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Oxygen Plasma Surface Modification Enhances Immobilization of Simvastatin Acid

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Running headline: Immobilization of Simvastatin Acid by O2-Plasma
ABSTRACT

Simvastatin acid (SVA) has been reported to stimulate bone formation with increased expression of BMP-2. Therefore, immobilization of SVA onto dental implants is expected to promote osteogenesis at the bone tissue/implant interface. The aim of this study was to evaluate the immobilization behavior of SVA onto titanium (Ti), O₂-plasma treated titanium (Ti+O₂), thin-film coatings of hexamethyldisiloxane (HMDSO), and O₂-plasma treated HMDSO (HMDSO+O₂) by using the quartz crystal microbalance-dissipation (QCM-D) technique. HMDSO surfaces were activated by the introduction of an OH group and/or O₂-functional groups by O₂-plasma treatment. In contrast, titanium surfaces showed no appreciable compositional changes by O₂-plasma treatment. The QCM-D technique enabled evaluation even at the adsorption behavior of a substance with a low molecular weight such as simvastatin. The largest amount of SVA was adsorbed on O₂-plasma treated HMDSO surfaces compared to untreated titanium, HMDSO-coated titanium, and O₂-plasma treated titanium. These findings suggested that the adsorption of SVA was enhanced on more hydrophilic surfaces concomitant with the presence of an OH group and/or O₂-functional group resulting from the O₂-plasma treatment, and that an organic film of HMDSO followed by O₂-plasma treatment is a promising method for the adsorption of SVA in dental implant systems.
Simvastatin, one of the liposoluble statins [inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase], has been widely used for lowering cholesterol levels. Recently, simvastatin was reported to stimulate bone formation both in vitro and in vivo in animal models of osteoporosis (25, 10). This phenomenon was associated with increased expression of the bone morphogenetic protein-2 (BMP-2) gene in bone cells. Therefore, immobilization of simvastatin onto titanium implants is expected to promote osteogenesis in the bone tissue surrounding the implants through its topical application. Furthermore, one distinct advantage of simvastatin for local stimulation of bone formation is its low manufacturing cost compared to the direct administration of recombinant proteins such as BMPs. There is also a reduced possibility of eliciting antibody responses.

Plasma-surface modification including plasma polymerization and plasma surface treatment is an effective and economical surface treatment technique for many materials. The technique is of growing interest in biomedical engineering because it can control the physicochemical properties of biomaterials (7, 23, 26).

Films formed by plasma polymerization are generally free of pinholes, have a strong adherence to a wide variety of materials, and have a greater degree of resistance to chemicals and physical treatment because of their cross-linked structure (19, 30). In particular, plasma polymerization using organosilicon monomers, hexamethyldisiloxane (HMDSO), has come into wide use (2, 16, 18, 24, 28, 31, 32). These monomers are of interest because of their high deposition rates and the ability of varying the deposition conditions to control their structure and properties. In addition, HMDSO is an easy and safe monomer to handle, especially in comparison with silane compounds. Our previous study demonstrated that the deposited HMDSO thin film firmly attached to the titanium and was stable in a phosphate buffered saline, and was useful for fibronectin adsorption due to the hydrophobic surfaces of the HMDSO (13). At the same time, plasma surface treatments using various gases such as Ar, O₂,
N₂, SO₂ have been utilized to modify blood compatibility, to influence cell adhesion and growth, and to control protein adsorption (5, 8, 15, 17, 33-35). Notably, O₂-plasma treatment was reported to be able to control hydrophilicity/hydrophobicity and to introduce various functional groups, leading to applications such as humidity sensors, enzyme immobilization, and polymer bonding without the use of adhesives (9, 36, 39).

Recent studies have confirmed that the quartz crystal microbalance-dissipation (QCM-D) technique is useful for evaluating surface-related processes in liquids. Nevertheless, the adsorption behavior of low molecular weight substances such as simvastatin has not been investigated.

Therefore, in the present study, we used the QCM-D technique to evaluate the immobilization behavior of simvastatin onto HMDSO-coated and O₂-plasma treated surfaces as well as to characterize these surfaces.

**MATERIALS AND METHODS**

*Preparation of specimens.* Commercially pure wrought titanium disks (JIS, Japan industrial specification H4600, 99.9 mass% Ti, Furuuchi Chemical Co., Tokyo) with a diameter of 6 or 15 mm were used. The 6-mm diameter disk was used for X-ray photoelectron spectroscopy (XPS) analysis and the 15-mm diameter disk was used for Fourier transform infrared reflection absorption spectroscopy (FT-IR-RAS) analysis. The disks were ground progressively finer down to 1200 grit, finely polished using colloidal silica at pH 9.8, and then ultrasonically cleaned with acetone to avoid affecting the surface conditions of the titanium after mirror polishing. A titanium-coated QCM-D sensor (14 mm diameter) was used for a simvastatin adsorption assay.

Four kinds of specimens, titanium (Ti), O₂-plasma treated titanium (Ti+O₂), an HMDSO-coated specimen by plasma polymerization (HMDSO), and O₂-plasma treated
HMDSO (HMDSO+O2) were prepared according to the method described below.

**HMDSO coating with plasma polymerization.** Hexamethyldisiloxane [HMDSO, (CH$_3$)$_3$SiOSi(CH$_3$)$_3$] monomers were used for plasma polymerization. Thin film coating by plasma polymerization was conducted using a commercially available plasma-surface modification apparatus (VEP-1000, ULVAC, Kanagawa, Japan). Mirror-finished titanium disks were positioned in a chamber at room temperature. The reservoir of HMDSO [(CH$_3$)$_3$SiOSi(CH$_3$)$_3$] was heated to 70°C and the pipe for introducing the HMDSO was heated to 80°C. The HMDSO gas was then introduced into the equipment chamber through the heated pipe at a gas flow rate of 45 sccm (mL/min). The plasma was generated using a radio-frequency generator operating at 13.56 MHz at a power level of 200 W. The chamber pressure was maintained at about 10 Pa throughout the plasma polymerization process. Plasma polymerization was performed for periods of 20 min. After the plasma was turned off, nitrogen gas was used to purge the chamber, which was then allowed to return to ordinary atmospheric pressure. The process resulted in the deposition of plasma-polymerized film on the titanium with a film approximately 100 nm thick as determined by a Dektak Stylus Profiler System (Veeco Instruments Inc., Woodbury, NY, USA).

**O$_2$-plasma treatment.** Using the same apparatus as the plasma polymerization, the HMDSO-coated specimens and titanium substrates were then treated with O$_2$-plasma for 10 min at room temperature with a gas flow rate of 50 mL/min and a chamber pressure of 1.5 Pa. Surface characterization and an SVA-adsorption assay were started 10-min after the O$_2$-plasma treatment.

The four kinds of specimens had mirror-like topographies with a mean surface roughness (Ra) of less than 0.1 µm.
Surface characterization. The contact angle with respect to double-distilled water was measured using a Contact Angle Meter (CA-P, Kyowa Interface Science Co. Ltd., Tokyo, Japan). Five measurements of 15-s each were made for each surface type; all analyses were performed at the same temperature and humidity.

XPS analysis (ESCA-750, Shimadzu, Kyoto, Japan) and FT-IR-RAS analysis (FT-IR-430, Jasco Corp., Tokyo, Japan) were conducted for surface characterization. The XPS analysis was carried out with Mg-Ka as the X-ray source at 8 kV and 30 mA to determine the intensity of Si2p, O1s, and C1s. For charging correction, the binding energy of each spectrum was calibrated with C1s of 284.6 eV. FT-IR-RAS analysis was conducted at a resolution of 4-cm⁻¹. The incident angle of the infrared ray to the specimen was 75 degrees. A bare Ti surface was used as the background while calculating the spectrum of the specimen in order to obtain a spectrum that was limited to the substances formed on the titanium by plasma polymerization. Each surface type was measured three times.

Adsorption assay of simvastatin acid. Commercially available simvastatin {(+)-(1S,3R,7S,8S,8aR)-1,2,3,7,8,8a-hexahydro-3,7-dimethyl-8-[2-[(2R,4R)-tetrahydro-4-hydroxy-6-oxo-2H-pyran-2-yl]ethyl]-1-naphthyl 2,2-dimethylbutanoate, S3449, Wako Chemicals, Japan} was used in this study. The chemical structures of simvastatin (SV, pro-drug; lactone) and simvastatin acid (SVA, open-acid; β-hydroxy acid) are shown in Fig. 1. Since only the open-ring or beta-hydroxy acid forms exhibit the efficacy of this medication, SV was hydrolyzed to β-hydroxy acid in the following manner. A convenient procedure for opening the lactone is to dissolve 4 mg of SV in 0.1 mL of ethanol (95-100%) and then add 0.15 mL of 0.1M NaOH. After being heated at 50°C for 2 h, the resulting solution is neutralized with HCl to a pH of approximately 7.2 and brought up to a
volume of 1 mL with distilled water.

Schematic illustrations of HMDSO coating, O₂-plasma treatment, and adsorption assay for QCM-D measurement are shown in Fig. 2. For the adsorption assay, a QCM-D instrument (QCM-D300, Q-Sense AB, Göteborg, Sweden) was operated with AT-cut single-crystal quartz sensors with a resonant frequency of 5 MHz. Ti, Ti+O₂, HMDSO and HMDSO+O₂ specimens were used as sensors. The crystal resonant frequency of the oscillator was measured simultaneously at a fundamental resonant frequency (5 MHz) and at a number of overtones, including 35 MHz. Monitoring the resonance of the piezoelectric oscillation enables measurements of mass adsorption at the surface in real time, usually as a function of a decrease in resonant frequency. The frequency shift is related to the adsorbed mass, and the adsorbed mass was estimated by the Sauerbrey equation (29). At 35 MHz, a frequency shift of 1 Hz corresponds to a mass change of approximately 2.6 ng/cm². A second measurement parameter, dissipation (D), gives qualitative information about the viscoelastic properties of the adsorbed layer.

SVA solution (0.2 mg/mL in double-distilled water) was introduced into an axial flow chamber consisting of a T-loop in order to thermally equilibrate the sample at 37±0.05°C. The sequence of injections into the QCM cell for an experimental run was as follows: 0.5 ml of double-distilled water, 0.5 mL of SVA solution, and 0.5 mL of double-distilled water. The results were expressed as the mean ± SD of five specimens (n = 5).

The data was analyzed for statistical significance using analysis of variance (ANOVA) followed by Scheffe’s test for multiple comparisons.

RESULTS

Surface characterization.

The contact angles were 68±5 and 100±3 degrees on the mirror-polished titanium (Ti) and the
HMDSO coatings, respectively. These values decreased dramatically after the O₂-plasma treatment on both the Ti and HMDSO-coated specimens of less than 5 degrees (p<0.01).

The XPS spectra of Ti, Ti+O₂, HMDSO, and HMDSO+O₂ specimens are shown in Fig. 3. The C1s peak of the outermost layer of the HMDSO surface appeared at 284.4 eV, and shifted to 285.9 eV on the HMDSO+O₂. These peak shifts indicate a change from the dominant CH₃-Si-O- group of the HMDSO surface to the Si-(O)₄ with OH group of HMDSO+O₂ surface (1,11, 12). The O1s peak of the outermost layer of the HMDSO surface was observed at 532.4 eV, and shifted to 534.3 eV on the HMDSO+O₂. On the Ti and Ti+O₂ specimens, O1s peaks appeared at around 533.0 eV and 530.5 eV, which corresponded to the Ti-OH and TiO₂, respectively. The Si2p peak of the outermost layer of HMDSO was observed at 101.3 eV, and shifted to 104.3 eV on the HMDSO+O₂.

The intensity ratios of the elements on Ti, Ti+O₂, HMDSO, and HMDSO+O₂ are shown in Fig. 4. The intensity of O compared to C and Si, and that of Si compared to C was increased by the O₂-plasma treatment on the HMDSO coatings (p<0.01). The intensity of O as compared to that of C was slightly increased by the O₂-plasma treatment on the Ti (p<0.05).

The FT-IR-RAS spectra of the HMDSO and HMDSO+O₂ specimens are shown in Fig. 5. A peak attributed to Si-O-Si bonds appeared at around 1080 and 1100 cm⁻¹ in the HMDSO and HMDSO+O₂ specimens, respectively, showing a higher shift in comparison with the reported wavelength of 1050 cm⁻¹ of the Si-O-Si bonds of the monomer. The O₂-plasma treatment caused this peak to broaden. The peaks at around 800 cm⁻¹, 850 cm⁻¹, 1250 cm⁻¹, and 1400 cm⁻¹ are attributed to the Si-(CH₃)ₓ bonds (3, 18, 28). The intensities of spectra attributed to the Si-(CH₃)ₓ were decreased by O₂-plasma treatment. No appreciable peaks were observed on the Ti+O₂ specimens.

SVA adsorption

Fig. 6 plots a typical example of a shift in frequency and dissipation against time for exposure
of HMDSO+O2 to SVA as obtained via QCM-D measurement. The frequency curve shows a decrease over time during the early stage of adsorption until it reached a certain frequency after about 180 min. A small amount of dissipation shift was observed on all specimens. The estimated amounts of SVA adsorption on the QCM sensors are shown in Fig. 7. The amount of SVA adsorption was estimated by the Sauerbrey equation (29) 3 h after injection of the SVA solution. The amount of SVA adsorbed onto the HMDSO+O2 sensor was more than three times greater than that onto other sensors (p<0.01). In contrast, there was no significant difference between the amount adsorbed by the Ti and Ti+O2 sensors (p>0.05).

**DISCUSSION**

The present study evaluated the immobilization behavior of SVA, which was reported to stimulate bone formation with increased expression of BMP-2, onto plasma-surface modified specimens by using the QCM-D technique.

Recent studies have confirmed that the QCM-D technique is useful for evaluating surface-related processes in liquids, including protein adsorption, complementary activity on biomaterials, and analysis of DNA hybridization (4, 14, 23, 26, 27.). Höök et al. reported the effectiveness of the QCM-D technique for analyzing the adsorption kinetics of three model proteins on titanium oxide surfaces compared with ellipsometry and optical waveguide lightmode spectroscopy (14). QCM-D measurement was performed to obtain more detailed information about the quantity of protein adsorption. They also pointed out that the mass calculated from the resonance frequency shift included both protein mass and water that was bound or hydrodynamically coupled to the protein adlayer. In this study, we evaluated the adsorption behavior of low molecular weight substances, in particular, SVA. The results showed a clear difference of frequency shift among the conditions. Consequently, it was shown that the QCM-D technique is able to evaluate the adsorption behavior of low molecular
weight substances such as chemicals like SVA as well as high molecular weight substances. The present study showed a small amount of change in dissipation (D), suggesting that the simvastatin adsorbed onto the specimens is rigid (29). Accordingly, the Sauerbrey equation can be used to estimate the amount of SVA adsorbed.

The largest amount of SVA was adsorbed on O

2

-plasma treated HMDSO surfaces compared to untreated titanium, HMDSO-coated titanium, and O

2

-plasma treated titanium. It was shown that the behavior of protein adsorption onto substrates depends largely on surface characteristics, such as wettability (hydrophilicity/hydrophobicity or surface free energy), chemistry, charge, roughness, and dynamics. Wettability is one of the most important parameters in the design of biomaterials and implant devices (38).

It is generally recognized that protein adsorption on more hydrophobic surfaces occurs via hydrophobic interaction. Hydrophobic surfaces support the adsorption of various surfactants and proteins from water because expulsion of solute from solution into the interphase between the bulk solid and solution phases is energetically favorable. Adsorption to hydrophobic surfaces is driven by the reduction of interfacial energetics concomitant with the replacement of water molecules at the surface by the adsorbed solute (surface dehydration) (21, 22). In contrast, it was reported that hydrophilic surfaces with a sulfonate group lead to greater adsorption of proteins from plasma (6), and that plasma protein adsorption on a wettability gradient surface is elevated with an increase in surface wettability. This suggests that more plasma protein adsorption at the hydrophilic positions of the gradient surface reduces platelet adhesion (20). These reports suggested that the adsorption characteristics of proteins depend on the residual groups of amino acid in proteins.

In this study, the largest amount of SVA was adsorbed on the O

2

-plasma treated HMDSO surfaces. Since SVA has both hydrophobic groups (-CH

3

) and hydrophilic functional groups (-COOH, -OH), as shown in Fig. 1, two possible adsorption mechanisms are conceivable. One
possible mechanism is due to hydrophobic interaction. The other is due to electronic interaction by hydrophilic groups and/or hydrogen bonds via functional groups. The contact angle measurement and XPS analysis showed that the O$_2$-plasma treated HMDSO had highly hydrophilic surfaces, and was dominant over the Si-(O)$_n$ with an OH group. In addition, the intensity of O was increased by the O$_2$-plasma treatment. FT-IR-RAS analysis showed that the O$_2$-plasma treatment reduced the intensity of Si-(CH$_3$)$_x$, and caused the peak attributed to the Si-O-Si bonds to broaden. These phenomena indicate that an introduction of OH groups and a slight oxidation reaction and/or O$_2$-functional groups occurred on the deposited films during the O$_2$-plasma treatment. The change of the HMDSO monomer structure caused by plasma polymerization and the O$_2$-plasma treatment that followed can be presumed to be as illustrated in Fig. 8. That is, the fragmentation of the Si-C and C–H bonds of the HMDSO monomers were caused by plasma polymerization, and changes in the hydrophilic surfaces were concomitant with introduction of an OH group and/or O$_2$-functional group during O$_2$-plasma treatment. Consequently, the latter mechanism, especially hydrogen bonds via functional groups, was considered dominant in the mechanism of SVA adsorption mediated by an OH group and/or O$_2$-functional groups.

In contrast, the O$_2$-plasma treated Ti showed less SVA adsorption, even though these surfaces exhibited hydrophilicity equal to that of the O$_2$-plasma treated HMDSO and were covered with oxide and hydroxide. It is reported that plasma treatment with Ar gas improves the initial adhesion of osteoblast-like cells to titanium surfaces because of increasing hydrophilicity (37). However, the relationship among cell adhesion, protein adsorption, and surface characteristics is still unclear. In this study, no appreciable compositional changes were observed on Ti surfaces by O$_2$-plasma treatment compared to the HMDSO surfaces. Therefore, the reason for lower SVA adsorption is considered the lack of the introduction of OH and/or O$_2$-functional groups because of their inorganic characteristics in contrast to
organic HMDSO surfaces. Thus, activation on organic surfaces by introducing functional
groups is considered to be responsible for the immobilization of SVA.

The clinical implications of this study are that hexamethyldisiloxane-coating followed by
O$_2$-plasma treatment is effective for the adsorption of SVA onto titanium implants in contrast
to simple O$_2$-plasma treatment on titanium. Further study through *in vitro* and *in vivo*
examination is necessary to determine the optimal concentration of SVA for maximum
effectiveness of this agent.

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Figure legends

Fig. 1 Chemical structures of simvastatin (SV) and simvastatin acid (SVA).
Fig. 2 Schematic illustrations of HMDSO coating, O$_2$-plasma treatment, and adsorption assay for QCM-D measurement.
Fig. 3 XPS spectra of Ti, Ti+O2, HMDSO, and HMDSO+O2 specimens.

Fig. 4 Intensity ratio of elements on Ti, Ti+O2, HMDSO, and HMDSO+O2 specimens under XPS analysis.
Fig. 5 FT-IR spectra of HMDSO and HMDSO+O2 specimens.

![FT-IR spectra of HMDSO and HMDSO+O2 specimens.](image)

- **HMDSO**
- **HMDSO+O2**

- Si-O-Si
- Si-O-C
- Si-(R)_{3}C-OH
- Si(CH_{3})_{x}
- Si(CH_{3})_{3}
- Si(CH_{2})_{2}
- Si-C
- Si-CH_{3}
- Si-O-C

**Absorbance (arb. units)**

**Wavenumber (cm\(^{-1}\))**

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<th>1000</th>
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**Species**

- **Si-O-Si**
- **Si-O-C**
- **Si-(R)_{3}C-OH**
- **Si(CH_{3})_{x}**
- **Si(CH_{3})_{3}**
- **Si(CH_{2})_{2}**
- **Si-C**
- **Si-CH_{3}**
- **Si-O-C**

**Notes**

- HMDSO: Hexamethyldisiloxane
- HMDSO+O2: Hexamethyldisiloxane with Oxygen
Fig. 6 Typical example of frequency and dissipation shift against time for exposure of a HMDSO+O2 specimen to SVA obtained via QCM-D measurement.

![Graph showing frequency and dissipation against time](image)

Fig. 7 Amounts of SVA-adsorption on Ti, Ti+O2, HMDSO, and HMDSO+O2 sensors.

The amount of SVA adsorbed onto the HMDSO+O2 sensor was more than three times greater than that onto other sensors (p<0.01). In contrast, there was no significant difference between the amount adsorbed by the Ti and Ti+O2 sensors (p>0.05).

![Bar chart showing amounts of SVA adsorption](image)
Fig. 8 Possible structure change of HMDSO monomers by plasma polymerization and following O\textsubscript{2}-plasma treatment.

The fragmentation of the Si–C and C–H bonds of the HMDSO monomers were caused by plasma polymerization, and changes in the hydrophilic surfaces were concomitant with introduction of an OH group and/or O\textsubscript{2}-functional group during O\textsubscript{2}-plasma treatment.