Extrusion-Based 3D Printing of Photocurable Hydrogels in Presence of Flavin Mononucleotide for Tissue Engineering

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3D printing became a widely used technique for tissue engineering applications. This additive technology enables easy fabrication of very complicated structures. However, selection and preparation of initial compositions for 3D printing satisfying high biocompatibility and processability requirements still remains challenging. One of the most promising materials for mimicking of the living tissues are hydrogels possessing properties close to native tissues. In this work, the printability of hydrogels based on hyaluronic acid and poly(ethylene glycol) derivatives dissolved in phosphate buffer saline in presence of flavin mononucleotide as an endogenous photosensitizer has been studied. To produce a hydrogel pattern, the extrusion of photocurable composition has been combined with its simultaneous photoinduced crosslinking under laser irradiation at 450 nm. Cytotoxicity of fabricated films and 3D scaffolds has been tested *in vitro* using human fibroblasts BJ-5ta.

Key words: extrusion; 3D printing; photocurable hydrogel; hyaluronic acid.

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Russian

Экструзионная 3D-печать фотоотверждаемых гидрогелей с использованием рибофлавина мононуклеотида для тканевой инженерии

В настоящее время в тканевой инженерии широко применяются методы 3D-печати, позволяющие формировать сложные пространственные структуры из различных материалов. Тем не менее создание перспективных композиций для 3D-печати, которые отвечают высоким требованиям, предъявляемым к их биосовместимости и технологичности, по-прежнему остается актуальной задачей. Одним из наиболее привлекательных материалов для копирования живых тканей являются биоактивные гидрогели, обладающие свойствами, близкими к свойствам нативных тканей организма. В работе исследована возможность применения гидрогелей

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на основе производных гиалуроновой кислоты и полиэтиленгликоля, растворенных в фосфатно-солевом буфере, с использованием рибофлавина мононуклеотида в качестве эндогенного фотосенсибилизатора для 3D-печати различных структур. Формирование слоя гидрогеля в процессе экструзии исходной композиции совмещалось с его одновременным фотоотверждением под действием лазерного излучения на длине волны 450 нм. Цитотоксичность полученных пленок и трехмерных скаффолдов была исследована *in vitro* с помощью человеческих фибробластов BJ-5ta.

Ключевые слова: экструзия; 3D-печать; фотоотверждаемый гидрогель; гиалуроновая кислота.

Introduction

Recent successes in regenerative medicine have made possible restoring of damaged tissues by assembling artificial mimicking constructs. Cartilage [1], bone [1, 2], blood vessels [3], skin [4], and liver [5] are only some examples of reproduced tissues that have been reported in the past decade. Fabrication of tissue engineering constructs usually begins with producing three-dimensional scaffolds representing a structural support for living cells. Mechanical properties, morphology, adhesion, and porosity of scaffolds can be varied in a wide range, depending on the mimicking tissue requirements. However, all materials for scaffolding as well as products of their biodegradation must be nontoxic both to the cells and surrounding tissues. One of the attractive candidates for those scaffolds are various hydrogels based on biocompatible macromolecules [6], such as hyaluronic acid and its derivatives [7], as hyaluronic acid (nonsulfated glycosaminoglycan) is one of the major native component of the human extracellular matrix for connective, epithelial, and neural tissues.

In this work hyaluronic acid has been modified to produce hyaluronic acid glycidyl methacrylate (HAGM) which is capable of crosslinking initiated by light. Methacrylation of biocompatible hyaluronic acid enables wide possibilities for its successful implementing in 3D printing as a key component of photocurable composition (PCC), representing a water solution of photocrosslinkable macromolecules with a photoinitiator and/or photosensitizer. PCC can also include biofunctional molecules, catalysts, and other additional moieties. It is essential that hydrogels obtained as a result of PCC crosslinking possess properties close to those of living tissues. The precise control of mechanical and swelling properties of hydrogels can be performed by simple adjusting concentrations of precursors in the initial PCCs. In particular, the addition of small amount of nontoxic poly(ethylene glycol) diacrylate (PEGDA) [8] in PCC allows to decrease dramatically the unnecessarily high swelling ratio (SWR) of HAGM-based hydrogel (SWR>2000%) and make it more elastic [9]. SWR of the sample is calculated according to the expression:

$$\mathrm{SWR}(\%)=\frac{W_s-W_d}{W_d}100\%, \qquad (1)$$

where W_s and W_d are the weights of the swollen and dried hydrogels, respectively.

It is vitally important to provide the biocompatibility of all components of PCC for tissue engineering including photoinitiator and/or photosensitizer. Therefore, in the present work, riboflavin (vitamin B2) in its water-soluble form, flavin mononucleotide (FMN), has been proposed and used as an endogenous photosensitizer. Vitamin B2 non-toxicity and goodness as a dietary supplement have been approved by Food and Drug Administration. FMN possesses absorption in ultraviolet A and nontoxic blue ranges of the spectrum [10]. Under irradiation FMN [11] can form either singlet oxygen via energy transfer to atmospheric oxygen or hydrogen peroxide and derivatives via radicalization [12]. All these reactive oxygen species are capable to activate the crosslinking process. Moreover, riboflavin photobleaching time exceeding 8 min [13] permits its effective implementation as a photosensitizer in the 3D printing process.

Herein we have developed a HAGM-based PCC containing FMN as a photosensitizer and small amount (ca. 5 wt%) of PEGDA to optimize mechanical properties of the final hydrogel. The hydrogel scaffolds have been produced in the shape of lattices by extrusion-based 3D printing combined with simultaneous laser irradiation at 450 nm. Scaffolds have been analyzed using optical microscopy. *In vitro* cytotoxicity tests of printed hydrogel structures have been performed using human fibroblasts BJ-5ta.

Materials and Methods

Chemical materials. Poly(ethylene glycol) diacrylate (PEGDA, M_n≈575), hyaluronic acid sodium salt (high molecular weight, ca. 5 MDa), glycidyl methacrylate, triethylamine, dimethylformamide, sodium azide, and acetone were purchased from Sigma-Aldrich (USA). Flavin mononucleotide (riboflavin-5′-phosphate) was obtained from Pharmstandard (Russia). Phosphate buffered saline (PBS) was purchased from Lonza (Switzerland). RPMI-1640 cell growth medium, L-glutamine, and penicillin-streptomycin (Pen Strep, 100×) were purchased from PanEko (Russia), fetal bovine serum (FBS) was from HyClone (USA).

Cell culture. Immortalized human fibroblasts BJ-5ta were grown in RPMI-1640 growth medium at 37°C in 5% CO_2 humidified atmosphere. RPMI-1640 was supplemented with 10% FBS, 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin. The medium was replaced every 3–4 days.

Photocurable composition preparation. Aiming to substitute sodium atoms in hyaluronic acid sodium salt by methacrylate units the reaction with glycidyl methacrylate was applied according to the method described in [14] and hyaluronic acid glycidyl methacrylate (HAGM) capable of crosslinking has been obtained as a result.

HAGM and PEGDA were placed in PBS containing FMN and subjected to sonication for 1 h. Then this mixture was left overnight and sonicated again to produce a homogeneous solution. To avoid spontaneous crosslinking, preparation of PCCs was performed under yellow light conditions. The prepared PCCs contained 20 wt% of HAGM and 5 wt% of PEGDA. The concentration of FMN was 0.01 wt%.

Scaffold fabrication. Self-designed extrusion-based 3D printer has been used to produce experimental hydrogel structures. Initially, PCC was loaded in the syringe where the open end was fitted with 5 mm long 250 μm inner diameter capillary. In order to start the extrusion, the plunger was pushed inside along the syringe. Plunger speed and positioning of the syringe in Z-direction, as well as the movement of the substrate in X-Y coordinates, were controlled using G-code in Repetier software. Acceleration parameters of plunger and translation stages were precisely adjusted in Repetier to ensure uniform thickness of extruded PCC layer deposited onto the substrate. Photocuring of the extruded PCC was performed by laser irradiation at 450 nm wavelength with intensity of 70 mW/cm^2. When the pattern was formed, the stage moved in Z-direction and the procedure was repeated. Inner structure and surface morphology of the scaffolds were analyzed by optical microscopy using HRM-300 microscope (Huvitz, Korea).

In vitro study. The cytotoxicity of the hydrogel samples was studied in the extract test as it was described earlier [15], and human fibroblasts BJ-5ta were used as model cells. Briefly, 3D printed hydrogel samples were incubated within RPMI-1640 cell culture medium supplemented with 10% of FBS at 37°C in 5% CO_2 humidified atmosphere, and the supernatants (extracts) were collected after 24 h. The BJ-5ta fibroblasts were seeded into 96-well plate (10^4 cells per well), and the plate was incubated overnight in CO_2-incubator. Then the medium in each well was replaced with 100 μl of the extracts. The cells cultivated in the medium without the extracts were used as a control. Cell viability was measured by a MTT assay, which is a colorimetric assay for assessing cell metabolic activity through the reducing of the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to its insoluble formazan. For this reason, after 24 h incubation with extracts, an MTT solution (0.5 mg/ml) was added to the wells, and the cells were incubated at 37°C for 3 h. Formazan crystals formed in the living cells were dissolved in 100 μl of dimethyl sulfoxide, and an optical density was measured at 570 nm using a Multiscan plate reader (Flow Laboratories, USA). Relative cell viability (V) was calculated according to the following expression:

$$V=(OD_{test}–OD_{background})/(OD_{control}–OD_{background})100\%, \quad (2)$$

where OD_{test}, $OD_{control}$, and $OD_{background}$ are optical densities of sample, control, and background, respectively.

To demonstrate cell growth on the hydrogel surface, printed samples were placed into the 24-well plates and seeded with BJ-5ta fibroblasts (20,000 cells per each sample), and successive gradual cell growth was observed within 7 days.

OHAUS Starter 3100 pH-meter (OHAUS, USA) was employed to control pH of solutions.

Results and Discussion

First, we performed methacrylation of hyaluronic acid to produce HAGM. The synthesized hyaluronic acid derivative combines advantages of its biocompatibility and capability to photocrosslinking. Therefore, we chose HAGM as the main component of PCC. In order to perform gelation of the entire volume of the initial multicomponent PCC after exposure process, the concentration of capable to photocrosslinking HAGM macromolecules must exceed the minimal threshold value of 16 wt% discussed in [9]. The formation of a disordered 3D polymer network threading through the entire exposed volume and triggering the appearance of elastic properties becomes possible only above this threshold concentration.

The water-soluble form of riboflavin was proposed as an endogenous photosensitizer in PCC. FMN used in our experiments is characterized by a broad UV-blue absorption band with two peaks illustrated in Figure 1 (*left*). Using FMN in HAGM-based PCCs makes possible photocuring at 450 nm wavelength. Additionally, in order to avoid the unnecessary high swelling of the final HAGM-based hydrogel in water solutions, we added linear polymer PEGDA to all PCCs. Thus, we prepared PCC for 3D printing containing 20 wt% of HAGM, 5 wt% of PEGDA (M_n≈575) and 0.01 wt% of FMN dissolved in PBS.

The scheme of our custom-designed extrusion-based 3D printer is presented in Figure 1 (*right*). The printer was equipped with 450 nm continuous wave semiconductor laser source. Owing to the focusing lens attached to the end facet of laser the divergent laser beam could expose the currently printed point or the whole sample. Depending on the irradiated area the power of the semiconductor laser was adjusted to provide 70 mW/cm^2 intensity.

To demonstrate the possibilities of our 3D printer, we fabricated scaffolds in the shape of lattices with period 1.4 mm as shown in Figure 2 (*left*). The lattices were formed using HAGM-based PCC by sequential printing of 25 layers. The printing speed was adjusted to 0.3 mm/s. During the extrusion process, the printing area was continuously irradiated at 450 nm wavelength to produce gelation of hydrogel layer. Finally, when the scaffold had been printed, we applied the additional exposure for 10 min in order to uniform the degree of crosslinking in

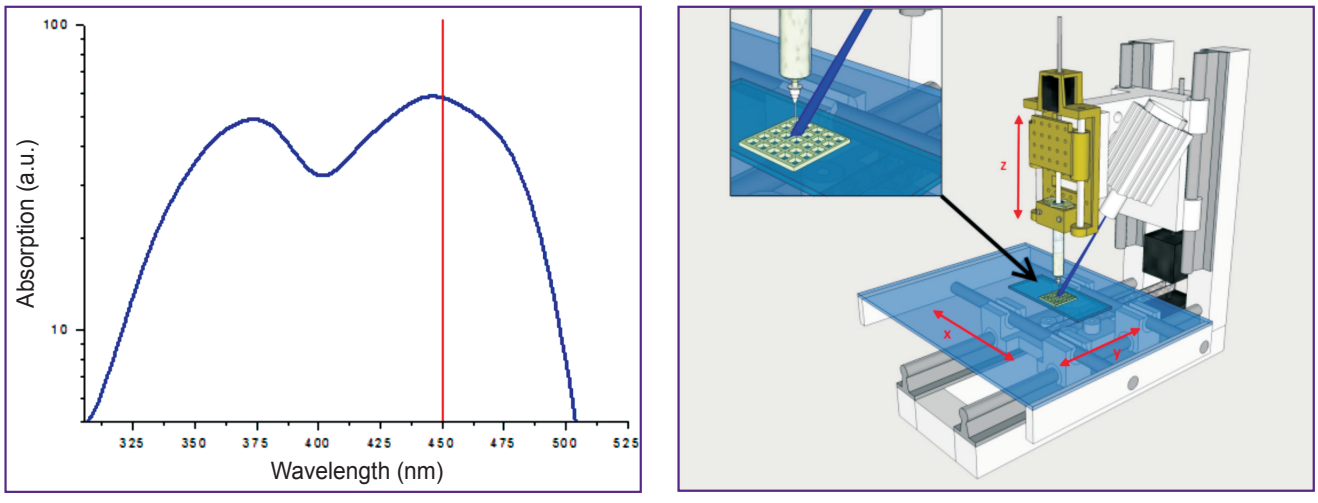

Figure 1. Absorption spectrum of flavin mononucleotide; the vertical line (*left*) indicates the blue light wavelength at 450 nm. Scheme of extrusion-based 3D printing (*right*), the inset shows zoomed image of photocurable composition flow squeezing out of the nozzle and divergent laser beam

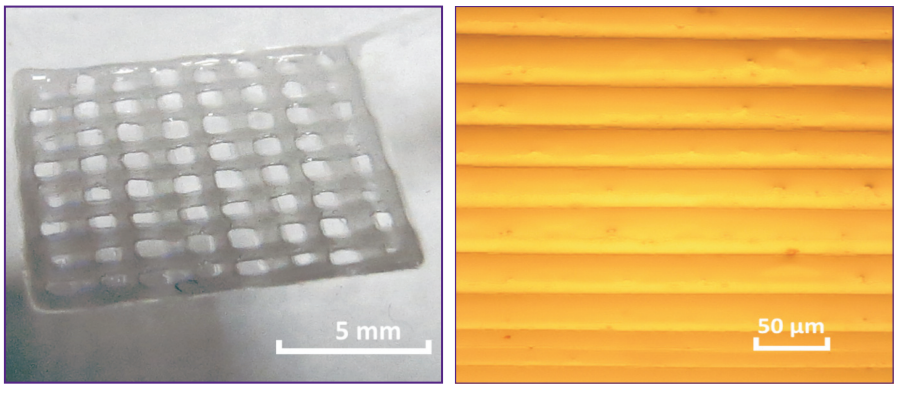

Figure 2. Scaffold structure on the surface of cover glass produced by extrusion-based 3D printing with period of lattice 1.4 mm (*left*) and zoomed image of side view of the printed scaffold obtained with the optical microscope Huvitz with 50× objective (*right*)

different hydrogel layers. Extrusion-based 3D printing of all structures was performed onto the smooth glass surface and the fabricated samples could be easily peeled out without destruction. Moreover, the scaffolds placed in PBS indicated stability for 3 weeks in PBS due to optimized swelling properties.

The surface of produced scaffolds was observed using optical microscope Huvitz with 50× objective (Huvitz, Korea). Figure 2 (*right*) illustrates the side wall of the scaffold. The wall represents uniform layered structure. The average height of every layer was measured as 30 µm. The total height of scaffolds was ca. 750 µm.

Prior to *in vitro* cytotoxicity study all printed samples were stored in PBS for 5 days to remove FMN and products of its photolysis from hydrogel structures.

The acute cytotoxicity of the hydrogel samples was evaluated using an extract test by MTT assay. It was found that hydrogel extracts slightly reduced the BJ-5ta cell growth (10–20% slower in comparison to controls) (see Figure 3). To clarify the possible reason of cell growth deceleration, the pH measurements of the extracts, both in RPMI-1640 and in de-ionized water, were conducted.

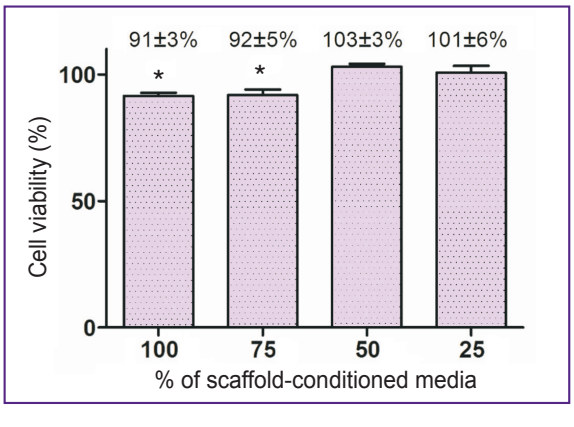

Figure 3. MTT assay data for the viability of BJ-5ta fibroblasts in the presence of hydrogel-conditioned media: 100% means hydrogel extract only; 75% means 75% of extract and 25% of non-conditioned RPMI-1640; 50% means 50% of extract and 50% of non-conditioned RPMI-1640; 25% means 25% of extract and 75% of non-conditioned RPMI-1640. Non-conditioned RPMI-1640 medium was used as a control. The data are the mean±SD. P-values were calculated by Mann–Whitney U test (* indicates a p-value <0.05)

Since no pH changes were observed, we suppose that cytotoxic effect could be caused by spontaneous daylight photoactivation of remaining FMN following by reactive oxygen species and free radical production [16]. Nevertheless, the fibroblasts were able to grow on the surface of hydrogels, confirming high biocompatibility of the resulted samples.

Conclusions

The printability of a new HAGM-based hydrogel photocurable composition has been studied. The elastic lattice structures have been produced using extrusion-based 3D printing combined with photocuring by laser light at 450 nm. We have demonstrated that human fibroblasts BJ-5ta were capable of attaching and growing on the surfaces of HAGM-based hydrogel scaffolds. Thus, we conclude that 3D printing of HAGM-based photocurable compositions containing flavin mononucleotide as an endogenous photosensitizer represents an attractive methodology for various tissue engineering applications.

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Conflict of interest. The authors confirm the absence of financial and other conflicting interests that could influence their work.

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