Microporous Hydroxyapatite Ceramic Composites as Tissue Engineering Scaffolds: An Experimental and Computational Study

Satish Kanhed, Shikha Awasthi, Swati Midha, Jitin Nair, Ambreen Nisar, Anup Kumar Patel, Aditi Pandey, Rajeev Sharma, Sneha Goel, Anish Upadhyaya, Sourabh Ghosh,* and Kantesh Balani*

Bone-tissue engineering mandates the development of multi-functional bioactive porous hydroxyapatite (HAp) scaffolds. Herein, microwave sintered HAp/ZnO and HAp/Ag composite scaffolds with ≈5–19% porosity are developed using 0–30 vol% graphite as a porogen. The mechanical properties of the porous scaffold are analyzed in detail, revealing that even being more porous, the reinforcement of ZnO (9% porosity, hardness of 2.8 GPa, and toughness of 3.5 MPa.m$^{1/2}$) has shown to have better hardness and fracture toughness when compared to Ag (5% porosity, hardness of 1.6 GPa, and toughness of 2.6 MPa.m$^{1/2}$). The flexural strength obtained experimentally are complemented with a finite-element technique that adopts microstructural features in visualizing the effect of porosity on stress distribution. The antibacterial efficacy and cytocompatibility of these composites are validated by increased metabolic activity and conspicuous cell-matrix interactions. The anticipation of the results reveal that HAp/ZnO (9% porosity) and HAp/Ag (5% porosity) composites can be used as a potential multi-functional bone implant scaffolds.

1. Introduction

Repairing of necrosis and neoplastic lesions of the osseous organs need implantation of porous scaffolds for bone tissue regeneration.[1] Load bearing regions of the body require the scaffolds that associate well with the mechanical strength of native tissue for functional regeneration of the bone.[2] Hydroxyapatite (HAp) and its composites have garnered interest due to their crystallographic and chemical similarity with inorganic components of natural bone.[3–7] Despite having several bio-functionality applications, extreme brittleness, limited contact with host tissue, and lack of antimicrobial activity are the various drawbacks of HAp scaffolds. In order to overcome these limitations, the inclusion of metals, ceramics, as well as polymers have been reinforced in HAp scaffolds.[1,8–13] Saha et al. fabricated HAp/ZnO ceramic composites by varying the ZnO concentration (0–30 wt%) and observed enhanced fracture toughness and hardness (1.7 MPa m$^{1/2}$ and 6.6 GPa, respectively), as well as optimal antimicrobial activity for the 30 wt% HAp/ZnO composite when compared to pure HAp (0.7 MPa m$^{1/2}$ and 5.8 GPa, respectively). This phenomenon was attributed to two factors: 1) the difference in phase assemblage, and 2) deflection of crack in the presence of second phase.[14] However, work on metallic inclusion in HAp with Ag prepared by Zamperini et al.[15] discussed antifungal activity against C. albicans planktonic cells with minimum fungicidal concentration (MFC) and minimum inhibitory concentration (MIC) values of 250 and 62.5 μg/ml, respectively. In addition to providing HAp biochemical inertness and an antibacterial effect, silver can potentially enhance the toughness and strength via ductile metal bridging mechanism, that is, crack bridging and plastic work of Ag which could lead to a remarkable absorption of energy of crack propagation.[8] Apart from this, due to high CTE of Ag (20 × 10$^{-6}$/°C) when compared to HAp, the matrix (HAp) will be under compressive which further provides resistance to crack propagation.[8] Additionally, chitosan/HAp bio-composite was developed by the freeze-drying technique with incorporated silver ions by reduction
phenomenon which enhanced antibacterial activity of chitosan/HAp/Ag composite (13.3 mm diameter of zone inhibition) compared to that of chitosan/HAp composite (4.7 mm diameter of zone inhibition). This was due to the synergetic effect of nano-Ag and micropores of scaffolds on cell adhesion, penetration, and spreading along with providing the mechanical strength.

Although the aforementioned reports were related to assessing the biocompatibility of nonporous HAp/ceramic or HAp/metallic composites, the incorporation of controlled porosity in hydroxyapatite scaffolds creates a strong bonding between scaffold and bone. Since the tissues can grow considerably faster in a particular size range of pores, porous hydroxyapatite scaffolds are considered to have better regenerative capacity. Porous HAp was already fabricated by several researchers, but the effect of porosity (generated by varying vol% of porogen) along with the addition of ceramic or metallic particles in the HAp scaffold on mechanical as well as biological performance remains largely unexplored.

Object oriented finite element method (OOF2) is an effective technique to predict mechanical and thermal properties; and stress distribution of a complex microstructure in terms of grains, grain boundaries, porosity, reinforcements etc. In literature, similar mesh free computations have been investigated to predict the properties of the material under loading conditions such as multi-length scale mechanical properties of Mg–Li alloys estimating the elastic modulus of turtle’s complex carapace multi-length scale thermal conductivity of Al-Si-CNT composites etc. Therefore, the current study has the following aims: 1) Preparation of porous HAp/ZnO and HAp/Ag bio-composites with varied pore sizes by varying concentration (0–30 vol%) of graphite (as a porogen), 2) Investigating the mechanical behavior of samples including hardness, elastic modulus, fracture toughness, and tensile strength, 3) Establishing the relationship between pore sizes and tensile strength of samples using a semi-empirical mathematical fitting, 4) Elucidating the biological behavior of porous scaffolds by mammalian cell culture, bacterial culture using confocal imaging, and zeta potential measurement in order to develop a mechanically robust biocomposite for bone tissue regeneration, 5) Complementing diametrical compression characteristics with the OOF2 modeling by mesh generation mechanism to predict the effect of reinforcement (ZnO and Ag) and porosity on the stress distribution, simulated at 1% compressive loading condition.

2. Materials and Methods

2.1. Synthesis of Porous HAp/ZnO and HAp/Ag Bio-Composites

HAp/ZnO and HAp/Ag were synthesized using solution precipitation route. In wet precipitation method, nano crystalline HAp powder can be prepared by adding (NH₄)₂HPO₄ (0.15 to 1 M) solution drop wise to Ca(NO₃)₂ (0.25 to 1.67 M) at 60 °C. The pH of the solution can be maintained at 9 by adding suitable amount of NH₄OH. After stirring for 30 min, the precipitate was filtered and dried at room temp for about 48 h. After that, the dried powder has been ball milled for 3 min in presence of acetone (with powder to ball weight ratio as 2:1). Then, ball milled powder has been sieved to get homogeneous nano-sized hydroxyapatite powder with good crystallinity. The spherical shaped particle size of the ball milled hydroxyapatite powder was in the range of 80–120 nm. Further, 0%, 5%, 10%, and 30% graphite powder (particle size 20–50 μm with 99.5% purity and purchased from Central Drug House Private Limited, New Delhi, India), 7.5 vol% ZnO (particle size 80–100 nm and purchased from Merck Specialities Private Limited, Mumbai, India) and 7.5 vol% Ag (particle size 10–20 nm and purchased from Modison Private Limited, Mumbai, India) were added separately in HAp matrix followed by compaction in the shape of cylindrical pellets (dimensions of 12 mm diameter and 6 mm height) using a uniaxial semiautomatic 50 T capacity hydraulic press (CTM 50, FIE Pvt. Ltd.).

Fabricated samples were further sintered in a microwave furnace with heating rate 100 °C min⁻¹ at 1100 °C for 10 min under ambient conditions. Porosity in both sets of the sample was measured by Archimedes principle (using water as an immersion medium).

2.2. Microstructure and Phase Characterization

Field emission scanning electron microscopy (SEM, FEI Quanta 200) was used to analyze the microstructure of the samples. The SEM images were analyzed using three images of the area around 100 μm² (both for grain size and porosity measurements). Computer-aided modeling has garnered interest in recent years for their use in the designing of porous materials. Such techniques are usually used for calculating center to center distance, uniformity index, and frequency of features (say phase, grain, or porosity). One such modeling technique is Voronoi Tessellation, wherein each identity (say grain or porosity) is associated with a region called Voronoi cell, which is formed by the line bisectors of the lines joining that particle to its nearest neighbors. The color conversion from grayscale SEM images into binary images was carried out by using a proper threshold value. Further, for the phase analysis, X-ray diffraction technique (Bruker D8 Focus, Germany) was carried out on the samples using copper Kα radiation (λ = 1.5405 Å) with a scan rate of 5° min⁻¹, step size of 0.5° min⁻¹, and 2θ value from 25° to 100°. The grain size of the samples was calculated from the SEM micrographs via line intercept method.

2.3. Mechanical Properties Evaluation

The hardness and elastic modulus of HAp/ZnO/Ag composites were assessed by an instrumented micro-hardness tester (Instrumented Micro-indentation, CSM international) at a load of 100 g at ramp rate of 66.67 mN s⁻¹ and hold at maximum load for 15 s followed by unloading at the same rate using Vickers’ indenter of type V–I 51. The reported value is an average of at least 15 indents in each composite pellets. Moreover, diametrical compression tests, widely known as Brazilian test and specifically
used for the testing of the ceramics (100 kN Universal Testing Machine, BiSS Ltd.) was carried out on the samples (12 mm diameter and 4 mm thickness) at maximum load of 2 kN with 0.5 mm min\(^{-1}\) speed of the cross head in order to estimate the fracture strength of these samples. Due to the limitation of the materials cost and processing techniques, only three samples were utilized for the diametrical compression tests.

Additionally, to visualize the stress distribution with varying levels of porosity, OOF2 was used. OOF2 is a finite element method (FEM) based tool which uses the SEM image as an input, builds a mesh on top of the image and according to the simulated conditions, and generates iso-stress contours.[26] For analysis, each phase (second phase or porosity) is assigned a pixel group by rigorous pixel selection techniques followed by assigning materials to each pixel group and at last assigning respective elastic properties (elastic modulus and Poisson’s ratio) to each phase. An image identical to the microstructure was formed after assigning material properties and color to the pixels belonging to different phases and is used to create the skeleton. The mesh of pixel group boundary was constructed by using different adaptive mesh refining techniques. The discretization of the image was facilitated by adaptive mesh option such that pixel boundary is confined by homogeneity and shape of the element. Post meshing, the stress contours were generated for a compressive loading condition where the strain was fixed to correspond to 1% compressive loading. The boundary condition was chosen to a fixed strain to avoid structural movement while applying the strain and simultaneously the deformation can be captured. The left boundary condition was kept fixed and the compressive strain was applied to the right boundary, however, the top and bottom boundaries were constricted (stress \(\sigma \neq 0\)). The homogeneity index of >0.95 was ensured in all the cases.

2.4. Wettability of the Scaffolds

Wettability of the porous HAp/ZnO and HAp/Ag scaffolds (using water) were analyzed using contact angle goniometer (VCA Optima, AST Products, Inc. USA).

2.5. Biological Response of HAp/ZnO and HAp/Ag Scaffolds

Cytocompatibility of porous HAp/ZnO and HAp/Ag scaffolds was investigated by culturing hFOB (human fetal osteoblast cells) cell line (procured from American type culture collection, ATCC, USA). The scaffolds were sterilized by autoclaving and incubated in standard culture media overnight in 24 well plate.[27] Next day, scaffolds were seeded with an initial density of \(\approx 2 \times 10^4\) cells followed by incubation of culture plates in a CO\(_2\) incubator (33.5°C, 5% CO\(_2\), 95% rel. humidity) for 1, 3, and 7 days, respectively.[28]

2.5.1. Metabolic Activity

Quantitative estimation of cellular metabolic activities was performed using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay.[29] After incubation at respective time points, MTT: PBS (phosphate buffer saline, to maintain pH which mimic physiological environment of the human body) in the ratio of 1:10 was added to each of the wells, and incubated for 4h at 37°C, following which formazan was dissolved by dimethyl sulfoxide (DMSO), and the absorbance was observed at 570 nm wavelength using microplate reader.

2.5.2. Immunostaining

For fluorescence microscopy, the cell-seeded constructs were washed with 1x PBS and subsequently fixed with 3.7% formaldehyde. AlexaFlour488 Phalloidin dye (Invitrogen)[30] was added on each sample to stain the actin filaments (red color), and then incubated in dark for 1 h. After 3 consecutive washes using 1x PBS, DAPI (4',6-diamidino-2-phenylindole) dye (fluorescent stain from Sigma-Aldrich) (Sigma-Aldrich) was added for 5 min to stain the nucleus. The stained cells were then examined under a fluorescence microscope (LSM 780NLO, Carl Zeiss).

2.5.3. Antibacterial Effect

The antibacterial tests were conducted using bacteria Escherichia coli (E. Coli, Gram-negative) and Staphylococcus aureus (S. aureus, Gram-positive). The experiment was performed in a 24-well plate and 100 \(\mu\)l of bacterial solution (with the measured absorbance of 0.1 optical density; OD) was used for seeding on each sample \(n=3\). After seeding, the samples were incubated for 4 h at 37°C. The attached bacterial cells were fixed with primary fixative 3% glutaraldehyde, 0.1 M sodium cacodylate, and 0.1 M sucrose providing three consecutive changes after 20, 15, and 15 min, respectively. After drying, the adhered bacterial cells were subjected to SEM analysis. The experiment was performed twice with each set of bacteria.[31]

The quantitative estimation of the adhered bacterial cells was performed 4 h post-seeding by MTT assay. MTT dye was added to the samples and incubated at 37°C for 2 h following dissolution by DMSO. The absorbance was measured at 570 nm wavelength using microplate reader.

2.5.4. Zeta Potential

There is a correlation between changes in Zeta potential with that of cell surface permeability (using Gram-positive and negative bacteria) which access the membrane damage and predicts alteration of cell viability. For that matter, the zeta potential of Ag and ZnO were measured in the solution in which these nanoparticles produced Coulomb explosion between the charges of nanoparticles by repelling each other.[31] Zeta potential for the HAp/ZnO and HAp/Ag composite powders was measured using a zeta potential analyzer (Zetasizer, Nano-ZS, ZEN 3600; Malvern Instruments, Malvern, UK) by diluting the liquid samples with distilled water.
3. Results and Discussions

3.1. Porosity, Microstructure, and Phase Analysis of HAp/ZnO and HAp/Ag Scaffolds

The nomenclature, vol% of graphite, grain size values, and porosity% of sintered pellets are shown in Table 1. It may be noted that graphite is used as a porogen (0–30 vol%), that is, which gets oxidized and leaves behind pores after microwave sintering. SEM images presented in Figure 1 shows that the porosity increases with the increasing porogen content for HAp/ZnO (Figure 1a–d) and HAp/Ag (Figure 1i–l) composites upon microwave sintering.\[32,33\] The porosity level in case of HAp/ZnO and HAp/Ag is marginally different with same graphite content (see Table 1) is due to the difference in vol% of ZnO (1.33) and Ag (0.71) as well as the unlike effect of microwave sintering on both the samples. Reduction in the grain size of samples is also observed, which may be attributed to the limited diffusion of atoms along the surface of the particle and increase in porosity. Similar features can be observed for both HAp/Ag (reduced from 0.19 to 0.11 μm) and HAp/ZnO (reduced from 0.26 to 0.21 μm) composites.

Additionally, Voronoi tessellation (VT) of HAp/ZnO (Figure 1e–h) and HAp/Ag (Figure 1m–p) samples also confirms the enhanced porosity in addition with more vol% of porogen

<table>
<thead>
<tr>
<th>Graphite vol%</th>
<th>Nomenclature</th>
<th>Grain size (μm)</th>
<th>Porosity %</th>
<th>Nomenclature</th>
<th>Grain size (μm)</th>
<th>Porosity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>HA0GZn</td>
<td>0.26 ± 0.08</td>
<td>5.1 ± 0.3</td>
<td>HA0GAg</td>
<td>0.19 ± 0.05</td>
<td>5.0 ± 0.5</td>
</tr>
<tr>
<td>5</td>
<td>HA5GZn</td>
<td>0.23 ± 0.10</td>
<td>9.0 ± 0.1</td>
<td>HA5GAg</td>
<td>0.15 ± 0.02</td>
<td>8.2 ± 0.2</td>
</tr>
<tr>
<td>10</td>
<td>HA10GZn</td>
<td>0.22 ± 0.21</td>
<td>13.2 ± 0.3</td>
<td>HA10GAg</td>
<td>0.14 ± 0.04</td>
<td>11.1 ± 0.4</td>
</tr>
<tr>
<td>30</td>
<td>HA30GZn</td>
<td>0.21 ± 0.08</td>
<td>19.1 ± 0.2</td>
<td>HA30GAg</td>
<td>0.11 ± 0.03</td>
<td>16.1 ± 0.3</td>
</tr>
</tbody>
</table>

Figure 1. SEM images and Voronoi tessellated images, respectively, of microwave sintered samples with 7.5 wt% ZnO reinforcement: a), e) HA0GZn, b), f) HA5GZn, c), g) HA10GZn and d), h) HA30GZn and 7.5 wt% Ag reinforcement: i), m) HA0GAg, j), n) HA5GAg, k), o) HA10GAg and l), p) HA30GAg samples.
(30% of graphite porogen) with decreased center to center distance (CTC) from 4.0 to 0.4 μm for HA0GZn, 3.2 to 0.8 μm for HA30GZn (Figure 2a), 2.5 to 0.5 μm for HA0GAg and from 0.9 to 0.2 μm for HA30GAg (Figure 2c).

Further, the uniformity index was also estimated by VT which is the ratio of pore area to the polygon area. Lower uniformity index implies a more uniform distribution of pores.[34] Figure 2a–d indicates that pores are more uniformly distributed in both HA30GZn and HA30GAg as compared with HA0GZn and HA0GAg. It can be observed that there are only fewer pores with pore to polygon area of more than 0.1 (i.e., porosity of <10% is uniformly distributed) for HAp/ZnO (Figure 2b) and HAp/Ag (Figure 2d), respectively.

The XRD pattern of HAp/ZnO and HAp/Ag composites is presented in Figure 3. The peaks corresponding to pure HAp matches well with ICDD number 04-008-4759. The characteristic 2θ peaks of HAp with (002), (210), (211), (300), (301), (310), (222), and (213) planes at 25.9°, 29.1°, 31.8°, 33.1°, 34.1°, 39.8°, 46.8°, 49.7° of 2θ, respectively,[35] can be observed along with the planes of ZnO as (101), (102), (103), and (200) at 36.4°, 47.6°, 63.3°, and 68.2°, respectively,[36] and the planes of Ag as (111), (200), and (311) at 39.5°, 43.5°, and 78.9°, respectively,[37,38] (shown in Figure 3). Thus, these observations indicate that the starting phases are retained, and no reaction has occurred between HAp and reinforced ZnO/Ag.

3.2. Mechanical Behavior of Porous HAp/ZnO and HAp/Ag Scaffolds

An enhanced hardness of porous HAp/ZnO was observed for HASGZn sample (2.8 GPa) which started to decline for

![Figure 3. XRD pattern of HAp/ZnO and HAp/Ag composites.](image)
Table 2. Mechanical properties of HAp/ZnO and HAp/Ag composites.

<table>
<thead>
<tr>
<th>Samples</th>
<th>H (GPa)</th>
<th>σ (MPa)</th>
<th>E (GPa)</th>
<th>KIC (MPa m(^{1/2}))</th>
<th>Samples</th>
<th>H (GPa)</th>
<th>σ (MPa)</th>
<th>E (GPa)</th>
<th>KIC (MPa m(^{1/2}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA0GZn</td>
<td>0.68 ± 0.04</td>
<td>6.2 ± 0.2</td>
<td>63 ± 3</td>
<td>3.4 ± 0.3</td>
<td>HA0GAg</td>
<td>1.66 ± 0.04</td>
<td>8.0 ± 0.3</td>
<td>89 ± 4</td>
<td>2.6 ± 0.1</td>
</tr>
<tr>
<td>HA5GZn</td>
<td>2.89 ± 0.03</td>
<td>8.7 ± 0.3</td>
<td>38 ± 2</td>
<td>3.5 ± 0.0</td>
<td>HA5GAg</td>
<td>0.58 ± 0.03</td>
<td>7.4 ± 0.4</td>
<td>49 ± 3</td>
<td>2.5 ± 0.1</td>
</tr>
<tr>
<td>HA10GZn</td>
<td>0.07 ± 0.02</td>
<td>5.6 ± 0.3</td>
<td>15 ± 1</td>
<td>3.3 ± 0.2</td>
<td>HA10GAg</td>
<td>0.34 ± 0.04</td>
<td>4.5 ± 0.2</td>
<td>16 ± 4</td>
<td>2.4 ± 0.2</td>
</tr>
<tr>
<td>HA30GZn</td>
<td>0.04 ± 0.03</td>
<td>0.2 ± 0.2</td>
<td>5 ± 2</td>
<td>3.1 ± 0.1</td>
<td>HA30GAg</td>
<td>0.26 ± 0.03</td>
<td>0.2 ± 0.2</td>
<td>5 ± 1</td>
<td>2.3 ± 0.1</td>
</tr>
</tbody>
</table>

HA30GZn (up to 0.04 GPa) as the porosity fraction increases from 5% to 19%. Thus, in case of HAp/ZnO scaffolds, the fractional porosity generated in HA5GZn sample is considered to be of optimal pore size, shape, and content. Such a distribution of porosity could successfully arrest the crack and provide maximum resistance to its propagation. However, for porous HAp/Ag scaffolds, improved hardness was observed for HA0GAg (1.66 GPa) scaffold followed by a declining trend (up to 0.26 GPa) for other HAp/Ag samples (Table 2). This waning trend for hardness is attributed to the increase in porosity and incorporation of softer Ag particles in HAp ceramic matrix. But, the silver inclusions may work as a cushioning/toughening agent, and the increase in porosity causes a reduction in strength of the pellet. The tensile strength and porosity of materials are observed to correlate by using the fitting equation:

\[
\sigma = aP^2 + bP^2 + c
\]

where, \(\sigma\), \(P\), and \(a\) are the tensile strength (MPa), fraction porosity, and center to center distance (\(\mu m\)) while \(a\), \(b\), and \(c\) implies the coefficient of porosity, distribution of porosity and absolute strength, respectively. The constants \(a\), \(b\), and \(c\) are, respectively, found to be -335.0, -0.9, and 13.0 for HAp/ZnO while -327.5, 0.6, and 8.0 for HAp/Ag samples. The degree of fit, \(R^2\), is closer to one (0.94 for HAp/ZnO and 0.97 for HAp/Ag samples). Thus, similar to the values of constants, HAp/ZnO exhibits higher tensile strength (up to 8.7 MPa for HA5GZn sample) when compared to that of Hap/Ag samples (8.7 MPa for HA0GAg sample, see Table 2).

Additionally, the fracture toughness (\(K_{IC}\)) can be calculated using Mandelbrot’s fractal equation for HAp/ZnO and HAp/Ag samples as mentioned below\(^{[39]}\):

\[
K_{IC} = \left\{ v_{HAp} \cdot \left( 2E_{HAp} \cdot \gamma_s \right)^{1/3} \right\} \left( \left( \log_{10} \left( \frac{r}{10} \right) \right) \right) \left( \frac{\left( \log_{10} \left( \frac{r}{10} \right) \right) - 1}{2} \right) + \left\{ v_{Ag/ZnO} \cdot \left( 2E_{Ag/ZnO} \cdot \gamma_s \right)^{1/3} \right\} \left( \left( \log_{10} \left( \frac{r}{10} \right) \right) \right) \left( \frac{\left( \log_{10} \left( \frac{r}{10} \right) \right) - 1}{2} \right) \right) \cdot \left( 1 - \gamma_{porosity} \right) \quad (2)
\]

where, \(v_{HAp}\), \(v_{Ag/ZnO}\), are the volume fractions of hydroxyapatite (79.67 to 93.67 vol% in HAp/ZnO while 83.29 to 94.29 vol% in HAp/Ag) Ag (0.71 vol%) and ZnO (1.33 vol%), respectively, \(E_{HAp}\), \(E_{Ag}\) are the elastic modulus for hydroxyapatite and silver, respectively, \(\gamma_s\) is surface energy, \(r\) is ratio of similarity. Fracture toughness is also observed to be maximum for HA5GZn sample (3.5 MPa.m\(^{1/2}\)) and HA0GAg (2.6 MPa.m\(^{1/2}\)) samples.

Conversely to the hardness, tensile strength, and fracture toughness, the elastic modulus decreases for both HAp/ZnO (from 63.5 to 5.0 GPa) and HAp/Ag (from 89.5 to 5.0 GPa) samples as the fractional porosity increases from \(\approx5%\) to \(19%\).\(^{[40,41]}\) Figure 4 shows the stress–strain plots for porous HAp/ZnO and HAp/Ag samples, where it can be observed that HA5GZn sample initiates cracking at higher stress (13.93 MPa) than any other composition during diametrical compression test.
(while in HAp/Ag composite, HA0GAg has high absorbed stress of 12.68 MPa, Figure 4).

For HA5GZn, the enhanced hardness, tensile strength, and fracture toughness (Table 2) is attributed to the toughening mechanism of ceramics (by introducing porosity, acting as a crack blunting phase). When the crack propagates, pores distributed in front of the crack results into a crack-blunting phase and absorb the energy for crack propagating and upshot in the enhanced dissipation of energy from the porous structure.14,42 But, for HAp/Ag, crack propagation is blocked by the soft, though tough, silver particles, and crack-propagation loses its energy by crack-blunting of metallic reinforcements. These silver particles absorb the energy by bridging the crack, thereby arresting it in addition to the subsequent effect of densification of HAp by silver particles.8,43 Nonetheless, such an intriguing observation of higher fracture toughness with ZnO reinforcement, when compared to that of Ag reinforcement, may be attributed to higher volume content of ZnO (1.33 vol%) when compared to that of Ag (0.71 vol%). Also, for higher porosity, the decreasing trend in strength is observed which can be attributed to the reduction in cross-sectional area of samples. Improper distribution, size and shape of porosity are also one of the reasons for the weakening of the ceramics. Thus, in this case, the sample without porogen shows better mechanical behavior.

The porosity generated in HA5GZn in HAp/ZnO composites and HA0GAg in HAp/Ag composites can be considered as having an optimum pore size, shape, and content. However, when comparing the hardness, strength, and toughness for HAp/ZnO and HAp/Ag composites, enhanced mechanical properties were observed for HAp/ZnO scaffold (see Table 2) due to the higher vol % (1.33) of ZnO in HAp matrix compared to that of the vol % of Ag (0.71) in HAp matrix for HAp/Ag scaffold.

Further, to complement the diametrical compression test results and to visualize the stress distribution with varying levels of porosity as well as reinforcements (Ag and ZnO), finite element method was used by mesh generation mechanism. The results of the simulation performed on Ag reinforced HAp samples using OOF2 are presented in Figure 5. The overall microstructural feature of the HAp/Ag composites with varying porosity is shown in the SEM micrographs, Figure 5a–d and the corresponding FEM meshes are shown in Figure 5e–h and the resultant stress distribution contours are shown in Figure 5i–l. The scale bar along the filled contour is calibrated in equal 20 such units (i.e., each unit is 250 MPa). It may be observed that increase in porosity results low stressed regions. Pores act as regions of stress-relief and cause the degradation in the load-bearing capability of materials. Among all the HAp/Ag composites, samples with 5 vol% porogen (i.e., HA5GAg) exhibited maximum stress build-up of up to 2 GPa.

Similarly, the effect of ZnO reinforcement on the stress distribution of HAp composites is shown in Figure 6. The SEM micrographs, corresponding FEM meshes and resultant stress counters of HAp/ZnO composites are shown in Figure 6a–l, respectively. Sample HA5GZn showed maximum stress development to the tune of 2.5 GPa among all microwave sintered 7.5 wt% ZnO reinforced HAp composites. However, a few traces of the blue dominant region (high-stress region/C25 4.5 GPa) was also observed in case of ZnO reinforced HAp composites, eliciting that the distribution of stress in HAp composites is non-uniform.

As observed from Figure 5i–j and 6i–j, the resultant stress distribution showed regions of relaxation around the pores. Also, the average stress distribution is in the range of 1.25 to 1.75 GPa,

![Figure 5](image-url)
respectively, for HAp/Ag and HAp/ZnO composites. Moreover, the distribution is more uniform in the HAp/Ag samples as there is no sudden drop in stress values unlike in the HAp/ZnO samples. The stress distribution reflects the trend shown in the stress–strain curves from Figure 4, that is, the stresses generated in the HAp/Ag samples are higher than the HAp/ZnO samples. Thus, the results of the OOF2 strongly corroborate the diametrical compression test results, which discusses crack propagation inhibition in case of HAp/Ag samples.

### 3.3. Biological Studies of HAp/ZnO and HAp/Ag Scaffolds

#### 3.3.1. Bacterial Viability Studies on Porous HAp/ZnO and HAp/Ag Scaffolds

The antibacterial behavior of porous HAp/ZnO (Figure 7a) and HAp/Ag (Figure 7b) is investigated by seeding with gram-negative *Escherichia Coli* (*E. coli*) and gram-positive *Staphylococcus aureus* (*S. aureus*). As the porosity increases in

![Figure 7](image-url)

**Figure 7.** Bacterial viability plot for HAp/ZnO and HAp/Ag for a) *E. coli* and b) *S. aureus*. 
HAp/ZnO and HAp/Ag biocomposites, surface area exposed to the seeded bacteria also increases hence the decreasing trend of bacterial viability is observed. However, in case of HAp/Ag, the bactericidal effect is more prominent than HAp/ZnO, due to the higher zeta potential of Ag nanoparticle (1.09 mV) than that of ZnO nanoparticle (0.01 mV). The overall bactericidal surface is negatively charged at pH 7 due to the polysaccharides of lipopolysaccharides, predominant over the amide.[44] The metal ions (Zn\(^{2+}\) or Ag\(^{+}\)) get enticed to the negatively charged surface of bacteria and moves into bacteria by transmembrane protein.[45] These ions change the thiol (-SH) group into S-Zn or S-Ag bonds along with acting as catalytic agent and initiate the formation of disulphide bonds (R-S-S-R) which subsequently deactivates the shape and function of the enzyme.[46,47] These ions also denature the DNA molecule of bacteria.[48]

3.3.2. Osteoblast Cell Viability of HAp/ZnO and HAp/Ag Biocomposites

Contact angle studies confirm the hydrophilic nature of porous hydroxyapatite scaffolds as shown in Figure 8a and b. Lower porosity scaffolds (HA0GZn or HA0GAg) show the hydrophilic behavior while scaffolds with increasing porosity (HA30GZn or HA30GAg) elicited the more hydrophilic nature (absorbed the water droplet within 200 ms). Since the contact angle is observed by reducing from 50° to almost 0° rapidly, it can be confirmed that the nature of porous hydroxyapatite scaffolds was indeed hydrophilic.

MTT results (Figure 8c, d) show the enhanced absorbance and hence cellular metabolic activity on HAp/ZnO and HAp/Ag scaffolds with increasing content of porosity. These porous...
hydroxyapatite pellets are seen to be cytocompatible as they demonstrate the increasing trend of absorbance with an increase in time in all the groups tested. The hydrophilic response can be well correlated with the MTT data which showed better absorbance for HA30GZn and HA30GAg samples. Nonetheless, a lower cell-density may be expected in Ag reinforced samples when compared to that of ZnO. The elongated cells with filopodial extensions and a prominent nucleus are also presented for the two optimum scaffolds; HA5GZn (Figure 8e) and HA0GAg (Figure 8f). Extensively flattened cellular morphology with prominent cell-cell and cell-matrix interactions highlighted by the conspicuous staining of actin stress fibers are visible, which further validates cytocompatible nature of the scaffolds.

In summary, it can be observed (Figure 9) that the biocomposites HA5GZn and HA0GAg with mechanical properties near to optimal with enhanced hardness (2.89 and 1.66 GPa, respectively), tensile strength (8.7 and 8.0 MPa, respectively), and fracture toughness (3.5 and 2.6 MPa m$^{1/2}$, respectively), may serve as potential biomaterials for load-bearing bone applications. This is due to the toughening mechanism of ceramics in case of HAp/ZnO and bridging as well as crack arrest by Ag, retaining the antibacterial efficacy and displaying cytocompatibility. Moreover, the porosity in samples enhances the exposure of ZnO and Ag particles for the bacterial surface followed by their dissociation into Zn$^{2+}$ and Ag$^{+}$ ions which get attracted by the negatively charged surface of bacteria. Subsequently, insertion of these ions inside the bacteria denatured the bacterial DNA and enzyme. Thus, HA5GZn and HA0GAg bio composites exhibited the cytocompatibility for bone tissue regeneration with antibacterial activity.

4. Conclusions

Porous HAp/ZnO and HAp/Ag biocomposites (by using 0–30% graphite as porogen) were fabricated using microwave sintering technique. A study of the mechanical behavior of porous HAp/ZnO and HAp/Ag scaffolds shows enhanced mechanical properties with the increase in porosity till 8% (HA5GZn sample) in case of HAp/ZnO as this porosity range is able to resist the crack propagation in the sample. Moreover, in case of HAp/Ag, improved mechanical properties were observed for 5% porosity (HA0GAg sample) wherein Ag particles act as cushioning agents. However, enhanced mechanical properties for HAp/ZnO over HAp/Ag scaffolds are attributed to the higher vol% (1.33) of ZnO in HAp matrix when compared to that of only 0.71 vol% of Ag in HAp matrix. Complementarily, OOF2 method is able to predict the behavior of material from the appearance of SEM image accurately revealing that the low stressed regions increase with an increase in porosity content in HAp composites. The distribution of stress in HAp/Ag composites was more uniform when compared to HAp/ZnO composites showing an average stress of 1.25–1.75 GPa, respectively. Additionally, the biological behavior of porous samples was validated by increased metabolic activity and conspicuous cell-matrix interactions with clearly defined actin stress fibers. Therefore, the combined effect ...
of mechanical and biological activities can provide the optimized scaffolds as HAG5Zn in HAp/ZnO and HA0GAg in case of HAp/Ag bio-composites for bone tissue replacement.

Acknowledgements

S. Kanhed and S. Awasthi have contributed equally as first authors. Authors acknowledge Advanced Centre for Material Science, and Department of Material Science and Engineering, IIT Kanpur for extending the micro-hardness, diametrical compression test and SEM facilities, respectively. KB acknowledges Swarnajayanti Fellowship from the Department of Science and Technology, Govt. of India. Fellowship from Council of Scientific and Industrial Research is also acknowledged by Shikha Awasthi. Mr. Felege Nekatibeb is also acknowledged for helping in the analysis of the curve fitting of mechanical properties. Funding from MHRD, Govt. of India, is kindly acknowledged.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

Antibacterial, Cytocompatibility, Diametrical Compression Test, Hydroxyapatite, Object-Oriented Finite Element Modeling (OOF2), Porosity, Silver, Zinc Oxide

Received: November 28, 2017
Revised: February 28, 2018
Published online: