Video Article

# Easy Manipulation of Architectures in Protein-based Hydrogels for Cell Culture Applications

Nicholas Bodenberger[1], Dennis Kubiczek[1], Frank Rosenau[1]

[1]Center for Peptide Pharmaceuticals, Faculty of Natural Science, Ulm University

Correspondence to: Nicholas Bodenberger at Nicholas.Bodenberger@uni-ulm.de

URL: https://www.jove.com/video/55813
DOI: doi:10.3791/55813

Keywords: Biochemistry, Issue 126, Hydrogel, material properties, pore sizes, cell culture, biopolymers, 3D architecture

Date Published: 8/4/2017

Citation: Bodenberger, N., Kubiczek, D., Rosenau, F. Easy Manipulation of Architectures in Protein-based Hydrogels for Cell Culture Applications. *J. Vis. Exp.* (126), e55813, doi:10.3791/55813 (2017).

## Abstract

Hydrogels are recognized as promising materials for cell culture applications due to their ability to provide highly hydrated cell environments. The field of 3D templates is rising due to the potential resemblance of those materials to the natural extracellular matrix. Protein-based hydrogels are particularly promising because they can easily be functionalized and can achieve defined structures with adjustable physicochemical properties. However, the production of macroporous 3D templates for cell culture applications using natural materials is often limited by their weaker mechanical properties compared to those of synthetic materials. Here, different methods were evaluated to produce macroporous bovine serum albumin (BSA)-based hydrogel systems, with adjustable pore sizes in the range of 10 to 70 µm in radius. Furthermore, a method to generate channels in this protein-based material that are several hundred microns long was established. The different methods to produce pores, as well as the influence of pore size on material properties such as swelling ratio, pH, temperature stability, and enzymatic degradation behavior, were analyzed. Pore sizes were investigated in the native, swollen state of the hydrogels using confocal laser scanning microscopy. The feasibility for cell culture applications was evaluated using a cell-adhesive RGD peptide modification of the protein system and two model cell lines: human breast cancer cells (A549) and adenocarcinomic human alveolar basal epithelial cells (MCF7).

## Video Link

The video component of this article can be found at https://www.jove.com/video/55813/

## Introduction

Hydrogels are materials that form insoluble 3D networks capable of binding large amounts of water. Such materials can provide excellent environmental conditions for living cells. Currently, there is increasing interest in the generation of three-dimensional hydrogel structures and in the development of processes to tailor their chemical and physical properties. Once this is achieved, a template for the growth of cells and the manipulation of cellular behavior can be generated[1,2,3,4]. These 3D structures not only create a more natural and realistic environment than conventional two-dimensional approaches, but they also reveal new possibilities for the growth of stem cells or tumor models[5]. Different materials possess a range of characteristics that mainly depend upon the pore size of the gel[6]. The pores play a crucial role in cell culture applications, tissue engineering, and the directed growth of stem cells. For example, oxygen and nutrients diffuse through the matrix, and adequate amounts must be able to reach the cells[7]. On the other hand, harmful metabolites must be removed as quickly as possible, and sufficient space for cell growth must be available[7]. Consequently, the properties of the material, and thus the pore size, severely influence the potential benefit and possible applications of the matrix. Depending upon the properties of the material, different cell-growth processes can occur in 3D cell culture, including the formation of neuronal structures; the growth and differentiation of skin or bone cells; and the directed growth of special stem cell lines, like hepatocytes or fibroblasts[2,3,8,9,10,11]. Another crucial point influencing the possible application of a material is its stability towards external stimuli[12]. For example, the hydrogel must maintain its mechanical integrity in cell culture media or the human body.

In recent years, research on 3D cell culture hydrogels intensified, and many studies were carried out to resolve the 3D architectures of the systems[13]. Hydrogels composed of chemically synthesized components are most commonly investigated because they can be easily synthesized and chemically modified and they exhibit high stability (see Zhu *et al.*, 2011 for a review)[5]. However, proteins have many beneficial properties: as so-called "precision polymers," they are biocompatible; they have a defined length; they are relatively easy to modify; and they have a large number of target sites[14,15]. In this regard, highly specific, innovative structures can be generated for application in many fields. In this study, a protein-based hydrogel[16] was used to demonstrate the ability of well-established methods to influence the 3D architecture of the material. Furthermore, the capability of and applicability to pore generation was also investigated.

Many different techniques are available to modify 3D structures, including both simple methods and sophisticated, highly specialized techniques from different fields of material science. A widespread technique is the use of electrospinning to generate well-defined structures[17]. Charged fibers are pulled from a solution by an electric field and then solidify upon exposure to oxygen. In this way, fibers in the range of several nanometers up to several microns can be produced. Additional techniques to tune the size, structure, and distribution of the pores within the matrix are soft lithography, photolithography, hydrodynamic focusing, electro-spraying, and bio-printing[18,19,20]. A significant drawback of these

 

techniques is their dependency upon specific, expensive equipment and special chemicals or materials. Furthermore, experience with these techniques is often not directly transferrable to protein-based materials, and many of the chemicals and methods are not cell compatible.

On the other hand, many techniques do not rely upon special equipment, making them easier and cheaper to apply and to reproduce. A widespread method for structure manipulation is solvent casting[21,22,23]. Particles are added prior to the polymerization reaction and are distributed homogenously to saturate the solution. After the polymerization, a change of conditions, such as a dilution or a pH change, leads to the solvation of the particles, while the pores remain within the material. The chemicals used in these techniques, such as salt, sugar, paraffin, gelatin, and chalk, are cheap and readily available. In freeze-drying, swollen hydrogels are frozen. The subsequent sublimation of the liquid phases under a vacuum is then performed[23,24,25]. Water sublimation from the network is gentle enough to maintain the specific 3D structures of the material. In gas foaming, a solution is streamed with a gas while the polymerization takes place, leaving pores within the gel[21]. The size and distribution of the pores can be adjusted depending upon the gas stream.

To form the protein hydrogel, BSA is reacted with tetrakis (hydroxymethyl) phosphonium chloride (THPC) in a Mannich-type reaction to allow for the formation of covalent bonds between primary amines and the hydroxy groups of the four-armed linker molecule[26]. Possible harmful intermediates are removed by excessive washing of the material after the reaction occurs.

This study demonstrates the possibility of treating a BSA-based material with different techniques to manipulate and tailor the size of the pores. Each of the techniques can be used in any laboratory worldwide, as no special equipment is necessary. In addition, different parameters, such as swelling ratio, enzymatic degradability, pH stability, and temperature sensitivity, were examined and compared to each other, especially respect to the influence of the different techniques on the generation of 3D architectures. Finally, the materials were functionalized with cell-adhesive peptides to investigate the possible application of the materials to cell culture. Two different model cell lines were used: A549 and MCF7.

## Protocol

# 1. Hydrogel Preparation

1. Mix 200 mg of BSA with 1 mL of deionized $H_2O$ to create 20% (w/v) BSA stock (stock solution A).
2. Mix 165 µL of THPC solution (134 mg/mL) with 4.835 mL of deionized water to create THPC stock solution (stock solution B).
3. Weigh 1 mg of KCSSGKSRGDS (1,111.1 g/mol) peptide (or an equivalent cell-adhesive peptide) and dilute it in 100 µL of sterile $H_2O$ to obtain a 10 mg/mL solution (stock solution C).
   NOTE: This step is optional and only needs to be included if the hydrogel is meant for cell culture application.
4. Remove the bottom of a 96-well plate and replace it with removable plastic wrap.
   NOTE: For the plates used here, the bottom can easily be removed by applying pressure to the bottom of each well; the thin plastic bottom will simply fall out.
5. Mix 100 µL of BSA stock solution (A) and 100 µL of THPC stock solution (B) (optional: 4 µL of stock solution C for functionalized, cell-adhesive hydrogels) in the 96-well plate to obtain 200 µL of hydrogel. Mix the components by pipetting up and down at least 5 times to guarantee a uniform hydrogel after polymerization.
6. Place the 96-well plate at room temperature (RT) for about 10 min until all hydrogels are properly polymerized.
7. Carefully and slowly remove the plastic wrap from the bottom of the plate.
8. Press the hydrogels out of the 96-well plate using a small stamp and transfer them to 1.5 mL tubes with sterile PBS, pH 7.4.
   NOTE: The hydrogels have a cylindrical shape, with a diameter of approximately 4 mm and a height of 8 mm.
9. Store the hydrogels in phosphate-buffered saline (PBS) at 4 °C for up to several months.

# 2. Freeze-drying the Hydrogels

1. Fill 1.5 mL tubes with 500 µL of sterile deionized $H_2O$ and remove the cap. Transfer the hydrogels to the 1.5 mL reaction tubes using a spatula.
2. Wrap the 1.5 mL tubes tightly with paraffin film-at least three layers for each vial. Use a needle to pierce small holes in the film to enable gas release from the tube.
3. **Proceed to one of the following steps:**
   1. Transfer the vial to liquid nitrogen solution for 5 min to guarantee that the water and hydrogel completely freeze. Immediately after removal from the liquid nitrogen, transfer the vials to the freeze dryer to prevent the material from thawing.
      NOTE: This procedure results in pores about 10-15 µm in radius.
   2. Keep the vials at -20 °C overnight to slowly freeze the hydrogels. Immediately after removal from -20 °C, transfer the vials to the freeze dryer to prevent the material from thawing.
      NOTE: This procedure results in pores about 50-60 µm in radius.
4. After 24 h (and the complete evaporation of the water in and around the hydrogel), thaw the material by removing it from the freeze dryer.
   NOTE: The pore sizes can be analyzed with confocal laser scanning microscopy (step 5, "Hydrogel Visualization").

# 3. Particle Leaching

1. Prepare the hydrogel as described in steps 1.1-1.5.
2. Directly after mixing the components, add NaCl until saturation occurs (36 mg/mL). Add salt until a salt crystal can be seen in the solution as a white precipitate.
3. Transfer the 96-well plate to a shaker and shake until polymerization takes place (about 10 min). Remove the hydrogels from the plate, as described in steps 1.7-1.8.

4. Incubate the hydrogels for at least 24 h at RT in sterile water on a shaker (20 rpm) to elute all salt from the hydrogel template. Store the hydrogels at 4 °C in PBS for up to several months.

## 4. Channel Formation

1. Prepare hydrogels as described in step 1. Remove a hydrogel from solution using a pincer, remove the excess water with highly absorbent paper, and place it on top of a block of dry ice.
2. Freeze the hydrogel for 30 s and carefully remove it from the block. Do not damage the hydrogel; carefully use a spatula to scrape it off the block. Transfer the hydrogel to a 1.5 mL tube and dry it overnight at 37 °C.

## 5. Hydrogel Visualization

1. Prepare a rhodamine B stock solution by diluting 1 mg of rhodamine B in 10 mL of PBS. Prepare a serial dilution by diluting the rhodamine stock solution in PBS until a concentration of 0.001 mg/mL is reached (dilution factor: 100)
2. Remove the hydrogel from the storage solution (*e.g.,* from step 2.4.) and transfer it to a 1.5 mL tube with 1 mL of 0.01 mg/mL rhodamine B solution. Stain the hydrogel overnight in rhodamine B solution at RT.
3. The next day, transfer the hydrogel that is to be visualized to 10 mL of PBS (pH 7.4) and wash for at least 3 h.
4. Transfer the hydrogel onto a µ-slide 8 well and cover it with PBS.
5. Cut small slices out of the hydrogel (about 0.5 mm in height) using a blade.
6. Using a confocal laser scanning microscope, visualize the hydrogel at a wavelength of 514 nm (objective: EC Plan-Neofluar 40x/1.30 Oil DIC M27, plane scan mode: 514 nm, and zoom: 1X).

## 6. Cell Culture Feasibility

1. Sterile-filter all stock solutions (A, B, and C) with a 0.45 µm filter.
2. Prepare the hydrogel as described in steps 1.1-1.4, including the cell-adhesive peptide prior to hydrogel polymerization.
3. After mixing the components, immediately pipette the mixture into a µ-slide 8 well until the bottom is completely covered.
   NOTE: This step must be performed quickly and directly after mixing the components, as polymerization takes place within minutes in the slide.
4. Culture adhesion cells and passage to obtain a single-cell suspension using standard cell culture techniques. Transfer the cells into pre-warmed, sterile DMEM cell culture medium supplemented with fetal bovine serum (FBS, 10% (w/v)), penicillin-streptomycin (1% (w/v)), and nonessential amino acid solution (MEM, 1% (w/v)).
5. Count the cells with a Neubauer counting chamber and carefully pipette 200 µL of the desired number of cells (2 x $10^5$ cells/$cm^2$) onto the hydrogel surface.
6. Cover the µ-slide 8 well with the lid and transfer it to an incubator (37 °C, 5% $CO_2$). Incubate for at least 4 h at 37 °C.
7. After at least 4 h of cellular attachment, wash the cells twice with 200 µL of sterile cell culture PBS.
8. Fix the cells with 200 µL of 3.7% (v/v) formaldehyde for 10 min at RT and wash twice with PBS (~200 µL).
   NOTE: Wear appropriate personal protective equipment when handling formaldehyde.
9. Permeabilize the cells with 200 µL of 0.1 % Triton X for 5 min. Wash twice with PBS.
10. Stain the cells with phalloidin-rhodamine by mixing 5 µL of methanolic stock with 195 µL of PBS and adding it to the cells at RT. Stain the cells for 20 min in the dark. Wash twice with PBS.
11. Investigate the cell adhesion properties using a confocal microscope at 514 nm (objective: EC Plan-Neofluar 40x/1.30 Oil DIC M27, plane scan mode: 514 nm, and zoom: 1X). Analyze the images using appropriate software (see the **Table of Materials**).

## 7. Hydrogel Properties

1. **Swelling ratio.**
   1. Completely dry the hydrogels at 37 °C for at least one day.
   2. Weigh each hydrogel and note the exact weight.
   3. Fill a 2 mL reaction tube with 1.5 mL of PBS.
   4. Transfer the hydrogel into this 2 mL reaction tube and completely immerse it in PBS.
   5. Leave the hydrogel for at least two days in PBS at RT to reach equilibrium with the PBS.
   6. After three days, remove the hydrogel from the solution and dry it with a paper tissue to remove excess water from the hydrogel surface.
   7. Weigh the hydrogel.
   8. Calculate the swelling ratio using the following formula:

$$Swelling\ ratio = \frac{Ws - Wd}{Wd}$$

   where $W_d$ is the weight of the dried gel (step 7.1.2.) and $W_s$ is the weight of the wet gel (step 7.1.7). Multiply with 100 to get the swelling ratio percent.

2. **pH and temperature stability.**
   1. Transfer 5 mL of PBS to a 15-mL reaction tube.
   2. Adjust the solution to the appropriate pH (*e.g.,* pH 2, 7, or 10) with NaOH and HCl. Bring the solution to the appropriate temperature (*e.g.,* RT, 37 °C, or 80 °C).
   3. Transfer the hydrogel that is to be investigated for its stability into the appropriate solution. Readjust the pH if necessary.

NOTE: All hydrogels should be completely swollen at this point (see the swelling ratio) to prevent the incorrect interpretation of the weight due to the swelling of the material.

4. After certain time intervals, remove the hydrogel from the solution (*e.g.,* each hour for up to 2 days), dry it with a highly absorbent paper tissue to remove excess water, and weigh it.

3. **Enzymatic degradation.**
    1. Prepare a stock enzyme solution of 300 U trypsin and pepsin, as per the manufacturer's instructions.
    2. Transfer the hydrogel (*e.g.,* from step 1.8) to the appropriate solution.
    NOTE: All hydrogels should be completely swollen at this point to prevent the incorrect interpretation of the weight.
    3. After certain time intervals, remove the hydrogel from the solution (*e.g.,* each hour), dry it with a highly absorbent paper tissue to remove excess water, and weigh it.

## Representative Results

Hydrogel development has become one of the most prominent fields in material research-related biological studies, with thousands of entries indexed in scientific research archives. Although the behavior of many systems is well studied, the manipulation of 3D networks, especially of sensitive protein-based materials, is often a major issue in material science. Another commonly underestimated challenge is the correct measurement of the native structure of a material using cryo electron microscopy. This is because the sample preparation (*i.e.,* drying) process often changes the hydrogel properties. To overcome this problem, the material samples here were analyzed using confocal microscopy, a method that allows for the characterization of the material in its native, water-swollen state. Rhodamine B can bind to the hydrogel backbone via electrostatic interaction and facilitates the visualization of the material. To investigate the feasibility of a freeze-drying approach to modify this protein-based system, hydrogels were subsequently polymerized and frozen at 196 °C in liquid nitrogen and at -20 °C. The results show a clear influence of the freezing temperature on the resulting pore sizes. At -196 °C, small pores with a radius of about 10 µm and a narrow size distribution were produced. On the other hand, freezing at higher temperatures leads to the production of materials with much larger pores, whose radii are between 50 and 70 µm, as seen in **Figure 1**. The slow freezing process leads to the formation of larger ice crystals, which results in bigger pores within the material after the sublimation of the ice.

Another technique for pore generation, which is well-described for synthetic materials, is particle leaching. Solid particles like paraffin, gelatin, salt, or another solid-state material, are incorporated into the hydrogel prior to solidification. They are then eluted from the solid hydrogel by changing external conditions, such as temperature, Ph, or buffer composition by dilution. The BSA-based hydrogel was polymerized in the presence of high salt concentrations. After the sol-to-gel transition, salt crystals were removed from the material by adding an excess of water to dilute the crystals. This was followed by the confocal analysis of the resulting materials. The size of the salt crystals was changed by grinding NaCl with a mortar and pestle. As shown in **Figure 2**, the pore size can be tremendously changed using this method. Depending upon the crystals used (untreated or ground for a certain time), pore sizes from 10 to 70 µm could be produced. Appropriately sized pores are essential for many applications that feature the incorporation of cells into a hydrogel. However, more sophisticated structures might be needed when the creation of more complex structures is desired (*e.g.,* to provide structural guidance for neuronal cells along a gradient). Complex architectures can often only be produced with special equipment (*e.g.,* spin coating, electrospinning, lithographic, or bioprinting techniques). However, the production of linear channels can be realized with a simple freeze-drying approach and the use of dry ice. The channels produced have radii of about 20-30 µm and lengths of several hundreds of micrometers, as shown in **Figure 3**.

Another crucial feature of hydrogels is the biodegradability of the template matrix, both *in vivo* and *in vitro*[1]. Protein hydrogels offer the advantage of being biodegradable. However, many systems lack fast degradation due to the restricted target sites within the material. Proteolytic agents must work their way from the outside of a material to the inside, resulting in very slow degradation times. In contrast, macroporous hydrogels are normally well-diffusible. This allows enzymes to target protein structures throughout the whole template simultaneously, which greatly reduces degradation times. For the presented protein-based hydrogel, the proteolytic degradation time depends mainly on the pore sizes within the matrix. A representative degradation kinetic is shown in **Figure 4**. Pore generation reduces the time from several days to a few hours for trypsin and pepsin degradation, as shown in **Table 1**.

The main benefit of hydrogels is the high water content within the materials, which is desirable to mimic the extracellular matrix[27]. The swelling ratio represents the amount of water a gel can absorb and hold after drying and is a valid indicator of the free water content of a macroporous hydrogel. Furthermore, diffusion depends strongly upon the free water content and is required for the transport of nutrients towards the cells and the removal of toxic metabolites. The swelling ratio correlates directly with the pore size within the material, as shown in **Table 1**. By changing the pore size using the demonstrated methods, it is possible to alter the diffusion behavior in the template and thus to influence cell fate by manipulating their feed.

The stability of a hydrogel to external stimuli, such as pH and temperature changes, severely limits or expands the possible applications of a system. Particularly for application in a cell culture-related area, resistance to changes in the external parameters is essential, as cells and cell culture matrix mutually influence their properties and behavior (*e.g.,* the acidification of the medium might lead to hydrogel degradation, and hydrogel degradation leads to cell release or death)[22]. To demonstrate the feasibility of the methods described here for pore generation, all hydrogel stabilities were determined for increasing temperatures (37 °C and 80 °C) and changing pH values (pH 2, 7, and 10). The residual weights of the hydrogels, which were treated using different methods, are summarized in **Table 1**. In conclusion, the presented protein-based hydrogels are stable over a wide range of conditions, while the residual weights of the hydrogels at high temperatures only reduce gradually. For the swelling ratio, it is important to consider that a partial unfolding might occur at higher temperatures, even if most BSA molecules should be held in place by the four-armed linker.

Whenever a macroporous hydrogel is intended for use in cell culture, the adhesion properties of the material play a crucial role in the individual application. Some materials bear inherent adhesion properties (*e.g.,* heparin, short, extracellular matrix-derived peptide structures, or fibronectin)[5]. For those that do not have these features, the possibility to efficiently modify the material is desirable in order to introduce cell adhesion properties. One major advantage of protein hydrogels is the presence of a variety of accessible functional groups on the surface, which can be targeted by specific reactive linker molecules. Another option is the incorporation of cell adhesive peptides during polymerization, which can further facilitate the handling of the material and the production process, as only a single step is necessary to produce cell-adhesive materials. By using a four-armed, amine-reactive crosslinking agent[26], the cell adhesion mediating peptide (in this case, RGD) can be directly co-polymerized during hydrogel formation. This procedure results in the proper adhesion of the cells, as shown in **Figure 5**. Two model cell lines, human breast cancer cells (A549, CCL-185) and adenocarcinomic human alveolar basal epithelial cells (MCF 7, HTB-22) were used to investigate the general feasibility of using this modified hydrogel in 3D cell culture. Both cell lines showed very good adhesion potentials on the modified hydrogels.

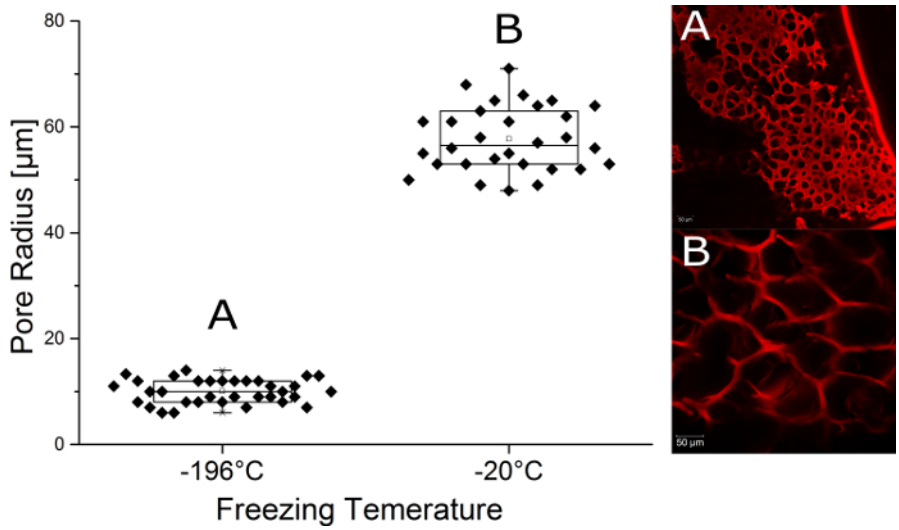


**Figure 1: Freeze-drying the hydrogels.** After hydrogel polymerization, the gels were transferred to -196 °C (liquid nitrogen) or -20 °C. The material was stained with fluorescent rhodamine B and analyzed with confocal laser scanning microscopy. The size and distribution of the pores within the material were analyzed with imaging software and were plotted for the different temperatures. On the right side, representative pictures of the materials frozen at (A) -196 °C and (B) -20 °C are shown. The error bars represent the standard deviation. Scale bars = 50 µm. Please click here to view a larger version of this figure.

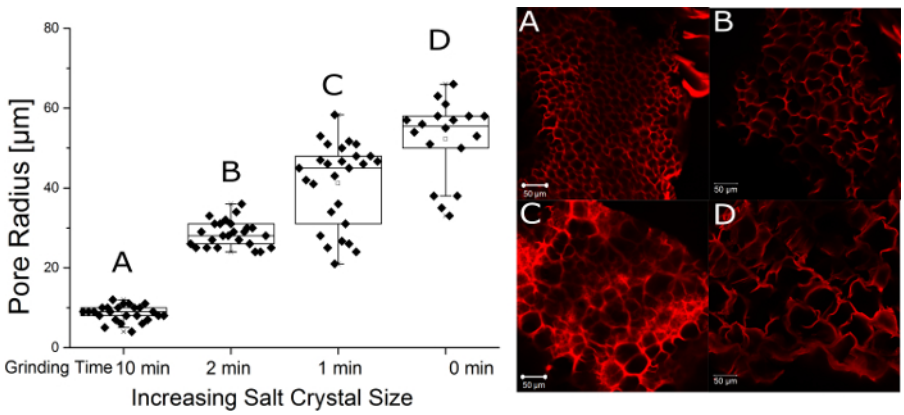


**Figure 2: Hydrogel particle leaching.** Salt crystals are ground with a mortar and pestle for (**A**) 10 min, (**B**) 2 min, (**C**) 1 min, and (**D**) 0 min. Both hydrogel components are mixed, and the solution is saturated with salt crystals. After the polymerization of the materials, salt is eluted from the material by diluting the template in large amounts of deionized water. The material is stained with fluorescent rhodamine B and analyzed with confocal laser scanning microscopy. The size and distribution of the pores within the material were analyzed with imaging software and plotted for the different grinding times. Images A to D show the pores within representative hydrogels, where salt crystals of varying sizes were used. The error bars represent the standard deviation. Scale bars = 50 µm. Please click here to view a larger version of this figure.

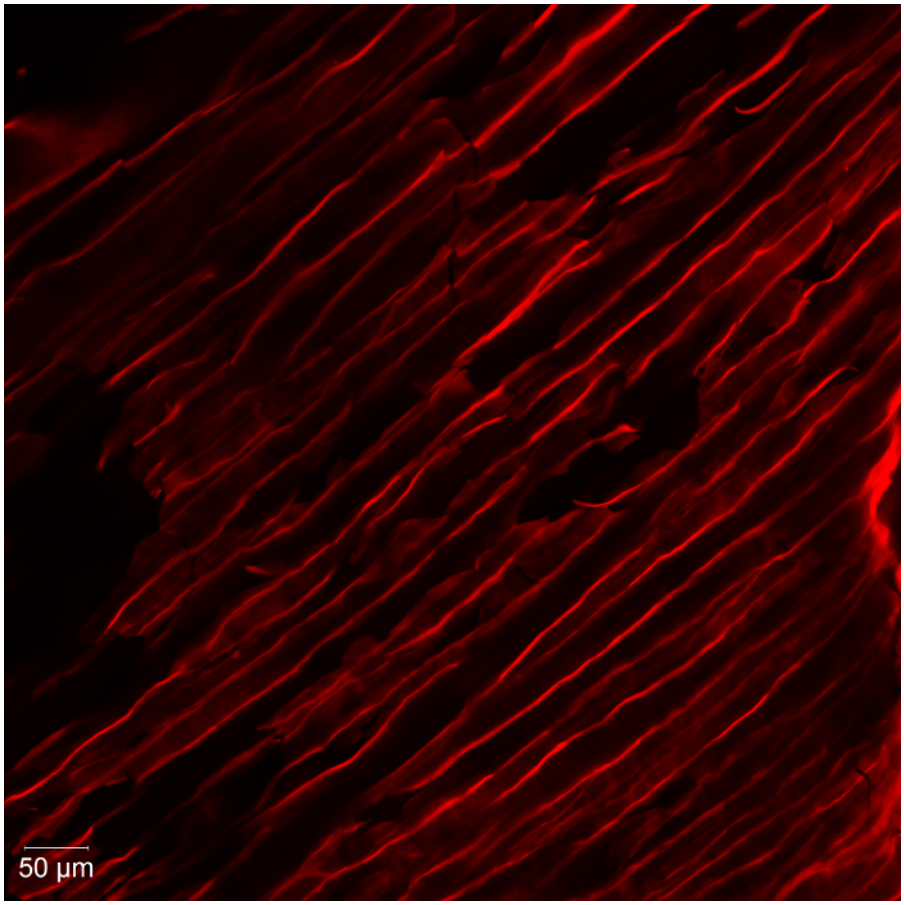


**Figure 3: Channel formation.** The polymerized hydrogels were transferred to a block of dry ice for 30 s. This was followed by the sublimation of the ice crystals from the structure at 0.05 mbar and -85 °C. The material was stained with fluorescent rhodamine B and analyzed with confocal laser scanning microscopy. The channels were several hundred microns long and had diameters of about 20 µm. Scale bars = 50 µm. Please click here to view a larger version of this figure.

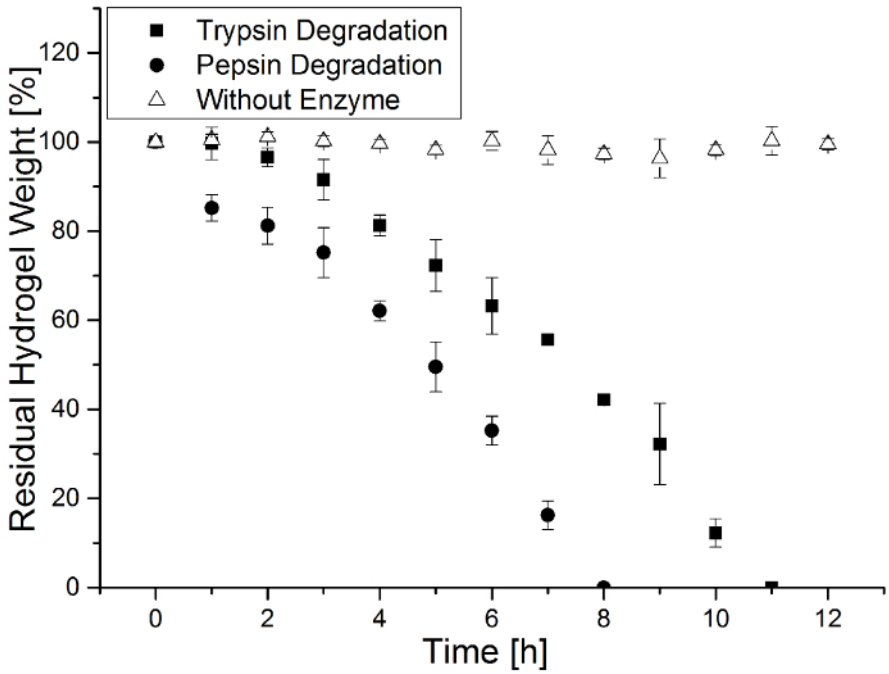


**Figure 4: Enzymatic degradation.** Representative degradation pattern of a macroporous hydrogel treated using a salt-leaching approach with ground salt crystals (10 min). Degradation took place in solution with 300 U trypsin and pepsin and was compared to an untreated hydrogel in PBS by measuring the residual weight of the material every hour. The error bars represent the standard deviation. Please click here to view a larger version of this figure.

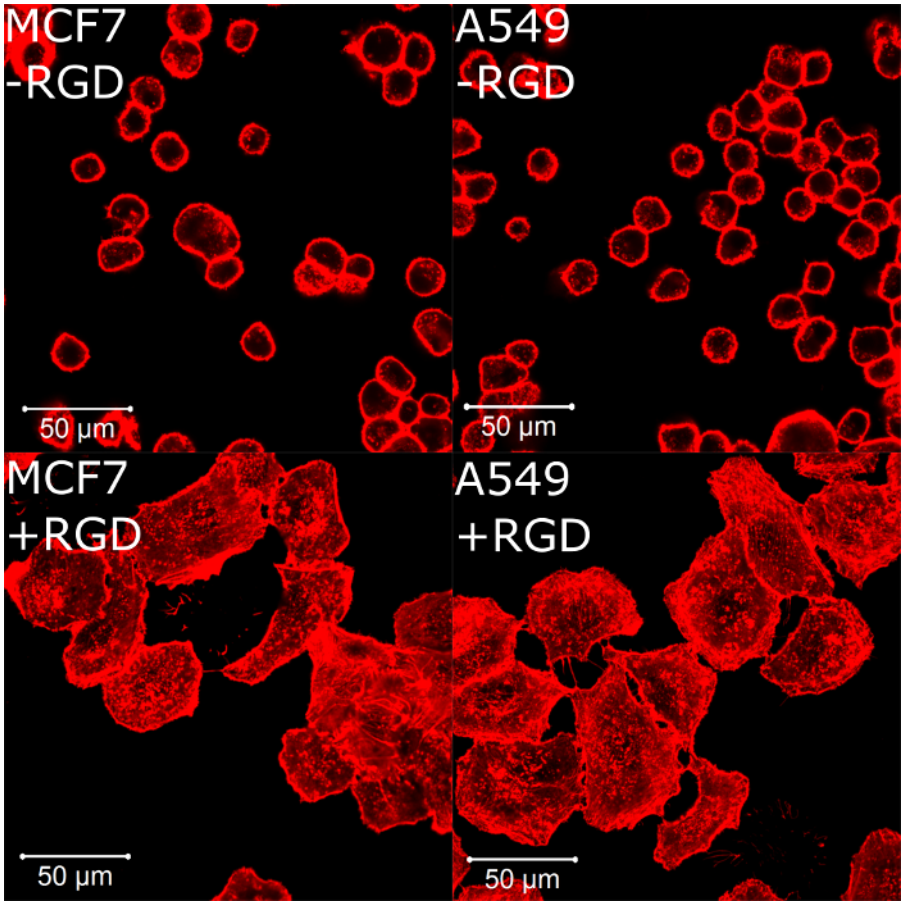


**Figure 5: Cellular behavior on hydrogels.** To investigate the potential of the material in cell culture, hydrogels were functionalized with a cell-adhesive RGD peptide. The hydrogels were polymerized into a µ-slide 8 well and seeded with 2 x $10^5$/$cm^2$ A549 and MCF7 cells. The cells were allowed to grow and adhere to the material for 24 h. After fixation with 3.7% formaldehyde and permeabilization with 0.1% Triton X-100, the cells were stained with cell-specific phalloidin-rhodamine and were visualized with inverted confocal laser scanning microscopy at a wavelength of 514 nm. Scale bars = 50 µm. Please click here to view a larger version of this figure.

| | Salt leaching | Salt leaching (grinded salt, 10 min) | Frozen at -20°C | Frozen at -196°C | Gradient freezing | Non-porous hydrogels |
|---|---|---|---|---|---|---|
| **pH 2 [%]** | 95.3 ± 3.8 | 96.2 ± 3.3 | 94.4 ± 3.4 | 96.2 ± 5.2 | 89.7 ± 4.3 | 69.5 ± 4.4 |
| **pH 7 [%]** | 95.6 ± 2.3 | 94.4 ± 4.2 | 95.3 ± 5.6 | 94.2 ± 3.2 | 94.1 ± 3.2 | 98.2 ± 1.6 |
| **pH 10 [%]** | 82.1 ± 4.4 | 86.1 ± 3.2 | 76.3 ± 5.5 | 83.2 ± 4.3 | 84.2 ± 4.5 | 42.3 ± 4.1 |
| **RT [%]** | 97.4 ± 4.4 | 95.4 ± 0.42 | 91.3 ± 2.2 | 94.3 ± 4.1 | 97.1 ± 1.9 | 99.1 ± 2.2 |
| **37°C [%]** | 95.3 ± 4.2 | 97.4 ± 0.4 | 93.2 ± 3.3 | 96.2 ± 1.9 | 95.3 ± 4.3 | 98.3 ± 3.4 |
| **80°C [%]** | 81.2 ± 4.4 | 83.6 ± 4.5 | 84.2 ± 4.9 | 83.5 ± 3.4 | 91.4 ± 8.1 | 70.2 ± 6.2 |
| **Swelling ratio [%]** | 1153 ± 110 | 534 ± 45 | 1312 ± 91 | 834 ± 78 | 823 ± 163 | - |
| **Trypsin [h]** | 4.5 ± 0.23 | 7.4 ± 0.29 | 3.2 ± 0.21 | 6.5 ± 0.13 | 4.2 ± 0.13 | 55.0 ± 2.48 |
| **Pepsin [h]** | 3.1 ± 0.19 | 4.0 ± 0.22 | 2.4 ± 0.13 | 3.8 ± 0.14 | 3.5 ± 0.19 | 46.5 ± 3.02 |

**Table 1: Hydrogel properties.** To investigate the influence of the pore formation methods used here (top line), different types of hydrogels were investigated for their pH and temperature stabilities, swelling ratio, and enzymatic degradation pattern (left column). For pH values of 2, 7, and 10 and temperatures of RT, 37 °C, and 80 °C, the average residual weights of the hydrogels after 7 days are displayed as percentages. For enzymatic degradation, the half-lives (h) of the materials in 300 U trypsin and pepsin are shown. For the swelling ratios, the water uptakes based upon the dried gels are shown are percentages.

## Discussion

The production of macroporous matrices can be beneficial to many different fields. It has high technical and economic potential due to the defined structure of the hydrogel and the ability to control and tune specific material properties. However, the introduction of supramolecular

structural elements, such as pores or channels, to a 3D template might influence the overall properties of a material, such as the swelling ratio or the stiffness. This can result in the undesired decomposition, degradation, or breakdown of the network, thus rendering the material useless or simply inapplicable for the desired application[12]. Proteins possess a known potential for application in hydrogels due to their defined structure, their intrinsic biocompatibility (because they are derived from natural sources), and their many available modification sites (*e.g.,* reactive amino-, hydroxyl- or carboxyl- groups). However, many protein-based hydrogels lack sufficient mechanical stability compared to their synthetic counterparts[16]. Because the introduction of large 3D architecture may lead to the complete structural disintegration of a hydrogel, the feasibility of methods to tune 3D structures must be critically evaluated to obtain the desired material properties and to achieve the full potential of the protein material. This was one reason for the evaluation of the freeze-drying and particle leaching processes and of the effect those procedures had on the resulting properties of the protein hydrogels in this study.

A critical point while working with hydrogels is the visualization of the materials on the macro-scale. Quite often, electron microscopy-based methods are used to examine the 3D structure of a hydrogel[28]. While doing this, it is important to consider that many procedures used to prepare the sample for electron microscopy might interact with the material and significantly alter its properties and integrity *(e.g.,* the freezing of the sample). To overcome this problem, confocal laser scanning microscopy was used here to evaluate the produced 3D architectures and the size and distribution of the pores within the hydrogels. As opposed to electron microscopy, only a simple staining step in the native hydrated environment of the swollen state of the material is necessary to analyze the micro-scale structure of the matrix.

Freeze-drying is a very well-established method in soft matter research and biochemistry. It allows for the production of pores of a certain size and regular distribution throughout the matrix due to ice crystal formation in the network. By applying very low pressure *(i.e.,* ~0.05 mbar) and temperatures below 0 °C, the ice sublimates from the material, leaving pores behind without further disintegrating the surrounding network. In the swollen state, pores of about 10 to 70 µm were detected in the template (**Figure 1**), with a narrow size distribution for smaller pores and a wider distribution for larger pores. Those sizes are within a suitable range for most cell culture-related applications, like hematopoietic stem cell incorporation[3] and the ingrowth of osteoids and fibroblasts[21]. However, this method also has certain limitations. For some applications, the creation of larger pores might be beneficial[22]. The size limit that could be achieved with freeze drying was approximately 70 µm in this material, but larger pores have already been developed by different groups[21].

The dried hydrogels must be handled with care; due to their fragile structure, they tend to break apart when slight pressure is applied to the dried material. The critical step in the preparation of the material is the polymerization. Both components must be mixed properly and quickly to guarantee the production of a uniform hydrogel. If the hydrogels are not mixed immediately, a partial polymerization might occur, leading to an uneven pore distribution within the matrix.

Another technique that is well-established for the modification of synthetic polymer materials is the so-called particle leaching. Solid but dispersible particles are incorporated into the material prior to the gel-sol transition and are later eliminated from the polymerized material by changing external conditions, leading to the solubilization of the particles[13,29]. Paraffin and gelatin can be melted by increasing the temperature[29], while crystalline particles, like sugars and salts, are dissolved by changing the buffer composition or dilution[13]. For this protein-based material, the radii of the pores could be changed 10-fold within the 3D matrix. Untreated salt crystals created the largest pores, with radii about 50 to 70 µm (**Figure 2**). One limitation of this method is the maximum size of the pores: above 70 µm, a considerable population of the pores exhibited fractures in their backbones, thus destabilizing the matrix. To overcome this problem, salt crystals were ground with a mortar and pestle, and significantly smaller pores, without damaged structural backbones, were produced. To improve the method, the crystals can be sieved to achieve a specific size[3]. Another critical step is the saturation of the solution: the hydrogels must be completely liquid when adding the salt. If the hydrogel is already partly polymerized, uneven pores will appear in the mesh, or the gel will break. If too little salt is added, the solution might be too diluted, causing no pores appear. To overcome this problem, a test series must be made prior to the experiment to find the exact amount of salt that is necessary for saturation.

Channels within a material can be useful for certain applications that require scaffold material (*e.g.,* the ingrowth of axons into a template)[11], but they are often difficult to produce in soft materials due to the lack of stability and rigidity. We established a process that includes the use of a block of solid carbon dioxide to produce linear-oriented channels within the protein hydrogel, with a length of several hundred microns and a radius of approximately 20 µm (**Figure 3**). During their formation, the time that the hydrogel remains on the block of dry ice is essential and must be evaluated individually for each material. Short freezing times do not lead to the formation of structures, while longer freezing times break the network. When removing the material, damage to the hydrogel can be avoided by pressing a spatula against the block of ice and scratching some of the ice off the block along with the hydrogel.

All procedures influence the structure of the material and may thus severely reduce the beneficial properties of the material. To address this effect, different basic gel parameters were evaluated. The swelling ratio is one of the most important features with respect to the use of a hydrogel in cell culture, as it directly correlates with the free water content, an essential factor for the growth and survival of cells within a 3D template. All treated hydrogels were dried and brought to swelling equilibrium prior to the measurements. The swelling ratios for the different methods varied from 534% to 1,153% (**Table 1**). The free water content is crucial to prevent the accumulation of harmful intermediates in the environment of the cell. At the same time, nutrients, growth factors, and oxygen should be able to diffuse rapidly throughout the gel to fulfill the demands of a growing and dividing cell[7]. With both freeze-drying and salt leaching, a high water content could be achieved for the protein-based macroporous hydrogels. Furthermore, the swelling ratio has a significant influence on the differentiation of stem cells within a matrix and can be further improved by incorporating PEG into the material[8]. This is easy to do in protein hydrogels with a variety of target sites.

Many hydrogels are intended for use in biotechnological fields, such as cell culture[2], tissue engineering[30], or transplantation medicine[31]. The material must deal with harsh external conditions, including temperature or pH changes and the presence of degradation agents, like proteases or catalytic active substances. Especially in materials intended for use in the human body, such as coatings for medical devices[32] or injectable hydrogels[33], the systems might mutually interact with the host system, thus influencing their stability and possibly limiting their use. As a variety of methods to tune 3D architecture severely influence the material stability, all procedures used in this study were investigated for their influence on the mechanical and chemical properties of the hydrogel. The reaction of the BSA protein hydrogel mainly depends upon the applied stimuli. For example, a temperature increase from RT to 37 °C or a pH shift into the acidic range did not alter the protein stability, while a shift to basic

conditions reduced the residual weight after a week (**Table 1**). More importantly, none of the procedures used here influenced the stability of the matrix in comparison to one another (**Table 1**).

For many applications, a cleavable backbone is desirable. Most protein hydrogels are biodegradable. However, many hydrogels require extended decomposing times, as agents must often work from the outside to the inside of the material. This further leads to uneven degradation patterns, resulting in the complete breakdown of the network after a certain, undefined time. Untreated BSA hydrogel was degraded after several days, with the complete breakdown and disintegration of the network after a certain time. Hydrogels treated with freeze-drying or particle leaching approaches degraded in a predictable manner within hours when in the presence of enzymes, as shown in **Figure 4**. For macroporous hydrogels, enzymes can freely diffuse throughout the matrix and cleave the backbone of the whole template, leading to an degradation and predictable degradation times. The degradation time was found to be dependent upon the sizes of the pores within the material. For example, hydrogels frozen at -20 °C, with an average pore radius of 60 µm, could be degraded to half of their weight within 3.2 h using 300 U of pepsin. Hydrogels frozen at -196 °C, with a significantly smaller average pore radius of 10 µm, had a half-life of 6.5 h at the same pepsin concentration (**Table 1**).

A major concern for cell culture applications is the adhesion potential of the matrix. Many materials facilitate adhesion due to their inherent adhesion properties, while other systems must be provided with proper cellular adhesion through the incorporation of cell-adhesive structures. One prominent example of guaranteed cell adhesion is a cell-adhesive peptide, with RGD one of the most prominent and well-characterized[34]. Protein-based materials are normally easy to modify due to the vast amount of accessible target sites within the template. To prove the general feasibility of this BSA-based protein hydrogel for cell culture applications, the material was functionalized with a cell-adhesive RGD peptide. To facilitate the process, a specific KCSSGKSRGDS peptide was designed with a primary amine in the lysine moiety, which can be targeted by the crosslinking agent during polymerization. A549 and MCF7 cells were grown on the surface of the protein hydrogel and showed distinctive adhesion after 24 h of growth, with high viability[16]. The growth of the cells and the strong adhesion, which could not be reversed by washing with PBS, show the viability of the cells on the matrix and the general feasibility of the produced material for cell culture application. The direct incorporation of the peptide is both an advantage and a limitation of this system: on the one hand, it simplifies the procedure and eliminates the need for an additional reaction step. On the other hand, many different cell-adhesive peptides have been developed and used over the last decade[5], and some of them bear primary amines in their structure. Those amines will readily react with the hydroxy groups of the linker molecule, thus potentially disabling the adhesion properties of the peptide. The peptide must be chosen according to this system. Another option is to add the peptide of choice after the polymerization. This way, it can be linked to different moieties in the protein backbone, whichever is best for the desired application.

For further use in 3D cell culture, the system must be optimized and adapted for each specific cell line. By changing the protein or crosslinker concentration, the properties can already be tuned, especially the mechanical properties of the material. The presented methods offer a range of tools for the manipulation of hydrogels, with no need for special equipment and with low costs. The techniques maintain the general properties of the protein-based hydrogel, making them useful for the initial evaluation of 3D cell culture applications.

## Disclosures

The authors declare that they have no competing financial interests.

## Acknowledgements

The authors would like to thank Baden-Württemberg Stiftung for their financial support in the "Bioinspired Material Synthesis" framework (BioMatS-14).

## References

1. Geckil, H., Xu, F., Zhang, X., Moon, S., Demirki, U. Engineering hydrogels as extracellular matrix mimics. *Nanomedicine.* **5** (3), 469-484 (2011).
2. Liu, Y., Chan-Park, M. B. A biomimetic hydrogel based on methacrylated dextran-graft-lysine and gelatin for 3D smooth muscle cell culture. *Biomaterials.* **31** (6), 1158-1170 (2010).
3. Raic, A., Rödling, L., Kalbacher, H., Lee-Thedieck, C. Biomimetic macroporous PEG hydrogels as 3D scaffolds for the multiplication of human hematopoietic stem and progenitor cells. *Biomaterials.* **35** (3), 929-940 (2014).
4. Wong Po Foo, C. T. S., Lee, J. S., Mulyasasmita, W., Parisi-Amon, A., Heilshorn, S. C. Two-component protein-engineered physical hydrogels for cell encapsulation. *Proc Nat Acad Sci USA.* **106** (52), 22067-22072 (2009).
5. Zhu, J., Marchant, R. E. Design properties of hydrogel tissue-engineering scaffolds. *Expert Rev Med Devic.* **8** (5), 607-26 (2011).
6. Samaryk, V., *et al.* Versatile Approach to Develop Porous Hydrogels with a Regular Pore Distribution and Investigation of their Physicomechanical Properties. *J Appl Polym Sci.* **114**, 2204-2212 (2009).
7. Li, X. J., Valadez, A. V, Zuo, P., Nie, Z. Microfluidic 3D cell culture: potential application for tissue-based bioassays. *Bioanalysis.* **4** (12), 1509-25 (2012).
8. Park, H., Guo, X., *et al.* Effect of Swelling Ratio of Injectable Hydrogel Composites on Chondrogenic Differentiation of Encapsulated Rabbit Marrow Mesenchymal Stem Cells In Vitro. *Biomacromolecules.* **10** (3), 541-546 (2010).
9. Stokols, S., Tuszynski, M. H. The fabrication and characterization of linearly oriented nerve guidance scaffolds for spinal cord injury. *Biomaterials.* **25** (27), 5839-5846 (2004).
10. Sung, K. E. *at al.* Understanding the Impact of 2D and 3D Fibroblast Cultures on In Vitro Breast Cancer Models. *PLoS One.* **8** (10), 1-13 (2013).
11. Tsai, E. C., Dalton, P. D., Shoichet, M. S., Tator, C. H. Synthetic hydrogel guidance channels facilitate regeneration of adult rat brainstem motor axons after complete spinal cord transection. *J Neurotrauma.* **21** (6), 789-804 (2004).

 

12. Shoichet, M. S., Li, R. H., White, M. L., Winn, S. R. Stability of hydrogels used in cell encapsulation: An in vitro comparison of alginate and agarose. *Biotechnol Bioeng.* **50** (4), 374-381 (1996).
13. Chiu, Y.-C., Kocagöz, S., Larson, J. C., Brey, E. M. Evaluation of physical and mechanical properties of porous poly (ethylene glycol)-co-(L-lactic acid) hydrogels during degradation. *PloS One.* **8** (4), e60728 (2013).
14. Jonker, A. M., Lo, D. W. P. M., Hest, J. C. M. Van Peptide- and Protein-Based Hydrogels. *Chem Mater.* **24**, 759-766 (2012).
15. Bodenberger, N., *et al.* Beyond bread and beer: whole cell protein extracts from baker's yeast as a bulk source for 3D cell culture matrices. *Appl Microbiol Biot.* **101** (5), 1-11 (2016).
16. Bodenberger, N., Paul P., Kubiczek D., Walther P., Gottschalk K.E., Rosenau F. A novel cheap and easy to handle protein hydrogel for 3D cell culture applications : a high stability matrix with tunable elasticity and cell adhesion properties. *Chem Sel.* **1** (7), 1353-1360 (2016).
17. Agarwal, S., Wendorff, J. H., Greiner, A. Use of electrospinning technique for biomedical applications. *Polymer.* **49** (26), 5603-5621 (2008).
18. Hong, J., deMello, A. J., Jayasinghe, S. N. Bio-electrospraying and droplet-based microfluidics: control of cell numbers within living residues. *Biome Mater.* **5** (2), 21001 (2010).
19. Jayasinghe, S. N., Irvine, S., McEwan, J. R. Cell electrospinning highly concentrated cellular suspensions containing primary living organisms into cell-bearing threads and scaffolds. *Nanomedicine.* **2** (4), 555-567 (2007).
20. Selimović, Š., Oh, J., Bae, H., Dokmeci, M., Khademhosseini, A. Microscale strategies for generating cell-encapsulating hydrogels. *Polymers.* **4** (3), 1554-1579 (2012).
21. Annabi, N., Nichol, J. W., *et al.* Controlling the porosity and microarchitecture of hydrogels for tissue engineering. *Tissue Eng Part B Rev.* **16** (4), 371-383 (2010).
22. Lee, J., Cuddihy, M. J., Kotov, N. A. Three-dimensional cell culture matrices: state of the art. *Tissue Eng Part B Rev.* **14** (1), 61-86 (2008).
23. Bodenberger, N., *et al.* Evaluation of methods for pore generation and their influence on physio-chemical properties of a protein based hydroge. *Biotech Rep.* ***12,*** 6-12 (2016).
24. Raja, S. T. K., Thiruselvi, T., Mandal, A. B., Gnanamani, A. pH and redox sensitive albumin hydrogel: A self-derived biomaterial. *Sci Rep.* **5**, 15977 (2015).
25. Hennink, W. E., van Nostrum, C. F. Novel crosslinking methods to design hydrogels. *Adv Drug Deliver Rev.* **64** (SUPPL.), 223-236 (2012).
26. Chung, C., Lampe, K.J., Heilshorn, S.C. Tetrakis (hydroxylmethyl) phosphonium chloride as a covalent crosslinking agent for cell encapsulation within protein-based hydrogels. *Biomacromolecules.* **13** (12), 3912-3916 (2008).
27. Calo, E., Khutoryanskiy, V. V. Biomedical applications of hydrogels: A review of patents and commercial products. *Eur Polym J.* **65**, 252-267 (2015).
28. Huang, H., Herrera, A. I., Luo, Z., Prakash, O., Sun, X. S. Structural transformation and physical properties of a hydrogel-forming peptide studied by NMR, transmission electron microscopy, and dynamic rheometer. *Biophys J,.* **103** (5), 979-988 (2012).
29. Draghi, L., Resta, S., Pirozzolo, M. G., Tanzi, M. C. Microspheres leaching for scaffold porosity control. *J Mater Sci.* **16** (12), 1093-1097 (2005).
30. Whang, K. *et al.* Engineering Bone Regeneration with Bioabsorbable Scaffolds with Novel Microarchitecture. *Tissue Eng.* **5** (1), 35-51 (1999).
31. Ziv, K. *et al.* A tunable silk-alginate hydrogel scaffold for stem cell culture and transplantation. *Biomaterials.* **35** (12), 3736-3743 (2014).
32. Butruk-Raszeja, B. A. *et al.* Athrombogenic hydrogel coatings for medical devices--Examination of biological properties. *Colloid Surface B.* **130**, 192-8 (2015).
33. Lü, S., Li, B., Ni, B., Sun, Z., Liu, M., Wang, Q. Thermoresponsive injectable hydrogel for three-dimensional cell culture: chondroitin sulfate bioconjugated with poly(N-isopropylacrylamide) synthesized by RAFT polymerization. *Soft Matter.* **7** (22), 10763 (2011).
34. Ruoslahti, E., Pierschbacher, M. D. New perspectives in cell adhesion: RGD and integrins. *Science.* **238** (4826), 491-497 (1987).


 