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Author(s)	Manchinasetty, Naga Vijaya Lakshmi
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Ph.D. thesis (Abstract and Summary)

Preparation and characterization of calcium phosphate ceramics and polymer composites as potential bone substitutes

Naga Vijaya Lakshmi Manchinasetty

Supervisor: Prof. Masanori Kikuchi

Graduate school of Information Science and Technology, Hokkaido University

Abstract

Hydroxyapatite (HAp), β -tricalcium phosphate (β -TCP) and their composites with natural polymers are widely used as filler materials for their biocompatibility and osteoconductivity. A three dimensional interconnection of both macro- and micro-pores (bimodal pores) is important for a porous scaffold, because macropores allow cell, blood vessel and tissue ingrowths and micropores serve as an effective pathway for exchange of fluid in which nutrition and wastes are dissolved. Marine derived biomaterials from corals, cuttlebones, sea urchin spines etc., have shown good potential as bone substitutes due to their interconnected porous structure and ease of conversion to calcium phosphate without any change in their original porous structure. However, corals are considered as reservoir of carbonate gas to decrease greenhouse effect and not recommended to collect from sea, cuttlebones consist of only macropores and sea urchin spines consist of only micropores. Contrarily, gonads of sea urchins are consumed as food in Japan and their skeletons are discarded as waste. Their skeletons consist of bimodal pores and hence utilization of sea urchin skeletons would reduce the waste, encourage Japanese fishery. In this study, calcium phosphate granules were prepared by hydrothermal phosphatization of sea urchin tests in an aqueous phosphate solution. The obtained calcium phosphate (CP1/CP2) was found to be biphasic in nature with 82 % Mg containing β -TCP and 18 % non-stoichiometric carbonate containing HAp (biphasic calcium phosphate, BCP) retaining their their original porous

structure.

Scaffolds, CP1Col/CP2Col (collagen as binder) and CP1Gel_GA/CP2Gel_GA (gelatin as binder) were prepared by mixing the obtained BCP granules with collagen or gelatin solution. The scaffolds obtained exhibited open porous structure and had sufficient strength for good operability during surgery. The *in vitro* evaluation of scaffolds using human osteoblast-like cell line, MG63 cells under static conditions showed negligible toxicity, higher distribution, proliferation and osteogenic activity in comparison to control collagen or gelatin sponges. From *in vitro* evaluation of scaffolds under pressure/perfusion cell culture condition, which mimics the biological conditions of bone, significant increase in proliferation (from total DNA analysis) and osteogenic activity from (gene expression analysis) was observed in scaffolds compared to control. The homogeneous, bimodal porous structure enhanced the cell distribution and the Mg^{2+} containing BCP granules promoted effective osteogenic activity in the scaffolds in both static and dynamic cell culture conditions. The scaffolds prepared by sea urchin-derived calcium phosphates with collagen or gelatin as binder could be a potential candidate for artificial bone filler in non-load bearing defects.

The supplementation of calcium carbonate and calcium citrate to an injectable hydroxyapatite/collagen (HAp/Col) paste prepared with sodium alginate increased its anti-washout property and pH controllability. This is due to coordinate effects of initial washout inhibition by weak but rapid formation of long-range network by citric acid followed by long term anti-washout inhibition by strong but slow network formation by Ca^{2+} ions. The paste also showed good cytocompatibility, MG63 cells proliferated with the culture time without any significant difference with the HAp/Col dense bodies. The HAp/Col paste is expected to be utilized in minimally invasive surgery of bone defect to fit irregular bone defects. The

calcium phosphate and natural polymers composites investigated in this study are expected to be utilised as filler materials for replacement and reconstruction of bone defects.

Summary

With the aim of developing novel functional biomaterials with calcium phosphate and their composites, preparation and characterization of scaffolds utilizing test of sea urchin with biocompatible polymers or calcium phosphate polymer composites for artificial bone fillers were studied in this thesis. The summarized description for each other is now discussed.

Skeletons of sea urchins namely *strongylocentrotus nudus* (SU1) and *strongylocentrotus intermedius* (SU2) were used to obtain calcium phosphate (CP1/CP2) by hydrothermal conversion in a phosphate containing solution and naked eye observation of them is shown in **Fig. 1**. The composition of skeletons of sea urchin was found to be Mg containing calcite (Mg-CaCO_3) by XRD analysis and the skeletons were porous by nature with macropores in the range of 200-300 μm and micropores in the range of 20-50 μm by SEM (**Fig. 2**) analysis. The obtained calcium phosphate was found to be biphasic in nature consisting of magnesium containing tricalcium phosphate (Mg β -TCP, 82%) with small amount of hydroxyapatite (HAp, 18%) from XRD result, also corroborated by The FT-IR analysis results. The SEM evaluation revealed that the skeletons retained their original porous structure after conversion where the diameter of the macropores ranged from 200 to 250 μm and micropores 20 to 40 μm (**Fig. 3**). From the calcination of the obtained calcium phosphate at 1000 $^\circ\text{C}$, and FT-IR analysis, the hydroxyapatite in the CP1/CP2 was found to be carbonate (CO_3^{2-}) containing Ca deficient hydroxyapatite. It was found that the presence of Mg^{2+} ion promoted the formation of β -TCP, and small amount of HAp due to the decrease in the Mg^{2+} ion concentration. The results suggested that the obtained biphasic calcium phosphate (BCP)

from skeletons of sea urchin can be used in the fabrication of three dimensional (3D) scaffolds with the help of biocompatible binders.



Figure 1 Naked eye observation of (a) SU1 (b) SU2 (c) CP1 and (d) CP2

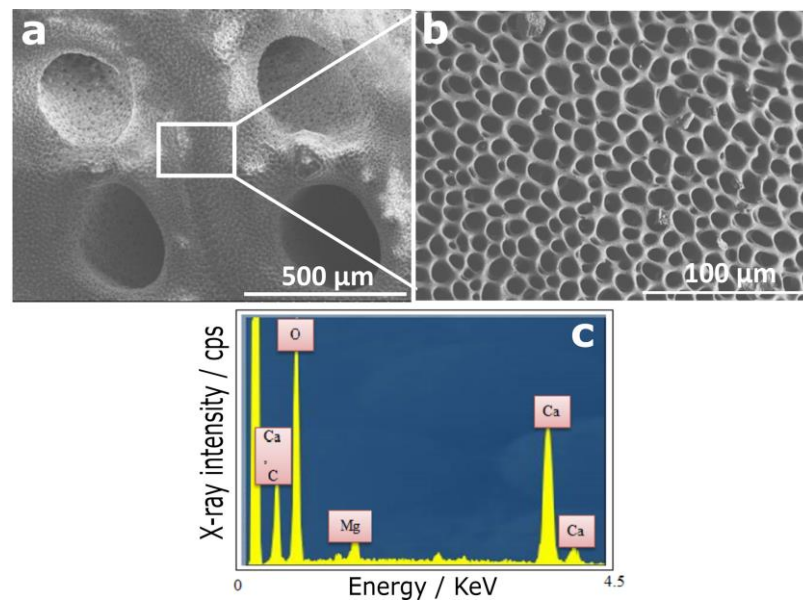


Figure 2 SEM images of SU2 (a-b) and (c) shows the EDS pattern of sample surface

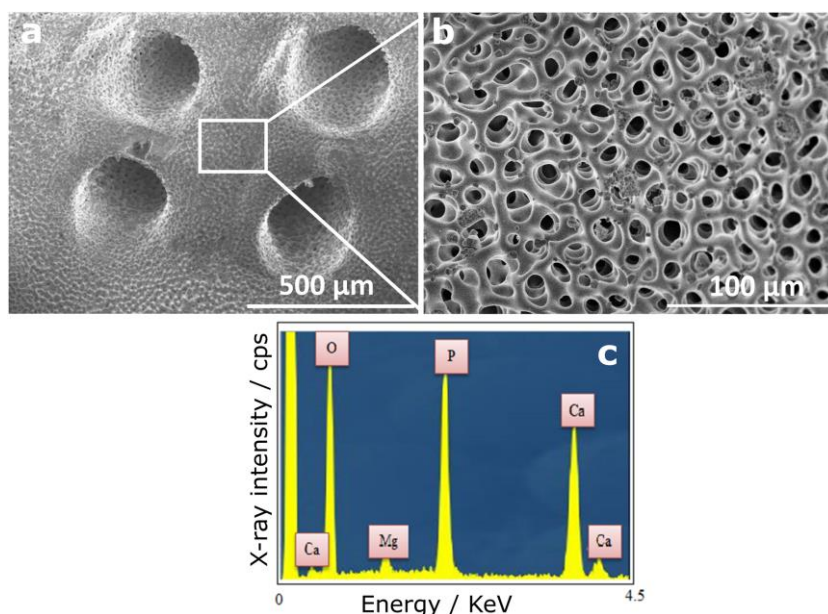


Figure 3 SEM images of CP2 (a-b) and (c) shows the EDS pattern of the sample surface

Fabrication of shape controllable and flexible 3D scaffolds with the help of biocompatible polymers collagen or gelatin was described. The scaffolds were fabricated by simple mixing of the CP1/CP2 granules with collagen or gelatin in a desired shape and size, freeze-drying and crosslinking. The scaffolds with collagen as binder, CP1Col/CP2Col were crosslinked dehydrothermally and found to be stable at 37 °C in PBS solution from stability analysis. The scaffolds with gelatin as binder (CP1Gel_GA/CP2Gel_GA), were stable only with chemical crosslinking at 37 °C in PBS. From the cross-sectional images of scaffolds using SEM, it was found that macropores and micropores were well maintained even after some pores were blocked by the polymer coating. The granules in the scaffold helped in the formation of homogeneous and interconnected porous structure. They showed good open porosity for cell migration and fluid infiltration, and adequate mechanical strength for handling operations. Overall, the binder collagen or gelatin helped in maintaining the shape and integrity of the scaffolds, whereas the granules reserved the space for bone formation. The scaffolds could be a potential candidate for non-load bearing defects.

Biocompatibility of the scaffolds under 2D cell culture with osteoblast cell line MG-63 cells was evaluated. The cytotoxicity/viability evaluation showed that the scaffolds were non-cytotoxic in nature when compared to control. In CP1Gel_GA/CP2Gel_GA, very few dead cells were found when compared to control, where many dead cells were found. From cell adherence/distribution analysis, the mid vertical cross-sectional images by SEM, showed that cells adhered well onto the surface and penetrated into the inner layer of the scaffold. In control gelatin sponge, cells were seen only on the peripheral area. From cell proliferation analysis, scaffolds proved to be significantly higher in the proliferation rate compared to their respective controls. The scaffolds fabricated with porous biphasic CP1/CP2 granules and collagen or gelatin provided essential porosity, bioactive surface for cell attachment and proliferation. Also, the scaffolds created a favorable environment for differentiation of cells, which can favor increased bone formation in comparison with control collagen and gelatin sponge. The scaffolds proved to be biocompatible under 2D culture conditions and have the potential to be utilized as artificial bone filler.

Biocompatibility of the scaffolds under 3D pressure/perfusion cell culture conditions, with MG-63 cells was evaluated. The scaffolds CP2Col showed higher proliferation ability evaluated by the total DNA quantification, high cell distribution to the inner layers of the scaffold evaluated by staining decalcified vertical cross-section by DAPI compared to control collagen sponge. The control collagen sponge, cell grew only on the outer layer during the initial stages and shrunk in size, whereas, CP2Col maintained its integrity and shape throughout the culture time. In the scaffold CP2Gel_GA higher cell proliferation and distribution ability was seen compared to control gelatin sponge. Higher osteogenic potential was seen in CP2Col and CP2Gel_GA compared to control collagen and gelatin sponge respectively. The homogeneous porous structure and Mg containing BCP granules in the scaffolds facilitated good cell distribution and enhanced the osteogenic activity under 3D

culture conditions. These results suggest that the scaffolds could be a potential candidate as artificial bone filler.

An injectable and anti-washout paste of hydroxyapatite/collagen (HAp/Col) nanocomposite was prepared utilizing sodium alginate by supplementation of calcium carbonate (CaCO_3) and calcium citrate (Ca-Cit). Combined supplementations of Ca-Cit and CaCO_3 improved the anti-washout property and pH controllability of the injectable HAp/Col paste. The improvements were caused by a competitive reaction that occurred co-ordinately in the pastes. From *in vitro* cell culture studies, all combinations showed good cytocompatibility without any significant suppression of cell proliferations. Hence, the presently prepared HAp/Col pastes could be good candidates for injectable artificial bone, which has the potential for incorporation into bone remodelling process.

In future work, biocompatibility of the scaffolds and the injectable paste should be evaluated with primary cell line and later under *in vivo* conditions for more accurate analysis, to be considered for non-load bearing defects.