Evaluation of protein adsorption to diamond-like carbon (DLC) and fluorine-doped DLC films using the quartz crystal microbalance method

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The aim of the present study was to evaluate albumin adsorption to stainless steel (SUS), diamond-like carbon (DLC) and fluorine-doped DLC (F-DLC) films using the quartz crystal microbalance (QCM) method. Each sensor was characterized using atomic force microscopy, surface roughness and surface wettability measurements and surface free energy calculations. Adsorbed amounts of bovine serum albumin on DLC and F-DLC were significantly lower than that on SUS ($p < 0.05$). The apparent first-order reaction rate, k_{obs} , of F-DLC was significantly larger than those of SUS and DLC ($p < 0.05$). Moreover, significantly lower total surface free energies of DLC and F-DLC influenced the albumin adsorbed amounts and k_{obs} . Furthermore, a clear correlation was found between the albumin adsorbed amounts and the hydrogen bond component of the total surface free energy. Thus, DLC or F-DLC coating is effective for preventing protein adsorption on orthodontic appliances.

Keywords: Diamond-like carbon, Albumin adsorption, Quartz crystal microbalance, Contact angle, Surface free energy

INTRODUCTION

In orthodontics, orthodontic brackets and wires are commonly used to treat malocclusion. An important factor influencing tooth movement is static friction that is present between brackets and wires. Lower friction enhances effective tooth movement due to more efficient force transmission to the teeth, which can allow the treatment period to be shortened without undesirable anchorage loss or pain to the patient^{1,2}. Numerous studies regarding the reduction of the friction between brackets and wires have been reported^{1,3,4}.

Recently, diamond-like carbon (DLC) has been used in various industrial applications⁵ because of its excellent physical properties, such as extreme surface hardness, low friction coefficient, chemical inertness, high wear resistance and superior biocompatibility^{6,7}. In orthodontics, application of DLC coating on brackets and wires has been reported to reduce the friction between the bracket and wire^{8,9}. Previously, we reported that homogeneous DLC-coated orthodontic brackets significantly reduced static friction between brackets and wires under both dry and wet conditions¹⁰. Moreover, fluorine-doped DLC (F-DLC) and silicon-doped DLC (Si-DLC) coatings were deposited onto brackets and frictional properties between coated brackets and wires under dry and wet conditions were characterized¹¹. DLC, F-DLC and Si-DLC coatings provided a significant reduction in static friction, and F-DLC-coated brackets exhibited the significantly lowest static friction between the bracket and wire under wet conditions, which was lower than that under dry conditions, among the three

tested coatings.

Orthodontic appliances used in the oral cavity are continually exposed to salivary proteins and oral bacteria. Friction increases and orthodontic brackets and wires deteriorate in the oral environment because of protein adsorption, which subsequently leads to an acquired pellicle and plaque formation on the orthodontic appliance¹²⁻¹⁵. Furthermore, it is presumed that salivary protein adsorption or adhesion is the initial stage of biofilm formation^{16,17}. Therefore, orthodontic appliances that are resistant to protein adsorption are desirable. However, evaluation of oral protein adsorption on DLC and F-DLC films has not yet been conducted.

Several methods are used to analyze protein adsorption, such as infrared reflection spectroscopy, ellipsometry and surface plasmon resonance¹⁸. Among them, the quartz crystal microbalance (QCM) technique is a straightforward method for detecting protein adsorption onto a material surface by measuring differences in the oscillating frequency of the quartz cell¹⁹. When molecules bind to the oscillating quartz crystal, the oscillating frequency decreases in relation to the amount of protein bound to the crystal surface. Yoshida and Hayakawa evaluated the adsorption of pellicle proteins to dental materials by the QCM method^{20,21}. They found that the adsorption behavior of pellicle proteins were influenced by the kinds of proteins and materials.

The aim of the present study was to evaluate protein adsorption to stainless steel (SUS)-, DLC- and F-DLC-coated surfaces using the QCM method. Albumin is a major component of oral saliva. Thus, the adsorption of albumin was evaluated. The influence of surface free energy on albumin adhesion was mainly analyzed.

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The null hypothesis of the present study was that the different surfaces, namely, SUS, DLC and F-DLC, do not influence albumin adsorption.

MATERIALS AND METHODS

Sample preparation

Au sensors for QCM measurements were coated with stainless steel 304 (SUS), DLC or F-DLC (Table 1). The SUS sensor was prepared by sputter coating (CS200, ULVAC, Kanagawa, Japan) with the use of a SUS 304 target. Each DLC coating was performed by the direct current-pulsed plasma-enhanced chemical vapor deposition method (DC-pulsed PECVD; NPS-330, Nanotec, Chiba, Japan)¹¹. The source gas for DLC and F-DLC coating was C₂H₂ and C₆F₆, respectively, and the base substrate under DLC and F-DLC layers is gold. During the coating process, the source gas was dissociated and ionized by plasma discharge. The resulting positively charged ions were attracted to the negatively biased substrate. The thickness of SUS, DLC and F-DLC demined by stylus profilometer (Dektak150, Bruker Nano, for SUS, SURFCOM E-RM-S01A, Tokyo Seimitsu for DLC and F-DLC) was 150, 20–30 and 20–30 μm, respectively.

Surface characterization

Atomic force microscopy (AFM; Nanosurf Easyscan 2, Nanosurf, Liestal, Switzerland) observation was performed to identify the surface morphology and surface roughness of each sensor. AFM images were captured in air by the tapping mode. Tapping mode silicon probes (Tap190AL-G, force contact 48 N/m; BudgetSensors, Sofia, Bulgaria) with resonance frequencies of approximately 190 kHz were used for imaging. AFM images were obtained in an area measuring 5×5 μm². Surface roughness (arithmetic mean height) was calculated using software (Nanosurf Easyscan 2) supplied with the AFM. All measurements were performed three times.

The surface wettability of each sensor surface was measured using a contact angle meter (DMe-201, Kyowa Interface Science, Tokyo, Japan), and surface free energies were obtained by measuring the contact angle with double-distilled water (WCA) and diiodomethane (DCA). Each sensor was irradiated by ultraviolet radiation (UV ozone cleaner, BioForce Nanosciences Holdings, Ames, IA, USA) for 20 min before contact angle measurement. The liquid drop volume was maintained at 0.5 μL. Measurements were performed three times at

the same room temperature and humidity.

The total surface free energy (γ^{total}), as well as dispersive surface (γ^{d}) and hydrogen bond (γ^{h}) components, were calculated by the Owens-Wendt theory according to the following equation^{22–24}:

$$\gamma_L(1+\cos\theta)=2(\sqrt{\gamma_L^{\text{d}}\gamma_S^{\text{d}}}+\sqrt{\gamma_L^{\text{h}}\gamma_S^{\text{h}}})$$

where γ_L and γ_S are liquid surface tension and solid surface energy, respectively, γ^{d} of water and diiodomethane are 21.8 and 49.5 mN/m and γ^{h} of water and diiodomethane are 51 and 1.3 mN/m, respectively^{22–25}.

QCM measurements

A 27-MHz QCM (AT-cut shear mode; AFFINIX QNμ, ULVAC) with a 500-μL sensor cell was used²⁶. The sensor cell of each sensor was mounted in the cell socket of the QCM apparatus. The temperature control system and stir bar were then equipped. The temperature was maintained at 25±1°C and the solution in the cell was stirred at 1,000 rpm during the measurement.

Bovine serum albumin (BSA; Wako Pure Chemical Industries, Osaka, Japan) was dissolved in a phosphate-buffered saline (PBS) solution (pH=7.4) at a concentration of 1 mg/mL. Before mounting the sensor cell, each sensor was cleaned with a 1% sodium dodecyl sulfate solution and then irradiated with ultraviolet light (BioForce Nanosciences Holdings) for 20 min. After mounting the sensor cell, 500 μL of PBS was added to the cell. After stabilization of the frequency shift, 1 mg/mL BSA solution (0.5 μL) was injected into the PBS solution in the cell. The frequency decrease was monitored until 120 min after BSA injection.

The albumin adsorbed amount at 120 min after BSA injection was calculated as follows by the Sauerbrey equation²⁷:

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

where ΔF is the measured frequency shift (Hz), Δm is the mass change (g), F_0 is the fundamental frequency of the quartz crystal (27×10⁶ Hz), A is the electrode area (0.049 cm²), ρ_q is the density of quartz (2.65 gcm⁻³) and μ_q is the shear modulus of quartz (2.95×10¹¹ dyncm⁻²). At 27 MHz, a frequency decrease of 1 Hz corresponds to a mass change of approximately 0.62 ng/cm² according to the Sauerbrey equation²⁷. By curve fitting for the ΔF curve against the adsorption time, the apparent reaction rate, k_{obs} , during 10 min of injection, was obtained using

Table 1 Sensors for QCM measurements

Sensor	Target/source gas	
SUS	SUS 304	sputter coating
DLC	C ₂ H ₂	DC-pulsed PECVC
F-DLC	C ₆ F ₆	DC-pulsed PECVC

the following equation:

$$\Delta F_t = \Delta F_\infty (1 - e^{-k_{\text{obs}} \cdot t})$$

where ΔF_∞ is the frequency shift at infinite time. Three runs of QCM measurements for protein adsorption to each sensor were performed that each lasted 120 min.

After QCM measurements, AFM images of each sensor were also observed as described above.

Statistical analysis

Statistical analyses were performed using SPSS (version 16.0J for Windows, IBM Japan, Tokyo, Japan) software. The mean and standard deviation (SD) of surface roughness, contact angle, surface free energy and QCM data were calculated. The results for each sensor were compared using a one-way analysis of variance (ANOVA) and Tukey's tests, with $p < 0.05$ considered to indicate statistical significance.

RESULTS

Surface characterization

Needle-like images were observed on the SUS surface and spherical particles were observed on DLC and F-DLC surfaces by AFM observation (Fig. 1). There were significant differences in surface roughness between SUS and DLC ($p < 0.05$) and SUS and F-DLC ($p < 0.05$)

(Table 2). However, no significant difference in surface roughness was observed between DLC and F-DLC ($p > 0.05$).

The results of contact angle measurements and the calculation of surface free energies are shown in Tables 3 and 4, respectively. There were significant differences in the WCA among the three surface types ($p < 0.05$). However, for the DCA, no significant difference was observed between SUS and DLC ($p > 0.05$). F-DLC showed significantly greater WCA and DCA values than SUS and DLC ($p < 0.05$).

Regarding the dispersive surface component (γ^d), DLC showed significantly greater values than SUS ($p < 0.05$), and F-DLC showed significantly smaller values than DLC ($p < 0.05$, Table 4). There was a significant decrease in the hydrogen bond surface component (γ^h) of DLC and F-DLC compared with that of SUS ($p > 0.05$). Moreover, total surface free energies (γ^{total}) of DLC and F-DLC were significantly smaller than that of SUS ($p > 0.05$).

QCM measurements

A frequency decrease was observed after albumin injection. As shown in Fig. 2, SUS exhibited a more rapid and greater frequency decrease than DLC and F-DLC. The calculated BSA absorbed amounts are listed in Table 5. The BSA absorbed amounts on DLC and F-DLC were significantly lower than that on SUS ($p < 0.05$). However, there was no significant difference in BSA absorbed

Fig. 1 AFM images of (a) SUS-, (b) DLC- and (c) F-DLC-coated QCM sensors.

Table 2 Surface roughness (Sa) of each sensor

Sensor	Sa (nm)
SUS	4.38 (0.18) ^a
DLC	3.02 (0.39) ^b
F-DLC	2.48 (0.29) ^b

Data are expressed as means (SD).

Groups that do not share the same superscript letter are significantly different ($p < 0.05$).

Table 3 Water and diiodomethane contact angles of each sensor

Sensor	Water contact angle (°)	Diiodomethane contact angle (°)
SUS	54.3 (3.7) ^a	39.6 (1.0) ^a
DLC	71.9 (2.7) ^b	34.8 (2.2) ^a
F-DLC	87.7 (1.7) ^c	51.0 (2.2) ^b

Data are expressed as means (SD).

Groups that do not share the same superscript letter are significantly different ($p < 0.05$).

Table 4 Dispersive surface component, hydrogen bond surface component and total surface free energy of each sensor

Sensor	Dispersive surface component (mN/m)	Hydrogen bond surface component (mN/m)	Total surface free energy (mN/m)
SUS	32.5 (1.8) ^a	19.0 (3.2) ^a	51.4 (1.5) ^a
DLC	37.9 (0.3) ^b	5.9 (0.8) ^b	43.8 (0.6) ^b
F-DLC	28.7 (2.4) ^a	3.3 (1.1) ^b	32.0 (1.3) ^c

Data are expressed as means (SD).

Groups that do not share the same superscript letter are significantly different ($p < 0.05$).

Table 5 Albumin adsorption amounts and k_{obs} of each sensor

Sensor	Albumin adsorption amounts (ng/cm ²)	k_{obs} ($\times 10^3$)
SUS	621.2 (62.0) ^a	2.2 (0.6) ^a
DLC	461.2 (35.1) ^b	2.9 (0.7) ^a
F-DLC	419.7 (43.3) ^b	4.2 (0.3) ^b

Data are expressed as means (SD).

Groups that do not share the same superscript letter are significantly different ($p < 0.05$).

Fig. 2 Typical change in frequency for each sensor after protein injection.

amounts between DLC and F-DLC ($p > 0.05$).

The apparent first-order reaction rate, k_{obs} , indicates the albumin adsorption rate after injection. As shown in Table 5, k_{obs} of F-DLC was significantly larger than

those of SUS and DLC ($p < 0.05$). However, there was no significant difference in k_{obs} between SUS and DLC ($p > 0.05$).

DISCUSSION

In the present study, protein adsorption to SUS, DLC or F-DLC was evaluated by using the QCM method. We revealed that differences in materials influenced protein adsorption behaviors. DLC and F-DLC showed significantly smaller albumin adsorbed amounts on their surfaces. Therefore, the null hypothesis—the different surfaces, namely, SUS, DLC and F-DLC, do not influence albumin adsorption—was rejected.

Ishihara *et al.* reported that the surface free energy of F-DLC is lower than that of DLC and antibacterial activity of DLC was improved by fluorine incorporation²⁸. In the present study, surface free energy also decreased in the order of SUS, DLC and F-DLC.

Hydrophobic interactions play an important role as a driving force in pellicle formation, and higher amounts of salivary proteins adsorb onto hydrophobic surfaces²⁹. Moreover, Wei *et al.* reported that albumin showed greater adsorption to hydrophobic surfaces³⁰. DLC and F-DLC have significantly hydrophobic surfaces.

Fig. 3 Relationship between albumin adsorbed amounts and total surface free energy, hydrogen bond component and dispersive surface component.

However, albumin adsorbed amounts on DLC and F-DLC were significantly lower than that on SUS.

It is well known that the surface free energy of materials influences protein adsorption. In the present study, lower amounts of albumin adsorbed onto DLC and F-DLC, which had lower surface free energies. Baier and Dutton reported that minimum interaction between biomolecules and materials was obtained when the surface free energy of the material was 20–30 mN/m³¹⁾. The total surface free energy of DLC and F-DLC is approximately 30–40 mN/m. Therefore, the interaction between albumin and DLC or F-DLC is likely weak, resulting in decreased adsorption.

In this study, the relationship between surface free energy and albumin adsorbed amounts was analyzed. As shown in Fig. 3, a positive correlation was found between total surface free energy and albumin adsorbed amount, but the correlation was not linear ($r^2=0.83$). However, a linear correlation was recognized between the hydrogen bond component and albumin adsorbed amount ($r^2=0.99$). No correlation existed between the dispersive surface component and albumin adsorbed amount. Michiardi *et al.* investigated the influence of surface free energy on protein adsorption on oxidized nickel-titanium alloy surfaces³²⁾. They found that surfaces with higher free energies produced higher amounts of albumin adsorption, and albumin adsorption was directly related to the surface energy with excellent linear correlation with the polar component of the surfaces studied. Our results correspond with their results. The surface of SUS is more hydrophilic than DLC and F-DLC. It is presumed that hydrophilic surface will easier to make hydrogen bond interaction with albumin compared to hydrophobic surface, although the clear reason is still under investigation. It is suggested that hydrogen bond interaction may contribute to albumin adsorption onto

Fig. 4 Relationship between k_{obs} and total surface free energy, hydrogen bond component and dispersive surface component.

the three different surfaces, SUS, DLC and F-DLC.

A larger value for k_{obs} indicates a more rapid reaction rate, namely, more rapid albumin adsorption. We observed more rapid albumin adsorption onto F-DLC than DLC and SUS. The analysis of the correlation between k_{obs} and surface free energy indicated a linear correlation existed between k_{obs} and total surface free energy ($r^2=0.99$, Fig. 4). In this case, a slightly linear correlation existed between k_{obs} and the hydrogen bond surface component ($r^2=0.75$), but not with the dispersive surface component ($r^2=0.30$).

Surface roughness also affects protein adsorption³³⁾. Generally, a roughened surface increases protein adsorption on the material surface. The smoother surface of DLC and F-DLC compared with SUS is one potential explanation for decreased albumin adsorption on their surfaces.

Various factors such as surface charges or chemical compositions also influence protein adsorption onto materials. Yoshida and Hayakawa²¹⁾ measured the apparent zeta potential of tested materials and speculated that lactoferrin adsorption onto titanium or zirconia was mainly influenced by electrostatic interaction. On the contrary, the concentration of albumin in oral saliva is generally estimated to be approximately 0.014–0.064 mg/mL³⁴⁾. The concentration employed in our study is higher than that in oral saliva. It is suggested that the concentration of protein may also influence the adsorption behaviors of the protein. Further detailed analysis of protein adsorption is needed.

CONCLUSIONS

Albumin adsorption to SUS, DLC and F-DLC films was evaluated by using the QCM method. The adsorbed amounts of BSA on DLC and F-DLC were significantly

lower than that on SUS. Furthermore, the apparent reaction rate, k_{obs} , of F-DLC was significantly larger than those of SUS and DLC. Our results revealed that the significantly lower total surface free energies of DLC or F-DLC influenced albumin absorbed amounts and k_{obs} . A clear correlation was found between albumin absorbed amounts and the hydrogen bond component of total surface free energy. Therefore, DLC or F-DLC coating is effective for preventing the protein adhesion on orthodontic appliances and may consequently decrease friction between the orthodontic wire and bracket due to the reduction of pellicle accumulation.

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