



In-Vitro Bioactivity Investigation of Sol-Gel Derived Alumina-Bovine Hydroxyapatite (BHA) Composite Powders

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Highlights

- Synthesis of the AlOOH gel via the sol-gel procedure was described in detail.
- Production steps of the AlOOH-bovine hydroxyapatite (BHA) mixtures were explained.
- Preparation of Al₂O₃-BHA composite powders was achieved through the heat-treatment process.
- Chemical, microstructural and physical analyses of the composite powders were performed.
- In-vitro bioactivity (MTT assay) characterization of the composite powders suggested great promise.

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Abstract

Alumina (α -Al₂O₃) and hydroxyapatite (HA, Ca₁₀(PO₄)₆(OH)₂) are well-known for being clinically successful bioceramic materials. In this work, in-vitro biological characterization of the sol-gel alumina-bovine hydroxyapatite composite powders was realized. Alumina powders were synthesized through the sol-gel process. First, boehmite (AlOOH) sol was prepared utilizing aluminium isopropoxide (Al(OC₃H₇)₃, AIP) as the starting precursor. Bovine hydroxyapatite (BHA) powders, which can be defined as naturally derived calcium phosphate powders were added as 10, 20, 30, and 50% wt. of AIP to each AlOOH sol. Homogeneous dispersion of the BHA powders in the AlOOH sol was managed due to employing Na-alginate as a kind of thickener. Gelation of the AlOOH-BHA mixtures was carried out at 110 °C for 3h. After drying, AlOOH-BHA mixtures were heat-treated at 1300 °C for 2h. Chemical, microstructural, thermal, and physical properties of the precursors/process products were characterized with X-Ray Diffraction (XRD), Fourier Transform Infrared Spectroscopy (FT-IR), X-Ray Fluorescence Spectroscopy (XRF), Differential Thermal Analysis (DTA), and Scanning Electron Microscopy - Energy Dispersive Spectroscopy (SEM-EDS) analyses. Indirect MTT assay was done to evaluate the biocompatibility of the Al₂O₃-BHA based biocomposite extracts using the L929 cell line. It is found that all Al₂O₃-BHA composite extracts with varying doses of 25% and 50% had no negative effect on the cell viability. In addition, % cell viability decreased with the increasing of the extract concentration. It can be concluded that the prepared Al₂O₃-BHA composites can be a good candidate for biomedical applications.

1. INTRODUCTION

Bioceramic materials are commonly used for hard tissue replacement and healing purposes. They can be in either ceramic, glass or glass-ceramic form. “Bioceramics” is the general name for the biocompatible ceramics and/or ceramic composite materials that are used in various prostheses and implants. Bioceramic materials can function in orthopedic surgery (vertebrae implants, total hip implants, maxillofacial constructions, etc.) and dental surgery applications thanks to their significant properties like excellent biocompatibility, chemical stability against body fluids such as blood and saliva, high mechanical strength,

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and aesthetical appearance. Specific interactions occur between the tissue and material surfaces, when a bioceramic implant material is placed into the body. These interactions can be categorized in three types depending on the implanted bioceramic material: bioinert, bioactive and bioresorbable. Alumina (α -Al₂O₃) and hydroxyapatite (HA, Ca₁₀(PO₄)₆(OH)₂) are well-known for being clinically successful bioceramics. Despite its high wear and corrosion resistance and high compression strength characteristics, the limited tissue bonding capacity of α -Al₂O₃ makes it a bioinert material. Conversely, HA, a bioactive ceramic, is found as crystals in the bone structure and has a superior capability of forming biochemical bonds with the tissues when inserted as an implant [1-3]. HA is a popular biomaterial in orthopedics for its proven biocompatibility and biodegradability. However, HA has a brittle character and its load bearing capacity is low. Chondrocyte loaded HA can be a good graft model to stimulate endochondral ossification. Mineralized cartilaginous tissue is formed when chondrocytes are cultured in combination with HA [4,5]. In this study, the target is to investigate the in-vitro behavior of the alumina-bovine hydroxyapatite (BHA) composite powders, where α -Al₂O₃ is predicted to provide strength to the composite structure while BHA is responsible for the bioactivity.

It is possible to encounter with studies and reports based on synthesis of Al₂O₃-HA composites and their properties [6-11]. Sol-gel process is ideal to produce high chemical purity powders. It is an efficient and economical way to obtain inorganic and/or organic/inorganic composite solids and advanced ceramic materials. Sol-gel method assures several important advantages such as obtaining nano-sized high purity powders, enabling a homogeneous mixture in molecular level, working at low reaction temperatures, having not hazardous side-effects on human health and environment, and requiring a reasonable experimental set-up. These benefits keep sol-gel technique preferred in many applications like high-strength ceramics, conductivity, multi-functional coatings, insulating structures, and also biomaterials [12-18].

Pure and highly porous α -Al₂O₃ powders were obtained via the sol-gel process in the experimental studies that our research group previously carried out [17,18]. In this work, aluminum isopropoxide (AIP) was used as the metal alkoxide starting material to synthesize pure α -Al₂O₃ powders via the sol-gel process [12,19-21]. Derivation of the BHA powders was realized from natural sources, i.e. bovine bones [8,22]. Investigation the biocompatibility of the biocomposite powders composed of α -Al₂O₃ and BHA bioceramic components was the goal of the current research. Besides the X-Ray Diffraction (XRD), Fourier Transform Infrared Spectroscopy (FT-IR), X-Ray Fluorescence Spectroscopy (XRF), Differential Thermal Analysis (DTA), and Scanning Electron Microscopy - Energy Dispersive Spectroscopy (SEM-EDS) analyses, bioactivity determination tests were also performed. Cytotoxicity of the Al₂O₃-BHA composite samples was evaluated by conducting Indirect MTT assay on L929 cell line.

2. MATERIALS AND METHODS

2.1. Preparation of the Al₂O₃-BHA Composite Powders

Al₂O₃-BHA composite powders were prepared through the following procedure; primarily, AlOOH (boehmite) sol was produced through the hydrolysis of aluminum isopropoxide (Al(OC₃H₇)₃, $\geq 98\%$, AIP, Aldrich) precursor. 100 was selected as the molar ratio of distilled water/AIP considering the Yoldas approach [12], i.e. 40 moles of distilled water/0.4 moles of AIP. Hydrolysis is the initial step of the sol-gel procedure, where 0.4 moles of AIP was reacted with distilled water, whose temperature was reached to 90 °C by employing a magnetic stirrer with hot plate. Peptization, the second stage of the sol-gel process, was performed by adding 30 ml of HCl (37% diluted up to 10%, Merck) in equal periods to the solution while hydrolysis reactions were still continuing. HCl solution was added to the system to accelerate the hydrolysis reactions. pH and temperature values of the AIP solution were continuously kept under control during the hydrolysis and peptization steps. pH of the synthesized AlOOH sol was recorded as ~2.5 at the end of the hydrolysis and peptization reactions and the sol has a gel-like appearance. Then the AlOOH sol was mixed with the BHA powders derived from a natural source, i.e bovine bones, by Oktar et al [8]. Deproteinizing the fresh bovine bones with NaOH, washing, calcination, and grinding with the mortar grinder are the stages that were respectively realized to obtain the BHA powders [8,23]. In order to achieve the Al₂O₃-BHA composite powders, BHA powders with the amount of 10, 20, 30, and 50% wt. of AIP were added to each AlOOH sol and mixed for 1h utilizing an overhead stirrer. Sodium alginate (NaC₆H₇O₆, Na-alg, Aldrich)

was also added as wt. 1, 2, 3, and 5% of its own molecular weight to the AlOOH-BHA mixture to overcome the precipitation tendency of the BHA powders and enable partially homogeneous dispersion of them in the AlOOH sol. AlOOH-BHA mixtures were gelated at 110 °C for 3h to let the AlOOH phase lose the residual water and alcohol groups in its structure and form a more rigid mixture. Finally, the dried/gelated AlOOH-BHA mixtures were heat-treated at 1300 °C for 2h with 10 °C/min heating rate in the Nabertherm LHT 08/17 furnace [17].

1300AH10, 1300AH20, 1300AH30, and 1300AH50 are the codes for the heat-treated composite samples. In these codes, 1300 is the heat-treatment temperature value, A depicts α -Al₂O₃, and H denotes BHA. 10, 20, 30, and 50 present the (wt.%) amount of BHA that was added to AlOOH. At the last part of the experimental procedure, formation of the cylindrical shaped pellets from the composite powders was executed with a hydraulic manual press. Sintering of the prepared pellets was done at 1300 °C for 2 h with 10 °C/min heating rate.

2.2. Characterization of the Al₂O₃-BHA Composite Powders

Various characterization studies were performed to analyze the produced Al₂O₃-BHA powders. Ca/P wt. ratio of the BHA powders was identified with the Panalytical Axios Minerals branded XRF. DTA of the Na-alginate was performed with TA SDT Q600 model device by using dry air with a heating rate of 10 °C/min and gas flow speed of 100 ml/min conditions. Chemical phase characterization of the heat-treated composite powders was carried out with the Rigaku D/Max-2200/PC model XRD through monochromatic Cu-K α radiation ($\lambda = 0.154$ nm). FT-IR graphs were obtained by benefitting from the Perkin Elmer Precisely Spectrum One device and applying the K-Br pellet technique. Microstructure details of the composite powders were observed by employing Jeol JSM 6335F (field emission) SEM. Elements that exist in the imaged areas were discovered with the Inca branded semi-quantitative EDS elemental analysis system that works concurrently with SEM. Determination of the apparent porosity (%) and bulk density features of the pellet samples were implemented considering the well-known Archimedes' Principle.

2.3. Biocompatibility Experiments

2.3.1. Preparation of Al₂O₃-BHA Composite Extracts

Al₂O₃-BHA composites and 1300Al₂O₃ powders were firstly incubated in cell culture medium (1g/10mL) at 37 °C for 48h. Then, the undiluted extracts (100%) were sterilized by filtrating with 0.22 μ m syringe filters (Isolab). In order to enable a dose-response relationship, the extracts were serially diluted in complete Dulbecco's Modified Eagle Medium with high glucose (DMEM, Gibco) to obtain with varying doses of 25% and 50%.

2.3.2. Cell Culture

Mouse fibroblast (L929) cell line was used to perform the biocompatibility experiments. The L929 cells were cultured in Low Glucose Dulbecco's modified Eagle's medium (L-DMEM) with high glucose supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 1% Penicilin-Streptomycin (Sigma, USA) at 37 °C with CO₂. When the L929 cells reached 70-80% confluence, they were separated from the flask with 0.05% trypsin / 0.02 EDTA solution (Gibco, USA), suspended by DMEM, and centrifuged at 1500 rpm for 5 min. After removal of the supernatant, cells were suspended by DMEM, and counted by thoma slide and seeded into 96-well plates at a density of 5x10³ cells/well. After allowing attaching overnight, the medium in each well was replaced with 200 μ L/well of the extraction and standard culture medium as the control group. Then the cells were incubated for 48h without changing the cell culture medium. Cells of the 5th passage were used for the experiments.

2.3.3. MTT Assay

In here, biocompatibility of Al₂O₃-BHA composites extracts with varying doses of 25, 50, and 100% were investigated. The biocompatibility of the Al₂O₃-BHA composite powder samples was tested by measuring cell viability with the MTT assay L929 cell line. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay is one of the most used methods for the detection of sample biocompatibility. In this study, indirect MTT assay was conducted to determine the cytotoxicity of the samples [24]. After 48h, incubation period of the sample extracts with cells, 200 µl of DMEM and 20 µl of MTT solution (500 µg/ml, diluted with PBS) per well were added, and incubated for a further 4h for formazan crystal formation. Then, in order to dissolve formazan crystals, the supernatant was replaced with 200 µl of DMSO. After incubation for 20 min. at 37 °C, the supernatant solution was measured at 570 nm utilizing a microplate reader (BMG Spectroster, Germany). DMEM cell culture medium (without sample extracts) was used as positive control. Viability of cultures exposed to only DMEM cell culture medium was set a 100% to make a comparison with the responses of the control [25]. The number of replicates was five. Data points were given as mean ± standard deviation. One-way ANOVA followed by Tukey's test was performed for each group using the GraphPad Prism 6.0 Software.

3. RESULTS AND DISCUSSION

(Ca/P) wt. ratio of the BHA powders used in the composites was calculated as 1.98 depending on the results of the XRF analysis, where wt. Ca% and wt. P% were found as 39.12 and 19.74, respectively. As the natural HA powders employed in the production process were derived from the bovine bones, (Ca/P) wt. ratio of the BHA powders (1.98) is different but close to that of the stoichiometric HA (2.15). XRD results (Figure 1) demonstrate that the composite powders include α -Al₂O₃ and CaP based phases, i.e. HA and tricalcium phosphate (Ca₃(PO₄)₂, Whitlockite, β -tri-calcium phosphate, β -TCP). Since Na-alginate, which was used for thickening the AlOOH-BHA mixtures is an organic polysaccharide [26,27], it disappeared from the system between ~200 °C and ~600 °C according to the DTA curve (Figure 2). Therefore, it did not exist in the XRD results of the heat-treated composite powders. The PDF numbers of the detected phases were determined as 74-1081 and 99-0036 for α -Al₂O₃, 76-0694 and 89-6438 for HA, and 09-0169 for β -TCP. HA and β -TCP belong to the same compound family, hence it was a difficult issue to separate these two phases. Although the diffraction angle values of the strongest peaks of HA (31.715° for (211)) and β -TCP (31.026° for (0210)) phases are very close to each other and some of the main peaks may be overlapped and/or shifted, there are distinctive peaks that helped us to detect HA and β -TCP and remark them on the XRD graphs. It is assumed that the β -TCP phase appeared in the composite powders due to the high heat-treatment temperature (1300 °C), which influenced the stability of the HA phase and caused its decomposition to β -TCP. However at least 1300 °C is required for the transformation of unstable (γ - δ - θ -) Al₂O₃ phases to the stable α -Al₂O₃ phase [12,17]. Thus, 1300 °C was chosen and applied as the heat-treatment temperature of the AlOOH-BHA gel mixtures.

Decomposition reaction of the HA phase realized through the oxyapatite (Ca₁₀(PO₄)₆O) phase. As HA dehydrates at ~1300 °C, oxyapatite forms. Aqueous environment conditions enhance the transformation tendency of oxyapatite to the HA phase. TCP and tetracalcium phosphate (TTCP) formed after the decomposition of oxyapatite but TTCP did not appear in the presented composite samples [28, 29]. It is interpreted that the decomposition process of HA was partially completed so that HA and TCP phases were identified together in the structure instead of TTCP and TCP [6]. As it is well known from the literature, α -Al₂O₃, HA and TCP phases take place in the bioceramics class and are intensely used in biomedical applications in terms of prostheses and implants. Moreover β -TCP shows bioresorbable interactions with the body tissues and it may increase the biochemical bond forming capacity of the intended Al₂O₃-BHA biocomposites [1,2]. Therefore, this triple composite material is thought to be advantageous for the biomedical studies.

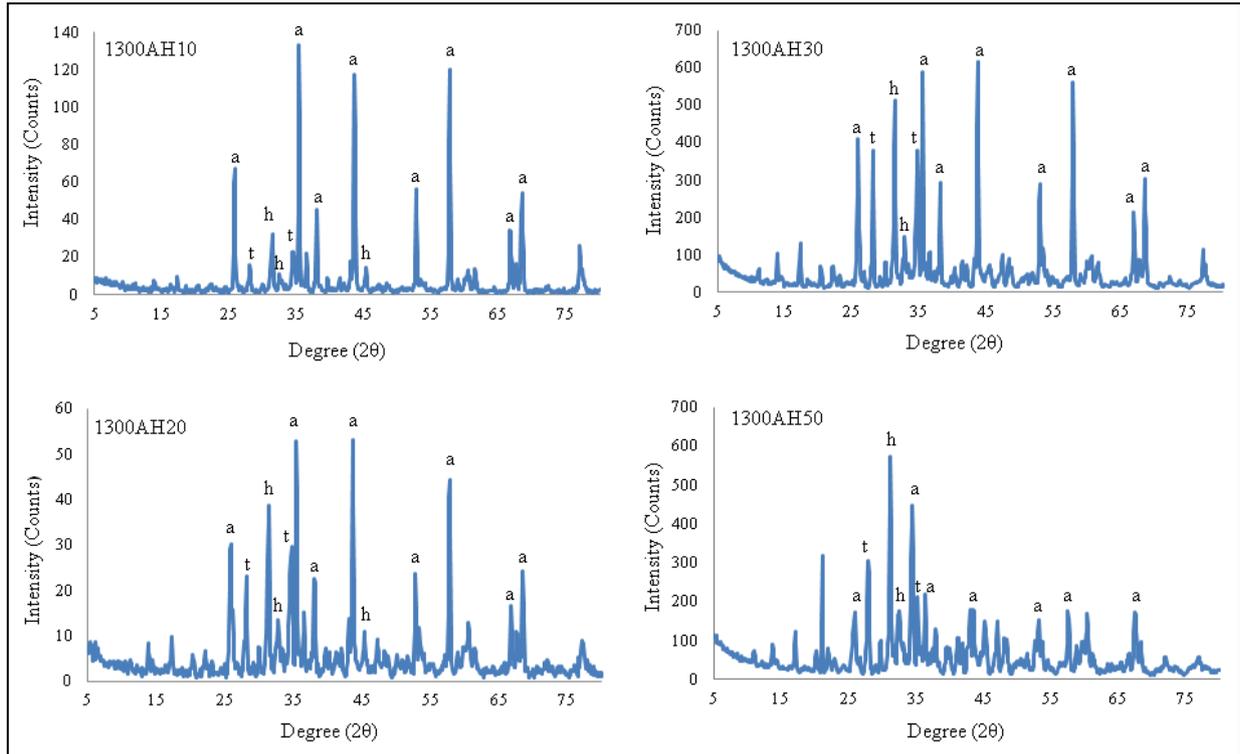


Figure 1. XRD results of the Al_2O_3 -BHA composite powder samples (a: α - Al_2O_3 , h: HA, t: β -TCP)

FT-IR spectra of pure BHA and pure α - Al_2O_3 phases are given in Figure 3a and Figure 3b, respectively, while Figure 4 exhibits the FT-IR analyses of the composite powders. Figure 4 includes the main bands that refer to the P-O, CO_3^{2-} and Al-O vibrations as can be also recognized from the FT-IR graphs of the pure BHA and α - Al_2O_3 phases. The peak chain between 1120 cm^{-1} and 947 cm^{-1} indicated as (1) on the spectra was attributed to the P-O stretching vibrations of the PO_4^{3-} ions in HA. The band marked as (2) between 835 cm^{-1} and 702 cm^{-1} represents the vibrations of the CO_3^{2-} ions, which may arise from the other ions that replaced in the HA structure due to the utilization of natural sources, i.e. bovine bones. The characteristic band noted as (3) between 602 cm^{-1} and 587 cm^{-1} was referred to the P-O bending vibrations of PO_4^{3-} ions in the HA structure. Al-O bond vibrations that are signed as (4) on the spectra were detected at $602\text{-}603\text{ cm}^{-1}$ and $503\text{-}468\text{ cm}^{-1}$ [17, 30-33].

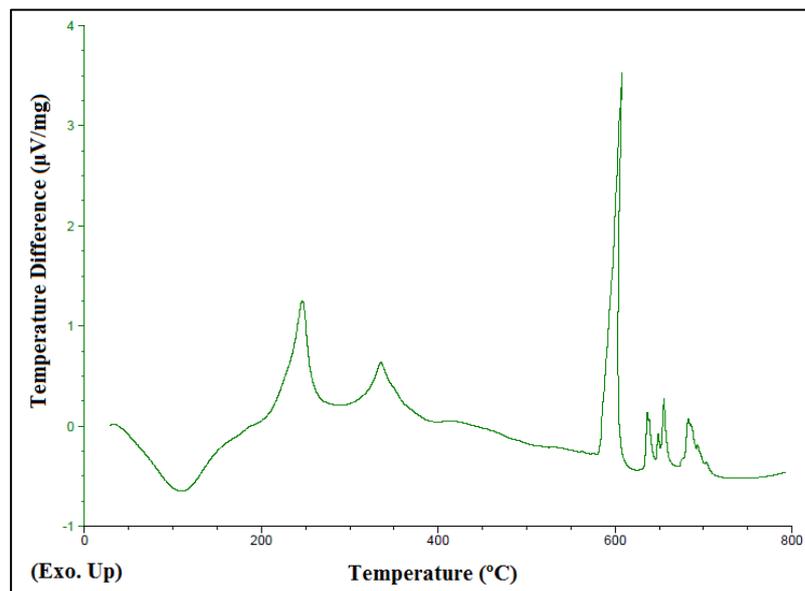


Figure 2. DTA curve of Na-alginate

composite system is naturally derived BHA and as stated previously, BHA partially decomposed to the TCP phase as a result of sintering the Al_2O_3 -BHA composites at 1300 °C. These facts are assumed to be influential on the lower \sim (Ca/P) molar ratio values of the composite samples with the lower BHA content.

The needle-like shaped particles belong to the α - Al_2O_3 phase while HA has a spongy structure. It is possible to notice that there are no well-developed neck formations between the particles of different phases owing to the OH removal from AlOOH that occurred while AlOOH was transforming to the stable α - Al_2O_3 . Such porous structures are also encountered in the researches reported by Bartonickova et al. [6] and Sakka et al. [34] who studied the HA- Al_2O_3 composites and β -TCP- Al_2O_3 composites, respectively. Pores are favorable sites for transmitting the necessary nutrition and body fluids to the other parts of the biomaterial. By this way, pores enable better material-tissue interactions and accelerate the healing process. Hence porosity becomes a crucial point for the composite powders and yields the usage of the material to other potential areas such as filling materials or tissue engineering scaffolds, etc. Average apparent porosity (%) values were measured by using 5 pellets for each composite sample group and found as 38.29% for 1300AH10, 43.43% for 1300AH20, 43.33% for 1300AH30, and 39% for 1300AH50.

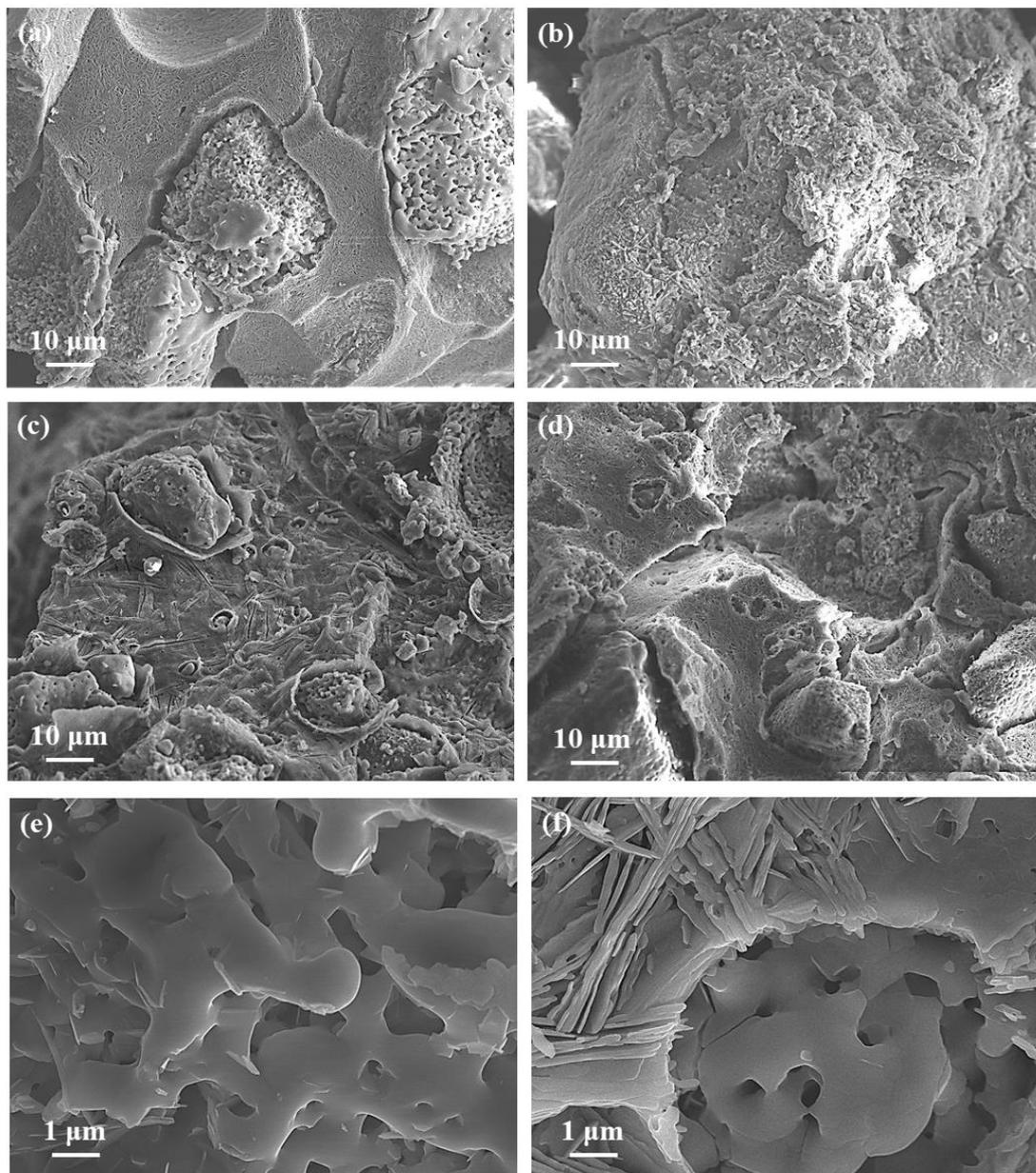


Figure 5. SEM micrographs of (a) 1300AH10 - (b) 1300AH20 - (c) 1300AH30 - (d) 1300AH50 (x500), and (e) 1300AH20 - (f) 1300AH50 (x5000) powder samples

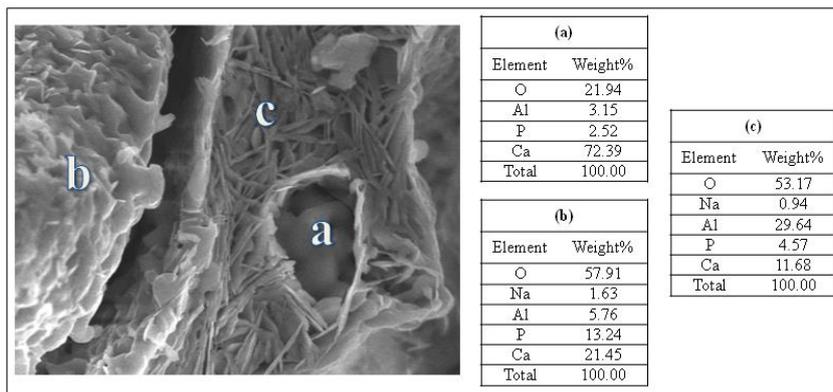


Figure 6. EDS analysis of the 1300AH50 powder sample

Table 1. ~ (Ca/P) molar ratios of the composite powders

Sample Code	~ (Ca/P) Molar Ratio (EDS)
1300AH10	1.30
1300AH20	1.41
1300AH30	1.52
1300AH50	1.61

Figure 7 shows cell viability results according to the effect of treatment with Al_2O_3 -BHA composite extracts with varying doses of 25, 50, and 100%. It is found that all Al_2O_3 -BHA composite extracts with varying doses of 25% and 50% had no negative effect on the cell viability. As known, α - Al_2O_3 has been extensively applied as reinforcing component for HA owing to enhancing especially the mechanical properties of HA and making it cost-effective. In addition, α - Al_2O_3 has considerable advantages such as great biocompatibility in its composites [35]. Liu et al. [36] presented a study on graphene-reinforced Al_2O_3 nanocomposites. They found that graphene- Al_2O_3 composites showed superior biomedical properties. Beyzay et al. [37] examined the cytotoxicity of the α - Al_2O_3 on macrophages. Cytotoxicity was observed at higher concentrations ($\geq 1000 \mu\text{g/ml}$) as cell viability reduced to 75%. Interestingly, the important result is that 1300AH20 extracts 100% exhibit statistically higher cell viability in comparison to 1300 Al_2O_3 . Cell viabilities of 1300AH20 extracts concentrations of 100%, 50%, and 25% of culture were $103.4 \pm 18.4\%$, $112.2 \pm 12.4\%$, and $141.5 \pm 12.3\%$, respectively. Furthermore, it can be understood that the % cell viability of all samples was dose-dependent, which decreased with the increasing of the extract concentration. These findings are compatible with literature [36,38]. The lower HA addition amount resulted in the higher cell viability due to presence of less and smaller pores as also expressed by Song et al. [39]. Although all samples displayed cell viability, it is assumed that the pore size played a significant role in the degree of it. As a matter of fact, it can be concluded that the prepared samples, especially 1300AH20, have a great cytocompatibility, which make Al_2O_3 -BHA composites suitable to be used in biomedical applications.

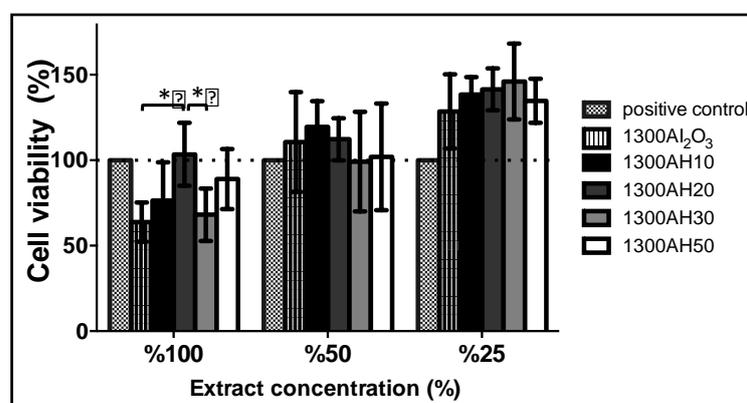


Figure 7. The effect of treatment with Al_2O_3 -BHA composites extracts with varying doses of 25, 50, and 100% on cell viability of L929 fibroblast cells assessed by MTT. Data points were presented as mean \pm SD (* $p < 0.05$ $n = 5$)

4. CONCLUSION

Porous composite powders that comprise α -Al₂O₃, HA and TCP phases were produced by mixing the sol-gel derived ALOOH sol with the BHA powders obtained from the bovine bones. Chemical phase analyses exhibited the existence of the aforementioned bioceramic phases together in the structure. FT-IR analyses indicated the characteristic vibrations of P-O, CO₃⁻² and Al-O bands. SEM-EDS characterization studies showed that the CaP based phases and α -Al₂O₃ did not form necks between each other and loosely located so that a highly porous structure occurred, which may be beneficial for several biomedical demands. The biocompatibility of the Al₂O₃-BHA composite powder samples was tested by Indirect MTT assay on L929 cell line. All results underline the good biocompatibility of all Al₂O₃-BHA composite extracts with varying doses of 25 and 50%. Moreover, 1300AH20 extracts 100% exhibit statistically higher cell viability in comparison to 1300Al₂O₃. In the light of all these results, it can be deduced that Al₂O₃-BHA composites have considerable potential for biomedical applications.

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CONFLICTS OF INTEREST

No conflict of interest was declared by the authors.

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