Original Article

Polyanhydride copolymer and bioceramic composites as bone substitutes

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ABSTRACT

Background/Purpose: Durable mechanical strength and biocompatibility are the two major requirements for osteogenic scaffolds. Polyanhydrides are a class of biodegradable polymers characterized by anhydride bonds that connect repeating units of the polymer backbone chain. Hydroxyapatite (HAP) is the main component of human bone and is a good osteoinductive factor that promotes bone mineralization. This work validates the combination of polyanhydrides and HAP for biomedical application.
Methods: Polyanhydride copolymers were fabricated from sebacic acid (SA) and 1,6-bis(p-carboxyphenoxy)hexane (CPH). HAP was surface-modified by polycaprolactone (PCL), and testing tablets were made using different ratios of copolymers and surface-grafted HAP (g-HAP). Degradation tests were performed to evaluate mechanical strength, pH, and weight loss. Biocompatibility was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and live/dead stain test. Cell affinity was measured using scanning electronic microscopy (SEM).
Results: The favorable surface erosion property of polyanhydrides prevented marked changes in the mechanical properties over time. In addition, the degradation byproducts of the copolymer did not cause a serious decline in pH and were less harmful to the cells. g-HAP increased cell affinity for the polymer surface.
Conclusion: The research team synthesized polyanhydride/g-HAP composites with high mechanical strength, slow degradation, and excellent biocompatibility. The result showed that a CPH/SA ratio of 7:3 in combination with 10 wt% g-HAP was optimal as bone substitute.
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1. Introduction

In current clinical applications, bone screws for internal fixation are mostly made of metal, such as titanium or 316L.[1–3] Removing metal implants after bone union requires a second surgery, which causes further injury to the patient. In order to overcome this shortcoming of metal bone implants, degradable polymer bone implants have been studied.[4,5] However, the use of polymer bone implants has been limited due to their inferior mechanical strength, rapid degradation, and local tissue acidity.[6,7]

Polyanhydrides are a class of biodegradable polymers characterized by anhydride bonds that connect repeating units of the polymer backbone chain. Polyanhydrides, which degrade slowly through the process of surface erosion,[8] can last between 1 month and 3 years. They exhibit better mechanical strength, payload release, moderate pH microenvironments,[9] and superior protein stabilization capabilities.[10] The US Food and Drug Administration has approved polyanhydrides for clinical use,[11] but the hydrophobic characteristics of polyanhydride copolymers have limited their use in biomedical applications.

Composite materials made of polymers and ceramics have been used as scaffolds for osteogenesis. Some authors have advocated the use of polylactic acid/hydroxyapatite (HAP)[12] and poly(lactide-co-glycolide) (PLGA)/tricalcium phosphate (TCP)[13] as bone substitutes. Polymers act as drug carriers and scaffolds, whereas ceramics provide improved cell affinity.[14] However, defects and cracks can form at the polymer/ceramic junction, resulting in uneven distribution of stress and subsequent inferior mechanical strength. Research into surface modification of ceramics[15] has shown that polymer-grafted ceramics can improve the incorporation of ceramic and polyanhydride.[16]

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In this research project, we fabricated and validated a novel polyanhydride/ceramic composite and performed *in vitro* analysis of chemical properties, mechanical strength, biocompatibility, and degradation time. Our results show the composite material exhibits high mechanical strength, good biocompatibility, and a slow rate of degradation with the potential to serve as a bone substitute.

2. Materials and methods

2.1. Materials

Sebacic acid (SA), acetic anhydride, hydroxyapatite (HAP) nanopowder, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma (Shanghai, China). Sodium hydroxide was purchased from J.T. Baker (Chu-Bei, Taiwan), 1,6-dibromohexane was purchased from Acros (New Jersey, US), and p-hydroxybenzoic acid was supplied by Lancaster (Ward Hill, MA). The live/dead cell double-staining kit and ε-caprolactone was obtained from Fluka (Shanghai, China). All other reagents used in this work were of analytical grade and were used as received.

2.2. Synthesis and characterization of polyanhydride copolymers

The synthesis of 1,6-bis(p-carboxyphenoxy)hexane (CPH) was carried out using a substitution reaction with 1,6-dibromohexane as reported previously.[17] Prepolymers of CPH and sebacic acid (SA) were synthesized as described in the literature.[18] The synthesized prepolymers were then copolymerized at different molar ratios by melt condensation and were characterized as described earlier. CPH—SA copolymer with molar ratio of 7:3 exhibits the best mechanical properties,[18] therefore, we used copolymer in this molar ratio throughout the study.

2.3. Grafting of polycaprolactone onto the surface of HAP nanopowders

A suspension of HAP nanopowder (2 g) in dry toluene (50 mL) was heated by a reflux system at 130 °C under nitrogen. Caprolactone monomer (5 g) and stannous octoate (0.045 g) were added subsequently and allowed to react for 6 hours. Then the reaction mixture was cooled to room temperature. The polycaprolactone (PCL)-grafted HAP nanopowders (g-HAP) were precipitated by centrifugation at 2000 rpm and washed with an excessive amount of chloroform three times to completely remove the free caprolactone oligomers. Finally, the separated precipitate was dried for 24 hours in a vacuum oven at room temperature to remove the residual chloroform.[19] The surface-modified reaction of HAP nanopowders is shown in Fig. 1.

2.4. Preparation and characterization of composite tablets

For each tablet (8 mm diameter, 4 mm height), different weight ratios of g-HAP (5 wt%, 10 wt%, and 20 wt%) were added to the copolymers and 300 mg of the mixture was placed into a mold. The mixture in the mold was heated for 120 minutes at 150 °C in a high-temperature furnace. Air bubbles in the mixture were removed by applying a pressure spring on the mold. The composite tablets were

Fig. 1. The scheme of surface modification of hydroxyapatite (HAP) nanopowder.

given the following designations: H7A3-05 (5 wt% g-HAP), H7A3-10 (10 wt% g-HAP), and H7A3-20 (20 wt% g-HAP).

The melting point (Tm), enthalpy changes (ΔH), and degree of crystallinity of the composites were measured using a differential scanning calorimeter (Diamond DSC, Perkin Elmer). Thermogravimetric analysis was carried out on a thermogravimetric analyzer (SDT Q600, TA Instruments) at a heating rate of 5 °C/minute from room temperature to 400 °C under nitrogen gas flow.

To study the degradation rate of composite implants, the composite tablets of three different weight ratios were placed in separate release bottles. Phosphate-buffered saline (PBS, 9 mL, pH 7.4) was added to each bottle, and the bottles were placed in a shaking bath (37 °C, 100 rpm). Composite tablets were retrieved at 0, 1, 2, 4, 6, 9, 12, or 15 weeks, dried under vacuum at room temperature, and weighed. The residual weight percent was calculated as $(Wd/Wo) \times 100\%$, where Wd represents the residual weight at the predetermined time and Wo is the original weight of the dried copolymer tablet. Acidity was assessed by measuring the accumulated pH of the supernatant at the predetermined time points using a pH meter (Shindengen).

To measure the mechanical properties of the composites, sample tablets were retrieved at each time point (0, 1, 2, 4, 6, 9, 12, and 15 weeks) and dried under vacuum at room temperature. To measure compressive strength and elastic modulus, composite tablets were mounted on a universal testing machine (AGS-2000G, Shimazu) and subjected to an axial loading at a compression speed of 0.5 mm/minute. The load versus displacement data was recorded.

2.5. In vitro cytotoxicity studies

The *in vitro* cytotoxicity and degradation products of the g-HAP composite tablets of different weight ratios were evaluated using the MTT assay and live/dead stain. C2C12 mouse cells were cultured in a 24-well plate (5×10^3 cells/well) using Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. After allowing 1 day for cell adhesion, testing samples were added to the wells. The cells were incubated for 1 week at 37 °C in a 5% CO$_2$ incubator, and the medium was changed every 2 days. The control group was cultured without the addition of testing materials. After incubation, 100 μL of freshly prepared MTT reagent (2.5 mg/mL) was added to each well. The plate was shaken gently and incubated again for another 2 hours. Following incubation, the supernatant was discarded, and 200 μL of isopropyl alcohol was added and swirled gently. The absorbance was measured using a microplate reader at 570 nm.
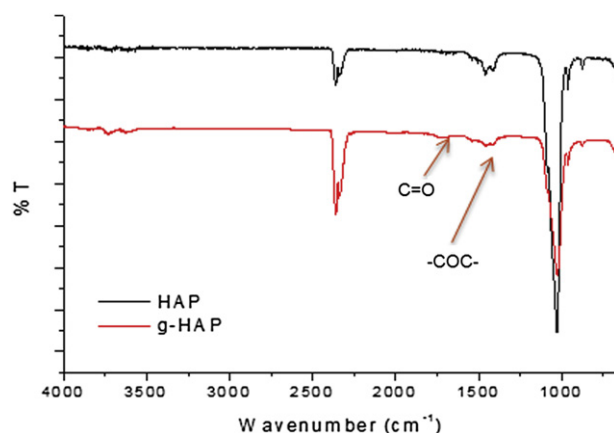

Fig. 2. Fourier transform infrared spectroscopy (FTIR) of hydroxyapatite (HAP) and polycarbonate-grafted hydroxyapatite (g-HAP). Compared with HAP, g-HAP has absorption peaks at 1575 cm^{-1} and 1730 cm^{-1}.

Table 1
Thermodynamic properties of composite materials.

Copolymer	T_m (°C)	ΔH (J/g)	T_d (°C)	X_{DSC} (%)
H7A3-05	115.09	19.52	270.66	26.36
H7A3-10	100.12	18.60	308.48	25.12
H7A3-20	108.81	14.98	340.41	20.23

Cell viability was also assessed by fluorescence, using the live/dead stain. Stock solutions of the green (calcein-AM) and red (ethidium homodimer) fluorescent dyes were diluted 1:500 and 1:1000, respectively, using sterile PBS. The cells were stained with the diluted fluorescent dye (1 mL/well) for 30 minutes in the dark. The dye solution was removed, after which the live cells (green-stained) and dead cells (red-stained) were observed using inverted fluorescence microscopy (Axiovert 200, Zeiss).

2.6. The cell adhesion profiles

C2C12 (5×10^3 cells/well) were seeded above each tablet, and the cells and tablet were co-cultured for 5 days. The medium was changed every 2 days, as above. After 5 days, the cell-seeded composite tablets were washed twice with PBS and then immersed overnight in 2.0% glutaraldehyde solution at 4 °C. The samples were washed three times with deionized water, and then dehydrated by immersion (10 minutes/cycle) in a series of solutions of increasing ethanol concentrations (50%, 70%, 95%, 100%). Finally, the samples were dried under vacuum overnight. The dried samples were then coated with platinum–palladium sputtering and examined with scanning electronic microscopy (SEM; Hitachi S-5000).

3. Results

The Fourier transform infrared spectroscopy (FTIR) spectra of HAP nanopowders before and after grafting are shown in Fig. 2. After surface grafting, a new adsorption band appeared at 1730 cm^{-1} (C=O) belonging to the carbonyl group of PCL on the surface of g-HAP. A new band at 1575 cm^{-1} originated from the ester bone –COC– vibration. The results indicated that PCL was grafted successfully on the HAP surface.

Table 1 shows the thermodynamic properties of the composites. The melting points of composites ranged from 100 °C to 115 °C, which were slightly higher than the melting point for polyanhydride copolymer without g-HAP.[20] The degree of crystallinity, measured by differential scanning calorimetry, declined with the addition of g-HAP. The pyrolysis temperature Td was defined, as the weight loss was up to 10%.

The degradation profiles of the composite tablets were determined from *in vitro* testing in PBS at 37 °C. Weight loss for the three kinds of composites was measured after 15 weeks (Fig. 3A). The degradation of the composites occurred mainly via hydrolytic processes. H7A3-05 and H7A3-10 exhibited similar degradation rates. The H7A3-20 composite, with a weight loss of 6.42 ± 0.61 % after 15-week degradation, showed a faster degradation rate compared with the H7A3-05 and H7A3-10 composites, which showed weight losses of 5.71 ± 0.52 % and 5.88 ± 0.32 %, respectively. The accumulated pH values of the composites in PBS were measured after 15 weeks. The results are shown in Fig. 3B. The pH of all three composites decreased over time. The pH values for the H7A3-05, H7A3-10, and H7A3-20 composite supernatants after 15-week degradation were 4.77 ± 0.07, 4.87 ± 0.08, and 5.00 ± 0.04, respectively.

The compressive strengths of the composite tablets are shown in Fig. 4. The initial average compressive strength of the composite tablets was about 105 MPa. The average compressive strengths of H7A3-05, H7A3-10, and H7A3-20 tablets after the 15-week degradation study were 80.73 ± 3.21 MPa, 73.22 ± 2.17 MPa, and 62.32 ± 2.76 MPa, respectively. Fig. 5 shows the plot of the elastic moduli of the composite tablets. The initial elastic modulus reached a maximum of 1358 MPa. The mean elastic moduli of H7A3-05, H7A3-10, and H7A3-20 after the 15-week degradation were 812.32 ± 9.88, 785.43 ± 18.22, and 712.32 ± 21.44 MPa, respectively. The elastic moduli and compressive strengths had the same tendency. The faster degradation of the H7A3-20 resulted in an unstable structure and decreased mechanical strength and elastic modulus.

The MTT test showed the cell viabilities of the three composites were higher than 97% (Fig. 6A). H7A3-05 and H7A3-10 had better cell viability compared with both the control group and H7A3-20. In the live/dead test (Fig. 6B), more green spots (living cells) were visible for the three composites than for the control group. There were no obvious detectable red spots (dead cells) among the four groups. The results indicated good biocompatibility of the composite material.

Scanning electron micrographs revealed that cells spread well on composite tables containing g-HAP of any weight ratio. Cellular protrusions (filopodia) with higher biocompatibility of the composite tablets were apparent in H7A3-10 (Fig. 7). By contrast, fewer cells were visible on the tablet surface of H7A3-05 and H7A3-20 and the cells were more spherical in shape.

4. Discussion

Compared with PLGA or PCL, polyanhydrides have slower degradation rates and produce fewer acidic byproducts.[21] Polyanhydrides maintain better mechanical properties than polyesters
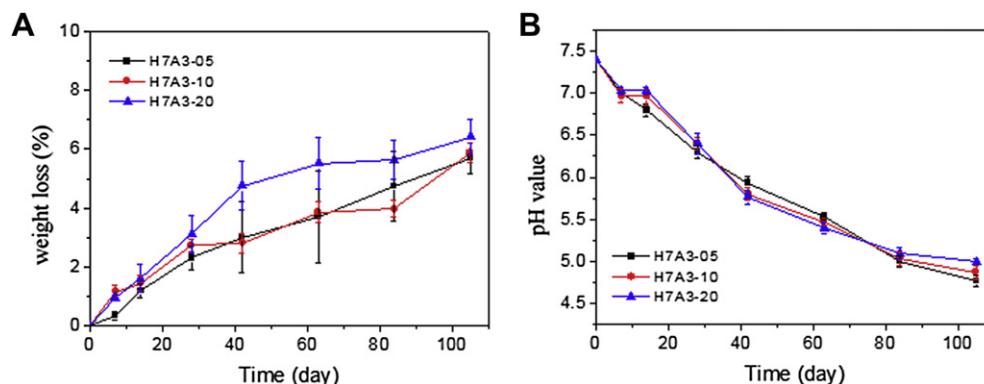

Fig. 3. (A) The weight loss of tablets [in phosphate-buffered saline (PBS)] during a 15-week period. The H7A3-05 and H7A3-10 tablets degraded slower than H7A3-20. (B) The pH profiles of tablets during a 15-week period. The accumulated pH values of the three tablets remained above 4.5.
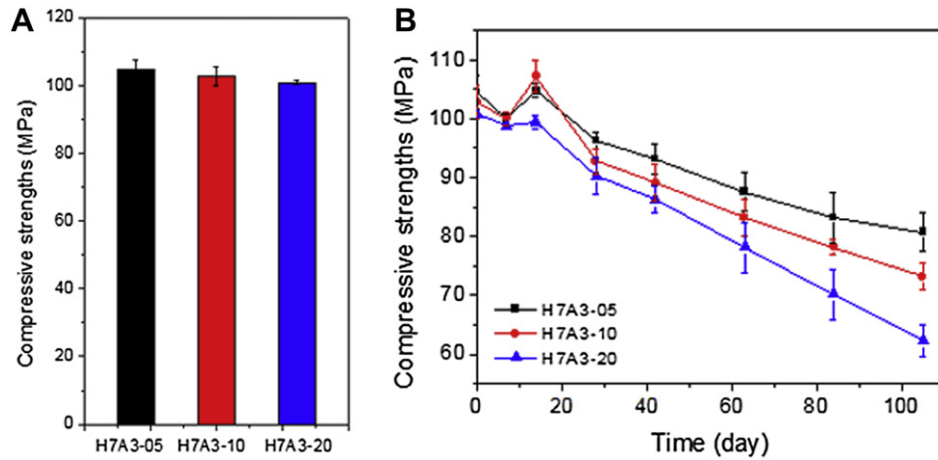
Fig. 4. The compressive strengths of the composite tablets. (A) The initial compressive strength; (B) the compressive strength during the degradation process in a 15-week period. The compressive strength of H7A3-05, H7A3-10, and H7A3-20 decreased 22.8%, 28.8%, and 38.2%, respectively.

during the degradation process. Our composite tablets exhibited a compressive strength two times greater than that of PLA and an elastic modulus slightly greater than that of PLA.[22]

Because the molding temperatures of the three composites were between the melting point and pyrolysis temperatures, the tables could remain stable in the process of heat molding. Mixing g-HAP into polyanhydride copolymers affected the thermal properties. The pyrolysis temperature increased as the g-HAP composition of the copolymers increased. However, g-HAP interfered with the arrangement of the polymer fibers and resulted in reduced crystallinity.

The degradation study showed that the mechanical properties decreased in proportion to the addition of g-HAP. An excessive amount of g-HAP lead to inferior compressive strength and elastic modulus. The incorporation of more than 10% g HAP had a significant influence on the degradation rate. It was more difficult to maintain the mechanical strength during the process of degradation.
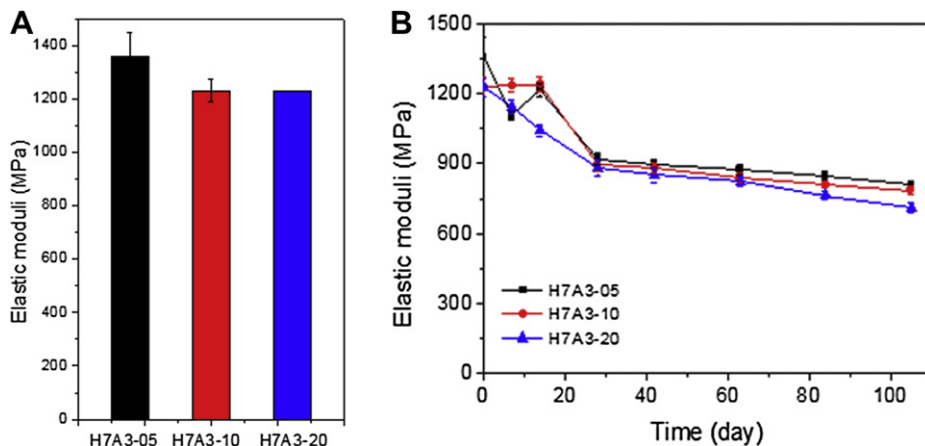

Fig. 5. The elastic moduli of the composite tablets. (A) The initial elastic moduli; (B) the elastic moduli during the degradation process in a 15-week period. The elastic moduli of H7A3-05, H7A3-10, and H7A3-20 decreased 35.6%, 36.2%, and 42.0%, respectively.
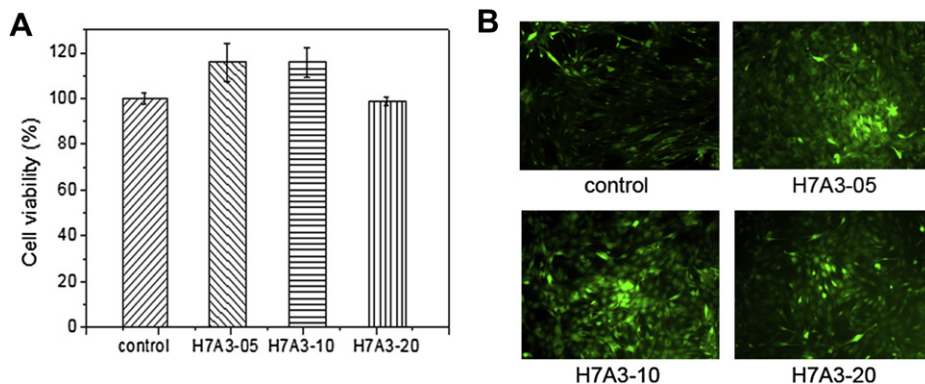

Fig. 6. (A) Cytotoxicity test [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay] of the composite tablets. The cell viability was greater than 97% in each case. (B) The live/dead cell assays. The amount and distribution of green spots indicate that the cell survival rates of the composites tablets are all better than the control group.
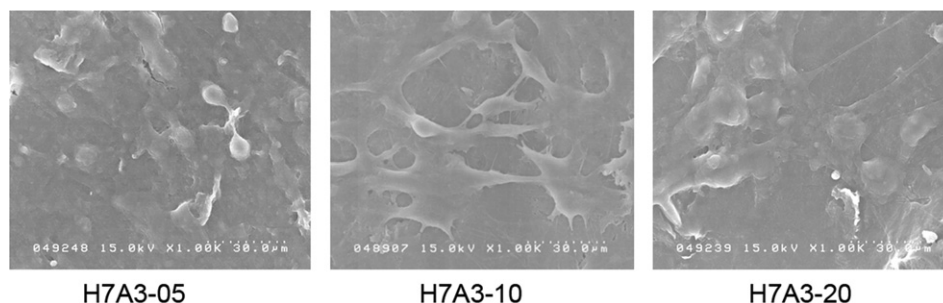
Fig. 7. Scanning electron microscopy (SEM) micrographs of C2C12 cells on the composite tablets. The cells spread and attach on the composite tablets. H7A3-10 exhibits better cell affinity after 7 days of seeding.

One of the key concerns with biodegradable biomaterials is their influence on the surrounding pH value. Acidic environments are harmful to the local tissues. Hydrolysis of polyanhydride copolymer generates fewer acidic byproducts than PLA or PLGA.[23] In addition, g-HAP can release alkaline ions in the degradation process, neutralizing some acidic byproducts. The final accumulated pH value remained above 4.5 in the present study.

The MTT assay revealed that cell survival rates did not decrease after the incorporation of g-HAP with polyanhydride copolymers. The live/dead staining assays showed that composite tablets and their degraded byproducts were nontoxic to C2C12 cells. Biocompatibility assessments using the MTT assay and live/dead stain implied that the degradation byproducts of the composite tablets are nontoxic. Cell affinity was confirmed by the identification of direct contact of cellular protrusions (filopodia) with the tablets. The C2C12 cells proliferated well on the surfaces of H7A3-10 and H7A3-20 tablets, exhibiting polygonal shapes. By contrast, there were fewer cells on the H7A3-05 tablet, and the cells were spherical in shape, indicating less cell adhesion. The incorporation of g-HAP allowed cells to attach to the hydrophobic surfaces of copolymers.

In this research, we identified H7A3-10 as the optimal composition for biomedical application. H7A3-10 composite tablets had a slower degradation rate, more stable mechanical strength, and better cell adhesion. These properties are appealing for the potential use of the composite as bone substitutes.

5. Conclusion

In this work, we validated the biocompatibility of polyanhydrides and g-HAP. The results showed that g-HAP can increase cell attachment to the surface of CPH—SA polyanhydride copolymers. When present in an excessive amount, g-HAP can lead to a faster degradation rate and a decrease in mechanical strength. With 10 wt% g-HAP in the copolymers, the composite exhibited a balanced condition. In conclusion, CPH—SA polyanhydride copolymers incorporated with g-HAP are a potential biodegradable bone substitute.

Acknowledgments

References

1. F.B. Christensen, M. Dalstra, F. Sejling, S. Overgaard, C. Bunger. Titanium-alloy enhances bone-pedicle screw fixation: mechanical and histomorphometrical results of titanium-alloy versus stainless steel. Eur Spine J 9 (2000) 97—103.
2. B. Sanden, C. Olerud, C. Johansson, S. Larsson. Improved bone-screw interface with hydroxyapatite coating: an in vivo study of loaded pedicle screws in sheep. Spine 26 (2001) 2673—2678.
3. P. Reynders, K. Reynders, P. Broos. Pediatric and adolescent tibial eminence fractures: arthroscopic cannulated screw fixation. J Trauma 53 (2002) 49—54.
4. B.L. Eppley, L. Morales, R. Wood, J. Pensler, J. Goldstein, R.J. Havlik, M. Habal, et al. Resorbable PLLA-PGA plate and screw fixation in pediatric craniofacial surgery: clinical experience in 1883 patients. Plast Reconstr Surg 114 (2004) 850—857.
5. M. Vert. Degradable and bioresorbable polymers in surgery and in pharmacology: beliefs and facts. J Mater Sci Mater Med 20 (2009) 437—446.
6. S.K. Nandi, S. Roy, P. Mukherjee, B. Kundu, D.K. De, D. Basu. Orthopaedic applications of bone graft and graft substitutes: a review. Indian J Med Res 132 (2010) 15—30.
7. O. Bostman, H. Pihlajamaki. Clinical biocompatibility of biodegradable orthopaedic implants for internal fixation: a review. Biomaterials 21 (2000) 2615—2621.
8. A.K. Burkoth, J. Burdick, K.S. Anseth. Surface and bulk modifications to photocrosslinked polyanhydrides to control degradation behavior. J Biomed Mater Res 51 (2000) 352—359.
9. D.S. Katti, S. Lakshmi, R. Langer, C.T. Laurencin. Toxicity, biodegradation and elimination of polyanhydrides. Adv Drug Deliv Rev 54 (2002) 933—961.
10. C. Manoharan, J. Singh. Evaluation of poly (1, 6-bis-p-carboxyphenoxy) hexane-co-sebacic acid microspheres for controlled basal insulin delivery. Pharm Res (2012). http://dx.doi.org/10.1007/s11095-012-0880-8.
11. H. Miao, Y. Fan, Y. Liu, Y. Liu, J.Y. Hao, X.M. Deng. Biodegradable poly(sebacic anhydride-co-caprolactone) multi-block copolymers: synthesis, characterization, crystallinity and crystalline morphology. Eur Polymer J 43 (2007) 1055—1064.
12. T. Kaito, A. Myoui, K. Takaoka, N. Saito, M. Nishikawa, N. Tamai, H. Ohgushi, et al. Potentiation of the activity of bone morphogenetic protein-2 in bone regeneration by a PLA-PEG/hydroxyapatite composite. Biomaterials 26 (2005) 73—79.
13. O.D. Schneider, F. Weber, T.J. Brunner, S. Loher, M. Ehrbar, P.R. Schmidlin, W.J. Stark. In vivo and in vitro evaluation of flexible, cottonwool-like nanocomposites as bone substitute material for complex defects. Acta Biomater 5 (2009) 1775—1784.
14. S.S. Kim, K.M. Ahn, M.S. Park, J.H. Lee, C.Y. Choi, B.S. Kim. A poly(lactide-co-glycolide)/hydroxyapatite composite scaffold with enhanced osteoconductivity. J Biomed Mater Res A 80 (2007) 206—215.
15. X.F. Lu, X.Q. Lv, Z.J. Sun, Y.F. Zheng. Nanocomposites of poly(L-lactide) and surface-grafted TiO$_2$ nanoparticles: synthesis and characterization. Eur Polymer J 44 (2008) 2476—2481.
16. F.Q. Ma, X.L. Lu, Z.M. Wang, Z.J. Sun, F.F. Zhang, Y.F. Zheng. Nanocomposites of poly(L-lactide) and surface modified magnesia nanoparticles: fabrication, mechanical property and biodegradability. J Phys Chem Solids 72 (2011) 111—116.
17. D.W. Hong, T.H. Liu, I.M. Chu. Encapsulation of curcumin by methoxy poly(ethylene glycol-b-aromatic anhydride) micelles. J Appl Polym 122 (2011) 898—907.
18. P.L. Lai, D.W. Hong, T.H. Liu, Z.T. Lai, M.H. Cheng, L.H. Chen, W.J. Chen, et al. Validity of poly(1,6-bis-(p-carboxyphenoxy hexane)-co-(sebacic anhydride)) copolymer in biomedical application. J Appl Polym (2012). http://dx.doi.org/10.1002/app.38597.
19. S.F. Yan, J.B. Yin, Y. Yang, Z.Z. Dai, J. Ma, X.S. Chen. Surface-grafted silica linked with L-lactic acid oligomer: a novel nanofiller to improve the performance of biodegradable poly(L-lactide). Polymer 48 (2007) 1688—1694.
20. E. Mathiowitz, E. Ron, G. Mathiowitz, C. Amato, R. Langer. Morphological characterization of bioerodible polymers. 1. Crystallinity of polyanhydride copolymers. Macromolecules 23 (1990) 3212—3218.
21. A.A. Weiner, D.M. Shuck, J.R. Bush, V.P. Shastri. In vitro degradation characteristics of photocrosslinked anhydride systems for bone augmentation applications. Biomaterials 28 (2007) 5259—5270.
22. P.L. Lin, H.W. Fang, T. Tseng, W.H. Lee. Effects of hydroxyapatite dosage on mechanical and biological behaviors of polylactic acid composite materials. Materials Letters 61 (2007) 3009—3013.
23. T.G. Park, W.Q. Lu, G. Crotts. Importance of in-vitro experimental conditions on protein release kinetics, stability and polymer degradation in protein encapsulated poly(D, L-lactic acid-co-glycolic acid) microspheres. J Control Release 33 (1995) 211—222.