Biochemical surface modifications to titanium implants using the tresyl chloride-activated method

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INTRODUCTION

Titanium implants are widely used as dental and orthopedic implants. Various surface modifications to titanium dental implants including mechanical treatments, such as blasting, or chemical treatments, such as an alkaline treatment or hydroxyapatite coating, have been used to accelerate and improve the bone healing process. Biochemical surface treatments, such as surface coating or immobilization of bioactive molecules, have also been employed. The first biological reaction that occurs at the implant-tissue interface following implantation into the living body is the adsorption or attachment of body fluid proteins. These adsorbed or attached proteins are known to control subsequent biological responses at the implant-tissue interface. The chemical immobilization of peptides or proteins has been attempted in order to enhance cell attachment, stimulate cellular responses, and promote tissue healing and remodeling processes.

Covalent immobilization using silane coupling reagents has been widely employed to immobilize cell adhesive proteins. For example, heparin was grafted onto titanium surfaces using 3-aminopropyltriethoxysilane and bone morphogenic protein (BMP)-2 was then immobilized onto the heparin-grafted titanium surfaces. However, using a silane coupling agent requires multiple treatment steps, for example, the acidic or alkaline pretreatment of titanium surfaces to produce free surface hydroxyl groups and a condensation reaction between the silane coupling agent and proteins. Moreover, unreacted silane coupling agents should be removed during the cleaning steps because of their physiological adverse effects. Electrochemical methods have also been applied. DNA was fixed onto titanium by an anodic polarization technique and BMP were then bound to the anchored DNA. Nilsson et al. reported a direct and simple technique for immobilizing enzymes to various OH groups using the highly reactive sulfonyl chloride, 2,2,2-trifluoroethanesulfonyl chloride (CF₃CCH2SO2Cl, tresyl chloride). The OH groups of silica or agarose were activated with the reaction of tresyl chloride, and an enzyme, such as a-chymotrypsin, was then coupled to tresylated-agarose. This enzyme could be immobilized onto agarose according to the scheme shown in Fig. 1.

Titanium possesses two types of OH groups: a basic terminal OH and acidic bridge OH group. If basic terminal OH will react with tresyl chloride, resulting in the formation of tresylated titanium, proteins could be immobilized onto titanium using a similar method to that described by Nilsson et al. The tresyl chloride-activation method was herein introduced as a useful protein immobilization technique.

PROTEIN IMMOBILIZATION ONTO TITANIUM USING TRESYL CHLORIDE-ACTIVATION METHOD

The tresyl chloride-activation method

An easy method to immobilize cell adhesive proteins onto a titanium surface, called the tresyl chloride-activated method was originally developed. It
is a modified version of that described by Nilsson et al.\textsuperscript{16,17}. Figure 2 shows the procedure used in the tresyl chloride-activated method. Tresyl chloride (2,2,2-trifluoroethanesulfonyl chloride) was applied to a titanium surface without the use of any solvent in order to prepare tresylated titanium, which was then immersed in a protein solution. Proteins were subsequently immobilized directly onto the titanium surface\textsuperscript{19,20}.

**X-ray photoelectron spectroscopic analysis**

First, the reaction between tresyl chloride and the titanium surface and further protein immobilization to the tresylated titanium surface were analyzed by X-ray photoelectron spectroscopy (XPS)\textsuperscript{19,20}. Three different types of titanium surfaces were prepared: mirror polished, pretreated with 0.1 M H\textsubscript{2}O\textsubscript{2}/phosphate buffered saline (PBS) solution (H\textsubscript{2}O\textsubscript{2}/PBS) at 37°C for 2 h, and pretreated with 0.1 M H\textsubscript{2}O\textsubscript{2}/0.1 M NaOH aqueous solution (H\textsubscript{2}O\textsubscript{2}/NaOH) at 37°C for 10 min.

Figure 3 shows the XPS analysis of the O1s spectra of the mirror polished, H\textsubscript{2}O\textsubscript{2}/PBS-, and H\textsubscript{2}O\textsubscript{2}/NaOH-pretreated titanium surfaces. No significant differences were observed in the patterns of the O1s spectra between the three titanium surfaces. The O1s peak at 530.3 eV corresponded to the bulk oxygen in titanium dioxide\textsuperscript{21,22}, while that at 532 eV corresponded to basic terminal OH\textsuperscript{23,24}. The intensities of the O1s spectra at 532 eV were almost half that at 530.3 eV on each surface.

Tresyl chloride was directly dropped in a liquid form onto the titanium surface. The titanium surface was completely covered with tresyl chloride without any solvent. Nilsson and Mosbach attempted to induce an activation reaction between tresyl chloride and the agarose in pyridine, which was used as a dehydrochloride reagent\textsuperscript{16,17}. However, a preliminary experiment revealed that tresyl chloride did not react with the OH groups of titanium in pyridine. Therefore, the reactivity...
of the terminal OH groups was presumed to be less than that of dehydrochloride reagents such as pyridine. The reaction between tresyl chloride and pyridine mainly will proceed during the activation procedure. The direct application of tresyl chloride to the titanium surface without any dehydrochloride reagents was more effective for introducing a tresyl group onto the titanium surface.

The reaction between tresyl chloride and titanium was conducted at two different reaction temperatures: 37ºC or 70ºC for 2 days. Tresylated titanium was then reacted with protein. Tresylated titanium disks were immersed in fibronectin/PBS solution at 37ºC for 24 h, which resulted in fibronectin-immobilized titanium.

Figure 4 shows the F1s spectra of the titanium surface after the reaction with tresyl chloride. A F1s peak, which was derived from the trilfluorocarbon of tresyl chloride, was observed at 688.6 eV. The intensity of the F1s peak at a reaction temperature of 37ºC was approximately three-fold higher than that at 70ºC on each surface. These findings indicated that the condensation reaction of tresyl chloride was more dominant than the reaction between tresyl chloride and the basic terminal OH groups on the titanium surface at 70ºC. Therefore, the reaction between tresyl chloride and the basic terminal OH of titanium was performed at 37ºC.

The presumed activation scheme of the titanium basic terminal OH groups by tresyl chloride is shown in Fig. 5. Tresyl chloride reacts with the basic terminal OH on the titanium surface to form a Ti-O-S bond. The reactivity of the oxygen atom of the basic terminal OH group was presumed to be affected by the formation of a Ti-O-S bond, and was later confirmed by a frontier molecular orbital study, as described later.

Figure 6 shows the XPS analysis following the reaction between fibronectin and the tresylated titanium disks. An N1s peak, which was derived from the amide groups of immobilized fibronectin, was monitored at 399.9 eV on the three different titanium surfaces. No F1s peak was observed. These findings confirmed that a coupling reaction between tresylated titanium and fibronectin occurred through the terminal basic OH group of titanium following cleavage of the Ti-O-S bond.

![Figure 4](image1.png)

**Fig. 4**  F1s spectra of a tresylated titanium specimen by XPS analysis. The titanium surface was mirror polished, H$_2$O$_2$/PBS-, and H$_2$O$_2$/NaOH-pretreated. A F1s peak at 688.6 eV was derived from the trifluorocarbon of tresyl chloride$^{20}$.

![Figure 5](image2.png)

**Fig. 5**  Activation scheme of the terminal OH group of a titanium surface treated with tresyl chloride. Tresyl chloride reacts with the basic terminal OH on the titanium surface forming a Ti-O-S bond$^{20}$. 

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significant differences were observed in the intensities of the N1s peaks of the three different titanium surfaces. These findings revealed that fibronectin could be immobilized on different titanium surfaces.

Tresylated and non-tresylated titanium (without tresyl chloride activation) were reacted with fibronectin to confirm the efficacy of tresyl chloride activation. Figure 7 shows the N1s and O1s peaks of the XPS analysis after the reaction between fibronectin and tresylated titanium. The titanium surface was mirror polished. The N1s peak derived from the amide group of immobilized fibronectin still remained on the argon-ion sputtering.

**Fig. 6** XPS N1s spectra of fibronectin-attached titanium with the tresyl chloride treatment after rinsing. The titanium surface was mirror polished, H2O2/PBS-, and H2O2/NaOH-pretreated. An N1s peak was detected at 399.9 eV, which was derived from the amide groups of fibronectin. The N1s peak did not diminish after 20 s of argon-ion sputtering.

**Fig. 7** XPS N1s and O1s spectra of fibronectin-attached titanium with the tresyl chloride treatment after rinsing.
sputtered surface after 60 s. Prior to argon-ion etching, the O1s peak at 530.3 eV, which was derived from the bulk oxygen in titanium dioxide, was not clearly visible and the intensity of the O1s peak at 532 eV, derived from the amide group of fibronectin, was higher than that of the O1s peak at 503.03 eV. The intensity of the O1s peak at 532 eV decreased, whereas that of the O1s peak at 530.3 eV increased as the argon-ion etching time increased.

In contrast, a reaction was conducted between fibronectin and titanium without the tresyl chloride activation treatment. As shown in Fig. 8, the N1s peak at 399.9 eV and O1s peak at 532 eV derived from the amide group of fibronectin were also detected. However, both peaks almost disappeared after 10 s of argon-ion sputtering. Moreover, the ratio of intensity between O1s at 532 eV and O1s at 530.3 eV was almost equal in the outermost layers of fibronectin. These findings clearly indicated that small amounts of fibronectin were present on the titanium surface when titanium was not treated with tresyl chloride. In contrast, the tresyl chloride activation treatment improved the reactivity of the titanium surface to fibronectin and greater amounts of fibronectin could be immobilized onto the tresylated titanium surface.

The reaction between tresyl chloride and the basic terminal OH of the titanium surface as well as the immobilization of fibronectin were also confirmed by Fourier transform infrared (FT-IR) spectroscopy. Figures 9 and 10 show the FT-IR spectra of tresylated titanium and fibronectin-immobilized titanium, respectively. The peaks at approximately 1,100–1,200 cm⁻¹ were

![Fig. 8 XPS N1s and O1s spectra of fibronectin-attached titanium without the tresyl chloride treatment after rinsing](image)

![Fig. 9 FT-IR-RAS spectra of a tresylated titanium specimen](image)

![Fig. 10 FT-IR-RAS spectra of a fibronectin-immobilized titanium surface after the tresyl chloride treatment](image)
attributed to the C-F bonds of the trifluorocarbon of tresyl chloride, while peaks at approximately 1,400–1,480 cm⁻¹ were attributed to the -O-S-O₂⁻ bonds formed by the reaction between tresyl chloride and the terminal basic OH group (Fig. 9). In Fig. 10, two amide groups derived from immobilized fibronectin were identified at approximately 1,450–1,550 cm⁻¹ and 1,600–1,800 cm⁻¹.

Fibronectin-immobilized specimens were ultrasonically cleaned for 60 min to confirm the stability of immobilized fibronectin. As shown in Fig. 11, no significant differences were observed in the intensity of the N1s peak after ultrasonic cleaning. The N1s peak did not diminish after 20 s of argon-ion etching. Immobilized fibronectin was not detached from the titanium surface by ultrasonic cleaning and the immobilization of fibronectin onto titanium was stable.

As described above, the H₂O₂ or NaOH pretreatment did not influence the immobilization of fibronectin onto titanium. Thus, polished titanium was employed for the following immobilization experiments.

MECHANISM OF THE PROTEIN IMMOBILIZATION

Frontier molecular orbital study

Frontier molecular orbital (FMO) calculations were performed to examine the mechanism responsible for the tresyl chloride activation method. The FMO theory was proposed by Fukui et al. According to this theory, the reactivities or selectivities of some organic reactions are controlled by interactions between the highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO).

FMO calculations were carried out with a personal computer using Quantum CAChe software (Fujitsu). It was impossible to calculate the molecular orbitals for titanium and proteins using the personal computer due to the large number of electrons. Methanol (CH₃OH) and methyl amine (CH₃NH₂) were used as model compounds for titanium and protein, respectively.

The energy diagrams for the HOMO and LUMO of methanol and tresyl chloride are shown in Fig. 12. The energy difference between the HOMO of methanol and LUMO of tresyl chloride, 9.004 eV, was smaller than that between the LUMO of methanol and HOMO of tresyl chloride, 15.855 eV. HOMO energy levels indicated reactivity towards the electrophile, whereas LUMO energy levels indicated reactivity towards the nucleophile. Smaller energy differences indicated an easy reaction pass. Thus, these findings revealed that the reaction between tresyl chloride and methanol was dominantly controlled by the interaction between the HOMO of methanol and LUMO of tresyl chloride.

Figure 13 shows the energy diagrams for the HOMOs and LUMOs of methanol, tresylated methanol, and methyl amine. Tresylated methanol was a reaction product of tresyl chloride and methanol. The energy difference between the LUMO of tresylated methanol and HOMO of methyl amine, path a: 8.559 eV, was smaller than that between the LUMO of methanol and HOMO of methyl amine, path b: 12.909 eV. This finding indicated that the nucleophilic reaction between methyl amine and tresylated methanol was...
proceeded more easily than that of the reaction towards methanol. Therefore, the tresylation of methanol improves its reactivity towards methyl amine by reducing its LUMO energy levels.

The present FMO calculations were a simple simulation for the tresyl chloride-activated method. The electronic properties of the basic hydroxyl group of titanium are different from those of methanol, and other factors such as the steric conformation of proteins will influence the reaction between titanium and tresyl chloride as well as the subsequent coupling reaction with protein. However, the present simple FMO calculations suggest that tresylated titanium may be more reactive towards proteins than titanium due to a reduction in LUMO energy levels.

Quartz-crystal microbalance-dissipation analysis

The quartz-crystal microbalance-dissipation (QCM-D) technique was employed for analyzing the mechanism between fibronectin and tresyl chloride-activated titanium. The QCM-D technique can estimate the amounts of proteins adsorbed by calculating the resonance frequency shift according to the Sauerbrey equation. Moreover, the viscoelastic properties of adsorbed proteins can be evaluated by dissipation (D). The surface of a titanium sensor was reacted with tresyl chloride at 37°C for 2 days. The adsorption behavior of fibronectin to an untreated or tresyl chloride-activated titanium sensor was monitored using the QCM-D technique.

Figure 14 shows the frequency shifts for the untreated and tresyl chloride-activated titanium sensors during the adsorption of fibronectin. The decrease in frequency was faster and greater for the tresyl chloride-activated titanium sensor (b) than for the untreated titanium sensor (a) during a 120-min adsorption, which indicated that the former (b) had adsorbed a greater amount of fibronectin. The amount of adsorbed fibronectin estimated by the Sauerbrey equation was approximately 600 g/cm² for untreated titanium and 1,000 g/cm² for tresyl chloride-activated titanium sensor. These findings revealed that the tresyl chloride treatment enhanced the adsorption of fibronectin onto titanium.

The relationship between frequency shifts and dissipation shifts (D-f plot) has provided an insight into the adsorption mechanism. If the adsorbed layer is rigid, a low ΔD value will be obtained. The greater increase

Fig. 12 HOMO and LUMO energy levels for methanol and tresyl chloride (Cl-O-SO₂CH₂CF₃)[30].

Fig. 13 HOMO and LUMO energy levels for methanol, tresylated methanol (Cl-O-SO₂CH₂CF₃) and methyl amine[30].

Fig. 14 Frequency shift as a function of time for the exposure to fibronectin of (a) an untreated titanium QCM-sensor and (b) a tresyl chloride-activated titanium QCM-sensor[30].
observed in the dissipation shift in the case of untreated titanium (Fig. 15) indicated that fibronectin formed a softer and more elastic layer on untreated titanium than on tresyl chloride-activated titanium. This was attributed to a difference in the binding mechanism; in the first stage of adsorption, fibronectin forms a stronger bond with tresyl chloride-activated titanium than with untreated titanium. After this initial stage, i.e., once the titanium surfaces are covered with fibronectin, the two types of titanium surfaces exhibited the same adsorption behavior, as evidenced by their overlapping D-f plots (Fig. 15).

The following three coupling mechanisms between fibronectin and the hydroxyl groups of titanium were proposed, as shown in Fig. 16: a) direct bond formation between titanium atoms and the nitrogen of fibronectin, b) covalent bond formation between the oxygen of titanium's terminal hydroxyl group and the nitrogen of fibronectin, and c) an ionic interaction between the oxygen of titanium's terminal hydroxyl group and the nitrogen of fibronectin. In the reaction between the hydroxyl group of silica or agarose and tresyl chloride, the hydroxyl group was tresylated. The tresylated hydroxyl group (-C-O-SO2Cl) then reacted with the NH2 group of the enzyme to form —C-NH-enzyme. However, it was difficult to form a direct Ti-N or Ti-O-N bond for Ti-OH, and mechanisms a) and b) were unlikely to occur. Based on the D-f plots, the tresyl chloride treatment appears to have increased the ionic characteristics of the oxygen in titanium's terminal hydroxyl group and also that the ionic interaction between the oxygen of the terminal hydroxyl groups of titanium and the nitrogen of fibronectin was important for the immobilization of fibronectin (mechanism c) in Fig. 16.

**BIOLOGICAL RESPONSES TOWARDS THE PROTEIN-IMMOBILIZED TITANIUM**

Biological responses towards the protein-immobilized titanium using tresyl chloride-activation method were evaluated by cell attachment, differentiation, and gene expression with GeneChip technology.

**Initial cell attachment and cell morphology**

First, the initial attachment of osteoblast-like cells (MC3T3-E1) on a fibronectin-immobilized titanium (Fn-Ti) surface was evaluated. Cells were cultured for 30 min on Fn-Ti and untreated Ti disks. As shown in Fig. 17, the rate at which MC3T3-E1 cells attached to Fn-Ti was significantly (p<0.05) higher than that of untreated disks.

The initial attachment of human gingival fibroblasts was also evaluated. In this study, fibronectin and collagen were immobilized onto
titanium sputter-coated glass using the tresyl chloride activation method. The number of attached cells after a 90-min cell culture was listed in Table 1. Collagen immobilization provided a significantly greater number of initially attached cells. The reason why only collagen immobilization was effective for initial cell attachment is not clear. It may be presumed that the difference of assayed cells, namely between MC3T3E-1 and human gingival fibroblast, may be one of the factors.

**Proliferation and attached cell morphology**

The proliferation of MC3T3-E1 cells cultured on Fn-Ti was evaluated. Two different titanium surfaces, *i.e.*, nanometer-smoothing (Ra: approximately 2.0 nm) and sandblasting (Ra: approximately 1.0 µm) surfaces were employed. Fibronectin was immobilized onto smooth and sandblasted surfaces by the tresyl chloride activation method. Fibronectin could be immobilized onto both nanometer-smooth sandblasted surfaces.

Figure 18 shows the cell viability of the MC3T3-E1 culture on days 1 and 11 as determined by the MTT assay. On day 1 of the cell culture, no significant differences were observed among the four different titanium surfaces. On day 11, the immobilization of fibronectin onto the sandblasted surface only significantly enhanced cell viability.

DNA levels during the 52-day cell culture were determined, as shown in Fig. 19. The immobilization of fibronectin produced slightly higher DNA levels; however, no significant differences were observed between nanometer-smooth or sandblasted surfaces with and without the immobilization of fibronectin. The alkaline phosphatase (ALP) activity results are shown in Fig. 20. ALP activity was significantly greater on sandblasted surfaces with the immobilization of fibronectin than on those without after 52 days. However, ALP activity was significantly reduced with the immobilization of fibronectin on nanometer-smooth surfaces. Wu et al. reported that ALP level of a polished surface with nanometer smoothness was much higher than that of grit-blasted surfaces at 16 days of cell culture. Although the detailed mechanism will be further investigated, the effect of combining sandblasting and FN immobilization may contribute to the improvement of ALP activity for T-sand/FN. Classical medium without any bone differentiation components, such as dexamethasone or β-glycerophosphate, was employed in the above described study.

Surface roughness, in addition to the immobilization of fibronectin, influenced cell activities, and only the immobilization of fibronectin enhanced the initial attachment of cells, but not their proliferation. Fibronectin is a major extracellular matrix protein that cultured cells produce by themselves during the cell assay; therefore, this may have been the reason for above findings.

SEM images of the attached cells cultured on days 1 and 3 were shown in Figs. 21 and 22. More cells attached to fibronectin-immobilized surfaces, and these cells were well-arranged. These findings suggest that cells can recognize immobilized fibronectin and, as a result, the arrangement of attached cells is aligned according to the presence of immobilized fibronectin.

**Gene expression**

The expression of genes by osteoblast-like cells (MC3T3-E1) on fibronectin-immobilized titanium...

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**Table 1**  Number of initially attached fibroblasts cultured on the various substrates

| Substrate                                           | Number of cells     |
|-----------------------------------------------------|---------------------|
| Titanium sputter-coated glass                       | 5,890 (884)         |
| Fibronectin immobilization onto titanium sputter-coated glass | 3,055 (1,768)       |
| Collagen immobilization onto titanium sputter-coated glass | 18,241 (8,205)     |
| Tissue culture polystyrene                          | 5,151 (2,472)       |

( ): standard deviation

Mean values with the same superscript were not significantly different at p<0.05.
Fig. 20 Change in the ALP activity of MC3T3-E1 cells cultured on nanometer-smooth (Ti-smooth), fibronectin-immobilized nanometer-smooth (Ti-smooth/FN), sandblasted (Ti-sand), and fibronectin-immobilized sandblasted (Ti-sand/FN) titanium disks after 1, 3, 15, and 52 days of cultivation. Connected bar: significant difference ($p<0.05$).

Fig. 19 DNA contents of MC3T3-E1 cells cultured on nanometer-smooth (Ti-smooth), fibronectin-immobilized nanometer-smooth (Ti-smooth/FN), sandblasted (Ti-sand), and fibronectin-immobilized sandblasted (Ti-sand/FN) titanium disks after 1, 3, 15, and 52 days of cultivation. Connected bar: significant difference ($p<0.05$).

(Fn-Ti) and fibronectin-derived peptide (GRGDSP)-immobilized titanium (GRGDSP-Ti) was evaluated using the GeneChip system. As shown in Figs. 23 and 24 (Fn-Ti and GRGDSP-Ti), differences were observed in the gene expression profiles of MC3T3-E1 on Fn-Ti, GRGDSP-Ti, and untreated control titanium. The immobilization of fibronectin or GGDSP altered the expression of many genes from that of the control. The number of genes that were up-regulated (more than 2-fold) and down-regulated (less than 0.5-fold) relative to control untreated titanium were 62 and 56 genes for the immobilization of fibronectin, and 31 and 51 genes for that of GGDSP immobilization, respectively (Tables 2 and 3). For example, Fn-Ti markedly increased the mRNA levels of bone sialoprotein (BSP) and osteomodulin (OMD), but reduced those of sulfatase 1 (SULF1). Some up-regulated (more than 2-fold) and down-regulated (less than 0.5) genes were selected and listed in Tables 1 (Fn-Ti) and 2 (GRGDSP-Ti).

BSP is a non-collagenous protein member of the
small integrin-binding ligand N-linked glycoprotein family, and its expression pattern has been temporospatially associated with the early stages of the mineralization process. BSP can promote bone formation and is localized in mineralizing tissues such as bone, dentin, cementum, and hypertrophic cartilage. Expression of the BSP gene, which is induced in newly formed osteoblasts, is up-regulated by cytokines that promote bone formation and down-regulated by factors that suppress bone formation.

OMD is a keratan sulfate proteoglycan that belongs to the small leucine-rich repeat proteoglycan family. It is found exclusively in mineralized tissues mouse osteoblasts, odontoblasts, ameloblasts, and human odontoblasts. OMD is also localized extracellularly in the alveolar bone, predentin, and enamel matrices of rat and mouse teeth. OMD has high affinity for hydroxyapatite and functions as a structural component of the bone matrix. It is also considered to be an early marker for terminally differentiated osteoblasts.

SULF is a group of enzymes that catalyze the hydrolysis of sulfate ester bonds from a wide variety of substrates, ranging from complex molecules such as glycosaminoglycans and sulfolipids to steroid sulfates. The degradation of glycosaminoglycans was shown to decrease interfacial strength between cultured...
Table 2  Gene expression of MC3T3-E1 cells on fibronectin-immobilized titanium disks relative to untreated disks

| Gene ID   | Gene name                                           | Fold change | Spot on plot |
|-----------|-----------------------------------------------------|-------------|--------------|
| NM_007566| Baculoviral IAP repeat-containing 6 (Birc6)         | 12.19       | A            |
| NM_011499| Serine/threonine kinase receptor associated protein (Strap) | 3.54       | B            |
| NM_008318| Integrin binding sialoprotein (Ibisp)              | 3.54        | C            |
| NM_011504| Syntaxin binding protein 3A (Stxbp3a)              | 3.27        | D            |
| NM_020559| Aminolevulinic acid synthase 1 (Alas1)             | 3.25        | E            |
| NM_029404| PHD finger protein 14 (Phf14)                      | 3.04        | F            |
| NM_020009| FK506 binding protein 12-rapamycin associated protein 1 (Frap1) | 2.79 | G            |
| NM_025848| Succinate dehydrogenase complex, subunit D (Sdhδ) | 2.68        | H            |
| NM_021518| RAB2, member RAS oncogene family (Rab2)            | 2.36        | I            |
| NM_012050| Osteomodulin (Omd)                                | 2.08        | J            |
| NM_021716| Fidgetin (Fign)                                   | 2.08        | K            |
| NM_010331| GPI anchor attachment protein 1 (Gpaa1)            | 2.08        | L            |

<0.5 fold

| Gene ID   | Gene name                                           | Fold change | Spot on plot |
|-----------|-----------------------------------------------------|-------------|--------------|
| NM_028973| Leucine rich repeat containing 15 (Lrrec15)         | 0.29        | M            |
| NM_024436| RAB22A, member RAS oncogene family (Rab22a)        | 0.36        | N            |
| NM_199468| Zinc finger, CCHC domain containing 5 (Zcchc5)     | 0.36        | O            |
| NM_134156| Actinin, alpha 1 (Actn1)                           | 0.41        | P            |
| NM_172294| Sulfatase 1 (Sulf1)                                | 0.42        | Q            |
| NM_175836| Spectrin beta 2 (Spm2)                             | 0.43        | R            |
| NM_011607| Tenascin C (Tnc)                                  | 0.44        | S            |
| NM_026144| Dehydrodolichyl diphosphate synthase (Dhdds)      | 0.47        | T            |
| NM_016799| Serine/arginine repetitive matrix 1 (Srrm1)        | 0.47        | U            |
| NM_007707| Suppressor of cytokine signaling 3 (Socs3)         | 0.48        | V            |
| NM_028188| RUN and SH3 domain containing 1 (Rusc1)            | 0.49        | W            |

mineralized tissue and titanium\(^{40}\). The down-regulation of sulfatase in this study indicated a decrease in the degradation of glycosaminoglycan, which further implied the early promotion of matrix mineralization in osteoblastic cells.

Regarding GRGDSP-Ti, the up-regulated expression of osteocalcin (OC), in addition to that of BSP and OMD, was monitored. OC is a major non-collagenous protein that is incorporated into the bone matrix during bone formation. A vitamin K deficiency resulted in an increase in under-carboxylated OC, leading to low biological activity, which has been associated with low bone mineral density and an increase in the frequency of bone fractures\(^{40}\).

The expression of many genes was found to be altered in MC3T3-E1 cultured on Fn-Ti or GRGDSP-Ti, including BSP and OC. The up-regulation of the mRNA levels of BSP and OC was also confirmed by RT-PCR and real-time PCR. Since BSP and OC gene products are useful markers for the bone formation process by osteoblasts, the increased transcription of BSP and OC genes suggests that Fn-Ti and GRGDSP-Ti may be useful for accelerating the formation of bone.

Animal experiments

The in vivo efficacy of the immobilization of biological molecules, such as cell adhesive proteins and cytokines, onto titanium using the tresyl chloride
Table 3  Gene expression profiles in MC3T3E1 cells on GRGDSP-immobilized titanium relative to untreated disks

| Gene ID    | Gene Name                                         | Fold |
|------------|--------------------------------------------------|------|
| NM_010008  | Cytochrome P450, family 2                        | 5.8  |
| NM_133903  | Spondin 2, extracellular matrix protein          | 4.3  |
| NM_008812  | Peptidyl arginine deiminase, type II             | 4.3  |
| AV025588   | Gelsolin                                         | 4.1  |
| NM_008318  | Bone sialoprotein                                | 3.5  |
| BB150720   | Glutaminyl-peptide cyclotransferase              | 3.3  |
| W91024     | Histone 1, H2ae                                  | 2.5  |
| BB291769   | Component of oligomeric golgi complex 8          | 2.3  |
| BM244014   | E3 ubiquitin protein ligase 2                    | 2.3  |
| BC027285   | Interferon induced transmembrane protein 1       | 2.2  |
| NM_011637  | Three prime repair exonuclease 1                 | 2.2  |
| BG065877   | High density lipoprotein (HDL) binding protein   | 2.1  |
| NM_012050  | Osteomodulin                                     | 2.1  |
| AV094567   | Golgi reassembly stacking protein 1              | 2.1  |
| BB400581   | N-ethylmaleimide sensitive fusion protein        | 2.1  |
| BB064885   | WD repeat domain 48                              | 2.0  |
| AI327038   | PFTAIRE protein kinase 1                         | 2.0  |
| NM_023223  | Cell division cycle 20 homolog                  | 2.0  |
| NM-199173  | Bone Gla Protein (osteocalcin)                   | 2.0  |
| AF458089   | Adenylate cyclase 3                              | 0.3  |
| BC005679   | Syndecan                                         | 0.3  |
| BB207105   | Phosphatidylinositol membrane-associated 1       | 0.4  |
| BB699415   | Minichromosome maintenance deficient 2           | 0.4  |
| NM_008580  | MAP kinase kinase kinase 5                      | 0.4  |
| AV330726   | Nucleophosmin 1                                  | 0.4  |
| BB800078   | MRV integration site 1                           | 0.4  |
| AW553715   | Endothelin converting enzyme 1                   | 0.4  |
| AU020421   | Jumonji, AT rich interactive domain 2            | 0.5  |
| BC004060   | Ras and a-factor-converting enzyme 1             | 0.5  |
| BI694945   | Ribosomal protein L41                           | 0.5  |
| NM_030888  | C1q and tumor necrosis factor 3                  | 0.5  |
| NM_054072  | Protocadherin alpha 11                          | 0.5  |
| AK017369   | Expressed sequence AI956758                     | 0.5  |
| BC027319   | ATPase, Na+/K+ transporting                      | 0.5  |
| BB389081   | Nemo-like kinase                                 | 0.5  |
| AW536432   | Heparan sulfate 6-O-sulfotransferase 2           | 0.5  |
| BB291816   | Zinc finger protein, subfamily 1A, 2 (Helios)    | 0.5  |
activation method was evaluated in animal experiments. Transforming growth factor \( \beta_2 \) (TGF-\( \beta_2 \)) was selected as a biological molecule\(^{49}\).

TGF-\( \beta_2 \) was immobilized to rectangle titanium implants using the tresyl chloride activation method. TGF-\( \beta_2 \) immobilized titanium (TGF-\( \beta_2/Ti \)) implants were inserted into rat femur defects. Histological and histomorphometrical analyses were performed 4 weeks after the implantation by examining non-decalcified thin sections. The histological appearances of bone formation around the untreated titanium and TGF-\( \beta_2/Ti \) implant were shown in Fig. 25. The new formation of bone was observed around the titanium and TGF-\( \beta_2/Ti \) implants. TGF-\( \beta_2/Ti \) implant showed more bone formation than titanium implant.

Measurements of bone-to-implant contact (BIC) and bone mass (BM) around the implants revealed that the BIC and BM of the TGF-\( \beta_2/Ti \) implants were significantly higher than those of the Ti implants 4 weeks after the implantation. TGF-\( \beta_2/Ti \) implants effectively enhanced bone regeneration around implants.

In conclusion, tresyl chloride-activated method is useful for immobilizing biological molecules onto titanium surface and subsequent biological response. It is also expected that immobilization of biological molecules onto titanium by using tresyl chloride-activated method will be applicable for bone reconstruction in dental clinics.

CONFLICTS OF INTEREST

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