

Comparison of Morphology and Cytotoxicity of Cellulose Composites with Nano- and Microhydroxyapatite for Bone Tissue Engineering

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Scaffolds for bone tissue regeneration must be precisely designed as they support cell attachment, proliferation, differentiation and blood vessel in-growth. Moreover, the materials which are implanted in human body must be non-cytotoxic, absolutely harmless.

In this work cellulose scaffolds with nanohydroxyapatite and microhydroxyapatite were prepared. The results obtained in this work revealed that the morphology of the scaffolds depended on the size of hydroxyapatite particles. The porosity of the scaffolds varied from 66 to 72%. The pores were interconnected with the average diameter 0.49 and 0.54 mm for scaffolds with nano- and microhydroxyapatite, respectively. Biocompatibility and potential toxicity of the experimental cellulose/hydroxyapatite scaffolds were tested. It was determined that the scaffolds containing nanohydroxyapatite particles showed slight cytotoxic effect.

Keywords: Scaffolds, Cellulose, Hydroxyapatite, Cytotoxicity, Bone tissue engineering.

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1. INTRODUCTION

Three-dimensional (3D) scaffolds fabricated from polymers are rapidly growing materials for bone regeneration procedures [1–3]. Saturated aliphatic polyesters are usually applied for this purpose [4]. Promising results have been obtained using naturally derived polymers, especially chitosan, alginate, starch, agarose or bacterial cellulose [5–9]. In order to enhance mechanical properties and bioactivity, several materials, such as hydroxyapatite, β -tricalcium phosphate, bioactive glass are embedded in polymer matrices [10–15].

Beside the choice of suitable material, the morphology is also very important. The structure of the scaffolds must be accessible for cell attachment, proliferation, differentiation and for blood vessel in-growth. The permeable pore network is required for nutrient delivery and waste exchange [16].

Hydroxyapatite being a component of natural bone is very often used for the preparation of bone scaffolds. Commercial hydroxyapatite is of different particle size, morphology and crystallinity.

The aim of this work was to prepare 3D cellulose based scaffolds with nano- and microhydroxyapatite particles and to compare their morphology as well as cytotoxicity.

2. MATERIALS AND METHODS

Cellulose/hydroxyapatite composites were produced mechanically immobilizing nanohydroxyapatite (nHA, 100 nm) and microhydroxyapatite (μ HA, 20 μ m) particles during the formation of cellulose scaffold. Composites were formulated with HA particles of ~ 50 wt. %, close to the percentage of inorganic part in natural bone. The micro-computed tomography (micro-CT) analysis was performed using a μ CT40 micro-CT system (Scanco Medical AG, Switzerland) in order to evaluate the morphology of the prepared scaffolds.

Cytotoxicity of the investigated scaffolds was studied using ex-vivo hepatocytes isolated from 3-month old

Wistar rats' liver. Immediately after isolation, cell aliquots were stained with trypan blue and counted in hemacytometer. Viability of the isolated hepatocytes before incubation in all experiments was not less than 95%. After isolation, cells were incubated in Petri dishes at 37°C through 90 minutes with milled composite samples 50 mg/ml of cell suspension for solid composites and 10 mg/ml for HA powders. After incubation aliquots for cell count and viability testing were dispensed. Cell viability was valued by staining with trypan blue dye. ≥ 600 cells for every sample in the set of experiments were tested. In all experiments control cells viability was over than 92%.

Membrane integrity was tested on isolated rat hepatocytes and *extensor digitorum longus* (EDL) muscle tissue by evaluating lactate dehydrogenase (LDH) and aldolase activity in incubation medium. LDH and aldolase outcome from the cells indicates membrane damage, and both these analyzes are widely used in clinics. For evaluating membrane damage in hepatocytes, cells after incubation with composite samples were centrifuged, and supernatants were collected for LDH assay. To investigate potential myocyte membranes damage, isolated EDLs from 3-month old Wistar rats were incubated at 37°C for 90 minutes in O₂ enriched Krebs saline buffer with composite samples. Incubation medium was used for aldolase assay.

Metabolic effects of cellulose/ hydroxyapatite composites were studied in isolated hepatocytes and EDLs of 3-month old rats by evaluating hormonal sensitivity and glucose uptake rate after 90 minutes incubation with composite samples. [³H]-D-glucose uptake under the insulin action was investigated in the EDLs, preincubated with composite samples. [¹⁴C]-glucose was added into the hepatocyte incubation medium after preincubation with samples to evaluate the basal levels of [¹⁴C]-glycogen synthesis, and insulin-stimulated [¹⁴C]-glycogen synthesis.

3. RESULTS AND DISCUSSION

For the morphological characterisation of the prepared scaffolds, micro-computed tomography was chosen. 2D images showed that HA particles affected the morphology of the prepared scaffolds: differences in pore size, framework thickness, and their distribution appeared (Fig. 1, Table 1).

The scaffolds comprised non-symmetrical interconnected pores. Such arrangement of the pores is particularly important for cellular activity and achieving the optimum rate of the new tissue growth.

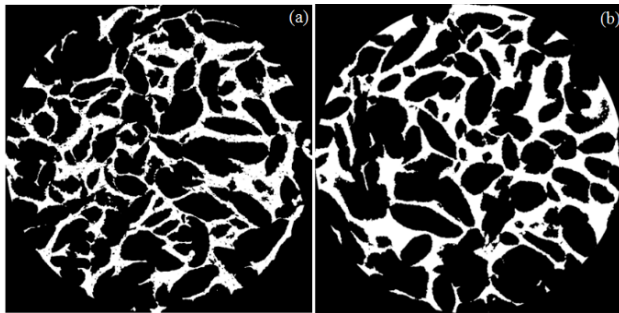


Fig. 1 – 2D images: cellulose/nHA scaffold (a), cellulose/μHA scaffold (b)

The structural parameters, such as the percent framework volume (Xv), porosity (P), specific surface (SS), mean framework thickness (L), mean pore diameter (D) within the scaffold were determined from 3D images (Fig. 2).

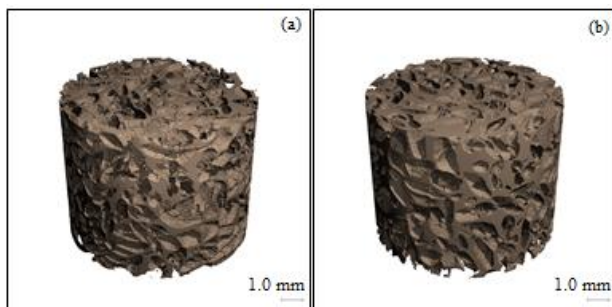


Fig. 2 – 3D images: cellulose/nHA scaffold (a), cellulose/μHA scaffold (b)

The structural parameters of the scaffolds are summarized in Table 1. The microcomputer tomography data showed that the porosity of cellulose/nHA scaffold was larger, leading to a reduced percentage of the framework volume, as compared with the cellulose/μHA scaffold. The framework thickness with the immobilized HA particles of the different composite scaffolds was different (Table 1). The frameworks of the cellulose/μHA scaffold were almost twice thicker, as compared with the frameworks of the cellulose/nHA composite. It was also observed in the 2D images (Fig. 1). The specific scaffold surface was found to be larger for the cellulose/nHA due to the thinner frameworks and a higher number of them per millimeter, as supposed. Thus, the scaffold had smaller pores. The mean pore diameter was of 0.49 mm.

Table 1 – Structural parameters of the scaffolds

Samples	Structural parameters				
	Xv (%)	P (%)	SS (mm ¹)	L (mm)	D (mm)
Cellulose/nHA	28	72	19	0.12	0.49
Cellulose/μHA	34	66	13	0.21	0.54

Generally, the morphology of the prepared cellulose/HA scaffolds for bone tissue engineering meets the requirements of materials which are implanted in the bone defect [17].

Besides the suitable morphology, the scaffolds should be absolutely harmless to body. Thus, the biocompatibility and cytotoxicity of the cellulose/HA scaffolds were tested. Trials were divided into three main stages, where cytotoxicity, membrane integrity and metabolic effects of the scaffolds were studied.

All investigated composites, including scaffolds containing μHA particles have demonstrated insignificant changes in cell viability after incubation, except cellulose/nHA scaffolds, where cell viability demonstrated significant 5 to 8% viability drop in comparison to control cells. Furthermore, hepatocyte incubation with HA powder have shown that μHA particles made no significant changes in cell viability, at the time when nHA decreased cell viability by about 10% (Fig 3).

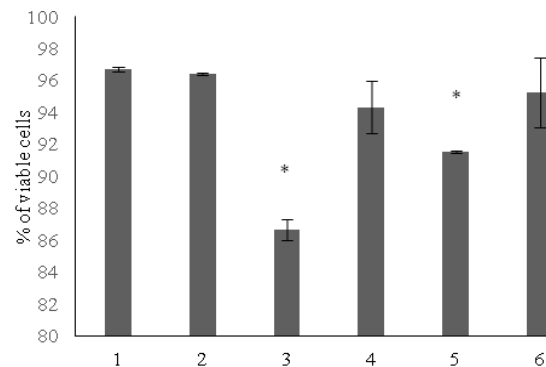


Fig. 3 – effect of cellulose/HA composite scaffolds and HA powdered particles on hepatocytes viability. 1 – untreated cells, 2 – control cells, 3 – nHA particles, 4 – μHA particles, 5 – cellulose/nHA, 6 – cellulose/μHA. *-p<0,05

Comparison of nHA and μHA particles incorporated into cellulose also demonstrated cytotoxicity of nHA, while μHA were harmless.

45% rise in LDH activity in hepatocyte incubation medium was found after preincubation of the cells with cellulose/nHA scaffolds. Other composite samples have demonstrated no significant LDH release from the cells in regard to control (Fig. 4a). When incubated with EDLs, cellulose/nHA scaffolds also significantly increased aldolase activity in the culture medium: about 3-fold compared to control (Fig. 4b). At the same time, whole-cellulose scaffolds without HA addition did not change the LDH or aldolase release from the cells (Fig. 4). Enzymatic activity, elevated in incubation medium of cells and muscle tissue after preincubation with cellulose scaffolds, containing nHA particles indicate membrane damage, caused by composite, which may lead to metabolic disregulation and cell death.

Significant [¹⁴C]glycogen synthesis failure under the action of insulin, but not in basal glycogen synthe-

sis rate, was found in cells incubated with cellulose/nHA scaffolds and whole-cellulose scaffolds without HA additives. Cellulose/nHA scaffolds have decreased [¹⁴C]glycogen synthesis rate by about 20%, compared to control at the time when whole-cellulose scaffolds decreased [¹⁴C]glycogen synthesis rate by about 10%. Other cellulose-based composites have demonstrated no significant decrease in [¹⁴C]glycogen synthesis under the insulin action. At the same time, cellulose scaffolds containing nHA induced 2-fold [³H]glucose uptake failure under the action of insulin in isolated EDLs in comparison to control, while other composite samples caused no significant changes in [³H]glucose uptake rate.

4. CONCLUSIONS

The prepared nanohydroxyapatite/cellulose and microhydroxyapatite/cellulose scaffolds showed well designed structures. The morphological parameters were in the range of required. All composite samples, except nanohydroxyapatite carrying cellulose scaffolds, during incubation period have demonstrated acceptable levels of cell integrity and viability, and were not cytotoxic or damaging to native primarily isolated liver and muscle cells. At the same time cellulose/nanohydroxyapatite scaffolds, according to the set of assays, have demonstrated slight cytotoxic influence and downregulation of insulin sensitivity in hepatocytes and EDL muscle tissue models. Moreover, nanohydroxyapatite powder, compared to control and to microhydroxyapatite particles, have demonstrated cytotoxic effects.

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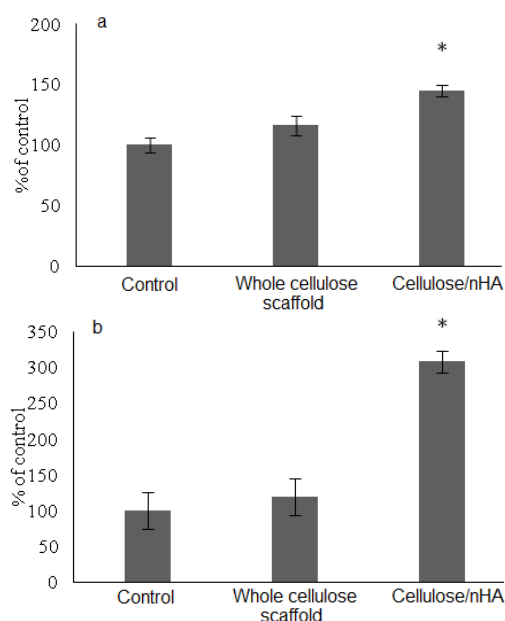


Fig. 4 – effect of cellulose/HA composite scaffolds on LDH and aldolase release in the incubation medium of cells. a – LDH release from hepatocytes; b – aldolase release from EDLs. * - $p < 0,05$

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