Influence of mucin pre-adsorption for lipoteichoic acid adsorption on titanium surfaces

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We investigated the adsorption of lipoteichoic acid (LTA) on titanium surfaces by using the quartz crystal microbalance (QCM) method, and confirmed the influence of mucin (MUC) pre-adsorption on LTA adsorption. Two injection methods were evaluated. Namely, Method A: single-step injection of LTA solution to the Ti sensor, and Method B: MUC solution was injected to the Ti sensor followed by LTA injection on the MUC pre-adsorbed surface. QCM measurement revealed that the adsorbed amount of LTA by Method A was low and constant regardless of the increase in LTA concentration. In contrast, the adsorption amount of LTA in Method B increased according with increasing concentration of LTA and was significantly higher than that of Method A. The hydrophobic surface after MUC pre-adsorption was presumed to contribute to the enhancement of LTA adsorption. Our results revealed that MUC pre-adsorption to Ti is necessary for LTA adsorption in living tissue.

Keywords: Titanium, Dental biofilm, Lipoteichoic acid, Mucin

INTRODUCTION

Dental implants are widely used as a repair method for tooth loss, and high success rates of dental implant treatment are reported. However, a few cases of fracture or loosening of dental implants have been reported. Notably, peri-implant diseases may affect the peri-implant mucosa and supporting bone, and may cause loosening of the implants by bone resorption, which is classified as peri-implantitis. Biofilm formation on the implant material is a possible cause of peri-implantitis. Biofilms are a complex of attached bacterial and salivary macromolecules, and form not only on the teeth and mucosal surfaces but also the surfaces of dental restorations in implants. In recent years, it was confirmed that bacterial colonization on Ti implants occurred immediately following transmucosal implant placement. The adherence of pathogenic oral bacteria on Ti implant surfaces has been directly linked to peri-implant inflammatory changes.

Biofilm formation is a multi-stage process. The initial stage of biofilm formation is the adsorption of salivary proteins. The surfaces of materials exposed to the oral environment are directly covered by adsorbed protein films, known as the acquired pellicle. Mature plaque biofilm formation occurs after pellicle formation by the attachment and colonization of oral bacteria.

Previously, we investigated the initial adsorption of pellicle proteins onto different dental materials, i.e., gold, silica and Ti, by using the quartz crystal microbalance (QCM) method. Among several adsorption analysis methods for proteins, the QCM method is a simple and straightforward technique to detect molecular behavior on a surface at nano-scale. No chemical or physical preparation of the proteins is needed, and the adsorption behavior of proteins can be directly monitored in real time. QCM can not only estimate the adsorption amounts of proteins but can also calculate binding and dissociation rates. Chemical binding and isolation points of protein on the material surface can also affect the adsorption amount or Ki.

We confirmed the differences in adsorption behaviors of salivary pellicle proteins. As pellicle proteins, lactoferrin, lysozyme, defensin and mucin (MUC) were evaluated. Among them, MUC has been identified in several additional types of dental biofilm, such as the salivary pellicle on hydroxyapatite, and on denture-acquired pellicle. The presence of MUC on dental material surfaces is thought to affect bacteria attachment and accumulation, which will lead to biofilm formation. Lori and Nok investigated the adsorption of MUC to Ti surfaces and found that pre-treatment of Ti with calcium or magnesium increased the adsorption of MUC.

Lipoteichoic acid (LTA) is a cell wall component of streptococci, and is suggested to play a central role in the pathogenesis of streptococci infections. Teichoic acids are exposed on the surface of intact bacteria. Many gram-positive bacteria produced LTA polymers, and LTA is reported to be an essential molecule for the viability of Staphylococcus aureus (S. aureus). The oral cavity is filled with saliva, and implanted Ti will be covered with adsorbed pellicle proteins. MUC is a representative pellicle protein in the oral cavity. In the present study, we stimulated LTA adsorption on Ti covered with MUC in the oral cavity by using the QCM method, and investigated the influence of MUC pre-adsorption for LTA adsorption on the Ti surface.

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MATERIALS AND METHODS

QCM apparatus and sensors
A 27MHz QCM (AT cut shear mode, AFFINIX QNµ, Initium, Tokyo, Japan) with a 500 µL cell, temperature control system and stirring bar was used. The temperature was maintained at 25±1°C and the solution in the cells was stirred during the measurements. The Ti sensor was prepared by sputter coating Ti onto an Au electrode. A Ti disk (99.99 mass%, ULVAC, Kanagawa, Japan) was used as the target. Using sputtering deposition equipment, Ti was sputter coated at a pressure of 0.2 Pa for 30 min. The Ti sensor was irradiated by ultraviolet radiation (BioForce Nanosciences Holdings, Ames, IA, USA) for 20 min before QCM measurement.

QCM measurement of LTA (Method A)
*S. aureus* lipoteichoic acid (LTA; L2515, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in phosphate-buffered saline (PBS) solution (pH=7.4, P3813, Sigma-Aldrich) at concentrations of 0.1, 0.3, 0.5, 1.0 and 2.0 mg/mL. Five-hundred microliters of PBS was added to the sensor cell, and 5 µL of LTA solution was injected into the PBS solution in the sensor cell. The frequency decrease was monitored until 2 h after protein injection.

QCM measurement of LTA after the MUC pre-adsorption (Method B)
Bovine submaxillary gland mucin (MUC; Wako Pure Chemical Industries, Osaka, Japan) was dissolved in PBS solution (pH=7.4) at a concentration of 0.5 mg/mL. Five-hundred microliters of PBS was added to the cell, and 5 µL of MUC solution was injected into the PBS solution in the cell. Two hours after MUC solution injection, the PBS solution including MUC was removed and fresh PBS solution added. The removal of PBS solution including MUC and addition of fresh PBS was repeated three times. The MUC pre-adsorbed Ti surface was thus obtained. Afterwards, 5 µL of LTA solution was injected into the cell. The frequency decrease was monitored until 2 h after the LTA injection. The concentrations of LTA solution used were 0.1, 0.3, 0.5, 1.0 and 2.0 mg/mL.

Equilibrium analysis of LTA adsorption behavior
The LTA adsorption behavior was analyzed by AQUA software (Initium). Where ΔF is the measured frequency shift (Hz), Δm is the mass change (g), F₀ is the fundamental frequency of the quartz crystal (27×10⁶ Hz), A is the electrode area (0.049 cm²), ρ is the density of quartz (2.65 g/cm³), and μ is the shear modulus of quartz (2.95×10¹¹ dyn/cm²). At 27 MHz, a frequency decrease of 1 Hz corresponds to a mass change of approximately 0.62 ng/cm² according to the Sauerbrey equation (eq.1)²⁰.

$$\Delta F = \frac{2F_0^2\Delta m}{A\sqrt{\rho\mu}}$$  \hspace{1cm} eq. 1

The dissociation constant (Kd) value for LTA adsorption was obtained by equilibrium analysis with reciprocal linear-fitting. The reciprocal linear-fitting was calculated by the eq.2 It is the correlation between the injected LTA molecule concentration divided by ΔF and the injected LTA molecule concentration. Bmax was defined the maximum adsorption constant.

$$\frac{\text{[Injected LTA]}}{\Delta F} = \frac{Kd}{B_{\text{max}}} + \frac{1}{B_{\text{max}}} \cdot \text{[Injected LTA]} \quad \text{eq. 2}$$

Contact angle measurements of sensors
The contact angles of the Ti and MUC pre-adsorption surfaces against millipore pure water were measured (DMe-201, Kyowa Interface Science, Tokyo, Japan). The Ti sensor was treated by ultraviolet irradiation before contact angle measurement. To prepare the MUC-coated Ti sensor for contact angle measurement, 0.5 mg/mL of MUC solution was dropped onto the Ti sensor. After 2 h, the MUC solution was removed and the MUC covered sensor was dried in air for 24 h. The water drop volume was maintained at 0.5 µL for contact angle measurement, and 5 measurements of 10 s each were made for each surface type. Measurements were performed at the same room temperature (20±1°C) and humidity (43±1%).

Atomic force microscope (AFM) observation of different sensors
AFM (Nanosurf Easyscan 2, Nanosurf, Liestal, Switzerland) observation was used to assess the surface condition and surface roughness (Sa: three-dimensional arithmetic mean height) of the different sensors before and after QCM measurement. AFM images were captured in air in static mode. Static mode silicon probes (ContAl-G, force contact 0.2 N/m Budget sensors, Sofia, Bulgaria) with resonance frequencies of approximately 13 kHz were used for imaging. The AFM images were obtained for an area of 2×2 µm². Surface roughness (Sa) was also measured on each surface.

Statistical analyses
The non-paired t-test was employed to compare data obtained from surface roughness measurements and in QCM measurements. Significant differences were determined by one-way analysis of variance (ANOVA) using statistical analysis software (GraphPad Prism, GraphPad Software, San Diego, CA, USA). Significance was set at p<0.05.

RESULTS

Protein adsorption behaviors of Methods A and B
Figures 1(a) and (b) show typical ΔF values for the LTA adsorption behavior of proteins to the Ti sensor by QCM measurements. For Method A [Fig. 1(a)], a rapid decrease in ΔF was observed immediately after the injection, and ΔF hardly changed thereafter. For Method B [Fig. 1(b)], the ΔF with MUC adsorption gradually decreased in the Ti sensor. After washing with PBS solution, the ΔF with
LTA adsorption further gradually decreased compared with Method A.

Kinetic analysis of LTA adsorption behavior
The relationships between the amount of LTA adsorption and LTA concentration are shown in Fig. 2. The adsorbed amount of Method A was almost constant at approximately 7–8 ng/mm², irrespective of the increase in LTA concentration. The adsorption amount of Method B increased with increasing LTA concentration on the MUC pre-adsorption surface. The absorbed amount of Method B was significantly higher than on the untreated Ti surface (p<0.05).

Equilibrium analysis using a reciprocal plot of LTA concentration
The Kd values were estimated from reciprocal plots as shown in Fig. 3. The Kd of Method B was significantly larger than that of Method A (p<0.05).

AFM analyses of sensor surfaces
Three-dimensional images of each sensor are shown in Fig. 4 (a–d). Spherical particles with a diameter of 0.2 to 0.3 µm were observed on the Ti surface before QCM measurement [Fig. 4(a)]. In Fig. 4(b), large spherical particles are seen in addition to the small LTA particles (0.1–0.3 µm). Very tiny particles less than 0.1 µm were observed after Method A measurement [Fig. 4(b)]. These LTA particles partially covered the Ti spherical particles. The MUC particles of around 0.1 µm were observed on the MUC pre-adsorption surface [Fig. 4(c)]. MUC particles completely covered the Ti spherical particles. After Method B measurement, LTA particles were adsorbed on the MUC particles, as shown in Fig. 4(d).

Roughness of sensor surfaces
Table 1 compares the data on the surface roughness of each sensor. There were no significant differences
Fig. 4 Results of AFM analysis of each surface. The AFM images were obtained for an area of 2×2 µm² (Nanosurf Easyscan 2, Nanosurf). (a) Ti before adsorption QCM method, (b) LTA adsorbed Ti in Method A, (c) MUC pre-adsorption surface in Method B, (d) LTA on MUC pre-adsorption surface in Method B.

Table 1 Surface roughness of each sensor

| Surface                  | LTA on Ti surface | MUC pre-adsorption surface | LTA on MUC pre-adsorption surface |
|-------------------------|-------------------|-----------------------------|-----------------------------------|
| Ti surface              | 3.4±0.4 nm a       | 2.9±0.1 nm a                | 4.7±0.3 nm b                      |
| LTA on Ti surface       | 3.1±0.4 nm a       | 2.9±0.1 nm a                |                                    |

Four kinds of data are displayed as mean±S.D. Significant differences between the different character (p<0.05). Surface roughness was determined by AFM analysis.

Table 2 Contact angle measurement

| Surface                  |   |
|-------------------------|---|
| Ti surface              | 4.5±0.5 a |
| MUC pre-adsorption surface | 74.1±0.5 b |

Two kinds of data are displayed as mean±S.D. Significant differences between the different character (p<0.05). Contact angle was measured by DMe-201 (Kyowa Interface Science).

DISCUSSION

In this study, we found that MUC pre-adsorption remarkably enhanced LTA adsorption onto the Ti surface. It is well-known that biofilm formation is one of the major factors in peri-implantitis. It is important to understand the adsorption of saliva proteins onto the surface of implant materials. Many factors such as surface roughness, electrostatic interactions or hydrophilicity influence the adsorption of proteins onto materials. In the present study, surface roughness did not influence the LTA adsorption because there were no significant differences in surface roughness between the Ti and the MUC-coated Ti surfaces. The isoelectric points (PI) of LTA and MUC are reported to be 5.9–6.2 and 3.18, respectively. Thus, MUC on LTA was negatively charged in the present buffer condition of pH=7.4. The zeta potentials of bulk Ti plates measured by streaming current measurement method were approximately −87 mV at pH=7.4. Ti surfaces are also negatively charged. Electrostatic repulsion occurred between the negatively charged Ti and LTA. The lower amount of adsorbed LTA on the Ti surface was due to the electric repulsion.

In contrast, MUC pre-adsorption increased the
adsorbed amount of LTA. The reason for this is not clear, but it is presumed that the zeta potential of pre-adsorption Ti surface was reduced. Thus, the electric repulsion between LTA and the MUC pre-adsorption surface was reduced. Electrical evaluation of the MUC coated Ti surface is needed. Hydrophilicity is another factor controlling protein adsorption. The contact angle of Ti surface was increased with the MUC pre-coating. The MUC pre-adsorption surface was hydrophobic. It is presumed that LTA is adsorbed more readily on a hydrophobic surface. It has been reported that hydrophobic interactions play an important role as a driving force in acquired pellicle formation, and that higher amounts of salivary proteins are adsorbed on hydrophobic surfaces20. In the present study, we also evaluated the Kd value for LTA adsorption. Smaller Kd values indicate tighter bonding or attachment of molecules. The present results suggested that LTA showed tighter attachment to the MUC pre-adsorption surface than to the Ti surface. The MUC pre-adsorption surface not only enhanced the adsorption amount of LTA but also improved the attachment of LTA.

Ultimately, we concluded that LTA does not directly adsorb to Ti surfaces. In living tissue, MUC pre-adsorption treatment of Ti will be necessary for LTA adsorption, to prevent the formation of biofilms on Ti. Adsorption analyses of other pellicle proteins or glycoproteins are needed, to better understand biofilm formation.

CONCLUSIONS

We investigated the adsorption of LTA on a Ti surface by the QCM method. The adsorbed amounts of LTA and the dissociation constant (Kd) for LTA adsorption to Ti were evaluated. We found that the adsorption amount of LTA to a MUC pre-adsorption surface increased with increasing concentration of LTA, and was significantly higher than that of an untreated surface. The Kd value for LTA adsorption to the untreated Ti surface without MUC pre-adsorption was significantly lower than that to the MUC pre-adsorption surface. We also revealed that MUC pre-adsorption influenced the LTA adsorption on the Ti surface, and conclude that MUC pre-adsorption to Ti will be necessary for LTA adsorption in living tissue.

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CONFLICTS OF INTEREST

The authors declare that they have no financial conflict of interest.