

Divalent Metal Ions Induced Osteogenic Differentiation of MC3T3E1

Guoshou Wang^a, Wenta Su^{b*}, Pohung Chen^b and Teyang Huang^a

^aDepartment of Orthopedic Surgery Mackay Memorial Hospital, Taipei, Taiwan

^bDepartment of Chemical Engineering and Biotechnology, National Taipei University of Technology, Taipei, Taiwan

1 Sec. 3, Chung-Hsiao E. Rd, Taipei 10608, Taiwan

*Corresponding author. Tel.: +886-2-27712171 ext. 2554; Fax: +886-2-27317117

E-mail: f10549@mail.ntut.edu.tw

Abstract: Biomaterial scaffolds blended with biochemical signal molecules with adequate osteoinductive and osteoconductive properties have attracted significant interest in bone tissue engineering regeneration. The divalent metal ions can gradually release from the scaffold into the culture medium and then induced osteoblastic differentiation of MC3T3E1. These MC3T3E1 cells expressed high activity of alkaline phosphatase, bone-related gene expression of collagen type I, Runx2, osteopontin, osteocalcin, and significantly enhanced deposited minerals on scaffold after 21 days of culture. This experiment provided a useful inducer for osteogenic differentiation in bone repair.

1. Introduction

Biomedical material containing inducing agent is an implant substitutes placed into body for damaged tissue to repairing. Many natural and synthetic polymers are employed as tissue scaffolds, which are biodegradable and bioresorption by the body after being implanted.

Bone mineral containing 36.7% calcium and 16% phosphorus, and seventy percent of bone consists of hydroxyapatite and the tooth enamel composed of almost ninety percent hydroxyapatite. However, various divalent ions such as magnesium, strontium, zinc can also promote bone cell maturation, increase the amount of new bone, and can inhibit the activity of osteoclasts [1-3].

In generally, ceramic or polymer composite scaffolds believed to mimic the natural bone tissue. The complex requirements for clinical bone regeneration including biocompatibility, mechanical support, osteoconduction and osteoinduction have been widely investigated [4]. Chitosan has structural characteristics similar to glycosamines, and derived from the alkali-deacetylation of chitin. Chitosan is biocompatible, non-antigenic, nontoxic and biodegradation in vivo, and had studied for many biomedical applications [5].

In this study, we investigate the possible influence of different divalent metal ions for the osteogenic differentiation of MC3T3E1 cells. Various *divalent metal* phosphates synthesized by wet precipitation and then blended with chitosan solution to form 3D scaffolds. Cellular proliferation was measured by MTT assay, alkaline phosphatase (ALP) activity assay, real-time polymerase chain reaction and Alizarin red S staining were performed to determine cellular matrix maturation and mineralization.



2. Materials and Methods

2.1. Synthesis of Divalent Metal Phosphate

Every *divalent metal* phosphate was synthesized via wet precipitation using 0.3M MgCl₂, 0.8 M SrCl₂, 0.33 M Ba(NO₃)₂, and 0.1 M Zn(NO₃)₂ with 0.2 M Na₂HPO₄ as agitation solution for one day, rinsed with distilled water, and dried in the oven. Resulting synthesized products were magnesium phosphate (MgP), strontium phosphate (SrP), barium phosphate (BaP), and zinc phosphate (ZnP). Calcium phosphate (CaP) was purchased from Sigma.

2.2. Fabrication of porous chitosan scaffolds containing *divalent metal*/phosphate

Chitosan (MW 400 000; 98.5% deacetylation) was obtained from C&B Industrial Co., Ltd. (Taiwan). Porous chitosan scaffolds were prepared using the freeze-drying method. A 3% (w/v) chitosan solution was prepared by dissolving chitosan in 0.167 N acetic acid. Divalent metal phosphate scaffolds were prepared by adding 1.0% divalent metal phosphate into the chitosan solution. All solutions were poured into a cylinder mold and kept in a freezer at -20 °C for one day. The frozen samples were then lyophilized in a freeze dryer (FDU-1200 EYELA, Japan) at -50 °C and 11.2 Pa for 8 h. Subsequently, all scaffolds were neutralized with 1.25 N sodium hydroxide solution in a shaker for 30 min and then thoroughly rinsed with deionized water.

2.3. Cellular proliferation by MTT assay

To evaluate the cellular viability of SHEDs in chitosan scaffolds with or without divalent metal phosphate, MC3T3E1 cells were seeded into the scaffolds at 2×10^4 cells/scaffold with DMEM medium supplemented with 10% FBS and 1% penicillin and streptomycin. The medium was replaced every 3 days for 21 days. For measured proliferated cells, the whole scaffold was treated with 5 mg/mL MTT at 37 °C for 24 h. The cultivated medium was removed fully, and formazan was solubilized in dimethyl sulfoxide. The metabolized MTT was evaluated in terms of its optical density in a spectrophotometer at 570 nm (Thermo Scientific Multiskan FC).

2.4. Alkaline phosphatase assay

Approximately 500 µL PBS containing 0.1 M glycine, 1 mM MgCl₂, and 0.5% Triton X-100 (pH 10.5) was added to each sample to rupture the cell membranes and release the ALP molecules. The samples incubated for 1 h; after which, 100 µL of the supernatant extracted and transferred into microcentrifuge tube wrapped in aluminum foil, and added 200 µL of p-nitrophenyl phosphate solution (Sigma) to each tube. The tubes were placed in a water bath at 37 °C for 30 min, followed by an ice bath at 0 °C for 10 min to reduce the reaction rate. Approximately 50 µL of 3N sodium hydroxide solution was added to each microcentrifuge tube to stop the reaction. The collected solutions were placed into a 96-well microplate for ALP assay using an ELISA reader (Thermo Scientific Multiskan FC) at a wavelength of 405 nm.

2.5. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA from differentiated cells was extracted with Trizol reagent (Ambion®, Life Technologies™, USA). First-strand cDNA was synthesized from 500 ng of the RNA by using a Super Script®III reverse transcriptase kit (Invitrogen). RT-PCR was performed according to the protocols of Invitrogen™ Corporation (USA). RT-PCR was performed using a Smart Quant Green Master Mix with dUTP and ROX according to the protocols of Protech Technology Enterprise Co., Ltd. (Taiwan). Sixty cycles of quantitative RT-PCR (Lightcycler 480, Roche) were performed for the target genes. The control experiments were conducted with the housekeeper gene GAPDH. The PCR primers of collagen type I, Runx2, osteopontin (OPN), osteocalcin (OCN), and GAPDH listed in Table 1.

Table1. Primer sequence.

Gene name	F: 5'- TGCTTGAATGTGCTGATGACAGGG -3' R: 5'-TCCCCTCACCCCTCCCAGTAT-3'
Collagen type I	F: 5- CTGAGGTAACCTTGCTAACG -3' R: 5'-ATCAATACACTAAGAAATGTTTCAAGG -3'
Osteocalcin	F: 5'- CTCTGCCTTAAACACACATTG -3' R: 5'- TTCCCTTTGCCCACCTC -3'
Osteopontin	F: 5- AGCCAATGATGAGAGCAA -3' R: 5'- TTCAGGTGTTTATCTTCTTCCTTAC-3'
GAPDH	F: 5'-ATGAGAAGTATGACAACAGCC- 3' R: 5'-AGTCCTTCCACGATACCAA -3'

2.6. Calcium quantification

The calcium (Ca) deposited for bone nodule formation was stained using Alizarin red S dye. All samples washed twice with PBS and immersed into 95% ethanol solution for 30 min, and then stained with 1% Alizarin red S for 10 min, and then solubilized with 10% cetylpyridinium chloride. Images of stained cells were captured using a confocal microscope (Leica, TCS-SP5-AOBSK), the amount of Ca was calculated using standard solutions, and absorbance at 570 nm was measured (Thermo Scientific Multiskan FC).

2.7. Statistical analysis

All results were performed at least in triplicate experiments for different samples. Data were analyzed by ANOVA and are presented as means $\pm$ standard deviation (SD). Statistical comparisons performed in which $p < 0.05$ was considered significant.

3. Results

All fabricated chitosan scaffolds containing divalent metal phosphate have an 8 mm diameter and 6 mm height and exhibited a 3D porous fibrous structure. The SEM image of the scaffold cross-section exhibits a homogenous structure with regular porosity, a pore size of approximately 150 μm to 200 μm , and excellent pore interconnections, and all tested metal ions are continuously and steadily released from the scaffold (data not shown).

3.1. Cell proliferation and ALP activity

MC3T3E1 cells proliferating in the chitosan scaffolds containing divalent metal phosphate were measured by MTT assay, and pure chitosan scaffold was used as control. Figure 1 shows the graph of cellular viability on the scaffolds after 1, 7 and 14 days of culture with and without divalent metal phosphate supplement. The number of living cells in the scaffolds increased continuously with time, indicating that the scaffolds are biocompatible for cellular growth. Based on the result, Mg, Ca, Sr, and Zn can enhance cellular proliferation, but Ba supplement slightly inhibited cellular proliferation compared with control.

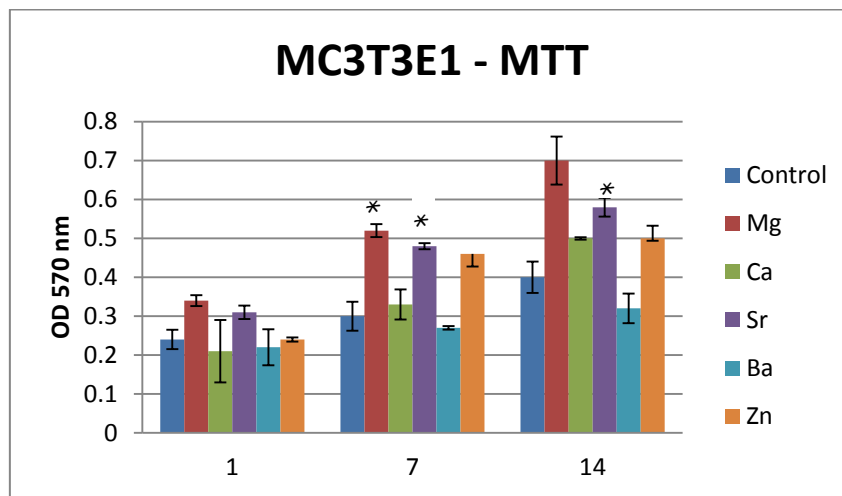


Figure 1. Proliferation of MC3T3E1 cells seeded on the scaffolds with and without divalent metal phosphate supplement after 1, 7, and 14 days of culture. (* $P < 0.05$)

ALP is among the most widely recognized functional markers of early osteoblastic differentiation. ALP activity was measured in MC3T3E1 differentiation among scaffolds containing different divalent metal phosphates cultured for 14 days. Among all tested scaffolds, Mg, Sr, and Zn significantly upregulated ALP activity, showing high expression for 14 days of culture; however, Ba had slower ALP expression than that of control during cultivation period (Fig. 2).

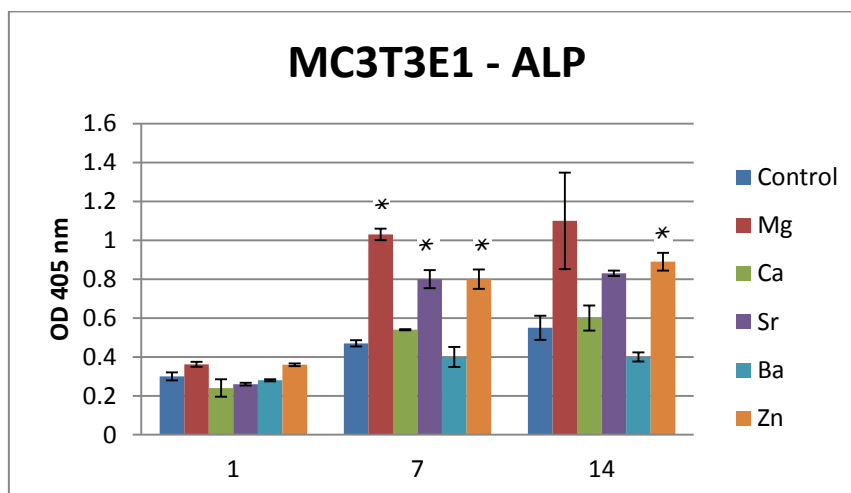


Figure 2. ALP activity expression from MC3T3E1 on various chitosan scaffold with and without divalent metal phosphate supplement during osteogenic differentiation. Values are expressed as mean $\pm$ SD ($n=3$), * $P < 0.05$

3.2. Osteoblastic gene expression

The RT-PCR results show that bone-related genes can be detected in culturing system. Figure 3 shows the expression levels quantified in the expression folds by the housekeeping gene GAPDH as control for bone specific proteins in the scaffolds after 1, 7 and 14 days culture. The MC3T3E1 differentiated cells produce maximal levels of type I collagen on 7 days cultivation, after which they appear to decrease gradually (Fig. 3a). The highest expression of Runx2 (21.9 folds) was noted after 7 days of culture (Fig. 3b). The highest OPN expression (30.6 folds) and terminal marker gene OCN (26.3 folds) were observed in Mg samples after 14 days of culture (Fig. 3c and Fig. 3d).

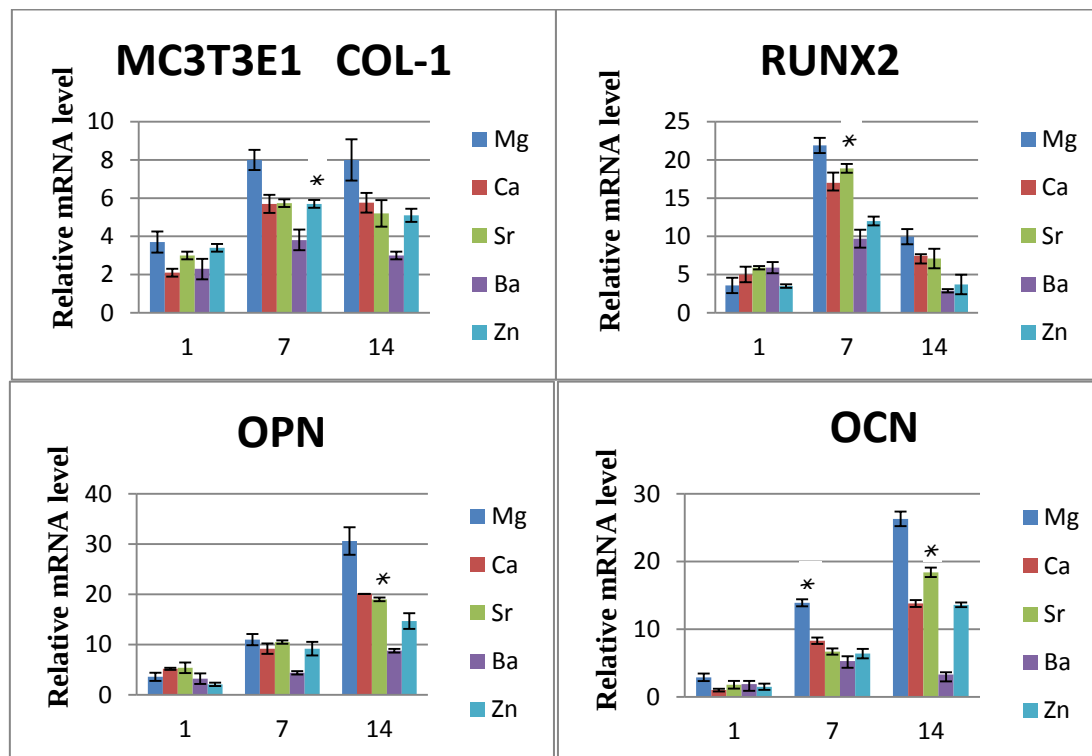


Figure 3. Relative mRNA expression level of COL-I (a), RUNX2 (b), OPN (c) and OCN (d) on various chitosan scaffolds during osteogenic differentiation of SHEDs. Values are expressed as mean $\pm$ SD (n=3), * $p < 0.05$.

3.3. Calcium quantification in the mineralized matrix

Alizarin red S was used to stain the intracellular Ca as well as the Ca-binding proteins and proteoglycans, and this method is useful in evaluating bone differentiation. Images of Alizarin red S staining on various chitosan scaffolds with and without divalent metal phosphate after 1, 7, and 14 days of culture are shown in Fig. 4a. Quantitative result shown in Fig. 4b indicated that Ca content increased continuously during 14 days of differentiation. Both Mg and Zn exhibited the largest increment among all the scaffolds. Ca depositions in Mg, Ca, Sr, and Zn-chitosan scaffolds containing divalent metal phosphate were higher than the depositions in pure chitosan scaffold, but Ca depositions in Ba scaffold is lower level and a negative effect. The formation of CaP salts or mineral deposition is a primary function of osteoblast cells, and the scaffold containing divalent metal phosphate can produce highly mineralized deposition during osteogenic differentiation, except for Ba.

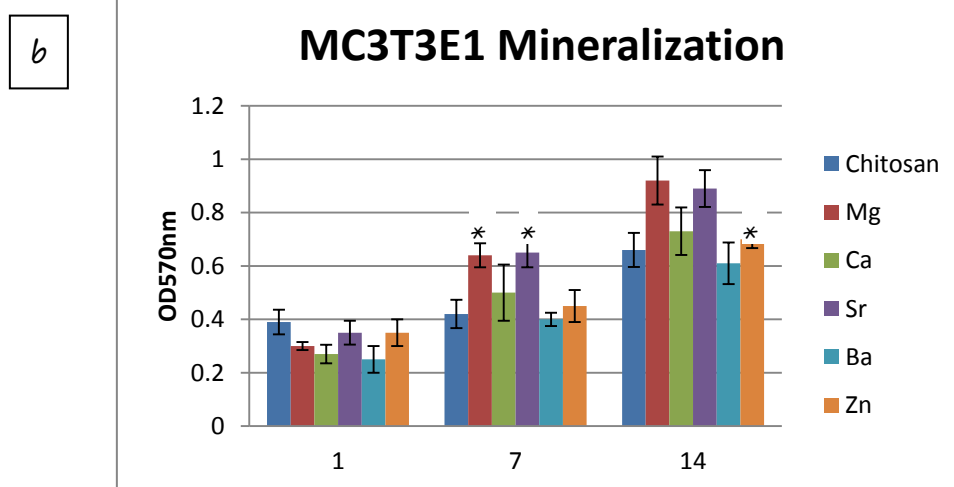
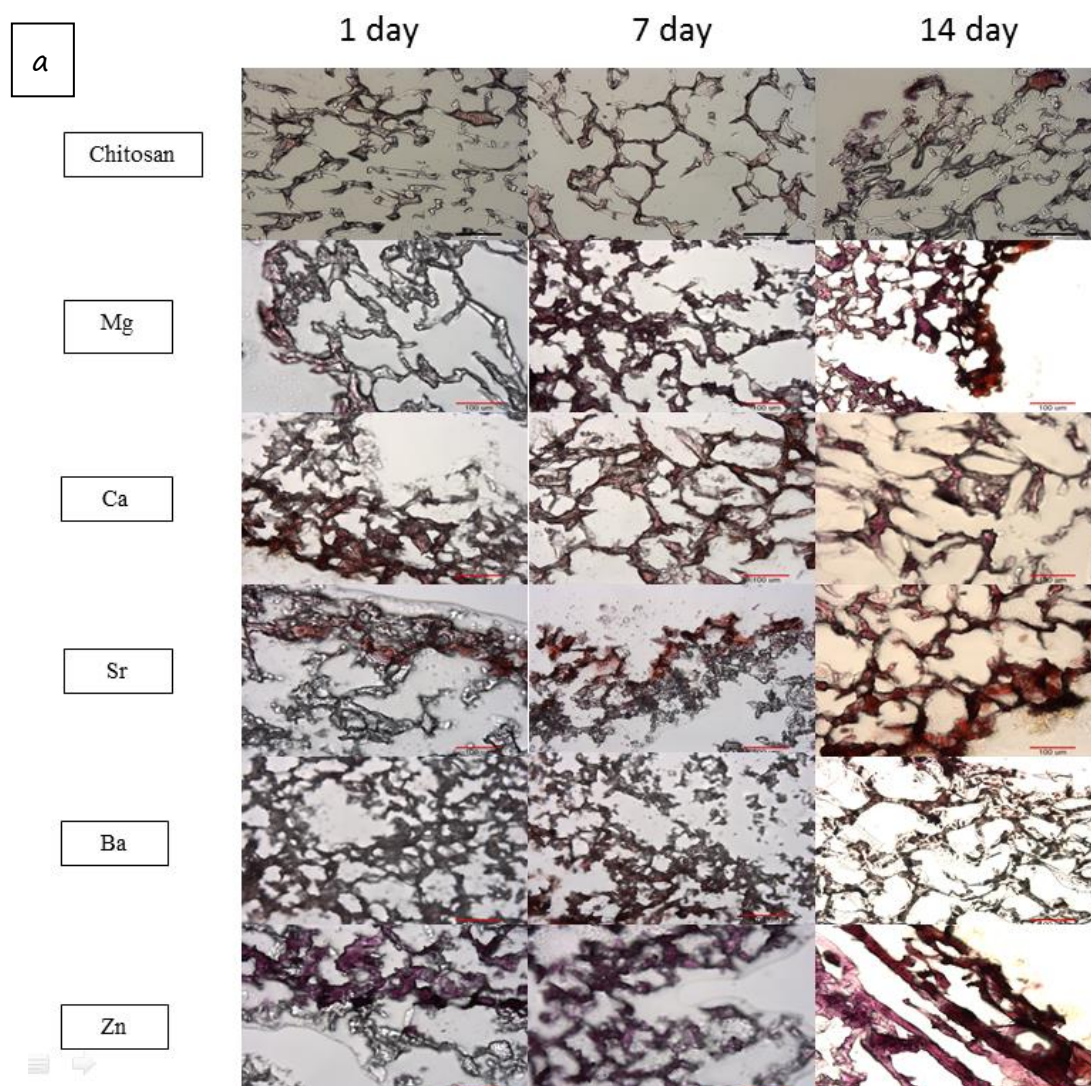


Figure 4. Alizarin red S stained on various chitosan scaffolds (a) and quantification of calcium deposition during osteogenic differentiation (b). Values are expressed as mean $\pm$ SD (n=3), *P<0.05. Scale bar is 20 μ m.

4. Discussion

The implants possess osteoconduction capabilities, allowing bone cell migration and bone deposition; however, osteoconductive materials cannot completely regenerate larger scale of bone defects. Therefore, osteoinductive material stimulation for osteogenesis of mesenchymal stem cells is needed for bone tissue engineering [6, 7]. Chitosan scaffold containing divalent metal phosphate mimicked bone mineral chemistry, and the trace ions released from scaffold can regulate bone metabolism [8].

ALP is a major osteogenic marker with a crucial function in mineralization; it is considered as an early-stage marker of osteogenic differentiation [9]. Biomineralization, which occurs in the late stages of osteogenesis, is a marker of fully differentiated stem cells [10]. All the tested samples had appeared that divalent metal phosphates can induce MC3T3E1 to osteogenic differentiation especially Mg. In addition, qualitative assay of mineralization was performed through Alizarin red S staining, and these Ca depositions on scaffold were higher than that on pure chitosan scaffold, but Ba inhibited the Ca deposition, as Fig. 4b.

In this study, Mg, Sr, and Zn showed higher osteoconductivity over culture time, high gene expression of bone-related, and higher degree of mineral deposition than conventional Ca supplement. However, the Ba appeared a negative effect in osteogenic differentiation.

5. Conclusions

This study demonstrated that divalent metal phosphate had been confirmed can induce effectively MC3T3E1 to osteogenic differentiation continuously into mature bone. Especially, Mg is greatest inducer for osteogenic differentiation and Ba is a negative agent. Therefore, understanding these different differentiated behaviors will be useful for the design and development of strategies for tissue engineering.

6. Acknowledgment

The authors would like to thank the National Science Council of Taiwan MOST 105-2622-E-027-031-CC3 and NTUT-MMH Joint Research Program NTUT-MMH-106-08 for their financial support in this study.

7. Reference

- [1] Luo T, Wu C, Zhang Y. The in vivo osteogenesis of Mg or Zr-modified silicate-based bioceramic spheres. *J Biomed Mater Res A* 2012; 100:2269-2277.
- [2] Wu C, Zhou Y, Lin C, Chang J, Xiao Y. Strontium-containing mesoporous bioactive glass scaffolds with improved osteogenic/cementogenic differentiation of periodontal ligament cells for periodontal tissue engineering. *Acta Biomater* 2012; 8:3805-3815.
- [3] Zhu Y, Li X, Yang J, Wang S, Gao H, Hanagata N. Composition–structure–property relationships of the $\text{CaO-MxOy-SiO}_2\text{-P}_2\text{O}_5$ ($\text{M} = \text{Zr, Mg, Sr}$) mesoporous bioactive glass (MBG) scaffolds. *J Mater Chem* 2011; 21:9208-9218.
- [4] Su WT, Pan YJ. Stem cells from human exfoliated deciduous teeth differentiate toward neural cells in a medium dynamically cultured with Schwann cells in a series of polydimethylsiloxanes scaffolds. *J Neural Eng* 2016; 13:46005.
- [5] Su WT, Wu PS, Ko CS, Huang TY. Osteogenic differentiation and mineralization of human exfoliated deciduous teeth stem cells on modified chitosan scaffold. *Mater Sci Eng C* 2014; 41:152-160.
- [6] Kim TG, Shin H, Lim DW. Biomimetic scaffolds for tissue engineering. *Adv Funct Mater* 2012; 22:2446-2468.
- [7] Hoppe A, Gldal NS, Boccaccini AR. A review of the biological response to ionic dissolution products from bioactive glasses and glass-ceramics. *Biomaterials* 2011;

- 32:2757–2774.
- [8] Su WT, Shih YA, Ko CS. Effect of chitosan conduit under a dynamic culture on the proliferation and neural differentiation of human exfoliated deciduous teeth stem cells. *J Tissue Eng Reg Med* 2016; 10:507-517.
 - [9] Hall SL, Dimai HP, Farley JR. Effects of zinc on human skeletal alkaline phosphatase activity *in vitro*. *Calcif Tissue Int* 1999; 64:163-172.
 - [10] Su, W.T.; Chou, W.L.; Chou, C.M. Osteoblastic differentiation of stem cells from human exfoliated deciduous teeth induced by thermosensitive hydrogels with strontium phosphate. *Mater. Sci. Eng. C* **2015**, 52, 46-53.