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***In Vitro* and *In Vivo* Evaluation of Self-Mineralization and Biocompatibility of Injectable, Dual-Gelling Hydrogels for Bone Tissue Engineering**

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Abstract

In this study, we investigated the mineralization capacity and biocompatibility of injectable, dual-gelling hydrogels in a rat cranial defect as a function of hydrogel hydrophobicity from either the copolymerization of a hydrolyzable lactone ring or the hydrogel polymer content. The hydrogel system comprised a poly(*N*-isopropylacrylamide)-based thermogelling macromer (TGM) and a polyamidoamine crosslinker. The thermogelling macromer was copolymerized with (TGM/DBA) or without (TGM) a dimethyl- γ -butyrolactone acrylate (DBA)-containing lactone ring that modulated the lower critical solution temperature and thus, the hydrogel hydrophobicity, over time. Three hydrogel groups were examined: (1) 15 wt% TGM, (2) 15 wt% TGM/DBA, and (3) 20 wt% TGM/DBA. The hydrogels were implanted within an 8 mm critical size rat cranial defect for 4 and 12 weeks. Implants were harvested at each timepoint and analyzed for bone formation, hydrogel mineralization and tissue response using microcomputed tomography (microCT). Histology and fibrous capsule scoring showed a light inflammatory response at 4 weeks that was mitigated by 12 weeks for all groups. MicroCT scoring and bone volume quantification demonstrated similar bone formation at 4 weeks that was significantly increased for the more hydrophobic hydrogel formulations - 15 wt% TGM and 20 wt% TGM/DBA - from 4 weeks to 12 weeks. A complementary *in vitro* acellular mineralization study revealed that the hydrogels exhibited calcium binding properties in the presence of serum-containing media, which was modulated by the hydrogel hydrophobicity. The tailored mineralization capacity of these injectable, dual-gelling hydrogels with hydrolysis-dependent hydrophobicity presents an exciting property for their use in bone tissue engineering applications.

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Keywords

thermogelling; tissue engineering; bone; poly(*N*-isopropylacrylamide)

1. Introduction

More than 500,000 bone graft procedures are performed every year to address bone fractures and other orthopedic-related injuries resulting from a variety of surgical, degenerative and traumatic causes [1, 2]. Injuries to the oral and maxillofacial complex are particularly challenging to repair as morphologically complex structures are damaged. Current treatment options such as autologous or allograft bone inadequately address the functional and aesthetic reconstruction of craniofacial bone due to limited supply, lack of contouring, donor-site morbidity, and other associated surgical complications [3-5].

Thus, injectable, *in situ* forming hydrogels have emerged as promising craniofacial tissue engineering strategies due to the ability to form three-dimensional, highly hydrated scaffolds after injection in aqueous form [6-8]. Therefore, these materials enable minimally invasive and simple, localized delivery of cells and biomolecules without the need for and the associated risks of surgical implantation. Injectable materials also allow for the ability to fill and create mechanically stable scaffolds that conform to irregular-shaped defects, avoiding the difficulty of prefabricating patient-specific shapes. Work by a number of groups has examined injectable hydrogels in the rat cranial defect model with varying degrees of success. Two studies demonstrated that acrylated hyaluronan or chitosan in combination with mesenchymal stem cells (MSC) and bone morphogenetic protein-2 supported new bone formation and MSC osteogenic differentiation [9, 10]. Synthetic hydrogels such as poly(ethylene glycol) or oligo(poly(ethylene glycol) fumarate) have also been examined, and show improved response *in vivo* when coupled with a potent osteogenic factor or inorganic materials [11, 12]. However, in many of these cases, the blank hydrogel alone was insufficient in promoting full bony union.

There is particular interest in synthetic thermoresponsive hydrogels, which undergo a sol-gel transition from an aqueous solution to an insoluble hydrogel at the lower critical solution temperature (LCST) without the aid of additional chemical reagents. One of the most widely investigated thermoresponsive materials is poly(*N*-isopropylacrylamide) (PNiPAAm), which has an intrinsic LCST of ~ 32°C. Although PNiPAAm-based hydrogels have been evaluated for cell sheet engineering [13, 14], drug delivery [15-17], and soft tissue engineering [18-20], they have not been previously investigated for craniofacial regeneration *in vivo*. Early *in vitro* work by our group using a PNiPAAm-based hydrogel system has suggested that these materials can self-mineralize in the presence of proteins and inorganic and organic phosphates [21]. In this work, we evaluated the *in vivo* mineralization capabilities and biocompatibility of acellular injectable, dual-gelling hydrogels based on PNiPAAm for craniofacial tissue engineering. The hydrogel system consists of two components, a thermogelling macromer (TGM) copolymerized with PNiPAAm for physical gelation and glycidyl methacrylate for chemical crosslinking and a diamine-functionalized polyamidoamine (PAMAM) crosslinker, and forms *in situ* and degrades hydrolytically over

time [22], as illustrated in Figure 1. The TGM can also be copolymerized with hydrolyzable moieties that present carboxyl and hydroxyl groups to modulate hydrogel resorbability over time [23]. Previous studies by our group with this versatile class of injectable hydrogels have established their rapid gelation and dimensional stability [22], tunable physicochemical characteristics [23, 24], favorable cytocompatibility [23, 24], and ability to support encapsulated cell viability [23, 25].

We hypothesized that the injectable, dual-gelling hydrogels based on PNiPAAm would promote hydrogel mineralization and bone formation across the defect *in vivo*, the extent of which would differ based on hydrogel polymer content. Additionally, the various hydrogel formulations were hypothesized to be biocompatible and demonstrate a mild tissue response *in vivo*. To test the hypotheses, the hydrogels were implanted in an 8 mm critical size rat cranial defect for 4 and 12 weeks. Bony union, bone formation, and tissue response were evaluated at each timepoint using microcomputed tomography (microCT), histological staining, and histomorphometric scoring. The mechanisms of implant mineralization were further probed through complementary acellular *in vitro* investigations.

2. Materials and Methods

2.1 Materials

N-isopropylacrylamide (NiPAAm), dimethyl- γ -butyrolactone acrylate (DBA), glycidyl methacrylate (GMA), acrylic acid (AA), 2,2'-azobis(2-methylpropionitrile) (azobisisobutyronitrile, AIBN), *N,N'*-methylenebisacrylamide (MBA), and piperazine (PiP) were purchased from Sigma Aldrich (Sigma, St. Louis, MO) and used as received. Anhydrous 1,4-dioxane, dimethylformamide, diethyl ether, and acetone in analytical grade; water, acetonitrile, chloroform, and methanol in HPLC-grade; and 1 N sodium hydroxide were purchased from VWR (Radnor, PA) and used as received. Phosphate buffered saline (PBS) solution was obtained from Gibco Life, Grand Island, NY (powder, pH 7.4). Ultrapure water was obtained from a Millipore Super-Q water system (Millipore, Billerica, MA).

2.2 In Vivo Experimental Design

Three groups of acellular hydrogels were examined in an 8 mm critical size rat cranial defect, as outlined in Table 1. Two experimental groups ("15 wt% TGM/DBA") and ("20 wt % TGM/DBA") consisted of the P(NiPAAm-*co*-GMA-*co*-DBA-*co*-AA) TGM with the DBA-containing hydrolyzable lactone ring at 15 or 20 wt% polymer. The polymer content of hydrogel formulations was selected on the basis of mechanical stability in the defect while remaining injectable. The amount of AA incorporated was experimentally determined as the amount needed to compensate for the hydrophobicity of the DBA and GMA comonomers and tune the initial LCST for handling at room temperature. To further reinforce the role of hydrophobicity in hydrogel mineralization, a group consisted of hydrophobic P(NiPAAm-*co*-GMA) TGM at 15 wt% polymer without hydrophilic comonomers was also evaluated. Based on previous work with rat cranial defects, the time periods chosen were 4 and 12 weeks [26, 27]. At both timepoints, the samples were evaluated with microCT and histology after harvest. All groups consisted of *n* = 6-7 rats.

2.3 TGM Synthesis and Characterization

Synthesis of P(NiPAAm-*co*-GMA) and P(NiPAAm-*co*-GMA-*co*-DBA-*co*-AA) TGMs were performed according to established protocols [22, 23]. In a typical reaction, 20 g of NiPAAm, GMA, DBA and AA was dissolved in 200 mL of either anhydrous dimethylformamide or 1,4-dioxane under nitrogen at 65°C for the TGM and TGM/DBA groups, respectively. AIBN pre-dissolved in the solvent was added at 0.7% of total mol content to thermally initiate free radical polymerization, and the reaction mixture was stirred for 16 h. After solvent removal by rotary evaporation, the material was re-dissolved in either pure acetone or a 95:5 (v/v) mixture of acetone:methanol for the TGM/DBA and TGM groups, respectively and purified twice via dropwise precipitation in at least 10X excess diethyl ether. The recovered polymer was air-dried overnight and transferred to a vacuum oven for several days prior to elemental analysis. The chemical composition of the TGMs was determined by proton nuclear magnetic resonance spectroscopy (¹H NMR, Bruker, Switzerland). The polymer was dissolved in D₂O at a concentration of 20 mg/mL that contained 0.75 wt % 3-(trimethylsilyl)propionic-2,2,3,3-d₄ acid, sodium salt as an internal shift reference (Sigma-Aldrich, St. Louis, MO) and the data were analyzed using the MestRe-C NMR software package (Mestrelab Research S.L., Spain). Acid titration was performed in conjunction with ¹H NMR to determine the AA content of the TGMs before hydrolysis. Aqueous gel permeation chromatography (GPC) using a Waters Alliance HPLC system (Milford, MA) and differential refractometer (Waters, model 410) equipped with a series of analytical columns (Waters Styragel guard column 20 mm, 4.6 × 30 mm; Waters Ultrahydrogel column 1000, 7.8 × 300 mm) was used to determine the molecular weight distributions of the synthesized TGM/DBA polymers. The TGM/DBA polymer was first hydrolyzed in accelerated conditions to remove its thermogelling properties. The weight average molecular weight (M_w), number average molecular weight (M_n), and polydispersity index ($PDI = M_w/M_n$) of the hydrolyzed polymer were determined by comparison to commercially available narrowly dispersed molecular weight poly(ethylene glycol) (PEG) standards (Waters, Mississauga, ON). The LCSTs of the TGMs were determined by differential scanning calorimetry (DSC). 14 μ L of each polymer solution was pipetted into an aluminum volatile sample pan (TA Instruments, Newcastle, DE) and capped/crimped. Thermograms were recorded on a TA Instruments DSC 2920 equipped with a refrigerated cooling system against an empty sealed pan as reference. The oven was equilibrated at -5°C for 10 min and then heated to 80°C at a heating rate of 5°C/min. The LCST was determined both as the onset and peak temperature of the endothermic peak in the thermogram using the Universal Analysis 2000 software provided with the DSC system. For this study, P(NiPAAm_{92.6}-*co*-GMA_{7.4}) with a M_n = 9.2 kDa and PDI of 3.12 [22] and P(NiPAAm_{80.4}-*co*-GMA_{10.3}-*co*-DBA_{6.3}-*co*-AA_{3.0}) with a M_n = 54.5 kDa and PDI of 1.84 were used [23].

2.4 PAMAM Synthesis and Characterization

PAMAM was synthesized by the polyaddition of piperazine (PiP) and methylene bisacrylamide (MBA) at a stoichiometric molar ratio of [MBA]/[PiP] = 0.75 following previously reported protocols [22, 24]. Molecular weight distributions of the synthesized PAMAM crosslinkers were analyzed using time-of-flight mass spectroscopy with positive-mode electrospray ionization on a Bruker microTOF ESI spectrometer (Bruker Daltonics,

Billerica, MA) equipped with a 1200 series HPLC (Agilent Technologies, Santa Clara, CA) to deliver the mobile phase (50:50 HPLC-grade water and methanol). After data acquisition, all peaks (including degradation and secondary reaction products) were identified using microTOF Control software (Bruker). The peaks were corrected for charge state (generally with H^+ or Na^+ and rarely K^+ ions), and quantified for calculation of M_n , M_w , and PDI. PAMAM with $M_n = 1440$ Da and PDI = 1.38 was used for this study.

2.5 Hydrogel Fabrication

Hydrogels (8 mm in diameter, 2 mm in height) were fabricated by combining the TGM and PAMAM crosslinker. The TGM and PAMAM crosslinker were first sterilized via UV-irradiation for 2 h. Individual solutions of TGM and PAMAM crosslinker were then prepared at twice the desired concentrations (Table 1) in sterile PBS pH 7.4 and placed on a shaker table at 4°C until dissolved. Under sterile conditions, the PAMAM solution was pipetted into the TGM solution using cold pipet tips and the resulting solution was manually mixed in the glass vial. 110 μ L injections were transferred to 8 mm diameter $\times$ 2 mm height cylindrical Teflon molds at 37°C either immediately for TGM/DBA groups or after a 20 minute delay for the TGM group, covered with a glass slide, and subsequently allowed to gel for 24 h prior to implantation.

2.6 Animal Surgeries and Euthanasia

This work was done in accordance with protocols approved by the Rice University Institutional Animal Care and Use Committee. After hydrogel fabrication with PBS pH 7.4 under sterile conditions in 8 mm $\times$ 2 mm Teflon molds, the hydrogels were implanted within an 8 mm rat cranial defect. All hydrogel groups were statistically randomized to minimize operative- or animal-related error. 11-12 week Fischer 344 rats weighing 176-200g (Harlan, Indianapolis, IN) were placed under 4% isoflurane and maintained at 2% isoflurane/ O_2 gas mixture for the duration of the operation. The incision site and surrounding area were shaved, sterilized with povidine-iodine swabs, and subcutaneously injected with 500 μ L of 1% lidocaine for local anesthesia. A linear incision was performed from the nasal bone to the mid-sagittal crest, and the skin and periosteum were exposed from the underlying bone. An 8 mm craniotomy was performed with a dental surgical drilling unit with a trephine burr and saline irrigation, and the calvarial disk was carefully removed to prevent dural tearing. After cleaning and removal of bone fragments, the hydrogel was implanted and the periosteum and skin were each separately closed with interrupted braided Vicryl stitches. The rats were given an intraperitoneal injection of saline (1 mL/100 g/h of anesthesia) to counteract blood loss and aid recovery. Additionally, intraperitoneal injections of buprenorphine (0.05 mg/kg) were given at 12, 24, and 36 h as post-operative analgesia. After surgery, the rats were placed under pure O_2 until awakened from anesthesia and individually housed in soft-bedding cages. Animals were given free access to food and water and monitored for complications. The implants and surrounding tissue were harvested 4 or 12 weeks post-surgery. The rats were euthanized by CO_2 inhalation after anesthesia under 4-5% isoflurane, followed by a bilateral thoracotomy.

2.7 Implant Retrieval

The implants were harvested by making an incision between the medial canthi of the eyes down to the bone using a 701 burr attached to a Stryker Total Performance System straight handpiece at 40,000 rpm with water irrigation. Similar cuts were made along the left and right temporal bone and posterior aspect of the cranial vault, resulting in a rectangular section of the cranium containing the defect site and implant. The implants were fixed in 10% formalin (Formalde-Fresh, Fisher) for 3 days at 37°C and transferred to 70% (v/v) ethanol for microCT and histological analysis.

2.8 Microcomputed Tomography

MicroCT analysis with a Skyscan 1172 High-Resolution Micro-CT (Aartselaar, Belgium) with 10 µm resolution, 0.5 mm aluminum filter, and voltage of 100 kV and current of 100 µA was used to examine the morphology and mineralization of the implants and bony union across the defect. Volumetric reconstruction and analysis was conducted using Nrecon and CT-analyser software provided by Skyscan. The percent of bone formation, including hydrogel mineralization, within the defect was determined by centering a cylindrical volume of interest (VOI) of 8 mm in diameter and 2 mm in height at the bottom of the defect. Data are reported as the % binarized object volume measured within this VOI within thresholding gray values (70-255) with the CT-analysis software [28]. The extent of bony bridging and union within the defect were scored according to the grading scale in Table 2. These scores were determined from maximum intensity projections of the samples generated from the microCT datasets. 3D models of each sample were also generated to visualize the distribution of mineral deposits within the hydrogel implants.

2.9 Histological Processing

After microCT scanning, the samples were sent to the MD Anderson Cancer Center Bone Histomorphometry Core Laboratory (Houston, TX) for dehydration and embedding in poly(methyl methacrylate). 5 µm coronal cross-sections were taken from the center of the defect of each sample and staining was performed with von Kossa, hematoxylin and eosin, and Goldner's trichrome.

2.10 Histological Scoring

The three histological sections were evaluated via light microscopy (Eclipse E600, Nikon, Melville, NY with attached 3CCD Color Video Camera DXC-950P, Sony, Park Ridge, NJ) and scored using histological scoring analysis. The histological evaluation was performed along random implant-tissue interfaces within central coronal cross-sections to assess: (1) tissue response at the bone-hydrogel interface following the rubric outlined in Table 3 [26], (2) mineralization within the hydrogel following the scoring guide outlined in supplementary figure 1A, and (3) extent of bony bridging across the defect following the rubric outlined in supplementary figure 1B. Additionally, the thickness and quality of the fibrous capsule was scored using high magnification images (AxioImager Z2, Carl Zeiss, Germany) taken at four predetermined locations along the sample-periosteal border and sample-dural side interface as outlined in Table 4 [29]. The evaluations were performed blindly with randomized samples by three reviewers.

2.11 Acellular Mineralization

8 × 2 mm acellular hydrogels (n = 4 per group) were fabricated as described above. The hydrogels were incubated in complete osteogenic medium without fetal bovine serum (FBS), complete osteogenic medium with 10% FBS (Cambrex Bioscience), PBS pH 7.4, and 1× simulated body fluid for 0, 1, 7, 14, and 28 days at 37°C in 12-well plates. The simulated body fluid was prepared according to published protocols [30]. Each solution was changed every 2-3 days. At timepoints, the hydrogels were soaked in ultrapure water for 30 min, cut in half, blotted and weighed. Sample halves (n = 4 halves) for the calcium assay were placed in 500 µL of ddH₂O, homogenized through three freeze/thaw/sonication cycles, and digested overnight in equal parts of 1N acetic acid for a final concentration of 0.5 N acetic acid. The assay was performed with a commercially available kit (Sekisui Diagnostics, Tokyo, Japan) according to the manufacturer's instructions. The remaining sample halves were fixed in 10% formalin, dehydrated in 70% ethanol, placed in Histoprep frozen tissue embedding media (Fischer Scientific, Waltham, MA), and frozen at -20°C.

2.12 Statistics

The microCT and histological scoring data were analyzed via one-way analysis of variance followed by the Kruskal-Wallis test (p<0.05) for n = 6-7 samples. The microCT bone volume for n = 6-7 samples and *in vitro* mineralization data for n = 4 were presented as means ± standard deviation, unless otherwise stated. The acellular mineralization data were analyzed by Tukey's post-hoc test using JMP v11 statistical software.

3. Results

3.1 MicroCT Analysis

Three different groups of acellular, dual-gelling hydrogels were implanted: one group without the DBA monomer containing a hydrolyzable lactone ring for LCST modulation (15 wt% TGM), and two groups with the DBA monomer at different polymer wt% concentrations (15 and 20 wt% TGM/DBA). MicroCT was used for nondestructive, quantitative analysis of bony bridging, union, and bone volume, as well as 3D visualization of the extent and spatial distribution of mineralization. For bony bridging of the defects, maximum intensity projections (MIPs) were generated from each of the microCT datasets and scored by three blinded reviewers using the 0 - 4 grading scale detailed in Table 2, which provides respective examples of actual MIPs for each score. Figure 2 shows the results from the scoring at 4 and 12 weeks. At the 4 week timepoint, the average union microCT score for 15 wt% TGM, 15 wt% TGM/DBA, and 20 wt% TGM/DBA was 2.4 ± 0.5, 1.9 ± 0.7, and 2.0 ± 0.0, respectively, indicating that bony bridging is only observed at the defect borders. At the 12 week timepoint, the 15 wt% TGM and 20 wt% TGM/DBA demonstrated an increase in bony bridging, leading to a statistically significant increase in the average union microCT score compared to the 4 week timepoint.

Figure 3 shows the results from the quantification of bone volume using microCT. The data are reported as the % binarized object volume within a given VOI above a critical threshold grayscale value of 70, and include mineralization within the implant and bone formation across the defect. Both the 15 wt% TGM and 20 wt% TGM/DBA groups demonstrated a

significant increase in the bone volume from 4 to 12 weeks. Additionally, the bone volume of the 15 wt% TGM group was significantly higher at 12 weeks compared to the two other groups.

To examine the spatial distribution and nature of mineralization in detail, 3D models of each sample were generated via microCT using the critical threshold value. Two representative samples in the 15 wt% TGM and 20 wt% TGM/DBA groups demonstrating the most mineralization at 12 weeks are shown in Figure 4. Mineralization across the defect at 12 weeks primarily extended underneath the hydrogel for both groups. Mineralization additionally occurred throughout the hydrogel for the 15 wt% TGM group. This is in contrast to samples in all the groups at 4 weeks, which demonstrated mineralization as nodules above the hydrogel (Figure 4 A, D, G) or other groups at 12 weeks, which showed bone formation at the defect borders.

3.2 Descriptive Light Microscopy

The samples were grossly examined after histological staining with hematoxylin & eosin (H&E), von Kossa, and Goldner's trichrome, and representative images from each group and time point are shown in Figure 5. For all sections, the implants were visible, although shrinkage and delamination artifacts generally associated with hydrogel histology were observed in many samples. For both the 4 and 12 week samples, a thin fibrous capsule was observed around the hydrogel, particularly around the periosteal border, as shown in the high magnification subsets in Figure 5. At 4 weeks, the tissue at the hydrogel-defect interface was generally loose and fibrous in nature and minimal bone formation was observed. The inflammatory response, characterized by the presence of neutrophils and macrophages, was also minimal and occurred primarily at the periosteal border. At 12 weeks, the inflammatory response was generally mitigated and the tissue at the hydrogel-defect interface was more organized. Although all the hydrogels did not demonstrate measurable material fragmentation, instances of cell-mediated bone formation across the defect were more pronounced in the 15 wt% TGM group compared to the other groups. With H&E and Goldner's trichrome staining, cell-mediated bone formation was generally observed on the dural side of the defect, although in certain 15 wt% TGM samples, significant mineralization was also seen throughout the center of the hydrogel with von Kossa staining. Additionally, promising direct bone-implant contact was observed in several of the 15 wt% TGM samples at 12 weeks, a representative example of which is shown in Figure 5D.

3.3 Quantitative Histological Analysis

Samples were evaluated for bony bridging across the defect and mineralization of the hydrogel (Supplementary Figure 1) according to the inlaid scoring rubrics. Supplementary Figure 1A shows the scoring results for mineralization within the hydrogel. The average score for the all the groups did not differ from each other at 4 and 12 weeks. Supplementary Figure 1B shows the scoring results for bony bridging across the central coronal cross-section of the hydrogel along the dural side of the implant. The 15 wt% TGM group showed significantly increased scores in bony bridging from 4 to 12 weeks, but the scores were only significant from the 15 wt% TGM/DBA group at 12 weeks. The samples were also scored

for the tissue response with two independent scoring guides for the bone-hydrogel interface and fibrous capsule surrounding the periosteal and dural borders. The tissue response at the bone-hydrogel interface showed a significant difference between the 4 and 12 week timepoints only for the 20 wt% TGM/DBA group (Table 3). Histological analysis of the fibrous capsule demonstrated that, on average, the TGM/DBA groups showed significantly better capsule quality scores at 12 weeks. However, for all the groups, the thickness and quality scored above a 2, indicating a fibrous-like capsule of no more than 10 layers (Figure 6). The 15 wt% TGM group showed significantly increased scores for both thickness and quality from 4 to 12 weeks, indicating an improvement in both parameters, whereas the 15 wt% TGM/DBA showed only a significant difference in the capsule quality. When examining the scores for the fibrous capsule at the top or bottom of the implant, there were no significant differences in terms of location except for the capsule thickness for the 20 wt% TGM/DBA group. Capsule thickness and quality at the top improved significantly over time for the 15 wt% TGM and 15 wt% TGM/DBA groups, while only capsule quality improved over time for the 15 wt% TGM group. Representative images of each score for the fibrous capsule thickness and quality scoring can be found in Supplementary Figure 2.

3.4 In Vitro Acellular Mineralization

To investigate the mechanism behind hydrogel mineralization, an acellular *in vitro* study was performed by immersing the hydrogels in four solutions: PBS pH 7.4 (PBS), SBF 1× (SBF), complete osteogenic media without serum (NS), and complete osteogenic media with serum (S). Figure 7 shows the calcium content of the hydrogels over the 28 day period. All of the groups demonstrated significantly increased calcium content over time after culture in complete osteogenic media with serum. This corresponded with increased von Kossa histological staining of hydrogel cross-sections over time for all groups, which detects the presence of phosphate groups (data not shown). However, the amount of calcium in the 15 wt% TGM group was significantly larger than that of the 15 wt% and 20 wt% TGM/DBA groups at the 7, 14 and 28 day timepoints. The 15 wt% TGM group also displayed significantly increased calcium content in the SBF 1× and complete osteogenic media without serum (NS) conditions; however, these values were not statistically different from the calcium content of complete osteogenic media with serum (S) conditions.

4. Discussion

The objective of this study was to evaluate the mineralization capacity and biocompatibility of acellular injectable, dual-gelling hydrogels based on PNiPAAm for the healing of an 8 mm critical size rat cranial defect. We hypothesized that the degree of hydrogel hydrophobicity modulated by the increase of the hydrogel polymer content would result in different capacities for mineralization and bony bridging in the absence of any delivered cells or growth factors. We also hypothesized that the material would present a compatible tissue response in the *in vivo* environment. Hydrogel implants copolymerized with the hydrolyzable lactone ring at two different polymer wt% contents were placed in the 8 mm critical size rat cranial defects. An additional group, 15 wt% TGM without the hydrolyzable lactone ring as a hydrophobic control, was also evaluated. The implants were harvested at 4

and 12 weeks, and analyzed for cell-mediated bony union, bone volume within the defect, and tissue response with MicroCT and histological staining.

MicroCT data at 4 weeks showed that there were no significant differences in the scores for bony union for all of the groups. No differences were also observed in the microCT quantification of bone volume, which included mineralization within and across the hydrogel. It is likely at this early stage that the injectable hydrogel has not undergone sufficient degradation to generate enough porosity to allow for tissue infiltration and growth. A number of studies in this defect have achieved some tissue infiltration when using porous scaffolds [31, 32]. Additionally, without the incorporation of stem cells or growth factors, there would be limited osteoinduction to promote an early osteogenic response [9, 11].

At 12 weeks, however, there was a significant difference in the scores for bony union for the 15 wt% TGM and 20 wt% TGM/DBA groups compared to the 15 wt% TGM/DBA group. This was supported by the significant increase observed in the bone formation quantification for both groups. Although there is a similar trend over time, the 15 wt % TGM group showed overall higher scores and cell-mediated bone formation at the 12 week timepoint, with full bridging in two out of six rats. This was also reflected in the histological data, where the 15 wt% TGM group demonstrated clear mineralization within the hydrogel space with von Kossa staining and significant new bone formation between the implant and the dura. However, statistically significant differences in the histological mineralization scores were not observed.

Because there are relatively few studies examining PNiPAAm-based hydrogels for bone tissue engineering in an orthotopic site, it is difficult to make direct comparisons. Results regarding PNiPAAm and *in vivo* bone regeneration have only been previously observed with combined cellular or growth factor delivery. An early study by Gao et al. exploring P(NiPAAm-*co*-*N*-acryloxysuccinimide) conjugated with bone morphogenetic protein-2 (BMP-2) in a rat ectopic muscle pocket model demonstrated that these copolymers could support BMP-2 osteoinductive activity, leading to increased calcium content, alkaline phosphatase activity, and bone neotissue in the explants after three weeks [33]. Another study by Na et al. demonstrated that P(NiPAAm-*co*-AA)/hydroxyapatite hybrid scaffolds delivering BMP-2 could promote the osteogenic differentiation of encapsulated rabbit MSCs in a subcutaneous nude mouse model. Von Kossa histological staining and collagen type I immunohistochemistry of the explants after eight weeks showed mineral deposition through the hydrogel and maintenance of the MSC osteogenic phenotype, respectively [34]. In both of these studies, the blank PNiPAAm-based hydrogel was not examined, but the acellular hydrogel with loaded growth factor or hydroxyapatite was capable of some mineralization and tissue ingrowth. In contrast, the hydrogels examined in this study were capable of mineralization and bone formation without cells, growth factors, or initial mineral content.

To understand these results, the general approaches toward hydrogel mineralization must be considered. Since the hydrated polymer network of hydrogels is poorly mineralizable, incorporation of inorganic phases, creation of nucleation sites, and chemical modification with anionic functional groups are commonly employed acellular strategies [35, 36]. The latter involves the functionalization of hydrogels with negatively charged side chains, or

anionic phosphate, carboxyl, or hydroxyl groups that can bind calcium, mimicking the natural process in bone tissue [35]. Both vinylphosphonic acid and silanol groups have been copolymerized with PNiPAAm to create hydrogels exhibiting calcium-binding abilities and apatite formation *in vitro* [37, 38]. However, with these considerations in mind, the presence of anionic chemical groups is not dominantly affecting mineralization in this study since the carboxyl and hydroxyl presenting-TGM/DBA hydrogels presented less bone formation and mineralization than the TGM only hydrogels.

The observed differences in acellular mineralization *in vitro* and *in vivo* are likely due to matrix hydrophobicity, which facilitates calcium binding and mineralization via enhanced protein adsorption. The TGM hydrogels consist of PNiPAAm and GMA, both of which are hydrophobic monomers. In contrast, the TGM/DBA hydrogels are copolymerized with DBA and AA, both of which present carboxyl and hydroxyl groups that increase the hydrophilicity of the polymer. Although all the groups underwent significant *in vitro* mineralization only in the presence of serum-containing media, the change in hydrophobic state resulted in an increase of the calcium content by an order of magnitude in the TGM hydrogels compared to the TGM/DBA hydrogels, which was reflected in the degree of acellular mineralization *in vivo*. This mineralization likely presented a favorable environment by which to promote osteogenic differentiation and increased mineralized matrix deposition, leading to greater bony union. Additionally, the increase in polymer content from the 15 wt% to 20 wt% hydrogels increased hydrogel hydrophobicity, which led to significantly more mineralization and bone formation. This is in agreement with previous studies that showed a strong correlation between calcium binding and the chain length of hydrophobic alkyl groups in ionic hydrogels [39]. Further work upon this phenomenon with poly(ethylene glycol) diacrylate hydrogels with varying lengths of *N*-acryloyl amino acid side chains in SBF with and without serum suggested that protein adsorption onto the more hydrophobic hydrogels mimicked the natural functions of bone sialoprotein as a nucleating protein. Using scanning electron microscopy and X-ray diffraction, hydrogels were observed to possess an apatite-like mineralization with calcium/phosphate ratios reminiscent of synthetic hydroxyapatite [40]. These studies also provide explanation for the increasing calcium content only observed in the 15 wt% TGM hydrogels in the SBF and NS conditions, since the more hydrophobic hydrogels demonstrated improved cooperative binding with calcium even in the absence of nucleating proteins. This suggests that modulating the hydrophobicity of the hydrogel through the timescale of DBA hydrolysis would improve the performance of the TGM/DBA groups in future studies in this defect model. Studies by other groups have previously established that hydrolysis sufficient enough to drive the LCST of a DBA-containing thermogelling hydrogel above body temperature (enabling for hydrogel degradation) occurred on the order of days depending on the DBA content, incorporation of AA, and environmental conditions [41]. However, in the dual gelling system, the DBA hydrolysis occurs within minutes, due to greater hydrogel stabilization and higher swelling from the additional chemical crosslinking [23]. By decreasing the crosslinking density or the PAMAM crosslinker length, the hydrolysis timescale may be lengthened and thus, maintain hydrogel hydrophobicity for a longer period of time.

In terms of tissue response, the hydrogels were characterized by some inflammation at 4 weeks mainly on the periosteal border with an unorganized implant-tissue interface. The inflammation was mitigated at 12 weeks and the implant-soft tissue interface showed more organized tissue. Two independent scoring guides were used to assess the bone-hydrogel interface and fibrous capsule surrounding the rest of the implant. Although no significant differences were observed in the scoring at the bone-hydrogel interface due to the lack of direct bone-implant contact, it was shown with the fibrous capsule scoring that the capsule thickness and quality improved over time, indicating that the hydrogels induced a favorable tissue response marked by little inflammatory cells and organized capsule formation.

5. Conclusion

This study evaluated the mineralization capacity, bone formation capability, and tissue response of acellular PNiPAAm-based hydrogels in an 8 mm critical size rat cranial defect. Although minimal bone formation and hydrogel mineralization was observed at 4 weeks for all groups, significantly higher mineralization within and across the implant was observed for the 15 wt% TGM and 20 wt% TGM/DBA groups after 12 weeks. The results, coupled with an *in vitro* acellular mineralization study, suggest that these hydrogels undergo matrix hydrophobicity-dependent mineralization. These mineralizable and biocompatible injectable hydrogels possess great potential as acellular strategies or localized delivery vehicles for stem cells or growth factors for craniofacial tissue engineering applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AA	Acrylic acid
BMP-2	Bone morphogenetic protein-2
DBA	Dimethyl- γ -butyrolactone acrylate
DSC	Differential scanning calorimetry
FBS	Fetal bovine serum
GMA	Glycidyl methacrylate
GPC	Gel permeation chromatography
LCST	Lower critical solution temperature

MBA	Methylene bisacrylamide
MicroCT	Microcomputed tomography
MSC	Mesenchymal stem cell
NMR	Nuclear magnetic resonance
NS	Complete osteogenic medium without serum
PAMAM	Polyamidoamine
PEG	Poly(ethylene glycol)
PBS	Phosphate buffered saline
PiP	Piperazine
PNiPAAm	Poly(<i>N</i> -isopropylacrylamide)
S	Complete osteogenic medium with serum
SBF	Simulated body fluid
TGM	Thermogelling macromer
VOI	Volume of interest

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Figure 1.

Scheme of injectable, biodegradable, dual-gelling system. Black, blue and orange lines represent the TGM, PAMAM crosslinker and DBA lactone ring, respectively. Upon temperature elevation to 37°C, the TGM and PAMAM crosslinker combine to form an insoluble, physically and chemically crosslinked hydrogel. After time, the PAMAM crosslinker, and in the case of the TGM/DBA hydrogels, the DBA lactone ring, are hydrolyzed, enabling hydrogel degradation through fully soluble degradation products.

Figure 2.

Results of microCT scoring for bony bridging and union for 15 wt% TGM hydrogels and 15 and 20 wt% TGM/DBA hydrogels at 4 and 12 weeks. Error bars represent standard deviations for $n = 6-7$. (*) indicates significant change from 4 week timepoint ($p < 0.05$).

Figure 3.

Results of microCT quantification of % bone volume within the cranial defect at 4 and 12 weeks using an 8 mm VOI with thresholding gray values (70-255). Error bars represent standard deviation of $n = 6-7$ hydrogels. (*) and (#) indicate significant change between timepoints or across groups, respectively ($p < 0.05$).

Figure 4.

Representative top (A-C) and side (D-F) views of microCT generated three dimensional models showing mineralization on a binary threshold (70-255) and raw coronal cross-sections from the center of the defects (G-I) of 15 wt% TGM at 4 (A,D,G) and 12 weeks (B,E,H) and 20 wt% TGM/DBA hydrogels at 12 weeks (C,F,I).

Figure 5. Histological staining of 15 wt% TGM (left column), 15 wt% TGM/DBA (middle column), and 20 wt% TGM/DBA hydrogels (right column) at 4 (A-C) and 12 (D-F) weeks. One set of representative images from each timepoint show von Kossa (top image) and hematoxylin and eosin staining (bottom image) at 2× magnification. Subsets at bottom demonstrate the tissue response on the periosteal border at 4 (left) and 12 (right) weeks at 20× magnification within the boxed regions. Scale bars for hydrogel slices represent 1 mm and 8 mm, respectively. Scale bars for high magnification images represent 50 μm.

Figure 6.

Top, bottom, and combined histological scores of fibrous tissue thickness and quality of the implants at 4 and 12 weeks. Error bars indicate standard deviation for 6-7 samples with 4 images per sample (n=12-14 for top and bottom, n=24-28 for total). (*) refers to significant differences within groups from 4 to 12 weeks ($p < 0.05$), (#) refers to significant differences within timepoints across groups compared to the 15 wt % TGM group ($p < 0.05$), and (^) refers to significant differences within groups and timepoints from images at different locations ($p < 0.05$).

Figure 7.

Calcium content of 15 wt% TGM and 15 and 20 wt% TGM/DBA acellular hydrogels ($n = 4$) over 28 days in phosphate buffered saline (PBS), $1\times$ simulated body fluid (SBF), complete osteogenic media without serum (NS), and complete osteogenic media with serum (S). 15 and 20 wt% TGM/DBA hydrogels correspond to the right y-axis, and 15 wt% TGM hydrogels correspond to the left y-axis. (*) refers to significant difference across timepoints within group and media ($p < 0.05$).