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# Silica-chitosan hybrid coating on Ti for controlled release of growth factors

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**Abstract** A hybrid material composed of a silica xerogel and chitosan was coated on Ti for the delivery of growth-factors. Fibroblast growth factor (FGF) and green fluorescence protein were incorporated into the coatings for hard tissue engineering. Silica was chosen as a coating material because of its high surface area as well as its good bioactivity. Chitosan provides mechanical stability and contributes to the control of the release rate of the growth factors. When the chitosan composition was 30% or more, the hybrid coating was stable physically and mechanically. The release of the growth-factors, observed in phosphate buffer solution at 37°C, was strongly dependent on the coating material. The hybrid coating containing FGF showed significantly improved osteoblast cell responses compared to the pure xerogel coating with FGF or the hybrid coating without FGF. These results indicate that the hybrid coating is potentially very useful in enhancing the bioactivity of metallic implants by delivering growth-factors in a controlled manner.

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## 1 Introduction

Various metallic implants have been employed to reconstruct impaired human tissues in the orthopaedic and dental fields because of their excellent mechanical properties. The bioactivity of metallic implants is often improved by surface treatments, especially through surface coating with osteoconductive substances. Inorganic substances, such as silica-based glasses or calcium phosphate compounds, have been widely used as the coating materials on the surface of metallic implants because of their excellent osteogenic cellular responses [1, 2].

Recently, there have been many attempts to further enhance the performance of osteoconductive coatings through the use of growth factors. Growth factors, including bone morphogenic protein (BMP), transforming growth factor-$\beta$ (TGF-$\beta$) and fibroblast growth factor (FGF), have the advantage of enhancing the efficacy and safety of the procedure used for the regeneration of the surrounding bone tissue. Many studies have demonstrated significant improvements in the osteoconductivity of implant materials through the use of growth-factors in combination with coating substances [1–7]. However, it is difficult to incorporate growth factors into inorganic coating materials, because coating processes generally require high-temperature heat treatment which would denature the growth factors.

In this respect, silica xerogels are attractive coating materials for use in combination with growth-factors, since they are synthesized at room-temperature using the sol–gel process. Silica xerogels have high porosity, nanostructured pores and bioresorbability. In addition, the sol–gel processing allows for the uniform distribution of the growth-factor in the silica xerogel. However, silica xerogels have some disadvantages when it comes to utilizing them as

coating materials on metallic substances, due to their brittleness and the initial rapid release of the growth-factors [8–13].

Chitosan has been widely studied for biomedical applications due to its biological compatibility and degradability. It is also suitable as a coating material because it is easily formed into thin layers as well as spheres, fibers and porous scaffolds. Therefore, in this study, chitosan was hybridized with silica xerogel for use as a coating layer, in order to control the release behavior of growth-factors and improve the physical stability of the coating layers [4, 14–17].

A hybrid sol of silica with 30% chitosan was prepared and then growth factors were added to the sol to incorporate them during the synthesis of the coating layer in an in situ manner. As the model growth factor, fibroblast growth factor 2 (FGF 2) (22 kDa), which is known to stimulate osteoblast cell proliferation and migration, was used [18–21]. Green fluorescent protein (GFP) (27–30 kDa) was also used to visualize the incorporation and release behaviors of the growth factor from the coating layers [22, 23].

## 2 Materials and methods

### 2.1 Synthesis of hybrid sols with $\beta$-FGF

Tetramethylorthosilane (TMOS, Sigma-Aldrich Chem. Co., USA), $CaCl_2$, and triethyl phosphate (Sigma-Aldrich Chem. Co., USA) were chosen as the precursors of the silica sol containing Ca and P (15 and 5 wt%, respectively), which was synthesized in distilled water using HCl as a catalyst [24]. 2 g of chitosan powder (85% deacetylated, Sigma-Aldrich Chem. Co., USA) was dissolved in 100 ml of 2 wt% acetic acid. The silica sol was mixed with the chitosan solution (30 vol% chitosan) and the mixtures were stirred for 10 min. After the hybrids were prepared, 100 μg/ml of FGF or GFP was added to the sols in situ and the mixtures were stirred for 1 h. The growth-factors were produced by *Escherichia coli* (more than 95% purity) following the established procedure [25, 26]. The FGF and GFP, which were recombinant histidine-tagged (His-tagged, located at the amino-terminal end of the protein) fusion growth factors, were verified by western blot analysis by using anti-His polyclonal antibodies (Santa Cruz Biotechnology, Inc, USA).

NaOH treated titanium (20 × 20 × 2 mm for the differentiation tests or 10 × 10 × 1 mm for the other tests) was used as a substrate, as described elsewhere [27]. 200 and 80 μl of the prepared hybrid sols were spin-coated on the substrates at 3,000 rpm for 1 min (WS-400-6NPP-LITE spinner, Laurell Technologies, North Wales, PA) and then dried at 37°C in a humid atmosphere. The hybrid coatings were treated with 0.1 N NaOH and then washed with distilled water and phosphate buffer solution (PBS) (pH 7.4) to remove the residual acetic acid.

The cross-section and surface morphologies of the coatings were observed by scanning electron microscopy (SEM) (JSM 5600, JEOL, Tokyo, Japan). The specimens for cross-sectional observation were obtained by fracturing them in liquid nitrogen.

### 2.2 Release of growth-factors in vitro

The GFP and FGF loaded hybrid coatings (10 × 10 × 1 mm) were immersed in 2 ml of PBS at 37°C and pH 7.4 for 28 and 35 days, respectively. The solutions were exchanged at specific intervals ranging from 1 day (initial 1 week) to 2–3 days (8–35 days).

The surfaces of the GFP loaded hybrid coatings after the GFP was released from them in PBS for 0, 14 and 28 days were observed by confocal laser scanning spectroscopy (CLSM). The green intensities of the GFP from the hybrid coatings were measured (n = 3).

The amounts of FGF released were measured by UV-spectroscopy (ICP-AES, Optima-4300 DV, USA) at 220 nm. A calibration curve was obtained by measuring the optical absorbance of FGF dissolved in PBS with concentrations in the range of 0.010–10 μg/ml. The curve has a linear relationship of FGF concentration (y) = 6.5142 × optical absorbance (x) – 0.1346 (μg $ml^{-1}$). By means of this relationship, each absorbance value was directly converted to the amount of FGF released. The relative amounts of FGF released were normalized to that in the initially loaded coating layer (n = 6).

### 2.3 Biological properties

The in vitro cellular responses of the in situ FGF loaded hybrid coatings were evaluated using pre-osteoblast cells (MC3T3-E1; ATCC, CRL-2593; Rockville, MD) and compared to those of the hybrid coatings without FGF. Prior to seeding the cells, the specimens were sterilized by ultraviolet irradiation for 30 min. The cells were grown in α-modified minimum essential medium (α-MEM; Welgene, Korea) containing 1% Fetal Bovine Serum (FBS) and 1% antibiotics (100 U $ml^{-1}$ penicillin and 100 μg $ml^{-1}$ streptomycin; FBS; GIBCO, Grand Island, NY) for 2 days before the examination of the cellular responses. The cells were seeded on the samples at a density of 50,000 cells/ml for initial cell attachment, 30,000 cells/ml for cell proliferation and 20,000 cells/ml for cell differentiation. The cells were cultured in a culture medium containing 3% FBS and 1% antibiotics at 37°C in a humidified atmosphere incubator with 5% $CO_2$.

The morphology of the cells adhered to the coatings after 2 h of culture was investigated by CLSM. The cells on the hybrid coatings with and without FGF were fixed in 4% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100 in PBS, and stained with fluorescent phalloidin.

The cell viability was examined using a cell proliferation assay kit (Cell Titer 96® Aqueous One Solution, USA). After harvesting the cells on the specimens for 5 days, the hybrid coatings were moved to 700 µl of medium in a new 24-well plate and 70 µl of MTS solution was added to each well. After incubating them at 37°C for 2 h, the colorimetric measurement of the samples was performed using 200 µl aliquots of each solution by measuring the optical density at 490 nm on a spectrophotometer.

The cell differentiation of the samples was evaluated using the alkaline phosphatase (ALP) activity test. After culturing the cells for 10 days, the cells on the samples were detached by trypsinization and washed with PBS by centrifugation at 1,500 rpm for 3 min. The cell pellets were resuspended in 0.1% Triton X-100 and treated using a cyclical freezing and thawing process. The ALP activity levels of the samples were assessed by the detection of *p*-nitrophenol (*p*NP) in the presence of ALP from a *p*-nitrophenyl phosphate (*p*NPP) substrate using a Sigma Kit 104 commercial kit. The absorbance was measured at 405 nm using a micro-reader and the ALP activity levels were calculated from a standard curve after normalizing to the total protein contents.

All experiments involving the in vitro test were repeated 2–3 times. The data are represented as means ±1 standard error deviation (SED) of triplicate samples (n = 3). Statistical differences were analyzed using one-way analysis of variance (ANOVA) with the statistical significance set at $P < 0.05$.

## 3 Results

The GFP and FGF used in this experiment expressed strong single bands at 30 and 22 kDa, respectively, by western blot analysis, as presented in Fig. 1. No other bands were observed, thus confirming the absence of any other proteins in the solution of the growth-factor.

The cross-section and surface morphology of the hybrid coatings with the growth factors on the Ti substrates were observed by SEM. When the pure silica xerogel was coated on the Ti substrate, the coating layer was severely cracked after drying, as shown in Fig. 2a. With the addition of chitosan to the xerogel, the formation of cracks in the coating layer became less severe. When 30% or more of chitosan was added to form the hybrid coating layer, no cracks were formed, as shown in Fig. 2b. The cross-sectional view of the hybrid coating layer also demonstrated that it was dense without any defects, as shown in Fig. 2c.

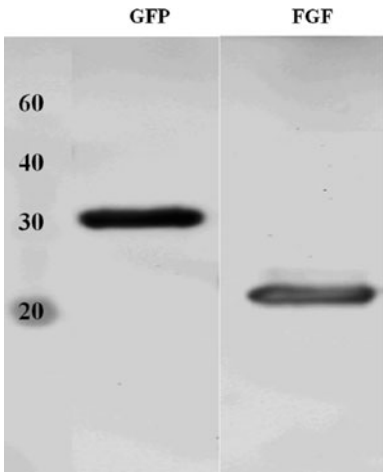


**Fig. 1** Expression image of the GFP and FGF by western blot analysis

GFP was loaded onto the coating layers and its release behavior in PBS was monitored by CLSM. When the same amount of GFP was loaded onto the pure xerogel and the hybrid coating layers, the intensities of the green fluorescence were about the same, as shown in Fig. 3. However, after 14 days of immersion in PBS, the green fluorescence observed from the pure xerogel coated specimen was significantly lower than that observed from the hybrid coated specimen. After 28 days of immersion, there was still a trace of green fluorescence from the hybrid coated specimen, while none was observed from the xerogel coated one.

The intensity of the green fluorescence observed from the specimens immersed in PBS solution is quantified in Fig. 4. The intensity from the hybrid coated specimen decreased steadily, while that from the xerogel coated specimen decreased rapidly.

The release of FGF in PBS was observed for up to 35 days. Similar amounts of FGF were loaded onto the pure xerogel and hybrid coatings (1.99 µg for pure xerogel and 2.03 µg for hybrid coating). The cumulative amounts of FGF released from the coatings were measured as a function of the release time, as shown in Fig. 5a. FGF was released rapidly from the pure silica xerogel coating during the initial 7 days and then slowly thereafter. After 28 days, little FGF was released. On the other hand, the release of FGF from the hybrid coating was relatively steady, so that appreciable amounts of FGF were released even after 28 days. The steady release of FGF from the hybrid coating compared to the pure xerogel coating was well illustrated by the estimated release rates, as shown in Fig. 5b.

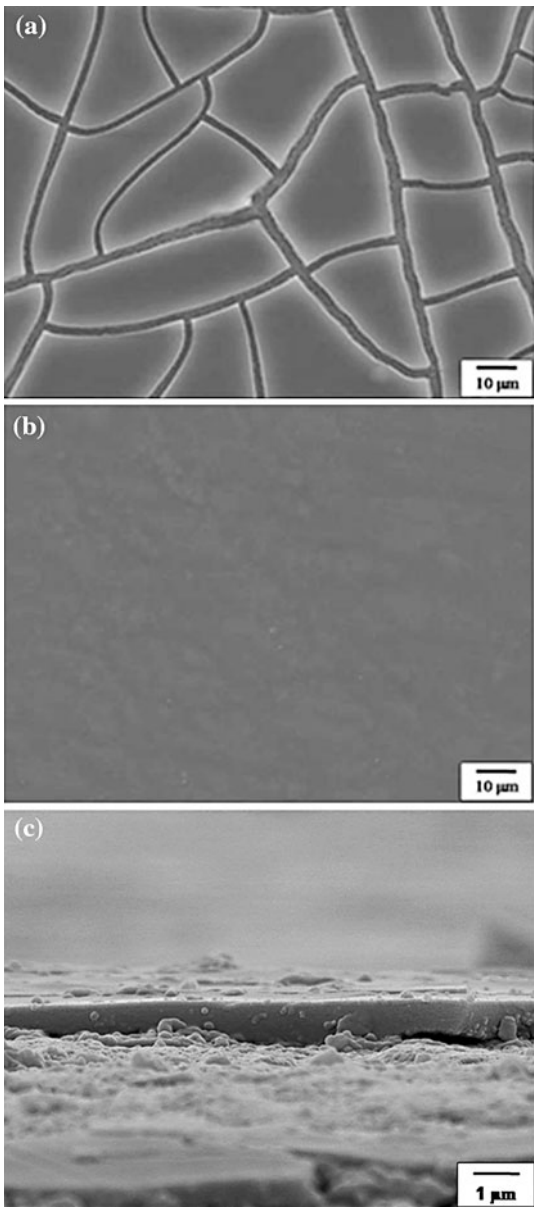


**Fig. 2** SEM micrographs of growth-factor loaded **a** pure xerogel, **b** silica xerogel/chitosan hybrid coating and **c** typical cross-section view of the hybrid coating

The stability of the coating layers in aqueous solution was estimated by observing the morphology of the coatings immersed in PBS solution. When the pure xerogel coating was soaked in PBS at 37°C for 28 days, most of the coating layer was dissolved away, thus exposing the titanium substrate, as shown in Fig. 6a. On the other hand, the surface of the hybrid coated specimen changed only slightly (Fig. 6b).

The in vitro cellular responses to the FGF loaded coatings were examined in terms of the initial attachment, proliferation and differentiation levels of osteoblast cells. Figure 7 shows the CLSM images of the MC3T3-El cells adhering to the coatings after culturing for 2 h. On the specimen coated with pure xerogel containing FGF, the cells adhered well, as shown in Fig. 7a. The cells adhered and spread even better on the specimen coated with the hybrid containing FGF (Fig. 7b). For the purpose of comparison, a specimen coated with the hybrid, but without FGF, was also tested. As shown in Fig. 7c, the cells adhered well on the coated surface, but not as well as on the specimen containing FGF.

The cell viability, quantified by the MTS assay after culturing for 5 days, and the differentiation levels, estimated by measuring the ALP activity after culturing for 10 days ($n = 3$), are illustrated in Fig. 8. The cell viability was influenced very little by the coating layer when FGF was included. However, the ALP activity of the cells on the specimens coated with the hybrid containing FGF was significantly higher than that on the specimens coated with the pure xerogel containing FGF, as shown in Fig. 8. When FGF was not used in the hybrid coating layer, both the cell viability and ALP activity were lower than those of the hybrid coating with FGF.

## 4 Discussion

The use of osteoconductive coatings consisting of growth factors on metallic implants is one of the major issues in hard tissue engineering for effective bone regeneration. Therefore, there is a need to develop a delivery vehicle for the growth factors that would allow for their controlled and sustained release [1–4, 28]. Silica xerogels are known to have the potential to be used as drug or growth-factor delivery vehicles. However, the burst effect resulting from their instability in aqueous solution is one of the most serious obstacles to their use in practical applications. In the present study, a hybrid coating layer consisting of a silica xerogel and chitosan was developed and its capability of delivering growth factors (FGF and GFP) was evaluated. The silica xerogel/chitosan hybrid was synthesized by a sol–gel method at ambient temperature. A low processing temperature has the advantages of allowing for the easy hybridization of the organic materials and growth factors without the denaturation of the latter. When the hybrid containing 30% or more of chitosan was used as the coating material, the coating layer was uniform and free of cracks, while the pure silica xerogel coating layer was severely cracked, as shown in Fig. 2. The stability of the hybrid is attributed to the increase in strength and the reduction of the shrinkage rate during the drying process as a result of the addition of chitosan to the silica xerogel.

The hybridization of the silica xerogel and chitosan was an effective method of fabricating physically stable coatings, which was in turn very useful for controlling the

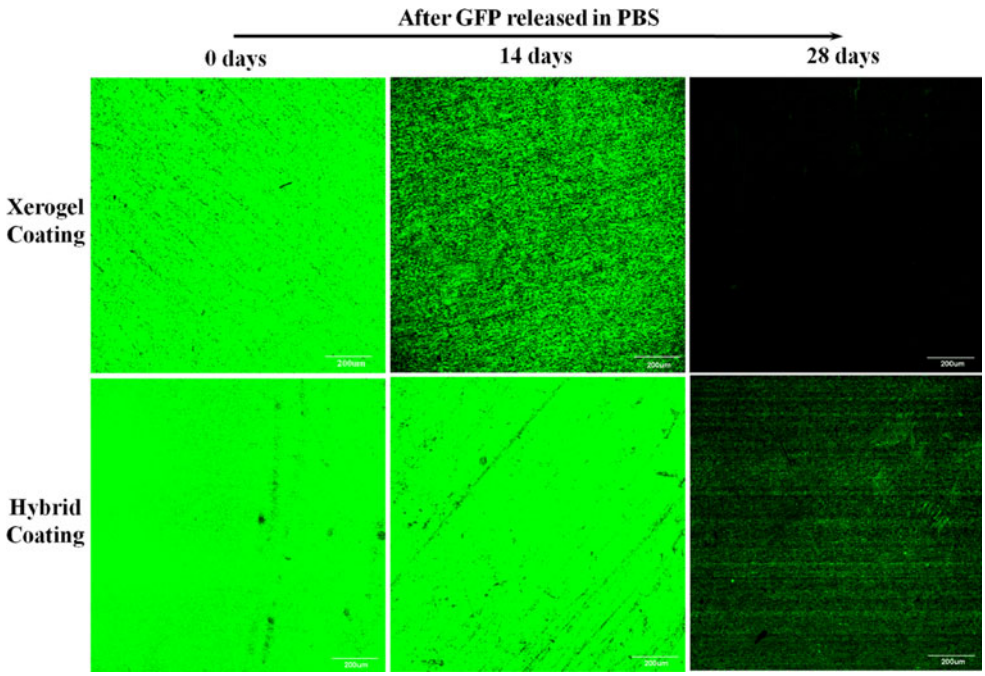


**Fig. 3** Typical CLSM images of GFP loaded pure xerogel and the hybrid coatings after GFP was released from them for up to 28 days

release behavior of the growth factors. Silica xerogels synthesized by the sol–gel process are known to act as carriers for growth factors due to their nano-porous structure. The release behaviors of the growth factors from the silica xerogel are controlled by the diffusion mechanism, which explains their rapid initial release [12, 29–31]. On the other hand, some studies reported that the silica xerogel coatings had a different nanostructure from that of the bulk materials. The sol–gel transformation of silica overlaps with and occurs immediately during the formation of the coating layer, so that the silica xerogel coating has a lower porosity than that of the bulk xerogel. Due to its less porous structure, the penetration of the solution used for the

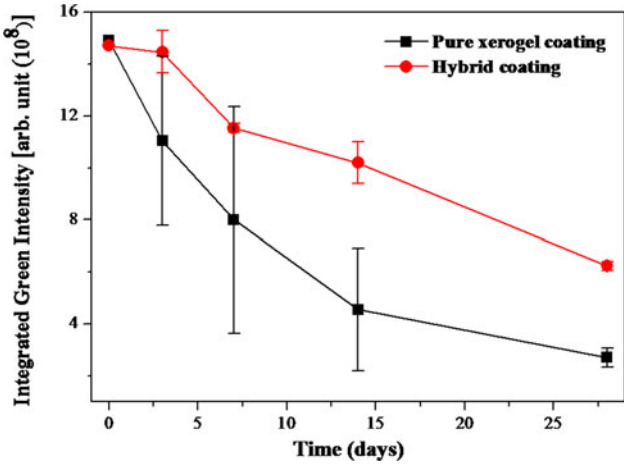


**Fig. 4** The integrated green intensity of the GFP loaded pure xerogel and hybrid coatings as a function of the release time in PBS solution (n = 3)

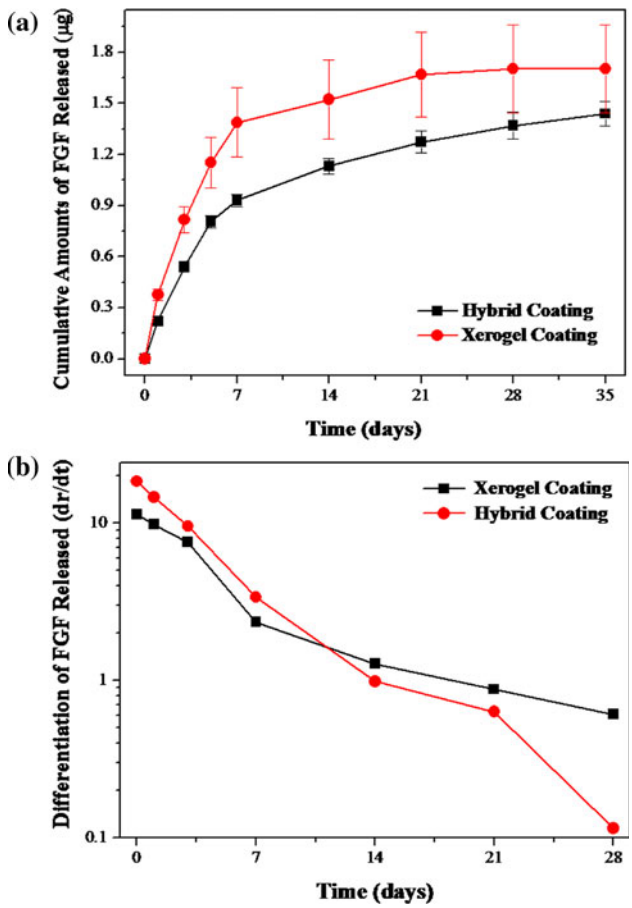


**Fig. 5** **a** Cumulative amounts of FGF released from pure xerogel and hybrid coatings as a function of time in PBS solution and **b** the differentials of the amount of FGF released (n = 6)

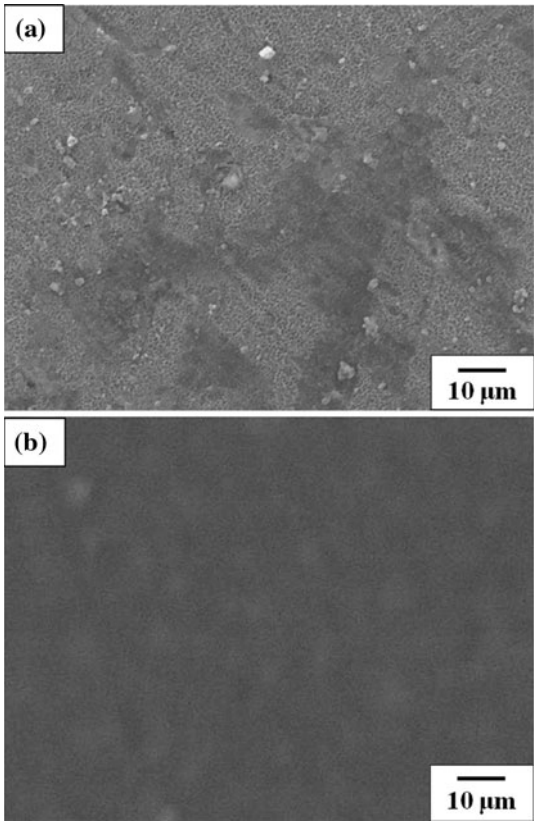


**Fig. 6** SEM micrographs of growth-factor released from **a** pure xerogel and **b** hybrid coatings after 28 days immersion in PBS

release of the growth factor is hindered and the diffusion of the growth factor is limited compared to that of other forms of silica xerogels [10, 12, 17, 32, 33]. In this study, the GFP was released from the xerogel coating layer rapidly in the early stage and then slowly after about 14 days (Fig. 4). On the other hand, the coatings hybridized with 30% of chitosan showed a steady release of GFP for a longer period of time. In addition, it is of note that the GFP release data from the hybrid coating had much smaller standard deviations compared to those from the pure xerogel

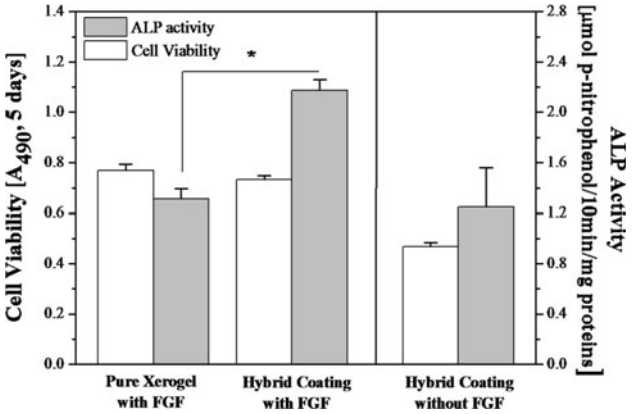


**Fig. 8** Cell proliferation level and ALP activity level of MC3T3-E1 cells of pure xerogel coating with FGF, hybrid coating with FGF and the hybrid coating without FGF

coating. This discrepancy is deemed to be closely related to the physical stability of the coating layers.

A similar trend was observed for the FGF release behavior. The FGF was released quickly from the pure xerogel coating layer in the early stage and then slowly after about 7 days. The FGF from the hybrid coating was released relatively steadily, so that it was still being released even after 35 days. The stability of the coating layer is also reflected in the morphology of the coatings after the release tests. As shown in Fig. 6a, the pure xerogel coating was severely damaged after being immersed in PBS for 28 days. On the other hand, the surface morphology of the hybrid coating was changed very little, as shown in Fig. 6b, and the green fluorescence of the hybrid coating with GFP was still observed after the same period of time (Fig. 3). These results indicate that the steady release of the growth factors from the hybrid coating is attributable to the enhanced stability of the xerogel containing chitosan. Besides, it is considered that the incorporated growth factors retain their properties without any denaturation after the growth factor has been released for a long period of time.

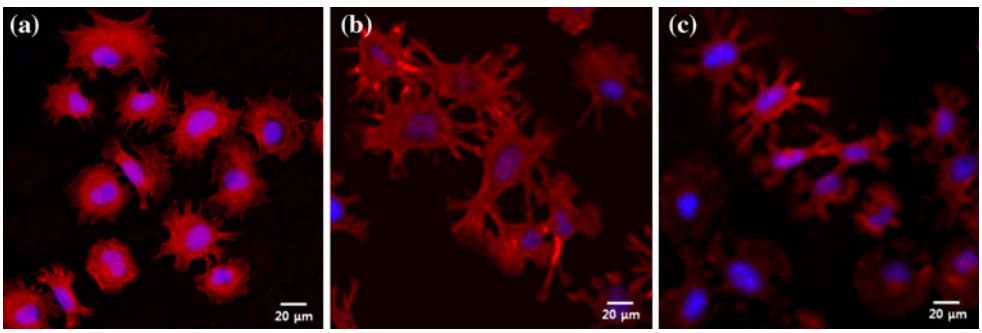


**Fig. 7** CLSM images of cellular attachment of **a** the pure xerogel coating with FGF and the silica xerogel-chitosan (70/30) hybrid coating **b** with and **c** without FGF after culturing for 2 h

FGF, an osteostimulative factor, is known to improve the bone healing process and the demineralization of the bone matrix by promoting the proliferation of the cells [18–21, 34, 35]. Especially, FGF-2 is a single-chain polypeptide which acts as a mitogen and chemoattractant for various cells; as a result, it plays a key role in angiogenesis and the bone formation process. Previous studies have also reported that a high bone formation rate is associated with early angiogenesis [36]. Both the coating layers (pure xerogel and hybrid) containing FGF showed excellent in vitro cell responses. The osteoblastic cells attached and spread well on the coating layers with FGF, as shown in Fig. 7a, b. Even without FGF, the cells attached well on the hybrid coating layer (Fig. 7c), indicating the good bioactivity of the silica xerogel-chitosan hybrid [26, 37]. The effects of the coating material and FGF on the proliferation and differentiation of the osteoblastic cells were estimated by observing the cell viability and ALP activity, respectively. The cell viability after culturing for 5 days was not influenced very much by the coating material when FGF was included in the coating layer, as shown in Fig. 8. However, the ALP activity of the cells cultured for 10 days on the hybrid coating layer with FGF was significantly higher ($P < 0.05$) than that on the xerogel coating layer with FGF for the same period of time. The beneficial effect of the steady release of the growth factors on the osteoblastic cells was well illustrated by this ALP activity. Therefore, it is clear that the incorporation of FGF further enhanced the bioactivity of the silica xerogel-chitosan hybrid coating layer.

## 5 Conclusion

The silica xerogel/chitosan hybrid was used as a coating material for delivering growth-factors to enhance the bioactivity of Ti. The growth factors (GFP and FGF) were incorporated into the coating layer during the coating process at ambient temperature. The coating layer was uniform and physically stable when 30% or more of chitosan was contained in the hybrid. The growth factors were distributed homogeneously in the coatings and were released for a long period of time ($\sim$30 days) from both of the coating layers. However, the growth factors were released more steadily from the hybrid coating layer. The in vitro cellular bioactivity of the hybrid coating with FGF was higher than that of the pure xerogel coating with FGF, because of this steady release of the growth factor.

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