Society For Biomaterials

Synthesis, characterization, and biological properties of composites of hydroxyapatite and hexagonal boron nitride

Semra Unal,[1,2] Nazmi Ekren,[2,3] Ahmet Z. Sengil,[2,4] Faik N. Oktar,[1,2] Ster Irmak,[5] Ozlem Oral,[6] Yesim M. Sahin,[7] Osman Kilic,[2,8] Simeon Agathopoulos,[9] Oguzhan Gunduz[2,10]

[1]Department of Bioengineering, Faculty of Engineering, Marmara University, Istanbul, Turkey
[2]Advanced Nanomaterials Research Laboratory, Marmara University, Istanbul, Turkey
[3]Department of Electrical and Electronic Engineering, Faculty of Technology, Marmara University, Istanbul, Turkey
[4]Department of Medical Microbiology, School of Medicine, Medipol University, Istanbul, Turkey
[5]Department of Nutrition and Dietetics, School of Health Sciences, Istanbul Bilgi University, Istanbul, Turkey
[6]Sabanci University Nanotechnology Research and Application Center, EFSUN-Center of Excellence for Functional Surfaces and Interfaces for Nano Diagnostic, Istanbul, Turkey
[7]Department of Biomedical Engineering, Istanbul Arel University, Istanbul, Turkey
[8]Department of Electrical and Electronic Engineering, Faculty of Engineering, Marmara University, Istanbul, Turkey
[9]Department of Materials Science and Engineering, University of Ioannina, Ioannina, Greece
[10]Department of Metallurgy and Materials Engineering, Faculty of Technology, Marmara University, Istanbul, Turkey

Received 1 July 2017; revised 29 September 2017; accepted 16 October 2017
Published online 23 November 2017 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/jbm.b.34046

Abstract:: Hydroxyapatite (HA), obtained from bovine bones, was successfully reinforced with hexagonal boron nitrite (h-BN). h-BN/HA composites, with BN content up to 1.5 wt %, were sintered at various temperatures between 1000 and 1300°C, in air. Well-sintered samples were obtained after sintering at 1200 and 1300°C. The presence of h-BN contributed to dense, fine, and well-crystallized microstructure. The results of X-ray diffraction analysis and FT-IR spectroscopy showed that the produced composites comprised biphasic β-TCP/HCA (HCA: carbonate partially substituted HA). High values of mechanical properties were achieved, namely compression strength 155 MPa for the sample 0.5% h-BN/HA and Vickers microhardness of 716 HV for the samples 1.5% h-BN/HA, both sintered at 1300°C. U2OS human bone osteosarcoma proliferation and cell viability showed no adverse effect in the presence of h-BN/HA, suggesting the potential use of the produced materials as safe biomaterials in bone tissue engineering. © 2017 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 106B:2384–2392, 2018.

Key Words: composites, hydroxyapatite, bovine bone, hexagonal boron nitride, bioceramic

How to cite this article: Unal, S, Ekren, N, Sengil, AZ, Oktar, FN, Irmak, S, Oral, O, Sahin, YM, Kilic, O, Agathopoulos, S, Gunduz, O 2018. Synthesis, characterization, and biological properties of composites of hydroxyapatite and hexagonal boron nitride. J Biomed Mater Res Part B 2018:106B:2384–2392.

INTRODUCTION

On a dry-weight basis, the bone consists of 65–70% hydroxyapatite and 30–35% organic compounds. Collagen is the main organic compound present in the natural bone (95%); there are also other organic compounds in small concentrations, such as chondroitin sulphate, keratin sulphate, and lipids (e.g., phospholipids, triglycerides, fatty acids, and cholesterol).[1] Hydroxyapatite (HA; $Ca_{10}(PO_4)_6(OH)_2$) is derived either from natural or synthetic sources. It is regarded as a bioactive substance and is recognized as a good bone graft material because it spontaneously forms a strong chemical bond with host bone tissues, which is attributed to its chemical composition and crystalline structure that is similar to the bone mineral. HA is not only bioactive but also osteoconductive, nontoxic, and nonimmunogenic.[2]

These attractive properties resulted in HA being widely used in dental, maxillofacial, and orthopedic surgery as a scaffolding material to repair damaged parts of the human skeleton and deter any further damage from happening. HA bioceramics generally feature biodegradability, simplicity of synthesis, and the ability to achieve highly controlled morphologies. Moreover, nanosized HA are proposed to be used as delivery vehicles for proteins, antibiotics, drugs, radioisotopes, genes, and even antigens for vaccines and anticancer medicines for human hepatocellular carcinoma cell, ovarian carcinoma cell, cervical cancer HeLa cell, anthropogenic urinary tumor cells, and leukemia cells. The biocompatibility of HA materials is undeniable, which is reflected in their wide applications in clinical medicine, such as bone reconstruction.[3]

Correspondence to: O. Gunduz; e-mail: ucemogu@ucl.ac.uk
Contract grant sponsor: Marmara University; contract grant number: FEN-C-YLP-110117–0021

Nowadays, the modern aspects of medicine, where the focus is gradually shifted from the chemically synthetic biomaterials to materials which pay careful attention to the Mother Nature guidelines, such as tissue engineering, have taken a great interest in the production of bioceramics of naturally derived HA rather than in synthetic HA. The former type of biologically derived HA is an attractive alternative, which can be produced by calcination of biowastes, for example, fish bones, bovine bones and teeth, and pig bones. Moreover, the production of HA from biowastes is more economical and an environmentally friendly technology.[4]

Nevertheless, the intrinsic weak mechanical properties of HA limit its use in load bearing applications. One way to overcome this limitation is to decrease the grain size and to achieve high density. To achieve these goals, only a few sintering temperatures have been used, specifically 1000, 1100, 1200, and 1300°C. The addition of a secondary phase can also favor sintering behavior and improve the densification of the resultant sintered HA materials.[5]

However, in biomaterials, the doping of HA matrix with a secondary phase might also be proposed to potentially satisfy another purpose. More specifically, in addition to the restrictions mentioned above related to the poor mechanical properties, in the biomedical field, postsurgical infection arising from the implanted synthetic biomaterials reveals one more problem of these types of implants. Such infections often cause severe pain, bone tissue loss and eventually require implant removal, which consequently raises morbidity.[6] As the use of antibiotics is limited to prevention and treatment of these infections, as they might lead to highly resistant bacteria, alternative antibacterial composite materials are investigated to overcome this big problem encountered currently. There are many studies which have reported alternative chemical elements, for example, cerium (Ce), copper (Cu), zinc (Zn), silver (Ag), or gold (Au), in the form of nanoparticles (NPs), introduced into biomaterials to achieve antimicrobial effects.[7,8] In particular, Ag-NPs with various morphologies are very popular because of their strong inhibition effect in bacterial growth.[8]x

This study focuses its interest on the addition of hexagonal boron nitride (h-BN) in HA matrix, as a material that can favor sintering of HA and also have a beneficial biological effect, which should potentially suppress the postsurgical infection. h-BN low-dimensional materials are among the most promising inorganic nanosystems explored so far. h-BN is highly thermo-conductive and mechanically strong due to hexagonal layers that are bound to each other with strong covalent bonds and weak Van der Waals forces. Boron nitride (BN) molecule contains an equal number of boron (B) and nitrogen (N) atoms. BN is isostructural to carbon (C) and the layered structure in its hexagonal form (h-BN) is similar to graphite.[9] However, unlike graphite, BN is a very good electrical insulator. h-BN has a high thermal conductivity and good thermal shock resistance. Si_3N_4 ceramics increase their thermal shock resistance when BN is added to them, which is attributed to microcracks that exist between the basal planes in h-BN.[10] BN does not (naturally) exist in the nature but it is produced synthetically. Pure BN material, which contains no binders, is used at extreme temperatures and when purity is important. BN is stable and inert in reducing atmospheres up to 2000°C, and in oxidizing atmospheres up to 850°C.[11]

In recent years, BN is also commonly used as functional filler in a wide variety of cosmetic applications. In this case, the small particle size is important because a high surface area is provided, which increases the amount of encapsulated cosmetics actives. The excellent performance in the dispersion and the nontoxic, transparent, and chemically inert qualities make possible and advantageous the use of h-BN in cosmetics.[12] Furthermore, the use of h-BN is also proposed in nanomedicine on account of the good biocompatibility and biodegradation properties.

It is worth noting that new generations of semiconducting materials with adjustable band gap have been produced through doping of other elements into BN structures. Nanostructures through surface functionalization of BN nanomaterials with nanoparticles of other materials have also been utilized at manufacturing hybrids. These advanced BN materials can also be considered for uses in biomedicine. For instance, functionalized BN nanotubes (BNNTs) have been tested in vitro on fibroblast cells, and demonstrated optimal cytocompatibility even at high concentrations in the culture medium.[13]

In literature, there are few studies on the microstructure and the mechanical properties of HA–boron composites, but there is poor documentation on the reinforcement of HA with h-BN.[13,14] To investigate the potential of such composites in orthopedic applications, where mechanically strong and well-sintered materials are required, in this study, h-BN/HA composites were sintered and their structural features and mechanical properties were experimentally measured. The biocompatibility of the produced composites using human bone osteosarcoma cells (U2OS) and their antibacterial activity against *Enterococcus faecalis* were also evaluated.

MATERIALS AND EXPERIMENTAL PROCEDURE

The materials and reagents used were as follows. HA was produced from femoral bovine bones, obtained from an international abattoir (Carrefour SA, Erenkoy, Istanbul, Turkey). h-BN powder was obtained from American Elements, USA, with an average particle size of <1 μm. NaOH 1% solution was used for deproteinization of femoral bovine bones (Sigma Aldrich, Germany). Hoechst 33342 (H3570) and Calcein AM (C3099) were purchased from Life Technologies (USA). Dulbecco's modified Eagle's medium (DMEM) was from SIGMA-Aldrich (D5671-Germany). L-glutamine (BIO3–020-1B), penicillin/streptomycin (BIO3–031-1B), and trypsin-EDTA (BIO3–050–1A) were purchased from Biological Industries (Israel). Fetal bovine serum (FBS) was purchased from BioWest (S1810-USA). Phosphate-buffered saline (PBS-17–516F) without calcium or magnesium was purchased from Lonza (USA). Human bone osteosarcoma cell line (U2OS-HTB-96) was purchased from American Type Culture Collection (ATCC, USA).

The femoral bovine bones were cut down to small pieces and cleaned from bone marrow components by manual scraping. The specimens were washed with tap water and then deproteinized using an alkali solution (NaOH, 1%), next they were dried, and finally calcinated at 850°C in air. The obtained specimens were crushed and ground in an alumina mortar and balls for 4 h (Planetary Ball Mill PM 200 Retsch, Haan, Germany). The resultant HA powder was sieved through a sieve of 100 μm. The produced fine HA powder was mixed with 0.5, or 1.0, or 1.5 wt % of h-BN powder. The resultant powder batches were ball milled for 4 h. Cylindrical samples, with dimensions of 11 mm diameter and 11 mm height, were prepared by compression of the powder mixtures under a pressure of 350 MPa, according to the British Standard 7253.[10] The green samples of h-BN/HA were sintered at different temperatures, specifically 1000, 1100, 1200, and 1300°C for 4 h in air in an electrical furnace (Nabertherm HT 16/17, Lilienthal, Germany).

Compression strength measurement tests of the produced materials were conducted in a universal tensile testing machine DVT (50 kN, Devotrans Inc., Istanbul, Turkey), with a displacement speed of the head at 2 mm/min. The presenting results of compression strength are the mean values from 6 different samples. Vickers microhardness (HV) tests were carried out at 200 g load with 20 s of dwell time in Shimadzu HMV-2 (Kyoto, Japan) equipment. The density of sintered samples was measured by the Archimedes immersion method (Precisa Gravimetrics AG, XB 220A). The presenting results for both microhardness and density are the mean values from 10 different samples.

The microstructure of the fracture surface of the sintered specimens was observed in scanning electron microscope (SEM, JEOL 590, Tokyo, Japan; accelerating voltage 20.0 kV), equipped with EDS device (JEOL 590) for chemical analysis. FTIR spectra were recorded at a wavenumber range of 400–4000 cm^{-1} (Shimadzu, AIM-9000; resolution 2 cm^{-1}). Crystallographic analysis was carried out through X-ray diffraction analysis (XRD, D8 Advance, Bruker-AXS, Germany) using Cu K_α ($\lambda = 1.542$ Å) radiation.

The in vitro antibacterial activity of the h-BN/HA composites was investigated against a prokaryotic strain, *E. faecalis* ATCC 29212, by using a quantitative analysis. *E. faecalis* test strain (10^4–10^5 CFU/mL) and powder samples of h-BN/HA at 10 mg/cm^3 concentration were added to 9 mL Muller Hinton broth and incubated at 37°C for 24 h. One milliliter of the sample was taken from this mixture and was analyzed for microbial counts at the beginning of the experiments, after 6 h and 24 h. The microbial analysis was performed three times in this study.

Cell viability assay with osteosarcoma was also conducted. U2OS human bone osteosarcoma cells were cultured in DMEM, supplemented with 10% FBS, 1% penicillin–streptomycin, and 1% L-glutamine. 1.5×10^6 cells were seeded on 10 cm culture plate in 10 mL cell culture medium and incubated at 37°C in a 5% CO_2 humidified incubator. At 24 h post-treatment, cells were stained by addition of 1 μg/μL Hoechst 33342 and Calcein AM, and further incubated for 30 min, at 37°C and 5% CO_2 (protected from light). After washing 3 times with PBS, cells were analyzed by an inverted fluorescent (Olympus IX70) and a confocal microscope (Zeiss, LSM 710).

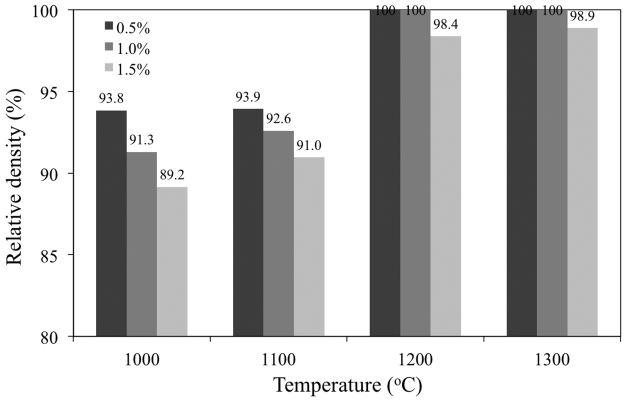

FIGURE 1. Values of relative density (i.e., % of the calculated theoretical density) of the produced h-BN/HA composites with different amounts of h-BN (0.5, 1.0, and 1.5 wt %), sintered at different temperatures (SD < 1%).

Cells treated with 0.1 g of 0.5 wt % h-BN/HA, 1.5 wt % h-BN/HA, h-BN, and HA (produced in this study, as mentioned above from the femoral bovine bones) were evaluated for cell viability. Nontreated cells were used as a control and handled in the same way as their treated counterparts. Cells were harvested at 24 h post-treatment and viability was assessed using the trypan blue exclusion assay. Estimation of viability by trypan blue exclusion relies on the loss in membrane integrity, determined by the intake of a trypan blue dye to which cells are normally impermeable. Quantification was expressed as the percentage of trypan blue positive cells, and the relative cell viability (%) was expressed as the percentage relative to the untreated control cells. The statistical significance of differences between groups was assessed by two-tailed Student's *t* test. Data are presented as mean values ±S.D. of 3 independent experiments. Values with $p < 0.05$ were considered as significant.

RESULTS AND DISCUSSION

Densification and crystallographic characterization

The influence of the amount of BN and the sintering temperature on the densification of the produced h-BN/HA samples is clearly seen in the results of relative density (defined as the ratio of the experimentally measured density to the calculated theoretical density, which was calculated assuming that the densities of h-BN and HA are 2.1 and 3.16 g/cm^3,[14] respectively, Fig. 1) and the microstructure (observed by SEM) of fracture surfaces (Fig. 2). First of all, h-BN incorporation in HA results in lighter material (as h-BN is a considerably lighter material than HA). The results suggest that densification is rather poor after sintering at 1000 and 1100°C, but it considerably improves after sintering at 1200 and 1300°C.

The elemental analysis with EDS (Fig. 2) detected phosphor and calcium as major elements and boron, nitrogen, oxygen, sodium, and magnesium. However, both B and N

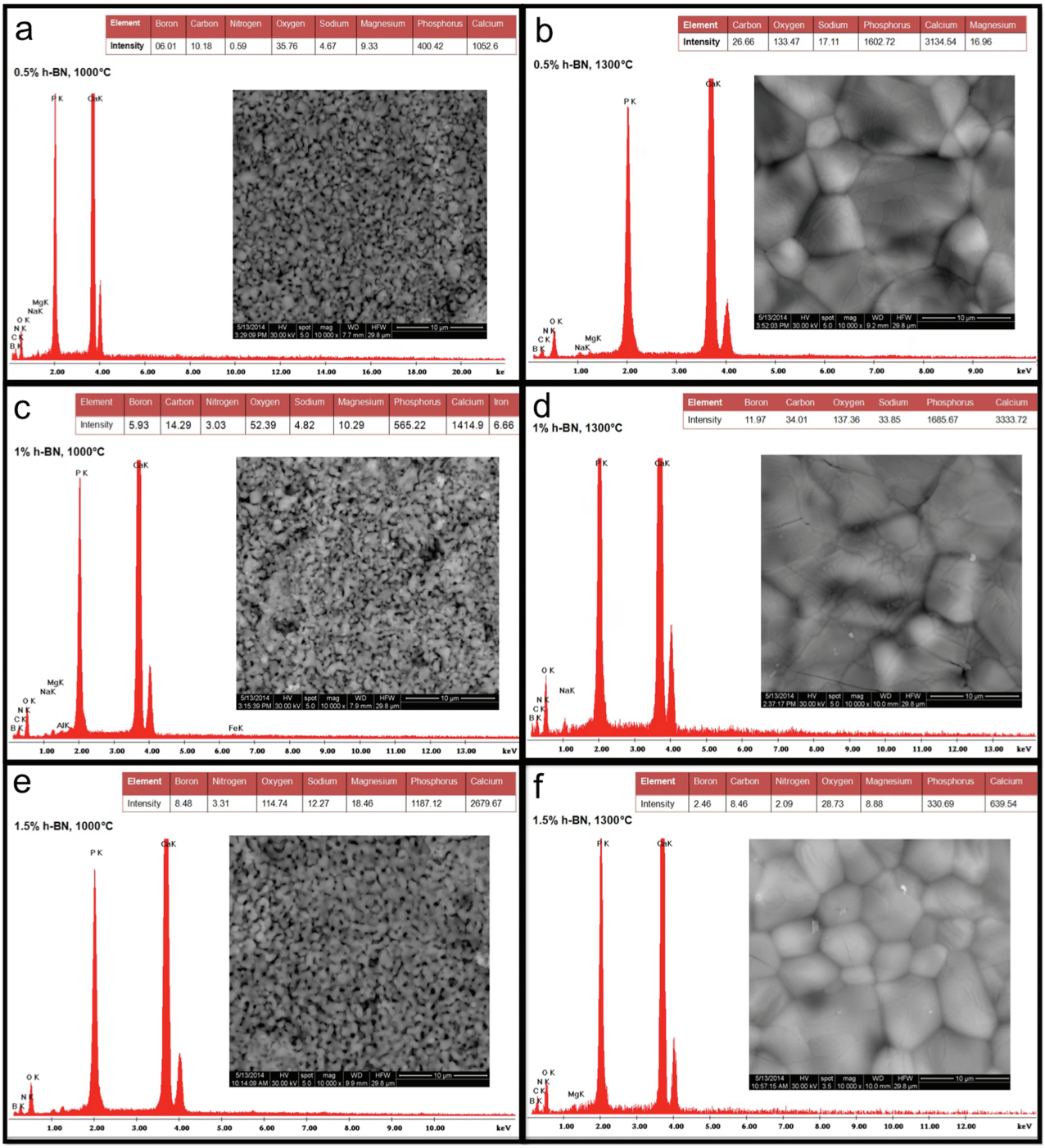

FIGURE 2. SEM images at fracture surfaces and elemental analysis (by EDS) of h-BN/HA composites: 0.5 wt % h-BN/HA sintered (a) at 1000°C and (b) at 1300°C; 1 wt % h-BN/HA sintered (c) at 1000°C and (d) at 1300°C; 1.5 wt % h-BN/HA sintered (e) at 1000°C and (f) at 1300°C.

are light elements and thus, they are not securely detected quantitatively by the EDS analysis.

However, the role of h-BN in the sintering behavior of HA is somehow controversial. More specifically, the microstructure of the well-sintered samples (Fig. 2b,d,f) is seemingly better than that developed in sintered samples of pure HA, which were produced by the authors and reported in our previous studies.[15,16] The microstructure of the samples with 1.5% h-BN sintered at 1300°C is apparently more homogeneous (in the size and the shape of the grains) than the samples sintered at the same temperature with lower amount in h-BN. Earlier studies reported that the presence of BNNTs favors grain enhancement in Al_2O_3, Si_3N_4, HA, and Al-based composites.[17] A similar microstructure has been also reported in sintered stainless steel with added boron-containing powders.[18] On the other hand, according to

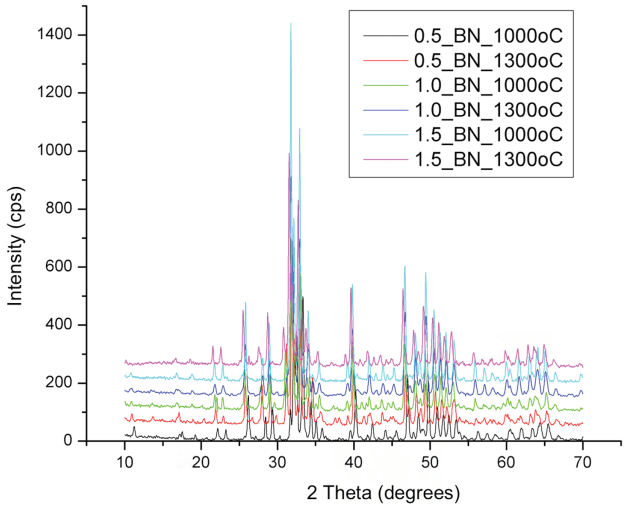

FIGURE 3. X-ray diffractograms of h-BN/HA composites with different amounts of BN sintered at 1000 and 1300°C.

Figure 1, the presence of h-BN seemingly reduced (slightly) densification, in all temperatures.

The X-ray diffractograms of the samples with 0.5 and 1.5% h-BN sintered at 1000 and 1300°C, shown in Figure 3, suggest that the produced h-BN/HA composites were well crystallized. Besides the dominant peaks of HA, the peaks of the basal plane of h-BN at ~30°, attributed to the (002) plane of h-BN, are clearly observed. The peaks of β-TCP (beta tricalcium phosphate) are also observed in the X-ray diffractograms at 1300°C, attributed to the partial decomposition of HA to β-TCP, which usually occurs in synthetic HA at temperatures above 1200°C.[19] The resistance of HA to extensively transform to β-TCP even at 1300°C in this study is attributed to the biological origin of HA, which has been observed many times in our previous studies.[7,15,16] However, it is worthy of noting that this thermal resistance was not annulled by the presence of h-BN in the matrix of HA. Moreover, biphasic HA/β-TCP materials are, undoubtedly, bioceramics which are in increased demand (these days). Meanwhile, there is no evidence for formation of reaction products between h-BN and HA or formation of glassy phase.

The FTIR spectra for all the produced samples are shown in Figure 4. The major bands correspond to the typical vibrations of the phosphate groups in HA.[20,21] The peak of the OH group of HA at ~635 cm^{-1} is generally more pronounced in the spectra of the samples sintered at lower temperatures than in the spectra of the samples sintered at higher temperatures. This is probably due to the partial decomposition of HA to β-TCP, already mentioned above.[19] Moreover, the two small bands which peak at ~745 and ~785 cm^{-1} strongly resemble vibrations due to carbonate groups in HA. This possibly suggests the formation of carbonate partially substituted HA (HCA), probably due to the biological origin of HA. HCA is the form of HA which is in greatest demand among bioceramics.[20]

Mechanical properties

The influence of sintering temperature on the mechanical properties, namely Vickers microhardness and compressive strength, for the produced samples is shown in Figures 5 and 6, respectively. The values of both properties are rather low in the samples sintered at low temperatures, for example, at 1000 and 1100°C. Apparently, the improvement of densification, discussed in the previous section, caused a considerable increase of the values of both properties in the samples sintered at 1200 and 1300°C. Moreover, assuming that β-TCP is, in general, mechanically weaker than HA,[19] the fact that there was suppressed transformation of HA to β-TCP after 4 h sintering at 1300°C, also discussed in the

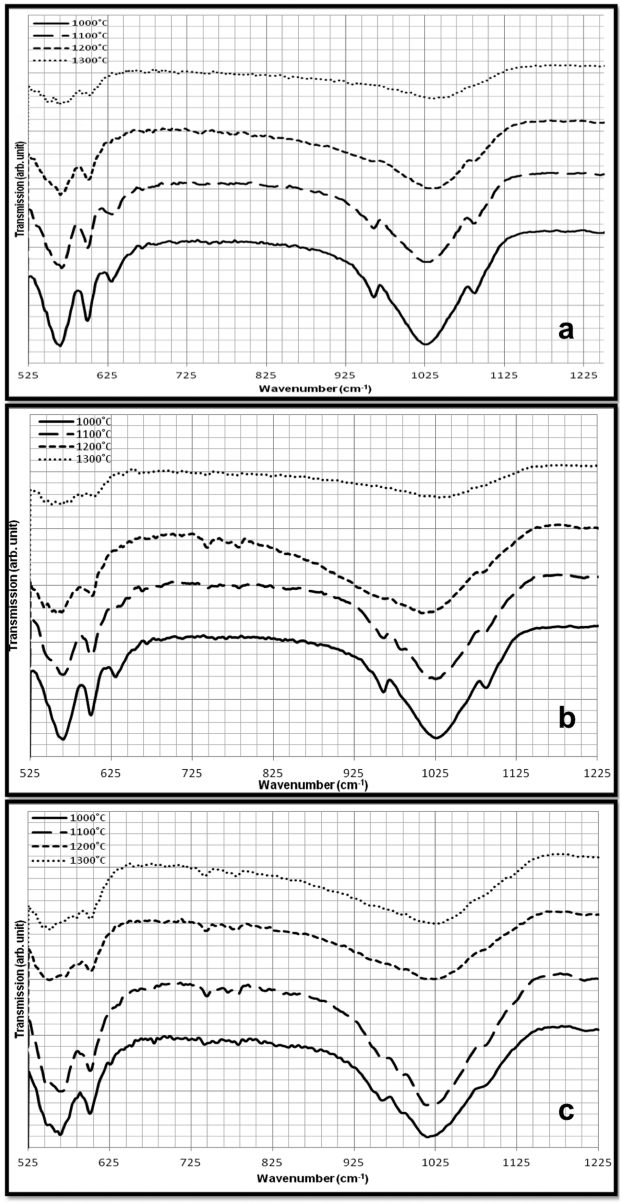

FIGURE 4. FTIR spectra of the produced h-BN/HA composites sintered at different temperatures: (a) 0.5% h-BN/HA, (b) 1.0% h-BN/HA, and (c) 1.5% h-BN/HA.

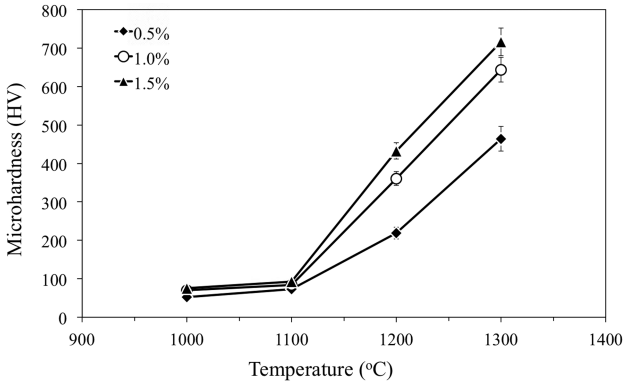

FIGURE 5. Influence of sintering temperature on Vickers microhardness of the produced h-BN/HA composites with different amounts of h-BN.

previous section, caused the further increase of the values of these properties from 1200 to 1300°C. If β-TCP was formed extensively at 1300°C, then a dramatic decrease of mechanical properties would perhaps be recorded in the experimental results. The considerable increase of microhardness in the samples with a high h-BN content (1 and 1.5%), compared to the sample containing a lower amount of h-BN (0.5%), sintered at high temperatures, agrees fairly well with the improvement of the microstructure (it becomes more fine and grain enhancement of HA occurs) of the final composite h-BN/HA composite.

The microhardness of sintered pure HA with biological (bovine) origin (BHA) was measured in our earlier study as 145 HV for samples sintered at 1300°C,[16,19] which is considerably lower than the values found in this study. Many researchers have tried to reinforce HA. For instance, BNNTs,[19] carbon nanotubes,[22] and stainless steel[23] have successfully been used to increase the microhardness of HA without having a negative effect on its biocompatibility. In our previous study, it was found that the best reinforcement of HA was achieved by the addition of commercial inert glass (CIG) to HA.[15] In that study, the highest value of microhardness of the CIG–BHA composites was 507 HV, which is evidently lower than the highest values measured

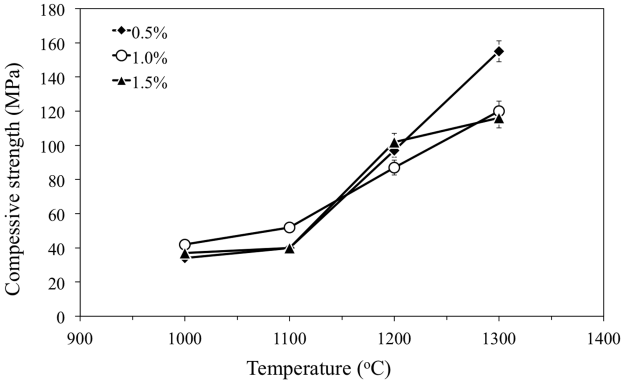

FIGURE 6. Influence of sintering temperature on the compressive strength of the produced h-BN/HA composites with different amounts of h-BN.

TABLE I. Antimicrobial Activity of h-BN/HA Composites, Analyzed for Microbial Counts at the Beginning of the Experiments, After 6 h, and After 24 h

	Antimicrobial activity (CFU/mL)		
Time (h)	h-BN (0.5%)	h-BN (1.5%)	Control (*E. faecalis*)
0	7.4×10^4	7.0×10^4	5.6×10^4
6	9.8×10^7	8.3×10^7	1.1×10^8
24	1.3×10^9	3.0×10^8	1.8×10^9

in this study (Fig. 5). The improvement in microhardness in the produced h-BN/HA composites might be because of the finer grain size. The increase of microhardness following the reduction of the grain size in the HA composites can be explained by the Hall–Petch mechanism.[19] Moreover, the crystalline h-BN may also have a positive influence on the microhardness in HA composites, better than the amorphous glasses (from CIG) used as sintering aids in our previous studies.

The maximum values of compression strength (Fig. 6) are higher than the values in HA samples which contained no h-BN, by over 30 MPa.[19] Gunduz et al. measured the compression strength in CIG-BHA composites as 133, 104, and 106 MPa for samples sintered at 1200°C with 10 wt % CIG, at 1300°C with 5 wt % CIG, and at 1300°C with 10 wt % CIG, respectively.[15] It is also worthy to be noted that CIG composites seemingly suffer by over-firing effect (i.e., formation of air bubbles in the bulk of the sintered sample when it is sintered at very high temperatures, which causes a dramatic degradation in mechanical properties). In this study, h-BN may also cause a negligible over-firing effect (mainly at 1300°C), as might be suggested by the plots of Figure 6 (the sample with the lowest amount in BN sintered at 1300°C achieved the highest compressive strength value) and Figure 1 (the samples with the highest amount in BN did not reach 100% densification); however, over-firing effect was not perceptible either visibly or in the SEM analysis (Fig. 2). Oktar et al. have also reinforced BHA by adding 0.25, 0.50, 1, and 2 wt % La_2O_3 and the best value of compressive strength was measured as 88 MPa for the sample with 2.0 wt % La_2O_3 sintered at 1300°C.[24]

Biological properties

The antibacterial activity of h-BN/HA composites was investigated by the plate counting method. The microbial counts of *E. faecalis* ATCC 29212 strains in the mixture are shown in Table I. According to these results, the antibacterial effect of h-BN/HA composites is easily observed by adding different quantities of h-BN. More specifically, the antibacterial durability effect of 1.5% h-BN/HA composites was better than that of 0.5% h-BN/HA and the control group. This provides evidence that the produced h-BN/HA materials, in general terms, should have a good antibacterial performance in situations close to a real infection.

Then, the in vitro evaluation of the toxicity effect of h-BN followed. U2OS was treated with a variety of h-BN composites, and then they were morphologically analyzed using

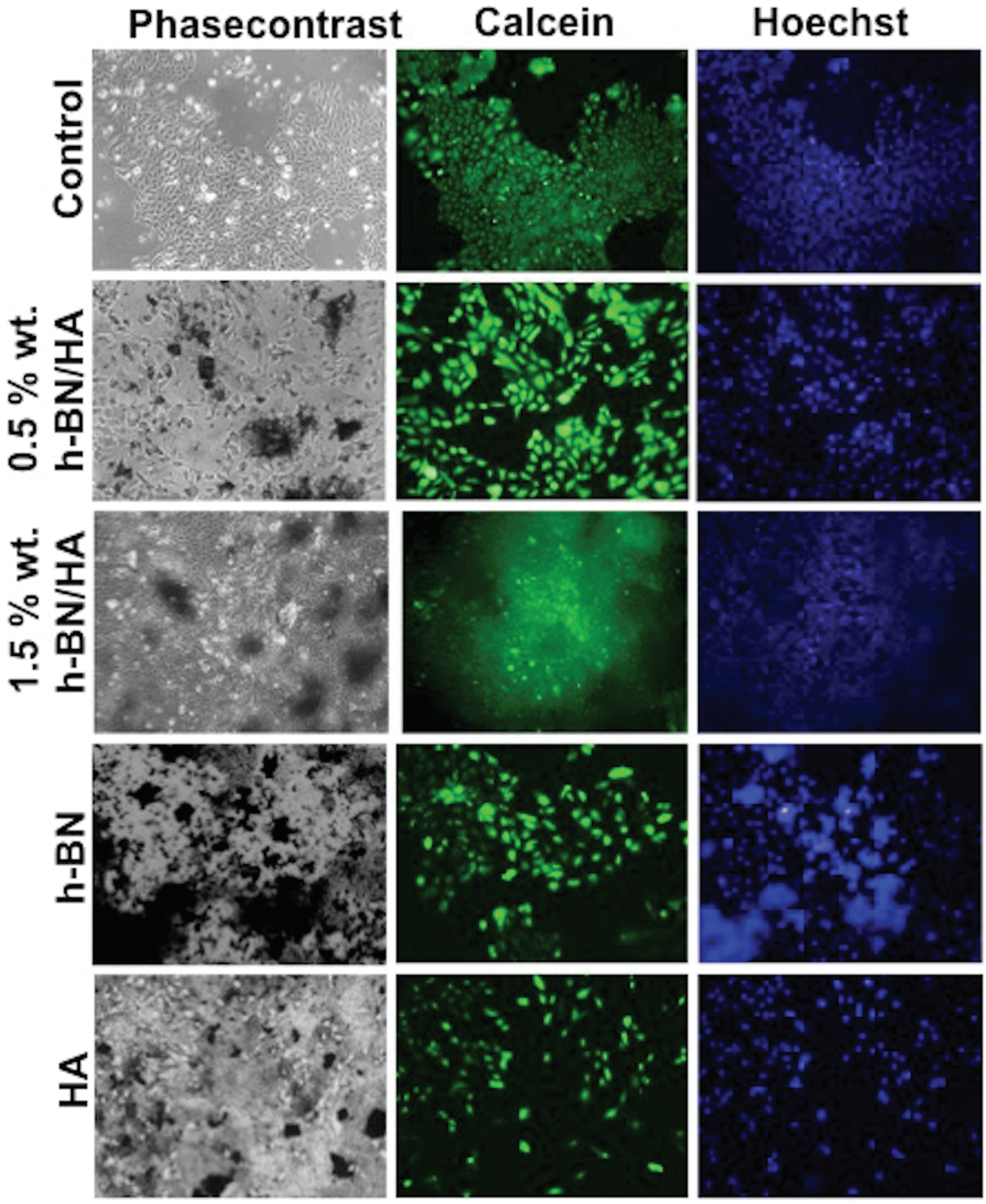

FIGURE 7. Inverted fluorescent images of BN-treated U2OS cells. Cells were treated with 0.1 g of material's powder for 24 h and then counterstained with 1 μg/μL of Hoechst 33342 and Calcein AM for 30 min. Images were taken under 10× magnification. h-BN, hexagonal boron nitride; HA, hydroxyapatite.

Calcein AM and Hoechst staining. According to the results shown in Figures 7 and 8, there was no evidence of signs of death, which is observed by the conversion of the nonfluorescent Calcein AM to a green-fluorescent calcein after acetoxymethyl ester hydrolysis by intracellular esterases. Hoechst 33342 is a popular cell-permeant nuclear stain that emits blue fluorescence when bound for dsDNA and is often used to distinguish condensed pycnotic nuclei in apoptotic cells. Counterstaining of cells with Hoechst showed no evidence of significant nuclear changes after treatment with the h-BN composites.

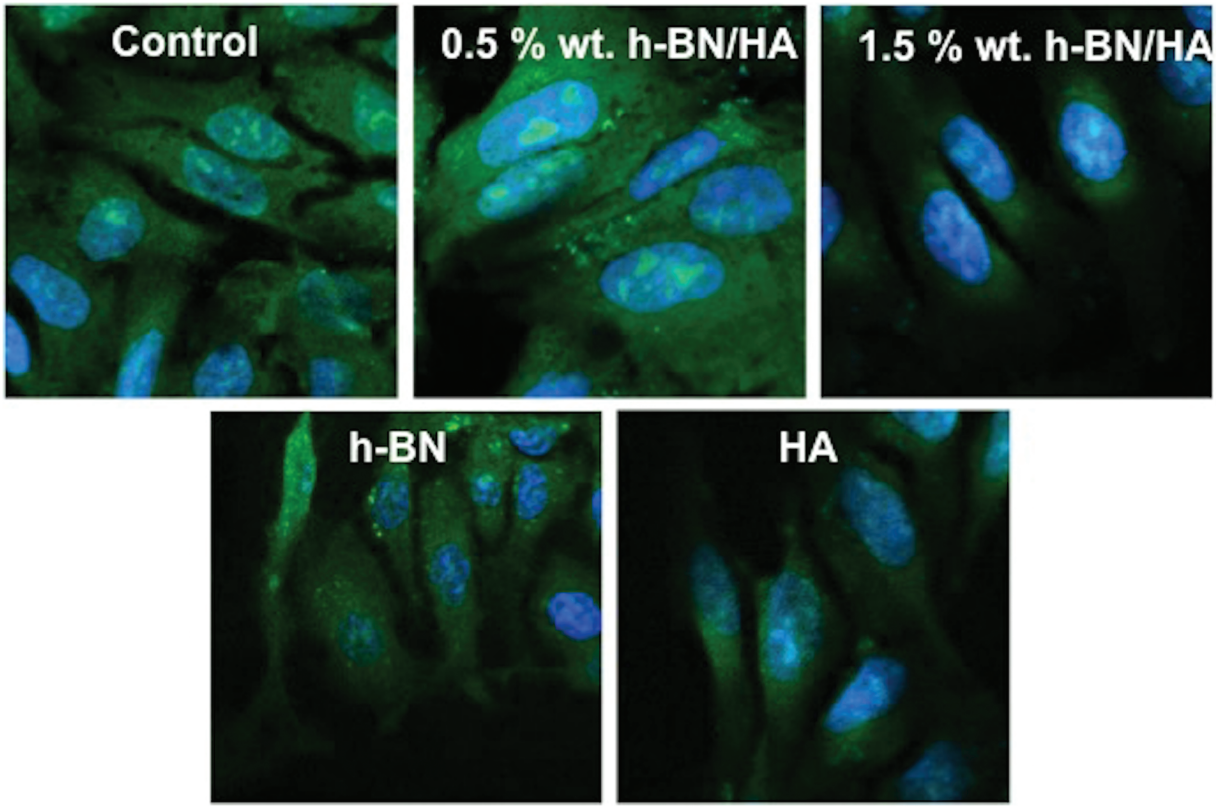

FIGURE 8. Confocal images of BN-treated U2OS cells. Cells were treated with 0.1 g of material's powder for 24 h and then stained with 1 μg/μL of Hoechst 33342 and Calcein AM for 30 min. Images were taken by Zeiss LSM 710 confocal microscope under 63× magnification. h-BN, hexagonal boron nitride; HA, hydroxyapatite.

Furthermore, the toxicity effect of h-BN was quantitatively analyzed by trypan blue exclusion assay, which determined by the intake of a trypan blue dye in dying cells. Compared to control, there was no efficient change in the viability of cells exposed to the HA only and 0.5% h-BN/HA, which can be seen in the plot of Figure 9. Although, h-BN and 1.5% h-BN/HA treatment showed slight decrease in the cell viability, these cells did not show any signs of death proved by morphological analyses in Figures 7 and 8. In general, stress-induced environmental factors cause changes to the cellular membrane and mitochondrial conditions in early stages of death signal. Morphological changes, such as nuclear fragmentation, chromatin condensation, or chromosomal DNA fragmentation, occur lately in the execution stages. In our study, exposition of cells to h-BN or 1.5% h-BN/HA seems to be effective, according to the quantitative analysis, which relies on the membrane changes; however, morphological analysis indicates that these cells did not go to execution stages.

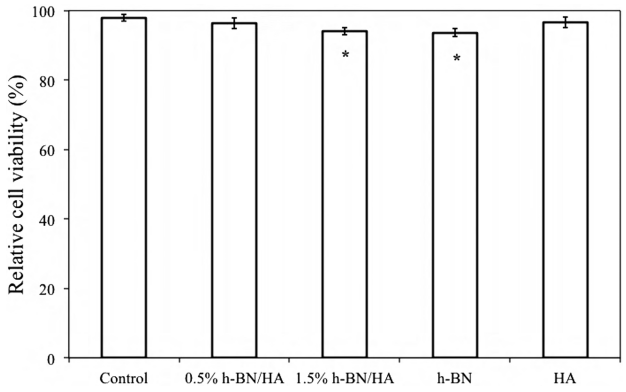

FIGURE 9. Quantitative analysis of toxicity effect of the h-BN/HA composites. $p = 0.189003658$ for 0.5% h-BN/HA, $p = 0.008049893$ for 1.5% h-BN/HA, $p = 0.007966202$ for h-BN, and $p = 0.274576629$ for HA (all versus control); (* statistically significant difference).

Overall, analysis of early and late death signals of h-BN composites indicates that h-BN is qualified for being considered as a promising compound, which can be used as an efficient and safe material in medical applications.

CONCLUSIONS

Composites of BHA reinforced with 0.5, 1.0, and 1.5 wt % h-BN were successfully produced via consolidation of green bodies. The samples were sintered at different temperatures between 1000 and 1300°C for 4 h in air. The experimental results showed that sintering of HA successfully occurs in the presence of h-BN, without forming any reaction products between h-BN and HA. The presence of h-BN maintains the intrinsic high thermal stability of BHA against transformation to β-TCP at 1300°C, and increases the Vickers microhardness and compression strength, whose best values were achieved at the highest sintering temperature, attributed to

the fine microstructure of the sintered h-BN/HA composites. The XRD and IR analyses revealed that biphasic β-TCP/HCA was formed, which is in greatest demand among other bioceramic materials. The results of the biological tests suggest that the produced h-BN/HA materials should have a good antibacterial performance in situations close to real infections. Accordingly, both material properties and biological/antibacterial performance qualify the produced h-BN/HA composites for further consideration and experimentation as potential novel and safe biomaterials in bone tissue engineering.

REFERENCES

1. Gibson IR, Rehman I, Best SM, Bonfield W. Characterization of the transformation from calcium-deficient apatite to β-tricalcium phosphate. J Mater Sci Mater Med 2000;11:799–804.
2. Samuel L, Turek MD. Orthopaedics: Principles and Applications, 2nd ed. J.B. Lippincott; 1985. pp 113–136.
3. Murugan R, Ramakrishna S. Nano-structured biomaterials. In Nalwa HS, editor. Encyclopedia of Nanoscience and Nanotechnology. California: American Scientific Publishers; 2004. p 595.
4. Barakat NA, Khil MS, Omran AM, Sheikh FA, Kim HY. Extraction of pure natural hydroxyapatite from the bovine bones bio waste by three different methods. J Mater Process Technol 2009;209: 3408–3415.
5. Thuault A, Savary E, Hornez JC, Moreau G, Descamps M, Marinel S, Leriche A. Improvement of the hydroxyapatite mechanical properties by direct microwave sintering in single mode cavity. J Eur Ceram Soc 2014;34:1865–1871.
6. Jiang H, Liu JK, Wang JD, Lu Y, Zhang M, Yang XH, Hong DJ. The biotoxicity of hydroxyapatite nanoparticles to the plant growth. J Hazard Mater 2014;270:71–81.
7. Gunduz O, Gode C, Ahmad Z, Gökçe H, Yetmez M, Kalkandelen C, Sahin YM, Oktar FN. Preparation and evaluation of cerium oxide-bovine hydroxyapatite composites for biomedical engineering applications. J Mech Behav Biomed Mater 2014;35:70–76.
8. Sharma VK, Yngard RA, Lin Y. Silver nanoparticles: Green synthesis and their antimicrobial activities. Adv Colloid Interface Sci 2009;145:83–96.
9. Pakdel A, Zhi C, Bando Y, Golberg D. Low-dimensional boron nitride nanomaterials. Mater Today 2012;15:256–265.
10. Trice RW, Halloran JW. Investigation of the physical and mechanical properties of hot-pressed boron nitride/oxide ceramic composites. J Am Ceram Soc 1999;82:2563–2565.
11. Ciofani G, Genchi GG, Liakos I, Athanassiou A, Dinucci D, Chiellini F, Mattoli V. A simple approach to covalent functionalization of boron nitride nanotubes. J Colloid Interface Sci 2012;374:308–314.
12. Ansaloni LM, de Sousa EM. Boron nitride nanostructured: Synthesis, characterization and potential use in cosmetics. Mater Sci Appl 2013;4:22.
13. Lahiri D, Singh V, Benaduce AP, Seal S, Kos L, Agarwal A. Boron nitride nanotube reinforced hydroxyapatite composite: Mechanical and tribological performance and in-vitro biocompatibility to osteoblasts. J Mech Behav Biomed Mater 2011;4:44–56.
14. Prajatelistia E, Han Y-H, Kim B N, Kim Y-M, Lee K, Jeong Y-K, Kim D-I, Kim K-H, KIM S. Characterization of boron nitride-reinforced hydroxyapatite composites prepared by spark plasma sintering and hot press. J Ceram Soc Jpn 2013;121: 344–347.
15. Gunduz O, Ahmad Z, Ekren N, Agathopoulos S, Salman S, Oktar FN. Reinforcing of biologically derived apatite with commercial inert glass. J Thermoplast Compos Mater 2009;22:407–419.
16. Gunduz O, Daglilar Z, Salman S, Ekren N, Agathopoulos S, Oktar FN. Effect of yttria-doping on mechanical properties of bovine hydroxyapatite (BHA). J Compos Mater 2008;42:1281–1287.
17. Lahiri D, Hadjikhani A, Zhang C, Xing T, Li LH, Chen Y, Agarwal A. Boron nitride nanotubes reinforced aluminum composites prepared by spark plasma sintering: Microstructure, mechanical properties and deformation behavior. Mater Sci Eng A 2013;574: 149–156.
18. Uzunsoy D. Investigation of dry sliding wear properties of boron doped powder metallurgy 316L stainless steel. Mater Des 2010;31: 3896–3900.
19. Goller G, Oktar FN, Agathopoulos S, Tulyaganov DU, Ferreira JM, Kayali ES, Peker I. Effect of sintering temperature on mechanical and microstructural properties of bovine hydroxyapatite (BHA). J Sol Gel Sci Technol 2006;37:111–115.
20. Rehman I, Bonfield W. Characterization of hydroxyapatite and carbonated apatite by photo acoustic FTIR spectroscopy. J Mater Sci Mater Med 1997;8:1–4.
21. Liao CJ, Lin FH, Chen KS, Sun JS. Thermal decomposition and reconstitution of hydroxyapatite in air atmosphere. Biomaterials 1999;20:1807–1813.
22. Sarkar SK, Youn MH, Oh IH, Lee BT. Fabrication of CNT-reinforced HAp composites by spark plasma sintering. Mater Sci Forum 2007;534:893–896.
23. Mahathanabodee S, Palathai T, Raadnui S, Tongsri R, Sombatsompop N. Effects of hexagonal boron nitride and sintering temperature on mechanical and tribological properties of SS316L/h-BN composites. Mater Des 2013;46:588–597.
24. Faik N. Oktar, L.S. Ozyegin, Onur Meydanoglu, H. Aydin, Simeon Agathopoulos, G. Rocha, B. Sennaroglu, Eyup Sabri Kayalı, Sintering effect on mechanical properties of composites of hydroxyapatite lanthanum oxide (HA-La_2O_3). Key Eng Mater 2006;309: 101–104.