Nanohydroxyapatite - calcium Fructoborate Composites
Synthesis and characterization

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This work presents a study on the possibility of obtaining new bionanocomposites based on hydroxyapatite nanopowders doped with calcium fructoborate using the sol-gel method. The hydroxyapatite - calcium fructoborate bionanocomposites were characterized by scanning electron microscopy (SEM) coupled with energy dispersive X-ray spectroscopy (EDX), Fourier transformed infrared (FTIR) spectroscopy and biological tests. The results obtained have shown that the active substance, calcium fructoborate, is well dispersed between hydroxyapatite crystals. Moreover, in vitro assays demonstrated that these composites have good cell compatibility with skeletal myoblast and have potential to be used as biomaterials (bone cements, dental filling materials, and resorbable materials) used in osteosynthesis.

Keywords: hydroxyapatite, calcium fructoborate, bionanocomposite, bone remodelling

There is variety of biomaterials used in the applied therapy of the bone tissue's reconstructive surgery. These biomaterials must meet a number of characteristics related to their physical and chemical structure, the interaction with the physiological environment where they will be used in and to their properties to allow cell adhesion and proliferation. Because of the important properties required, such as biocompatibility, biodegradability, mechanical properties and osteoconductivity, the number of materials that are suitable for such applications is limited. Therefore, the ideal solution would be to obtain composite biomaterials that would combine synergistic the properties of the constituents of which they are formed of, thus generating new biomaterials with superior properties [1]. Another aspect to be taken into account in designing a bone substitute is the fact that the biocomposite must imitate as closely as possible the physical and chemical properties of the bone tissue [2].

The synthetic hydroxyapatite is as basic component of the composites because of a similar structure to that of the primary mineral from the bone tissue composition and for its remarkable properties such as biocompatibility, bioactivity, non-toxicity, osteoinduction, osteoconduction and osseointegration, which were demonstrated in several *in vitro* and *in vivo* studies [2, 3]. The high purity hydroxyapatite (HA, $Ca_{10}(PO_4)_6(OH)_2$) has been used so far in various forms for various biomedical use, such as: dense sintered ceramics (implants for middle ear, alveolar usage), porous or granular filling of the bone defects (cavities), materials for depositing on the surface of implants, vector type materials for releasing the biologically active principles, etc. [3]. The hydroxyapatite, even with no superior mechanical properties, but with osteoinductive properties instead and a non-toxic property for human body, represents an ideal alternative to be used as transporting materials.

The chemical structure of the calcium fructoborate (FB) is $Ca[(C_6H_{10}O_6)_2B]_2 \cdot 4H_2O$ and it is known as a boron-based nutritional supplement. Calcium fructoborate is a superoxide ion scavenger and anti-inflammatory agent such as revealed in several *in vitro* studies [4]. It was shown that calcium fructoborate administrated to humans in a dose range of 1-7 mg calcium fructoborate (0.025-0.175 mg elemental boron)/kg body weight per day do not have a side effect and proved a good anti-inflammatory activity. Many studies performed have reported that calcium fructoborate interacting with other nutrients has an important role in regulating mineral metabolism, such as calcium metabolism, and implicitly, bone metabolism [5, 6]. Calcium fructoborate plays an important role in the growth and development of bone, soft tissues, the formation of antibodies and collagen [7]. Also, it promotes the absorption of calcium by helping in this way the maintenance of healthy bones and skin, as well as in the treatment of osteoporosis [8, 9].

Given these considerations, combining apatite materials (hydroxyapatite) with active principles such as calcium fructoborate may lead to new bone substitutes that successfully combine the properties of these classes of materials.

In this context, the present study was intended to create a new biocomposite system based on hydroxyapatite and calcium fructoborate with possible applications in "drug-targeted" therapy. These new biocomposites are intended to be administered as topical systems with drug's controlled release [10-12]. There is still a problem in finding to identify and develop anabolic agents in order to treat osteoporosis, which is a major public health problem nowadays. For the treatment of osteoporosis most drugs which are usually used are bone resorption inhibitors, including either bisphosphonates or estrogenic and related compounds. Nevertheless there is no prove that these types of drugs increase or restore bone mass, even if they seem to stabilize bone mass and prevent further bone loss [13]. To increase or restore bone mass of the composite based on hydroxyapatite we adopted the introduction in its structure the calcium fructoborate, as a biologically active principle [14].

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Experimental part

Materials and methods

The calcium nitrate $Ca(NO_3)_2 \cdot 4H_2O$, ammonium phosphate dibasic $(NH_4)_2HPO_4$, NaOH were purchased from Sigma-Aldrich (Germany). Calcium fructoborate $Ca[(C_6H_{10}O_6)_2B]_2 \cdot 4H_2O$ (fig. 1) was purchased from FutureCeuticals (USA). All chemicals were of analytical grade and used as received without further purification. Experiments were performed in triply distilled and deionized water.

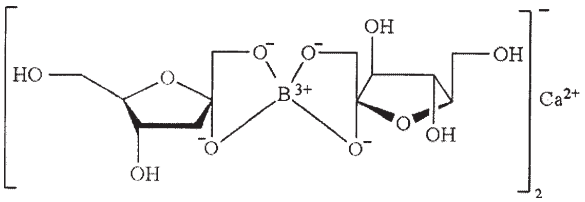

Fig. 1. Calcium fructoborate structure

Synthesis of nanohydroxyapatite

The hydroxyapatite (HA) nanoparticles were synthesized by a sol-gel method from $Ca(NO_3)_2 \cdot 4H_2O$ as calcium source and $(NH_4)_2HPO_4$ as phosphorous source. The hydroxyapatite powder was prepared adding drop-wise 250 mL $Ca(NO_3)_2 \cdot 4H_2O$ (0.01 M) aqueous solution to an appropriate amount of $(NH_4)_2HPO_4$ (0.006 M) aqueous solution to achieve predetermined Ca/P atomic ratio of 1.67, under magnetic stirring for 1 hour at 40°C. The solution was kept constant at *p*H = 10.5 by further adding small amounts of NaOH conc. The suspension was aged for 24 h and then it was filtered and washed with triply distilled water. After mixing complete, the white powder was removed from the solution by deionized water and dried at 40°C for 48 hours [15-19].

Synthesis of nanohydroxyapatite-calcium fructoborate composite.

The biocomposites was obtained with the same method like hydroxyapatite powder but at the maturation of suspension was an added calculated amount of active substance. The HA:FB molar ratio added was 1:0.25.

Characterization

The morphology and chemical composition of samples were studied by scanning electron microscopy (SEM) coupled with energy dispersive X-ray spectroscopy (EDX) with QUANTA 200 3D microscope (FEI, Netherlands). Gold sputtering was used to make the coating surfaces conductive for the SEM investigations. The coating formed on the support was characterized by X-ray diffraction (XRD) with X'PERT PRO MRD diffractometer (PANalytical, Netherlands), operated with CuKá radiation. The FTIR spectra of all samples were recorded on a DIGILAB SCIMITAR-SERIES spectrophotometer with an attenuated total reflectance (ATR) accessory.

Biological assay

Cell survival analysis

Two different culturing media were used: a basic medium (BM) and an osteogenic medium (OM) consisting of BM supplemented with 10 nM dexamethasone (Sigma-Aldrich).

The mice skeletal myoblasts C2C12 cell line can be induced under special conditions to differentiate to the osteoblasts or myoblasts. Therefore, this cell line is often used *in vitro* evaluation of biomaterials for the proliferation and differentiation of cells influenced by biomaterials. C2C12 cells (mouse C3H muscle myoblasts ECAXX 91031101) were maintained in Dulbecco's Modified Eagles Medium (DMEM, Sigma-Aldrich) supplemented with 10% fetal bovine serum (Lonza), 100 U/mL penicillin and 100 mg/mL streptomycin (Gibco). Medium was refreshed every 2-3 days. Cells were harvested at approximately 80% confluence for subculture. All experiments were performed in a 5% CO_2 humid atmosphere at 37°C.

Differentiation media

Osteogenic differentiation medium (OM) consisted of BM containing Dulbecco's Modified Eagles Medium (DMEM, Sigma-Aldrich) supplemented with 10% fetal bovine serum (Lonza), 100 U/mL penicillin and 100 mg/mL streptomycin (Gibco), 10nM dexamethasone (Sigma-Aldrich), 0.2 mM ascorbic acid (Sigma-Aldrich).

Cell differentiation experiments

For differentiation experiments, passage 7 C2C12 cells were seeded at 18.000 cells/cm^2 and allowed to adhere overnight in biomaterials. The next day, medium was changed to the experimental culture conditions. Medium was refreshed every 2-3 days.

SEM imaging

Samples were washed with phosphate buffer saline, PBS (Gibco) for 2-3 times, fixed with 10% formalin (Sigma-Aldrich) for 45 min. The fixated sample was dehydrating with ethanol gradient series (45 min in 70, 80, 90, 95, 97 and 100% ethanol). The goal of dehydrating fixated samples is to change the samples from a watery state to a waterless state. This is to prevent water to interfere in different kinds of tissue processing steps. To lose all the water which a sample or a cell layer contains the sample (or cell layer) will be taken through ethanol gradient with increasing percentages of ethanol. In this way, in each step water will be pulled out of the cells and extracellular matrix and will be replaced by ethanol. Ethanol can then easily be replaced by liquid CO_2 or embedding material for further processing of the samples.

The dehydrating fixated samples were transferred to tissue bags and further dehydrated using the CPD 030 Critical Point dryer (Balzers). Samples were gold sputtered using a 108 auto sputter coater (Cressington) at 30mA in a cycle of 50 s. Subsequently, the samples were analysed in vacuum mode by using the XL30 ESEM-FEG (Philips).

Results and discussions

The sol-gel method has been shown to be a good way to obtain nanohydroxyapatite-calcium fructoborate composites which are homogeneous and would be more favorable to biological interaction. The SEM images (fig. 2) illustrate the morphology of the samples. It can be seen

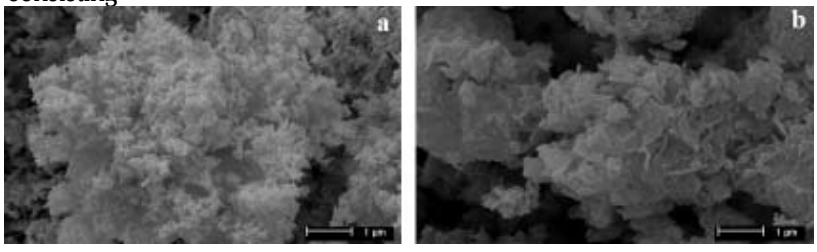

Fig. 2. SEM micrographs of the hydroxyapatite (a) and nanohydroxyapatite - calcium fructoborate biocomposite (b)

Fig. 3. Optical images of the hydroxyapatite (a) and nanohydroxyapatite - calcium fructoborate biocomposite (b)

that the hydroxyapatite powder is composed of nanosized primary particles that tend to form agglomerates with intergranular micropores. These aggregates of individual crystallites were probably formed by the coalescence of the crystals or by direct initiation at the contacting surfaces. The SEM measurements indicated that the individual nanometric particles (with an average size of about 40 nm) predominate over the particle agglomerations (with an average size of about 150 nm). The nanohydroxyapatite-calcium fructoborate composites contain agglomerates of lamellar particles (with an average size of about 250 nm), due to the calcium fructoborate presence in the composites. Based on the average size determined by SEM, the composite materials can be considered as true hybrid nanocomposites.

Optical images (fig. 3) of the hydroxyapatite and nanohydroxyapatite - calcium fructoborate biocomposite indicate that hydroxyapatite material (fig. 3a) is a powder which consists of sub-micrometer grains. In contrast, the fructoborate biocomposite exhibits coarser particles characterized by a sponge like structure (fig. 3b).

The SEM-EDX analysis was performed in order to determine the surface elemental composition of the hydroxyapatite and nanohydroxyapatite-calcium fructoborate biocomposite powders [20, 21]. Figure 4 shows the EDX spectra of hydroxyapatite (a) and nanohydroxyapatite-calcium fructoborate biocomposite (b) samples, and for the latter the characteristic peaks of boron are well evidenced. The EDX analysis confirms the presence of calcium or/and boron, phosphorous, oxygen and hydrogen in certain content. The Ca/P molar ratios were of 1.682 and 1.665 for the hydroxyapatite and nanohydroxyapatite-calcium fructoborate biocomposite, respectively, which corresponds to the stoichiometric hydroxyapatite [21].

The FTIR spectra (fig. 5) obtained for calcium fructoborate, hydroxyapatite and nanohydroxyapatite - calcium fructoborate composite provide a number of spectral details demonstrating the formation of hydroxyapatite phase [22-24]. The spectrum of the pure hydroxyapatite (HA) sample shows the bands at 1093, 1028 and 962 cm^{-1} due to the stretching mode of P"O, whereas the bands at 601, 567 and 472 cm^{-1} are due to the bending mode of O"P"O. The bands at 2146-1996 cm^{-1} are attributable to the PO_4^{3-} ions. A significant concentration of OH^- groups exists in the apatite structure as observed from the intensity of the bands at 3572 and 632 cm^{-1}. Molecular and adsorbed water bands are also noticed at 1654 and 3445 cm^{-1} (as a broader band) [25].

The IR spectrum of calcium fructoborate and nanohydroxyapatite - calcium fructoborate composite contains in the 1050-600 cm^{-1} range the vibrational bands characteristic to the specific groups of fructoborate, like: CO, CH_2OH, C–CH, CH_2, C–C and C–O–C [26].

On *biological assay* it was investigated SEM images of hydroxyapatite and nanohydroxyapatite - calcium fructoborate composite, on whose substrate C2C12 cells were cultured during 7 days (fig. 6).

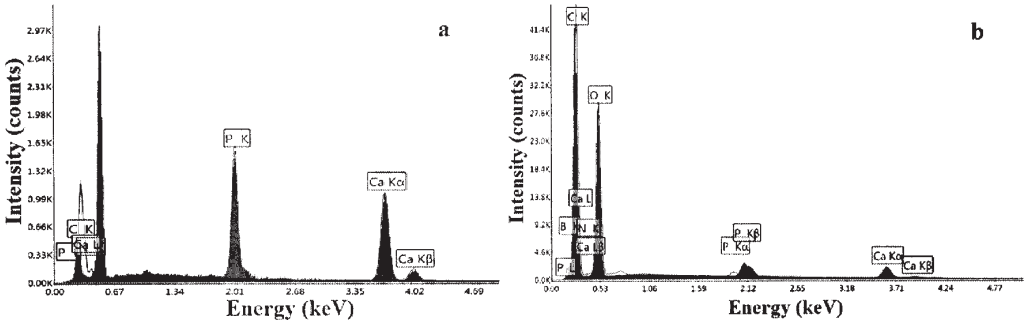

Fig. 4. EDX spectra of the hydroxyapatite (a) and nanohydroxyapatite - calcium fructoborate biocomposite (b)

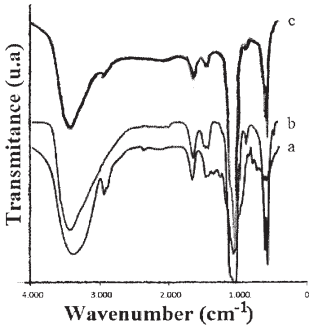

Fig. 5. FTIR spectra of the calcium fructoborate (a), hydroxyapatite (b) and nanohydroxyapatite - calcium fructoborate biocomposite (c)

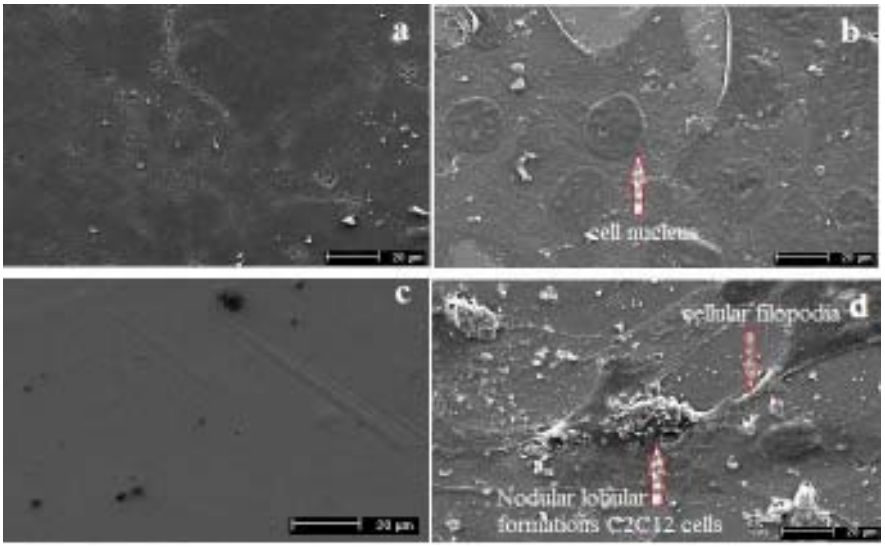

Fig. 6. SEM images of hydroxyapatite (a) and nanohydroxyapatite - calcium fructoborate composite (c) without cells culture; hydroxyapatite (b) and nanohydroxyapatite - calcium fructoborate composite (d) with C2C12 cells culture.

SEM images of the biocomposites of which surface was used to culture C2C12 cells revealed good adhesion and spreading of cells, although generally they are prone to adhere and to attach much better to rough surfaces compared to the smooth ones. There are still some differences in the degree of cell attachment according to the concentration of calcium fructoborate incorporated in biocomposite. Most of the cells grown on the surface of these biomaterials have a phenotypic morphology: osteoblasts were flattened in the form of nodular/lobular configuration, showing frills on the rear side. The cells are firmly attached to the substrate via cellular extensions (filipods).

The cells cultured on hydroxyapatite surface (HA, figure 6b) have a polygonal shape and the degree of distribution on the surface of the material is relatively low.

The cells cultured on the substrate surface of the nanohydroxyapatite - calcium fructoborate composite biocomposite (HA-FB, fig. 6d) are much better distributed on its surface, the osteoblasts being more flattened and having several cytoplasmic extensions (filipods). From the SEM images shown in Figure 6d one can see specific changes in cells morphology, namely the appearance of some globular formations - nodules which are disposed randomly on the surface of the biocomposite. It is possible that these nodular formations may contain mineral deposits of calcium (calcium carbonate) as a result of osteoblastic cell activity. This process can be accelerated as well by introduction in the hydroxyapatite's structure of fructoborate calcium. Also, it can easily notice the appearance of a uniform and dense layer of cells, which may represent the emergence of extracellular matrix generated by the activity of osteoblasts.

In vitro assays demonstrated that these new nanohydroxyapatite-calcium fructoborate biocomposites have good cell compatibility with skeletal myoblast and have potential to be used as biomaterials used in osteosynthesis, such as bone cements, dental filling materials, and resorbable materials.

Conclusions

In summary, an alternative method it was elaborated which is advanced in preparing of bioactive materials with anti-inflammatory ability for biomedical devices. New nanohydroxyapatite-calcium fructoborate biocomposites were obtained by a sol-gel method, based on the reaction between aqueous mixtures of calcium nitrate and ammonium phosphate dibasic as calcium and phosphorus precursors, respectively. Structural studies revealed the formation of biocomposites as lamellar agglomerates with intergranular micropores.

The results obtained in biological assay suggest that nanohydroxyapatite-calcium fructoborate biocomposites are potential materials which can prevent further bone loss and are promising to increase or restore bone mass, with that might be used as implantable biomaterials.

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