Novel Fluorapatite-Forsterite Nanocomposite Powder for Oral Bone Defects

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Failing implants lead to osseous defects. Guided tissue regeneration made of bioactive ceramics has been used to promote bone formation in osseous deformation. The aim of this study was to prepare and to characterize the fluorapatite/forsterite nanocomposite powder for treatment of oral bone defects. In this study, these composite powders with different contents of forsterite nanopowder were prepared via sol-gel process. Characterization of prepared nanocomposite powders and their cytotoxicity evaluation were done and compared with pure forsterite and fluorapatite powders. Results showed that nanocomposite powders with crystallite size of about 21–24 nm were fabricated successfully by gel calcination at 600°C. Besides the non-toxicity effects of powders, nanocomposite containing 20 wt% forsterite significantly increased cell viability compared with control groups. According to these results, these nanocomposite powders might be suitable as bioactive material for oral bone defect.

Introduction

Bone defects around dental implants are often seen in extraction sockets, or around failing implants when implants are placed in areas with inadequate alveolar bone. In these defects, bone regeneration could improve the long-term prognosis implant by means of substitutes or bone grafts.

Bone fillers and guided tissue regeneration made of bioactive ceramics such as bioactive glass have been
used to promote bone formation in osseous deformation. Literature has shown that the release of ions such as Na, Ca, and Si ions from bioactive glass could control the cell progress leading to the differentiation and proliferation of bone cells, modulation of the expression of genes that regulate osteogenesis, and the synthesis of growth factors. Hydroxyapatite (HA) has been widely used in medical applications especially as bone filler. However, studies showed that substituting of hydroxylic groups (OH) by F ions could improve the mechanical strength, decrease the dissolution rate of HA and enhance bone tissue growth. In addition, results confirmed that presence of F ion could be suitable for dental application. 

Recent study shows that nanoscale forsterite (Mg$_2$SiO$_4$) is a bioactive and biocompatible ceramic. According to recent studies, forsterite has better mechanical properties than other bioactive ceramics that promote the use of this bioceramic in the oral bone defects treatment. In addition, similar to bioactive glass, forsterite contains essential ions that release in the biological environment leading to hydroxi-carbonate apatite layer on the surface of particles which have positive effects on the bone calcification. So, forsterite nanopowder is expected to be a suitable candidate as filler for oral bone defect treatment. According to the above-mentioned points, nanocomposite powder of fluorapatite and forsterite could be good candidate for oral bone defects.

Sol-gel technique is a unique method for fabrication of nanoparticles. This method has many advantages such as high reactivity of initial materials, lower sintering temperature compared with other powder preparation methods, and low cost. Therefore, the aim of this work was fabrication and characterization of fluorapatite-forsterite nanocomposite powder via sol-gel method and studying the effects of forsterite contents on the structural and morphological properties and cytotoxicity of fluorapatite nanopowder.

Experimental Procedures

Preparation of Fluorapatite-Forsterite Powder

To fabricate composite powder, forsterite nanopowder was fabricated, separately. The forsterite nanopowder was fabricated by sol-gel method using organic precursors; sucrose, and PVA described in a previous report. Briefly, water-based solutions of the magnesium salts and colloidal silica were prepared. The aqueous solution of sucrose (sucrose-to-metal ratio = 5:1 mol) was added to the precursor solution. After continuous stirring, PVA solution was added and the pH value was adjusted to 1 using nitric acid. Then, the solution was stirred at 80°C and aged for 24 h. After that, it was dried on a hot plate at 100°C and finally, the dried gel was calcined up to 800°C in a furnace for 2 h.

Calcium nitrate tetrahydrate (Ca(NO$_3$)$_2$·4H$_2$O, Merck, Whitehouse Station, NJ) as Ca-precursor, phosphoric pentoxide (P$_2$O$_5$, Merck) as P-precursor, hexafluorophosphoric acid, (HPF$_6$; Sigma-Aldrich, St-Louis, MO) and ethanol (Merck) were used for preparing fluorapatite powder. A predetermined amount of calcium nitrate was dissolved in ethanol on the stirrer. Simultaneously, phosphoric pentoxide was dissolved in ethanol on the stirrer. After that, the first solution was added into the second one while stirring. HPF$_6$ as a fluorine-containing reagent was added into the mixture. After about 15 min, forsterite powder was directly added in the stirred sol. In this way, three solutions with different amounts of forsterite nanopowders (10, 20 and 30 wt %) were prepared. Finally, the mixtures were continuously stirred for about 24–48 h (depending on the amount of forsterite) with the stirrer at room temperature to form a gel, aged for about 24 h, and dried in an oven at 80°C. As-dried gel was calcined at 600°C to remove polymeric materials and calcination. To study the cytotoxicity, pure fluorapatite nanopowder was fabricated similarly according to the above method.

Characterization of Composite Powder

The phase analysis was carried out using X-ray diffraction (XRD) (CuKα radiation: λ = 0.154056 nm at 40 kV and 30 mA). The obtained experimental patterns were compared to the standards collected by the Joint Committee on Powder Diffraction and Standards, which include cards for forsterite and fluorapatite.

The Scherer’s equation (Eq. (1)) was considered for calculating the crystallite size of the obtained composite. For this goal, three picks of each phase were selected for measuring in the XRD pattern.

$$B \cos \theta = \frac{0.89 \lambda}{t}$$

where $t$ is the apparent crystallite size (nm), $\lambda$ is the wavelength of the X-ray (for Cu tube is about
Transmission Electron Microscopy (TEM) technique was utilized to evaluate morphology and particle size of synthesized forsterite. Scanning Electron Microscope (SEM, Philips XL 30) equipped with energy dispersive spectroscopy (EDS) and X-ray map were used to characterize the morphology and agglomerates size of the powders and elemental mapping was used to investigate distribution of elements in the structure.

The functional groups of prepared powders were analyzed using Fourier Transform Infrared spectroscopy (FT-IR) in the range of 400–4000 cm$^{-1}$.

Cell culture

Human Osteoblast-like cells were isolated from bone by an enzymatic digestive process: The bones were washed three times in phosphate-buffered saline (PBS, pH = 7.4) and then minced into fragments. After washing the bone fragments three times with PBS, the chips of calvaria were treated with osteoblast-special cell culture for 90 min at 37°C to release osteoblast-like cells from the calvaria. The supernatants were centrifuged at 1000 rpm for 10 min, and then suspended in the DMEM F12 medium containing 10% fetal calf serum (FCS) with 1% penicillin/streptomycin, and incubated in a 75 cm$^2$ flask at 37°C under a humidified atmosphere consisting 5% CO$_2$. Culture media were refreshed every 3 days until the cells reached confluency. The cells were routinely subcultured by trypsinization (0.05% [w/v] trypsin and 0.02% [w/v] EDTA in PBS). The cells used in the study were second passage.

Cytotoxicity Assay

The cells proliferation on different substrates was determined by using the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. This method was carried out with a dilution of forsterite, fluorapatite, and composite nanopowder extract in contact with human osteoblast-like cells. Prior to test, nanopowders were washed in 75% ethanol solution, sterilized for 20 min under ultraviolet light, and autoclaved for 30 min at 120°C. For preparation of solution containing the composite extracts, the powders with the ratio of 200 mg/mL (the ratio of the powder weight and the PBS) were added to the PBS. After 24 h-incubation at 37°C, the mixture was centrifuged and the supernatant was collected.

The cell suspension with the density of 1 x 10$^4$ cells/mL was prepared and 180 µL of it was added to each well of a 96-well plate and incubated at 37°C and 5% CO$_2$. After 24 h cell incubation, 50 µL of extract solution of nanopowders was added to each well of the plate, respectively. The positive control was prepared by the cells in the medium supplemented with 10% FCS without the addition of diluted extracts in each well and the negative control was also prepared by 50 µL of cell medium supplemented with 10% FCS and 50 µL solution without cells.

After 7 days of incubation, cells were washed with PBS and then media were replaced with a basal medium containing MTT solution (400 µL DMEM + 40 µL MTT). After 4 h incubation at 37°C, in 5% CO$_2$, the medium was discarded and formazan was solubilized using DMSO. The plates were incubated for 5–10 min, aliquots were pipetted in to a 96-well plate and finally the absorbance of each well was measured at 540 nm using a spectrophotometric plate reader (Bio Rad, Madrid, Spain).

Statistical Analysis

All data were collected with $N=3$ and expressed as means ± standard deviation (SD) in each experiment. Statistical analysis was done by two-way ANOVA with Duncan test. Differences were considered statistically significant at $P < 0.05$.

Results and Discussion

Figure 1 shows the TEM micrograph of forsterite nanopowder. The particles of forsterite powder are homogenous and nearly spherical mainly with a size of 18–40 nm. In addition, clear boundary and smooth surface can be observed.

Figure 2 illustrates X-ray diffraction patterns of the forsterite nanopowder along with composite powders and fluorapatite calcined at 600°C. According to standard card of fluorapatite (JCDP#15–0876), fluorapatite diffraction peaks were observed at pure fluorapatite and composite samples. At this temperature, the well-crystalline fluorapatite was fabricated without any impurity. By adding forsterite powder, the new peaks were observed. In comparison with the pure forsterite pattern
and according to the standard card of forsterite (JCPDS#34–0189), they belonged to the forsterite phase. By increasing the amount of forsterite nanopowder, the intensity and number of forsterite peaks increased. No other peaks were observed, which shows that there was no other reaction between the fluorapatite precursors and forsterite powder. The crystallite sizes of fluorapatite and forsterite powder estimated by Scherer’s equation were about 21–24 nm which shows two powders are nanostructured and no grain growth happened during calcination.

The SEM micrographs of nanocomposite powders along with pure forsterite powders are shown in Fig. 3. Flourapatite powder showed the accumulated fine particles which were strictly agglomerated and interconnected into the surface of flake-like larger agglomerates. The nano scale nature of the sol-gel derived nanopowders causes intense agglomeration of particles with size of about 2–5 μm. As shown in Fig. 3, the agglomerated particles are composed of very fine particles. However, by increasing the amount of forsterite nanopowder in nanocomposites, the agglomeration decreased.

To estimate the distribution of forsterite in the flourapatite matrix, powders were mounted and BSE micrograph of powders were studied. Figure 4 shows the BSE micrograph and EDS results of nanocomposite powders. Fluorapatite particles appear brighter than forsterite ones because of their higher molecular weight. The agglomerate sizes of fluorapatite phase in the composites containing 10, 20, and 30 wt% of forsterite were about 5–20 μm, 5–15 μm, and 1–6 μm, respectively, which shows the positive effects of forsterite on reducing the agglomeration of florapatite.

The EDS results illustrate that the particles of composite are composed of calcium, phosphorus, and also Mg and Si. Mg and Si ions originate from the forsterite and two other elements are related to fluorapatite particles. No other peaks are identified in this spectra revealed any impurity in fabricated composites. Furthermore, the molar raitos of Mg and Si ions were about 2 in all samples, which corresponds to forsterite. Also, the molar ratio of Ca and P is almost likely 1.67 which is approximately equal to this ratio in fluorapatite. The stoichiometry Ca/Mg weight ratios for composite samples containing 10, 20, and 30 wt% forsterite were 10.48, 4.65, 2.71, respectively. These ratios for mentioned composites, according to the spectra, were about 10.21, 4.67, 2.86, respectively, which confirmed the correct weight ratios of fluorapatite and forsterite contents in different composites.

Figure 5 shows the results of elemental mapping of fluorapatite-20 wt% forsterite nanopowder. It can be seen that the elements have almost covered the entire surface of the sample, homogenously. The Si and Mg spots partially overlapped, which corresponds to the dispersed forsterite in the structure. These results show homogenous distribution of both forsterite and fluorapatite powder in the composite.

The FT-IR spectra of the prepared composite powders with different forsterite contents are shown in Fig. 6. In all the spectra, the characteristic peaks of forsterite and fluorapatite could be noticed. The P–O stretching 𝜈₁ is observed at 960 cm⁻¹ and O–P–O
Fig. 3. SEM micrographs of (a) flourapatite and flourapatite-forsterite composite containing (b) 10, (c) 20 and (d) 30 wt% forsterite.

Fig. 4. BSE micrographs and EDS results of flourapatite-forsterite composite powders containing (a) 10, (b) 20 and (c) 30 wt% forsterite.
bending $\nu_2$ is observed at 470 cm$^{-1}$, respectively. The other important characteristic peak of $\text{PO}_4^{3-}$ tetrahedral groups is the P–O stretching $\nu_3$ vibration mode, which was visible at 1026 and 1082 cm$^{-1}$. The bands at 572 and 601 cm$^{-1}$ are the $\nu_4$ vibration mode of the phosphate group. In addition, a doublet appears at 1420 and 1462 cm$^{-1}$ corresponding to the $\nu_3$ vibration mode and the band centered at 869 cm$^{-1}$ for $\nu_2$ vibration mode of the carbonated groups. These peaks showed that the prepared fluorapatite contained some carbonated groups in $\text{PO}_4^{3-}$ sites of apatite lattice. As a result of decomposition of used alcohol hydrocarbons as solvent, carbonates imported into the hydroxy apatite structure and in the wet chemical methods for fabricating fluorapatite such as sol-gel make carbonate hydroxyapatite. As carbonates are constituents of bone tissue structures, its presence could improve the similarity and biological response of the repaired fluorapatite to the bone structure.

According to this spectrum, the absence of OH$^-$ band at 630 cm$^{-1}$, and the presence of the band at 738 cm$^{-1}$ corresponding to the shifting of OH$^-$ liberation mode indicate complete transformation of HAp into fluorapatite. By increasing the amounts of forsterite contents, forsterite peaks appeared or their intensity increased. For example, the intensity of the absorption peaks at 873 cm$^{-1}$ and, 507 cm$^{-1}$ was related to SiO$_4$ groups and at 475 cm$^{-1}$ attributed to MgO$_6$ octahedral it increased. However, other peaks such as those at 961 cm$^{-1}$ and, 616 cm$^{-1}$ appeared on increasing the amount of forsterite up to 30 wt%. $\text{PO}_4^{3-}$ tetrahedral groups appeared in the broad spectrum.

In addition to these characteristic peaks, another peak was observed at 1360 cm$^{-1}$ and its intensity increased by increasing the forsterite content. This peak most likely is due to weak bonding between both the existing ceramics.

Figure 7 shows the results of the MTT assay of nanocomposite and pure forsterite and fluorapatite powder after 3–7 days of cell culturing. The OD values are indicators of the relative number of cells. According to other reports, if the OD value has no significant differences with the positive control, it means that samples
have less growth inhibition effects on cells and, on the contrary, if the value is close to the negative control, growth inhibition effects on cells is expected. It could be observed that the cell density increased by increasing incubating time in all samples, which proved that pure forsterite and fluorapatite nanopowders along with their composite are non-cytotoxic and their composite could support human osteoblast proliferation. Generally, the dissolution extracts of composite powder show higher osteoblast proliferation than pure ceramic powders. It could be seen that the cell proliferation was significantly higher than the negative control ($P < 0.05$) after incubating for 5 days in the nanocomposite containing 20 wt% forsterite nanopowder. As can be seen, it has cell viability approximately two times of the positive samples, after 3 days of incubation. Furthermore, by increasing forsterite nanopowder concentration, the stimulatory effect decreased. Although the proliferation rate of cells in contact with positive control after 7 days of cell incubation was higher than that of the other samples, no significant differences with the positive control ($P > 0.05$) were observed. The higher rate of proliferation at 7 day of incubating might be the result of reduction of ion released in the solution.

Recent studies have published the stimulatory effects of Ca-, Si-, and Mg-containing ionic products from the dissolution of MgO-SiO$_2$-CaO ceramic systems at a certain concentration range on the osteoblast-like cells. Results showed that the released Si ions could promote mineralized nodule formation of human primary osteoblasts. In addition, it could stimulate gene expression and osteoblast proliferation. Mg ion is the other ion released during the immersion of forsterite nanopowder in solutions. Results showed that, Mg ion closely associated with mineralization of calcined tissues and indirectly influenced mineral metabolism. In spite of the important role of Mg ion dissolution in the bioactivity of inorganic biomaterials, at high concentration of extracts, the inhibitory effect on cell proliferation might be observed. These results are reported previously in the cytotoxicity evaluation report of forsterite nanopowder. Besides forsterite, presence of fluorapatite in the composite powder led to the release of Ca and P ions in the solution. Reports showed that the released Ca ions could induce osteoblast proliferation and chemotaxis through binding to a G-protein coupled with extracellular calcium sensing receptor. According to the above results, the release of inorganic ions, such as Ca, Mg, and Si ions together could stimulate the cell proliferation which might be one of the evaluation criteria for bioactivity of the biomaterials. However, to identify the mechanisms of the specific stimulatory effects of different ions and ion combinations, further investigations are required. So, Si ions from composite powder containing 20 wt% forsterite nanopowder significantly stimulated osteoblast proliferation.

Conclusion

In this study fluorapatite-forsterite nanocomposite powder was fabricated and characterized for oral bone defects. Nanopowder forsterite (18–40 nm) fabricated using sol-gel method was used for this purpose. Composite nanopowder with 10, 20, and 30 wt% forsterite nanopowder was prepared using sol-gel method. Results showed that calcined gel at 600°C produced powders with forsterite and fluorapatite without impurity. The crystallite size of fluorapatite and forsterite reported about 21–24 nm. Presence of forsterite decreased powder agglomeration. In addition, the composite powders and the pure ceramic powders are non-cytotoxic and nanocomposite powder containing 20 wt% powder showed the best biocompatibility. In conclusion, fluorapatite-forsterite nanocomposite powder possessed good biocompatibility and might be suitable for oral hard tissue repair.

Acknowledgment

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References


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