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Superior Calvarial Bone Regeneration using Pentenoate-Functionalized Hyaluronic Acid Hydrogels with Devitalized Tendon Particles

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Abstract

Traumatic brain injury (TBI) is a life-threatening condition defined by internal brain herniation. Severe TBI is commonly treated by a two-stage surgical intervention, where decompressive craniectomy is first conducted to remove a large portion of calvarial bone and allow unimpeded brain swelling. In the second surgery, spaced weeks to months after the first, cranioplasty is performed to restore the cranial bone. Hydrogels with paste-like precursor solutions for surgical placement may potentially revolutionize TBI treatment by permitting a single-stage surgical intervention, capable of being implanted with the initial surgery, remaining pliable during brain swelling, and tuned to regenerate calvarial bone after brain swelling has subsided. The current study evaluated the use of photocrosslinkable pentenoate-functionalized hyaluronic acid (PHA) and non-crosslinking hyaluronic acid (HA) hydrogels encapsulating naturally derived tissue particles of demineralized bone matrix (DBM), devitalized cartilage (DVC), devitalized meniscus (DVM), or devitalized tendon (DVT) for bone regeneration in critical-size rat calvarial defects. All hydrogel precursors exhibited a yield stress for placement and addition of particles increased the average material compressive modulus. The HA-DBM (4–30%), PHA (4%), and PHA-DVT (4–30%) groups had 5 ($p < 0.0001$), 3.1, and 3.2 ($p < 0.05$) times greater regenerated bone volume compared to the sham (untreated defect) group, respectively. *In vitro* cell studies suggested that the PHA-DVT (4–10%) group would have the most desirable performance. Overall, hydrogels

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containing DVT particles outperformed other materials in terms of bone regeneration *in vivo* and calcium deposition *in vitro*. Hydrogels containing DVT will be further evaluated in future rat TBI studies.

Graphical Abstract

Keywords

Bone; Calvarial; Hyaluronic Acid; *In Situ*; Hydrogel; Traumatic Brain Injury

1. Introduction

Traumatic brain injury (TBI) is a life-threatening condition characterized by internal brain swelling, the degree of which can vary greatly. TBI inflicted by a closed head injury or stroke can result in severe brain swelling requiring surgical intervention.[1] The current surgical procedure to treat severe TBI involves a two-stage surgery. In the first surgery, decompressive craniectomy (DC) is performed to remove a large portion of the calvarial bone and allow un-impaired brain swelling.[2] The size of calvarial bone removed during DC is considered a critical size defect as the bone will not naturally heal by itself. After brain swelling has subsided, a second surgery—termed cranioplasty—is performed to close the cranial vault.[3] The average time between DC and cranioplasty has been reported up to 80 days; during this time the brain is left unprotected. Syndrome of the trephined (SoT), also known as sinking skin flap syndrome, is a severe neurological condition associated with mood changes, fatigue, dizziness, motor skill problems, and concentration issues.[4] The occurrence of SoT has been connected to patients following DC for the treatment of TBI.[5] The cause of SoT has been attributed to various factors such as changing intracranial pressure,[6] and physical distortion of the brain from the weight of the scalp.[7] Immediate relief from SoT has been observed directly after cranioplasty,[5] and it is recommended that cranioplasty be performed as soon as clinically possible to mitigate the occurrence of SoT. [7] The current two-stage surgical treatment is non-advantageous as it prolongs patient recovery by requiring two separate surgeries and potentially results in the neurological condition SoT.

Various research groups have reported new approaches to treat TBI. Martin *et al.*[8] previously studied the effect of a single-surgical approach combining DC and autologous bone flap cranioplasty in a pediatric patient population and found high complication rates. A potential issue with combining DC and cranioplasty in a single surgical intervention is that current methods, such as autologous bone flap cranioplasty, do not allow the brain to swell as the constructs are rigid bodies. Currently there is an unmet need for a material that can be implemented in a single-stage surgery to treat TBI, capable of remaining flexible during brain swelling, then transitioning to bone after brain swelling has ceased. Most research into TBI treatment has focused on methods to improve brain tissue healing [9–13] or blocking tissue growth to ease the transition between DC and cranioplasty.[14] Currently available commercial bone void fillers cannot be used for cranioplasty following DC as the treatments are unable to regenerate sufficient bone across the critical size defect and do not remain pliable after implantation. The first step in designing a material for TBI treatment after DC is to identify a pliable material capable of regenerating bone across a critical size defect. Once a pliable material that can sufficiently regenerate bone has been identified, the next logical step would be testing in a TBI animal model.

The objective of the current study was to evaluate the use of a photocrosslinking hyaluronic acid (HA) hydrogel encapsulating devitalized tissue particles for calvarial bone regeneration in a critical size calvarial defect. The use of a photocrosslinking hydrogel for calvarial bone regeneration represents a next-generation biomaterial following our previous work.[15, 16] In the current study, hydrogels encapsulating tissue particles were created by combining pentenoate functionalized hyaluronic acid (PHA) or non-crosslinking hyaluronic acid (HA) with demineralized bone matrix (DBM), devitalized cartilage (DVC), devitalized meniscus (DVM), or devitalized tendon (DVT) particles. PHA hydrogels are an attractive material choice as the crosslinking time is on the range of 1–2 minutes. Compared to other options like methacrylated HA, which has a crosslinking time on the range of 5–10 minutes, a faster crosslinking time is desirable for the addition of cells or for surgical use.[17–21] The use of hydrogels by surgeons is often overlooked during the hydrogel design process. Hydrogel precursor handling properties can be tuned specifically with the surgeon in mind, developing paste-like precursor solutions to aid in material placement to ease clinical translation.[22] A Disadvantage of hydrogels for bone regenerative medicine include low stiffness and thus they cannot be used in high load bearing applications. Further research is needed to improve hydrogel mechanical performance.

The use of different extracellular matrix (ECM) materials for calvarial bone regeneration attempts to apply and recapitulate part of the endochondral ossification process by delivering an intermediate material to accelerate healing.[23] Cartilage, meniscus, and tendon tissues were selected as they are comprised of varying amounts of collagen I or collagen II and may provide additional bioactive signals to promote healing. Crosslinking (i.e., PHA) and non-crosslinking (i.e., HA) tissue hydrogels with a single colloidal loading of 30% were evaluated in a critical size rat calvarial defect model and groups with significant bone regeneration were refined explored *in vitro*. We hypothesized that ECM from sources other than bone would promote comparable or superior bone regeneration in a critical-size calvarial defect.

2. Materials and Methods

Tissue Preparation

Cartilage, meniscus, and tendon tissues were harvested from the knees of 10 castrated male Berkshire hogs (7–8 months of age, 120 kg) purchased from the local abattoir (Bichelmeyer Meats, Kansas City, KS). Tissue devitalization was described in our previous publication. [24] Briefly, harvested tissues were rinsed, strained, and chopped into small pieces. Tissues were coarse ground using a cryogenic tissue grinder (BioSpec Products, Bartlesville, OK) then frozen and lyophilized. Tissues were then cryoground using a freezer-mill (SPEX 6775, SamplePrep, Metuchen, NJ). Devitalized cartilage (DVC), devitalized meniscus (DVM), and devitalized tendon (DVT) particles were stored at -20°C for later use. DBX® was procured and was not an industry gift.

Synthesis of Pentenoate Functionalized Hyaluronic Acid (PHA)

Pentenoate functionalized hyaluronic acid (PHA) was synthesized as previously described. [25, 26] hyaluronic acid (HA, Mw = 1.01–1.8 MDa, Lifecore Biomedical, Chaska, MN) was dissolved in DI water at a concentration of 0.5% (w/v). Once fully dissolved, dimethylformamide (DMF) was slowly added to the solution to achieve a final ratio of 3:2 (water:DMF). The pH of the solution was then adjusted to 9.5 using 1M NaOH. Pentenoic anhydride (Cat# 471801, Sigma-Aldrich, St. Louis, MO) was added in 5 M excess relative to HA and the pH was maintained between 8–9 for approximately 2 hours. Afterwards, NaCl was added to the solution to achieve a final concentration of 0.5 M. The PHA was then precipitated in four volumes of acetone and centrifuged at $7,000 \times g$ to separate the PHA from solution. PHA pellets were then dissolved in DI water and transferred to dialysis packets (MWCO: 6–8 kDa). PHA was dialyzed against DI water for 48 hours, performing DI water exchanges every 12 hours. After dialysis, the PHA solution was frozen and lyophilized. Dry PHA was stored at -20°C for later use.

Hydrogel Preparation

Hydrogels were prepared as previously described.[15] Briefly, HA or PHA and tissue particles were weighed dry and combined. HA groups were resuspended in phosphate buffered saline (PBS, Cat# P3813, Sigma-Aldrich) solution, and PHA groups were resuspended in PBS solution containing 2.3 mM of 2-Hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure 2959, Cat# 410896, Sigma-Aldrich) and 1% dithiothreitol (DTT, Cat# D0632, Sigma-Aldrich). Dry material combinations were sterilized using ethylene oxide gas (AN74i, Anderson Anprolene, Haw River, NC) and the PBS-I2959-DTT solution was sterile-filtered prior to resuspending PHA groups for use *in vivo* or *in vitro*. Hydrogel precursor solutions were allowed to reach ambient conditions for 2 h before implanting. Groups are presented as the material contents followed by weight per volume (in PBS) percentages of each in parentheses (i.e. PHA-DVC (4–30%) = 4% PHA + 30% DVC in PBS).

Rheological Testing of Hydrogel Precursor

Hydrogel precursor yield stress ($n = 3$) was determined using a DHR-2 controlled stress rheometer (TA Instruments, New Castle, DE). Measurements were performed using a 20-mm diameter crosshatched stainless steel plate geometry and a crosshatched Peltier plate cover at 37°C and a gap distance of 500 μm . The Peltier solvent trap was used to ensure the material did not dehydrate during testing. An oscillatory shear stress sweep from 10–5000 Pa was used to measure hydrogel precursor yield stress. Material yield stress was defined as the cross-over point of the storage (G') and loss modulus (G''). [27]

Mechanical Testing of Crosslinked Hydrogel

Crosslinked hydrogel compressive moduli ($n = 5$) were determined using an RSA III dynamic mechanical analyzer (TA Instruments). Crosslinked hydrogels were prepared using a previously published protocol.[22] Briefly, hydrogel precursor solutions were loaded into 2-mm thick Teflon molds sandwiched between glass microscope slides and secured using clamps. Hydrogels were then crosslinked using a 312 nm UV-light at 9 mW/cm² for 2 minutes (EB-160C, Spectrolite, Westbury, NY). Post-crosslinking, circular hydrogel cylinders were cut using a sterile 3 mm punch and swelled in PBS for 24 hours before proceeding with mechanical testing. Swollen hydrogels were measured using a stereomicroscope and micrometer to determine the diameter (3.75 ± 0.1 mm), and hydrogel height (2.75 ± 0.1 mm) was measured using the RSA III. Hydrogels were compressed at a constant rate of 0.005 mm/s until 30% strain. The compressive modulus was calculated from the linear portion slope of the stress-strain curve between 5–15% strain.

Animal Model and Surgical Method

Animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center (protocol #2015-2303). Male Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA) at an age of 7–8 weeks; surgeries were conducted between 8–9 weeks of age. The calvarial bone was exposed by creating an incision on the posterior periphery of the skull to pull back the skin and periosteum. Using a dental trephine, a critical-size 7.5 mm diameter defect was created on the center of the calvarial bone. The circular piece of calvarial bone was carefully removed, leaving the dura mater intact. Approximately 50 μL of hydrogel paste was then syringed into the space and smoothed/shaped to the defect using a sterile spatula. Groups containing PHA hydrogel were then crosslinked using a handheld 312 nm UV-light using a 312 nm UV-light at 9 mW/cm² for 2 minutes (EB-160C, Spectrolite). The skin and periosteum were carefully draped over the defect site and sutured in place. The sham (untreated defect) group received the same surgical procedure without the addition of material, the DBX® group received approximately 50 μL of DBX® Putty, and the uninjured group received no surgical intervention. Post-surgery the rats returned to a normal physical activity state. Calvarial bone was harvested after 8 weeks post-implantation ($n = 5$).

Micro-Computed Tomography (μCT)

Micro-computed Tomography was performed using a Quantum FX imaging system (PerkinElmer, Waltham, MA) with a 50-kV X-ray source at 160 μA . μCT imaging was

conducted on harvested rat calvarial bone after 8 weeks post-implantation (n = 5). Scans were analyzed using Avizo computational software (FEI Company, Hillsboro, OR) to quantify regenerated bone volume. Regenerated bone was confirmed using pre-existing peripheral bone as the threshold. Total bone was determined using uninjured rat calvarial bone for determination of total bone regeneration percentage.

Histology and Immunohistochemistry (IHC)

After fixing in 10% phosphate-buffered formalin for 48 hours and μ CT imaging, explanted cranial bone samples were submerged in 70% ethanol for long-term storage. Embedding, sectioning, and staining of tissue was performed by the Stephenson Cancer Tissue Pathology Core at the University of Oklahoma Health Sciences Center. Briefly, cranial implants with surrounding host bone were embedded in paraffin and sectioned in the sagittal plane following a standard protocol. Paraffin blocks were cut to a thickness of 4 μ m and affixed to glass microscope slides. The SelecTech hematoxylin and eosin (H&E) staining system (Leica Biosystems, Wetzlar, Germany) was performed using a Leica ST5020 multistainer (Leica Biosystems) following the manufacturer's protocol (n = 5).

In Vitro Cell Culture and Experimental Design

Pre-osteoblast mouse calvarial cells (MC3T3-E1, ATCC, Manassas, VA) were cultured in minimum essential medium- α (Cat# 12561072, Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS, Cat# 16000044, Thermo Fisher Scientific) and 1% penicillin/streptomycin (Cat# 15140-122, Thermo Fisher Scientific). Medium was exchanged every other day and cells were cultured to a passage number of 2 before being used in the study. Experimental groups from the 8-week *in vivo* study that significantly outperformed the negative control ($p < 0.05$) were chosen to be refined *in vitro* to determine the minimum tissue particle concentration to achieve a desirable cellular response. Following the results of the *in vivo* study; PHA, PHA-DBM, and PHA-DVT groups were selected to evaluate the tissue particle concentration from 10 to 30%. Approximately 50 μ L of material was injected by syringe into each well of a 96-well plate, centrifuged at $1000 \times g$ for 5 minutes to evenly coat the bottom of the well, then crosslinked using the handheld UV light for 2 minutes. MC3T3-E1 cells were seeded at a concentration of 10^4 cells per well (150 μ L volume) directly on top of the crosslinked material in each well of the 96-well plate (n = 5).

Biochemical Assays

After 2 and 10 days of cell culture, seeded cells were lysed with 200 μ L of lysis buffer (Cat# R1060-1-50, Zymo Research, Irvine, CA) and DNA content was determined using the Quant-iTTM PicoGreenTM assay (Cat# P7589, Thermo Fisher Scientific) according to the manufacturer's protocol (n = 5). Hydrogels without seeded MC3T3 cells served as controls for the study, designated as the tissue culture plastic (TCP) group. Hydrogel constructs were then dissolved in 100 μ L of 1M hydrochloric acid to determine the amount of deposited calcium. Calcium was assayed using the QuantiChromTM calcium assay kit (BioAssay Systems, Hayward, CA) according to the manufacturer's protocol (n = 5).

Statistical Methods

GraphPad Prism (GraphPad Software Inc, La Jolla, CA) software was used to conduct all statistical analyses. A one-way analysis of variance with groups of factors was used for analyzing mechanical testing and μ CT results, and a two-way analysis of variance with groups of factors was used to analyze biochemical assays. Tukey's post-hoc analysis was used to compare between groups, where $p < 0.05$ was considered significant. Yield stress testing had $n = 3$ samples per group. Compressive modulus, μ CT, DNA, and calcium testing had $n = 5$ samples per group; all data are reported as the mean $\pm$ the standard deviation.

3. Results

Rheological Analysis of Hydrogel Precursor

All material formulations tested exhibited a yield stress ($\tau_y > 200$ Pa, Fig. 1A). The addition of ECM particles to PHA significantly increased the material yield stress in all groups except for the PHA-DVT (4–10%) group. PHA-DVC (4–30%), PHA-DVM (4–30%), PHA-DBM (4–30%), PHA-DVT (4–20%), and PHA-DVT (4–30%) had 23.1, 12.8, 14.8, 8.4, and 10.4 times greater yield stresses compared to the control PHA (4%) group (206 Pa) ($p < 0.0001$). PHA-DBM (4–10%) and PHA-DBM (4–20%) had 6.3 and 5.8 times greater yield stresses compared to the PHA (4%) group ($p < 0.05$). The PHA-DVC (4–30%) group (4760 Pa) had the highest yield stress, which was 1.8, 3.7, 4.0, 1.6, 5.3, 2.8, and 2.2 times greater than those of the PHA-DVM (4–30%), PHA-DBM (4–10%), PHA-DBM (4–20%), PHA-DBM (4–30%), PHA-DVT (4–10%), PHA-DVT (4–20%), and PHA-DVT (4–30%) groups, respectively ($p < 0.0001$). The PHA-DVM (4–30%) group (2644 Pa) had a yield stress that was 2.0, 2.2, and 2.9 times greater than those of the PHA-DBM (4–10%), PHA-DBM (4–20%), and PHA-DVT (4–10%) groups, respectively ($p < 0.0001$), and 1.5 times greater than that of the PHA-DVT (4–20%) group ($p < 0.05$). The PHA-DBM (4–30%) group (3060 Pa) had a yield stress that was 2.4, 2.6, and 3.4, times greater than those of the PHA-DBM (4–10%), PHA-DBM (4–20%), and PHA-DVT (4–30%) groups, respectively ($p < 0.0001$), and 1.8 times greater than that of the PHA-DVM (4–20%) group ($p < 0.001$), and 1.4 times greater than that of the PHA-DVT (4–30%) group ($p < 0.05$). The PHA-DVT (4–30%) group (2154 Pa) had a yield stress 1.7, 1.8, and 2.4 times greater than that of the PHA-DBM (4–10%), PHA-DBM (4–20%), and PHA-DVT (4–10%) groups, respectively ($p < 0.05$).

Mechanical Analysis of Crosslinked Hydrogel

The addition of ECM particles increased the average compressive modulus of all hydrogels tested (Fig. 1B). The PHA-DVC (4–30%), PHA-DVT (4–20%), and PHA-DVT (4–30%) groups respectively had 7.6, 3.2, and 2.5 times greater compressive moduli compared to the PHA (4%) group (7.4 kPa) without ECM particle ($p < 0.01$). PHA-DVC (4–30%) had a compressive modulus that was 2.5 times greater than that of the PHA-DVM (4–30%) group ($p < 0.05$), and 4, 2.7, and 3 times greater than those of the PHA-DVT (4–10%), PHA-DBM (4–10%), and PHA-DBM (4–30%) groups, respectively ($p < 0.001$), and 2.4 times greater than that of the PHA-DBM (4–20%) group ($p < 0.01$). The PHA-DVT (4–20%) group had a compressive modulus that was 3.2 times greater than that of the PHA-DVT (4–10%) group ($p < 0.05$). The addition of ECM particles in DBM groups did not significantly increase the

compressive modulus; however, a statistically significant increase in compressive modulus was observed in PHA-DVT groups with increasing ECM particles.

Micro-computed Tomography (μ CT) Analysis

Significant bone regeneration was not observed in non-crosslinking HA-ECM formulations, except for the HA-DBM (4–30%) group (Fig. 2). Large bone island formation was observed in all PHA groups; however, minimal bone-bridging was observed. The HA-DBM (4–30%) group (67.1%) had 5, 4.2, 6.4, 6.3, 3.6, and 2.9 times greater percent bone volume regeneration compared to the sham, HA-DVC (4–30%), HA-DVM (4–30%), HA-DVT (4–30%), PHA-DVC (4–30%), and PHA-DVM (4–30%) groups, respectively ($p < 0.0001$). HA-DBM (4–30%) also had 2.4 times ($p < 0.01$) and 1.8 times ($p < 0.05$) times greater percent bone volume regeneration compared to the DBX® and PHA-DBM (4–30%) groups, respectively. The PHA (4%) group (41.9%) had 3.1, 4, and 3.9 times greater percent bone volume regeneration compared to the sham, HA-DVM (4–30%), and HA-DVT (4–30%), respectively ($p < 0.05$). The PHA-DVT (4–30%) group (42.4%) had 3.2, 4.1, and 4 times greater percent bone volume regeneration compared to the sham, HA-DVM (4–30%), and HA-DVT (4–30%), respectively ($p < 0.05$).

Histological Analysis

Bone growth at the defect periphery was observed in all samples and formed toward the dural side of the defect (Fig. 3). A noticeable soft tissue formation was observed in the defect space where bone formation was absent. Regenerated bone observed within the defect site was thicker and spanned more of the defect on average for groups containing PHA compared to groups containing HA. Peripheral bone growth spanning into the defect site was observed at a higher rate in PHA hydrogel groups. Disjoined DBM particles were observed in the defect space for both the HA and PHA hydrogels and did not appear to integrate with the regenerated bone. DVC, DVM, and DVT tissue particles were not observed in any group after the 8 week recovery time.

Biochemical Analysis from In Vitro Study

After 2 days of cell growth, DNA content was by far the highest for the PHA-DVT (4–30%) group (14.2 μ g/mL), significantly outperforming all other groups (Fig. 4A). Specifically, this PHA-DVT (4–30%) group at day 2 had 6.9, 18.1, 19, 15.6, 13, 6.4, and 3.5 times greater DNA content compared to the TCP, PHA (4%), PHA-DBM (4–10%), PHA-DBM (4–20%), PHA-DBM (4–30%), PHA-DVT (4–10%), and PHA-DVT (4–20%) groups at day 2, respectively ($p < 0.0001$). After 10 days of cell culture on hydrogel groups, the only group with a significant increase in DNA content was the TCP group. The TCP group (12.7 μ g/mL) at day 10 had 6.1 times greater DNA content compared to TCP at day 2 ($p < 0.0001$). The TCP group at day 10 had 21.5, 19.3, 11.7, 10.6, 17.5, and 3.9 times greater DNA content compared to the PHA (4%), PHA-DBM (4–10%), PHA-DBM (4–20%), PHA-DBM (4–30%), PHA-DVT (4–10%), and PHA-DVT (4–20%) groups, respectively ($p < 0.001$). The PHA-DVT (4–30%) group (18.4 μ g/mL) at day 10 had 31.2, 28, 16.9, 15.4, 25.4, and 5.6 times greater DNA content compared to the PHA (4%), PHA-DBM (4–10%), PHA-DBM (4–20%), PHA-DBM (4–30%), PHA-DVT (4–10%), and PHA-DVT (4–20%) groups, respectively ($p < 0.0001$).

Calcium assayed at days 2 and 10 showed a significant increase of calcium content in hydrogels containing devitalized tendon. The TCP group had a near zero result (Fig. 4B). PHA-DVT (4–10%) (8.44 mg/dL), PHA-DVT (4–20%) (8.78 mg/dL), and PHA-DVT (4–30%) (6.82 mg/dL) at day 10 had increases of 2.8, 2.7, and 3.3 times compared to day 2, respectively ($p < 0.0001$). At day 10, The PHA-DVT (4–10%) group had 2.2, 1.6, 3.1, and 6.3 times greater calcium content compared to the PHA (4%), PHA-DBM (4–10%), PHA-DBM (4–20%), and PHA-DBM (4–30%), respectively ($p < 0.0001$). The PHA-DVT (4–20%) group at day 10 had 2.3, 1.7, 3.2, and 6.6 times greater calcium content compared to PHA (4%), PHA-DBM (4–10%), PHA-DBM (4–20%), and PHA-DBM (4–30%), respectively ($p < 0.0001$). The PHA-DVT (4–30%) group at day 10 had 1.8, 2.5, and 5.1 times greater calcium content compared to the PHA (4%), PHA-DBM (4–20%), and PHA-DBM (4–30%) groups, respectively ($p < 0.05$). The PHA-DBM (4–10%) group (5.17 mg/dL) at day 10 had 3.9 times greater calcium content compared to the PHA-DBM (4–30%) group ($p < 0.001$). No significant differences were observed among groups at day 2.

4. Discussion

The current study was the first to evaluate multiple sources of extracellular matrix materials for bone regeneration in a critical size calvarial defect. Although xenogenic tissues were used in the current study, allogenic tissues would be proposed for human clinical translation. Hydrogels presented in the current study demonstrated desirable paste-like handling properties for surgical placement and fast crosslinking times. The current study represents the next generation of materials following our previous work using micron-sized decellularized cartilage and hydroxyapatite for calvarial bone regeneration *in vivo*. [15, 28] In designing hydrogels for the current study, aspects such as mechanical performance, both solid (post-crosslinking) and fluid (pre-crosslinking), were determined to be of great importance for clinical translation and future application to TBI treatment. [29] Mechanical performance of the hydrogel precursor solution and the crosslinked hydrogel, specifically the precursor solution yield stress and the crosslinked hydrogel compressive modulus, respectively, were determined to be vital parameters to demonstrate feasibility for material placement and retention. All hydrogel precursors (prior to crosslinking) exhibited adequate yield stress ($\tau_y > 200$ Pa) for material placement, and the addition of tissue particles increased the average yield stress of all groups. For context, the yield stress of common household items such as mayonnaise and Play-Doh are 200 and 3000 Pa, respectively. [30] The addition of 30% cartilage particles had the greatest effect on material yield stress, potentially due to the presence of the charged proteoglycan, aggrecan. [31] Only PHA-containing groups were evaluated for yield stress as the rheological differences between HA and PHA were assumed to be negligible. After material crosslinking to form hydrogels, the average compressive modulus increased for the groups containing particles; however, the result was only significant in the DCC and some DVT-containing groups. The significant increase in compressive modulus for the DVC and DVT groups was speculated to be attributed to material interaction between the PHA hydrogel and the tissue particle. Although the compressive moduli of the hydrogels tested in the current study were relatively low, the stiffness does not necessarily need to meet that of native bone (i.e., GPa) due to the TBI application requiring flexibility of the biomaterial. [32]

One of the more striking results of the current study was that *in vivo* bone formation differed greatly between the HA- and PHA-containing groups. Both HA and PHA were evaluated due to the speculation that hydrogel crosslinking may influence cellular infiltration. For every group besides the DBM containing group, average bone formation was greater when the tissue was combined with PHA rather than non-crosslinking HA. The difference in bone regeneration for the HA-DBM group compared to the PHA-DBM group was interesting and potentially due to leftover, non-integrating bone particles as observed by H&E staining. Alternatively, hydrogel formation may potentially aid in regeneration by providing a three-dimensional framework; however, the hydrogel may also impede access to the colloidal material. Mechanical testing of the regenerated bone would have been useful in the current study for determining the integrity of the regenerated bone and is recommended for future studies. The speculation that the PHA hydrogel crosslinking may potentially impede cellular infiltration was debunked for the PHA hydrogels in the current study as demonstrated by H&E staining. Interestingly, the positive control of DBX® had less bone regeneration compared to both HA-DBM and PHA-DBM. DBX® contains approximately 31% cortical bone and 4% hyaluronic acid ($M_w = 1$ MDa) by weight and would be expected to have similar bone regeneration compared to the non-crosslinking HA-DBM (4–30%) group that had approximately 22% cortical bone by weight.[33] The major difference of DBX® comparatively to HA-DBM (4–30%) and PHA-DBM (4–30%) being the hyaluronic acid molecular weight and bone concentration. The PHA (4%) control group similarly demonstrated greater average bone regeneration compared to DBX®; however, the finding was not significant. Another study used an 80 kDa, 30 wt% maleimide-modified HA (MaHA) hydrogel tested in a critical size rat calvarial defect and reported less bone regeneration on average compared to the current study.[34] Noting differences of the MaHA hydrogel compared to the current study of HA molecular weight, concentration, and crosslinking mechanism, histological analysis demonstrated that the MaHA gel remained after 8 weeks of recovery. No hydrogel persisted after 8 weeks of recovery in the current study as demonstrated by H&E staining. Further investigation into the relationship between hyaluronic acid molecular weight and bone regeneration is recommended to fully understand the use of the polymer in bone regeneration.

Other groups have evaluated the use of different extracellular matrices for bone regeneration. One such group demonstrated desirable bone regeneration in a mouse calvarial defect model using scaffolds derived from decellularized bovine Achilles tendon seeded with human adipose-derived stem cells.[35] Another group proposed the use of different ECM materials for general hydrogel applications, in which photocrosslinking hydrogels composed of methacrylated gelatin and solubilized-methacrylated-ECM (i.e., cartilage, meniscus, and tendon) were created and tested for *in vitro* gene expression.[36] Perhaps the majority of focus into new ECM materials for bone regeneration has been targeted at using cartilage as a material for bone regeneration.[15, 37–39] Although other groups have demonstrated promise for cartilage as a material to facilitate bone regeneration, including our own group, the current study demonstrated minimal regeneration compared to previous studies as indicated by μ CT and H&E staining.

Groups with significantly greater bone regeneration compared to the sham were selected for further refinement *in vitro* to determine the most attractive tissue particle concentration for

future application to TBI treatment. PHA (4%), PHA-DBM (4–30%), and PHA-DVT (4–30%) were selected for further refinement. Although the HA-DBM (4–30%) group had the largest bone regeneration in the current study, a crosslinking hydrogel is necessary for application to TB treatment, thus the PHA-DBM (4–30%) group was included due to the large bone regeneration observed in the HA-DBM (4–30%) group. Material crosslinking is necessary for potential application to a single surgical intervention to treat TBI, thus refining the material to determine the minimum particle concentration to achieve maximum crosslinking was explored. *In vitro* studies demonstrated significantly greater proliferation on the PHA-DVT (4–30%) group, potentially attributed to greater initial cellular adhesion during seeding. Interestingly, cellular number did not significantly change over 10 days on the hydrogel groups. Calcium content was relatively the same for hydrogel groups after 2 days of culture, but significantly increased on hydrogels containing DVT after 10 days. Calcium deposition at day 2 was unexpected and caution should be employed as further research is necessary to confirm calcium deposition over the short period of time. *In vitro* calcium deposition and *in vivo* bone regeneration results were inconsistent for the PHA (4%) group, potentially due to differing environmental conditions between the two situations. Overall, DVT hydrogels outperformed other crosslinked hydrogels and calcium deposition suggested that the most desirable particle concentration would be 10%. Future studies will focus on using the refined particle concentration in a rat TBI model.

5. Conclusion

The current study demonstrated the feasibility of PHA hydrogels encapsulating ECM tissue particles for use in calvarial bone regeneration. Desirable paste-like handling properties of the pre-crosslinked hydrogels, and mechanical performance after crosslinking was demonstrated in the current study for relevance to TBI. The addition of tissue particles increased both the yield stress and compressive modulus of the material on average, with the cartilage particles leading to the greatest yield stress. The PHA (4%) and PHA-DVT (4–30%) groups led to the greatest bone regeneration, and the tendon particles likewise demonstrated superior calcium deposition by MC3T3 cells *in vitro*, with 10% DVT appearing to be the most desirable concentration. Future studies will attempt to evaluate leading materials in an *in vivo* rat TBI model.

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Statement of Significance

Traumatic brain injury (TBI) is a life-threatening condition characterized by severe brain swelling and is currently treated by a two-stage surgical procedure. Complications associated with the two-stage surgical intervention include the occurrence of the condition termed syndrome of the trephined; however, the condition is completely reversible once the secondary surgery is performed. A desirable TBI treatment would include a single surgical intervention to avoid syndrome of the trephined altogether. The first hurdle in reaching the overall goal is to develop a pliable hydrogel material which can regenerate the patient's bone. The development of a pliable hydrogel technology would greatly impact the field of bone regeneration for TBI application and other areas of bone regeneration.

Figure 1.

A) Hydrogel precursor yield stress determined by the crossover point of the storage (G') and loss (G'') modulus ($n = 3$). Note increasing yield stress with DVT concentration. The highest yield stress observed in the DVC group. α = significantly larger value compared to PHA (4%) ($p < 0.0001$). β = significantly larger value compared to PHA (4%) ($p < 0.05$). μ = significantly larger value compared to all other groups ($p < 0.0001$). θ = significantly larger value compared to PHA-DVT (4-20%) ($p < 0.05$). λ = significantly larger value compared to PHA-DVT (4-20%) ($p < 0.001$). γ = significantly larger value compared to PHA-DVT (4-30%) ($p < 0.05$). π = significantly larger value compared to PHA-DBM (4-10%), PHA-DBM (4-20%), and PHA-DVT (4-10%) ($p < 0.05$). B) Crosslinked hydrogel compressive modulus determined by the slope of the stress-strain curve between 5–15% ($n = 5$). Note higher compressive moduli for hydrogels containing DVT or DVC. α = significantly larger value compared to PHA (4%) ($p < 0.01$). β = significantly larger value compared to PHA-DVM (4-30%) ($p < 0.05$). γ = significant larger value compared to PHA-DVT (4-10%), PHA-DBM (4-10%), and PHA-DBM (4-30%) ($p < 0.01$). δ = significantly larger value compared to PHA-DBM (4-20%) ($p < 0.05$). μ = significantly larger value compared to PHA-DVT (4-10%) ($p < 0.05$). PHA = pentanoate-functionalized hyaluronic acid, DBM = demineralized bone matrix, DVC = devitalized cartilage, DVM = devitalized meniscus, DVT = devitalized tendon.

Figure 2.

Microcomputed tomography (μ CT) analysis using Avizo software after 8 weeks post hydrogel implantation ($n = 5$). A) Representative μ CT scans of calvarial defect groups. Orange coloring indicates the space in which the original defect was created. Scale bar = 5 mm. Note greater bone regeneration for PHA hydrogel groups. B) Percent regenerated bone volume determined by μ CT. α = significantly larger value compared to the sham, HA-DVC (4–30%), HA-DVM (4–30%), HA-DVT (4–30%), PHA-DVC (4–30%), and PHA-DVM (4–30%) ($p < 0.0001$). β = significantly larger value compared to the sham, HA-DVM (4–30%), and HA-DVT (4–30%) ($p < 0.05$). γ = significantly larger value compared to DBX® ($p < 0.01$). δ = significantly larger value compared to PHA-DBM (4–30%) ($p < 0.05$). HA = Hyaluronic acid, PHA = pentanoate-functionalized hyaluronic acid, DBM = demineralized bone matrix, DVC = devitalized cartilage, DVM = devitalized meniscus, DVT = devitalized tendon.

Figure 3.

Histological analysis of rat calvarial defects after 8 weeks post implantation ($n = 5$). Sections were taken from the sagittal plane in which the dural side of the calvaria is the bottom of each image. Sections were stained with hematoxylin and eosin (H&E) and magnified images shown below each overall section correlate to the box above. Note thicker bone formation in hydrogels containing PHA. Scale bar = 1 mm. HA = hyaluronic acid, PHA = pentanoate-functionalized hyaluronic acid, DBM = demineralized bone matrix, DVC = devitalized cartilage, DVM = devitalized meniscus, DVT = devitalized tendon.

Figure 4.

Biochemical analysis of hydrogels for 10 consecutive days of *in vitro* cell culture (n = 5). A) DNA content after 2 and 10 days of cell culture. Note high DNA content on PHA-DVT (4–30%) hydrogels after 2 days of culture. α = significantly greater DNA content compared to all other day 2 groups ($p < 0.0001$). Δ = significantly greater increase in DNA content between day 2 and day 10 ($p < 0.0001$). β = significantly larger value compared to all day 10 groups except PHA-DVT (4–30%) ($p < 0.01$). μ = significantly larger value compared to all other day 10 groups except the TCP group ($p < 0.0001$). B) Deposited calcium content after 2 and 10 days of cell culture. Note increased calcium deposition on DVT hydrogels after 10 days of cell culture. γ = indicates a near zero result. Δ = significantly greater increase in calcium content between day 2 and day 10 ($p < 0.0001$). α = significantly larger value compared to all day 10 groups excluding those containing DVT ($p < 0.0001$). μ = significantly larger value compared to all day 10 groups excluding those containing DVT and the PHA-DBM (4–10%) group ($p < 0.05$). β = significantly larger value compared to PHA-DBM (4–30%) ($p < 0.001$). PHA = pentanoate-functionalized hyaluronic acid, DBM = demineralized bone matrix, DVT = devitalized tendon, TCP = tissue culture plastic.