In vitro and *in vivo* biocompatibility of D-amino acid/ polyurethane polymeric biocomposites for bacterial biofilm prevention in bone defects

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Statement of Purpose: Infectious complications of open bone fractures significantly contribute to patient morbidity and poor healing outcomes. Despite treatment, up to 30% of open wound fractures, progress to a chronic form of infection, which is often associated with high rates nonunion and extremity amputation [1]. The development of microbial biofilms is recognized as a significant factor contributing to chronicity of human infections, including device and non-device related orthopeadic infections [2,3]. The nature of biofilms, including resistance to antibiotics and host-mechanisms of clearance, makes it extremely difficult to treat infections involving this phenotype. Given the limitations of current therapies, the use of agents that can prevent and/or disperse biofilms have gained considerable interest. D isoforms of amino acids (D-AAs) have previously been shown to prevent and disperse biofilms in a broad range of pathogens and reduce contamination *in vivo* when incorporated in polyurethane (PUR) grafts [2]. Given the potential therapeutic application of D-AAs, herein we investigated the biocompatibility of D-AAs and their affect on osteoblast differentiation, *in vitro*, as well as *in vivo* using a large animal sheep femoral condyle plug model. We hypothesized that D-AAs released from PUR biocomposites would not hinder osteoblast differentiation *in vitro* or new bone formation *in vivo*.

Methods: Composites were made from a lysine triisocyanate– poly(ethylene glycol) prepolymer, polyester triol (Mn ~450 g mol^{-1}), triethylene diamine catalyst, 40 wt% Mastergraft® (MG) Mini Granules (0.5-1.6mm), with and without (+/-) 5 total wt% of D-AAs (equal weight D-Methionine: D-Proline: D-Phenylaline). *In vitro* biocompatibility of D-AAs was evaluated using human osteoblast cells (Promocell). Cells were seeded at 5×10^4 cells per well in 24-well tissue culture plates. For osteogenic differentiation cells were cultured in α-MEM supplemented with ascorbate-2 phosphate (50μM), β-glycerolphosphate (10mM), dexamethasone (0.1μM) (Sigma-Aldrich, St. Louis, MO). Two treatment groups were evaluated: collected samples of released D-AAs from prefabricated PUR composites (r-D-AAs) in α-MEM and exogenously prepared D-AA. Both groups were collected/prepared in a-MEM, supplemented with osteogenic inducing agents, and added to cells for up to 7 days (n=4 per group). At days 1, 3, and 7, cell viability was assessed by measuring total DNA. Osteogenic differentiation in the presence of D-AAs was evaluated by measuring ALP activity and the expression of alkaline phosphatase, collagen-I, osteocalcin, and bone-morphogenic protein 2 using the p-nitrophenyl phosphate colorimetric assay and by performing quantitative real-time polymerase chain reaction , respectively. Osteoblast mineralization was evaluated at day 7 by alizarin Red S staining. *In vivo* biocompatibility of D-AAs was evaluated by a 11mm diam X 18mm length unicortical defect created in the sheep femur diaphysis, filled with +D-AA MG/PUR, and allowed to cure. After 16 weeks defects were harvested, and evaluated by x-ray microtomography (mCT) as well as histological analysis with Sanderson's Rabid Bone Stain and Von Gieson Solution.

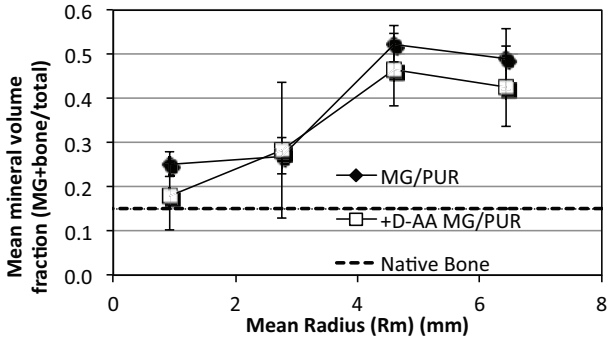

Figure 1. Radial mCT analysis of MG/PUR biocomposites +/- D-AA, 16 wks post implantation. R_m from center of defect and composite/defect interface originally at R_m =5.5 mm).

Results: During days 1-7, exposure of osteoblasts to either r-D-AA or the D-AA mixture did not result in significant decreases in osteoblast viability. Exposure of osteoblasts to r-DAA was not observed to alter differentiation and mineralization, whereas moderate, albeit insignificant, decreases in ALP and gene expression were observed following exposure to the prepared D-AA mixture. The bone growth in defects treated with MG/PUR biocomposites +/- D-AA were compared in order to evaluated the effect of D-AAs on biocompatibility of *in vivo* bone remodeling. After 16 weeks, the sheep defect with +D-AA MG/PUR contained a mean mineral volume fraction (total volume of MG and bone/total volume) that was not significantly different then those filled with MG/PUR alone. As shown in Fig. 1, this holds true for each mean radius (R_m) analyzed via mCT. The presence of bone growth in the biocomposites and defects was confirmed by histological analysis. Qualitative observations paralleled mCT radial analysis that more bone growth was present at outer edge of implanted composite.

Conclusions: Collectively, maintenance of osteoblast activity and differentiation (+/- r-D-AA or D-AA) *in vitro* as well as the presence of bone growth in +D-AA MG/PUR, comparable to MG/PUR, supports the original hypothesis that local delivery of D-AAs is a safe and effective anti-biofilm strategy for orthopaedic defects.

References:

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