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In vivo bone formation by and inflammatory response to resorbable polymer-nanoclay constructs

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Abstract

The development of synthetic bone grafts with requisite mechanical and morphological properties remains a key challenge in orthopaedics. Supercritical carbon dioxide ($scCO_2$)-processed nanocomposites consisting of organically-modified montmorillonite clay dispersed in poly-D-lactide (PDLA) have shown structural and mechanical properties similar to corticocancellous bone. Using quantitative undecalcified histology and micro-computed tomography (μCT), time and material-dependent influences on *in vivo* bone formation, and inflammatory response were characterized. This represents the first *in vivo* evidence of the ability of scCO2-processed PDLA-nanoclay constructs to support osteogenesis, while eliciting an inflammatory response comparable to PDLA-hydroxyapatite materials. Histologic analyses demonstrated that the *in vivo* performance of nanoclay-containing PDLA constructs was similar to pure PDLA constructs, though nanocomposites demonstrated more radiodense bone at all time points (μCT analysis), and higher bone volume at 6 weeks. Taken with previous structural and mechanical studies, these *in vivo* analyses suggest that scCO2-processed, polymer-clay nanocomposites may be suitable structural bone graft materials.

From the Clinical Editor: With advances in science, orthopedic researchers have devoted significant amount of time in developing synthetic bone graft materials. Many of which are indeed currently in clinical use. In their previous studies, the authors described and studied supercritical carbon dioxide (scCO2)-processed nanocomposites consisting of organically modified montmorillonite clay dispersed in poly-D-lactide (PDLA) in in-vitro experiments. Here, in-vivo experiments were performed to investigate if this new material had improved mechanical properties, as well as the induction of inflammatory response. The overall positive findings may mean that this material could be used for future bone graft substitute applications.

Key words: Supercritical CO_2; Bone graft; Nanocomposites; Nanoclay; Bone scaffold

Owing to the disadvantages associated with autologous and allogenic bone grafts (autograft and allograft, respectively), there is a significant interest in developing synthetic substitute materials to enhance *in vivo* bone growth in clinical applications, such as fracture healing, joint arthroplasty and spinal fusion.[1-4] Resorbable polymers, based on polymerized lactic and glycolic acid have a nearly four-decade history of clinical use, predictable degradation kinetics and myriad options of processing.[2,4-13] As such, these polymers have served as a platform for bone graft substitute and hard tissue engineering applications. In order to support *in vivo* bone formation these polymers must possess a three dimensional porous morphology. Average pore diameters in the range of 100-300 μm are necessary to support cellular infiltration and neovascularization, which are both integral components to osteogenesis.[1-3,14]

Methods such as thermal- or pressure-induced phase separation, particulate leaching, supercritical fluid processing and three dimensional printing have been used to create porous constructs from resorbable polymers.[8-10,12,14-22] While these methods are effective in terms of induction of an appropriate porous morphology, poor static mechanical properties of the constructs preclude their use in load bearing applications. To enhance the mechanical performance of these constructs, researchers have attempted to reinforce the polymeric matrices with a variety of micro- and nano-structured filler materials, including hydroxyapatite, phosphate glass and carbon nanotubes.[2,4,6,7,10,14,16,18,21-26] Only modest gains in compressive mechanical properties have

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been observed to date, which are often attributed to poor dispersion of the filler in the matrix, as well as poor interaction between the filler and matrix.[10] Supercritical carbon dioxide ($scCO_2$) processing of organo-modified Montmorillonite nanoclays with poly-D-lactide (PDLA) polymers has shown promise in the rapid production of porous resorbable nanocomposites for structural bone graft substitute applications.[6] In addition to inducing an interconnected porous structure to the polymer matrix, $scCO_2$ processing increases the spacing between silicate platelets allowing for the infiltration and intercalation of polymer chains. With as little as 2.5 wt% of nanoclay, the compressive mechanical properties of the nanocomposite are nearly twice that of pure polymer constructs. Compressive mechanical properties of $scCO_2$-processed resorbable polymer nanocomposites also compare favorably to native corticocancellous bone. Similarly, the interconnected porous structure of the nanocomposite core and the dense outer region of the constructs mimic the morphology of clinically used autologous and allogenic bone graft materials, such as the corticocancellous grafts harvested from the antero-superior region of the iliac crest.[1-3,5,6,14]

Recent *in vitro* experimentation characterizing the response of human osteoblasts to $scCO_2$-processed porous PDLA-clay nanocomposite constructs demonstrated increased alkaline phosphatase and osteoprotegerin expression.[6] Electron microscopy of osteoblasts cultured on PDLA-clay nanocomposites discs displayed cellular infiltration throughout the three dimensional porous structure of the constructs, as well as the deposition of calcium phosphate-rich extracellular matrix. While the structure, mechanical properties and *in vitro* osteoblastic response to $scCO_2$-processed PDLA-clay nanocomposites have been characterized, their ability to support *in vivo* bone formation has not been investigated.

In addition to the ability of bone graft substitute materials to support bone formation, the extent to which these materials elicit an inflammatory response *in vivo* is also an important aspect to characterize. The nanoclay used in the nanocomposites is a Montmorillonite clay that has been modified with alkylammonium salts to increase the basal spacing between the silicate platelets that comprise the nanoclay particles, which enhances dispersion within polymer matrices.[27-29] The biocompatibility of Montmorillonite clay-containing materials have been characterized in their bulk form.[18,25,30-32] To date, however, little has been done to understand the inflammatory response to polymer-clay composite materials in their particulate form. It is hypothesized that throughout the duration of implantation of highly porous, resorbable materials, the combination of polymer degradation and mechanical loading may lead to the generation of particulate debris. Numerous biomaterials in particulate form elicit an inflammatory response, which can ultimately lead to focal osteolysis.[33-40]

The purpose of this manuscript is to characterize the *in vivo* inflammatory response to polymer-nanoclay materials, as well as the ability of $scCO_2$-processed polymer-nanoclay constructs to support bone formation. Given the significant reinforcing effect of nanoclay on PDLA matrices, the goal of the present experimentation was to determine if the filler within the matrix led to any negative biologic effects. A series of *in vivo* experiments were undertaken and comprehensive methods were employed to determine the biologic suitability of these nanocomposites constructs to function as structural bone graft substitutes.

Methods

Preparation of PDLA and PDLA-Nanoclay Debris for Inflammation Study

The inflammatory response to, and osteolytic potential of PDLA-nanoclay particulate debris was investigated, and compared to both pure PDLA, as well as PDLA filled with hydroxyapatite. Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$, Sigma-Aldrich) is a calcium phosphate material which has been traditionally used as a functional filler material in hard tissue engineering constructs and bone graft substitute materials.[4,7,13,16,23,24,26,41] As a secondary objective, two different types of nanoclay (Cloisite 93A and Cloisite 30B, Southern Clay Products, TX) were utilized as fillers for PDLA, to characterize the effect of organic modifier chemistry on the inflammatory response to nanocomposite material. The nanoclays differ in the type of organic modifier used to increase the basal spacing of silicate platelets that make up the nanoclay particles. Cloisite 93A employs a methyl dehydrogenated tallow ammonium, while Cloisite 30B uses methyl-tallow bis-2-hydroxyethyl quaternary ammonium.

Cryo-grinding of supercritical CO_2-processed materials failed to reliably produce particulate of a size conducive to implantation in a murine air pouch. As such, solvent casting was used to create bulk PDLA, PDLA-93A, PDLA-30B and PDLA-hydroxyapatite materials. The solvent casting method consisted of dissolving 2.0 g of PDLA in 50 mL of chloroform under constant agitation at 25 °C. Hydroxyapatite was loaded into the polymer at 45 wt%, while Cloisite 93A and Cloisite 30B were loaded at 2.5 wt%. The disparity in filler content is reflective of the high hydroxyapatite content used by other researchers to achieve adequate increases in mechanical properties, and bioactivity.[2,4] Pure PDLA particulate without filler was created using similar methods to be used as a positive control. Solutions were cast and dried in a sterile laminar flow hood for 24 hours, and lyophilized for 48 hours. Lyophilized materials underwent cryogrinding, followed by ultrasonication in sterile 70% ethanol to remove bound endotoxin. Samples from each particle lot underwent scanning electron microscopy (SEM, JSM 6400, JEOL Ltd.) to analyze particle size and morphology.

In Vivo Inflammatory Response to Nanoclay-Polymer Composite Particulate

An *in vivo* assessment of the inflammatory response of nanocomposites particulate was performed in accordance with a protocol approved by our Institutional Animal Care and Use Committee (IACUC), which assured that all animal procedures were performed in a humane fashion with adherence to local and federal regulations. The murine air pouch model is a common technique utilized to assess the inflammatory response to particulate debris generated by orthopedic devices.[40,42,43] Twenty-five female Balb/C mice underwent serial injections of air (1 mL each) on the dorsum for a period of five days. On day