Adsorption study of pellicle proteins to gold, silica and titanium by quartz crystal microbalance method

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Initial stage of biofilm formation is the adhesion of salivary pellicle proteins on the material surfaces. The aim of the present study was to evaluate the adsorption behaviors of saliva pellicle proteins onto a gold, silica and titanium by using the 27 MHz quartz crystal microbalance method. As pellicle proteins, lactoferrin, lysozyme, defensin and mucin were evaluated. Adsorption amount of lactoferrin to silica was significantly lower than gold and titanium. Significant differences were detected between titanium and silica for the adsorption amounts of lysozyme. Chemical bond formation of sulfur atom of lysozyme and gold could be suggested. There were no significant differences of the adsorption amount of β -defensin among each substrate. For mucin adsorption, gold showed the highest adsorption amount. It is presumed that electrostatic repulsion caused less adsorption amounts of mucin to titanium and silica. In conclusion, the differences of the adsorption behaviors of pellicle proteins could be clearly identified.

Keywords: Biofilm, Salivary pellicle proteins, Quartz crystal microbalance, Adsorption

INTRODUCTION

Biofilm formation in the oral cavity is now postulated to cause oral diseases such as dental caries, periodontal diseases, denture-induced stomatitis and peri-implantitis etc. Biofilm includes a complex of attached bacterial and salivary macromolecules, and forms on not only teeth and the mucosal surface but also the surface of metal or ceramic prostheses, orthodontic brackets, resin restoratives and titanium implants¹⁻³.

Biofilm formation is a multiple-stage process. The initial stage of biofilm formation is the adhesion of salivary pellicle proteins. The surface of materials exposed to the oral environment is directly converted by spontaneous adsorption of protein-dominated films, denoted by the acquired pellicle^{4,5}. Mature plaque biofilm formation occurs after pellicle formation by the attachment and colonization of oral bacteria.

Numerous researches have been reported to prevent biofilm formation, and most focused on the reduction or diminishing of bacterial adhesion to dental materials. For example, silver is the most commonly used antimicrobial agent and has been incorporated into restorative composite resin or coated on titanium implants^{2,3,6}.

In contrast, few studies have analyzed the adsorption/adhesion of pellicle proteins onto the natural and artificial surfaces of materials. Lori *et al.* investigated the adsorption of mucin to titanium and found that pretreatment of titanium with calcium or magnesium increased adsorption of mucin to titanium⁷. Nezu *et al.* evaluated the adsorption of lysozyme on gold compared with the adsorption of collagen⁸.

The quartz crystal microbalance (QCM) is recently employed as a sensing device for monitoring protein adsorption. When molecules bind to the oscillating quartz crystal, the oscillating frequency decreases in

relation to the amount of protein bound to the crystal surface. The amount of protein adsorbed onto the biomaterial can be estimated by the Sauerbrey equation⁹. There have been several reports on QCM monitoring for protein adsorption to titanium¹⁰⁻¹². Photocatalytic decomposition of the pellicle on anatase surfaces was also investigated using the QCM method¹³; however, most QCM studies of protein adsorption were carried out using 5 MHz AT-cut crystals. The higher fundamental frequency of QCM produces higher sensitivity, but higher fundamental frequency quartz crystals are very thin and fragile to handle, and thin quartz crystal plates are susceptible to water pressure. As a result, the signal-to-noise ratio (S/N) tends to decrease^{14,15}.

Recently, a 27 MHz QCM with a very low noise level of ± 0.05 Hz has been developed¹⁶⁻¹⁸. The adsorption behavior of albumin, fibronectin and collagen to titanium was monitored by 27 MHz QCM and differences in the adsorption amount and adsorption rate among the three proteins could be clearly identified¹⁹.

In the present study, the adsorption behavior of pellicle proteins onto gold, silica and titanium surfaces using 27 MHz QCM was investigated. By using 27 MHz QCM, the measurement with higher sensitivity and less noise will be possible. Gold and silica are the main components of the prosthesis such as metal inlay or porcelain crown, respectively, and titanium is widely used as a material of dental implant. As pellicle proteins, lactoferrin, lysozyme, defensin and mucin were evaluated.

MATERIAL AND METHODS

QCM apparatus

The QCM apparatus used was a 27-MHz AFFINIX Q_N μ (Initium Co., Ltd, Tokyo, Japan) with 500 μ L cells,

equipped with a temperature control system and stirring bar. The temperature was maintained at $25 \pm 1^\circ\text{C}$ and the solution in the cells was stirred during the measurements. AT-cut quartz crystal is sandwiched by gold electrode on both sides. The surface area of the Au electrode was 4.9 mm^2 . In addition to the gold (Au) sensor, silica (SiO_2) and titanium (Ti) sensors were used.

The SiO_2 sensor was prepared by sputter coating SiO_2 onto an Au electrode. An SiO_2 disk (Quartz 4N; ULVAC, Inc., Kanagawa, Japan) was used as a target. Using sputtering deposition equipment (CS200; ULVAC, Inc.), SiO_2 sensor was deposited at a pressure of 0.1 Pa for 24 min.

The Ti sensor was also prepared by sputter coating titanium onto an Au electrode. A titanium disk (99.99 mass%, ULVAC, Inc.) was used as the target. Using sputtering deposition equipment, titanium was sputter coated at a pressure of 0.2 Pa for 30 min.

QCM measurement

Lactoferrin (molecular weight, MW=80 kDa), lysozyme (MW=14 kDa), pig stomach mucin (MW=1,000–10,000 kDa) (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and human β -defensin-3 (MW=4 kDa; Peptide Institute, Inc., Osaka, Japan) were dissolved in phosphate-buffered saline (PBS) solution (pH 7.4) at a concentration of $50 \text{ }\mu\text{g/mL}$. The molecular weight of mucin was assumed as 5,000.

First, $500 \text{ }\mu\text{L}$ PBS was added to the cell. After stabilization of the cell, $5 \text{ }\mu\text{L}$ protein solution was injected into the PBS solution in the cell. The frequency decrease was monitored until 30 min after the protein injection. The amount of protein adsorbed onto each surface at 30 min after the protein injection was calculated by Sauerbrey's equation⁹.

$$\Delta F = \frac{2F_0^2 \Delta m}{A \sqrt{\rho_q \mu_q}}$$

Where ΔF is the measured frequency shift (Hz), Δm is the mass change (g), F_0 is the fundamental frequency of the quartz crystal ($27 \times 10^6 \text{ Hz}$), A is the electrode area (0.049 cm^2), ρ_q is the density of quartz (2.65 gcm^{-3}), and μ_q is the shear modulus of quartz ($2.95 \times 10^{11} \text{ dyn cm}^{-2}$). At 27 MHz, a frequency decrease of 1 Hz corresponds to a mass change of approximately 0.62 ng/cm^2 according to the Sauerbrey equation. By curve-fitting for the ΔF curve against the adsorption time, the apparent reaction rate, K_{obs} , in the following equation was obtained. ΔF_∞ is the frequency shift at infinite time.

$$\Delta Ft = \Delta F_\infty (1 - e^{-K_{obs} \cdot t})$$

Three runs of QCM measurements for each protein to each sensor were performed during 30 min, respectively.

Contact angle measurements

The contact angle of the sensor surface with respect to double-distilled water was measured using a contact

angle meter (CA-P; Kyowa Interface Science Co. Ltd., Tokyo, Japan) at 20°C with 45% relative humidity. The volume of water drop was maintained at $2 \text{ }\mu\text{L}$, and three measurements for 15 s each were made for each surface type. Measurements were performed at the same room temperature and humidity.

Statistics

Significant differences were determined by one-way analysis of variance (ANOVA) using statistic analysis software (GraphPad Prism, GraphPad Software Inc., San Diego, CA, USA). Statistical significance was set at $p < 0.05$.

RESULTS

Contact angle measurements

Table 1 shows the results of contact angle measurements of each sensor against water. The contact angle of Ti sensor was the highest, and the SiO_2 sensor had the lowest contact angle. That means that Ti sensor was the most hydrophobic and SiO_2 was the most hydrophilic.

QCM measurements for protein adsorption to each sensor

Figure 1 shows the typical ΔF for the adsorption of proteins to each sensor by QCM measurements. The decrease in ΔF after the protein injection could be monitored. The greater degree of the decrease in ΔF corresponds to a greater degree of adsorption of proteins to each sensor.

For the adsorption of lactoferrin (Fig. 1-a), a rapid decrease of ΔF was observed immediately after the injection and little change of ΔF was recognized subsequently. The degree of frequency decrease with Au and Ti sensors was greater than that of SiO_2 sensor.

The adsorption behavior of lysozyme was influenced by the difference in QCM sensor (Fig. 1-b). The ΔF to Au sensor was more rapidly decreased after lysozyme injection than Ti and SiO_2 . The ΔF to Ti and SiO_2 sensors gradually decreased and Ti sensor produced a greater degree of decrease in the ΔF .

For β -defensin adsorption (Fig. 1-c), prompt decrease of ΔF to Au sensor was also observed. Ti and SiO_2 sensors showed a relatively rapid decrease in ΔF at an early point. A stable ΔF was recognized subsequently. The ΔF with mucin adsorption gradually decreased in the three types of sensors (Fig. 1-d).

Figure 2 shows the estimated amounts of proteins adsorbed on each sensor 30 min after injection estimated by the Sauerbrey equation⁹. Adsorption

Table 1 The result of contact angle measurements of each sensor against double-distilled water

	Au	Ti	SiO_2
Contact angle (deg)	34.6 (3.1)	52.0 (4.0)	7.3 (0.6)

(): SD

Fig. 1 Typical ΔF s for the adsorption of proteins to each sensor by QCM measurements (a: lactoferrin, b: lysozyme, c: β -defensin, d: mucin).

Fig. 2 Estimated amounts of proteins adsorbed on each sensor 30 min after injection estimated by the Sauerbrey equation. (a: lactoferrin, b: lysozyme, c: β -defensin, d: mucin). —: Presence of significant difference ($p < 0.05$).

Table 2 The *Kobs* (1/s) values for the adsorptions of the proteins to each sensor during 30 min

Protein	Au	Ti	SiO ₂
Lactoferrin	0.079 (0.011)	0.033 (0.006)	0.098 (0.035)
Lysozyme	0.175 (0.069)	0.005 (0.006)	0.007 (0.004)
β-defensin	0.060 (0.012)	0.025 (0.005)	0.022 (0.013)
Mucin	0.004 (0.001)	0.002 (0.001)	0.003 (0.001)

—: no significant difference ($p > 0.05$) (): SD

amount of lactoferrin to SiO₂ sensor was significantly lower than to Au and Ti sensors, as shown in Fig. 2-a ($p < 0.05$). In Fig. 2-b, significant differences were analyzed between Ti and SiO₂ sensors for the adsorption of lysozyme ($p < 0.05$). No significant difference in the adsorption amounts of lysozyme existed between Au and Ti sensors. Figure 2-c shows that there were no significant differences in the adsorption amount of β-defensin among sensors ($p > 0.05$). For mucin adsorption, Au sensor showed the highest adsorption amount of mucin ($p > 0.05$), and no significant difference was detected between Ti and SiO₂ sensors.

Table 2 lists the *Kobs* values for the adsorption of the proteins to each sensor during 30 min. A large value of *Kobs* corresponds to rapid rate for reaching a saturated adsorption of protein to the sensor. *Kobs* of Ti for lactoferrin adsorption, *Kobs* of Ti and SiO₂ for lysozyme and β-defensin adsorption were significantly smaller than the other sensors in same protein adsorption ($p < 0.05$). There were no significant differences in *Kobs* of mucin among Au, Ti and SiO₂ sensors ($p > 0.05$).

DISCUSSION

In this study, we evaluated that adsorption of pellicle proteins to Au, Ti and SiO₂ using 27 MHz QCM.

Among several adsorption analyses for protein, the QCM method is a simple technique to detect molecular behavior on a surface in nano scale and the adsorption behavior of protein could be directly monitored in real time¹⁹. QCM can estimate not only the adsorption amount of protein but also calculate binding and dissociation rates.

The surfaces of dental materials acquire a salivary pellicle immediately after exposure to the oral cavity. Understanding the protein adsorption process on a material surface is very important regarding biofilm formation. In the present study, four types of pellicle proteins, lactoferrin, lysozyme, β-defensin and mucin, were investigated. These four proteins were reported to be present in the biofilm layer and to play a role in biofilm formation, for example, specific adsorption to *Streptococcus mutans*²⁰.

The adsorption behavior of the four pellicle

proteins to Au, Ti and SiO₂ is complicated. Many factors influence the adsorption of proteins to material surfaces. For example, chemical bond formation, ionic and hydrophobic-hydrophilic interactions and steric conformation of proteins contribute to the formation of the proteinaceous pellicle layer^{21–23}. Surface roughness of the material surface also influences adsorption behavior. More proteins adsorb to a rough surface²⁴.

In the present study, the employed QCM sensors had the same surface smoothness at nano scale level. The influence of surface roughness could therefore be ignored. The adsorbed amount was only estimated by Sauerbrey's equation. The differences of stiffness or elasticity of adsorbed proteins were neglected and simple comparison of estimated amounts of adsorbed proteins was performed as a first step of our QCM experiments.

Lysozyme and β-defensin had sulfur atoms of cysteine groups in their molecule. For the adsorption of lysozyme and β-defensin to Au sensor, chemical bond formation between Au and sulfur contributed to the prompt decrease in ΔF ²⁵.

Electrostatic interaction has been reported to be dominant in the protein adsorption to titanium¹⁹. The isoelectric point (PI) of Ti and SiO₂ was approximately 5–5.5 and 2.0–3.0, respectively^{26–28}. As for proteins, PI of lactoferrin, lysozyme, β-defensin and mucin was approximately 7.8, 10.5–11, 8.9–9.7 and 4.8–5.1, respectively^{29,30}. Thus, the surfaces of Ti and SiO₂ were negatively charged at physiological pH (7.4). In contrast, lactoferrin, lysozyme and β-defensin were positively charged. Mucin was negatively charged under the present condition. Adsorption of albumin (PI=4.7) and fibronectin (PI=5–6) to Ti was suppressed compared with collagen (PI=9.5) due to electrostatic repulsion¹⁹. It is suggested that less adsorption of mucin to Ti and SiO₂ sensors was mainly due to the electrostatic repulsion because mucin, Ti and SiO₂ were negatively charged. Some researches pointed out that difference of pH value contributed the adhesion of proteins to materials^{10,31}. The influence of pH for the adhesion of pellicle proteins to dental materials should be further investigated.

Hydrophobic-hydrophilic interaction is another powerful candidate for controlling the adsorption behavior of proteins to material surfaces. It has been reported that hydrophobic interactions played an important role as a driving force in pellicle formation, and that higher amounts of salivary proteins adsorbed on hydrophobic surfaces³². The SiO₂ surface was the most hydrophilic. It is presumed that the less adsorption of lactoferrin and lysozyme to SiO₂ sensor was due to the hydrophilic property of the SiO₂ surface. Hydrophilic and hydrophobic properties of proteins should be more clearly elucidated.

Kobs is the apparent reaction rate and was easily obtained by curve-fitting the ΔF curve. The large value of *Kobs* of lysozyme to Au sensor was due to chemical bond formation between Au and a sulfur atom of lysozyme. For mucin, *Kobs* for mucin was relative smaller. Although the detailed mechanism is still not clear, the higher molecular weight of mucin than other pellicle will cause

steric influence for adsorption behavior.

Analysis of adsorption behaviors of other proteins or biomolecules such as glucan-binding protein or lipoteichoic acid should be needed. Further, the development of anti-biofilm surfaces will be investigated as a next series of our experiments.

In conclusion, it revealed that the adsorption behaviors of pellicle proteins to gold, silica and titanium material surfaces could be influenced by many factors, such as electric repulsion or chemical bond formation and the differences of the adsorption behaviors of pellicle proteins could be clearly identified.

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