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NANO-HYDROXYAPATITE COMPOSITES FOR DENTAL USE

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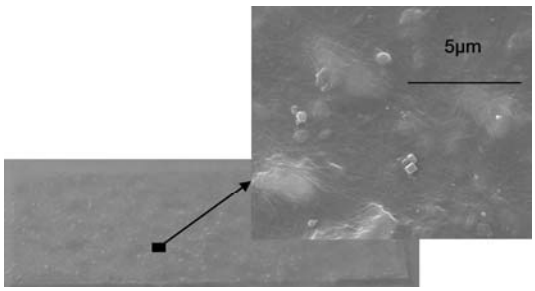
This work proposes the preparation of some nano-composites materials for dental use. The bio-resorbable materials have the potential to reduce some issues that occur with metallic implants, such as strain, shielding, and corrosion. The type I collagen was extracted from pig skin using acetic acid 0.5M and pepsin at 4°C. Hydroxyapatite was extracted from bovine bones at different temperatures with water and NaOH 0.1M, using microwave technology. The mimicked collagen-nano-hydroxyapatite and chitosan- nano-hydroxyapatite membranes were prepared by physical mixing of components in ultrasonic field at 59 kHz and 20±2°C, during 1 h. The mixtures were spread in thin layer by Dr. Blade technique. Characterization of materials was performed by transmission and scanning microscopy, X-ray diffraction, FT-IR and BET techniques. These materials should help in reducing the problems of graft rejection and drug therapy costs.



INTRODUCTION

Nowadays, many people manifest more attention for health, perfect teeth and a beautiful smile. Generally, there are some treatments for bone repairing like: patching, replacing the missing tissue using allografts or xenografts, or self - healing initiated by materials containing signal molecules for tissue remodeling.[1] In dentistry, there are many possibilities to correct some natural mistakes or to repair the defects arising after different accidents. A novel procedure consists in the use of fresh extracted teeth, grinded (Smart Dentin Grinder) and mixed with the centrifuged blood of patients (A-PRF or I-PRF), for grafting matrix - autogenous dentin particulate.[2] The main constituent of bone matrix - collagen type I can be prepared using different techniques from various raw materials.[3-5] Collagen can be purified at high levels (≥90%). Hydroxyapatite is known as the mineral component of bones. Stiff crystals are responsible for imparting an appropriate compressive strength, whereby collagen fibers, able to dissipate energy effectively, provide superior elastic properties, thus ameliorating the brittleness of the hydroxyapatite base.[6-11] Indeed,

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the bone is the most widely investigated from among all tissues in the body by tissue engineering due to its high potential for regeneration. By storing minerals within, mostly calcium and phosphate, it presents the main mineral reservoir for the body. Absorption and release of salts is the mechanism by which bones buffer the blood and prevent the excessive pH changes. Some bones like skull and ribs serve to physically protect of internal organs (brain, lungs, heart) and other act as "plants" for production of red and white blood cells.[6] The regenerative medicine and tissue engineering is a multidisciplinary research field that uses principles of chemistry, biology and engineering sciences towards growth development and regeneration of damaged tissues or organs.[11] Many research efforts were carried out in order to find substitutes for teeth and bones.[3] Hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$ is an excellent material for its biocompatibility, bioactivity and osteoconductivity and is widely used as powders, particulate or porous, and solid scaffold forms. Collagen is the most preferred protein used in tissue engineering due to the presence of several functional groups that can enhance osteoblast adhesion and migration. Chitosan is a polysaccharide produced from marine crustacean shells and is produced in forms commercially available by deacetylation of chitin. Chitosan is a cationic polymer composed of randomly distributed N-acetylglucosamine and D-glucosamine, varying in composition, sequence, and molecular chain length and has developed in diverse forms like films, fibers, foams, hydro gel, and particles for applications in bone and cartilage tissue engineering and wound healing due to excellent properties like biocompatibility, biodegradability, ability for cell ingrowths, and its intrinsic antibacterial nature.[9] Membranes with chitosan should absorb fluid of the body and should permit a good distribution of nutrients, metabolites, growth factors through extracellular media.[3] The aim of this work is the preparation of composite materials with dental use from roumanian bio-waste, bovine bones and pig skin.

RESULTS

For collagen extraction, the skin was treated using a combined method: 0.5M acetic acid and pepsin (3%) mixture solution, in different ratio, 1:8 (w:v), (step 1) and 1:5 (step 2), at 4°C, during 48h. After filtration of exhausted skin, the samples were combined and were centrifuged at 5000g, during 0.5h (Sigma 3-30KS centrifuge, Germany) for elimination of other compounds with large molecules. Then, the solution was treated under stirring with solid NaCl (final concentration ~1M). The mixture was leaved at 4°C for 12-24h. The collagen was separated by filtration and was subjected to other treatments. The obtained collagen was characterized by spectrophotometric method of hydroxyproline determination[1, 15] using a Jasco 530 UV-Vis apparatus (Abble&Jasco, Germany) and by denaturation temperature. The collagen amount was determinated indirectly from hydroxyproline content after complete hydrolyses in acidic media (5h). We used the relation:

$$m_{collagen} = [m_{HYP}/W_{HYP}] \times 100 \quad (1)$$

where: $m_{collagen}$ is amount of collagen in mg, m_{HYP} is amount of hydroxyprolyne, W_{HYP} is the percentage (13%) of hydroxyproline in collagen. Results indicated 9-9.2% hydroxyproline. The yields of extraction were small (2-2.5%). The collagen was characterized by thermal denaturation temperature. The degradation of about 50% of collagen by viscosity variation into a 0.8% solution in 0.5M acetic acid using a Fungilab SMART Series apparatus equipped with Standard Spindle R2 rotor was followed (at 100 rpm; variation of temperature was assured with a Julabo F12 Thermostat). Denaturation temperature was established at 36°C (Figure 1).

Nano-hydroxyapatite particles were obtained by extraction of hydroxyapatite from bovine bones using microwave irradiation method. Technical conditions were presented in Table 1(only the best results are presented).

The best results were obtained at 230°C and 190°C with natrium hydroxide, NaOH 0.1M as extracting agent.

Nano-hydroxyapatite particles were mixed by ultrasonic technique with collagen or chitosan, indifferent ratios, in order to obtain mimetic natural membranes for dental use. The mimetic natural membranes were characterized by scanning microscopy and the aspects of them were presented in Figure 2, 3.

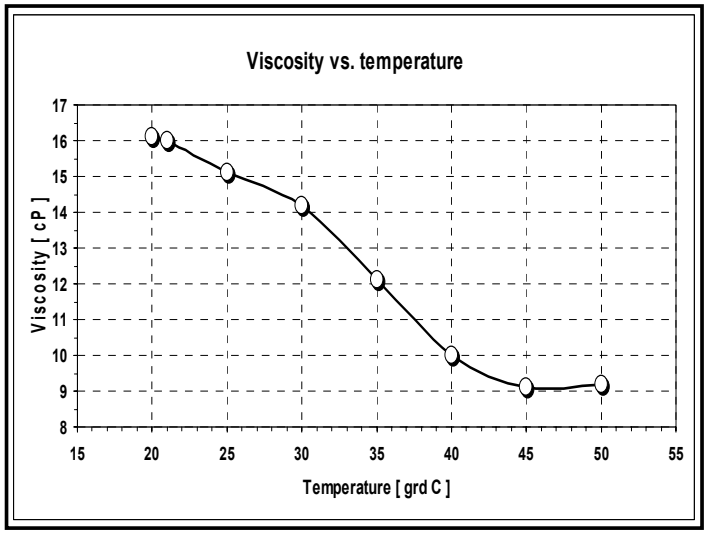


Fig. 1 – Viscosity variation vs. temperature in collagen denaturation process.

Table 1

Extraction conditions of hydroxyapatite in microwave field

Sample	Bone/ NaOH 0.1M ratio (w/v)	Temperature (°C)	Pressure (bar)	Yield (%)
Sample bone 1	1:40*	230	<40	72.45
Sample bone 2	1:40	230	<40	74.50
Sample bone 3	1:40	150	<40	65.26
Sample bone 4	1:40	190	<40	79.60

*water was used

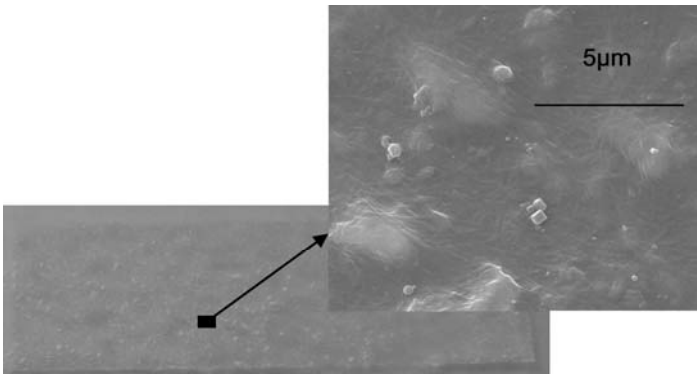


Fig. 2 – Resorbable membrane with collagen and nano-hydroxyapatite.

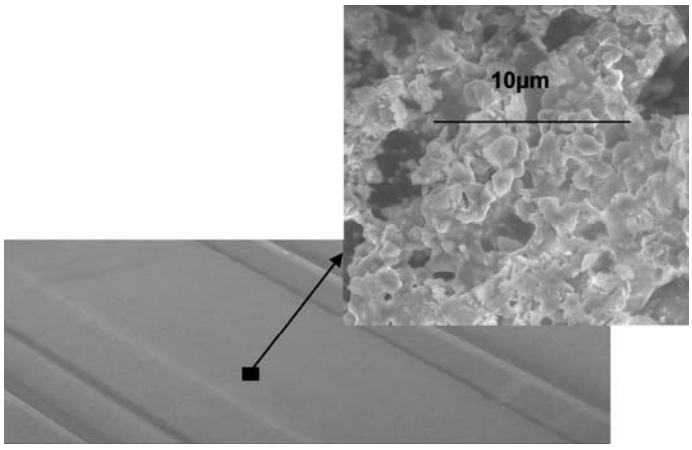


Fig. 3 – Resorbable membrane with chitosan and nano-hydroxyapatite from bones.

DISCUSSION

The structures of the nano-hydroxyapatite were characterized using XRD and FT-IR methods (Figure 4, 5). X ray diffractions were performed by X'Pert PRO MPD, PANalytical (Holland) apparatus. All the patterns were indexed as hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$ (JCPDS card no. 01-072-1243). It was observed that the increasing of temperature in the extraction process has determined the developing of (3 0 0) crystal plane, and an effect on (1 1 2) crystal plane. According to (2 1 1) crystal plane and TEM distance between planes measurement, the highest crystallinity was obtained for sample bone 2.

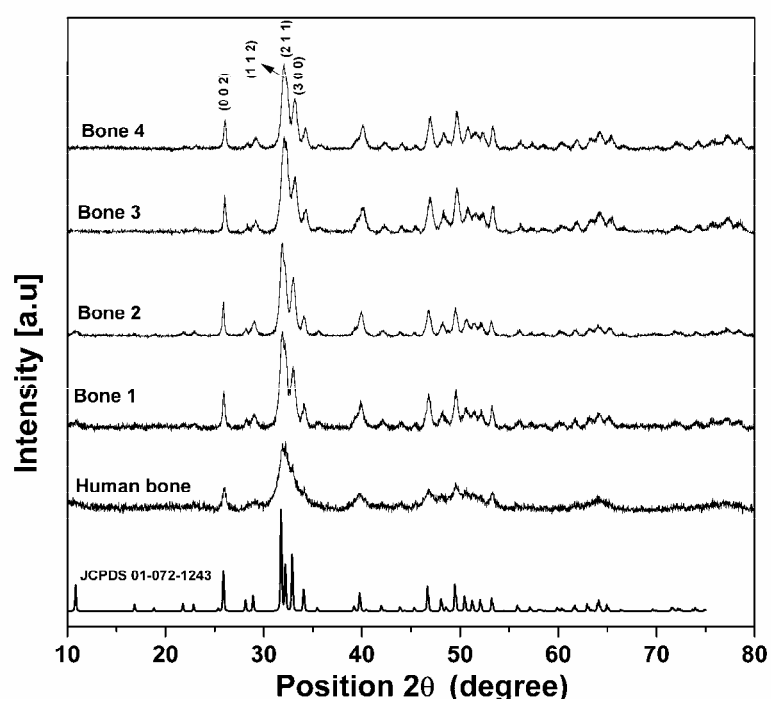


Fig. 4 – X-ray diffraction patterns of human bone and nano-hydroxyapatite obtained by microwave technology from bovine bones at different temperatures.

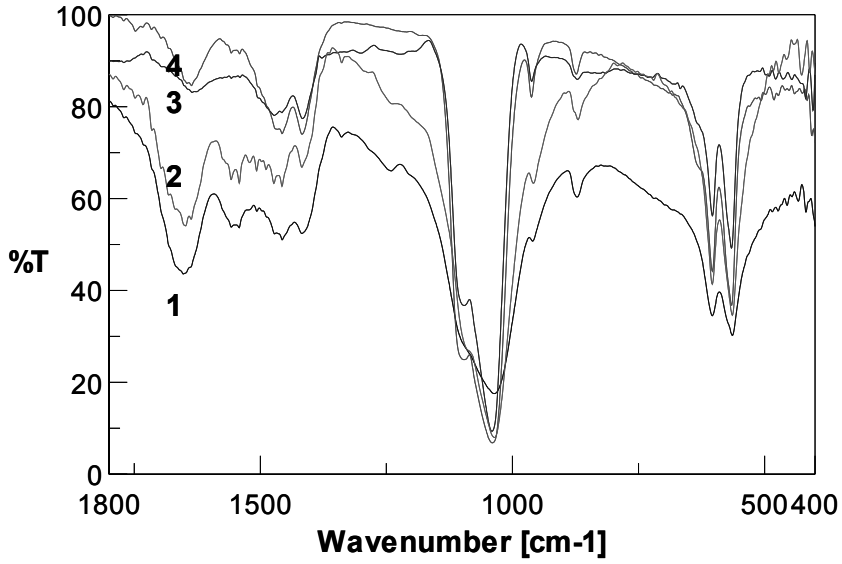
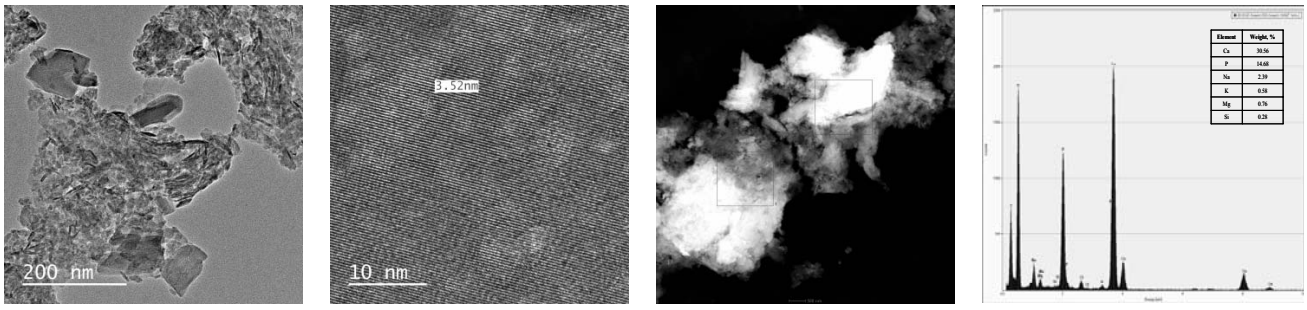


Fig. 5 – FT-IR spectra of samples: 1 - human bone; 2 - human tooth; 3 - sample bone 2 (at 230°C); 4 - sample bone 4 (at 190°C).

FT-IR spectra have been registered with a spectrophotometer Jasco FT/IR-430 at 4 cm-1 resolution and 2 mm/sec scanning speed (KBr pills). The characteristic adsorption bands for stretching (–OH) (3570-3440 cm^{-1}), stretching ν (P–O) for PO_4^{3-} (950-1100 and 374-605 cm^{-1}), bending (–OH) (1630-1650 and 635 cm^{-1}), stretching (C–O–C) (1406, 1478 cm-1), bending (P–O) for PO43- (1045 and 565 cm-1) were registered for all the samples. Figure 5 reveled the main adsorption bands on characteristic interval 1800-400 cm^{-1}, for human bone, a human tooth and samples 2 and 4, obtained by microwave NaOH 0.1M treatment at 230°C and 190°C. The structure analyses confirmed the existence of pure hydroxyapatite. Further characterization of nano-hydroxyapatite particles was realized by TEM and SEM microscopy and BET analysis.

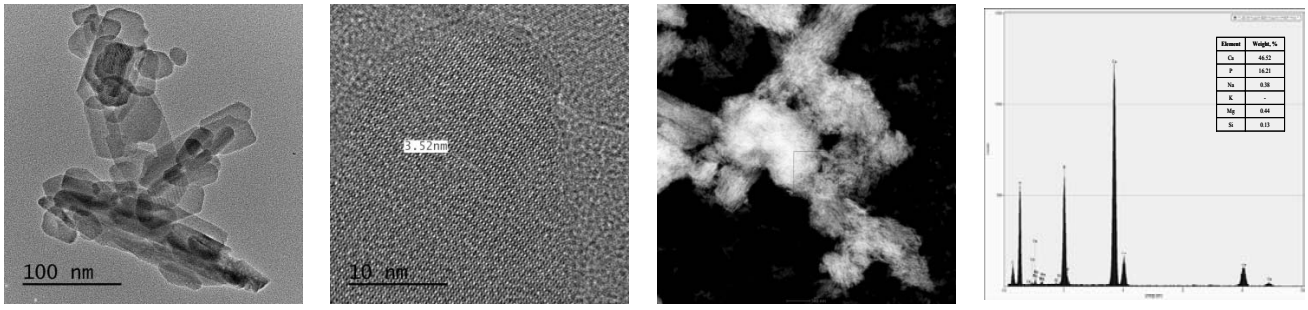
The TEM, STEM-EDX analyses for human bone and microwaving samples bone 1, 2, 3 and 4 were performed by an electron transmission microscope Titan™ G2 80-200 with ChemiSTEM™ - HRS/EDX (FEI, Holland). For TEM analyses, the samples were mixed with ethanol in ultrasonic field, using a Wisd Wise Clear apparatus (Witeg, Germany) and, then 0.2-0.3μL of suspension was putted on the grille (Carbon Coated 300 mesh Copper Grids). After the samples were fixed in the holder, they were introduced in vacuum Plasma Clear 1020 (Fischione, UK). Degassing process was finished in 40-45 min. and the holder was placed in TEM apparatus. The results were presented in figures 6, 7. Human bone was considered as standard and particles aspect, distance between planes and surface composition EDX are presented in Figure 6.

Higher temperatures of the microwave process have determined higher ratios Ca/P: 2.33 and 2.87, respectively, for samples bone 1 and bone 2. Treatment with NaOH 0.1M was very efficient and determined at 230°C a Ca/P a high ratio 2.87 and at 190°C a Ca/P ratio 2.18. Particles diameters were varied between 50-200 nm. Distance between crystalline planes was varied between 3.52 nm (standard and sample 2) and 3.57 (sample 1). At lower temperature (≤150°C) all the characteristics of the samples were unsuitable for dental purpose (data for all samples can be presented at request).



a. **b.** **c.** **d.**

Fig. 6 – TEM (a), distance between crystalline planes (b), STEM image (c) and EDX spectrum for zone 2 of human bone (Ca /P ratio is 2.08).



a. **b.** **c.** **d.**

Fig. 7 – TEM (a), distance between crystalline planes (b), STEM image (c) and EDX spectrum for sample bone 2 (Ca /P ratio is 2.87).

The BET analyses were performed on ASAP 2020 apparatus (Micromeritrics, USA). Degassing of bone samples was made in N_2 flux, at 90°C (2h) and 300°C, during 12h. The BET surface area was 71.36-77.46 m^2/g and adsorption average pore width (4V/A by BET) was about ~21.22 nm. This surface can assure possibilities for 'well soaking' of hydroxyapatite nano-material. Also, this type of analysis revealed very clearly that water is not a good extractive agent: bone 1 sample became brown after treatment, due to possible residues of fats.

On the dentistry market, different types of commercial membranes are available. The resorbable ones with the longest lasting membrane stability, easily drapes over the ridge, but not so flimsy that it collapses into defects are preferred. The high tensile strength allows membrane stabilization with sutures. Mixtures with collagen or/and chitosan were made for the obtaining some nano-composite materials like membranes with specific use in dentistry. The samples obtained were relatively elastic and were appropriate as characteristics determined *in vitro* to other commercial products. The thickness and homogenity of the films will be controled by using another technique, electrospinning.

EXPERIMENTAL

Materials

The fresh pig skin and bovine bones were achieved from a private farm from Arad County, Roumania, which developed a chain of pig and bovine products stores in Timişoara. Acetic acid, sodium hydroxide, acetone, ethanol, sodium chloride, chitosan and pepsin were purchased from Sigma-Aldrich, Germany. Standard of human bone was achieved from German Dental Clinic (Timişoara, Roumania).

Collagen

The collagen-type I was extracted from fresh pig skin.[1,3-6] The pig skin was cut into small pieces (0.5x1 cm) and was washed with 1:2 (w:v) ethanol under stirring during 2 h (magnetic stirrer Falc F60, Italy). After filtration, the skin was washed in 1:2 ratio (w:v) with 4% NaOH solution at room temperature during 2 h for noncollagenic proteins elimination. Then, the pig skin was washed with double distillated water until neutral pH. For collagen extraction the skin was treated with 0.5 M acetic acid and solution 3% pepsin at 4°C, in different ratio, step I: 1:8 (w:v) acetic acid and pepsin solution during 24h; step II: 1:5 (w:v) acetic acid and pepsin solution during 24h. Finally, the extracts were reunited. The sample was centrifuged at 5000g (EBA20 Hettich Zentrifugen, Germany), during 30 min. and then treated with NaCl powder (final NaCl concentration was 1M) for collagen precipitation. The material was kept for 24h at 4°C. Thus, obtained collagen was filtrated and washed with cold double distillated water.

Nano-hydroxyapatite

The bovine bones were boiled with water during 2 h for tendons and fats elimination. Then, they were mechanically cleaned with a knife and washed with acetone: water mixture (90:10 v/v). After this treatment, the bones were dried at 140°C during 12h. The small particles of bones for experiments were obtained by drilling. Only those with d<250 μm were used for microwave extraction with water or alkaline solution. An oven MWS-2 Berghof (1000 W) with DAP-60K TFM vessels was used.

Nano-hydroxyapatite composites/mimetic membranes

Nano-hydroxyapatite particles were mixed by ultrasonic technique with collagen or chitosan in order to obtain mimetic natural membranes for dental use. An ultrasonical bath (Falc Instruments, Italy) at 59 kHz and 20±2°C, during 60 min. was used. The mixtures were spread using Dr. Blade technique. The thickness of membranes was variable.

CONCLUSIONS

The work proposes the preparation of the collagen and nano-hydroxyapatite by extraction from by-products of food industry from Romania in order to obtain some nano-hydroxyapatite composites for developing films/membranes with dental use. The by-products from food industry, pig skin and bovine bones can be exploited to obtain superior materials used in dentistry. Nano-hydroxyapatite with special characteristics was obtained from bovine bones using the microwave technology and synthetic membranes from natural products (collagen, chitosan and nano-hydroxyapatite) were obtained. The by-products are cheap and available anytime. Collagen was extracted by a combined method: 0.5M acetic acid and pepsin 3%. The best nano-hydroxyapatite by microwave extraction was obtained at 230°C with NaOH 0.1M as extracting agent. Crystallinity of samples was increased with the increasing of process temperature. This nano-material can be used with success in mixture with A-PRF or I-PRF membranes (from patients). In order to obtain permeable natural membranes for use in dental treatment, films/membranes were realized in laboratory by Dr. Blade technique. Future experiments will approach the electrospinning technology.

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