Fabrication of Tissue Engineering Scaffold from Hydroxyapatite/Alginate Composite

Masoumeh Haghbin Nazarpak and Farzaneh Pourasgari

***Abstract*—Scaffolds for bone tissue engineering must meet the functional requirements, porosity, biocompatibility, and biodegradability. In this study, hydroxyapatite was prepared by wet chemical method and incorporated into the alginate gel solution to improve both the mechanical and cell-attachment properties of the scaffolds. Next, composite scaffolds were fabricated from hydroxyapatite/alginate with different weight ratios by freeze drying method and then some of them coated with triblock copolymer and compared with others. Microstructure observation with SEM suggests the formation of about 50 micrometer size porous structure and interconnected porosity. Then, human mesenchymal stem cells were cultured on the composite scaffolds. Cells adhesion to the scaffolds was observed after three days by DAPI fluorescence microscopy in which more cells adhesion to the coated scaffolds and cells diffusion into the pores are visible. Also, cell adhesion within the structure was observed by SEM in which showed cell attachment was well in depth which confirms DAPI results. These results suggest that the triblock-coated HA/Alg porous scaffolds could provide enhanced cell adhesion and proliferation which may be a promising approach for bone tissue-engineering applications.**

***Index Terms*—Alginate, cell adhesion, composite, freeze-drying, human mesenchymal stem cells, hydroxyapatite, porous scaffold, tissue engineering.**

I. Introduction

Tissue engineering is the science of design and fabrication of new tissues for functional restoration of impaired organs and replacement of lost parts due to cancer, disease and trauma [1], [2]. The three key ingredients for tissue engineering and tissue regeneration are signals, stem cells and Scaffolds [3]. Scaffolds could provide a solid framework for cell growth and differentiation, allowing cell attachment and migration [4]-[6]. Several requirements must be considered in the design of three-dimensional (3D) scaffolds for tissue engineering [7]-[9]. High porosity is a major factor that is desired to increase the specific surface area for cell attachment and tissue in-growth in scaffolds [10]. The pore size must be large enough to allow accommodation of cells so that interconnected pores may facilitate uniform distribution of cells, diffusion of oxygen and nutrient, and waste exchange by cells deep within the construct [11]-[14].

Polymer/ceramic composites may improve mechanical properties compared with polymers, and better structural integrity and flexibility than brittle ceramics. In fact, the combination of ceramics and polymers could provide reinforced porous structures with enhanced bioactivity and controlled resorption rates [15], [16].

Several conventional methods have been used to fabricate 3-D scaffolds [17]. In particular, freeze drying is a technique including solvent casting method, in which the solvent removing may be accompanied by freeze drying to a more porous structure to be obtained. In this procedure, cavities is produced in the scaffold due to the space occupied by the solvent after drying, and the smaller pores arising from sublimation of the solvent serves as interconnection between the macropores [18].

Natural and synthetic materials can be used for fabrication of porous scaffolds for bone regeneration. Alginate is a naturally occurring anionic and hydrophilic polysaccharide. It is one of the most abundant biosynthesized materials [19], [20], and is derived primarily from brown seaweed and bacteria. Unfortunately some drawbacks to alginate include mechanical weakness and poor cell adhesion. In order to overcome these limitations, the strength and cell behavior of alginate have been enhanced by mixtures with other materials, such as ceramics [20]. In this way, production of composite scaffolds containing ceramics and synthetic polymers provided a scaffold with desirable properties. In this way, a biodegradable Alg/HA composite scaffolds with different weight ratio for efficient bone tissue engineering were prepared in the present work. On the next step, we compared cell adhesion and proliferation on the triblock copolymer coated scaffolds with uncoated one. The results showed better cell adhesion on the modified samples.

It is demonstrated in the present work that porous Alg/HA scaffolds can be successfully prepared by the proposed freeze-drying method. The prepared scaffolds are highly porous, and have interconnected pores about 50 μm. Study of the cell–scaffold interaction also demonstrated the ability of the scaffold to support hMSC adhesion and proliferation that suggests its potential application in bone tissue engineering.

II. Materials and Methods

A. Synthesis of Hydroxyapatite Powder

Orthophosphoric acid, calcium hydroxide and NH_4OH were obtained from Aldrich Chemical Co. Hydroxyapatite powders were synthesized by a wet chemical method, based on the precipitation of HA particles from aqueous solution. The synthesis procedure involved drop by drop addition of the H_3PO_4 solution (0.3 M) into an aqueous suspension of $Ca(OH)_2$ (0.5 M) while stirring vigorously for about 24 h. Simultaneously, ammonia hydroxide solution was added to adjust pH at 10-11. Then, the obtained white precipitate was aged for 7 days, decanted, rinsed with deionized water, and

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filtered. After filtration, the precipitate was dried in an oven at 70 ℃ for 24 h. Finally, it was heated at 1000 ℃ for 1 h in a conventional furnace under air atmosphere.

B. Scaffold Fabrication

Sodium alginate (29 cP for 1% at 25 ℃) was obtained from Sigma Chemical Co., triblock copolymer were prepared in our laboratory [21], [22]. To prepare the composite scaffolds, different weight ratios hydroxyapatite suspension were prepared using aqua-sonication for 20 min and added into the prepared alginate solution. Then, the gelation process was started by spraying 1.0 M $CaCl_2$ on the mixture.

Next, the gels were cast in moulds (12 mm diameter) and frozen in freezer overnight. Next, the frozen samples were freeze dried. Some of the samples are used as prepared. For the coated ones, the triblock copolymer synthesized in laboratory according to procedure previously reported [21], [22] dissolved in ethanol was used as coating on some of samples and finally air dried.

III. Results

A. X-Ray Diffraction

X-ray diffraction was performed to determine phase structure of the scaffolds using XRD (INEL, EQuinox 3000, France). The XRD pattern of the sample indicated the presence of hydroxyapatite phase as shown in Fig. 1. In addition, there was no evidence of formation of other unwanted phases, which confirms the purity of the powder is appropriate for biomedical applications. Also the sharpness of the peaks revealed its high crystallinity.

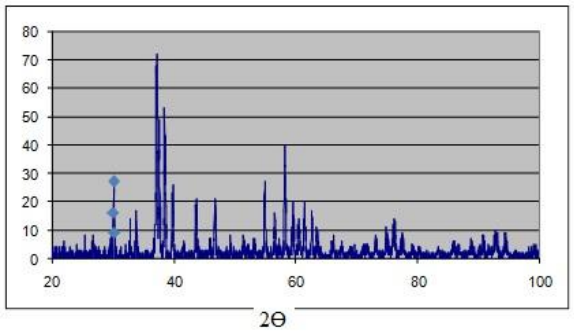


Fig. 1. XRD pattern of hydroxyapatite powder prepared in wet chemical method compared with the reference hydroxyapatite pattern.

B. FTIR Analysis

FTIR analysis (BRUKER VECTOR 33, Germany) was performed using KBr. The result is shown in Fig. 2. As it is seen the 635 and 3570 cm^{-1} bands correspond to OH^- group, to strongly adsorbed and/or bound H_2O. H_2O band was also observed at 1640 cm^{-1}. A strong band of PO_4^{3-} group was also seen at 1046, 962, 602 and 571 cm^{-1}. The bands obtained for respective phosphate and hydroxyl groups of pure HA, were in agreement with other published data.

C. Particle size Distribution (PSD) Analysis

The mean diameter and size distribution of hydroxyapatite particles were measured by NANOPHOX particle size analyser (Sympatec GmbH, Germany) which is shown in Fig. 3. As can be seen the average particle size of powder is between 1-5 μm and the 50% of the particles are about 2.5 μm.

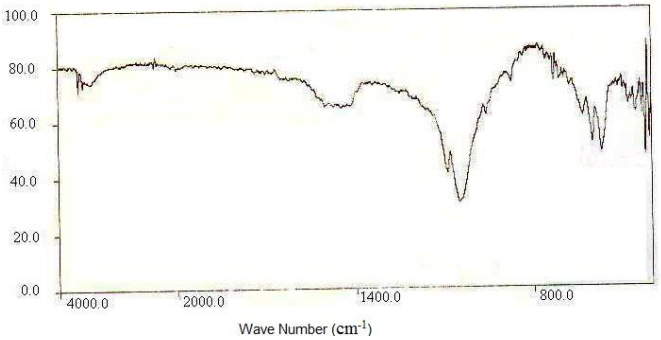


Fig. 2. FTIR patterns of hydroxyapatite powder.

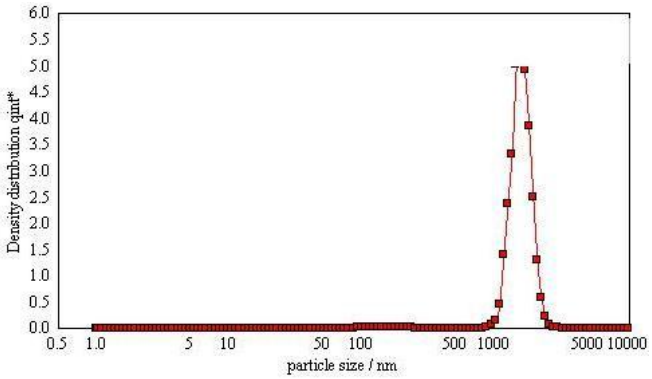


Fig. 3. Particle size distribution of hydroxyapatite particles.

D. Surface Area of Scaffolds

The specific surface area was measured by the chromatographic method using Brunauer, Emmett and Teller (B.E.T) model by nitrogen adsorbtion (Bellsorp II, Japan). The result showed that the surface area of the scaffolds were about 57 m2/g which reveals their relatively high porosity.

E. Microstructure of the Scaffolds

Microstructure of the scaffolds was studied by scanning electron microscopy (SEM) (XL30 Philips) at an accelerating voltage of 15 kV. Before observation, the scaffolds were coated with gold using a sputter coater (BAL-TEC, SCDOOS, Switzerland).

Morphology of the porous scaffolds was characterized using scanning electron microscope (Fig. 4) showed the diameter of pores about 50 micrometer using image analysis software in which a value of 10 μm has been suggested as necessary for cellular infiltration.

F. Cell Culture

Dulbecco Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), phosphate buffered saline (PBS) and trypsin–EDTA were purchased from Gibco, Singapore. The samples were sterilized by UV exposure under a laminar flow hood for 10 min on each side and placed in DMEM for one day in order to be compatible. Cells used for culturing were human mesenchymal stem cells, originally isolated from bone marrow. The cells were cultivated in DMEM, supplemented with 10% FBS and 100 U/ml penicillin–streptomycin–amphotercin, at 37 ℃ in 5% of CO_2. Then, scaffolds were placed and immobilized in culture dishes. HMSCs suspended in culture medium (104 cells/ml) were then added in the dishes to allow the in growth of cells

to the scaffolds. The culture medium was changed every two days. After incubation in various periods, cells attached on the scaffolds were harvested for analysis.

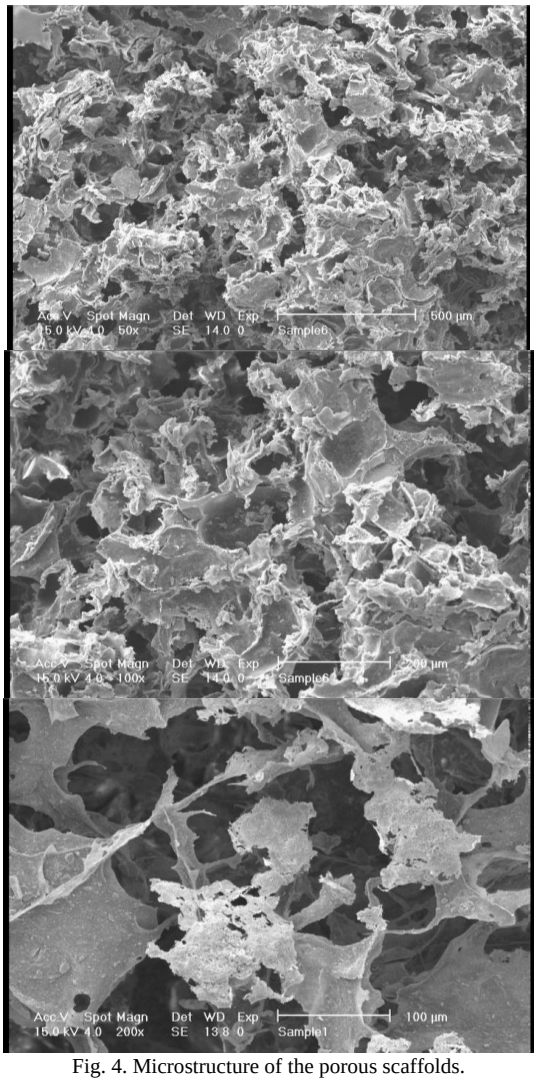
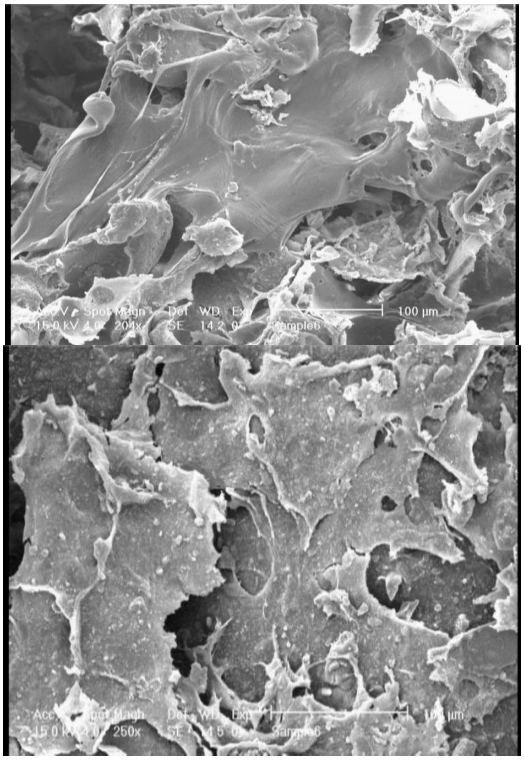


Fig. 4. Microstructure of the porous scaffolds.



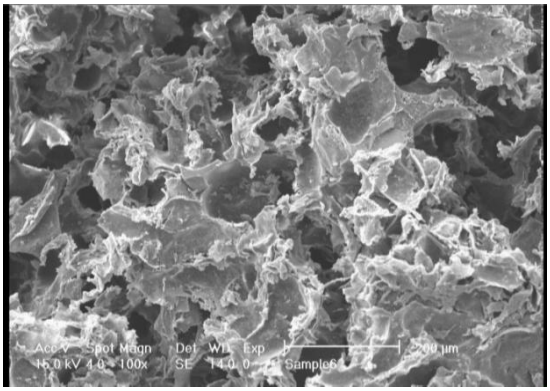


Fig. 5. SEM photographs of cells morphology on the scaffolds.

G. Morphology of Cells

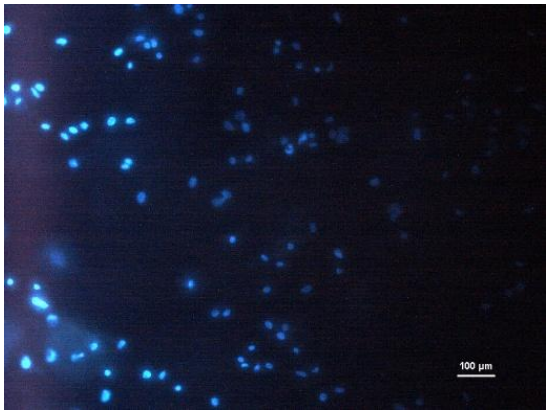
Cell morphology on the scaffold was also investigated by scanning electron microscopy that is shown in Fig. 5. The cell-loaded scaffolds were rinsed with PBS after 3 day of cell seeding and fixed in glutaraldehyde 2.5% for 1 h. For dehydrating the scaffolds were placed in a series of gradient of alcohol concentration and then dried.

The results indicated that the mesenchymal stem cells cultured in scaffolds can be seen not only in the surface of the scaffold, but also inside the pores. The images showed the perfect adhesion of cells to scaffold surface outside and inside the pores. Adhesion structures resembling tight junctions were present. The cells elongations and their interconnection forming a cell net were observed clearly. Cells that attach themselves to the scaffold, but spread little might show lower proliferative rates than those with greater spreading. These scaffolds allowed flattening and spreading of the cells, showing adequate cell shape for proliferation and secretion functions.

H. DAPI Fluorescent Staining

The cells on the scaffolds were fixed with 4% paraformaldehyde. Samples were then washed twice with PBS, incubated with 4, 6-diamidino-2-phenylindole (DAPI;Sigma Chemical Co.) for 30 seconds to label nuclei of the cells and again were rinsed twice with PBS. The immunofluorescence images were obtained by using a fluorescence microscope (Nikon, Eclipse).

DAPI fluorescent staining was carried out after 3 days cultivation which is shown in Fig. 6. In DAPI staining, bright fluorescence revealed the presence of nuclei. As can be seen, the samples with more hydroxyapatite percent and/ or coated with triblock copolymer had better cell adhesion.



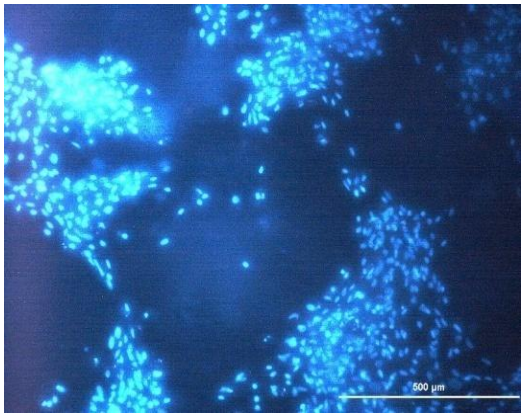


Fig. 6. DAPI staining results of coated samples 3% hydroxyapatite suspension & 2% alginate solution (up) and 6% hydroxyapatite suspension & 2% alginate solution (down).

IV. Conclusion

In the present work, it is demonstrated that porous Alg/HA scaffolds was successfully prepared by the proposed freeze-drying method. The prepared scaffolds were characterized, which the results showed their high porosity, and interconnected pores about 50 µm. Also, the cell culture results revealed that the matrix was not cytotoxic and the cells were strongly adhered to the substrate in the first hours of cell/ substrate contact. The ability of these scaffolds to support hMSC adhesion and proliferation suggests its potential application in tissue engineering.

It is concluded that these scaffolds are promising materials for tissue engineering, providing a good environment for the adhesion and proliferation of cells. However, in the next step, the tests will be focused on the osteogenic differentiation capability of mesenchymal stem cells seeded on these scaffolds for bone tissue engineering.

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