The Effects of Hydroxyapatite Coating on Poly(caprolactone) Micromechanics and Mesenchymal Stem Cell Behavior

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Introduction

Robust osseointegration is a critical component in the success of many orthopaedic interventions from fracture healing to spinal fusion. Of particular interest to our group is the osseointegration of our endplate-modified disc-like angle ply structure (eDAPS), a tissue engineered total disc replacement designed for the treatment of end-stage disc degeneration[1]. Hydroxyapatite (HA) coating has long been an established method to improve osseointegration. However, disagreement exists as to whether scaffold macro- or micromechanical properties dictate successful bone tissue regeneration. Some studies report increases in scaffold stiffness following HA coating[2,3]. Other studies report only increases in local stiffness[4,5], indicating that stiffening at the cellular level may primarily drive osteogenesis. In this study, we investigated the effect of two different HA coatings on the mechanical behavior of salt-leached poly(caprolactone) (PCL) porous scaffolds as well as the scaffolds' ability to induce osteogenesis of MSCs *in vitro*, with or without growth factors.

Methods

PCL scaffolds (5 mm in diameter x 1.5 mm in height) were fabricated according to a previously established salt-leaching protocol[6]. Constructs were hydrolyzed in 2M NaOH for 28 hours and immersed in 10x simulated body fluid (SBF) for up to 7 days (0.25, 1, 3, and 7 days) to create materials with varying degrees of hydroxyapatite surface functionalization. The formation of hydroxyapatite crystals was characterized using μCT and SEM imaging (n = 9). To characterize the scaffolds' macromechanical properties, constructs were compressed for 3 cycles up to 3 N (n = 5). To characterize the scaffolds' micromechanical properties, constructs were indented using the Optics11 Piuma system and a 4.33 N/m probe with a radius of 51.5 μm (n = 3).

PCL scaffolds hydrolyzed for 28 hours and subsequently incubated in SBF for 1 or 7 days were selected for *in vitro* experimentation. Prior to cell seeding, PCL only scaffolds and HA-coated PCL scaffolds were hydrated and sterilized through an ethanol gradient and coated overnight in fibronectin. Each construct was seeded with 41,500 P2 bovine MSCs on both the top and bottom surfaces and then divided into groups fed with either basal or osteogenic media. Every other week, cellular metabolism was quantified using an Alamar Blue assay (n = 6). At 5 and 10 weeks, ± scaffolds from each group were removed from culture. Half of the sample was used to quantify alkaline phosphatase (ALP) activity (n = 3-6). Cryosections from the second half of the scaffold were utilized for immunohistochemistry (IHC) using osteocalcin and osteopontin primary antibodies, or Von Kossa and Draq5 staining (n = 3-6). Data was analyzed using parametric or nonparametric One-Way ANOVAs based on normal/non-normal distribution. Significance was defined as $p < 0.05$.

Results

Longer periods of SBF immersion correlated with greater depths of HA crystal infiltration into the scaffolds' interior (Figure 1B) as well as increases in surface crystal size (Figure 1A). Macromechanically, hydrolyzed scaffolds experienced a reduction in linear modulus followed by a significant increase in stiffness after both 1 day and 7 days of HA coating with no differences between coated groups (Figure 1A). Micromechanically, PCL only scaffolds were significantly stiffer than 7 day HA-coated constructs. *In vitro* studies revealed an upregulation in cellular proliferation on scaffolds fed basal media compared to scaffolds fed osteogenic media (Figure 3A), in addition to cell migration further into the depth of the scaffold.

By 10 weeks, 7 day osteogenic HA-coated scaffolds had significantly increased ALP activity compared to both basal HA-coated scaffolds and PCL only scaffolds. Minimal differences in ALP activity existed between 1 day HA-coated scaffold groups (Figure 3B). Von Kossa staining

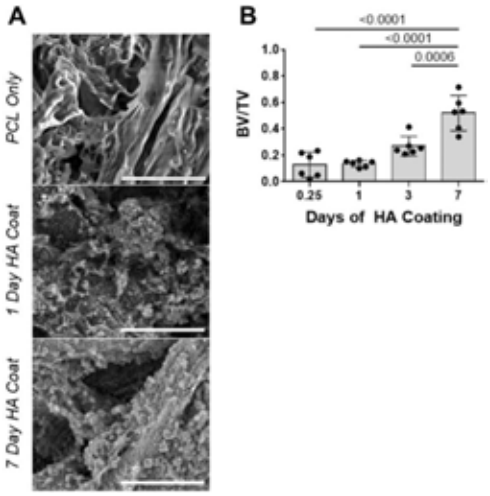

Figure 1 (left and above). (A) Representative SEM images of PCL scaffolds immersed in SBF for 0, 1, and 7 days. **(B)** Scaffolds hydrolyzed for 28 hours and immersed in SBF for 0.25, 1, 3, and 7 days (scale bar = 15 μm).

suggested an increase in calcium deposition homogeneously throughout the osteogenic HA scaffolds and concentrated around the very exterior of osteogenic PCL scaffolds for both coating durations. Von Kossa staining was heterogeneous in basal HA constructs and minimal in basal PCL scaffolds. Osteocalcin IHC in 1 day HA-coated scaffolds revealed an upregulation only in osteogenic constructs, while 7 day HA-coated scaffolds also showed an upregulation of osteocalcin in basal HA scaffolds (Figure 3C).

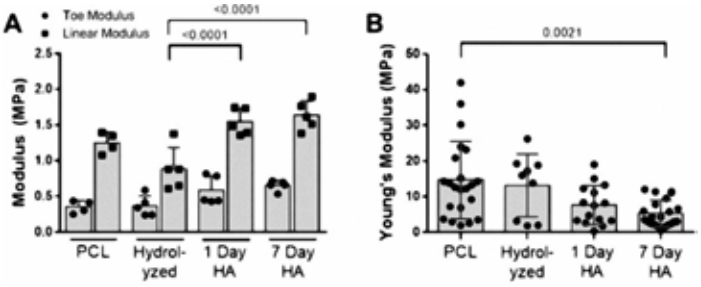

Figure 2 (right). (A) Macromechanics and **(B)** microcmechanics (5-9 indentations of each scaffold, n = 3) of PCL only, hydrolyzed, and HA-coated scaffolds.

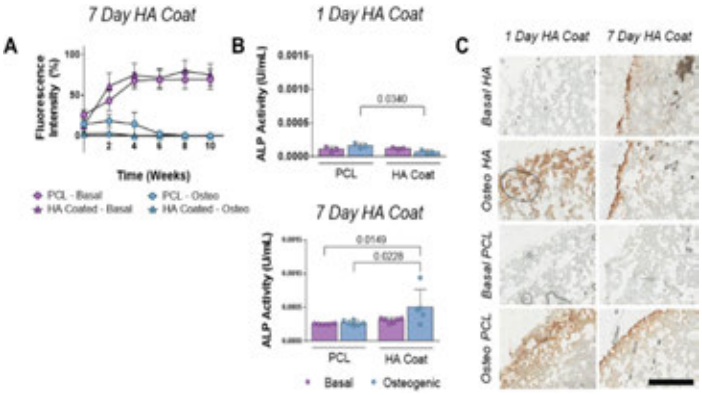

Figure 3 (above and right). For MSC-seeded PCL only and HA-coated PCL scaffolds at 10 weeks: **(A)** Alamar Blue assay, **(B)** ALP activity, and (C) osteocalcin IHC (scale bar = 500 μm).

Discussion

The immersion of scaffolds in SBF for 7 days led to increased osteogenic behavior of MSCs, both without and in concert with osteogenic media. Overall, MSCs on PCL only scaffolds fed basal media exhibited minimal to no osteogenic behavior, whereas PCL only scaffolds fed osteogenic media exhibited an upregulation of osteogenic markers at their external edge. Decreased cellular metabolism in osteogenic media scaffolds suggests a decrease in proliferation that most likely correlates with cell specialization. For HA-coated scaffolds cultured in basal media, increases in osteocalcin and Von Kossa staining were observed primarily in the 7 day coating group. It is unlikely that macroscopic mechanics are driving this upregulation, as no significant differences in compressive modulus between coating groups were observed. Although there was a trend of decreasing micromechanical stiffness as coating time increased, this may be attributed to the heterogeneous surfaces of PCL only and hydrolyzed PCL scaffolds in addition to the limited indentation depth achieved with the 4.33 N/m stiffness probe utilized. The increase in osteogenic behavior of MSCs between the 1 day and 7 day HA-coated scaffolds may be attributed to the mechanobiologic effects of larger, more homogeneously distributed HA crystals. Although osteogenesis was more significantly upregulated in HA-coated scaffolds fed osteogenic media, our data suggests that the coating alone (after 7 days of SBF immersion) can have an osteogenic effect. This supports our group's strategy to implant HA-coated PCL scaffolds, as part of our eDAPS, to drive osseointegration with native bone *in vivo*.

Significance

Understanding how hydroxyapatite influences cellular differentiation is critical to the successful osseointegration of our lab's tissue-engineered disc replacement and can inform approaches for improved osteogenesis for other applications, such as fracture healing and spinal fusion.

References

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