Bone Healing in the Presence of a Biodegradable PBS-DLA Copolyester and Its Composite Containing Hydroxyapatite

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ABSTRACT: The healing process of the fractured bone in a presence of poly(butylene succinate-butylene dilinoleate) (PBS-DLA) copolymer containing nanosized hydroxyapatite (HAP) particles has been investigated. The PBS-DLA material containing PBS hard segments and DLA soft segments (50:50 wt %) was used to prepare a polymer/ceramic composite with 30 wt % HAP. A new PBS-DLA copolymer showed a high elasticity of 500% and 15 MPa tensile strength. Addition of HAP improved tensile strength up to 25 MPa while high elasticity has been preserved going down only to 300% of elongation at break. A polymer nanocomposite was fabricated into small elastic polymer rods 15 mm long and 1 × 2 mm in cross section and used for tibia bone fixation in rats. Mallory trichrome staining indicated that new biodegradable copolymers and its composite containing HAP have triggered the most advanced bone healing of all tested materials, thus indicating their high potential for bone tissue engineering and repair.

INTRODUCTION

Biodegradable polymeric materials are important class of materials frequently used in biomedical technologies as resorbable sutures for soft tissue repair.1,2 Controlled degradation time and safe, nontoxic degradation products of biodegradable polymers, including poly(dioxanone), poly(glycolic acid), or poly(lactic acid) (PLA), triggered their biodegradable polymers, including poly(dioxanone), poly(glycolic acid), or poly(lactic acid) (PLA), triggered their development toward other biomedical products such as screws, plates, or scaffolds for bone tissue engineering.1–5 Among numerous biodegradable polymers, a biodegradable poly(butylene succinate) (PBS) is an interesting aliphatic polyester with excellent mechanical properties and thermoplastics processability.6,7 It has promising properties for applications as bioabsorbable/biocompatible materials for medical purposes as its properties can be easily tailored by copolymerization with other comonomers.4,8,9 Recently, we used PBS for the synthesis of copolyesters constituting the hard segments in multiblock copolymers, while dimer linoleic acid (DLA) or dimer linoleic diol (DLA-OH) units were used as the soft segment component.8,10 We demonstrate that PBS-based copolymers underwent controlled biodegradation and showed good biocompatibility in vitro.8,11 By varying the PBS to DLA or DLA-OH (hard to soft segment) ratio, poly(butylene succinate-co-butylene dilinoleate) (PBS-DLA) or poly(butylene succinate-dilinoleic succinate) copolymers, covering a wide range of mechanical properties and degradation rates, can be synthesized.10,12 Ceramic materials are commonly used in bone implantation.13,14 Particular interest is focused on bioactive ceramics, including sintered hydroxyapatite (HAP) which bonds to the living bone through the bone-like apatite layer being formed on ceramic surfaces in the biomimetic process of the living body.7 Such approach holds the promise for biodegradable and biocompatible polymer/ceramic composite materials able to mimic the bone healing process.15,16 Recent works on poly(e-caprolactone) (PCL)/HAP materials showed that the organic/inorganic composite is suitable for sustainable bone implants, as it had good interaction with the natural tissue and cells.5,17 In our earlier study, it was demonstrated that segmented copolyester, poly(butylene terephthalate-butylene dilinoleate) (PBT-DLA), mixed with HAP nanoparticles and implanted subperiosteally into rabbits has triggered bone remodeling after 180 days of observation.18 Thus, it is clearly demonstrated that organic/inorganic (polymer/ceramic) materials are needed to mimic the natural bone structure19 and thus promote the bone healing. Therefore, the objective of this work was to screen for the

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first time the bone healing and remodeling process after implantation of new PBS-DLA/HAP composites into tibia bone in rats. PBS-based copolymers, where long chain fatty acid or fatty glycol is used as soft segment components, are relatively new and not well-explored materials, which already demonstrated a great potential as biodegradable drug delivery vehicles or elastomeric scaffolds for soft tissue engineering.

From the series of PBS-DLA copolymers where the ratio of hard to soft segments was varied from 40:60 to 80:20, we selected the PBS-DLA 50:50, as this material showed the highest elasticity at good tensile strength of all materials. Moreover, according to our earlier work on similar copolyesters, where dilinoleic acid sequences were used as the building block, we found out that adding 30% of ceramic materials to the polyester matrix did not significantly altered mechanical properties while triggered very good in vitro cell response. Here, the bone tissue repair after implantation of polyester−HAP composites is discussed based on histological analysis of tibial bones.

## RESULTS AND DISCUSSION

### Material Characterization

Biodegradable polymers and their composites containing HAP—a calcium-phosphate ceramic mimicking natural bone mineral components, are widely tested and already used for bone tissue engineering. This modern approach is much favored than using metals or allografts as they can trigger the risk and are much expensive. Among many biodegradable polymers, including PCL, PLA, and their copolymers, PBS is triggering increasing interest in biomedical applications.

Poly(butylene succinate-butylene dilinoleate) (PBS-DLA) copolymers are relatively new and not well-explored materials, and the bone tissue response after implantation to such biodegradable copolymer and its composite containing HAP (Figure 1) is reported here for the first time.

PBS-DLA copolymers have been recently developed and showed already great potential for drug delivery systems and heart tissue engineering. A broad range of possible applications is arising from combined properties of constituents used for the synthesis of these multiblock copolymers, where glassy or semicrystalline thermoplastic components are combined with soft elastomeric units, thus showing a biphasic morphology of thermoplastic elastomers and properties ranging from soft elastomers to hard solids. We already demonstrated that PBS-based copolymers undergo controlled biodegradation and show good biocompatibility in vitro.

The chemical structure of PBS-DLA copolymer containing 50:50 wt % of hard to soft segments is depicted in Scheme 1.

The material characteristics summarized in Table 1 show thermal and mechanical properties, illustrating its thermoplastic elastomer behavior as revealed by two distinct temperature transitions: low $T_g$ ($-45.3 \degree \text{C}$) and high $T_m$ (88 °C). The molecular material characteristic is similar to commercially available PCL widely used in manufacturing various biomedical applications.

Despite similar molecular mass of PBS-DLA and PCL, the materials show extremely different mechanical characteristics as depicted in Figure 2. The stress−strain curve of PBS-DLA copolymer clearly shows plastic deformation below 50% strain (typical for thermoplastic) and then an elastic (typical for rubbers) region exceeding 500% strain. PCL shows similar plastic deformation with yielding at 25% strain, and then only

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**Scheme 1. Chemical Structure of PBS-DLA Copolymer Containing 50:50 wt % of Hard to Soft Segments; DPh—Degree of Polycondensation of Hard Segments, DPh = 4.0**

**Table 1. Characteristic Properties of PBS-DLA Copolymers**

| material | $M_n$ (g/mol) | $D$ | η (dL/g) | $T_g$ (°C) | $T_m$ (°C) | $T_c$ (°C) |
|----------|---------------|-----|----------|-------------|------------|------------|
| PBS-DLA  | 35 000        | 1.88| 1.08     | -45.3       | 88.0       | 10.9       |
| PCL      | 43 000        | -61.2| 59.0     | 24.9        |            |            |

$M_n$—number average molecular weight, $D$—dispersity of molar masses ($M_w/M_n$), η—intrinsic viscosity, $T_g$—glass transition temperature, $T_m$—melting temperature, and $T_c$—crystallization temperature.
small elongation leading to material fracture at 100% strain and indicating brittle character of this material. Addition of HAP nanoparticles is leading to increase of elastic modulus for the PBS-DLA material (from $E = 98 \pm 4$ to $E = 178 \pm 7$ MPa) while preserving still good elasticity (strain at break reduced from 500% to only 280%). In contrary, addition of HAP nanoparticles to the PCL material resulted in increase of elastic modulus (from $E = 380 \pm 28$ to $E = 890 \pm 90$ MPa) but elongation at break decreased to 70%. An increase of modulus of elasticity for both polymer matrices after introducing HAP nanoparticles is because of the nanoreinforcement.25

Histology of Bone Tissue after Fixation with New Materials. Biodegradable polyesters are important group of materials used in tissue engineering as already indicated by numerous studies, including bone tissue engineering.5 The process of tissue regeneration especially after bone fracture is complex and depends mainly on vascularization and stable fixation.26 Therefore, the PBS-DLA copolymer and its composite containing 30 wt % HAP have been used in this study for fracture fixation for the first time, and the healing process of the bone after the fracture, stabilized with implanted biomaterials (Figure 3), has been analyzed from histological examinations of the explanted tissue.

The histology of bone tissue reconstructed with the PBS-DLA copolymer is shown in Figure 4A–D. For the PBS-DLA copolymer, we found that in the endochondral ossification process,19 the woven bone (primary or temporary) callus was formed with osteocartilaginous trabeculae formation. As can be seen from Figure 4, the fibrocartilaginous callus (violet arrows) with the areas of woven bone (red arrows) around the cartilage tissue (blue arrows). Staining: H–E: A,B; Mallory trichrome: C,D. Original magnification of the objective: A,C—×5; B,D—×10.
ossification, the woven bone (primary or temporary) callus was formed (Figure 4A−D; red arrows) with osteocartilaginous trabeculae (Figure 4C,D; orange arrows). This tissue response is clearly indicating normal healing process.

The inorganic HAP, which is an important component of natural bone tissue, has triggered much stronger stimulation of bone regeneration compared to the neat copolymer after 8 weeks of implantation. The results observed for the PBS-DLA/HAP nanocomposite used in this work are comparable in term of bone regeneration to PCL/HAP27 or poly(lactic-co-glycolic acid)/HAP28 nanocomposites and showed much faster healing compared to the neat polymers. As can be seen from Figure 5, for the PBS-DLA/HAP composite material containing 30 wt % of HAP nanoparticles, the cartilaginous phase was also present, however the process of primary callus formation was more advanced. Only small amount of cartilaginous material was observed in a periosteal region (Figure 5—blue arrows; cancellous bone—red arrows). However, larger amount was visible in deeper parts of the callus, between the places of endochondral ossification and formation of woven bone (Figure 5—blue arrows; woven bone—red arrows). It can be clearly seen that incorporation of HAP into the PBS-DLA copolymer enhances bone remodeling, thus clearly indicating that the presence of calcium and phosphate ions from the composite material strongly stimulates bone regeneration.

PCL/HAP composites are well-explored materials for bone implants and bone tissue engineering and were prepared in current study as the reference material. The examination of tissue response to PCL/HAP composite implant revealed the cartilaginous tissue formation typical for the fracture repair process (Figure 6). The subperiosteal region contained a new formed cortical bone in the form of woven bone (Figure 6A,B,D; red arrows) and also the cartilaginous calitus with deeper located hypertrophic chondrocytes (Figure 6A−D; blue arrows). Just under this, the osteocartilaginous trabeculae (Figure 6A−D; orange arrows) lined with a layer of osteoblasts (Figure 6B,D; dark blue) were visible and deeper a meshwork of trabeculae—bony callus (Figure 6A,B,D; red arrows). The tissue response to the PCL/HAP composite material is comparable to our new PBS-DLA/HAP composite material, however the bone tissue remodeling was somehow slower.

The experimental data obtained for the bone fracture healing process with the use of polymeric and composite (containing HAP nanoparticles) implants were compared to the results of fracture healing without the polymer/composite, only with the use of Kirschner wire stabilization (Figure 7). As can be seen from Figure 7, in the subperiosteal bone surface, two stages are
clearly seen: the cartilage callus with the proliferating phase (Figure 7C; blue asterisk) and hypertrophic chondrocytes (Figure 7A,B,D; blue arrows) and the endochondral ossification that in the cartilage (Figure 7A–D; blue arrows) surrounded by the woven bone (Figure 7A–D; red arrows). Between them, the osteoblast/chondrocyte junction was visible (Figure 7A,C,D; dark blue arrows). The endosteal surface was formed by the compact bone, with the not fully organized bone lamellae (Figure 7A–D; red arrows). The results obtained with Kirschner wire were comparable to bone stabilization with the PCL material (data not shown here). The advantage of using the biodegradable polymer/HAP nanocomposite over the conventional approach used in clinics, which is bone stabilization with Kirschner wire, is clearly demonstrated when the results of in vivo bone healing were compared. Histological observations clearly indicated that the effect of such stabilization is comparable to the bare PCL polymer, and less pronounced bone healing was observed as compared to polymer/ceramic nanocomposites.

Finally, in the experiments no polymer/composite implant nor Kirschner wire stabilization has been used (Figure 8), at

The subperiosteal surface, the cartilage (cartilaginouscallus) tissue was present (Figure 8A–D; blue arrows). Around the tissue, where hypertrophic chondrocytes were located, the osteocartilaginous trabeculae (Figure 8A–D; orange arrows) and woven bone (Figure 8A–D; red arrows) were observed. In the endosteal surface, the new but not fully organized cortical bone was visible (Figure 8A–D; yellow asterisk), and this clearly indicate that mechanical fixation (either with polymer/ceramic implant or Kirschner wire) is important for the healing process of natural bone. It can also be noticed that at the central part (between periosteum and bone near to the endosteme), the granulation tissue (procallus) could be recognized (Figure 8A,B), that included blood vessels (Figure 8B; white asterisks), fibrocartilaginous callus (Figure 8C; violet arrow), and woven bone (Figure 8A–D; red arrows).

## CONCLUSIONS

In this work, we demonstrated a polymer/ceramic slurry preparation and solvent evaporation process for obtaining the PBS-DLA/HAP composite. We showed that 30 wt % of HAP nanoparticles were successfully incorporated into the PBS-DLA matrix forming materials of well-preserved elastomeric properties (elongation at break of 280%), high strength at break of 25 MPa, and an elastic modulus of ~170 MPa, thus providing physically stable, strong, and elastic materials for bone fixation. New PBS-DLA/HAP nanocomposite materials triggered the most advanced bone remodeling of all tested materials indicating that the presence of calcium and phosphate ions from the composite material strongly stimulates bone regeneration. Collectively, our results demonstrate high potential of the developed composite material for bone tissue engineering and repair.

## EXPERIMENTAL SECTION

### Materials Preparation.

The neat PBS-DLA copolymer containing 50:50 wt % of hard/soft segments, synthesized as described in, and PCL (Capa 6430, M$_n$ = 43 000 g/mol, Perstorp) as a reference material were used. Polymer/ceramic composites containing 30 wt % of HAP (purity ≥ 90% as Ca$_3$(PO$_4$)$_2$, KT, Sigma-Aldrich, Germany, Figure 1a,b) were prepared for implantation study. Polymers were dissolved first in dichloromethane (POCh, Poland) to prepare 10 w/v solutions, and then HAP was added and dispersion was sonicated at 0.5 cycle and 50% amplitude for 30 min. Polymer/ceramic composite slurry was placed in a Petri dish, and the solvent was slowly evaporated for 24 h. Samples (0.5 mm thick, 4 mm wide and 20 mm long) (Figure 1c) were EtO sterilized and used for implantation.

### Materials Characterization.

Differential scanning calorimetry (DSC) was performed using the Q100, TA Instruments apparatus to determine thermal properties. The samples were dried in vacuum at 70 °C for 24 h. The DSC process was carried out in a triple cycle, “heating—cooling—heating,” in the temperature range of ~100 to 300 °C. The heating—cooling rate was 10 °C/min. The glass transition temperature (T$_g$) was determined from the second run in order to eliminate the thermal history from the sample as the temperature corresponding to the upper inflection point or maximum of the curve. The melting point (T$_m$) and crystallization temperature (T$_c$) were determined as the values corresponding to the maximum of endothermic curve and the minimum of exothermic curve, respectively. The quasistatic tensile data were collected at room temperature with an Instron 3366 tensile tester equipped with a 500 N load cell, employing a crosshead speed of 100 mm/min. The strain was measured using the clamp displacement, according to DIN 53 455. The starting clamp distance was 25 mm. The obtained results were averaged from six specimens with a cross-section of 0.5 × 4 mm.

### Bone Fixation with Polymer Composite Materials.

The study was performed on 28 male Wistar rats weighing 300–350 g. Anaesthesia was performed by intramuscular injection of ketamine at a dose of 100 mg/kg body weight. The animals were divided into four groups of seven animals each. After anaesthesia, right tibial bones were unveiled, cut through,
and joined together (Figure 3). Right tibial bones were joined with the PBS-DLA, PCL polymers, and their composites containing 30 wt % of HAP. Each material was anchored in seven right tibias in seven rats. Additionally, left tibial bones in eight animals (2 from each group) were used to study the healing process after bonding with Kirschner’s steel wire, which is routinely used for bone anastomosis. Another eight left tibia bones of animals (2 from each group) were cut and left without anastomosis to check the natural bone healing process. The remaining left tibia bones were not surgically exposed, and the wound was closed. The technique of bone anastomosis was based on the use of material rods 15 mm long and 1 × 2 mm in cross-section. The material specimens were placed along the cut bone and attached to each splinter with one loop of unabsorbed Prolen 4.0 seam. Kirschner’s wire used for anastomosis had a diameter of 1 mm and was cut into 15 mm lengths. The wound was closed with skin sutures 5.0. Animals were observed for 8 weeks. During this period, they received standard food and water without restrictions. During the 8 weeks of experiment, the animals showed normal activity. Samples of polymers were retrieved after 8 weeks after euthanasia by intramuscular injection of sodium pentobarbitone at a dose of 200 mg/kg body weight, and tibial bones were collected for examination. Animal studies were performed at the Pomeranian Medical University in Szczecin (P. Prowans, MD, N. Czapla, MD) based on the permission from the Local Ethical Commission 9/2014 from 06.06.2014. Ethical principles and guidelines for scientific experiments on animals were respected throughout this study. The maintenance and handling of the experimental animals followed EU Council Directive 86/609 EEC, and the animals were treated in accordance with the principles of care and use of animals.

**Histology Analysis.** To study the new bone formation around the implant material, tibia of animals were cleaned of paraﬁn-embedded specimens were cut into series slides (3–5 μm). For the morphological analysis, slides were stained with hematoxylin–eosin31 and Mallory trichrome (Bio Optica Milano, Italy).

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The manuscript was written through contributions of all the authors. All the authors have given approval to the final version of the manuscript.

**Notes**

The authors declare no competing financial interest.

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