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Preparation and Evaluation of Biocompatible Composite for Bone Tissue Engineering

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Abstract
Bone tissue engineering with cells and synthetic extracellular matrix represents a new approach for the regeneration of mineralized tissue than bone transplantation. Hydroxyapatite (HA) and its composite with a biopolymer are extensively developed and applied in bone tissue regeneration. This study's main aim was to fabricate and characterize HA apatite-based biocompatible scaffold for bone tissue engineering. Scaffolds with different polymers (chitosan & alginate) ratios and a fixed amount of synthetic HA were prepared using in situ co-precipitation method. The mineral to polymer ratio was 1:1 to 1: 2. A crosslinker agent, 2-Hydroxylmethacrylate (HEMA), was added at a different percentage (0.5-2%) into the selected composition and irradiated at 5- 25 kGy to optimize the proper mixing of components at the presence of HEMA. Fabricated scaffolds were analyzed to determine porosity, density, biodegradability, morphology, and structural properties. The prepared scaffold's porosity and density were 75 to 92% and 0.21 to 0.42 g/cm3, respectively. However, the swelling ratio of the fabricated scaffolds was ranged from 133 to 197%. Nonetheless, there had a reasonable in-vitro degradation of prepared scaffolds. Flourier transform infrared spectroscopy (FTIR) analysis showed intermolecular interaction between components in the scaffold. A scanning electron microscope measured the scaffold's pore size, and the value was 162-510 µm. It could be proposed that this scaffold fulfills all the main requirements to be considered as a bone substitute for biomedical applications shortly.

Keywords: *Bone tissue engineering, Scaffold, Chitosan, Alginate, Hydroxyapatite.*

I. INTRODUCTION

The human body is a complex network of different organs and tissues with specific functions. 8% of healthcare spending globally accounts for organ replacement, and nearly a quarter of patients die before receiving their transplant. The risk of disease transmission and immune-rejection in allogeneic transplantation also limit the transplantations. Therefore, alternative biomedical solutions need to be developed to address the above serious health care systems' issues. Tissue engineering offers to bypass these difficulties by replacing and restoring various tissues by delivering the cells derived from the patient into an engineered platform to pre-engineer custom tissue construct for implantation [1].

Thus, the design and selection of scaffold fabrication materials is the first important step for intended tissue engineering. While structural design depends on various fabrication methods including phase separation, freeze-drying, foaming, particle leaching, electrospinning, and sintering, material selection dictates the material's biocompatibility and biodegradability, support for cell adhesion, proliferation, migration and differentiation, and finally, adaptability to scaffold manufacturing techniques [2]. Some other criteria such as chemistry, molecular weight, solubility, hydrophobicity/ hydrophilicity, surface energy, water absorption, etc. also play a role in the material selection process. Among all, natural materials are more biocompatible, readily available in abundance, and processed easily compared to others. On the other hand, the properties (e.g., mechanical properties, porosity, biodegradability, density, swelling kinetics, etc.) of synthetic biomaterials can be tailored for specific applications. In some cases, synthetic biomaterials are cheaper than natural ones, and can be produced in large quantities with uniform properties, have long shelf life [3].

Despite all these, natural biomaterials are preferable in tissue engineering due to their superior biological properties. The most commonly used natural biomaterials in tissue engineering are collagen, gelatin, alginate, chitosan, hyaluronic acid, and fibrins - all of which are polymers [2]. Among them, chitosan has higher mechanical properties and easily available in abundance and can be processed easily. Commonly produced from crab shells, chitosan is non-toxic and biodegradable; its hydrophilic surface promotes cell adhesion and proliferation [4,5], unlike the natural polymers derived from costly mammalian proteins and chitosan evokes minimal foreign body response and fibrous encapsulation. [6-8].

Moreover, chitosan is a semi-crystalline polymer so that mechanical properties can be tailored according to the

requirement in scaffold fabrication. Alginate is a naturally occurring anionic and hydrophilic polysaccharide. It is one of the most abundant biosynthesized materials [9] and is derived primarily from brown seaweed and bacteria. Alginate is of particular interest in a broad range of applications as a biomaterial, especially as the supporting matrix or delivery system for tissue repair and regeneration. Due to its outstanding properties in terms of biocompatibility, biodegradability, non-antigenicity, and chelating ability, alginate has been widely used in various biomedical applications, including tissue engineering, drug delivery, and in some formulations preventing gastric reflux [11]. Another important biomaterial for scaffold fabrication is hydroxyapatite (HA). HA has a chemical composition similar to human mineral tissue and can be synthesized from natural sources with calcium-based structures, such as bovine bone, mollusk shell, and coral [12].

This study focuses on fabricating and characterizing composite scaffold synthesized from three natural-based materials – chitosan, alginate, and HA for bone tissue engineering. In combination with the right amount of chitosan-alginate concentration and in-situ co-precipitation method, we prepared high strength porous HA scaffolds with a wide range of properties, including pore size, density, swelling ratio, and in-vitro degradation and thus could be suitable for bone tissue regeneration as well as other biomedical applications.

II. MATERIAL AND METHOD

A. Fabrication of scaffold

Scaffolds were fabricated according to the protocol of [13] with a simple modification. HCA (hydroxyapatite, Chitosan, Alginate) complex scaffolds with different ratios (1:1 and 1:2) were prepared through in situ co-precipitation technique. In short, 1:1 ratio (HCA-1), 1.0 g chitosan was dissolved in 1 wt % acetic acid solution under agitation, and 1 g of alginate powder was dissolved and thoroughly mixed in 25mL of deionized water. Then 80 mL of 0.1 mol/L $CaCl_2$ solution and 48 mL of 0.1mol/L KH_2PO_4 solution were added to the aqueous chitosan and alginate solutions, respectively. Again concisely like HCA-1 sample in 1:2 ratio (HCA-2), 2.0 g chitosan was dissolved in 1 wt % acetic acid solution under agitation, and 2 g of alginate powder was dissolved and thoroughly mixed in 25 mL of deionized water. Then 80 mL of 0.1mol/L $CaCl_2$ solution and 48 mL of 0.1 mol/L KH_2PO_4 solution were added to the aqueous chitosan and alginate solutions sequentially. In the above-mentioned methods, the mineral concentration in both samples was constant. After that, the alginate solution was mixed with the chitosan solution under continuous stirring in a blender for 1 h at room temperature. Then the samples were frozen at -40℃ for 24 h. The samples were then lyophilized in a freeze dryer at -50˚C for 30 h to obtain the HCA composite scaffolds.

B. Crosslinker

After observing physicochemical properties, the HCA-2 sample was found to be more porous, and also considering the degradation properties. It was selected for further study for adding cross liker. In this experiment, 2-Hydroxyethyl Methacrylate (HEMA) was used as a cross-linking agent, and 0.5%, 1%, 1.5 %, and 2% HEMA was added to the HCA-2 sample and radiated at 25 kGy, 20 kGy, 15 kGy, 10 kGy, and 5 kGy. Then the solutions were frozen at -40˚C for 24 h. Then samples were lyophilized in a freeze dryer at –50˚C for 30 h to obtain HCA composite scaffolds.

C. Characterization of scaffold

a) Porosity and Density

The density and porosity of the produced 3D scaffolds were measured by liquid displacement. The liquid used in this study was ethanol. A sample with a known weight (W) was immersed in a graduated cylinder in a known volume of ethanol (V1) for 5 min. The total volume of ethanol in the cylinder and ethanol - impregnated scaffold was V2. The ethanol-impregnated scaffold was removed from the cylinder, and the residual ethanol volume was recorded (V3). Each sample was measured in triplicate. The density of the porous samples (d) and the porosity of the scaffolds (Є) are expressed as follows:

$$d = W/(V2 - V3)$$

$$Є = (V1 - V3)/(V2 - V3)$$

b) Swelling ratio evaluation

The swelling ability was determined by the percentage of water absorption. The dry weight of the scaffold was denoted as W_0. Then, porous scaffolds were immersed in PBS buffer solution with pH 7.4 at 37°C for 24 hours. Afterward, the scaffolds were taken out from the PBS buffer solution, and its wet weight was measured, denoted as WW. The ratio of swelling was calculated using the equation.

$$\text{Swelling ability (\%)} = \frac{Ww - Wo}{Wo} \times 100$$

c) In-vitro Biodegradability

The biodegradability of the scaffolds was characterized by in vitro study. The scaffolds were immersed in PBS medium containing lysozyme (10,000 U/ml) at 37 °C for 7, 14, and 21 days. The initial scaffold weight was denoted as W_0. After calculated days, the scaffolds

were washed in deionized water to remove ions adsorbed on the surface and then freeze-dried. The dry weight of the scaffold was denoted as Wt. The degradation of the scaffold was calculated using equation-

$$\text{Biodegradability} = \frac{Wo-Wt}{Wt} \times 100$$

d) Fourier transform infrared spectroscopy (FTIR) analysis

FTIR Spectroscopy is an analytical technique used to identify organic, polymeric, and in some cases, inorganic materials. The FTIR analysis method uses infrared light to scan test samples and observe chemical properties. FT-IR 8400S Shimadzu Spectrophotometer in the range 4000-700cm-1, Resolution 4cm-1,No of scan : 20 times.

e) Pore morphology

The pore size and surface morphology of the biocomposite scaffolds were studied using scanning electron microscopy (SEM JSM-6490LV, JEOL, Tokyo, Japan). Briefly, scaffold samples were cut into small pieces and fixed on carbon tape, then dried under vacuum and platinum-coated before examining under SEM.

RESULT AND DISCUSSION:

Scaffold development

The scaffolds produced through the lyophilization method and cross-linking steps using HEMA and radiation produced a scaffold structure that was found to be ideal for bone tissue grafting purposes. In addition to structure produced with the electrostatic interaction of chitosan and alginate, the choice of this fabrication technique produced scaffolds with excellent porosity that is contributed by the formation of ice crystals during freezing and its subsequent removal during lyophilization. The scaffolds produced were found to be rigid structures that had a sponge-like appearance. In this study, scaffolds (HCA-1 and HCA-2) were prepared at two different ratios where the mineral's ratio was constant for both composite scaffold, but the polymer's ratio was altered (1:1 and 1:2).

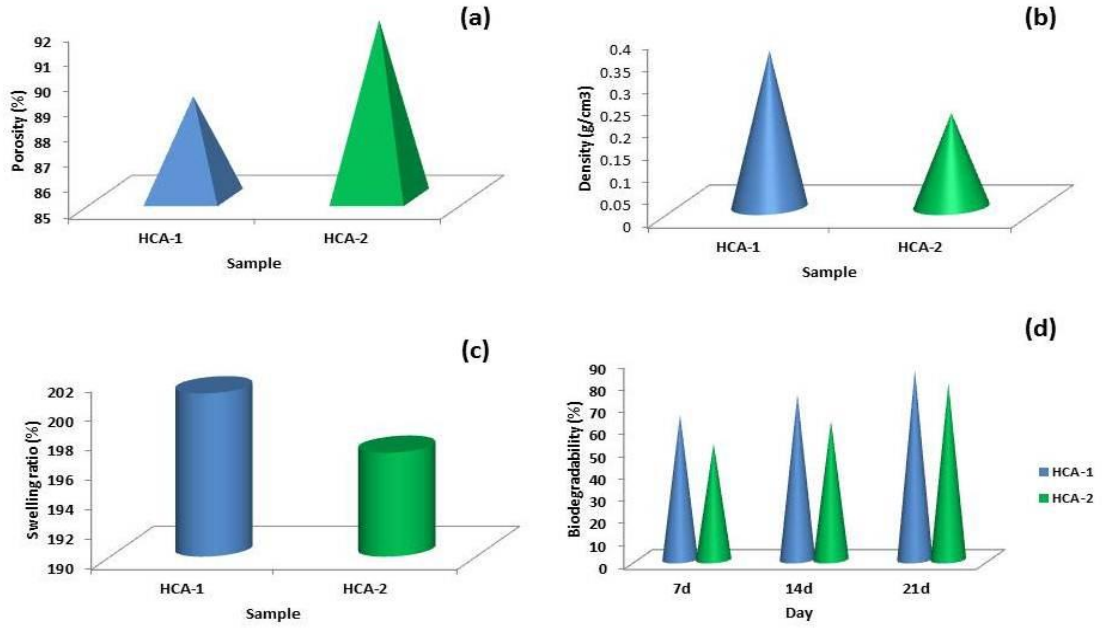

Figure-01: Properties of fabricated scaffold

The total porosity and density of the scaffold were measured through the liquid displacement method using ethanol. The highest and lowest porosity value was measured respectively at sample HCA-2 (92%) and HCA-1 (89%) (Figure 1a). The highest density was found in sample HCA-1 (0.35 g/cm^3), and the lowest density was found in sample HCA-2 (0.21 g/cm^3) (Figure 1b). In the case of the swelling kinetics rate, the sample showed a higher rate of 201% and 197% swelling in HCA-1 and HCA-2, respectively (Figure 1c). To

determine the biodegradability result, HCA (HCA-1, HCA-2) samples were soaked into the PBS buffer solution with containing lysozyme for sequentially 7, 14 & 21 days, the remaining weight of the scaffold after 7, 14 and 21 days for sample HCA-2 were found 52, 62 and 79% sequentially, which was comparatively higher

than sample HCA-1 that was 65, 74 and 85% respectively (Figure 1d).

Characterization of cross-linked scaffold

Based on porosity, density, and biodegradability test, from the HCA (HCA-1 and HCA-2) samples, Scaffold sample HCA-2 was selected and crossed linked with HEMA (2-Hydroxyethyl Methacrylate), at different percentages (0.5%, 1%, 1.5%, and 2%) and irradiated at different doses (5 kGy, 10 kGy, 15 kGy, 20 kGy, and 25 kGy). In the beginning, it found it difficult to dissolve components in the presence of HEMA, and the slurry was continuously formed when the solutions were irradiated at10-25 kGy.

Table 01: Radiated scaffold (5 kGy) with a different percentage of cross-linking agent

Sample ID		**% of Crosslinker (HEMA)**
HCA-2	CR0H0	No HEMA and Radiation
	CR5H0	No HEMA
	CR5H1	0.5
	CR5H2	1
	CR5H3	1.5
	CR5H4	2

But when the components were dissolved in irradiation at 5 kGy at the presence of HEMA, the optimum radiation dose for dissolving components in the presence of cross linker HEMA was found (Table-01). So, it can be hypothesized from this analysis, that the increased radiation dose (>5 kGy) might be effecting the crosslinker for better dissolving with the components that are subjected to the chemical bond breakdown of the cross linker.

Porosity and density of scaffold

The porosity of the cross-linked scaffolds was ranged from 75-92% (Figure-2a), while cancellous bone porosity ranges from 30% to 90 % (mostly in the range 75%-95%)[14]. Among the different cross-linked scaffolds, the highest value was a non cross-linked scaffold, and the lowest value was the higher amount of HEMA. Greater than 80% of total porosity was observed for the polymeric scaffold, which could be an added advantage for tissue engineering purposes [15]. This high degree of porosity would allow cells to migrate into and populate within the scaffold. The density of the scaffolds was measured by using the liquid displacement method using ethanol. Among the samples, the highest density was in the CR5H4 sample (0.42 g/cm^3), and the lowest was the CR0H0 sample (0.21 g/cm^3) (Figure-2b). The apparent density of trabecular bone ranges from 0.14 to 1.10 g/cm3. The density value increased from due to the denser packing of the polymer network and, as expected, led to a decrease in porosity [16-17] (Sehaqui *et al*., 2010; Sehaqui *et al*., 2011).

Swelling Evaluation

The swelling behavior of a scaffold is an important aspect that causes an increase in pore sizes that determines its practical use in facilitating the attachment and growth of cells and the subsequent new tissue regeneration [18]. The swelling ability of the scaffold was studied by immersing these scaffolds in 1× PBS solution (Figure 2c). The results showed that there are differences in the swelling behavior among the scaffolds, where the water uptake ability of the non-cross-linked HCA scaffold was higher when compared to the cross-linked scaffold. It has been reported earlier that alginate absorbs water quickly and holds 200–300 times its own weight of water [19]. The surface generally increases upon swelling of the scaffold, which is suitable for more cell adhesion and infiltration.

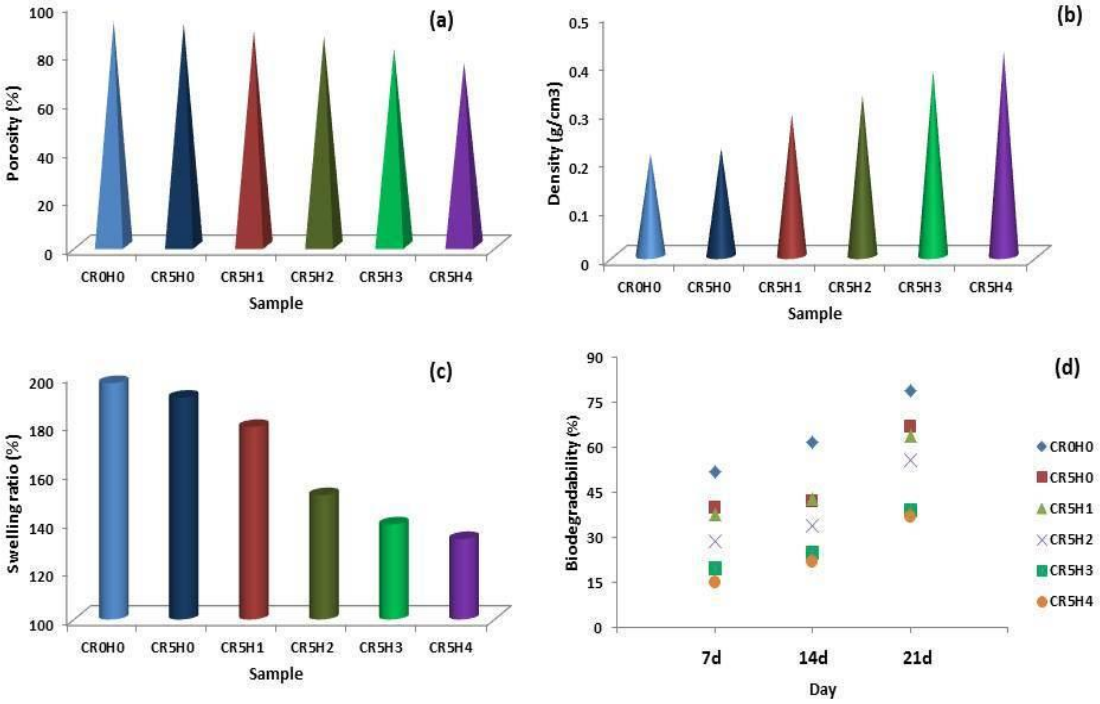

Figure-02: Properties evaluation of cross-linked scaffold

Degradation Evaluation

An important aspect in the field of tissue engineering is often attributed to the degradation behavior of a polymer-based scaffold. The biodegradation of scaffolds provides space for tissue growth and matrix deposition. An ideal rate would be that which corresponds to the regeneration of a new bony structure in order to provide a smooth transition [20]. The enzymatic degradation study was performed as a short term observation based upon the nature of lysozyme enzymes to break down the glycosidic linkage of the alginate network. In observing the enzymatic degradation study, it could be justified that the breakage of linkage within the alginate network occurs at an accelerated rate with the increase in fluid uptake, thus causing the loss of network structure within a few days of soaking. The high concentration of HEMA in the scaffold results in its structural integrity and stability. The rate of degradation gradually was increased at a time with the addition of a lower amount of HEMA at the fixed radiation dose (5 kGy), and the highest degradation was observed in the non-cross-linked scaffold (Figure-2d). A desirable characteristic of a scaffold would be a degradation rate that is neither too fast nor too slow for it to be rendered workable.

Physico-chemical characterization of scaffold

Alginate is an anionic polymer that possesses the ability to form strong electrostatic interaction with cationic polymers. In this case, the cationic charged amine group of the chitosan unit interacted electrostatically with the negatively charged alginate to form a polyelectrolyte mixture. The addition of HA in the Chi-Alg may make more complex ionic interactions possible. Several studies suggested that hydrogen bonding or ion-ion pair interaction between the components usually increases the uniform dispersion [21]. These molecular chemical interactions between Chi-Alg and HA-Chi-Alg were studied by FT-IR. FT-IR spectra were used to confirm the functional groups and interactions of HA, chitosan, alginate, Chi-Alg, and different cross-linked HA-Chi-Alg scaffolds, and the spectra are depicted in Figure 3.

It has shown the asymmetric stretching vibration of phosphate (v^1 PO_4), (v^3 PO_4), and (v^4 PO_4) present in HA appeared at 962 cm^{-1}, 570-603 $cm^{-1,}$ and 1028-1058 cm^{-1}, respectively. Whereas the band at 877cm^{-1}& 572 cm^{-1} suggests the presence of HPO_4^{2-} group. The characteristic bands for OH^- appeared at 3452, 3782cm^{-1}

The band at 1028 and 1083 cm-1 has corresponded to the C-O-C stretching. The broadband in 1409 and 1597

was attributed to the presence of -COO group. The characteristics peak -CH stretching was observed at 815cm$^{-1.}$ The characteristic bands for OH$^-$ was appeared at 3365cm^{-1}.

In chitosan, the bands at 2845 cm^{-1} have corresponded to the CH stretching. The broad bands at 1637 and 1543 cm^{-1} were attributed to the presence of amide I and amide II groups. The sharp band at 1408 cm^{-1} was assigned to stretching of carbonyl from the COO$^-$ group. The low intense bands at 1382 and 1321 cm^{-1} corresponded to CH bending vibrations of the ring. The characteristics peaks of C-O-C glycosidic linkage were observed in the region of 1153-1021cm^{-1} (Figure 3.2).

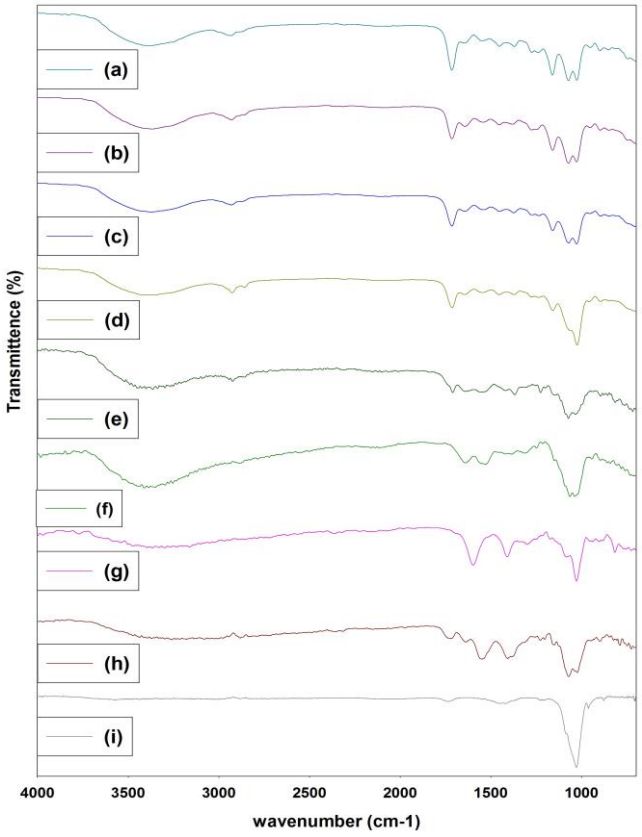

Figure 03: FTIR spectra of a) CR5H4 b) C R5H3, c) CR5H2, d) CR5H1, e) CR5H0, f) CR0H0, g) Alginate, h) Chitosan, and i) HA.

On the other hand, in the case of Chi-Alg, an intense peak was observed at 1613 cm−1, corresponding to the superposition of the bands assigned to the carboxylate group of alginate and the amine group of chitosan. The interaction from electrostatic interaction between the carboxylate group of alginate and the amine group of chitosan forms a polyelectrolyte complex. The results are consistent with previous studies [22]. The lower stretching frequency in –OH was observed from 3433 cm−1 to 3420 cm−1. This suggests that intermolecular hydrogen bonds exist in the chitosan-alginate system.

When the bands of the scaffold were compared with the HA, chitosan, and alginate banding pattern, some peak of scaffolds were found to shift from the original peak position due to the formation of new bonds among them. The asymmetric stretching vibration of phosphate (v3 PO4) was found in 1024-1037 cm^{-1}, whereas the characteristic bands for OH$^-$ appeared at 3354 to 3782cm^{-1} in the different scaffold, which was indicated in HA. The HPO_4^{2-}bands at 875 cm^{-1} was shifted to 894-896 cm^{-1}in different percentages of the cross-linked sample. The peak position 1382, 2883, and 2926 cm^{-1} for the band of C-H were shifted to 1311-1381, 2858-2881, and 2924-2933 cm^{-1} in different scaffolds containing crosslinker sequentially. Peak position 1153 cm^{-1}& 1637 cm^{-1} for band of C=O & amide-I were shifted into 1159-1161 cm^{-1} and 1641-1645 cm^{-1} consequently in different scaffold(Figure 03).

Pore morphology analysis

Scanning electron microscopy (SEM) is one of the most crucial and precise tools that can image bio nanostructure, and it can also track bio-materials in nano-materials, and finally measure physical properties and determine composition. SEM images surface with a beam of electrons, which produce a three-dimensional image of a sample. So SEM micrograph is useful for understanding the structure of the surface. The surface morphology, pore distribution, and pore size of HA/Chi-Alg was directly measured in a scanning electron micrograph and found to be highly porous with a pore size of 162–510 μm for the CR5H3 scaffolds (Figure 04).

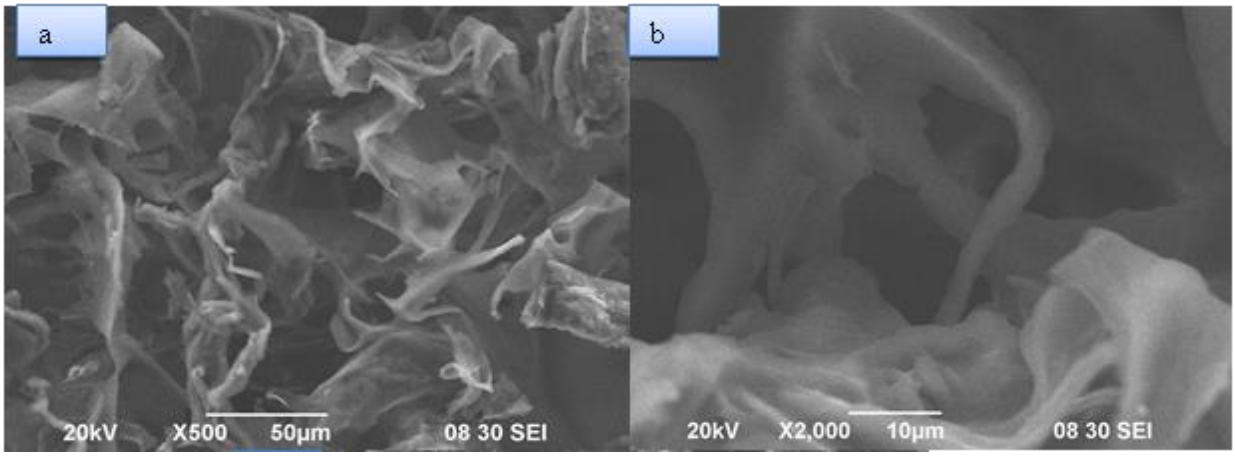

Figure 04: SEM micrographs of scaffold (a) CR5H3 (low magnification) (b) CR5H3 (High magnification)

The optimum pore size for bone tissue engineering remains unclear; however, investigations that sought to identify the optimum pore size for bone tissue engineering found pore sizes ranging from 80 to 500 µm to be viable [23]. The depicted pore size enables the scaffolds to allow for cell adhesion, proliferation, and also nutrient supply, which will enable proper bone tissue growth. The optical microscopic images inferred that the dispersion of the components is uniform within the scaffolding network for scaffolds.

Conclusion

In this study, three-dimensional, open cells, the porous scaffold was prepared through a freeze-dried technique and cross-linked with HEMA at the presence of radiation. Firstly, Hydroxyapatite/Chitosan-alginate (HCA) scaffold was fabricated by the in-situ co-precipitation method. Different percentages of 2-Hydroxylmethacrylate (HEMA) were applied as a cross-linking agent; meantime, radiation dose was also revealed for proper mixing of components in the presence of HEMA. Among the different percentages of HEMA, 1.5% was the optimum concentration at 5 kGy radiation dose for proper mixing. The results showed that the prepared scaffolds are porous, with a porosity of about 86%, and biodegradability of the scaffold was decreased with increasing of polymer concentration. Scanning Electron Microscope (SEM) analysis showed an open pore structure of scaffolds appropriate for blood supply and cell attachment and pores ranging from 162 to 510 µm. Fourier transform infrared spectroscopy (FTIR) results showed intermolecular interaction between components in the scaffold.

Acknowledgments

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Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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