Quartz-Crystal Microbalance Monitoring for the Adsorption of Cell Adhesive Proteins onto a Titanium Surface

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The quartz crystal microbalance (QCM) method can detect the amounts of proteins adsorbed on biomaterials by a frequency shift of an oscillating quartz crystal. In the present study, we observed the adsorption behavior of proteins onto a titanium surface by using 27-MHz QCM. As proteins, two cell adhesive proteins, fibronectin and collagen, and albumin were evaluated.

The QCM apparatus used was a 27-MHz AFFINIX Qμr (Initium Co., Ltd., Tokyo, Japan) with 500-μL cells. The temperature was maintained at 25±1°C and the solution in the cells was stirred during the measurements. A titanium sensor was prepared by titanium sputter-coating onto an Au electrode. Bovine albumin, human plasma fibronectin and atelocollagen were dissolved in phosphate-buffered saline (PBS) solution (pH 7.4) at a concentration of 50 μg/mL. Each protein solution was injected into the PBS solution in the cells of the QCM apparatus. The decrease of frequency was monitored and the amounts of proteins adsorbed onto the titanium surface at 30 min after the injection were calculated by Sauereby’s equation. The apparent reaction rate, Kobs, was also obtained.

A slight frequency decrease was observed upon injection of albumin, and the frequency decrease for fibronectin adsorption was larger than that for albumin adsorption. Collagen significantly showed the largest decrease of frequency shift. Significant differences in the adsorbed amounts were seen among the three proteins. Namely, collagen exhibited the significantly largest adsorption amount on titanium, and albumin showed the significantly smallest amount. Kobs for collagen was significantly the smallest, and that of albumin was significantly the

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Introduction

Titanium implants are widely used as dental and orthopedic implants. The adsorption of body-fluid proteins including extracellular matrix components at the implant-tissue interface is the first biological response to titanium implants after implantation. The initial adsorption of proteins is known to affect some biological responses such as cell proliferation and growth, microbial colonization, etc. A clear understanding of the protein adsorption process is very important for implant dentistry.

There are several methods of detecting and quantifying the protein adsorption directly on biomaterial surfaces, including radiolabeling, ellipsometry, Fourier transform infrared spectroscopy, surface plasmon resonance, atomic force microscopy, etc. The quartz crystal microbalance (QCM) is now also used as a sensing device for monitoring protein adsorption.

The QCM method is a simple technique to detect molecular behavior on a surface. The principle of QCM is shown in Fig. 1. AT-cut quartz crystal is sandwiched by gold (Au) electrodes on both sides. When an alternating electric current is applied across the quartz, thickness-shear mode oscillation of the quartz crystal occurs. When molecules bind to the oscillating quartz crystal, the oscillating frequency decreases in relation to the amount of protein bound on the crystal surface. The amount of protein adsorbed onto the biomaterial can be estimated by Sauerbrey’s equation:

\[ \Delta F = \frac{2F_0^2 \Delta m}{A \sqrt{\rho_s \mu_s}} \]

where \( \Delta F \) is the measured frequency shift (Hz), \( \Delta m \) is the mass change (g), \( F_0 \) is the fundamental frequency of the quartz crystal (27 \( \times \) 10^6 Hz), \( A \) is the electrode area (0.049 cm^2), \( \rho_s \) is the density of quartz (2.65 g cm^{-3}), and \( \mu_s \) is the shear modulus of quartz (2.95 \( \times \) 10^{11} dyn cm^{-2}).

Okahata et al. reported on a pioneering study using the QCM technique to detect the interactions of various biomolecules such as deoxyribonucleic acid (DNA), peptide, and carbohydrate. Hayakawa et al. also monitored the complex formation between DNA and basic fibroblast growth factor by using the QCM method. Yoshinari et al. reported that the QCM technique was useful for determining the adsorption behavior of simvastatin acid or titanium binding peptide to a titanium surface.

There have been several reports on QCM monitoring of protein adsorption on titanium. Hemmersam et al. compared fibronectin adsorption on gold, titanium oxide and tantalum oxide by QCM and found that the adsorption on gold is higher than those on titanium and tantalum oxide surfaces. A QCM study by Nezu et al. revealed that lysozyme adsorbed more rapidly than collagen on a gold surface. Hayakawa et al. evaluated the effectiveness of the tresyl chloride-activation technique, which was their original protein-immobilization technique, and reported that fibronectin adsorption on titanium was enhanced by tresyl-chloride activation.

However, most of the previous studies on QCM for protein adsorption were carried out using 5-MHz...
AT-cut crystals. Higher fundamental frequency QCM produces higher sensitivity, and so QCMs with higher frequencies have been developed. The development of 16.6-MHz, 50-MHz, and 170-MHz QCMs has been reported\(^{16-18}\). However, high fundamental frequency quartz crystals are very thin and fragile to handle, and thin quartz crystal plates are susceptible to water pressure, and so the signal-to-noise ratio (S/N) tends to decrease. For example, the 170-MHz QCM apparatus has a noise level of ±50 Hz. Okahata and co-workers developed and recommended a 27-MHz QCM with a very low noise level of ±0.05 Hz\(^{19,20}\).

In the present study, we observed the adsorption behavior of proteins onto a titanium surface using 27-MHz QCM. Two cell adhesive proteins, fibronectin and collagen, were evaluated, along with albumin.

**Materials and Methods**

1. **27-MHz QCM apparatus**

   The appearance of the QCM apparatus and titanium sensor is shown in Fig. 2. The frequency changes were monitored on a personal computer. The QCM apparatus used was a 27-MHz AFFINIX \(Q_{\text{et}}\) (Initium Co., Ltd., Tokyo, Japan) with 500 µL cells and equipped with a temperature control system and stirring bar. The temperature was maintained at 25 ± 1°C and the solution in the cells was stirred during the measurements. The surface area of the Au electrode was 4.9 mm\(^2\).

   The titanium sensor was prepared by sputter-coating titanium onto Au electrodes. A titanium disk (99.99 mass%, ULVAC, Inc., Kanagawa, Japan) was used as the target. Using sputtering deposition equipment (CS200, ULVAC, Inc.), sputter-coating of titanium was performed at a pressure of 0.2 Pa for 30 min.

2. **QCM measurement**

   Bovine albumin (Wako Pure Chemical Industries, Ltd., Osaka, Japan), human plasma fibronectin (Harbor Bio-Products, MA, USA) and atelocollagen (Atelocell, IPC-50, Koken Co., Ltd., Tokyo, Japan) were dissolved in phosphate-buffered saline (PBS) solution (pH 7.4) at a concentration of 50 µg/mL.

   First, 500 µL of phosphate-buffered saline (PBS) solution at pH 7.4 was added to the cell. After stabilization of the cell, 5 µL of protein solution was injected to the PBS solution in the cell. The frequency in PBS was calibrated as zero, and the frequency decrease from zero was monitored until 30 min after the injection of protein. The amount of proteins adsorbed onto the titanium surface at 30 min after the injection of protein was calculated by Sauerbrey’s equation: At 27 MHz, a frequency decrease of 1 Hz corresponds to a mass change of approximately 0.62 ng/cm\(^2\) according to this equation\(^{21}\).

   By curve-fitting the frequency shift curve against adsorption time, the apparent reaction rate, \(K_{\text{obs}}\)\(^{21}\), in the following equation was obtained. \(\Delta F_\infty\) is the
frequency shift at infinite time.
\[ \Delta F_t = \Delta F_\infty |1 - \exp(-K_{obs}^* t)| \]

Three runs of QCM measurements were performed for each protein.

### 3. Statistics

Significant differences were determined by one-way analysis of variance (ANOVA) using GraphPad software (Graphpad Prism, GraphPad Software Inc., San Diego, California, USA), then Mann-Whitney tests were performed. Statistical significance was set at \( p < 0.05 \).

### Results

Figure 3 shows the typical frequency shifts of the adsorption of albumin, fibronectin and collagen to the titanium sensor by QCM measurements. An immediate frequency decrease was observed after the injection of protein. The rapid decrease in frequency is related to rapid adsorption of proteins.

A slight frequency decrease was observed upon injection of albumin and the frequency shift remained almost constant from the injection time until 30 min. The decrease in frequency for fibronectin adsorption was observed until approximately 5 min after the injection and was larger than that for albumin adsorption. Collagen showed the largest decrease of frequency shift, and the frequency shift decreased gradually until 30 min.

Figure 4 shows the estimated amounts of proteins adsorbed on the titanium sensor. Significant differences in the adsorbed amounts were seen among the three proteins. Namely, collagen exhibited the significantly largest adsorption amount on titanium, and albumin the significantly smallest.

Table 1 lists the \( K_{obs} \) values for the adsorptions of the three proteins on titanium. There were significant differences among the three \( K_{obs} \) values \((p < 0.05)\). \( K_{obs} \) for collagen was the significantly smallest, and that of albumin was the significantly largest. A small value of \( K_{obs} \) corresponds to rapid adsorption of protein on titanium.

### Discussion

In this study, we evaluated the adsorption of three proteins on titanium by the QCM method. Two cell adhesive proteins, fibronectin and collagen, and bovine serum albumin were evaluated. Fibronectin and collagen have RGD sequences for cell adhesion. Fibronectin is a large extracellular matrix glycoprotein.
Fibronectin is a dimer with a molecular weight of approximately 440 kDa. Collagen is a typical fibrous protein. Collagen itself is insoluble, so atelocollagen was employed in the present study: atelocollagen is a collagen solubilized by protease in which telopeptide, a non-spiral part present at the end of collagen, is removed. The molecular weight is approximately 300 kDa.

Albumin is the most abundant plasma protein and is known to discourage the adsorption of proteins that may stimulate inflammation and bacterial colonization. Albumin is thus a cell adhesion inhibiting protein. The molecular weight of bovine serum albumin is approximately 6–7 kDa, which is smaller than those of fibronectin or collagen.

It has been suggested that titanium has two types of hydroxyl groups: basic terminal OH and a bridge acidic OH. The isoelectric point of titanium is reported to be approximately 5–5.5, and is negatively charged in the phosphate buffered condition of pH = 7.4. The isoelectric points of albumin, fibronectin and collagen are reported to be 4.7, approximately 5–6 and approximately 9.5, respectively. Thus, albumin and fibronectin were negatively charged and collagen was positively charged in the present buffered condition of pH = 7.4, respectively.

It is presumed that electrostatic repulsion was present in the case of the adsorption of albumin and fibronectin because titanium, albumin and fibronectin were negatively charged at pH 7.4. Wei et al. investigated the adsorption of albumin on titanium by time-of-flight secondary ion mass spectroscopy and reported that the electrostatic repulsive force between albumin and titanium hindered the adsorption at higher pH than their isoelectric points.

In contrast, the attractive force was present between collagen and titanium because collagen was positively charged at pH = 7.4. Thus, albumin and fibronectin showed significantly less adsorption on titanium compared with collagen.

The apparent reaction rate $K_{obs}$ was obtained in the present study. A smaller $K_{obs}$ means a more rapid reaction rate. The order of $K_{obs}$ agreed with the order of the isoelectric points of the proteins: positively-charged collagen showed the significantly most rapid reaction rate because of the electrostatic attraction between collagen and titanium. The reaction rate of albumin was the significantly slowest because of the increasing electrostatic repulsion.

Hayakawa et al. previously reported that approximately 1,000 ng/cm$^2$ of fibronectin adsorbed on titanium during 120 min of adsorption. They employed fibronectin at a concentration of 20 ng/mL in PBS solution using 5-MHz QCM. The concentration of fibronectin in the present sensor cell was 5 ng/mL and the adsorbed amount of fibronectin was approximately 440 ng/cm$^2$. Thus, the adsorbed amount detected in the present study was a factor of approximately 1.8 larger. It is presumed that this difference was caused by the difference of sensor cells and/or fundamental frequency of QCM, 27 MHz vs 5 MHz, although the reason is still not clear.

Many factors affect the adsorption of proteins onto biomaterials, including hydrogen bonding and hydrophobic interaction, in addition to electrostatic interaction. Wei et al. reported that the hydrophilicity and hydrophobicity of materials influenced the adsorption of albumin and fibronectin. It is also suggested that the differences of molecular weight of proteins will influence the adsorption behavior. The present study examined the adsorption behavior only at a concentration of 50 ng/mL. Varying the concentration of protein will elucidate the influence of molecular weight of proteins for the adsorption. Moreover, detailed studies of the titanium surface and protein structures such as steric conformation should be conducted.

Finally, we established that the QCM method is a useful tool for monitoring not only the adsorption amounts of proteins but also adsorption behaviors including the adsorption rate. The advantages of the QCM method are that it is simple and offers direct in situ detection of the chemical reaction. A titanium sensor was used in the present study, but various
kinds of sensors will need to be evaluated. For example, adsorption of proteins on metals such as gold and stainless steel, ceramics such as silica and hydroxyapatite, and resins such as polymethyl methacrylate will be monitored using this QCM method in future studies.

**Conclusion**

The adsorption behavior of proteins onto a titanium surface was monitored using 27-MHz QCM. The frequency decrease for fibronectin adsorption was larger than that for albumin adsorption. Collagen showed the significantly largest decrease of frequency shift. According to Sauerbrey’s equation, collagen exhibited the largest adsorbed amount. The adsorbed amount of albumin was the smallest. The apparent reaction rate \( K_{obs} \) for collagen was significantly the smallest and that of albumin was the largest.

It is presumed that electrostatic interaction was dominant in the protein adsorption on titanium. Both albumin and fibronectin had more electrostatic repulsion to titanium based on their isoelectric points. In contrast, the attractive force increased between collagen and titanium because of the positively-charged collagen. The QCM method was shown to be a useful tool for monitoring not only the adsorption amounts of proteins but also adsorption behaviors including the adsorption rate.

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**References**

水晶発振子マイクロバランス（QCM）法は、共振している水晶発振子の振動数の減少からタンパク質の生体材料への吸着量を検出できる方法である。本研究では、27 MHz QCMを使用して、チタン表面へのタンパク質の吸着挙動を観察した。タンパク質としては、細胞接着タンパク質であるフィブロネクチンとコラーゲン、およびアルブミンについて検討した。

QCM装置として、500 μLのセルを備えた27-MHz AF-FINIX QnQ（イニシアム、東京）を使用した。温度は25 ± 1℃に設定し、セル中の溶液を攪拌しながら測定した。チタンセンサーは、金電極にスパッタコーティングを施して作製した。牛アルブミン、ヒト血漿フィブロネクチンおよびアテロコラーゲンをそれぞれ50 μg/mLの濃度でリン酸緩衝液（PBS、pH 7.4）に溶解した。それぞれのタンパク質溶液を、PBS溶液を満たしたQCM装置付属のセル中に注入した。振動数の減少を観察し、注入30分後のタンパク質吸着量をSauerbreyの式から算出した。さらに、見かけの反応速度定数Kobsを得た。

アルブミンを注入した時は振動数のわずかな減少がみられ、フィブロネクチン吸着時の振動数減少のほうが、アルブミン吸着時よりも大きかった。コラーゲンが統計学的に有意に最も大きな振動数の減少を示した。観察した3種類のタンパク質の吸着量はそれぞれ統計学的に有意差があり、コラーゲンのKobsは、統計学的に最も小さい値であり、アルブミンが統計学的に最も大きい吸着量を示した。統計学的に最も小さい値である小さいKobsの値は、タンパク質のチタンへの吸着速度が早いことを意味している。

タンパク質のチタンへの吸着は静電的な相互作用が主要因であると推察される。アルブミンおよびフィブロネクチンはその等電点からチタンとの間に静電的な反発力が働いている。一方、コラーゲンとチタンの間には、コラーゲンが正に荷電しているために吸引力が増加した。以上、QCMはタンパク質の吸着量だけでなく、吸着速度も含めた吸着挙動の観察に非常に重要なツールであることが判明した。