POROUS COMPOSITE MATERIALS CHITOSAN - CALCIUM COMPOUND PARTICULATE FOR BONE DEFECT FILLING AND REPAIR

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Abstract

In this study we developed techniques to fabricate porous composites (scaffolds) chitosan (Ch) – various calcium materials such as hydroxyapatite (HA), carbonated HA (CHA), and calcium carbonate (CC) in the nano-powder state. The scaffold's properties are dependent on the porosity of the foam, the quantity and the kind of powder fillers. The solubility, morphology and factor of compression of the foams were studied. Also the biological tests in vitro were performed showing cells adhesion and viability, and absence of cytotoxicity.

Keywords: hydroxyapatite, carbonated hydroxyapatite, calcium carbonate, chitosan, scaffold, composite

INTRODUCTION

Porous calcium phosphate ceramics are used in surgery to repair bone defects. The number of medical applications of the ceramics is limited for the following reasons: fragility, relatively low fracture toughness, difficulty to obtain exact size and shape of final material to be the defect hole replica, and impossibility to produce materials of high resorption rate required to rapid regeneration of bone tissue. Composite chitosan-based materials with ceramic reinforcing particles possess elasticity that gives a possibility to overcome the deficiencies of the ceramics.

Chitosan is a multifunctional compound, possessing a lot of unique properties: high compatibility with living tissues, biodegradation, absence of toxicity, bactericidal properties, hemocompatibility, linkage and deducing radionuclides, heavy metals, etc. [1].

In medicine chitosan is used both in a pure state (film, fibre, capsule, sponge), and as a composite material (chitosan – calcium phosphates). Chitosan films are applied as dividing ion-exchange membranes, medical and protective coatings. Surgical sutures and capsules for drug delivery are received from chitosan. Advantages of porous composite materials chitosan - calcium phosphates (in comparison with porous ceramics) are higher porosity, elasticity and resorption rate. The elasticity allows filling of the bone defect of any complex form, thus there is no gap between a bone and implant [2]. The high resorption allows a new bone tissue to be formed quickly. There are some ways to obtain chitosan-based composite materials, such as freeze drying, foaming and leaching.

Bone regeneration usually employs the interconnected structure of porous materials. It provides the necessary support for cells to proliferate and maintain their differentiated function, and its architecture defines the ultimate shape of the new bone [3].

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Porosity and interconnective pore structure are also critical to provide sufficient opportunity for cell migration and expansion, while maintaining transport of nutritious [4, 5]. The aim this work was to develop manufacturing of chitosan-base composites and investigate the properties of the composites.

MATERIALS AND METHODS

Powder synthesis

Hydroxyapatite powder was precipitated in an aqueous medium by slow addition of a diammonium phosphate ($(NH_4)_2HPO_4$) solution into a calcium nitrate $Ca(NO_3)_24H_2O$ solution, containing NH_4OH, under constant stirring. The Ca/P molar ratio was 1.67. The pH of the mixture was about 9. After total addition of the reactants the suspension was filtered and washing in ethanol. Then it was filtered, dried at 80°C and sintered at 700°C for 2 hours [6].

Carbonated hydroxyapatite was produced by a solid-state reaction [7] from calcium oxide, ammonium hydrophosphate and ammonium carbonate. The synthesis was carried out in a planetary-type mill for 1 hour. CHA was sintered at 700°C to stabilize it.

Calcium carbonate was produced by a solid-state reaction from calcium oxide and ammonium carbonate in the same planetary-type mill for 1 hour.

The phase compositions of the dried and lightly calcined HA, CHA and CC powders were investigated by powder X-ray diffraction (XRD) using a Shimadzu XRD-6000 diffractometer (CuK_α radiation), and infrared (IR) spectra using an IR spectrometer Nicolet Avatar in the wavenumber range of 7800-350 cm^{-1} with a resolution of 0.9 cm^{-1}.

Preparation of chitosan matrices

The medium molecular weight Ch (50-190 kDa, Aldrich) was used. A Ch solution was prepared by dissolving 2 g of Ch in 50 g of 8% solution acetic acid and mixing it at room temperature for 5 hours until the Ch was entirely dissolved. A filler was added to the suspension with ratio Ch/filler equal 1/4 during mixing. Next step was adding a foaming agent (ammonium carbonate) that also increased the pH value of the solution. The foamed mixture was frozen at -18°C. After that, it was soaked in ethanol to remove water and acetic acid. The foam obtained was dried at room temperature. Porous structure of scaffolds resulted from both the foaming by carbon dioxide and the removing of crystallized water.

Measurement of solubility

Test for solubility of the Ch scaffolds was carried out. Scaffolds of 1 g weight were soaked in distilled water at temperature 37°C for 1, 3, 7, 14 and 28 days. The scaffolds were washed out, dried at temperature of 60°C and their weights were measured after specified periods of time.

The solubility of Ch scaffolds was estimated using the formula:

$S = (m_1-m_2)/m_1$,

where: m_1- is weight of a dry scaffold prior to the beginning of tests for solubility; m_2 - is weight of the dried scaffold after tests for solubility.

Estimating compression ratio

The samples of 14 mm diameter (d) and 20 mm in height (h) were prepared. The samples were immersed in distilled water and maintained for one day. Then the compression test was carried out in the following way: sample immersed in distilled water was cyclically deformed with increase in deformation at 5% till the moment when

the sample ceased to be restored completely to the initial size (limiting elastic deformation (D_{lim})). Times of recovery of the sample were 3-5 seconds. The compression ratio was defined by the following formula:

$C_r = D_{lim}/h$

The tests for compression were carried out using an Instron 5581 apparatus.

In vitro test

Composite materials were washed in distilled water up to pH=7 of solution. Dried materials were sterilized by γ-radiation up to 2 mega rad. The nutrient medium and human fibroblasts (HF) were further added. The nutrient medium was refreshed twice a week. Then the cells were treated on 1, 7, 14, 21 and 28 days by dimethiltetrazolium (MTT) [8]. MTT transformation caused by mitochondria in cells resulted in formasan crystallization. Finally the formasan was dissolved with isopropanol and the transmission density of the solution was measured, which is proportional to the number of viable cells.

RESULTS AND DISCUSSION

High porous scaffolds with porosity 96% (Ch) and 90-92% (Ch/Filler) were obtained. The morphology of so-obtained composites was studied. Figure 1 shows the microstructure of Ch and Ch/filler composite with ratio of components equals to 1/4. The structure of Ch scaffolds without filler is cellular, pores are interconnected and have circular form. The size of pores is in the range from 100 to 300 microns. Thickness of walls between pores is up to 50 microns. The structure of scaffolds reinforced by nanoparticles of HA, CHA and CC was different: the size of pores increases from a surface of the sample toward depth, pores have channel form with length up to 2-3 mm and diameter 0.2-0.3 mm.

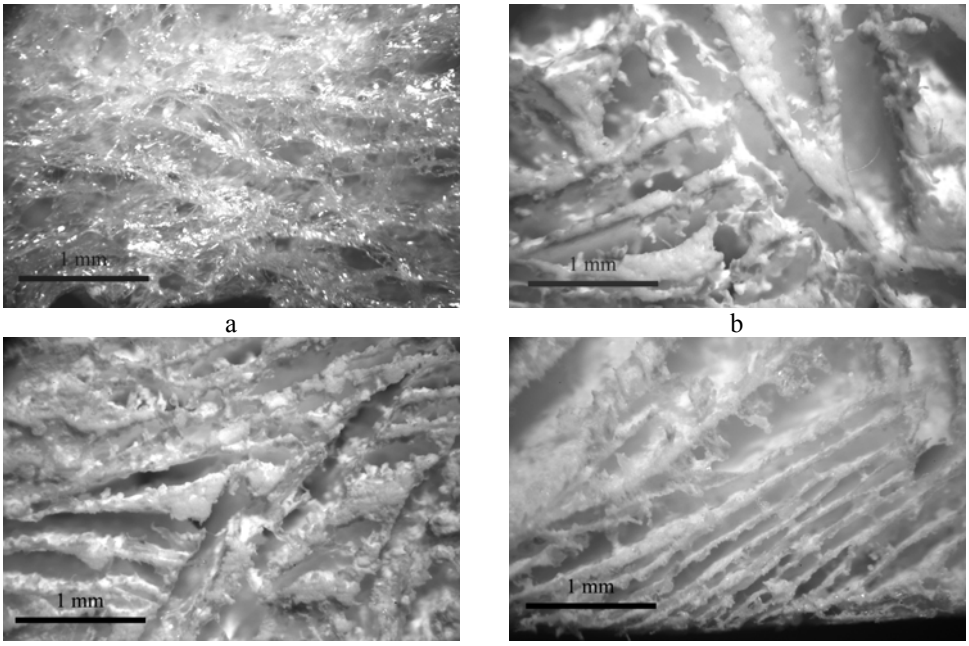

Fig.1. a - chitosan scaffold; b - Ch/HA scaffold; c - Ch/CHA scaffold; d - Ch/CC scaffold (x40).

This can be due to influence of nanopowders on the crystallization in the form of needle ice crystals. Pores are formed due to removal of the ice crystals. Porosity decreased from 96% to 90-92% with an increase of filler content.

Solubility

Figure 2 shows that the highest solubility is obtained for Ch scaffolds without fillers. Solubility of the composite materials decreases by factor of 2-4 due to introduction of nanoparticle HA, CHA and CC. The solubility of the scaffolds in distilled water at 37°C increases in the next sequence: Ch/HA < Ch/CC < Ch/CHA < Ch.

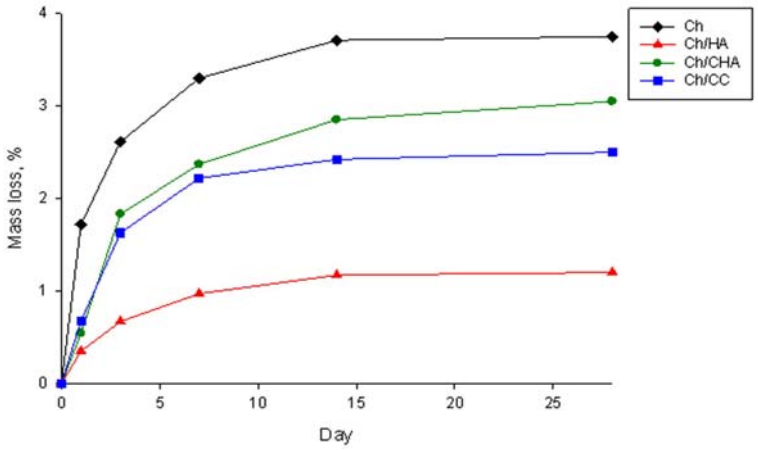

Fig.2. The solubility of scaffolds.

After 28 day of the dissolution experiment, the dissolution process was practically stopped. This can result from establishment of a balance between water and scaffold, i.e. the water becomes saturated by chitosan and ions of calcium.

Compression ratio

Ch scaffolds possess the highest compression ratio $C_r = 0.85$ (Fig.3). Elasticity of materials decreases owing to reinforcing nanoparticles (in case of: HA up to 0.8, CHA up to 0.75 and CC up to 0.7). It can be explained by decrease in continuity of an organic skeleton that leads to partial destruction at smaller deformations. The scaffold of Ch/CC possesses the lowest compression ratio. It is probably due to CC having an alkaline nature. It gives additional rigidity to Ch matrix.

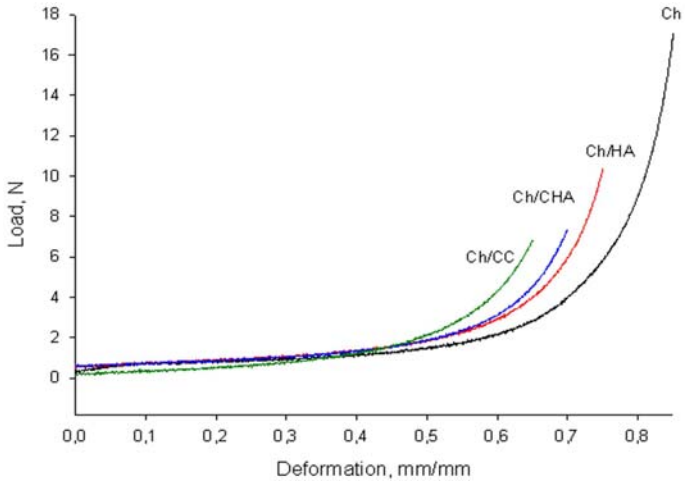

Fig.3. Compression ratio of scaffolds.

In vitro tests

The test on acute cytotoxicity was carried out in 24 hours cultivation. The composite materials are not toxic for HF: the optical density of a formasan solution is comparable to that of the control, therefore a pool of survived cells on samples, in comparison with the control (polystyrene) was 90-105% (Figs.4 and 5).

It is shown, that in the first two weeks HF incubation on various porous Ch matrix, reinforced by nanopowders of HA, CHA and CC, there was an insignificant lag in processes of cellular expansion of scaffolds from the control (cultivation on polystyrene). So, in all cases the optical density of formasan solution was a little below that of the control. As a whole, by the end of cultivation this parameter did not differ from control values for all the samples.

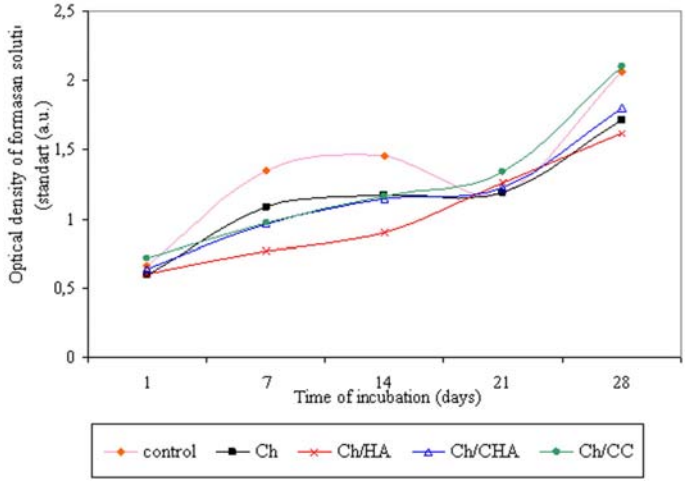

Fig.4. The dynamics of formasan solution optical density (a.u.) at HF cultivation on Ch scaffolds, reinforced nanopowders of HA, CHA and CC.

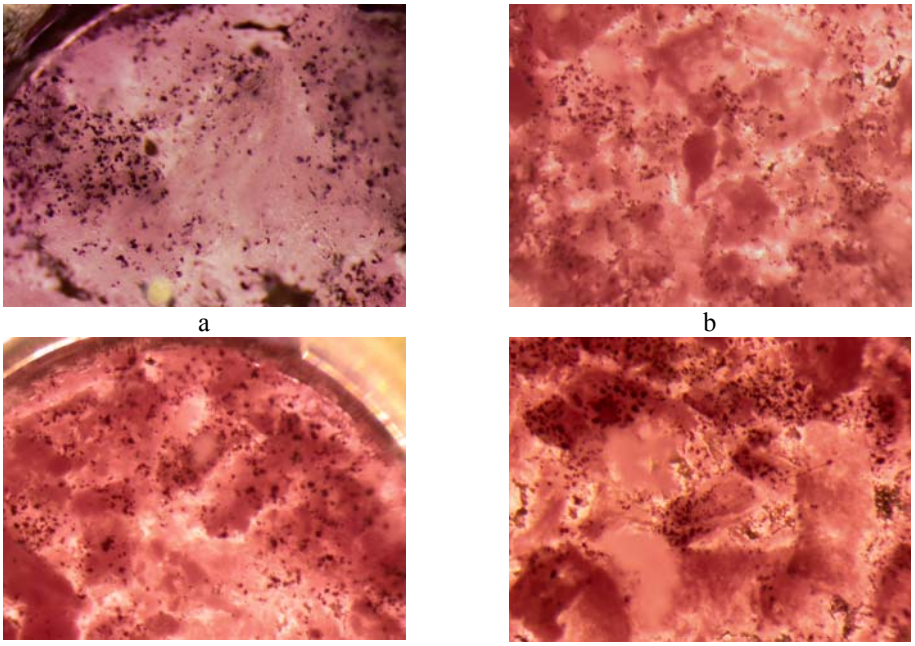

Fig.5. Distribution of a HF pool in wells with materials after 24 hours: a – chitosan scaffold; b – Ch/HA scaffold; c – Ch/CHA scaffold; d – Ch/CC scaffold (x16).

CONCLUSIONS

1. A new method to manufacture high pours composite materials with Ch matrix reinforced by nanoparticles of HA, CHA and CC was developed.
2. The reinforcing nanoparticles of HA, CHA and CC affect the microstructure of composites: the size of pores increases from the surface of the sample towards depth, pores have channel form of length up to 2-3 mm and diameter 0.2-0.3 mm. Porosity decreases from 96% to 90-92% with an increase of a filler content.
3. The solubility of scaffolds in distilled water at 37°C increases in the following sequence: Ch/HA < Ch/CC < Ch/CHA < Ch.
4. Composites possess high elasticity of 70-85%. That allows filling of a bone defect of complex form without a gap between bone and implant.
5. The biological tests in vitro were performed showing cells adhesion and viability, and an absence of acute cytotoxicity.

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