

## Osteogenic Differentiation of Mesenchymal Stem Cells on Novel Three-dimensional Poly(L-lactic acid)/Chitosan/Gelatin/ $\beta$ -Tricalcium Phosphate Hybrid Scaffolds

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### ABSTRACT

The concept of combining synthetic polymers with naturally derived biopolymers and resorbable bioceramics has been recently considered as an effective solution for orthopedic tissue engineering. In the present study, based on a biomimetic approach, a novel 3D biodegradable porous hybrid biomaterial consisting of poly(L-lactic acid) (PLLA), chitosan, gelatin, and  $\beta$ -tricalcium phosphate ( $\beta$ -TCP) was developed. Macroporous PLLA/ $\beta$ -TCP scaffolds were prepared by freeze-extraction/particulate leaching method and embedded with porous chitosan/gelatin/ $\beta$ -TCP sponges. The influences of chemical, physical, and structural properties of the scaffolds prepared on the attachment and differentiation of mesenchymal stem cells into osteoblasts and the proliferation of the differentiated cells were investigated. Osteogenically induced cultures revealed that cells were well-attached, penetrated into the scaffold and uniformly distributed. The expression of early and late phenotypic markers of osteoblastic differentiation was upregulated in the scaffolds cultured in osteogenic medium.

### Key Words:

bone;  
chitosan;  
hybrid scaffold;  
tissue engineering;  
stem cell.

### INTRODUCTION

Bone defects resulting from tumors, diseases, infections, trauma, biochemical disorders, and abnormal skeletal development raise significant different health problems. Traditional biological procedures such as autografts, allografts, and grafts with nondegradable materials have been used to

overcome bone defect problems. Bone transplantation by these curative methods, however, is often limited by donor scarcity and highly associated with the risk of rejection and disease transfer [1-4].

In recent years, tissue engineering as a new discipline integrating concepts of the life sciences, such

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as biology, chemistry, and engineering, with surgical techniques, can develop approaches for the regeneration of skeletal tissues [5-7]. It has been believed that appropriate strategies for orthopedic tissue engineering should ideally contain osteoprogenitor stem cells, osteoinductive growth factors, and biodegradable osteoconductive scaffolds [8]. A successful strategy involves in vitro seeding and attachment of human osteoprogenitor stem cells onto a scaffold. These cells then proliferate, migrate, and differentiate into the osteoblasts that secrete the mineral extracellular matrix required for the creation of the bone. It is evident that the choice of the most appropriate scaffolding material is crucial to enable the cells to behave in the manner required producing bone of the desired shape and size [9-11].

From engineering and material science points of view; however, no single biomaterial provides all acceptable chemical, physical, mechanical, and biological properties especially, osteogenesis, osteoinduction, and osteoconduction [12,13]. Therefore, the concept of hybridization of synthetic polymers with biopolymers and/or bioresorbable bioceramics seems very effective to combine their advantages to provide ideal 3D porous biomaterials for tissue engineering [14].

Numerous cell types are available for engineering organized musculoskeletal tissues. The first, and most obvious choice for bone tissue engineering because of their non-immunogenicity is the isolating osteoblasts from biopsies taken from the patients (autologous cells), followed by limited expansion in vitro. However this methodology has several limitations: it is time consuming, relatively few cells are available after the dissociation of the tissue and their expansion rates are relatively low, limiting in this way the number of cells available to be seeded on the scaffolds. Furthermore, in certain bone related diseases osteoblasts may not be appropriate for transplantation because their protein expression profile is under the expected values. In contrast, bone marrow derived stem cells are potentially an autologous cell source with the proliferative and regenerative capacity to be used in tissue engineering, particularly of the musculoskeletal system. It should be mentioned that adult bone marrow is a major source of hematopoietic stem cells responsible for renewing circulating blood com-

ponents; it also contains non-hematopoietic stem cells, termed mesenchymal stem cells (MSCs), which contribute to the regeneration of mesenchymal tissues such as bone, cartilage, muscle, ligament, tendon, adipose, and stroma [15]. MSCs are thought to be recruited in the body for repairing injured tissues, and therefore are good candidates for cell-based therapies for musculoskeletal tissue regeneration. MSCs can be easily isolated and expanded in vitro while maintaining their ability to differentiate into chondrogenic, osteogenic, and adipogenic lineages.

In the present study, based on a biomimetic approach, a novel 3D biodegradable porous hybrid scaffold composed of poly(*L*-lactic acid) (PLLA), chitosan (an osteoconductive biodegradable natural polymer), gelatin (a partially denatured derivative of collagen and a major component of skin, bone, and connective tissues), and  $\beta$ -tricalcium phosphate ( $\beta$ -TCP) (an osteoinductive bioresorbable bioceramic) was developed for trabecular bone tissue engineering, especially for non-load bearing applications. Utilizing four different fabrication methods simultaneously, chitosan/gelatin/ $\beta$ -TCP microsponges were formed in the pores of PLLA/ $\beta$ -TCP scaffold. Moreover, the influence of chemical, physical, and structural properties of the scaffolds prepared on the differentiation of MSCs into osteoblasts and the proliferation of the differentiated cells were the main focuses of this study. It should be noted that the main objective of the study was to prepare a completely biodegradable hybrid scaffold with highly interconnected macroporosity, but with improved strength and stable dimensions in biologically relevant testing conditions. The scaffolds reported have promising perspective for their use in engineered bone tissue.

## EXPERIMENTAL

### Materials

Chitosan powder with a viscosity average molecular weight of  $4.0 \times 10^5$  g.mol<sup>-1</sup> and a deacetylation degree of 85% was obtained from Fluka Biochemika and the gelatin powder (porcine) was purchased from the Sigma Chemical Co. PLLA with an intrinsic viscosity of 1.5 dL/g (Boehringer-Ingelheim Inc., Germany) and  $\beta$ -TCP powder, with an average particle diameter

of <26  $\mu\text{m}$ , (Biovision GmbH, Germany) were used as received. 1,4-Dioxane, ethanol, and glacial acetic acid were obtained from Merck. Distilled and deionized water was used throughout this study. All other reagents were of analytical grade and used as received from the manufacturer.

### Preparation of Macroporous PLLA/ $\beta$ -TCP Matrices

Macroporous PLLA/ $\beta$ -TCP matrices were prepared by combining PLLA and  $\beta$ -TCP cement particles with sugar crystals (average particle diameter:  $\sim 2$  mm) as porogen through the process of freeze-extraction/particulate leaching technique [16-19]. Briefly, PLLA and  $\beta$ -TCP cement particles at a mass ratio of 2:1 were dissolved in dioxane to form a 10 wt% solution and sieved sugar particles were added (90 wt% sugar). The vortexed dispersion was cast in a 5 cm glass petri dishes and frozen at  $-20^\circ\text{C}$ . The frozen PLLA/ $\beta$ -TCP/sugar solutions were immersed in an ethanol aqueous solution (80 wt%) that was pre-cooled to  $-20^\circ\text{C}$ . Due to the miscibility between dioxane and the ethanol aqueous solution, the solvent was extracted out and replaced with ethanol molecules, a non-solvent for PLLA. After extraction, drying at room temperature was performed to remove the ethanol aqueous solution contained in the PLLA/ $\beta$ -TCP/sugar matrices. After the drying stage, the PLLA/ $\beta$ -TCP/sugar composite matrices were immersed in 500 mL deionized water on a shaker at  $25^\circ\text{C}$  for 48 h (the water was changed every 6 h) to leach out the sugar particles. The porogen-free PLLA/ $\beta$ -TCP matrices were air-dried for 72 h and stored in a desiccator at room temperature until use.

### Preparation of Chitosan/Gelatin/ $\beta$ -TCP Microsponges

Chitosan/gelatin/ $\beta$ -TCP microsponges were prepared by solid-liquid phase separation and subsequent lyophilization [20-25]. Briefly speaking, solutions with total concentration of 2, 3, and 5 wt% were prepared by dissolution of chitosan, gelatin, and  $\beta$ -TCP in a 0.25 M acetic acid aqueous solution at a mass ratio of 2:2:1 under agitation at  $40^\circ\text{C}$  for 12 h. The homogeneous mixtures were put into 24-well polystyrene culture dishes and then rapidly transferred into a freezer at the preset temperature ( $-20^\circ\text{C}$ ) to

solidify the water and induce solid-liquid phase separation. The solidified mixtures were maintained at that temperature overnight. In the final stage, frozen mixtures were lyophilized at 0.02 mbar and freeze-drying temperature of  $-20^\circ\text{C}$  for 48 h.

### Fabrication of PLLA/Chitosan/Gelatin/ $\beta$ -TCP Hybrid Scaffolds

The macroporous PLLA/ $\beta$ -TCP matrices were immersed in a 3 wt% chitosan/gelatin/ $\beta$ -TCP aqueous solution under vacuum so that the PLLA/ $\beta$ -TCP scaffold pores were filled with biopolymer solution [26]. The chitosan/gelatin/ $\beta$ -TCP solution-containing PLLA/ $\beta$ -TCP matrices were frozen at  $-20^\circ\text{C}$  to induce solid-liquid phase separation and then freeze-dried to completely remove the solvent and enable the formation of chitosan/gelatin/ $\beta$ -TCP microsponges in the pores of PLLA/ $\beta$ -TCP matrices.

The porosity value of the hybrid scaffolds prepared was measured by liquid displacement, similar to a published method [27]. In this method water has been used as the displacement liquid. However, water was difficult to work with since it did not penetrate very easily into the pores, probably due to the hydrophobicity of PLLA. Thus ethanol was used in this procedure because it penetrated easily into the pores and did not induce shrinkage or swelling as a non-solvent of the polymers. A hybrid scaffold was immersed in a graduated cylinder containing a known volume ( $V_1$ ) of ethanol. The sample was kept in the ethanol for 2 h. Afterward, the total volume of ethanol and the ethanol-impregnated scaffold was recorded as  $V_2$ . The volume difference ( $V_2 - V_1$ ), was the volume of the hybrid scaffold. The ethanol-impregnated scaffold was removed from the cylinder and the residual ethanol volume was recorded as  $V_3$ . The quantity of ( $V_1 - V_3$ ), i.e. the volume of the ethanol held in the hybrid scaffold was determined as the void volume of the scaffold. Thus the porosity of the scaffold,  $\varepsilon$ , was obtained by:

$$\varepsilon = \frac{(V_1 - V_3)}{(V_2 - V_3)} \quad (1)$$

### Mechanical Properties of PLLA/Chitosan/Gelatin/ $\beta$ -TCP Hybrid Scaffolds

Compressive moduli of PLLA/chitosan/gelatin/ $\beta$ -TCP hybrid scaffolds were determined using a SAN-

TAM SMT-20 mechanical testing system. Cylindrical samples with an external diameter of 12 mm were compressed between platens with a constant deformation rate of 1 mm/min at room temperature. A small preload was applied to each sample to ensure that the entire scaffold surface was in contact with the compression plates prior to testing, and the distance between plates prior to each test was equal to the measured thickness of the scaffold being tested. Compressive modulus was calculated as the slope of the initial linear portion of the stress-strain curve. Four specimens at each PLLA/chitosan/gelatin/ $\beta$ -TCP hybrid scaffold were tested and the results were the average of four measurements.

### Culture Methods

Bone marrow mesenchymal stem cells (MSCs) were isolated from the femurs of young adult Wistar rats as described by Maniopoulos et al. [28], and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 15% fetal bovine serum (FBS; Gibco). Determining the MSCs' proliferation potentiality, they were cultured in humidified atmosphere of 95% air with 5% CO<sub>2</sub> at 37°C, extensively propagated, and passaged from 15 to 20 times in vitro. The adherent MSCs, with passage number of 3 to 5, were detached from tissue culture flask with 0.05% trypsin containing 1 mM EDTA (Gibco) and suspended to  $3 \times 10^5$  cells/mL in culture medium. The cell suspension was applied to seed on sterilized hybrid scaffolds placed in 12-well tissue culture plates at a density of  $1.5 \times 10^5$  cells/scaffold. Following the incubation of the cell seeded scaffolds at 37°C for 3-4 h, 2 mL of culture medium was then added to each well. MSCs were treated with osteogenic medium containing DMEM supplemented with 15% FBS, 10 nM dexamethasone (Sigma-Aldrich), 10 mM  $\beta$ -glycerophosphate (Merck), and 0.28 mM ascorbic acid two-phosphate magnesium salt n-hydrate (Sigma-Aldrich). The culture media were changed three times a week during the two-week differentiation treatment.

### Scanning Electron Microscopy and DAPI Staining

The morphology of 3D macroporous hybrid PLLA/chitosan/gelatin/ $\beta$ -TCP scaffolds, with and without cells, was observed by means of scanning electron microscopy at an accelerating voltage of

20 kV. Before the observation, samples of cell-scaffold constructs were fixed in 2.5% glutaraldehyde, dehydrated through a graded series of ethanol, and vacuum-dried. All samples were coated with gold using a sputter coater.

To assess nuclear morphology, cultures were fixed for 10 min in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.2, stained in 4,6-diamidino-2-phenylindole (DAPI, 2.5  $\mu$ g/mL) for 5 min, and washed once in water, after which coverslips were placed with Aquamount (Polysciences Inc.). Photographs were taken using a Nikon camera mounted on a Nikon Diaphot 300 microscope and UV illumination. It is worth mentioning that DAPI is a fluorescent stain that binds strongly to DNA. It is used extensively in fluorescence microscopy. Since DAPI will pass through an intact cell membrane, it may be used to stain live and fixed cells. For fluorescence microscopy, DAPI is excited with ultraviolet light. When bound to double-stranded DNA its absorption maximum is at 358 nm and its emission maximum is at 461 nm. DAPI will also bind to RNA, though it is not as strongly fluorescent. Its emission shifts to around 400 nm when bound to RNA.

### Reverse Transcription Polymerase Chain Reaction Analysis

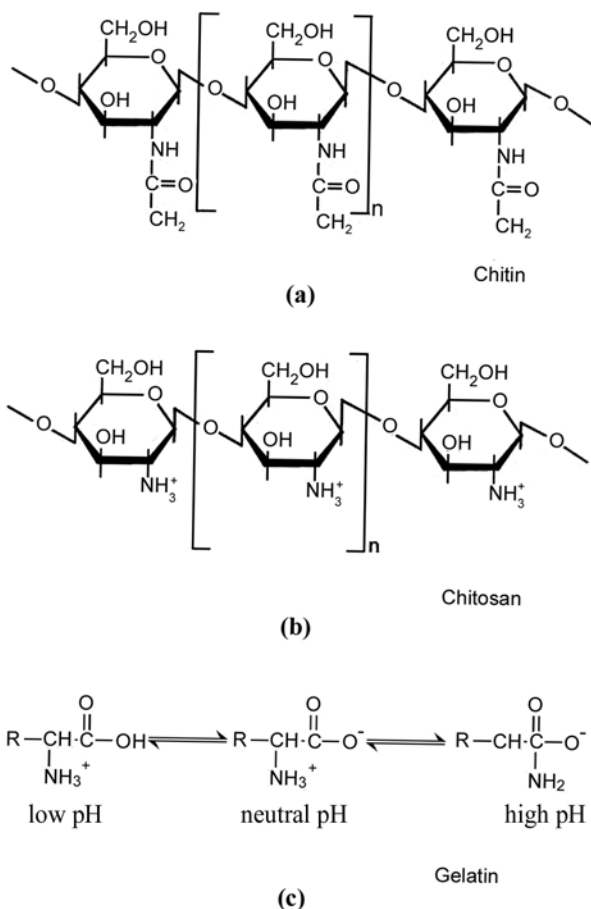
For the reverse transcription polymerase chain reaction (RT-PCR) analysis, total cellular RNA was extracted using TRI-reagent (Sigma T-9424) according to the manufacturer's protocol. Synthesis of cDNA was carried out with M-MuLV reverse transcriptase (RT) and random hexamer as primer, according to the manufacturer's instructions (Fermentas Inc., Hanover, MD, USA). PCR Amplification was performed using a standard procedure with Taq DNA polymerase (Fermentas Inc., Hanover, MD, USA) with denaturation at 94°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s. Depending on the abundance of the particular mRNA, the number of cycles varied between 25 and 30. The primers and product lengths are listed in Table 1.

## RESULTS AND DISCUSSION

The natural extracellular matrix (ECM) is a complex

**Table 1.** RT-PCR Primers for bone specific gene expression analysis.

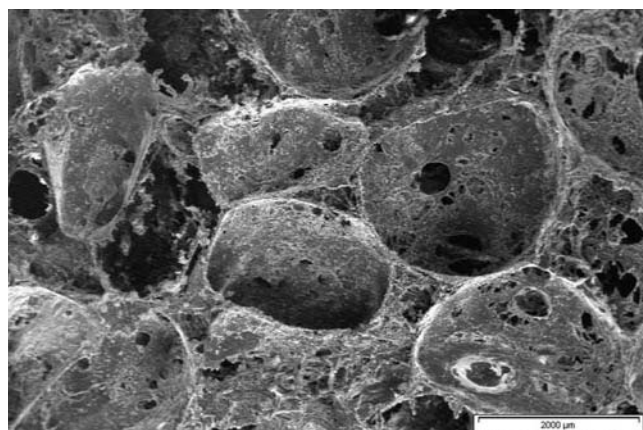
Gene	Primer sequences (Sense: top, antisense: bottom)	Size (bp)
<b>Housekeeping gene</b>		
$\beta$ -2-Microglobulin ( $\beta$ 2M)	TGGAAAGAAGATACCAAATATCGA GATGATTCAGAGCTCCATAGAGCT	201
<b>Bone specific genes</b>		
Alkaline phosphatase (ALP)	TTAAGGGCCAGCTACACCAC GATAGGCGATGTCCTTG CAG	401
Bone sialoprotein (BSP)	CGCCTACTTTTATCCTCCTCTG CTGACCCTCGTAGCCTTCATAG	795
Osteocalcin (OC)	CTAGCAGACACCATGAGGACC ATACTTTCGAGGCAGAGAGAGG	404
Osteopontin (OP)	AGAGGAGAAGGCGCATTACA GCAACTGGGATGACCTTGAT	497



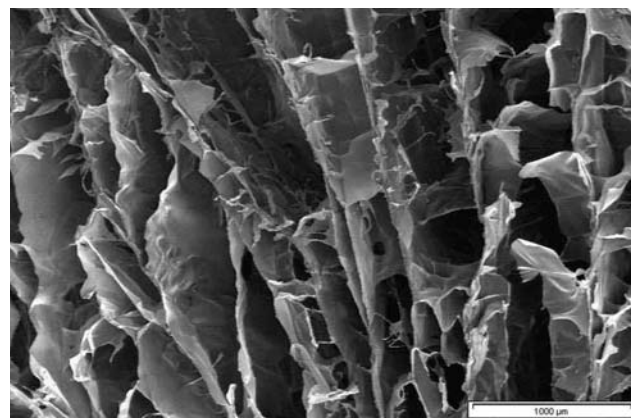
**Figure 1.** Schematic representation of the molecular structure of (a) chitin, (b) chitosan, and (c) gelatin.

mixture of structural and functional proteins, glycoproteins, and proteoglycans arranged in a unique, tissue specific 3D ultrastructure. Structural proteins such as collagen, the most abundant protein within the ECM, and GAGs are especially important in mechanically supporting tissue reconstruction and providing attachment sites for cell surface receptors. GAGs play important roles in binding growth factors, water retention, and gel properties of ECM. It is worth mentioning that chitosan and gelatin have chemical structures similar to a repeating unit of GAGs (Figure 1). Hence, in the present study, PLLA, chitosan, and gelatin were chosen as the substitutes of collagen and GAGs in ECM. Moreover, bone is a biomineralized tissue, i.e. its ECM is composed of structural protein, type I collagen fibrils on which crystalline apatite is deposited, regularly associated with other extracellular components, for example, non-collagenous proteins, GAGs, and water, etc. Animal implantation experiments have shown the need for bone substitutes to present macroporous structures with pore diameters in the range of 100-800  $\mu\text{m}$  [29]. This is to facilitate the connection with the connective tissue to avoid displacement between implants and tissues and promote bone ingrowth.

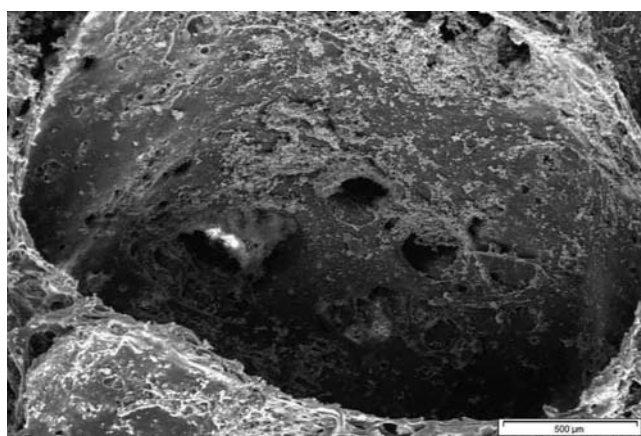
From SEM micrographs shown in Figure 2, it is observed that porous PLLA/ $\beta$ -TCP scaffolds can be



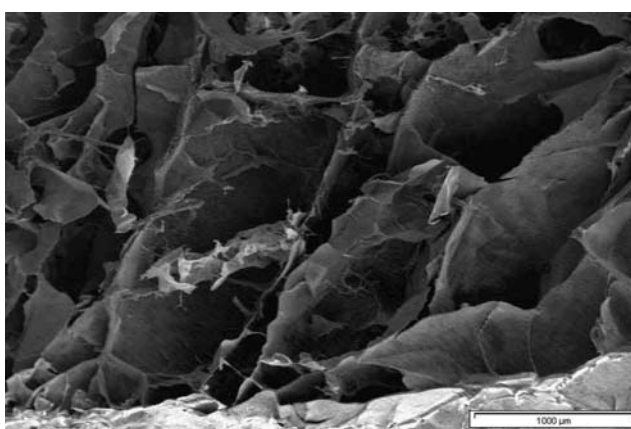
(a)



(a)



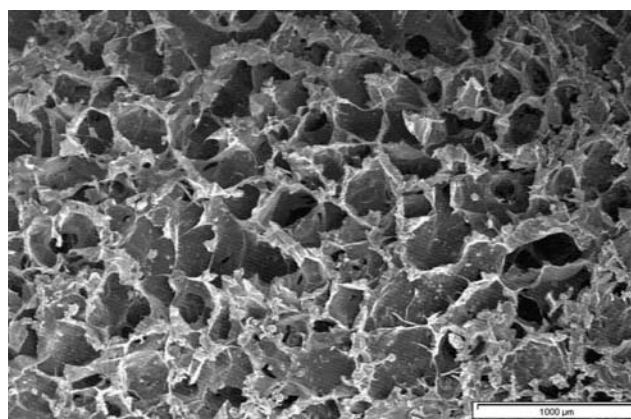
(b)



(b)

**Figure 2.** SEM Micrographs of PLLA/β-TCP scaffolds (PLLA/β-TCP:2/1) prepared from a 10 wt% PLLA/β-TCP/dioxane solution (freezing temperature: -20°C). Original magnifications: (a) 10 wt% PLLA/β-TCP/dioxane, ×15; and (b) 10 wt% PLLA/β-TCP/dioxane, ×50.

successfully fabricated by using the freeze-extraction/particulate leaching method. It is obvious that the pores are interconnected ranging between 1 to 2 mm. Therefore, the scaffolds prepared by freeze-extraction/particulate leaching method, possess suitable pore size for bone tissue regeneration. In addition, the interconnected pore structure can provide enough space for possible neo-vascularization when being implanted. Meanwhile, the porosity of the scaffolds was also measured by liquid displacement method. The data obtained indicate that the porosity of the prepared scaffolds is high enough (~87%) to be used in bone tissue engineering. Recently, Davies et al. have created macroporous poly(lactide-glycolic acid) (PLGA) scaffolds having a pore size of approximate-



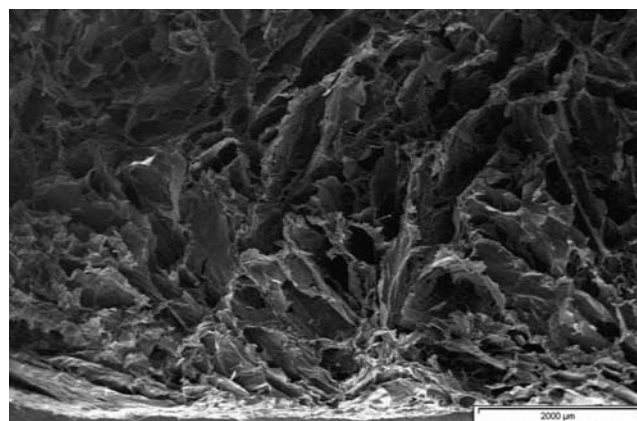
(c)

**Figure 3.** SEM Micrographs of chitosan/gelatin/β-TCP microsponges (chitosan/gelatin/β-TCP:2/2/1) fabricated from chitosan/gelatin/β-TCP/aqueous acetic acid mixtures with different total concentrations (freezing temperature: -20°C). Original magnifications: (a) 2 wt% chitosan/gelatin/β-TCP/aqueous acetic acid, ×30; (b) 3 wt% chitosan/gelatin/β-TCP/aqueous acetic acid, ×30; and (c) 5 wt% chitosan/gelatin/β-TCP/aqueous acetic acid, ×30.

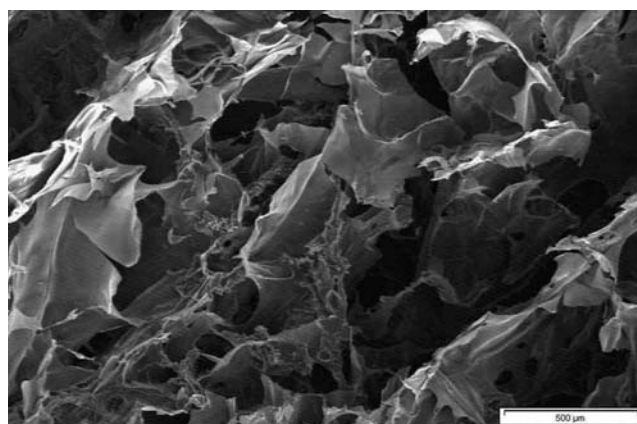
ly 0.85 to 1.18 mm in diameter [19,30]. Their results have shown that accelerated early healing of critical-sized segmental osteotomies in the rabbit femur can be achieved by combining autologous marrow cells with these macroporous biodegradable PLGA scaffolds.

It is worth mentioning that in freeze-extraction method, the idea was to remove the solvent by extraction with a non-solvent. After the removal of the solvent, the space originally occupied by 1,4-dioxane was taken by the non-solvent and the polymer was then surrounded with ethanol molecules. Under this circumstance, even at room temperature, the polymer would not dissolve. Hence, drying and leaching at room temperature could be carried out to remove the non-solvent and sugar crystals, leaving some space, i.e. pores in the scaffolds. It should be noted that the extraction was performed at a temperature lower than the freezing point of PLLA solution to assure that the polymer would not redissolve during the extraction process. Hence, the non-solvent should have a freezing point lower than that of PLLA solution, so it could be kept at a liquid state during extraction. As mentioned above, the non-solvent used in this study was ethanol aqueous solution of which the freezing point can be changed by adjusting its composition. With a suitable composition of ethanol aqueous solution (80 wt%), the non-solvent bath (ethanol aqueous solution) was in liquid state during the extraction of 1,4-dioxane out of the frozen PLLA solutions.

Figure 3 illustrates representative cross-sectional structures of chitosan/gelatin/ $\beta$ -TCP hybrid scaffolds fabricated using a thermally induced phase separation and subsequent lyophilization technique with different concentrations of the initial solution. As it is observed, the concentration of the initial solution has a significant effect on the pore structure of the hybrid scaffolds, i.e. the smaller and closer pore structures were enhanced with the increase in initial concentration. Furthermore, raising the initial concentration yielded scaffolds with more homogeneous microstructures and larger wall thicknesses. It is believed that the viscosity of the initial solution significantly affects the pore structure during the phase separation process. The higher the concentration of initial solution, the larger its viscosity, which is unsuitable to water migration and prevents large ice



(a)



(b)

**Figure 4.** SEM Micrographs of PLLA/chitosan/gelatin/ $\beta$ -TCP hybrid scaffolds prepared from a 10 wt% PLLA/ $\beta$ -TCP/dioxane and a 3 wt% chitosan/gelatin/ $\beta$ -TCP/aqueous acetic acid solutions (freezing temperature:  $-20^{\circ}\text{C}$ ). Original magnifications: (a)  $\times 15$ ; and (b)  $\times 50$ .

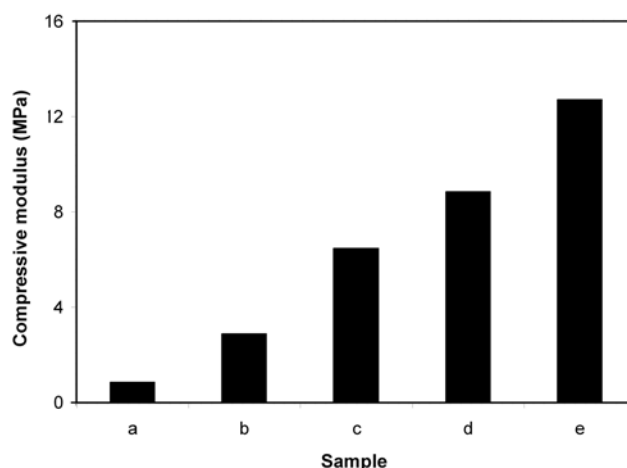
crystalline growth during freezing, thus reducing larger pores in the hybrid scaffolds. It should be noted that the trends observed are in good agreement with the results published in the literature [20-23].

SEM Images of 3D macroporous PLLA/chitosan/gelatin/ $\beta$ -TCP hybrid scaffolds are shown in Figure 4. The results obtained indicate that chitosan/gelatin/ $\beta$ -TCP microsponges were formed in the pores of PLLA/ $\beta$ -TCP macroporous scaffolds and the pore surfaces were covered with biopolymers. It should be mentioned that the mean pore size of chitosan/gelatin/ $\beta$ -TCP microsponges was  $\sim 500\text{ }\mu\text{m}$ . It is well accepted that for bone tissue engineering purposes, pore size should be within the 200-900  $\mu\text{m}$  range [31-33]. However, as mentioned above, Davies et al.

reported a different concept. In the referred case the authors believe that bone reconstruction will only be achieved by having a 3D temporary matrix with a large macroporous interconnected structure with pore size ranging from 1.2-2.0 mm. The latter approach has distinct advantages due to its high surface to volume ratios that will facilitate cell, tissue and blood vessels in-growth. However, this affects the mechanical properties avoiding its use in areas which are very demanding from the mechanical point of view.

As mentioned above, the mechanical property of the scaffold is another critical feature that must be considered for bone tissue engineering applications. Scaffolds need good mechanical properties, especially compressive modulus, to maintain their shape during culturing and the surgical procedures of transplantation [34-35]. Because scaffolds made from pure chitosan or gelatin are soft and weak, they may not provide the necessary mechanical properties during in vitro and in vivo assays. Using  $\beta$ -TCP as a reinforcing material to fabricate chitosan/gelatin/ $\beta$ -TCP hybrid scaffolds solves this problem. In addition, direct implantation of bioresorbable bioceramics has resulted in the dislocation of material within the tissue; therefore,  $\beta$ -TCP powders are often mixed with other materials, such as collagen, gelatin, alginate when used as artificial grafts, not only to reinforce the mechanical properties of scaffolds, but also to eliminate undesired mobility of the grafts [36-40]. Evidently, in the present study, the skeletal PLLA/ $\beta$ -TCP scaffold provided the PLLA/chitosan/gelatin/ $\beta$ -TCP with good mechanical properties and easy formation into desired shapes, while the chitosan/gelatin/ $\beta$ -TCP microsponges gave the scaffold good cell interaction as well as a good degree of wettability for cell seeding.

Figure 5 shows the mechanical properties of the hybrid scaffolds prepared in this study. As can be observed, the compressive modulus of the chitosan/gelatin/ $\beta$ -TCP hybrid sponges created here generally increased with increasing the concentration of the initial solution and  $\beta$ -TCP content. Moreover, as shown in this figure, the compressive modulus of the PLLA/chitosan/gelatin/ $\beta$ -TCP hybrid scaffolds was increased about six times more than those of chitosan/gelatin/ $\beta$ -TCP scaffolds fabricated from a 3 wt% composite solution at a mass ratio of 2:2:1. It should

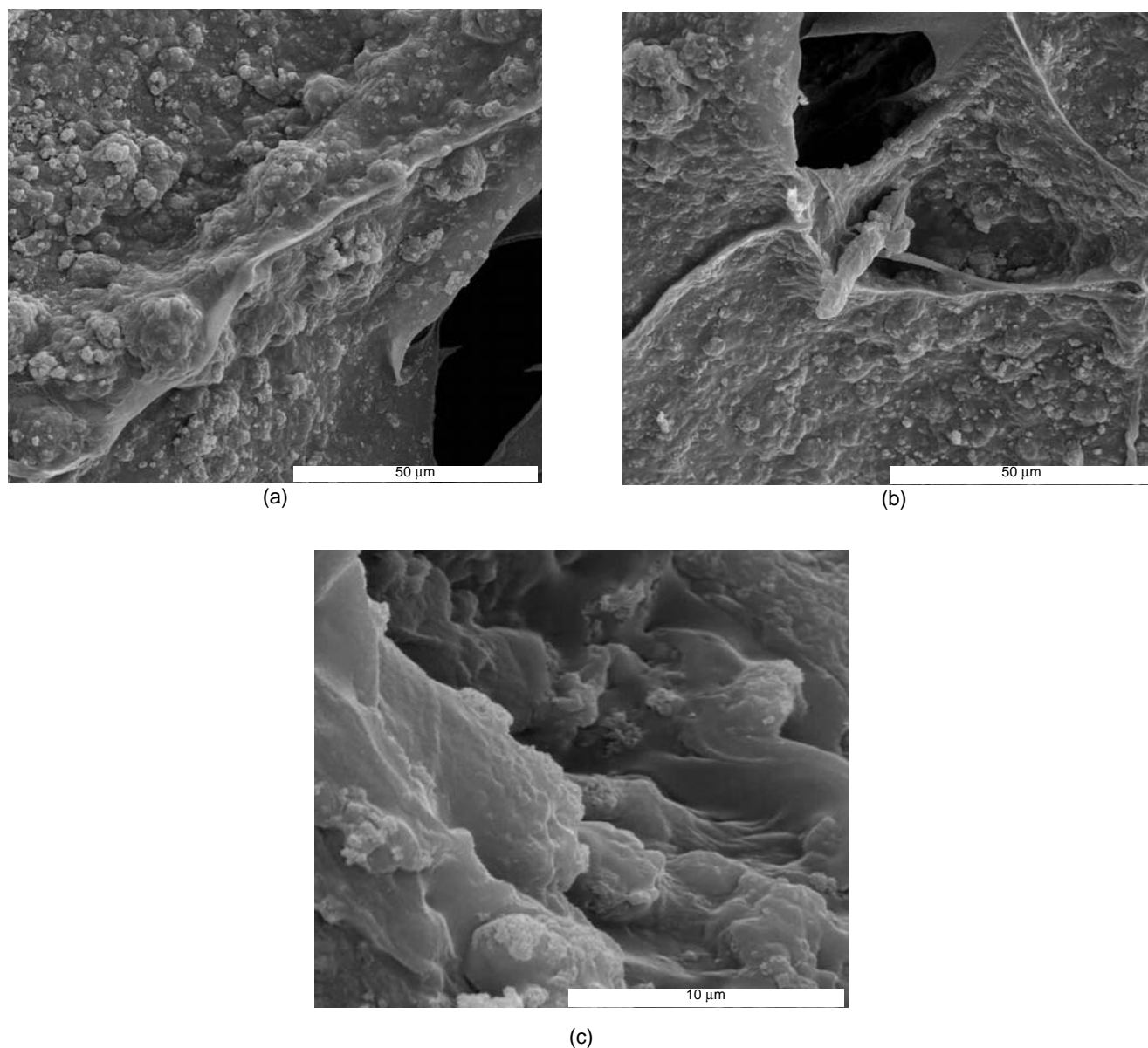


**Figure 5.** Compressive modulus of biodegradable hybrid scaffolds: (a,b,c) 2, 3, and 5 wt% chitosan/gelatin/ $\beta$ -TCP solutions at a mass ratio of 2:2:1, respectively; (d) 5 wt% chitosan/gelatin/ $\beta$ -TCP solution at a mass ratio of 2:2:4; (e) PLLA/chitosan/gelatin/ $\beta$ -TCP hybrid scaffold prepared from 10 wt% PLLA/ $\beta$ -TCP solution at a mass ratio of 2:1 and 3 wt% chitosan/gelatin/ $\beta$ -TCP solution at a mass ratio of 2:2:1.

be mentioned that the compressive modulus of PLLA/chitosan/gelatin/ $\beta$ -TCP hybrid scaffolds is comparable with trabecular bone.

In this work, osteogenic differentiation of adult rat bone marrow derived MSCs was investigated on the hybrid scaffolds. Cell-scaffold constructs cultured for two weeks in osteogenic medium containing  $\beta$ -glycerophosphate, ascorbic acid and dexamethasone, were analyzed using SEM (Figure 6). Osteogenically induced cultures revealed that cells were well-attached, penetrated into the constructs, and uniformly distributed. In the constructions cultured in presence of osteogenic medium, the formation of mineralized nodules in the MSC-derived osteoblast-like cells was observed. As it is noticed in Figure 6, the high surface-area-to-volume ratio and the high porosity of 3D hybrid scaffolds support attachment and migration of anchorage-dependent MSCs inside the scaffolds with interconnected pores and their differentiation into osteoblast-like cells as well. In contrast with tissue culture plates, the cells grow and develop more extensively with appropriate interactions inside the 3D medium of porous scaffolds mainly due to their chemical and morphological similarity to natural extracellular matrices. These results are consistent with previous





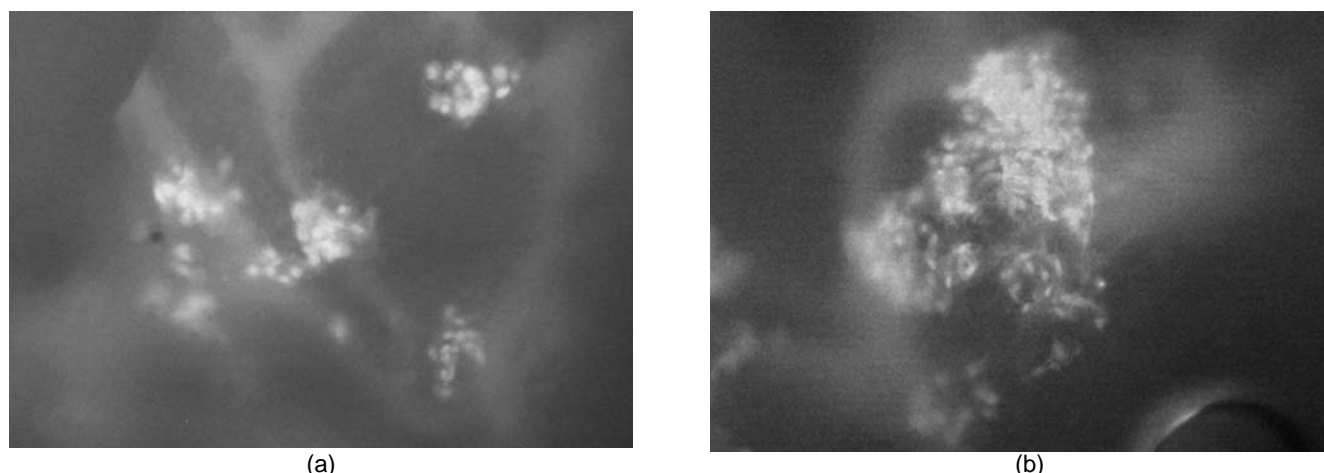
**Figure 6.** SEM Micrographs of MSCs seeded and differentiated on 3D PLLA/chitosan/gelatin/ $\beta$ -TCP hybrid scaffolds: (a) magnification  $\times 1.36$  k and (b) low magnification view of the cell-polymer constructs after 2 weeks of culture in a differentiated medium (the scaffold surface is partially covered by a layer of attached cells) magnification  $\times 1.30$  k; and (c) high magnification view of the cell-polymer constructs after 2 weeks of differentiation treatment, magnification  $\times 6.56$  k.

reports demonstrating the ability of 3D osteoconductive and/or osteoinductive biomaterials in osteogenic differentiation of MSCs [41-47].

It is noticed in Figure 6 that the cells are covered by natural ECM produced by differentiated cells. To confirm the existence of osteoblast-like cells inside the porous scaffolds, the existence and distribution of cells were studied on the hybrid scaffolds by using fluorescence microscopy (DAPI staining). According to Figure 7, examination of cell distribution, under

fluorescence microscopy, showed that cells were clearly adherent and spread out along the sides of the pores of the PLLA/chitosan/gelatin/ $\beta$ -TCP hybrid scaffolds and were observed in the centre of a 12 mm diameter, 10 mm high scaffold.

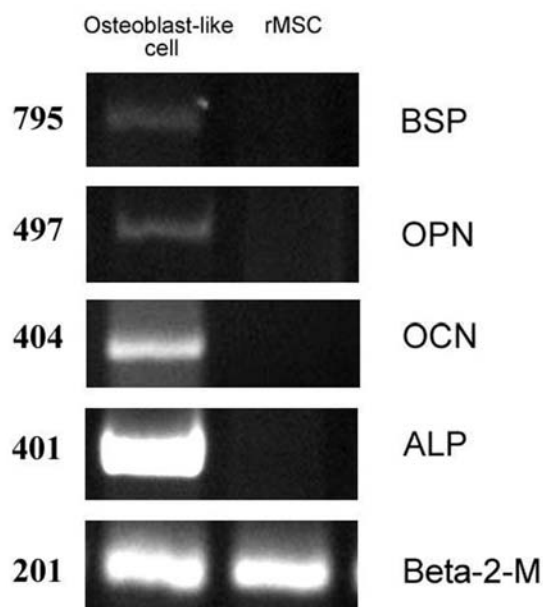
In addition, to confirm the osteogenic differentiation of MSCs on PLLA/chitosan/gelatin/ $\beta$ -TCP hybrid scaffolds, RT-PCR (reverse transcription-polymerase chain reaction) analysis was utilized to detect the expression of mRNA transcripts of bone specific



**Figure 7.** The existence and distribution of differentiated cells on PLLA/chitosan/gelatin/β-TCP scaffold by using fluorescence microscopy (DAPI staining).

molecules by the second week of culture. In fact, this technique is sensitive enough to enable quantitation of RNA from a single cell [48]. According to Figure 8, the expression of markers of osteoblastic differentiation, including alkaline phosphatase (ALP), osteocalcin (OC), osteopontin (OP), and bone sialoprotein (BSP) was upregulated in constructs cultured in osteogenic medium (Table 1). It should be mentioned

that ALP is a protein localized on the cellular membrane of the osteoblasts and has been used as a marker for early osteogenic differentiation cascade. Osteocalcin is a bone-specific protein which has been used as a late marker of osteogenic differentiation. This protein is exclusively produced by osteoblasts and accumulated in the bone matrix, which is also produced by the osteoblasts. Furthermore, Osteopontin and BSP are other late phenotypic markers of osteoblastic differentiation. By contrast, untreated control cultures failed to express either genes by the second week.



**Figure 8.** RT-PCR Analysis of osteogenic differentiation of rMSCs seeded inside the 3D hybrid scaffolds. The addition of osteogenic supplements to cellular constructs led to increased expression of ALP, OC, OP and BSP compared to control cultures.

## CONCLUSION

A novel approach for the fabrication of biodegradable PLLA/chitosan/gelatin/β-TCP hybrid scaffolds was developed in this research. Utilizing freeze-extraction/particulate leaching as well as lyophilization techniques, chitosan/gelatin/β-TCP microsponges were formed in the pores of PLLA/β-TCP scaffolds. The prepared scaffolds were highly porous, with porosities larger than 87%, and had interconnected pores. Moreover, in vitro assay suggested that these 3D hybrid scaffolds were fully capable of supporting attachment and osteogenic differentiation of rat MSCs. The ability of cultured stem cells to self-renew and differentiating into lineage-committed osteoblast-like cells on these biocompatible and biodegradable scaffolds may be beneficial for bone tissue engineering.

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