Asian Biomedicine Vol. 6 No. 5 October 2012; 753-758 DOI: 10.5372/1905-7415.0605.117

Brief communication (Original)

Preparation and evaluation of porous alginate/hydroxyapatite composite scaffold coated with a biodegradable triblock copolymer

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Background: The scaffolds for bone tissue engineering must meet the functional requirements such as porosity, biocompatibility, and biodegradability. Composite materials could improve mechanical properties compared with polymers, and structural integrity and flexibility compared with brittle ceramics.
Objective: The effect of a biodegradable triblock copolymer in the cell attachment into the alginate/hydroxyapatite composite scaffold was evaluated.
Methods: Scaffolds were fabricated by freeze-drying method in different alginate/hydroxyapatite weight ratios. Hydroxyapatite was incorporated into the alginate gel solution to improve both the mechanical and cell-attachment properties of the scaffolds. The scaffolds were then coated with triblock copolymer (as a surface modifier) and sterilized by ultraviolet light. Then, human mesenchymal stem cells were cultured on the scaffolds, which are an attractive cell source for tissue engineering.
Results: Cell adhesion to the scaffolds was observed after three days by 4, 6-diamidino-2-phenylindole (DAPI) fluorescence microscopy. In addition, microstructural observation with SEM suggests the formation of about 50 micrometer size pores and interconnected porosity so that cell adhesion within this structure is well in depth as also observed in DAPI results.
Conclusion: These results suggest that the triblock-coated alginate/hydroxyapatite porous scaffolds could provide enhanced cell adhesion and proliferation, which may be a promising approach for tissue-engineering applications.

Keywords: Alginate, cell adhesion, freeze-drying, human mesenchymal stem cells, hydroxyapatite, porous scaffold, tissue engineering

Tissue engineering has emerged as a promising alternative approach for organ repairing and tissue reconstruction [1-6]. The scaffold provides a solid framework for cell growth and differentiation at a local site, allowing cell attachment and migration. In this approach, scaffolding plays a vital role and a porous scaffold is needed to guide cell attachment, growth, and tissue regeneration in three dimensions [7-9]. To accommodate large number of cells the scaffold need to be highly porous with large surface to volume ratio. The porosity, pore size and pore structure of the scaffold is important for nutrient supply of cells [10, 11].

It is proved that polymer/ceramic composites have improved mechanical properties compared with polymers, and they have better structural integrity and flexibility than brittle ceramics. In fact, the combination of ceramic and polymer could provide reinforced porous structures with enhanced bioactivity and controlled resorption rates [12, 13]. Alginate is a hydrophillic, biodegradable natural linear polysacharide with a long history of use in many clinical applications [14]. In addition, it is a non-toxic, biocompatible and anionic polysaccharide obtained from marine brown algae [15, 16]. Alginates consist of varying amounts of 1, 4 linked β-D-mannuronic acid and α-L-guluronic acid residues and can be ionically crosslinked by the

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addition of divalent cations such as calcium in aqueous solution [17, 18].

There are several conventional methods to fabricate 3-D scaffolds [19]. Freeze-drying is a technique including the solvent casting method in which the solvent removing may be accompanied by freeze-drying to obtain a more porous structure. In this way, solvent was frozen and space occupied by solvent made cavities in the scaffold. The smaller pores arising from sublimation of the solvent served as interconnection between the macropores [20].

In this work, we have developed the triblock copolymer coated alginate/hydroxyapatite scaffolds and investigated human mesenchymal stem cells behavior on them.

Materials and methods

Synthesis of HA 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 hours. Simultaneously, ammonia hydroxide solution was added to adjust pH at 10-11. Then, the obtained white precipitate was aged for seven days, decanted, rinsed with deionized water, and filtered. After filtration, the precipitate was dried in an oven at 70°C for 24 hours. Finally, it was heated at 1000 C for 1 hour in a conventional furnace under air atmosphere.

Scaffold fabrication

Sodium alginate (29 cP for 1% at 25 C) was obtained from Sigma Chemical Co. Triblock copolymer PEG-co-P(FA/SC)-co-PEG was prepared as previously described [21, 22]. To prepare the composite scaffolds, hydroxyapatite suspension was prepared using aqua-sonication for 20 minutes 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. After that, the triblock copolymer dissolved in ethanol used as coating. Finally, the samples were air-dried.

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 minutes 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°C in 5% of CO_2. Then, scaffolds were placed and immobilized in culture dishes. HMSCs suspended in culture medium (10^4 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.

DAPI 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, Tokyo, Japan).

Scanning electron microscopy

Microstructure of the scaffolds was studied by scanning electron microscopy (SEM) (XL30 Philips, Eindhoven, The Netherlands) at an accelerating voltage of 15 kV. The samples were washed twice with PBS and fixed in 1.5% v/v glutaraldehyde in 0.14 M sodium cacodylate (pH 7.4) for 30 minutes at room temperature. Dehydration was performed by sequential immersion in serial diluted ethanol solutions (50, 60, 70, 80, 90, and 100% v/v). Then, the scaffolds were coated with gold using a sputter coater (BAL-TEC, SCDOOS, Balzers, Switzerland).

Cell morphology

Cell morphology on the scaffold was investigated by scanning electron microscopy. The cell-loaded scaffolds were rinsed with PBS after three days of cell seeding and fixed in glutaraldehyde 2.5% for 1 hour. Then, the scaffolds dehydrated with a graded ethanol series (30%, 50%, 70%, 90%, and 99.5%) for

15 minutes, and lyophilization was carried out. After the deposition of a thin gold layer, the cell adhesion was evaluated using SEM.

Results

XRD

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 **Figure 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.

Microstructure

Morphology of the porous scaffolds was characterized using scanning electron microscope (**Figure 2**) showed the diameter of pores about 50 micrometer using image analysis software in which a value of 10 m has been suggested as necessary for cellular infiltration. This result shows that the pore size of the scaffold is sufficient for cell adhesion and proliferation.

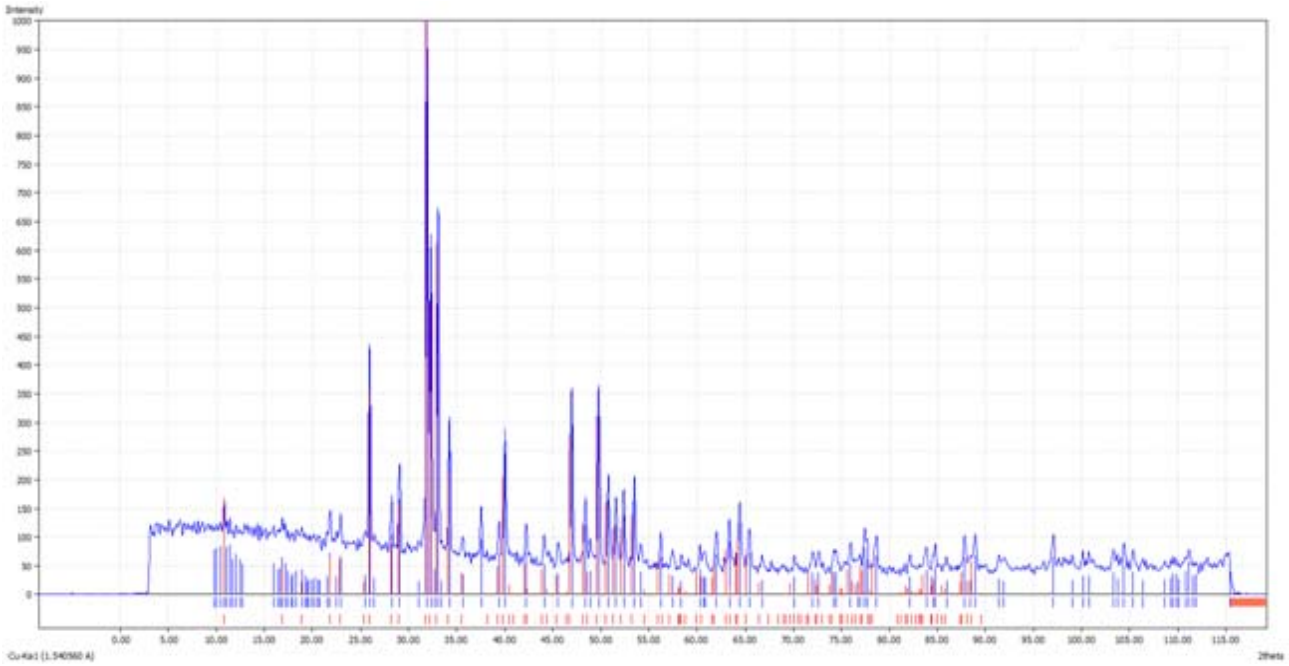

Figure 1. XRD pattern of hydroxyapatite powder prepared in wet chemical method compared with the reference hydroxyapatite pattern

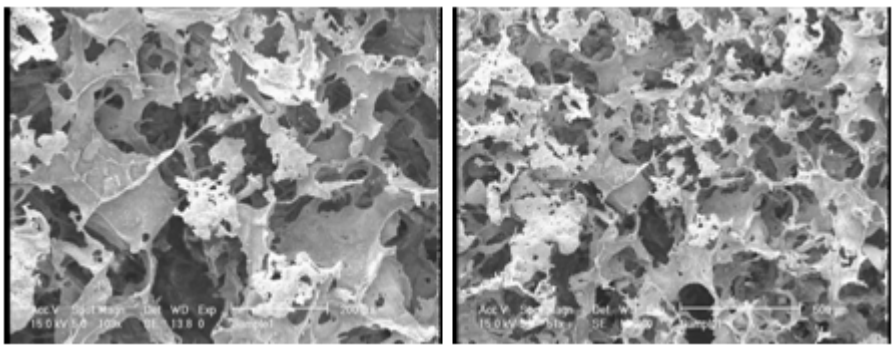

Figure 2. Microstructure of porous scaffolds

Cell attachment

DAPI fluorescent staining was carried out after three days cultivation. As can be seen, the samples coated with triblock copolymer (**Figure 3A, B**) and with more hydroxyapatite percent (**Figure 3C, D**) has more attached cells which confirms the SEM results and prove that both the hydroxyapatite and the triblock copolymer have a positive effect in cell adhesion as we have expected.

Cell morphology on the scaffolds

Morphology of cells on porous scaffolds was studied by scanning electron microscopy (SEM) that is shown in **Figure 4**. The results indicated that the mesenchymal stem cells cultured in scaffolds could be seen not only in the surface of the scaffold, but also inside the pores. This cells infiltration is very functional parameter in tissue engineering scaffolds that could provide cells nutrition in the porous structures.

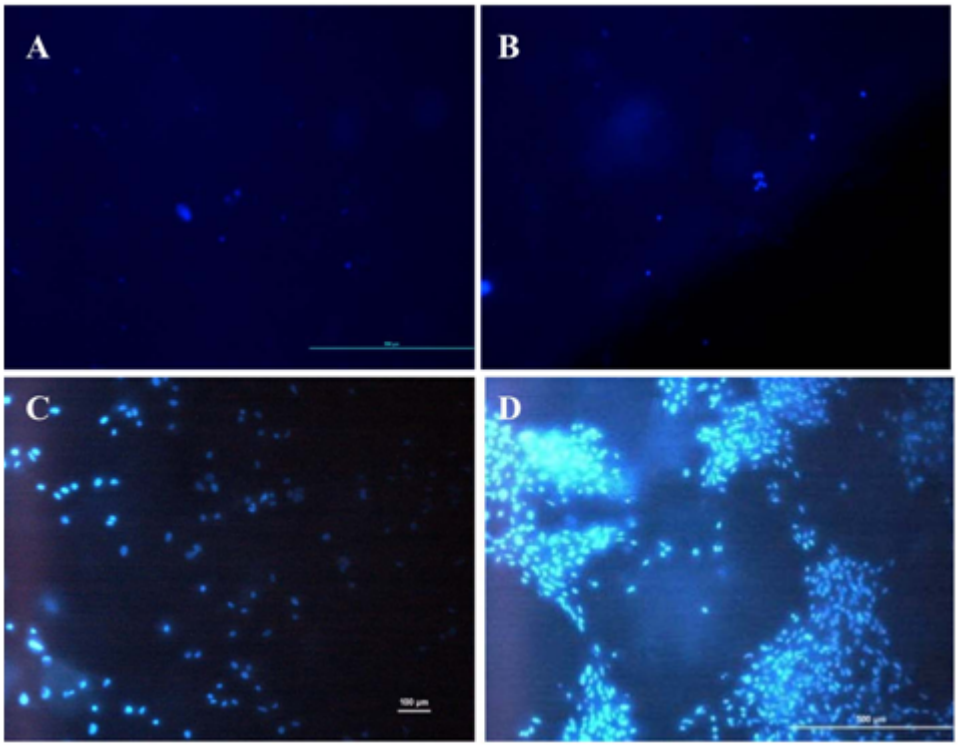

Figure 3. DAPI staining results of samples 2A (**A**), 2B (**B**), 32B (**C**), and 62B (**D**)

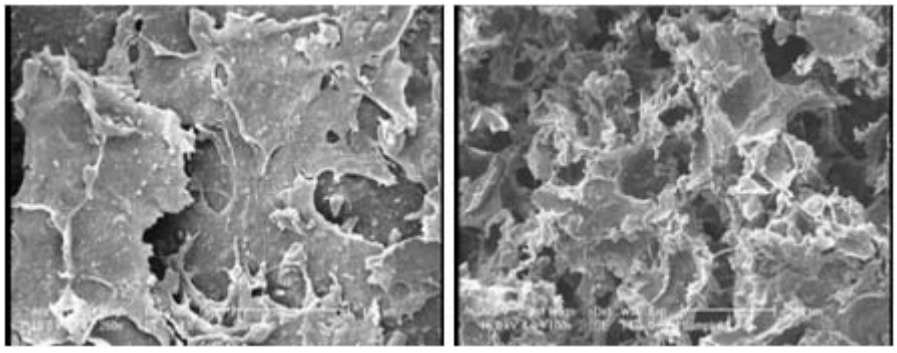

Figure 4. SEM photographs of cells morphology on the scaffolds

Discussion

Natural and synthetic materials can be used for fabrication of porous scaffolds for bone regeneration. However, preparation of composite scaffolds containing ceramics and synthetic polymers provide a scaffold with desirable properties. In the present work, biodegradable alginate/hydroxyapatite composite scaffolds with different weight ratios were prepared for efficient bone tissue engineering. Then we compared cell adhesion and proliferation on the triblock copolymer coated scaffolds (as surface modification) with uncoated one. The results showed better cell adhesion on the modified samples.

In addition, the pore size of the scaffolds of this study was sufficient to allow nutrients and wastes to permeate while preventing the migration of undesirable cells and tissues to the healing site. The data from SEM showed that our samples have appropriate porosity for cell penetration into the scaffold.

For functional scaffold, cells should attach efficiently to the structure. Therefore, we examined cell adhesion and proliferation using SEM and the results show that the cells can properly adhere and grow on alginate/hydroxyapatite composite scaffolds. Moreover, coating of scaffolds with triblock copolymers could improve cell proliferation on alginate/ hydroxyapatite composite scaffolds.

In addition, cells penetration into the inner layers of the scaffold could improve its functionality for bone tissue engineering and develop its applicability. The results obtained from DAPI staining and SEM indicated that the mesenchymal stem cells cultured in scaffolds can attach, spread, and proliferate, not only in the surface of the scaffold, but also inside the pores (**Figures 3, 4**) suggesting its potential application as bone tissue engineering scaffolds. However, more detailed in vitro and in vivo studies are recommended.

Conclusion

In the present work, the porous alginate/ hydroxyapatite scaffolds coated with triblock copolymer was prepared by freeze-drying method. The scaffolds are highly porous, and have interconnected pores about 50 m. Moreover, the study of the cell–scaffold interaction demonstrates the ability of the scaffold to support hMSC adhesion and proliferation. These results suggest that the matrix was not cytotoxic and the cells could strongly attach to the substrate in the first hours of cell/ substrate contact. The scaffold fabricated may have a promising structure for tissue engineering and facilitate cells adhesion and proliferation**.**

Acknowledgement

The authors would like to thank the Iran National Science Foundation (INSF) (Grant No. 87040193) for financial support. The authors declare no conflict of interest to report.

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