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Osteogenic activity of nanonized pearl powder/poly (lactide-*co*-glycolide) composite scaffolds for bone tissue engineering

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Abstract. Numerous materials have been proposed for bone tissue engineering. In this study, a newly designed hybrid composite scaffold composed of poly (D,L-lactide-co-glycolide) and a naturally bioceramic hybrid material, nanonized pearl powder, were prepared and the biological activities and physical properties of the scaffold for bone tissue engineering were evaluated. It is a composite consisting calcium carbonate crystal in an aragonite structure, embedded in an organic matrix. Peral contains one or more signal molecules capable of stimulating bone formation. The nanonized pearl powder is considered as a promising osteoinductive biomaterial. This biomaterial is biocompatible and shows osteogenic activity. In this study, the designed biohybrid of nanonized pearl powder/poly (lactide-*co*-glycolide) (NPP/PLGA) biocomposite scaffolds would employ biodegradable material as MC3T3-E1 cells seeded scaffolds. Therefore, the biocomposite scaffolds would be used to culture with MC3T3-E1 cells under spinner bioreactor *in vitro*. Furthermore, it also detailed how these tissues were characterized, qualitatively and quantitatively, with scanning electron microscopy and biochemical testing. The identity and the mode of action of these molecules on the osteoblast differentiation were analyzed. This study indicates that the efficiency of nanonized pearl powders in bone cell differentiation are certainly different from that of proteins. Further sudy will look forward to manufacturing the promising new generation bone substitute, three dimensional biocomposite scaffolds to replace the implant and autogeneous bone graft, which combines basic research and clinical application.

Keywords: nanonized pearl powder, poly (D, L-lactide-co-glycolide), osteoblast, bio-mineralization, bone tissue engineering

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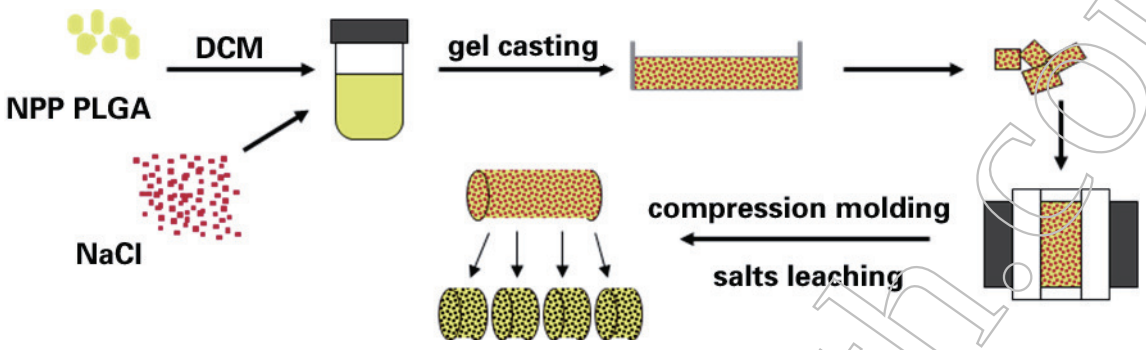
1. Introduction

Numerous materials have been proposed, modified and used for biomedical applications such as scaffolds for bone tissue reconstruction. Bone disorders are of significant concern due to the increase in the median age. Traditionally, bone grafts have been used to repair damaged bones. Synthetic biomaterials are now being used as bone graft substitutes [1], among which, polyimide, polyamide, polyelectrolyte, polyacrylate, polyurethane, polycarbonate, polynorborene, hydroxyapatite, poly (lactide-*co*-glycolide), polyglycolide and polylactide have been used either singly or in combination with naturally derived materials. These materials are designed to serve as bone substitutes or as enhancement for the bone-healing process. Among the several commercially available bone graft materials, collagen-hydroxyapatite composite scaffold is currently the most commonly used material clinically. Recently, a cell-based approach has been proposed as a new concept in bone tissue regeneration. While many biomaterials serve as a scaffold that augments the body's ability to heal itself, a tissue engineering approach uses cells added to a scaffold to achieve formation of bone tissue. Some materials may not be suitable for cells due to their physical and structural configuration. However, poly (lactide-*co*-glycolide) has been used as a scaffold for bone tissue engineering due to its favorable physical properties. A pearl is rich in minerals, especially in high quality calcium, and amazing special compounds called "signal proteins" [2–5]. "Signal proteins" act as messengers by emitting signals that control cellular growth and repair. What the pearls' signal proteins can do are stimulating skin and bone regeneration, enhancing skin tissue repair and increasing the existing bone density [6]. Additionally, these signal proteins can play a powerful role in the maintenance of youthful skin tone and the promotion of vital skeletal and bone health. The pearl can facilitate bone cell differentiation and speed up their mineralization [7]. Flausse et al. reported on the capability of nacre to induce differentiation of human bone marrow mesenchymal stem cells (hBM-MSCs) and the production of extracellular matrix [8]. Here the authors developed a composite scaffold of nanonized pearl powder/poly (lactide-*co*-glycolide) (NPP/PLGA) utilizing the gel molding and particulate leaching method designed in our laboratory. The bio-hybrid composite scaffolds were designed to accommodate cells and provide adequate structural support. The biological activities and structural properties of the bio-hybrid composite scaffolds (NPP/PLGA) were studied to evaluate the application of bone tissue regeneration.

2. Materials and methods

2.1. Preparation of designed bio-hybrid composite scaffolds, nanonized pearl powder/ poly (lactide-co-glycolide)

Newly designed bio-hybrid composite bone scaffolds were prepared by nanonized pearl powder (NPP) and poly (lactide-*co*-glycolide) (PLGA, Lactide/Glycolide=85/15, Mw=190000, PURAC), which were dissolved and mixed with various ratios of NPP and PLGA in dichloromethane afterwards. In brief, fine PLGA particles were dissolved in dichloromethane at concentrations of 20% w/v. Pore sizes were controlled by using sieved NaCl particulates with a diameter of 210-350 μm. Polymeric gels mixtures were put into a cylindrical mold and compressed to obtain a disk-shaped scaffold. The polymeric scaffold was immersed in water for 48 h to leach out the salts and then freeze-dried overnight [9,10].



Scheme 1. 3-D porous PLGA and NPP/PLGA scaffolds fabrication using gel casting and salts leaching methods

2.2. Cell culture

MC3T3-E1 cells (ECACC 99072810) at passage 6 were grown for 1, 4, 7, 14 and 28 days at 37°C under 5% CO_2 in α-MEM medium containing 50 U/mL of penicillin and 50 μg/mL of streptomycin, 10% of fetal bovine serum, and 2 mM L-glutamine. Cells were seeded in 24-well plates at a density of 6×10^4 cells per well. Near the confluence, cells were treated with the NPP containing scaffolds. The culture medium was supplemented by mineralization inductors, which was composed of 10 mM β-glycerophosphate (Sigma) and 50 μg/mL ascorbic acid (Sigma). Media were changed every 3 days.

2.3. Biological evaluation of the bio-hybrid composite scaffolds, NPP/PLGA

After 14 days, the seeded scaffolds were fixed with 2.5% glutaraldehyde in 0.1-M sodium cacodylate buffer (pH 7.4) for 1 h and stored in buffer overnight. Specimens were postfixed in 1% osmium tetroxide for 1 h, washed, dehydrated in graded ethanol, critical-point dried, sputter coated with platinum for 200 s in vacuum, and observed under scanning electron micrographs (SEM).

Bone related markers of type I collagen, osteopontin and osteocalcin expression on NPP/PLGA scaffolds were analyzed by immunofluorescence staining. The MC3T3-E1 cells were cultured during various days in the absence or presence of NPP in α-MEM medium. To assess protein expression, the authors used specific polyclonal antibodies followed by fluorescein isothiocyanate-conjugated secondary antibody. Hoechst 33258 was used to counterstain the nuclei. Confocal laser scanning photomicrographs were obtained.

MC3T3-E1-seeded scaffolds were harvested from the bioreactor after 7 or 14 days, lyophilized under a vacuum, and weighed. The calcium content was determined using a colorimetric assay with cresolphthalein complexone.

3. Results and discussion

3.1. Fabrication and architecture of the NPP/PLGA composite scaffolds

Current bone-repair strategies use autogenous or allogeneic grafts, which are limited by donor-site morbidity, bone supply, or risk of inducing transmissible diseases. NPP is a promising natural bioceramic, which consists of the internal lustrous "mother of pearl" layer of many molluscan shells. It is a composite consisting calcium carbonate crystal in an aragonite structure embedded in an organic

matrix and forms biohybrid materials. NPP contains one or more signal molecules capable of stimulating bone formation. NPP/PLGA scaffolds with porous structures were prepared and biodegradable material was selected as MC3T3-E1 cells seeded scaffolds were studied. The NPP/PLGA composite scaffolds, fabricated by the solvent casting/particulate leaching process, exhibited highly porous and uniform interconnected structures. Cross-sectional observation of the NPP/PLGA composite scaffold is shown in Fig.1 (A). NPP/PLGA composite scaffold showed the architecture with uniform porous structures. Scale bar indicates 600 μm. The exterior surface, serial cross-section and side wall morphologies of the NPP/PLGA composite scaffolds and PLGA scaffolds exhibited a highly porous structure with interconnectivity that would support adequate cell seeding, adhesion and proliferation. The pore size of the NPP/PLGA composite scaffolds was about 150-200 μm. The NPP/PLGA composite scaffold exhibited uniformly distributed-interconnecting pores in its inner microstructures, which were successfully achieved by a particulate leaching process. Furthermore, the NPP/PLGA biocomposite scaffolds were studied to culture with MC3T3-E1 cells under spinner bioreactor *in vitro* and the morphology of NPP/PLGA with MC3T3-E1 cells is shown in Fig.1 (B). The significant difference between NPP/PLGA and NPP/PLGA with MC3T3-E1 cells could be observed.

3.2. Characterization of the NPP/PLGA composite scaffolds

This study indicates that the efficiency of pearl powders in bone cell differentiation is certainly different from that of proteins, and could be useful for *in vivo* bone treatment. Through this study, further study will look forward to manufacturing the promising new generation bone substitute, three dimensional NPP/PLGA scaffolds incorporated with MC3T3-E1 cells to replace the implant and autogeneous bone graft, which combines basic research and clinical application. Cell proliferation and DNA contents of PLGA scaffold and NPP/PLGA composite scaffold up to 14 days culture are determined by MTT assay as shown in Fig. 2. The cells seeded on NPP/PLGA composite scaffolds show a higher proliferation rate when compared to PLGA scaffolds. Particularly, the cell numbers and DNA contents of NPP/PLGA composite scaffold after 14 days show more than twice of the values during 7 days. However, the cell numbers and DNA contents of PLGA scaffold decrease with culture time, which means that NPP/PLGA composite scaffold is good for generation of bone.

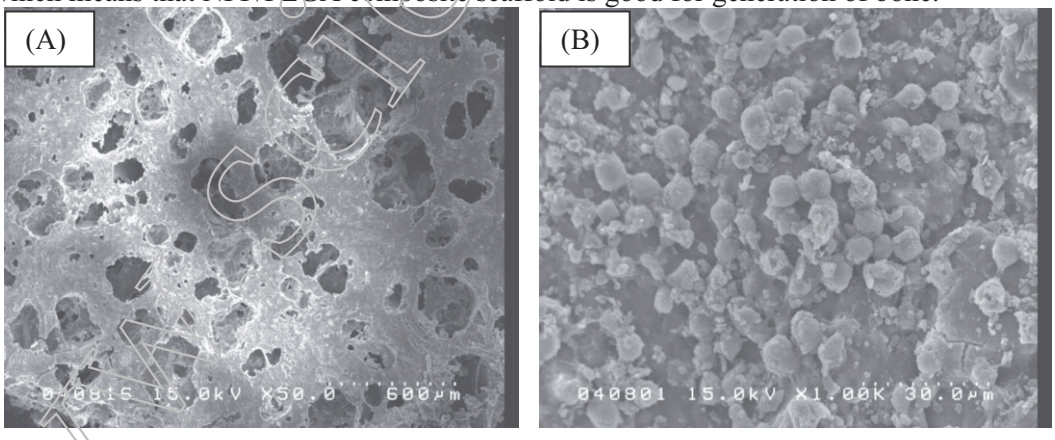


Fig 1. Cross-sectional scanning electron micrographs (SEM) of (A) NPP/PLGA and (B) MC3T3-E1 cells seeded onto the NPP/PLGA after 14 days of culture.

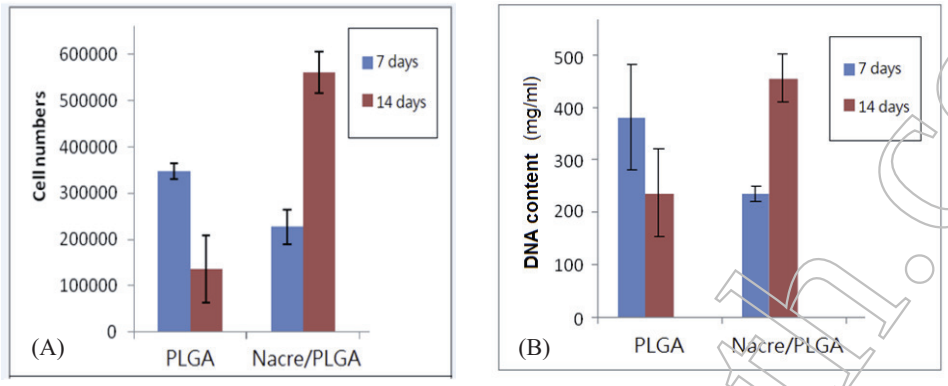


Fig. 2 (A) Cell proliferation and (B) DNA contents of PLGA scaffold and NPP/PLGA composite scaffold up to 14 days culture, as determined by MTT assay.

3.3. Immunofluorescence staining of type I collagen, osteopontin and osteocalcin in NPP/PLGA

The effects of NPP on type I collagen, osteopontin and osteocalcin expression in MC3T3-E1 cells were analyzed by immunofluorescence staining. The MC3T3-E1 cells were cultured during various days in the absence or presence of NPP in osteogenic medium. Fig. 4 shows confocal micrograph exhibited the architecture of NPP/PLGA composite scaffold and strongly positive expression of type I collagen, osteopontin and osteocalcin around the structure of NPP/PLGA composite scaffold.

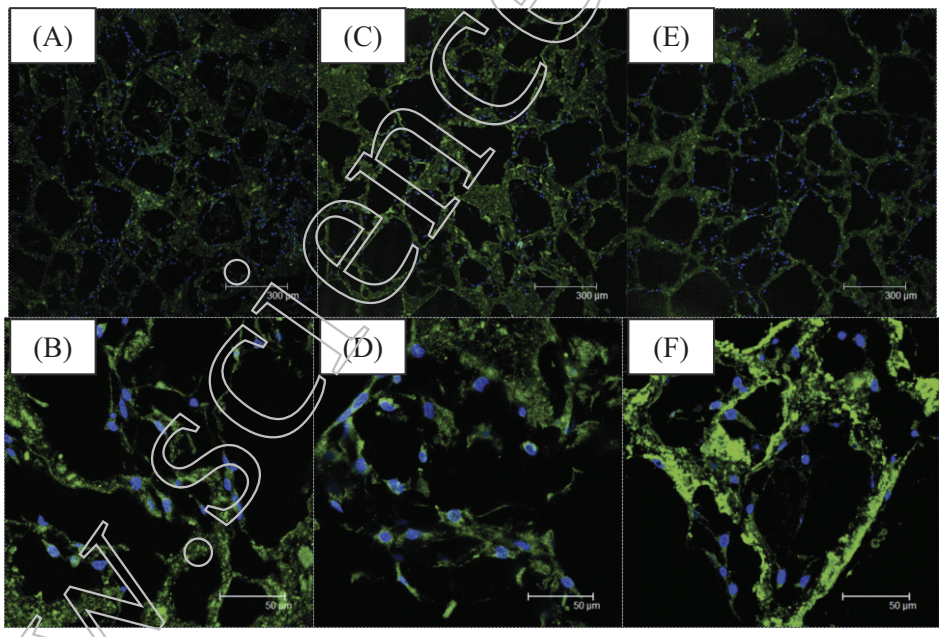


Fig. 3. Scanning confocal photomicrographs of osteogenesis-relevant markers of type I collagen, osteopontin and osteocalcin expression on NPP/PLGA scaffolds, wherein (A) and (B): type I collagen; (C) and (D): osteopontin; (E) and (F): osteocalcin for 14 days culture.

3.4. Biomineralization and biodegradation of the NPP/PLGA biocomposite scaffolds

The incorporation of NPP, in the microstructure provides original calcium contents of NPP/PLGA biocomposite scaffolds as shown in Fig. 4. As the MC3T3-E1 cells were cultured in the PLGA scaffold for 14 days, no additional calcium concentration could be observed. In the NPP/PLGA scaffolds, a relatively higher calcium concentration was observed because of the original NPP incorporation (0 day in Fig. 4). During the initial culture time (<7days), calcium contents in the scaffold would be partially dissolved. When the induced biomineralization occurred, the calcium contents of NPP/PLGA biocomposite scaffolds would increase with culture time during 7-14 days. Finally, the increased calcium concentration was obtained. These results implied that induced biomineralization could occur in the MC3T3-E1 cells cultured with NPP/PLGA biocomposite scaffolds within 14 days.

The degradation ratios of NPP/PLGA biocomposite scaffold and PLGA scaffold could be studied within 30 days as shown in Fig. 5. The degradation ratio of PLGA scaffold increased rapidly with the degradation time, particularly within 5-30 days. After 30 days, 60% of degradation ratio of PLGA scaffold would occur. A relatively lower degradation ratio could be controlled in NPP/PLGA scaffold. The incorporation of nacre could avoid degradation of scaffold.

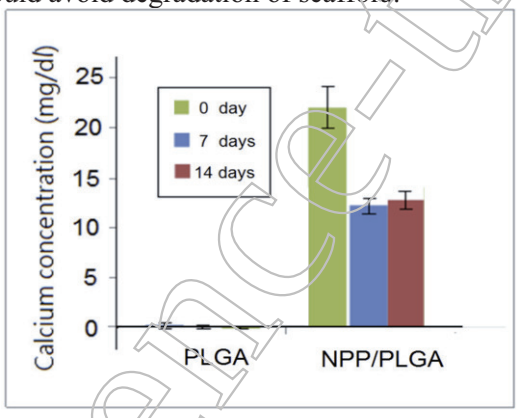


Fig.4 Calcium concentration of PLGA and NPP/PLGA scaffolds cultured with MC3T3-E1 cells

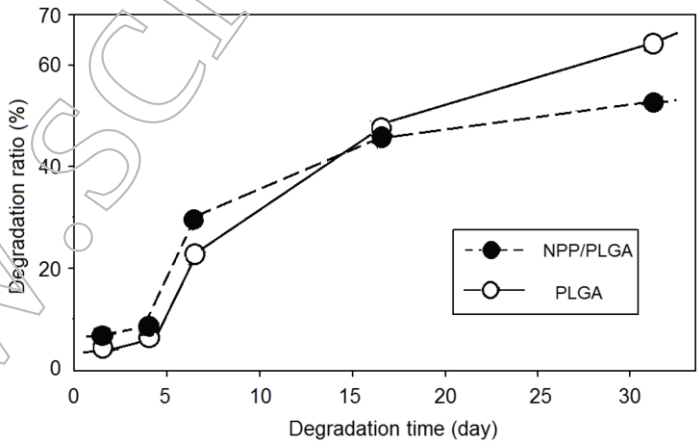


Fig. 5 The biodegradation ratios of NPP/PLGA biocomposite scaffold and PLGA scaffold within 35 days

4. Conclusion

In this study, new bio-hybrid bone scaffolds composed of nanonized pearl powder and poly (lactide-*co*-glycolide) that possess necessary characteristics for bone tissue engineering was successfully designed and prepared. The NPP/PLGA composite scaffolds are hydrophilic and possess porous structures with a consistent interconnectivity throughout the entire scaffold, which contributes to uniform cell seeding, adhesion and proliferation. The NPP/PLGA composite scaffolds are non-toxic, easily fabricated and can provide structural features that may enhance the formation of bone tissue. Cytochemical assay and scanning electron microscopy analysis were successfully employed to evaluate the activity of osteoblasts which determines osteogenic activity.

5. Acknowledgements

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