



Fabrication and characterization of silver nanorods incorporated calcium silicate scaffold using polymeric sponge replica technique

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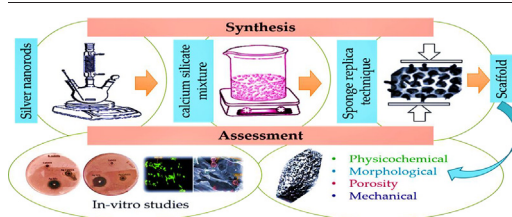
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HIGHLIGHTS

- Silver nanorods have been produced using a reflux method.
- Silver nanorods incorporated calcium-silicate scaffold has been fabricated using polymeric sponge replica technique.
- Excellent porosity has been detected in the newly nanocomposite formed.
- The nanocomposite exhibited good antibacterial properties against *B. subtilis*, *S. aureus*, *E. coli* and *S. typhi* strains.
- The scaffold obtained revealed good biocompatibility with MG-63 human osteosarcoma cells.

GRAPHICAL ABSTRACT



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ABSTRACT

The risk of infection in bones after surgical procedures can be reduced by using antibacterial scaffolds. This study was aimed at preparing a silver nanorods (Ag-nr) incorporated wollastonite (CaSiO₃) scaffold using the polymeric sponge replica method. The powdered Ag-nr sample shows an ultrafine microstructure with a minimum rod diameter of 20.8 nm. The Ag nanorods were fabricated by a reflux method and the sol-gel derived Ag-incorporated-CaSiO₃ samples (Ag-CaSiO₃) were characterized through FTIR, XRD, TEM, SEM, and BET analytical techniques. The electron microscopic results of the scaffold revealed a well-defined porous construction with pore sizes varying from 103 to 164 μm which are suitable for cell infiltration. The Ag-CaSiO₃ scaffold showed a brittle behavior with compressive strength of 11.7 MPa. The in-vitro degradation and mineralization behavior of the Ag-CaSiO₃ scaffold was investigated using SBF. The Ag-CaSiO₃ samples present sound antibacterial effects against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella typhi* without negotiating the formation of apatite layer in simulated body fluid (SBF). The in-vitro cell viability and attachment studies with MG-63 cells using Alamar blue assay confirmed the cytocompatible nature of Ag-CaSiO₃. The rationally designed Ag-CaSiO₃ sample can generate a healthier substitute for bone tissue engineering compared to other similar materials.

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1. Introduction

Hard tissues in a human body have limited self-healing abilities, and any crushed part of such tissues is usually treated by surgical intervention [1,2]. The surgical procedures include local or distant autologous tissue substitutions but are restricted by the lack of a tissue donor and other tissue site complications [3]. The demand for hard tissue engineering has been rising with an alternative being a scaffold or implant-based treatment [4,5]. Scaffolds used for tissue engineering perform mechanically supportive functions as well as serve to guide and increase the healing process of the surrounding bone tissue [6] and enhance in-vivo tissue regeneration that is a primary requirement [7,8]. The materials used to produce an ideal scaffold must tightly bond with bone tissues, promote new bone growth, and may have to bear higher mechanical loads. Bioceramic materials such as tricalcium phosphate [9], hydroxyapatite (H.A.), and bioglass (B.G.) are commonly used for bone regeneration [10,11]. These have shown significant osteoinductive and osteoconductive properties required for bone regeneration [12]. In recent years, calcium silicate (CaSiO_3) has been studied as a biocompatible material for hard tissue restoration [13] and has attracted attention for use in applications in regeneration of bone tissue, due to its high bioactivity compared with hydroxyapatite [14]. CaSiO_3 has various advantages such as low cost of production with excellent biomechanical characteristics [15]. Bone damage or loss and surgical treatment produce various changes such as bacterial infection, which may affect the functionalities of tissues or even tissue loss [16]. A bacterial infection causes inflammation, which may also lead to implantation failure, and become a severe threat to the bone tissue [17,18]. Therefore, antibacterial/anti-inflammation activity as an additional prevention measure is necessary for bone transplantation [19]. Many researchers have used silver in scaffolds such as mesoporous bioactive glass loaded with *in situ* grown silver [20], Ag-doped CaSiO_3 with glycine as fuel [21], GO-Ag nanosystem [22], and Ag-decorated barium titanate [23] for biomedical applications. However, they are expensive procedure and pose problems in terms of antimicrobial performances. The antimicrobial effect of just the bioceramics is limited, and a small quantity of secondary phase materials is not sufficient to achieve useful antibacterial properties [24]. Due to their unique antimicrobial, anticancer, and biological properties with limited side effects, silver nanostructures have been getting considerable attention from researchers since ancient times [25,26]. It is highly desirable to develop a functional 3D scaffold with antibacterial properties that have the potential to control infection and inflammation rates during bone regeneration.

A cost effective sponge replica method was selected for scaffold fabrication using commercial grade sponge-like templates. This economical method fulfills the need for a proper pore size distribution produced by burning-off the organic foam from the powder compact through a sintering process [27]. In the present work, to prove the viability of this method, Ag nanorods incorporated CaSiO_3 scaffold was fabricated using a sponge replica technique.

2. Experimental

2.1. Synthesis of Ag nanorods

The pure Ag nanorods were synthesized by reducing silver nitrate (AgNO_3 ; $\geq 99\%$ Sigma Aldrich) with ethylene glycol (99.8% Sigma Aldrich) in the presence of palladium (II) chloride seeds (99% Sigma Aldrich) and polyvinyl pyrrolidone. The process of nanorods synthesis was carried out as follows: a 250 ml three-necked round-bottom flask attached with a magnetic stirrer used to reflux 50 ml of ethylene glycol at 160°C . Afterward, 20 ml of palladium (II) chloride solution ($1.5 \times 10^{-4}\text{ M}$) was added dropwise to the ethylene glycol solution and allowed for 10–15 min stirring. 0.1 M solution of silver nitrate and 50 ml solution of 0.35 M polyvinyl pyrrolidone were simultaneously added dropwise into the refluxing solution. The final mixture solution was allowed for 1 h. of heat treatment at 160°C . After that, the cooled

solution was diluted with acetone and centrifuged at 5000 rpm for 10 min to get precipitate remained [28].

2.2. Scaffold synthesis

Ag- CaSiO_3 powder was synthesized through the sol-gel method. $\text{Na}_2\text{SiO}_3 \cdot 9\text{H}_2\text{O}$ ($\geq 98\%$ Sigma Aldrich) and $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ ($\geq 99\%$ Sigma Aldrich) were considered as the source of Si and Ca, respectively [15,29]. The above mentioned raw materials were dissolved in enough quantity of deionized water separately using a magnetic stirrer. By using an ammonia solution, the pH of sodium silicate mixture was raised to 12 and mixed with calcium nitrate solution with vigorous agitation. In order to make the composite, 2% w/w of Ag-nanorods were dispersed in 100 ml distilled water and mix within the above mentioned homogenous mixture with continuous stirring of 15 h.

The reactant mixture was filtered, and then washed three times using distilled water and dried at 80°C , followed by manual grinding. A commercial-grade sponge consisting of 30 pores/in. and PVA used to make the Ag- CaSiO_3 scaffold through the polymeric replica sponge method. After successful synthesis, the samples were molded in different shapes and further cut into desired sizes for further analysis. Fig. 1 displayed the schematic illustration for the fabrication of Ag nanorods incorporated CaSiO_3 scaffold.

2.3. Characterization

The phase identification and compositional information of the sample were investigated using XRD (Rigaku Ultima IV, USA), and FTIR (Perkin Elmer Frontier FTIR, USA). The morphology was scrutinized through TEM (TECNAI 200 KV, USA) and SEM (JEOL, JSM 6100, Japan) while surface area and porosity measured by BET (BELSORP mini II, Japan) procedure. The compressive strength of the scaffold was tested at room temperature using a universal test machine stable, according to ASTM D-695 testing protocols.

2.4. In-vitro degradation and mineralization studies

Simulated body fluid (SBF) prepared with ion concentrations approximately equal to human blood plasma [30], used for in-vitro assessment of mineralization and degradation behavior of the Ag- CaSiO_3 scaffolds [31]. For the mineralization behavior analysis, samples were immersed in SBF for fourteen days and characterized through SEM and XRD. On the other hand, for degradation analysis, the samples were immersed in the above mentioned synthetic fluid for 24–96 h separately. The sample scaffold started to absorb SBF, and it may degrade the surface constituents in this physiological fluid that was observed in terms of weight loss [32]. In this process, the generation of the apatite layer on the scaffold surface was observed after fourteen days [33]. Eq. 1 was used for the measurement of the percentage of degradation of the scaffolds [38]. The preliminary weight of the scaffold was considered as W_i and W_f is considered as the concluding weight of the sample.

$$\text{Degradation} = \frac{W_f - W_i}{W_i} \quad (1)$$

2.5. Antimicrobial activity study

Ag-incorporated CaSiO_3 sample was tested for their antibacterial activity against gram-negative (*E. coli* and *S. typhi*) and gram-positive (*B. subtilis* and *S. aureus*) bacterial strain. For the antimicrobial study, the disk diffusion method was used [35]. These bacteria were grown in Lysogeny broth (L.B.) for 24 h, and 100 μL of the L.B. culture used to spread over nutrient agar. Paper discs containing the sample at a 20 μL concentration were added to the agar plates along with discs containing Ag- CaSiO_3 , silver nanorods (SNP), and distilled water (D.W.), which

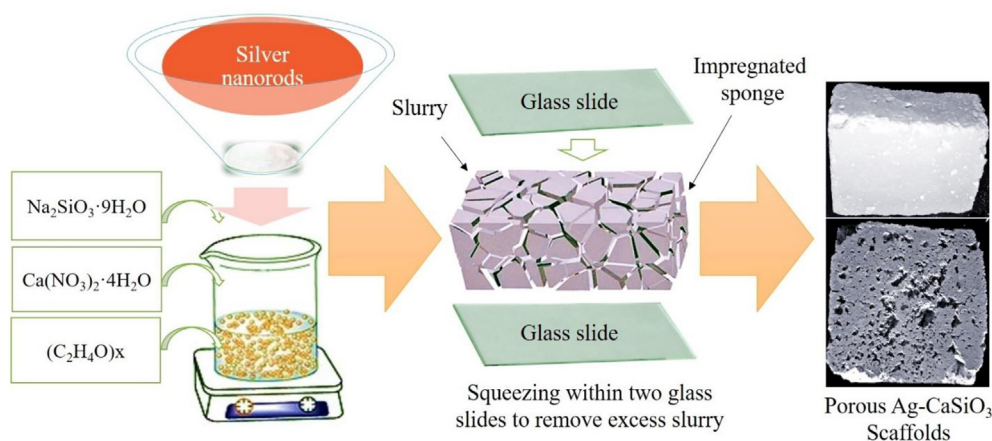


Fig. 1. Ag-CaSiO₃ scaffold synthesis via polymeric replica sponge method.

were incubated for 24 h and checked their zone of inhibition via using a transparent ruler. These cultures were procured from Microbial Type Culture Collection, IMTECH, Chandigarh (India). The cultures were maintained on nutrient agar for further use as per the condition is given in the MTCC protocol.

2.6. Cell viability and attachment studies

MG-63 Human osteosarcoma cells viability after growth in scaffolds was estimated through the Alamar assay [36]. The specimen scaffold was sterilized by ethylene oxide gas, and cells (1×10^5 cells/100 μ l of the medium/scaffold) were seeded on it. The cell-seeded scaffold was stained with 10% Alamar blue and incubated 4 h at 37 $^{\circ}$ C [37]. Calcein AM (Thermo Fisher Scientific, India) is a cell-permeant dye that was also used to determine cell viability on the surface of the scaffold. In order to study cell attachment on the scaffold, samples in triplicate were analyzed using SEM. The cells were seeded on the surface of the sterilized scaffold with the help of 2.5% glutaraldehyde and cultured for 72 h [38].

3. Results and discussion

3.1. Physicochemical analysis

Biosynthesized Ag nanorods scanned in between the range of 30 to 80 degrees that provide information when conditions satisfy Bragg's Law ($n\lambda = 2d \sin\theta$). By scanning the sample, all possible diffraction

directions (Fig. 2A) shows major peaks at 38.45 $^{\circ}$, 44.83 $^{\circ}$, 64.7 $^{\circ}$, and 78.06 $^{\circ}$, which confirm the presence of Ag phases in the sample, verified with the standard PDF database card no. 76-1393. In the second XRD spectra (Fig. 2B), the distinct peaks of the phase of Ag-CaSiO₃ were identified and matched with the standard database card no. 00-901-1950 and PDF 40-0393 [39].

FTIR gives information regarding the present functional groups in the synthesized materials. Fig. 3 shows spectrum data with major peaks at 3868, 3426, 2930, 2850, 1634, 1380, 1127, 1022, 572, and 452 cm^{-1} . The FTIR plot revealed O—H stretching vibrations observed at 3868 and 3426, and the peak at 2850 cm^{-1} could be assigned to C—H stretching of alkanes, and the peak at 1634 could be attributed to C=O stretching or amide due to amide bending [40]. The peak intensity at 1380 cm^{-1} could be assigned to nitro bending N—O bending and 1127, 1022 cm^{-1} could be attributed to C—O— stretching aromatic ring. The peak at 572 and 452 cm^{-1} could be related to alkyl halides, specifically the C—Cl bond.

The FTIR spectrum of the synthesized sample confirms the functional group of Ag-CaSiO₃. In the spectra, the major peaks at 3640, 2140, 1800, 940, 650, 487, and broadband between 1340 and 1260 cm^{-1} are observed. The peak at 940 cm^{-1} indicated the presence of the Ag phase in the Ag-CaSiO₃ sample. The intense bands within 450, 650 cm^{-1} correspond to Si—O—, and SiO₄⁴⁻, while the absorption bands at 1800 cm^{-1} can be assigned to the vibration mode of the Si—O—Si bond [39,40]. The absorption bands at 940 cm^{-1} represent Si—CH₃ functional groups. The band between 1340 and 1260 cm^{-1} is associated

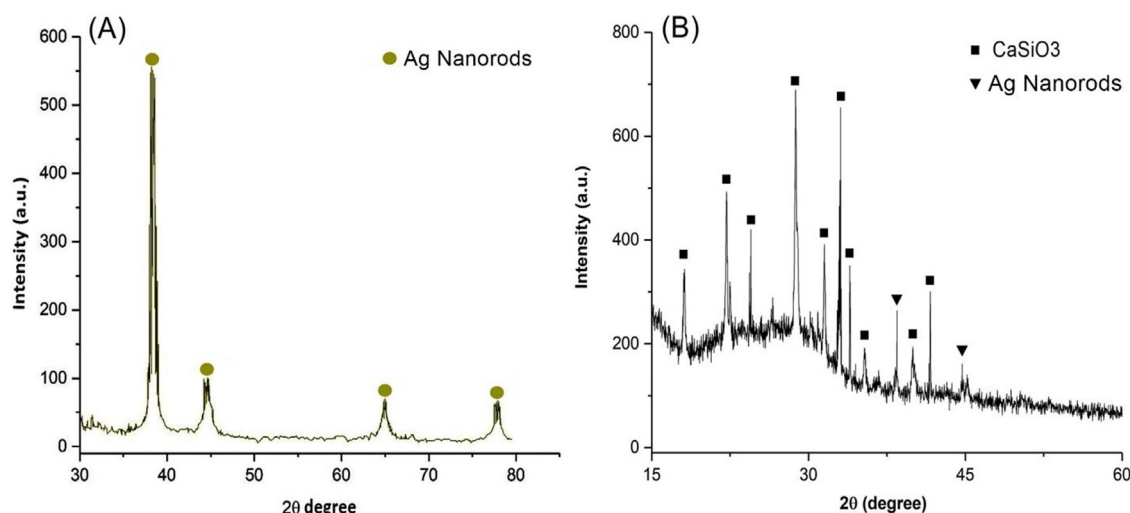


Fig. 2. X-ray diffraction pattern of (A) Ag nanorods, (B) Ag-CaSiO₃.

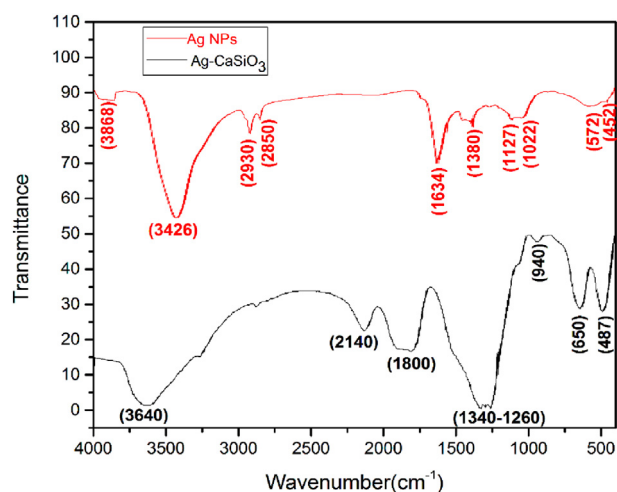


Fig. 3. FTIR analysis of Ag nanorods, and Ag-CaSiO₃.

with the Si-CH₃ and SiO₄⁴⁻ functional groups or Si—O— stretching [40]. The standard stretching vibration was detected at 1320 cm⁻¹ due to the C=O group.

3.2. Morphological analysis

TEM and SEM images (Fig. 4A and 4B) revealed small black spots that were attributed to nanorods. The minimum particle size of Ag nanorods was found 26.4 nm (diameter) with a little agglomeration. SEM images (Fig. 5) showed a well interconnected porous microstructure in the Ag-CaSiO₃ scaffold sample. The Ag nanorods may be uniformly dispersed throughout the Ag-CaSiO₃ composite scaffold. Pore size varied from 54.3 to 196.1 μm in the Ag-CaSiO₃ scaffold as measured by SEM. Bone formation and vascularization were reported for scaffolds requiring a minimum pore size between 100 and 150 μm [41]. Pore size smaller than 10 μm helps to support fast osteoinduction in the scaffold because the size of osteoblasts cells is ≤30 μm in diameter [42,43]. They can easily attach on the rough surface and proliferate effectively within small pores ~100 μm [44]. Although relatively large pores allow high vascularization and oxygenation that involved in early-stage bone formation [45], while small pores cause results in osteochondral ossification; however, the type of bone tissue ingrowths that develops depends on the engineering of biomaterials and pores [46].

3.3. Surface area and porosity

The presence of the high surface area and porosity in the Ag-CaSiO₃ scaffold was confirmed by BET analysis, see Fig. 6. Ag-CaSiO₃ scaffold showed a specific surface area of 476.2 m²/g. Mean pore size and pore size distribution were analyzed using Image-J software. The software analysis revealed mean pore size 93.3 μm. An increased surface areas by porosity can offer more protein adsorption sites and accelerate the release of degradation products, which facilitate the interactions between scaffolds and bone-related cells [43]. Microporosity related characteristics of scaffolds depend on their host materials and process parameters such as pH, and temperature along with the sample's synthesis technique [47]. The cell attachment, cell viability, and micro-nutrient diffusion also rely on the surface area and porosity of the scaffold [48].

3.4. Degradation

The porous structure, surface state, and surface area existing between these pores along with the physicochemical nature of the constituents, decide the degradation of the scaffolds. After 96 h, the Ag-CaSiO₃ scaffold showed 2.3% less degradation than the CaSiO₃ scaffold that was due to the addition of Ag nanorods, see Fig. 7. Degradation is essential for the release of Ca, Si, and Ag moieties from the interior regions of the scaffold. Ionic degradation products of scaffolds facilitate the apatite or bone regeneration process [49,50]. The porous structure provides a higher surface area to the body fluid adsorption on the surface of the scaffold. During the in-vitro cell culture, the porous structure can facilitate cell infiltration through the scaffolds in a 3-D fashion. The growth in adsorption also allows the scaffold to avail of nutrients from culture media more effectively.

3.5. Mechanical testing

The 10 × 10 × 4 mm fragment of the scaffold was cut with the help of a scalpel for mechanical testing. The compression test (ASTM D-695) was performed until the specimen fractured. In this experiment, observations are obtained in triplicate [51]. Fig. 8 (stress, vs. strain curve) revealed the compressive strength of Ag-CaSiO₃ and CaSiO₃ scaffolds, i.e., 11.7 ± 0.82 MPa and 10.9 ± 78 MPa, respectively. The resultant values can be useful for the trabecular bone (compressive strength: 0.1–16 MPa) tissue engineering [52]. The synthesized scaffolds need improvement for the application in cortical bone regeneration because cortical bone

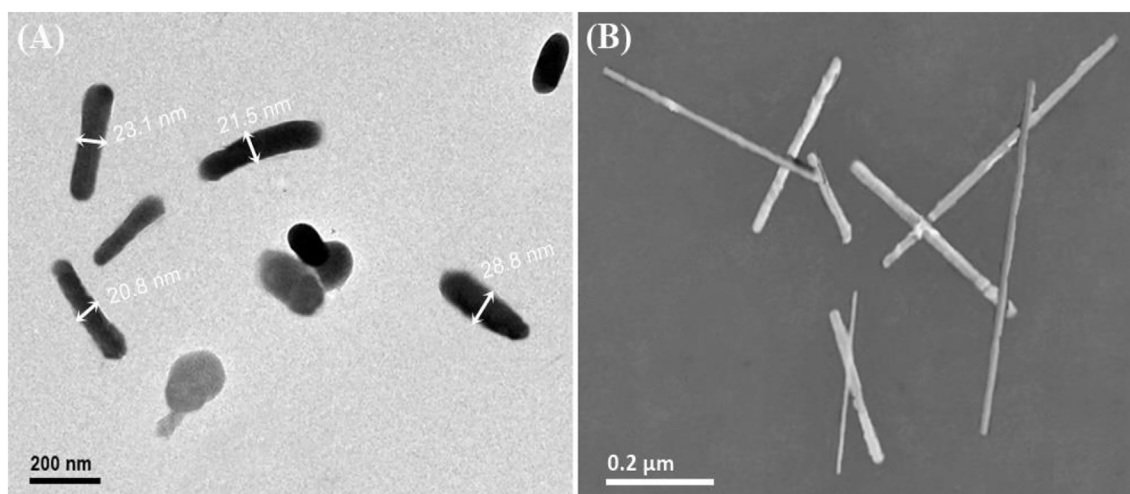


Fig. 4. TEM image (A) and SEM image (B) of Ag nanorods.

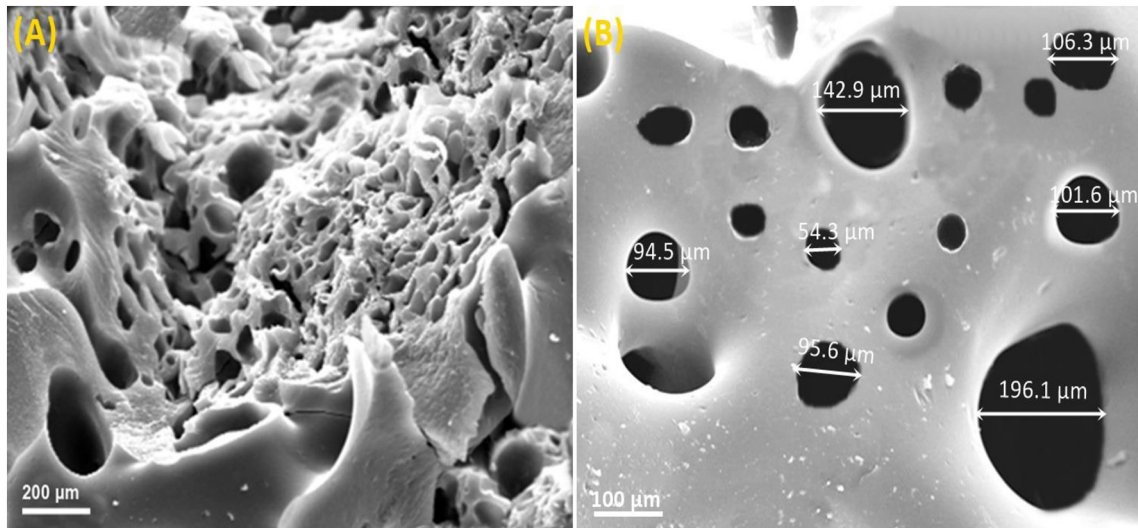


Fig. 5. Morphological examination of Ag-CaSiO₃ scaffold through SEM images.

revealed compressive strength 100–150 MPa [53]. More than 20 calcium silicate bioceramics with a broad range of compositions have been fabricated with different mechanical properties in the last decade [54]. We speculate that the mechanical strength of Ag-CaSiO₃ and CaSiO₃ scaffolds is primarily affected by the porosity of the scaffolds.

3.6. Mineralization behavior

In tissue engineering, the mineralization of the scaffolds with time is a significant parameter to check the bioactivity [55]. Ideally, the scaffolds should release ions or minerals as new tissue formation takes place. The sample was immersed in a beaker holding simulated body fluid at 37 °C for fourteen days in an incubator because SBF facilitates the same environment as blood plasma [34]. The SBF solution was prepared according to the procedure described in the literature [56]. After the dissolution of a scaffold, it may release minerals/ions in the SBF solution that starts to generate the apatite layer, confirmed by SEM and XRD [57,58], as shown in Figs. 9 (A and B) and Fig. 10. The release of Ca and Si ions will support the new bone formation because cortical

bone contains 99% of the Ca in the human body while Si improves bone matrix quality and facilitates bone mineralization [59]. The dissolution of the sample and release of ions also affected by some factors, such as pH, ion concentration, and synthesis parameters [34]. Fig. 9 (A and B) showed an amorphous structure that confirms the activity of CaSiO₃. This testing suggested that Ag doping would not affect the ability of CaSiO₃ to form an apatite layer and direct bonding within the damaged living bone part if implanted in a body [46].

3.7. Antimicrobial activity study

The antibacterial activity of the Ag-CaSiO₃ composite is not equivalent to pure Ag nanorods. The presence of other elements such as Ca and Si, may slow-down the fast action of Ag nanorods. Although Ag-CaSiO₃ revealed better antibacterial activity against *S. Typhi* and *E. coli*, while intermediated action was shown against *B. subtilis* and *S. aureus*, as shown in Fig. 11. The inhibitory activities in the culture media of Ag-CaSiO₃ (R.D.) reported in Table 1 were compared with standard viz. silver nanorods (SNP). Distilled water (D.W.) was used as a dummy, which has not shown any antibacterial activity. Silver is an

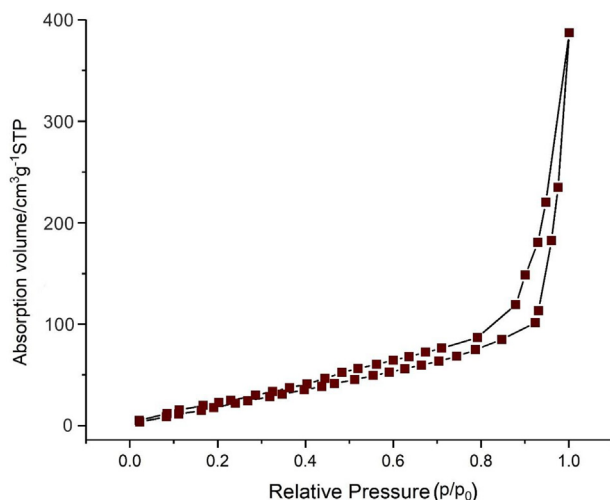


Fig. 6. Surface area analysis through N₂ adsorption-desorption isotherm.

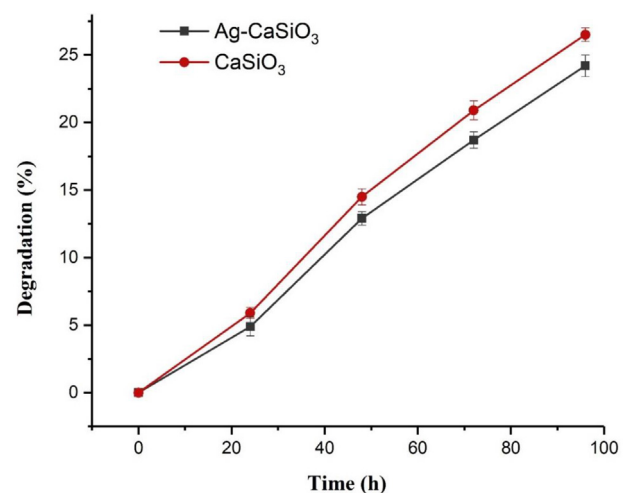


Fig. 7. Degradation behavior of CaSiO₃ and Ag-CaSiO₃ scaffold.

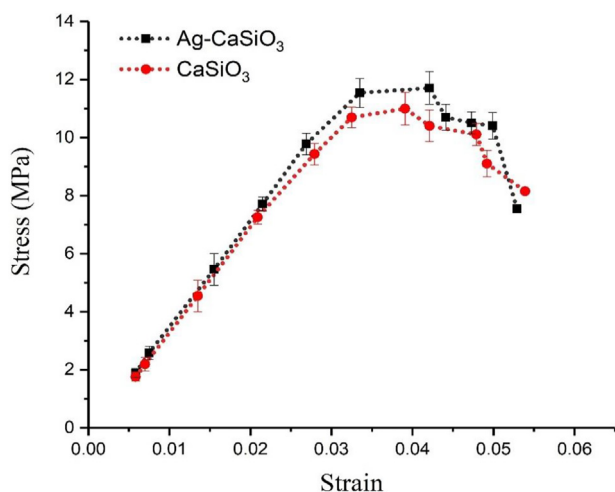


Fig. 8. Stress vs. strain graph of the nanocomposite scaffold.

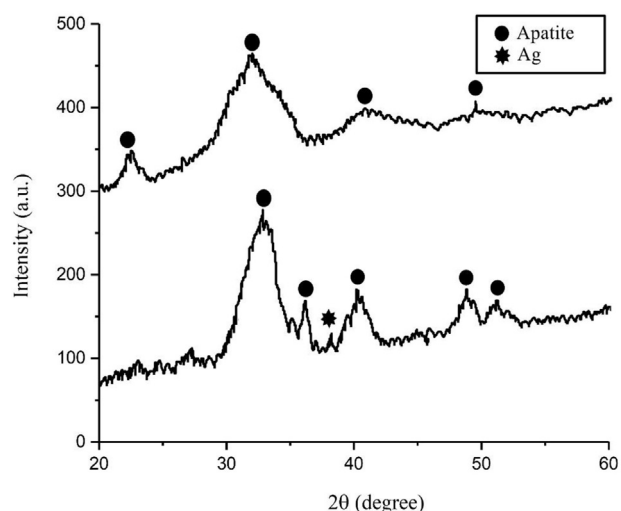


Fig. 10. XRD analysis of mineralized CaSiO_3 and Ag-CaSiO_3 after 14 days.

inert material, but nano-Ag has shown fast action as a fungicide against broad-spectrum fungi such as *Candida*, *Saccharomyces*, and *Aspergillus* [60]. Due to the small size of Ag nanorods, it crosses the cell wall of bacteria and may affect the intercellular process [61]. Metallic silver is inert in the presence of human tissues, but when nano-silver were exposed to moisture and body fluids, it may be oxidized and releases Ag^+ ions [24,62], and it shows a strong affinity for negatively charged biomolecules in the cell wall membrane through the electrostatic attraction [63]. These Ag ions interact with nucleic acid, DNA, RNA, and make complexes with them, which inhibit protein synthesis that generates reactive oxygen species and disrupting the cell [64]. The mechanism of action of Ag nanorods on bacteria is not entirely known. Ag ions can affect the function and structure of protein existing in the cell membrane

and create a functional defect in the microorganism [65,66]. The Minimum Inhibitory Concentration (MIC) of the Ag-CaSiO_3 sample can be appreciated between 6.6 and 13.2 μM . The antibacterial analysis was performed in triplicate, and then the inhibition zone was calculated.

3.8. Cell viability and attachment

Fig. 12A revealed the result obtained from the Live/Dead staining assay, while Fig. 12B (SEM image) displayed the indication of cell attachment on the surface of the scaffold. The surface characteristics of the scaffold can influence the cells' attachment and proliferation [67]. Surface dissolution or mineralization of scaffold release Ca^{2+} ions in the culture medium, which can persuades apoptosis of the cells [46].

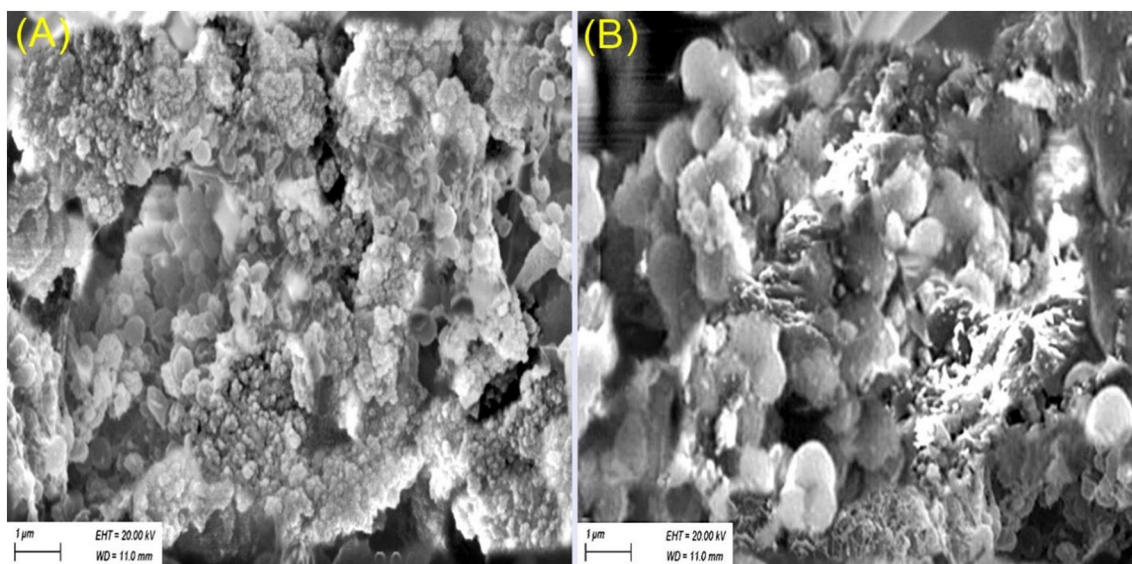


Fig. 9. Mineralization of CaSiO_3 (A) and Ag-CaSiO_3 (B) after 14 days.

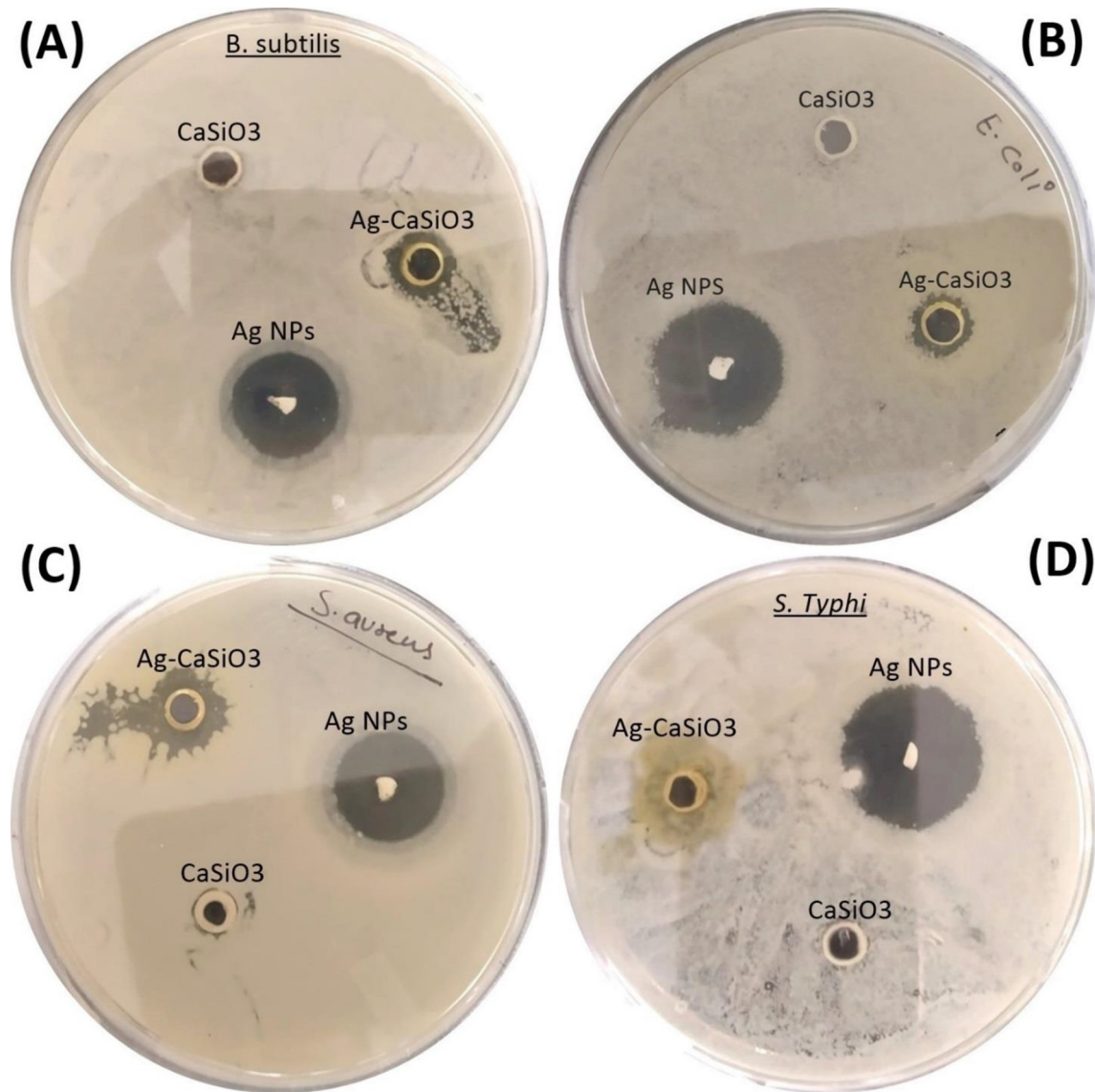


Fig. 11. Antibacterial activity of Ag-CaSiO₃ against *B. subtilis* (A), *E. coli* (B), *S. Aureus* (C), and *S. Typhi* (D).

The in-vitro study here has demonstrated that the presence of Ag moiety in the sample did not affect the biocompatibility of scaffold and did not produce significant toxicity. After the reports on successful use of nano-Ag in wound dressing and surface modification of implants [68–70], it can be used as an Ag-CaSiO₃ scaffold for hard tissue regeneration.

Table 1
Zone of inhibitions (ZOI) with standard deviations (S.D.)

Sr. no.	Name of bacteria	Accession No.	ZOI of Ag-CaSiO ₃ (cm) with SD	ZOI of SNP (cm) with SD
1.	<i>B. subtilis</i>	MTCC 2616	1.1 ± 0.1	2.6 ± 0.12
2.	<i>E. coli</i>	MTCC 443	1.5 ± 0.1	2.6 ± 0.1
3.	<i>S. aureus</i>	MTCC 3160	1.2 ± 0.15	2.8 ± 0.2
4.	<i>S. typhimurium</i>	MTCC 3224	1.2 ± 0.1	2.2 ± 0.15

4. Conclusion

The current research work confirmed the successful synthesis of silver nanorods and the Ag-CaSiO₃ scaffold using the sponge replica method which is a cost effective method. The electron microscopic studies enabled to interpret the microstructure of samples. From the SEM micrographs results, a well interconnected porous structure observed in this scaffold. The cell viability and attachment on the sample's surface were assessed using MG-63 cells; furthermore, the in-vitro mineralization behavior of the scaffold confirmed the bioactivity of the scaffold. The result of the antimicrobial activity of Ag-CaSiO₃ displayed good results against *B. subtilis* (+ve), *E. coli* (–ve), *S. Aureus* (+ve) and *S. Typhi* (–ve) bacteria. Ag-CaSiO₃ sample has the prospective capacity to control the cellular damage from oxidative stress and bacteria. The antibacterial activity of the Ag-CaSiO₃ scaffold makes it very useful substitute for the regeneration of damaged hard bone tissue.

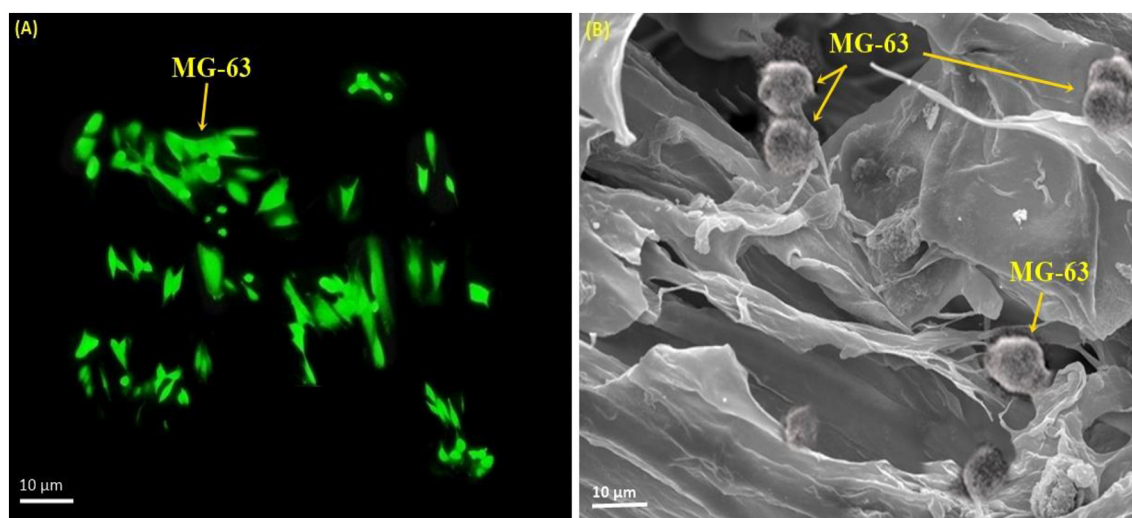


Fig. 12. Images of cell viability after 72 h (A) and Cell attachment (B).

Data availability

The raw/processed data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study.

Author statement

The authors confirm that this work has not been published elsewhere and also it has not been submitted simultaneously for publication elsewhere.

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Declaration of Competing Interest

The author declares that they have no competing interests.

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