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Development of apatite-based composites by a biomimetic process for biomedical applications

Ayako OYANE†

Nanotechnology Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Central 4, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8562

A new biomimetic process for apatite coating on polymeric materials has been developed. In this process, the surface of a polymer is modified with amorphous calcium phosphate (ACP), and then the polymer is immersed in a supersaturated calcium phosphate solution. The new biomimetic process has the advantages of safety, simplicity, and applicability to various types and forms of polymeric materials. By adding a biomolecule (such as protein, antibacterial agent, or DNA) to the supersaturated solution, immobilization of a biomolecule into the apatite coating while retaining the intrinsic biological activity of the biomolecule is possible. As a result of this, the base polymer would possess biological activity to control cell behaviors (such as adhesion, proliferation, and differentiation), in addition to good biocompatibility owing to the apatite. Hence, the new biomimetic process and the resulting composites have a wide variety of biomedical applications including tissue engineering scaffolds, percutaneous devices, and gene delivery carriers.

Key-words : Biomimetic process, Apatite, Composite, Supersaturated solution, Surface modification, Laminin, DNA

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1. Introduction

Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) is the main inorganic component of human hard tissues.[1] Hence, apatite is capable of bypassing a host's foreign body response and thereby integrating with the surrounding bone tissue in vivo,[2] unlike general artificial materials. Owing to these biocompatible and osteoconductive properties, apatite has long been used clinically in important hard tissue substitutes such as artificial iliac crests, artificial vertebrae, and artificial intervertebral discs, mainly in the form of dense ceramics. However, apatite ceramics has critical disadvantages in its intrinsic mechanical properties, i.e., low fracture toughness and high Young's modulus,[3] which limit the scope of biomedical applications. Surface coating of an apatite layer on artificial materials is an effective approach to compensate this mechanical disadvantage of apatite ceramics and provide the material with good biocompatibility and osteoconductivity. As a base material for the apatite coating, we focus on polymeric materials because of their large variations in mechanical and chemical properties, and excellent workability and formability. The resulting apatite-coated polymers have a great variety of current and potential biomedical applications including hard tissue substitutes, soft tissue substitutes, tissue engineering scaffolds, percutaneous devices, and gene delivery carriers.

It is well known that apatite could have wide-ranging nonstoichiometric compositions, and this leads to variation in its physicochemical and biological properties.[4] Accordingly, it is significantly important to adjust the composition and structure of apatite depending on the intended biomedical application. For example, in bone substitutes and bone tissue engineering scaffolds, bonelike apatite, i.e., calcium-deficient and carbonate-containing apatite with low crystallinity and defective structure, would be suitable in terms of biological performance.[5]

Among conventional apatite coating processes for polymers, such as spraying,[6] silane-coupling,[7] alternate dipping,[8] and biomimetic[9)–22)] processes, the biomimetic process is particularly useful for obtaining apatite with adjustable composition and structure. In this process, apatite coating is generally carried out by immersing a surface-modified polymer in a neutralized calcium phosphate aqueous solution, which is supersaturated with respect to apatite similarly to body fluid (**Fig. 1**). The composition and structure of the thus-obtained apatite is adjustable according to the condition of the supersaturated solution, such as

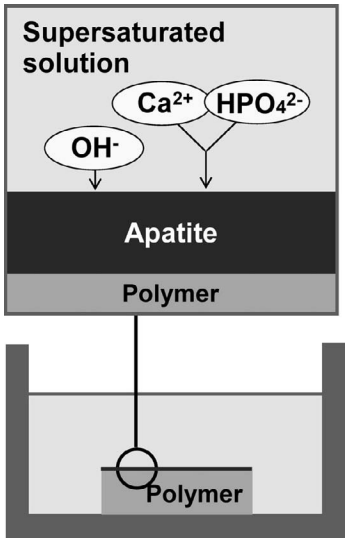

Fig. 1. Schematic illustration of biomimetic process for apatite coating on polymer.

† Corresponding author: A. Oyane; E-mail: a-oyane@aist.go.jp

ion concentrations, pH, and temperature.[23),24)] For example, bonelike apatite can be obtained from a simulated body fluid (SBF)[25)–27)] with ion concentrations, pH, and temperature approximately equal to those of human blood plasma.[23),24)].

An additional and noteworthy advantage of this biomimetic coating process is its capability to immobilize biomolecules such as proteins,[28)–40),45)–47)] antibacterial agents,[41),42)] coenzymes,[40),43)] and DNA[44)–47)] in the apatite layer. Owing to the mild coating condition, the biomolecule immobilized in the apatite layer retains its inherent biological activity. Consequently, the resulting material coated with a biomolecule–apatite composite layer could exhibit a wide variety of biological activities as well as good biocompatibility. This type of composite material has recently been attracting much attention as a third-generation biomedical material.[48)]

In this article, we review the biomimetic process for apatite (Section 2) and biomolecule–apatite composite (Section 3) coatings on the surfaces of polymeric materials with an emphasis on our research outcomes. Potential biomedical applications of the coatings and the resulting apatite-based composites are also described on the basis of our recent in vitro and in vivo results.

2. Biomimetic process for apatite coating

The biomimetic apatite coating process for polymeric materials was first proposed in the early 1990s.[9)] This original biomimetic process was carried out by placing a polymer on $CaO–SiO_2$-based glass particles in SBF, and then immersing it in another supersaturated solution. In the first treatment, silicate ions released from the glass particles attach to the polymer surface, and Si–OH groups in the silicate ions induce heterogeneous nucleation of apatite on the surface. The catalytic effect of Si–OH groups of inducing apatite nucleation has been confirmed by the experimental result that a sol–gel-derived silica gel rich in Si–OH groups forms apatite on its surface in SBF.[49),50)] In the second treatment, apatite nuclei grow spontaneously into an apatite layer on the polymer surface by consuming component ions of apatite and/or calcium phosphate clusters[51)–53)] in the supersaturated solution.

In this original biomimetic process, however, apatite is formed only on the polymer surface facing the glass particles and is not formed on the other side of the polymer. To form apatite on the entire surface of a polymer, even in a three-dimensional structure, we have modified the original biomimetic process. In this modified biomimetic process, functional groups effective in inducing apatite nucleation are introduced on the entire surface of a polymer through sol–gel surface modification without using glass particles.[10)–13)] As the functional groups, Si–OH[10)–12)] and Ti–OH[13)] have been employed according to previous fundamental studies on sol–gel-derived silica[49),50)] and titania[49),54)] gels. Utilizing the modified biomimetic process, an apatite layer was successfully formed on the entire surface of a polymer even in the form of fibers.[12),13)] Extensive and systematic research based on this modified biomimetic process has proven that the induction period required for the heterogeneous apatite nucleation strongly correlates with the type, density,[11)] and arrangement[13)] of the surface functional groups, and the calcium ion release from the polymer into a supersaturated solution,[12)] which is in good agreement with the previous fundamental results[49),50),54),55)] and recent results on other functional groups: COOH,[14),15)] PO_3H_2,[16)] and SO_3H.[17)]

The weakness of the modified biomimetic process described above is the complex and unsafe surface modification procedure which requires toxic reagents in most cases such as a metallic catalyst, alkoxy silane, and acidic solution. In the 2000s, we established a new biomimetic process[18)–22)] with the advantages of simplicity and safety in the surface modification procedure by employing nanoparticulate amorphous calcium phosphate (ACP) as a nucleating agent instead of the functional groups. The nanoparticles of ACP are introduced onto a polymer surface by a simplified alternate dipping treatment,[8)] dipping the polymer in calcium and phosphate ion solutions only three times.[56)] When the ACP-modified polymer is subsequently immersed in a supersaturated calcium phosphate solution such as SBF, the ACP acts as a precursor of apatite and induces apatite nucleation within only 3 h.[34)] This is because apatite has the lowest solubility and has thermodynamically the most stable phase among all the calcium phosphates in a neutral solution.[4)] Once apatite is nucleated, it grows spontaneously into an apatite layer.

Similarly to the modified biomimetic process, the new biomimetic process is applicable to various forms of polymeric materials (**Table 1**); three-dimensional porous scaffolds (**Fig. 2**(a)),[18),19)] fibers (Fig. 2(b)),[22)] and sponges (Fig. 2(c))[57)] as well as two-dimensional tabular plates. A primal advantage of the new biomimetic process over the modified biomimetic process is the simple and safe surface modification procedure that basically

Table 1. Polymeric Materials Applied to New Biomimetic Process for Apatite Coating

Polymer	Form of tested polymer	References
Ethylene-vinyl alcohol copolymer	Plate, Film, Fiber	22,31,58
Polyethylene	Plate	33
Poly(ethylene terephthalate)	Plate	33
Poly(ε-caprolactone)	Plate, Porous scaffold	18,19
Poly(L-lactide) (PLL)	Plate, Sponge	21,57
Poly(DL-lactide-co-glycolide)	Sponge	57
Polyglycolide	Sponge	57
Polystyrene (PS)	Plate	20

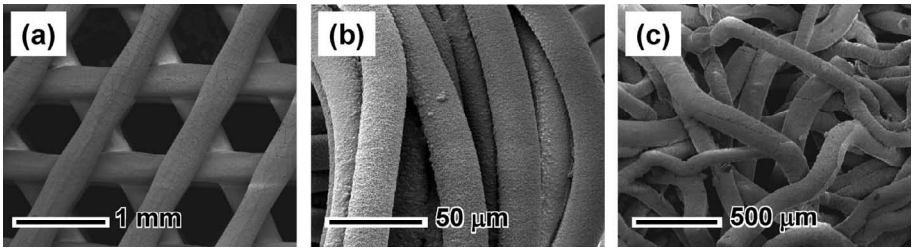

Fig. 2. Scanning electron microscopy images of bonelike apatite-coated poly(ε-caprolactone) scaffold (a), bonelike apatite-coated ethylene-vinyl alcohol copolymer fibers (b), and apatite-coated poly(DL-lactide-co-glycolide) sponge (c), all of which were prepared by new biomimetic process.

does not require the use of any toxic reagents, carried out at room temperature on an ordinary laboratory table, and accomplished easily within a short period. In addition, the new biomimetic process has a secondary advantage of applicability to various types of polymeric material including implantable polymers (Table 1), if the polymer surface is intrinsically hydrophilic or made to be hydrophilic by pretreatment.[18)–22)] For example, poly(L-lactide) (PLL) forms a bonelike apatite layer on its surface when it is treated with oxygen plasma prior to coating using SBF. The strength of adhesion between the polymer and the apatite layer formed on its surface can be improved by regulating the pretreatment conditions.[21),22)] In the case of PLL, its strength of adhesion to the bonelike apatite layer increases up to approximately 6 MPa by optimizing plasma power density in the oxygen plasma pretreatment (**Fig. 3**).[21)]

PLL is a biodegradable polymer used in bone fixation devices. If PLL is coated with a bonelike apatite layer by the process described above and implanted in a bone defect, it would integrate firmly with the surrounding bone tissue through the surface apatite layer. Hence the bonelike apatite-coated PLL has high potential as a bone fixation device with osteoconductivity.

Additional and representative examples of apatite-coated polymers with promising biomedical applications are as follows. A bonelike apatite-coated poly(ε-caprolactone) with a three-dimensional interconnecting porous structure (Fig. 2(a))[18),19)] would be useful as a bone tissue engineering scaffold with biodegradability, high affinity with bone cells, and osteoconductivity. An apatite-coated poly(DL-lactide-co-glycolide) sponge (Fig. 2(c)) has been found to produce calcified chronic total coronary occlusion lesions in animal coronary/peripheral arteries;[57)] hence, it would support the development of new treatments and surgical training for these challenging lesions. An apatite-coated polystyrene (PS) plate retains high transparency of the base PS and is hence useful as a cell culture substrate for observing in situ cell behaviors on the surface apatite layer.[20)]

3. Biomimetic process for biomolecule–apatite composite coating

A biomimetic process is useful not only for apatite coating, as was described in the previous section, but also for biomolecule–apatite composite coating.[28)–47)] We have applied our new biomimetic process to the biomolecule–apatite composite coating to develop third-generation biomedical materials.[48)] The immobilization of a biomolecule in the apatite layer is achieved by adding an intended biomolecule to the supersaturated calcium phosphate solution. For example, an apatite layer immobilizing a cell adhesion protein laminin (LAp layer) is formed if an ACP-modified polymer is immersed in a supersaturated calcium phosphate solution containing laminin.[31),33)] The biomolecule–apatite composite layer is formed heterogeneously on a polymer surface most likely through the spontaneous growth of apatite crystals and simultaneous adsorption of biomolecules onto the growing apatite crystals in the supersaturated solution.[34)] Consequently, the content of the biomolecule immobilized in the apatite layer strongly correlates with the adsorption affinity between the biomolecule and apatite; the content increases with increasing adsorption affinity.[42)]

We have paid particular attention to the LAp layer and examined its physicochemical and biological properties in detail. As determined by transmission electron microscopy observation, the laminin molecules were not localized but dispersively immobilized in a matrix of apatite crystals on the nanoscale in the LAp layer (**Fig. 4**).[34)] As determined by a mechanical test, the laminin molecules immobilized in the LAp layer increased the layer's shear strength under wet condition, probably owing to electrostatic interactions with the surrounding apatite crystals.[36)] As determined by an in vitro test, the laminin molecules retained their inherent cell adhesion property even in the LAp layer; they apparently enhanced adhesion and spreading of various cells on the layer (**Fig. 5**).[30),31),45),47)] Such a biological effect of laminin

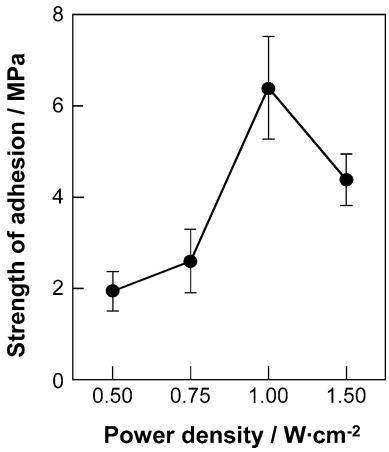

Fig. 3. Strength of adhesion between PLL plate and bonelike apatite layer formed on its surface by new biomimetic process, as function of power density of oxygen plasma pretreatment.

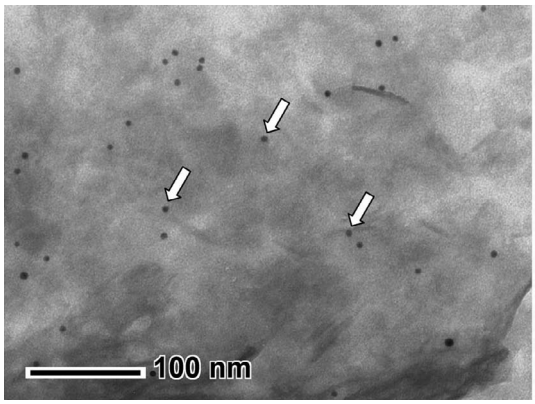

Fig. 4. Transmission electron microscopy image of ultrathin section prepared from LAp layer (laminin was immunologically stained with 5 nm colloidal gold (see arrows)).

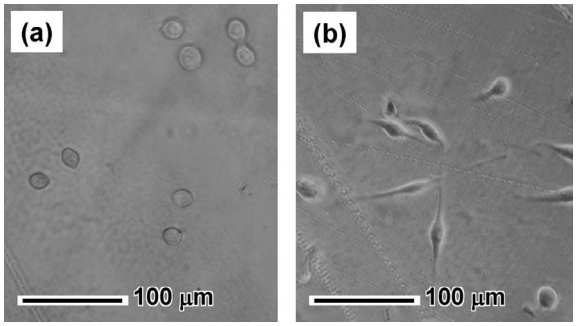

Fig. 5 Optical microscopy images of BHK–21 cells cultured on surfaces of apatite (a) and LAp (b) layers for 1 d.

is sustainable, because the surface concentration of laminin on the LAp layer does not decrease even after partial dissolution of the apatite matrix.[30] This is most likely due to the above-mentioned nanocomposite structure (see Fig. 4).

The LAp coating would be useful for percutaneous devices such as catheters and cannulas, because it is expected to facilitate integration between the device and the surrounding skin tissues, thereby reducing the risk of bacterial infection through the device–skin interface. As determined by a preliminary in vivo test,[58] the LAp-coated percutaneous implant successfully suppressed the host's foreign body response and integrated with the surrounding skin tissue that corresponds to a previously developed percutaneous device[59] made of apatite ceramics. Application studies of anti-infective percutaneous devices are currently in progress not only on this LAp coating but also on apatite coatings immobilizing antibacterial agents,[42] fibroblast growth factor-2,[38)–40] and/or ascorbate.[40),43]

Application studies of gene carriers for safe and efficient gene transfer into cells are also in progress using an apatite layer immobilizing both DNA and laminin (DLAp layer).[45),47] As determined by an in vitro gene expression assay, the DLAp layer showed excellent gene transfer efficiency as a nonviral gene delivery carrier; its efficiency was one to two orders of magnitude higher than that mediated by a previously developed DNA-immobilized apatite layer,[44] and equivalent to or greater than that mediated by a conventional lipid-based carrier.[45] The cell adhesion property of laminin in the DLAp layer is considered to be responsible for the increased efficiency of gene transfer. This consideration was confirmed by the experimental findings that gene transfer efficiency increased proportionally to the laminin content in the DLAp layer[45),47] and that the immobilization of fibronectin (another cell adhesion protein besides laminin) also increased gene transfer efficiency on the layer[46] whereas that of albumin (with no cell adhesion property) did not.[47] The role of the immobilized cell adhesion protein increasing gene transfer efficiency is considered to be as follows. Cell adhesion proteins enhance cell adhesion and cell spreading on the layer, as in the case of the LAp layer (see Fig. 5).[45)–47] The DNA molecules released from the layer are condensed in the thus-generated static interspace between the cell and the layer. Taking advantage of the high DNA concentration at the vicinity of the cell and the enlarged area of contact between the cell and the layer, DNA is efficiently transferred into the cell.

Utilizing the above-mentioned biomolecule–apatite composite coating, a multifunctional and customizable tissue engineering scaffold would be available. For example, with the DLAp coating, the scaffold is considered to gain good biocompatibility from apatite, cell adhesion property from laminin, and function as a gene carrier from DNA. In fact, a DLAp layer prepared using cDNA of nerve growth factor enhanced adhesion and neuronlike differentiation of PC12 cells on the layer.[47] This finding demonstrates that the DLAp coating is useful for a nerve tissue engineering scaffold. By selecting suitable DNA and cell adhesion proteins for immobilization in the apatite layer, the resulting composite can be customized to an appropriate scaffold for a variety of tissues and organs. A preliminary in vivo study on this type of composite has already started.

4. Conclusions

A safe and simple biomimetic process for apatite and biomolecule–apatite composite coatings on polymeric materials has been developed. The resulting composite possesses good biocompatibility owing to apatite and/or biological activity owing to the biomolecule. The new biomimetic process and the resulting composites have a wide variety of promising biomedical applications, as in the examples described above.

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Ayako Oyane is a senior researcher at the Nanotechnology Research Institute, AIST. She graduated from Kyoto University in 1997, received her Master of Engineering degree from Kyoto University in 1999, and received her Doctor of Engineering degree from Kyoto University in 2002. Then, she joined AIST as a researcher. Her current research interests lie in the development of apatite-based composites for biomedical applications.