OPEN ACCESS
Journal of Medical Materials and Technologies
Vol 1, No 2, Special Issue Proceedings of 4th Euro BioMAT, Page No. 26-29 (2017)
http://journal.medmat.online

med:mat
ISSN: 2366-9136

Cryogels as potential scaffolds for wound healing applications

[1]*Annika Wartenberg, [1]Jürgen Weisser, [1]Susanne Thein, [2]Henri-Pierre Suso, [2]Ralf Schmidt, and [1]Matthias Schnabelrauch

[1]INNOVENT e.V., Biomaterials Department, Prüssingstr. 27B, 07745 Jena, Germany
[2]Biovotec AS, Hoffsveien 21-23, Oslo 0275, Norway
*Corresponding author (aw@innovent-jena.de)

Abstract – **Various cryogels have been manufactured from methacrylated biopolymer-derived hyaluronan and dextran as well as from synthetic macromer polyethylene glycol diacrylate (PEGDA) with and without addition of egg shell membrane protein as potential mediator of the cell regeneration capacity of the final scaffolds. Among the studied crosslinkable macromers, PEGDA has been found to provide cryogels with excellent mechanical stability, inter-connecting porosity and an encouraging in vitro cytocompatibility. Furthermore, the addition of egg shell membrane protein particles to the cryogels considerably increased the ability of cells to adhere onto the cryogel surfaces or even migrate into the porous structure. Overall, the synthesized cryogels are promising candidates for wound dressings that support tissue regeneration.**

Keywords – cryogel, PEGDA, hyaluronan, dextran, collagen, eggshell membrane, human fibroblasts, wound dressing

1. INTRODUCTION

Cryogels are 3D sponge-like networks with interconnected macropores [1]. Their unique properties make cryogels attractive for numerous biomedical applications [2], especially as scaffolds for tissue engineering, drug carriers or wound healing devices. Cryogelation is an effective, low-cost process occurring in aqueous media only requiring cross-linkable functionalities within the starting materials.

Due to their excellent biocompatibility combined with potential biodegradability, biopolymeric cryogels based on polysaccharides like dextran [3], hyaluronan [4], and chitosan [5], or proteins like collagen or gelatine [6] are promising starting materials. In addition the polyfunctionality of those biopolymers allows a large variety of derivatization to initiate cross-linking reactions. The combination of biopolymers with stabilizing synthetic macromers can influence the cryogel properties and therefore extend their application potential. The easy production process allows high flexibility in size and shape. Different active molecules, like drugs or proteins can be incorporated into those gels.

Among natural macromolecular ingredients known for its tissue regeneration potential, eggshell membrane has recently gained attention [7]. Chicken eggshell membrane is a protein rich fibrous structure with a composition similar to human skin. It consists of 2-10 % polysaccharides and 1-3 % fat [8]. The main proteins are different types of collagen and elastin [9]. Also, different GAGs are present in the membrane [10]. Specific membrane proteins are responsible for its antibacterial, anti-inflammatory and antiviral activity [11]. Recently, an efficient process was established by the company Biovotec AS (Oslo, Norway) to separate eggshell membrane from the egg [12] and provide high-purity eggshell membrane in larger quantities.

Aim of the study: The core idea of the work is to develop a new three-dimensional scaffold for wound regeneration based on a porous hydrogel carrier containing egg shell membrane protein as an active ingredient to promote tissue regeneration.

2. MATERIAL AND METHODS

Materials

Purified eggshell membrane protein (PEP) was manufactured/prepared by *Biovotec, Oslo, Norway* to be used for the fabrication of the scaffolds.

700-Polyethylen-glycol-diacrylat (700-PEGDA) was purchased from *Sigma Aldrich, Taufkirchen, Germany*, lithium phenyl(2,4,6-trimethylbenzoyl)phosphinate (LAP) from *TCI, Eschborn, Germany* and collagen type I (rat tail) from *Amedrix, Esslingen, Germany*.

Native hyaluronan with a molecular weight of 1,000 kDa was purchased from *Kraeber, Ellerbek, Germany* and was degraded thermally according to a previously published protocol [4] down to a molecular weight of ~100 kDa.

Hyaluronan methacrylate (HyaMA) synthesis was performed according to literature [4]. Briefly, 2.63 g of hyaluronan was dissolved in borate buffer at pH 8.5. After cooling to 5 °C, 14.6 ml of methacrylic acid anhydride were added drop wise. The pH was kept constant between 8-9 (with 1 N NaOH) while stirring the mixture at 5 °C over

night. Afterwards the resulting milky-white solution was precipitated in cold acetone (1 l). The residue was filtered and dissolved in distilled water. After adjusting the pH to 7 and dialysis followed by freeze-drying, 2.1 g of a white, fluffy solid were obtained. The DS was determined via ^{1}H-NMR to be 0.3-0.5.

Dextran methacrylate (DexMA) synthesis was performed as previously reported [4]. Briefly, 10 g dextran (*Sigma Aldrich*, M 15-25 kDa) were dissolved in DMSO. A spatula tip of 4-Dimethylaminopyridine was added and the mixture stirred for 15 min. Afterwards, 8.2 ml of glycidyl methacrylate were added slowly to the stirring mixture. The mixture was stirred for 48 h at RT in the dark. The reaction was stopped by adding concentrated HCl and stirring for further 15 min. After adjusting the pH to 7 and dialysis (7 days) followed by freeze-drying, 10.4 g of a white-brownish, fluffy solid were obtained. The DS was determined via ^{1}H-NMR to be 0.5.

Cryogel formation

Different polymer methacrylate solutions (1, 2, 10, 20 mg/ml) in water were prepared and mixed with the photo-crosslinker LAP (0.1-1 mg/ml in biopolymer solution). Collagen I (0.5 or 2 mg/ml) was dissolved in 0.5 % w/v acetic acid. To the polymer methacrylate solutions or to the mixtures of PEGDA with collagen, 12.5 or 50 mg of PEP particles were added to 1 ml of solution and the mixtures intensively vortexed. The suspensions were pipetted into pre-cooled silicone moulds of ~6 mm diameter and frozen at −20 °C over night. After freezing, samples were immediately UV-irradiated at 365 nm for 20-30 min at RT, whereupon the methacrylated endgroups of the biopolymers crosslinked. The ice crystals were allowed to thaw and the cryogels were washed several times with distilled water, before being stored in water for at least 48 h to ensure that all toxic side products were washed out. Finally, the gels were freeze-dried and the morphology (see FIGURE 1 for dextran methacrylate-based cryogel) visualized by SEM (Supra 55 VP, *Carl Zeiss, Jena, Germany*) at an accelerating voltage of 5 kV. All samples were sputtered with gold before analysis.

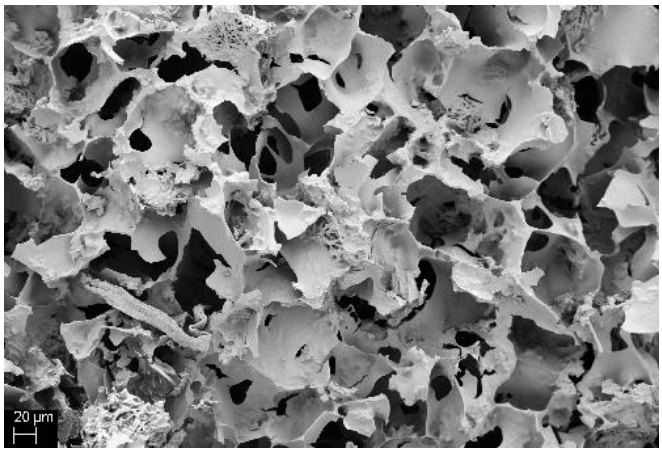

FIGURE 1
SEM IMAGE OF A DEXTRAN METHACRYLATE CRYOGEL CONTAINING 5 % W/V PEP.

Cryogel properties

In order to investigate mechanical properties, cryogels were compressed at room temperature using a Texture Analyzer TA-XT2i (*Stable Micro Systems*), while measuring the maximum force and the deformation behavior. Each sample was penetrated with a speed of 0.1 mm/sec up to 50 % of its original height using a 3.5 cm diameter cylindrical stainless steel plunger attached to a 5 or 50 kg cell connected to the crosshead. The stress is defined as the force per area (N/mm² or MPa). The compression modulus is measured as the slope in the linear elastic region of the stress/strain curve.

Swelling behavior of cryogels was determined by inserting different gels into distilled water and measuring their weight increase after 1 and 24 hours.

Biological evaluation

To evaluate cytotoxicity of different cryogel materials, prepared cylindrical scaffolds (size 12x3 mm) were transferred into a 24 well cell culture plate and fixed by adjustable rings (CellCrown culture inserts, *Scaffdex Oy, Tampere, Finland*). The scaffolds were disinfected with 70 % EtOH for 30 min, and then the EtOH was substituted by PBS, which was changed against new PBS after about 1 h. After being stored in sterile PBS over night followed by an additional PBS change, PBS was replaced after 2 h by 1 ml of cell culture medium (DMEM without phenol red, supplemented with 10 % FCS and 50 U/ml penicillin, 50 µg/ml streptomycin, *Biochrom, Berlin, Germany*). The medium was changed after 2 h, filling the new cell culture medium only to the upper edge of the scaffolds. 0.5 ml of a primary human fibroblasts cell suspension (100,000 cells/ml, passage 6, *PromoCell*) was added into each well resulting in a cell density of about 25,000 cells/cm^2. After 7, 14, and 28 days, scaffold samples were withdrawn and after viability staining (with 15 µg/ml fluorescein diacetate (FDA, *Fluka, Germany*) and GelRed® (diluted 1:10,000 from concentrated stem solution, *VWR International, Darmstadt, Germany*), images from the cell layers at the surface were taken by fluorescence microscopy (AxioObserver.Z1m, *Carl Zeiss microscopy, Jena, Germany*). To monitor cell growth into the scaffolds, samples were cut vertically through the centre with a scalpel and a small slide of about 2-3 mm thickness was harvested, stained and examined under the microscope.

3. RESULTS AND DISCUSSION

Eggshell membrane containing 3D-constructs were produced via cryogelation technique with pore sizes between 10 and 100 µm. For crosslinking LAP was used, which is known as a non-cytotoxic photoinitiator. Because the used polysaccharides do not contain readily crosslinkable groups, photochemically cross-linkable methacrylate groups were introduced into the polysaccharides by partial chemical modification of their hydroxyl groups with either methacrylic acid anhydride or glycidyl methacrylate using common literature procedures. Cryogels made from PEGDA containing PEP were rather stable and flexible like foam rubber with PEP homogenously distributed all over the scaffold. From literature it is known, that PEGDA modified with bioactive molecular components may act similar to the native ECM, mimicking a suitable microenvironment for the infiltration of cells. [13]

In contrast to PEGDA-derived gels, cryogels made from biopolymers were deformable. Hyaluronan-based cryogels were extremely soft, whereas dextran-derived gels were more rigid and stable. Depending on the origin, the degree of substitution and the concentration of the precursors, properties can be adapted to the desired application.

A typical stress-strain-curve from cryogels was not recordable, since there was no real break visible. We thus have developed another method for testing mechanical stability and elasticity. The gels were compressed to 50 % deformation. Pure PEGDA scaffolds maintained the highest mechanical stress, whereas dextran-based cryogels exhibited the highest compression moduli (see TABLE 1). Interestingly, the addition of eggshell membrane resulted in an increase in stability.

TABLE I
COMPARISON OFMECHANICAL STABILITY OF DIFFERENT CRYOGELS WITH AND WITHOUT PEP

	cryogels without PEP			PEP-containing cryogels		
	PEGDA	HyaMA	DexMA	PEGDA PEP	HyaMA PEP	DexMA PEP
stress at 50% deformation [MPa]	3.67 ± 0.20	0.03 ± 0.01	1.01 ± 0.01	0.40 ± 0.01	0.12 ± 0.01	3.02 ± 0.14
compression modulus [N/mm²]	3	0.05	43 ± 10	5	2	150 ± 43

The swellability of cryogels is an important factor for their evaluation as wound healing devices. The graph below (FIGURE 2) shows the x-fold weight increase of different cryogels with regard to non-swollen gel. Hyaluronan-derived cryogels took up the most water, whereas the addition of PEP reduced the swelling ratio of those gels drastically. Cryogels derived from the synthetic precursor PEGDA took up the least amount of water. Swelling of all gels occurred quickly. There was no visible difference between the swelling after 1 hour and after 24 hours. For use in wound healing, swelling is favorable, since those gels might absorb wound exudates better. But since hyaluronan-derived gels are rather unstable, mixtures of hyaluronan with more dimensionally stable polymers should be favored.

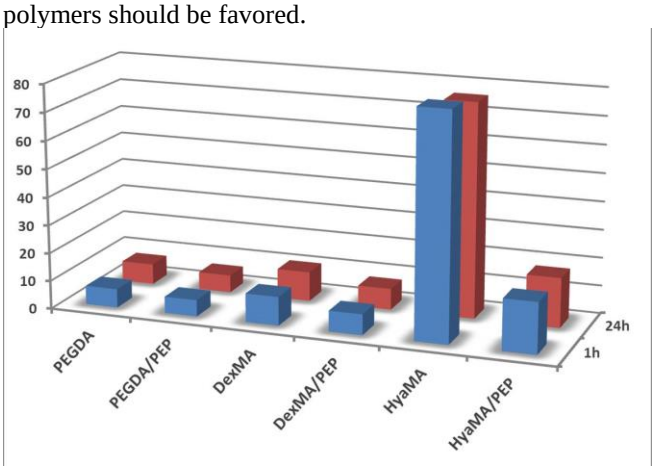

FIGURE 2
WATER UPTAKE AFTER 1 AND 24 HOURS AND RESULTING WEIGHT INCREASE OF DIFFERENT CRYOGELS WITH AND WITHOUT PEP.

As an essential prerequisite for applications in wound tissue regeneration, the prepared cryogels need to have a high cytocompatibility. In previous studies [4, 14] we could show that HyaMA and DexMA-based cryogels after careful purification of the final products do not show cytotoxic effects under in vitro conditions using 3T3 fibroblast cells.

To test the ability of cells to grow on the scaffolds and to migrate into the pores, human fibroblasts cultured on the scaffolds were detected and analysed by the live/dead staining. Viable cells developed a green fluorescence by cleavage of fluorescein diacetate and dead nuclei appeared in red from binding of GelRed to DNA. Pure PEGDA-derived scaffold was not a suitable carrier to culture human fibroblasts on its surface. It was not cytotoxic but obviously the cells could not adhere effectively. Even after 14 and 21 days only some rounded (but viable) cells were detected at the surface. Only a few cells were found somewhat deeper in the surface layer.

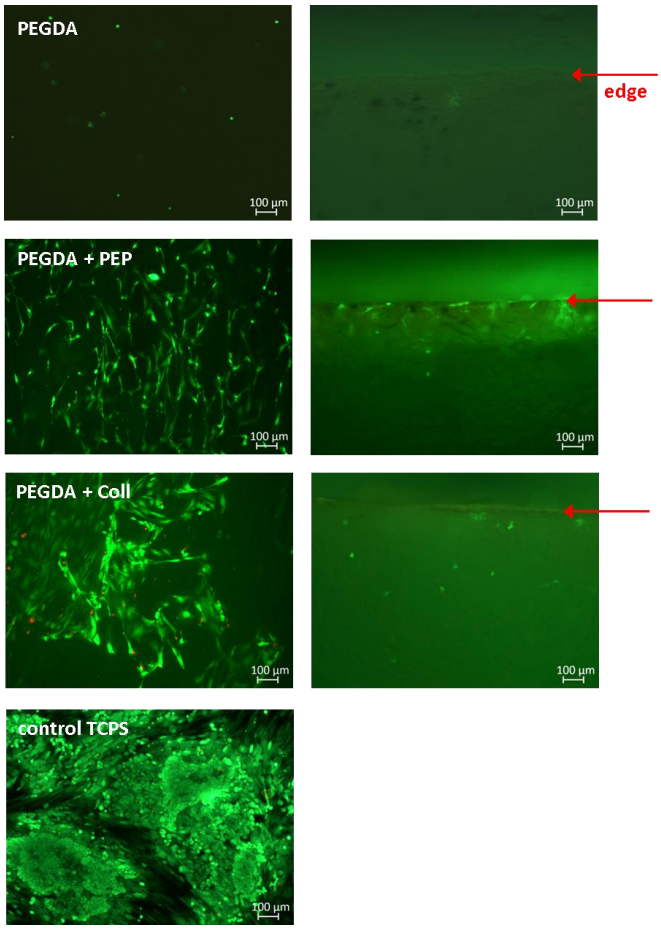

FIGURE 3
FLUORESCENCE MICROGRAPHS FROM THE SURFACES (LEFT, INCLUDING TISSUE CULTURE POLYSTYRENE CONTROL) AND FROM THE CROSS SECTIONS (RIGHT) OF CRYOGELS CONTAINING ONLY PEGDA, PEGDA MIXED WITH PEP AND PEGDA MIXED WITH COLLAGEN

The addition of PEP to PEGDA improved the adhesion of the cells at the surface, especially after 14 days and later, a layer of adherent cells was found. In the cross-sectional views single cells were found in the deeper layers of the scaffolds (see FIGURE 3).

Addition of collagen to PEP containing PEGDA material clearly improved cell adhesion and spreading already at

7 days of culture. The cell layer showed the highest cell density at this material consisting of typically elongated fibroblasts (see FIGURE 3). In the cross-sectional view living cells located some hundreds of micrometers in the depth were found. Artificial displacement of the cells could be excluded.

The cell viability was difficult to be evaluated due to strong background fluorescence of eggshell membrane in the scaffold. Obviously some loss of cells occurred during the staining procedure due to weak adherence of the cells on the scaffold material. Remarkably, even after 4 weeks of culture, a closed cell layer of viable cells developed in most of the tested PEP-containing scaffolds. Whereas pure PEGDA was not really supporting the cell attachment of primary human fibroblasts, the addition of PEP promoted their adherence and proliferation. Furthermore, the addition of collagen enabled the cells to grow into pores in deeper zones of the material. From the experiments it could be concluded that all of the cryogel scaffolds were not cytotoxic. It could be further concluded that collagen and eggshell membrane might be comparable in case of fibroblast attachment and migration.

4. CONCLUSIONS

Cryogels of various sizes and shapes have been prepared from both synthetic methacrylate-terminated polyethylene glycols and methacrylated natural polysaccharides. The resulting continuously porous materials varied in their mechanical and swelling properties dependent on the nature and composition of the used macromer.

Whereas PEGDA-derived cryogels showed rubber foam-like properties, biopolymer-derived cryogels were deformable after compression. All cryogel materials were cytocompatible under in vitro conditions. The addition of the naturally occurring protein material PEP increased the mechanical stability of the biopolymer cryogels, but lowered the swelling ability compared to pure biopolymer cryogels. Remarkably, the addition of PEP to the crosslinkable mixture as undissloved microparticles seems to positively influenced cell adhesion and migration into deeper areas of scaffolds comparable to collagen type I. Future work is focused on further optimization of the macromer composition of the cryogels to tailor the scaffold materials with regard to wound regeneration and on in vivo testing of the cryogel materials.

Acknowledgment

Authors gratefully acknowledge financial support provided by the *Research Council of Norway* (project no. 235545). We would like to thank Dr. Enda Kenny from Biovotec, Oslo, Norway and Drs. Mona Pederson and Tram Thu Vuong from Nofima, Oslo, Norway and Prof. Maxwell T. Hincke and Dr. Tamer A.E. Ahmed from the University of Ottawa, Canada for helpful feedback and discussion.

5. REFERENCES

[1] Lozinsky, V., Galaev, I.Y., Plieva, F.M., Savinal, I.N., Jungvid, H., Mattiasson, B., "Polymeric cryogels as promising materials of biotechnological interest"; *Trends Biotechnol.*, Vol. 21, 2003, 445-451

[2] Henderson, T.M.A., Ladewig, K., Haylock, D.N., McLean, K.M., O'Connor, A.J., "Cryogels for biomedical applications", *J. Mater. Chem. B*, Vol. 1, 2013, 2682–2695

[3] Zhou, D., Shen, S., Yun, J. et al., "Cryo-copolymerization preparation of dextran-hyaluronate based supermacroporous cryogel scaffolds for tissue engineering applications", *Front. Chem. Sci. Eng.*, Vol. 6, 2012, 339-347

[4] Reichelt, S., Becher, J., Weisser, J., Prager, A.,, Decker, U., Möller, S., Berg, A., Schnabelrauch, M., " Biocompatible polysaccharide-based cryogels", *Materials Science and Engineering C*, Vol. 35, 2014, 164–170

[5] Nikonorov, V.V., Ivanov, R.V., Kil'deeva N. R., Bulatnikova, .L.N., Lozinskii, V.I., "Synthesis and Characteristics of Cryogels of Chitosan Crosslinked by Glutaric Aldehyde", *Polymer Science, Ser. A*, Vol. 52 (8), 2010, 828–834

[6] Fassina, L., Saino, E., Visai, L., Avanzini M.A., Cusella De Angelis, M.G., Benazzo, F., Van Vlierberghe, S., Dubruel, P., Magenes, G., "Use of a gelatin cryogel as biomaterial scaffold in the differentiation process of human bone marrow stromal cells", *Conf Proc IEEE Eng Med Biol Soc.*, 2010, 247-250

[7] Baláz M. "Eggshell membrane biomaterial as a platform for applications in materials science*", Acta Biomaterialia*, Vol. 10, 2014, 3827-3843

[8] Baker, J.R., Balch, D.A., "A Study of the Organic Material of Hen's-Egg Shell", *Biochem. J.*, Vol. 82, 1962, 352-361

[9] Wong, M., Hendrix, M.J.C., von der Mark, K., Little, C., Stern, A., "Collagen in the Egg Shell Membranes of the Hen", *Developmental Biology*, Vol. 104 (1), 1984, 28-36

[10] Liu, Z., Zhang, F., Li, L., Li, G., He, W., Linhardt, R.J., "Compositional analysis and structural elucidation of glycosaminoglycans in chicken eggs", *Glycoconj J*, Vol. 31, 2014, 593-602

[11] Hincke, M.T., Gautron, U.J., Panheleux, M., Garcia-Ruiz, J., McKee, M.D:, Nys; Y., "Identification and localization of lysozyme as a component of eggshell membranes and eggshell matrix", *Matrix Biology*, Vol. 19, 2000, 443-453

[12] Schmidti, R. Suso, H.P. Kenny, E., "Micronized Eggshell Membrane Particles and the Use thereof to Promote the Healing of Wounds", WO 2015/058790 A1 (Biovotec AS)

[13] Zhu, J., "Bioactive Modification of Poly(ethylene glycol) Hydrogels for Tissue Engineering", *Biomaterials*, Vol. 31 (17), 2010, 4639–4656

[14] Reichelt, S., Naumov, S.,Knolle, W., Prager, A., Decker, U., Becher, J., Weisser, J., Schnabelrauch, M.; "Studies on the formation and characterization of macroporous electron-beam generated hyaluronan cryogels", *Radiation Physics and Chemistry*, Vol. 105, 2014, 69–77