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Resorbable, amino acid-based poly(ester urea)s crosslinked with osteogenic growth peptide with enhanced mechanical properties and bioactivity

Kimberly Sloan Stakleff [a,*], Fei Lin [b], Laura A. Smith Callahan [b], Mary Beth Wade [a], Andrew Esterle [a], James Miller [b], Matthew Graham [c], Matthew L. Becker [b,d,*]

[a] Calhoun Research Laboratory, Akron General Medical Center, Akron, OH 44307, USA
[b] Department of Polymer Science, The University of Akron, Akron, OH 44325-3909, USA
[c] Akron Polymer Systems, Akron, OH 44305, USA
[d] Center for Biomaterials in Medicine, Austen Bioinnovation Institute, Akron, OH 44308, USA

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ABSTRACT

Materials currently used for the treatment of bone defects include ceramics, polymeric scaffolds and composites, which are often impregnated with recombinant growth factors and other bioactive substances. While these materials have seen instances of success, each has inherent shortcomings including prohibitive expense, poor protein stability, poorly defined growth factor release and less than desirable mechanical properties. We have developed a novel class of amino acid-based poly(ester urea)s (PEU) materials which are biodegradable in vivo and possess mechanical properties superior to conventionally used polyesters (<3.5 GPa) available currently to clinicians and medical providers. We report the use of a short peptide derived from osteogenic growth peptide (OGP) as a covalent crosslinker for the PEU materials. In addition to imparting specific bioactive signaling, our crosslinking studies show that the mechanical properties increase proportionally when 0.5% and 1.0% concentrations of the OGP crosslinker are added. Our results in vitro and in an in vivo subcutaneous rat model show the OGP-based crosslinkers, which are small fragments of growth factors that are normally soluble, exhibit enhanced proliferative activity, accelerated degradation properties and concentration dependent bioactivity when immobilized.
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1. Introduction

Synthetic, degradable polymers have been used in a myriad of ways for regenerative medicine and orthopedic applications. However, they generally lack the mechanical properties necessary for load-bearing surgical interventions [1]. Numerous examples are found in the literature where degradable polymers have been used successfully in orthopedic applications, including poly(L-lactic acid) (PLLA) [2–5], polycaprolactone and poly(propylene fumarate) [6–10]; however, the maximum reported mechanical properties for these polymers hover near 3.0–3.5 GPa (Young's modulus) [11–13]. For comparison, the elastic modulus of cortical bone within the mid-diaphysis of a long bone, along the axis of the bone, is approximately 18 GPa [14,15]. Most of the clinical community has acknowledged that PLLA has insufficient mechanical properties to sustain load-bearing applications. Resorbable biomaterials that possess high moduli are needed for numerous regenerative medicine and orthopedic applications [16,17].

Ideally the mechanical properties of the scaffold must be appropriate to regenerate bone in load-bearing sites [18]. It is unlikely that stand-alone polymers will attain those numbers. Composite and blending approaches have increased the mechanical properties of degradable materials, yet producing engineering polymeric materials with sufficient mechanical properties that retain the ability to degrade fully has remains a challenge. Traditional methods to mechanically reinforce the polymers, including covalent crosslinking, generally limit or prevent the biodegradation. One strategy has been to use naturally occurring amino acids as building blocks for monomer precursors [19,20]. However, conventional poly(α-amino acids), despite their biological origin, possess distinct physical, chemical and biodegradation properties which limit their synthetic utility [21]. However, the poly(ester urea) materials described herein are a significant step in the right direction in that they are both strong, yet degradable.

Significant limitations in bringing new materials to the clinic include the findings that fully synthetic materials lack cell specific receptors and have poorly defined serum adsorption properties, which can vary widely depending on the amount and nature of the adsorbed layer [22]. Recent advances in synthetic polymer chemistry have enabled the synthesis of polymeric materials designed to elicit specific cellular functions and to direct cell–cell

* Corresponding authors. Address: Department of Polymer Science, The University of Akron, Akron, OH 44325-3909, USA (M.L. Becker).
E-mail addresses: Kimberly.Stakleff@akrongeneral.org (K.S. Stakleff), becker@uakron.edu (M.L. Becker).

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interactions [19,23–25]. Biomimetic approaches based on polymers derivatized with adhesive receptor-binding peptides, glycoproteins and tethered growth factors have been reported to enhance interactions at the biological–synthetic interface [26–29]. Potential solutions have included doping with proteins or peptides or decorating the polymer with covalently tethered peptides that mimic the extracellular matrix or growth factors have been employed over the years [30,31]. One of this example is osteogenic growth peptide (OGP). OGP is a naturally occurring 14-mer peptide growth factor found in serum at micromolar concentrations [32,33]. As a soluble peptide, OGP regulates proliferation, differentiation and matrix mineralization in osteoblast lineage cells [33,34]. The active portion of OGP, the OGP(10–14) region, is cleaved from the peptide and binds to the OGP receptor which activates multiple signaling pathways including the MAP kinase, Src and RhoA pathways [35–37]. When administered intravenously to animals, OGP and OGP(10–14) promote increased bone density and stimulate healing, suggesting a potential use in bone-tissue-engineering applications [38]. We, and others, have shown that OGP retains the ability to initiate potent osteoinductive activity when tethered to surfaces and scaffolds [39,40].

Third-generation tissue-engineering materials are designed to stimulate specific cellular responses at the molecular level, mimic the dimensionality of the native tissue, and degrade at the rate in which the tissue is repaired with by-products that are benign and resorbable [41]. Further advances in both synthetic methodology and scaffold fabrication are needed to drive these efforts forward. While bioactive approaches have been demonstrated to aid biochemical signaling and integration into host tissues, they generally reduce the mechanical properties of the material. This report describes our efforts to develop a new class of crosslinked, mechanically robust polymeric material for orthopedic applications. Our methods include enhanced mechanical properties in addition to imparting specific osteogenic signaling motifs. To mechanically reinforce our polymers and stimulate specific biological activity we have incorporated OGP-based crosslinkers. Peptide-crosslinked phenylalanine and leucine-based poly(ester urea) (PEU) homopolymers were synthesized and tethered with 0.5% and 1.0% OGP(10–14). In addition, the semicrystalline nature of poly(ester urea)s afford non-chemical methods in which the mechanical properties, chemical stability and biodegreadation rates can be tailored. This report describes in detail the chemical, mechanical, in vitro and in vivo data which demonstrate enhanced moduli, biocompatibility and resorption of the PEU materials. Furthermore, the data herein highlights the many opportunities that the clinical community will find for these materials in regenerative medicine applications.

2. Materials and methods

2.1. Materials

Unless listed otherwise, all solvents and reagents were purchased from Sigma-Aldrich and used as received. Fluorenylmethyloxycarbonyl (FMOC)-protected amino acids and preloaded Wang-resins were purchased from CEM Corp. Alpha minimum essential medium (α-MEM) and ultraculture media were purchased from Lonza. All other cell culture reagents were purchased from Invitrogen Corp. All reagents were used as received.

2.2. Synthesis of di-p-toluenesulfonic acid salts of bis-L-phenylalanine and bis-L-leucine esters

Di-*p*-toluene sulfonic acid salts of bis-L-phenylalanine and bis-L-leucine esters were prepared using procedures published previously [42], as shown in Fig. 1. Briefly, L-leucine (1.31 g, 10 mmol), 1,6-hexanediol (0.48 g, 4 mmol), *p*-toluenesulfonic acid (1.92 g, 10 mmol) and toluene (20 ml) were mixed in a 250 ml three-neck flask equipped with Dean Stark trap and a magnetic stirrer bar. The system was purged with nitrogen for 30 min after which the reaction mixture was heated at 135 °C under nitrogen for 20 h. The reaction mixture was allowed to cool to ambient temperature and the crude product was isolated by vacuum filtration. The organic residue was recrystallized four times using 25 ml water to yield 2.26 g (82%) of compound 1, the di-*p*-toluenesulfonic acid salt of bis-L-leucine ester, as a white power. The product was characterized with ^{1}H and ^{13}C nuclear magnetic resonance (NMR), and melting point measurements.

Di-*p*-toluenesulfonic acid salt of bis-L-leucine hexane-1,6-diester (monomer 1, 1-LEU-6): mp: 186–188 C; ^{1}H-NMR (300 MHz, DMSO): 0.90 (d, 12H) 1.34 (s, 4H) 1.45–1.80 (m, 8H) 2.29 (s, 6H) 3.99 (t, 2H) 4.15 (d, 4H) 7.13 (d, 4H) 7.49 (d, 4H) 8.31 (s, active H); ^{13}C-NMR (75 MHz, DMSO): 169.91, 145.34, 137.35, 129.10, 125.48, 65.52, 50.62, 27.76, 24.75, 23.79, 23.13, 21.92, 20.79.

Di-*p*-toluenesulfonic acid salt of bis-L-phenylalanine hexane-1,6-diester (monomer 2, 1-PHE-6): mp: 215–217 C; ^{1}H-NMR (300 MHz, DMSO): 0.90–1.15 (m, 4H) 1.38 (s, 4H) 1.25–1.50 (m, 4H) 2.23 (s, 6H) 2.91–3.09 (m, 2H) 3.10–3.21 (m, 2H) 4.01 (t, 4H) 4.30 (t, 2H) 7.11 (d, 4 H) 7.19–7.40 (m, 10H) 7.49 (d, 4H) 8.43 (s, active H); ^{13}C-NMR (75 MHz, DMSO): 169.06, 145.00, 138.12, 134.70, 129.32, 128.55, 128.21, 127.54, 125.53, 65.45, 53.34, 36.20, 27.63, 24.70, 20.82.

2.3. Interfacial polycondensationof 1-LEU-6 and 1-PHE-6

A general scheme for PEU synthesis is given in Fig. 1. Monomer 1-LEU-6 (6.89 g, 10 mmol), sodium carbonate (3.18 g, 30 mmol) and water (150 mL) were mixed in 500 ml three-neck flask equipped with an overhead mechanical stirrer and a thermometer. The mixture was heated with a warm waterbath at 40 °C for 30 min. The waterbath was removed and replaced with an ice-salt bath. When the inside temperature decreased to about 0 °C, pre-prepared triphosgene solution (1.035 g, 3.30 mmol in 30 ml chloroform) was added to the reaction system quickly (5 s) with fast mechanical stirring. The reaction was allowed proceed for 30 min and then additional aliquots of triphosgene (0.108 g, 0.330 mmol, total) were dissolved in chloroform (5 ml) and 1 ml aliquots were added into the reaction system every 10 min. After the addition of the triphosgene, the organic phase was precipitated into hot water, filtered and dried in vacuum to yield a white solid (3.2 g, 74.5% yield). The product was characterized by ^{1}H-NMR, ^{13}C-NMR, Fourier transform infrared (FT-IR) spectroscopy, size exclusion chromatography (SEC), thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC). The molecular weights and thermal properties of the polymers are listed in Table 1. Poly (1-LEU-6): FT-IR (cm^{-1}): 1740 [–C(CO)–O–], 1648, 1542 [–NH–C(O)–NH–], 3283 [–NH–C(O)–NH–]; ^{1}H-NMR (300 MHz, DMSO): 0.91 (d, 12H) 1.20–2.00 (m, 14H) 4.21 (t, 4H) 4.45 (d, 2H) 5.35–5.80 (m, active H); ^{13}C-NMR (75 MHz, DMSO): 174.64, 157.08, 65.00, 51.60, 65.00, 51.00, 42.31, 28.20, 25.29, 24.74, 22.61, 22.12. Poly (1-PHE-6): FT-IR (cm^{-1}): 1736 [–C(CO)–O–], 1649, 1553 [–NH–C(O)–NH–], 3384 [–NH–C(O)–NH–]; ^{1}H-NMR (300 MHz, DMSO): 0.91 (d, 12H) 1.20–2.00 (m, 14H) 4.21 (t, 4H) 4.45 (d, 2H) 5.35–5.80 (m, active H); ^{13}C-NMR (75 MHz, DMSO): 174.64, 157.08, 65.00, 51.60, 65.00, 51.00, 42.31, 28.20, 25.29, 24.74, 22.61, 22.12.

2.4. Peptide crosslinker

A symmetric vinyl functionalized OGP(10–14) was synthesized using the (Aloc)KYGFGGK(Aloc) sequence by solid-phase FMOC

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