

## In Vitro Biodegradation and In Vivo Biocompatibility of Forsterite **Bio-Ceramics: Effects of Strontium Substitution**

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ABSTRACT: The present study investigates the potential use of forsterite as an orthopedic biomaterial along with the role of strontium oxide (SrO) as a dopant. The *in vitro* degradation behavior was measured as a function of immersion time in simulated body fluid (SBF) for up to 8 weeks and was analyzed by micro computed tomography ( $\mu$ -CT) and scanning electron microscopy (SEM). All the doped samples showed higher degradation than pure sample. The in vitro cytocompatibility study showed good cytocompatibility and proliferation of MC3T3-E1 cells on Sr-doped MgS samples. The in vivo experiments were carried out by implanting the



ceramics in a rabbit femur for 30 and 90 days. The 3D  $\mu$ -CT and SEM images of 2 and 3 wt % Sr-doped MgS showed increased bone regeneration around the implant materials compared with pure and 1 wt % Sr-doped MgS, which was further confirmed by quantitative oxytetracycline labeling. The histological examination of three major organs of heart, kidney, and liver confirmed that the degradation product of the MgS ceramics, with or without doping, had no toxicological side effects. These results indicate that Sr-doped MgS bioceramics exhibit enhanced degradability with the potential to be used for temporary bone regeneration.

**KEYWORDS:** forsterite, bone regeneration,  $\mu$ -CT, cell culture, in vivo

#### INTRODUCTION

In the past decade, magnesium (Mg)-based degradable bioceramics have gained significant interest because of their good solubility and biocompatibility.<sup>1,2</sup> Among the class of Mg-based bioceramics, forsterite, (Mg<sub>2</sub>SiO<sub>4</sub>) (MgS) has been investigated due to the beneficial effects of both Mg and Si ions on osteogenesis and vascularization.<sup>3,4</sup> The *in vitro* studies have shown that Mg ions, released from forsterite bioceramics, increases osteoblast cell proliferation over a period of 7 days. Similar studies by Naghiu et al. confirmed that the dissolution products of forsterite powders promoted osteoblasts proliferation of U20S-type cells.<sup>6</sup> Previous in vivo studies in rabbit model has shown that MgS can regenerate bone after 90 days of implantation.

One of the primary advantages of degradable bioceramics is its tunable degradation kinetics to match the bone regeneration rate suitable to the patients' need. Literature suggests two important pathways that are involved in in vivo degradation of bioceramics, namely, solution-mediated dissolution and cellmediated resorption.<sup>8</sup> These two mechanisms can be controlled by the addition of essential inorganic ions found in the bone tissue. Several researchers have reported that the addition of trace elements such as zinc (Zn), manganese (Mn),

iron (Fe), strontium (Sr), sodium (Na) in CaP-based materials plays a significant role in enhancing the mechanical properties, controlling the degradation rate and improving the biological response.<sup>9–12</sup> Among all these ions, strontium ions have drawn special interest because of their synergistic effects on bone remodelling. It is well reported that Sr, an essential therapeutic ion, plays a dual role in bone remodelling by stimulating bone forming osteoblast cells and inhibiting bone resorbing osteoclast cells.<sup>13,14</sup> Yang et al. showed that Sr-substituted hydroxyapatite scaffolds improved the osteogenic differentiation of mesenchymal stem cells (MSCs) and enhanced the bone regeneration through  $Wnt/\beta$ -catenin signaling pathway when implanted in the calvarial defect rat model.<sup>15</sup> Studies have shown that Sr incorporated bioactive glass (BG) increased the osteoblast proliferation and ALP activity compared with undoped BG. Additionally, the released Sr ions from BG inhibited osteoclast differentiation and resorption.<sup>16</sup> Thus, considering all the beneficial effects of Sr in bone growth, we have substituted Sr in forsterite

Received: July 11, 2018 Accepted: December 26, 2018 Published: December 26, 2018

bioceramics in order to make it an effective biomaterial for bone tissue engineering. To best of our knowledge, for the first time, we have compared the *in vitro* degradation behavior and *in vivo* biocompatibility of undoped and Sr-doped MgS bioceramics in the rabbit model.

In the present research, we have prepared pure and Sr-doped crystalline MgS by the solid state sintering method. A comparative degradation study of MgS with HA and TCP, for 8 weeks, was carried out in SBF. A detailed analysis of porosity and pore volume was performed using  $\mu$ -CT. The cytocompatibility of all the bioceramics was analyzed using Murine MC3T3-E1 preosteoblast cells. *In vivo* osteogenesis of pure and Sr-doped MgS was studied by implantation in rabbit femoral condyle. New bone formation around the implant was confirmed by SEM, 3D  $\mu$ -CT and quantified through fluorochrome oxytetracycline labeling.

#### MATERIALS AND METHODS

**Materials Processing.** Forsterite (MgS) and strontium oxide (SrO) doped forsterite powder was prepared using silicon-dioxide (SiO<sub>2</sub>) (99.9%, Loba Chemie), magnesium oxide (99.98%, Himedia, India), and SrO (99.98% Loba Chemie) by the solid-state method. Four compositions of forsterite were prepared for this study. Pure forsterite, forsterite doped with 1, 2, and 3 wt % SrO (from now on labeled as MgS, MgS-1Sr, MgS-2Sr, and MgS-3Sr, respectively). The milling was carried out in a tungsten carbide container with tungsten carbide balls in toluene media. In all the milling runs, the ball-to-powder weight ratio was 10:1 and the rotational speed was 300 rpm for 9 h. After ball milling, the powders were dried in an oven at 70 °C for 24 h and then pressed into circular discs (8 mm diameter and 2.0 mm thickness) and sintered at 1200 °C for 2 h in a muffle furnace.

Commercially available hydroxyapatite (HA) and  $\beta$ -tricalcium phosphate ( $\beta$ -TCP) (Sigma-Aldrich Chemical Co., USA) were used for comparative study. The powders were pressed into circular discs of 8 mm diameter and 2.0 mm thickness, respectively, and sintered at 1200 °C for 2 h in a muffle furnace.

**Material Characterization Methods.** Crystalline phases of MgS were identified using X-ray diffraction (XRD, Bruker-D8 ADVANCE) equipped with Cu K $\alpha$  radiation:  $\lambda = 0.154$  nm at 40 kV and 40 mA over the  $2\theta$  range of  $20^{\circ}$ – $80^{\circ}$  at a scan rate of  $0.02^{\circ}$ /sec. The phase identification of all the samples, before and after immersion, was analyzed using ICDD (International Centre for Diffraction Data) database. The surface morphologies of all the compacts, before and after immersion in simulated body fluid (SBF),<sup>17</sup> were observed using scanning electron microscopy (SEM) ZEISS, EVO 60 system attached with energy-dispersive spectroscopy (EDS) (Oxford INCA Penta EETX3). Samples were sputter coated with gold–palladium alloy for 120 s. The fractographic features of all the samples were analyzed using scanning electron microscopy (SEM) (MERLIN, S.NO. 6105, CARL, ZESIS, Germany).

**Micro-Computed Tomography.** Internal structure, porosity, and interconnected pores of the compacts before and after immersion were evaluated by  $\mu$ -CT (Phoenix Vltomelxs, GE, Germany). The scan was carried out at a voltage of 100 kV and a current of 90  $\mu$ A. No filter was applied. The voxel size is fixed as 12  $\mu$ m. For one complete rotation, the time fraction was maintained as 500 ms with 1000 images. The obtained data were reconstructed into 2D images and all the 2D images were compiled to make 3D models using VG studio MAX 2.2, from which porosity and pore size distribution were calculated. Pore size distribution has been obtained by plotting the equivalent pore diameters having the same pore volume in the histogram and fitted with an appropriate function.

*In Vitro* Dissolution Study with SBF. To investigate the *in vitro* bioactivity of undoped and doped MgS samples, the compacts were immersed in SBF at pH 7.43 buffer using tris (hydroxymethyl)-aminomethane/1 M HCl. All the disc compacts were immersed in SBF at 37 °C for 2, 4, 6, and 8 weeks at a solid/liquid ratio of 150 mg  $L^{-1}$ . The SBF solution was changed twice a week. At preselected time

points, the compacts were removed, rinsed, with deionized water and dried at 150  $^{\circ}$ C in an oven, after which the final weight of each disc was measured using Analytical Balance (AUY series, Shimadzu). The concentration of the released magnesium, strontium and silica ions was measured by inductively coupled plasma mass spectroscopy (ICP-MS, Agilent/7500ce, USA). Change in pH of the dissolution media was determined with a pH meter (SYSTRONICS-Type 335). The percentage weight loss of the samples was calculated using the following equation:

weight loss (%) =  $(W_{\rm t} - W_{\rm o})/(W_{\rm o}) \times 100\%$ 

where  $W_0$  is the initial dry weight and  $W_t$  is the weight after a specific immersion time t.

Cytocompatibility. Live/Dead Assay and Cell Proliferation Study. Murine osteoblast cell line MC3T3-E1 (American Type Culture Collection (ATCC), Manassas, VA, U.S.A.) was used to study the cell materials interactions following a previously published protocol.<sup>18</sup> Briefly, cells were cultured in culture flasks with growth medium that consists of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Atlanta Biological, Lawrenceville, GA) and 1% penicillin streptomycin (P/S; Gibco, Grand Island, NY) at 37 °C in a 5% CO2 incubator. The culture medium was refreshed every 2 days. Confluent cells were trypsinized and resuspended in fresh medium for passaging. The samples were sterilized under ultraviolet (UV) light for 2 h and placed in 12-well plates. Further, cells were seeded on the substrates at a seeding density of 80 000 cells well<sup>-1</sup>. Cells cultured on the samples for 1 and 3 days were subjected to live/dead assay. Cells were washed twice with phosphate-buffered saline (PBS; Lonza Bio Whittaker) and incubated with the appropriate amounts of fluorescent dye for 45 min. Later, the cells were then rinsed twice using PBS and visualized using an inverted microscope with a fluorescence illuminator (CKX41, Olympus, Olympus America Inc.) attached with a digital camera (Olympus DP25 Microscope Camera, Olympus, Olympus America Inc.). The images were captured from four to six various locations.

The ceramic samples were placed in 12-well plates, at a seeding density of 80 000 cells well<sup>-1</sup> which were plated on the ceramic samples, and cultured for 3 days prior to changing to the differentiation medium. The medium was made by adding 50  $\mu$ M ascorbic acid, 10 mM  $\beta$ -glycophosphate, and 100 nM dexamethasone to  $\alpha$ -minimum essential medium ( $\alpha$ -MEM, Invitrogen, U.S.A.). After 1 and 3 days of culture, cells were lysed and the quantity of DNA was measured using Quant-iT PicoGreen dsDNA Reagent and Kits (Invitrogen) following the manufacturer's instructions. The fluorescent analysis was then performed at a wavelength of 480 nm for excitation and 520 nm for emission using Synergy 2 Multi-Mode Microplate Reader (BioTek, U.S.A.).

In Vivo Study. The animal experiments were performed in West Bengal University of Animal and Fishery Science (WBUAFS), West Bengal, India as per standard protocol and guidelines of the Institutional Animal Ethics Committee (IAEC) (IAEC permit no. Pharma/IAEC/460(iv) dated 4.8.2016)of WBUAFS. For the current research, 16 white New Zealand rabbits were used weighing approximately 1.5-2 kg. Cylindrical shaped samples of 4 mm × 2 mm were implanted in the rabbit's distal metaphysis femur.<sup>19</sup> The 16 rabbits were randomly divided into two groups to receive different types of implants. In the first group (I) (8 animals) pure MgS scaffolds were placed centrally in the left lateral femoral condyle (A1), whereas MgS-1Sr scaffolds were placed in the right lateral femoral condyle (A2). In the second group (II) (8 animals), MgS-2Sr and MgS-3Sr scaffolds were placed in the left (B1) and right lateral femoral condyle (B2). Postoperatively, all the rabbits were allowed to move freely without any external support, and daily clinical observations were performed.

Radiography,  $\mu$ -CT, and SEM Analysis. The rabbits were sacrificed after 30 and 90 days, and the radiographs of the femur bones were recorded at regular intervals postoperatively using medical diagnostic X-ray machine (300 mA, M.E. X-ray, India) to study the nature of the implant and the host bone-implant interactions. To evaluate the new bone growth the postoperated bone samples were scanned using  $\mu$ -



Figure 1. (a) Weight loss, (b) pH, and (c) in vitro dissolution of released ion concentration at different time points.

CT (Phoenix Vltomelxs, GE, Germany). All the bone samples were completely dried at room temperature, and scans were made at a source voltage of 85 kV and beam current of 70  $\mu$ A. The scanning resolution was fixed as 27  $\mu$ m to analyze the defined region of interest (ROI). Time fraction was 500 ms per image with 1000 images for one complete rotation. The constructed 2D images were compiled to obtain 3D models using VG studio MAX 2.2 (Volume Graphics) software. The bone-implant interface was studied using scanning electron microscopy (SEM) ZEISS, EVO 60 system. Samples were embedded in polymer resin. Then the mounted samples were polished to make the dust free surface, dried and sputter coated with gold–palladium alloy.

Oxytetracycline Labeling, Histology, and Toxicological Study. In order to analyze the new bone formation, fluorochrome oxytetracycline dihydrate (Pfizer India, 25 mg/kg) was injected 25 days before sacrificing the 30- and 90-day animals. After the animals were sacrificed, the implanted segments of the bone were collected. The samples were then ground using different grade sand papers. The ground sections were examined under the microscope for imaging the structural details of the bone. The ground-undecalcified sections were observed under UV light with Leica DM 2000 to find out the amount and source of new bone formation. The new bone formation is represented as golden yellow color and old bone as sea green color. The amount of new bone formation was calculated using the ImageJ software.

The implanted ceramics along with the surrounding bones were collected after 30 and 90 days postoperatively. The sections collected from both the normal and implanted area were cut, washed with normal saline, and stored in 10% formalin. Decalcification of the bone tissues were carried out using Goodling and Stewart's fluid containing formic acid (15 mL), formalin (5 mL), and distilled water (80 mL) solution. The decalcified tissues were cut and stained with

hematoxylin-eosin (H&E). The stained sections were viewed under the optical microscope.

In order to study the toxicity of the implant materials, histology of three major organs namely heart, kidney, and liver were analyzed. At the end of 30 and 90 days, the three vital organs (heart, kidney and liver) were collected and preserved in 10% formalin buffer. The sections were processed, stained with H&E, and examined under an optical microscope.

Statistical Analysis. The weight loss, pH, and DNA assay are reported as means  $\pm$  standard errors. The percentage of new bone regeneration is analyzed statistically using the SPSS software. The significance level among all the bone samples was analyzed using Tukey's posthoc method of one-way ANOVA test. The statistical analysis was performed using OriginPro 8 software, U.S.A., considering p < 0.05 [n = 5] as significant.

## RESULTS

**Comparative Degradation of HA**,  $\beta$ -TCP, and MgS. Degradation behavior of pure MgS was studied in comparison to HA,  $\beta$ -TCP in SBF for 8 weeks. To get a proper understanding of the degradation behavior, wt % loss and change in pH of SBF was measured, and the results are shown in Figure 1. At 8 weeks, almost 9 wt % loss was noticed for MgS compared to marginal 1 wt % loss for  $\beta$ -TCP (Figure 1a). In contrast, HA samples gained nearly about 2.5 wt % at 8 weeks. Similar weight gain for HA has previously been reported.<sup>9</sup> Change in pH of the SBF dissolution media for HA,  $\beta$ -TCP, and MgS are shown in Figure 1b. For MgS samples, the pH was found to increase up to 7.55 before coming down to 7.5. The pH decreased for both HA and  $\beta$ -TCP samples initially and stabilized around 7.35. The



**Figure 2.** (A) XRD patterns of forsterite containing various amount of strontium dopant: (a) MgS, (b) MgS-1Sr, (c) MgS-2Sr, and (d) MgS-3Sr. (B) XRD patterns of forsterite at  $2\theta$  range of  $35^{\circ}$ -37. (C) XRD patterns of undoped and Sr-doped forsterite before and after immersion in SBF for 8 weeks

	Table 1.	Rietveld	refinements	of	pure	MgS	and	Sr-do	ped	forsterite	samp	oles
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	phase composition (wt %)						
sample	$Mg_2SiO_4$	MgSiO <sub>3</sub>	MgO	$MgSr_2Si_2O_7$	a (Å)	b (Å)	c (Å)
MgS	100	0	0	0	4.750	10.191	5.978
MgS-1Sr	95.84	2.03	1.23	0.90	4.751	10.197	5.980
MgS-2Sr	91.60	3.14	2.46	2.8	4.759	10.198	5.984
MgS-3Sr	87.84	2.08	4.1	5.98	4.750	10.191	5.976



Figure 3. SEM images showing the surface morphology of pure, Sr-doped MgS before immersion (a,b,c,d), and after immersion (e,f,g,h) in SBF for 8 weeks

cumulative release profile of MgS (Mg),  $\beta$ -TCP (Ca), HA (Ca) ions in SBF at 37 °C are shown in Figure 1c. The Ca and Mg ion concentration in SBF are represented as dotted line.

Mg<sup>2+</sup> ion concentration steadily increased over the period of 8 weeks for MgS whereas, the release profile for  $\beta$ -TCP (Ca<sup>2+</sup>),

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Figure 4. SEM micrographs of fracture surface: (a) MgS, (b) MgS-1Sr, (c) MgS-2Sr, and (d) MgS-3Sr.



Figure 5. EDS pattern of undoped, Sr-doped MgS before immersion (a,b,c,d) and after immersion (e,f,g,h) in SBF for 8 weeks.

HA ( $Ca^{2+}$ ) ions was found to be much lower compared to MgS ( $Mg^{2+}$ ).

**Phase Analysis using XRD and Rietveld Refinements.** Figure 2A shows the XRD patterns of undoped and Sr-doped MgS ceramics sintered at 1200 °C. Primary peaks at  $2\theta$  = 35.98°, 36.78° were identified to be forsterite phase (JCPDS card no. 34-0189). Addition of Sr resulted in the formation of secondary phases; such as MgO (JCPDS card no. 45-0946), MgSiO<sub>3</sub> (JCPDS card no. 19-0768), and MgSr<sub>2</sub>Si<sub>2</sub>O<sub>7</sub> (JCPDS card no. 79-8255). Peak shifts of the primary forsterite phases were noticed due to Sr doping (Figure 2B). The primary (131) peak shifted from  $2\theta = 35.98$  to  $2\theta = 35.80$ , and  $2\theta = 35.76$  for MgS-2Sr and MgS-3Sr, respectively. No such peak shift was noticed for MgS-1Sr sample. Quantitative phase analysis has been performed from Rietveld refinements studies. The phase composition (wt %) and lattice parameters of Mg<sub>2</sub>SiO<sub>4</sub> (a,b,c) were analyzed and are shown in Table 1. From the Table 1 it can be observed that for pure MgS, only characteristic forsterite phase was formed, whereas incorporation of Sr leads to formation of other secondary phases such as MgSiO<sub>3</sub>,



Figure 6.  $3D \mu$ -CT visualizations of the pore along with materials' distribution of undoped and doped MgS before immersion (a,b,c,d) and (e,f,g,h) after immersion in SBF.

MgO, and MgSr<sub>2</sub>Si<sub>2</sub>O<sub>7</sub> depending on the percentage of strontium addition. The peak at  $2\theta = 31.9^{\circ}$  in the XRD pattern (Figure 2C) of MgS and MgS-1Sr represents formation of HA (211) on these sample surfaces after 8 weeks of immersion, whereas the XRD pattern of MgS-2Sr and MgS-3Sr did not show any HA peaks or any other Ca–P deposits.

In Vitro Dissolution Studies. SEM and  $\mu$ -CT Analysis. SEM micrographs of the as-prepared MgS and Sr-doped MgS samples are shown in Figure 3a-d. Grain sizes were found to be 913  $\pm$  291 nm, 585  $\pm$  246 nm, 216  $\pm$  57 nm, and 126  $\pm$  42 nm for MgS, MgS-1Sr, MgS-2Sr, and MgS-3Sr, respectively. Figure 4a-d shows the SEM micrograph of the fracture surface of pure MgS and Sr doped MgS. Grain sizes of the fracture surface were found to be 901  $\pm$  179 nm (MgS), 513  $\pm$  139 nm (MgS-1Sr), 209 ± 43 nm (MgS-2Sr), and 115 ± 36 nm (MgS-2Sr)3Sr). Presence of dopants was confirmed by EDS and is shown in Figure 5a-d. After immersion in SBF for 8 weeks, degradation at the sample surfaces were evident as shown in Figure 3e-h. MgS-3Sr sample showed the highest degradation among all the samples. The grain size increased after immersion for MgS-2Sr (298 ± 48 nm) and MgS-3Sr (245  $\pm$  53 nm), whereas the grain size of pure MgS and MgS-1Sr cannot be calculated due to the deposition of hydroxyapatite (HA) on the surface. Presence of Ca and P on the surface was confirmed by EDS (Figure 5e-h). Flower-shaped HA was noticed on pure MgS samples, whereas HA globules were found in Sr-doped MgS samples, and in addition to that, trace amount of calcium and phosphorus peaks were also observed.

The  $\mu$ -CT tomographs were used to understand the porosity. The 3D  $\mu$ -CT images (Figure 6a–d) of undoped and doped MgS revealed total internal porosity and its distribution inside the sample. Porosity was found to be the lowest for pure MgS (1.15%) and increased subsequently with increasing % of Sr doping. Maximum porosity of 3.06% was seen for MgS-3Sr samples. After immersing the samples in SBF for 8 weeks, increased porosity was noticed for pure as well as Sr-doped MgS samples (Figure 6e–h). Porosity increase (1.47%) was maximum for MgS-3Sr samples and minimum for pure MgS samples (0.88%). Porosity in MgS-1Sr and MgS-2Sr samples had increased by 0.93% and 1.21%, respectively.

Weight Loss and pH Variation. The *in vitro* degradation of all the ceramics, immersed in SBF for various time points, is shown in Figure 7. When soaked in SBF, both doped and undoped MgS degraded gradually with soaking time. The



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**Figure 7.** Weight loss of undoped and Sr-doped Mgs with different soaking time (\* statistically significant, n = 5).

degradation rate increased with an increase in the dopant wt %. Up to 2 weeks, MgS-3Sr sample showed highest weight loss among all the samples. After 2 weeks of immersion, Sr-doped ceramics degraded much faster with weight (wt.) loss of 6.2% (MgS-3Sr), 5.8% (MgS-2Sr), 6% (MgS-1Sr) and 3% for MgS. However, after 8 weeks of soaking time, the degradation was found to be maximum for MgS-3Sr with 12% wt loss compared to 9% for pure MgS. Figure 8 presents the changes of pH value of SBF during 8 weeks soaking of pure and Sr-doped MgS. An initial rise in pH from 7.4 to 7.5 was noticed for pure as well as doped MgS samples. Thereafter, the pH steadily increased for doped MgS samples with highest increase noticed for MgS-3Sr samples. Degradation media pH for MgS-2Sr and MgS-3Sr was found to be nearly 7.78 after 8 weeks of immersion in comparison to 7.58 for pure MgS samples.

*lonic Concentration.* Figure 9a–c shows the time-dependent cumulative ion release profile of Mg, Si, and Sr ions in SBF at 37 °C. During the initial incubation period, a rapid rise in Mg, Si, and Sr ions were noticed. Release of  $Mg^{2+}$  steadily increased over the period of 8 weeks for MgS-3Sr samples. MgS-2Sr and MgS-1Sr samples showed a lower rate of  $Mg^{2+}$  release after a period of 72 h (Figure 9a). For MgS samples, saturation was noticed after nearly about 200 h. Similar release profile was noticed for Si<sup>4+</sup> and is shown in Figure 9b. Maximum release of nearly 300 ppm of Si ions was found for MgS-3Sr after 8 weeks of immersion in SBF. Release profile of



Figure 8. pH of undoped and Sr-doped MgS in SBF solution for various time periods.

Sr ions from pure and doped MgS are shown in Figure 9c. A steady release of Sr ions from MgS-3Sr, reaching a maximum of 550 ppm, was noticed. Sr ion concentration in SBF also increased for MgS-1Sr and MgS-2Sr, however, much less compared to MgS-3Sr, which is in line with previously published literature.<sup>20</sup>

In Vitro Cell-Material Interactions. Cytocompatibility of the undoped and Sr-doped MgS samples was studied using MC3T3 cells and verified by live/dead imaging. Figure 10 depicts the fluorescence images of MC3T3 cells after 1 day (Figure 10a-e) and 3 days (Figure 10f-j) of culture. At day 1, cell attachment was found to be higher in Sr-doped MgS samples compared with pure MgS samples. There was no apparent difference in cell attachment among the Sr-doped MgS samples. At day 3 of post culture, a significant increase in live cells was noticed for MgS-1Sr and MgS-2Sr. Cells were found to be confluent in these samples. In comparison, MgS and MgS-3Sr samples showed a moderate increase in live cells at day 3, and complete coverage of the sample surface was not noticed.

MC3T3 cell proliferation on pure MgS and Sr-doped MgS samples were studied through DNA quantification as a function of culture time and is shown in Figure 11. At day 1, cell attachment was significantly higher on all of the Sr-doped MgS samples. Addition of Sr increased the cell proliferation rate at day 3. MgS-1Sr and MgS-2Sr samples showed maximum proliferation compared to other samples. The results are also in accordance with live/dead imaging at day 3, which showed a maximum of live cells on MgS-1Sr and MgS-2Sr samples.

In Vivo Osteogenesis. Radiological and  $\mu$ -CT Analysis. All the ceramic samples are well fitted inside the bone tissue without any complication such as infection or bleeding. The implants inside the bone were externally visualized by soft X-



Figure 9. In vitro dissolution of released ion concentration at different time points: (a)  $Mg^{2+}$  ions release, (b)  $Si^{4+}$  ions release, and (c)  $Sr^{2+}$  ions release

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Figure 10. Live/dead images of MC3T3-E1 cells after day 1 (a-e) and day 3 (f-j) (scale bar = 300  $\mu$ m).



Figure 11. DNA assay normalized to day 1 of control (# indicates statistical significance).

ray and are shown in Figure 12. The zeroth day radiograph of undoped and Sr-doped implants showed a horizon of hyperdense cylindrical material. The density, shape, size, and contour of the pure MgS material showed no appreciable changes even after 90 days of implantation, whereas the MgS-1Sr depicted the irregular outline of the implant denoting resorption with gradual decrease in radiographic density of the intraimplanted area. The MgS-2Sr radiograph after 30 days showed an appreciable reduction in the volume as well as density of the material, indicating early "take-up" of the implant by the host tissue compared with the MgS-1Sr and MgS-3Sr samples. The 90th day radiograph showed progressive loss of radio opacity of the implant allowing more radiation energy to pass through it rather than getting absorbed, indicating a significant level of implant resorption and advanced stage of implant "take-up" by the host tissue.

The  $\mu$ -CT images of undoped (Figure 13a1,a2) and Srdoped MgS (Figure 13b1-d1,b2-d2) depicts gradual dissolution of the implant. The 30 days postimplant  $\mu$ -CT images have characteristic new bone growth surrounding the implant material which is irregular in shape and partially covers it. MgS-2Sr has a remarkable amount of newly formed bony tissue surrounding it, and the thickness of the newly formed bone has also increased (Figure 13c1). The 90 days postimplant images showed higher amount of bony tissue that has been newly formed (marked by white arrow-implant material, red arrow-new bone formation around the implant) (Figure 13c2).The new bone has undergone remodelling in 90 days, which is evident from their regularity in arrangement and the structural stability. The implant material has gradually been degraded or has undergone resorption with time for all the implants, but the resorption process in the case of MgS-2Sr group is mostly noticeable with a thick newly formed bony tissue covering it.

Scanning Electron Microscopy. The SEM images of boneimplant-interfaces of undoped and Sr-doped MgS for 30 and 90 days are shown in Figure 14a1-d1,a2-d2. The boneimplant-interface (BII) was projected here to study the bridging gap between the implant and bone by new bone formation. Pure MgS has not shown much integrated BII bridging in the given time span, whereas in the Sr-doped series, the result was indicative of stronger bone formation and almost covers the bridging area. MgS-1Sr image (Figure 14b1) reveals a fissure gap between the bone and implanted the material, which by 90 days (Figure 14b2) heals up with an irregular bony deposition and thus binds the mature bone to hold the implant inside with new bone formation at the interface. Similarly, MgS-2Sr gives a far better evidence of bone regeneration in the interface. Figure 14c1 shows a wellstructured interfacial gap, which has been filled up with bridging callus and has shown thick and extravagant new bone formation surrounding the implant at 90 days (marked by red arrow-showing the interface between the bone and implant) (Figure 14c2). The thick interfacial newly formed bone was indicative of the fact that the implant (MgS-2Sr) has sufficient bone binding properties. The MgS-3Sr also has significant interfacial new bony regeneration in 90 days image (Figure 14d2).

Fluorochrome Labeling and Histological Analysis. The oxytetracycline (OTC)-labeled new bone emitted distinctive bright golden yellow fluorescence, while the old bone emitted a deep sea green fluorescence under UV radiations. Figure 15 shows the OTC-labeled MgS implanted bone specimens, collected after sacrificing the animals at 30 and 90 days. The new bone formation was quantitatively studied and are shown in Table 2. At 30 days, MgS scaffold implanted bone sections showed less area of golden yellow fluorescence  $(25 \pm 4\%)$  and mostly old bone was present, whereas MgS-1Sr showed (37  $\pm$ 1%) double tone golden yellow fluorescence in a narrow zone. The MgS-2Sr ( $52 \pm 2\%$ ) and MgS-3Sr samples showed almost similar new bone formation at 30 days ( $52 \pm 2\%$ ). At 90 days, all the bone samples showed increased new bone formations as compared with the baseline records. However, more coverage of new bone formation was noticed for MgS-2Sr samples (80  $\pm$ 2%) and MgS-3Sr (75  $\pm$  2%) compared to MgS-1Sr (48  $\pm$ 3%).

Histological analysis of bone (Figure 16) showed the role of implants in bone healing. It has been observed that the newly

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Figure 12. X-ray radiographs of undoped and Sr-doped MgS for various days (yellow arrow showing the implant material).



Figure 13. 3D micrographs of bone implant interface of undoped and Sr-doped MgS for 30 days (a1-d1) and 90 days (a2-d2) (red arrow showing the new bone regeneration around the implant material and white arrow representing the implant).

formed osteoids, haversian system along with the proliferative population of osteoblasts and angio-invasion are predominant in the case of MgS-2Sr implant (Figure 16c1), followed by MgS-3Sr and MgS-1Sr, respectively. At 90 days numerous osteoblast proliferations in association with homogeneous matrix deposition, controlled osteoclastic resorption and organization of bony callus are seen throughout the section mostly in the case of MgS-2Sr and MgS-3Sr. The postoperative histological images of the three major organs such as heart, kidney and liver at the end of 90 days are shown in Figure 17. Histological examination of the cardiac tissue conducted after 90 days of implantation showed normal musculature and

MgS MgS-1Sr MgS-2Sr MgS-3Sr (a1 (b1) 30 Days 10µm a2) (c2)(d2)Days 06 10µm 10µm 10µm

Figure 14. SEM images of bone implant interface of undoped and doped MgS for 30 days (a1-d1) and 90 days (a2-d2) (red arrow showing the interface between the bone and the implant).



**Figure 15.** Fluorescence images of host bone-implant interface of undoped and Sr-doped MgS for 30 (a1-d1) and 90 (a2-d2) days (bright yellow region showing new bone formation and green region showing the local bone) (scale bar = 500  $\mu$ m).

# Table 2. Percentage of New Bone Formation in Defect Site of Pure and Sr-doped MgS Ceramics<sup>a</sup>

composition	30 days	90 days
MgS	$25^{a} \pm 4$	$42^{a} \pm 3$
MgS-1Sr	$37^{b} \pm 1$	$48^{b} \pm 3$
MgS-2Sr	$52^{c} \pm 2$	$80^{\circ} \pm 2$
MgS-3Sr	$52^{\circ} \pm 2$	$75^{\circ} \pm 2$

"Means (n = 4) along with different letters (a-c) above each value differ significantly (p < 0.05) among the treatments (One way ANOVA).

intricately arranged branching myofibrils in the tissue sections of all the animals. There is no inflammatory cells or cellular secretion present in the cardiac tissue and the structural integrity of the cells are ideal to maintain organ vitality. The renal microstructure of 90 days histology of all the samples showed intact and healthy renal tubular endothelium, a regular pattern of luminal cellular organization and nonspecific degenerative changes. There is no evidence of organ toxicity in the kidney. The 90-day liver sections showed no physiological or pathological alteration in the tissue micromorphology in case of MgS-2Sr and MgS-3Sr implanted animals, whereas in the MgS-1Sr implanted animal the liver architecture has gone through a slight perivascular cuffing as the only inflammatory change observed.

#### DISCUSSION

In the present work, we have demonstrated the suitability of forsterite (MgS) as a potential new degradable material for bone replacement. To begin with, we have compared the degradation behavior of MgS to that of the widely used bioceramics such as HA and  $\beta$ -TCP. It was found that MgS lost almost 9 wt % after 8 weeks of immersion in SBF, which is much higher than HA or  $\beta$ -TCP. The higher degradation of MgS is also supported by increased pH of the dissolution media. MgS partially reacts with water to form (OH) ions that increases the pH of the solution. To the best of our knowledge, we for the first time report a comparative degradation behavior of forsterite with that of HA and  $\beta$ -TCP.

We have synthesized forsterite ceramics through the solidstate route with and without Sr doping. XRD of MgS confirms the formation of pure forsterite phases at 1200 °C. Formation of forsterite is a diffusion-controlled mechanism in which MgO diffuses into the surface of SiO<sub>2</sub> to produce enstatite (MgSiO<sub>3</sub>), an intermediate phase, and upon further diffusion this enstatite phase transforms into pure forsterite.<sup>21</sup> Strontium addition results in incomplete transformation of enstatite to

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Figure 16. Representative histological H&E stained micrographs of the implanted bone at 30 (a1-d1) and 90 (a2-d2) days (scale bar = 100  $\mu$ m).



Figure 17. Representative histological sections of heart, kidney, and liver after 90 days implantation using (H&E) staining (scale bar =  $100 \ \mu m$ ).

forsterite and remains as a secondary phase. The reaction can be explained as below:

$$MgO + SiO_2 \rightarrow MgSiO_3$$
 (1)

$$MgSiO_2 + MgO \rightarrow Mg_2SiO_4$$
 (2)

$$MgSiO_3 + SiO_2 + 2SrO \rightarrow Sr_2MgSi_2O_7$$
(3)

Formation of enstatite in Sr doped MgS silicate samples indicate that SrO reduces the diffusivity of MgO in MgSiO<sub>3</sub>. It may also be possible that  $Sr_2MgSi_2O_7$ , which forms subsequent to MgSiO<sub>3</sub> formation, acts as a diffusion barrier for Mg<sub>2</sub>SiO<sub>4</sub> formation. Formation of intermediate enstatite phase results in unreacted MgO. Moreover, formation of  $Sr_2MgSi_2O_7$  consumes SiO<sub>2</sub> that also leaves out unreacted MgO. As a result, we have noticed MgO phase in the XRD pattern of Sr-doped MgS samples. Peak shift for MgS-2Sr and MgS-3Sr indicates partial substitution of Sr in the forsterite along with formation of  $Sr_2MgSi_2O_7$ . The peak shift of (131) and (211) plane could be due to substitution of bigger size of Sr atoms than Mg which leads to an increase in lattice parameters of Mg<sub>2</sub>SiO<sub>4</sub> (a, b, and c) for Sr doped forsterite. However, addition of 1% Sr into forsterite does not influence the cell parameters significantly. It might be possible that out of 1% Sr, only a fraction substitutes Mg in Mg<sub>2</sub>SiO<sub>4</sub> and rest of it reacts to form MgSr<sub>2</sub>Si<sub>2</sub>O<sub>7</sub> (0.90%). With such small substitution, no apparent peak shift was noticed. Addition of higher amount of Sr (3%) decreased the lattice parameters which is in line with previously published literatures. Gheitanchi et al. has also reported similar behavior where Sr addition up to 0.2 at. % increased the lattice parameters then decreased at 0.4 at. %. They explained it by the phenomenon of partial substitution of Sr at high Sr doping along with the formation of secondary phases.<sup>20</sup> After 8 weeks of immersion in SBF, the peak at  $2\theta$ =31.9° in the XRD pattern of MgS and MgS-1Sr represents formation of HA (211) on these sample surfaces. During the immersion period, the released Mg<sup>2+</sup> ions exchange with H<sup>+</sup> or H<sub>3</sub>O<sup>+</sup> from the SBF solution resulting in the formation of (Si-OH) silanol bonds, which gradually increased the pH value due to increase in the concentration of OH<sup>-</sup> ions. Further, movement of Ca<sup>2+</sup>, PO<sub>4</sub><sup>2-</sup>, and OH<sup>-</sup> ions to the surface of the ceramics which induced the formation of bone-like apatite. This apatite formation gradually decreased the pH value due to consumption of OH<sup>-</sup> ions. The EDS showed some amount of Ca and P peaks on all the samples, independent of strontium content. The XRD pattern of MgS-2Sr and MgS-3Sr did not showed any HA peaks or any other Ca–P deposits due to incoherent and insufficient HA deposition to be detected by XRD.

One of the primary advantages of forsterite bioceramics is its enhanced degradation.<sup>22</sup> Degradation study in SBF is a wellaccepted technique in which wt change, pH change, and ionic concentrations are measured as a function of time. In the present work, rapid initial weight loss for MgS-3Sr samples is a result of its smallest grain size  $(126 \pm 42 \text{ nm})$  among all the samples along with highest initial porosity. Sr being larger in size compared with Mg ions results in expansion of the unit cell parameters (supported by the peak shift in the XRD pattern) and results in lower sintered density of Sr-doped MgS samples. Similar results have been reported in metal-ion-doped CaP systems.<sup>10</sup> The faster weight loss also results in highest concentrations of Mg, Si, and Sr in the dissolution media for MgS-3Sr samples. Preferential release of Mg ions below pH 8 is reported earlier that explains the continuously increasing Mg ions in dissolution media compared to Si ions. Release of Si ions are preferred above the pH  $8.^{23}$  In the present study, pH of the degradation media remained well below pH 8.Sr ion concentration steadily increased in the degradation media in wt %-dependent manner. After 8 weeks of immersion SEM images of forsterite showed irregular degraded morphology and the volume of all the doped MgS decreased due to degradation of ceramics as determined by  $\mu$ -CT. The addition of Sr dopant increased the degradation property of the forsterite ceramics due to distortion of the MgS crystal structure.

Because of its controlled and enhanced degradation, forsterite bioceramics are gaining importance as degradable bioceramics. As explained before, degradation of these materials increases the metal ion concentrations and the pH of the media. Therefore, it is essential to understand the forsterite-bone cell materials interactions to assess the cytocompatibility of these materials with or without Sr doping. Figure 10 shows that MC3T3-E1 cells have proliferated considerably to reach confluency on MgS-1Sr and MgS-2Sr samples from day 1 to day 3. Similarly, DNA quantification (Figure 11) indicated that MgS-1Sr and MgS-2Sr are most potent for cell attachment as well as cell proliferation. The results can be directly correlated to the degradation behavior of pure and Sr-doped MgS samples. Higher degradation of MgS-1Sr and MgS-2Sr leads to increased Mg, Si, and Sr ions in the degradation media. These elements are specifically known to enhance osteoblastic cell proliferation in vitro. The research findings of Zhu et al. indicated that Sr doped calcium silicate ceramics increases MC3T3-E1 cell proliferation and differentiation marker such as alkaline phosphatase (ALP) activity.<sup>24</sup> The in vitro studies of Lin et al. proved that the released Si and Sr ions from Sr-substituted calcium silicate (CS) increased cell viability and stimulated angiogenesis process.<sup>25</sup> It has been reported that Si doped hydroxyapatite up-regulated the osteoblastic proliferation by activating Wnt/ $\beta$  MAPK signaling pathways.<sup>26</sup> Similarly, the presence Mg ions up to 3 mM

increased the osteoblastic activity by inducing the gap junction intercellular communication in osteoblasts.<sup>27</sup>Thus, the multifunctional role of Mg, Si, and Sr ions increased the cell proliferation in MgS-1Sr and MgS-2Sr compared with pure MgS. On the contrary, MC3T3-E1 cells failed to proliferate on MgS-3Sr samples. The higher initial degradation of MgS-3Sr increased the pH along with the excess release of Mg, Si, and Sr ions in the static culture media, thereby reducing cell proliferation.<sup>28–33</sup>

In vivo osteogenesis in animal model provides a better understanding about the suitability of a degradable bioceramics. In the present work, we have evaluated, for the first time, the in vivo osteogenesis of forsterite in rabbit femur defect model using 3D  $\mu$ -CT imaging, sequential radiograph, fluorochrome labeling, and histology. In an ideal situation, the degradation rate of the degradable bioceramics must be coordinated with the growth rate of the new bone tissue surrounding the implant. From the sequential radiographs and  $\mu$ -CT images, it is evident that MgS bioceramics can actively take part in osteogenesis process. Presence of bone struts on the MgS surface in abundance, post 30 day surgery, indicates successful bone-implant integration.  $\mu$ -CT results are also confirmed by sequential radiographs which showed the implant uptake by the host bone. The thick bone struts at 90 day indicates continued osteointegration for MgS. We have also tried to understand the effects of Sr doping in MgS on any possible enhancement in in vivo osteogenesis. Addition of 2 and 3 wt % Sr in MgS results in thicker bone struts at 90 days post implantation and when quantified through fluorochrome labeling, shows nearly 80% higher bone formation compared to pure MgS, indicating beneficial effects of Sr in enhanced osteointegration of MgS bioceramics. The enhanced osteointegration of MgS-2Sr and MgS-3Sr can be correlated to its degradation kinetics. As explained before, an increased degradation of MgS-2Sr bioceramics results in increased Mg, Si, and Sr ion concentration at the vicinity of the implantation site. These elements are specifically known to induce osteogenesis and angiogenesis as proven by published literatures. For example, Sr release from HA stimulated hFOB osteoblast cell proliferation and differentiation through various signaling mechanisms.<sup>34</sup> It is well-established that Sr can open up a parallel Ca sensing pathways and cbfa1 gene expression, leading to higher bone regeneration rate.<sup>35</sup> Similarly, Si ions can also independently enhance osteoblast proliferation; however, it has a more profound effect on angiogenesis. It has been shown that Si release from bioglass and calcium silicate increases VEGF (vascular endothelial growth factor) and bFGF (basic fibroblast growth factor), which are essential for neovascularization that ensures proper nutrient supply to the defect site.<sup>36</sup> The positive role of Sr and Si ions can also be seen in the histological images, which show an abundance of the actively proliferating osteoblasts in MgS-2Sr and MgS-3Sr compared with pure MgS ceramics. Similar results are also reported by Gu et al., where they showed that the Sr-substituted calcium polyphosphate accelerated the bone formation in a rabbit model by stimulating the secretion of angiogenic growth factors (VEGF and bFGF) from the osteoblasts.<sup>37</sup> Li et al. observed the effect of Sr ions in ovariectomized goats showing significant increase in insulinlike growth factor (IGF)-1 and runt-related transcription factor 2 (Runx2). The results confirmed that addition of Sr increases the osteogenic gene expression and stimulated new bone formation.<sup>38</sup> In addition to the Sr and Si ions, Mg also plays as

essential role in in vivo bone formation. The release of adequate amount of Mg from MgS-2Sr implants stimulates the extracellular matrix formation and mineralization, which results in early healing and higher bone formation in MgS-2Sr implantation site compared to pure MgS.<sup>39</sup> In a similar way, the in vivo studies of Cheng et al. in a rabbit model proved that presence of Mg elevated collagen type 1 and OPN expression, thereby leading to higher bone regeneration.<sup>40</sup> This excellent bone formation in MgS-2Sr and MgS-3Sr are due to the synergistic stimulation of the released Mg, Sr, and Si ions, which triggered the differentiation of osteoblasts and heal bone defect through angiogenesis and neovascularization process.<sup>25</sup> Although MgS-3Sr samples showed a decrease in cell proliferation in vitro studies due to excessive metal ion release, and no such detrimental effects are seen in in vivo results due to the dynamicity of the in vivo studies.

A degradable bone replacement material poses an inherent challenge of systemic organ toxicity that can possibly arise from the degradation products. In the present scenario, also the degradation of MgS bioceramics leads to increase in metal ion (Mg, Si, Sr) concentration in the body fluid. Therefore, it was of paramount importance that any toxicity of vital organs be evaluated. The histological analysis of the heart, liver, and kidney however, showed no major pathological changes in the organs after 8 weeks of implantation. The results indicate that the degradation products of pure and Sr doped MgS are indeed well tolerated by the vital organs, and thus, any indications of systemic toxicity can be eliminated demonstrating the potential of these doped systems.

#### CONCLUSIONS

In summary, undoped and Sr-doped forsterite ceramics were synthesized from magnesium oxide and silicon dioxide by the solid-state method. Addition of 2 wt % Sr significantly increased the weight loss and pH of MgS after 8 weeks of immersion in SBF. The in vitro dissolution study revealed that with an increase in time, magnesium release increased along with Si and Sr ions. SEM and 3D  $\mu$ -CT analysis showed with increase in dopant concentration the degradation property of the ceramics increased. The in vitro cytocompatibility of MgS-2Sr bioceramics revealed that synergistic stimulation of the released Mg, Sr, and Si ions provided a suitable environment for improved cell proliferation and differentiation of MC3T3-E1 cells compared to pure MgS. The effect of strontium doping on the in vivo bone regeneration in the rabbit model was studied. The 3D  $\mu$ -CT and SEM results showed enhanced bone regeneration around the MgS-2Sr implants in the early days of implantation which was further confirmed by oxytetracycline fluorochrome labeling. Quantitative analysis showed 80  $\pm$  2% of new bone formation for MgS-2Sr samples compared with that of 42  $\pm$  3% for pure MgS. No major degenerative changes in the vital organs was noticed as indicated by histological analysis. Thus, all these observations collectively suggest that SrO-doped MgS ceramics may be a potentially viable approach for bone regeneration therapies where a faster degradation rate is required.

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The authors declare no competing financial interest.

### ACKNOWLEDGMENTS

M.R. would like to acknowledge the financial assistance from Science and Engineering Research Board (SERB-SB/FTP/ ETA-0114/2014), Department of Science and Technology (DST), India and technical personnel at the Central Research Facility, IIT-Kharagpur, for their support in  $\mu$ -CT analysis. S.K.N. and B.T. would like to acknowledge the support from the Honorable Vice Chancellor, West Bengal University of Animal and Fishery Sciences, Kolkata. P.N.K., A.R. and B.L. acknowledge the support of the National Science Foundation, Engineering Research Center (ERC) entitled, "Revolutionizing Metallic Biomaterials", grant no. EEC-0812348. Additionally, P.N.K. would like to acknowledge the Edward R. Weidlein Chair Professorship Funds and the Center for Complex Engineered Multifunctional Materials (CCEMM) for use of equipment employed for characterization.

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