

Synthesis of fully interconnected multiporous hydroxyapatite block for orthopedic application

CHANDRANI SARKAR^{1,2} and ARVIND SINHA^{1*}

¹Advanced Materials and Processes Division, CSIR-National Metallurgical Laboratory, Jamshedpur-831007, Jharkhand, India

²Department of Chemistry, Mahila College, Kolhan University, Chaibasa- 833201, Jharkhand, India

Abstract : In the present work, three dimensional multiporous hydroxyapatite block has been synthesized by a novel process which comprises of two steps. At first, three dimensional polymer-hydroxyapatite nanocomposite was synthesized by mimicking matrix mediated in situ biomineralization process. Second is sintering step where synthesized polymer-hydroxyapatite composite was sintered at 1200°C under atmospheric pressure. After sintering, a structurally stable fully interconnected multiporous hydroxyapatite block was obtained. Detailed structural and chemical characterization revealed the role of polymer matrix in the formation of three dimensional multiporous hydroxyapatite block. Cytocompatibility of sintered hydroxyapatite block was evaluated by MTT assay and cell adhesion test. Results evidenced the non-toxic nature of sintered hydroxyapatite block.

Keywords: *Hydroxyapatite; Three dimensional; Biomimetic; Block; Sintering*

INTRODUCTION

Hydroxyapatite [HA, Ca₁₀(PO₄)₆(OH)₂], a calcium phosphate ceramic is extensively researched in water treatment plant, fertilizers and pharmaceuticals industries. Since decades, it has received tremendous attention in biomedical field because the chemical composition is very similar to the inorganic component of bone. Moreover, it has excellent biological properties such as highly biocompatible and bioactive. On material point of view, HA is stiff, brittle and fragile in nature^[1, 2]. So, it is very difficult to contour as three dimensional (3D) structure. Hence, its clinical applications are limited as powder material especially for cavity filler and coating material of alloy or metallic implants^[3, 4].

To be used as bone grafts or bone implants, HA must be in stable 3D shaped with porous structure. Porous structure is required for cell motility and survivability, tissue infiltration and vascularization^[5, 6]. Many attempts have been made by researchers to develop 3D porous HA blocks. Most of these developed processes are gel casting, foaming, replication and sacrificial templating^[4, 6-8]. Mbarki et. al. have demonstrated gelatin as a pore forming agent and synthesized 3D porous HA block^[9]. The common limitations in above mentioned methods are formation of isolated pores, lack of interconnectivity in pores and non-uniform dispersion of pores/ HA^[6-8]. Chen et. al. fabricated 3D HA scaffold with hierarchical porosity by extrusion deposition with porogen foaming technique. In this technique, they had used various chemicals particularly for dispersing HA particles^[10].

On the other side, our group synthesized interconnected macroporous HA granules by biomimetic mineralization process coupled with sintering^[11]. This is a powerful approach which is basically based on in situ nucleation and growth of HA particles in polymeric matrix that leads uniform distribution of HA particles in the matrix resulted in the generation of complex hierarchical macroporous granular structure after sintering^[11]. In recent years, researchers try to develop synthetic strategies that mimic this process. The aim of present work is also to develop an interconnected multiporous HA block by adapting this process. In this work, we have selected carboxymethyl cellulose (a cellulose derivative) and polyethylene glycol (a polyether) as organic matrix. Both the polymers are hydrophilic and nontoxic in nature. They are approved by the U.S. Food and Drug Administration (FDA) for use in different pharmaceutical formulations^[12-13]. These features contribute to their broad application in biomedical research particularly in drug delivery and tissue engineering field. Researchers also demonstrated that

* Corresponding Author E-mail: arvind@nmlindia.org

polyethylene glycol (PEG) efficiently crosslinked with cellulose and formed a homogenous stable structure^[14, 15]. In our study, we aimed to use this homogenous stable CMC-PEG blend as hybrid polymeric matrix for in-situ mineralization of HA. By this simple polymer matrix mediated biomimetic route, three dimensional polymer-HA nanocomposite has been synthesized. Finally, interconnected multiporous HA block was obtained by removal of polymers after sintering. The detailed structural and chemical characterizations along with the mechanical performance of the synthesized block have been studied. Results showed that a fully interconnected multiporous HA block has been successfully synthesized by this process.

EXPERIMENTAL DETAILS

Materials

Polyethylene glycol (PEG), carboxymethyl cellulose [CMC, DS (degree of substitution) ~ 0.9], calcium salt (calcium nitrate tetrahydrate), phosphate salt (diammonium hydrogen phosphate) and ammonia (30%) were procured from Merck, India.

Synthesis of three dimensional hydroxyapatite block (s-HA)

In this work, multiporous hydroxyapatite block (s-HA) has been simply synthesized by sintering 3D polymer-hydroxyapatite composite (p-HA) at 1200°C under atmospheric pressure. At first, 3D polymer-hydroxyapatite composite was synthesized by biomineralization process^[16, 17]. Details of synthetic procedure are as follows- 600 mL CMC (1 wt%) and 300 mL PEG (1 wt%) aqueous solutions were prepared in different vessels. After that, PEG solution was slowly added to CMC solution with constant stirring for 15 min, and a homogenous CMC-PEG blend was formed. Later, 400 mL calcium nitrate solution (0.99 M) was gently added to polymers blend and stirred for 15 min. The pH of the mixture was maintained at 10 with ammonia solution and kept ageing for 24 h at 30 ± 2°C. Next day, 200 mL phosphate solution (0.56 M) was prepared and gradually added to the calcium containing polymer solution. The final mixture was allowed to age for a week at a temperature of 30 ± 2°C. After a week the white slurry was washed with deionized (DI) water and dried in an oven at 60°C for 72 h. At the final step, dried polymer-HA blocks were sintered at 1200°C for 2 h (rate ~4°C min⁻¹) in a tubular furnace under atmospheric pressure, and finally 3D multiporous HA block was obtained.

Characterization

X-ray diffraction [XRD Bruker, D8 Discover, Cu K α radiation with $\lambda=0.154$ nm, 40 kV, 40 mA] was used to characterize the crystallographic phases present in the synthesized samples. Fourier transform infrared (FTIR) spectra of CMC, PEG, p-HA and s-HA were recorded in the range of 4000-400 cm⁻¹ at a resolution of 4 cm⁻¹ using JASCO-FTIR, Model 410. JEOL 2100 transmission electron microscope (TEM) at 200 kV was used for microstructural evaluation. Nova nano scanning electron microscope (SEM) equipped with the analyzer of energy dispersive spectroscopy (EDS) was used for morphological characterization and element investigation. Dissolution study was carried out in simulated body fluid (SBF), the ionic concentrations of SBF is (Na⁺ 142.0 mM, K⁺ 5.0 mM, Mg²⁺ 1.5 mM, Ca²⁺ 2.5 mM, Cl⁻ 147.8 mM, HCO₃³⁻ 4.2 mM, HPO₄²⁻ 1.0 mM and SO₄²⁻ 0.5 mM) nearly equal to those of human blood plasma^[4, 17]. Sintered HA blocks were immersed in 30 mL SBF (pH 7.40) for different time periods (7 days and 14 days). After that, samples were withdrawn, rinsed with DI water and dried for characterization. The Mercury Porosimeter, Pascal 140/440 was used to measure the porosity and pore size distribution of the sintered HA block. Density is measured by Sartorius balance and the data is used for the computation in mercury porosimetry. The compressive mechanical properties of the sintered HA blocks were tested using Universal Mechanical Testing Machine [Hounsfield H10KS-0290] at 0.2 mm min⁻¹ crosshead speed. The dimensions of the blocks were 20 mm × 10 mm × 10 mm.

Cell culture

In-vitro cell study of sintered HA was carried out in Shree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India. Bone marrow mesenchymal stem cells (BMSCs) at third passage were used for in-vitro experiments.

MTT assay and cell adhesion study

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay was performed with extract method as per ISO-10993-5 [16, 17]. The extract of sintered blocks [2 mm × 2 mm × 0.2 mm] was prepared by incubating in 1 mL culture medium [α -MEM containing FBS and antibiotic] at 37°C for 24 h. In the meantime, BMSCs (5×10^3 cells per well) were seeded in 96-well plates and maintained at 37°C under humidified atmosphere containing 5% CO₂ for 24 h. After that, 200 μ L of the sample extract was added to the cell-seeded wells and incubated for 24 h and 48 h. At the day of test, 50 μ L MTT solution (1 mg mL⁻¹) was added into each well and left it for 2 h at 37°C. After 2 h, MTT solution was discarded and isopropanol (100 μ L) was added to each well for dissolving the formazan crystals. The developed color was quantified by measuring absorbance at 570 nm using a micro plate reader (Power Wave-XS micro plate spectrophotometer BioTek -Reader).

For In-vitro cell adhesion test, the test materials (0.5 cm × 0.5 cm × 0.5 cm) were conditioned in culture medium. BMSCs were seeded on pre-conditioned materials at a density of 5×10^3 and incubated for 48 h at 37°C under humidified atmosphere containing 5 % CO₂. After 48 h of incubation, cells with test materials were stained with working FDA solution (10 μ g mL⁻¹) and examined under fluorescent microscope (Leica DMI6000B).

STATISTICAL ANALYSIS

Experiments were performed three times and data represented as mean standard deviation. Data were statistically processed using one way analysis of variance (ANOVA); $p \leq 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

The present study demonstrated a simple biomimetic process coupled with sintering for the synthesis of tailor-made 3D HA block for orthopedic application. It is based on in situ biomineralization of HA particles in CMC-PEG polymeric matrix, and then its conversion to fully interconnected multiporous HA block after sintering at 1200°C. Scheme of synthetic procedure is given in Fig.1. At 1200°C the polymers decomposed out but left their impression, as a result sintered HA with interconnected multiporous three dimensional structure was obtained.

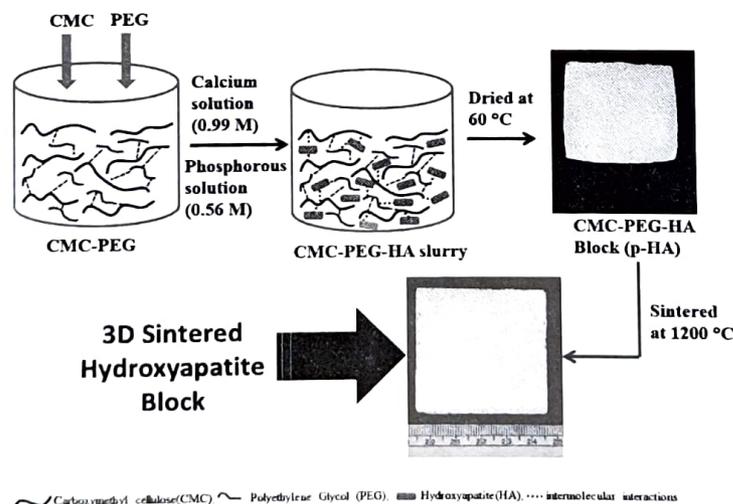


Fig.1. Scheme of synthetic procedure of fully interconnected multiporous hydroxyapatite block.

X-ray Diffraction

XRD patterns of CMC-PEG-HA (p-HA) and sintered HA (s-HA) are shown in Fig. 2. The diffraction peaks in the XRD spectrum of p-HA are assigned as (002), (102), (211), (202), (310), (222), (004), (213) and (511) planes of hydroxyapatite as per the JCPDS card number: 09-432 [4, 11]. All the above mentioned diffraction peaks of hydroxyapatite are also found in s-HA pattern, however, few extra peaks are observed at $2\theta \sim 28.9^\circ, 32.2^\circ, 32.9^\circ, 34.1^\circ, 35.5^\circ$ correspond to (120), (112), (300), (202) and (301) planes of HA. It is clearly seen that the peaks became sharpened and distinctly separate from each other after sintering at 1200°C , indicating the formation of well-developed HA crystals in s-HA [18, 19, 20]. Besides these, very small peaks are observed at $2\theta \sim 25.4^\circ, 31.5^\circ$ indexed as (1010), (0210) planes of β -TCP [20-22].

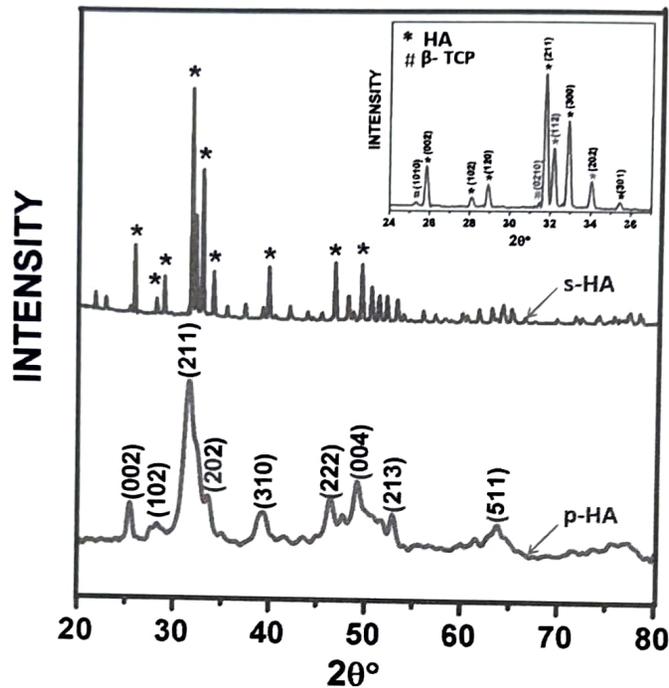


Fig.2. XRD patterns of synthesized samples.

FTIR spectroscopy

FTIR spectra of PEG, CMC, CMC-PEG-HA [p-HA] and sintered HA [s-HA] are presented in Fig. 3. In Fig. 3a and 3b, the broad band of $-\text{OH}$ vibration was found at 3440 cm^{-1} in the spectrum of both PEG and CMC [15, 16]. In addition, two bands at 2880 cm^{-1} and 1352 cm^{-1} were observed in the FTIR spectrum of PEG (Fig. 3a) assigned to $-\text{C}-\text{H}$ stretching and CH_2 wagging, respectively. Moreover, the bands associated with $\text{C}-\text{C}$, $\text{C}-\text{O}$, $\text{C}-\text{O}-\text{C}$ and $\text{C}-\text{H}$ vibrations of PEG (Fig. 3a) were detected at 1287 cm^{-1} , 1250 cm^{-1} , 1108 cm^{-1} and 951 cm^{-1} [14, 15, 19]. In Fig. 3b, the major vibrational bands related to carboxylate (COO^-) stretching of CMC were observed at 1598 cm^{-1} (asymmetric) and 1417 cm^{-1} (symmetric). The $-\text{C}-\text{OH}$ band and β -1,4 glycoside bands of CMC were detected at 1062 cm^{-1} and 599 cm^{-1} , respectively [14, 15]. In the spectrum of CMC-PEG-HA (p-HA) [Fig. 3c], the $-\text{O}-\text{H}$ vibration gave an overlapped broad band at 3155 cm^{-1} , which was slightly shifted from the position of hydroxyl band of CMC and PEG [Fig. 3a,b]. Similarly, asymmetric and symmetric stretching bands of COO^- group of CMC were also shifted to 1633 cm^{-1} and 1398 cm^{-1} , respectively. These shifting in vibrational bands implied the existence of intermolecular interactions among the three different constituents (CMC, PEG and HA) in CMC-PEG-HA composite [14-16, 18, 19]. The vibrational bands of phosphate (PO_4^{3-}) groups were occurred at 1035 cm^{-1} (stretching), 954 cm^{-1} (stretching), 604 cm^{-1} (bending) and 566 cm^{-1} (bending), confirming the formation of in-situ HA in CMC-PEG matrix [16, 18-19]. In addition, a small band appeared at 829 cm^{-1} corresponds to HPO_4

group^[23]. In the FTIR spectrum of sintered HA [Fig. 3d], it was observed that the stretching vibration of -OH group shifted to higher wavenumber at 3570 cm^{-1} , and phosphate (PO_4^{3-}) bands were occurred at 1088 cm^{-1} , 1053 cm^{-1} , 602 cm^{-1} , 571 cm^{-1} and 474 cm^{-1} . However, no characteristic vibrational bands of CMC and PEG were found, indicating the complete decomposition of polymers during sintering^[9].

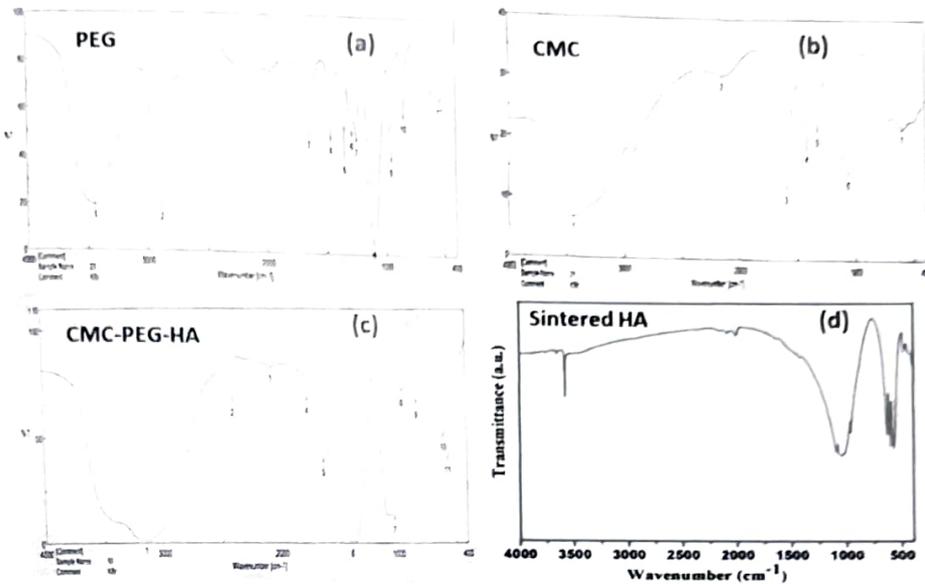


Fig.3. FTIR spectra of (a) PEG, (b) CMC, (c) CMC-PEG-HA and (d) sintered HA.

TEM analysis

The TEM images of CMC-PEG-HA (p-HA) and sintered-HA (s-HA) are presented in Fig.4. The morphology of p-HA consists of clustered spherical particles [Fig. 4a], approximately 20–40 nm in size. On the other hand, the s-HA morphology [Fig. 4b] is highly fused and agglomerated with individual particle size ranging from 200 nm to 400 nm. After sintering, it was noticed that the spherical particles had diffused to bigger particles (almost 10 times in size) and formed agglomerated HA particles. It can be concluded that during sintering, HA particles approached to each other and neck formations occurred between them and the heat treatment temperature favored the particles to enlarge. Hence, particles grow and the grain boundaries became clearer in sintered HA^[24, 25].

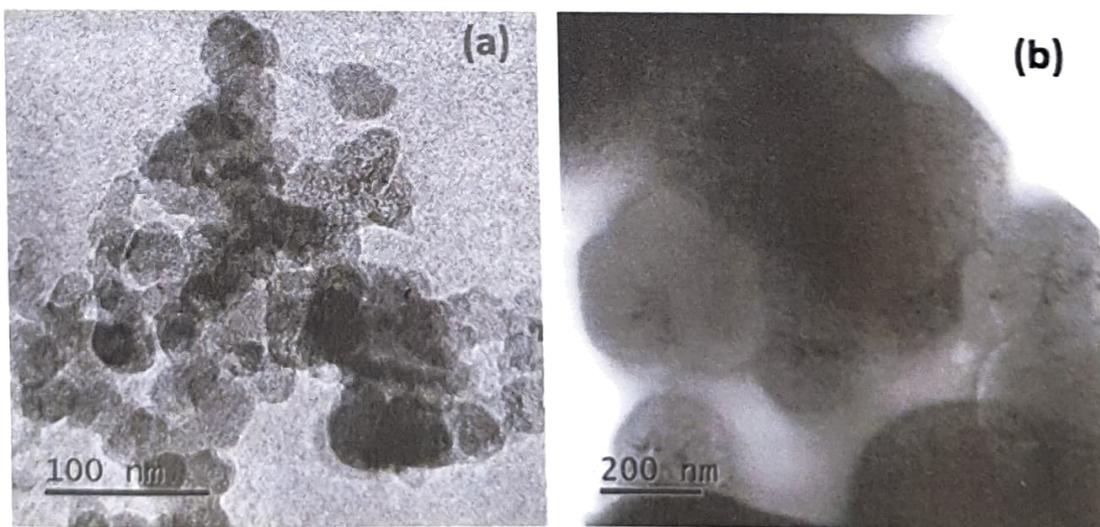


Fig.4. TEM image of (a) CMC-PEG-HA [p-HA] and (b) sintered HA [s-HA].

Microstructural analysis

The SEM microstructure of the as-synthesized CMC-PEG-HA (p-HA) composite from biomimetic process and sintered HA (s-HA) are shown in Fig. 5a-c. Biomimetic p-HA composite (Fig. 5a) consists of irregular shaped small and large aggregates. These aggregates were made up with nanosized HA particles (20- 40 nm) as shown in TEM image (Fig. 4a). This aggregation of nanoparticles is due to the intermolecular interactions among CMC, PEG and HA^[14-16, 19]. Moreover, the surface properties associated with HA nanoparticles caused these particles to clump together and formed different size of agglomerates^[3, 18, 26].

Fig. 5b exhibits the interconnected porous structure of sintered sample. This porous structure may be attributed to the elimination of polymers during sintering^[11]. A closer look at the microstructure evidenced the presence of a hierarchy in pores [Fig. 5c], all the primary pores consists of secondary pores in their volume. This study confirmed that the fully interconnected multiporous HA block was formed by this simple process (biomimetic method coupled with sintering). In Fig. 5c, few small and immature clusters of particles were found at the edge of grain boundaries. The elemental analysis (EDS) data showed that these clusters are made up with calcium (Ca), Phosphorous (P), Oxygen (O) and Carbon (C), mainly deficient calcium phosphate minerals (Ca/P ~1.60). It is known that calcium deficient minerals are generally water soluble^[4]; hence, the solubility of sintered sample was checked in SBF (simulated body fluid) for 7 days and 14 days [Fig. 5e, f]. At day 7, calcium deficient parts were started to solubilize, however, at day 14 those were completely solubilized.

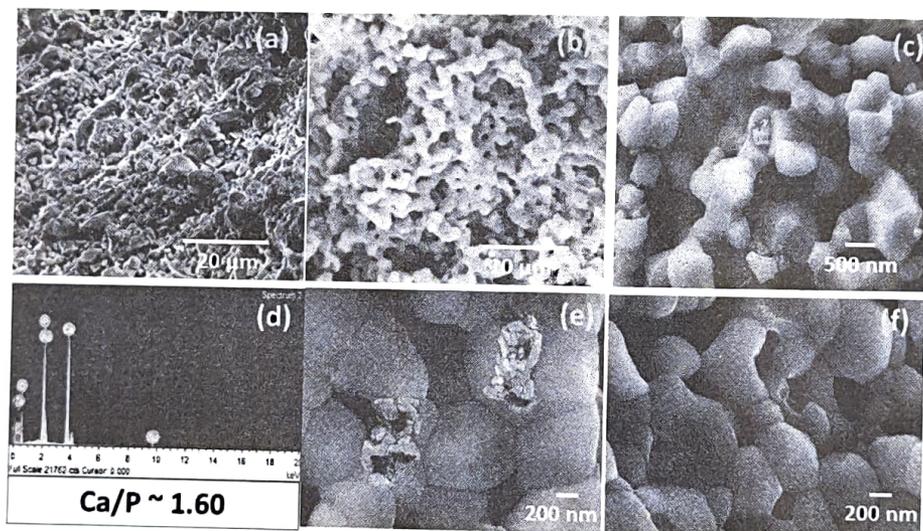


Fig. 5. SEM image of (a) CMC-PEG-HA [p-HA]; (b) sintered HA [s-HA]; (c) high resolution image of s-HA; (d) point EDS of s-HA; images of simulated body fluid treated s-HA [(e) 7 days and (f) 14 day].

Porosity and mechanical properties of sintered HA

The performance of bone implant mainly depends on its structure and strength, generally porous structure having wide range of pores with sufficient strength is more effective after implantation^[8-9]. So, the porosity and compressive strength of sintered HA block have been evaluated. The pore size distribution pattern of sintered HA are presented in Fig. 6a. It shows that the pores diameter ranges from 4 -100 μm and the porosity is ~ 55%. The compressive stress-strain curve of sintered HA is shown in Fig. 6b. The curve exhibits a typical behavior of porous ceramic material, and sample failed at 3.6 MPa (stress) with lower strain percentage (6.3%) under compression^[4]. Porous sintered HA block possesses compressive strength ~ 3.22 ± 0.35 MPa and elastic modulus ~ 242 ± 15 MPa, which are both in the range of human cancellous bone^[4, 14]. These results showed that the sintered multiporous HA block can support bone tissue regeneration at the site of implantation, and can maintain sufficient integrity.

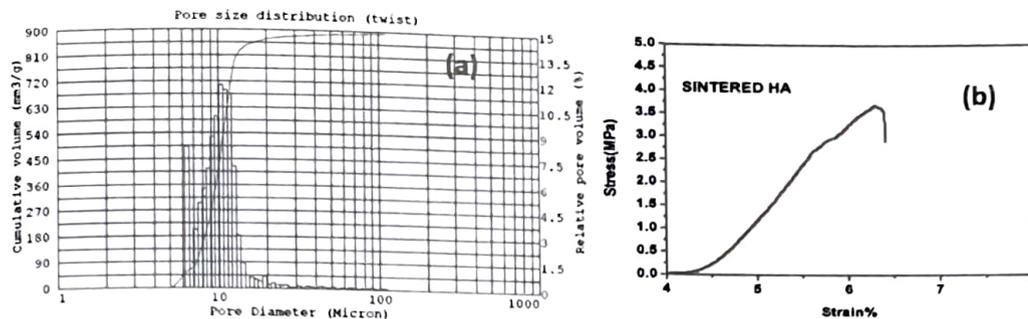


Fig.6. (a) Pore size distribution curve and (b) stress-strain curve of sintered HA.

MTT assay and cell adhesion study

Sintered HA (s-HA) was subjected to MTT assay with BMSCs. This study exhibits the cell metabolic activity where MTT absorbance corresponds to the number of viable cells [6-7, 17]. Results evidenced the proliferation of cells with culture time, and no significant toxicity was observed. Cell adhesion study revealed the behavior of cells on direct interaction with test material [16]. A representative image of cell adhesion test is shown in Fig. 7b, number of cells was observed on the surface of sintered HA which revealed that the sintered HA block do not interfere in cellular mechanism suggesting its cytocompatible nature [27]. Therefore, it can be concluded that sintered multiporous HA block may be used as an efficacious bone construct in future.

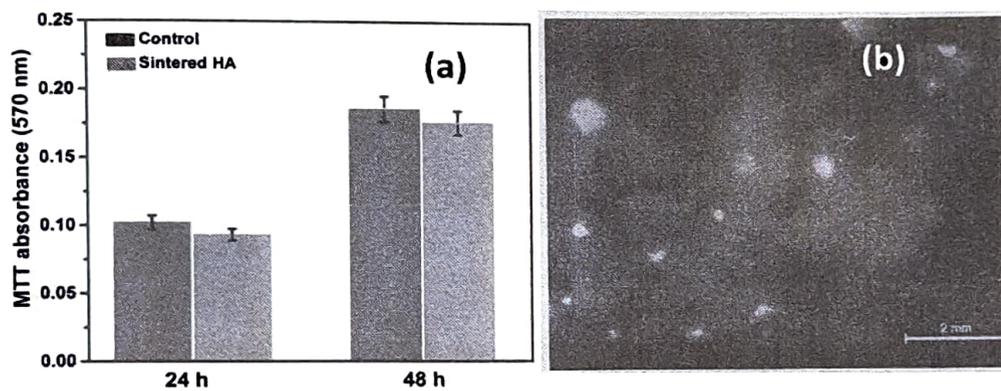


Fig.7. (a) MTT assay of sintered HA scaffold, (b) Fluorescent microscopy image after BMSCs were cultured with sintered HA for 48 h

CONCLUSION

In this study, fully interconnected multiporous hydroxyapatite block was successfully synthesized by a simple and cost effective process. The process is based on the concept of biomineralization, which demonstrates good control over the microstructural features of the block. Structural characterization clearly evidenced the formation of fully interconnected multiporous HA block. The microstructural features of sintered HA is a function of hybrid polymers matrix where the nucleation, growth and aggregation of HA nanoparticles were occurred. During sintering, polymers decomposed out and left their impression in sintered sample, as a result fully interconnected multiporous HA block was formed.

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