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Synthesis, scanning electron microscopy (SEM) and biocompatibility study of SLA 3D printable biopolymer hydrogel

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Purpose: The demonstration of the production, SEM investigation and study of the biocompatibility of a biopolymer-based 3D printed hydrogel.

Materials and methods: Hydrogel samples with 1 and 2 mm thickness were planned by Ansys SpaceClaim (Ansys Inc, USA) 3D modeling software. The biodegradable methacrylated-poly-γ-glutamic-acid (MPGA) polymer-based hydrogel were produced by a stereolithographic (SLA) type Formlabs Form 2 (Formlabs Inc.) 3D printer. The surface and structure of the hydrogels were studied by stereo and scanning electron microscopy (SEM) respectively. The biocompatibility of the 3D printed samples was investigated by Alamar blue viability test using MG63 cells. The actual cells growing on the surface of the samples were also examined by SEM.

Results: Our results showed that the MPGA based hydrogels were 3D printable by SLA technique. The printed hydrogels are constructed by few hundred diameter nanofibers and web-like structures. The Alamar blue test showed that, however, after 1 day of seeding, the numbers of the MG63 cells were significantly reduced at the hydrogel surface, after another 3 days we could not detect any alteration in the cell number compared to that of the control. Additionally, the SEM examination demonstrated the attachment of the cells to the surface of the hydrogel samples.

Conclusions: Our MPGA based polymer system were 3D printable by SLA technique. The prepared nanostructured and biocompatible hydrogels might be promising vehicles for biologically active components in tissue engineering.

Keywords: 3D printing, Scaffold, Biopolymer, Hydrogel, Scanning electron microscopy

Introduction

The development of digital technologies is creating more and more opportunities in medicine, including dentistry. Nowadays CAD-CAM (Computer Aided Design-Computer Aided Manufacturing) technologies involve a combination of complex processes, including the use of specific devices and new material systems. These workflows involve three basic stages. In the first work phase, the data input prepared e.g. by CT (computer tomograph) or scanner, then in the second phase the digital data are processed and the required form of the material is designed, and finally, the previously designed products are produced [23]. The two main types of rapid prototyping (RP) are the subtractive and additive techniques. In the first case, the final shape of the product can be formed from a block of prefabricated material (based on high-speed CNC (Computer Numerical Control) milling technology), while in the second case the final product is built up directly from raw powder or liquid materials [6, 9, 17].

Tissue engineering is a discipline dealing with the replacement or supplementation of different organs and tissues using biocompatible materials (scaffolds) as basis for cell growth and tissue development. Scaffolds play an important role in tissue engineering, and various 3D technologies can offer great potential for their production. In these systems, alloplasts, artificially produced materials can be used, which can be processed by different technologies. Currently, more than 30 RP technologies have been developed, of which more than 20 are suitable for biotechnological applications [5]. The most important of these are liquid-based (e.g. Stereolithography (SLA), Polyjet technology, Bioplotter technology) and solid-based systems (e.g. Fused Deposition Modeling (FDM), Laminated Object Manufacturing (LOM), Multi-jet Modeling Systems (MJM)), and Powde r-Based Systems (e.g. Selective Laser Sintering (SLS), Three-Dimensional Printing (3DP), Selective Laser Melting (SLM)) [10]. In these systems, the most commonly used raw materials are biodegradable polymers (e.g. polyglycolic acid (PGA), polylactic acid (PLA), polycaprolactone (PCL), poly-γ-glutamic acid (γPGA)), or polymers of natural origin (e.g. collagen, gelatin, alginate, fibrin), bioceramics (e.g. Bioactive ceramics: bioactive glasses, hydroxyapatite (HA), tricalcium phosphate (TCP) and metals (e.g. titanium).

Hydrogels are 3D structures of hydrophilic polymer chains, which are able to mimic the extracellular matrix.

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These physically or chemically crosslinked constructions are actively applied in the field of tissue engineering [16]. The photopolymerization is a way of the creation of the covalent bonds and provided a stable but soft and resilient matrix which could be similar to soft tissues. The mechanical properties of these photocrosslinked hydrogels are controllable by the type or the dose of the irradiations [11]. 3D printing offers a new approach to the fabrication of functional structures. The diversity of the useable biopolymers and the available 3D printing techniques ensure the chance of rational design and the fabrication of patient-specific medical devices. Although the extrusion-based 3D printing methods (such as the FDM) have been widely used because of their low cost, the photopolymerization-based bioprinting systems have more advantages e.g. the better accuracy or milder producing parameters which could be critically important in the cases of the cell-laden, or biomolecule containing 3D bioprinting [7, 17].

The aim of this work was to demonstrate the synthesis of a biopolymer-based 3D printed hydrogel produced by Formlabs Form II 3D printer and the examination of the structure and biocompatibility of the prepared samples that might be used as a scaffold material in tissue engineering in the future.

Materials and methods

Preparation of 3D printed hydrogels

An aqueous solution of a 1/3:1/3:1/3 mixture of methacrylated-poly-γ-glutamic acid (MPGA) (the fabrication way was written earlier in detailed [4]) and 2-hydroxyethyl methacrylate (HEMA) (≥99%) with polyethylene glycol dimethacrylate (Mw: 550 Da) (PEGDMA) was custom developed in our institute/laboratory to 3D printing. A multicomponent initiator system was created by the combination of a high efficiency component the lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) (≥95%) and Irgacure 2959 (~99%) in addition to L-arginin (≥99%). The materials were obtained from Sigma-Aldrich Ltd. (USA) and were used without further modification. The swelling of the polymer mixture and the homogeneity of the whol e system was achieved with continuous mixing for 72 hours. The prepared base material was printed using a Form2 (Formlab Inc., USA) 3D device using a Form2 Resin Tank LT tray, in an "open mode" with a layer thickness of 25 microns, set directly on the platform. The specimens were designed in Ansys SpaceClaim (Ansys Inc, USA) and were printed as grids with a diameter of 1 cm, and 1 and 2 mm thickness respectively. The 3D layer-by-layer structures were removed from the platform according to the specifications of the manufacturer. Finally, the samples were washed for 30 min with 2-propanol (VWR International Ltd.) and then for 3 × 10 min with phosphate buffer solution, pH: 7.4 (PBS) (Gibco, Life technologies, UK), after which the samples were stored in PBS. Images were taken from prepared samples using an Olympus SZ61 (Olympus Inc, Japan) stereomicroscope *(Figure 1).*

Scanning electron microscopy studies

The studies were performed using a scanning electron microscope (JEOL JSM-IT500HR, Japan). Samples were fixed in a 2% glutaraldehyde solution (Sigma-Aldrich, USA) for 2 h and then in a 1% OsO_4 solution (Sigma-Aldrich, USA) for 1 h. Samples were dehydrated in ethanol solutions (10, 30, 50, 70, 80, 90 and 100% EtOH) for 15 min each step, then dried using CO_2 at critical point and covered with a gold layer of about 12 nm thickness before microscopic investigation. The accelerating voltage was 10 kV.

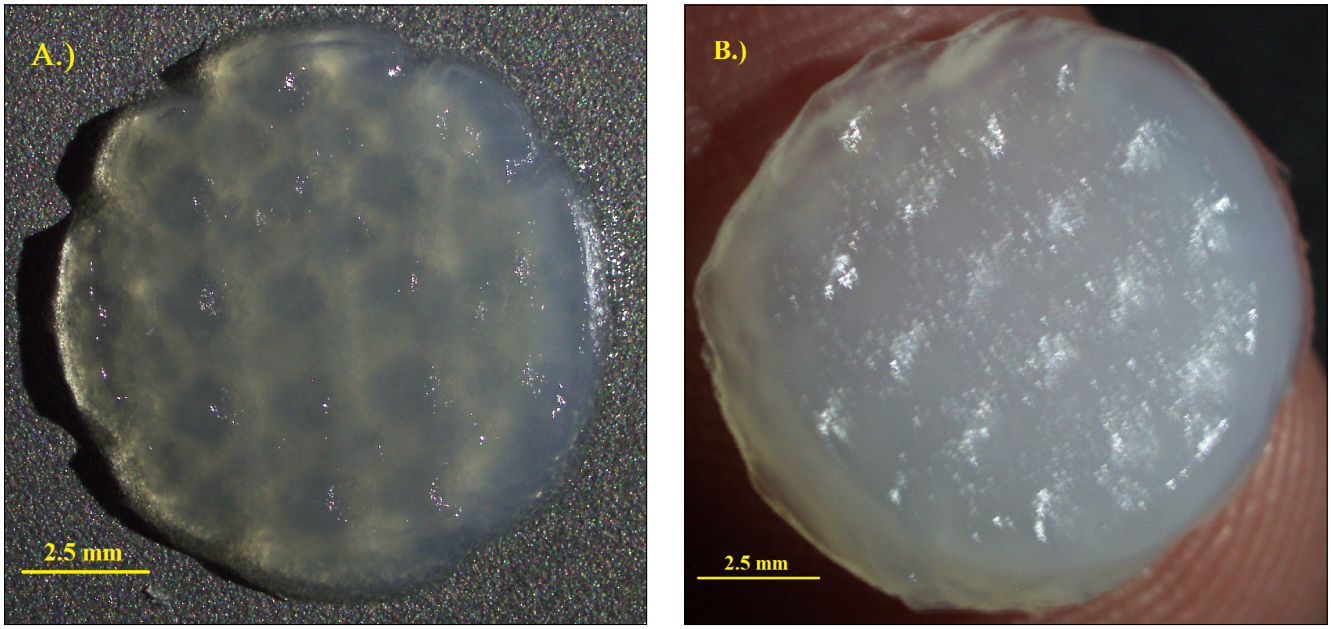


Figure 1: A) 1 mm, and *B)* 2 mm thickness of 3D printed hydrogels at 8× magnification.

Biocompatibility assay

Cell viability studies were performed on MG63 cell line (ATCC, USA). The cells were grown in Dulbecco's Modified Eagle's Medium – low glucose (Sigma-Aldrich, USA) containing 10% fetal bovine serum, 1% Antibiotic-Antimycotic solution and 1% Glutamax (all Gibco, Japan) at 37 °C with 5% CO_2.

To perform the AlamarBlue viability assay, sterility of the samples was achieved by 5 minute of in-situ generated ozone treatment (Ozone DTA, Taiwan) it was followed by 20 minutes of UV light illumination. After that, 4×10^4 cells were plated onto the surface of the hydrogel samples in 24-well cell culture dishes for the assays, and into wells without hydrogels which were used as controls for the experiment. After 1 and 4 days, the number of live cells was measured using the AlamarBlue assay (Thermo Fisher Scientific, USA). Experiments were performed with three parallel samples and each sample was measured with three technical replicates. The assays were executed according to the manufacturer's instructions, and finally the fluorescence of the samples was determined using a Hidex Sense microplate reader (Hidex, Finland) at 544 nm excitation/595 nm emission wavelengths.

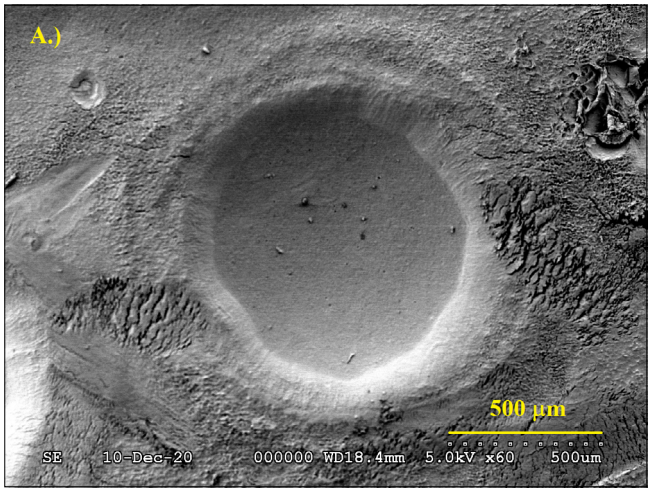
Statistical analysis

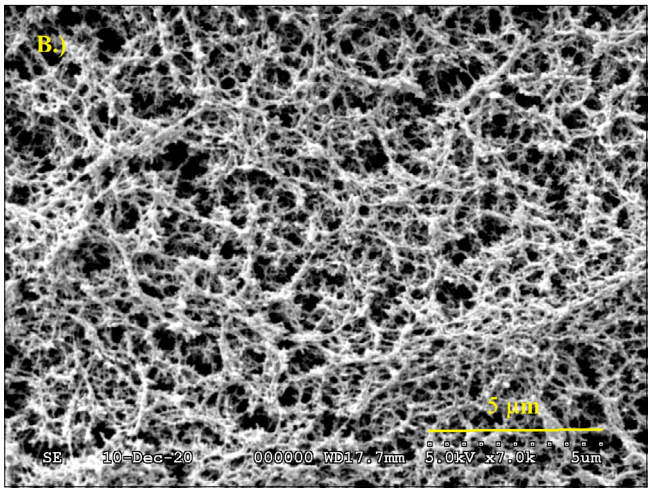
Statistical analysis was carried out using the Student's test to determine the statistical significance of differences between of experimental groups. $P \leq 0.05$-issued minimum to determine significance. Microsoft Analysis ToolPak for Excell (Microsoft, USA) was used for the investigations.

Results

The images of the hydrogel samples *(Figure 1)* show, that the printer was not able to establish the grid structure as designed by the Ansys SpaceClaim software. However, the grid structures are clearly identifiable, the system was not able to form the holes inside. This visible light polymerizable polymer matrix was able to utilize the advantage of the illumination time that is given by the factory parameters of the 3D printer. The well-defined edges of the hydrogels demonstrate that it was possible to produce stable gels even under this extremely short illumination time. The 3D printed hydrogels we have created show form-stable, flexible features and have a consistent structure.

According to the SEM images *(Figure 2A)*, the intended holes appear as depressions with a diameter of 0.5–1 mm, which are not fully formed due to the uncompleted regulation of the reaction front, however, their position and size are quite similar to the expected ones, while further magnification of the images B) and C), revealed a network structure typical of the fine structure of hydrogels. The images also show mesh points of different sizes and densities, and fiber structures of different





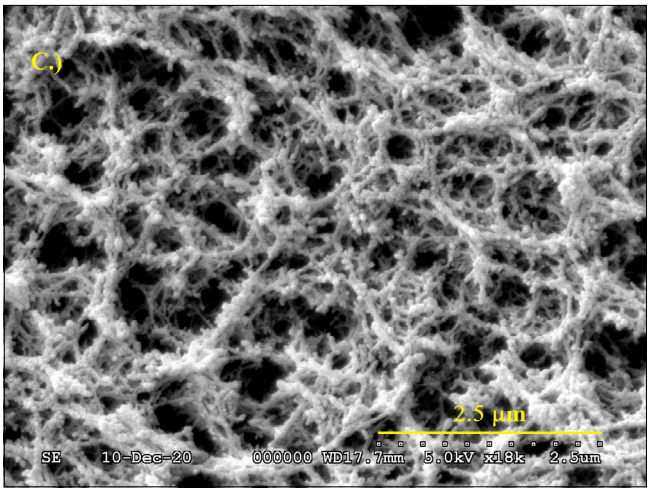


Figure 2: Scanning electron microscopy (SEM) pictures of 3D printed hydrogels
A.) magnification of 60×,
B.) magnification of 7000×,
C.) magnification of 18000×.

lengths and thicknesses connecting them in different directions. It is clearly recognizable, that this macroscopically homogeneous structure is formed by a random network of interconnected polymers linked by different cross-links, but still seem to resemble the digital design at macroscopic scales. The few hundred nanometer diameter pores of the resulting structure are clearly visible on the images, suggesting that they could be utilized to control the delivery of different growth proteins or drugs.

The Alamar blue assay showed that 1 day after seeding, the viability of cells grown with the gels decreased by 35% compared to the control *(Figure 3)*, however, after another 3 days this difference disappeared completely, and the viability of cells grown with the gels did not show significant ($p \leq 0.05$) alteration compared to that of the control.

The SEM image at 900× magnification *(Figure 4)* shows MG63 cells after fixation on the hydrogel surface, covered with a gold layer. ($p \leq 0.05$)

This image also shows that the surface of the hydrogels is suitable for the attachment and growth of the cells.

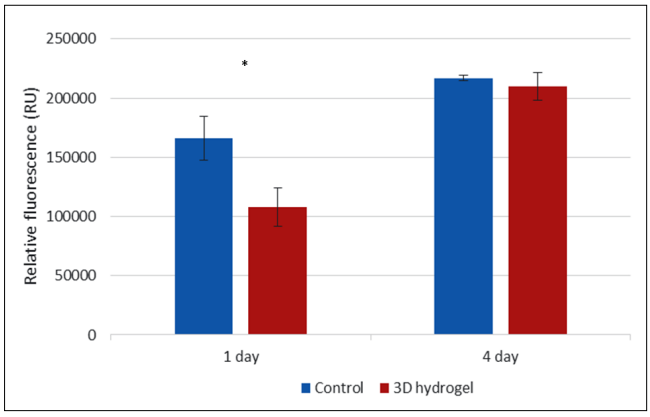


Figure 3: Results of the biocompatibility tests of the 3D printed hydrogels.

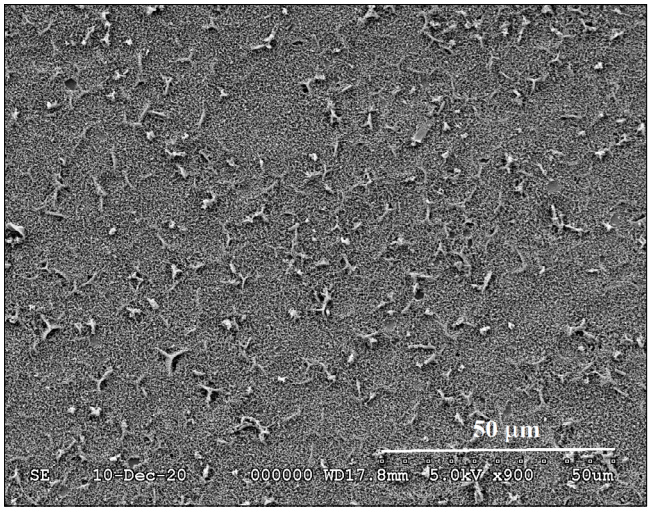


Figure 4: SEM image of MG63 cells on the surface of a 3D printed hydrogel at 900× magnification.

Discussion

Due to the rapid development of 3D printing techniques, there are a growing number of alternatives available for dental applications [6, 10]. A wide range of options offer faster, and in some cases, cheaper way to achieve the pursued objectives, from master or working casts to temporary crowns, surgical templates and surgical fixation splints to suit individual patient needs. The diversity of printing technologies, capable of molding metals, ceramics, polymers and composite materials, can provide us the most efficient ways to achieve this goal. There are examples of 3D printing applications of even zirconium ceramics, where yttrium-stabilized (3Y-TZP) ceramic powder is mixed with acrylates and methacrylates to form a material that can be photo-polymerized by stereolithography [154]. Among metals used in dentistry, the most commonly used techniques of selective laser sintering (SLS), selective laser melting (SLM) or electron beam additive (EDM) 3D printing are now available for CoCr, Ti and Ta powders [3, 20, 26, 31]. In the case of composites and polymers, especially for dental applications, photo-polymerization has played a significant role, but with the emergence of 3D printing, its importance is increasing [1, 22]. Moreover, there is also a need for drug delivery systems, one of which might be 3D printing to produce “bio-ink” systems combined with stem cells e.g. to promote pulp regeneration supported by modified calcium phosphate cements and different growth factors [17, 21, 28]. The most active area for the development of drug delivery systems and the design of hydrogels or composites today is tissue engineering as a part of complex tissue repair. Biopolymers, such as methacrylated-gelatin-based systems have been used as wound healing materials, or silk-based controllable porosity hydrogels have been developed for tissue engineering, moreover there are also examples of studies on combinations of other polymers [8, 25, 29].

In the recent years, a negatively charged biocompatible and biodegradable polymer the poly-γ-glutamic acid (PGA) has been in the focus of an increasing number of investigations, also due to the development of its biotechnological production and its increasing availability [11, 18]. The ability of the polymer to form nanofibers, nanoparticles, monolithic hydrogels, as well as structures similar to the extracellular matrix and its very good combinability with other modifying components (HA, BTCP) have allowed promising results in drug delivery and tissue engineering [12, 13].

Technologies using biopolymer-based 3D printing processes can already be found in the literature, but most of them are still based on FDM and require post-treatment, often with UV light, to achieve the required stability. The advantage of this method is that a wider range of flow properties can be applied, and the shear stress is limited only by the viability of the cells. However, the relative inexpensiveness of the method also opens the way for the development of further alterna-

tives due to the disadvantages of post-treatment and limited accuracy [19, 24].

In the course of our work, we have developed an MPGA-based polymer system that is suitable for SLA-based 3D printing. By reducing the polymerization time to sub-second range, we achieved a polymer blend capable of forming a solid structure according to the shapes defined by the digital designs, which does not require additional post-illumination. However, other hydrogels based on biocompatible or biodegradable polymers can be found in the literature, the more common pressure printing techniques are used for their production [2, 16].

In the hydrogel-based systems, there are continuous improvements in the initiators, raw materials, excipients, and appropriate ratios of the materials used, to achieve the next level of control for further increasing the accuracy of structure construction [7, 17]. Based on the SEM imaging we can state, that our MPGA-based, macroscopically homogeneous hydrogels are composed of nanostructured webs with interconnected structures of a few hundred nanometers, so that they can already be used to develop a retaining effect for proteins molecules or drugs. The advantage of the visible-light initiated polymerization, that it uses much softer energy transfer to form hydrogels than the γ-ray or even UV light source, so the biomolecules can remain active inside the polymer structure. This is the reason why SLS technology provides a good opportunity to apply in sensitive biological systems to best meet individual needs. There are also recent examples of PGA-based hydrogels, which form mainly micron-scale internal structures by photo-polymerization or enzymatic processes to develop load-bearing systems that can be used for drug delivery or tissue engineering purposes [15, 27, 30]. 3D printing has undergone extraordinary advancement in both the materials systems and technologies over the past decades and able to produce structures that are increasingly close to that are available in nature. In the future, these developments expected to continue, and the development of different systems that respond to external or internal effects could be one of the directions. In this way the 3D printing can be completed in the 4-dimensional material systems by developing a capability to dynamically respond to environmental changes.

Conclusion

Based on the experience of our previous studies, we have succeeded in the development of a polymer system suitable for MPGA-based 3D printing. The polymerizability has been designed to enable the polymer to realize the shapes defined by the digital designs in millisecond time. By SLA 3D printing technique, we have produced stable hydrogels, which follow the designed contours appropriately, and in which the grid structure can be clearly identified. The biocompatibility of the formulated gels was proved by cell viability studies, and the attachment of the cells to the hydrogel surface were demonstrated by scanning electron microscopy. We believe, that due to the nanostructured architecture of the hydrogel, our system is able to store different bioactive materials or drugs, and so it might be a promising candidate for the generation of a controlled drug delivery platform, which enhanced by the customizability through 3D printing.

Acknowledgement

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Conflict of interest

The authors deny any conflicts of interest related to this study.

Author contribution

JB contributed to data conception design, data acquisition, analysis, interpretation and drafted the manuscript. FT contributed to data acquisition, analysis, and reviewed the manuscript. CsL contributed to data acquisition and revised the manuscript. LD contributed to data acquisition and drafted the manuscript. CsH contributed to conception design, interpretation and critically revised the manuscript.

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Eredeti cikk

Bakó J, Tóth F, Csámer L, Daróczi L, Hegedűs Cs

SLA 3D nyomtatható biopolimer alapú hidrogél előállítása, pásztázó elektronmikroszkópos és biokompatibilitási vizsgálata

Célkitűzés: Munkánk célja biopolimer bázisú 3D nyomtatható hidrogél előállításának, pásztázó elektronmikroszkópos (PEM) vizsgálatainak és biokompatibilitásának bemutatása.

Anyagok és módszerek: Ansys SpaceClaim (Ansys Inc, USA) 3D modellező szoftver segítségével 1 és 2 mm vastagságú mintákat terveztünk, majd biodegradábilis metakrilált poli-γ-glutaminsav (MPGA) alapon hidrogéleket állítottunk elő sztereolitográfia (SLA) típusú Form 2 (Formlabs Inc, USA) 3D nyomtató alkalmazásával. A hidrogélek felületét és szerkezetét sztereo- és elektronmikroszkóp segítségével vizsgáltuk. A 3D nyomtatott hidrogélek biokompatibilitását MG63 sejtvonalon Alamar blue teszt felhasználásával bizonyítottuk, és a minták felületén növesztett sejtek PEM felvételein keresztül mutattuk be.

Eredmények: Eredményeink bizonyították, hogy az MPGA alapú hidrogélek nyomtathatóak SLA technikájú 3D nyomtató segítségével. A nyomatás útján kialakított hidrogélek néhány száz nanométeres hálószerű struktúrájúak. Az Alamar blue teszt bizonyította, hogy ugyan 1 nap elteltével csökkent az MG63 sejtek száma a felületen, de 3 nappal később a kontrollhoz viszonyítva különbség már nem volt kimutatható. Mindezen túl a PEM felvételek is bizonyítják a sejtek kötődését a hidrogél felületéhez.

Következtetés: Az általunk előállított MPGA alapú polimer rendszer SLA technikával nyomtathatónak bizonyult. A kialakított biokompatibilis, nanostruktúrált hidrogélek ígéretes jelöltek a biológiailag aktív komponensek szállítására a szövettervezés területén.

Kulcsszavak: 3D nyomtatás, Vázanyag, Biopolimer, Hidrogél, Pásztázó elektronmikroszkóp