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Brief communication (Original)

Preparation of a novel porous scaffold from poly(lactic-*co*-glycolic acid)/hydroxyapatite

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Background: Scaffolds for bone tissue engineering must meet functional requirements, porosity, biocompatibility, and biodegradability. Different polymeric scaffolds have been designed to satisfy these properties. Composite materials could improve mechanical properties compared with polymers, and structural integrity and flexibility compared with brittle ceramics.
Objective: Fabricate poly (lactic-co-glycolic acid) (PLGA) /hydroxyapatite (HA) porous scaffolds by freeze-extraction method, and evaluate the possibility for optimizing their biocompatibility by changing their HA content.
Methods: Porous PLGA/HA composites structure were prepared by freezing a polymer solution, and then the solvent was extracted by a non-solvent and subsequently air-dried. The scaffolds were coated with triblock copolymer and sterilized by ultraviolet light. Human mesenchymal stem cells were cultured on the prepared scaffolds and were studied after three days by 4, 6-diamidino-2-phenylindole (DAPI) fluorescence microscopy.
Results: Microstructural studies with SEM showed the formation of about 50 micrometer size porous structure and interconnected porosity so that cells were adhered well into the structure of the coated samples. DAPI fluorescence microscopy showed more cell adhesion to the coated scaffolds and cell diffusion into the pores are visible. Direct assay of cell proliferation performed with MTT test showed cell growing on the scaffold similar to or more than on control samples.
Conclusion: The triblock-coated PLGA/HA porous scaffolds may provide cell adhesion and proliferation, demonstrating their potential application in bone engineering.

Keywords: Cell adhesion, composite, freeze-extraction, human mesenchymal stem cells (hMSC), porous scaffold, tissue engineering

Tissue engineering scaffolds provide a solid framework for cell growth and differentiation, allowing cell attachment and migration [1-3]. Several requirements must be considered in the design of three-dimensional (3D) scaffolds for tissue engineering [4-6]. High porosity is a major factor that is desired to increase the specific surface area for cell attachment and tissue in-growth in scaffolds [7]. 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 [7-10].

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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 [11, 12].

Several conventional methods have been used to fabricate 3-D scaffolds [13]. 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 are 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 [14]. To overcome these

problems, 3D porous scaffolds have been fabricated by solvent exchange methods. Using these methods, efficiency of the manufacturing process can be increased because samples are immersed in a non-solvent bath after freezing and samples are dried [15].

In the present study, we developed a novel method for fabrication of biodegradable composite scaffolds for efficient tissue engineering. Applying this method, we produced porous triblock coated poly(lactic-co-glycolic acid) (PLGA)/hydroxyapatite (HA) scaffolds, and investigated human mesenchymal stem cells behavior on these scaffolds.

Materials and methods

HA, PLGA, triblock copolymer were prepared as previously described [16-18]. Dimethyl sulfoxide (DMSO) and ethanol were obtained from Aldrich Chemical Company (Milwaukee, USA). To prepare the composite scaffolds, HA suspension (with different weight ratios) was prepared using aqua-sonication for 20 minutes, and then added into the prepared PLGA solution with two different weight ratios in DMSO. It was then stirred for two hours at room temperature. The mixture was cast in moulds (12 mm diameter) and frozen in a freezer overnight. The frozen samples were immersed in a non-solvent bath of 30% ethanol solution cooled in -4°C to allow the exchange of solvent and non-solvent at a temperature lower than the freezing point of the polymer solution. Then, the samples were air-dried, and the triblock copolymer dissolved in ethanol was used as coating. Finally, the samples were air-dried.

Dulbecco Modified Eagle Medium (DMEM), fetal bovine serum (FBS), phosphate buffered saline (PBS) and trypsin-EDTA were purchased from Gibco (Singapore). The samples were sterilized by ultraviolet (UV) light exposure under a laminar flow hood for 10 minutes on each side, and placed in DMEM for one day to be compatible. Human mesenchymal stem cells (HMSCs) were used for culturing. The cells were cultivated in DMEM, supplemented with 10% FBS and 100 U/mL penicillin-streptomycin-amphotercin, at 37°C in 5% CO_2. Then, the scaffolds were placed and immobilized in culture dishes. Then, HMSCs suspended in culture medium (10^4 cells/mL) were 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.

The MTT assay was used for measurement of the cell viability and proliferation. The hMSC cells cultured on the scaffolds after day 1, 3, 5, and 7 of cell seeding were firstly trypsinized, treated by 25 μL 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole (MTT), and incubated four hours at 37°C with 5% CO_2. After incubation and adding DMSO, the viable cells were detected by measuring the absorbance (A_{570}) of the cell lysates was measured at 570 nm by a microplate reader). The cell viability was expressed by optical density (OD) of A_{570} of cells cultured on scaffolds.

The cells on the scaffolds were fixed with 4% paraformaldehyde after three days of cell seeding. Then, samples were then washed twice with PBS, incubated with four, 6-diamidino-2-phenylindole (DAPI) (Sigma Chemical Co, USA) for 30 seconds to label nuclei of the cells, and rinsed twice with PBS. The immunofluorescence images were obtained by using a fluorescence microscope (Nikon, Eclipse, Tokyo, Japan).

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 on the scaffold was investigated using SEM. The cell-loaded scaffolds were rinsed with PBS after three days of cell seeding, and fixed with glutaraldehyde 2.5% for one hour. Then, the scaffolds dehydrated with a graded ethanol series (30%, 50%, 70%, 90%, 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

Figure 1 shows the morphology of the porous scaffolds using SEM. The diameter of pores was about 50 μm. We note that this pore size is larger than the critical size of 10 μm to support cellular infiltration [19]. The overall interconnected pore architecture by a network of fine channels was demonstrated in SEM studies.

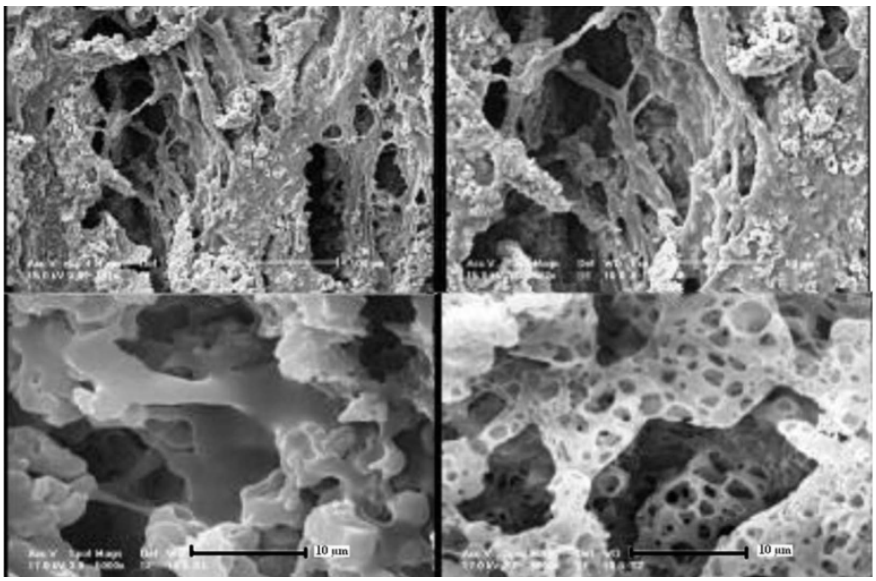

Figure 1. Scanning electron microscopic images showing the microstructure of interconnected porous scaffolds

DAPI staining after three days cultivation showed that cells adhesion and intrusion to the porous structure and their morphology was spread. In DAPI staining, bright fluorescence revealed the presence of nuclei. **Table 1** shows results of cell nuclei on the scaffolds based on florescence microscopic images.

Table 1. Florescence microscopic results of cells' nuclei on the scaffolds stained with DAPI and sample conditions for PLGA/HA scaffolds

Sample code	PLGA/HA weight ratio	PLGA (w/v) %	HA (w/v) %	Coating	Quantity of DAPI stained cells
11	30/3	30	3	———	Average, on the surface and into the interior of the construct
12	20/2	20	2	———	Average, on the surface and into the interior of the construct
13	20/10	20	10	———	High, on the surface and into the interior of the construct
14	30/10	30	10	———	Average, on the surface and into the interior of the construct
15	20/15	20	15	———	High, on the surface and into the interior of the construct
16	30/15	30	15	———	High, on the surface and into the interior of the construct
21	20/2	20	2	Triblock	Low on the surface but penetrated the inner region of the scaffold
22	30/3	30	3	Triblock	High, on the surface and into the interior of the construct
23	20/10	20	10	Triblock	High, on the surface and into the interior of the construct
24	30/10	30	10	Triblock	Low but penetrated the inner region of the scaffold less
25	20/15	20	15	Triblock	High, on the surface and into the interior of the construct
26	30/15	30	15	Triblock	Low on the surface but penetrated the inner region of the scaffold

PLGA: poly(lactic-co-glycolic acid), HA: hydroxyapatite, DAPI: 4, 6-diamidino-2-phenylindole

MTT assay was performed after culture for 1, 3, 5, and 7 days. The cells on scaffolds were viable and proliferated compared with control samples. **Figure 2** shows MTT results at 1, 3, 5, and 7 days cell seeding. Interestingly, on day 1, OD of triblock coated scaffolds was higher than control and uncoated samples, about two times of uncoated samples. On day 2, they were a little more than control but again about two times of uncoated samples. Each day, triblock coated scaffolds showed an OD higher than uncoated scaffolds. On day 3, the OD of coated scaffolds decreased in comparison to controls, but it was more than uncoated ones again. On day 7, the coated scaffolds conserved the increase against uncoated ones.

Figure 3 shows morphology of cells on porous scaffold after cell seeding. The mesenchymal stem cells cultured in scaffolds could 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. Our scaffolds allowed flattening and spreading of the cells, showing adequate cell shape for proliferation and secretion functions.

Discussion

We have developed a novel method of fabrication of biodegradable composite scaffolds for efficient tissue engineering, and fabricated porous PLGA/HA scaffolds coated with triblock copolymer. On the next step, we evaluated cell adhesion and proliferation on the scaffolds. The results indicated that the pores formed by solvent exchange method were effective for the cell intrusion into the scaffold.

Pore morphology such as size, shape, and interconnectivity is of clinical importance because it affects cellular growth and tissue regeneration [20]. In our study, pore size was optimized to achieve cell adhesion and proliferation on the scaffold surface. As shown in **Figure 1**, the cells intruded through the pores (about 50 mm) that were formed by solvent exchange.

MTT assay is a quantitative assay for cell survival. Increasing of OD in MTT assay showed more cell attachment in the coated samples than uncoated ones (**Figure 2** and **Table 1**). More nuclei were seen in coated scaffolds in microscopic observation after

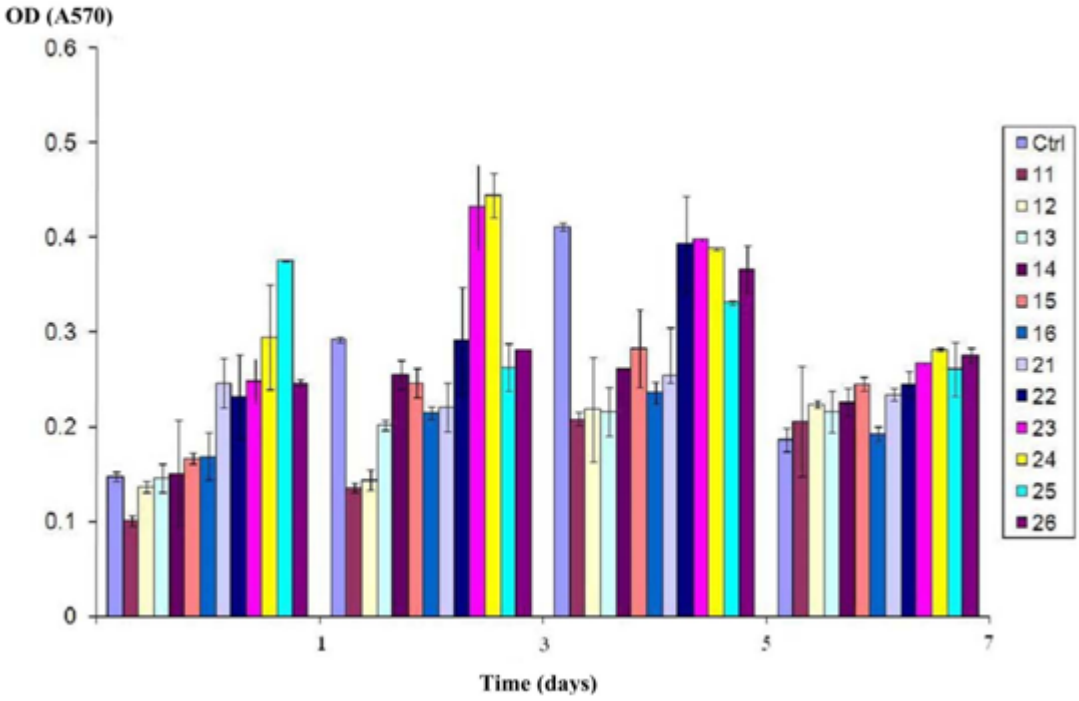

Figure 2. Results of MTT assay at 1, 3, 5, and 7 days cell seeding (Sample codes are shown in **Table 1)**

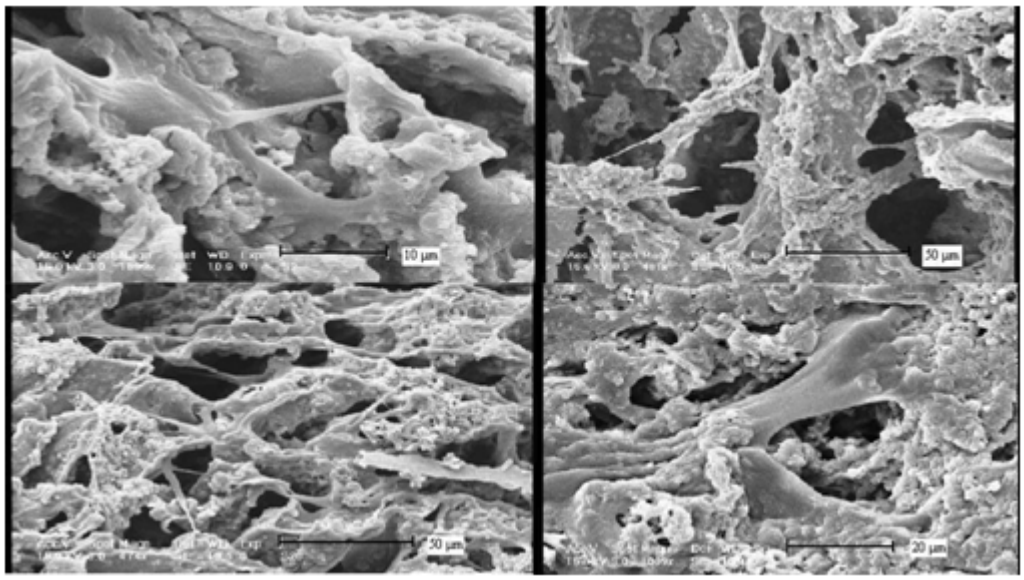

Figure 3. Scanning electron microscopic images showing the morphology of cells on surface of scaffold (left) and in depth (right) after three days of cell seeding

DAPI staining in day 3. This indicated that not only the cells could properly attach and grow on PLGA/HA composite scaffolds, but also they could attach to triblock scaffolds stronger than to uncoated ones. Therefore, coating of scaffolds with triblock copolymers improved cell attachment and proliferation on PLGA/HA composite scaffolds. When cells on the scaffolds were analyzed by MTT assay, the cells showed more OD than controls. This might be because of the scaffolds effect on cells.

Cells penetration into the inner layers of the scaffold could improve its functionality for tissue engineering and develop its applicability. Our microscopic studies showed that the porosity and pore interconnectivity of the scaffolds were adequate for cell migration. SEM observations of cell cultures showed that the scaffolds had a good ability to support the attachment and spreading of cells. The results from DAPI staining and SEM indicated that the mesenchymal stem cells cultured in scaffolds can attach, spread, and proliferate, not only on the surface of the scaffold, but also inside the pores as shown in **Figures 2** and **3**. This suggests its potential application as tissue engineering scaffolds.

In conclusion, the present porous PLGA/HA scaffolds were prepared by the proposed freeze-extraction method. The prepared scaffolds were highly porous, and had interconnected pores of about 50 μm. The matrix was not cytotoxic and the cells 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. The scaffold produced may be a promising material for tissue engineering, providing a good environment for the adhesion and proliferation of cells

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