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GROWTH AND DIFFERENTIATION OF MARROW STROMAL CELLS ON SCAFFOLDS CONTAINING POLYVINYL ALCOHOL IMPRINTED POLYCAPROLACTONE MICROSPHERES

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ABSTRACT

A new technique for increasing hydrophilicity of polycaprolactone (PCL) was recently reported. PCL was prepared as microspheres by using the single emulsion technique. Polyvinyl alcohol (PVA) in the emulsion was imprinted on the microspheres while they were being formed. To fabricate a scaffold, the mixture of PVA-imprinted PCL microspheres and hydroxyapatite (HA) at a weight ratio of 1:1 was casted by using trisodium polyphosphate (TPP)/chitosan cross-linked networks. Apatite layers on scaffold surfaces were greatly generated following immersed in a physiologic solution for 3 weeks. Rat marrow stromal cells (rat MSCs) were grown on this scaffold in DEME–F12 medium supplemented with $1x10^{-9}$ M triamcinolone and $2.8x10^{-4}$ M ascorbic acid for 5 days. Growth and differentiation of these cells were considerably improved regarding MTT and alkaline phosphatase (ALP) activity assays. In compared with scaffolds containing an ordinary PCL, those consisting of PVA-imprinted microspherical PCL were likely hydrophilic and bioactive. The established procedure would be extended for manipulation of scaffolds containing other synthetic polymers, and might be applicable for bone tissue engineering in the future.

1.0 Introduction

Bone fracture is a medical condition in which a break in the continuity of bones occurs. It can be the result of high force impact or stress, or conditions that weaken the bone, such as osteoporosis, bone cancer, etc. Bone tissue has no sensors of the pain pathway. But, its break is extremely painful and poses a significant risk to the patient. A surgical procedure called bone grafting is commonly employed to repair severe bone fracture by replacing with a new bone. The bone used in bone grafting can come from the patient own body (autograft), a donor (allograft), or could be entirely manmade. There are problems associating autografts and allografts, such as donor site morbidity, disease transmission and/or host response reaction. Nevertheless, these dilemmas can be resolved or reduced by using an artificial bone graft. Generally, synthetic bone grafts are designed to perform as frameworks for which new living bone can grow. By taking physical, biochemical, and mechanical properties of the damaged bone into account, its structure and composition can be chosen to resemble as

much as possible of the bone extracellular matrix. Biodegradable property is of additional requirement to eliminate the secondary surgery of graft removal.[1] To meet most of the descriptions, scaffolds containing synthetic biodegradable polymers have been widely constructed and tested.[2,3] PCL is of special interest among others for preparing long-lasting implantable devices, due to its gradual degradation.[4] However, the polymer is biologically inactive as caused by its native hydrophobicity, leading to cell growth interference.[5] There have been procedures for improvement of the polymer bioactivity. For examples, PCL nanofibers are impregnated with Tween 80. The resulting composite is highly effective for guiding bone regeneration compared to the unmodified specimens.[6] Scaffolds composing of PCL and polyethylene glycol (PEG) are fabricated to obtain surfaces that are more optimal for cell proliferation than those lacking PEG.[7] Interestingly, incorporation of HA in PCL scaffolds is elicited as a simple way for increasing scaffold bioactivity.[8] The expression of marker proteins for osteogenesis by bone progenitor cells grown on these HA/PCL scaffolds has found to be higher than of those cultured on PCL scaffolds without HA addition.

In this study, a technique for improving PCL hydrophilicity was recently developed. The polymer was manipulated to form microspheres by using the single emulsion method. The existing PVA in the emulsion was imprinted onto surfaces of the microspheres while being formed. Scaffolds consisting of HA powder and the PCL microspheres at a weight ratio of 1:1 were fabricated.[9] The *in vitro* bioactivity was determined by measuring ALP activity as produced by rat MSCs grown on the scaffolds. For the control samples, parts of the PCL microspheres were replaced by PCL pellets, and the compression molding/salt leaching technique was employed for scaffold preparation. The research goals are to obtain scaffolds which greatly support osteogenesis of MSCs, and a technique that is profitable for bone tissue engineering applications.

2.0 Materials and methods

2.1 Preparation of Scaffolds

Two techniques, namely the compression molding/salt leaching and the microsphere/chitosan cross-linking, were used for the preparation of scaffolds as follows.

2.1.1 The compression molding/salt leaching technique

Five grams of PCL pellets (Sigma-Aldrich, USA) were dissolved in 25 ml of chloroform (Merck, Germany), followed by the addition of 2.5g of HA (Fluka) and 1g of sodium chloride (Loba Chemie). The salt was used as a pore forming agent. The mixture was stirred until obtaining viscous slurry and placed in a fume hood for 24 h to remove the organic solvent, and a granulated mixture was obtained. Two grams of the granule were poured into a mold of 12 mm diameter and pressed at 0.3 Newton/mm^2 by a hydraulic pump to obtain a scaffold with 4-mm thick and 12-mm diameter. Then, the scaffold was soaked in distilled water for 5 days with gentle shaking, while the medium was changed every 6 h. After subjected to vacuum drying, a porous scaffold was acquired and called as “Sample 1”.

2.1.2 The microspheres/chitosan cross-linking technique

Firstly, PCL microspheres were prepared by using the single emulsion method. In brief, 5 g of PCL pellets were dissolved in 25 ml of chloroform and the resulting solution was poured into a beaker containing 100 ml of PVA solution (0.5% w/v in water). The mixture was homogenized (WiseTis, HG-15D, Korea) at 6,500 rpm for 3 min to form an emulsion and stirred around in a fume hood at room temperature for 12 h to remove the organic solvent. PCL microspheres were collected by centrifugation at 1,000 rpm for 20 min (Universal 16R, Hettich, Germany), washed with distilled water (3 times, 5 min each), and freeze-dried at −40 °C for 24 h (OPERON, Korea).

Next, a scaffold was fabricated by triturating 400 mg of the PCL microspheres and 400 mg of HA with 1 ml of chitosan solution (1.5% w/v in 3% v/v acetic acid). The mixed slurry was crushed into an opened ends-cut plastic tube (5 mm diameter x 5 mm length) and allowed to dry at room temperature for 4 h. The packed tube was subsequently immersed in 5 ml of 1% w/v TPP solution for 24 h at room temperature during which linkages between chitosan and TPP occurred. The wrapped plastic tube was trimmed to take out the scaffold. The scaffold was washed 3 times with distilled water, freeze-dried, and stored in dry place until use. It was named as “Sample 2”.

The commercially preformed scaffolds consisting of calcium phosphate from BD Bioscience were used for evaluating *in vitro* performance of the recently prepared scaffolds.

2.2 Scanning Electron Microscopy (SEM)

Surface morphologies of PCL microspheres, “Sample 2” scaffolds, and scaffolds with cultured cells were examined by using SEM (FEI Quanta 400, Czech Republic). Before examination, samples were separately fixed on a double adhesive carbon tape, which was stuck on aluminum stubs, and then coated with goldunder an argon atmosphere. The samples were visualized by SEM with an accelerating voltage of 8-20 kV.

2.3 In vitro Bioactivity

2.3.1 Induction of apatite formation

Phosphate buffered saline (PBS) was prepared as described previously.[10] Five grams of the scaffolds were immersed in 20 ml PBS at 37 ^{O}C for 3 weeks under static condition. After that the scaffolds were gently washed with distilled water and air-dried. The amounts of the formed apatite were semi-quantitatively evaluated by using SEM and compared with the un-soaked samples.

2.3.2 Cell culture experiment

Rat MSCs were kindly obtained from Miss Paweena Wongwitwichot, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand. The cells were routinely grown in Dulbecco's Modified Eagle-F12 medium (DMEM-F12) supplemented with 6% fetal bovine serum (FBS) at 37 ^{O}C in a CO_2 incubator. The medium was changed every 3 days. All of the chemicals were purchased from Gibco (Life Technologies, NY, USA).

The test scaffolds were sterilized by soaking in 70% ethanol for 12 h. The residual ethanol was removed by washing several times with sterile water, followed by with FBS-free DMEM-F12 medium. The inducible culture medium was prepared, composing of DMEM–F12 supplemented with FBS (1%), ascorbic acid ($2.8x10^{-4}$ M), and triamcinolone ($1x10^{-9}$ M).[11] The suspension of rat MSCs in PBS with a density of $1x10^{7}$ cells ml^{-1} was prepared by using standard cell culture technique. A 100-µl cell suspension was seeded drop wise onto each of the scaffolds placed on a well of 6-wells plate. The seeded scaffold was incubated further for 1 h at 37 ^{O}C in a CO_2 incubator to allow cell attachment. This was followed by the addition of 1 ml of the inducible medium and cultured for another 5 days before the measurement of ALP activity was carried out. The cells directly cultured in a well were used as the control.

The ability in producing ALP by cells of osteoblastic origin was called "differentiation". The ALP activity was determined according to the previously established method.[11] Briefly, 200 µl of the cultured supernatant was added to a tube containing 200 µl of ALP substrate buffer [$7.5x10^{-2}$ M p-nitrophenyl phosphate and $6.7x10^{-3}$ M $MgCl_2$ in 0.7 M glycine buffer pH 8.5], and the volume was adjusted to 1 ml by using 0.7 M glycine buffer. After incubation for 90 min at room temperature, 100 ml of 0.5 M NaOH was added to stop the reaction and the OD_{410} was measured. To report the ALP activity, the measured OD_{410} was normalized by the OD_{560} of the corresponding sample as acquired from MTT assay.

The procedures for the MTT assay were in accordance with the previous study.[12] In brief, the cell-seeded scaffold was washed twice with PBS and incubated in an excess amount of MTT reagent ($1.2x10^{-2}$ M in PBS) for 4 h at 37 ^{O}C in a CO_2 incubator. Then, the excess reagent was removed and 1 ml of dimethylsulphoxide (DMSO) was added to dissolve the formed formazan product, followed by the measurement of the OD_{560}. For the control, the cells grown directly on a well using the previously described conditions were determined for the OD_{560} according to the MTT assay. The sample OD_{560} was normalized by that of the control before reporting as relative viability.

2.4 Statistical Analysis

All experiments were performed in triplicate. Results were given as means ± SD. Statistical analysis was performed by using one-way ANOVA with significance reported when $P < 0.05$.

3. Results

3.1 SEM Images

The representative SEM image of the scaffolds prepared by the compression molding/salt leaching technique or "Sample 1" was shown in Figure 1a. The embedded sodium chloride crystals were not observed. In addition, chloride ions in the lastly changed leaching medium were not detected by using silver nitrate as a precipitant (data not shown). The resulting 3D-construct of the scaffold was slightly porous. The pores were of irregular shape and classified as the closed pore type. HA particles were distributed over the scaffold and coated by thin films of PCL.

The sample SEM micrograph of PCL microspheres was demonstrated in Figure 1b. The microspheres' diameters were found to range between 5 and 50 µm. The microspheres with smooth, rough or cracked surfaces were obtained. However, for small sized microspheres, the corresponding surfaces were fairly smooth.

The typical SEM picture of the scaffolds prepared by the microsphere/chitosan cross-linking method or "Sample 2" was displayed in Figure 1c. The scaffold's internal structure was distinctly inhomogeneous and loose. The pores were unevenly distributed and extremely irregular in shape, which were classified as the open pore type. HA particles and the microspheres were uniformly dispersed over the scaffold matrix. After immersed in PBS for a period of 1 month, the scaffold structure was not deformed or disintegrated (data not shown).

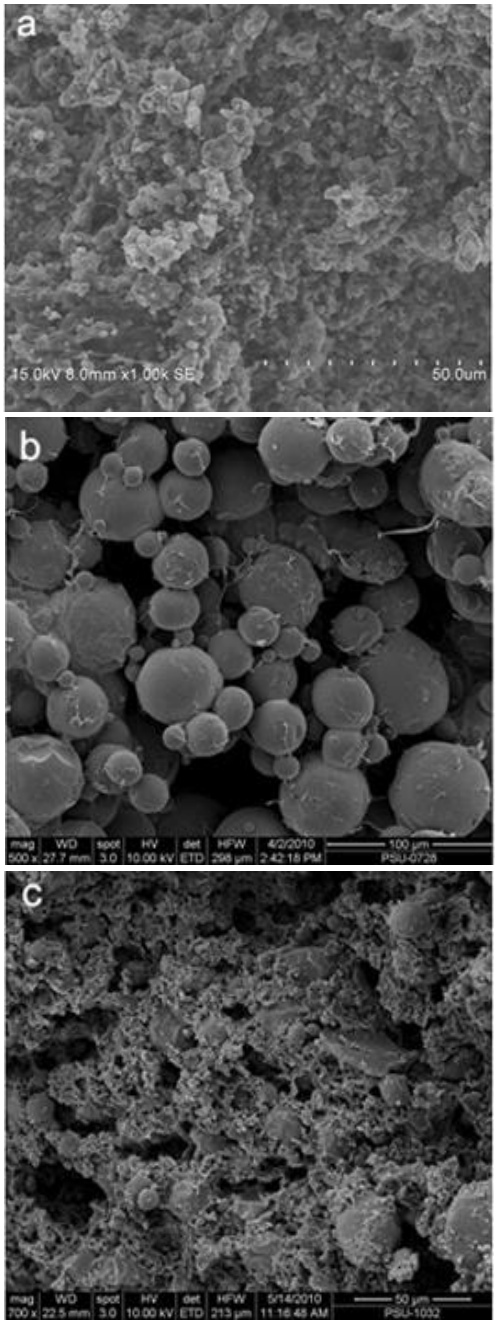

Fig. 1: The representative SEM images of scaffolds prepared by the compression molding/salt leaching technique (a); PCL microspheres (b); scaffolds prepared by the microsphere/chitosan cross-linking (c)

The illustrative SEM image of cell-seeded scaffolds after cultured in the inducible medium for 5 days was shown in Figure 2. In the beginning of incubation, the scaffolds might be absorbed by proteins present in the medium. For longer incubation, the proteins' adsorption was expected to increase by layering onto the prior layers during which sheets and/or fibers of proteins were formed, as observed in Figure 2a and 2b, respectively. However, the exact location and morphology of the cells adhered on the scaffolds could not be indicated.

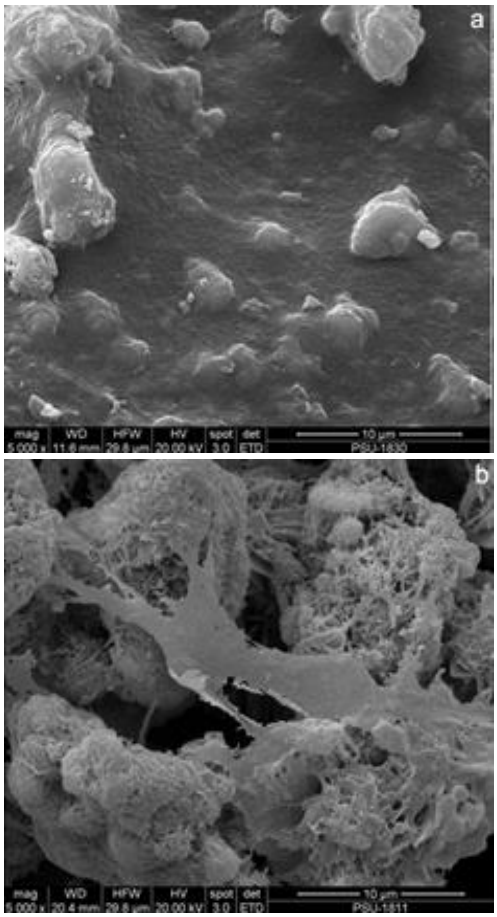

Fig. 2: The SEM micrographs of cell-seeded scaffolds cultured in the inducible medium for 5 days on "Sample 1" (a); and "Sample 2" (b)

3.2 *In vitro* Bioactivity

By soaking in PBS for 3 weeks, apatite layers were induced to form on the scaffold surfaces, as demonstrated in Figure 3. Certainly, the amount of the apatite formed on "Sample 2"was greater than that detected on "Sample 1".

Growth and differentiation of rat MSCs seeded on the test scaffolds were determined after cultured in the inducible medium for 5 days. In Figure 4, active proliferation was observed for the cells on "Sample 2", in compared to that of "Sample 1". The number of viable cells on BD-scaffolds was somewhat lower than that of "Sample 2", but significantly greater than that of "Sample 1". In corresponding to the growth, the ALP activity determined for the cells grown on "Sample 2" was relatively higher than that indicated by the cells of BD-scaffolds and of "Sample 1", respectively.

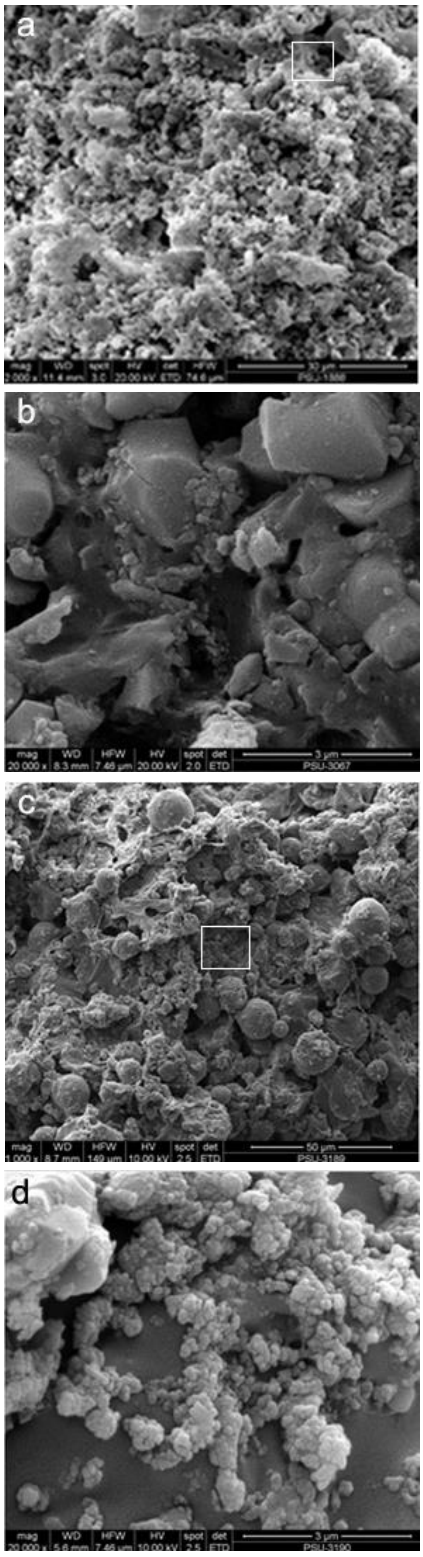

Figure 3 Apatite layers formed on scaffold surfaces after immersed in PBS for 3 weeks: (a) "Sample 1", (c) "Sample 2", (b) and (d) the magnifications of (a) and (c), respectively; the corresponding un-immersed samples of (a) and (c) were respectively demonstrated in Figure 1a and 1c.

4. Discussion

Recently, several methods for preparing porous scaffolds containing biodegradable polymers are reported. These are fiber bonding,[13] solvent casting/particulate leaching,[14] particle sintering,[15] three-dimensional printing,[16] gas foaming,[17] emulsion freeze drying,[18] and phase separation.[19] Nonetheless, the solvent casting/particulate leaching is most popular among others, due to its simple operation with the ease in controlling pore size and porosity of scaffold products. But, this method is generally applied for 2D-films with a thickness of less than 2 mm.

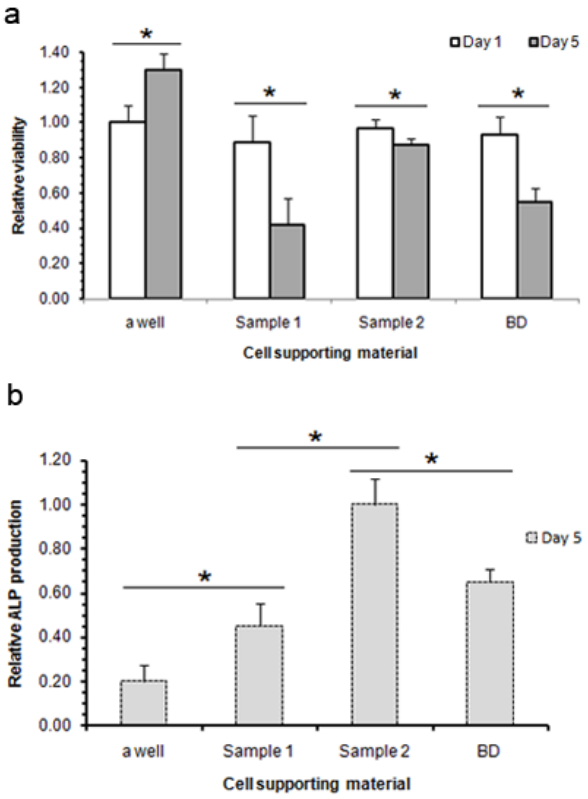

Fig. 4: (a), Relative viability of the cells grown on a well or the scaffold of "Sample 1", "Sample 2" and "BD" in the inducible medium after seeding for 1 and 5 day(s); (b), Relative ALP production of the cells grown on a well or the scaffold of "Sample 1", "Sample 2" and "BD" in the inducible medium for 5 days; * indicates $P < 0.05$.

To construct other 3D-structures like scaffolds, a complicated procedure has been applied by layering a number of films to form a laminated assembly.[14] In this study, the generally existing hydraulic pump equipped with a mold was used to compress granules of PCL coated HA into scaffolds of "Sample 1". Several advantages were attributed by this manipulation. For examples, the volume of chloroform used for

dissolving the polymer was reduced, and simple processing was carried out in acquiring porous scaffolds with uniform and homogeneous microstructure.[20] Regrettably, the obtained scaffolds were biologically inactive because of being coated by PCL film, which is hydrophobic (Figure 1). Its consequence is to primarily prohibit cell adhesion that adversely affecting cell proliferation (Figure 4). In accord with previous studies, a distinctive limitation of synthetic biodegradable polymers for applications as tissue engineering scaffolds is their hydrophobic trait. Such feature is disadvantageous regarding parameters of cell culture experiment, such as lowered density of cell seeding, non-uniformity of cell distribution, and/or diminution of cell growth. Moreover, flows of oxygen, nutrients and metabolic wastes seemed to be restricted because of the existing closed-pores in the scaffolds (Figure 3). Totally, these would cause an inadequacy of cell culture medium circulating into the scaffolds that impeded any cellular activities.[21,22] For the compression molding/salt leaching technique, its potential in bone tissue engineering is thus limited.

To improve cell adhesion, spreading, and proliferation on scaffolds, the associating surfaces are suggested to be moderately hydrophilic.[23,24] Hydrophilization is an approach to be used for increasing the hydrophilicity of synthetic polymers.[22] In this study, the hydrophilization of "Sample 2" scaffolds was achieved by two subsequent steps, i.e., production of PVA-imprinted PCL microspheres and inclusion of HA particles in the scaffolds. Both components were casted to form 3D-constructs by using TPP/chitosan cross-linked networks. The acquired scaffolds were highly porous (Figure 1), helping an infiltration of PBS and/or cell culture medium into the scaffolds. Consequently, large amounts of apatite layers were generated (Figure 2), in compared to those formed on the scaffolds "Sample 1". Moreover, in response to great characteristics of large pores and loose structure of the scaffolds (see Figure 3), the seeded cells would readily approach to oxygen and nutrients, or be easy to eradicate metabolic waste products, resulting in increased proliferation and ALP production by the growing cells (Figure 4). In fact, the micro-structure of BD scaffolds has been highly porous,[9] and the scaffolds are very hydrophilic due to consisting of only calcium phosphate. Instead, these parameters were not beneficial in supporting cell growth and differentiation. It was suggested that the decrease of ALP production by the cells grown on them would be because of decreased cell adhesion and activities in respond to the unsuitable hydrophilic surfaces.[23,24] The method used for cell seeding was proven to be efficient, since the numbers of viable cells on the well and the test scaffolds were insignificantly different when cultured in the inducible medium after seeding for 1 day, and there were a bit of cells detached from the seeded scaffolds (data not shown). The cells on the well greatly proliferated after 5 days of culture, whereas those remaining adhered on the scaffolds had reduced cell viability with the relative cell numbers of "Sample 2" > "BD" > "Sample 1" (Figure 4a). At the end of induction, the ALP activity produced by the proliferative cells on the well was very low. The highest ALP activity was detected for the cells of "Sample 2", followed by "BD" and "Sample 1", respectively (Figure 4b). Regarding to Figure 2, it was uncertain where on the scaffolds being adhered by the cells. It was likely that the cells were embedded in the coated protein sheets/fibers because of positively detected by MTT assay. In summary, the moderately hydrophilic surfaces of "Sample 2" scaffolds, their 3D-structure, and some micro-structural parameters including pore type and porosity were important for adhesion, growth and differentiation of the MSCs. In using the microspheres/chitosan cross-linking technique, the obtained scaffolds were biologically active, in compared to those obtained by using the compression molding/salt leaching method. These procedures were newly established for fabricating scaffolds, which might be profitable for bone tissue engineering applications.

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