The present invention relates to material engineering and could be applied in odontology or other kind of surgery for bone tissue regeneration in a defect site. The aim of this invention is to prepare a porous three dimensional cellulose-based biocompatible scaffold of good osteoconductive and mechanical properties with the structure like that of a natural bone. To achieve the above object, the present invention provides a composite comprising cellulose and biogenic bone particles at a ratio (w/w) 1:0.12-6.0, wherein the diameter of the particles is 0.01-2000 microns. In a separate case a bone material which comprises inorganic compounds and polysaccharide chitin could be used as a biogenic bone. In a separate case the surface of the scaffold could be coated with collagen, platelet- rich fibrin, various growth factors, therapeutic additives, stem cells. The three dimensional porous scaffold of the natural bone morphology is prepared by inserting biogenic bone particles into a cellulose gel during its formation from cellulose acetate at a ratio 1:0.12-6.0, afterwards loading the gel with an aqueous 10-30 % ethanol solution and freeze-dried or extracted with carbon dioxide at supercritical conditions at 24-70 MPa and 35-80°C temperature. The resulting scaffold could be ground to granules of a desired size and/or the pasta could be prepared.
A 3D POROUS CELLULOSE SCAFFOLD FOR BONE TISSUE ENGINEERING
AND THE METHOD FOR ITS PREPARATION

The present invention relates to material engineering and could be applied in odontology or other kind of surgery for bone tissue regeneration in a defect site.

A 3D porous biocompatible cellulose scaffold which possesses biocompatibility, good osteoinductive and mechanical properties, is prepared as a composite comprising cellulose, as a natural polymer, and a biogenic source of calcium, such as particles of an autogenic (own), allogenic (of the same species, i.e. human, but of different person), or xenogenic (of other species) bone. A structure of the composite, applicable for bone tissue regeneration, is formed by means of lyophilization or supercritical extraction of the samples presoaked with alcohol aqueous solutions. The composites could be in block or granular forms and also a paste could be prepared.

Bone grafts used for bone tissue regeneration in odontology are as follows:

- autogenic bone grafts, i.e. an own bone of the patient from the other place. Advantages: no immune response. Disadvantages: an additional operation is needed; lack of a bone; complicated shaping;
- allogenic bone grafts, i.e. a bone of the same species (human), but of a different person. It fits by its physical properties, but there is a risk of disease transmission;
- xenogenic bone grafts, i.e. a bone of other species, usually a bullock, after its deproteinization. The morphology does not fit to that of a human bone. Too quick resorption in the organism;
- synthetic grafts. Among them the most popular are tricalcium phosphate (TCP) and hydroxyapatite (HA).

Most grafts are in powder or granule form (0.5-2 mm in size). The main disadvantage is that they often conglomerate what hinder vascularization. The resulted product is of insufficient strength and crumbles when screwing a metallic implant.

In order to get a porous 3D structure, solid aggregates from bone-derived elements bonded to each other are prepared by means of different methods, e.g. agglutination, pressing, or caking. Different bone particles, TCP, HA (Winterbottom J.M. et al. Implant, method of making same and use of the implant for the treatment of bone defects. Patent No. US 6478825 Bl, 2002-11-02), or those minerals together with polymeric particles

However, synthetic polymers are of lower biocompatibility than natural, they often cause necrosis of tissues, their degradation products, e.g. glycolic acid or other acidic compounds can increase local acidity and cause tissue lesions. Moreover, toxic metabolites in the organism can form during degradation of the polymers. The methods described are complicated and do not give desirable porosity, because the particles are agglutinated or caked. An important disadvantage is also the application of synthetic minerals TCP and HA as calcium raw materials.

The prototype of this invention is a cellulose-based three dimensional scaffold, with the surface mineralized in a simulated body fluid (SBF) (Petrauskaite, O. et al. Biomimetic mineralization on a macroporous cellulose-based scaffold for bone regeneration. BioMed Research International. ISSN 2314-6133. 2013, vol. 2013, p. 1-9). In this case a porous scaffold is formed by lyophilization of a regenerated cellulose gel. The morphology of the porous scaffold obtained corresponds to that of a natural bone.

The substantial disadvantage of the scaffold is that cellulose is not bioactive, it does not ingrow with bone tissues, possesses very low osteoconductive properties. To enhance those properties the scaffold surface is mineralized in SBF. However synthetic minerals possess lower biocompatibility in comparison with natural ones. Moreover, while mineralized in SBF, just the surface of the scaffold is coated, therefore the content of
minerals in the composite reaches just up to 12 %, calculating from the cellulose weight. Thus, the osteoconductive properties increase unremarkably. Moreover, the scaffold possesses poor mechanical properties (the Young’s modulus is 4 MPa).

The aim of this invention is to prepare a porous three dimensional cellulose-based biocompatible scaffold of good osteoconductive and mechanical properties with the structure like that of a natural bone.

To achieve the above object, the present invention provides a composite comprising cellulose and biogenic bone particles at a ratio (w/w) 1:0.12-6.0, wherein the diameter of the particles is 0.01-2000 microns. In a separate case a bone material which comprises inorganic compounds and polysaccharide chitin could be used as a biogenic bone. In a separate case the surface of the scaffold could be coated with collagen, platelet-rich fibrin, various growth factors, therapeutic additives, stem cells. The three dimensional porous scaffold of the natural bone morphology is prepared by inserting biogenic bone particles into a cellulose gel during its formation from cellulose acetate at a ratio 1:0.12-6.0, afterwards loading the gel with an aqueous 10-30 % ethanol solution and freeze-dried or extracted with carbon dioxide at supercritical conditions at 24-70 MPa and 35-80°C temperature. The resulting scaffold could be ground to granules of a desired size and/or the pasta could be prepared.

The advantages of the resulting scaffold are the following: natural components, such as cellulose and biogenic bone particles, are used. Cellulose is biocompatible, not cytotoxic. Its degradation products are not toxic. As a biogenic bone, an autogenic, allogenic, or xenogenic (of any animal) bone could be used. In a separate case a milled bone material which comprises inorganic compounds and polysaccharide chitin could be used. It possesses antimicrobial and coagulation properties what give additional advantages. The ratio (w/w) of cellulose to bone particles in the composite is equal to 1:0.12-6.0.

Brief description of the drawings.

Fig. 1 Cross-section of the scaffold described in the 1st example, obtained by means of microcomputed tomography.

Fig. 2 3D image of the scaffold described in the 1st example, obtained by means of microcomputed tomography.
Fig. 3 Cross-section of the scaffold described in the 2nd example, obtained by means of microcomputed tomography.

Fig. 4 3D image of the scaffold described in the 2nd example, obtained by means of microcomputed tomography.

Fig. 5 Proliferation of cells on the scaffolds.

The method for preparing the scaffolds

Ground bone particles are dispersed in the solution of cellulose acetate from which the gel with the particles is formed. The particle size is in the range of 0.01-2000 microns, preferably up to 200 microns. The porous structure which corresponds to the morphology of a natural bone is formed by lyophilization of the cellulose gel with the inserted bone particles loaded with the 10-30% alcoholic solution or by extraction with carbon dioxide at supercritical conditions at 24-70 MPa and 35-80°C temperature. Different water soluble alcohols could be used, preferably ethanol. The resulting composites possess good mechanical properties, the Young's modulus is at least 8 MPa. The morphology of the scaffolds corresponds to that of a natural bone (see Table). The pore size is suitable for the vascularization and cell proliferation. The tests with mice showed that during two weeks a network of blood vessels was formed. The pores being interconnected are suitable for the nutrient and metabolite transport.

Table. The structural parameters of the resulting scaffolds and of a natural bone

<table>
<thead>
<tr>
<th>Bone grafts, obtained according the examples</th>
<th>Structural parameters</th>
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<tbody>
<tr>
<td></td>
<td>Framework volume, %</td>
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<tr>
<td></td>
<td>Porosity, %</td>
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<tr>
<td></td>
<td>Specific surface, mm$^{-1}$</td>
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<tr>
<td></td>
<td>Mean framework thickness, mm</td>
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<tr>
<td></td>
<td>Mean pore diameter, mm</td>
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<tr>
<td>Example no.1</td>
<td>25</td>
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<td>Example no.2</td>
<td>27</td>
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<tr>
<td>Example no.3</td>
<td>28</td>
</tr>
<tr>
<td>Jawbone (depends on the site)</td>
<td>7-49</td>
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</table>
The studies of human osteoblast MG-63 proliferation showed that obtained grafts possess better osteoconductive properties in comparison to the graft obtained according to the prototype (Fig. 5).

The method for the evaluation of osteoconductive properties.

The osteoconductive characterization of the scaffolds was conducted with human osteoblastic cells MG-63 (ATCC® CRL-1427™) (American Type Collection Culture, USA). The cells were seeded on the samples sterilized with UV rays for 24 h. The samples were soaked for 1 h in culture media, put to the 24 well plates (one sample in a plate) and covered with a suspension of the cells. The scaffolds with the cells were kept at 37°C in a 5% CO₂ atmosphere. Colonized materials were evaluated throughout the culture time by the DNA content after 1, 3, and 7 days using a fluorochrome Quant-iT™ PicoGreen® (Life Technologies, USA) according to the manufacturer's instructions.

The surface of the scaffolds could be coated with collagen, platelet-rich fibrin, various growth factors, therapeutic additives, stem cells. The resulting scaffold could be in a block form easy shaped to an implant; could be ground to granules of a desired size; and/or a paste could be prepared.

The paste could be obtained by mixing the ground scaffold with glycerol, polyethylenglycol (molecular weight 400-600), or another hydrogel.

1st example
To a solution of 25 g of cellulose acetate in 261 mL of an acetone-ammonia solution (v/v 1:0.45) 15 g of an allogenic graft (the particle size less than 200 microns) is added. The resulting dispersion is poured into the vessels of a desired volume and shape and kept until a solid gel is formed. The gel is thoroughly washed with distilled water and afterwards is kept in an aqueous 25% ethanol solution for 24 hours and then freeze-dried.

The Young's modulus of the resulting composite is 8 MPa. The cross-section image obtained by means of microcomputed tomography is presented in Fig. 1, whereas 3D image in Fig. 2. The results of cells proliferation on the graft are demonstrated in Fig. 5.
2nd example
To a solution of 25 g of cellulose acetate in 267 mL of an acetone-ammonia solution (v/vl:0.5) 12 g of a ground cuttlebone (the particle size less than 100 microns) is added. The resulting dispersion is poured into the vessels of a desired volume and shape and kept until a solid gel is formed. The gel is thoroughly washed with distilled water and afterwards is kept in an aqueous 25% ethanol solution for 24 hours and then freeze-dried. Before using, proteins are washed out from the cuttlebone. For this the cuttlebone powder is kept in a 0.5 M sodium hydroxide solution for 5 hours at 80°C temperature. Afterwards the powder is thoroughly washed with distilled water, dried and sieved. The Young's modulus of the resulting composite is 10 MPa. The cross-section image obtained by means of microcomputed tomography is presented in Fig. 3, whereas 3D image in Fig. 4. The results of cell proliferation on the graft are demonstrated in Fig. 5.

3rd example
To a solution of 10 g of cellulose acetate in 101.5 mL of an acetone-ammonia solution (v/v 1:0.45) 12 g of a xenogenic bullock graft (the particle size less than 200 microns) is added. The resulting dispersion is poured into the vessels of desired volume and shape and kept until a solid gel is formed. The gel is thoroughly washed with distilled water and extracted with supercritical carbon dioxide at 30 MPa and 80°C temperature. The Young's modulus of the resulting composite is 20 MPa. The results of cell proliferation on the graft are demonstrated in Fig. 5.

4th example
The surface of resulting grafts is coated with collagen. For this purpose the collagen of type 1 (human, pig, rat, cow or recombinant) was dissolved in 0.1 M acetic acid according to Sigma Aldrich recommendations, diluted 10 times with a phosphate buffer and titrated with 0.1 M sodium hydroxide till pH 7. The graft was put into a centrifuge vessel, covered with the collagen solution and centrifuged at 4000-6000 rpm min⁻¹ for 10 minutes. After that the graft was freeze-dried.
Claims

1. A three dimensional porous cellulose-based scaffold for bone tissue engineering possessing the morphology similar to a natural bone structure characterized in that it is composed of a cellulose composite with biogenic bone particles at the ratio (w/w) 1: 0.12-6.0, wherein the diameter of the particles is in the range of 0.01-2000 microns.

2. A three dimensional porous cellulose-based scaffold according to claim 1, wherein composed of cellulose with a bone material, which comprises inorganic compounds and polysaccharide chitin, 0.01-2000 micron particles.

3. A three dimensional porous cellulose-based scaffold according to claim 1 and 2, whereas its surface is coated with collagen, platelet-rich fibrin, various growth factors, therapeutic additives, stem cells.

4. A method for preparing a three dimensional porous scaffold based on regenerated cellulose characterized by the formation of a gel from a cellulose acetate solution with inserted particles of a biogenic bone at the ratio (w/w) 1: 0.12-6.0.

5. A method for preparing a three dimensional porous scaffold by lyophilization of the regenerated cellulose gel obtained according to claim 4, wherein the cellulose gel with inserted biogenic bone particles is loaded with a 10-30 % alcoholic solution before its lyophilization.

6. A method for preparing a three dimensional porous scaffold, wherein the cellulose gel with inserted bone particles is loaded with a 10-30 % alcoholic solution and extracted with supercritical carbon dioxide at 24-70 MPa and 35-80 °C temperature.

7. A method for preparing a three dimensional porous scaffold, wherein the resulted block was ground to the desired particle size and/or the pasta was formed.
**INTERNATIONAL SEARCH REPORT**

**A. CLASSIFICATION OF SUBJECT MATTER**

INV. A61L27/36 A61L27/44 A61L27/56

**ADD.**

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

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<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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  **Z** document member of the same patent family

Date of the actual completion of the international search

10 December 2015

Date of mailing of the international search report

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Heck, Georg

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<td>US 6340477 B1</td>
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