ISSN No:-2456-2165

Biomimetic Scaffolds from Hydroxyethyl Cellulose/Calcium Phosphate for Bone Tissue Engineering Applications

[1]Fathima Shahithaa, [2] Hassanal Harrisb, [3]Mohammed Al-Sibania
[4] Ab. Rahim, Mohd Hasbib, [5] Ahmed Al Harrasic, [6]Asiya Obaid Abdallah Al Saadia

The College of Arts and Sciences, Department of Biological Sciences and Chemistry, University of Nizwa Initial Campus, Birkat Al Mouz, P.O. Box 33, PC 616, Nizwa, Sultanate of Oman, bFaculty of Industrial Sciences & Technology, Universiti Malaysia Pahang, Lebuhraya Tun, Razak, 26300 Gambang, Kuantan, Pahang, Malaysia, bNatural and Medical Sciences Research Center, University of Nizwa Initial Campus, Birkat Al Mouz, P.O. Box 33, PC 616, Nizwa, Sultanate of Oman.

Abstract:- Hydroxyapatite (HA) crystals inside the pores of hydroxyethyl cellulose (HEC) sponges have been developed using simulated body fluid (SBF) in a green chemistry approach and has been used as a scaffold material. Porous HEC sponges were prepared by freeze-drying method. HEC is a water soluble polymer which is non-toxic and biocompatible. The crystallization of hydroxyapatite (HA) was done by immersing HEC sponges (1 wt%, 3 wt% and 5 wt%) into various concentration of simulated body fluid (SBF) for different time periods. For the analysis of these materials, various bulk and particle level characterization techniques have been employed, which includes Field transmission scanning electron microscopy (FESEM), Scanning Electron Microscope (SEM), Fourier transform infrared spectroscopy (FTIR), X-ray diffraction spectroscopy (XRD) and mechanical testing. Energy dispersive X-ray (EDX) analysis was used to confirm the deposition of apatite on the surface of HEC sponges. It was found that porosity, pore size and pore inter connectivity depends upon the concentration of the HEC. The size of the HA crystals increased with the concentration of the HEC. The sponges had pores with diameter~2-200µm and pores were interconnected. In-vitro testing with human dental pulps stem cell (DPSC) was also conducted to assess its biocompatibility. In-vitro bioactivity and biodegradability studies showed that the HEC/HA scaffolds were bioactive as well as bioresorbable. Combining hydroxyapatite into hydroxyethyl cellulose may generate a composite with favorable mechanical and chemical properties that are appropriate for various medical applications.

Keywords:- *Hydroxyapatite, Hydroxyethyl Cellulose, Scaffolds, Sponges, Tissue Engineering.*

I. INTRODUCTION

A key component in tissue engineering for bone regeneration are the scaffold that serves as a template for cell interactions, and the formation of bone-extracellular matrix to provide structural support to the newly formed tissue. Scaffolds for bone regeneration should meet certain criteria to serve this function, including mechanical properties similar to those of the bone repair site, biocompatibility and biodegradability at a rate commensurate with remodelling. Scaffolds serve primarily as osteoconductive moieties, since new bone is deposited by creeping substitution from adjacent living bone (Groeneveld et al. 1999). Scaffolds for osteogenesis should mimic bone morphology, structure and function in order to optimize integration into surrounding tissue. Bone is a structure composed of hydroxyapatite ($Ca_{10}(PO_4)6(OH)_2$) crystals deposited within an organic matrix ~95% is type I collagen (Marks et al. 2002). The requirements for a synthetic bone substitute appear deceptively simple, that is, to supply a porous matrix with interconnecting porosity that promotes rapid bone ingrowth, yet possesses sufficient strength to prevent crushing under physiological loads during integration and healing. The ideal bone substitute is not a material that interacts as little as possible with the surrounding tissues, but one that will form a secure bond with the tissues by allowing, and even encouraging new cells to grow and penetrate. One way to achieve this is to use a material that is osteophilic and porous, so that new tissue, and ultimately new bone, can be induced to grow into the pores and help prevent loosening and movement of the implant. Resorbable bone replacements have been developed from inorganic materials that are very similar to the apatite composition of natural bone (Groeneveld et al. 1999). Porosity is defined as the percentage of void space in a solid (Leon et al. 1998) and it is a morphological property independent of the material. Porosity and pore size of biomaterial scaffolds play a critical role in bone formation in vitro and in vivo. Porous hydroxyapatite (HA) and related calcium phosphate ceramic materials with open pore structures are of particular interest, since they have excellent permeability and a large surface area as well as excellent biocompatibility (Suchanek et al. 1998) and (Deville et al. 2006). These properties allow them to be used in biomedical engineering applications, such as bone scaffolds and drug carriers (Deville et al. 2006). In recent years, considerable attention has been given to the development of fabrication methods to prepare porous ceramic scaffolds for osseous tissue regeneration (Deville et al. 2006; Almirall et al. 2004; Charriere et asl. 2003; del Real et al. 2002; Kawata et al. 2004; Milosevski et al. 1999; Ramay et al. 2003; Tian et al. 2001). The ideal fabrication technique should produce complex-shaped scaffolds with

controlled pore size, shape and orientation in a reliable and economical way. Although extensive efforts have been put into the development of porous scaffolds for bone regeneration, with encouraging results, all porous materials have a common limitation: the inherent lack of strength associated with porosity. Hence, the development of porous hydroxyapatite scaffolds has been hindered to low-stress locations, such as broken jaws or fractured skulls. Therefore, the unresolved dilemma is how to design and create a scaffold that is both porous and strong.

Several technologies exist today for manufacturing porous bioceramics. Important advantages have been found in the production of macroporous ceramics by freeze drying methods. Freeze casting is a simple technique to produce porous complex-shaped ceramic or polymeric parts (Fukasawa et al. 2001). In freeze casting, a ceramic slurry is poured into a mold and then frozen. The frozen solvent acts temporarily as a binder to hold the part together for demolding. Subsequently, the part is subject to freeze drying to sublimate the solvent under vacuum, avoiding the drying stresses and shrinkage that may lead to cracks and warping during normal drying. After drying, the compacts are sintered in order to fabricate a porous material with improved strength, stiffness and desired porosity. The result is a scaffold with a complex and often anisotropic porous microstructure generated during freezing. By controlling the growth direction of the ice crystals, it is possible to impose a preferential orientation for the porosity in the final material (Kang et al. 1999). The technique was applied more specifically to polymeric materials, for tissue engineering. A wide variety of materials have already been investigated, including chitin (Madihally et al. 1999), gelatin (Kang et al. 1999) and (Huang et al. 2005) collagen (Schoof et al. 2000) PLA (Zhang et al. 1999; Ho et al. 2004), PDLLA, PLGA ((Zhang et al. 1999; Maquet et al. 2004), poly(HEMA) (Oxley et al. 1993), agarose (Stokols et al. 2004), sericin (Tao et al. 2005) and alginate (Stokols et al. 2004; Shapiro et al. 1997; Zmora et al. 2002; Chung et al. 2002; Tampieri et al. 2005). Although not stiff and strong enough for load-bearing applications, all these materials have in common an homogeneous structure with open porosity, favorable for rapid cell proliferation. In particular, pore size and its structure can be controlled by heat transfer rate. We show here how freeze casting can be applied to hydroxyapatite (HAP), an osteophilic ceramic related to the inorganic component of bone, to process bone substitute materials with suitable physical and mechanical properties. In particular, we describe here how the processing conditions (concentration, freezing rate, sintering) affects the scaffold characteristics (size and amount of porosity, compressive strength) and discuss the limits of the technique. Scaffold properties, depend primarily on the nature of the biomaterial and the fabrication process. The nature of the biomaterial has been the subject of extensive studies including different materials such as metals, ceramics, glass, chemically synthesized polymers, natural polymers and combinations of these materials to form composites. Properties and requirements for scaffolds in bone tissue engineering have been extensively reviewed. Calcium hydroxyapatite (HA: Ca10(PO4)6(OH)2), the main inorganic component of the hard tissues in bones , is a member of the `apatite' family, including compounds with similar structure but not necessarily of identical composition.

The biologically beneficial characteristics of HA, not just the fact that it is the major inorganic component of bone matrix, such as its specific affinity to many adhesive proteins and its direct involvement in the bone cell differentia-tion and mineralization processes, make HA especially suited for utilization in the bone regeneration field. Formation of apatite on surface of biomaterials by the treatment of simulated body fluids (SBF) is frequently used. SBF is an electrolyte solution, has inorganic composition similar to human blood plasma. Initially SBF designed to test the bioactivity of artificial bone material in vitro because its composition is very close to human blood plasma. SBF have the ability to form calcium phosphate containing apatite on the surface of artificial materials (Kokubo et al. 1990). There is a technological need for porous polymer/hydroxyapatite (HA) composites to be produced in an environmentally friendly manner.

In this study, hydroxyethyl cellulose (HEC) which is one of the polymers is used for the formation of hydroxyapatite (HA) on it through crystallization process. The effect of the concentration of HEC sponges on crystallization of HA and cell growth will be determined. These can be used to compare with the previous study that used other polymer or synthetic method.

II. METHODOLOGY

A. Materials and methods

HEC with molecular weight of 250000 was purchased from Sigma Aldrich, Glutaraldehyde, 25% in H_2O was purchased from Sigma-Aldrich, USA. Phosphoric acid was purchased from R&M Chemical, UK. Analytical reagent grade acetone was purchased from Bendosen. Sodium chloride (NaCl) was purchased from Merck KGaA, Germany, calcium chloride dehydrate, ($CaCl_2.2H_2O$) was purchased from Fisher Chemical, UK, sodium bicarbonate ($NaHCO_3$), potassium chloride (KCl), magnesium chloride hexahydrate ($MgCl_2.6H_2O$) and sodium dihydrogen phosphate ($NaH_2PO_4.H_2O$) were purchased from Sigma-Aldrich, USA. Human Dental Pulp Stem Cell, DPSCs were obtained from Department of Biology of University Malaya. The cells were cultured and maintained in Molecular and Cellular Biology Lab, Integrated Centre for Research Animal Care and Use (ICRACU), IIUM.

B. Preparation of Hydroxyethyl Cellulose Solutions

Three sets of different HEC concentration solutions: 1 wt%, 3 wt%and 5 wt% were prepared by dissolving 1g, 3g, and 5g of HEC powders in 100ml of deionized water, taken in beakers. After that, all these five beakers were covered by aluminium foil and stirred for 24 hr at room temperature to ensure the formation of homogenous HEC solution.

C. Preparation of HEC sponges

The HEC sponges were prepared by the freeze drying process. Freeze drying is a process of removing frozen solvents from a material through sublimation and desorption. Before the samples were placed into the freeze dryer, all HEC solutions were put into a freezer with the temperature of -80°C for five days until the samples were completely turned into solid form. Then, the samples were put into the freeze dryer for four days to remove the water contained in the samples and formed a HEC sponges. All samples were crosslinked by using glutaraldehyde in the presence of acetone and phosphoric acid.

D. Preparation of Hydroxyapatite coated Hydroxyethyl Cellulose (HEC/HA) sponges

In order to form the HA crystal on HEC sponges, SBF solution is required. In this research, two different sets of SBF concentration solution (10xSBF solution and 20xSBF solution) were prepared based on Tas and Bhaduri(Chalal et al., 2013). The purpose of preparing two different sets of SBF solutions were used to study effect of SBF concentration solution on crystallization of HA on HEC sponges.

E. *Characterization*

The surface morphology of HEC/HA crystals were observed by using ZEISS EVO 50 Scanning Electron Microscopy. The functional groups present in HEC/HA sponges were recorded by using a Perkin Elmer Fourier Transform Infrared Spectrometerin the range of 400 cm^{-1} to 4000 cm^{-1} with resolution of 4 cm^{-1}. Thermal Gravimetric Analysis (TGA). The samples werethen undergoing "Vickers hardness test" using a 136 diamond pyramid for mechanical testing.

F. Cell expansion and seeding

The DPSC cells were cultured in DMEM containing 10% fetal bovine serum and 5% penicillin/ streptomycin in 75 cm2 cell culture flasks. The DPSC cells were incubated at 37 ◦C in a humidified atmosphere containing 5% CO2 for 3 days. The HEC/HA sponges were cut into minuscule disks with 10 mm in diameter marinated in 100% ethanol for 24 h, and then sterilized under UV light for 3 h. These scaffolds were again sterilized with 70% ethanol for 30 min then washed with phosphate buffer saline, PBS for 15 min three times and subsequently immersed in cell culture medium overnight. The scaffolds were washed thrice with sterile PBS and transferred into 96-well tissue culture plate. DPSC cells grown in 75 cm^2 cell culture flasks were detached on confluency by integrating 1 ml of 0.25% trypsin containing 0.1% EDTA. Detached cells were centrifuged and counted by Trypan blue utilizing heamocytometer, seeded on HEC sponges scaffold at a density of 1×10^4 cells/cm^2 and incubated to facilitate cell magnification. The medium was refreshed every 3 days.

All cell cultures were examined customarily and routinely microscopically. The cell morphology, colour, and turbidity were checked by microscope to ascertain there is no presence of contaminants. Media (DMEM) was transmuted once for each day to eschew the cells from depleting, depriving of concrete nutrients, or becoming acidic. For media transmuting, the spent media was abstracted and equal volume of fresh media (5 ml) was integrated to the cell culture flasks.

The flasks were stored and maintained in 5% carbon dioxide (CO2) humidified atmosphere at 37 ºC in the incubator. The cell cultures were passaged when they have occupied 80-90% confluence of the surface area of a flask to maintain salubrious magnification. Cell culture media (DMEM) were abstracted and discarded. TrypLE was integrated to each flaskto detach the cells. The solution was swirled across the adhered cells to ascertain TrypLE reaches all cells. The flasks were returned to the incubator for 5 minutes.

The detachments of the cells were checked by utilizing microscope. Once the cells were detached from the flask, the cell suspensions were transferred into 15mL tube and centrifuged at 800rpm for 5 minutes. Then, TrypLE were abstracted and fresh cultured media (DMEM) containing serum was then integrated to inactivate the TrypLE in the cell suspension. The aliquot of cell suspensions was placed into an incipient flask containing 5 mL of cell culture medium (DMEM), then stored in the incubator. After 24 hours, the cultures were checked to ascertain that cells were reattached. The medium in each flask was transmuted as indispensable until the next subculture.

G. Cell proliferation study

To determine cell viability, the colorimetric MTT metabolic activity assay was utilized. DPSCs (1×10^4 cells/well) were cultured in a 96-well plate at 37 °C, and exposed to 3 wells of variant of HEC cellulose sponges for 72 h. Cells treated with medium only accommodated as a negative control group. After abstracting the supernatant of each well and washing twice by PBS, 20 µl of MTT solution (5 mg ml-1 in PBS) and 100 µl of medium were then introduced at 37 ◦C for 4 h. 100 micro liter of DMSO were integrated to each well to dissolve the MTT formazon crystals. The absorbance was recorded at reference wave length 620 nm by spectrophotometer. Since the absorbance reference wave length 620 nm is proportional to cell viability, all experiments were performed in quadruplicate, and the relative cell viability (%) was expressed as a percentage relative to the untreated control cells. Relative viability is then expressed as percent of control and calculated as in Eq. 1:

% viability = mean OD sample x 100 / mean OD controls (1)

III. RESULTS AND DISCUSSION

A. Morphological structure of HEC-HA sponges

FESEM was done to determine the morphology of the HA crystals. Fig. 1 and shows the FESEM images of HA crystals formed on 1 wt%, 3 wt% and 5 wt% of HEC sponges respectively. From the images obtained, the pores of the HEC sponges cannot be seen clearly at certain area, this is because the surface of the HEC sponges are covered

by the HA crystals and the pores were blocked by the HA crystals. The results revealed that the HA crystals formed were in spherical and polyhedral shapes. The average size the HA crystals formed on HEC sponges were in the range of 0.1 – 1.0 μm, for spherical particles and 0.10-1.5 μm for the polyhedral shapes. The size of the polyhedral shaped HA crystals were increasing with the concentration of the HEC sponges.

Zhang et al. (2009) stated that the crystal growth in HEC is strongly depends on the interaction between the HEC molecules and the crystal surface. HEC with higher concentration may lead to the interaction between the HEC molecules and crystal surface sufficient to lower the crystal surface energy as well as to stabilize the crystals in metastable phase. The difference in concentration of HEC will affect the morphology and size of the crystals formed. When the HEC concentration increased, there is less space between the HEC molecules and become entangled or aggregated. This will alter the interaction between the HEC molecules and HA crystals. Polar hydroxyl side groups of the HEC molecules tend to interact with Ca^{2+} during the HA crystallization process, to create the nucleation sites. The interface energy is then reduced and eases the formation of metastable phase.

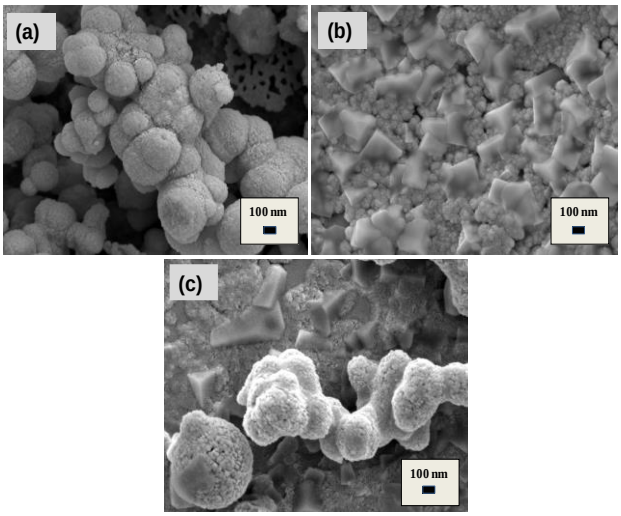


Fig 1: FESEM images of porous scaffolds with hydroxyapatite (a) 1 wt% HEC, (b) 3 wt% HEC and (c) 5 wt% HEC.

B. ATR-FTIR spectroscopy analysis

Fig.2 shows the FTIR spectra of SBF treated HEC sponges. The carbonate group (CO_3^{2-}) peaks of HA appear at 1428.73, 1431.46 and 1427.78 cm^{-1} for 1 wt%, 3 wt%, and 5 wt%, respectively, this suggests that the HA is type B carbonate apatite (CO_3 substituting PO_4) The characteristic absorption bands of PO_4 are formed as P-O stretching at 1012.82 (v_3) cm^{-1} and 1135.91 cm^{-1} for 1 wt% HEC, 1012.85 (v_3) and 1132.25 cm^{-1} for 3 wt% HEC, and 1024.91 (v_3) cm^{-1} and 1132.40 cm^{-1} for 5 wt% HEC. These bands correspond to the commercial HA. The other characteristic peaks of PO_4 are seen between 962-959 cm^{-1} (v_1), 609.40 cm^{-1} (v_4) and also the absorption band of PO_4 was seen at 611.49 cm^{-1}. The O-H bending for water molecules existing in the unit cell of dicalcium phosphate dehydrate is seen at 3500-3400 cm^{-1} and around 1650 cm^{-1}. From the ATR-FTIR result we confirmed the present of hydroxyapatite without contamination.

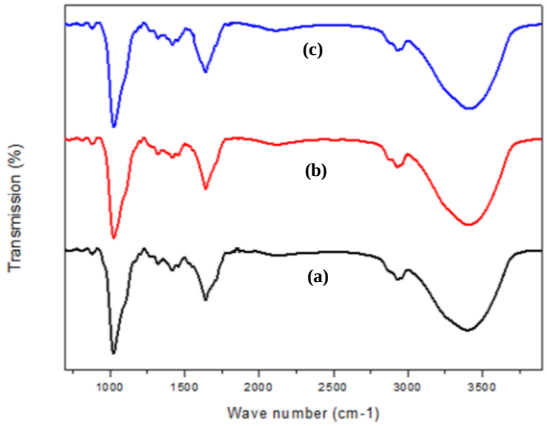


Fig 2: ATR-FTIR analysis for HEC/HAP at (a) 1 wt%, (b) 3 wt%, and (c) 5 wt%.

C. EDX analysis

EDS were performed to confirm the mineral phase. Fig. 3 shows the EDS image of images of porous scaffolds with hydroxyapatite. The major elements in the mineralized scaffolds consisted of carbon (C), oxygen (O), phosphorus (P), and calcium (Ca). The peaks from carbon and oxygen could be from HEC and the phosphorus and calcium could be from the mineral phase. This observation suggested that the mineral deposited on the porous scaffolds might be similar to hydroxyapatite.

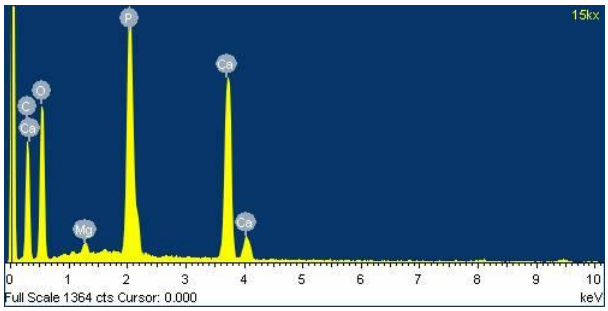


Fig. 3. EDS image of porous scaffolds with hydroxyapatite

D. Mechanical Study

One of the most important characteristic of a scaffold to be used in tissue engineering is that it has particular strength to maintain their integrity while maintaining their shape for cell ingrowth and matrix production until later neo-tissue formation.

Mechanical properties such as tensile strength and elongation of hydroxyethyl cellulose sponge scaffolds were evaluated during this study. Typical nonlinear stress–strain curves of hydroxyl ethyl cellulose sponge scaffolds at different ratio are shown in figure below. The calculated Young's modulus based on slope shown in the elastic region was measured and found to be approximately 0.135 GPa for 5wt% HEC, 0.125 GPa for 3wt% HEC and 0.12 GPa for 1 wt% HEC as shown in Fig. 4.

The stress at breakage was found to be 5 MPa, 4.7 MPa and 4.6 MPa, with elongation at break of 83%, 79% and 77% for sample 5 wt% HEC, 3 wt% HEC and 1 wt% HEC respectively. Higher tensile stress and strain values were obtained with the increasing of weight percentage of hydroxyethyl cellulose value. So, the 5 wt% of HEC scaffolds are more mechanically stable and could serve scaffold as compared with 1wt% and 3wt% HEC. Plotted graph below show stress vs strain behavior of 3 different concentration of wt % of hydroxy ethyl cellulose in the sample.

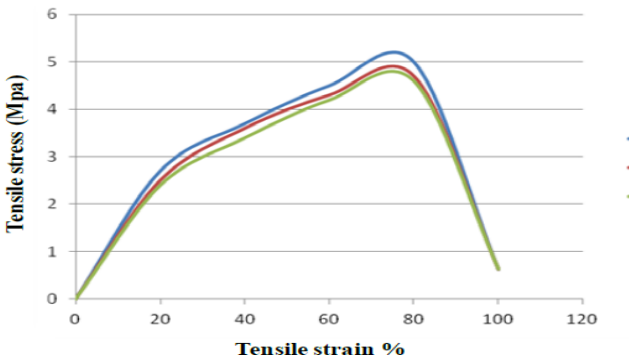


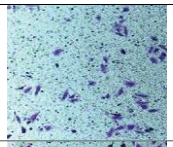
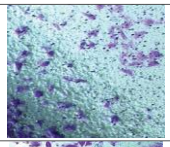
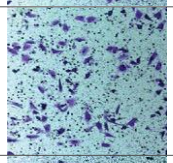
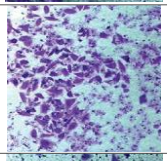
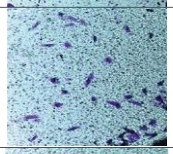
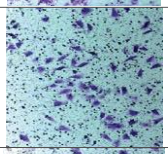
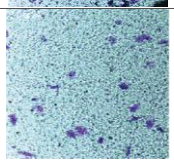
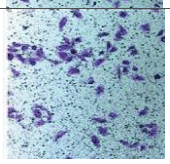
Fig. 4: Stress-strain curves for porous HEC/HA scaffolds

E. Cell proliferation

In vitro is one of the famous method uses to study orgasm cell demeanor. It is a process of analysis on biological minuscule structures and cells outside its mundane biological environment/ body. It mundanely run on artificially fabricated medium. Some researcher calls it as "test tube experiment" because of the way we run it artificially. Anteriorly, in vitro analysis has been run on test tube, petri dishes, flasks and many more opportune types of equipment. The sample needs to be analyzing far away in its isolated artificial medium that has virtually the same with its mundane biological medium. This let the researcher to have more accommodation space in fixating on certain angle of the respective research field. On another hand, there is an virtually homogeneous term that mundanely use erroneously by people. It is in vivo. Authentically in vivo additionally is an analysis of biological molecules and cells same as in vitro but the different is the in vivo analysis transpired in the authentic human/animals or mundane biological environment not in the environment fabricated by the researcher.

Table 1 shows the morphology of human Dental Pulps Stem Cells DPSC on different scaffolds wt% (5 wt%, 3wt%, 1wt%) on days 1 and 3 observed by SEM. Seeded DPSC cells were adhered and had spread well on the scaffolds in a time depended manner. It was observed that at 3 days time point, the cells started to adhere and attracted at the surface of sponges in a flat morphology. The attached cells become rounded and appeared in globular morphology on the scaffolds after day 3 (Mei et al.,2012). These cells were growth with effective spreading, indicating that scaffolds exhibited excellent biocompatibility.

Table 1: Cell Proliferation activity after 3 days of culture.

Sample	Day 1	Day 3
Control		
HEC (1wt%)		
HEC (3 wt%)		
HEC (5 wt%)		

IV. CONCLUSION

In this present work, HEC sponges were prepared using freeze drying method. All the initial characterizations were performed. The HEC sponges showed good porosity and had excellent inter connected pores. The sponges had diameter in the range of 2-200 µm. The pore size decreased with increase in the concentration of HEC. Among the various concentration of HEC, 1wt % showed good porosity. The 10 wt % HEC was too viscous, too thick and did not show much interconnected pores. For further studies 1 wt%, 3 wt % and 5 wt % HEC were chosen. The HEC sponges were cross linked and made water insoluble and the initial characterizations were made. The hydroxyapatite coating was done on the HEC sponges using SBF solution. The FTIR and FESEM-EDX confirmed the formation of apatite minerals on the HEC sponges. The chemical, mechanical properties and in-vitro tests show that HEC sponges are suitable for bone tissue engineering in the future.

ACKNOWLEDGEMENTS

The authors acknowledge the support from the College of Arts and sciences, University of Nizwa and TRC, sultanate of Oman.

REFERENCES

[1]. Almirall, A., Larrecq, G., Delgado, J. A., Martínez, S., Planell, J. A., & Ginebra, M. P. (2004). Fabrication of low temperature macroporous hydroxyapatite scaffolds by foaming and hydrolysis of an α-TCP paste. *Biomaterials*. https://doi.org/10.1016/j.biomaterials.2003.10.066

[2]. Charrière, E., Lemaitre, J., & Zysset, P. (2003). Hydroxyapatite cement scaffolds with controlled macroporosity: Fabrication protocol and mechanical

properties. *Biomaterials*. https://doi.org/10.1016/S0142-9612(02)00406-4
[3]. Chung, T. W., Yang, J., Akaike, T., Cho, K. Y., Nah, J. W., Kim, S. Il, & Cho, C. S. (2002). Preparation of alginate/galactosylated chitosan scaffold for hepatocyte attachment. *Biomaterials*. https://doi.org/10.1016/S0142-9612(01)00399-4
[4]. Fukasawa, T., Deng, Z.-Y., Ando, M., Ohji, T., & Goto, Y. (2001a). Pore structure of porous ceramics synthesized from water-based slurry by freeze-dry process. *Journal of Materials Science*, *36*(10), 2523–2527. https://doi.org/10.1023/A:1017946518955
[5]. Fukasawa, T., Deng, Z. Y., Ando, M., Ohji, T., & Goto, Y. (2001b). Pore structure of porous ceramics synthesized from water-based slurry by freeze-dry process. *Journal of Materials Science*. https://doi.org/10.1023/A:1017946518955
[6]. Ghosh, P. (2006). Rapid Uplift of the Altiplano Revealed Through 13C-18O Bonds in Paleosol Carbonates. *Science*. https://doi.org/10.1126/science.1119365
[7]. Groeneveld, E. H. J., van den Bergh, J. P. A., Holzmann, P., ten Bruggenkate, C. M., Tuinzing, D. B., & Burger, E. H. (1999). Mineralization processes in demineralized bone matrix grafts in human maxillary sinus floor elevations. *Journal of Biomedical Materials Research*, *48*(4), 393–402. https://doi.org/10.1002/(SICI)1097-4636(1999)48:4<393::AID-JBM1>3.0.CO;2-C
[8]. Ho, M. H., Kuo, P. Y., Hsieh, H. J., Hsien, T. Y., Hou, L. T., Lai, J. Y., & Wang, D. M. (2004). Preparation of porous scaffolds by using freeze-extraction and freeze-gelation methods. *Biomaterials*. https://doi.org/10.1016/S0142-9612(03)00483-6
[9]. Huang, Y., Onyeri, S., Siewe, M., Moshfeghian, A., & Madihally, S. V. (2005). In vitro characterization of chitosan-gelatin scaffolds for tissue engineering. *Biomaterials*. https://doi.org/10.1016/j.biomaterials.2005.05.036
[10]. Kokubo, T., & Takadama, H. (2006). How useful is SBF in predicting in vivo bone bioactivity? *Biomaterials*. https://doi.org/10.1016/j.biomaterials.2006.01.017
[11]. León y León, C. A. (1998). New perspectives in mercury porosimetry. *Advances in Colloid and Interface Science*. https://doi.org/10.1016/S0001-8686(98)00052-9
[12]. Maquet, V., Boccaccini, A. R., Pravata, L., Notingher, I., & Jerome, R. (2004). Porous poly(α-hydroxyacid)/Bioglass® composite scaffolds for bone tissue engineering. I: Preparation and in vitro characterisation. *Biomaterials*. https://doi.org/10.1016/j.biomaterials.2003.10.082
[13]. Mei, L., Hu, D., Ma, J., Wang, X., Yang, Y., & Liu, J. (2012). Preparation, characterization and evaluation of chitosan macroporous for potential application in skin tissue engineering. *International Journal of Biological Macromolecules*. https://doi.org/10.1016/j.ijbiomac.2012.08.004
[14]. Milosevski, M., Bossert, J., Milosevski, D., & Gruevska, N. (1999). Preparation and properties of dense and porous calcium phosphate. *Ceramics International*. https://doi.org/10.1016/S0272-8842(99)00003-6
[15]. Ramay, H. R., & Zhang, M. (2003). Preparation of porous hydroxyapatite scaffolds by combination of the gel-casting and polymer sponge methods. *Biomaterials*. https://doi.org/10.1016/S0142-9612(03)00171-6
[16]. Schoof, H., Bruns, L., Fischer, A., Heschel, I., & Rau, G. (2000). Dendritic ice morphology in unidirectionally solidified collagen suspensions. *Journal of Crystal Growth*. https://doi.org/10.1016/S0022-0248(99)00519-9
[17]. Shapiro, L., & Cohen, S. (1997). Novel alginate sponges for cell culture and transplantation. *Biomaterials*. https://doi.org/10.1016/S0142-9612(96)00181-0
[18]. Stokols, S., & Tuszynski, M. H. (2004). The fabrication and characterization of linearly oriented nerve guidance scaffolds for spinal cord injury. *Biomaterials*. https://doi.org/10.1016/j.biomaterials.2004.01.041
[19]. Xiao Huang, & Xigeng Miao. (2007). Novel Porous Hydroxyapatite Prepared by Combining H_2O_2 Foaming with PU Sponge and Modified with PLGA and Bioactive Glass. *Journal of Biomaterials Applications*, *21*(4), 351–374. https://doi.org/10.1177/0885328206063905
[20]. Zhang, R., & Ma, P. X. (1999). Poly (α - hydroxyl acids) / hydroxyapatite porous composites for bone - tissue engineering . I . Preparation and morphology. *J Biomed Mater Res*, *44*, 446–455. https://doi.org/10.1002/(SICI)1097-4636(19990315)44:43.3.CO;2-6
[21]. Zmora, S., Glicklis, R., & Cohen, S. (2002). Tailoring the pore architecture in 3-D alginate scaffolds by controlling the freezing regime during fabrication. *Biomaterials*. https://doi.org/10.1016/S0142-9612(02)00146-1