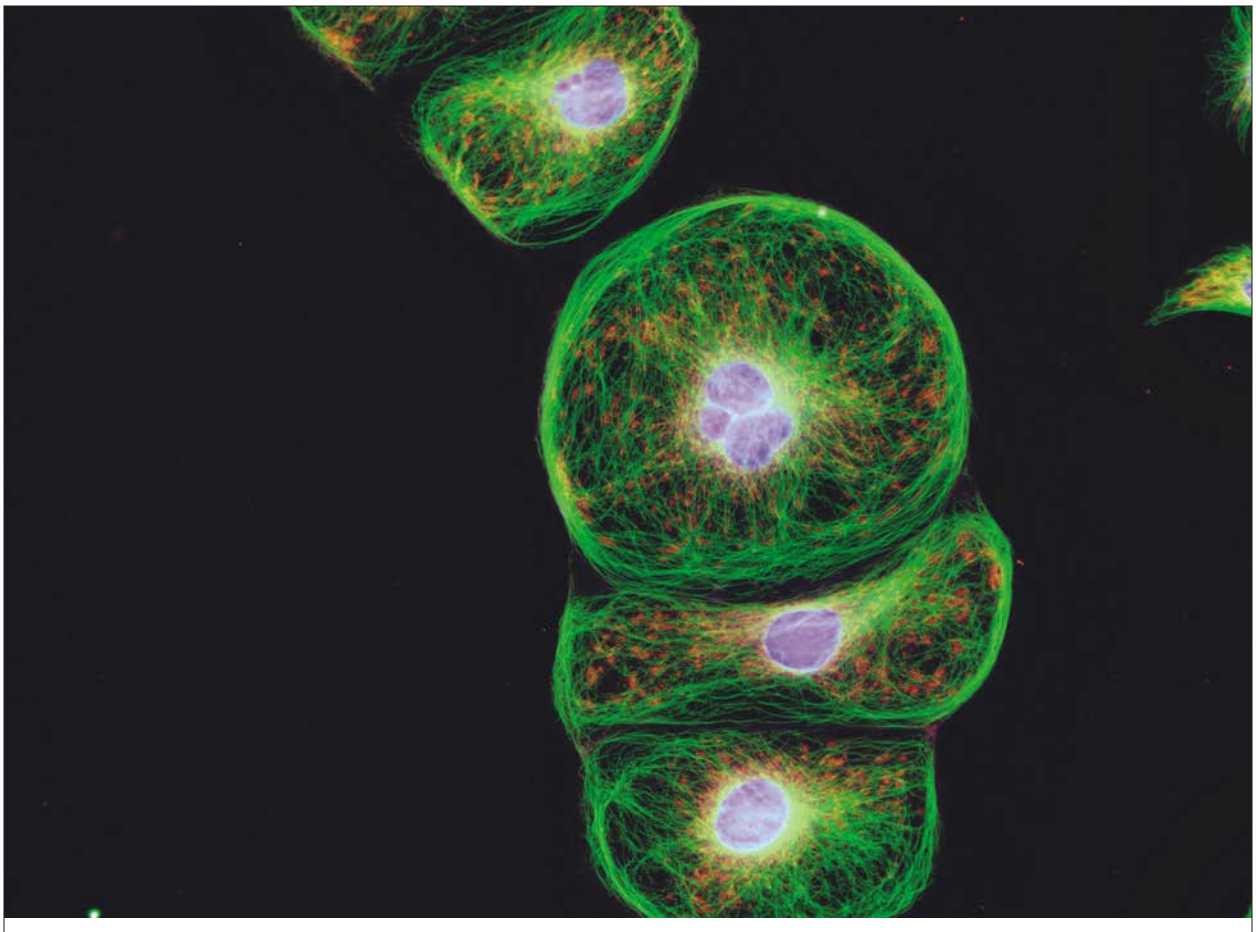

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Osteogenic Expression of Bone Marrow Stromal Cells on PCLTF Scaffold: *In vitro* Study

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Abstract: This study was conducted to evaluate the potential of poly(caprolactone-trifumarate) (PCLTF) as scaffold for *in vitro* Bone Marrow Stromal (BMS)-derived osteoblasts growth, proliferation and differentiation. Cylindrical porous scaffold was fabricated by free radical initiated crosslinking of PCLTF macromere been synthesized in our laboratory. BMS cells was isolated from Sprague-Dawley rats, cultured and expanded in osteogenic medium. BMS-derived osteoblasts were then seeded on PCLTF porous scaffolds and the osteogenic phenotypes expression were evaluated by Scanning Electron Microscopy (SEM), detection of Alkaline Phosphatase (ALP) secretion using p-nitrophenyl phosphate substrate and demonstration of mineralization by von-Kossa staining. Histological structure of cells seeded scaffold was demonstrated by H & E staining. Cells seeded PCLTF scaffold transformed from yellow-brownish into whitish bone tissue-like appearance after been cultured in osteogenic medium for 28 days. SEM revealed osteoblasts adhesion and proliferation on PCLTF scaffold after 3 days culture. At day 14, we found fibrillar collagen network as well as calcified globuli was deposited on PCLTF scaffold and at 28 days we observed massive mineralization on PCLTF. ALP secretion was detected in both cells seeded PCLTF scaffold and positive controls and their total ALP fold production were significantly no different. Von-Kossa staining demonstrated calcium mineral (dark-brownish) pigments on cells seeded PCLTF scaffold. Bone-like tissue structures (i.e., bone matrix with osteocytes resided in lacunae) were found on H & E stained slides of cells seeded PCLTF scaffold after 28 days of incubation. BMS-derived osteoblasts growth, proliferation and differentiation were observed on synthesized PCLTF porous scaffold. Thus, we believe PCLTF has the potential to be used as scaffold for bone tissue engineering.

Key words: poly (caprolactone-trifumarate), bone marrow stromal, alkaline phosphatase

Introduction

Development of novel biocompatible material as substrate for osteoprogenitor cells growth, proliferation and differentiation is very much desired. Poly(e-caprolactone) (PCL) is an FDA-approved biodegradable polymer for use as resorbable sutures. It has been studied as scaffold for skin (Dai *et al*., 2004), heart muscle (Shin *et al*., 2004) and bone (Kweon *et al*., 2003) tissue engineering. Various modifications such as addition of crosslinkable functional groups

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(e.g., Fumarate segment) have been made on PCL to ease fabrication of three-dimensional scaffolds (Turunen *et al*., 2001). Jabbari *et al*. (2005) reported the synthesis and biocompatibility of linear polycaprolactone fumarate (PCLF) and also it's application in nerve tube regeneration (Jabbari *et al*., 2005; Wang *et al*., 2005). They reported the possibility of PCLF to be used as injectable material to treat skeletal defect when the material (in semi-crystalline form) was brought to melting temperature, i.e. ~40-45°C.

In this study, PCL-based polymeric macromere with three incorporated fumarate groups i.e., (polycaprolactone-trifumarate) (PCLTF), was developed and was evaluated as substrate for bone marrow derived osteoblasts growth, proliferation and differentiation. The PCLTF was synthesized into viscous liquid form, with excess of unreacted hydroxyl groups (-OH) exist within the PCL-backbone. It is believed that hydroxyl groups could contribute towards enhancing hydrophilicity of the newly synthesized polymeric macromere, which subsequently could facilitate cells attachment onto the scaffold.

Materials and Methods

Poly(caprolactone-trifumarate) (PCLTF) macromere was synthesized as described elsewhere (Chai *et al*., 2004). PCLTF porous scaffolds were fabricated by crosslinking PCLTF macromere using benzoyloxide radicals inside Teflon molds. The scaffolds were cut into discs (6 mm in diameter x 2 mm in height), sterile in 70% ethanol overnight and treated with DMEM media containing 10% fetal calf serum for 1 h prior to cell seeding. Sieved NaCl particles (215-325 μm, ratio of 1 : 1 g to PCLTF) were added to create porous structure.

Bone marrow stromal cells (BMSCs) were isolated aseptically from tibia of Sprague Dawley rats (6 weeks old, 150-170 g) and induced osteogenically using DMEM containing 10 mM ,-glycerophosphate, 50 μg mL^{1} L-ascorbic acid and 10 nM dexamethasone. Second passage BMS-derived osteoblasts were trypsinized at 80% confluent and approximately 3×10^{3} cells were seeded onto the top of each pre-treated PCLTF porous scaffold placed individually in 24-well plate. Fresh osteogenic medium was added to each well and changed every two days.

The osteogenic phenotypes expression of BMS-derived osteoblasts on PCLTF scaffolds were evaluated using following methods:

Scanning Electron Microscopy (SEM)

After 3, 14 and 28 days, environmental scanning electron microscopy was employed to reveal cells morphology on PCLTF scaffolds. Briefly, cells seeded PCLTF scaffolds were fixed with 2.5% glutaraldehyde in PBS followed by 1% osmium tetroxide in the same buffer. They were then dehydrated in ethanol, critical-point dried with CO_2 and finally coated with gold before examined under scanning electron microscope (Philips, SEM 515), with accelerating voltage of 9.5 kV.

Alkaline Phosphatase (ALP) Detection

Production of alkaline phosphatase (ALP) enzyme in cell culture is a widely recognised early marker for osteogenic cells. In this study, after incubated for 7, 14 and 21 days, the ALP level secreted from the cells seeded on PCLTF scaffolds was measured colorimetrically using p-Nitrophenyl Phosphate Liquid Substrate System (Sigma, product No. N7653). The absorbance was read at wavelength of 405 nm. Cells cultured directly onto 24-well plate were used as positive control, whereas unseeded PCLTF porous scaffolds were used as negative control. Graph shows mean

absorbance (mean + standard deviation) versus every incubation time was plotted. Student's paired t-test was performed to compare the mean absorbance values of samples and positive control.

Von-Kossa Staining: Mineralization

Formation of calcium-containing minerals on cell seeded PCLTF scaffolds is a prominent phenotype of well-differentiated BMS-derived osteoblasts. von-Kossa staining is based on the principle that when tissue sections are treated with silver nitrate solution, the calcium originated from the sections is reduced by the strong UV light and replaced with silver deposits that visualized as metallic silver or black deposit within the sections. Briefly, after 28 days, cell seeded PCLTF scaffolds were processed into paraffin block, sectioned and stained with silver nitrate solution before exposed under direct sunlight for 1 h. Sections were then washed with 5% sodium thiosulphate and counter stained with Nuclear-fast Red solution.

H & E staining

The histological structures of cell seeded scaffolds were revealed using H & E staining. After incubated for 14, 28 and 35 days, cell seeded PCLTF scaffolds were fixed with 10% neutral buffered formalin and then processed into paraffin blocks. They were then sectioned, stained with Haematoxylin and eosin, mounted onto glass slides and observed under microscope.

Results

After been incubated in osteogenic medium for 28 days, we found mineralized tissue structure was formed on entire BMSCs seeded PCLTF scaffold's surface with no significant cells growth at the bottom (Fig. 1). But the gross appearance of unseeded scaffold remained unchanged in yellowish color.

At day 3 (Fig. 2a), significant cells growth was observed on the scaffold's surface as cell layers (*) were prominently formed. Filopodia extensions (as indicated by arrows) were clearly seen bridging into the pores (*p*) that were created using sieved NaCl salt particles. But no extracellular matrix or mineral nodules were observed. On the other hand, at day 14 (Fig. 2b), cells (*Ost*) with round morphology (~30 μm in diameter) were found to be secreting extracellular matrix (*ecm*) which revealed as fibres-like network. We believed these cells were the well-differentiated osteoblasts derived from BMSCs when cultured using osteogenic medium. Beside that, we also found massive calcified globuli (*glb*, diameter ranges from 6-14 μm) deposited on the scaffolds. Finally, at day 28, we can see the entire scaffold's surface turned into mineralized tissue-like structure. Cells with round morphology and fibres network were not present as in day 14, but cells with trabeculae morphology (*trab*) were observed (Fig. 2c).

Secretion of Alkaline phosphatase enzyme was detected in both cell seeded PCLTF scaffolds and positive control, but not in negative control. The colorless substrate solution turned into yellowish color after incubated with cell seeded scaffolds for 30 min, at 37°C. The absorbance versus incubation time (i.e., at 7, 14 and 21 days) for both cell seeded scaffolds and positive control were plotted in Fig. 3. Results showed that the absorbance for cell seeded scaffolds were significantly lower than that of positive control at day 7, 14 and 21 days.

Von-Kossa staining demonstrated the present of calcium mineral nodules on cell seeded PCLTF scaffolds (Fig. 4). After been incubated for 28 days, we found dark-brownish pigments at the peripheral of the seeded scaffold, but no pigments were found within the scaffold. Furthermore, H & E staining of cell seeded PCLTF section revealed individual nucleated cells (as indicated by arrows)

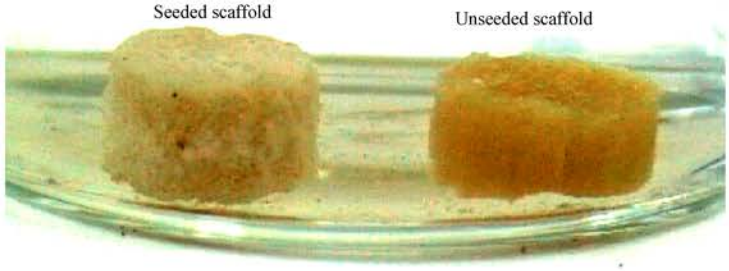

Fig. 1: Gross appearance of seeded and unseeded PCLTF scaffolds after been incubated for 28 days in osteogenic medium. Mineralized tissue structure was found covering the entire scaffold's surface. Unseeded scaffold remained yellowish color. In: 10% neutral buffered formalin fixative

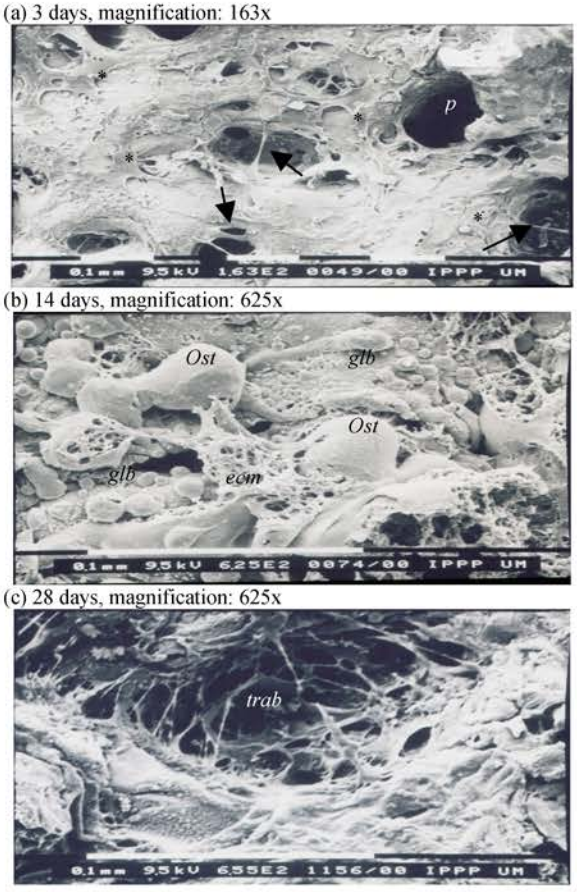

Fig. 2: Representative scanning electron micrographs of cell seeded scaffolds after incubated in osteogenic medium for (a) 3 days, (b) 14 days and (c) 28 days. Cells filopodia are indicated by arrows. (*) cell layer; (p) macropores; (Ost) BMS-derived osteoblasts; (ecm) extracellular matrix; (glb) calcified globuli; cells with trabeculae morphology (*trab*)

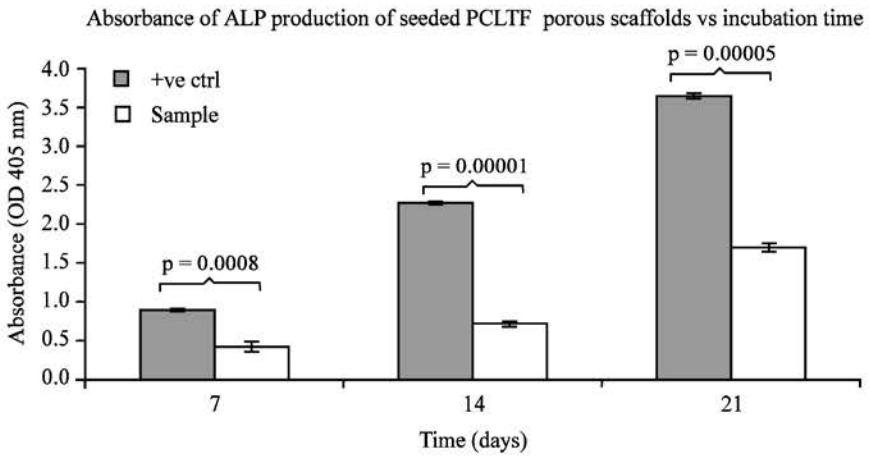

Fig. 3: Absorbance of ALP production of cell seeded PCLTF scaffolds versus incubation time (7, 14 and 28 days)

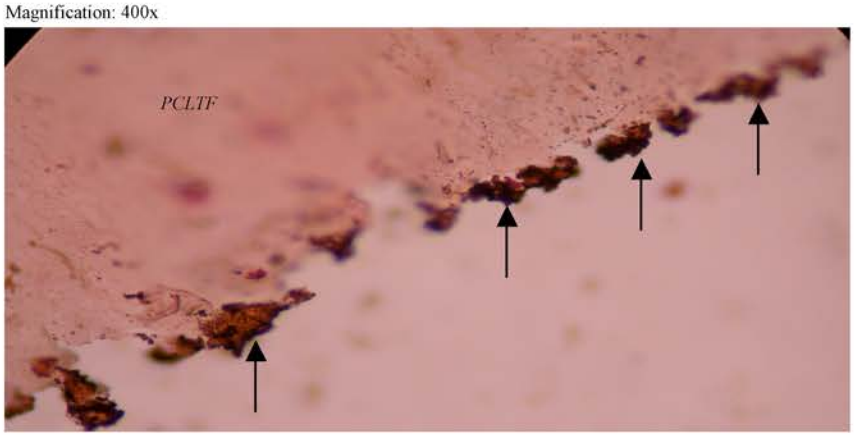

Fig. 4: Vertical section of cell seeded PCLTF scaffold after incubated in osteogenic medium for 28 days. Calcium mineral nodules and cells (as indicated by arrows) were prominent at the peripheral of the section and demonstrated as dark-brownish pigments. No significant pigments were found within the section. Von-Kossa staining

aligning the peripheral of the scaffold after 14 days of incubation (Fig. 5a). Few cells were found within the scaffold (*). Significant extracellular matrix (as indicated by arrows) was observed at the peripheral of the scaffold after 28 days of incubation (Fig. 5b). At day 35 (Fig. 5c), we were able to find bone matrix-like structure (bm) among some sections attached loosely to the scaffold. This bone matrix-like structure containing cells reside in lacuna-like spaces (indicated by arrows)

Discussion

PCLTF scaffolds fabricated from the macromere showed relatively hydrophobic property. This is partly due to the long hydrocarbon chain of polycaprolactone triol (the monomer). Treatment

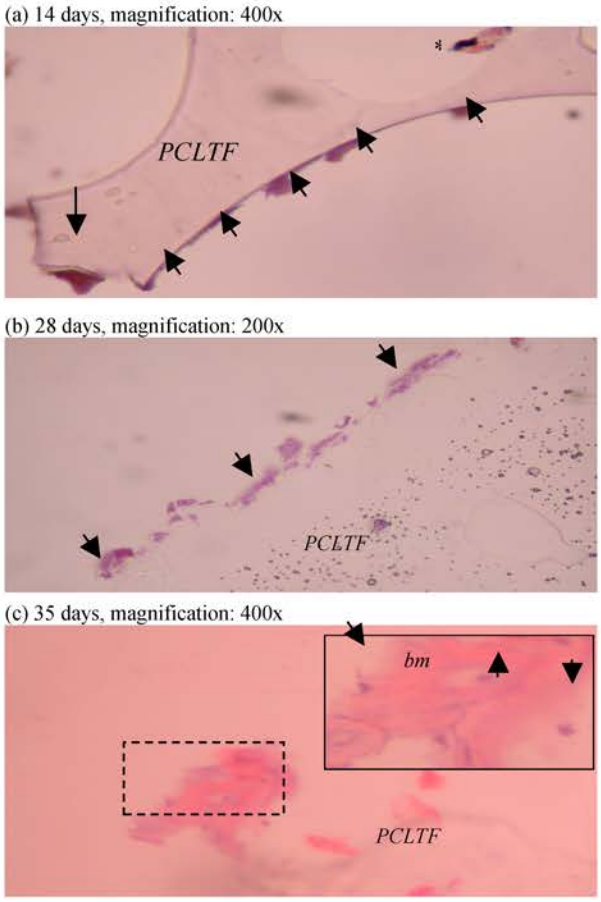

Fig. 5: Vertical sections of cell seeded PCLTF scaffold after incubated in osteogenic medium for 14, 28 and 35 days. At day 14, individual cells were seen at the peripheral of the scaffold, and significant extracellular matrix was formed after 28 days. Bone matrix-like structure (bm) was found attached loosely to the scaffold after incubated for 35 days. Cells reside in lacuna-like spaces (indicated by arrows) were prominent within the scaffold. Cell nuclei were clearly seen in some cells. Relatively fewer cells were found within the scaffold (*)

with 70% ethanol would enhance the water uptake of the scaffolds besides sterilizing the scaffolds prior to cell seeding. The scaffolds were found containing macropores (about 100-400 μm) interconnected by micropores (about 10-40 μm) as reported earlier but the porosity was not determined (Chai *et al*., 2004) in this study. BMSCs cultured in osteogenic medium were subcultured and only the second passage and onwards were seeded onto PCLTF scaffolds. This was to ensure more homogenous osteogenic cells were seeded onto the scaffolds after treated with osteogenic medium.

The white, mineralized tissue-like structure appearance of cell seeded scaffolds demonstrated the compatibility of PCLTF scaffold to allow the cells growth, proliferation and even differentiation. Microscopically, significant flatted cells were attached and proliferated on the scaffolds after 3 days

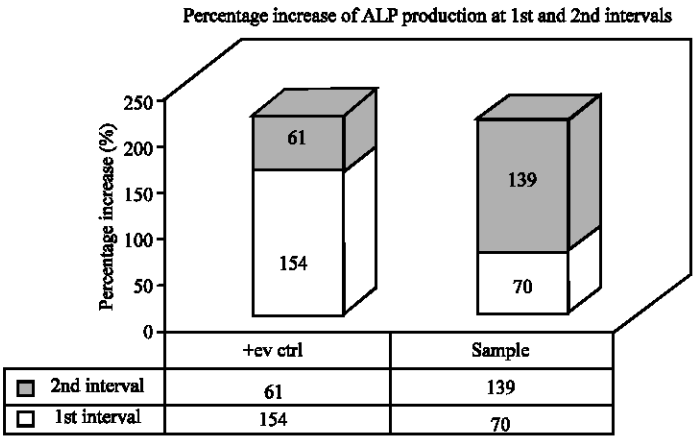

Fig. 6: Fold increase of ALP level for 1st and 2nd incubation weeks

of incubation. The cells differentiated to round osteoblastic cells that able to produce fibrillar collagen-like extracellular matrix and calcified globuli on the scaffold at day 14. Mineralization of the scaffold was occurred after 28 days of incubation.

The ALP levels of cell seeded scaffolds were significantly lower than that of positive control at day 7, 14 and 21 days. This was probably due to the lower initial number of cells seeded on the porous scaffold as compare to cells seeded directly on 24-well plate. The high porous structure of PCLTF caused most of the cells were not properly seeded onto the scaffolds but collected at the bottom of the plate. This will contribute to inaccurate comparison of ALP production between the cells seeded scaffolds and the positive control later on inaccurate. However, by comparing the percentage increase in ALP production between positive control and the seeded scaffolds, they were significantly no different over the two weeks of incubation (starts on day 7 to day 21) (Fig. 6).

H & E staining showed significant nucleated cells aligned along the peripheral of the PCLTF scaffold after been incubated for 14 days. But these cells were merely found within the scaffolds. This probably was attributed to the static culture condition employed in this study that prevented well nutrient diffusion into the inner part of the scaffold, thus limiting cells penetration and growth inside the scaffold. In future, dynamic culture techniques such as perfusion bioreactor should be employed to overcome this limitation. Extracellular matrix was found along the peripheral of the scaffold after 28 days of incubation, however, relatively fewer cells were present. We believed the used of excessive solvent during histological processing had caused the lost of dedicate cells from the scaffolds. Nevertheless, in this study, we were able to observe bone matrix-like structure from the scaffold after 35 days of incubation. Furthermore, cells reside in vacuole were present which resemble the osteocytes and lacunae structure of the bone. This finding was complemented by the demonstration of calcium-containing minerals nodules secreted on the scaffold by seeded BMS-derived osteoblasts.

Conclusions

In this study, scaffolds fabricated from PCLTF macromere showed biocompatibility and supported BMS-derived osteoblasts growth, proliferation and differentiation as well as expression of osteogenic marker such as ALP and mineralization. Thus, we believe PCLTF has the potential to be used as scaffold for bone tissue engineering.

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