In vivo and in vitro biological efficacy of double-layer coating of titanium with gelatin and calcium phosphate

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This paper presents a new type of surface modification of titanium to promote osseointegration. A double-layer coating was designed to endow titanium with enhanced osteoconductivity; a gelatin layer, that offers an environment for the attachment of osteoblast cells, was covalently bonded to the surface of titanium, after which a calcium phosphate layer composed of octacalcium phosphate and low-crystallinity hydroxyapatite was deposited onto specimens at 310 K with the mediation of urease. Although it has been difficult to combine gelatin and calcium phosphate because there has never been an appropriate technique to do so, a newly developed technique employing urease has made possible to deposit calcium phosphate at 310 K. The calcium phosphate layer was approximately 20 to 30 μm thick and adhered well to the titanium/gelatin substrate. The gelatin/calcium phosphate layer promoted the proliferation of osteoblast-like MC3T3-E1 cells and subsequent calcification. Additionally, an implant test in GK rats suggested that the layer may enhance the formation of new bone and new blood vessels. The surface modification presented herein may be a promising technique for dental and orthopedic implants.

Key-words: Titanium, Gelatin, Calcium phosphate, Calcification, Bone formation

1. Introduction

Titanium and titanium alloys are popular metals used in orthopedic and dental implants. However, these metals are not bioactive in nature, and therefore, their surfaces are occasionally modified to accelerate integration with the surrounding tissues. For example, thermal spraying of calcium phosphate (CaP) layers[1,2] has been carried out on clinically used medical implants. Alkaline/heat treatment[3,4] has also been employed to prepare such metals for implantation. Furthermore, numerous types of surface modifications have been utilized[5,6] to impart bioactivity to titanium, including coating with bioactive glasses,[6,7] titanium coating by H2O2 treatment[8,9] or anodic oxidation,[10] surface modification with bioactive polymers,[11-14] collagen coating,[15] and combinations of these techniques.

Because collagen and hydroxyapatite (HA) are the primary constituents of natural bone, it is believed that the concurrent coating of any substrate with collagen/HA or collagen/CaP layers would provide good bioactivity. As far as titanium and titanium-based alloys are concerned, deposition of collagen by covalent bonding[16-18] or physical adsorption[19-24] has been attempted by many researchers. Collagen coatings on titanium have been shown to promote cell adhesion[11,12,19,20] and osteogenesis.[6,18,21,22] However, concurrent coating of titanium with collagen and CaP is difficult. One reason for this difficulty may be that collagen inhibits apatite formation in SBF.[25] Accordingly, physical adsorption of the HA-collagen mixture[26] and concurrent

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or sequential electrochemical deposition of CaP and collagen[27,28] have been attempted.

Gelatin, which is derived from collagen and considered to play a similar role to collagen in living organisms, is easier to handle than collagen. As a result, gelatin/CaP coatings should also be beneficial as collagen/CaP coatings. Xiao et al.[29] fabricated gelatin/HA coatings on Ti6Al4V alloy by the dip-coating method. Aradit et al.[30] have shown that gelatin/HA coatings applied on poly(e-caprolactone) by the alternate soaking process stimulated the proliferation and differentiation of bone marrow stromal cells.

In the present study, another process for fabrication of gelatin/ CaP is proposed. In this method, gelatin is bonded to the substrate covalently rather than by physical adsorption, after which CaP is coated on top at near room temperature in a process mediated by urease. In this double-layer coating, the gelatin offers a preferable environment for tissue-regenerating cells such as osteoblasts to proliferate and function, while the CaP layer acts as a reservoir of inorganic ions that are necessary for the cells.[31,32] In this paper, the preparation and characterization of a gelatin/CaP layer on titanium is presented and the biological efficacy determined from a MC3T3-E1 cell culture and implant tests in diabetic GK (Goto−Kakizaki) rats is described.

2. Materials and methods

2.1 Sample preparation

The gelatin coating process was a modification of the process described in Ref. 15. Commercially pure titanium discs 20 mm in diameter by 2.0 mm thick and 4.0 mm in diameter by 2.0 mm thick were polished with #700 sandpaper and then sequentially sonicated in acetone, pure water, concentrated nitric acid, and
water for 10 min each before they were air-dried. Next, the discs were immersed in an ethanol solution of 20 mass % aminopropyl-triethoxysilane (APTS, Shin-Etsu Chemical Co., Ltd., Tokyo, Japan) for 24 h at room temperature before being dried at 400 K for 30 min to functionalize their surfaces with amino (-NH$_2$) groups. The discs were then transferred to an aqueous solution containing 3.0 g/L of water-soluble carbodiimide (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) (WSC; Dojindo Labs., Kumamoto, Japan) and 200 mg/L of gelatin (RM-100B, originated from pig skin, kind gift from Jellice Co., Ltd., Sendai, Japan) at 310 K for 24 h to covalently bond gelatin to titanium surfaces through amide (-NH-CO-) bonds. The gelatin-coated titanium discs are hereafter referred to as Ti/gel.

After the gelatin coating was applied, a CaP layer was coated onto the discs using the urease-mediated deposition method described by Utsuna et al. The Ti/gel discs were modified with urease by immersion in a solution containing 600 mg/L of urease (5000 U/g, prepared from Jack bean, Kariito Chemical Co., Inc., Tokyo, Japan) and 3.0 g/L of WSC at 310 K for 24 h. Next, the urease-bearing discs were transferred to a solution containing 10 mmol/L of Ca(NO$_3$)$_2$, 6 mmol/L of NH$_4$H$_2$PO$_4$, and 110 mmol/L of urea, with a pH that was adjusted to 5.8 by the addition of ammonium. The samples were then incubated at 310 K for 1 h. During the last step, urease was enzymatically hydrolyzed to ammonia in the vicinity of the surfaces of the discs, after which a CaP layer was preferentially precipitated on the discs. The resultant discs coated with gelatin and CaP are hereafter referred to as Ti/gel/ CaP. All the discs used in the cell culture tests and animal implantation tests were sterilized by γ-ray irradiation.

Ti/gel/CaP was characterized by the X-ray diffraction analysis (XRD, Miniflex, Rigaku Corp., Tokyo, Japan), scanning electron microscopy (SEM, e-SEM, Shimadzu Rika Corp., Tokyo, Japan and VE-9800, Keyence Corp., Osaka Japan). The CaP layer was scraped off the substrate and observed by transmission electron microscopy (TEM, JEM-2100F, JEOL Ltd., Tokyo, Japan). The Ca/P ratio of the CaP layer was analyzed using a chemically coupled plasma analyzer (ICP, ICP-7000, Shimadzu Corp., Kyoto Japan) after dissolving the layer into 0.1 mol/L nitric acid.

2.2 Cell culture tests

Mouse calvaria-derived osteoblastic cells (MC3T3-E1) were obtained from the Riken Cell Bank (Tsukuba, Japan). Titanium discs 20 mm in diameter coated with highly crystalline HA 3 μm in thickness were used as a control. These control samples, custom-made by Platon Japan Co., Ltd., Tokyo, Japan, were used to examine the effects of differences in the crystalline phase and crystallinity of calcium phosphate on the cell response because it has been reported that some commercially available dental implants coated with highly crystalline HA promote osseointegration. Ti/gel/CaP and the control disc specimens (each 20 mm in diameter) were placed in a mixture of 2 mL of culture medium (α-MEM + 10% FBS, Gibco, Invitrogen, Carlsbad, CA, USA) and 1 mL of cell solution containing 1.0 × 10$^5$ cells of mouse osteoblast-like MC3T3-E1. The culture dishes were then stored at 310 K for 40 days in an atmosphere composed of 5% CO$_2$/95% air. During incubation, the culture medium was replaced every four days. Following incubation on Ti/gel/CaP and the control, the samples were washed three times with PBS. The adhered cells were then fixed with 1% glutaraldehyde for 15 min, rinsed three times with PBS, and fixed with 2% osmium tetroxide for 15 min. The fixed cells were subsequently dehydrated in a series of ethanol (50, 70, 80, 90, 95, and 100%) for 5 min each, after which they were dried using a critical drying apparatus. The morphology of the cells was observed with a Keyence scanning electron microscope and an optical microscope (VHX-1000, Keyence Corp.).

2.3 Animal implantation test

All animal experiments were conducted in accordance with the Guidelines for Animal Research at the Kanagawa Dental College, Japan. GK rats were obtained at 30 weeks of age and maintained under a specific pathogen-free condition at controlled temperature and humidity. Rats were given a standard laboratory diet and water ad libitum. All rats were exposed to a light/dark cycle for four weeks before the experiment. Thirty male GK rats were divided into two control groups (untreated Ti and Ti/gel) and one experimental group (Ti/gel/CaP) that each contained ten animals. Diabetic rats were used because they have poor bone formation ability. If new bone formation is promoted with our material even in diabetic rats, the superiority of osteogenesis capability is to be demonstrated.

Animals were anesthetized by intraperitoneal injection with 8% trichloroacetaldehyde monohydrate (Chloral hydrate; 0.5 ml/100 g, Wako Pure Chemical Industries, Ltd., Osaka, Japan). The cranium was then shaved, after which the surgical site was prepared with an iodine tincture scrub and the animals were placed in a sterile surgical “sock”. Next an incision was made using a sterile scalpel with a No. 15 blade from the left to right auricle and the full-thickness flaps was reflected to expose the calvarial bone. A trephine drill with 4.2 mm outside diameter was subsequently used to create a surgical defect approximately 0.8 mm deep in the calvarium, after which the bone was carefully removed to avoid injury to the underlying area. Untreated Ti, Ti/gel, or Ti/gel/CaP specimens 4.0 mm in diameter were implanted in the bone defect. Untreated Ti was used as a control for this experiment because the HA-coated Ti discs 4.0 mm in diameter were not available. After four weeks, the animals were sacrificed by administering an excessive amount of Nembutal (40 mg/kg, Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan). The implanted portions were then excertated and soaked in a 10% formalin solution for ten days, decalcified with formic acid, sliced into thin sections and dyed with hematoxylin-eosin stain for histological observations.

3. Results

3.1 Materials characterization

Figure 1 shows the XRD pattern of the CaP layer of Ti/gel/CaP. The CaP layer consisted of octacalcium phosphate (OCP).
and low-crystallinity HA, both of which are known to be readily soluble in water. Although the diffraction patterns of OCP and HA were similar to each other, the diffraction around $2\theta = 4.7^\circ$ is characteristic of OCP. The Ca/P ratio of the CaP layer was 1.50, which also indicates that the layer was a mixture of OCP (Ca/P = 1.33) and HA (Ca/P = 1.67).

SEM pictures of the surface and cross-section of the CaP layer of Ti/gel/CaP are shown in Fig. 2. The outermost portion of the CaP layer consisted of randomly oriented crystals with flake-like morphology characteristic of OCP [Fig. 2(a)]. The thickness of the layer was found to be approximately 20 to 30 $\mu$m from the cross-sectional view [Fig. 2(b)]. Although the gelatin layer was too thin to be observed by SEM, it was essential for the CaP layer to be adhered to titanium; when the gelatin layer was absent, the CaP layer easily detached.

A TEM picture of the particles in the CaP layer of Ti/gel/CaP is shown in Fig. 3. TEM analysis revealed two types of particles with distinctly different morphologies, ribbon-like crystals and spherical particles. The authors assume that the former particles may correspond to OCP and the latter to HA because OCP has a tendency to grow into ribbon-like crystal flakes.

3.2 Cell culture test
The surfaces of Ti/gel/CaP and the control before and after the cell culture are shown in Fig. 4. On the control after the 40-day...
culture [Fig. 4(b)], a number of calcified spheres approximately 10 μm in diameter were observed to be sparsely distributed on the surface. These spheres were white in color under an optical microscope (data not shown), and were assumed to be the calcification products. Conversely, an innumerable number of calcified spheres were observed on Ti/gel/CaP after culture [Fig. 4(d)].

3.3 Animal implantation test

Figure 5 shows the histologic micrographs of the thin sections of the cranial bones after removal of the implants. When Ti/gel/ CaP had been implanted [Fig. 5(a)], extensive new bone formation was observed in the gap between natural bone and the disc. Also, new blood vessels were observed in the new bone. In contact with the bottom surface of the Ti/gel/ CaP disc [Fig. 5(b)], newly formed bone is observed. The bone-implant contact ratio was calculated to be 47.8 ± 8.3% from 10 pieces of thin sections. It is commonly accepted that diabetes hinders the ability of the patient to form bone. Nevertheless, the abovementioned results imply that Ti/gel/ CaP can promote osteogenesis in diabetic rats.

When Ti/gel or untreated Ti were implanted [Figs. 5(c) to 5(f)], the gaps between the natural bone and the discs were occupied with fibrous tissue. The bone-implant contact ratios were 0% in both cases.

4. Discussion

As was shown in Fig. 4, there is a possibility that the surface of Ti/gel/ CaP helped the proliferation of MC3T3-E1 and calcification. To be more definitive, however, quantitative analysis has to be made including gene expression analysis, which is a future work. The difference in the extent of calcification between the control and Ti/gel/ CaP may be attributed to (1) the difference in the dissolution kinetics between highly crystalline HA on the control and the CaP layer of Ti/gel/ CaP and (2) the presence of a gelatin layer on which the cells easily proliferate. Owing to the readily soluble nature of the CaP layer of Ti/gel/ CaP, the osteoblasts are expected to have utilized necessary calcium ions for differentiation[11,12] and subsequent calcification.

The results of the animal tests, Fig. 5, seem to imply that coating titanium with gelatin and calcium phosphate, OCP and low-crystallinity HA in this case, promotes bone formation.
course, in order to clarify the biological efficacy of Ti/gel/CaP with statistical significance, a larger number of tests will have to be iterated. After those analyses are done, Ti/gel/CoP would become a promising candidate material for bone plates, bone screws, dental implants, and other titanium-based medical devices that can reduce the duration required for therapy.

5. Conclusions

Ti was sequentially surface-modified with gelatin and calcium phosphate by a technique in which urea is utilized as the mediator for the calcium phosphate deposition. The calcium phosphate layer was 20 to 30 μm thick and consisted of a mixture of octacalcium phosphate and low-crystallinity hydroxyapatite, both of which are readily soluble in the surrounding water. The gelatin/calcium phosphate layer extensively promoted the proliferation of osteoblast-like MC3T3-E1 cells and subsequent calcification in the cell culture test. When implanted in the defect in the cranial bone of diabetic rats, the coating layers seemed to enhance the formation of new bone and new blood vessels. This implies that titanium coated with gelatin/calcium phosphate is a promising material for orthopedic and dental implants. Quantitative analysis will have to be made to show statistical significance of the efficacy of the present material.

References