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Preparation of nanocrystalline zinc-substituted hydroxyapatite films and their biological properties

Tania Guadalupe Peñaflor Galindo [a], Takuya Kataoka [a], Shuji Fujii [a], Mitushiro Okuda [b,c], Motohiro Tagaya [a,d,*]

[a] Department of Materials Science and Technology, Nagaoka University of Technology, 1603-1 Kamitomioka, Nagaoka, Niigata 940-2188, Japan
[b] CIC nanoGUNE, Tolosa Hiribidea 76, E-20018 Donostia-San Sebastian, Basque Country, Spain
[c] Ikerbasque, Basque Foundation for Science, Alameda Urquijo 36-5, Plaza Bizkaia, E-48011 Bilbao, Basque Country, Spain
[d] Top Runner Incubation Center for Academica-Industry Fusion, Nagaoka University of Technology, 1603-1 Kamitomioka, Nagaoka, Niigata 940-2188, Japan

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ABSTRACT

Nanocrystalline zinc (Zn)-substituted hydroxyapatite (HAp) films were prepared and electrically-plated on a titanium-coated silicone to investigate the biological properties. The hydroxyapatite nanocrystals with the different initial molar (Zn + Ca)/P ratios (1.67 and 2.00) and Zn ion concentrations were synthesized and subsequently coated by an electrophoretic deposition method to successfully form homogenous thin films. The nanocrystalline films provided bioactive properties based on the fibroblast ingrowth as well as the reduction in the number of viable *Escherichia coli*. Therefore, the optimized cytocompatible and antibacterial properties of the films by the effective Zn ion substituted in the HAp will be useful as a silicone surface modification technique.

Bacterial infections frequently occur in medical patients who use catheters. The catheters are made of silicone (poly(dimethylsiloxane)) and are low bio-affinity with skin tissues. The non-adhesion parts caused by the low affinity make the interspaces between the catheters and skin tissues, which enable the bacteria to easily permeate into the spaces and finally to infect the patients. Thus, the surface modification of the silicone has been demanded to effectively bind with the human tissues by improving the biocompatibility in addition to the antibacterial property.

For improving the biocompatibility, it has been reported the chemical coating of hydroxyapatite (HAp; $Ca_{10}(PO_4)_6(OH)_2$) on the catheters by a chemical synthetic process using silane coupling agents [1]. The HAp is extremely indispensable bioceramic due to the high biocompatibility to be implementing the defective places and to improve the implant biointegration in animal bodies to encourage the healing process through the binding with living tissues [2]. For example, the HAp nanocrsytals on a gold substrate can provide the effective serum protein adsorption and biological apatite growth [3,11]. For further catheter applications, the functionalization of antibacterial property is necessary to restrict the unexpected bacterial growth. Although a lot of antibacterial reagents have been researched, zinc (Zn) ion is well-known as an effective chemical reagent to inhibit bacteria growth. Small amount of Zn ion in living hard tissues restricts the bacterial attachment. To achieve antibacterial property in a long time, it would be useful to stabilize the Zn ions in biocompatible materials by the mineralization. Therefore, we can suggest the Zn-substituted HAp (Zn:HAp) nanocrystals as a biocompatible and antibiotic material by dense coating on the silicone.

For the dense coating, an electrophoretic deposition (EPD) technique has been attracted. It is because the technique has strong merits, i.e., the deposition with preserving crystalline structure and purity, follow-up coatings on the complex shapes with uniform film thickness, and no requirement for binder chemical reagents such as silane coupling agents [4–6]. Therefore, the EPD technique would be useful for the coating of the functional materials on silicone. The EPD is a colloidal process and can shape directly on the substrates from the stable alcoholic suspension by a direct current (DC) electric field [4,5]. The EPD is a process due to the motion of charged particles in a suspension under the influence of an electric field [4–7]. Thus, we can propose the importance of the EPD coating of Zn:HAp nanocrystals on the silicone.

In this study, the films of Zn:HAp and carbonated HAp (Zn:CHAp) nanocrystals, which were chemically synthesized at the initial (Ca + Zn/)P molar ratios at 1.67 and 2.00 with the different initial Zn concentrations, respectively, were formed on titanium-coated silicone (Ti-silicone) by an EPD technique. Furthermore, the cytocompatible and antibacterial properties of the nanocrystalline films were

* Corresponding author at: Department of Materials Science and Technology, Nagaoka University of Technology, 1603-1 Kamitomioka, Nagaoka, Niigata 940-2188, Japan.
E-mail address: tagaya@mst.nagaokaut.ac.jp (M. Tagaya).

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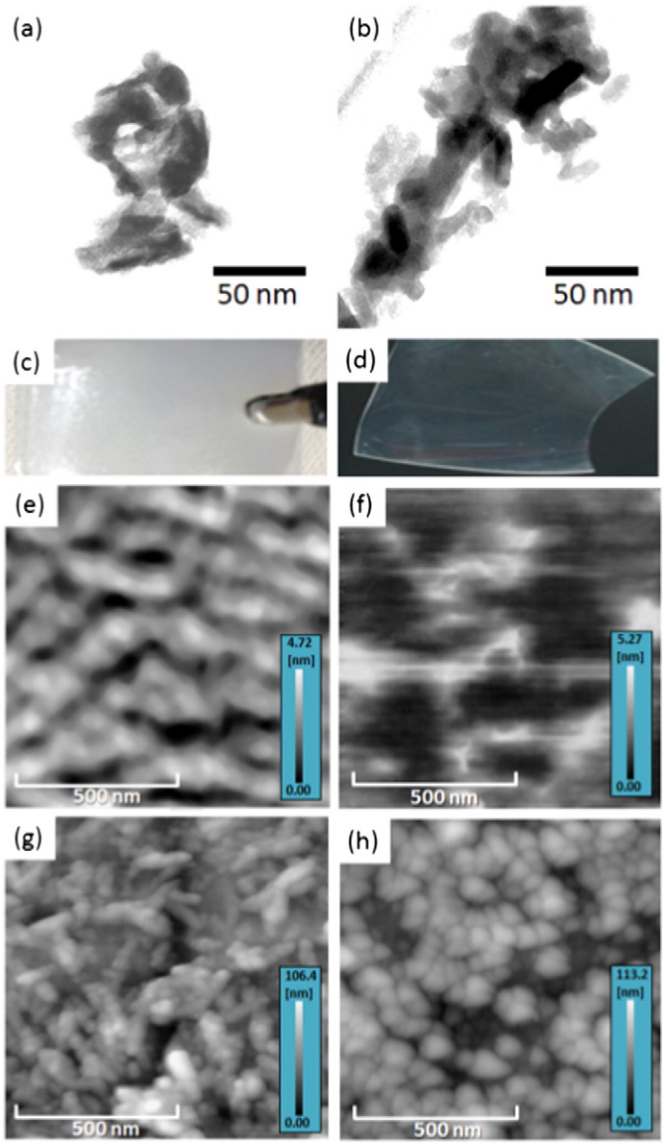

Fig. 1. TEM images of (a) 5.0-Zn:HAp and (b) 5.0-Zn:CHAp nanocrystals, photographs of the deposited films (c) before and (d) after the removal of the surplus nanocrystals in the case of 5.0-Zn:HAp, and (e–h) AFM topographic images (area: 1.0 × 1.0 μm^2) of (e) silicone, (f) Ti-silicone and the nanocrystalline (g) Zn:HAp and (h) Zn:CHAp film surfaces.

investigated using NHI3T3 fibroblasts and *Escherichia coli* (*E. coli* DH5α), respectively.

The synthetic process of the nanocrystals followed the modified procedure from our previous report [18]. $K_2HPO_4 \cdot 3H_2O$ was dissolved in ultrapure water. The aqueous solution containing both $CaCl_2$ and $ZnCl_2$ was added into the phosphate solution and the pH was adjusted up to 12 using 1 N-NaOH aqueous solution. Subsequently, the mixed solution was refluxed at 40 °C for 24 h. The resulting sediment was washed with ultrapure water and ethanol, and ultrasonically dispersed in ethanol at the HAp concentration of 1 wt.%. On the synthesis, the molar ratios of (Ca + Zn)/P at 1.67 and 2.00 with the different initial Zn concentrations to (Ca + Zn) at 0.0, 2.5, 5.0 and 10 mol%, and the samples with the ratios of (Ca + Zn)/P at 1.67 and 2.00 with the different initial Zn concentrations at *Y* mol% were abbreviated as *Y*-Zn:HAp and *Y*-Zn:CHAp ($Y = 0.0, 2.5, 5.0, 10$), respectively. The characterization of the nanocrystals was conducted using a X-ray diffraction (XRD) meter and transmission electron microscope (TEM). The resulting Zn contents in the nanocrystals were determined using a wavelength dispersive X-ray fluorescence spectrometry (XRF).

The silicone surfaces were pre-coated with a thin Ti layer using a DC sputtering as follows: Before the pre-coating, the silicone surfaces were treated by an O_2-plasma. Then, the sputtering equipment (J Sputter, ULVAC Co. Ltd., Japan) was used to apply the power (100 W) at the distance of 12 cm between the silicone and Ti target. The Ar flow amount is 20 sccm to rapidly form the Ti thin layer at the thickness of ca. 5 nm on the silicone. The resultant substrate was abbreviated as Ti-silicone.

For the EPD, the Ti-silicone and aluminum substrates were used as the working and counter electrodes, respectively, and the DC voltages of 10, 50 and 100 V/cm were applied for 1 min to deposit the nanocrystals. The surplus nanocrystals were removed by ultrasonic treatment (28 kHz, 100 W) for 1 min in ethanol.

The nanocrystalline film thicknesses, surface structures and coverages of the nanocrystals were analyzed by an atomic force microscope (AFM). The film surface roughness (R_{rms}) was calculated by the root mean squares in the Z-range images. The dissolution behavior of the nanocrystalline films was examined by the immersion into 2 mL of Dulbecco's minimum essential medium (DMEM) and incubated at 37 °C under 5%-CO_2 for 3 days, and the surface structural changes were observed by the AFM.

The cytocompatibility was examined as follows: The films were kept in phosphate buffered saline (PBS) and sterilized with ethanol/water solutions (50 and 70 vol.%), and then rinsed twice with PBS and fetal bovine serum (FBS)/DMEM solution. The NIH3T3 fibroblast suspension was seeded at the density of 8000 cells/cm^2 on the films, and cultured at 37 °C under 5%-CO_2 for 3 days, and rinsed with 1 mL of FBS/DMEM and PBS. The washed cells were immediately fixed with formaldehyde and then were observed by an optical microscopy to obtain the adhered cell density and aspect ratio. Here, the aspect ratio was defined as the

Table 1
Summarized numerical results of the surface coverage and roughness values of nanocrystalline Zn:HAp and (h) Zn:CHAp films analyzed by the AFM topographic images (area: 5.0 × 5.0 μm^2), and the viable number of *E. coli* DH5α after the second culture at the lower and higher seeding concentrations.

Sample	Nanocrystalline surface properties		Antibacterial properties	
	Surface coverage (%)	Surface roughness, R_{rms} (nm)	Viable number of *E. coli* DH5α at the lower seeding concentration	Viable number of *E. coli* DH5α at the higher seeding concentration
0.0-Zn:HAp	95.2	2.6 ± 1.2	80	800
2.5-Zn:HAp	95.6	6.1 ± 0.7	70	700
5.0-Zn:HAp	98.8	6.3 ± 1.5	60	638
10-Zn:HAp	94.8	5.6 ± 1.2	48	575
0.0-Zn:CHAp	96.9	4.3 ± 0.6	102	1038
2.5-Zn:CHAp	94.4	6.3 ± 0.9	83	790
5.0-Zn:CHAp	97.6	6.5 ± 2.1	81	775
10-Zn:CHAp	94.5	5.8 ± 1.3	50	463
Reference	–	0.7 ± 0.2	38	375