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Laser-assisted biomimetic process for surface functionalization of titanium metal

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ABSTRACT

Biomimetic calcium phosphate (CaP) precipitation processes using supersaturated CaP solutions are useful in surface functionalization of biomedical materials. We applied our laser-assisted biomimetic (LAB) process to successfully achieve rapid single-step CaP precipitation on the surface of titanium metal, which is an important metallic biomaterial, by applying pulsed laser irradiation to the titanium substrate immersed in a supersaturated CaP solution. Precipitation occurred via the combined effect of laser surface modification and ambient heating. Moreover, we demonstrated immobilization of various contents of osteogenic substances (zinc and fibronectin components) on the titanium surface together with CaP by supplementing the CaP solution with these substances. The LAB process is expected to be a facile and effective surface functionalization technique for titanium-based biomaterials to provide them with osteoconductivity because of CaP and stimulatory effects on bone formation due to osteogenic substances.

Certain types of calcium phosphate (CaP) ceramics such as hydroxyapatite and β-tricalcium phosphate have long been used as orthopedic and dental implants owing to their good biocompatibility and osteoconductivity. However, CaP ceramics are hard and brittle; hence, they are utilizable only under low load-bearing conditions.

Under high load-bearing conditions, implants made of tough and strong metals including titanium metal and its alloys have been used. To provide these metallic implants with osteoconductivity, various CaP deposition techniques such as plasma spraying, sputtering, laser ablation (pulsed laser deposition), and biomimetic processes have been proposed. Among these techniques, biomimetic processes [1–5] utilizing supersaturated CaP solutions as both the CaP source and growth media have recently attracted increased attention. Biomimetic processes are capable of precipitating CaP and concurrently immobilizing biologically functional substances, such as antimicrobials, cytokines, trace elements, and nucleic acids, by taking advantage of pseudo-physiological reaction conditions [6,7].

The weaknesses associated with conventional biomimetic processes are the relatively long processing time required because of the slow CaP precipitation/growth rate in supersaturated CaP solutions and the necessity of subjecting the substrate materials to a prior surface modification step. Examples of surface modification methods required for metallic materials include grit-blasting followed by CaP-precoating [1], NaOH treatment [2], H_2O_2 treatment [3], anodic oxidation [4], and focal laser irradiation [5]. The resulting metals with modified surfaces induce CaP precipitation on their surfaces when subsequently immersed in a supersaturated CaP solution, typically for as long as one day or more.

Recently, we achieved rapid and single-step CaP precipitation on polymer substrates by a laser-assisted biomimetic (LAB) process [8,9]. In this process, pulsed, unfocused laser irradiation was applied to a polymer substrate that was immersed in a metastable supersaturated CaP solution (denoted herein as the CP solution [10]) in accordance with Lee's system [11]. Within 30 min of irradiation, CaP precipitated onto the laser-irradiated polymer substrate surface by the combined effects of laser surface modification and ambient heating at the solid–liquid interface [8,9]. Therefore, the LAB process is expected to be a facile and effective tool for surface functionalization of biomedical materials.

The first aim of the present study was to apply the LAB process for CaP precipitation on the surface of titanium metal. The second aim of this study was to immobilize biologically functional substances onto the titanium surface along with CaP precipitation by the LAB process. The biologically functional substances selected were zinc (Zn), an essential trace element and fibronectin (Fn), a protein facilitating cell adhesion. Both Zn [12,13] and Fn [14] could act as osteogenic substances and are expected to stimulate bone regeneration.

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To achieve the first aim, the LAB process was carried out on titanium metal substrates using the CP solution, as described in our previous reports [8,9]. Briefly (see Supplementary information for experimental details), a single titanium substrate (1 mm × 10 mm × 10 mm) was immersed in 10 mL of the CP solution maintained at 25 °C using a water bath. Pulsed, unfocused laser irradiation (30 Hz, 355 nm, 4 W/cm^2) was applied to the titanium substrate for 30 min while immersed in the CP solution. The laser-irradiated surface ($\phi = 5$ mm) of the substrate was examined.

In addition to polymer substrates, as reported previously [8,9], the applicability of the CaP precipitation technique using the LAB process was verified for titanium metal substrates as well. As revealed by energy-dispersive X-ray analysis (EDX), Ca and P were newly detected on the titanium surface after conducting the LAB process (Fig. 1a), suggesting the precipitation of CaP compounds. As shown in the scanning electron microscopy (SEM) images in Fig. 1b, the titanium surface deformed into a micro-scale grainy structure with sub-microscale asperity faces after conducting the LAB process. To isolate the morphological effects on the metal surface owing to laser surface modification and CaP precipitation, we examined an equivalently laser-irradiated titanium surface in ultrapure water, which revealed a similar micro-scale grainy structure to that of the surface subjected to the LAB process but without sub-microscale asperity (Fig. S1). Therefore, the microscale and sub-microscale deformations of the substrate are expected to be due to laser surface modification and CaP precipitation, respectively. According to the results of thin-film X-ray diffraction (TF-XRD) shown in Fig. 2a and selected area diffraction by transmission electron microscopy (TEM) shown in Fig. 2b, the CaP precipitates contained hydroxyapatite as a major crystalline phase. The obtained CaP precipitates might also contain the amorphous CaP phase, which was difficult to be identified from the background intensity in Fig. 2(a).

In the LAB process, laser absorption by the titanium substrate should be an essential first step for CaP precipitation, as suggested from our previous results on polymer substrates [8,9]. Absorption of 355 nm light by the titanium substrate was experimentally confirmed by absorption spectroscopy using an ultraviolet–visible–near-infrared (UV–VIS–NIR) spectrophotometer (Fig. S2). The absorbed laser light energy is assumed to be transformed into thermal energy thereby causing not only surface modification of the substrate, but also ambient heating during the LAB process, as detailed in the following paragraph.

As briefly discussed previously, the laser surface modification in the LAB process was elucidated by a control experiment that evaluated an equivalently laser-irradiated titanium surface in ultrapure water. The untreated titanium substrate used in the present study had a naturally-oxidized titanium surface (Fig. S3b). Laser irradiation in ultrapure water caused not only micro-scale deformation (Fig. S1) but also further oxidization of the substrate surface to a rutile phase (Fig. S3). The laser-irradiated titanium surface showed higher hydrophilicity than the untreated surface; the water contact angle on the substrate decreased from 94.8° ± 1.2° to 12.8° ± 1.7° (n = 3) after laser irradiation in ultrapure water. UV light-induced photochemical reaction [15] and/or laser ablation might be involved in these surface reactions, and similar surface reactions are expected to occur in the CP solution as well. The resulting highly-oxidized titanium surface with increased hydrophilicity and roughness is expected to have increased affinity with the CaP components (e.g., Ca, P ions, and CaP nanoclusters [16,17]) in the CP solution, thereby inducing CaP precipitation heterogeneously at the laser-irradiated solid–liquid interface. The above expectation about heterogeneous CaP precipitation is supported by the experimental results that the equivalently laser-irradiated titanium surface in ultrapure water formed a CaP layer on its surface when it was subsequently immersed in the CP solution for 24 h (Fig. S4). On the other hand, when the subsequent immersion period in the CP solution was 30 min (the same as the LAB process), neither Ca nor P was detected by EDX on the laser-irradiated titanium surface in ultrapure water (Fig. S4). This result indicates that the CaP precipitation on the titanium surface by the LAB process is accelerated by the effect of ambient heating due to laser irradiation. To amplify the heating effect, the LAB process was performed on a titanium substrate in the absence of a temperature-controlled water bath. The temperature of the CP solution increased by approximately 9 °C (from 25 to 34 °C) after conducting the LAB process. This increased temperature must be due to the light-to-heat energy conversion at the titanium surface because the CP solution itself exhibits very little light absorption at 355 nm [8], and the temperature rise of the CP solution was negligibly small (<1 °C) in the absence of a titanium substrate. The ambient heating accelerates CaP precipitation by increasing the mass transfer rate and the degree of supersaturation with respect to the CaP compounds [18,19].

Next, we attempted to immobilize the selected biologically functional substances, Zn and Fn, on the titanium surface together with CaP by the LAB process. For this purpose, we supplemented the CP solution with $ZnCl_2$ or Fn at various concentrations. Using the

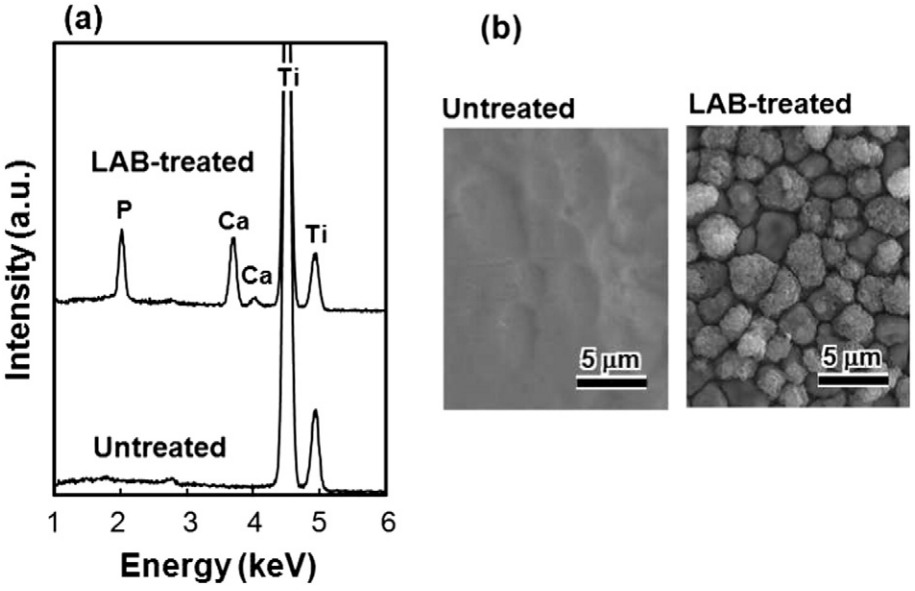

Fig. 1. (a) Energy-dispersive X-ray analysis (EDX) spectra and (b) scanning electron microscopy (SEM) images of the untreated titanium substrate surface and that after conducting the LAB process.

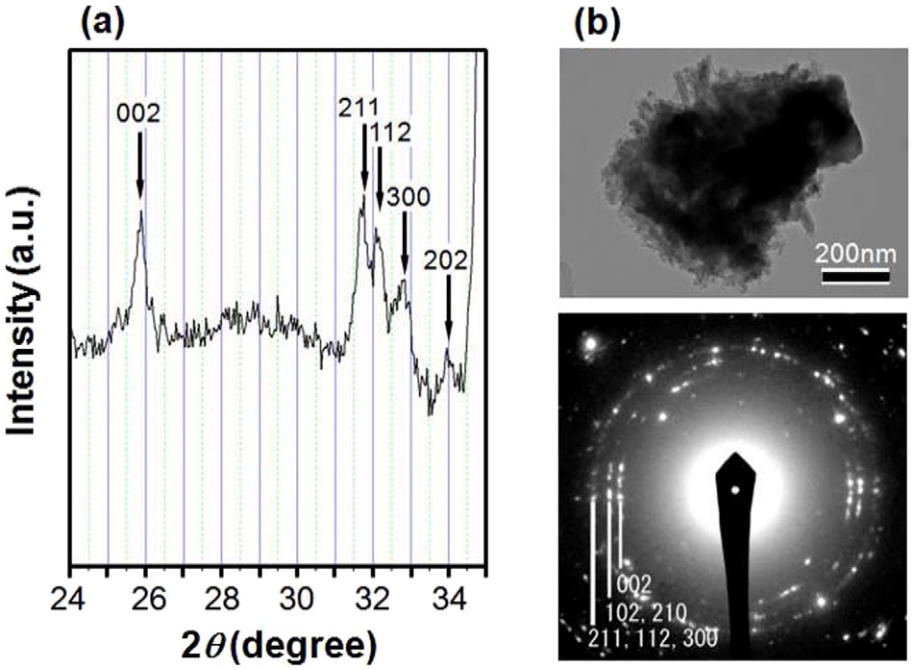

Fig. 2. (a) Thin-film X-ray diffraction (TF-XRD) pattern of the titanium substrate surface after conducting the LAB process. (b) Transmission electron microscopy (TEM) image (upper) and the selected area electron diffraction pattern (lower) of a CaP precipitate collected from the titanium surface after conducting the LAB process. The observed peaks and rings were indexed to hydroxyapatite (JCPDS No. 09-0432).

modified CP solutions, the LAB process was carried out according to an equivalent protocol as described above, except that the solution volume was decreased from 10 to 3 mL. The resulting samples are denoted as Z1, Z10, Z100, and Z1000 according to the $ZnCl_2$ concentrations: 1, 10, 100, and 1000 μM, and as Fn10, Fn20, and Fn40 according to the Fn concentrations: 10, 20, and 40 μg/mL of the modified CP solutions. The control sample prepared using the CP solution (3 mL) in the absence of $ZnCl_2$ or Fn was denoted as CP0.

Employing $ZnCl_2$-supplemented CP solutions in the LAB process, Zn of various contents was successfully immobilized on the titanium surface. In the X-ray photoelectron spectroscopy (XPS) spectra shown in Fig. 3a, peaks attributable to Ca and P were observed for all samples, whereas Fig. 3b indicates that peaks attributable to Zn (i.e., the Zn_{2p} peaks) were detected for only samples Z1, Z10, Z100, and Z1000. In addition, Fig. 3b demonstrates that the Zn_{2p} peak intensities increased with the increasing $ZnCl_2$ concentration of the modified CP solution, suggesting that the Zn content on the titanium surface follows an order corresponding to CP0 < Z1 < Z10 < Z100 < Z1000. Selected Zn-rich samples (Z10, Z100, and Z1000) were further subjected to chemical analysis to quantify the Zn content on their surfaces. For chemical analysis, the precipitates were completely dissolved in a 6 M HCl solution, and the Zn concentration in the solution was determined by inductively coupled plasma atomic emission spectrometry (ICP). The ICP results (Fig. S5) quantitatively support the XPS results in that the Zn content

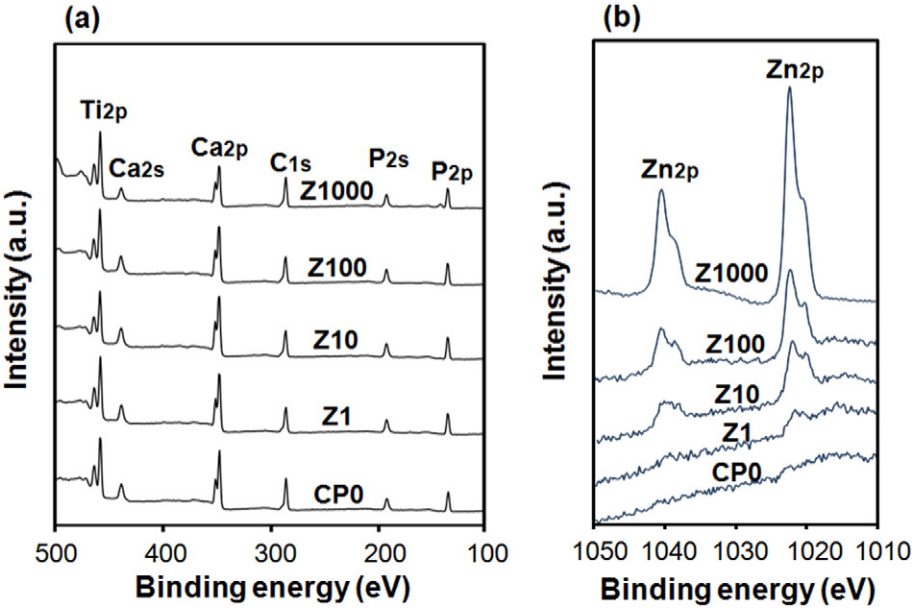

Fig. 3. X-ray photoelectron spectroscopy (XPS) spectra (a: wide range, b: narrow range) of the surfaces of the CP0, Z1, Z10, Z100, and Z1000 samples.

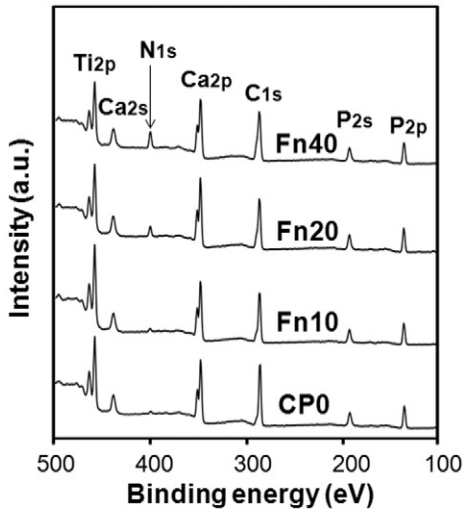

Fig. 4. X-ray photoelectron spectroscopy (XPS) spectra (wide range) of the surfaces of the CP0, Fn10, Fn20, and Fn40 samples.

on the sample surface increased in the order corresponding to Z10 < Z100 < Z1000. The results indicate that the Zn content immobilized on the titanium surface is controllable by tuning the $ZnCl_2$ concentration in the CP solution. The Zn content of Z100 (~0.2 μg/cm^2; Fig. S5) is comparable to that of a Zn–apatite composite layer (prepared by a conventional biomimetic process) that is noncytotoxic and effective for enhancing osteogenic differentiation in vitro [13]. According to the TF-XRD results, crystallinity of hydroxyapatite for Z100 was lower than that for CP0 (Fig. S6). This is attributed to an inhibitory effect of zinc ions on hydroxyapatite crystal growth [13]. Zn substitution for Ca sites of hydroxyapatite might also be involved in the lowered crystallinity [20].

Fn molecules and/or their derivatives of various contents were also immobilized together with CaP on the titanium surface by the LAB process using the Fn-supplemented CP solutions. As shown in Fig. 4, immobilization of the Fn components on the surfaces of the Fn20 and Fn40 samples was verified by the detection of N, which is a constituent element inherent in Fn, by XPS. The intensity of the N peak is observed to increase relative to the Ca and P peaks with the increasing Fn concentration of the modified CP solution. Thus, this suggests that the content of Fn components is controllable, as was found for Zn. This controllability is significant for medical applications because the osteogenic effects of Zn and Fn are dependent on their concentration [12–14]. In addition, overdose of these biologically functional substances may cause undesirable side effects such as cytotoxicity and homeostatic imbalance.

The potential exists for damage of Fn molecules in the CP solution during the LAB process owing to the photothermochemical effects of UV laser light. To investigate this issue, laser irradiation was applied to a titanium substrate immersed in a physiological salt solution supplemented with Fn (20 μg/mL), and the Fn concentration in the residual solution was measured by the Bradford method employing UV–VIS spectrophotometry. Because the physiological salt solution is free of calcium and phosphate ions, CaP precipitation is unexpected in this case. As shown by the center bar in Fig. S7, the Fn concentration of the physiological salt solution clearly decreased after laser irradiation for 30 min. This can be attributed not only to Fn adsorption onto the titanium surface, but also to Fn aggregation, denaturalization, and/or decomposition due to the photothermochemical effects of laser irradiation. In fact, when the titanium substrate was immersed for 30 min in an equivalent salt solution without laser irradiation, the decrease in the Fn concentration was considerably less than that occurring with laser irradiation, as shown by the gray bar at left in Fig. S7.

Note that the Fn concentration decrease observed after laser irradiation of the titanium substrate immersed in the Fn-supplemented CP solution (20 μg/mL) for 30 min was greater than that occurring in the case of the physiological salt solution (as demonstrated by the two black bars in Fig. S7). It is reasonable to expect that this difference is attributable to the facilitated immobilization of Fn components onto the titanium surface owing to the affinity binding between the Fn components and CaP. It has been reported that some proteins exhibit good affinity with CaP precipitates, and they can be incorporated within a CaP layer via protein–CaP coprecipitation in the CP solution [21]. However, in the LAB process, it remains unclear whether the Fn components are incorporated within the interstices of the CaP nanocrystals or adsorbed onto the precipitated CaP surface.

We performed cell adhesion assay using Chinese hamster ovary-K1 (CHO-K1, RIKEN BioResource Center, Japan) cells (5×10^4 cells/0.5 mL RPMI/well) for the Fn20 sample with immobilized Fn components. As shown in Fig. 5, after culturing for 24 h, Fn20 exhibited enhanced cell adhesion on its surface compared with the CP0 sample without Fn. This observation suggests that the Fn components provided active binding sites on the Fn20 surface despite the partial damage of the Fn molecules in the CP solution during the LAB process. It is possible that these active binding sites on the Fn20 surface were derived from the simple adsorption of Fn components from the residual CP solution onto the CaP-precipitated substrate after turning off the laser and before removing the substrate from the solution. In fact, surface adsorption of Fn took place when sample CP0 (with CaP precipitate) was dipped in a Fn solution (Fn 20 μg/mL, NaCl 142 mM, buffered to pH = 7.40 at 25 °C with 40 mM trishydroxymethylaminomethane and HCl) for 5 min. As shown in the rightmost image of Fig. 5, the Fn-adsorbed CP0 sample also enhanced cell adhesion on its surface, similarly to the F20 sample. Therefore, from the perspective of providing active binding sites for cell growth, posterior protein adsorption onto the LAB-treated substrate would also be effective and probably more practical in terms of the prevention of protein damage. The immobilization methods and conditions should be carefully chosen and optimized depending on the type of biologically functional substances employed and the intended applications.

This study provides a new method for rapid surface functionalization of titanium-based biomaterials through CaP precipitation and

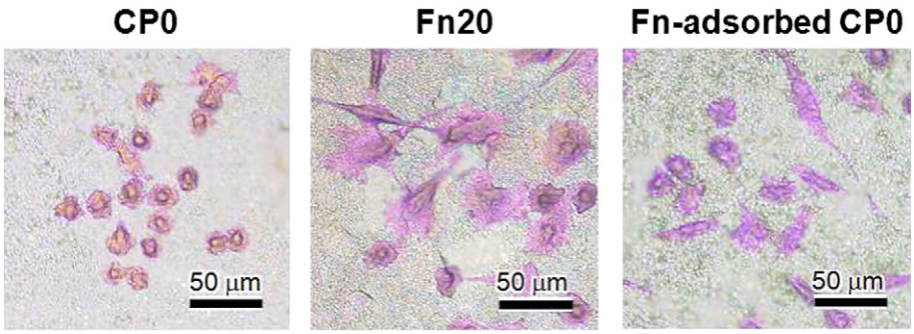

Fig. 5. Optical microscopy images of the Chinese hamster ovary-K1 (CHO-K1) cells after culturing for 24 h on the surfaces of samples CP0, Fn20, and CP0 with posterior adsorption of Fn.

immobilization of biologically functional substances using supersaturated CaP solutions. Various surface modification methods including chemical treatments [2,3] and anodic oxidation [4] have been proposed for inducing CaP precipitation on metallic substrates in supersaturated CaP solutions. Because such surface modification processes are carried out prior to the immersion process in a supersaturated CaP solution, conventional biomimetic processes generally required multiple steps with the overall processing time of one day or more. In our LAB process, the surface modification and immersion processes are combined into a single-step process: pulsed laser irradiation in the CP solution. During this LAB process, CaP precipitates in situ on the laser-irradiated titanium surface within only 30 min. The rapid and single-step LAB process would be useful in surface functionalization of titanium-based biomaterials. Considering the current irradiation area (with 5-mm diameter), the present LAB process would be suitable for small-sized biomaterials such as dental implants, bone screws, pins, and wires. For larger-sized biomaterials, the irradiation area should be enlarged by arranging the laser irradiation system (e.g., beam expansion, spreading, and scanning). Further studies are needed to fully understand the laser-mediated solid–liquid interactions occurring in the LAB process and the biological responses to the functionalized surfaces.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.colcom.2015.03.003.

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