Video Article

Three-dimensional Biomimetic Technology: Novel Biorubber Creates Defined Micro- and Macro-scale Architectures in Collagen Hydrogels

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URL: https://www.jove.com/video/53578
DOI: doi:10.3791/53578

Keywords: Bioengineering, Issue 108, Biomimetic, Collagen, BSA, Tissue engineering, Biofabrication, Biomaterials, Molding, Casting, Hydrogels, computer-aid software, CAD, 3D scaffolds

Date Published: 2/12/2016

Citation: Rodriguez-Rivera, V., Weidner, J.W., Yost, M.J. Three-dimensional Biomimetic Technology: Novel Biorubber Creates Defined Micro- and Macro-scale Architectures in Collagen Hydrogels. *J. Vis. Exp.* (108), e53578, doi:10.3791/53578 (2016).

Abstract

Tissue scaffolds play a crucial role in the tissue regeneration process. The ideal scaffold must fulfill several requirements such as having proper composition, targeted modulus, and well-defined architectural features. Biomaterials that recapitulate the intrinsic architecture of *in vivo* tissue are vital for studying diseases as well as to facilitate the regeneration of lost and malformed soft tissue. A novel biofabrication technique was developed which combines state of the art imaging, three-dimensional (3D) printing, and selective enzymatic activity to create a new generation of biomaterials for research and clinical application. The developed material, Bovine Serum Albumin rubber, is reaction injected into a mold that upholds specific geometrical features. This sacrificial material allows the adequate transfer of architectural features to a natural scaffold material. The prototype consists of a 3D collagen scaffold with 4 and 3 mm channels that represent a branched architecture. This paper emphasizes the use of this biofabrication technique for the generation of natural constructs. This protocol utilizes a computer-aided software (CAD) to manufacture a solid mold which will be reaction injected with BSA rubber followed by the enzymatic digestion of the rubber, leaving its architectural features within the scaffold material.

Video Link

The video component of this article can be found at https://www.jove.com/video/53578/

Introduction

In the tissue engineering field the ability to fabricate tissue scaffolds is vital. A suitable tissue scaffold has a 3D structure, is composed of biocompatible materials, and mimics *in vivo* tissue architecture to facilitate cell and tissue growth and remodeling. This scaffold must allow the transport of nutrients and the removal of wastes[1-4]. One of the main obstacles in the production of these scaffolds is the ability to recapitulate specific geometrical features into a biocompatible material. Several biofabrication techniques have been reported to control the geometrical features of these scaffolds, examples are electrospinning[5-8], solvent-casting[9], stereolithography[10], and 3D-printing[11], among others. These techniques fall short in providing a relatively easy transfer of controllable internal and external architectural features, are expensive, are limited by their resolution and printability (*e.g.,* nozzle gauge, material restriction), or require post-fabrication techniques which demands a long period of time to produce viable scaffolds[12].

In many commercial fabrication systems, the creation of internal voids, channels, and features is achieved using sand or other suitable removable or sacrificial materials. The metal or plastic part is formed around the sand mold, and once it is solidified, the sand is removed. In much the same manner, the next generation of biomaterials needs the biosand equivalent. Therefore, the BSA rubber was developed as a substitute for biosand. The BSA rubber is a newly formulated material that consists of bovine serum albumin crosslinked with glutaraldehyde. The ultimate goal is to recreate specific architectural features into a biodegradable collagen scaffold. The characteristics of the sacrificial biorubber that maintains dimensional fidelity with the mold of the original tissue are described.

Several combinations of BSA and glutaraldehyde concentrations were tested using a variety of solvents. This material was created by the reaction between BSA and glutaraldehyde. BSA rubber can be reaction injected into the intricate geometries of the tissue molds. Crosslinked BSA is trypsin labile and readily digested by the enzyme at mild pH and temperature conditions. Conversely, intact type I collagen is very resistant to trypsin digestion. These features were capitalized to selectively remove the BSA rubber leaving the collagen behind. The present work consisted of determining the ideal parameters needed to obtain a labile mold that can deliver specific architectural features to a biocompatible scaffold. The specific features that were evaluated included mixability, enzyme digestion, load bearing, and ability to be reaction injected into a negative mold. The combination of 30% BSA and 3% glutaraldehyde fulfills these requirements. This protocol provides the necessary guidelines to create these three-dimensional scaffolds. The prototype consists of a collagen scaffold that represents a branched architecture with one inflow and two outflow channel with diameters of 4- and 3-mm, respectively. This technique has the potential to mimic macro- and micro-environments of the tissue of interest. This technology provides a viable technique to deliver a specific geometrical instructive

to a biodegradable material in a relatively easy and timely matter with high fidelity, which can be tuned to mimic the *in vivo* tissue elasticity and other characteristics of the tissue of interest.

Protocol

1. Determine the Percentage of Solids in the Collagen Batch

1. Extract the collagen following a previously published procedure[13]. Thaw a minimum of 20 ml of collagen. Determine the initial percentage of collagen solids in the batch in order to manipulate the collagen concentration in the formed hydrogels.
    1. Cut three pieces of aluminum foil (about 6 x 6 cm) and shape each one as a pan by using the bottom of a 25 ml beaker. Record the weight of each pan.
    2. Add a small amount of collagen to each pan and record the total weight of the aluminum pan and collagen. Add 0.5 - 0.8 g of collagen on each aluminum pan.
    Note: After this step, there are three aluminum foil pans and each one should have a small amount of collagen. Make sure to record the weight of each (total 3) empty aluminum pan and the weight of the pan after the addition of collagen.
    3. Calculate the weight of the collagen in each pan using the following formula:
    Collagen weight = Pan and collagen weight - Pan weight
    4. Place the three aluminum pans that hold the collagen in the oven at 100 °C for 24 hr.
    5. After 24 hr, record the weight of each aluminum pan and the dehydrated collagen.
    6. Calculate the weight of the dehydrated collagen using the following equation:
    Dehydrated collagen weight = Pan and dehydrated collagen weight - Pan weight
    7. Calculate the percentage of solid for the three samples to determine the collagen solid concentration using the formula below:

$$\text{Percentage of Collagen Solid} = \frac{\text{Dehydrated collagen weight}}{\text{Hydrated collagen weight}} \text{x } 100$$

    8. Calculate the average collagen solid content of the batch using the percentage of collagen solids for each of the three samples.
    Note: The collagen that will be used is what is left of the hydrated collagen. None of the dehydrated collagen will be used.
    9. After determining the percentage of collagen solid (initial collagen concentration) of the batch, continue using the remaining hydrated collagen. Use a calibrated pH meter to adjust the pH of the collagen batch to 3.
        1. Add small amounts (2-5 µl at a time) of 12 N Hydrochloric Acid (HCl). Keep on ice at all times. Do not add the hydrochloric acid directly to the collagen — add the acid to the side of the tube. After adding the acid, use the spatula to push the acid to the collagen and quickly stir the mixture.
    10. Once it reaches a pH of 3, let the collagen sit O/N at 4 °C.

2. Preparation of the BSA Rubber

1. Prepare the Bovine Serum Albumin (BSA) solution following the procedure listed below.
    1. Prepare 2x Phosphate Buffered Saline solution (PBS). Add two PBS tablets to 100 ml of water to make 0.02 M PBS solution.
    2. Combine BSA with 2x PBS to create a 30% BSA solution using the procedure listed below.
        1. For example, to make a 30% BSA with 30 ml of 2x PBS, use 12.9 g of BSA. Add 1/3 of the 2x PBS (*e.g.,* 10 ml) to a flask with a stir bar. Add 1/2 of the BSA (*e.g.,* 6.45 g BSA) to the flask and using a spatula, wet the dry solute.
        2. Repeat this process of adding PBS and then BSA until all the solute and solvent is in the flask. Use the spatula to wet all the solute. It will look like a mixture of some liquid and clumps. Let it sit for approximately 30 min.
        3. Then, turn the stirrer on a low cycle and make sure that there are no clumps around the stir bar. It should take 90 min to get everything in solution or it can be left stirring O/N at 4 °C. After the solute is all dissolved, place the solution in a 20 ml syringe and capped with a 0.20 µm syringe filter. Press the plunger to expel liquid through filter and collect the sterilized solution in a new tube. Store at 4 °C.
    3. Prepare 3% glutaraldehyde solution by diluting 25% glutaraldehyde solution with sterile filtered 2x PBS. For example, for 10 ml of 3% Glutaraldehyde solution, use 2 ml of 25% glutaraldehyde and 8 ml of 2x PBS. Store at 4 °C.

3. Molds Treatment

Note: The prototype described in this paper uses a custom made stainless steel Y mold piece. The mold contains an inflow and two outflow channels of 4 and 3 mm, respectively. First, clean molds, spray them with unsaturated lard, and sterilize them. Prepare the molds following the procedure described below.

1. Clean stainless steel molds using sonicator at a frequency of 35 kHz. Place the molds in the sonicator and submerge them with water and ice. Keep the molds cold at all times while the sonicator is running. Run the sonicator for 2 periods of 90 min.
    1. After each period, use a needle to make sure that there is no material in the Luer lock stainless steel or brass connector. Use soap and water to clean the entire surface of the two sides of the molds. Verify that there is no obstruction in the channels.
2. Place the molds, screws, and Luer lock connector in an autoclave bag and autoclave it.
3. Fill the bottle that attaches to an air sprayer half way with commercially available lard (mixed fatty acid release agent). Replace the cap with a regular cap bottle. Place it in an autoclave bag and autoclave it.

Note: Lard is used to facilitate the release of the material that will be reaction injected later on (BSA rubber). Do not place the sprayer bottle lid in the autoclave- it can damage the internal seal.

4. Warm up the lard for 45 sec or until its clear and liquid in a microwave. Screw the air sprayer lid to the lard bottle. Connect the lid with the sprayer. Attach the sprayer to the air source at the lab bench. Open the air valve, and open the nozzle of the sprayer until it starts wetting the surface of a paper towel.
5. Spray lard perpendicular to the mold surface until the surface is fully covered. After each piece has been sprayed, place them in a Petri dish and seal it. Place the molds at 4 °C for 2 hr.
6. Proceed to sterilize the molds by exposing the surface to UV light for 30 min. Place them back at 4 °C until they are ready to be reaction injected.

4. Reaction Injection of the BSA Rubber

Note: All the materials and solution should be keep cold until ready to use to prevent premature setting of the BSA rubber in the next steps.

1. Prepare the dispenser for delivering the BSA rubber to the molds following these steps.
    1. Sterilize all of the mixing components (two O rings, syringe cap, double syringe, mix tip, and 4:1 dispenser) by exposing them to UV light for 30 min in the polymerase chain reaction (PCR) hood.
    Note: A PCR hood was used because this procedure involves fixatives. These chemicals cannot be used in the cell/tissue culture hood due to the risk of exposure and toxicity to the cells. Any other hood that contains a UV light will be suitable.
    2. Place the tip cap on the solution holder.
    3. Perform the mixing and injection at a 4:1 ratio of BSA:Glutaraldehyde. Add the 30% BSA to the double syringe chamber that will deliver the highest amount of solution (It will take approximately 4 ml to fill). Make sure to leave enough space to place the O ring in order to prevent overflowing and contamination of the adjacent chamber.
    4. Add 3% glutaraldehyde solution to the other chamber (it will take about 1 ml to fill). Make sure to leave enough space to place the O ring to prevent overflowing and contamination of the adjacent chamber.
    5. Place the double syringe on the dispenser. Tilt the assembly vertically so that the syringe cap is on top. Replace the cap with the mixing tip.
    6. Screw the two stainless steel mold pieces together.
    7. Place the assembly inside of an autoclave bag.
    8. To remove any air in the dispenser, hold it in the upright position and quickly squeeze the handle one time to release a small amount of the BSA mixed with the glutaraldehyde. Then quickly attach the stainless steel Y mold's Luer lock connector to the syringe tip.
    9. Hold the stainless steel Y mold with the left hand and the BSA-Glutaraldehyde mixture dispenser on the right. Alternate covering each of left and right exhaust of the outflow channels by pressing the exhaust to the sides of the autoclave bag to make sure the inside voids are filled with solution. Then, place the molds horizontally and inject again.
    10. Detach the molds from dispenser and place in a 25 mm Petri dish.
    11. Place Parafilm around the Petri dish to prevent dehydration of the rubber.
    12. Place the mold in the 4 °C fridge O/N.

5. Adjusting the Collagen Concentration

Note: The collagen should be kept on ice at all times during the process.

1. Modify the collagen concentration using the percentage of collagen solids.
    1. Make 10 ml of 1.75% collagen by adjusting the initial collagen concentration with cold water.
    2. Using a calibrated pH meter, adjust the pH to 3 using 12 N Hydrochloric Acid. Do not add the hydrochloric acid directly to the collagen- add the acid to the side of the tube. After adding the acid, use the spatula to push the acid into the collagen and quickly stir the mixture.
    3. Weigh 4 g of collagen in a separate conical tube.
    4. Centrifuge the collagen to remove air at 4 °C and 9,343 x g for 20-30 min.
    5. UV sterilize the cell culture hood for 30 min, and add 14 µl of laminin to the 4 ml collagen. This will result in a final laminin concentration of 10 µg/µl. Note: Laminin provides structural integrity, adhesion, and promotes various cellular responses.
    6. Turn the PCR UV light on for 20-30 min prior to using the hood for sterilization.
    7. Place the Luer lock cap, to attach to a 20 ml syringe, in ethanol for a 2 hr. Then, allow it to dry and place it in UV light.
    8. Gamma irradiate the collagen for 8.6 min to reach 1,200 cGy.
    Note: The time will depend on the decay of the Cesium source. Adjust the time to reach the same dosage.

6.Casting Collagen on BSA Rubber

1. To polymerize the collagen, use an 8:1:1 ratio (collagen:HEPES:MEM). The following procedure is based on an initial 4 g of acidified collagen (from Step 5.1.8).
    1. Make a 0.2 N HEPES solution in water, and adjust the pH to 9 by adding small amounts (1-5 µl) of 1-5 M sodium hydroxide (NaOH) solution. Using a calibrated pH meter, monitor the pH of the solution after each addition. Store at 4 °C or keep on ice.
    2. Turn on the UV light of the tissue culture hood for 20-30 min to sterilize the hood.
    3. Autoclave forceps, spatula, and scalpel for sterilization.
    4. Mix 1.5 ml of 0.2 N HEPES (pH 9) and 1.5 ml of 10x MEM using the tissue culture hood. Make sure to keep it on ice and vortex it for 5 sec prior to use. Store at 4 °C or keep on ice.
    5. To sterilize the PCR hood, turn on the UV light for 30 min.

6. Place a 12 well plate and a 20 ml syringe in -20 °C for 10 min, or until ready to continue. Note: Keep all materials cold until ready to use. The increase in temperature induces premature collagen fibrillogenesis.
7. Spray all tubes and well plates that are going to be in the hood with 70% ethanol and let them dry for sterilization. Place a 20 ml syringe on ice to cool for later use.
8. In the PCR hood, open the stainless steel molds to release the BSA rubber and using a scalpel, cut the exhaust channels of the BSA rubber mold.
9. Under the PCR hood, open the sterile collagen tubes and add 1 ml of the HEPES-MEM solution (make sure that before extracting the HEPES-MEM solution, that it is well mixed and there are no solid deposits).
10. Using the sterile spatula, thoroughly mix the collagen and buffer solution.
11. Close and vortex it quickly to ensure a well-mixed hydrogel.
12. Transfer to a cold 20 ml syringe.
13. With one hand, hold the BSA Rubber inside of the well and, using the other, dispense half of the collagen hydrogel solution onto the bottom of the well.
14. Using the sterile tweezers, ensure that the rubber inflow and outflow ends are touching the sides of the well.
15. Pour collagen solution on top of the rubber until is completely covered.
16. Ensure that the BSA rubber is suspended within the collagen and that there are no bubbles, especially near the ends of the rubber.
17. Place the cover and wrap Parafilm around the circumference of the well.
18. Put in the incubator for 1 hr at 37 °C. Keep the PCR UV light on.

2. After the polymerization of the collagen, UV crosslink the hydrogel via the following procedure.
   Note: The crosslinking of the collagen will be done using a UV crosslinker apparatus in which the amount of energy can be controlled.
   1. Turn on the UV crosslinker and use the energy setting to irradiate the empty chamber with 630,000 μJ/cm^2.
   2. Remove the gels from the incubator.
   3. Spray hands with ethanol, and, inside the chamber, remove the lid as quickly as possible.
   4. Close the chamber and UV crosslink the hydrogels by selecting the energy setting and irradiating 630,000 μJ/cm^2.
   5. After the crosslinking cycle, turn off the UV light on the PCR hood
   6. Spray hands with ethanol and open the chamber, quickly placing the lid back onto the well plate. Move the well plate to the PCR hood.
   7. Using the sterile spatula, gently loosen and remove the gel from the well. Flip the gels under the hood to crosslink the bottom of the hydrogel. Repeat step 6.2.3 and 6.2.4.

7. Enzyme Digestion of the BSA Rubber

1. In order to have a hollow collagen scaffold, remove BSA Rubber in a way that does not affect the dimensions embedded in the hydrogel. The procedure is described below.
   1. Turn on the UV light for 20-30 min to sterilize the tissue culture hood.
   2. Make 0.25% trypsin solution pH 7.8. For example, for 15 ml of water, add 0.0376 g of trypsin in a 50 ml conical tube. Adjust the pH to 7.8 by adding small amounts (2-5 μl) of 1 M NaCl. Place the solution in a 20 ml syringe and capped with a 0.20 μm syringe filter. Press the plunger to expel liquid through filter and collect the sterile solution in a new tube.
   3. Turn on the water bath and set the temperature to 30 °C.
   4. After 30 min, turn off the UV light. Spray ethanol on all the tubes and materials that will be used in the hood.
   5. Transfer the collagen hydrogel under the hood and place in separate conical tube.
   6. Add around 3-5 ml (just enough to cover the gels) of 0.25% trypsin solution with a pH of 7.8 to each tube.
   7. Seal the tubes with Parafilm and vortex lightly for approximately 1 min.
   8. Place in the 30 °C water bath for 15-24 hr. While in the water bath, lightly vortex the gels frequently until the BSA rubber has been digested or removed from the hydrogel.
      Note: In order to determine if the BSA rubber has been removed, either the rubber is floating in the trypsin solution or there are broken-down pieces. There must be no visual dark areas within the hydrogel.

2. To ensure that all the BSA rubber and trypsin has been removed from the hydrogels, rinse it as described below.
   1. Turn on the PCR hood UV light for 20-30 min.
   2. Prepare Mosconas solution. Combine potassium chloride (KCl, 28.6 mM), ($NaHCO_3$, 11.9 mM), glucose (9.4 mM) and (NaH_2PO_4, 0.08 mM) in water. Adjust pH to 7.4 with 1 M NaOH or 12 M HCl solution. Place the solution in a 20 ml syringe and use a syringe filter of 0.20 μm to sterilize the solution.
   3. Spray everything with ethanol prior placing them on the hood and allow the ethanol to dry.
   4. Open the tube and transfer 5-10 ml of sterile Mosconas solution to a new 50 ml conical tube.
   5. Transfer the collagen hydrogel to the conical tube that contains the enzyme solution. Make sure that the hydrogel is completely covered with the solution. Leave the tube in the shaker in the fridge at 4 °C for 30 min.
   6. Aspirate the Mosconas solution and repeat step 7.2.5 twice.
   7. Store at 4 °C.

Representative Results

The results demonstrate that this biofabrication technique is efficient in generating 3D scaffolds that can mimic the spatial arrangement seen in *in vivo* tissue. The architectural features are vital parameters for tissue engineering application, playing a crucial role in the *in vivo* cell interaction and functionality of the tissue.

The consistency and mixability of the BSA rubber was an important parameter in producing a BSA rubber that is homogeneous and is able to maintain its intended shape. The solubility of proteins is determined by intermolecular effects, such as the protein-protein interaction, and the interaction with the solvent, which induces changes on the overall protein behavior. The conductivity of the BSA solution was measured, which is an indication of the salt concentration of the solutions. **Table 1** lists the combinations of BSA, solvent, and glutaraldehyde tested. As expected, the samples that had the highest conductivity (2x PBS solvent) facilitated the solubility of the BSA.

Another parameter used to determine the appropriate condition for the development of this sacrificial material was the reaction rate. The reaction time of the BSA decreased as the concentration of glutaraldehyde increased, as expected. The fixative reacts with the α-amino groups of the amino acids, the N terminal amino group of peptides, and the sulfhydryl group of cysteine. The glutaraldehyde reacts predominantly with the BSA through the amino groups of lysine to form the intermolecular covalent bonds (**Figure 1A**) [14]. After an incubation period, the samples showed a color change from pale yellow to dark yellow and brown, increasing in intensity with increased glutaraldehyde concentration (**Figure 1B).** The 20%, 30%, and 40% BSA with 2% glutaraldehyde in water did not form a rubber. The 40% BSA solution, due to its high viscosity and the highly reactive fixative, resulted in varying strength along the rubber. This behavior can be caused by the difficulty of the glutaraldehyde in penetrating the protein chains homogeneously. The solvent greatly influenced the solubility of the protein as well as its reaction with the fixative. The 2x PBS solutions were easily mixable. The BSA solution with water was difficult to mix. BSA solubility is greatly affected by the conductivity of the solvent (**Table 2**), causing conformational changes in the protein. The most promising samples were the 30% BSA with 3% glutaraldehyde in 1x PBS and 2x PBS.

To ensure that the rubber was able to sustained loading forces, a compression test was performed. The mechanical properties of four samples of BSA rubber were measured: 30% BSA 3% glutaraldehyde in 2x PBS, 30% BSA 3% glutaraldehyde in 1x PBS, 20% BSA 3% glutaraldehyde in 2x PBS and 20% BSA 2% glutaraldehyde in 1x PBS. The sine waves showed a very small phase change between the load and displacement curves (**Supplement Figure 2**) that are transferred to the stress and strain curves (**Supplement Figure 3**). Based on the stress and strain curves, the first three samples showed hysteresis in between loading and unloading (**Figure 2A-2C**). These three specimens behaved as a viscoelastic material that contains elastic and viscous properties when forces were applied. The 20% BSA 2% glutaraldehyde showed signs of permanent deformation (**Figure 2D**). The 30% BSA 3% glutaraldehyde in 1x PBS and 2x PBS showed a similar behavior (**Figure 2E**-one loading and unloading cycle). The elastic modulus was determined from the linear portion of these four samples (**Figure 2F**). The concentration of the phosphate solvent significantly increased the elastic modulus at the range tested ($p=0.03$). The 20% BSA 2% glutaraldehyde in 1x PBS deformed easily, showing a lower elastic modulus.

To evaluate the enzymatic digestion of the rubber, the reaction rate was calculated based on the disappearance of the BSA rubber when placed in contact with the enzyme at specific time point. The enzymatic digestion process was treated as a batch reactor. A comparison between the starting rubber concentration prior to treatment and the rubber left after being lyophilized was made to obtain the kinetics of the digestion. **Figure 3** shows the rate of reaction for each sample in relation to the concentration of glutaraldehyde and BSA, solvent, and the residence time. A clear trend was observed between the crosslinker concentrations and the reaction rate of dissociation of the entity. Statistical analysis was performed at each time point. For the 15-hr time point, the glutaraldehyde concentration significantly affected the reaction rate resulting in a p value of 0.02 (**Supplement Table 4**). After that time point, both the glutaraldehyde and the BSA concentration significantly affected the rate (**Supplement Table 5-7**). The most influential factor overall was the glutaraldehyde concentration, indicated by a more significant p value. The increase in glutaraldehyde concentration decreased the reaction rate of the digestion of the rubber entity.

The amount of protein dissolved by trypsin was determined using a BCA assay (**Figure 4**). A common trend was observed: the lower the concentration of the fixative, the more protein was digested from the BSA rubber. Trypsin interacted with the rubber sample by cleaving the BSA and the newly created covalent bonds formed by the glutaraldehyde, thus dissolving the overall structure over time. It seems that with the 1x PBS there is more solubilized protein at an earlier time point compared to the 2x PBS. Over time, there is an increase of proteins in solution at 15 hr, which continued to increase until 48 hr and then it decreased. This might be due to the trypsin constantly cleaving the proteins and, thus, creating smaller peptides and amino acids. It can also be attributed to the assay's limitations, which can only read peptides that are composed of three or more amino acids. Statistical analysis showed that the BSA and glutaraldehyde concentration significantly affected the release of the protein from the BSA rubber ($p<0.05$). An increase in BSA concentration caused an increase of protein in the supernatant, while an increase in glutaraldehyde caused a decrease in dissolved protein.

To measure the dissociation of this sacrificial material, the rubber was weighed (wet basis) before placing it in contact with the trypsin. The equivalent of dry weight of the rubber placed in the enzyme digestion solution was determined using the values shown in **Supplement Figure 1.** The enzyme solution reacted with the BSA rubber, and thus, solubilized the protein. The rubber remaining after the treatment was lyophilized O/N and weighed. **Figure 5** shows that the solvent influenced the dissociation of the rubber. At the same concentration of BSA and glutaraldehyde, the 2x PBS solvent rubbers retained more of their material compared to the 1x PBS.

Three solid mold pieces were fabricated: Loop Mold (**Supplement Figure 4A**), Stability Piece (**Supplement Figure 4B**), and Y Mold (**Figure 6A**, left). The stainless steel Y mold piece was created using the Microlution machine (**Figure 6A**, right). This mold was reaction injected with 30% BSA and 3% glutaraldehyde in 2x PBS (**Figure 6B**, left). The rubber was allowed to react O/N at 4 °C. The rubber was casted with collagen (**Figure 6B**, center) and then enzyme digested (**Figure 6B**, right). Preliminary data suggested that at pH 7.8 and a temperature of 30 °C for 15 hr, the BSA rubber can be digested with minimal impact on the collagen scaffold. After 15 hr, the rubber is weakened by the enzyme and loose enough that it leaves the channels without affecting the geometrical features of the collagen. A 3D collagen scaffold was created that has specific geometrical features. **Figure 6B** (right) shows a 4 mm diameter channel inside a collagen hydrogel after enzyme digestion of the BSA rubber. The channel was measured with a caliper to ensure that the original dimension was maintained. Indeed, the new channel in the collagen hydrogel was 4 mm. The BSA rubber molds can hold dimensions as small as 300 µm, which was tested using the stability mold (**Figure 7**). These scaffolds were tested for residual glutaraldehyde and we found no residue after the Mosconas washes.

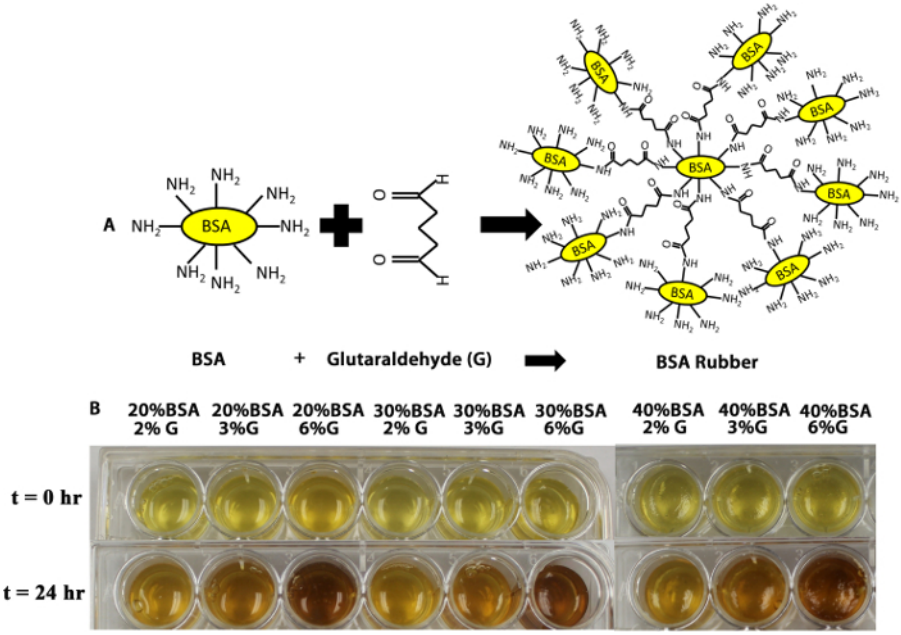


Figure 1. BSA rubber. (**A**) BSA rubber reaction. The glutaraldehyde crosslinks the BSA by creating covalent bonds. (**B**) BSA Rubber. Different concentrations of BSA, concentrations of glutaraldehyde, and type of solvent were casted on 24-well plates and reacted O/N at 4 °C. Please click here to view a larger version of this figure.

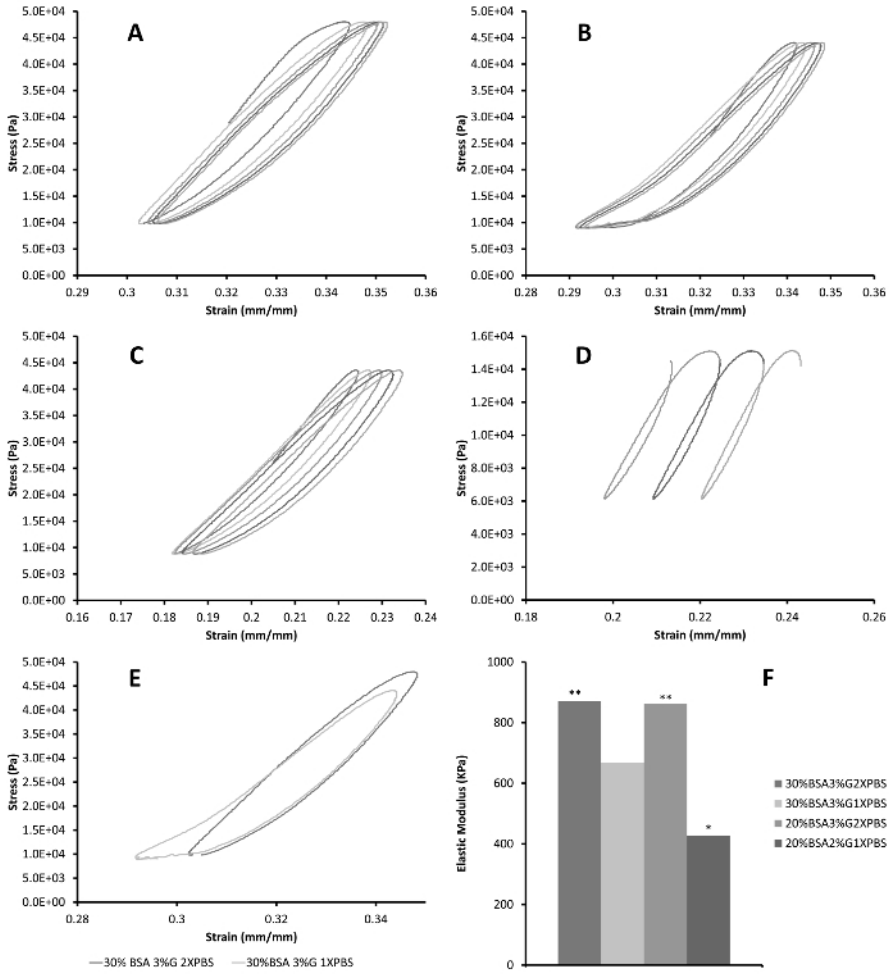


Figure 2. Stress-Strain curves of BSA rubbers. Stress-Strain curves of BSA rubbers. (**A**) 30% BSA 3% glutaraldehyde in 2x PBS; (**B**) 30% BSA 3% glutaraldehyde in 1x PBS; (**C**) 20% BSA 3% glutaraldehyde in 2x PBS; and (**D**) 20% BSA 2% glutaraldehyde in 1x PBS (3 cycles). The curves A-C show rubbers that have some hysteris, but return to their original shape. Sample D shows a rubber with a very low elastic modulus that easily deforms permanently during the loading and unloading processes. Sample A and B showed a very similar behavior as seen in one loading and unloading cycle on graph E (representative of one cycle of each sample). The elasticity was influenced by the concentration of the fixative and the solvent used (F). The samples displayed a significant increase in modulus with an increase of salts (**p<0.05). A higher fixative concentration caused a significant increase in the elasticity of the BSA rubbers (*p<0.05). Please click here to view a larger version of this figure.

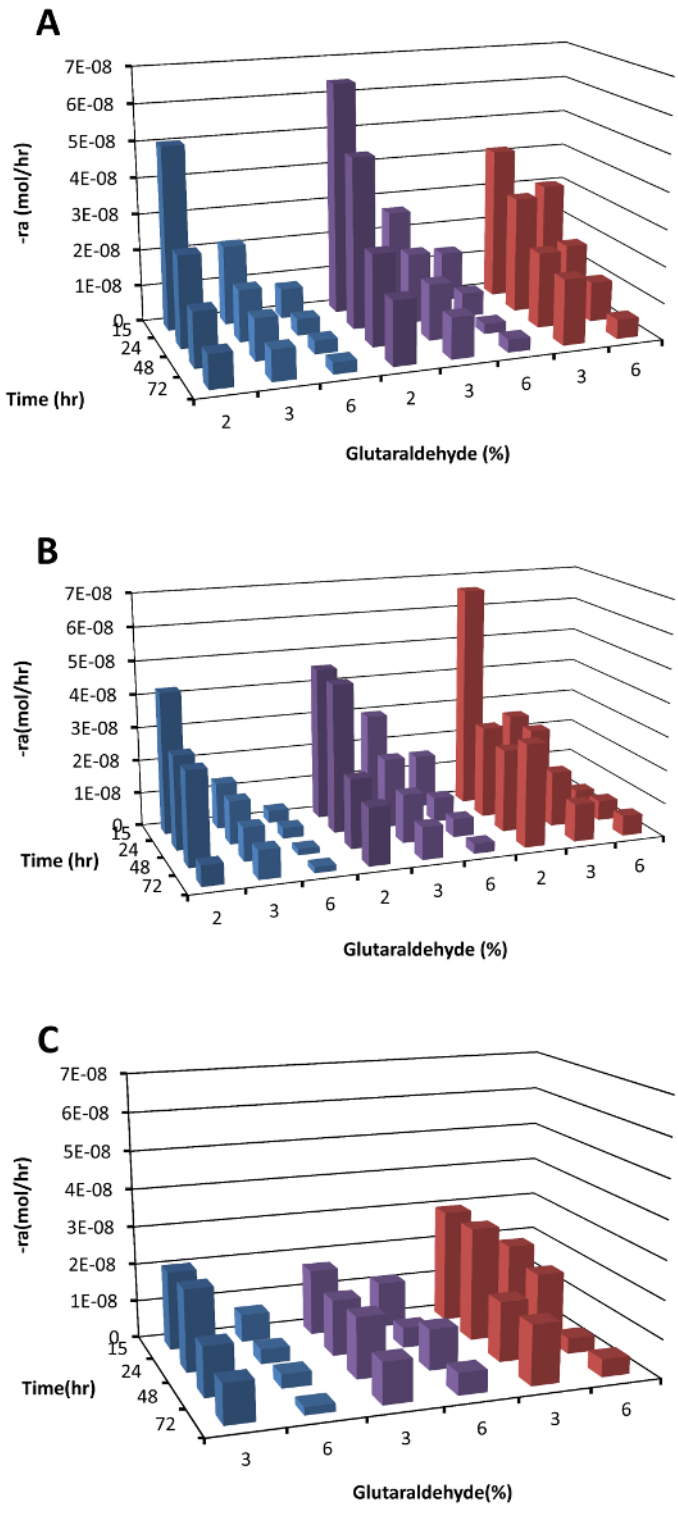


Figure 3. Reaction rate of the disintegration of the BSA rubber. As the fixative increases, the reaction rate decreases for all samples in 1x PBS (**A**), 2x PBS (**B**), and water (**C**). The 40% BSA 2% glutaraldehyde in 2x PBS shows one of the highest rates of reaction. This is due to the difficulty encountered in making the BSA protein homogeneous. (blue: 20% BSA, purple: 30% BSA, and red: 40% BSA). Please click here to view a larger version of this figure.

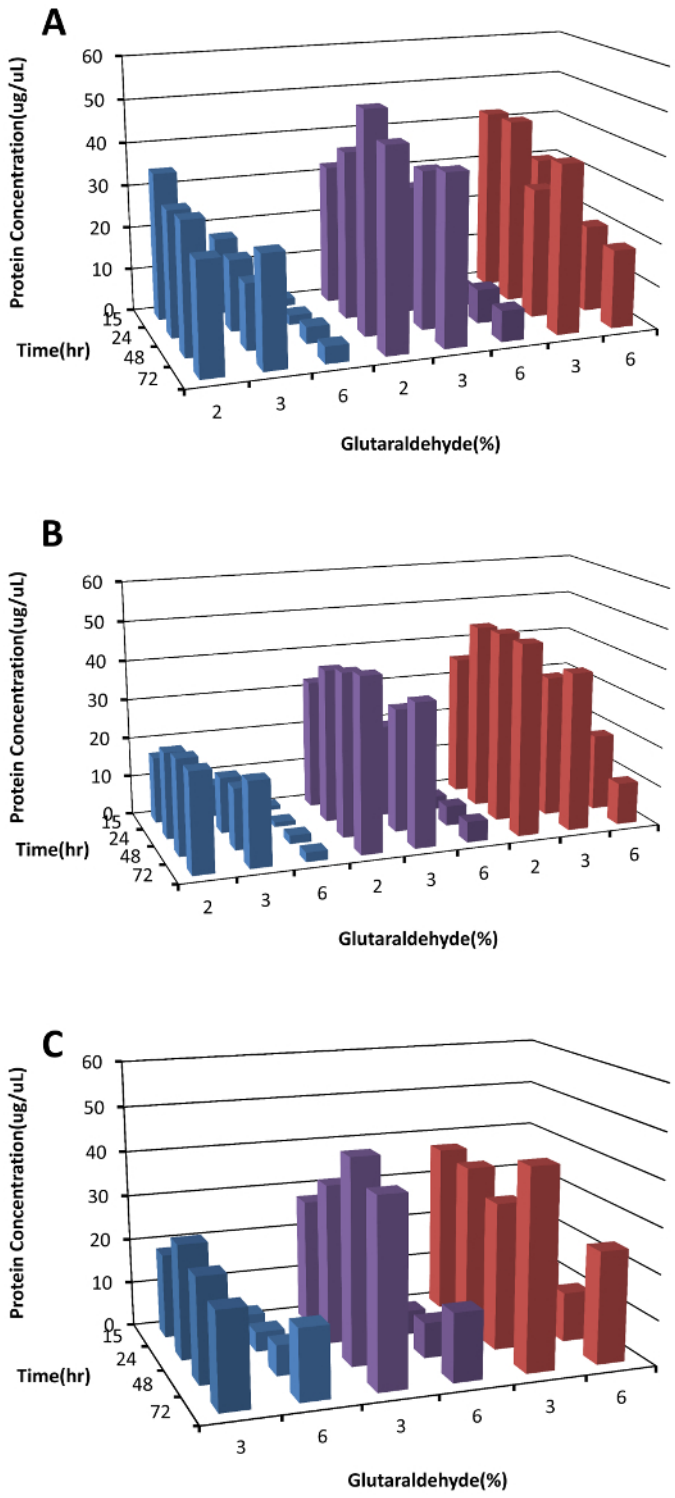


Figure 4. Protein quantification after enzyme digestion. As the fixative increases, the protein dissolved from the rubbers decreases for all samples in 1x PBS (**A**), 2x PBS (**B**), and water (**C**). (blue: 20% BSA, purple: 30% BSA, and red: 40% BSA). Please click here to view a larger version of this figure.

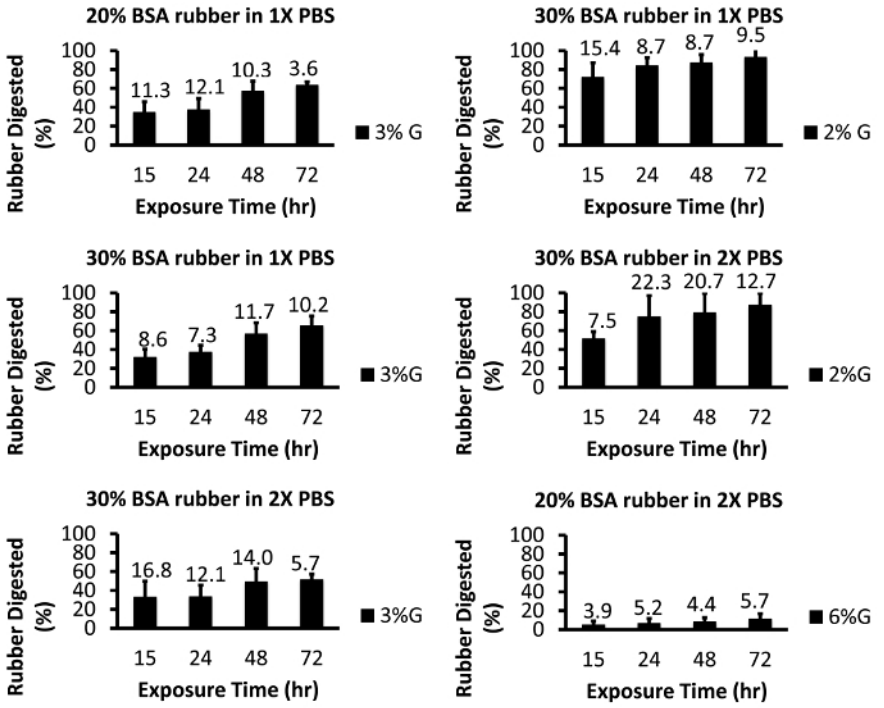


Figure 5. Rubber digestion. We determined the amount of rubber digested by comparing the starting and end dry basis products. We obtained the least amount of digestion on the 6% glutaraldehyde sample and the most at 30% BSA 2% glutaraldehyde in 1x PBS. Please click here to view a larger version of this figure.

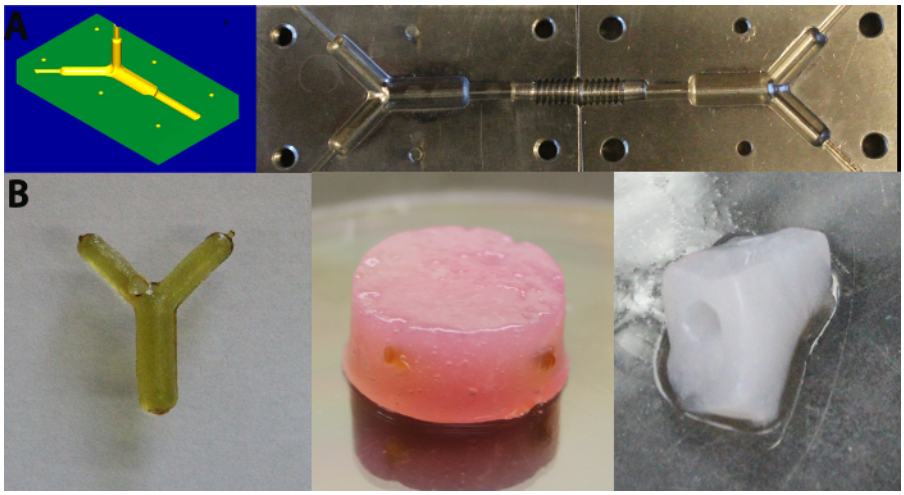


Figure 6. Branched prototype. (**A**) Representation of branch vasculature. Shown on the left is the solid created in Mastercam which was converted to G Code and fabricated using the Microlution 363-S as seen on the right. The stainless steel mold piece represents a 4 mm inflow channel with two-3mm outflow channels. (**B**) 3D collagen scaffold. Shown on the left is the BSA rubber made using the mold shown above. The center shows the rubber embedded in the collagen hydrogel. Shown on the right are the channels left within the collagen scaffold after the rubber was enzyme digested. Please click here to view a larger version of this figure.

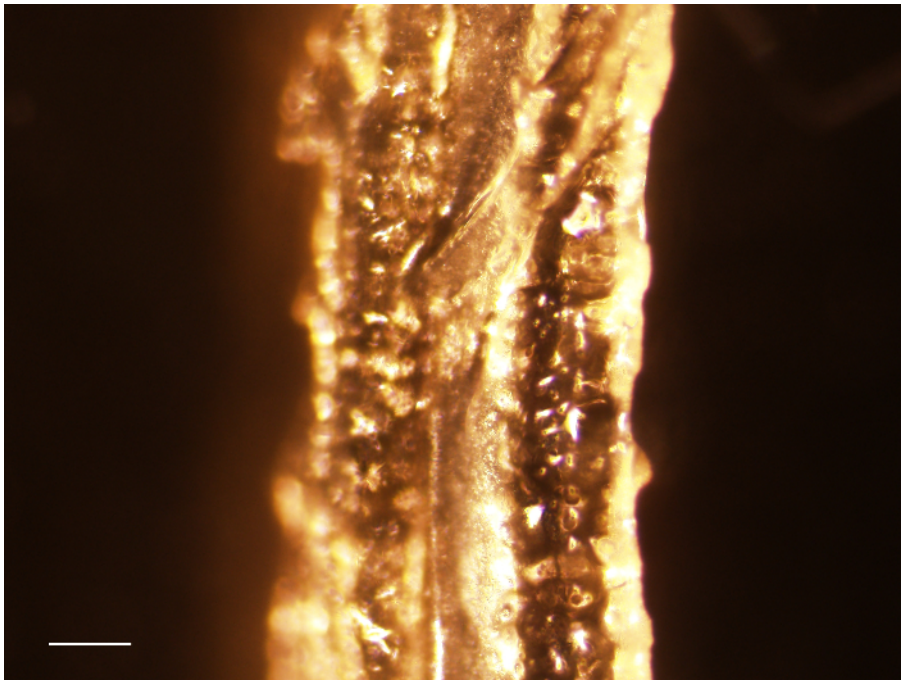


Figure 7. BSA channel. A 300 µm BSA channel was removed from the stability piece mold. There is a thin layer of mold release agent around the channel. The additional area is clearly distinguished under a microscope and can be easily removed. Please click here to view a larger version of this figure.

BSA (%)	Glutaldehyde (%)
20	2
20	3
20	6
30	2
30	3
30	6
40	2
40	3
40	6

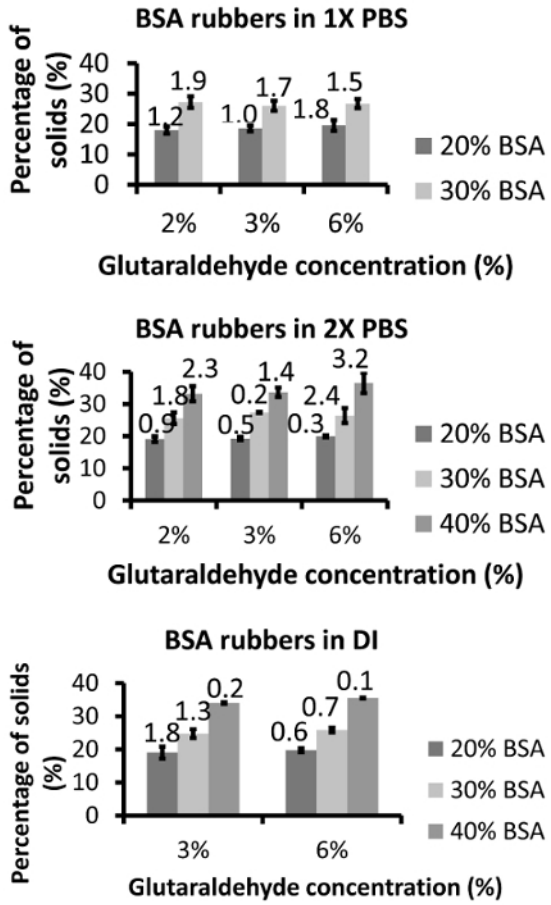
Table 1. BSA rubber parameters. BSA and fixative were mixed at a 4:1 ratio using three different solvents.

Sample		Conductivity (mS/cm)	pH
BSA (%)	Solvent		
30	2x PBS	11.43	7.06
30	1x PBS	6.35	7.05
30	DI	2.39	6.76
20	2x PBS	13.00	6.92
20	1x PBS	8.67	7.09
20	DI	2.08	6.90

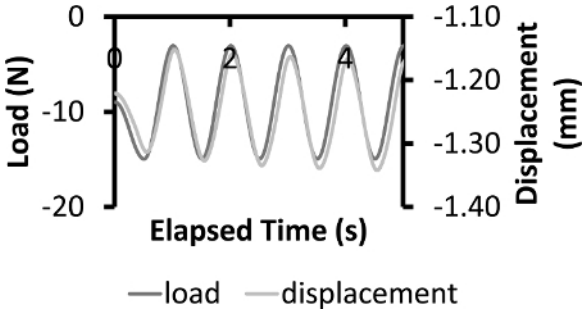
Table 2. Conductivity and pH of BSA samples. The conductivities and pH of the BSA solutions was measured in the presence of different solvents. An increase in conductivity is shown between 2x and 1x PBS.

BSA (%)	Glutaraldehyde (%)	Solvent	Observations
20	**2**	**1x PBS**	Soft, easy deformable material
20	**3**	**1x PBS**	Soft but more sturdy than the 2% glutaraldehyde
20	**6**	**1x PBS**	Stiff, a little brittle
30	**2**	**1x PBS**	Good consistency
30	**3**	**1x PBS**	Good consistency
30	**6**	**1x PBS**	Brittle
40	**2**	**1x PBS**	Very inconsistent in creating a gel/rubber consistency
40	**3**	**1x PBS**	The consistency vary along the sample
40	**6**	**1x PBS**	Brittle
20	**2**	**2x PBS**	Soft, easy deformable material but more sturdy than the 1x PBS sample
20	**3**	**2x PBS**	Soft but stiffer than the 2%
20	**6**	**2x PBS**	Good consistency
30	**2**	**2x PBS**	Good consistency, more studry than with 1x PBS
30	**3**	**2x PBS**	Good consistency, more studry than with 1x PBS
30	**6**	**2x PBS**	Good mixability but britle
40	**2**	**2x PBS**	The consistency vary along the sample
40	**3**	**2x PBS**	The consistency vary along the sample
40	**6**	**2x PBS**	The consistency vary along the sample and it was brittle
20	**2**	**Water**	Did not form a gel/rubber material
20	**3**	**Water**	Formed a gel but seem stiffer than the 1x PBS and 2x PBS, inconsistent
20	**6**	**Water**	Formed a gel but seem stiffer than the 1x PBS and 2x PBS, inconsistent
30	**2**	**Water**	Did not form a gel/rubber material
30	**3**	**Water**	Formed a gel but seem stiffer than the 1x PBS and 2x PBS, inconsistent
30	**6**	**Water**	Formed a gel but seem stiffer than the 1x PBS and 2x PBS, inconsistent
40	**2**	**Water**	Did not form a gel/rubber material
40	**3**	**Water**	The consistency vary along the sample — stiffer on top
40	**6**	**Water**	The consistency vary along the sample — stiffer on top

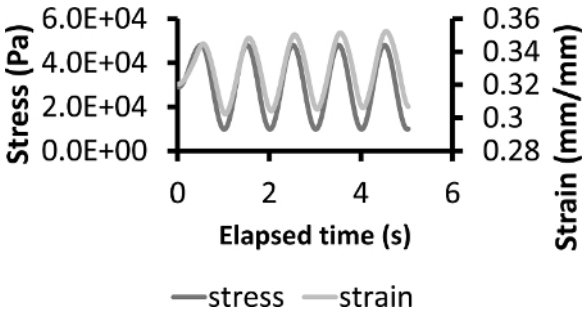
Table 3. BSA rubber. After the BSA rubber reacted O/N, an 8-mm biopsy punch hole of the sample was taken. This table contains some visual observations of the consistency and appearance of the samples.



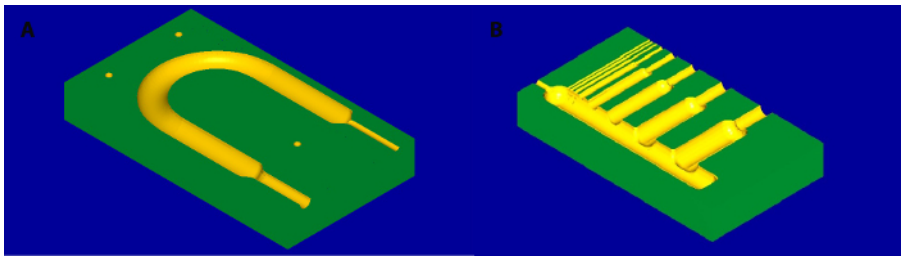
Supplement Figure 1. Solid percentage. The percentage of solids from each rubber was determined (dry weight/wet weight). The solvent did not affect the percentage of solids ($p>0.05$). Please click here to download this file.



Supplement Figure 2. Sine wave of 30% BSA 3% glutaraldehyde 2x PBS. The compressive load and displacement of the sample are shown as functions of the elapsed time. Please click here to download this file.



Supplement Figure 3. Sine wave of 30% BSA 3% glutaraldehyde 2x PBS. The stress and strain of the sample were calculated and plotted as a function of elapsed time. There is a small phase shift, indicative of the viscoelastic behavior of the rubber. Please click here to download this file.



Supplement Figure 4. Mastercam solids. (**A**) Loop and (**B**) stability pieces. After designing these using Mastercam, the G code was imported and a stainless steel or brass piece using the Microlution machine or a PLA piece using the Makerbot 3D Replicator. Please click here to download this file.

Limits	**Min**	**Max**		
Load (N)	-17	3		
Displacement (mm)	-1.5	0.3		
Wave	**Level 1**	**Level 2**	**Frequency (Hz)**	**Cycle**
Sine	-15	-3	1	5,000
Data Acquisition				
Scan time	1.008			
Scan points	360			
Number of Scans	5			
Subsequent Scan	1.008 sec between scan			

Supplement Table 1. Compression testing parameters. Using a sine wave, the relationship between the load and displacement was determined for the four types of rubber. Please click here to download this file.

ANOVA Results					
	df	**SS**	**MS**	**F**	**Significance F**
Regression	3	9.85E+02	3.28E+02	4.70E+02	1.06E-18
Residual	20	1.40E+01	6.99E-01		
Total	23	9.99E+02			
Regression Analysis					
	Coefficients	**Standard Error**	**t Stat**	**P-value**	
Intercept	1.74E+00	8.23E-01	2.12E+00	4.70E-02	
BSA (%)	7.82E-01	2.09E-02	3.74E+01	5.49E-20	
Glutaraldehyde (%)	3.17E-01	1.03E-01	3.09E+00	5.71E-03	
Solvent	2.61E+01	2.22E+01	1.18E+00	2.53E-01	

Supplement Table 2. Statistical analysis of the percentage of solids in the BSA rubber. The BSA and glutaraldehyde significantly affected the percentage of solids. The solvent did not influence the percentage of solids. Please click here to download this file.

ANOVA Results					
	df	**SS**	**MS**	**F**	**Significance F**
Regression	3	3.06E+05	1.02E+05	1.18E+01	4.00E-03
Residual	7	6.07E+04	8.67E+03		
Total	10	3.67E+05			
Regression Analysis					
	Coefficients	**Standard Error**	**t Stat**	**P-value**	
Intercept	6.50E+02	4.25E+01	1.53E+01	1.23E-06	
BSA (%)	4.67E+00	3.80E+01	1.23E-01	9.06E-01	
Solvent	1.02E+02	3.80E+01	2.67E+00	3.20E-02	
Glutaraldehyde (%)	1.16E+02	5.70E+01	2.03E+00	8.21E-02	

Supplement Table 3. Statistical analysis of the elastic modulus related to the PBS concentration. The PBS concentration significantly affected the elastic modulus. The increase of salts in the solvent caused an increase in the elastic modulus. Please click here to download this file.

ANOVA Result					
	df	**SS**	**MS**	**F**	**Significance F**
Regression	3	6.15E-15	2.05E-15	3.68E+00	1.67E-02
Residual	60	3.34E-14	5.57E-16		
Total	63	3.96E-14			
Regression Analysis					
	Coefficients	**Standard Error**	**t Stat**	**P-value**	
Intercept	3.74E-08	1.37E-08	2.74E+00	8.03E-03	
BSA (%)	3.89E-10	3.62E-10	1.07E+00	2.88E-01	
Glutaraldehyde (%)	-5.92E-09	1.83E-09	-3.23E+00	2.02E-03	
Solvent	-6.78E-08	3.76E-07	-1.80E-01	8.58E-01	

Supplement Table 4. Statistical analysis of the reaction rate after 15 hr of enzyme digestion. The glutaraldehyde concentration significantly affected the reaction rate of the digestion of the rubber by decreasing at higher glutaraldehyde concentrations. Please click here to download this file.

ANOVA Results					
	df	**SS**	**MS**	**F**	**Significance F**
Regression	3	7.36E-15	2.45E-15	3.62E+01	1.21E-13
Residual	62	4.20E-15	6.78E-17		
Total	65	1.16E-14			
Regression Analysis					
	Coefficients	**Standard Error**	**t Stat**	**P-value**	
Intercept	2.98E-08	4.77E-09	6.25E+00	4.22E-08	
BSA (%)	4.56E-10	1.26E-10	3.62E+00	6.03E-04	
Glutaraldehyde (%)	-6.04E-09	6.15E-10	-9.82E+00	3.00E-14	
Solvent	-6.57E-08	1.31E-07	-5.02E-01	6.17E-01	

Supplement Table 5. Statistical analysis of the reaction rate after 24 hr of enzyme digestion. The BSA and glutaraldehyde concentration significantly affected the reaction rate of the digestion of the rubber. At higher glutaraldehyde concentrations, there is a decrease in the reaction rate. At higher BSA concentrations, there is an increase in the reaction rate. Please click here to download this file.

ANOVA Results					
	df	**SS**	**MS**	**F**	**Significance F**
Regression	3	3.10E-15	1.03E-15	2.74E+01	1.64E-11
Residual	64	2.42E-15	3.78E-17		
Total	67	5.52E-15			
Regression Analysis					
	Coefficients	**Standard Error**	**t Stat**	**P-value**	
Intercept	2.17E-08	3.50E-09	6.20E+00	4.55E-08	
BSA (%)	2.13E-10	9.20E-11	2.31E+00	2.39E-02	
Glutaraldehyde (%)	-3.94E-09	4.53E-10	-8.70E+00	1.90E-12	
Solvent	3.04E-08	9.57E-08	3.18E-01	7.51E-01	

Supplement Table 6. Statistical analysis of the reaction rate after 48 hr of enzyme digestion. The BSA and glutaraldehyde concentration significantly affected the reaction rate of the digestion of the rubber. At higher glutaraldehyde concentrations, there is a decrease in the reaction rate. Please click here to download this file.

ANOVA Results					
	df	**SS**	**MS**	**F**	**Significance F**
Regression	3	2.19E-15	7.29E-16	3.05E+01	3.56E-12
Residual	61	1.46E-15	2.39E-17		
Total	64	3.64E-15			
Regression Analysis					
	Coefficients	**Standard Error**	**t Stat**	**P-value**	
Intercept	1.05E-08	2.84E-09	3.69E+00	4.80E-04	
BSA (%)	3.61E-10	7.48E-11	4.83E+00	9.48E-06	
Glutaraldehyde (%)	-3.07E-09	3.69E-10	-8.33E+00	1.21E-11	
Solvent	3.97E-08	7.80E-08	5.09E-01	6.12E-01	

Supplement Table 7. Statistical analysis of the reaction rate after 72 hr of enzyme digestion. The BSA and glutaraldehyde concentration significantly affected the reaction rate of the digestion of the rubber. At higher glutaraldehyde concentrations, there is a decrease in the reaction rate. At higher BSA concentrations, there is an increase in the reaction rate. Please click here to download this file.

Discussion

Biofabrication is a highly multidisciplinary field in which biology and engineering principles merge to generate complex materials that mimic native tissue. In order to achieve this, there is a need to develop techniques that use the information gathered from *in vivo* tissue and translate it into an *in vitro* scaffold. In this way, a platform can be engineered that closely resembles the architectural, functional, and mechanical properties of the *in vivo* tissue. The optimal scaffolding material should possess certain properties, such as being biocompatible, mimic the mechanical properties of the tissue of interest, be capable of controlled degradation, able to support cell viability, and capable of allowing tissue remodeling[2,3].

A multitude of fabrication techniques have been developed to generate viable, three-dimensional constructs. These technologies fall into two major categories: conventional and advanced. The conventional techniques include the use of synthetic and natural traditional materials to make porous structures. Some examples are solvent-casting, freeze drying, and melt molding. Disadvantages of these techniques include poor control of porosity within the structure (pore size and pore interconnectivity) and difficulty making internal channels within the scaffolds. Advanced techniques include stereolithography, molding, 3D printing, and electrospinning, among others[1]. These techniques have drawbacks such as the lack of long-range microarchitecture channels, material selection to provide optimal mechanical strength while being capable of dispensing through small-diameter nozzles, optimization dependent on the material, require extensive post-processing that might be toxic, and restriction in the design of inner architectures in an *in vitro* construct. A major drawback in 3D printing is the availability of biomaterials that are adequate cell carriers, while also having the mechanical properties required to maintain a defined architectural organization[12]. The technology presented here incorporates both conventional and advanced fabrication techniques. The best of both worlds is derived from the convergence of computer-aided manufacturing to create, or import, the desired architecture with the development of an enzyme-labile rubber. The stainless steel molds were created using a milling machine, but their fabrication isn't limited to this technique. Using a 3D Printer (*e.g.*, Makerbot), the same molds were fabricated from polylactic acid (PLA). The negative molds were milled using architectural directives designed by the CAD program, which provides solid molds that create an easy and reliable transfer of features to any material. This technology is significant in that it allows not only the control of the external tissue composition, but also of the highly complex internal architecture. The work presented here focuses primarily on the characterization of the BSA rubber, which can be mixed homogeneously, is easily digested in a reasonable amount of time, was resistant to alterations in its structure, and is able to mimic minute features, while holding its stability during the casting process. The limitation of this technique was tested and the sacrificial material's resolution can maintain dimensions as small as 300 µm in diameter. However, **Figure 7** show

that the channel is surrounded by a thin layer of mold release agent. Using a microscope, this additional area is clearly distinguished and can be removed to have the desired dimension. This biofabrication technique allows the replication of a wide variety of internal structures that range from macro- to micro-scale.

Serum albumin is the most abundant protein in the circulatory system. Since the proteins are polyelectrolytes, solubility was determined by electrostatic interactions[15-17]. It has been shown that at low salt concentrations, there is a salting-in effect on the protein facilitating its solubility[18]. Glutaraldehyde is a crosslinking agent that causes changes in the properties of albumin. The color change seen in **Figure 1** is attributed to the formation of the aldimine linkages[19-21]. The glutaraldehyde reacts predominantly with the BSA through the amino groups of lysine to form the intermolecular covalent bonds[14]. Data indicates that the increase of salts improved the elasticity of the rubber (from 1x PBS to 2x PBS). The higher glutaraldehyde concentration produces stiffer gels that set earlier compared to low concentration ones. However, they are more difficult to dispense, mix homogenously, and they form brittle gels. To deliver the appropriate amount of BSA rubber into the molds, every part of the assembly must be kept cold because higher temperatures accelerate the glutaraldehyde and BSA reaction. The ideal rubber (30% BSA 3% glutaraldehyde in 2x PBS or 1x PBS) behaves as a viscoelastic material that can withstand loading without permanently deforming. This becomes very important when handling and casting material around the BSA rubber structure.

Trypsin is a serine protease that hydrolyzes proteins. Trypsin is a widely used enzyme that has high cleavage specificity. It cleaves the peptide chains mainly at the carboxyl side of the amino acids lysine and arginine[22]. BSA is readily soluble at low concentrations in water, and the trypsin readily digested the BSA rubber leaving the collagen intact and relatively untouched. The material will not have any contact with cells. The construct was tested for the presence of free glutaraldehyde and there were no traces of this fixative. This demonstrates the efficacy of the BSA rubber as a sacrificial material for biofabrication. In this protocol, collagen was used as the scaffold material, but any other material that is resistant to trypsin digestion could be used.

Future work will be focused on reconstituting the vascular components within the hydrogels by seeding the scaffold with endothelial and fibroblast cells. One of the challenges is to create a uniform distribution of cells throughout the inside of the channels that mimic the native 3D distribution. To address this issue, a strategy is being developed that allows the sealing or plugging of the channels and the injection of the cell suspension. The material tested is pluronic F127, which is a thermoreversible gel, liquid at 4 °C and a solid above 30 °C[23,24]. A high concentration of pluronic was successful in creating the necessary temporal seal. After the cell suspension is within the channels of the scaffold, the entity is rotated for a specific amount of time until the cells adhere to all sides of the 3D structure. The inside channel will have adequate media for maintaining cell survival. Pluronic maintains its gel form and is readily soluble in an aqueous environment. Once the cells adhere, the hydrogel will be flooded with media, and can be cultured in stationary or flow conditions, depending on the purpose of the study. This methodology will be further assessed and will become the follow up to this publication. The biofabrication technique described herein is currently being used to develop a scaffold replica of a human renal artery. The same approach could be done with other types of tissues, such as cardiac, to expand the applicability of this technique to a wide range of clinical applications.

The biofabrication technique developed here is a step forward in the generation of *in vitro* scaffolds that can recapitulate intrinsic geometrical features quickly and reliably. A natural material, such as collagen, was selected because it offers optimal chemical and physical cues to cells over synthetic materials. These natural materials can be used for therapeutic research, as *in vitro* models of development, malformation, and disease tissue, as well as for replacement of damaged tissue.

Disclosures

The authors have nothing to disclose.

Acknowledgements

This work was supported by NIH-NIDCR IRO1DE019355 (MJ Yost, PI), and NSF-EPSCoR (EPS-0903795).

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