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Preparation of hydroxyapatite/hyaluronan biomimetic nano-hybrid material for reconstruction of critical size bone defects

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Sintered bi-phase calcium phosphate material was subjected to dry high-energy milling for 40 hours to obtain crystalline nano-metric hydroxyapatite. During milling the tertiary calcium phosphate got fully amorphous. Powder XRD analysis was used for phase control and crystallite size measurements. The morphological studies were performed by SEM and TEM and showed formation of granulated agglomerates. The obtained material was additionally milled with 1% hyaluronan for times of 2, 5, and 10 hours and the initially formed agglomerates were subsequently disintegrated to form well homogenized nano-hydroxyapatite particles of a hybrid hydroxyapatite/hyaluronan material, which was used for filling of bone defects with critical sizes in cases of apical periodontites. Test clinical studies showed good healing process.

Keywords: hydroxyapatite, high-energy milling, hybrid hydroxyapatite/hyaluronan, apical periodontites.

INTRODUCTION

The development of bone–like materials with optimized function are general problems and have been developed to meet and provide promising ways to repair and replace damaged bone areas [1, 2]. As it is well known human bone is a nanocomposite with organic/inorganic structural components. In regenerative processes the extracellular matrix plays a critical role in supporting and directing bone cells adhesion, proliferation and differentiation. Therefore, in development of bone-like materials it is desirable to create biomimetic nanostructured engineering scaffolds providing structural support for initial cell adhesion [3, 4]. Therefore, recently, research has been focused towards bone-like biomaterials, which might substitute bone as a natural functional nanostructure.

In this regard, calcium orthophosphates have substantial potential due to their chemical similarity with natural bone mineral – they are biocompatible, bioactive, and biodegradable and have the ability to form a direct chemical bond with the surrounding tissues and are non-toxic, non-inflammatory and non-immunogenic agents [5–8]. Hydroxyapatite (HA) has been preferably proposed as substitute for defective bones or teeth.

Nowadays, significant research effort has been dedicated to develop inorganic nano-sized crystalline hydroxyapatite (nHA) because of crystallite size, active surface and potential biological activity [9–11]. Moreover, nHA particles will provide an increased ratio of high surface area to volume and their ultrafine structure similar to biological apatite can suitably affect their biological activity in biomedical application. Recently, HA at nano-level has playing increasing role in various biomedical applications [12, 13]. Thus, with the development of nanotechnologies, considerable efforts are focused on controlling the morphology and size of materials [14–17]. Moreover, nHA with ultrafine structure imitates the natural bone mineral as an extracellular matrix component and can cause an increased binding capacity of macromolecules, optimized bone cell–surface protein interaction, promoting the attachment and growth of osteoblast-like cells [18, 19, 20–23]

Even after extensive investigations related to bio-mineralization many intermediate stages in synthesis and preparation of nHA are still unclear.

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Therefore, various parameters that affect size, shape, and crystallinity of nHA still need to be further studied.

Biomimetic composite materials have recently drawn considerable attention [24, 25]. In investigation of critical size bone defects (intraosseous wounds of size that do not heal by natural regenerative processes) they are subjects of repair and regeneration and require substitute materials as scaffolds, to provide osteogenic processes and formation of new bone [26].

Strategies in bone tissue regeneration nowadays are related with scaffolds mimicking the natural extracellular matrix as templates for cells. Hence, of particular interest are nanocomposite and organic/inorganic hybrid biomaterials based on selective combination of biodegradable polymers and bioactive inorganic nanomaterials [27]. Generally, the focus is based on nHA as filler in combination with biodegradable natural or synthetic polymers. It was demonstrated that a HyA matrix is a promising biomimetic material [28, 29]. These studies suggest that HyA-containing scaffold materials are efficacious in bone repairing. HyA is well known as hyaluronan or hyaluronate (Na salts of hyaluronic acid) [30], being linear glycosaminoglycan polysaccharide consisting of repeated disaccharide units of negatively charged high–molecular–weight linear polymer, with repeated unites of N-acetyl glucosamine and glucuronic acid and in water they form three dimensional (3D) network [31]. The long chains of HyA macromolecules with a large number of carboxyl, hydroxyl and amino groups have a strong capacity to bind various ions [32]. As a consequence of this specificity they are ideal network of mineralizing template [33]. Furthermore, through HyA complex interactions with extracellular matrix components and cell surfaces regulation of cellular behavior controlling the tissue macro and micro environments occurs [34] and could induce osteoblast differentiation and bone formation [35]. In presence of endotoxins hyaluronic acid is produced by fibroblasts and is a potential anti-inflammatory agent [36]. The anti-inflammatory effect may be due to the action of HyA as a scavenger draining prostaglandins, metalloproteinases and other bioactive molecules [37, 38, 39]. Additionally, HyA is essential component of the periodontal ligament and plays role in cell adhesion, migration and differentiation modulated binding proteins and cell–surface receptors such as CD44 [40] and is a significant factor in growth, development and repair of tissue [41, 42]. Relating to the unique properties of HyA it is possible that nano-hybrid with hydroxyapatite can produce more effective bone filler-like materials that provide the necessary features for more efficient healing of bone defects.

The choice of hyaluronic acid (HyA) is chiefly based upon its natural abundance in soft tissues and high chemical affinity with hydroxyapatite (HA) and the ability to enhance anisotropic growth in bio-mineralization [43]. Moreover, hyaluronic acid based materials have been intensively investigated for tissue engineering repair of bone, cartilage and in reconstructive dentistry [44–46]

In oral and maxillofacial surgery, bone defects are always challenging problems for reconstructive orthopedic craniofacial procedures, and various treatments have been proposed in this consideration [47, 48]. Synthetic bone grafts are among the promising approaches in this field. It is of interest to refer clinical results for Endodontic Orthograde treatment of apical bone defect in chronical apical periodontal lesions (49–52).

As a result of all above mentioned about the biological activity of nHA, HyA and composites of HyA/nHA, we hypothesized that it will be of theoretical, practical and biomedical clinical interest to prepare nHA, and nano hybrid of HyA/nHA and to be tested as new filling materials in endodontics treatment of critical size apical bone defects.

The purpose of the present study is to prepare nano–hybrid of nano-hydroxyapatite (nHAp) and hyaluronan (Na salt of Hyaluronic acid – NyA-Na) in varying ratio of nHAp/HyA in attempt to optimize their physical-chemical and mechanical properties, and to be used as a test filling material for orthograde endodontic treatments.

MATERIALS AND METHODS

Nano hydroxyapatite was prepared by top down method using high energy milling process of the sintered bi-phasic calcium phosphate. The obtained material was used to prepare nano-hybrid of nHA/HyA.

1. Synthesis and methodology

Biphasic calcium phosphate was prepared by sintering of precursor precipitate sample obtained by chemical wet precipitation method in a typical process by double decomposition of 1M calcium nitrate solution (prepared from $Ca(NO_3)_2x4H_2O$) in de-ionized water and di-ammonium hydrogen phosphate (prepared with $(NH_4)_2HP0_4$, 0.6 M), in stoichiometric ratios for hydroxyapatite Ca/P ~1.67 under controlled temperature and pH = 10.5–11 conditions [56] (the used salts of calcium nitrate and ammonium hydrogen phosphate are of analytical grade Merck). However, this method has some inherent disadvantage such as difficulty to control the pH value over pH = 9, to avoid the formation of

Ca-deficient HA, which during the sintering forms tertiary calcium phosphate (TCP).

At the end of the maturation for period of 24 h the precipitate was centrifuged, filtered and washed 3 times with de-ionized water. The resulting crystalline apatite was dried at 100 °C for 10 h to use as a starting material. Afterwards, this material was sintered stepwise at 1100 °C for 1 h. at a heating rate 50 °C min^{-1}. It is well known that depending on the degree of Ca deficiency of the precipitate the molar composition of the resulting bi-phase composition of TCP/HA can vary [53, 54] and for these reasons, we have characterized the processes in a step-like manner to follow calcination in the interval 800–1100 °C (more details are presented in our work [56]).

The obtained sintered material subsequently was dry milled in agate planetary ball mill (‘pulverisette’6 Fritsch) at 600 rev/min, working in a programmed manner with agate bolls (10 mm diameter) in standard mass of 30 g of sample. Nanocrystalline HA (nHA) was obtained after 40 h milling and was used to prepare nano-hybrid from HyA-Na solution in different ratios of HA/HyA-Na, as it is described below.

2. Characterization of the nano-sized apatite material

The concentration of calcium in the sintered material was determined potentiometrically and by EDTA complex-metrically titration in the presence of Cu EDTA at pH = 10, using a Cu ion-selective electrode and Ag/AgCl reference electrode. The concentration of P-PO_4^{3-} was measured spectrophotometrically by NOVA 60 equipment, using Merck and Spectroquant® test kits.

For SEM study, nano-metric materials were suspended in water, transferred on specimen stubs, dried and then coated with conductive material (Au) for observation in SEM JEOL JSM-5510 at accelerating voltage of 10 kV and electron beam current of 2–20 nA to reveal the textural details of nano-metric agglomerations after dry milling for 40 hours.

For TEM study, nano-metric materials were suspended in de-ionized water, sonicated for 15 min and then transferred on formvar coated TEM grids and observed in TEM JEOL JEM-2100 at 200 kV.

Powder nano-metric samples were ground in an agate mortar for 10 min, suspended in de-ionized water and sonicated for 15 min. After allowing setting for 20 min the supernatant was collected and was collected and redropped on formvar coated grids and observed in TEM JEOL JEM-2100 at 200 kV.

The crystalline phases in all samples were determined by powder X-ray diffraction (XRD) with a Bruker D8 diffractometer, operating at 40 kV and 40 mA with Cu Ka radiation. Scans were performed over the range of 2Θ 10–80° (step size 0.01°, counting time 1 s). The crystallite sizes were determined using the Scherrer equation, $D = K\ \lambda/(\beta_{1/2} \cos \Theta)$, where D is the crystal size in nanometers, K is the Scherrer constant, λ is the x-ray wavelength, and β is the experimental full-width at half-maximum intensity of the diffraction measured peak.

3. Preparation of nano-hybrid HA/HyA-Na

Nano hybrid samples prepared from 40h milled HA and HyA in ratio 3:1 were subjected to second wet milling for 2 and 5 h and then characterized by powder XRD for phase content and crystallite sizes.

Then, hyaluronan with 1% concentration (denoted as L) was mixed with nHA milled material (denoted as S) in different experimentally chosen ratios S/L from 0.2 to 3.3. From the obtained mixed materials, as dense pastes there were molded samples in rubber-molds with diameter of 10 mm and height of 5 mm, dried in air for 24 h at room temperature. The initial and final setting times of the nano hybrid material prepared in the rubber-molds were determined by the Vicar Needle method (ASTM C191-9 1993). The formation features are shown in Table 1.

Table 1. Formation mechanisms of the prepared nano hybrid materials

Solid to liquid ratio	Liquid phase	Formation features	
		Initial setting time, min	Final setting time, min
3.3	HyA/HA	6	60
2.8	Hya/HA	15	60
2.0	HyA/HA	20	60
1.4	HyA.HA	27	60

4. Micro environmental tested behavior of hybrid HA/HyA

The prepared samples of hybrid **HA/HyA** were used for testing their environmental behavior. The air-dried solid samples of HA/HyA were placed in distilled water and in simulated body fluid (SBF) (prepared according to Kukubo, 1990). The solid samples taken out from the rubber-molds were air-dried for 24 h, and then were immersed in a solution at ratio of 1:20 S/L. All experiments were conducted at room temperature under static conditions. The changing values of pH in the solution were kinetically followed for a period of 30 h at room temperature. The same procedure was used in the case of distilled water.

5. Test-clinical studies

For testing biogenic repair capability of the obtained nano-hybrid of nHA/HyA through healing processes on the critical size bone defects there were carried out chronical periodontal bone lesions on clinical compromised teeth with periapical lesions and ill prognosis. Orthograde root canal filling process was used. Clinical treatment was carried out after obtaining the patient's consent to the guidelines of ethical committee of Faculty of Dental Medicine of the Medical University of Sofia.

Preparation of nano-hybrid of HA/HyA for clinical use was obtained by mechanical mixing in ratio 4:1 of nHA/HyA. (The nano-HA was autoclave sterilized and the used HyA is a pharmaceutical product for injection application).

RESULTS AND DISCUSSION

Synthesis and modification

The synthesis procedure and reaction conditions strongly influence the type of the precipitated calcium phosphate phases and the morphology, size and specific surface area of the particles [57–60].

The elemental chemical analysis of the obtained material gave for calcium and phosphate the ratio of Ca/P 1.63.

The morphological characterization of the obtained nHA material after 40 h milling showed continuous decrease of particle sizes until some lower size limit when many defects in the crystal lattice are accumulated and the phase of TCP is almost fully transformed in amorphous phase, as it was shown earlier in our work [56].

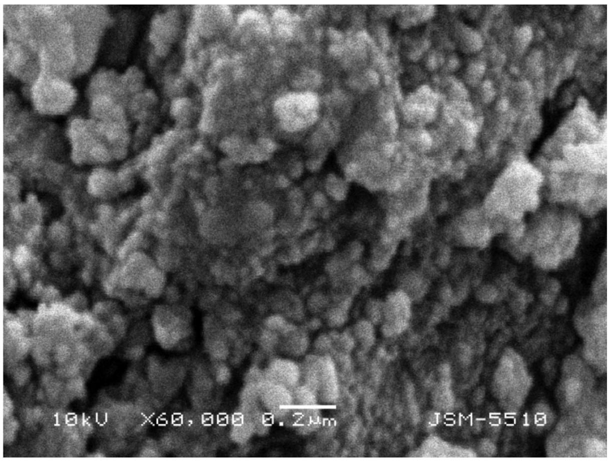

Fig. 1. SEM image of nHA milled for 40 hours. Bar – 0.2 µm.

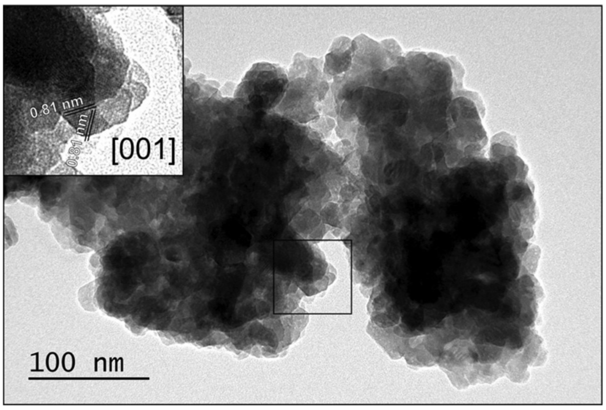

Fig. 2. TEM bright-filed image of nHA aggregates (after milling for 40 hours). The insert shows enlarged area marked in the image center with indicated d-spacing typical for the zone [001] of apatite.

Scanning and transmission electron microscopy (SEM, TEM)

The SEM study of the sample after 40 hour milling showed the presence of 20–100 nm nanoparticles self-organized into aggregates with size 200–800 nm (Fig. 1). The aggregates are randomly shaped being occasionally connected with each other to form much larger and porous agglomerates. These agglomerates most probably are secondary assemblies being formed due to the preparation procedure for SEM study.

The sonication procedure used for the TEM sample preparation allows to substantially exclude the secondary aggregation and to observe the actual microstructure of the milled material. This case is well illustrated in Fig. 2 where two steady aggregates with size 300×500 and 200×300 nm are shown. The aggregates consist mainly of platy (thin) hexagonally shaped nanocrystals with size 10–30 nm. The most developed face of the crystals is {001}. The insert on the left-top part of the figure shows an enlarged fragment from the central part of the image (delineated as square) where the hexagonal nHA crystal demonstrates two-dimensional lattice fringes (high-resolution image) corresponding to d-spacings equal to 0.81 nm and angle of 60°, which is typical for the zone [001] of apatite. A part of nHA is presented by slightly elongated platy nanocrystals with the aspect ratio close to 2 and the length varying between 30 and 60 nm. The crystal structure of the studied in TEM nanocrystals is not perfect. This fact is well demonstrated in Fig. 3, where high-resolution image of defect and comparatively large HA nanocrystal in zone [010] is shown. It is well visible that the two-dimensional lattice fringes are characterized by different contrast and clarity.

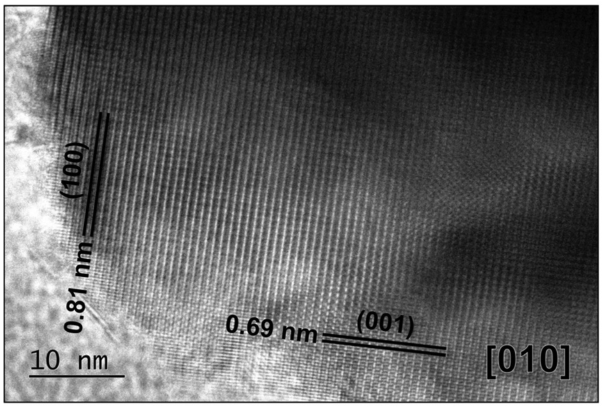

Fig. 3. HRTEM image of defect HA nanocrystal whose [010] zone axis is parallel to the electron beam. d-spacing 0.81 nm for (100) and 0.69 nm for (001) typical for the [010] zone of apatite are drawn in the image. Defect structure of the nanocrystal is characterized by the presence slightly oriented domains with size of about 10 nm.

In some places of the image, the two-dimensional lattice is transformed into one-dimensional lattice. There are light and dark areas. All these data indicate the presence of differently oriented domains constructing the nanocrystal.

Powder X-ray diffraction

The prepared nano-apatite by stepwise dry milling process for 40 h and the hybrid material of nano-HA/HyA was subject to powder X-ray diffraction analysis. In the model studies hyaluronan was used as crystal modifier controlling shape and size. The results from powder XRD analysis are shown on the Figure 4.

The crystallite size determination gave about 70 nm for the as-syntesized sample and about 30 nm for the milled sample for 40 h, which value is preserved for the mixed HA-HyA sample milled for 5 hours.

The lowest pattern (Fig. 4a) shows presence of major hydroxyapatite and whitlockite (TCP phase) in the as-synthesized ceramic. After milling for 40 h the phase TCP gets amorphous (Fig. 4b). It can be stated that the mixed HA-HyA sample after additional milling for 5 hours contains three components – nano-crystalline (HA), amorphous (TCP) and HyA (hyaluronan) (Fig. 4c). Possible disaggregation due to hyaluronan addition is expected and can e registered by granulometric methods in future.

Micro environmental behavior of samples

The obtained series of nano-materials from the tested ratios (Table 1) were molded in rubber-molds were used for testing their microenvironment behavior and mechanochemical properties.

The micro environmental behavior of the test air dried samples were studied in distilled water

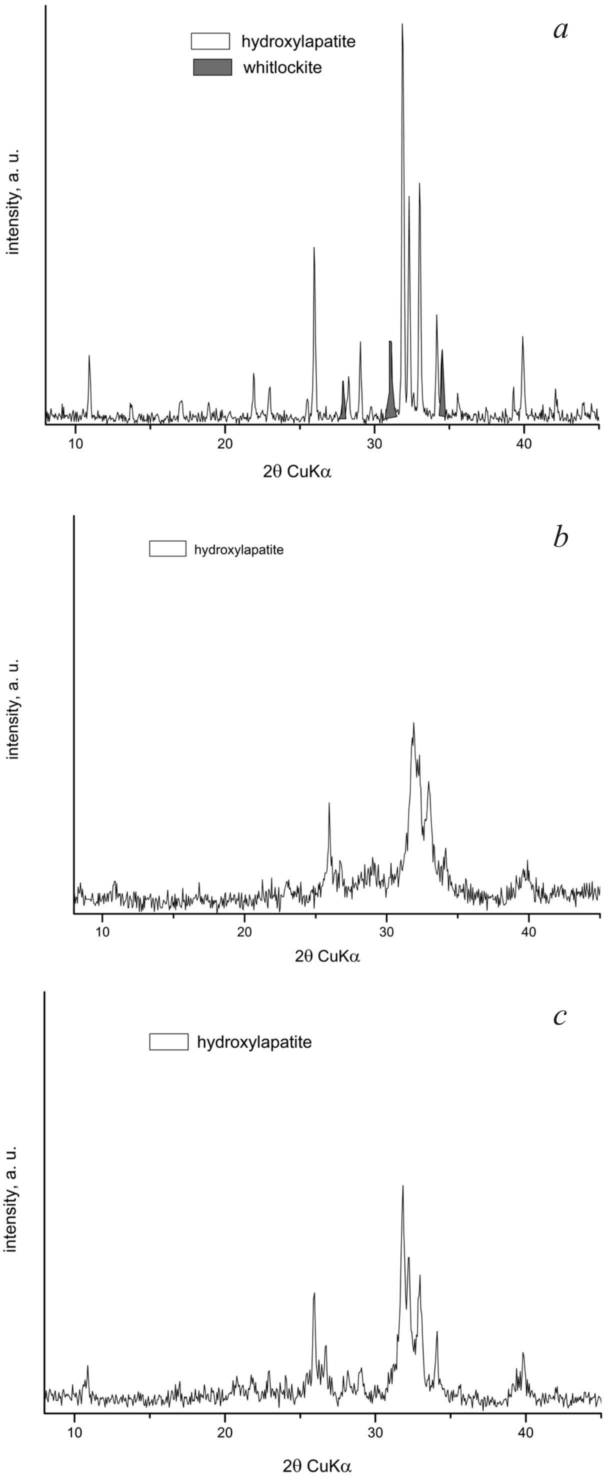

Fig. 4. Powder XRD patterns of the used materials: *a*, XRD pattern of as-synthesized Ca-P ceramics; *b*, XRD pattern of Ca-P ceramic milled for 40 h; *c*, XRD pattern of Ca-P ceramic milled for 5 h with hyaluronan.

with pH = 6.9 and in simulated body fluid (SBF) prepared according to Kokubo (1990) at pH = 7.2–7.4. The test samples were immersed in liquid and the pH value of the liquid phase was measured for a period of 30h. The results revealed that the finer the powder the higher is the amount of the liquid needed for preparation of plastic material that remains compact after drying (solid to liquid ratio of 3.3). The results from the *in vitro* micro environmental behavior testing of compact dried sample moulds in SBF and in distilled water are presented on Fig. 5.

The results of the microenvironmental behavior show the tendency for keeping pH values in the interval 8.4–9.2 for the entire studied time.These values of pH are relevant to biological reactions and bio-compatibility.

Test clinical studies – a case report

A 37 years old female was referred for endodontic retreatment on tooth #36. The radiographic examination revealed a tooth with unsatisfied endodontic treatment and large periapical lesion – PAI5 (critical size bone defect). The patient felt pain on percussion. The endodontic treatment plan included the removal of old root canal filling, precise inspection and preparation of apical zone into the canals. The canals were prepared using ProTaper Universal instruments (Dentsply Maillefer) and classic endodontic irrigating protocol – 2.5% NaOCl, 17% EDTA and distilled water. Final preparation of the endodontic space with apical bone lesion was filled with hybrid HA/HyA and after that was obturated with bioceramic sealer TotalFill (FKG, Switzerland) and gutta-percha.

It is know [62] that hyaluronic acid and synthetic calcium phosphate have osteoconductive and biodegradable properties and that was confirmed bu our presented clinical case (Fig. 6).

SUMMARY OF THE OBTAINED RESULTS

The most commonly used technique for the formation of HA is the precipitation of precursors, involving wet chemical reaction between calcium and phosphate salts under controlled pH and temperature. Having in mind the complexity of the processes involved in the precipitation synthesis it is obvious that understanding the mechanism in the individual history of the samples used is an intriguing task.

This work confirmed that synthesis procedures and reaction conditions strongly influence the char-

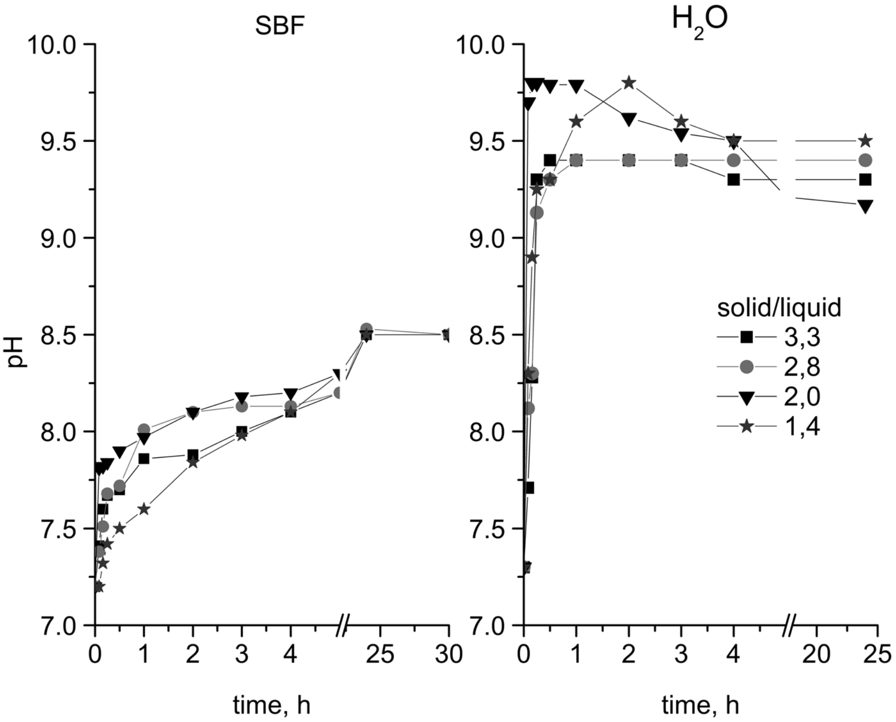

Fig. 5. pH changes in SBF and in distilled water at *in vitro* testing of molds of CaP nano-crystalline particles with sodium hyaluronate (solid/liquid 3.3; SBF pH = 7.2–7.4; water pH = 7).

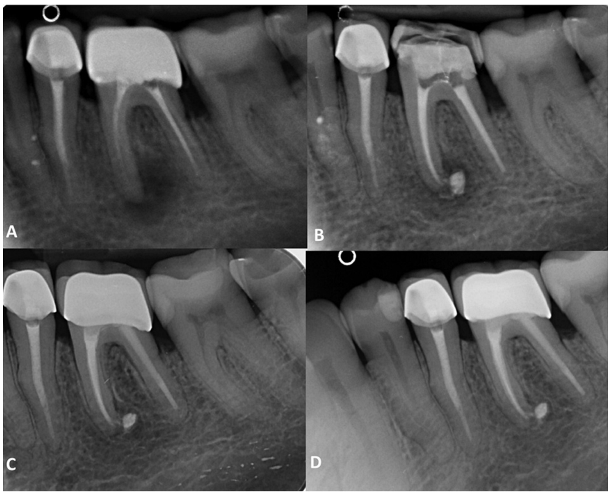

Fig. 6. *A*, Diagnostic radiograph of tooth #36; *B*, Control radiograph after definitive root canals obturation; *C*, Control radiograph after 6 months follow up period; *D*, Control radiograph after 12 months follow up period.

acteristics of nanoparticles obtained by top-down milling processes – their particles morphology, size and specific surface area, etc. [55–61].

In these contexts interesting features of the mechanical high energy milling processes is to be used for top-down preparation of nanoscale hydroxyapatites with particle and crystallite sizes relevant to the bio mineralized structures. As a result, the milling process is one of the pathways of choice to prepare nano-sized bone substituted material. There is a great clinical need for advanced biomaterials with enhanced functionality in order to improve quality of life of patients and to reduce the healthcare burden of a global aging population.

Furthermore, the various parameters that affect size and shape of crystallinity of nano scale HA (1–100 nm) still need further study. We hypothesize that this material has higher potential to be used as bone building blocks in repair processes and probably to be self-organized matrix with biological activity.

Another problem posed the results from wet milling of nano-HA with HYA-Na solution receiving materials with nano-particles of 20–25 nm or smaller. These results also would be subjects of more detailed future experimental studies and crystallographical analysis.

The vantage point to prepare nanocrystals is a great challenge maximizing in vivo targeting efficiency. Applications of macromolecules for functionalization of mineral nano-particles have been widely investigated and it was proven to be effective for bone repair strategy.

The obtained results are related to the new challenges in 21 century of endodontics studies on the clinical application of new materials that can adopt to ever-changing microenvironment of the root canal system (composite-like HA/HYA materia). Our test-clinical study in the cases of apical bone lesions as critical bone size defects show healing with nano-hydroxyapatite as an orthogonal filling material.

CONCLUSION

In this study a series of nano-hybrid materials of NyA/nHA with nano-hydroxyapatite and hyaluronan (sodium HyA) in different chosen experimental ratios of the components were investigated. The obtained material was chemically, morphologically and structurally studied. The composition of the liquid phase and the solid/liquid ratio on the formation features and mechanical strength of the materials were investigated. It was found that high energy milling stimulates the binding capacity of the received nano-material. The performed studies revealed that the obtained nano-hydroxyapatite by high-energy milling is with crystallite size about 30 nm. SEM and TEM investigations show aggregates of nHA crystallites. Hybrid material of nHA and 1% hyalorunan additionally milled was applied in test treatment of bone defects with critical dimensions, which lead to good clinical results.

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ПОЛУЧАВАНЕ НА ХИДРОХИЛАПАТИТ/ХИАЛУРОНОВ БИОМИМЕТИЧЕН НАНОХИБРИДЕН МАТЕРИАЛ ЗА РЕКОНСТРУКЦИЯ НА КОСТНИ ДЕФЕКТИ С КРИТИЧЕН РАЗМЕР

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(Резюме)

Синтерован бифазна калциевофосфатен материал е подложен на високоенергийно смилане в продължение на 40 часа за получаване на нанокристален хидроксилапатит. По време на смилането трикалциевият фосфат напълно се аморфизира. Прахов рентгенов анализ е използван за фазов контрол и определяне на размера на кристалитите. Морфоложките изследвания са направени със СЕМ и ТЕМ анализи, които показаха формирането на агломерати от гранули. Полученият материал беше допълнително млян с 1% хиалурон за времена 2, 5, и 10 часа, при което първоначалните агломерати бяха дезинтегрирани до формиране на добре хомогенизирани нанохидроксилапатитови частици в хибриден хидроксилапатит/хиалуронов материал, който беше използван за запълване на костни дефекти с критичен размер в случаите на апикални пародонтити. Тестовите клинични изследвания показаха добър лечебен процес.