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Review

Calcium phosphate composite layers for surface-mediated gene transfer

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ABSTRACT

In this review, the surface-mediated gene transfer system using calcium phosphate composite layers is described. Calcium phosphate ceramics are osteoconductive bioceramics used typically in orthopedic and dental applications. Additionally, calcium phosphate particles precipitated by a liquid-phase process have long been used as a safe and biocompatible transfection reagent in molecular biology. Recently, calcium phosphate composite layers immobilizing DNA were fabricated on the surfaces of base materials through a biomimetic process using supersaturated solutions. These composite layers possess useful characteristics of both osteoconductive bioceramics and transfection reagents; they thus provide a biocompatible surface to support cell adhesion and growth, and can stimulate the cell effectively via surface-mediated gene transfer. By modifying the fabrication conditions, physicochemical and biological properties of the composite layers can be varied. With such an approach, these composite layers can be designed to have improved affinity for cells and to exhibit increased gene transfer efficiency over that of conventional lipid transfection reagents. The composite layers with the increased gene transfer efficiency induced specific cell differentiation and tissue regeneration in vivo. These composite layers, given their good biocompatibility and the potential to control cell behavior on their surfaces, have great potential in tissue engineering applications.

1. Introduction

Calcium phosphates such as hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$; hereafter apatite) have been used as materials for osteoconductive bioceramics in orthopedic and dental applications. In addition, calcium phosphates have long been used as nonviral gene delivery carriers (transfection reagents) in molecular biology. The research on calcium phosphates in the fields of biomaterials and molecular biology has recently converged through the immobilization of DNA in calcium phosphate layers. The resulting calcium phosphate composite layers are endowed with useful characteristics of both osteoconductive bioceramics and transfection reagents; they thus provide a biocompatible surface to support cell adhesion and growth, and can stimulate the cell effectively via surface-mediated gene transfer.

This article summarizes a surface-mediated gene transfer system using calcium phosphate composite layers. In the first half of the article, we review the background research that is relevant to calcium phosphates in the fields of biomaterials (Section 2) and molecular biology (Section 3); in the second half (Section 4), we present a new gene transfer system using calcium phosphate composite layers and its potential application to tissue engineering, and comment on our recent research outcomes. Throughout the article, calcium phosphates synthesized by a liquid-phase process (without sintering) are considered to be "bioceramics" in a broad sense.

2. Calcium phosphates in bioceramics

2.1. Bioceramics: from the first to the third generation

When artificial materials are implanted inside the human body, they are generally recognized by the body as foreign substances and encapsulated by fibrous tissues [1]. First-generation biomaterials, developed during the 1960s and 1970s, were designed to achieve a suitable combination of physical properties to match those of the replaced tissue with a minimal toxic response in the host [1]. Examples of first-generation ceramic biomaterials are bioinert alumina and zirconia.

In the early 1970s, Hench et al. first discovered that some ceramics composed of Na_2O–CaO–P_2O_5–SiO_2, later named Bioglass®, spontaneously bond to living bone tissue without forming fibrous tissues [2,3]. Since then, other ceramics, including sintered apatite [4–6], sintered β-tricalcium phosphate [7], octacalcium phosphate [8,9] and glass–ceramic A-W containing apatite and wollastonite [10,11], have been shown to bond to living bone.

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Ceramics that exhibit bone-bonding ability (referred to as osteoconductivity or bioactivity) have emerged as an important subset of second-generation biomaterials [12]. Second-generation biomaterials are defined as biomaterials producing bioactive components that could elicit a controlled action and reaction in the physiological environment [12].

Significant efforts have been made to improve the performance of these osteoconductive bioceramics as hard tissue substitutes, including their osteoconductive activity, chemical stability (with the goal of making them permanently stable or bioresorbable at a suitable resorption rate) and mechanical properties. Earlier approaches were based mostly on physicochemical techniques such as porosity control, stoichiometry control and electrochemistry control. During the past few decades, biological approaches utilizing biofunctional substances, such as bone morphogenic protein-2 (BMP-2, cytokine effective in bone formation [13,14]), have also been extensively studied by taking advantage of the remarkable progress in molecular biology. In such biological approaches, a technique to immobilize biofunctional substances in or on bioceramics should be designed to minimize deactivation of the biofunctional substances and to allow their controlled release at the site of implantation. Bioceramics manufactured or modified to activate genes that stimulate regeneration of living tissues are categorized as third-generation biomaterials [15]. Third-generation biomaterials are defined as biomaterials stimulating specific cellular responses at the molecular level [15].

2.2. Calcium phosphate bioceramics

Human bone is mainly composed of apatite crystals deposited on organic collagen fibers that have been woven into a three-dimensional structure [16]. Sintered apatite [4–6] and sintered β-tricalcium phosphate [7], which are similar in composition to bone mineral, are currently the most often used osteoconductive bioceramics in orthopedic and dental applications. Both sintered apatite and β-tricalcium phosphate exhibit good biocompatibility and good osteoconductivity, and they have an established safety record in clinical applications. The critical difference between them lies in their solubility in the body's internal environment: sintered apatite is stable almost permanently (it is still present after many years), whereas sintered β-tricalcium phosphate is resorbable when implanted into bone defects [17].

However, similarly to other general ceramic materials, these calcium phosphate ceramics have significant disadvantages in their intrinsic mechanical properties (i.e. low fracture toughness and high Young's modulus [17,18]), which limits the scope of biomedical applications for which they are suitable. Due to this limitation, these calcium phosphate ceramics in the form of dense or porous bulk material have not been used under high load-bearing conditions, but have been used under low load-bearing conditions for repair and replacement of damaged hard tissues such as iliac crests, vertebrae, intervertebral discs and cranial bones [17].

2.3. Calcium phosphate coating

Although calcium phosphate in bulk ceramic form has a mechanical disadvantage (as described in Section 2.2), coating an artificial material with a thin calcium phosphate layer is an effective compensatory approach to provide the base material with good biocompatibility and good osteoconductivity. Physical calcium phosphate coating processes such as plasma spraying [19], sputtering [20] and pulsed laser deposition (laser abrasion) [21–23] are useful for thermally durable base materials; moreover, some of them are practical for use in metallic implants under high load-bearing conditions. Although the deposition rates of these physical processes are relatively high, it is difficult to obtain a uniform coating because of the thermally unstable character of calcium phosphates. In contrast, a biomimetic process [24–33] is a low-temperature chemical process that is useful for obtaining a uniform coating on various base materials, including organic polymeric materials.

Usually, biomimetic processes have involved surface modification of the base material with calcium phosphate seeds and/or functional groups that are effective for inducing calcium phosphate nucleation, such as Si–OH [24–27], Ti–OH [28,29], COOH [30,31], PO_3H_2 [32] and SO_3H [33]. The surface-modified material is then immersed in a supersaturated calcium phosphate solution, such as a simulated body fluid (SBF) [34–36], with ion concentrations, pH and temperature approximately equal to those of human blood plasma. As a result of this immersion step a dense and uniform calcium phosphate layer, typically composed of low-crystalline apatite, appears on the surface of the base material. Conventional biomimetic processes developed during the 1990s and 2000s have several drawbacks: limitations in the types or configurations of the base materials that can be used, the complex and time-consuming surface modification procedure and/or the necessity of toxic reagents for the surface modification steps. For example, with the biomimetic process first developed in the early 1990s [24,25], it was difficult to obtain a uniform coating over entire surface throughout the porous materials. This original biomimetic process was carried out by placing a base material on $CaO–SiO_2$-based glass particles in one SBF, then immersing it in another SBF. In the first step (the surface modification step), silicate ions released from the glass particles attach to the material surface, and Si–OH groups in the silicate ions induce heterogeneous nucleation of calcium phosphate on the surface. As a result of this, calcium phosphate seeds are formed on the material surface facing the glass particles. Accordingly, the coating is not formed on the other side of the material, nor is it formed on the entire surface throughout the porous materials.

Recently, we have developed a new biomimetic process [37–42] in which a base material is precoated with calcium phosphate seeds that consist of nanoparticles of amorphous calcium phosphate (ACP) [43] by a simplified alternate dipping treatment [44] before immersion in a supersaturated calcium phosphate solution. The ACP-assisted biomimetic process has the advantages of safety, simplicity and applicability to various types and configurations of base materials [42]. Fig. 1 shows representative examples of materials coated with an apatite layer using the ACP-assisted biomimetic process. As demonstrated in these images, the ACP-assisted biomimetic process can be applied not only to dense materials, but also to three-dimensional porous bodies (Fig. 1(a)) [37,38], knitted fibers (Fig. 1(b)) [41] and sponges (Fig. 1(c and d)) [45]. Even on such porous materials, a calcium phosphate layer is formed on the entire surfaces if the porosity is high enough to allow sufficient ion diffusion into internal pores. For example, when a poly(lactide-co-glycolide) sponge rod, with a porosity of 87% and a rod diameter of 3 mm, was subjected to the ACP-assisted biomimetic process, each individual fiber constituting the sponge was fully covered with an apatite layer, while the three-dimensional porous structure of the sponge was retained [45]. However, when the porosity was decreased to 60%, apatite formation inside the sponge declined due to the reduced ion diffusion into the internal pores. Generally, in a liquid-phase coating process as the ACP-assisted biomimetic process, the lower the porosity and the larger the bulk size of a material, the harder it is to obtain a uniform coating over the entire surface throughout the porous material.

A noteworthy feature of the biomimetic processes is its capability to immobilize biofunctional substances such as trace elements [46–48], proteins [49–56], antibacterial agents [57–59], coenzymes [60,61], DNA [62–68] and lipids [64,65,68] within the calcium phosphate layer on the nanoscale. More importantly, even

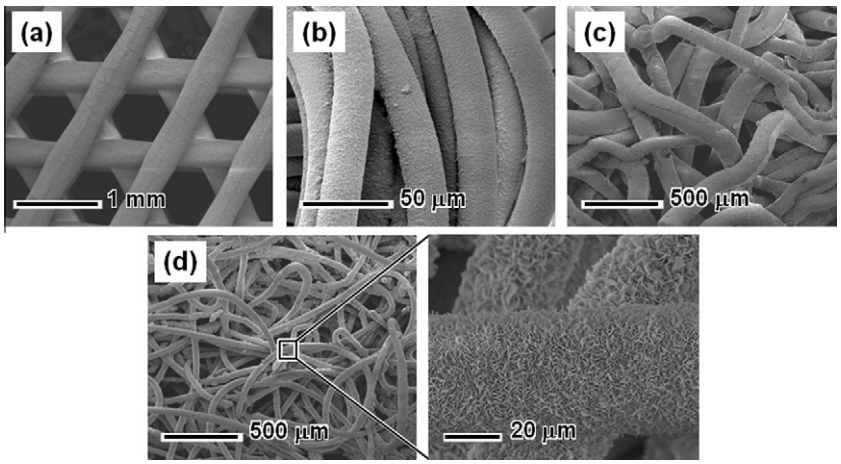

Fig. 1. Scanning electron microscopy images of (a) a poly(ε-caprolactone) porous body [37,38], (b) ethylene–vinyl alcohol copolymer (EVOH) fibers [41], (c) a poly(lactide-co-glycolide) sponge [45] and (d) a poly(L-lactide) sponge [45] at low (left) and high (right) magnifications, all of which were coated with an apatite layer by the ACP-assisted biomimetic process [42].

organic substances are not deactivated completely in the resulting composite layer, owing to the mild coating condition, which is similar to the physiological environment [42,50–69,71,72]. In addition, calcium phosphates formed by the biomimetic process have a significantly lower crystallinity and higher dissolution rate compared with sintered apatite, which enables the controlled release of immobilized substances from the composite layer [49,53,54,57–59,62,66,69–72]. Hence, biomimetic processes meet the demand for a fabrication technique for third-generation bioceramics that immobilize biofunctional substances.

In this article, we describe the use of DNA as a biofunctional substance and explore the biomedical potential of the DNA–calcium phosphate composite layers prepared using biomimetic processes – typically the ACP-assisted biomimetic process. More details on the ACP-assisted biomimetic process and calcium phosphate layers that immobilize other biofunctional substances have been described in prior articles [42,69] (see also Section 4.4 for the ACP-assisted biomimetic process).

3. Calcium phosphate for gene transfer

3.1. Transfection reagents: materials for gene transfer

Gene transfer, or transfection, is a fundamental technique in molecular biology used to introduce foreign genes (DNA, RNA) into cells, to manipulate cells or to study gene function and protein expression in vitro. Initially, the gene transfer technique was envisioned to transform cells utilizing viral vectors [73,74] by inserting a functional gene into a nonspecific site within the genome. Although a viral system is the most efficient method of gene transfer, it suffers from a number of drawbacks, including toxicity (immunogenicity and oncogenicity), overexpression of the transgene, and difficulties in handling and large-scale production [74,75]. To overcome these drawbacks, a variety of nonviral transfection reagents, such as calcium phosphates [76,77], lipids (either liposomal or nonliposomal type) [78], and cationic polymers, including diethylaminoethyl dextran [79] and poly(ethylene-imine) (PEI) [80] have been developed since the late 1960s. Great efforts have been directed to increase the gene transfer efficiency and to minimize the toxicity of these nonviral transfection reagents by tuning their molecular structures and/or by combining them with other biofunctional molecules [77,81]. A variety of synthetic transfection reagents, including the liposomal transfection reagent Lipofectamine (Invitrogen, USA), the nonliposomal lipid transfection reagent FuGENE HD (Roche, Switzerland), the dendrimer-based transfection reagent Superfect (Qiagen, USA), the PEI-based transfection reagent jetPEI (Polyplus-transfection, USA) and the calcium phosphate transfection reagent Profection (Promega, USA), have been commercialized for in vitro gene transfer [82].

These nonviral transfection reagents are generally less effective in vivo than in vitro due to adverse reactions that occur in the body's internal environment, such as inflammation, interaction with serum proteins and DNA degradation. Recently, some nonviral transfection reagents optimized for in vivo gene transfer have also been commercialized (e.g. in vivo jetPEI). However, the use of such in vivo transfection reagents, which are based on synthetic organic molecules, is still controversial owing to their toxicity and insufficient efficiency, i.e. the larger the amount of transfection reagent used to increase transgene expression, the greater the adverse side effects on the surrounding tissues. Safer and more efficient nonviral transfection reagents are still being sought.

3.2. The gene transfer system: methodology of gene transfer

Tissue engineering aims to regenerate or even create tissues in vitro or in vivo utilizing three essential components: the cell, a scaffold and a biofunctional substance [83]. Nonviral gene transfer techniques can be applied in tissue engineering as a tool to regulate cell behavior such as proliferation and differentiation utilizing genes as the biofunctional substance. In such applications, the construction of a suitable system of gene transfer, i.e. how, where and when to mediate gene transfer, is necessary to ensure efficient and effective interactions among the cell, scaffold and gene [84].

Conventional gene transfer systems are mediated by particulate transfection reagents in complex with genes (usually DNA). The gene complexes are either added onto cells that have been precultured in a medium (in vitro gene transfer, see Fig. 2, left) or injected into intended sites of the body by parenteral administration (in vivo gene transfer). Some of the gene complexes are then taken up by cells upon contact with the cell surfaces via endocytosis and/or membrane fusion. However, such particle-mediated systems lack spatial control of genes. For example, these systems are not effective for all the cells on a three-dimensional porous scaffold, nor do they allow area-specific gene transfer at an intended location of a scaffold because particulate transfection reagents easily disperse in a culture medium or in body fluids. Furthermore, the dispersion of transfection reagents is problematic, especially in in vivo gene transfer, because of its association with the risk of undesired gene transfer to unintended cells and of the accumulation of transfection reagents within organs such as the liver, spleen and lung.

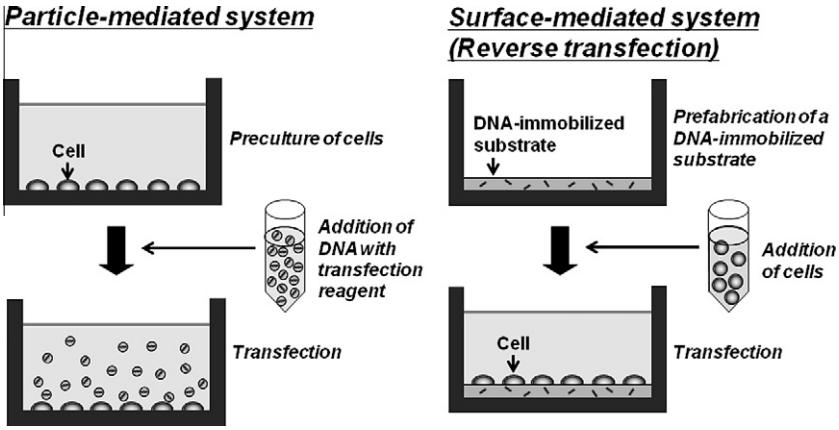

Fig. 2. Schematic illustration of particle-mediated (left) and surface-mediated (right) gene transfer systems using calcium phosphates.

Since the early 1980s, gene transfer systems mediated not by transfection reagents but by physical stimulations have been developed [85–87]. Such physical gene transfer systems allow genes to penetrate directly through cell membranes into the cell utilizing physical stimulations. For example, electroporation [85], microinjection [86] and biolistic particle delivery [87] are carried out utilizing stimulations of electric pulses, fine needle puncture and high-pressure gas (in combination with gold particles), respectively. These physical systems are effective for single or multiple target cells at an intended location, and carry little risk of dispersion of transfection reagents. However, they present several drawbacks, including damage to cells, a difficulty in large-scale manipulation, labor-intensive protocols and/or the necessity of costly apparatus.

Since around 2000, new gene transfer systems mediated by substrates immobilizing DNA alone or in combination with transfection reagents have been developed [62–68,88–96]. In this system, cells are added onto prefabricated substrates that immobilize DNA and, in most cases, the transfection reagent as well (see Fig. 2, right). DNA released from the substrate is then taken up by cells adhering to the substrate. These systems are called surface-mediated gene transfer systems, or reverse transfection systems (the latter term reflecting that the order of the addition of DNA and cells is reversed compared to the conventional system [89]). DNA can be adsorbed or bonded onto the surfaces of substrates utilizing physicochemical and biological interactions [89,92,94,97]. DNA can also be impregnated into polymer hydrogels (collagen, alginate, chitin, etc.) [90,93,95,96] or immobilized into biodegradable matrixes, such as bioresorbable polymers [88,91] and low-crystalline calcium phosphate layers [62–68]. Some of these surface-mediated systems have been applied in transfection microarrays used for the assessment of gene and cell functions (e.g. Transfection Array™, CytoPathfinder, Inc., Japan).

In this article, we focus on a surface-mediated gene transfer system using low-crystalline calcium phosphate layers as the immobilization matrix for DNA. Surface-mediated gene transfer systems using PEI and lipid transfection reagents are described in more detail in a recent review by He et al. [84].

3.3. The conventional calcium phosphate gene transfer system

The conventional calcium phosphate gene transfer system is mediated by particulate calcium phosphate coprecipitated with DNA. In the early 1970s, Graham et al. first conducted systematic research on this calcium phosphate gene transfer system [76]. Since then, the calcium phosphate system has been widely used as an easy-to-use, cost-effective and highly safe gene transfer technique [77]. Critical disadvantages of the calcium phosphate gene transfer system are insufficient efficiency and poor reproducibility compared with other nonviral systems [77].

The conventional fabrication process [98] of calcium phosphate transfection reagents is as follows. First, DNA is added to a calcium ion solution to make complexes of negatively charged DNA molecules and positively charged calcium ions. Second, the prepared complex solution is mixed with a phosphate ion solution at neutral pH in a controlled manner to allow the precipitation of calcium phosphates. The reaction mixture is then incubated for a certain period of time (several tens of minutes) to age of the calcium phosphate precipitates before adding them to precultured cells for transfection. In general, homogeneous nucleation of calcium phosphate is induced in the solution promptly after mixing the calcium and phosphate ion solutions because the concentrations of calcium (e.g. 125 mM [98]) and phosphate (e.g. 0.75 mM [98]) ions in the reaction mixture are much higher than the saturation level of calcium phosphates. The initial calcium phosphate precipitated under such a highly supersaturated condition is likely to be in a nonapatitic phase [99,100]. The nonapatitic calcium phosphate precipitate spontaneously grows and undergoes several steps of transformation and maturation during the aging and/or transfection processes, as has been observed by transmission electron microscopy [101]. These reactions spontaneously occur due to the highly supersaturated condition of the reaction mixture and the cell culture medium with respect to multiple calcium phosphate phases, including ACP, octacalcium phosphate and apatite. Regardless, all of the calcium phosphates spontaneously convert into apatite with time because apatite is thermodynamically the most stable phase under physiological conditions [102,103]. The initially formed apatite is most likely to be low-crystalline and calcium-deficient apatite, which then undergoes further maturation into a more highly crystalline apatite with a more stoichiometric composition [102,103].

During the sequential reactions of homogeneous nucleation, growth, phase transformation and maturation during the aging and/or transfection processes, the calcium phosphate precipitate increases in size. In addition, different calcium phosphate phases have different physicochemical properties, such as solubility and lattice structure [102,103]. Therefore, the low reproducibility of the calcium phosphate system is quite natural. In fact, the gene transfer efficiency of the calcium phosphate system is largely dependent on the mixing speed, composition, pH and temperature of the reaction mixture [77,98,104–120], all of which affect the kinetics of the calcium phosphate precipitation process. For example, a very small change in pH (±0.1) of the reaction mixture can compromise the efficacy of calcium phosphate transfection reagents [98,111]. The aging time of the reaction mixture before transfection also has a significant effect on gene transfer efficiency

because the efficiency is largely dependent on particle size [105–108,112,113].

To address the limitations of calcium phosphate transfection reagents described above, various approaches, such as the reverse emulsion technique [114,115] and supplementing the reaction mixture with ions [116,117] and block copolymers [118] that act to inhibit crystal growth, have been proposed to regulate the particle size and crystal structure. The combination of calcium phosphate particles with other nonviral transfection reagents [119] or cell adhesion proteins [120] have also been proposed to regulate cell–material interactions. An architectural change in the calcium phosphate transfection reagent from a particulate to a laminar configuration is another approach to accomplish efficient and stable gene transfer, as will be described in the next section.

4. Gene transfer using calcium phosphate composite layers

Calcium phosphate composite layers immobilizing DNA are categorized as third-generation bioceramics (see Section 2). These composite layers can serve as a substrate for the surface-mediated gene transfer system, as mentioned in Section 3.2. These composite layers with good biocompatibility and gene transfer capability have been the subject of increasing attention in tissue engineering applications as surface components of scaffolds. In this section, a surface-mediated gene transfer system using calcium phosphate composite layers and its potential application to tissue engineering will be reviewed.

4.1. DNA–calcium phosphate composite layers

Pioneering work on the surface-mediated gene transfer system using a DNA–calcium phosphate composite layer was reported by Shen et al. in 2004 [62]. In this system, cells are seeded on a composite layer that was previously fabricated on the well of a culture plate (Fig. 2, right). During the culture, the composite layer supports cell adhesion and growth on its surface with minimal toxicity. At the same time, the composite layer releases DNA molecules into the surrounding medium through interactions with the medium [62]. The interactions at the composite surface may involve the dissociation of the fractured composite layer and DNA desorption and diffusion associated with the phase transformation, maturation and/or partial dissolution of the calcium phosphate matrix. As a result of the DNA release, regions of high DNA concentration are provided locally near the surface of the composite layer, and this encourages DNA uptake by the cells adhering to the layer.

The significance of Shen et al.'s work is not only the novelty of the gene transfer system but also the relatively high gene transfer efficiency despite it being a nonviral system that does not use any other transfection reagents. Experimental results showed that the gene transfer system mediated on the DNA–calcium phosphate composite layer was as efficient as a conventional particle-mediated system using a commercial lipid transfection reagent [62]. The DNA molecules released from the composite layer should be in the form of complexes with calcium ions, nanosized calcium phosphate clusters and/or submicron- or micron-sized calcium phosphate particles. Nanosized calcium phosphate clusters have been detected in supersaturated calcium phosphate solutions, including SBF [121], using dynamic light scattering [121–123]. Particles with a larger size would be formed as a result of the dissociation of the fractured composite layer and/or the remineralization due to the elevated calcium and phosphate ion concentrations caused by the partial dissolution of the composite layer. These calcium-containing species should take a facilitating role in the cellular uptake of DNA by increasing the affinity of negatively charged DNA molecules to the negatively charged cell surface and by enhancing endocytosis [124–126].

Rao et al. [127] recently reported that the presence of a calcium phosphate layer increases the efficiency of gene transfer on its surface in the conventional particle-mediated system using PEI. This increase in gene transfer might be due to the aforementioned reactions, such as dissociation, dissolution and remineralization, which could occur on the calcium phosphate layer.

4.2. DNA–cell adhesion protein–calcium phosphate composite layer

The DNA–calcium phosphate composite layer described above has a relatively low affinity to cells, especially to non-bone cells. We have previously succeeded in improving the affinity of a calcium phosphate layer to cells by immobilizing a cell adhesion protein, laminin, in the layer [50,51]. According to the optical microscopic observation, the density of the cells adhering to the laminin–calcium phosphate composite layer after 2 h of culture was 3–10 times higher than that of the cells adhering to the calcium phosphate layer [50,51]. On the basis of this background knowledge, we immobilized both DNA and laminin in the calcium phosphate layer [63]. The immobilization of laminin in the DNA–calcium phosphate composite layer not only improved the affinity to cells (Fig. 3, see optical microscopy images), but also increased the gene transfer efficiency of the layer by 1–2 orders of magnitude [63]. For some cells, the gene transfer efficiency of the DNA-laminin–calcium phosphate composite layer was equivalent to, or even an order of magnitude higher than, a conventional particle-mediated system using a commercial lipid transfection reagent [63].

The cell adhesion property of laminin is considered to be responsible for the increased gene transfer efficiency of the DNA-laminin–calcium phosphate composite layer over that of the laminin-free composite layer. This consideration was confirmed by our own experimental results showing that the immobilization of fibronectin (another cell adhesion protein) into the composite layer also increased gene transfer efficiency by 1–2 orders of magnitude [67], whereas that of albumin (a non-cell adhesion protein) did not [66]. Moreover, the gene transfer efficiency increased proportionally with the laminin [66] or fibronectin [67] content of the composite layer.

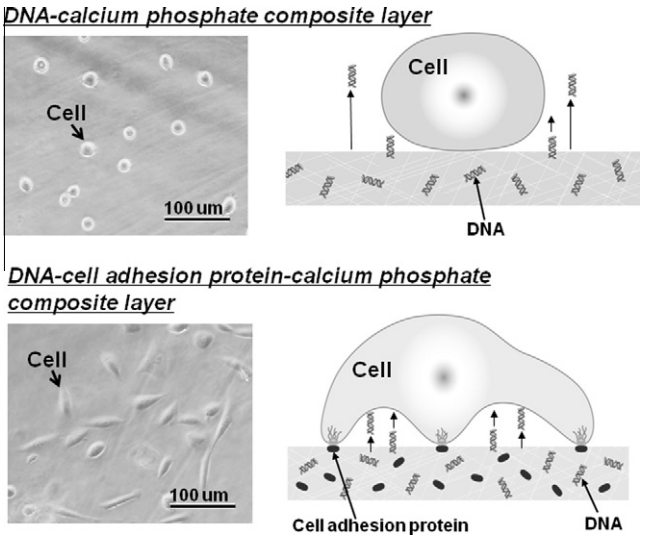

Fig. 3. Optical microscopy images (left) and schematic illustrations (cross-section view) (right) of the CHO-K1 cells cultured for 1 day on the surfaces of DNA–calcium phosphate composite (upper) and DNA–cell adhesion protein (fibronectin)–calcium phosphate composite (lower) layers [67].

The mechanism by which the cell adhesion proteins affect the efficiency of gene transfer has been proposed as follows [66]. Without cell adhesion protein in the composite layer, the area of contact between a cell and the layer is relatively small (Fig. 3, top). Consequently, the DNA molecules released from the composite layer easily diffuse into the medium surrounding the cell. In this situation, it is difficult to retain the released DNA molecules within regions near the cell surface for a long time. On the other hand, with cell adhesion proteins in the composite layer, cells are well spread and flattened on the layer through interactions with integrins and nonintegrin receptors on the cell surface (Fig. 3, bottom). As a result, a static microenvironment with low fluidity is generated at the interface between the cell and the composite layer. Thus, the DNA molecules released from the composite layer are condensed with time in this static microenvironment because the release of DNA continues (for longer than 24 h [62,66]) even after the cells have fully adhered and spread (within a few hours). As a consequence of the enlarged area of contact between the cell and the layer and the increased DNA concentration in this interfacial microenvironment, a higher gene transfer efficiency was accomplished on the composite layer co-immobilizing cell adhesion proteins. Cell adhesion proteins released together with DNA from the composite layer might also have a facilitating role in gene transfer by enhancing the cellular uptake of DNA in a manner similar to that reported for conventional particle-mediated systems using calcium phosphate [120], virus [128–130] or lipid [131] in combination with cell adhesion molecules.

4.3. DNA–lipid–calcium phosphate composite layers

Both Sun et al. [64] and Luong et al. [65] have shown that the immobilization of lipid transfection reagents into the DNA–calcium phosphate composite layer is also effective in improving the gene transfer efficiency of the layer. For example, the gene transfer efficiency of the DNA–lipid–calcium phosphate composite layer was shown to be approximately 7% (the ratio of stained cells showing β-galactosidase expression to the total number of initially seeded cells), whereas that of the DNA–calcium phosphate composite layer was less than 0.1% [65]. Such an increase in gene transfer efficiency is likely caused by the lipids complexed with DNA: the lipids can bind to DNA, protect the DNA from degradation and enhance the cellular uptake of DNA when released from the composite layer [65]. It has been shown that lipid transfection reagents enhance the cellular uptake of DNA because of their strong ability to fuse with the endosomal membrane of a cell [132].

Luong et al. [65] demonstrated colocalization of DNA and the lipid transfection reagent in the composite layer by fluorescent staining. This colocalization occurs because lipid transfection reagents form stable complexes (lipoplexes) with DNA molecules through electrostatic interactions between positively charged lipids and negatively charged DNA molecules [133]. The authors also revealed that a DNA–lipid–calcium phosphate composite layer fabricated by a biomimetic process (a coprecipitation process) showed 3–7 times higher gene transfer efficiency than a calcium phosphate layer with a superficially adsorbed lipoplex [65]. This result might derive from the presence of lipoplexes through the thickness of the DNA–lipid–calcium phosphate composite layer fabricated by the biomimetic process, which would enable a slow and sustained release of lipoplexes from the composite layer (see also Section 4.5).

More recently, we fabricated DNA–lipid–calcium phosphate composite layers under different preparation conditions using various types of lipid transfection reagents [68]. The gene transfer efficiency of the DNA–lipid–calcium phosphate composite layer was found to be largely dependent on the type of lipid and the lipoplex content of the composite layer. The composite layer prepared under the optimized conditions showed approximately 4 and 1 orders of magnitude higher gene transfer efficiency than the DNA–calcium phosphate composite layer and the DNA–fibronectin–calcium phosphate composite layer, respectively [68]. The gene transfer efficiency achieved with the DNA–lipid–calcium phosphate composite layer was 2–3 orders of magnitudes higher than that achieved with a conventional particle-mediated system using commercial lipid transfection reagents in the presence of serum [68]. Given these results, the gene transfer system using the DNA–lipid–calcium phosphate composite layer is highly efficient as a nonviral system; hence, this system has great potential in tissue engineering applications. However, before applying this system to in vivo tissue engineering, one should take into account the toxicity of the lipid transfection reagents, although the risk of dispersion and accumulation in other parts of the body is relatively low compared with a conventional particulate-mediated system.

4.4. Fabrication of the composite layers: biomimetic process

Shen et al. [62] fabricated a DNA–calcium phosphate composite layer on the well surface of a tissue culture plate using supersaturated calcium phosphate solutions similar to SBF as the coating solutions (see also Sun et al. [64]). For the purpose of tissue engineering applications, we fabricated calcium phosphate composite layers on the surfaces of various base materials (i.e. not in a tissue culture plate) utilizing the ACP-assisted biomimetic process [42,63,66–68,134]. In addition, we employed a simplified supersaturated calcium phosphate solution (CP solution: 142 mM NaCl, 3.75 mM $CaCl_2$, 1.5 mM $K_2HPO_4{\cdot}3H_2O$, pH 7.40, 25 °C [50–52,63,66–68,134,135]) as the coating solution. The CP solution was designed to accelerate calcium phosphate precipitation by increasing the concentrations of calcium and phosphate and by reducing or excluding other additional ions, such as magnesium, sulfate and carbonate, from the conventional SBF [34–36]. In addition, the temperature of the CP solution was decreased from 36.5 °C (SBF) to 25 °C to minimize the denaturation of biofunctional macromolecules such as DNA and to stabilize the solution. In fact, the CP solution is a metastable solution that does not induce homogeneous calcium phosphate nucleation during the fabrication of calcium phosphate composite layers [136]. This is a critical difference from the fabrication process of particulate calcium phosphate transfection reagents, which is initiated by homogeneous nucleation.

Fig. 4 shows a schematic of the ACP-assisted biomimetic process of a DNA–cell adhesion protein–calcium phosphate composite layer onto an ethylene–vinyl alcohol copolymer (EVOH) plate. The ACP-assisted biomimetic process involves the ACP precoating step and the subsequent coprecipitation step: immersion in a supersaturated calcium phosphate solution. The ACP precoating onto the surface of the EVOH plate (usual size: 1 mm × 10 mm × 10 mm) is carried out by a simplified alternate dipping treatment [44] that involves dipping the plate for 10 s alternately in 200 mM $CaCl_2$ and 200 mM $K_2HPO_4{\cdot}3H_2O$ aqueous solutions (usual volume: 20 ml), three times each [43]. The ACP-precoated plate is subsequently immersed in a coating solution (usual volume: 3 ml): the CP solution supplemented with DNA (plasmid) alone or together with other biofunctional macromolecules, such as cell adhesion protein or lipid. Upon contact with the coating solution, which is supersaturated with respect to calcium phosphates, the ACP nanoparticles on the EVOH surface act as seeds: they spontaneously grow and/or induce secondary nucleation of calcium phosphates by consuming calcium and phosphate ions and/or calcium phosphate clusters [121–123] in the coating solution. Macromolecules such as DNA in the coating solution are immobilized onto the calcium phosphates during their growth through electrostatic interactions [137]. In addition, the immobilized macromolecules may induce secondary nucleation of calcium phosphates [138,139]. The formation of a

DNA–calcium phosphate composite layer may also involve the transformation of calcium phosphate phases [140,141], as was observed for particulate calcium phosphate transfection reagents [101]. As a result of such a sequence of reactions involving heterogeneous nucleation, growth and phase transformation of calcium phosphates and simultaneous immobilization of macromolecules onto the calcium phosphates, calcium phosphates and macromolecules are coprecipitated on the surface of the EVOH plate.

Besides the ACP-assisted biomimetic process described above, other biomimetic processes involving different surface modification techniques can be used in the fabrication of calcium phosphate composite layers on base materials. Note that surface modification is unnecessary for some osteoconductive materials (such as apatite ceramics) that intrinsically have nucleation sites for calcium phosphates on their surfaces [134,135]. For other general base materials without nucleation sites, surface modification is required to induce heterogeneous calcium phosphate nucleation at the surface during the subsequent immersion step in a supersaturated calcium phosphate solution. Various surface modification techniques have been proposed to create nucleation sites, i.e. calcium phosphate seeds and/or functional groups that act as nucleating agents (Si–OH [24–27], Ti–OH [28,29], etc.). For example, Luong et al. [65] precoated a polymer film with calcium phosphate seeds by etching the film with 0.5 M NaOH aqueous solution for 7 min and then immersing it for 3–4 days in a modified SBF that has calcium and phosphate ion concentrations that are twice as high as those of SBF. Uchida et al. [50,52] formed a sodium titanate layer rich in Ti–OH groups on the surface of a titanium plate using Kim et al.'s technique [28]: treating the plate with 5 M NaOH aqueous solution at 60 °C for 24 h and then heating it at 600 °C for 1 h. Such a surface modification technique should have a critical effect on the growth kinetics of a composite layer in the subsequent immersion step in a supersaturated calcium phosphate solution, especially at the initial stage. This is because calcium phosphate seeds and functional groups have different nucleation kinetics depending on their formula, arrangement and density at the surface [42,102,103]. Consequently, the resulting calcium phosphate composite layers could have different physicochemical properties (e.g. thickness, content of macromolecules) even when the coating condition after the surface modification was the same [134]. The surface modification technique also has a significant effect on the strength of adhesion between the base material and the calcium phosphate composite layer formed on its surface [40,41]. The effect of the surface modification on the gene transfer efficiency of the calcium phosphate composite layer is yet to be elucidated.

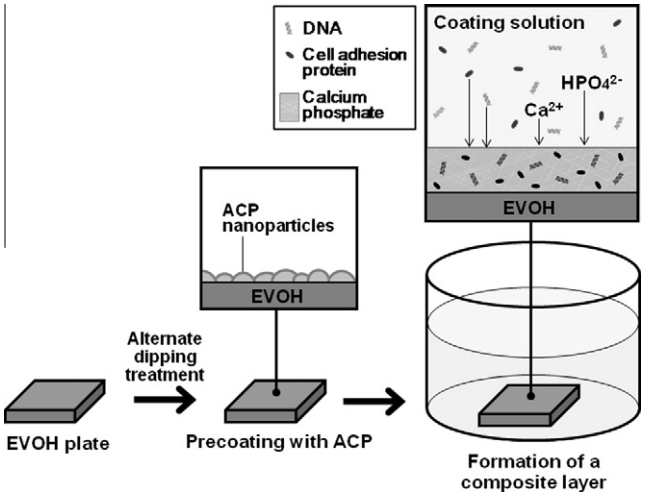

Fig. 4. Schematic illustration of the ACP-assisted biomimetic process to fabricate a DNA–cell adhesion protein–calcium phosphate composite layer on an EVOH plate [42].

The primary advantage of the ACP-assisted biomimetic process over other biomimetic processes is a simple and safe surface modification procedure that basically does not require the use of any toxic reagents, can be carried out at room temperature on an ordinary laboratory table and can be accomplished easily within a short period of time (several tens of minutes) [42]. Additional advantages are its applicability to various types and configurations of base materials, and the relatively high and controllable strength of adhesion between the base material and the calcium phosphate composite layer formed on its surface [42]. With appropriate adjustment of the ACP-precoating conditions, calcium phosphate composite layers can be fabricated on various types of base materials [42], including polyethylene [142], poly(ethylene terephthalate) [142], poly(ε-caprolactone) [37,38] and poly(L-lactide) [40], all of which are implantable biomaterials. Furthermore, these materials can take configurations of three-dimensional porous bodies [37,38], fibers [41] and sponges [45], which are all suitable as scaffold structures (see Fig. 1). In addition, the strength of adhesion between the base material and the calcium phosphate layer formed on its surface is tunable by regulating the precoating conditions [40,41]. For example, in the case of poly(L-lactide), the strength of adhesion to the apatite layer fabricated by the ACP-assisted biomimetic process was increased to approximately 6 MPa by optimizing the precoating conditions [40]. These findings show that the ACP-assisted biomimetic process is truly useful for the fabrication of calcium phosphate composite layers on tissue engineering scaffolds.

4.5. Structure of the composite layers

The DNA–calcium phosphate composite layer fabricated on an EVOH plate by a typical ACP-assisted biomimetic process (24 h after immersion in 3 ml of CP solution supplemented with 40 g ml^{-1} DNA) is a few micrometers thick, has a submicroscale cavernous structure (Fig. 5, left) and contains 14 ± 4 g cm^{-2} DNA, 120 ± 7 g cm^{-2} calcium and 64 ± 4 g cm^{-2} phosphorus [66]. The calcium phosphate phase was identified as low-crystalline apatite (Fig. 5, right [63,66–68]), with an octacalcium phosphate-like character [143], by thin-film X-ray diffraction and Fourier transform infrared spectroscopy.

Immobilization of cell adhesion proteins [63,66,67] and lipids [64,65,68] into the DNA–calcium phosphate composite layer is possible by adding these macromolecules to the coating solution at particular concentrations. It should be noted that the mechanism of immobilization of macromolecules (DNA, cell adhesion proteins, lipids) in the calcium phosphate layer is completely different from ion substitution, which is a well-known phenomenon for calcium phosphates: these macromolecules are not incorporated

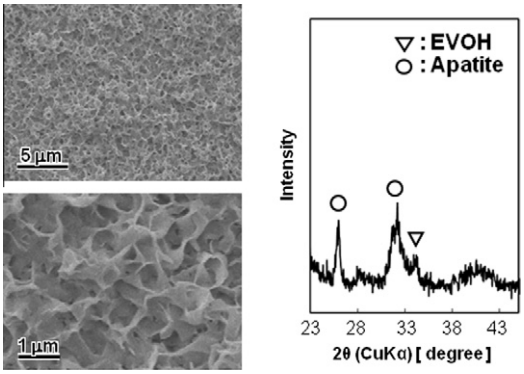

Fig. 5. Scanning electron microscopy images at low (top left) and high (bottom left) magnifications and the thin-film X-ray diffraction pattern (right) of the surface of the DNA–calcium phosphate composite layer, fabricated on an EVOH plate after immersion for 24 h in 3 ml of the CP solution supplemented with 40 μg ml^{-1} DNA [66].

within the lattice of calcium phosphate crystals because they are much larger than the component ions of calcium phosphates [144]. In fact, Okazaki et al. [145] reported that lattice parameters of DNA–apatite composites precipitated in mixture solutions of 0.5 l of 100 mM $Ca(CH_3COO)_2H_2O$, 0.5 l of 60 mM $NH_4H_2PO_4$ and 1 l of 1.3 M CH_3COONH_4 containing 0.1–1.0 g l^{-1} DNA were comparable to those of apatite precipitated in the DNA-free mixture solution. Macromolecules such as DNA are most likely present in the labile environment on the calcium phosphate crystal surface [146,147], and embedded in the interstices of calcium phosphate crystals in the composite layer. Additionally, it is considered that these macromolecules are immobilized within the layer through the thickness, and the composite layer has a matrix-filler-type nanocomposite structure [136,148]. This consideration is supported by our previous transmission electron microscopy [136] and X-ray photoelectron spectroscopy [50] studies on a laminin–apatite composite layer fabricated by the ACP-assisted biomimetic process. Transmission electron microscopy revealed that laminin molecules are distributed on the nanoscale throughout the entire matrix, which is composed of needle-like apatite nanocrystals in the laminin–apatite composite layer [136]. The X-ray photoelectron spectroscopy revealed no decrease in laminin concentration at the composite surface even after partial dissolution of the composite layer [50].

According to previous reports, calcium and phosphate ions of apatite interact with carboxyl (and/or phosphate groups in the case of phosphoprotein) and amino groups of proteins, respectively [144]. Calcium ions of apatite also interact with phosphate groups of DNA [144,145]. It is considered that such electrostatic interactions are involved in the coprecipitation of calcium phosphates and macromolecules during the immersion step in a supersaturated calcium phosphate solution, and the molecular structure of the macromolecules in the composite layer is stabilized through the chemical bonding with the surrounding calcium phosphate crystals. Possibly because of such electrostatic interactions and a matrix-filler-type composite structure, a relatively high content of macromolecules is stably immobilized in the calcium phosphate composite layer, and this may be responsible for the slow and sustained release of macromolecules from the composite layer. In the DNA–laminin–apatite composite layer, for example, DNA (12 ± 5 g cm^{-2}) was immobilized so stably that only approximately 7% of the immobilized DNA was released into a pseudophysiological solution even after incubation for 24 h [66].

4.6. Control of gene transfer on the composite layers

The gene transfer efficiency of the composite layer varies, depending on the type of cell lines, the culture conditions (presence of serum, culture period, etc.) and the fabrication conditions of the composite layer [62–68,143]. Sun et al. [64] fabricated DNA–calcium phosphate composite layers using six types of supersaturated calcium phosphate solutions, and optimized the gene delivery to the cell types of interest.

The fabrication conditions (besides the surface modification condition) affecting gene transfer efficiency include the concentrations of inorganic ions, DNA and other supplemental macromolecules (cell adhesion protein or lipid) in the coating solution (supersaturated calcium phosphate solution), as well as the immersion period in the coating solution [62–68,143]. These factors affect the gene transfer efficiency most likely by affecting the DNA conformation, the content of the DNA and the supplemental macromolecules in the composite layer, their release profile from the layer and/or the physicochemical properties (e.g. particle size, surface charge and dissolution kinetics) of the DNA complexes released from the layer [62–68,143]. It has been reported that a longer immersion period causes a change in the DNA conformation from the supercoiled to the nicked circular form (half of the supercoiled DNA changed into the nicked circular form within 48 h), and this could lead to lower gene transfer efficiency of the composite layer [65]. It has also been suggested that sufficient solubility of the composite layer near the phagosomal pH may support the release of endocytosed DNA complexes from phagosomes, thus leading to higher gene transfer efficiency [64]. Higher contents of DNA, cell adhesion protein and lipid in the composite layer generally lead to higher gene transfer efficiency [63,66–68]. According to our systematic study on a DNA–laminin–apatite composite layer, both the DNA and laminin content of the composite layer affected the gene transfer efficiency of the layer, but the laminin content had a more dominant effect [66].

Recently, we proposed a unique approach to control gene transfer on a calcium phosphate composite layer based on crystal chemistry [143]. In this approach, the gene transfer efficiency was controlled by incorporating carbonate or fluoride ions into the DNA–fibronectin–apatite composite layer via ionic substitution of the apatite crystals. Carbonate and fluoride ions have been known to be present in trace amounts in the apatite of the human bone tissue and to affect the dissolution rate of apatite. Carbonate and fluoride ions were incorporated into the composite layer by adding them to the coating solution (the CP solution supplemented with 40 g ml^{-1} DNA and 10 g ml^{-1} fibronectin) in the ACP-assisted biomimetic process [143]. The addition of 1 mM carbonate ions to the coating solution decreased the crystallinity of the apatite (Fig. 6, left) and increased the dissolution rate of the composite layer, thereby trebling the efficiency of gene transfer of the layer (Fig. 6, right) [143]. In contrast, the addition of 1 mM fluoride ions to the coating solution increased the crystallinity of apatite (Fig. 6, left) and decreased the dissolution rate of the composite layer, thereby decreasing the efficiency of gene transfer of the layer by 3 orders of magnitude (Fig. 6, right) [143]. It should be noted here that a higher dissolution rate of the composite layer does not always increase the gene transfer efficiency [62]; rather, there is an optimum range. The addition of fluoride ions to the coating solution also delayed the timing of gene transfer on the composite layer in a dose-dependent manner [143]. For example, the addition of 10 μM fluoride ions to the coating solution delayed the peak stage of gene expression in the cells cultured on the composite layer from 1 to 5 days [143]. The expression of specific transcriptional factors at appropriate times is important for the effective induction of cell differentiation [149,150]. Therefore, a gene transfer system using these calcium phosphate composite layers that has the advantage of controllable efficiency and timing would be useful in tissue engineering applications.

4.7. Area-specific gene transfer on the composite layers

The calcium phosphate composite layers can mediate area-specific gene transfer on their surfaces due to localized gene delivery at the cell–layer interface [67,68]. An area-specific gene transfer technique has been sought not only in tissue engineering, but also in gene therapy. This is because such a technique would enable control of the cell behavior at an intended location of a scaffold, and should thus increase the therapeutic effect on target cells with minimal side effects on the other parts of the body (see also Section 3.2). An area-specific gene transfer technique could also potentially be a useful tool for fabricating three-dimensional tissues comprising different types of cells, as described in the last paragraph of this section.

Area-specific gene transfer on calcium phosphate composite layers was successfully demonstrated by the following experiment. Two types of DNA–fibronectin–apatite composite layers were fabricated using genes of either firefly luciferase (FL) or renilla luciferase (RL). Epithelial-like CHO-K1 cells were cultured on these two layers

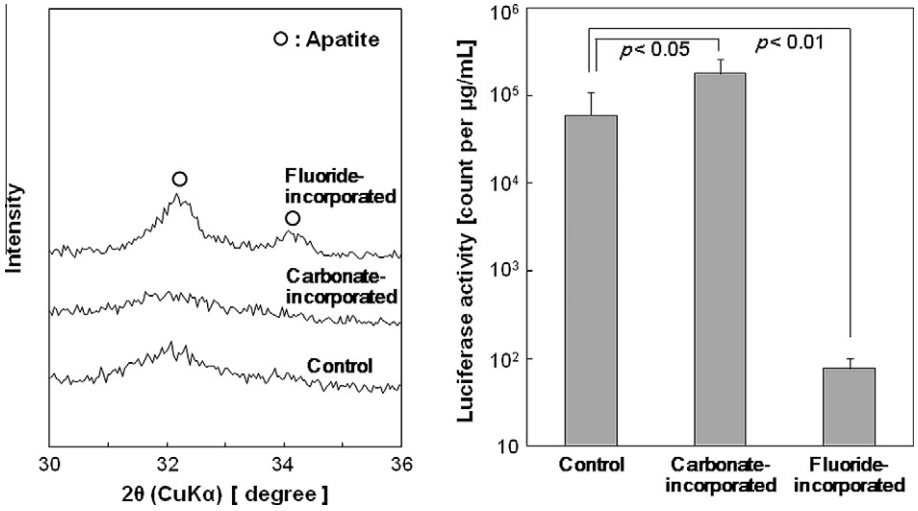

Fig. 6. Thin-film X-ray diffraction patterns (left) and gene transfer efficiency to the CHO-K1 cells (right) of the DNA–fibronectin–apatite composite layer (Control), and that incorporated with carbonate or fluoride ions. The composite layers were fabricated on an EVOH plate after immersion for 24 h in 3 ml of the CP solution supplemented with 40 μg ml^{-1} DNA and 10 μg ml^{-1} fibronectin, or that further supplemented with 1 mM Na_2CO_3 or NaF [143].

together in the same well. According to the dual luciferase assay results (Fig. 7), cells cultured on the composite layer that was prepared using the FL gene showed FL expression only (RL expression was at the background level). In contrast, the cells cultured on the composite layer that was prepared using the RL gene showed RL expression only (FL expression was at the background level). That is, DNA immobilized in the composite layer was locally transferred to the cells adhering to the layer but not to neighboring cells not in contact with the layer. Such an area-specific gene transfer has also been demonstrated for a DNA–lipid–apatite composite layer [68].

In the experiment described above, the cells on two composite layers were cultured together in the same medium in the same well, with a distance between the layers (center to center) of only 5 mm (Fig. 7). Normally in such situations it is difficult for conventional particulate transfection reagents to confine the area of gene transfer due to diffusion in the medium. Hence, a gene transfer system using such calcium phosphate composite layers is advantageous over conventional particle-mediated systems in terms of spatial control of gene transfer. In addition, the calcium phosphate composite layers carry little risk of dissipation from the intended site, which could lead to unfavorable gene transfer to cells outside the layer surface and accumulation within unintended organs.

Area-specific gene transfer would also be feasible with the physical systems [85–87] and the surface-mediated systems using other substrate materials, including DNA-immobilized biodegradable polymers [88,91] and substrates with adsorbing DNA molecules [89,92,94,97] (see Section 3.2). Compared with these systems, the surface-mediated system using calcium phosphate composite layers would be more beneficial for tissue engineering because of its safety, good biocompatibility, simple and easy fabrication process of the composite layers, and applicability to various scaffold materials. In addition, the efficiency and timing of gene transfer are finely tunable by varying the fabrication conditions of the composite layer, as described in Section 4.6 [62–68,143]. Furthermore, the area of gene transfer can be easily confined to the specific location of a scaffold with the use of an area-specific calcium phosphate coating technique. This is described below.

Very recently, we developed an area-specific calcium phosphate coating technique utilizing a laser-assisted biomimetic process [151,152]. In this process, laser light is irradiated onto a base material that is immersed in a supersaturated calcium phosphate solution. A calcium phosphate layer is formed only on the laser-irradiated surface of the base material within a short period of time (30 min in the case of EVOH [152]). With this technique, a calcium phosphate composite layer can be fabricated on an intended area/position of various materials. By utilizing such an area-specific coating technique, different calcium phosphate composite layers immobilizing different genes could be patterned on a scaffold surface. On the resulting scaffold, stem cells could differentiate into different types of cells, depending on the genes immobilized in the underlying composite layers. With this approach, it may be feasible to fabricate three-dimensional tissues comprising different types of cells, although this is a subject for a future study.

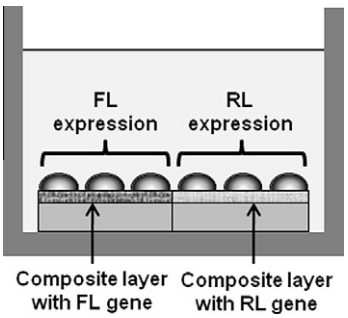

Fig. 7. Schematic illustration of the dual luciferase assay result obtained for the DNA–fibronectin–apatite composite layers fabricated using FL and RL genes.

4.8. Tissue engineering applications

The techniques to coat scaffolds with calcium phosphate composite layers and to design and modify these composite layers could lead to multifunctional and customizable tissue engineering scaffolds. For example, a base scaffold coated with a DNA–laminin–apatite composite layer would inherit good biocompatibility from the apatite, possess an affinity to the cell surface from the laminin and demonstrate gene transfer capability from the DNA. In fact, a DNA–laminin–apatite composite layer fabricated using the nerve growth factor (NGF) gene has been shown to enhance adhesion and NGF gene expression (six orders of magnitude higher than a laminin–apatite composite layer) of neuronal PC12 cells, thereby inducing their neuron-like differentiation [66]. This finding demonstrates that a scaffold coated with a DNA–laminin–apatite composite layer is useful in nerve tissue engineering.

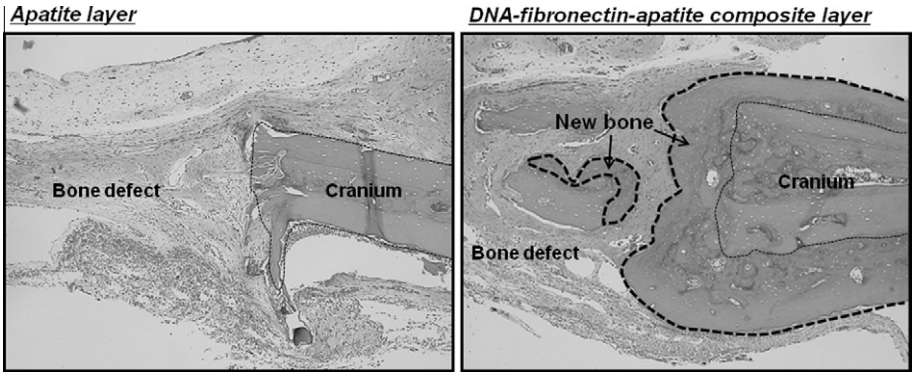

Fig. 8. Histological sections from tissues surrounding the apatite (left) and DNA–fibronectin–apatite composite (right) layers fabricated using BMP-2 gene 8 weeks after implantation in a rat cranial bone defect [134]. The thick dotted lines show the area of bone formation (indicated by new bone).

The calcium phosphate composite layer has even greater potential in bone tissue engineering than in nerve tissue engineering due to its high osteoconductivity. To demonstrate its potential for such application, we fabricated a DNA–fibronectin–apatite layer using the BMP-2 gene on dense and porous substrates composed of ceramic apatite [134,135]. BMP-2 is a cytokine effective in bone formation, and has been shown to induce the osteogenic differentiation of mesenchymal stem cells and to enhance bone development [13,14]. According to an in vitro study, the DNA–fibronectin–apatite composite layer enhanced osteogenic differentiation (as evaluated by alkaline phosphatase (ALP) activity) of preosteoblast MC3T3-E1 cells twice as much as apatite and DNA–apatite composite layers [134]. According to an in vivo study using a rat cranial bone defect model, the DNA–fibronectin–apatite composite layer increased the BMP-2 concentration in the surrounding tissue to 107 pg mg^{-1} (vs. the apatite layer, 58 pg mg^{-1}; and the DNA–apatite composite layer, 46 pg mg^{-1}), thereby promoting osteogenic cell differentiation (at 2 weeks) and bone regeneration (at 8 weeks) at the bone defect site (Fig. 8) [134]. Histological examination revealed no inflammation and no necrosis, indicating that the DNA–fibronectin–apatite composite layer has good tissue compatibility [134]. Moreover, even in an ectopic site (the dorsal subcutaneous tissue of rat), a 49-fold increase in BMP-2 gene expression and a 4-fold increase in ALP gene expression were observed for the subcutaneous cells on a DNA–fibronectin–apatite composite layer compared with those on an apatite layer 4 weeks after implantation [135].

In general, nonviral gene transfer systems are less efficient than viral systems, and have difficulty in attaining gene expression at therapeutically effective levels in vivo. The present gene transfer system using calcium phosphate composite layers was found to be effective in enhancing cell differentiation and bone tissue regeneration in vivo. By selecting appropriate genes and supplemental substances (cell adhesion proteins, lipids) for immobilization in the calcium phosphate layer, the resulting composite layer can be customized for a variety of target cells and tissues.

4.9. Comparison with protein delivery

Protein delivery is also possible using calcium phosphate composite layers [69]. For example, protein–calcium phosphate composite layers have been coated onto metallic implants using recombinant BMP-2 protein to accelerate and augment bone formation around the implant [153]. Many other delivery systems for proteins have been developed and extensively studied for tissue engineering applications [154]. However, these protein delivery systems have limitations associated with the diffusion, inactivation and degradation of the proteins. In general, growth factor proteins are rapidly inactivated after delivery into the body [155]. Therefore, these protein delivery systems often require high protein doses that range from micrograms to milligrams, leading to increases in medical cost and the risk of overdose [156,157]. The delivery of the plasmid encoding gene of a therapeutic protein rather than the protein itself might be an alternative and probably more effective approach because plasmids are more economical and are simpler to manufacture; moreover, they are more chemically stable in vivo compared with many therapeutic proteins [157–160]. In the nonviral gene delivery system, the transfected cells serve as local "bioreactors" because they express the exogenous genes and continue to secrete the fresh recombinant proteins for specific periods in a paracrine/autocrine manner [154]. Such an in situ protein secretion in the vicinity of the cell would be effective in ensuring the therapeutic effect.

Note that proteins exhibit therapeutic activity just after delivery into the body, whereas genes require three steps to exhibit therapeutic activity: transfection (transduction), transcription and translation [155,161]. Thus, genes are not effective unless they come into contact with cells and generate therapeutic proteins. In this respect, an efficient delivery system of genes to the intended cells at an intended location is of vital importance in clinical applications. The surface-mediated gene transfer system using calcium phosphate composite layers summarized in this review article would be a promising system for such delivery and merits further studies, including a longer-term in vivo study.

5. Conclusions

Calcium phosphate composite layers immobilizing DNA were recently fabricated on the surfaces of base materials by a biomimetic process. These composite layers support cell adhesion and growth on their surfaces, and stimulate the cell via surface-mediated gene transfer. By modifying the fabrication conditions, calcium phosphate composite layers can be designed to have improved affinity for cells and to exhibit increased gene transfer efficiency over that of commercial lipid transfection reagents. Such layers with increased gene transfer efficiency induced specific cell differentiation and tissue regeneration on the layer in vivo. These calcium phosphate composite layers are endowed with the useful characteristics of both osteoconductive bioceramics and transfection reagents, and have great potential in tissue engineering applications.

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