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Surface modification of coralline scaffold for the improvement of biocompatibility and bioactivity of osteoblast

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ABSTRACT

Coral is a marine invertebrate consisting of calcium carbonate and used as orthopedic implants due to porous structure and mechanical properties. Its chemical composition, however, can cause rapid degradation and inflammatory response upon implantation. In this study, a surface modification method is developed for coralline scaffold using calcium phosphate (CP) to improve the biocompatibility and osteoblast bioactivity. The physicochemical properties of the scaffolds were evaluated. *In vitro* investigation demonstrates that the CP coating promoted the osteoblastic differentiation and mineralization. *In vivo* histological assessment revealed not only reduction in inflammatory response but increase in collagen synthesis in the CP-coated coralline scaffold.

Introduction

Tissue engineering is a newly emerging field for the developments of biological substitutes based on the combined knowledge of biology, medicine, pharmacy, bio and material engineering. Porous degradable 3D scaffolds, in particular, are useful in hard tissue engineering for the replacement and restoration of damaged bone tissues because of bony diseases (*i.e.*, cyst and tumor), aging or accidents. In this study, we investigated the feasibility of a CP coating as the surface modifier for a coralline scaffold to improve its biocompatibility and osteoconductivity.

Biomaterials used as bone substitutes require specific properties to promote bone regeneration in addition to satisfying mechanical and biological requirements, closely related to bulk and surface properties, respectively. Metals such as titanium, alloys and ceramics have been widely used owing to their mechanical strength [1,2]. Use of polymeric materials in biomaterial fields is also increasing owing to their advantage such as easy fabricability into desired shapes, despite mechanical weakness [3,4]. *In vivo* degradability of biomaterials is also a very important factor because the implanted biomaterials can be gradually replaced by newly forming tissues ultimately enabling the complete recovery with natural tissues. In recent years, biodegradable ceramics such as β-tricalcium phosphate have attracted considerable interest owing to their excellent biocompatibility and biodegradability [5]. Unfortunately, their mechanical weakness such as brittleness limits their use to coating materials [6]. By far, various coating methods using different types of CP, such as plasma spraying, electron beam sputtering, and sol–gel method have been introduced [7–9]. However, the preparation of CP coating *via* these methods requires harsh conditions, for example high temperature, extreme pH, and long processing time. These conditions can cause structural dissimilarities to natural bone, *i.e.*, high crystallinity.

Coral is a marine invertebrate with a naturally highly porous structure with high interconnectivity among its pores. Its porous structure and mechanical strength are strikingly similar to the trabecular bone. The compressive strength of coral (9–14 Mpa) is comparable to that of trabecular bone (5–10 Mpa) [10,11]. Therefore, coral can be one of excellent alternates for bone substitutes [12,13]. Coral is mainly composed of calcium carbonate ($CaCO_3$, 97–99%), whereas the inorganic phase of a natural bone mainly consists of amorphous hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$). Therefore, coral degrades fast and can often cause inflammatory responses upon implantation [10,14,15]. Studies have been directed for the conversion of the coralline calcium carbonate into hydroxyapatite and the hydrothermal processing is one of representative examples in this aspect [16,17]. In this study, we developed a simple method to form a CP coating on the coralline

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scaffold without causing serious changes in structural and mechanical properties and examined its feasibility for the improvement of the biological function of the coralline scaffold. For this purpose, we performed *in vitro* assays, including OB proliferation, differentiation, and mineralization, and *in vivo* histological assessments of biocompatibility and biodegradation. Our results demonstrated that the CP coating on the scaffold substantially promoted the OB differentiation and biomineralization as well as *in vivo* biocompatibility.

Materials and Methods

Preparation of coralline scaffold

Coralline scaffolds were prepared from Porites coral, which inhabits the Pacific and Indian Oceans by cutting into the size of 7 mm × 7 mm × 2 mm and then washed with distilled deionized water (ddw) by sonicating for 30 min. The scaffolds were further washed with 4% NaClO solution (239205, Sigma–Aldrich, USA) by stirring for 3 days [17], followed by washing with ddw by stirring for 6 h, and finally dried. For the sterilization, the dried coralline scaffolds were autoclaved for 1 h followed by drying under UV for 24 h.

Surface modification of the coralline scaffold by the CP coating

The prepared coralline scaffolds were immersed in an ionic solution saturated with calcium and phosphate ions to form a CP coating on inner and outer surfaces of the coralline scaffolds. Calcium nitrate tetrahydrate ($Ca(NO_3)_2{\cdot}4H_2O$, Sigma–Aldrich, C4955, USA) and diammonium phosphate ($(NH_4)_2{\cdot}HPO_4$, Sigma–Aldrich, A5764, USA) were used for the preparation of the ionic solution. The coating procedure was similar to those reported in earlier reports [18,19]. The scaffolds were immersed in the solution and continuously mixed using a rotator mixer for 60 min at 4 °C and then for 90 min at 37 °C. Finally, the coated scaffold was removed from the tube and washed thrice with excess ddw, and then dried on a clean bench. The untreated and CP-coated coralline scaffolds were designated as the CR and CRC scaffolds, respectively.

Morphology and chemical composition analysis

The surface morphology of the CR and CRC scaffolds was monitored using a field emission electron microscope (FE-SEM, HITACHI, S-4300, Japan). Prior to the observation, the coralline scaffolds were sputter coated with platinum using an ion-sputtering coater (HITACHI, E-1045, Japan). The chemical elements constituting the surface of coralline scaffolds were measured by energy dispersive spectroscopy (EDX, HORIBA, EX-250, Japan).

Porosity measurement

The porosity and pore size of the CR and CRC scaffolds were measured using a mercury porosimeter (Micromeritics, AutoPore IV 9500, USA). After the complete dehydration, the scaffolds were cut into four pieces. The individual pieces after measuring the weight were kept into a measuring cylinder, which was subsequently filled with mercury. Then, the cylinder was pressurized to measure the porosity and pore size of the CR and CRC scaffolds.

Fourier transform infrared spectroscopic (FT-IR) analysis

The chemical state of the CP coatings was analyzed by a FT-IR spectroscopy (Spectrum One, Perkin Elmer, UK). The IR spectrum from the CR scaffold can be considered as that of coral. The CR scaffold was incubated in an aforementioned ionic solution for 0, 30, 60, 90, and 120 min. At desired incubation time, the scaffold was taken out from the solution and dried, followed by IR spectral acquisition.

Degradation of the CR and CRC scaffolds

To analyze the effect of the CP coating on the degradation of the CR and CRC scaffolds, the amount of calcium ions liberated from the scaffold because of the degradation was measured. The calcium content was determined by inductively coupled plasma-atomic emission spectrometry (ICP-AES, Perkin Elmer, OPTIMA, 4300 V, USA). For this purpose, weights of the CR and CRC scaffolds were measured prior to placing them in a 50 mL tube filled with PBS solution. The tubes were fixed to rotator for continuous mixing at 37 °C. Aliquots of 20 mL were collected from the tube at designated incubation time and used for the determination of the amount of calcium ions. An equal volume of PBS solution to that of the collected aliquot was added as soon as the aliquot was collected. The degradation experiment was conducted for 60 days.

Cell culture

Cellular responses of the prepared scaffolds were analyzed by using an MG63 human osteosarcoma cell line (ATCC). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% (v/v) antibiotic antimycotic solution (Gibco, 15240, USA) and 10% (v/v) fetal bovine serum (FBS, Gibco, 16000, USA) in a 5% CO_2 incubator at 37 °C. For cell seeding, the cells were harvested by trypsinization and prepared at the desired cell density. Prior to the cell seeding, the CR and CRC scaffolds were sterilized using 99% ethanol and washed with excess ddw and then dried. Then, the scaffold was placed in a 96-well plate followed by filling with DMEM and incubated in a CO_2 incubator. After 6 h of incubation, the scaffolds were taken out from the tube, and the DMEM remaining inside of the scaffold was removed using a Whatman paper, and then placed in a new culture plate. Next, 25 mL of the cell solution was prepared at the desired cell density, and the scaffold was added, followed by incubation for 2 h. To that solution, 200 μl DMEM was added, followed by adding another 800 μl after 24 h.

Cell proliferation

The MG63 cell proliferation on the CR and CRC scaffolds was evaluated using a cell counting kit (CCK) (Dojindo, CK04, Japan). The cells were plated on the scaffolds at a cell density of 15,000 cells/scaffold and cultured for 2, 3, 5, and 7 days. At desired culture periods, 300 μl of the mixture solution was prepared by mixing the culture media and CCK solution at a ratio of 9:1, and added to each scaffold, followed by incubating for 3 h. Aliquot of 200 μl was taken from each sample and kept into a 96-well plate for the measurement of the optical density (OD) at 450 nm wavelength.

Cell differentiation

To evaluate the effect of the CP coating on the scaffold for the osteoblastic differentiation, we examined the alkaline phosphatase (ALP) activity using the ALP staining method. MG63 cells were plated on the scaffold at a cell density of 20,000 cells/scaffold and cultured for 3, 5, 7, and 14 days in an osteogenic media supplemented with dexamethasone, ascorbic acid, and β-glycerol phosphate. At the desired culture periods, the cultured cells were fixed with 10% formaldehyde for 2 min, followed by washing with TBST solution (20 mM Tris-HCl, 0.15 M NaCl, 0.05% Tween 20, pH 7.4). Then, the scaffold was immersed in 150 μl NBT/BCIP solution (BM Purple ALP substrate precipitating, Roche) and stirred for 40 min in a dark room. The scaffold was rinsed with an excess ddw