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Evaluation of methods for pore generation and their influence on physio-chemical properties of a protein based hydrogel

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ABSTRACT

Different methods to create and manipulate pore sizes in hydrogel fabrication are available, but systematic studies are normally conducted with hydrogels made of synthetic chemical compounds as backbones. In this study, a hydrogel made of natural and abundant protein in combination with different, well-available techniques was used to produce different architectures within the hydrogel matrix. Pore sizes and distribution are compared and resulting hydrogel properties like swelling ratio, resistance towards external stimuli and enzymatic degradation were investigated. Porous hydrogels were functionalized and two cancer cell lines were successfully adhered onto the material. With simple methods, pores with a radius between 10 and 80 μm and channels of 25 μm radius with a length of several hundreds of μm could be created and analyzed with laser scanning confocal microscopy and electron microscopy respectively. Furthermore, the influence of different methods on swelling ratio, enzymatic degradation and pH and temperature resistance was observed.

1. Introduction

Hydrogels are insoluble networks that can maintain huge amounts of water while keeping their insoluble form and structure [1]. Because those materials can serve as an excellent growth substrate for cells many approaches have been made to produce artificial networks for 3D cell growth in order to trigger and alter cellular behavior within those structures [2–4]. This is not only important because it represents a more realistic environment for cells than standard 2D approaches, but it also offers an enormous amount of new possibilities for the growth of cells, especially for stem cells and tumor models [5]. Numerous types of materials have a variety of properties, especially pore sizes fluctuates strongly depending on the matrix. For cell culture or tissue engineering applications, certain pore sizes in the material are crucial for sufficient oxygen transport throughout the matrix, the removal of toxic compounds and the supply of enough space for cellular outgrowth [6]. The properties and hence pore sizes of the novel materials and play a tremendous role concerning the cell type which should be cultivated and the application, with differences for each intended use: neovascularization, skin or bone regeneration and growth of special cell types like stem cells, hepatocytes or fibroblasts [7–9]. Furthermore, materials stability to environmental parameters is crucial for the use of hydrogels in complex environments like the human body which is strongly influenced by and correlates with the pore size and distribution of pores within matrices [9,10].

In recent years, extensive studies have been conducted on 3D structures of hydrogels consisting of artificial precursors [11], which are often outstanding due to their high mechanical and chemical stability and their ability to be modified to generate pores of desired sizes. Proteins, on the other hand, offer a great potential for hydrogel applications as they consist of well-defined and characterized structures which are tunable in every possible way to produce highly specific, innovative materials. In this study, a protein based hydrogels was developed and the feasibility of different, well available strategies for the production of macro porous scaffolds for protein hydrogels and the properties of the resulting protein networks were compared. Furthermore, the feasibility for those materials in cell culture are evaluated.

A huge variety of techniques is available for tuning the 3D structures of hydrogel materials, ranging from rather simple approaches to sophisticated, new techniques from all different fields of material science. A popular technique is electrospinning to produce specific structures which can be controlled within a certain range [12]. Charged threads are formed with the help of a

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strong electric field from a polymer solution and solidification takes place when the polymer is drawn from the solution, resulting in threads in the range of nm up to μm [12]. Further techniques like soft lithography, photolithography, hydrodynamic focusing, electro-spraying and bio-printing have a high potential to properly tune the exact size of the hydrogels pores [8,9], their structures and their distribution within the matrix. However, most techniques have one major drawback: they need special equipment, are often very expensive in production or limited to a certain size, require expert knowledge in different fields and often are not applicable for protein based materials. Additionally, many of the needed chemicals or procedures used are not cell compatible [13].

On the other hand, there are several simple and robust methods which do not require special equipment, can be used in any lab at any time and are very robust and easy to reproduce. One major system is solvent casting, where particles of certain, well-controlled size are homogeneously distributed into a solution prior to solidification [13]. Salt is the most widely used material, due to its good availability at low costs. Apart from salt, affordable materials like sugar, gelatin, paraffin or chalk are good substitutes due to the potential to be eliminated from the polymer by heat, dilution or pH switches [14,15]. Another important physical approach to form pores is freeze drying, where ice crystals are formed in the matrix upon freezing of the material [16]. By drying the material in vacuum, water is sublimated leaving pores in the zones which were previously occupied by ice crystals. In gas foaming, a polymer solution is saturated with gas which is later released from the material. Sodium bicarbonate can be used to produce gas in solution which creates porous structures in the hydrogel [1].

Here, we investigate the feasibility of freeze-drying, particle-leaching and gradient freezing to control size and distribution of pores within a protein hydrogel [17]. All methods are applicable in every standard lab with cheap and well obtainable chemicals (NaCl, $CaCO_3$, liquid nitrogen, dry ice) and without special equipment. Furthermore, swelling ratio, proteolytic degradation and pH and temperature stability over a broad range were investigated to detect the influence of preparation methods on protein network properties. Finally, hydrogels were modified with a cell-adhesive peptide to investigate its feasibility in cell culture with two different cell lines.

2. Materials and methods

Bovine Serum Albumin (BSA) and Tetrakis (hydroxymethyl) phosphonium chloride (THPC) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA), dilutions were stored at RT. NaCl and $CaCO_3$ were obtained from Carl Roth (Carl Roth GmbH und Co. KG, Karlsruhe, Germany), Phosphate Buffered Saline was purchased from life technologies (Carlsbad, California, USA). Rhodamine-phalloidine was purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA).

Hydrogels were prepared by mixing 100 μl of a 20% (w/v) solution of BSA with the same volume of THPC to afford a 1:1 ratio of reactive amine to hydroxy groups and polymerized in an open 96 well plate at RT within minutes.

To generate pores via freeze-drying, protein hydrogels were transferred into Eppendorf-Tubes and lyophilized (FreezeDryer Epsilon 1-6D, Christ, Osterode am Harz, Germany) over night at −85 °C and 0.05 mbar.

For particle leaching, gels were polymerized in the presence of NaCl and $CaCO_3$ (Carl Roth GmbH und Co. KG, Karlsruhe, Germany), respectively. Particles were added to hydrogels right after mixing of protein with THPC, before polymerization could take place. NaCl and $CaCO_3$ were added until a saturation effect could be observed. Half of NaCl and $CaCO_3$ crystals were grinded to reduce salt crystal size, half was left untreated. Solidification took place within minutes, leading to an even distribution of crystals in the hydrogel matrix. Afterwards, polymerized hydrogels were transferred to 5 ml of H_20 (pH 7.4 for NaCl and 6.5 for $CaCO_3$) to dilute and remove salt crystals and obtain porous hydrogels.

For gradient freezing, 200 μl hydrogels were polymerized as described above and placed on a block of dry ice at 37° RT, leading to specific site-directed freezing and channel formation within hydrogel.

After pore generation in the matrix, gels were left at room temperature for 24 h to evaporate water to get dried gels for further use. The swelling ratio for different drying methods was determined by transferring dried gels into 5 ml PBS, pH 7.4 at RT. Swelling ratio was determined by the following equation as soon as swelling equilibrium was reached:

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

Where Ws is the wet weight of the gel and Wd the weight of the dried gel.

Thermal and pH resistance were determined by bringing untreated or porous gels (200 μl) to a swelling equilibrium by immersing gels in PBS. Afterwards, hydrogels were transferred to 5 ml solution at intended pH or temperature (pH 2, 7.4, 10 and temperatures of 37 °C and 80 °C). Weight was determined at fixed time points over 7 days to determine residual weight. All experiments were performed in triplicate.

In vitro degradation was determined for all modified hydrogels and compared to unmodified ones. Gels were brought to a swelling equilibration and immersed in enzyme solution of 300 Units trypsin and pepsin at pH 7.4 and 2 respectively. Hydrogels were removed from the solution, dried with a highly absorbent paper and weighted every hour over a period of 12 h to determine residual weight. All experiments were performed in triplicate.

In order to visualize three-dimensional structures of the hydrogel, hydrogels were co-polymerized with the fluorescent dye rhodamine B (0.05 mg/ml (w/v)) and treated as described earlier with different freezing or leaching techniques. Gels were observed with a Zeiss Confocal 248 Microscope (Carl Zeiss Ag, Oberkochen, Germany) with Zen software (Zen 2012 Sp1, 250 black edition, Version 8,1,0,484) at an excitation wavelength of 561 nm to reveal 3D structures. Different regions were cut to reveal pore distribution in the gel, and distribution and size of the pores within the matrix were analyzed using the GSA image analyzer software (GSA Image Analyzer, GSA, Version 419 3.8.7).

Peptide synthesis. A microwave synthesizer (CEM Cooperation, Matthews, NC, USA) with a standard fmoc solid phase was used. The fmoc protecting group was removed with 20% (v/v) piperidine in DMF and amino acid were added in 0.2 molar equivalent to the reactor, followed by 0.5 molar equivalent HBTU and 2 molar equivalent DIEA and repeated for all amino acids followed by subsequent deprotection. The peptide was cleaved in 95% (v/v) trifluoroacetic acid (TFA), 2.5% (v/v) triisopropylsilane (TIS), and 2.5% (v/v) H_2O for one hour, precipitated and washed with cold diethyl ether followed by vacuum drying.

Peptide purification. The peptide was purified via reverse phase preparative high performance chromatography in an acetonitrile/water gradient under acidic conditions on a Phenomenex C18 Luna column (5 mm pore size, 100 Å particle size, 250/21.2 mm).To determine the peptide mass, a liquid chromatography mass spectrometry approach was used. Finally, the peptide was freeze-dried (Labconco, Kansas City, MO, USA) and stored at −20 °C.

Cryo-scanning electron microscopy. Hydrogels were mixed with 30% isopropanol over night during freezing procedure. Samples were transferred into two low mass aluminum planchettes and high pressure frozen (Engineering Office M. Wohlwend