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Facile method of building hydroxyapatite 3D scaffolds assembled from porous hollow fibers enabling nutrient delivery

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Abstract

Nowadays, diffusion through scaffold and tissue usually limits transport, and forms potentially hypoxic regions. Several methods are used for preparation of 3D hydroxyapatite scaffolds, however, production of a scaffold including porous hollow fibers for nutrition delivery is difficult and expensive. In this study, we describe an easy and inexpensive method to create 3D hydroxyapatite structure containing porous hollow fibers via microtemplating. The fibers which are assembled into 3D scaffold and sintered, contain asymmetric membrane walls with flux suitable for nutrient delivery. These hollow fibers have good mesenchymal stem cell adhesion showing that the presented method has no negative influence on cell cytocompatibility. The proposed straightforward method for building 3D structures containing porous hollow fibers for nutritions can be suitable for in vitro bioreactors studies as well as for production tissue engineered or in vivo prepared bone grafts.

Keywords: A. Sintering; E. Membrane; Hydroxyapatite; Microtemplating; Cell adhesion

1. Introduction

The porosity and pore size are important morphological properties of biomaterial scaffolds, and play a critical role in vitro and in vivo tissue formation. For example, in bone tissue engineering – in vivo: lower porosity stimulates osteogenesis by suppressing cell proliferation and forcing cell aggregation. Contrary, in vivo, higher porosity and pore size result in greater bone ingrowth [1]. Then combination of macroscopic pores for cells growth and microscopic pores for cell adhesion and nutrition transport is necessary.

Synthetic foams are one solution for composition and structural control of bone filling materials [2,3]. The structure of foam is dependent on viscosity and surface tension, however, it is not easy to create macroscopic and microscopic porosity for the development of a network of blood vessels with the finer structure preferred for mechanical strength. Another possibility is to create scaffold using a rapid prototyping from fibers containing suitable material, therefore the 3D structure can be organized by orientation of fibers [4]. Both negative and positive scaffold biomaterials have been produced by rapid prototyping methods [5]. These established methods are dealing with polymer based materials allowing flexibility and solubility. Bioceramic materials have the rigid structure and direct formation of fibers is very limited. It is possible to design a hard tissue scaffold with hierarchies of pores, channels, and accompany smaller recesses which can host cells development as well as large voids structure for vascularization [6]. However, the vascular network is not separated from cells and only the size of voids differentiates vascular network from cells area. The separation of channels for nutrition delivery in hard tissue scaffold is desirable especially for construction of a bioreactor or in vitro preparation of a hard tissue. The absence of a nutrition delivery network capable of distributing gas, nutrients and removal

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of waste products is one major limiting factor in creating viable artificial tissues [7]. The concept of isolated microchannels (in hollow porous fibers) for nutrition delivery was already tested for a polymeric scaffold. The hollow fiber membranes integrated inside large three-dimensional (3D) scaffolds improved the cell density and cell distribution [8]. Nevertheless, the preparation of microchannels inside ceramic scaffolds is based on indirect building of channels via rapid prototyping techniques [9–11]. These techniques can build various shapes precisely, but the process is not facile and requires high investments. Furthermore, the obtained hollow structures are usually not rounded and a possibility of porosity tailoring is limited due to restrictions in selection of suitable powder materials.

Here we demonstrate an easy and economical method for production of porous and hollow fibers, which can be directly assembled for building the hydroxyapatite (HA) 3D scaffold. Polypropylene template fibers and other organic components were burn out during the thermal treatment and only inorganic HA structure remained. HA is bioactive ceramic material with relatively low hardness similar to human bone apatite and has been used as bone grafting material or active coating for dental implants [12,13].

The microchannels inside HA hollow fibers are tested for exchange of nutrients and the outer surface of the fibers can accommodate cells. Confirmation of scaffold cytocompatibility after processing was done by testing the metabolic activity of the cells on the hydroxyapatite fibers over the period of 7 days.

2. Materials and methods

2.1. Fabrication of the ceramic structure

Suspension for the dip coating was prepared by ball milling of following components: solvent (2-propanol), dispersant (solperse 20000), ceramic powder (hydroxyapatite), plasticizer (BBP), and binder (PVB) as listed in Table 1.

Polypropylene hollow and porous fibers were 4 times dip-coated in the suspension with withdrawing speed 0.29 m min^{-1}. The outside diameter of the micro template – polypropylene fiber was 300 μm, porosity and hollow space of the polypropylene fiber ensure collapse of the fiber shape before its thermal expansion may destroy green ceramic structure [14]. Each layer was fully dried between each dip-coating step and ambient conditions were always used for drying.

Table 1
Composition of suspension for the microtemplating [14], same suspension was used for connections of the green coated fibers.

Suspension components	Weight (%)	Volume (%)
2-propanol	52.8	77.3
Solperse 20000 (± 90% polymeric alkoxylate)	1.8	1.7
Hydroxyapatite (Sigma-Aldrich, BET 10–15 $m^2 g^{-1}$)	38.6	14.1
BBP (Benzyl butyl phthalate), S-160	0.9	1.0
PVB (Polyvinyl butyral) B-98	5.8	6.0

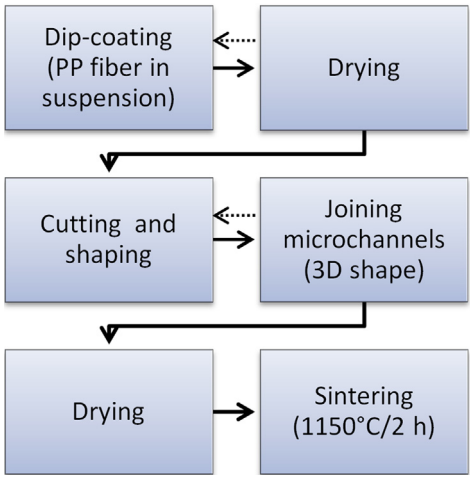

Fig. 1. Chart flow of describing the building process of ceramic 3D structure.

Furthermore, the prepared green fibers were cut, shaped and joined together. The cutting, shaping and joining of the coated fibers were done manually and distance between middles of the green fibers was 1 mm. Suspension with the identical composition (Table 1) was used to fix green coated fibers in intended positions (before sintering). Five vertical levels of the green coated fibers network were prepared, when the first layer contained eight parallel coated fibers and the following layers had seven parallel coated fibers. Sintering was done in air and subsequent heating or cooling steps were applied: 40–220 °C (1 °C min^{-1}), 220–600 °C (2 °C min^{-1}), 600–1150 °C (10 °C min^{-1}), 1150 °C/2 h, 1150–40 °C (5 °C min^{-1}). The chart flow in Fig. 1 describes the fabrication process of the 3D hydroxyapatite scaffold assembled from porous hollow fibers. The identical procedure, without 3D assembling step, was used for preparation straight self-supporting porous and hollow fibers for testing of membrane performance and biocompatibility.

2.2. Structural analysis and membrane characterization

Photography was used to demonstrate shape of the sintered hydroxyapatite structure. Hollow fibers after sintering were characterized with scanning electron microscopy (Jeol JSM 5600LV). SEM pictures of the membrane outer surface and cross sections were analyzed with image processing software to determine the porosity (ImageJ). Fluxes of gases (N_2, CO_2) and liquids (water, cell culturing medium) were measured in dead-end filtration mode using modules containing one single hollow fiber (microchannel inside). Gas permeability was measured using the manual low pressure gas permeation set up. The time needed to collect 25 cm^3 of gas was measured whereas the pressure was controlled at 1 bar, and temperature was 22 °C. The water flux of the HA fibers was determined by filtrating inside-out ultrapure water at the constant trans membrane pressure of 1 bar, volume increase of the filtrate was recorded as a function of time for at least 90 min.

The flux through the membrane (J, in 1 $m^{-2} h^{-1}$) was calculated from the slope of the filtrated volume (V) versus

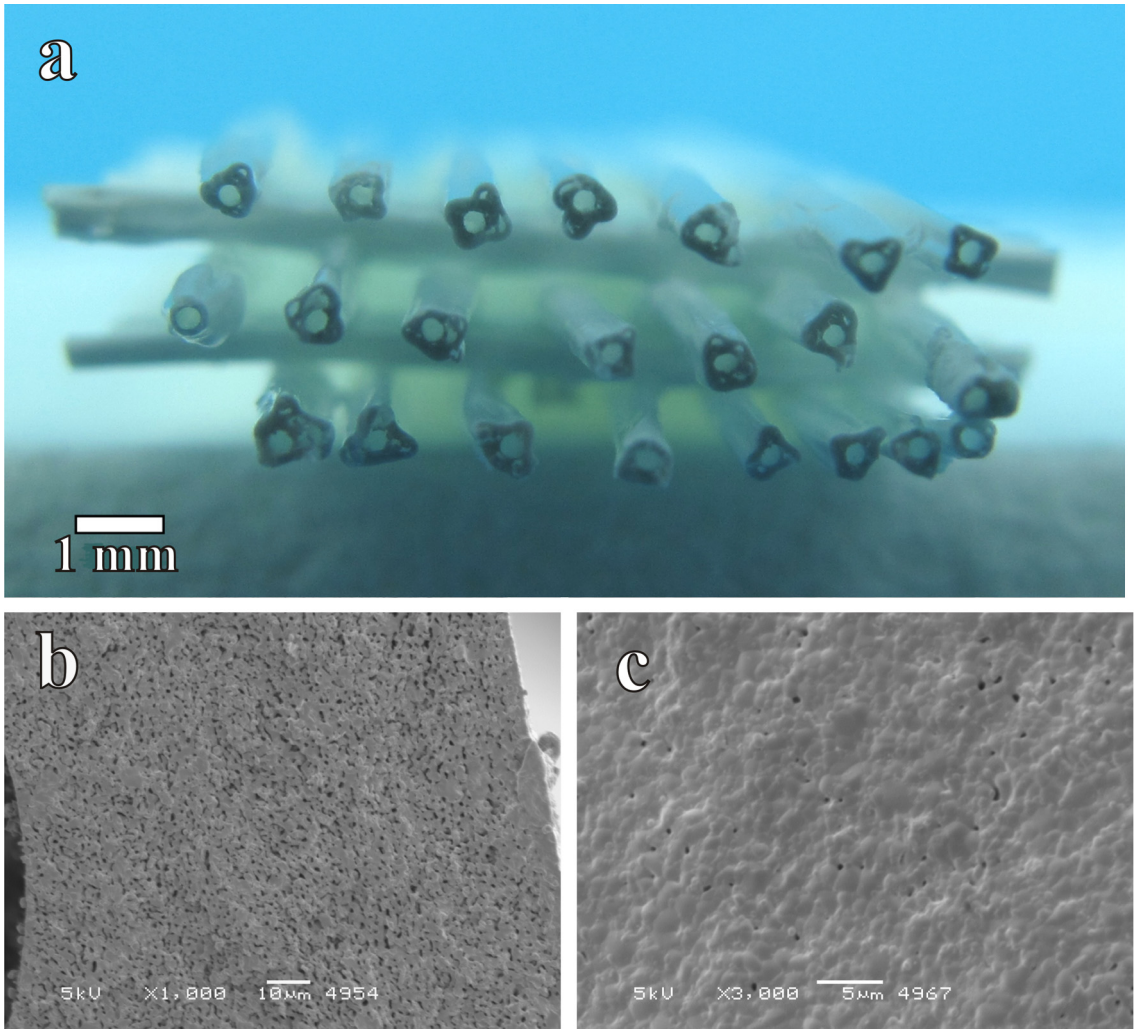

Fig. 2. Sintered hydroxyapatite scaffold assembled from hollow fibers with asymmetric membrane walls prepared by microtemplating technique. (a) Photo of the 3D hydroxyapatite structure. (b) SEM micrograph showing the porous cross-section of the hydroxyapatite hollow fiber. (c) SEM micrograph showing the porous outer surface of the hydroxyapatite hollow fiber.

time (t) graph using the following equation:

$$J = \frac{V}{A\,t} \tag{1}$$

where A is the membrane area (m^2).

Gas and water permeability were measured for five different hollow fiber samples to obtain the representative data. Transport of cell culturing media (D-MEM, Gibco) through the fibers was also measured to evaluate possible interaction of medium components with hydroxyapatite fibers. In this case the flux was measured for up to 840 min.

Bubble point analysis of the outside porosity of the fibers was performed by a Coulter porometer II (wetting fluid, trade name 'Porofil'). The presented measured data are average from four measurements of the four different fibers.

2.3. Cell culturing

Bone marrow aspirates (5–20 mL) were obtained from donors with written informed consent. Human mesenchymal stem cells (hMSCs) were isolated and proliferated as described elsewhere [15]. Cells were grown at 37 °C in a humidified atmosphere with 5% CO_2. After 2 days, medium was changed and non-adherent cells removed. Medium was refreshed twice a week until cells reached confluence. Upon confluence, cells were trypsinized with 0.25% trypsin—1 mM EDTA for 5 min at 37 °C and counted. hMSCs (passage 3) were seeded on the hydroxyapatite fibers at a density of 100.000 cells/cm^2 and incubated overnight to allow cell adherence. Afterwards, medium was carefully added and the fibers were cultured for 7 days in minimal essential medium (α-MEM, Life Technologies), 10% heat-inactivated fetal bovine serum (Gibco), 50 µg/mL ascorbic acid (Asap, Life Technologies), L-glutamine (Life Technologies) and 100 U/mL penicillin (Life Technologies). The culture medium was refreshed twice a week. All samples were analyzed at specific days of culturing, namely day 1, 3 and 7, respectively.

At each time point, the samples were processed and cell adhesion, distribution and morphology were assessed by cytotoxicity assay (MTS), DNA quantification and scanning

electron microscopy (SEM). Data are presented as the average of five replicates (mean ± standard deviation). All assays are described in detail below. Tissue culture polystyrene (TCPS) was used as a control in this study.

2.4. Cell characterization

2.4.1. MTS cytotoxicity assay

The MTS cytotoxicity assay (Cell Titer 96 Aq Non-Radioactive Cell Assay, Promega), an alternative method of the widely used MTT assay, was used to screen the viability of the cells cultured on the scaffolds. The MTT assay where the reaction product formazan is water insoluble, could not be used. Formazan was strongly attached to the surface of the scaffolds making impossible to obtain a correct measurement. In the MTS test (chapter 2.3), using a novel tetrazolium compound, the reaction product formed was soluble in the culture medium. Briefly, the MTS was added to the wells and incubated for 3 h; the blank used was culture medium with MTS, and the absorbance was measured at 492 nm in an ELISA 96 well-plate reader.

2.4.2. Cell proliferation assay

The cell proliferation was assessed by CyQUANT cell proliferation assay according to the manufacturer's instructions (Molecular Probes). Cell numbers were determined at different times points by incubation with CyQUANT dye and fluorescence was measured with filters for 480 nm excitation and 520 nm emission in a Tecan Safire.

2.4.3. Scanning electron microscopy

Hydroxyapatite scaffolds were washed twice with PBS and fixed in 1.5% v/v glutaraldehyde (Sigma) in 0.14 M sodium cacodylate (Sigma, pH 7.4) for 30 min at room temperature. Dehydration of the fibers was performed by sequential immersion in serial diluted ethanol solutions of 50, 60, 70, 80, 90, and 100% v/v. The samples were kept in absolute alcohol and taken to critical point, using CO_2. The samples were then sputtered with a thin gold film for SEM analysis.

3. Results

3D hydroxyapatite structure composed from porous hollow fibers suitable for nutrition delivery was successfully prepared via the microtemplating method. Fig. 2 shows the sintered 3D structure and the typical SEM micrograph of the porous walls of the hydroxyapatite hollow fiber. The inner diameter of fibers is 245 ± 2 (SD) μm and outer diameter is below 800 μm (except fibers connections). Sufficient mechanical stability necessary for a manipulation and flux tests was achieved after the sintering step. The mechanical load during the testing includes sealing of the fibers inside inlets, application of internal or external liquid pressure, and fixation for SEM analysis.

3.1. Characteristic of the hydroxyapatite membrane

The surface porosity of cross-section and outer surface of the fibers was significantly different. The cross-section has porosity 19.0 ± 0.1% and outer surface only 1.0 ± 0.9% (determined by image analysis of SEM figures, example can be seen in Fig. 2b and c), when macrodefects inside hydroxyapatite walls were not taken into account (Fig. 2a). The prepared hydroxyapatite hollow fibers have skin with low porosity. The thickness of the surface low porosity area is less than 10 μm. The low porosity of the outer hollow fiber surface compare with majority of the inner volume forms asymmetric membrane, when the flux limitations are connected with the surface porosity.

Fig. 3 presents the cumulative filter flow, as the result of the bubble point test method, and demonstrates that no single pore is bigger than 2.1 μm and 70% of the flow is through pores smaller than 0.7 μm. The presented data demonstrates the absence of large defects in the outside surface of the sintered hydroxyapatite structure.

Table 2 presents the transport of various gases and liquids through the hollow fibers. Measurement of fluxes demonstrated the ability of asymmetric ceramic membrane to transport gasses

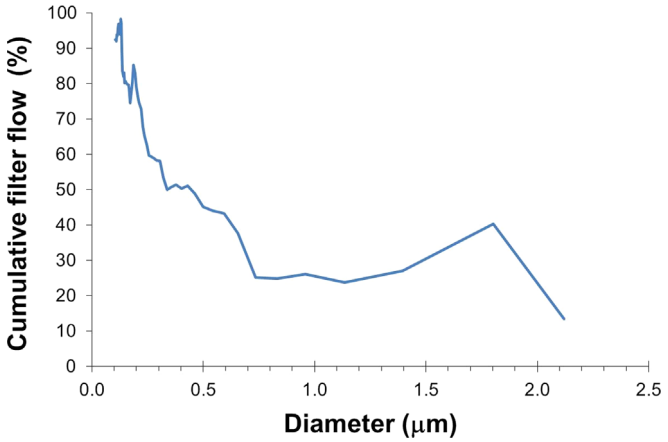

Fig. 3. Cumulative flow during bubble point meter test, which demonstrates that maximum pore diameter is 2.1 μm and 70% of the flow is through pores smaller than 0.7 μm in diameter.

Table 2
Flux of gases and liquids through the porous ceramic walls, the measurements were performed from inside to out.

	Average ($l\,m^{-2}\,h^{-1}$)	Standard deviation ($l\,m^{-2}\,h^{-1}$)
Flux N_2 (1 bar)	42730	12574
Flux CO_2 (1 bar)	40416	11735
Flux water (1 bar)	404	106
Flux cell culture medium (1 bar) 0–30 min	172	38
Flux cell culture medium (1 bar) 60–120 min	133	44
Flux cell culture medium (1 bar) 600–840 min	91	$R=0.9994^{*}$

*R-squared value of the flow ($l\,m^{-2}$) in time chart.

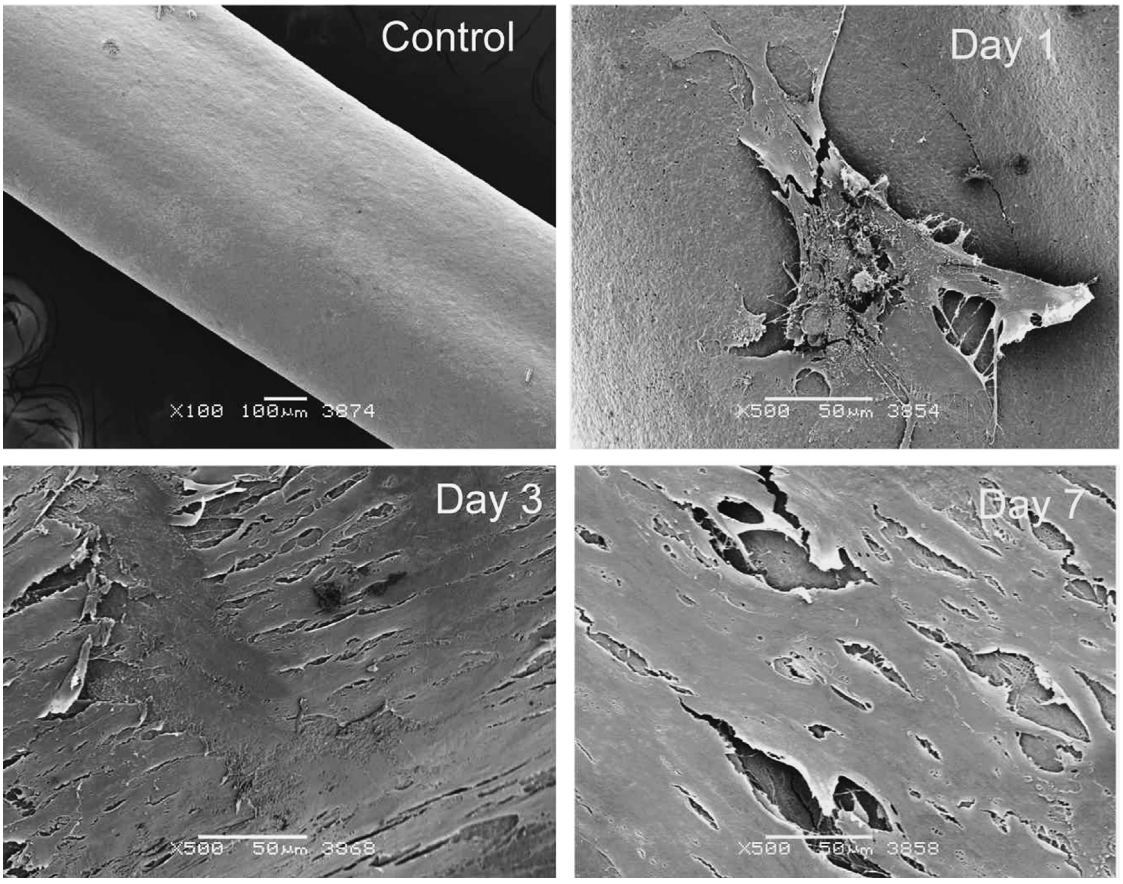

Fig. 4. SEM of the hydroxyapatite hollow fiber without cells (Control) and cells morphology visualization after 1, 3, and 7 days.

and fluids. The transport of culturing medium decreases in time to the stable value, this suggests that there is probably interaction between the fiber and proteins in the medium. Nevertheless, within 10 h flux was very stable and reached constant value of approximately 91 l m^{-2} h^{-1} and no significant decrease of the flux was further observed.

3.2. Cell culturing results

The ability of hydroxyapatite hollow fibers to support the adhesion and proliferation of hMSCs was evaluated. The hydroxyapatite fibers were cultured for 7 days and at each timepoint, the structures were processed to visualize cell morphology by means of SEM (Fig. 4).

On day 1, cell adhesion was slow, nevertheless, at day 3 cell proliferation was extensive in all tested samples. The adhered cells maintained their flat, well-spreaded morphology, while occupying all the available surface area and initiating the monolayers formation.

3.2.1. Cellular metabolic activity and proliferation

Cell metabolic activity and proliferation was evaluated by the MTS and the DNA quantification assay, respectively, as illustrated in Fig. 5. The cellular metabolic activity increased over time, demonstrating that the cells were viable in the presence of the tested hydroxyapatite structures. Cellular growth was higher in TCPS (2D structure) than on the 3D scaffolds. The cell proliferation demonstrated a similar trend, with an increased proliferation behavior for day 1, 3, and 7.

4. Discussion

Our work demonstrates a facile method of the 3D hydroxyapatite scaffold preparation. The scaffold is composed from HA hollow porous fibers. Each fiber is able to supply nutrients and remove the waste products, when both liquid and gas components can be efficiently supply or removed. hMSC cells were isolated and cultured on the hydroxyapatite fibers and their behavior was studied for 7 days in order to evaluate their biological response. The difference between two dimensional control sample and 3D hydroxyapatite is not unusual during the short experiment [16], and steady increase of cells proliferation and metabolic activity is obvious.

The presented method of the building 3D hydroxyapatite scaffold containing porous and hollow fibers is direct and easily processed without a significant investment (see, Fig. 1). Flexibility of the coated fibers allows to combine various supply for each layer of fibers in the constructed bioreactor. The total content of organic additive, which is necessary to burn out, is very low and the process does not require special heat treatment or controlled atmosphere. This microtemplating process can be easily handled in regular laboratory conditions,

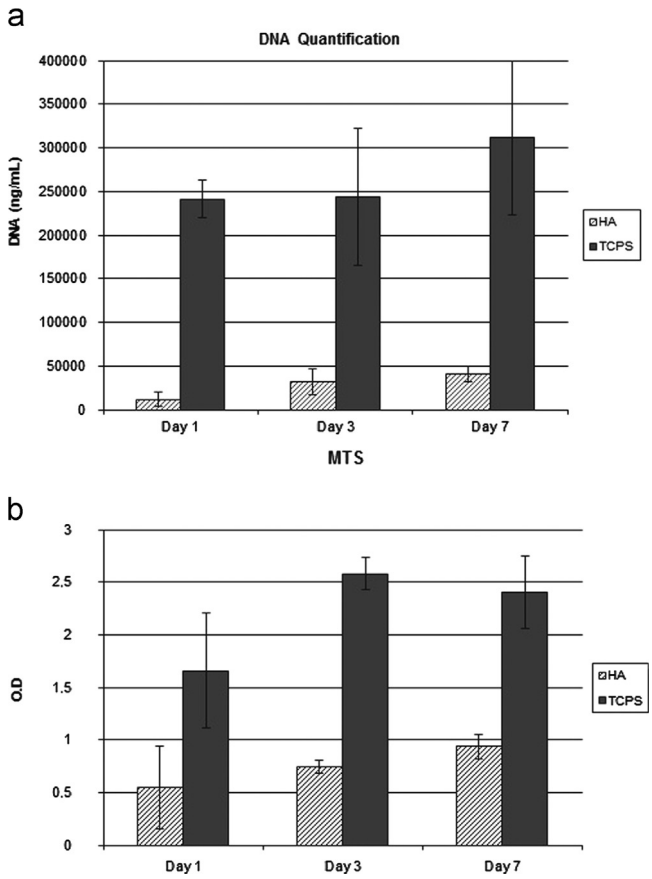

Fig. 5. (a) Cell proliferation in time. (b) Cellular metabolic activity in time.

but better control of the drying conditions may improve the shape control of the outer shape of the hollow fiber [14]. Drying conditions are also reason of the formation of denser layer close to outer surface. This density gradient in green body (not sintered ceramic powder) results in formation of asymmetric membrane, where porosity is significantly lower close to outer surface (1% vs. 19% in bulk). Despite this, the measured gas and liquid fluxes seems to be high enough for supply of nutrient and oxygen to the cells. Further tailoring of porosity is possible via control of sintering temperature or by choice of hydroxyapatite starting powder, but an increase of the porosity usually brings significant degradation of mechanical properties [17]. Mechanical stability of the hollow fibers was not measured in quantitative matter, but the fibers were strong enough to be handled during the flux experiments. Furthermore, multiple coating procedure brings benefits for the mechanical stability and additional tailoring can improve the mechanical stability [18].

The biological response of these materials was determined via the quantification of the cell metabolic activity, proliferation and morphology. The results indicate that our hydroxyapatite structures are biocompatible as expected, with cell adhesion and proliferation confirmed by MTS and DNA quantification assay, respectively. Even though – as seen by SEM – the cell adhesion was slower in the first analyzed time point (day 1), for the remaining time points, the cells proliferated well and cover the hydroxyapatite fiber as from day 3 onwards. The reason for slow start is that the proposed structure complex 3D shape has, when compared to 2D surfaces, low initial surface area leading to low initial cell adhesion. Nevertheless, the structure is intended for long term culturing since it can accommodate a high cell population in volume.

Previous studies, dealing with implementation of hollow fibers in a scaffold, were based on polymeric materials [8], or they were dealing with templated and rapidly prototyped structures [9,10]. Both hydroxyapatite processing methods are limited in variability of an additional functionalization, and hollow fibers for selective nutrition delivery are not present. The preparation of a membrane suitable for nutrient distribution and removal of waste products is difficult especially from a non-elastic material (hydroxyapatite). Nevertheless non-elastic materials have an advantage of very stable pore structure, which is not deformed by liquid pressure. The rapid prototyping methods allows multi-scale hierarchies of void structure as well as to ensure the mechanical strength [6], however an implementation of isolated microchannels for nutrition delivery requires micro-extrusion of hollow fibers with functional wall properties. This is technically very difficult task, when also rheology of the extruded paste has to be carefully controlled. Contrary, the microtemplating provides direct solution with flexibility in selection of materials and tailoring of the porosity. Hydroxyapatite is a good candidate for the preparation of biocompatible membranes, because it is mechanically and chemically stable material and nontoxic additives are needed during the microtemplating processing. The cell culture study showed that cell adhesion and proliferation are good on the fibers produced by the presented fabrication method (microtemplating). The production process of hollow fibers is safe and the developed fibers cytocompatible.

Our direct concept of building 3D hydroxyapatite structure has still some limitations. The outside shape is not fully controlled due to fast drying step, and further tailoring of drying conditions or suspension compositions is necessary. The building of the 3D shape was done manually and this processing step introduces imperfections into shape of the final structure. Complementary, further research of the shrinkage behavior during the sintering is necessary to precisely predict final dimension.

5. Conclusions

The 3D hydroxyapatite scaffold made from hollow porous fibers with functional properties was prepared via the direct processing method – microtemplating. The porous hollow fibers can be used for nutrition delivery and remove waste products in vitro. We have measured sufficient fluxes of carbon dioxide, nitrogen and water (40416 l m^{-2} h^{-1}, 42730 l m^{-2} h^{-1}, and 404 l m^{-2} h^{-1}, respectively) through the membrane walls. The flux of cell culture medium was decreasing in time, and after 10 h the flux has stable value of 91 l m^{-2} h^{-1}. The presented results confirm that the developed fibers are adequate to support adhesion and proliferation of MSCs.

Future studies will include evaluation of 3D scaffold in vitro using dedicated bioreactor. Generally, we expect potential applications in tissue engineering, when the size of the bio-construct is in level of centimeters and 3D structure is required.

Acknowledgments

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