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Effects of powder particle size on mechanostructure and cell viability of the synthesised bioceramic bone grafts

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Hydroxyapatite (HA) based biografts were syntesized at 0.68, 1.2 and 2.4 μm powder particle size by sol gel method. The effect of powder particle sizes on morphology, mechanical properties and in vitro cell viability of the biografts were analysed. Each composition at different particle sizes (0.68, 1.2 and 2.4 μm) were sintered at 1180 °C and quantitative phase analysis was characterized by FTIR, XRD and SEM-EDX. Mechanical properties were determined by axial compression and Vickers hardness tests. The highest compresion strengths and hardness values were obtained in samples having the particle size of 0.68 μm and such values were increased with decreased particle sizes. Furthermore, from in vitro Cell viability tests it was determined that no toxicity was detected at 0,1 and 0,3 μM concentrations at lower powder particle sizes. However, cell viability was significantly reduced in the syntesized samples having 2.4 μm particle size at 0,3 μM concentration.

Key words: Biograft, Hydroxyapatite, Mechanical properties, Cell viability.

Introduction

In orthopedics, defects in bone are generally replaced with natural bone (autografts), because artificial bone materials have some biocompatibility, bioaffinity and infection problems. Therefore, the studies are recently focused on fabricating better artificial grafts exhibiting the similar biological properties of natural bone that can be replaced with autografting [1]. Hydroxyapatite (HA) and β-TCP based biomaterials are widely used in hard tissue applications due to their good biocompatibility and osteointegration properties [2, 3]. It was reported that other ceramic based materials such as α-TCP tetracalcium phosphate, have promoted not as useful as bone replacement [4]. Hydroxyapatite (HA, $Ca_{10}(PO_4)_6(OH)_2$), is the main composition of bone and being widely investigated for possible medical applications due to its excellent biocompatibility and tissue bio-activity properties [5,6]. Therefore, various HA synthesizing techniques are worldwide used such as direct precipitation from aqueous solution, electrochemical deposition and sol-gel procedure, etc [7-14].

Systematic investigations on artificial biografts are still being investigated worldwide the to be used as hard tissue surgical applications in othopaedics. Sol-gel method is used extensively due to exhibiting some advantages such as simplicity of process, easy coating of complex shapes, low processing temperature with solid and thin films [14-17]. However, the major limitations of sol-gel processes are reported to the possible hydrolysis of phosphates and the repeatability problems encountered during coatings [18]. In this study, the influence of powder particle size on the HA-based syntesized bone grafts and the changes in morphology was analysed, and the mechanical properties and cell viability of the syntesized biografts have been evaluated with respect to different initial HA powder particle sizes.

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Materials and Methods

Sol-Gel / Graft Preparation

In order to syntesize Hydroxyapatite (HA) based biografts; 9.25 gr $Ca(OH)_2$ was dissolved in 27.9 ml distilled water and then 4.254 ml phosphoric acid (H_3PO_4, Merck, %99) was added into the prepared sol. The graft was determined by using the syntesized HA as starting powder at 1.67 Ca/P ratio. The final sol was stirred by a magnetic stirrer for two hours and then homogenized 20 minutes using an ultrasonic homogenizer (Cole Parmer-750W) until a white gel was obtained. The obtained jel was then filtered and aged over a night and finally the gel was dried in a furnace at 340 °C for three hours.

In order to fabricate different particle sized biografts, the mixed solution including the starting powders was adjusted by a buffer (44%wt NH_4OH + 56% wt H_3PO_4) with ethanol and deionized water and was stirred for 30 min. The pH of the solutions were ammended to 5 to 7 and 9, respectively by using an EDTA buffer. The

syntesized powders were pressed into the cylindrical dies (10 × 10 mm) at a pressure of 24 kN. To produce biograft discs at particle sizes of 2.4 µm (pH = 5), 1.2 µm (pH = 7) and 0.68 µm (pH = 9), respectively. The obtained gels are then dried in air for 24 h and prehated in oven at 120 °C, then all were sintered in a vacuum furnace with a heating rate of 5 °C/min at 1180 °C for 3 h. To show the influence of particle size on mechanostructure and cell viability on the syntesized HA-based biografts, a series of experiments were conducted using five specimens for each group including different particle sizes [14].

Characterization

FTIR(Alti Unicam Wattson1000) and XRD diffraction (Bruker D8 Advance, λ = 1,5406 Å) were used to identify the inorganic compunds and phases of fabricated biograft for various particle sizes measured by SEM. The syntesized biografts were characterized by Scanning Electron Microscopy (SEM, JSM-7001F) and XRD (Brucker).

Mechanical test

The syntesized powders at different particle sizes were isostatically pressed axialy into the cylindrical (10 × 10 mm) at a pressure of 24 N for 1 min. Four discs in each group were prepared and they were then sintered in air at 1180 °C for 3 h with a heating rate of 5 °C/min. The axial compression strengths of the sintered samples were measured via tensile tests (Shimadzu-5 kN) at 5 mm/min crosshead speed. Hardnesses of Vickers microhardnesses (Leica) of the syntesized biografts were measured at 20N load for 5 seconds. Four specimens and repeats were used for each group in hardness tests.

Cell Viability

Cell Viability tests was carried out on HA-based synthesized biograft samples with particle sizes having 0.68 µm, 1.2 µm and 2.4 µm. The concentration of the samples were reduced to 1 mg/ml with DMEM (Dulbecco's Modified Eagle Medium) using the osteoblast cells. The Cell Viabilities were performed by CellTiter 96 Aqueous One Solution Assay, uses the novel tetrazolium compound (3-(4, 5dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H -tetrazolium using 96-well plates. Then the cells (1 × 105 cells/ml) were seeded in 96-well plates and left to grow over night in humidified atmosphere containing 5% CO_2 at 37 °C and 90% humidity incubator and read at 490 nm. 10 µL of MTS solution (Sigma, USA) and 100 µL of culture environment was added into each well in the 96-well plate. The plates were incubated for selected periods with the extract 24 h, 48 and 72 hrs, respectively. The cell viabilities were measured for the syntesized and prepared samples after the incubation time periods for the selected extracts 24 h, 48 and 72 hrs, respectively.

Results

FTIR analyses

Fig. 1 shows the FTIR spectras of HA-based biograft samples at different particle sizes (0.68 µm, 1.2 µm and 2.4 µm). The combined FTIR spectrums of the dried gel shows broad peaks, typical of crystalized products with the characteristics bands of phosphate (961.95-1086.92 cm^{1-}) groups are distinguishable. The FTIR spectrums of HA-based biograft at different particle sizes shows characteristic peaks corresponding to PO_4^{3-} (1086.92, 1021.92, 961.95 cm^{1-}) vibrations, which indicates that the syntesized grafts are hydroxyapatite based biografts.

XRD analyses

The XRD patterns of HA based synthesized powders with different particle sizes, which was varied from 0.68 to 2.4 µm, are shown in Fig. 2. As shown in the XRD patterns, the peaks obtained after syntesizing processes correspond to HA and partially β-TCP implying the complete reaction between $Ca(OH)_2$ and phosphoric acid. In a comparison with the peaks given at the top of patterns, the crystalline phases were formed in the samples of biografts at different particle sizes.

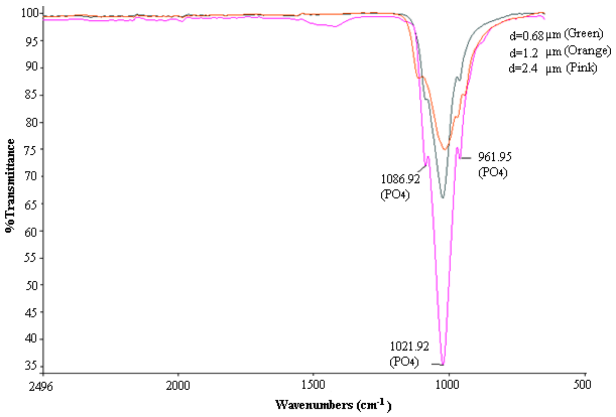


Fig. 1. FTIR spectrums for the syntesized biografts with different particle sizes.

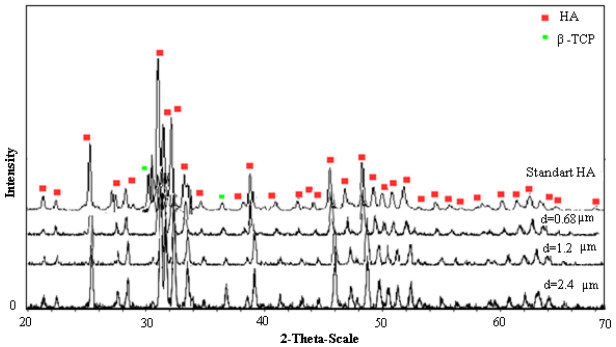


Fig. 2. XRD spectrums of the syntesized samples for different particle sizes.

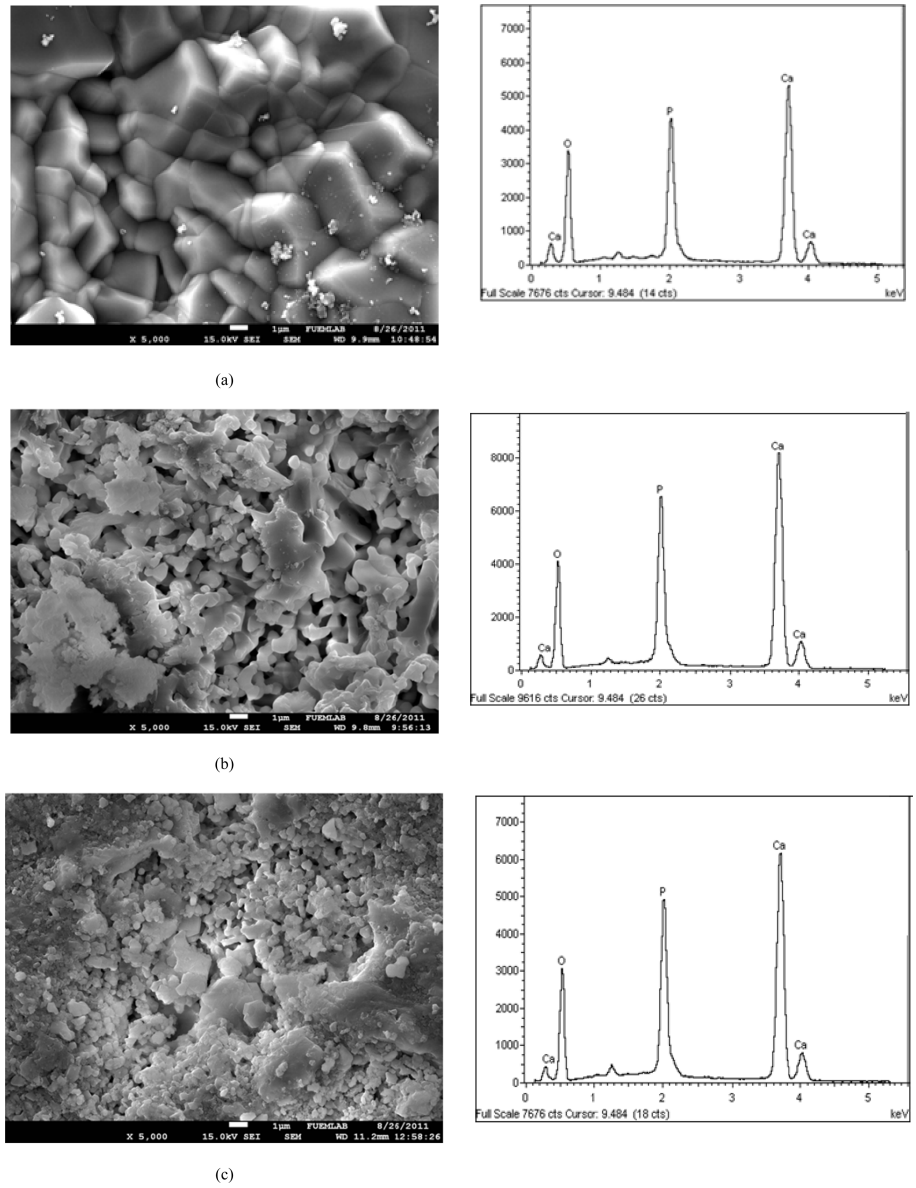


Fig. 3. SEM views and EDX results of the syntesized biografts at different particle sizes; a) 2,4 µm, (b) 1.2 µm, (c) 0,68 µm.

SEM/EDX

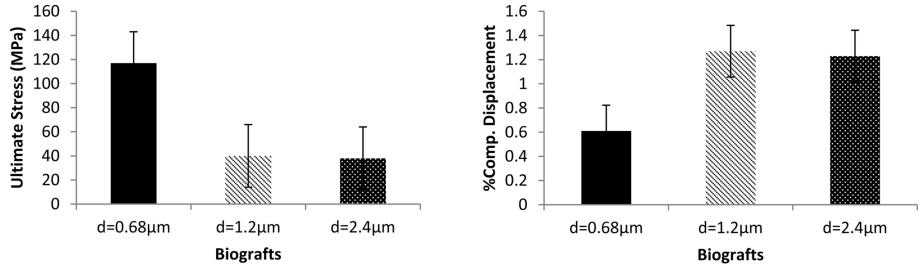
Fig. 3 shows the SEM images and EDX spectrums of the sol-gel synthesized HA samples obtained from the solutions corresponding to differrent particle sizes of 2.4 µm, 1.2 and 0.68, respectively. From the SEM images a high densification regime can be observed at lower particle sized biografts.

Mechanical Tests

In order to show the effect of particle size (d = 0.68, 1.2 and 2.4 µm) on the mechanical properties (compression strength and hardness) of the syntesized biografts. Five specimens and repeats were used for each group in both the compression and hardness tests. The influence of the synthesized biografts with different particle sizes (d = 0.68 µm, 1.2 µm and 2.4 µm) on compressive strength is plotted in Fig 4. It was seen from the ploted results that the stress and strain is inversely proportional. It was also observed that by applying higher pH during processing of these HA synthesis samples, it was possible to increase maximum strength together with decreasing particle sizes, which showed significant improvement in different particle sizes. Increase in particle sizes of these HA-based synthesed biograft samples indeed decreased the maximum strength.

Hardness

The microhardness of these HA synthesized samples having different particle sizes (d = 0.68 µm, 1.2 µm and 2.4 µm) were determined by a Vickers hardness tester from randomly five points on each samples. It can be observed from the Fig 5 that lower particle size caused an increase in hardness of HA the synthesed



(a) (b)

Fig. 4. Compression Stress (a) and Strain (b) variations for the syntesized biografts with different particle sizes.

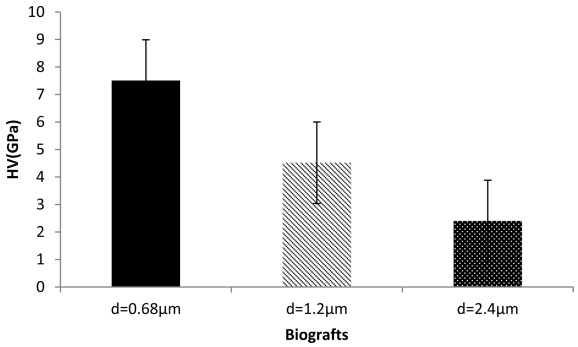


Fig. 5. Variation of hardnesses with different particle sizes.

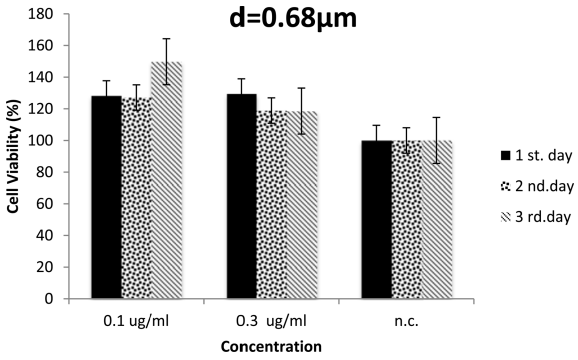


Fig. 6. The variation of cell viability with concentration and incubation times for the fabricated biografts. a) 0.68 µm b) 1.2 µm and c) 2.4 µm particle sizes (n.c: negative control).

samples. A maximum microhardness (7.51 GPa) was found for the sample having 0.68µm particle size, where the Vickers microhardness of HA samples increased with decreasing particle size.

Cell viability

HA synthesis with varying particle sizes at different concentration were cultured using the osteoblast cells for three days, followed by MTT assay. This assay was used to determine the number of living osteoblast cells on the matrices of each composition. The result found thorougout in vitro tests of cell viability is shown in Fig. 6a-c. At different concentration values (0.1-0.3 µM), the osteoblast cells grown well in the extract of all HA-based biograft samples with varying particle sizes. The results of MTT assays on matrices of different concentration values in the third day showed that the HA synthesized samples with varying particle sizes. Such MTT assay observation of the cell rate showed no cell viability at 0.1µM. When compared the cell viability of HA-based biograft samples (0.3 µM) with particle sizes (d = 0.68, 1.2 and 2.4 µm) were not observed (Fig. 6), whereas, cell viability of HA-based biograft samples having 2.4µm particle size was found to be generating lower cell viability.

Discussion

As shown in FTIR (Fig. 1) and XRD (Fig. 2) spectrums, after the syntesizing, it can be seen that the major peaks are belong to HA and minor ones to β-TCP phases. The crystalization and peak intensity was decreased at high powder particle sizes in the syntesized grafts. As seen from the Figure, no significant difference was detected from XRD patterns of HA synthesis samples at different particle sizes, however, the biograft with 2.4µm particle size generated poorer crystallinity. This was also reported in some studies in which different sintering temperature and additives promote formation of phases like HA and β-TCP [14, 19-22]. The formation of small amounts of β-TCP is advantageous as it allows ionic substitutions and thereby may enhance bioactivity of the material [23]. The crystallinity of the HA and β-TCP phases in biphasic chalcium phosphate (BCP) depends on the sintering temperature and the higher sintering temperature provided higher crystallinity [14, 24]. The typical spectrum for stoichiometric HA, showing PO^{4-}derived bands at 962-1087 cm^{1-} was also the present and no significant difference was observed in FTIR spectrums (Fig. 1).

SEM micrographs are shown in Figures 3a-c for different particle sizes and compositions. Such samples did not show clear demarcation in grain boundaries and their grain diameters could not be calculated. The SEM micrographs of 1.2 µm and 2.4µm particle size compositions does not correspond to complete sintering (Fig 3 b-c). In comparison of the syntesized biografts, depending upon the contribution rates (wt%), they showed different morphology and properties. From the morphologic views of HA-syntesized biografts at different particle sizes, the pore formations were

observed in between grains for the biografts at particle sizes of 1.2 μm and 0.68 μm, however, the biograft at 1.2 μm particle size gave a compact morphology. The pores were observed to be decreasing with increasing particle size.

From the compression tests, it was shown that, the compressive strength and Vickers microhardness increased with decreaed particle sizes (Figs 4 and 5). Additionally, the compressive strenght and Vickers microhardness of the sample having 0.68μm particle size is found to be the highest (Fig 5). It is well known that the compressive strength of HA depends on many parameters such as preparation conditions, methods of pre-sintering compaction, sintering time and temperature, porosity, microstructure (grain size and pore shapes) degree of high temperature decomposition of the HA [25]. In this study, at lower particle sizes, grain size of samples decreased but pore density in the structure increased. However, increased pore density of samples caused a decrease in both the Compression Strength and Vickers microhardness. The increasing grain size (1.2 and 2.4 μm) caused compression strength and microhardness to decrease because of sharp edges of grains. It was also shown that there was a positive correlation between the decreased particle sizes and mechanical behaviour of the HA synthesis samples shown in Figs 4 and 5. The average compression strength and Vickers hardness of human bone were reported to be 159MPa and 3.43GPa, respectively. The compression strength of the HA which is the main component of the human bone was also reported to be 249 MPa [18]. The mechanical properties such as compression strength and hardness of the syntesized HA-based biografts in this work were found to be between 38-119 MPa and 2.40-7.50 GPa, respectively. In overall results, the hardness and strength was increased with decreased particle size.

Through cell viability tests [14], it was observed that the osteoblast cells were nucleated and grown directly towards the samples. Fig 6 shows the cell viability and proliferations after three days of incubation time by the MTT assay. Statistical analysis revealed that no significant differences between the proliferation of osteoblastic cells was encountered on the all samples at 0.1 μM. However, the cell viability of the other samples were significantly increased at 0,3μM concentration with the exception in samples having 2.4μm particle size. It was also detected that the cell viabilty rates decreased in the first day of incubation time in 0.68 μm ve 2.4 μm particle sized biografts, however increased in the foloowing days when compared to the 1.2 μm samples. The results obtained from the cell viability tests performed on the syntesized biografts, the cell viability of the biografts having 0.68 and 1.2 μm powder particle size were better compared to 2.4 μm particle sized samples at 0,3 μM concentration.

Conclusions

HA powders with different particle sizes (0.68, 1.2 and 2.4μm) were synthesized via sol-gel method. Through the analysis, it was shown that the powder particle size was effective on the structure, mechanical property and cell viability. The HA-based biograft samples for particle sizes (0.68 and 1.2 μm) showed the best densification. A decrease in particle size enhanced the strength and hardness of the syntesized biografts. The maximum strength and microhardness values were found to be 119 MPa and 4.60 GPa, respectively for the sample having 0.68 μm particle size. Compression and hardness test results showed that increasing particle size decreased compressive strenght and microhardness of HA-based biograft sample. In vitro analyses carried out with a modified human osteoblast cell line showed that the syntesized graft compositions were non-toxic. The analysis also revealed that the best cell viability was obtained in the biografts syntesized at lower powder particle sizes e.g 0.68 μm and 1.2 μm.

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