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Published in:
Materials Science and Engineering C

Link to article, DOI:
10.1016/j.msec.2020.111611

Publication date:
2021

Document Version
Peer reviewed version

Link back to DTU Orbit

Citation (APA):

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PII: S0928-4931(20)33529-3
DOI: https://doi.org/10.1016/j.msec.2020.111611
Reference: MSC 111611
To appear in: Materials Science & Engineering C

Received date: 12 February 2020
Revised date: 1 October 2020
Accepted date: 7 October 2020

Please cite this article as: N. Golafshan, M. Alehosseini, T. Ahmadi, et al., Combinatorial Fluorapatite bioceramic substituted with strontium, magnesium and silicon ions for mending bone defects, Materials Science & Engineering C (2020), https://doi.org/10.1016/j.msec.2020.111611

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Combinatorial Fluorapatite bioceramic substituted with strontium, magnesium and silicon ions for mending bone defects

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Abstract

In bone tissue engineering, ionic doping using bone-related minerals such as magnesium (Mg) or strontium (Sr) is a promising strategy to make up for the inherent disadvantages (low solubility) of various apatite-based materials such as Fluorapatite (FAp) and Hydroxyapatite (HA). Therefore, number of studies in recent years have tried to address the lack-of-methodology to improve the properties of bioceramics in the field. Even though, the outcome of the studies has shown some promises, the influence of doped elements on the structures and properties of in vitro and in vivo mineralized FAp has not been investigated in detail so far, and thus, it is still an open question mark in the field. In this work, strontium modified fluorapatite (Sr-FAp), magnesium and silica modified fluorapatite (Mg-SiFAp) nanopowders were synthesized using a mechanical alloying methodology. Results showed that the doped elements could decrease the crystallinity of FAp (56%) to less than 45% and 39% for Sr-FAp and Mg-SiFAp, respectively. Moreover, in vitro studies revealed that Sr-FAp significantly enhanced osteogenic differentiation of hMSCs, after 21 days of culture, compared to Mg-SiFAp at both osteogenic and normal media. Then, in vivo bone formation in a defect of rat femur filled with a Sr-FAp and Mg-SiFAp compared to empty defect was investigated. Histological analysis revealed an increase in bone formation three weeks after implanting Sr-
FAp compared to Mg-SiFAp and the empty defect. These results suggest that compared to magnesium and silica, strontium ion significantly promotes bone formation in fluorapatite making it appropriate for filling bone defects.

1. Introduction

Bone disease is still one of the big burdens for healthcare systems worldwide which particularly impacts older people. One of the treatments for the healing of bone defects caused by trauma involves the implantation of synthetic bioceramics that could support bone regeneration. Among various bioceramics, synthetic hydroxyapatite [HA, Ca_{10}(PO_{4})_6(OH)_2] mimicking the structure of natural bone has received significant attention in the field [1–3] and could promote bone growth and improve bone induction [4,5][6]. However, in order to control the dissolution rate of HA which is stable in vivo, Fluoride (F\textsuperscript{-}) ion substituted OH ions in the chemical structure of HA has shown great promise. Błaszczyk et al [7] showed that partially or total substitution of hydroxyl groups (OH\textsuperscript{-}) in HA with small doses of fluoride (F\textsuperscript{-}) can improve HA solubility in vivo and improve the treatment of osteoporosis and various vertebral body fractures [7].

To enhance the bioactivity and biocompatibility of FAp, various ions can be doped in its crystal structures [8,9]. These ions which are exist in the bone such as strontium (Sr\textsuperscript{2+}), magnesium (Mg), silica (Si), zinc (Zn), and cobalt (Co) [10,11]. Even though the amount of these elements is low in natural bone, they do have a great impact on the physico-chemical properties and regeneration of bone [12]. For instance, Mg\textsuperscript{2+} deficiency restricts the growth of osteoblasts leading to a decrease in bone mass density. Thus, Mg\textsuperscript{2+} also plays a key role in mediating cell-extracellular matrix interaction [13]. In this respect, Cai et al. [14] demonstrated that the incorporation of Mg\textsuperscript{2+} ions to FAp bioceramics improved the ability to stimulate new bone formation. In addition, previous studies have demonstrated that Si ions
could promote bone growth [15,16]. In fact, Si could promote the proliferation and differentiation of rat bone marrow stromal cells (rBMSCs) and improve the collagen synthesis process of osteoblasts [17]. In order to enhance the osteoinductivity of FAp, it has been reported that Strontium (Sr$^{2+}$) could act as an inhibitor of osteoclasts resorption as well as a stimulus to induce osteogenesis [18].

Due to the above-mentioned properties and valuable aspects of Sr$^{2+}$ in bone formation, Sr-based bioceramics have gained extensive attention as a new class of bioceramic materials [19–21]. For instance, recently, it has been shown that Sr$^{2+}$ ions doped into calcium phosphate ceramics. The osteoprogenitor cells proliferate and differentiated at the relevant doses of Sr$^{2+}$ ions (2.21 at%) [18]. Accordingly, by incorporation of various ions, the proliferation and differentiation of the cells could be influenced. In a similar study, it was shown that doping the ions such as Mg, Zn, Si, and Sr in the hydroxyapatite structure, the chemical, physical, and biological properties are influenced [22].

Despite the wide interest towards using Sr in the field, the synergistic role of various ions on the ability to stimulate bone formation has not been fully investigated yet. For this reason, this study aimed to investigate the role Mg, Si and Sr ion substituted FAp. In this regard, two types of fluorapatite-based nanopowders were synthesized: Sr doped fluorapatite and Mg-Si doped fluorapatite and their potential as bone substitutes were investigated both in vitro and in vivo.

2. Materials and methods

2.1. Materials

Calcium hydroxide (Ca(OH)$_2$), calcium fluoride (CaF$_2$), phosphorous pentoxide (P$_2$O$_5$), strontium carbonate (SrCO$_3$) and magnesium hydroxide (Mg(OH)$_2$) were purchased from Merck, Germany and silica oxide (SiO$_2$) was obtained from Sigma-Aldrich. For the in vitro cell culture experiments, human Mesenchymal stem cells were supplied by the Pasteur
Institute in Iran (passage 5). Dulbecco's modified Eagle's medium (DMEM/F12), fetal bovine serum (FBS), penicillin/streptomycin and phosphate buffer saline (PBS) were bought from Bioidea, Iran. Dexamethasone, acetic acid, ascorbic acid, ammonium solution, b-glycerophosphate, Glutaraldehyde, MTT (3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide), Resazurin, Alizarin red S, Glutaraldehyde, and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich.

2.2. Synthesize of fluorapatite based powders

Two kinds of ceramic powders consisting of Sr-doped FAp (SrFAp) and Mg and Si-doped FAp (Mg-SiFAp) were synthesized via the mechanical alloying process, according to the protocol described elsewhere [23]. In this regard, the precursors of the SrFAp and Mg-SiFAp powders were weighted and mechanochemical activation for both powders was performed in a planetary ball mill (Fretch Pulverisette 5) for 12 h with the ball to powder ratio of 25:1 and the speed of 250 rpm. The precursor types and amounts are presented in Table 1.

2.3. Physical and chemical Characterization

Phase characterization of synthesized powders was evaluated by X-ray diffractometer, XRD (X’Pert Pro X-ray diffractometer, Phillips, Netherlands) performed with CuKα radiation (λ = 0.154 nm, 40 kV, 40 mA). Based on the XRD patterns, the crystallographic parameters, crystallite size, and crystallinity of each sample compared with FAp (24) as a reference value, were estimated. Fourier transform infrared spectroscopy (FTIR, Bruker tensor) was used to characterize the functional groups and chemical composition of the powder over a range of 650–2000 cm⁻¹ and resolution of 2 cm⁻¹. The morphology of the synthesized powders was evaluated using a scanning electron microscope (SEM, Philips XL30) at an operating voltage of 20 kV and electrical current of 10 mA. The particle size of ceramic powders was studied using a Transmission Electron Microscope (TEM) (Zeiss 100 kV). Before imaging, following
the dispersion of samples in acetone using ultrasonication for 15 min, the suspensions were put on the carbon-coated copper grid and the grids were dried at room temperature.

Apatite-formation ability of the synthesized powders was evaluated by immersing the ceramic discs in simulated body fluid (SBF). The synthesized powders were uniaxially pressed (N=50 for 5 min) to fabricate ceramic disks with 12 mm in diameter (n=3). The obtained disks were then soaked in SBF (pH=7.4) at 37°C for 7 days (n=3), while the ratio of disc surface area to SBF solution volume was kept 0.1 cm²/ml. The concentration of released Calcium and phosphorous ions in SBF was quantified using inductively coupled plasma atomic emission spectroscopy (ICP-AES).

2.4. Cell culture

Human mesenchymal stem cells (hMSCs) obtained from healthy donors (according to the existing regional and national ethical guidelines), were cultured in DMEM/F12 supplemented with 10 % (v/v) fetal bovine serum (FBS), 1 % (v/v) penicillin-streptomycin (passage number = 3). Prior to cell seeding, the compacted discs (diameter= 5 mm, n=3) were fabricated and soaked in 70 % (v/v) ethanol (30 min), rinsed with PBS (3x) and UV-sterilized for 1 h each side. Human MSC cells were seeded on the disks at a density of 1 × 10⁴ cells in a 96 well-plate. The disks were cultured in normal media for 7 days while the media was changed every 3 days. On day 7, a part of the ceramic discs was cultured in osteogenic media (normal media supplemented with 50 mg/ml ascorbic acid, 100 nM dexamethasone, and 10 mM b-glycerophosphate), while others have been cultured in normal media.

2.4.1. Proliferation and osteogenic differentiation of human MSCs

To evaluate the proliferation rate of MSCs on the discs, MTT colorimetric and Resazurin assays were performed. After 1, 5, 10 and 15 days of culture, the MTT and Resazurin assay were performed, according to the following. For MTT assay, the cell-discs samples (n = 3 per group) were incubated with 0.5 mg/ml MTT solution in PBS for 4 h. Finally, the absorbance
of dissolved formazan using dimethyl sulfoxide (DMSO) was measured at 570 nm in a microplate reader (Bio-Rad, Model 680 Instruments). The relative viability at each time-point was described as below: 

\[
\text{Relative cell viability (\%)} = 100 \times \frac{A_{\text{Sample}}}{A_{\text{TCP}}}
\]

The values indicated the cell proliferation relative to the control groups (TCP).

The Resazurin assay is based on the reduction of Resazurin, so after discarding the culture medium from samples, Resazurin solution (10 μg/ml) was added to each sample and kept in an incubator until the color of the Resazurin solution was changed. Subsequently, the absorbance of the solution was read at 630 nm using a microplate reader.

The osteogenic differentiation of MSCs in normal media (days 7, 14, 21, and 28 of culture) and differentiation media (days 14, 21, and 28 of culture) was assessed using alizarin red S staining (n=3). At the specific time point, after fixation of the cell-discs in 10%(v/v) formalin solution, the Alizarin red solution (400 mM in PBS) was added to each sample on a shaker for 20 min. After washing with water, they were studied using a stereoscope and their images were captured by a digital camera (Moticam 480). For quantitation of alizarin staining, the samples were immersed in 10%(v/v) acetic acid for 30 min at room temperature and then shacked for 30 min. Subsequently, the samples were kept at 80°C for 10 min and immediately in an ice bath for 5 min. Afterthought, the tubes were centrifuged for 15 min at the speed of 4300 rpm and the supernatants transferred to 10%(v/v) ammonium solution. The absorbance of samples was read by a microplate reader at 405 nm. In order to investigate the role of various ions released from samples on hMSCs behavior, the concentration of Sr, Ca, Mg and P ions were collected from culture media at different time-points (14 and 21 days) by ICP-AES, quantified and displayed in figure.

Cell morphology in normal and osteogenic media (day 28) was evaluated using SEM observation (n=3). The samples were fixed with 2.5 % (v/v) Glutaraldehyde solution for 2 h. After rinsing with water, the samples were further dehydrated through gradient
concentrations of ethanol for 5 min each solution. Finally, they were air-dried, gold-coated and evaluated by SEM. Additionally, to confirm the chemistry of the deposited matrix, SEM coupled with energy–dispersive spectroscopy (EDS) was performed after 28 days of culture in osteogenic medium.

2.5. In vivo studies

The surgical procedures have been precisely accomplished according to the provisions of the protocol of the Ethics Committee at Isfahan University of Medical Sciences on the care and use of animals for scientific purposes (ethical grant number = 52268). All in vivo study were performed in rat femoral defects according to the previous studies [23,24]. The ceramic powders (0.2 g) were compressed into discs and sterilized by gamma irradiation for 30 min before the surgery. Fifteen healthy Albino female rats with an average body weight of 300 gr were divided randomly into five groups and used in the animal experiment. The compressed bioceramics powder were implanted in both femurs of the same animal, and all animals were anesthetized after two and three weeks of implantation. Each animal was implanted with the same bioceramic combination using both femurs in the animal. Three of the animals were used as a control - meaning that nothing was implanted in the femur defects (Empty defects; the control group). Before the experiment, buprenorphine (0.1 ml/kg) was injected into the rats for pain relief. Consequently, the animals were anesthetized with an intraperitoneal injection of 3% coupled with oxygen prior to the surgery. A drill hole defect with a diameter of 2 mm and a depth of 3 mm was made in the lateral Femoral Condyles, perpendicular to the long axis of the femur. The defects were washed by physiological saline from the remaining bone fragments. The ceramic powders including Sr-FAp and Mg-SiFAp were pressed into the defect, while the defects in the third group were remained empty. Then, the wounds were rubbed with 5% povidone-iodine disinfectant to prevent infections. Animals were sacrificed by deep anesthesia, 2 and 3 weeks after operations. Bone formation was evaluated by
histology and immunohistochemistry. Samples were fixed in 4% paraformaldehyde before embedding in paraffin. The slides were stained with hematoxylin-eosin (H&E) and Masson’s trichrome methods according to standard protocols [25]. The slides were observed by a light microscope (HP cx21) and the amount of newly formed bone after 2 and 3 weeks were quantified using by ImageJ 1.52n based on the difference in the threshold of the histological images.

2.6. Statistical analysis

Results were expressed as Mean ± Standard deviation. Prior to the ANOVA tests (p_value < 0.05), the data passed the normality test (D'Agostino-Pearson Test, alpha=0.05). The data were subsequently analyzed using Prism 6 software with a 5% significance level (p < 0.05).

3. Results and discussion

3.1. Characterization of ceramics powders

In order to evaluate the role of ion substitution on the structural properties of powder, XRD patterns of samples were investigated (Fig. 1a). The characteristic peaks -(211), (300), (002)- were detected at both Sr-FAp and Mg-SiFAp powders were related to FAp phase, Ca₅(PO₄)₃F [26]. However, the position of these characteristic peaks changed, depending on the sample type. For instance, the characteristic peaks of Mg-SiFAp shifted to higher angles compared to FAp, while they were shifted to lower angles for Sr-FAp. This can be explained by the differences in ionic radius between Mg²⁺ (0.065 nm) and Sr²⁺ (0.113 nm) with Ca²⁺ (0.099nm) and Si⁴⁺ (0.042 nm) with P⁵⁺ (0.035 nm) [19,25]. It could be concluded that the lattice parameters changed due to the substitution of Ca²⁺ with Mg²⁺ or Sr²⁺ ions and P⁵⁺ with Si⁴⁺ ions in the lattice of FAp. Both samples, Sr-FAp and Mg-SiFAp, revealed nanostructures (crystallite size 55 and 40 nm) with a crystallinity of about 45% and 39%, respectively (Fig.
1b). We speculate, that the lower crystallinity of Mg-SiFAp could be caused by the substitution of two elements in the structure of FAp instead of one. Indeed, a number of studies have demonstrated similar behavior in previous studies entailing combinatorial ion substitution in apatite-coatings. Moreover, the incorporation of Sr in FAp crystal resulted in the formation of a stronger bond between Sr-OH compared to that of Ca-OH, which resulted in a decrease in the lattice energy. In addition, substitution of Mg and Si in FAp lattice led to a broadening and decrease in the intensity of the XRD peaks (Fig. 1a) compared to that of FAp and Sr-FAp patterns, which may be attributed the increase in the lattice energy of Fap (showed by red arrows).

FT-IR spectra of Sr-FAp and Mg-SiFAp (Fig. 1c) confirmed that presence of a crystalline FAp. The major characteristic absorption peaks of the $\text{PO}_4^{3-}$ group belonging to $\nu_3$ vibration peak appeared in the 1000-1100 cm$^{-1}$ region in spectrum of Mg-SiFAp [27], while it was detected as two separate absorption peaks at 1041 and 1092 cm$^{-1}$ in the spectrum of Sr-FAp. Moreover, the $\nu_1$ absorption peak observed as a shoulder at about 960 cm$^{-1}$ in both samples regarding $\text{PO}_4^{3-}$. In addition, the absorption peaks at 1421 and 1459 cm$^{-1}$ ($\nu_2$) and a small absorption peak at 872 cm$^{-1}$ ($\nu_3$) of Mg-SiFAp spectrum revealed the presence of carbonated groups in phosphate sites in FAp structure. There was no evidence of these peaks in Sr-FAp samples. Moreover, according to Fig. 1c, the intensity of $\text{CO}_3^{2-}$ and $\text{PO}_4^{3-}$ absorption peaks were decreased by incorporation of Si, confirmed the incorporation of $\text{SiO}_4^{4-}$ groups into the FAp lattice [28]. In addition, incorporation of Sr in FAp lattice created a broader P-O bonds than FA, as similarly reported previously [29].

To evaluate the morphology of ceramics powder, TEM image of Mg-SiFAp and Sr-FAp are presented in Fig. 1d. In this respect, Mg-SiFAp particles with spherical shape morphology with the size of 22 ±13 nm was seen to become agglomerated together. On the other hand, Sr-FAp powder consisted of relatively uniform spherical particles with average size of 44±15
nm. These results confirm that the incorporation of larger ions such as Sr$^{2+}$ into the FAp crystals led to greater distance of Sr-hydroxyl than that of Ca-hydroxyl, which is in accordance to previous works [30].

![Figure 1](image1.png)

**Figure 1.** (a) XRD patterns and (b) The table showed the crystallite size, crystallinity degree and lattice parameters (a and c) of Sr-FAp and Mg-SiFAp powders compared to FAp reference. (C) FT-IR spectra of Sr-Fap, Mg-SiFAp and FAp powders. (d) TEM images of Sr-FAp and Mg-SiFAp powder.

### 3.2 In vitro bioactivity evolution of bioceramics

The bioceramics applied for bone regeneration should represent high reactivity in a native environment to fulfill their promise and enable optimal bone-regeneration. In this regard, to evaluate the ability of bone-bonding capacity of the powders, the capability of apatite formation on its surface evaluated in a simulated body fluid (SBF). According to the SEM analysis, a layer of Ca-P deposition was deposited on the surface of both Mg-SiFAp and Sr-FAp disks after 7 days of immersion in SBF (Fig. 2a). The tiny particles scattered over the surface of Mg-SiFAp and Sr-FAp corresponded to bone-like apatite. There were no
morphological differences between apatite formed on the surface of the samples which could be due to the constant Ca/P molar ratio in all solutions. In addition, according to the results of ICP, the dissolution rate of Ca$^{2+}$ ions from Mg-SiFAp was higher than Sr-FAp at day 7 which is not statically significant (Fig. 2b). However, this might be due to the dissolution-precipitation reaction on Mg-SiFAp disks in SBF. As mentioned in some previous studies [31,32], high doses of metallic ions such as magnesium and strontium have been associated with toxicity in the human body. Fortunately, our ion release results reveal that the ions concentration releasing in the media are too low to facilitate any toxic responses [33,34].

3.3 Cell culture

The metabolic activity increased from day 1 to day 15, for both Sr-FAp and Mg-SiFAp (fig. 2c). Moreover, while the cells were active in both normal and osteogenic media, less cell proliferation capacity could be detected in osteogenic media compared to normal medium. For example, after 15 days of culture, the metabolic activity on the Mg-SiFAp significantly increased and reached to 123±5 % (control) and 100±13 % (control), in normal medium and osteogenic medium, respectively (P<0.05). Moreover, the metabolic activity on the Sr-FAp sample was enhanced to 137±15% and 117±10%, in normal medium and osteogenic medium, respectively (P<0.05). Moreover, the metabolic activity on the Sr-FAp sample was enhanced to 137±16% and 117±0%, in normal medium and osteogenic medium, respectively (P<0.05). The proliferation of human MSCs on Sr-Fap and Mg-SiFAp samples was also evaluated by Resazurin assay in normal and osteogenic mediums (Fig. 2d). This assay confirmed that the proliferation of cells increased on various samples with increasing culture time in normal media (P < 0.05). Moreover, the proliferation rate of hMSCs on Sr-FAp was greater than those on Mg-SiFAp. For example, after 15 days of culture in normal medium, the fluorescent Resazurin measurement for Sr-FAp and Mg-SiFAp were 133±8 and 119±5, respectively. Furthermore, the cell proliferation in osteogenic media was less than in normal
media which was due to that fact that the cells in osteogenic media were differentiating toward osteoblasts [35]. According to the data presented in Fig. 2, we can therefore confirm that various apatite substituted ions have different roles on the cell growth. Si is an initiator of mineralization and bound to glycosaminoglycan which plays a crucial role in forming cross-links between collagen and proteoglycan. In a similar vein, studies have shown that Si substitution into the HAp crystal lattice altered surface charge and enhanced cell proliferation and early cellular attachment of mesenchymal stem cells. Substitution of Si also effectively induced cell proliferation, adhesion, and differentiation as compared to pristine HAp – notably it was found that Si can accelerate bone healing [36]. Moreover, researches have demonstrated that Sr$^{2+}$, like calcium, can act as an agonist on the calcium-sensing receptor and in turn promote cell replication, differentiation, and survival [37]. Based on these results, despite the significant role of released ions on the cellular behavior, there was not significant difference between the results of cell proliferation. Therefore, these ions did not meaningfully change the significant characteristic property compared to each other.
Figure 2. SEM images of (a) Sr-FAp and Mg-SiFAp powders after 7 days soaking in SBF. (b) Ca, P, Sr, Mg and Si concentration of Sr-FAp and Mg-SiFAp after 7 days immersion in SBF. (c, d) Proliferation of MSCs cells on Sr-FAp and Mg-SiFAp substrated cultured through (c) MTT and (d) Resazurin assays evaluated in normal (-inducing) and osteogenic (+inducing) mediums.

To confirm the differentiation of MSCs, alizarin red staining was performed. Fig.3a shows the digital images of the alizarin red stained samples after 21 and 28 days of culture. Intense red dots were observed on the samples corresponded to the calcium deposition. After culturing in normal and osteogenic medium for 28 days, Alizarin red staining indicated that the cells could produce mineralized extracellular matrices, an important precursor of bone formation in vivo. Furthermore, the degree of staining quantified with a colorimetric analysis
(Fig. 3b) revealed that the differentiation of MSCs cultured on Sr-FAp was 1.4 and 1.1 times greater than that of cultured on Mg-SiFAp in normal and osteogenic culture medium, after 28 days of culture, respectively (p<0.05). According to a previous study, it could be argued that the calcium ions produced during ceramics degradation enhanced mineralization, which generally occurred in the last stage of osteogenic differentiation [38–42]. However, in addition to Ca ions, the release of other metallic ions may provide a stimulating environment for bone growth, which would be beneficial for important signals involved in stem cell differentiation towards the osteogenic lineage [43].

In order to evaluate the role of ions released from samples, the concentration of ions in culture medium was investigated. Results (Fig. 3c) showed that the concentration of Ca in culture medium for Sr-FAp and Mg-SiFAp were decreased from 3.90 mM and 8.08 mM after two weeks and reached 2.75 mM and 6.23 mM by after three weeks. This gradual decrease in Ca$^{2+}$ might be due to the consumption by the continuously forming apatite layer. Moreover, the release of Si and Mg ions reached to 2.11 mM and 3.31 mM, respectively, after 14 days of MSC culture with respect to Mg-SiFAp. This level was considered to be relatively non-toxic and within the doses stated to positively affect osteoblast-like cells in vitro (<10mM) [44]. Previous studies have also shown that such ions were able to impact on cell differentiation as well. For instance, the amount of Mg released from the Mg-SiFAp powder may also interact with integrins of osteoblasts, which are responsible for cell adhesion and stability, which could stimulate new bone formation [45]. The concentrations of Sr ions in culture medium increased from 2.66 mM in the second week to 3.17 mM in the third week of culture of SrFAp. Our results demonstrated that the concentration of Sr$^{2+}$ ions released in the culture medium could promote the proliferation and osteogenic differentiation of MSCs, which is in accordance with previous works [18,46].
Figure 3. Effects of Sr-FAp and Mg-SiFAp powders on the differentiation of MSCs: (a) The mineralized matrix was stained with Alizarin Red S and (b) quantified after following days of incubation in normal and osteogenic mediums. (c) Ca, P, Sr, Mg and Si concentration changes of Sr-FAp and Mg-SiFAp after 2- and 3-weeks immersion in culture medium.

The role of released ions on the osteogenic differentiation of stem cells was further investigated via evaluation of cell morphology (Fig. 4). To be able to distinguish the calcified structures from the mineral deposits from hMSCs, the images of bioceramics in SBF were also included after 28 days (Fig. 4a). The EDS analysis of the samples has shown the precipitation of Ca and P on the bioceramics powder (Fig. 4b). In this direction, after 28 days of culture in osteogenic medium, higher magnification SEM images showed that analogous particles densely covered the surface of samples, which were qualitatively identified as consisting of calcium and phosphate by EDS analysis (Fig. 4c). In addition, results demonstrated that large accumulation of calcium and phosphorous were deposited on both samples, which effectively revealed the capacity of the samples to form a bone-like
hydroxyapatite (Fig. 4d). From the EDX analysis, it is been concluded that the concentration of Ca and P after immersing in SBF and after osteogenic differentiation is less than those after immersing in SBF solution. Moreover, the Ca/P molar ratio (Fig. 4e) deposited on the cells (labeled by a yellow rectangle) was 1.51 and 1.54 for Sr-FAp and Mg-SiFAp samples, respectively, which was in the range of Ca/P ratio for bone-like apatite coatings (1.5–1.67) [47]. Overall, these results demonstrated the effective role of the substrates to support the differentiation of MSCs toward the osteogenic phenotype.

Figure 4. SEM images of MSCs on different bioceramics powder after 28th days of culture in SBF as a control (b) Energy dispersive X-ray spectrometer analysis of the bioceramics powder after 28 days immersing in SBF. (c) SEM images of the hMSCs after culturing on the bioceramics powder in normal and osteogenic medium. (d) Energy dispersive X-ray spectrometer analysis of calcified formation on scaffolds was showed in the right panel. (e) The amount of deposited Ca and P on the surface of the discs. The calculated ratio between the Ca and P also determine the purity of hydroxyapatite.

3.4. In vivo study

The local evolution of Sr-FAp and Mg-SiFAp after 2 and 3 weeks of in vivo implantation demonstrated significant differences in bone formation (Fig. 5 and 6). According to Fig. 5,
two weeks after implantation, the formation of connective tissue was detected in the defect sites in the presence of Mg-SiFAp and Sr-FAp samples. For Mg-SiFAp samples, the proliferative connective tissues and fibrous tissues were observed (Fig. 5). Moreover, the formation of bone was observed in defect sites of Sr-FAp samples after 2 weeks. From the quantification of histology staining, it is concluded that the amount of mineralized tissue after three weeks increased significantly both for Sr-Fap and Mg-SiFAp (p<0.05). In parallel, the amount of connective tissue after three weeks decreased for Sr-FAp. The mineralized tissue in both bioceramics powder is more than that in empty defects after 3 weeks implantation.

New bone tissue was detected after three weeks of implantation of Mg-SiFAp samples (Fig. 6). After three weeks of implantation, the formation of collagen fibers in immature trabecula of bone were observed by Masson’s trichrome staining (Fig. 6). The bone formed in defect site could be shown in Masson's trichrome staining image. On the other hand, for control samples, almost no new bone formation was observed in the defect site after three weeks (Fig. 6), except in small areas surrounding the defect site. These results have quantified and it shows the significant differences among all the samples (p<0.05).

These results were in accordance with previous literature where authors investigated the in vivo performance of a HA substituted with Si in a rabbit calvarial defect [36]. Along the same vein, another study demonstrated a faster new bone formation in Merwinitie (Ca₃Mg(SiO₄)₂)-filled-bone-defect than in HA models which was due to its superior degradation rate, proper biocompatibility and the existence of Mg and Si ions [48]. Besides, bone remodeling improved and established a good bonding with living bone and sufficient density in the presence of Sr-FAp samples. Also, the integration of Sr-FAp powder could significantly promote the differentiation and proliferation of osteoblast cells due to the osteoinductive properties of metallic ions like Sr²⁺ doped in the FAp structure.
Figure 5. Bone injury repair in bioceramics powder discs were implanted animals after 2 and 3 weeks. After 2 weeks, invasion of connective tissue elements into the site of injury for repair, but at this time there is no sign of bone formation (CT shows the connective tissue). After 3 weeks, for Mg-SiFAp, immature Bone (IB) and connective tissue (CT) and for Sr-FAp, invasion of connective tissue elements (CT) and formation of bone islands (MB) can be
distinguished. The quantification of mineralized and fibrous tissue for various bioceramics during 3 weeks.

Figure 6. Photomicrographs showing Masson-trichrome staining of samples from rats after 3 weeks. Higher magnifications of the boxed regions at right as Masson-trichrome staining, which showed collagen fibers were stained blue (arrows marked active osteoblast cells). H&E and Masson-trichrome staining of samples showed the initial stages of bone formation in empty bone defects in control group after 3 weeks. Sharp tips of compact bone in defect
sites are marked by white arrow and limited immature bone (IB) formed in the site of defects. The percentage of newly formed bone after 3 weeks of implantation.

In the present study, from histological analysis, larger regions of newly formed bone were identified in the Sr-Fap groups than Mg-SiFAp and control groups after 3 weeks. This potentially indicates a faster bone remodeling for the Sr-Fap. The released Sr from Sr-FAp powder was most likely responsible for the higher new bone formation compared to Mg-SiFAp powder.

**Conclusion**

In conclusion, we studied the role of various metallic ions Sr, Mg and Si, on the chemical, physical and biological properties of Fluorapatite (FAp) by comparing two powders compositions, Mg and Si doped FAp (Mg-SiFAp) and Sr doped FAp (Sr-FAp) produced through a procedure known as mechanical alloying. Results showed that both nanopowders enabled in vitro cellular proliferation and enhanced bone formation when compared to non-modified FAp. Both Mg-SiFAp and Sr-FAp revealed a significant enhancement of new bone formation compared to empty defects in a distal femur bone defect model in rats. Interestingly, Sr-FAp exhibited a significant higher new bone formation both at the biomaterial-bone interface and in the entire defect area compared to Mg-SiFAp. Taken together our study suggests that incorporation of Sr ions into FAp can result in improved bone growth, which has great potential as a new bone substitute or coating of metallic implants for bone replacement.

**Acknowledgements**

Authors wish to thank funding from the project PID2019-106094RB-I00 / AEI / 10.13039/501100011033.
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doi:https://doi.org/10.1016/j.msec.2016.05.043.


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738.
Table 1. The precures and the amount to synthesize Mg-SiFAp and SrFAp.

<table>
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<th>Table</th>
<th>$\text{Ca}_9\text{Mg}_0.5\text{(PO}_4\text{)}_5\text{(SiO}_4\text{)}_0.5\text{F}_2$</th>
<th>$\text{Ca}_9\text{Sr}_0.5\text{(PO}_4\text{)}_6\text{F}_2$</th>
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<td>gr</td>
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<tr>
<td>SrCO$_3$</td>
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<td>Mg(OH)$_2$</td>
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Declaration of competing interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
Graphical abstract
Highlights

- Bioceramics are the most reliable materials to promote bone growth.
- Fluoride ion substituted in the chemical structure of HA has shown to control the dissolution rate.
- Some trace elements can be doped in its crystal structures to increase the bioactivity.
- The synergic effect of magnesium, silica, and strontium ions have been investigated.
- Compared to magnesium and silica, strontium ion significantly promotes bone formation.