jove

Fragmenting Bulk Hydrogels and Processing into Granular Hydrogels for Biomedical Applications

Victoria G. Muir[1], **Margaret E. Prendergast**[1], **Jason A. Burdick**[1,2,3]

[1] Department of Bioengineering, School of Engineering and Applied Sciences, University of Pennsylvania [2] BioFrontiers Institute, University of Colorado Boulder [3] Department of Chemical and Biological Engineering, College of Engineering and Applied Science, University of Colorado Boulder

Corresponding Author

Jason A. Burdick
jason.burdick@colorado.edu

Citation

Muir, V.G., Prendergast, M.E., Burdick, J.A. Fragmenting Bulk Hydrogels and Processing into Granular Hydrogels for Biomedical Applications. *J. Vis. Exp.* (183), e63867, doi:10.3791/63867 (2022).

Date Published

May 17, 2022

DOI

10.3791/63867

URL

jove.com/video/63867

Abstract

Granular hydrogels are jammed assemblies of hydrogel microparticles (i.e., "microgels"). In the field of biomaterials, granular hydrogels have many advantageous properties, including injectability, microscale porosity, and tunability by mixing multiple microgel populations. Methods to fabricate microgels often rely on water-in-oil emulsions (e.g., microfluidics, batch emulsions, electrospraying) or photolithography, which may present high demands in terms of resources and costs, and may not be compatible with many hydrogels. This work details simple yet highly effective methods to fabricate microgels using extrusion fragmentation and to process them into granular hydrogels useful for biomedical applications (e.g., 3D printing inks). First, bulk hydrogels (using photocrosslinkable hyaluronic acid (HA) as an example) are extruded through a series of needles with sequentially smaller diameters to form fragmented microgels. This microgel fabrication technique is rapid, low-cost, and highly scalable. Methods to jam microgels into granular hydrogels by centrifugation and vacuum-driven filtration are described, with optional post-crosslinking for hydrogel stabilization. Lastly, granular hydrogels fabricated from fragmented microgels are demonstrated as extrusion printing inks. While the examples described herein use photocrosslinkable HA for 3D printing, the methods are easily adaptable for a wide variety of hydrogel types and biomedical applications.

Introduction

Granular hydrogels are fabricated through the packing of hydrogel particles (i.e., microgels) and are an exciting class of biomaterials with many advantageous properties for biomedical applications[1,2,3]. Due to their particulate structure, granular hydrogels are shear-thinning and self-healing, allowing for their use as extrusion printing (bio)inks, granular supports for embedded printing, and injectable therapeutics[4,5,6,7,8,9]. Additionally, the void space between microgels provides a microscale porosity for cell movement and molecular diffusion[8,10,11]. Further, multiple microgel

populations can be combined into a single formulation to allow for enhanced tunability and material functionality[8,10,12,13]. These important properties have motivated the rapid expansion of granular hydrogel development in recent years.

There is a range of methods available to form microgels towards granular hydrogel fabrication, each with its own advantages and disadvantages. For example, microgels are often formed from water-in-oil emulsions using droplet microfluidics[4,11,13,14,15,16,17], batch emulsions[7,18,19,20,21,22], or electrospraying[6,23,24,25]. These methods yield spherical microgels with either uniform (microfluidics) or polydisperse (batch emulsions, electrospraying) diameters. There are some limitations to these water-in-oil emulsion fabrication methods, including potentially low-throughput production, the need for low-viscosity hydrogel precursor solutions, and the high cost and resources for setup. Additionally, these protocols may require harsh oils and surfactants that must be washed from the microgels using procedures that add processing steps, and may be difficult to translate to sterile conditions for biomedical applications in many labs. Removing the need for water-in-oil emulsions, (photo)lithography can also be used, where molds or photomasks are used to control the curing of microgels from hydrogel precursor solutions[1,26,27]. Like microfluidics, these methods may be limited in their production throughput, which is a major challenge when large volumes are needed.

As an alternative to these methods, mechanical fragmentation of bulk hydrogels has been used to fabricate microgels with irregular sizes[19,28,29,30,31,32]. For example, bulk hydrogels can be pre-formed and subsequently passed through meshes or sieves to form fragmented microgels, a process which has even been done in the presence of cells within microgel strands[33,34]. Bulk hydrogels have also been processed into microgels with mechanical disruption using techniques such as grinding with mortar and pestle or through the use of commercial blenders[35,36,37]. Others have also used mechanical agitation during hydrogel formation to fabricate fragmented microgels (i.e., fluid gels)[31].

The methods herein expand on these mechanical fragmentation techniques and present a simple approach to fabricate microgels with extrusion fragmentation, using photocrosslinkable hyaluronic acid (HA) hydrogels as an example. Extrusion fragmentation uses only syringes and needles to fabricate fragmented microgels in a low-cost, high throughput, and easily scalable method that is appropriate for a wide range of hydrogels[19,32]. Further, methods to assemble these fragmented microgels into granular hydrogels are described using either centrifugation (low packing) or vacuum-driven filtration (high packing). Lastly, the application of these fragmented granular hydrogels is discussed for use as an extrusion printing ink. The goal of this protocol is to introduce simple methods that are adaptable to a wide variety of hydrogels and can be implemented in virtually any laboratory interested in granular hydrogels.

Protocol

1. Fabricating bulk hydrogels inside of a syringe using photocrosslinking

NOTE: An overview of bulk hydrogel fabrication inside a syringe using photocrosslinking is shown in **Figure 1**. This protocol uses norbornene-modified hyaluronic acid (NorHA) to fabricate bulk hydrogels using a photo-mediated thiol-ene reaction. Detailed procedures for the synthesis of NorHA are described elsewhere[38]. However, this protocol is highly adaptable to any photocrosslinkable hydrogel. See Discussion for more information.

1. Predetermine desired concentrations of polymer, crosslinker, and initiators for the bulk hydrogel formulation. In this protocol, the hydrogel precursor solution consists of NorHA (2 wt.%, ~25% degree of norbornene modification), dithiothreitol (DTT, 6 mM), and Irgacure D-2959 (I2959, 0.05 wt.%). Ensure that the components (1 mL) are fully dissolved in phosphate-buffered saline (PBS) within a microcentrifuge tube.
   NOTE: When preparing the hydrogel precursor solution, high molecular weight FITC-dextran (2 MDa, 0.1 wt. %) can be added to the solution to visualize microgels fabricated later in the protocol using fluorescent microscopy.
2. Load a 3 mL syringe with the hydrogel precursor solution.
   1. Remove the plunger from the back of an empty 3 mL syringe and add a tip cap to the top of the syringe barrel.
   2. Use a 1,000 µL pipette to transfer the hydrogel precursor solution into the syringe barrel with the tip cap.
   3. Hold the syringe barrel with hydrogel precursor solution in one hand, with the tip cap facing down and the open end of the barrel facing up. With the other hand, return the syringe plunger to the opening of the back of the syringe barrel. Gently push the syringe plunger into the barrel, just enough to seal the opening at the back of the syringe barrel.
   4. Carefully holding the plunger and syringe barrel together to ensure the back of the syringe barrel is sealed with the plunger, invert the syringe such that the plunger is facing down, and the tip cap is now facing up. Remove the tip cap and gently push the plunger into the syringe barrel until all the air is removed from the syringe (just hydrogel precursor solution remains).
   5. Reattach the tip cap to the syringe. Ensure that the hydrogel precursor solution is secured within the 3 mL syringe with a tip cap.
3. Form a bulk hydrogel within the 3 mL syringe.
   1. Ensure proper personal protective equipment (PPE) and safeguards are taken prior to turning on the UV lamp. This includes wearing UV-protectant eyeglasses and enclosing the lamp area to protect others from UV light.
   2. Calibrate the UV spot cure lamp to a light intensity of 10 mW/cm^2 using a radiometer.
      NOTE: There will be light attenuation through the syringe barrel. Prior to fabrication, determine the percentage of light attenuation present using a radiometer. Light intensity output from the spot curing system should be adjusted accordingly to account for such attenuation.
   3. Place the 3 mL syringe loaded with the hydrogel precursor solution under the UV spot cure lamp for a desired amount of time to fully photocrosslink. For the system described herein, NorHA hydrogel precursor solution is exposed to UV light for 5 min at an intensity of 10 mW/cm^2, which, based on prior studies[39], was sufficiently enough time and light intensity to ensure complete crosslinking as determined by photocrosslinking oscillatory shear rheology time sweeps.
      NOTE: To ensure complete photocrosslinking within the syringe, the syringe can be flipped halfway through the photocrosslinking period.

4. Turn off the UV lamp and remove the syringe. Ensure that the hydrogel is now photocrosslinked within the syringe. This can be done by pulling back the plunger and observing the hydrogel move as a solid block rather than a viscous liquid.

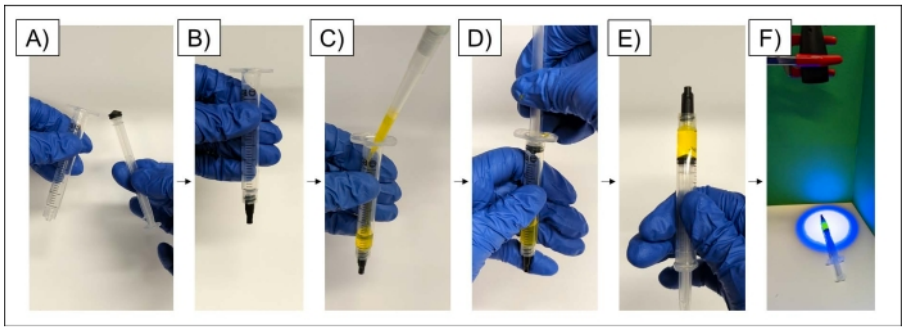

Figure 1: Overview of fabricating bulk hydrogels inside a syringe using photocrosslinking. The figure depicts (**A**) removing the plunger from the syringe, (**B**) securing the tip cap to the syringe barrel, (**C**) adding hydrogel precursor to the syringe barrel, (**D**) returning the plunger to the syringe, (**E**) removing excess air and securing the tip cap, and (**F**) photocrosslinking bulk hydrogel inside of the syringe. Please click here to view a larger version of this figure.

2. Fabricating microgels using extrusion fragmentation

NOTE: An overview of microgel fabrication using extrusion fragmentation is shown in **Figure 2**.

1. Remove the plunger from the back of an empty 3 mL syringe. Secure a tip cap to the Luer-Lock.
2. Remove the tip cap from the syringe containing the photocrosslinked bulk hydrogel. Line up the top of the hydrogel syringe with the opening of the barrel on the empty syringe.
3. Extrude the bulk hydrogel through the syringe opening (no needle attached) into the barrel of the empty syringe. Properly discard the syringe that is now empty (formerly contained the hydrogel) into the proper waste stream.
4. Hold the syringe that contains the extruded hydrogel such that the tip cap is facing down, and the barrel opening is facing up. Using a 1,000 µL pipette, add 1.5 mL of PBS to the syringe barrel.
5. Align the syringe plunger with the opening of the barrel, just barely pushing the plunger in enough to create a seal. Invert the syringe such that the plunger is now facing down and the tip cap is facing up, making sure to hold the plunger and syringe barrel together in place so that no hydrogel or PBS leaks out. Invert multiple times to mix the fragmented hydrogel with the PBS added.
6. Hold the syringe such that the tip cap is facing up and the plunger is facing down. Remove the tip cap. Very gently push the plunger upwards to remove any air from the inside of the syringe.

NOTE: There will likely be a groove in the back of the 3 mL syringe that will require extra force to push the plunger in. Very carefully push the plunger over the groove. Any sudden or harsh amount of force will cause the plunger to move too fast and possibly expel the fragmented hydrogel suspension.

7. Extrude the fragmented hydrogel suspension through a series of needles to create fragmented microgels.
    1. Secure a blunt-tip 18 G needle to the top of the syringe containing the fragmented hydrogel and PBS. Remove the plunger from a fresh 3 mL syringe and secure a tip cap to the empty syringe barrel.
    2. Extrude the fragmented hydrogel suspension through the 18 G needle into the back of the empty syringe barrel. Discard the empty syringe and needle into the proper sharps waste stream.
    3. Hold the syringe that contains the fragmented hydrogel suspension such that the tip cap is facing down and the barrel opening is facing up. Align the syringe plunger with the opening of the barrel, just barely pushing the plunger in enough to create a seal.
    4. Invert the syringe such that the plunger is now facing down, and the tip cap is facing up, making sure to hold the plunger and syringe barrel together so that no hydrogel or PBS leaks out.
    5. Hold the syringe such that the tip cap is facing up and the plunger is facing down. Remove the tip cap. Very gently push the plunger upwards to remove any air from the inside of the syringe. See the NOTE above regarding gently pushing the syringe plunger inward to prevent unwanted expulsion of hydrogel material.
    6. Repeat steps 2.7.1-2.7.5 with a 23 G, 27 G, and 30 G needle. Upon the last extrusion step (30 G needle), extrude the fragmented hydrogel suspension into microcentrifuge tubes. For the volumes described herein, the final fragmented hydrogel suspension volume will be ~2.5 mL, requiring two 1.5 mL microcentrifuge tubes (volume split equally).

       NOTE: No excessive force should be required to extrude fragmented hydrogel suspension through the needles. For best safety practices, it is recommended to perform all extrusion fragmentation steps inside of a chemical hood to provide protection in the event of syringe over-pressurization during extrusion. In addition, this process can be easily performed in a biosafety cabinet/laminar flow hood to maintain sterility during fabrication. See Discussion for additional troubleshooting suggestions.

8. Wash and isolate the fragmented hydrogel suspension.

   NOTE: Washing fragmented microgels will help to remove any unreacted polymer and crosslinker. In addition, centrifuging will help to isolate the microgels from the suspension by forming a pellet.
    1. Using a microcentrifuge, spin down the fragmented microgel suspension at 5,000 x *g* for 5 min.
    2. Use a pipette to remove the supernatant. Add 1 mL of PBS to each microcentrifuge tube containing fragmented microgels and vortex for 5-10 s.
    3. Repeat centrifuging and washing with PBS 3x.

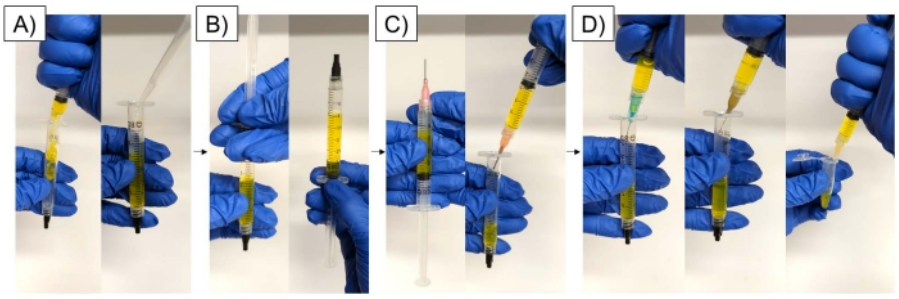

Figure 2: Overview of microgel fabrication using extrusion fragmentation. The figure depicts (**A**) extruding bulk hydrogel into an empty syringe barrel and adding PBS, (**B**) securing a plunger in the syringe with fragmented hydrogel, (**C**) attaching an 18 G needle and extruding fragmented hydrogel suspension into an empty syringe barrel, and (**D**) repeating extrusion fragmentation steps with 23 G, 27 G, and 30 G needles, collecting fragmented hydrogel suspension in microcentrifuge tubes on final extrusion. Please click here to view a larger version of this figure.

3. Characterizing fragmented microgels using ImageJ

NOTE: An overview of characterizing the fragmented microgels using ImageJ is shown in **Figure 3**, as well as representative results for describing size distributions and shapes within a batch of fragmented microgels. Microgels should be fluorescently labeled prior to visualization. For example, high molecule weight FITC-dextran (2 MDa) can be encapsulated in the bulk hydrogel prior to fragmentation to create fluorescein-labeled microgels.

1. Combine 20 µL of fragmented microgel suspension with 180 µL of PBS to create a dilute fragmented microgel suspension. Vortex to mix thoroughly.
2. Transfer 50 µL of dilute fragmented microgel suspension to a glass microscope slide.
3. Use an epifluorescent microscope to acquire images of fluorescently-labeled microgels at 4x or 10x zoom.

NOTE: The microgel suspension should be dilute enough such that neighboring microgels are not in contact with one another, yet concentrated enough such that dozens of microgels are visible in the viewport. The dilution of microgel suspension can be adjusted accordingly to achieve this.

4. Using ImageJ to analyze fragmented microgel particles. Additional information on using the **Analyze Particles** functionality in ImageJ can be found in the link provided in the **Table of Materials**.
    1. Open the images of microgels in suspension in ImageJ.
    2. Select **Analyze > Set Measurements**, Check **Area, Shape Descriptors**, and **Feret's Diameter**. Click **OK**.
    3. Select **Image > Type > 8-bit**.
    4. Select **Image > Adjust > Threshold**. Adjust the threshold such that microgels are covered by a red

mask, and the background remains black. Click on **Apply**.

NOTE: If any microgels are slightly overlapping, use the **Pencil Tool** to draw a thin (<5 pixels) black line between microgels to separate them in the black and white image.

5. Select **Analyze > Analyze Particles**. Set **Size** (pixel^2) from 50-Infinity to reduce background noise. Set **Circularity** to 0.00-1.00. Select **Show Outlines** from the Dropdown menu. Check **Display Results**, **Exclude on Edges**, and **Include Holes**. Leave the remaining boxes unchecked. Click on **OK**.

6. A results display will open, including the area, shape descriptors, and Feret's diameter information for each microgel identified. Copy and paste the results into a spreadsheet.

7. Determine the equivalent circular diameter for each particle.

    1. Obtain the image scale in μm/pixel from the scale bar or the instrument information. Create a column in the spreadsheet that converts the area of each microgel from pixel^2 to μm^2.

    2. Use the area in μm^2 to determine the equivalent circular diameter of the microgel in μm (i.e., take the square root of the area divided by pi, then double it).

8. Use the μm/pixel scale to convert the Feret's diameters (i.e., the longest distance between any two points on the particle boundary) for each microgel to a unit of μm.

9. Circularity ("Circ."), aspect ratio ("AR"), roundness ("Round"), and solidity values for each microgel can be used as is direct from ImageJ.

10. Analyze the microgel population as desired, considering the distribution of diameters (equivalent circular and Feret's), circularity, aspect ratio, roundness, and solidity.

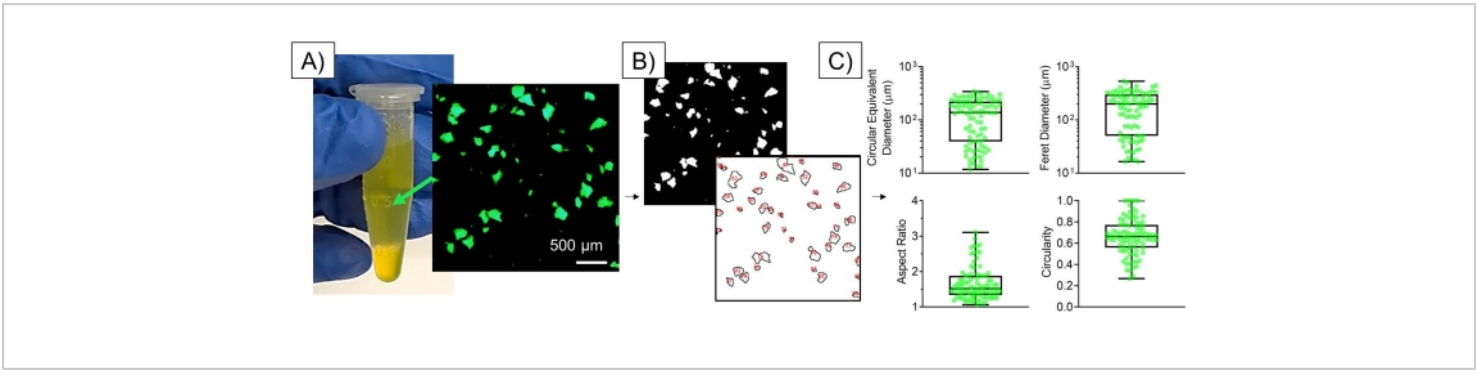

Figure 3: Overview of characterizing fragmented microgel particles using ImageJ. The figure depicts (**A**) creating a dilute suspension of fragmented microgel particles and using an epifluorescent or confocal microscope to image microgels in suspension (scale bar = 500 µm), (**B**) converting to a binary image in ImageJ and analyzing particles (count, shape descriptors, etc.), and (**C**) representative results. Error bars depict min and max with inner quartile ranges demarcated. A population size of n = 100 microgels is shown. Please click here to view a larger version of this figure.

4. Assembling fragmented microgels into granular hydrogels

NOTE: Two methods for the formulation of granular hydrogels from fragmented microgels are presented, using centrifugation and filtration. The method used will depend on the desired microgel packing (i.e., filtration packs particles more densely) and whether biological components are included (i.e., centrifugation will retain components between particles, whereas in filtration these may be lost). Prior work[40] thoroughly describes comparative outcomes (i.e., mechanics, porosity) for granular hydrogels formed from either centrifuge or vacuum-driven filtration.

1. Option 1: Jam fragmented microgels using centrifugation.
    1. After removing PBS supernatant from the last washing step, add 1 mL of PBS to each microcentrifuge tube and resuspend the microgels.
    2. Spin down the fragmented hydrogel suspension at 18,000 x *g* for 5 min.

        NOTE: Slower centrifuge speeds can be used for the jamming of microgels into granular hydrogels with less dense packing if desired.
    3. Remove the PBS supernatant.
    4. Obtain a fresh 3 mL syringe and remove the plunger. Use a metal spatula to scoop the fragmented granular hydrogel out of the microcentrifuge tube and transfer it into the back of the empty syringe barrel. A pipette tip can be used to assist in transferring the granular hydrogel into the syringe. Return the plunger to the syringe. Now load the fragmented granular hydrogel into the syringe, and it is ready for use.
2. Option 2: Jam fragmented microgels using vacuum-driven filtration. An overview of jamming by vacuum-driven filtration is depicted in **Figure 4**.

1. Assemble and test the vacuum-driven filtration apparatus.
    1. Secure a Buchner funnel inside of a filter flask, placing the filter adapter in between the funnel and the flask opening.
    2. Use tubing to connect the filter flask to a vacuum line.
    3. Place a membrane filter (0.22 µm) in the Buchner funnel cup.
    4. Turn on the vacuum line by opening the dial valve. Test the connection by pipetting ~0.5 mL of PBS onto the membrane filter and observe that all the PBS goes through the filter and collects in the bottom of the filter flask.
2. Turn on the vacuum line and ensure a complete seal. Vortex the fragmented hydrogel suspension so that microgels are suspended in PBS.
3. Using a 1,000 µL pipette, transfer the fragmented hydrogel suspension onto the membrane filter (0.22 µm). After transferring the entire microgel suspension, wait for ~30 s for the vacuum to pull PBS out of the fragmented hydrogel suspension. Turn off the vacuum line.

    NOTE: The time that the fragmented hydrogel suspension sits on the membrane filter while pulling vacuum can be varied. See Discussion for more information and troubleshooting suggestions.
4. Obtain a fresh 3 mL syringe and remove the plunger. Use a metal spatula to scoop the fragmented granular hydrogel from the filter and transfer it into the back of the empty syringe barrel. A pipette tip can be used to assist in transferring the granular hydrogel into the syringe. Return the plunger to the syringe. Load the fragmented granular hydrogel into the syringe, and it is now ready for use.

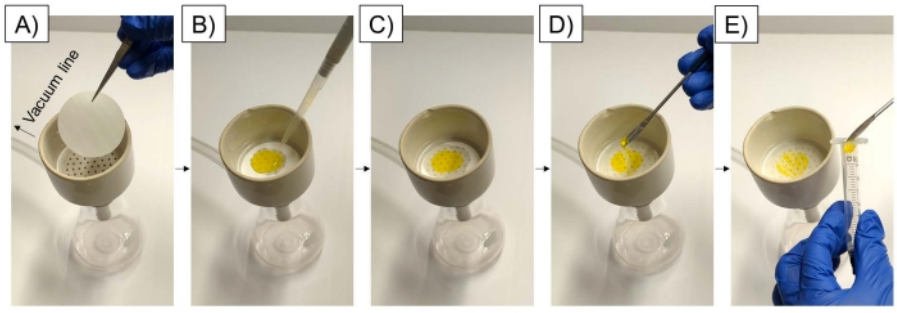

Figure 4: Overview of jamming microgels by vacuum-driven filtration to fabricate tightly-packed fragmented granular hydrogels. The figure depicts (**A**) placing a membrane filter on the vacuum filtration apparatus, (**B**) using a pipette to transfer fragmented microgel suspension onto the filter, (**C**) pulling the vacuum and waiting for microgels to jam and form a granular hydrogel, (**D**) turning off the vacuum and removing fragmented granular hydrogel using a metal spatula, and (**E**) using a metal spatula to transfer granular hydrogel to the syringe. Please click here to view a larger version of this figure.

5. Extrusion printing with granular hydrogel inks

NOTE: An overview of the extrusion printing process is shown in **Figure 5**, including a representative print of a star-shaped construct using fragmented granular hydrogels jammed with vacuum-driven filtration. The printing workflow consists of formulating an ink, planning the print design, and then printing the ink based on the desired design[41]. If desired, printed granular hydrogel constructs can be stabilized using photocrosslinking post-extrusion by adding excess DTT (5 mM) and I2959 (0.05 wt.%) to the fragmented microgel suspension prior to jamming. This will result in photocrosslinked covalent bonds formed between the microgels, leading to permanent stabilization of the granular hydrogel construct.

1. Ink formulation
    1. During the planning process, keep in mind the properties of the ink to be used. To characterize the ink, complete the rheological analysis of the fragmented hydrogels to help inform the print design process. Methods describing the rheological characterization of granular hydrogels are described elsewhere and can be adapted for this study[40].
    2. From the rheological analysis, select a printing platform and a series of initial print parameters.
    NOTE: Due to the overall high viscosity and shear-thinning properties of granular hydrogel inks, screw-based extrusion printers are typically used.
2. Print design
    NOTE: Repetier Host software (henceforth referred to as 3D printing software) is used for 3D printing applications (steps 5.2-5.3).
    1. Create the print designs through computer-aided design (CAD) software. Users can create novel designs from scratch or modify pre-existing designs, such as from patient tissue scans or from other users. For more information on creating CAD designs, please refer to the following references[41,42,43].
    2. To process CAD models into G-Code, ensure that the CAD file is saved in ".stl" format (**Supplemental File 1**) and uploaded to the 3D printing software by selecting the load button in the top panel or selecting **File > Load** in the menu bar. This G-code defines the printing path for the deposition of the ink. An example .stl file of a hollow cylinder has been included in the supplemental files.
    3. Once an STL file has been uploaded to the 3D printing software, navigate to the **Slicer** panel and select **Slic3r** as the slicer option. Here, settings such as nozzle diameter, layer height, print speed, and extrusion rate can be adjusted based on ink characterization and desired printing outcomes. In this protocol, an 18 G needle (inner diameter of 838 µm) is used. Layer height is set to 1 mm, print speed is set to 8 mm/s, and flow rate is set to 9 µL/s, based on previous optimization[39]. Numerical values of parameters may be adjusted by ± 20% to account for variations in the properties of granular hydrogel inks.
    NOTE: It is important to note that these settings and the print design may need to be adjusted through iterative experimental tests, depending on adjustments to the ink formulation, desired print resolution, or printing platform used. For

more information on these parameters, as well as on the characterization of print settings with a novel ink formulation, please refer to other references[40,44,45,46].

3. Extrusion printing with fragmented granular hydrogels

    1. For loading syringes with fragmented granular hydrogels, see 4.2.4, as well as **Figure 4** and **Figure 5**.

    2. Remove the tip cap and replace it with a needle of choice.

    3. Load the syringe into the printing platform of choice. Here, a custom-built screw-based extrusion printer is used.

        NOTE: For information on building custom bioprinters, please see other references[44,47].

    4. Load the prepared G-Code file from the planning phase into the 3D printing software. Navigate to the **Print Preview** panel and press **Print**.

    5. As soon as the printing deposition is complete, expose the fragmented granular hydrogel constructs to UV light for photocrosslinking and stabilization.

    6. Once crosslinking is complete, process the sample by washing it in PBS three times.

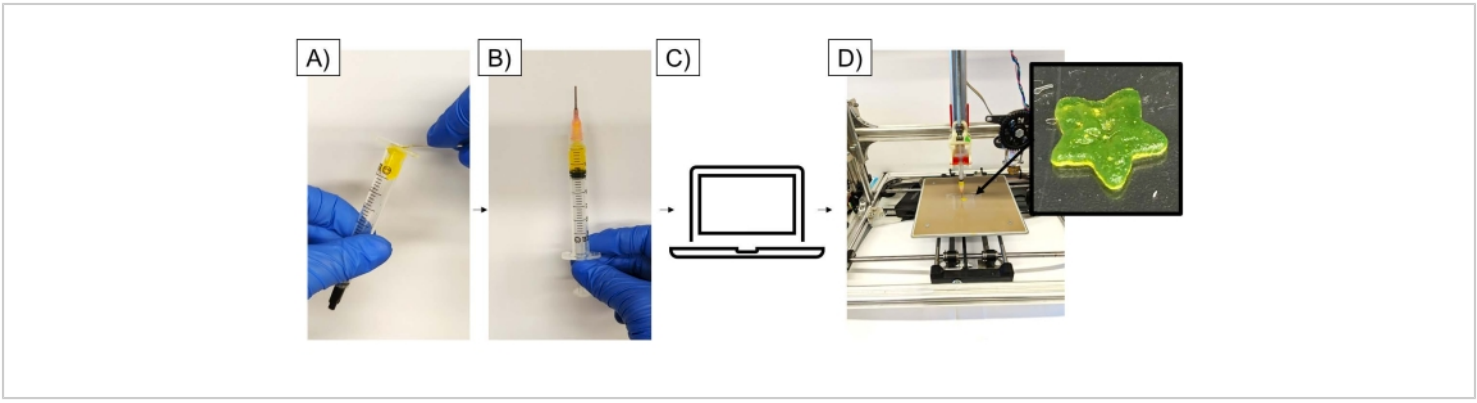

Figure 5: Overview of extrusion printing with fragmented granular hydrogels. The figure depicts (**A**) using a spatula to transfer fragmented granular hydrogel to a syringe barrel, (**B**) attaching a blunt-tip needle (18 G shown) and pushing the sample to the top, (**C**) a graphic representing the connection to computer software for printing, and (**D**) completing the printing of a star-shaped construct with fragmented granular hydrogel. Please click here to view a larger version of this figure.

Representative Results

Representative results from these protocols are shown in **Figure 3** and **Figure 6**. Extrusion fragmentation yields microgels with jagged, polygon shapes with diameters ranging from 10-300 µm (**Figure 3**). Further, circularity ranges from 0.2 (not circular) to almost 1 (perfect circle), and the aspect ratio ranges from 1-3 (**Figure 3**). These parameters describe the irregular and jagged microgel shapes formed by the fragmentation process.

When packed together using either centrifugation or vacuum-driven filtration, the assembled granular hydrogel is shear-

thinning and self-healing, as described in the previous work[39]. In addition, the fragmented granular hydrogel has high shape fidelity and mechanical integrity for an injectable hydrogel, as shown by the deposition of a hollow cylinder with a height of 2 cm being extrusion printed in **Figure 6**. Fragmented granular hydrogels fabricated with these simple and cost-effective methods are useful for many biomedical applications, including injectable therapeutics and 3D printing inks.

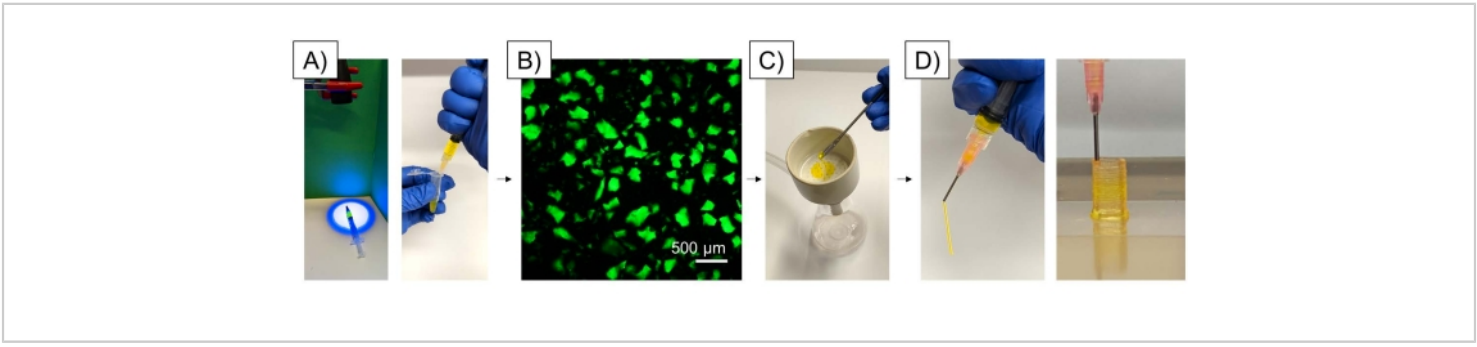

Figure 6: Protocol overview and representative results. The figure depicts (**A**) fragmentation, (**B**) microgels in suspension, (**C**) jamming by vacuum-driven filtration, and (**D**) jammed granular hydrogel being extruded through a needle and printed into a hollow cylinder. Please click here to view a larger version of this figure.

Supplemental File 1: Example .stl file Please click here to download this File.

Discussion

Herein, methods to fabricate granular hydrogels using extrusion fragmented microgels and packing by either centrifugation or vacuum-driven filtration are described. Compared to other microgel fabrication methods (i.e., microfluidics, batch emulsions, electrospraying, photolithography), extrusion fragmentation microgel fabrication is highly rapid, low-cost, easily scalable, and amenable to a wide variety of hydrogel systems. Further, this protocol is highly repeatable with minimal batch-to-batch variability, which was characterized in the previous work[39].

This protocol uses norbornene-modified hyaluronic acid (NorHA) to fabricate bulk hydrogels using a photo-mediated thiol-ene reaction. Detailed procedures for the synthesis of NorHA are described elsewhere[38]. However, many hydrogel chemistries can be used to fabricate fragmented microgels using the methods described herein if a bulk hydrogel can be formed within the barrel of a syringe. It is also useful to understand the bulk hydrogel mechanical properties (e.g., compressive modulus). The bulk hydrogels used in this protocol have a bulk compressive modulus of about 30 kPa[39]. A bulk hydrogel with a higher compressive modulus will require more force to extrude during the fragmentation steps, which could lead to increased clogging or over-pressurization of the syringes; thus, it is recommended to use hydrogels with compressive moduli less than 80 kPa. Further, a bulk hydrogel with compressive moduli lower than 10 kPa may deform during the fragmentation steps, making it challenging to fragment.

This protocol is optimized for a UV spot cure lamp. As an alternative to the UV light source and UV-

responsive photoinitiators, visible light sources can also be used along with visible light-responsive photoinitiators, such as water-soluble lithium phenyl-2,4,6-trimethylbenzoyl-phosphinate (LAP). Initiator concentration, light intensity, and sample volume will influence crosslinking times depending on the polymer and crosslinking system being used. Further, many lamp sources can be used as an alternative to spot cure systems.

The most critical step in the protocol is the serial extrusion through smaller and smaller needle gauges. In this procedure, it is suggested to use needle gauges from 18 G (838 µm inner diameter) down to 30 G (159 µm inner diameter). Adding PBS to the fragmented bulk hydrogel prior to extruding through needles is crucial to significantly reduce the force needed to extrude and fragment. No excessive force should be used to extrude the hydrogel, as excessive force can lead to back pressurization in the syringe and risk bursting the hydrogel out of the syringe back. Additional strategies to reduce the force required to extrude include using more needles in the series to reduce fragment size more gradually, as well as adding additional PBS between fragmenting steps.

When jamming the fragmented microgels using vacuum-driven filtration, there may be variability in the process. Some material systems may require more (or less) time to remove PBS and fully jam the microgels. It is suggested to record the time needed for individual material systems to ensure repeatability across experiments. The time to jam will also be dependent on the thickness and size of the sample added to the filter. Spreading the sample out evenly across the filter can help with uniform jamming.

The extrusion fragmentation microgel fabrication method can be adapted for many biomedical applications. For instance, therapeutics can be included in the hydrogel precursor solution and subsequently encapsulated within fragmented microgels to fabricate a shear-thinning, self-healing granular hydrogel for localized therapeutic delivery. In addition, fragmented microgels can be dried to allow for long-term storage and straightforward sterilization practices. However, one limitation to the extrusion fragmentation is the incorporation of cells within microgels. Due to the high shear rates during extrusion fragmentation, the method is likely not amenable to cell encapsulation within microgels, as the high shear may lead to significantly decreased cell viability. Still, cells and spheroids can easily be incorporated between microgels for *in vitro* culture and *in vivo* cell delivery.

Fragmented granular hydrogels are a promising biomaterial for biomedical applications. In recent years, granular hydrogels made from various fragmentation methods (i.e., mortar and pestle, blenders, and mesh graters) have been used as cell-laden 3D printing inks[48], therapeutic delivery vehicles[29], injectable tissue repair scaffolds[30], and spheroid-culture platforms[39]. Of the fragmentation methods previously reported, the extrusion fragmentation method described herein is one of the most simple and cost-effective methods with numerous advantages. Sharing the methods herein will increase accessibility to granular hydrogel fabrication and lead to significant advances in the growing field of granular hydrogel biomaterials, allowing more researchers to engineer innovative biomedical solutions with fragmented granular hydrogels.

Disclosures

The authors have no competing financial interests.

Acknowledgments

This work was supported by the National Science Foundation through the UPenn MRSEC program (DMR-1720530) and

graduate research fellowships (to V.G.M and M.E.P.) and the National Institutes of Health (R01AR077362 to J.A.B.).

References

1. Daly, A. C., Riley, L., Segura, T., Burdick, J. A. Hydrogel microparticles for biomedical applications. *Nature Reviews Materials.* **5** (1), 20-43 (2020).
2. Qazi, T. H., Burdick, J. A. Granular hydrogels for endogenous tissue repair. *Biomaterials and Biosystems.* **1** (November 2020), 100008 (2021).
3. Riley, L., Schirmer, L., Segura, T. Granular hydrogels: emergent properties of jammed hydrogel microparticles and their applications in tissue repair and regeneration. *Current Opinion in Biotechnology.* **60**, 1-8 (2019).
4. Highley, C. B., Song, K. H., Daly, A. C., Burdick, J. A. Jammed microgel inks for 3D printing applications. *Advanced Science.* **6** (1), 1801076 (2019).
5. Griffin, D. R., Weaver, W. M., Scumpia, P. O., Di Carlo, D., Segura, T. Accelerated wound healing by injectable microporous gel scaffolds assembled from annealed building blocks. *Nature Materials.* **14** (7), 737-744 (2015).
6. Xin, S., Chimene, D., Garza, J. E., Gaharwar, A. K., Alge, D. L. Clickable PEG hydrogel microspheres as building blocks for 3D bioprinting. *Biomaterials Science.* **7** (3), 1179-1187 (2019).
7. Hirsch, M., Charlet, A., Amstad, E. 3D printing of strong and tough double network granular hydrogels. *Advanced Functional Materials.* **31** (5), 2005929 (2021).
8. Seymour, A. J., Shin, S., Heilshorn, S. C. 3D printing of microgel scaffolds with tunable void fraction to promote cell infiltration. *Advanced Healthcare Materials.* **10** (18), 2100644 (2021).
9. Xin, S. et al. Generalizing hydrogel microparticles into a new class of bioinks for extrusion bioprinting. *Science Advances.* **7** (42), eabk3087 (2021).
10. de Rutte, J. M., Koh, J., Di Carlo, D. Scalable high-throughput production of modular microgels for in situ assembly of microporous tissue scaffolds. *Advanced Functional Materials.* **29** (25), 1900071 (2019).
11. Qazi, T. H. et al. Anisotropic rod-shaped particles influence injectable granular hydrogel properties and cell invasion. *Advanced Materials.* **34** (12), 2109194 (2021).
12. Darling, N. J., Sideris, E., Hamada, N., Carmichael, S. T., Segura, T. Injectable and spatially patterned microporous annealed particle (MAP) hydrogels for tissue repair applications. *Advanced Science.* **5** (11), 1-8 (2018).
13. Hsu, R. S. et al. Adaptable microporous hydrogels of propagating NGF-gradient by injectable building blocks for accelerated axonal outgrowth. *Advanced Science.* **6** (16), 1900520 (2019).
14. Sheikhi, A. et al. Microfluidic-enabled bottom-up hydrogels from annealable naturally-derived protein microbeads. *Biomaterials.* **192** (September 2018), 560-568 (2019).
15. Griffin, D. R. et al. Activating an adaptive immune response from a hydrogel scaffold imparts regenerative wound healing. *Nature Materials.* **20** (4), 560-569 (2021).
16. Pruett, L. J., Jenkins, C. H., Singh, N. S., Catallo, K. J., Griffin, D. R. Heparin microislands in microporous annealed particle scaffolds for accelerated diabetic wound healing. *Advanced Functional Materials.* **31** (35), 1-12 (2021).

17. Feng, Q. et al. Engineering the cellular mechanical microenvironment to regulate stem cell chondrogenesis: Insights from a microgel model. *Acta Biomaterialia.* **113**, 393-406 (2020).

18. Caldwell, A. S., Rao, V. V., Golden, A. C., Anseth, K. S. Porous bio-click microgel scaffolds control hMSC interactions and promote their secretory properties. *Biomaterials.* **232** (December 2019), 119725 (2020).

19. Muir, V. G., Qazi, T., Shen, J., Groll, J., Burdick, J. Influence of microgel fabrication technique on granular hydrogel properties. *ACS Biomaterials Science and Engineering.* **7** (9), 4269-4281 (2021).

20. Jivan, F. et al. Sequential thiol-ene and tetrazine click reactions for the polymerization and functionalization of hydrogel microparticles. *Biomacromolecules.* **17** (11), 3516-3523 (2016).

21. Truong, N. F., Lesher-Pérez, S. C., Kurt, E., Segura, T. Pathways governing polyethylenimine polyplex transfection in microporous annealed particle scaffolds. *Bioconjugate Chemistry.* **30** (2), 476-486 (2019).

22. Riederer, M. S., Requist, B. D., Payne, K. A., Way, J. D., Krebs, M. D. Injectable and microporous scaffold of densely-packed, growth factor-encapsulating chitosan microgels. *Carbohydrate Polymers.* **152**, 792-801 (2016).

23. Xin, S., Wyman, O. M., Alge, D. L. Assembly of PEG microgels into porous cell-instructive 3D scaffolds via thiol-ene click chemistry. *Advanced Healthcare Materials.* **7** (11), 1-7 (2018).

24. Isaac, A. et al. Microporous bio-orthogonally annealed particle hydrogels for tissue engineering and regenerative medicine. *ACS Biomaterials Science and Engineering.* **5** (12), 6395-6404 (2019).

25. Xin, S., Gregory, C. A., Alge, D. L. Interplay between degradability and integrin signaling on mesenchymal stem cell function within poly(ethylene glycol) based microporous annealed particle hydrogels. *Acta Biomaterialia.* **101**, 227-236 (2020).

26. Yao, M. H. et al. Directed self-assembly of polypeptide-engineered physical microgels for building porous cell-laden hydrogels. *Chemical Communications.* **50** (66), 9405-9408 (2014).

27. Han, Y. L. et al. Directed self-assembly of microscale hydrogels by electrostatic interaction. *Biofabrication.* **5** (3) 035004 (2013).

28. Gehlen, D. B. et al. Granular cellulose nanofibril hydrogel scaffolds for 3D cell cultivation. *Macromolecular Rapid Communications.* **41** (18), 2000191 (2020).

29. Kurt, E., Segura, T. Nucleic acid delivery from granular hydrogels. *Advanced Healthcare Materials.* 11 (3), 2101867 (2021).

30. Hsu, C. C. et al. Increased connectivity of hiPSC-derived neural networks in multiphase granular hydrogel scaffolds. *Bioactive Materials.* **9**, 358-372 (2021).

31. Feig, V. R. et al. Conducting polymer-based granular hydrogels for injectable 3D cell scaffolds. *Advanced Materials Technologies.* **6** (6), 2100162 (2021).

32. Zhang, H. et al. Direct 3D printed biomimetic scaffolds based on hydrogel microparticles for cell spheroid growth. *Advanced Functional Materials.* **30** (13), 1-10 (2020).

33. Sinclair, A. et al. Self-healing zwitterionic microgels as a versatile platform for malleable cell constructs

and injectable therapies. *Advanced Materials.* **30** (39), 1803087 (2018).

34. Kessel, B. et al. 3D bioprinting of macroporous materials based on entangled hydrogel microstrands. *Advanced Science.* **7** (18), 2001419 (2020).
35. Hinton, T. J. et al. Three-dimensional printing of complex biological structures by freeform reversible embedding of suspended hydrogels. *Science Advances.* **1** (9), e1500758 (2015).
36. Koetting, M. C., Guido, J. F., Gupta, M., Zhang, A., Peppas, N. A. pH-responsive and enzymatically-responsive hydrogel microparticles for the oral delivery of therapeutic proteins: Effects of protein size, crosslinking density, and hydrogel degradation on protein delivery. *Journal of Controlled Release.* **221**, 18-25 (2016).
37. Heo, D. N. et al. 3D bioprinting of carbohydrazide-modified gelatin into microparticle-suspended oxidized alginate for the fabrication of complex-shaped tissue constructs. *ACS Applied Materials and Interfaces.* **12** (18), 20295-20306 (2020).
38. Gramlich, W. M., Kim, I. L., Burdick, J. A. Synthesis and orthogonal photopatterning of hyaluronic acid hydrogels with thiol-norbornene chemistry. *Biomaterials.* **34** (38), 9803-9811 (2013).
39. Muir, V. G. et al. Sticking together: Injectable granular hydrogels with increased functionality via dynamic covalent inter-particle crosslinking. *Small.* **2201115** (2022).
40. Qazi, T. H., Muir, V. G., Burdick, J. A. Methods to characterize granular hydrogel rheological properties, porosity, and cell invasion. *ACS Biomaterials Science & Engineering.* In Press (2022).
41. Daly, A. C., Prendergast, M. E., Hughes, A. J., Burdick, J. A. Bioprinting for the biologist. *Cell.* **184** (1), 18-32 (2021).
42. Pakhomova, C., Popov, D., Maltsev, E., Akhatov, I., Pasko, A. Software for bioprinting. *International Journal of Bioprinting.* **6** (3), 41-61 (2020).
43. Junk, S., Kuen, C. Review of open source and freeware CAD systems for use with 3D-printing. *Procedia CIRP.* **50**, 430-435 (2016).
44. Bessler, N. et al. Nydus one syringe extruder (NOSE): A Prusa i3 3D printer conversion for bioprinting applications utilizing the FRESH-method. *HardwareX.* **6**, e00069 (2019).
45. Skardal, A. et al. Bioprinting cellularized constructs using a tissue-specific hydrogel bioink. *Journal of Visualized Experiments: JoVE.* **110**, 53606 (2016).
46. Thayer, P. S., Orrhult, L. S., Martínez, H. Bioprinting of cartilage and skin tissue analogs utilizing a novel passive mixing unit technique for bioink precellularization. *Journal of Visualized Experiments: JoVE.* **131**, 56372 (2018).
47. Pusch, K., Hinton, T. J., Feinberg, A. W. Large volume syringe pump extruder for desktop 3D printers. *HardwareX.* **3** (November 2017), 49-61 (2018).
48. Ding, A. et al. Jammed micro-flake hydrogel for 4D living cell bioprinting. *Advanced Materials.* **34** (15), e2109394 (2022).