Influence of strontium dopant on bioactivity and osteoblast activity of spray pyrolyzed strontium-doped mesoporous bioactive glasses.

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ABSTRACT
With the emerging development of bioactive materials, mesoporous bioactive glasses (MBGs) are regarded as potential candidates for bone and tissue engineering due to its high surface area. In the present work, spray pyrolyzed MBGs doped with Strontium (Sr), an ion which promotes osteoblast bone-forming activity, were prepared. While the phase information, particle morphology, porous structure, and specific surface area were characterized by X-ray diffractometer, scanning electron microscope, transmission electron microscope, and nitrogen adsorption/desorption isotherm, respectively. In addition, in vitro bioactivity was examined following Kokubo’s protocol while the osteoblast activity was examined by alkaline phosphatase (ALP) assays. Finally, the results indicate that MBG specimens with optimal Sr dopant can enhance bioactivity and osteoblast activity and corresponded mechanisms were discussed.

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1. Introduction
Bioactive glasses (BGs) has gained enormous attention since Hench’s first report in 1969 [1]. During the past few decades, SiO₂–CaO–P₂O₅ based BGs have become promising materials due to their superior properties (bioactivity, biodegradability, biocompatibility, etc.). Extensive researches have been conducted in the field of biomedical engineering [2,3], while potential applications such as drug carriers, bone implants, and dental fillers were also in progress [4–6]. In addition, mesoporous bioactive glasses (MBG) were developed to increase the specific surface area in order to achieve better bioactivity. For instance, various studies used nonionic copolymers surfactants P-123 (EO₁₀₀PO₇₀EO₁₀₀, where EO is poly-ethylene oxide and PO is poly-propylene oxide) or F-127 (EO₁₀₀PO₇₀EO₁₀₀) for the preparation of highly ordered mesoporous structure [7–9]. While Hong et al. proposed the use of H₂O₂ as a novel pore-forming agent for spray pyrolysis to replace the traditional carbon-based surfactants [10]. However, the increase of specific surface area is not sufficient when encountering applications that require antibacterial or osteoblast activity. Therefore, the incorporation of metal ions (Ag, Zn, Sr, etc.) to achieve different functions has attracted great interest [11–13].

Among these ions, Sr dopant is one of the most attractive owing to its role in enhancing osteoblast activity and promote bone cell differentiation [14]. According to previous studies, low doses of Sr are able to increase the activity of alkaline phosphatase, stimulate the formation of bone cells and osteocalcin, without affecting the mineralization of bone matrix [15,16]. Furthermore, Marie reported that a high concentration of Sr ions that were bound to hydroxyapatite tends to inhibit the effect of osteoclasts [17]. All these results indicate the significance of Sr for the development of bone implant applications.

Based on the previous studies, it has been demonstrated that conventional BG preparation methods, i.e. glass melting or sol-gel method, have been reported for synthesizing Sr-doped BGs. For example, Gorustovich et al. prepared melt-derived 45S5 glasses with SrO substitution and implanted into rat tibiae, demonstrating its potential in bone-bonding ability [18]. Moreover, Solgi et al. investigated the sol-gel-derived Sr-doped BGs with different concentrations of SrO and reported that Sr ions are able to promote cell proliferation and enhance osteoblasts differentiation [19]. However, the calcination temperature of 1400°C to 1600°C was frequently used for the melt-derived process, which may cause difficulty in maintaining high purity and structural homogeneity while hard to perform morphological modification [20]. Alternatively, the sol-gel method is able to decrease the calcination temperature to around 600°C, which allows much higher control over purity and morphology. Yet, the drawbacks of batch production and time-consuming make it difficult to be commercialized.

To deal with the above problems, in this work we aimed to synthesize Sr-doped MBG specimens using the spray pyrolysis technique. The technique provides advantages of fast fabrication, rapid calcination, and
continuous process as compared to glass melting and sol-gel method [21,22]. Preparation of undoped and Sr-doped MBG specimens were carried out, while the phase information, particle morphology, mesoporous structure, and specific surface area were characterized by X-ray diffraction (XRD), scanning electron microscopy (SEM), transmission electron microscopy (TEM), and Brunauer-Emmett-Teller (BET) method, respectively. Meanwhile, evaluation of in vitro bioactivity tests were carried out using both XRD and Fourier transform infrared spectroscopy (FTIR). The osteoblast activity was examined employing alkaline phosphatase (ALP) assay. Finally, the formation mechanisms of all MBG specimens and their related properties were discussed.

2. Materials and methods

2.1. Synthesis

In this work, Sr-doped MBG specimens were synthesized using the spray pyrolysis technique. To begin with, note that the composition of 76 S (SiO2: CaO: P2O5 = 80:15:5 (mol%)) was chosen for preparation of all MBG specimens in order to achieve a higher specific area. Initially, the precursor solution of undoped MBG specimen was prepared by adding 6.70 g tetraethyl orthosilicate (Si(OC2H5)4, 99.9%, Showa, Japan), 1.40 g calcium nitrate tetrahydrate (Ca(NO3)2.4H2O, 98.5%, Showa, Japan), 0.73 g triethyl phosphate ((C2H5)3PO, 99.0%, Alfa Aesar, UK), 1.00 g 0.5 M hydrochloric acid (HCl), and 40 vol% hydrogen peroxide (H2O2, 35%, Showa, Japan) into 40.00 g ethanol as the foundation of SiO2, CaO, P2O5, acid catalyst, and pore-forming agent, respectively. For the Sr-doped MBG specimens, additional strontium nitrate (Sr(NO3)2, 99%, Sigma-Aldrich, United States) with various concentrations of 1, 5, and 10 mol% were dissolved into the undoped precursor solutions. All precursor solutions were stirred at 25°C for 1 h for the complete dissolution of precursors, and then deionized water was added to 400 mL and stirred again for 24 h to ensure solution homogeneity. Finally, each precursor solution was atomized into droplets with an ultrasonic nebulizer (KT-100A, King Ultrasonic, Taiwan) operating at 1.65 MHz. The droplets were directed into a tube furnace (D-80, Dengying, Taiwan) equipped with three heating zones, the temperatures were set at 400°C, 700°C, and 500°C which corresponds to evaporation, calcination, and drying stages, respectively. At last, a 16 kV earthed stainless steel collector was located at the end for the collection of all calcined particles.

2.2. Characterization

First, examinations of phase information were carried out with XRD (D2 Phaser, Bruker, Germany) for undoped and Sr-doped MBG specimens. The XRD patterns were acquired with a wavelength of 1.54 Å using a Ni-filtered Cu-Kα source. Next, the particle morphologies and mesoporous structures were observed by both SEM (6500 F, JEOL, Japan) and TEM (Tecnai G2 F20, FEI, United States). Meanwhile, the particle sizes and shape proportions were calculated from several SEM images sampling around 300 particles to ensure its reliability. In addition, the BET method was used to measure the specific surface areas of all MBG specimens. By degassing the MBG specimens at 150°C for 3 h, the degassed specimens were placed on a nitrogen adsorption/desorption device (Novatouch LX2, Quantachrome Instruments, United States) and all isotherms were recorded at −196°C.

Next, the bioactivities of all MBG specimens were evaluated following Kokubo’s protocol [23]. By using the simulated body fluid (SBF), which has an ion concentration close to human blood plasma, the MBG specimens were immersed with a ratio of 0.02 g/mL to form the test solutions. All test solutions were kept in an orbital shaker (S3000R, Firstek Scientific, Taiwan) and held at 37°C for 7 d, note that SBF was changed once per day. The resulting specimens were cleaned with both deionized water and acetone for three times; then, placed in a 70°C oven to dry for 1 d. Finally, XRD and FTIR (FTS-1000, Digilab, United States) were used for the evaluation of the bioactivity of each specimen. The XRD patterns were collected following the above setup, while FTIR spectra were acquired ranging from 400 to 1600 cm⁻¹, covering the fingerprint regions of Si-O-Si and P-O vibration.

To measure the in vitro release of Sr, the test solutions were prepared by immersing the Sr-doped MBG specimens into phosphate-buffered saline (PBS) with a solid to liquid ratio of 0.02 g/mL. The pH of the test solutions were kept at 5.0 reflecting the pH value in inflammatory disease, and all solutions were placed in a 37°C orbital shaker. PBS was renewed once per day and all test solutions were examined for 42 d in total. A microplate spectrophotometer (Multiskan GO, ThermoFisher Scientific, United States) was employed to observe the release of Sr. By acquiring the absorption spectra of wavelength at 300 nm, which corresponds to the maximum absorbance of Sr ion, at various durations (1 h to 42 d), the Sr release curve was obtained by cumulative calculation.

Finally, evaluations of osteoblast activity were conducted by ALP assay on bone marrow stem cells (BMSCs). The substrate buffer was prepared by dissolving 0.19 g disodium 4-nitrophenyl phosphate hexahydrate, 0.49 g 2-Amino-2-Methyl-1-Propanol and 0.04 g magnesium chloride hexahydrate into 100 mL deionized water. The cells were cultured with the MBG specimens for 7 d and the lysate were reacted with the substrate buffer in 1.5 M Tris buffer and 1% Triton X-100. Then, the solutions were transferred to 96-well
plates and assayed spectrophotometrically at a wavelength of 405 nm.

3. Results

The XRD patterns of the undoped, and 1, 5, 10 mol% Sr-doped sprays pyrolyzed MBG specimens were shown in Figure 1. First, the graph shows that no diffraction peaks were observed from 2 theta = 20° to 80° for the undoped MBG specimen. In addition, a wideband between angles of 2 theta = 20° to 40° was observed, which indicates the phase of the undoped MBG specimen is amorphous. Next, for the Sr-doped MBG specimens, the specimens treated with 1 and 5 mol% showed similar results in the undoped MBG specimen. However, the wideband shifted to lower angles meaning that the increase of d-spacing in the specimens. This correlates well with the dopant of Sr substituting the Ca network, resulting in larger d-spacing in the structure. At last, unlike all other specimens,

![Graph showing XRD patterns of undoped, 1 mol%, 5 mol%, and 10 mol% Sr-doped MBG specimens.](image1)

**Figure 1.** XRD patterns of undoped, 1 mol%, 5 mol%, and 10 mol% Sr-doped MBG specimens.

![SEM images of (a) undoped, (b) 1 mol%, (c) 5 mol%, and (d) 10 mol% Sr-doped MBG specimens.](image2)

**Figure 2.** SEM images of (a) undoped, (b) 1 mol%, (c) 5 mol%, and (d) 10 mol% Sr-doped MBG specimens.
diffraction peaks at 2 theta = 25.5° and 31.0° were found in 10 mol% Sr-doped MBG specimen, which can be identified as Sr₃P₂O₇ (JCPDF 24-1011) and Sr₂SiO₄ (JCPDF 38-0271) phases. In brief, the results suggest that the dopant of Sr with 5 mol% or less did not affect the phase information of the MBG specimens and were successfully synthesized with an amorphous structure, while precipitations of strontium phosphate and strontium silicate were formed in the 10 mol% Sr-doped MBG specimen.

SEM images of all MBG specimens are shown in Figure 2. Initially, typical morphology of spherical shape can be found in the undoped MBG specimen as shown in Figure 2(a). In addition, the particles exhibit smooth surface without observations of concaved or roughed particles, and particle diameters of 0.2 to 1.9 µm were measured. As compared to the undoped specimen, all Sr-doped MBG specimens showed similar morphology and particle diameters as shown in Figure 2(b–d). Meanwhile, average particle sizes of all MBG specimens were statistically measured using numerous SEM images taking at least 300 particles into account, which results in 501 ± 292, 590 ± 317, 583 ± 358, and 506 ± 311 nm for the undoped, 1, 5, and 10 mol% Sr-doped MBG specimens, respectively.

Figure 3 shows TEM images of all MBG specimens for the observation of the mesoporous structure. To begin with, all micrographs demonstrated that all MBG particles are spherical, confirming the surface morphology with SEM images as shown in Figure 2. In addition, based on the image formation mechanism, factors of atomic mass and specimen thickness dominate the contrast in TEM micrographs. Yet, all specimens have a similar atomic mass of Si, Ca, O, and Sr, thus the change, in contrast, is only contributed by the thickness of the specimen. This indicates that the bright contrast corresponds to the thinner regions (pore) while the dark contrast are thicker regions (wall). Based on the above contrast mechanism, mesoporous structures were found in all MBG specimens. However, it is difficult to compute the pore size and specific surface area directly from the micrographs; hence, BET measurements were carried out and the results show that specific surface areas for undoped, 1, 5, and 10 mol% Sr-doped MBG specimens were 258.1 ± 10.3, 322.8 ± 6.4, 305.7 ± 6.8, and 227.9 ± 15.8 m²/g, respectively. Whereas the pore sizes for undoped, 1, 5, and 10 mol% Sr-doped MBG specimens were 7.0 ± 1.8, 12.0 ± 1.5, 11.4 ± 0.9, and 21.8 ± 0.6 nm, respectively. In summary, the SEM results show that all MBG specimens were successfully synthesized with spherical morphology, while the mesoporous structures were confirmed by both TEM and BET results.

Figure 4 shows the XRD patterns of undoped and Sr-doped MBG specimens after immersing in SBF for 7 d for the evaluation of in vitro bioactivity. Compared to the XRD patterns before immersion (Figure 1), a peak at
31.8°, which corresponds to the (211) plane of hydroxyapatite (Ca$_{5}$(PO$_{4}$)$_{3}$(OH), JCPDF 09–0432), can be observed from all MBG specimens. Meanwhile, for the Sr-doped MBG specimens, an additional (211) plane of Sr-substitute hydroxyapatite (Ca$_{5}$Sr$_{5}$(PO$_{4}$)$_{6}$(OH)$_{2}$, JCPDF 34–0479) were found at 31.2°. Yet, quantification based on the XRD patterns is hard owing to the need for background subtraction of amorphous structure. Thus, further bioactivity evaluations were carried out using FTIR and the spectra were shown in Figure 5. Initially, the Si-O-Si stretching and bending vibrations can be observed at 1080, 800, and 482 cm$^{-1}$, which corresponds to the silica network. In addition, P-O bending vibration at 566 cm$^{-1}$ can be found in all MBG specimens [24], which indicates the formation of hydroxyapatite. Moreover, following Hench’s protocol [20], quantification of bioactivity can be derived from the FTIR spectra by computing the ratio of peak intensity ($I_1/I_2$), where $I_1$ is the intensity of P-O bending (566 cm$^{-1}$) while $I_2$ is the intensity of Si-O-Si bending (482 cm$^{-1}$). Whereas the higher value of $I_1/I_2$ indicates more P-O vibrations were observed, which corresponds to higher hydroxyapatite formation and bioactivity. The resulting $I_1/I_2$ values computed were 0.15, 0.20, 0.19,

Figure 4. XRD patterns of (a) undoped, (b) 1 mol%, (c) 5 mol%, and (d) 10 mol% Sr-doped BG specimens after immersing in SBF for 7 d.

Figure 5. FTIR spectra of (a) undoped, (b) 1 mol%, (c) 5 mol%, and (d) 10 mol% Sr-doped BG specimens after immersing in SBF for 7 d.
and 0.12 a.u. for undoped, 1, 5, and 10 mol% Sr-doped MBG specimens, respectively. Hence, indicating the order of bioactivity is: 1 mol% Sr-doped BG specimen > 5 mol% Sr-doped BG specimen > undoped BG specimen > 10 mol% Sr-doped BG specimen. In brief, both XRD and FTIR results confirmed the bioactivity of all MBG specimens after immersed in SBF for 7 d.

Figure 6 shows the Sr release curve of all MBG specimens for 42 d. Initially, the undoped MBG specimens has no Sr release since there is no Sr dopant at all. Then, for the Sr-doped MBG specimens, the results show that the Sr release rapidly within the first day (24 h) of immersion. This corresponded to 24.6%, 25.6%, and 22.7% for 1, 5, and 10 mol% Sr-doped MBG specimens, respectively. From 1 to 7 d (24 to 168 h), Sr releases steadily till 39.5%, 41.4%, and 38.6%. After 7 d (168 h), Sr ions were slowly released, ending up with 51.0%, 55.8%, and 46.8% at 42 d (1008 h). These results show that the order of Sr release amounts was 5 mol% Sr-doped MBG > 1 mol% Sr-doped MBG > 10 mol% Sr-doped MBG.

Finally, for the osteoblast activity, ALP assays were carried out for undoped and 1, 5, 10 mol% Sr-doped MBG specimens, and the results are shown below. During bone cell differentiation, a large amount of ALP will be generated; thus, the osteoblast activity was measured as the percentage of ALP concentration against the control sample. The osteoblast activities of undoped and 1, 5, 10 mol% Sr-doped BG specimens were 99.0 ± 7.7, 100.6 ± 7.1, 101.3 ± 14.6, and 84.7 ± 11.0%, respectively. This indicates that the undoped MBG specimen has no effect on the process of osteoblasts, while the increased osteoblast activity of both 1 and 5 mol% Sr-doped MBG specimens can promote the differentiation of the bone cells. However, deterioration of the osteoblast activity was found in the 10 mol% Sr-doped MBG specimen.

4. Discussion

First, the phase information of the MBG specimens were discussed. Based on the XRD patterns, the excess of Sr-dopant will result in precipitations of strontium phosphate and strontium silicate as shown in the 10 mol% Sr-doped MBG specimen. In addition, a Sr dopant of 5 mol% or less will not affect the phase of the MBG specimens and can be successfully synthesized with an amorphous structure.

Next, the discussion of morphologies was carried out in two parts, the particle morphology, and the mesoporous structure. For the particle morphology, studies have shown that the morphologies of the spray-pyrolyzed particles might be influenced by various factors, such as the solution concentration, the precursor properties, or the sizes of the atomized droplets [25–27], while “one-particle-per-droplet” and “gas-to-particle” are the two main formation mechanisms that are often proposed [28]. As the SEM images shown in Figure 2, the results showed that all MBG specimens exhibit the morphology of smooth sphere with no observation of irregular shapes or nanosized particle (less than 100 nm) formed, this indicates that both undoped and Sr-doped MBG specimens went through the “one-particle-per-droplet” mechanism rather than “gas-to-particle” conversion.

Furthermore, since the preparation of all precursor solutions were kept at the same concentrations and the same frequency was applied during the spray pyrolysis process, the distributions of droplet size should be approximately the same, thus giving similar particle sizes, ranging from 0.2 to 1.9 µm, for all MBG specimens. In addition, for the mesoporous structure, Hong et al. has demonstrated that the addition of H2O2 is capable of forming pores within the particle during its deposition [10]. While the TEM micrographs (Figure 4)
and measured specific surface areas confirmed the successful synthesis of mesoporous structure within all MBG specimens. However, the specific surface areas and the pore sizes varied with different amount of Sr dopant, this is owing to the decomposition of the strontium nitrate precursor during the calcination stage [29]. Note that Messing et al. proposed that if the precursor melts prior to the solvent evaporation, it will trap the solvent to form a porous structure after calcination [25]. Thus, the low melting point of Sr(NO₃)₂·4H₂O will induce the secondary formation of the porous structure, leaving increased pore sizes when a higher concentration of Sr precursor is added. 

Then, the bioactivity of all MBG specimens were discussed. Based on the XRD results as shown in Figure 4, it is confirmed that all MBG specimens were bioactive owing to the formation of hydroxyapatite after SBF immersion. In addition, a report has demonstrated that the dopant of Sr in the glass network will increase the dissolution of Ca, thus resulting in the faster formation of hydroxyapatite (higher bioactivity) [30]. However, an excess of Sr ions will cause the formation of Sr-substitute hydroxyapatite, which results in a decrease in bioactivity. Moreover, the quantitative bioactivity results derived from the FTIR spectra (Figure 5) correlate well with both XRD and BET results, indicating that the order of bioactivity is 1 mol% Sr-doped BG specimen >5 mol% Sr-doped BG specimen > undoped BG specimen >10 mol% Sr-doped BG specimen. This shows that the factor of specific surface area dominates the bioactivity over the Sr-dopant effect.

Finally, the Sr release and osteoblast activity were discussed. Based on the XRD patterns shown in Figure 1, precipitations of strontium phosphate and strontium silicate were found in the 10 mol% Sr-doped MBG specimen which limits the release of Sr. The result correlates well with the Sr release curve as shown in Figure 6, showing the order of Sr release was 5 mol% Sr-doped MBG >1 mol% Sr-doped MBG >10 mol% Sr-doped MBG. In addition, since the Sr ions directly relates to osteoblast activity, a similar order of osteoblast activity can be observed from the ALP assays. In addition, the correlation between Sr release and osteoblast activity was plotted and shown in Figure 7. As shown in the graph, the order of Sr release at 7 d was 5 mol% Sr-doped MBG >1 mol% Sr-doped MBG >10 mol% Sr-doped MBG, which has a good agreement with the corresponding osteoblast activity. This is owing to the effect of Sr ions on cell differentiation, which has been reported in various studies [31–33]. Thus, it can be summarized that the excess dopant of Sr will result in precipitations of impurities which will affect the release of Sr meanwhile decrease the osteoblast activity.

5. Conclusions

In this study, the undoped, and 1, 5, and 10 mol% Sr-doped MBG specimens were successfully prepared by spray pyrolysis technique. The resulting particle morphologies and mesoporous structures of all specimens were characterized by SEM, TEM, and BET, and their formation mechanisms were discussed. In addition, all MBG specimens were confirmed to be bioactive after being immersed in SBF for 7 d. While quantitative results computed from FTIR show that, compared with the release of Sr, the specific surface area is the main factor affecting the bioactivity. Finally, the correlation between osteoblast activity and Sr release was discussed, which demonstrated
that the Sr-doped MBG with optimal Sr concentration can be considered as a candidate in bone implant applications.

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