR spectrum of CPH:SA copolymers of H3S7, H5S5, and H7S3. Peaks a–c represent SA and peaks d–h represent CPH. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

spectra, the peak of aliphatic–aromatic anhydride bond was at 1806 cm^{-1} (Figure 3).

Thermal Properties and Crystallinity

Table II shows the series of DSC thermograms obtained for each copolymer. The CPH:SA copolymer family of polyanhydrides melted at temperatures ranging from 56 to 103°C, which was lower than that reported for CPP:SA.[22] The lower melting point of CPH:SA is expected to be advantageous because this property reduces denaturation of incorporated drug/protein molecules in the processing of heat molding. Figure 4 shows the X-ray diffraction patterns of the copolymers. The integral of the peak at $2\theta = 22°$ increased in proportion to the ratio of CPH in the copolymer.[22]

Table I. Molecular Weight of Polymers

	M_n[a]	M_w[a]	PDI[b]	M_n[c]
H3S7	5,517	6,235	1.13	6,974
H5S5	8,562	9,291	1.09	9,880
H7S3	10,912	15,794	1.45	13,527

[a]Determined by GPC measurement in THF.
[b]Polydispersion (PDI, M_w/M_n).
[c]Calculated by [1]H-NMR.

The degree of crystallinity, measured by DSC, was calculated by the following equation.[23]

$$X_{DSC} = \frac{\Delta H_{copolymer}}{W_{CPH}\Delta H_{CPH,pure} + W_{SA}\Delta H_{SA,pure}}$$

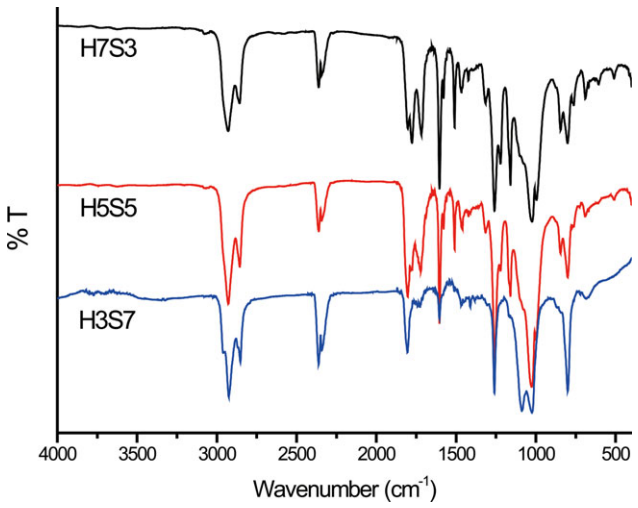

Figure 3. FTIR spectra of polyanhydride copolymers H3S7, H5S5, and H7S3.

Table II. Thermodynamical Properties of Copolymers

	T_m (°C)	ΔH (J/g)	T_d (°C)	X_{DSC} (%)
H3S7	65.23	44.06	295.10	30.42
H5S5	56.82	35.84	284.63	33.81
H7S3	102.09	23.32	334.08	31.50

Here, $\Delta H_{copolymer}$ is the heat of fusion of the copolymer and is obtained from DSC of the copolymer. W_{SA} and W_{CPH} are the mass fractions of the monomers in each copolymer and the value of 221.3 J/g was used for $\Delta H_{SA,\ pure,}$ 35.6 J/g was used for $\Delta H_{CPH,\ pure}$.[22] The X_{DSC} agreed with that obtained previously. The pyrolysis temperature is defined, as the weight loss is up to 10%. H7S3 had the highest thermogravimetric temperature; thus, of the three copolymers, H7S3 would be the most stable in the process of heat molding.

Characterization of Copolymer Tablets

To observe the morphological properties of the tablets, we took a series of photos with digital camera and examined the tablets by SEM (Figure 5). After degradation for 15 weeks, all three kinds of the copolymer tablets had smooth homogeneous surfaces without significant gaps. The findings confirmed that the degradation of polyanhydrides is a process of surface erosion.

The degradation profiles of the copolymer tablets were determined *in vitro*. Hydrolytic degradation of the copolymer tablets was carried out in PBS at 37°C. The weight losses of the three kinds of tablets were measured for 15 weeks [Figure 6(A)]. The degradation of the polyanhydride copolymers occurred mainly via hydrolytic processes. H5S5 and H3S7 exhibited similar degradation rates, showing ~10% weight loss over the 15-week study period. Compared with H5S5 and H3S7, copolymer H7S3 tablets showed a slower degradation rate, retaining 95% residual

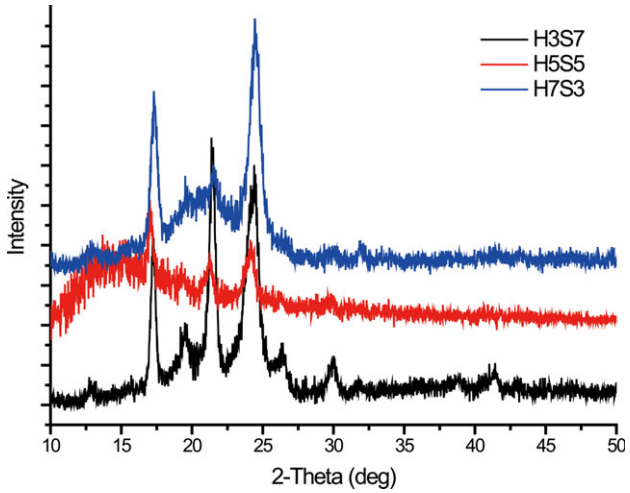

Figure 4. The X-ray diffraction of polyanhydride copolymers. The integral of the peak at 2θ = 22° represents CPH protons. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

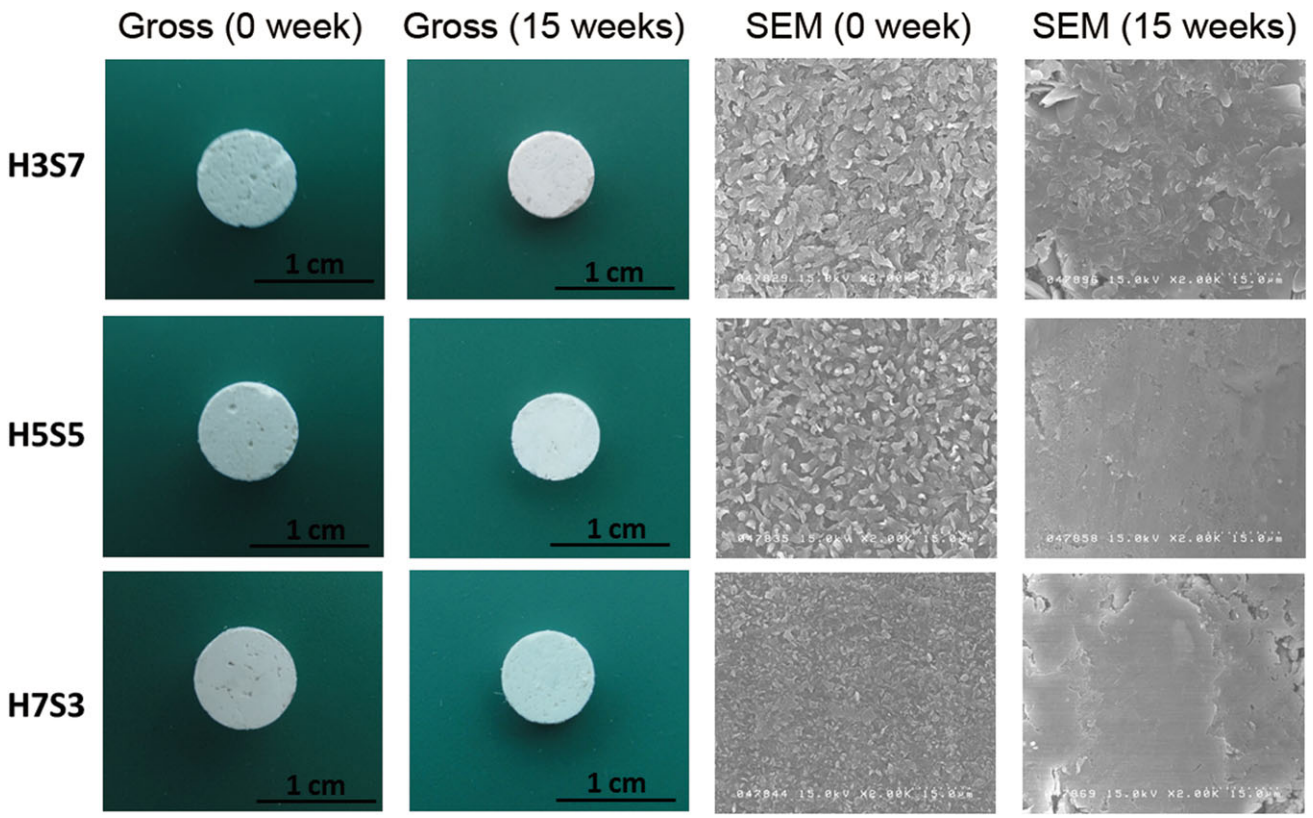

Figure 5. After degradation test in PBS for 15 weeks, the tablets of H3S7, H5S5, and H7S3 remain intact. The surfaces become smooth as shown in SEM. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

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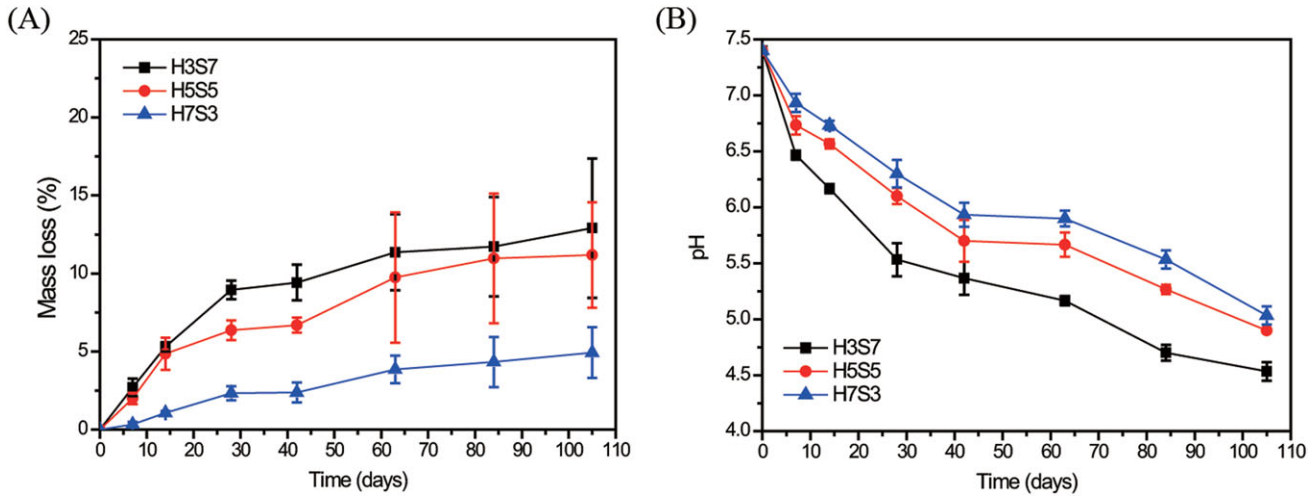

Figure 6. (A) The weight loss of tablets (in PBS) during a 15-week period. The H7S3 tablets degraded slowest and had 95% residual weight. (B) The pH profiles of tablets during a 15-week period. The H7S3 tablets showed a less decline in pH, compared with H3S7. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

weight during the study period. In addition, the degradation of H7S3 in the weight loss-time chart was linear regression. The value of R^2 was 0.94, and the process was a zero-order reaction.

Acidification of the surrounding environment due to degradation of biomaterials may cause harmful effects to the local tissues. We monitored the accumulated pH values of the tablets in PBS for 15 weeks. The results are shown in Figure 6(B). All three types exhibited progressive decreases in pH. Of the three, H7S3 showed the smallest decline in pH, with final pH remaining above 5. H3S7 exhibited the largest decline in pH, with final pH approaching 4.5. These differences may reflect difference in the degradation rate, because more polymer hydrolysis would generate more of the acid byproducts. The pH value of PLGA may lower to pH 3.[24] Compared with PLGA, which generates more acidic by-products, the degradation of anhydrides is less acidic and so is expected to be less harmful to the surrounding tissue.

Mechanical Properties

Figure 7 shows the ultimate compressive strengths of the copolymer tablets. These values increased in proportion to the fraction of CPH. The initial average compressive strength of H7S3 was 105 MPa, a value more than twice that previously reported for PLLA.[25] The mean compressive strengths of H7S3, H5S5, and H3S7 after the 15-week degradation study were 84.23 ± 1.33, 40.87 ± 2.17, and 20.22 ± 2.02 MPa, respectively. These losses of strength corresponded to decreases of 18.33%, 33.79%, and more than 50%, respectively. The relative magnitude of these decreases corresponded to the observed differences in the degradation rate (as indicated by weight loss). Presumably, the faster degradation of the H3S7 copolymer makes the structure

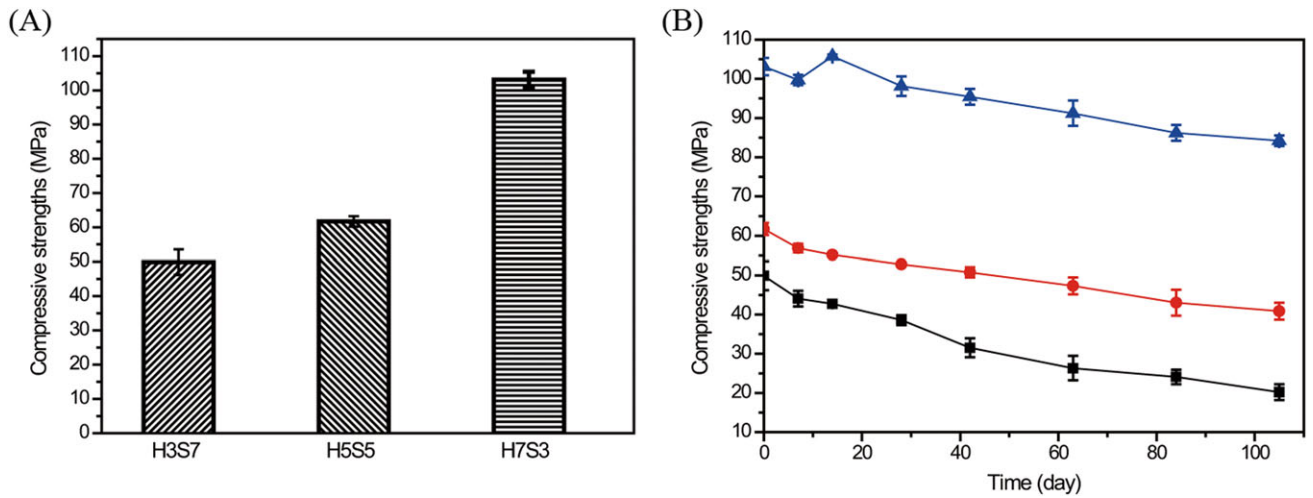

Figure 7. The compressive strengths of copolymer tablets. (A) The initial compressive strength (B) during the degradation process in a 15-week period. The compressive strength of H7S3, H5S5, and H3S7 decreased 18.3, 33.8, and 59.5%, respectively. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

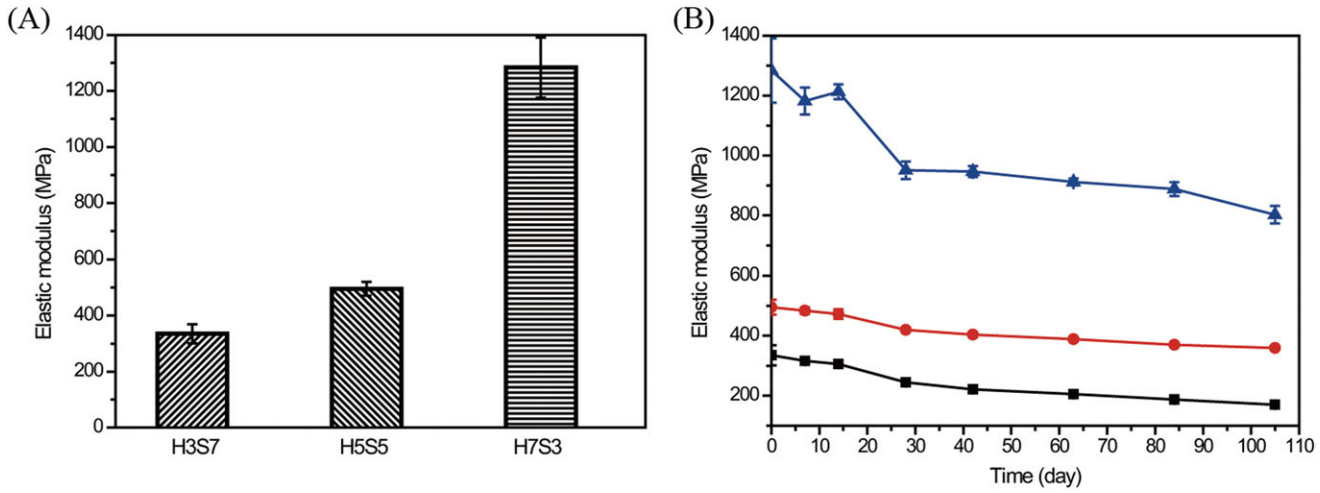

Figure 8. The elastic modulus of copolymer tablets. (A) The initial compressive strength (B) during the degradation process in a 15-week period. With an increasing amount of CPH, the compressive elastic modulus became larger. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

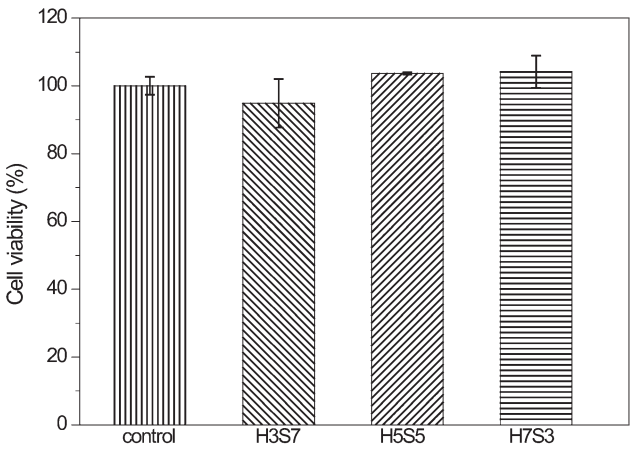

Figure 9. Cytotoxicity test (MTT assay) of different copolymer tablets. All the cell viability was greater than 95%.

of the tablet less stable and thus compromises the tablet's original mechanical strength.

Figure 8 shows a plot of the elastic moduli of the copolymer tablets. The value of the elastic modulus increased with the increased fraction of CPH content. When the ratio of CPH:SA was 7 : 3, the initial compressive elastic modulus reached a maximum of 1283 MPa. Comparison with previously published values for PLA [25] revealed that H7S3 exhibits a compressive strength two times that of PLA (43 MPa); the elastic modulus of H7S3 was slightly larger than that of PLA (1000 MPa). The mean compressive strengths of H7S3, H5S5, and H3S7 after 15-week degradation were 802.67 ± 29.10, 359.43 ± 5.83, and 170.25 ± 3.43 MPa, respectively. The elastic moduli of the three kinds of tablets did not decrease much during the 15-week study period. Overall, the polyanhydrides were able to maintain better mechanical properties than polyesters, such as PLA and PLGA. During degradation process, the mechanical properties of PLLA decreased 80% after 15 weeks.[26] However, H7S3 decreased less than 20%. The degradation of polyesters is known to occur via bulk erosion, whereas the degradation of polyanhydrides is known to occur via surface erosion.[27] This distinction would be a key factor for implanted biomedical materials, especially for bone tissue engineering.

Cytotoxicity of Tablets

Cytotoxicity of polyanhydride copolymer tablets was studied using mouse myoblast C2C12 via the MTT assay and live/dead staining. All observed cell viability data from the MTT test were greater than 95% (Figure 9). The results clearly demonstrated

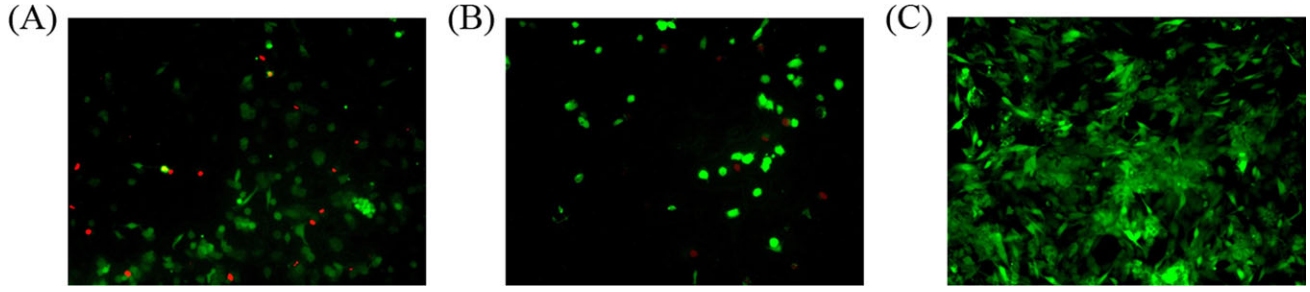

Figure 10. The live and dead cell viability assays: (A) H3S7, (B) H5S5, and (C) H7S3. The amount of green fluorescence was obviously higher in H7S3 copolymer tablet. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

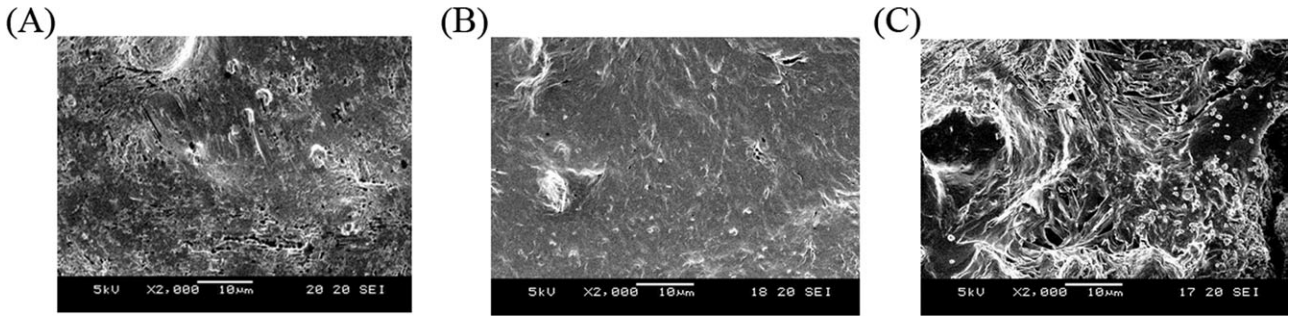

Figure 11. SEM micrographs of C2C12 cells on copolymer tablets of (A) H3S7, (B) H5S5, and (C) H7S3 after 7 days of seeding. The cells spread well on the copolymer tablets.

that the materials were of low cytotoxicity. Live/dead staining detected the toxic effect of copolymer tablets on the neighboring cells *in vivo*. Figure 10 shows that the amount of green fluorescence was obviously higher in wells harboring H7S3 copolymer tablets, although each well had been seeded with the same number of cells. This observation suggests that with more CPH in the copolymers, the cell survival rate increases. Increasing CPH content could decrease the degradation rate of CPH:SA copolymer. The value of pH would not decline abruptly due to the slow degradation rate. H7S3 tablets showed a slower degradation rate, retaining 95% residual weight during the degradation process. Because of the smallest decline in pH value, H7S3 provided more suitable environment to cells. Based on these results, H7S3 copolymer tablets would be the most appealing for biomedical applications.

The Property of Cell Adhesion

SEM micrographs revealed that cells spread well on copolymer tablets containing different mole ratios of CPH and SA (Figure 11). Direct contact of cellular protrusions (filopodia) with the tablets suggested biocompatibility of the copolymers. The photos demonstrate that the polyanhydride material had good biological adhesion properties. Cells could grow and proliferate on the surface. This property is appealing for the potential use of copolymers in biomedical applications.

CONCLUSIONS

In this study, three kinds of polyanhydride copolymers were prepared by melt condensation. All tablets had slow degradation rates, with weight losses ranging between 4.93 and 12.91% in the course of the 15-week study. A slight decline in pH was observed during the study period. This property is expected to reduce the damages caused by the acidification of surrounding tissues as seen in many polyester biomaterials. Maintaining a stable mechanical strength during degradation is another advantage in biomedical applications. With a higher portion of CPH, as with the H7S3 tablets in this study, only an 18.3% decrease in compressive strength and a 27.5% decrease in the elastic modulus were observed.

Biocompatibility of the polyanhydride tablets was confirmed by MTT assay and live and dead staining. The cell survival rate was more than 95%. Cultures grown in the presence of H7S3 tablets exhibited more green fluorescence than those grown in the presence of H5S5 and H3S7, suggesting that H7S3 provides a better environment for C2C12 cell growth. SEM micrographs revealed good cell spread over the surface of the copolymers. These results show that polyanhydride CPH:SA copolymers could find potential application as biodegradable implants. Notably, copolymers with higher molar ratios of CPH exhibited decreased degradation rate, and increased biocompatibility and mechanical strength.

ACKNOWLEDGMENTS

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