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Chemical Sintering Generates Uniform Porous Hyaluronic Acid Hydrogels

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Abstract

Implantation of scaffolds for tissue repair has been met with limited success primarily due to the inability to achieve vascularization within the construct. Many strategies have shifted to incorporate pores into these scaffolds to encourage rapid cellular infiltration and subsequent vascular ingrowth. We utilized an efficient chemical sintering technique to create a uniform network of polymethyl methacrylate (PMMA) microspheres for porous hyaluronic acid hydrogel formation. The porous hydrogels generated from chemical sintering possessed comparable pore uniformity and interconnectivity as the commonly used non- and heat sintering techniques. Moreover, similar cell response to the porous hydrogels generated from each sintering approach was observed in cell viability, spreading, proliferation in vitro, as well as, cellular invasion in vivo. We propose chemical sintering of PMMA microspheres using a dilute acetone solution as an alternative method to generating porous hyaluronic acid hydrogels since it requires equal or ten-fold less processing time as the currently used non-sintering or heat sintering technique, respectively.

Keywords

Hydrogel; Hyaluronic acid / hyaluronan; Porosity; Angiogenesis

1. Introduction

One of the overarching limitations of current approaches in developing scaffolds for tissue repair is the inability to achieve scaffold vascularization [1, 2]. Without blood vessels, the lack of oxygen and nutrient diffusion in and out of the scaffold does not allow for cells to infiltrate and survive deep within the scaffold for subsequent repair and new tissue formation. Therefore, much of current tissue engineering efforts have focused on developing scaffolds that promote vascular ingrowth to improve scaffold efficacy and overall success in aiding tissue repair.

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Although implantation of a completely pre-vascularized tissue or scaffold may appear as an attractive option, the time course of such an approach renders it impractical. For this reason, popular strategies have been developed to more rapidly fabricate scaffolds that focus on the promotion host vascularization and integration. One approach to improve scaffold vascularization is to rationally design the scaffold architecture to promote rapid cellular infiltration and blood vessel invasion. This can be achieved by designing a scaffold with a porous structure; pores allow for better cellular infiltration, migration, and proliferation, improving mass transport of oxygen and nutrients, as compared to non-porous scaffolds of similar composition, while the scaffold provides the necessary structural integrity for new tissue formation and vascular ingrowth [1]. Porous scaffolds have been formed via various techniques that include gas foaming [3, 4], salt leaching [5-8], heat sintering [9, 10], and lyophilization [11-14]. Even in the absence of pro-angiogenic factors, porous hydrogels formed via the previously mentioned techniques with pore sizes ranging from 30-150 μm have been shown to promote angiogenesis and tissue formation *in vivo* with poly(N-isopropyl acrylamide) (poly-NIPAM) [9], poly(2-hydroxyethyl methacrylate-co-methacrylic acid) (pHEMA-co-MAA) [10], and PEG hydrogels [7]. Although gas foaming, salt leaching, and lyophilization are successful in achieving porous scaffolds, these techniques often lack uniformity in interconnected pore structure and pore size which make it difficult to accurately attribute the observed effects to these parameters. For this reason, heat sintering polymethyl methacrylate (PMMA) microspheres has been an attractive alternative, where the hydrogel is formed around a uniformly packed PMMA microsphere template.

Although heat sintering requires manual PMMA microsphere handling, the technique produces a uniform network of slightly fused PMMA microspheres following sintering at 150 $^{\circ}\text{C}$ for 17-22 h for subsequent hydrogel formation [9, 15, 16]. Due to this lengthy processing time to produce a network of PMMA microspheres for porous hydrogel formation, we proposed to develop a more efficient sintering technique. The objectives of this study were to: (i) decrease the PMMA microsphere network processing time and (ii) create a technique that minimizes manual handling and resultant error propagation. We proposed to utilize a suspension of dilute acetone in ethanol to slightly mate the microsphere interfaces to generate a highly uniform network more efficiently. We compared the proposed chemical sintering technique to the commonly used heat sintering technique and another suspension method that utilizes only ethanol by other research groups [17, 18] to determine the uniformity and efficacy *in vitro* and *in vivo*. In our studies, we used PMMA microspheres ranging from 53-63 μm in diameter to form our porous hydrogels since scaffolds with pore sizes outside 20-60 μm result in avascular, fibrotic healing [19]. Moreover, we aimed to pair the advantages of a porous network with a hyaluronic acid (HA) scaffold since HA is a biocompatible glycosaminoglycan and key component in the extracellular matrix that has been shown to stimulate endothelial cell activity and its oligosaccharides have been shown to promote angiogenesis *in vivo* [20-23]. The HA backbone was chemically modified to contain acrylates to provide sites for the inclusion of cell adhesion peptides (RGD) and matrix-metalloproteinase (MMP) - degradable peptide crosslinkers to promote cell infiltration and cell-mediated scaffold degradation, respectively. MMPs are a family of proteinases that play critical roles in many physiological processes and are upregulated during the wound healing cascade [24, 25], therefore can be utilized as an initiator for scaffold degradation and a potential delivery mechanism of pro-angiogenic bioactive signals contained within the scaffold to stimulate vascularization.

2. Materials and methods

2. 1. Materials

Peptides Ac-GCRDGPQGIWGQDRCG-NH₂ (HS-MMP-SH) and Ac-GCGYGRGDSPG-NH₂ (RGD) were purchased from Genscript (Piscataway, NJ). Sodium hyaluronan (HA)

was a gift from Genzyme Corporation (60 kDa, Cambridge, MA). All other chemicals were purchased from Fisher Scientific (Pittsburgh, PA) unless otherwise noted.

2. 2. Hyaluronic acid-acrylate modification

Sodium hyaluronan was modified to contain acrylate functionalities as previously described [26]. Briefly hyaluronic acid (2.0 g, 5.28 mmol, 60 kDa) was reacted with 18.0 g (105.5 mmol) adipic acid dihydrazide (ADH) at pH 4.75 in the presence of 4.0 g (20 mmol) 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) overnight and purified through dialysis (8000 MWCO) in deionized (DI) water for 2 days. The purified intermediate (HA-ADH) was lyophilized and stored at -20 °C until used. Approximately 60% of the carboxyl groups were modified with ADH, which was determined using ¹H-NMR (D₂O) by taking the ratio of peaks at $\delta = 1.6$ and 2.3 corresponding to the eight hydrogens of the methylene groups on the ADH to the singlet peak of the acetyl methyl protons in HA ($\delta = 1.88$). HA-ADH (1.9 g) was reacted with N-acryloxysuccinimide (NHS-Ac) (1.33 g, 4.4 mmol) in HEPES buffer (10 mM HEPES, 150 mM NaCl, 10 mM EDTA, pH 7.2) overnight and purified through dialysis against a 100 mM to 0 mM salt gradient for 1 day, and then against DI water for 3-4 days before lyophilization. The degree of acrylation was determined to be ~10% using ¹H-NMR (D₂O) by taking the ratio of the multiplet peak at $\delta = 6.2$ corresponding to the cis and trans acrylate hydrogens to the singlet peak of the acetyl methyl protons in HA ($\delta = 1.88$).

2. 3. Design template using PMMA microspheres

Heat sintered (HS) microsphere templates for porous hydrogels were prepared as previously described [26]. Briefly, approximately 24 mg polymethyl methacrylate (PMMA) microspheres (53–63 μ m, which will be referred to as 60 μ m hereafter, Cospheric, Santa Barbara, CA) were added into glass-bottom silicon wells (6 mm $\times$ 1 mm, D $\times$ H). The microspheres were then packed by slight tapping for 1–2 min and examined for homogenous packing through phase microscopy. The filled glass-bottom silicon wells were then placed into an oven and the microspheres were sintered for 17–22 h at 150 °C. Non-sintered and chemically sintered microsphere templates were prepared by suspending PMMA microspheres in a sintering solution at 0.4444 mg/ μ l. 55 μ l of the PMMA suspension was added to flexiPERM molds (Sigma–Aldrich, St. Louis, MO) adhered to sigmacoted glass slides and placed in an incubator at 37 °C for 1–2 h to dry. The non-sintering (NS) suspension solution was optimized to be 70% ethanol for the 60 μ m PMMA microspheres. The “chemical sintering” (CS) solutions were optimized to be 1% acetone in 70% ethanol for the 60 μ m PMMA microspheres.

2. 4. Hydrogel formation

Hydrogels were formed by Michael-type addition of acrylate functionalized HA (HA-Ac) with bis-cysteine containing MMP peptide cross-linkers at pH 7.6–7.8. Prior to reaction, a hydrogel precursor solution was made by mixing HA-Ac with a lyophilized aliquot of cell adhesion peptide, RGD, for 30 min at 37 °C. After incubation, HA-RGD was mixed with the remaining HA-Ac and 0.3M triethanolamine (TEOA), pH 8.8 for a final gel concentration of 3.5 w/v% HA and 500 μ M RGD. Finally, lyophilized aliquots of the cross-linker (0.9 mg HS-MMP-SH) were diluted in 18 μ l of TEOA buffer pH 8.8 immediately before addition to the rest of the mixture. For porous hydrogels, 20 μ l of gel solution was then added directly on top of a PMMA microsphere template and perfused into the template by centrifugation at 500 g for 5 min at 4 °C. The slide was then incubated at 37 °C for 30–45 min to induce polymerization. Once complete, the gels were removed from the silicon wells and placed directly into 100% acetone for 48 h to dissolve the PMMA microsphere template (Fig. 1). The acetone solution was replaced 2–3 times during this incubation. The gels were then serially hydrated into sterile phosphate buffered saline (PBS) and left in PBS until ready for

use. For non-porous hydrogels, the gel solution was sandwiched between two sigmacoted slides using 1 mm thick plastic spacers and incubated at 37 °C for 30–45 min to induce polymerization. Once complete, the gels were placed directly into sterile PBS and left in PBS until ready for use.

2. 5. Hydrogel preparation for SEM imaging

Each hydrated hydrogel was cut in half with the vertical cross section facing up and placed onto a dry carbon tape-covered holder. Samples were imaged under low vacuum at 50 Pa, 10 kV, and spot size of 3.0 using an FEI Nova Nano 230 SEM in the UCLA Molecular & Nano Archaeology (MNA) facility.

2. 6. Characterization of hydrogel structural and mechanical properties

The storage and loss moduli were measured using a plate-to-plate rheometer (Physica MCR, Anton Paar, Ashland, VA) with an 8 mm (diameter) plate under a constant strain of 0.1% and angular frequency ranging from 0.1 to 10 s⁻¹. Hydrogels were generated as described above and cut to 8 mm in diameter using an 8 mm biopsy punch. To prevent the hydrogel from drying, a humidity hood was utilized and the stage was set to 37 °C. Hydrogel water content was quantified by taking the ratio of equilibrated swollen hydrogel weight, subtracting the lyophilized dry hydrogel weight to the swollen gel weight. Interconnected pore diameters were manually measured using ImageJ on SEM images taken at 500× magnification (2 images per condition). NHS-Alexa488 (Invitrogen, Grand Island, NY) was used to stain the HA hydrogel by binding to the free amines (following formation). Stained hydrogels were imaged with an inverted Observer Z1 Zeiss fluorescent microscope; orthogonal projections and 3D rendering was obtained using Zen software.

2. 7. Cell culture

Mouse bone marrow-derived mesenchymal stem cells (mMSCs) (D1, CRL12424) were purchased from ATCC (Manassas, VA) and cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Grand Island, NY) supplemented with 10% bovine growth serum (BGS, Hyclone, Logan, UT) and 1% penicillin/streptomycin (Invitrogen, Grand Island, NY) at 37 °C and 5% CO₂. The cells were passaged using trypsin following standard cell culture protocols every 2–3 days.

2. 8. Cell seeding onto porous hydrogels

mMSCs were seeded onto 3.5 w/v%, 500 μM RGD porous hydrogels as previously described [27]. Briefly, porous hydrogels were sterilized by 3 consecutive 10 min washes in sterile PBS. Hydrogels were then placed into dry low-binding plates and cells were seeded onto the hydrogels in two steps. First, 15,000 cells at a density of 15,000 cells/10 μl DMEM were added directly on top of each hydrogel and centrifuged down for 3 min at 700 rpm. Then, an additional 15,000 cells were seeded directly on top without further centrifugation. The hydrogels were incubated at 37 °C for 15min and then 180 μl of fresh media was added for a final volume of 200 μl/well.

2. 9. Cell viability and spreading

mMSC viability was studied with a LIVE/DEAD® viability/cytotoxicity kit (Molecular Probes, Eugene, OR). Briefly, 1 μl of ethidium homodimer-1 and 0.25 μl of calcein AM from the kit were diluted with 500 μl DMEM to make the staining solution. Each gel (2–3 gels per condition per time point) was stained with 150 μl of staining solution for 30 min at 37 °C in the dark before imaging. To better analyze cell spreading, gels (2–3 gels per condition per time point) were fixed for 30 min at RT using 4% paraformaldehyde (PFA), rinsed with PBS, treated with 0.1% triton-X for 10 min and stained for 90 min in the dark

with YOYO-1 for cell nuclei (1:250 dilution from 1 mM stock, Invitrogen, Grand Island, NY) and rhodamine-phalloidin (1:40 dilution, Invitrogen, Grand Island, NY) in 1% bovine serum albumin solution. The samples were then washed with 0.05% tween-20. For both cell viability and cell spreading, an inverted Observer Z1 Zeiss fluorescent microscope was used to visualize samples. To better visualize the distribution throughout the hydrogel, multiple z-stacks 1.9–2.3 μm thick were taken for each image, deconvolved to minimize background, and presented as orthogonal projections. Cell viability was assessed by quantifying the ratio of live cells to the total number of cells as manually counted from an orthogonal projection of z-stacks through 70 μm of hydrogel acquired from the LIVE/DEAD® assay. Cell spreading was quantified by manually measuring end-to-end cell length using Zen Blue 2011 (Carl Zeiss, Jena, Germany) for three images (orthogonal projections of z-stacks through 125 μm of hydrogel) per condition per time point.

2. 10. Cell proliferation

An alamarBlue® assay (AbD Serotec, Raleigh, NC) was used to quantify cell proliferation rate. 20 μl alamarBlue reagent with 200 μl DMEM was added to each well containing a hydrogel seeded with cells in a 96-well plate and incubated at 37 °C for 5 h. The solutions were transferred to a new plate and absorbance was measured at 570 nm and 600 nm using a standard plate reader. Three gels for each condition were analyzed at each time point and proliferation rate was calculated following the manufacturer's instructions.

2. 11. Subcutaneous implant model

All in vivo studies were conducted in compliance with the NIH Guide for Care and Use of Laboratory Animals and UCLA ARC standards. 6 to 8-week old female Balb/c mice, each 20–30 g, were used to study cellular infiltration and blood vessel formation in HA hydrogels since this strain and size has been previously used for wound healing and angiogenesis assays [28, 29]. Non-porous or porous hydrogels were made exactly as described above (3.5 w/v% HA, 100 μM RGD) and cut to 6 mm in diameter using a sterile biopsy punch, for final overall dimensions of 6 mm $\times$ 1 mm, D $\times$ H. All porous hydrogels were made using 60 μm beads. In fabricating the hydrogels, the starting reagents were sterilized through filtering with a 0.22 μm filter. After scaffold fabrication, the hydrogels were washed with sterile PBS and kept in PBS with 1% P/S. Immediately prior to surgery, mice were anesthetized with 4% isoflurane through an induction chamber. After anesthesia induction, the isoflurane concentration was lowered to 1.5–2.5% for the remainder of the surgery. The back of the mouse was subsequently shaved and washed with Betadine and 70% isopropyl alcohol. Two lateral incisions appropriate to the size of the implant were made in the skin (one on each side of the midline of the animal) using scissors. Two subcutaneous pockets were subsequently created by blunt dissection using rounded-end scissors. The hydrogels were inserted into each respective subcutaneous pocket and closed with a single wound clip. All animals were observed daily for signs of inflammation and pain and also administered carprofen injections for the first 48 h post-survival surgery. After 2 weeks, mice (n=4) were sacrificed with isoflurane overdose. Two 1 cm^2 pieces of tissue were collected from each mouse containing the implant and the surrounding tissue and skin, fixed in 2% PFA for 16 h at 4 °C, dehydrated in 70% EtOH, and finally paraffin embedded. A total of 12 mice were used in this study, with four mice per hydrogel condition.

2. 12. Immunohistochemistry and immunofluorescence

Paraffin embedded sections (5 μm thick) were deparaffinized by incubation in multiple xylene washes followed by serial hydration from 100% ethanol to 100% water. Cell membranes were permeabilized with a 15 min incubation at 37 °C in 0.1 mg/ml proteinase K solution. Sections were then washed with PBS and incubated in blocking buffer (1% goat serum (Jackson Immuno Research Labs, West Grove, PA) + 0.05% Tween-20 in PBS) for 1

h at RT before being incubated in primary antibody solution (1:100 dilution in blocking buffer of rat anti-mouse CD31 (BD Pharmingen, San Diego, CA)) overnight at 4 °C. Sections were again washed with PBS and incubated in blocking buffer for 10 min at RT before being incubated for 2 h at RT in secondary antibody solution (1:200 dilution in blocking buffer of goat anti-rat Alexa 568 (Invitrogen, Grand Islands, NY), which also contained α -smooth muscle actin-FITC (1:500 dilution, Sigma–Aldrich, St. Louis, MO) and DAPI nuclear stain (1:400 dilution, Invitrogen, Grand Island, NY). Sections were then washed twice in PBS, mounted and imaged using an inverted Zeiss fluorescence microscope. All hematoxylin and eosin staining of sections was conducted by the Translational Pathology Core Laboratory (TPCL) at UCLA.

2. 13. Quantification and characterization of cellular infiltration and blood vessels in vivo

Hydrogels were halved and embedded in paraffin with the vertical cross sections facing up. Each paraffin embedded section contained 2 halves (vertical cross section) of the hydrogel. Three separate sections at least 100 – 150 μ m apart were analyzed for each sample. 5 randomly chosen areas were imaged on each half gel, with 10 images total in per section. Cellular infiltration was quantified by using Fiji software by converting the image to an 8-bit file, applying a threshold sensitivity, and using the watershed tool to separate joined cells to analyze the cell number. Vessels were counted manually in each section, totaled from all 30 sections, and finally normalized to the total imaged area. The bar graph in Fig. 6D represents the average vessels/mm² from 4 different animals. The diameter of each vessel was manually measured using Zen imaging and analysis software (Zeiss). Percentages were determined using the entire vessel set.

2. 14. Statistical analysis

Statistical analyses were performed using Prism (GraphPad, San Diego, CA). Interconnected pore diameter, water content, rheology, cell viability, spreading, and proliferation data were analyzed using a one-way analysis of variance (ANOVA) followed by a Tukey post-hoc test. The results were represented as mean $\pm$ SD. The *in vivo* cellular invasion and blood vessel characterization data were analyzed using a Kruskal-Wallis one-way ANOVA followed by a Dunn's post-hoc test and represented as mean $\pm$ SEM. Single, double, and triple asterisks represent $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. A p value < 0.05 was considered statistically significant.

3. Results

3. 1. Effect of PMMA sintering technique on hydrogel formation

Prior to hydrogel formation, PMMA microsphere packing was investigated as a result of each sintering technique. SEM images show similar microsphere organization (Fig. 2A). 3.5 w/v% HA-MMP porous hydrogels were generated from heat sintering (HS), non-sintering (NS), and chemically sintering (CS) 60 μ m PMMA microspheres to compare the differences in hydrogel structure and architecture as a function of sintering technique (Fig. 3). SEM images taken at 255 $\times$, 500 $\times$, and 1000 $\times$ magnification demonstrated that 60 μ m pore hydrogels generated from chemical sintering had comparable uniform open pore structure and pore interconnectivity as the currently used HS and NS techniques. SEM images of non-porous gels are included as a comparison (Fig. 7A). Moreover, phase and fluorescent microscopy was used to investigate the hydrogel structure in its hydrated state. Porous hydrogels were stained with NHS-Alexa488 and imaged under fluorescent microscopy. Application of orthogonal projections and 3D rendering to multiple z-stack images showed apparent pores and high interconnectivity in all hydrogels generated with each sintering technique.

3. 2. Effect of PMMA sintering technique on structural and mechanical properties

Pore interconnectivity of SEM images was manually measured using ImageJ and defined as the interfacial, longitudinal distance between adjacent pores. Porous hydrogels generated via heat sintering, non-sintering, and chemical sintering had comparable interconnected pore diameters of 20.75 ± 8.93 , 18.29 ± 6.81 , and 20.08 ± 8.92 μm , respectively (Fig. 4A). In addition, the quantification of water content of hydrogels generated from each technique revealed that the sintering technique did not significantly affect the water retention capability, where the hydrogels were 98.38 ± 0.36 , 98.28 ± 0.32 , and $98.49 \pm 0.22\%$ water from with HS, NS, and CS techniques, respectively (Fig. 4B). Conversely, the PMMA microsphere sintering technique appeared to significantly affect the mechanical stiffness of the resultant porous hydrogels (Fig. 4C, D). Gels formed via heat sintering were comparable to those generated with chemical sintering, but significantly stiffer than when formed with non-sintering where the storage moduli were 713 ± 317.3 , 597.2 ± 140.5 , and 488 ± 129.6 Pa, respectively.

3. 3. Effect of PMMA sintering technique on cell behavior in vitro

To study the effect of sintering technique on cell morphology in vitro, mMSCs were seeded on 3.5 w/v% HA-MMP hydrogels functionalized with 500 μM RGD. Cell-seeded hydrogels were stained at days 2 and 7 with live/dead® (Fig. 5A) and rhodamine-phalloidin/dapi (Fig. 5B) to study cell viability and spreading, respectively. Quantification of viability at day 2 showed no significant differences among sintering techniques with greater than 82% viability (Fig. 5C). Morphological differences could not be observed in images obtained from staining the cell-seeded gels for f-actin and nuclei. This result was confirmed by quantifying cell spreading by manually measuring the end-to-end cell length (Fig. 5F), where cell spreading was comparable among the hydrogels generated from the different techniques (Fig. 5E). Cell proliferation was also monitored over time using an alamarBlue® assay and days 1, 4, and 7 post-seeding (Fig. 5D). For any time point, there was no difference among the hydrogels formed from the different sintering techniques. However, for each condition, cell growth peaked at day 4, and plateaued through day 7.

3. 4. Effect of PMMA sintering technique in vivo

Acellular 3.5 w/v% HA-MMP hydrogels functionalized with 100 μM RGD were implanted subcutaneously in the backs of balb/c mice for 14 days to study cellular infiltration. Samples were excised at day 14 for analysis. Hematoxylin and eosin (H&E) staining of the hydrogel cross sections showed that the porous hydrogel structure was maintained through 14 days and high cellular infiltration into the hydrogels was present (Fig. 6A), while host cells remained on the periphery of non-porous gels (Fig. 7B). Quantification of cellular invasion was obtained by counting nuclei normalized to gel area; this data supported H&E observations where the number of cells infiltrating the hydrogels generated from chemical sintering (2424 ± 292 nuclei/ mm^2) were comparable to hydrogels generated with heat or non-sintering (3037 ± 206 or 2672 ± 282 nuclei/ mm^2 , respectively) (Fig. 6C). Immunofluorescence staining for PECAM positive endothelial cells revealed that blood vessels were present in hydrogels formed from all three different sintering techniques (Fig. 6B). Quantification of the number of blood vessels present normalized to gel area showed that the PMMA microsphere sintering and resultant porous hydrogel did not significantly affect the vessel invasion (Fig. 6D). Moreover, characterization of the blood vessel diameters showed that approximately half of the vessels present were no more than 6 μm in diameter (diameter of an erythrocyte) (Fig. 6E).

4. Discussion

Several current approaches in generating porous hydrogels possess limited control over consistency, therefore using PMMA microspheres as a sphere template via heat sintering has offered better uniformity in pore structure and interconnectivity. However, the main limitations with heat sintering include the manual handling and long processing time (17-22 h) that carries over to delayed experimental investigation. For this reason, we proposed to chemically sinter the PMMA microspheres in a liquid suspension to (i) utilize a liquid handling technique to minimize manual handling and resultant error and (ii) slightly fuse the microspheres at its interfacial boundary to generate a neatly packed microsphere network. Since acetone is a known solvent for PMMA, it was used in a dilute concentration with the expectation that the PMMA microspheres would slightly mate the microspheres at each adjacent interface. Moreover, the dilute acetone was suspended in ethanol to pair the volatile state of these solvents with an incubation step to expedite the drying rate of the PMMA microsphere network. In this study, we compared our proposed chemically sintered PMMA microspheres to those suspended in a 70% ethanol solution (non-sintering) as used by Peyton et al. [17] and Stachowiak et al. [18] and to the commonly used heat sintered technique in order to investigate the resultant hydrogel pore structure and cell response *in vitro* and *in vivo*.

We report that similar uniform pore structure with the proposed chemical sintering technique was achieved when compared to hydrogels generated via heat sintering and non-sintering (Fig. 3). However, the difference herein lies with the processing time, where non- and chemically sintered hydrogels only required 1-2 h for incubation while heat sintering requires more than ten times this. Unexpectedly, our studies demonstrate no quantifiable difference among the three sintering techniques with regard to pore uniformity and interconnectivity, despite the vastly dissimilar approaches (Fig. 3, 4A). Further investigation of PMMA microsphere packing via SEM microscopy showed similar resultant microsphere networks generated with each technique (Fig. 2A). This was confirmed via quantification of PMMA microsphere diameter using ImageJ following each respective sintering solution where no marked difference among the techniques was present (Fig. 2B). This result indicates that the proposed chemical sintering suspension solution with dilute acetone does not significantly cause surface dissolution or fusion of the PMMA microspheres as previously hypothesized. This may be altered with slightly increasing the acetone concentration between 1-5%; optimization studies realized that acetone concentrations exceeding 5% acetone in ethanol resulted in complete PMMA dissolution (data not shown). Moreover, the interconnected pore diameter achieved from heat sintering at high temperatures was comparable to chemical and non-sintering. This result is likely due to the intrinsic viscous nature of hyaluronic acid; we believe that despite centrifugation of the hydrogel into the PMMA microsphere templates, the viscous hydrogel does not penetrate the void spaces or interfacial gaps as deeply as expected, rendering the porous hydrogel interconnectivity similar among all sintering techniques.

Despite similar porous hydrogel structure and interconnectivity, the effect of sintering technique on mechanical properties was apparent (Fig. 4C). Although the storage moduli of hydrogels formed via heat and non-sintering techniques were significantly different, it is important to note that hydrogels formed via chemical sintering were comparable to those generated by heat sintering. These data show that porous hydrogels can be generated via chemical sintering to achieve mechanical properties similar to the commonly used heat sintering technique with ten-fold less processing time. It was also observed that the storage moduli of the porous hydrogels varied at angular frequencies nearing 10 rad/s (Fig. 4D). This result may due in part to the large pores we have introduced into the scaffold; the thin

hydrogel walls between pores that may be experiencing variable deformation with increased angular frequencies.

Cell behavior in response to hydrogels generated from the three sintering techniques was evaluated via viability and proliferation assays, as well as immunofluorescent staining for cell morphology to confirm that porous hydrogels formed from the different techniques did not significantly vary in presentation to cells *in vitro*. Cell viability, spreading, and proliferation was not significantly different among the hydrogels generated from each sintering technique (Fig. 5A-F), suggesting that the differences observed in mechanical properties did not largely affect cell behavior *in vitro*. This result may be attributed to the scale of interaction between the cell and scaffold, as well as the relative difference in moduli. Despite observing a statistically significant difference in storage moduli, the difference is not extreme; the hydrogel moduli remain on the same order of magnitude. Since the cell-scaffold surface interaction more closely mimics a 2D presentation of cues, we believe that the overall cell behavior *in vitro* may not be significantly influenced by bulk mechanical stiffness with the small difference in modulus. Mechanical properties have been shown to influence mesenchymal stem cell behavior in 2D when moduli vary from 2 to 10 fold [30, 31]. The differences observed between our hydrogels are much smaller than this, where the stiffest hydrogel was 713 Pa and the softest was 488 Pa. Therefore, this small variation in moduli may not be sufficient to see differences in cellular proliferation, spreading, and viability.

To further investigate the effect of sintering *in vivo*, acellular porous hydrogels generated from heat, chemical, and non-sintering were implanted subcutaneously in the backs of balb/c mice to assess cellular infiltration and implant integration with host tissue at 14 days. H&E staining of hydrogel sections showed high cellular infiltration into the porous hydrogels generated from each technique (Fig. 6A). This observation was confirmed with quantification of nuclei normalized to hydrogel area via Fiji software (Fig. 6C), demonstrating that the host cells were able to invade the pores generated from each technique comparably. Moreover, staining for endothelial cells demonstrate that blood vessels were present at 14 d in porous hydrogels generated from each PMMA sintering technique (Fig. 6B). Characterization of the vessels present indicated that the density of blood vessels present were comparable in hydrogels formed with each technique, and of the vessels present, most were on the dimensional order of erythrocytes suggesting capillary formation. Immunofluorescent staining confirmed this, as no PECAM+ vessels stained positive for α -SMA, a smooth muscle cell marker that exists on larger, contractile vessels. These results highlight the therapeutic potential of these porous hydrogels for tissue repair for several reasons. First, the inclusion of pores by any of the described methods allow for rapid cellular infiltration when compared to a non-porous hydrogel of the same composition (Fig. 7A, B). Secondly, even without the inclusion of pro-angiogenic factors in the porous hydrogels, we observed blood vessel invasion into the hydrogel at 14 days. Blood vessel formation and maturation could potentially be encouraged further with the incorporation of pro-angiogenic growth factors or therapeutic plasmids. More importantly, although hyaluronic acid possesses many attractive qualities that include biocompatibility and biodegradability, it was strategically chosen for our hydrogel system because its degraded fragments of HA stimulates angiogenesis *in vivo* [20]. Interestingly, it appears as though the degraded HA alone was not a strong enough pro-angiogenic stimulus for the non-porous hydrogels to recruit blood vessel invasion. This observation may indicate that for a HA-MMP degradable hydrogel system to promote vascular ingrowth without the presence of pro-angiogenic growth factors or therapeutic plasmids, a porous structure is necessary.

5. Conclusions

In this paper, we report a chemical sintering method to generate porous hyaluronic acid hydrogels comparable to currently used non- and heat sintering techniques. More importantly, the proposed chemical sintering was able to achieve similar uniform pore structure and interconnectivity when compared to non- and heat sintering techniques with equal or less processing time at 1-2 h versus 1-2 h and 17-22 h, respectively. Chemical sintering was able to achieve similar *in vitro* and *in vivo* responses as ascertained by studying cell viability, spreading, proliferation, and cell and blood vessel invasion. These results indicate that porous hyaluronic acid hydrogels can be generated via chemical sintering or non-sintering comparable to the commonly used heat sintering technique with higher efficiency as these techniques minimize manual PMMA microsphere handling and significantly reduce processing time.

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Fig. 1.

Schematic representation of steps taken to generate porous hyaluronic acid hydrogels via chemical and non-sintering of PMMA microspheres. The appropriate sintering suspension solution is described in 2.3. The resultant porous hydrogel is achieved after step 6, following PMMA dissolution via acetone and serial hydration.

Fig. 2.

(A) PMMA microspheres packed with each respective sintering technique imaged via SEM demonstrated comparable microsphere structure and organization prior to hydrogel formation. (B) Quantification of PMMA microsphere diameter following packing showed no marked difference in microsphere size immediately following the various sintering methods.

Fig. 3. Porous hydrogels with 60 μm pores generated from HS, NS, and CS have apparent open pore and interconnected pore structure in SEM, phase, and fluorescent orthogonal projections and 3D renderings.

Fig. 4.

(A) Quantification of interconnected pore diameters indicates similar interconnectivity between hydrogels generated with HS, NS, and CS. (B) Water content was similar for hydrogels generated with the various techniques. (C, D) Conversely, PMMA microsphere packing technique affects gel stiffness, where gels generated via HS were comparable to CS but stiffer than NS-generated gels. (***) $p < 0.001$. Values represent the mean and standard deviation.

Fig. 5.

In vitro characterization and quantification of mMSCs seeded onto hyaluronic acid hydrogels following 7 days. (A) mMSC viability (green = live and red = dead cells) and (B) spreading (red = f-actin and blue = nuclei) was assessed at day 2 and 7 by fluorescent microscopy. (C) Quantification of day 2 viability and (D) cell growth at days 1, 4, and 7 showed no significant differences among groups. (E) Quantification of cell spreading by measuring end-to-end cell length (F) at days 2 and 7 showed no difference among the groups at each time point. (*) $p < 0.05$. Values represent the mean and standard deviation.

Fig. 6.

In vivo characterization and quantification of acellular hyaluronic acid hydrogels (60 µm pores) implanted subcutaneously in balb/c mice for 14 days. (A) H&E stained sections of porous hydrogels generated from the different techniques showed similar levels of cellular infiltration. (B) Staining for an endothelial cell marker showed PECAM positive cells within the hydrogels generated by all three sintering techniques. (C) Quantification of cellular infiltration showed comparable invasion within gels generated from all three techniques. (D) Vessels in 30 images over 3 sections separated by 100-150 µm were quantified and normalized to the total image area. The bar graph represents the average of 4 separate samples. (E) For those samples that contained vessels, vessel diameters were measured. Approximately half of the vessels in all samples were less than 6 µm in diameter. Red = PECAM positive staining (endothelial cells), yellow = erythrocytes, and blue = nuclei. Values represent the mean and standard error of the mean.

Fig. 7.
(A) Scanning electron micrographs of non-porous (n-pore) 3.5 w/v% hyaluronic acid hydrogels confirm that no pores are present. (B) H&E staining of samples of n-pore hydrogels implanted subcutaneously in balb/c mice demonstrate that host cells are only penetrate the hydrogel periphery at 14 days. The asterisk denotes the remaining n-pore hydrogel and (M) denotes muscle.