Forsterite/nano-biogenic hydroxyapatite composites for biomedical applications

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\textbf{ABSTRACT}

Recently, silicate materials have received attention as materials with promising applications in the bioceramics field. A recent study aimed to investigate the effect of forsterite (Mg\textsubscript{2}SiO\textsubscript{4}) addition to biogenic hydroxyapatite, Ca\textsubscript{10}(PO\textsubscript{4}\textsubscript{6})(OH)\textsubscript{2}, on the phase formation, physical and mechanical properties, and biocompatibility of the produced composites. Different proportions of forsterite, 10 to 40 mass\%, were added to hydroxyapatite obtained from fish bones to prepare the target composites. Various techniques, such as X-ray diffraction analysis (XRD), scanning electronic microscope (SEM), transmission electronic microscope (TEM), mechanical strength measurements and in vitro studies, were carried out to evaluate the composite properties. The results indicate that the addition of 20 to 40 mass\% forsterite led to the transformation of forsterite into protoenstatite and the formation of Mg-rich whitlockite at the expense of hydroxyapatite. It is concluded that 20 mass\% forsterite is the optimum addition amount to enhance the physical and mechanical properties of the produced composites. The cell culture tests and in vitro studies agree with the abovementioned results.

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1. Introduction

Bioceramics are well known for their use in bone and dentistry applications. Calcium phosphate ceramics, which exist in different phases, are the most common materials to be used due to their bioactivity and degradability [1]. Hydroxyapatite (HA) is a member of the calcium phosphate family and has extraordinary properties that make this material compatible with bone tissues and enhance bone growth [2–5]. HA has been used for many clinical applications, such as bone repair, bone growth, and coating of metallic implants [6]. The most common drawbacks of HA are its weak mechanical properties, which limit its use in load-bearing applications [3,7].

The addition of a second phase, as a reinforcement phase, is used to improve the mechanical properties of hydroxyapatite. Hannora et al. [8] prepared HA/titania composites by a high-energy ball milling technique and achieved a high crushing strength of more than 200 MPa in comparison to 50 MPa for pure hydroxyapatite. The addition of 15 wt\% zirconia-toughened alumina (ZTA) and 10 wt\% mullite to hydroxyapatite both improved the bending strength by \approx 240\% [9] and improved the compressive strength and microhardness [2].
Forsterite is a fascinating additive that can enhance the mechanical properties of hydroxyapatite. In addition to its remarkable bioactivity, forsterite possesses favorable mechanical properties compared to hydroxyapatite [10].

The preparation and sintering of forsterite have a great effect on the microstructure, purity, grain size, sinterability, etc., of this material. Sintering of forsterite is carried out using different techniques, such as solid-state reaction [11], spark plasma vitrification [12], and mechanical alloying [13]. Nanosized forsterite is an active biomaterial that is converted to apatite immediately upon immersion in simulated body fluid (SBF) solution, while micron-sized forsterite is biologically inert, undergoing only a slight reaction with the surrounding tissue [10].

Sebdani and Fathi [1] used forsterite prepared via the sol-gel technique and calcined at 600°C for the preparation of forsterite/hydroxyapatite composites, and the prepared composites were used for coating 316 L SS substrates. An increase in the forsterite content enhances the mechanical properties as well as the bioactivity of the produced composites. The ability of the apatite layer to form on the coated surface upon immersion in SBF solution increases with increasing forsterite content.

In the forsterite/hydroxyapatite composite that contains 10 wt% to 50 wt% forsterite and that was prepared via mechanical ball milling and traditional sintering, the hydroxyapatite was decomposed to β-tricalcium phosphate (TCP) regardless of the firing temperature and composition. Although all the composites showed a decrease in both the bulk density and Vickers hardness, they displayed a high fracture toughness in comparison to that of pure hydroxyapatite. The enhancement in the fracture toughness is attributed to the effect of forsterite on the grain size and the presence of β-TCP [3].

A literature survey showed that little research has been conducted on the effect of forsterite content on the physicomechanical and biological properties of the forsterite/hydroxyapatite composites. The present work is devoted to the study of the biocompatibility of forsterite/hydroxyapatite composites as well as their physical and mechanical properties.

2. Experimental procedures

2.1. Extraction of HA powder from fish bones

Natural hydroxyapatite (HA) powder was extracted from fish bones through heat treatment according to the method described by Naga et al. [14].

2.2. Forsterite synthesis

Predetermined amounts of tetraethyl orthosilicate (TEOS; C₄H₉O₄Si, M = 208.33 g/mol, Merck Schuchardt OHG, Germany) and chemically pure magnesium carbonate were carefully weighed. The TEOS was hydrolyzed in distilled water with stirring for 2 h at 80°C until completely mixed. The hydrolyzed TEOS was peptized with the addition of 3 ml nitric acid. A transparent sol was formed, which was allowed to cool. MgCO₃ was heated to 900°C for 1 h to convert into magnesium oxide. The obtained MgO was dissolved in a small quantity of nitric acid before mixing with the previously hydrolyzed TEOS. The formed gel was dried at 110°C, followed by calcination for 2 h at 700°C to completely remove organic and nitrate species. The calcined powder was ground in a ball mill to remove all particle agglomerations.

2.3. Preparation of forsterite/HA composites

Different amounts (10, 20, 30 and 40 mass%) of prepared forsterite powder were ball milled with HA (90, 80, 70 and 60 mass%) at 200 rpm for 6 h to ensure complete mixing. The batches were denoted MH₁, MH₂, MH₃ and MH₄, respectively. Pure hydroxyapatite (HA) and pure forsterite (F) batches were also prepared. The batches were dry pressed into discs 10 mm in diameter and 3 mm thick, to investigate the physical properties and into rectangles with dimensions of 5 x 5 x 60 mm to investigate the mechanical properties using a uniaxial press at 225 MPa. The formed samples were sintered in a static air electric oven at 1300°C for the HA, MH₁ and MH₂ specimens and at 1275°C for the MH₃ and MH₄ specimens with a heating rate of 5°C/min and a soaking time of 1 h.

2.4. Characterization

2.4.1. Phase composition, morphology and physical properties

The phase composition of the synthesis powders and the sintered composites were identified by means of X-ray diffraction (XRD) analysis with a Philips X-ray diffractometer, model PW1730, with a Cu target and Ni filter. The morphology and grain size of the starting materials were analyzed by transmission electron microscopy (TEM) analysis using a JEOL JEM-2100 Electron Microscope, HR-TEM, Japan. The apparent porosity of the fired samples was estimated according to the liquid displacement method ASTM (C-20-74). In addition, the linear shrinkage (LS) of the fired composites at their optimum sintering temperatures was calculated from length variation of the green body (L₁) and the fired samples (L₂) by Micrometer Digital Calliper by using the following Equation (1):

\[ LS = \left( \frac{L_1 - L_2}{L_1} \right) \times 100 \]  

(1)
2.4.2. Microstructure
For the microstructure examination, sintered samples were polished and thermally etched in the air at a temperature lower than their sintering temperature by 50 °C for 15 min. The polished etched samples were coated with gold (15 nm thickness) by means of electrodeposition in order to impart electric conduction. Scanning electron microscopy; SEM-Jeol JSM-T20 attached to an energy-dispersive X-ray spectroscopy (EDS) system was used for microstructure examinations.

2.4.3. Mechanical test
The Vickers hardness of the sintered samples was measured using a hardness tester (Omnimet automatic MHK system Model Micro Met 5114, Buehler USA). The average hardness was calculated according to the Equation (2) given by [15]:

\[ H = \frac{1.8544 \times P}{d^2} \]  

(2)

Where p is the load and d is the length of the impression diagonal.

The bending strength was measured using a three-point bending test on a universal testing machine (Model LLOYD LRX5 K), while compression test was carried out on a universal testing machine (LRLR10KPlus 10 KN type, Japan) with a speed of 0.1 mm/min.

2.4.4. Cell culture test
For the cell culture test, the osteoblast-like cell line MG 63 was used. MG 63 cells (American Type Culture Collection, Rockville, Maryland) were cultured in Dulbecco’s modified Eagle medium: Ham’s F12 (DMEM) (Gibco, Invitrogen™, Grand Island, New York, USA) with 10% fetal bovine serum (FBS) (Biochrom AG, Berlin) and penicillin (100 U/ml)/streptomycin (100 U/ml) (P/S) (Gibco, Invitrogen™, Grand Island, New York, USA) at 37°C and 5% CO₂. For the in vitro tests, MG 63 cells were cultured in DMEM F-12 nutrient mixture (Ham) (DMEM/F-12:1), with L-glutamine, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1% FBS and 1% P/S without phenol red. For WST-1 and LDH-assays, cells were seeded at 2 \times 10³ cells per well. The cell culture experiments were carried out for all the prepared batches (HA, MH₁, MH₂, MH₃, MH₄ and F). Moreover, the metabolic activity of MG 63 cells was determined after 3, 7 and 10 days via mitochondrial succinate-dehydrogenase activity (WST-1 test) (Roche Diagnostics GmbH, Mannheim) according to the manufacturer’s protocol. The conversion of tetracolium salt to colored formazan by mitochondrial succinate dehydrogenase from metabolically active cells was measured spectrophotometrically using an ELISA reader (Magellan Measurement Parameter Editor infinite M200, Tecan, Salzburg, Austria) at 450 nm.

2.4.5. Bioactivity test
For the bioactivity test, the simulated body fluid (SBF) was prepared according to the method described by Kokubo et al. [16]. The studied bodies for HA and MH₂ composites samples were immersed in SBF solution at a solid-liquid ratio of 150 mg/ml for different time periods (1,7,14,21 and 28 days) at 37°C. Both calcium and phosphorus ions concentration were analyzed with an inductively coupled plasma spectrometer (ICP) [model Ultima-2 JY 2000–2, France]. The formed apatite layer on the surface of studied bodies was characterized using SEM analysis and EDX analysis.

2.4.6. Biodegradability study
A biodegradability study was carried out by immersing the studied HA and MH₂ composites samples in a phosphate buffer solution (PBS) at pH 7.4 as reported by S.K. Motwani et al. [17]; at a solid-liquid ratio of 150 mg/ml for different time intervals (1,7,14,21 and 28 days) at 37°C. After the mentioned periods, the biodegradability was calculated according to the following Equation (3):

\[ WL = \frac{[(M₀ - Mt) / M₀] \times 100}{(M₀ - Mt) / M₀} \times 100 \]  

(3)

Where,

- M₀: the initial weight of each sample
- Mt: the dry weight of the same sample measured at time t.
3. Results and discussion

3.1. Characterization of the starting materials

The phase composition of the starting materials is shown in Figure 1(a,b). Figure 1(a) shows that hydroxyapatite powder that was heat treated up to 1050°C is composed entirely of pure hydroxyapatite phase. On the other hand, the forsterite XRD patterns shown in Figure 1(b) indicate the formation of the forsterite phase at 850°C. Increasing the calcination temperature up to 900°C did not affect the purity of the forsterite phase. Calcination at 950°C led to the formation of protoenstatite together with the forsterite phase.

The TEM image shown in Figure 2(a) indicates that the particle size of the hydroxyapatite powder ranged from 85.71 to 119 nm. The image shows that the small particles are agglomerated and materialized into large particles. The particle size of the forsterite powder, as shown in Figure 2(b), was approximately 18.0 nm. The hydroxyapatite powder manifested as homogeneously dispersed particles.

3.2. Characterization of the sintered composites

3.2.1. Physical properties

The apparent porosity and the bulk density of both of hydroxyapatite (HA) and various hydroxyapatite/forsterite composites are seen in Figure 3. It was observed that the apparent porosity of the hydroxyapatite sample was 1.68%, the addition of 10 and 20 mass% forsterite increased slightly the porosity, while the addition of 30 mass% forsterite caused a more substantial increase in the apparent porosity. Further forsterite addition up to 40 mass% partially improved the apparent porosity. In agreement with Vassal et al. and Castkova et al. [18,19], we believe that sintering at high temperature (1300°C) enhanced agglomeration for

Figure 1. X-ray diffraction analysis (XRD) patterns of the starting materials: (a) hydroxyapatite extracted from fish bone and (b) forsterite powder prepared via the sol-gel technique.

Figure 2. Transmission electronic microscope (TEM) image of the starting materials: (a) hydroxyapatite powder calcined at 1050 °C and (b) forsterite powder calcined at 950 °C.
MH₁ and MH₂ composites, which in turn minimized the sinterability and increased the porosity of the material. The slight improvement in the apparent porosity of MH₄ samples may be due to the effect of the higher content of forsterite on the formation of the glassy phase, which enhanced pore closure. The bulk density of the sintered bodies, as shown in Figure 3, corresponded to the apparent porosity. The bulk density decreased with the increase in the forsterite mass%, which may be due to the decomposition of hydroxyapatite and the formation of a new phase, Mg-rich whitlockite, which possesses a lower theoretical density than hydroxyapatite [3]. The variation in linear shrinkage for both HA and the HA/Forsterite composites sintered at their optimum sintering temperature is shown in Table 1. The result reveals that HA sample had higher shrinkage values (12.99%) in comparison to HA/Forsterite composite samples. This is returned to the high homogeneity of the HA bodies, which led to coalescence of the particles to each other and as a result pore elimination and sample densification [20]. On the other hand, with introducing the forsterite phase to the HA matrix, linear shrinkage decreased to reach 10.62% for MH₁, slightly increased to 10.85% for MH₂ composite fired at 1300°C, while it recorded its lower value for MH₃ and MH₄ composite samples fired at 1275°C (8.41, 9.71% respectively). The observed shrinkage variations of the fabricated composites maybe are due to the variance in the porosity values, sintering temperatures and newly formed phases.

### Table 1. Physical and mechanical properties of the studied samples sintered at their maturing temperatures.

| Symbol | Apparent porosity, % | Linear shrinkage, % | Bending strength, MPa | Crushing strength, MPa | Vickers hardness, MPa |
|--------|-----------------------|---------------------|-----------------------|------------------------|----------------------|
| HA     | 1.68 ± 0.6            | 12.99               | 28.20 ± 1.3           | 56.62 ± 3.9            | 681 ± 36.5           |
| MH₁    | 2.10 ± 0.8            | 10.62               | 31.23 ± 2.4           | 57.50 ± 3.3            | 746 ± 36             |
| MH₂    | 1.89 ± 0.7            | 10.85               | 35.50 ± 2.4           | 66.35 ± 2.7            | 891 ± 28             |
| MH₃    | 8.78 ± 0.6            | 8.41                | 22.80 ± 1.7           | 30.07 ± 2.9            | 329 ± 29.5           |
| MH₄    | 5.42 ± 0.9            | 9.71                | 25.73 ± 1.8           | 36.30 ± 3.6            | 577 ± 38             |

3.2.2. Phase composition and microstructure

The XRD patterns of the examined samples are shown in Figure 4. The hydroxyapatite sample XRD pattern revealed the strong presence of hydroxyapatite phase. The existence of minor β-tricalcium phosphate phase was also detected, which present as a result of high sintering temperature at 1300°C [21]. The MH₁ composite containing 10 mass% forsterite was composed mainly of hydroxyapatite as the main phase and forsterite and β-tricalcium phosphate as the minor phases. Increasing the forsterite content to 20 mass% led to a partial transformation of the forsterite phase into protoenstatite. Mg-rich whitlockite was also present. The presence of whitlockite as a secondary phase in the composites of hydroxyapatite and Mg-containing materials has been reported by many authors [3,22,23]. The microstructure micrograph and energy-dispersive X-ray spectroscopy (EDS) pattern shown in Figure 5(a,b) confirmed the formation of the Mg-rich whitlockite phase. The XRD patterns of MH₃ and MH₄ containing 30 and 40 mass% forsterite showed the presence of diopside phase together with protoenstatite and Mg-rich whitlockite with some remnant forsterite. The presence of diopside phase was affirmed by the SEM micrograph and EDS pattern shown in Figure 6(a,b). The figure indicates the presence of both forsterite and diopside. Diopside particles are shown as white cubic particles. We believe that the presence of protoenstatite in the samples containing more than 10 mass% forsterite
Figure 4. X-ray diffraction analysis (XRD) patterns for the studied samples sintered at their maturing temperatures.

Figure 5. (a) Scanning electron microscopy (SEM) micrograph of MH$_2$ confirming the formation of the whitlockite phase; (b) energy-dispersive X-ray spectroscopy (EDS) analysis pattern of forsterite.

Figure 6. (a) SEM micrograph of the MH$_4$ composite containing diopside and forsterite particles; (b) energy-dispersive X-ray spectroscopy (EDS) analysis of the diopside particles.
is attributed to the partial decomposition of the forsterite phase. When forsterite is present in a Si-gas atmosphere at a high temperature (985-1557°C), a reaction occurs between Si and forsterite to form protoenstatite [24]. At the same time, the excess Mg resulting from the above reaction reacts with HA to produce calcium magnesium phosphate phase (whitlockite rich in magnesium). The formation of the diopside phase in the composites containing more than 20 mass% forsterite, which were sintered at high temperature, is due to the reaction of CaO, which resulted from the decomposition of hydroxyapatite into whitlockite and forsterite [1].

3.2.3. Mechanical properties
The effect of forsterite addition on the Vickers hardness and bending and crushing strengths of hydroxyapatite is shown in Table 1. The results indicated an increase in the mechanical properties with the addition of forsterite up to 20 mass%, which was considered the optimum addition. The grain size of the composites is a significant factor that affects the mechanical properties. Figure 7 shows that the addition of 10 mass% forsterite led to a reduction in the particle size. Such a reduction in the particle size caused a reduction in the crack free path, which in turn improved the strength [2,25]. An increase in the forsterite content to 30 mass% led to a decrease in the mechanical properties. The results indicated that a decrease in the mechanical properties was observed for the samples that possessed low bulk density and high apparent porosity [26]. Excessive reaction between the hydroxyapatite and forsterite phases (upon adding forsterite with a high percentage of up to 30 mass%) resulted in an increase in the obtained Mg-rich whitlockite phase (XRD figure), which causes additional reduction in the bending and crushing strengths. The abovementioned reaction and the decomposition of hydroxyapatite are responsible for increasing the porosity and thus decreasing both the crushing and bending strengths [8,9].

3.2.4. In vitro study
3.2.4.1. Cell culture experiments.
3.2.4.1.1. Viability. To test cell viability during contact with samples, Live/Dead Cell Staining Kit II (Roche Diagnostics GmbH, Mannheim) was used, which stains the vital cells green using calcein AM and stains the dead cells red using ethidium homodimer III (EthD-III) together with an effective plasma membrane. The standard working solution (2 µM calcein AM, 4 µM Eth D-III) was continuously refreshed prior to light microscope assessments with Dulbecco’s phosphate-buffered saline (DPBS). Cell viability was analyzed after 3, 7, and 10 days. Samples were analyzed under a fluorescence microscope (Olympus BX51, Tokyo, Japan) and photographed slice-by-slice. Viable cells were stained green, and dead cells were stained red.

After 3 days, the cells in contact with HA, MH₂, MH₃ and MH₄ were all alive, while some cells in contact with MH₁ were dead. However, when the samples were in contact with the cells for 7 and 10 days, all of the cells remained alive (Figure 8).

3.2.4.1.2. Giemsa staining. This histological staining method is very well suited to assessing cell morphology and provided initial information on the cells’ behavior versus the abrasion particles. Giemsa staining (with the Giemsa solution diluted 1:10) was performed on days 3, 7, and 10. Histological analysis was performed via optical microscopy (Olympus BX51, Tokyo, Japan) at 10x and 20x magnification. Ten images each from various regions were examined for analysis.

Giemsa staining was used to visualize morphological alterations in the cells during contact with the samples. There were no morphological alterations for all samples and all days of incubation (Figure 9).

3.2.4.1.3. Metabolic activity. The results of the metabolic activity of MG 63 cells were determined after 3, 7 and 10 days shown in Figure 10. The conversion of tetrazolium salt to colored formazan by mitochondrial succinate dehydrogenase from metabolically active cells was measured spectrophotometrically using an ELISA reader at 450 nm. For measurement samples

![Figure 7. Scanning electron microscopy (SEM) micrographs of (a) HA and (b) MH1 composite showing fine particle size for MH1 composite.](image-url)
were removed and placed in new well plate. We measured both the metabolic activity of MG 63 cells on samples in the new plate Figure 10(a), and the metabolic activity of MG 63 cells without samples in the old plate Figure 10(b) (cells can migrate through samples and from the top of samples). New Plate (cells on samples). There was no significant difference between samples on proliferation of MG63 cells. There was a small reduction of proliferation on samples F.

3.2.4.1.4. Cytotoxicity. Figure 11 shows the cytotoxicity assessment after 24, 48 and 72 h. The LDH test is colorimetric and quantifies cell death and cell lysis by measuring LDH activity. Cell death is accompanied by damage to the plasma membrane, thus vital cells release little LDH into the medium. Results showed that only few cells died – which live/dead assay confirmed. A slightly higher cytotoxicity was observed compared to control group after contact with MH1 after 24 h and for HA after 48 h. These differences were not significant. On the other hand, the cytotoxicity after 72 h is very low for all compositions.

Based on the obtained results concerning the physical, mechanical properties and cell culture test of the studied samples, the MH2 composition which possessed adequate values in terms of porosity and mechanical properties and biocompatibility characteristics, was selected to estimate their bioactivity and degradability.

3.2.4.2. SBF test. To test the bioactivity, both the MH2 samples, which possess enhanced physical and mechanical properties and HA samples were immersed in SBF for different times (1, 7, 14, 21 and 28 days) at 37°C. Changes in the Ca, P, Mg and Si ion concentrations (mg/dl) for MH2 nanocomposite samples and HA

Figure 8. Test of cell viability during contact with samples. Viable cells are stained green, and dead cells are stained red.
Figure 9. Morphological alterations in the cells during contact with the samples.

Figure 10. Metabolic activity of MG 63 cells determined after 3, 7 and 10 days on samples in a new plate (a), via mitochondrial succinate-dehydrogenase activity (b).
during incubation in the SBF solutions for different time intervals are illustrated in Figure 12(a,b). In the case of the MH$_2$ samples, the Ca and Mg ion concentrations increased, whereas the P ion concentration decreased slightly during the early stages of soaking in the SBF solution (up to 3 days). A sharp decrease in the Ca, P and Mg ion concentrations was observed after 7 days of immersion in SBF. This attributed to the broken of the Mg-O and Ca-O bonds in MH$_2$ composition by ionization and its exchanged with H$^+$ and H$_3$O$^+$ in the SBF solution in the early stages, which led to increasing such ions in the solution. Subsequently,

**Figure 11.** Assessment of the cytotoxicity after: a) 24, b) 48 and c) 72 h.

**Figure 12.** Changes in Ca and P ion concentrations as a function of incubation time in SBF solution for 28 days [a: pure hydroxyapatite and b: MH$_2$ samples].
Si-O on the surface of the composite will bond with -H to form an amorphous silica-rich colloid layer. Furthermore, positively charged electrolytes (such as Ca$^{2+}$ and Mg$^{2+}$) could be attracted by negatively charged silica-rich colloid layer. As a result, the newly formed silica-rich colloid layer will adsorb the dissolved Ca$^{2+}$ and Mg$^{2+}$ ions from cations in SBF (the readsorption of Ca$^{2+}$ and Mg$^{2+}$ ions). Thereafter, the surface acquires a positive charge, which further attracts negatively charged OH$^-$ and PO$_4^{3-}$ ions from the surrounding SBF solution. Eventually, an apatite layer is formed. This reaction led to the consumption of such ions from the solution and as a result, a decrease in its content [27]. On the other hand, within the remaining immersion period from 14 to 28 days, the concentrations of Ca and Mg ions increased. The release of Ca, Mg and Si ions in the SBF solution indicated the slight dissolution of the MH$_2$ composite samples. The abovementioned increase in the Ca, Mg, and Si ion concentrations was accompanied by a small decrease in the P ion concentration, which indicates the formation of an apatite layer. Notably, throughout the incubation period, the Si ion concentration increased starting at the first day of incubation [27].

Furthermore, the results show that the Ca and P ion concentrations in hydroxyapatite as a function of the incubation time in SBF solution decreased from the 1st day of incubation until the 28th day. The decrease in the Ca and P ion concentrations reveals that the apatite layer formation rate is higher than the dissolution rate [28].

Figure 13 illustrates the variations in pH versus incubation time in the SBF solutions for the hydroxyapatite and 20 mass% forsterite/hydroxyapatite (MH$_2$) composite samples. A slight increase in pH was observed within the first 3 days of incubation in the SBF solution, followed by a sharp decrease until the 7th day for both hydroxyapatite and MH$_2$ samples. The decrease in the pH for the MH$_2$ samples was sharper than that for the hydroxyapatite samples. The different in the pH values could be affected by many internal and external factors, including chemical composition, crystal structure and the dissolution behavior.

Then, during the following 7 days, a sharp increase in the pH value was observed for MH$_2$, followed by a decrease in the pH value until the end of the experiment after 28 days. On the other hand, the pH increase for HA was moderate and continued until the 21st day, followed by a slight decrease until the 28th day. The decrease in the pH value of the SBF solution is attributed to the consumption of OH$^-$ ions during the formation of the apatite layer [29]. The final pH value was approximately 7.3, which was very adequate for cell growth [30].

To confirm the formation of the apatite layer on the surface of the samples incubated in the SBF solution, the morphology changes that occur on the surface of the samples were examined via Scanning electron microscopy (SEM) micrograph before and after incubation in SBF for 28 days (Figure 14(a,b)). A thick heterogeneous apatite layer was observed on the surface of the MH$_2$ samples that were immersed in SBF for 28 days. Formation of the apatite layer is thought to take place through the dissociation and replacement of the calcium and magnesium ions in the MH$_2$ samples by H$^+$ (or H$_3$O$^+$) ions in the SBF solution. Such replacement led to the creation of Si–OH silanol groups by hydrolysis and breaking of Si–O–Si bridges. The newly formed layer attracted both Ca and P ions from the solution to produce an amorphous calcium phosphate layer. This amorphous layer was then incorporated with OH$^-$ ions from the solution, and the
amorphous calcium phosphate layer was crystallized to a hydroxyapatite layer [29,31,32].

We believe that the formation of various hydroxyapatite morphologies with different particle sizes is attributed to the presence of silica, which was liberated by dissociation of silica-containing phases, such as forsterite, diopside and protoenstatite. Silica plays an essential role during the nucleation and growth of the apatite-like layer [33]. The energy-dispersive X-ray spectroscopy (EDS) pattern shown in Figure 14(e) confirmed the formation of the HA layer on the studied sample surface.

Moreover, Figure 14(c,d,f) illustrates the Scanning electron microscopy (SEM) micrograph and energy-dispersive X-ray spectroscopy (EDS) analysis results of the pure HA sample surface before and after 28 days of incubation in SBF. This figure indicates that after immersion in SBF solution for 28 days, the surface of pure hydroxyapatite was completely covered with an apatite layer, which refers to the bioactivity of the HA samples as described by many authors [20,34]. As shown in the figure, hydroxyapatite crystallized in two particle sizes, fine and coarse. It seems that the nucleation and growth of hydroxyapatite started with small grains, which developed into large particles that appeared as scattered particles over the fine particle matrix. This result means that the formation of the apatite layer occurred by hydroxyapatite nucleation, followed by particle growth during incubation in SBF [35]. The energy-dispersive X-ray spectroscopy (EDS) analysis, shown in Figure 14(f), confirmed that the Ca/P ratio of the formed apatite layer is close to that for HA, which has a Ca/P ratio = 1.83.

3.2.4.3. Biodegradability test. It is preferred that the biomaterials used for biomedical applications are degradable over time and are progressively substituted by newly created bone tissue [36]. The comparison between the biodegradability results of MH$_2$ composite samples and hydroxyapatite samples after immersion in PBS for 28 days is demonstrated in Figure 15. The results revealed that the mass loss (%) for MH$_2$ composites ranged from 0.51 to 1.86% during the 28 days. These composites possess higher mass loss than hydroxyapatite samples, which displayed mass loss % values from 0.12 to 0.92% during the 28 days. The high degradation rate for MH$_2$ samples is attributed to the high dissolution properties of this sample compared to those of hydroxyapatite. This difference in properties may be due to the presence of silicate minerals, such as forsterite, protoenstatite and diopside, in addition to whitlockite rich in magnesium, as the main components of MH$_2$. On the other hand, the degradation amounts of hydroxyapatite seems too high than generally prepared sintered hydroxyapatite, it is attributed to the presence of β-tricalcium phosphate phase that responsible for this increase in PBS solution. The abovementioned phases possess a high dissolution rate and different dissolution behavior relative to the hydroxyapatite phase [37–39]. Such a degradation rate difference influenced the acceleration of the degradation process.

4. Conclusions
This study evaluates the introduction of the forsterite phase into hydroxyapatite extracted from a biogenic...

Figure 14. Scanning electron microscopy (SEM) micrographs of MH$_2$ composites and pure HA samples before and after incubation in SBF solution for 28 days (a, b,c and d) and their energy-dispersive X-ray spectroscopy (EDS) analysis (e and f).
source to form forsterite/hydroxyapatite composites. The results demonstrated the following:

1. Addition of up to 20 mass% forsterite boosted both the physical and mechanical properties of the forsterite/hydroxyapatite composites.
2. The addition of more than 20 mass% forsterite to hydroxyapatite and sintering at high temperatures produced protoenstatite and Mg-rich whitlockite phases.
3. The formation of a Mg-rich whitlockite phase is linked to the increase in porosity, which in turn reduced both the bending and crushing strengths.
4. The cell culture study showed that forsterite has no toxic effect on cells, and the cells remained alive.
5. The in vitro study indicated the formation of an extensive heterogeneous apatite layer on the surface of the MH$_2$ samples after 28 days of immersion in SBF. We believe that the apatite layer is formed as a result of the dissociation and replacement of the Ca and Mg ions in the MH$_2$ samples by H$^+$ (or H$_3$O$^+$) ions in the SBF solution. Moreover, the hydrolysis and breaking of the Si–O–Si bridges produced Si–OH silanol groups. Such groups attract both Ca and P ions from SBF solution to form an amorphous calcium phosphate layer, which forms apatite as a final product.

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**Disclosure statement**

The authors state that there are no conflicts of interest.

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**Data availability**

The authors declare that all data supporting the findings of this study are available within the paper.

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