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Biofilm Bormation on Titanium and Hydroxyapatite Surface using Artificial Mouth System

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Abstract: Biofilm formation in the oral cavity is postulated to cause oral diseases such as dental caries, periodontal diseases, and peri-implantitis etc. Recently, an artificial mouth system (AMS) was developed to study oral biofilm formation on dental materials *in vitro* by simulating the human oral environment. The purpose of this study was to monitor the biofilm formation on titanium (Ti) and hydroxyapatite (HA) using AMS and to evaluate the biofilm morphology by scanning electron microscopy (SEM). As bacterial strains, *Streptococcus mutans* (*S. mutans*) and *Streptococcus sobrinus* (*S. sobrinus*) were employed. The number of bacterial cells on each specimen was evaluated after 5 and 18 hours assays. The number of *S. mutans* cells after 18 hours was significantly higher than after 5 hours ($p < 0.05$). No significant differences existed between Ti and HA specimens for both *S. mutans* and *S. sobrinus* cells in each assay period. The 18 hours assay specimens provided a significantly higher amount of water-insoluble glucan (WIG) by *S. mutans* than 5 hours specimens ($p < 0.05$). There were no significant differences between Ti and HA in the amount of WIG in each assay period. On SEM observation, biofilm formation of *S. mutans* and *S. sobrinus* was recognized on the HA specimen surface and no distinct differences were recognized. Higher magnification observation revealed the formation of pores with a diameter of approximately 5 μm . This was due to the pH decrease during biofilm formation. Ti surface could not be influenced by the decrease of pH. This was confirmed by acid etching of HA and Ti. Pores were also formed by acid etching on enamel but not on Ti. It is suggested that the formation of such pores is related with caries formation. In conclusion, biofilm formation on HA and Ti could be monitored using AMS.

Key words: Artificial mouth system, Biofilm, *Streptococcus mutans*, *Streptococcus sobrinus*, Hydroxyapatite, Titanium

Introduction

Dental caries is one of the most prevalent infectious diseases in humans. Bacteria that attach to and accumulate on the teeth are responsible for initiating dental caries. Dental plaque biofilm is the deposit of proteins, cell-free enzymes, and bacteria embedded in exopolysaccharides that adhere firmly to the tooth surface, and dental caries is now recognized as a biofilm-dependent oral disease, and prevention of the biofilm formation is a useful to protect against dental caries.

In addition to dental caries, biofilm formation in the oral cavity is postulated to cause periodontal diseases. Sometimes biofilm formation causes denture-induced stomatitis and peri-implantitis after denture setting or implant placement, respectively. Biofilm consists of 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 Ti implants¹⁻³⁾.

Some antibacterial resins or surface modifications for titanium (Ti) implants have been introduced and their antibacterial activities evaluated. However, there are few studies of biofilm formation on different substances, for example, enamel or Ti implants.

Recently, an artificial mouth system (AMS) was developed to study oral biofilm formation on dental materials *in vitro* by simulating the human oral environment^{4,5)}. Using the AMS, a study model was established to provide a better understanding of how the surface properties of dental materials could influence biofilm adherence and growth. Ono et al. and Ikeda et al.^{6,7)} investigated biofilm formation on various resin composites using AMS and found that the surface roughness and composition of resin composites influenced biofilm adherence. A primary artificial secondary caries model was also established using AMS⁸⁾.

The purpose of this study was to monitor biofilm formation on Ti and hydroxyapatite (HA) using AMS and to evaluate the biofilm morphology by scanning electron microscopy (SEM). As

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Figure 1. Schematic diagram of AMS (a). Images of specimens before (left) and 18 hours (right) after biofilm formation (b).

bacterial strains, *Streptococcus mutans* and *Streptococcus sobrinus* were employed because they are the major pathogenic bacteria associated with dental biofilms⁹⁾.

Materials and Methods

Specimen

Machined commercially pure wrought Ti disks (Ra = approximately 0.9 μm , Japan Industrial Specification H 4600, 99.9 mass %; Furuuchi Chemical Corp., Tokyo, Japan) with a diameter of 5 mm, and a polished high sintered HA pellet (Ra = approximately 0.24 μm , CELLYARD HA pellet; PENTAX Corp., Tokyo, Japan) with a diameter of 5mm were used in this study.

Contact angle measurements

The contact angle of each specimen surface with respect to double-distilled water was measured using a contact angle meter (CA-P; Kyowa Interface Science Co. Ltd., Tokyo, Japan). The volume of water drops was maintained at 2 μL and five measurements for 5 sec each were made for Ti and HA surfaces. Measurements were performed at the same room temperature and

humidity.

Preparation of bacterial suspension

Streptococcus mutans (*S. mutans*) ATCC 25175 and *Sterptococcus sobrinus* (*S. sobrinus*) ATCC 33478 were used in this study. A suspension of *S. mutans* and *S. sobrinus* in PBS (phosphate-buffered saline, pH7.2) at $\text{OD}_{500} = 2$ (approximately 2×10^7 colony-forming units/ml) was prepared from 16 hours fresh culture in TS (Bacto Tryptic Soy Broth; Becton Dickinson, Sparks, MD, USA) after washing with PBS and was stored at 4°C. For growth in the AMS, a solution of TS without dextrose (Becton Dickinson) but with 1% sucrose was used.

Biofilm formation in the AMS

Schematic diagram of the AMS is shown in Fig. 1. The AMS consisted of three identical columnar artificial mouths, a thermostatic incubator, multiple pH meter, peristaltic pump, and cooling stirrer (Fig. 1a). The artificial mouth (60mm in diameter, 140mm in height) and surrounding water jacket (140mm in diameter) were made from transparent vinyl chloride. Warm water

Figure 2. Number of bacterial cells on each specimen with different assay periods (5 and 18 hours). Bars represent means $\pm$ SD, * $P < 0.05$. a: *S. mutans*, b: *S. sobrinus*.

was circulated using the thermostatic incubator (Taitech, Saitama, Japan) to maintain the temperature of the artificial mouths at 37 °C. A pH electrode with a flat bulb (9 mm diameter; TOA-DKK, Tokyo, Japan) was set upside down in the center of the artificial mouth with a silicon plug.

Eight specimens were arranged on the flat surface of a special Teflon holder (28mm diameter) around the bulb of the electrode. Another silicon plug with five stainless steel tubes and a thermometer was set in the upper part of the artificial mouth to constantly supply TS medium supplemented with sucrose, PBS and bacterial cell suspension with a peristaltic pump (Furue Science, Tokyo, Japan). The cell suspension ($OD_{500} = 2.0$) was maintained at a low temperature with gentle stirring using a cooling stirrer (Iwaki Glass, Tokyo, Japan) during the experiment. Changes in pH underneath the artificial biofilm were continuously monitored with a multiple pH recorder (TOA-DKK). All procedures were conducted under aseptic conditions.

Evaluation of quantity of artificial biofilm

After stopping the operation of the AMS, the biofilm formed on each specimen and the flat bulb was carefully removed with silicon scraper before being immersed in a 0.5N NaOH solution to resolve the water-insoluble glucan (WIG) on ice (Fig. 1b). This was followed by centrifugation (3,000 rpm, 20 min) to separate the bacterial cells from the WIG. Bacterial cells were re-suspended in PBS and turbidity at 500nm was measured. In addition, the amount of WIG in NaOH solution was determined colorimetrically at 492nm by the phenol-sulfuric acid method. Both cell turbidity and the amount of WIG were used as indicators of the amount of biofilm.

Acid etching of specimens

Ti or HA was etched with 30% phosphoric acid for 3 minutes. After etching the specimens, each specimen was washed by rinsing

Table 1. Measurement of contact angles

	HA	Ti
Contact angle (°)	81.6 $\pm$ 4.98	59.6 $\pm$ 6.54

in water and was dried in a desiccator. Acid etching was performed to evaluate the acid corrosion effect of accumulated biofilm on Ti and HA.

Scanning electron microscopy observation

After 5 and 18 hours of biofilm formation in the AMS, each disk was rinsed thoroughly with PBS, mounted on a piece of cork and fixed with PBS containing 2.5% glutaraldehyde (pH 7.4). Following fixation, disks were dehydrated through a graded series of ethanol (50, 60, 70, 80, 90, 95 and 100 %) and then dried with tetramethylsilane. After ion coating with gold, the morphology of the cells was observed using SEM (JSM-5600LV; JEOL, Tokyo, Japan) at an accelerating voltage of 10 kV. Ti and HA before the assay and after acid etching were also observed after ion-plating under the same SEM conditions.

Statistics

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

Results

Table 1 shows the results of contact angle measurements of the Ti or HA surface against water. The contact angle of the HA disk was higher than the Ti disk, indicating that the HA disk was more hydrophobic than the Ti disk.

Fig. 2 shows the amount of bacterial cells on each specimen after different assay periods (5 and 18 hours). The amount of *S.*

Figure 3. Amount of WIG by phenol- H_2SO_4 method. Bars represent means $\pm$ SD, * $P < 0.05$. a: *S. mutans*, b: *S. sobrinus*.

Figure 4. Results of SEM observation before assays. a: HA, b: Ti, Bars: 1 μm .

Figure 6. Results of SEM observation in Ti specimen after 5h. a and b: *S. mutans*, c and d: *S. sobrinus*, Bars: a and c; 10 μm , b and d; 1 μm .

mutans and *S. sobrinus* cells after the 18 hours assay was significantly higher than after 5 hours ($p < 0.05$). No significant differences existed between Ti and HA specimens for both *S. mutans* and *S. sobrinus* in each assay period.

Fig. 3 shows the amount of WIG by the phenol- H_2SO_4 method. The 18 hours assay specimens provided a significantly higher amount of WIG by *S. mutans* than 5 hours specimens ($p < 0.05$). There were no significant differences between Ti and HA in the amount of WIG in each assay period. In the case of *S. sobrinus*, the amount of WIG was not affected by the assay time. The amount of WIG did not differ between HA and Ti at each assay time, as

Figure 5. Results of SEM observation in HA specimen after 5h. White arrow: pore, a and b: *S. mutans*, c and d: *S. sobrinus*, Bars: a and c; 10 μm , b and d; 1 μm .

shown in Fig. 3b.

The results of SEM observation of each specimen surface before the assay are shown in Fig. 4. Ti specimens had a very smooth surface. On the other hand, HA specimens showed HA graining with a diameter of approximately 0.5 μm .

Figure 5 shows the SEM appearance of the surface of HA specimens after 5 hours using AMS. Biofilm formation of *S. mutans* and *S. sobrinus* was recognized on the HA specimen surface, and no distinct differences were recognized between *S. mutans* and *S. sobrinus*. Higher magnification observation revealed the formation of pores with a diameter of approximately 5 μm . Pores were present close to or underneath the formed biofilm.

Biofilm formation of *S. mutans* and *S. sobrinus* was also clearly identified on the Ti specimen surface, as shown in Fig. 6. Similar appearances were observed between *S. mutans* and *S. sobrinus*. The Ti surface showed almost the same appearance as the HA surface. There were no pores on Ti surfaces with both *S. mutans* and *S. sobrinus*.

Fig. 7 shows the SEM observation of the HA surface after 18 hours. Growth of biofilm was confirmed. More aggregation of

Figure 7. Results of SEM observation in HA specimen after 18h. a and b: *S. mutans*, c and d: *S. sobrinus*, Bars: a and c; 10µm, b and d; 1µm.

Figure 8. Results of SEM observation in Ti specimen after 18h. a and b: *S. mutans*, c and d: *S. sobrinus*, Bars: a and c; 10µm, b and d; 1µm

Figure 9. SEM images of HA and Ti after acid etching. White arrow: pore, a: HA, b: Ti, Bars: 1µm.

bacterial cells was observed. Detachment of the biofilm of *S. sobrinus* could be partly observed. Pores could not be identified due to the over-layering formation of biofilm.

Fig. 8 shows the SEM appearances of the Ti surface after 18 hours. Growth of biofilm was also observed. In the case of *S. sobrinus*, most of the aggregated biofilm detached from the Ti surface during sample preparation, especially the dehydration procedure. Thus, less aggregation of biofilm was observed for *S. sobrinus*.

Fig. 9 shows SEM images of HA and Ti after acid etching. Pores are observed on the HA surface, but not on the Ti surface.

Discussion

HA and Ti are now widely used as bone substitute materials or dental implant materials. However, there are few reports of biofilm formation on HA or Ti. Thus, in the present study, the biofilm formation of *S. mutans* and *S. sobrinus* on hydroxyapatite or Ti was evaluated using an AMS.

The AMS simulates the complex oral environment and provides a continuous or intermittent supply of nutrients to bacterial plaque or biofilm. Among the various kinds of AMSs, our AMS was originally developed by Hinode *et al.*⁽⁴⁾ in 1984. The aim of this system is to assess the important parameters responsible for causing dental caries, such as biofilm formation, the pH underneath biofilm and enamel demineralization. Usui *et*

al. reported that diammine silver fluoride had a strong anticarcinogenic effect on dentin using the present AMS⁽¹⁰⁾. Kamoda *et al.*⁽¹¹⁾ reported that disaccharide xylosylfructoside partly inhibited the cariogenicity of sucrose using the AMS. They also showed good reproducibility of the pH curves. Effect of bovine lactoferrin on enamel demineralization and acid fermentation was also evaluated using AMS⁽¹²⁾. These results suggested that the present AMS is reliable as an *in vitro* system.

The amount of bacterial cells and the amount of WIG were detected and there were no distinct differences in biofilm formation between HA and Ti. It is widely known that WIG, which is produced by glucosyltransferase of bacteria, plays a significant role in the adhesion of biofilm to substances^(13,14). On SEM observation, easy detachment of *S. sobrinus* biofilm was recognized during the preparation of SEM samples, as mentioned above. It is presumed that the adhesion of the biofilm of *S. sobrinus* to HA and Ti is lower than that of *S. mutans*.

Among the numerous factors affecting biofilm formation, surface roughness has been regarded as one of the most important factors. Many studies have reported that a rougher surface resulted in the increased adhesion of bacteria⁽¹⁵⁻¹⁷⁾. Ikeda *et al.*⁽⁷⁾ reported that a smooth resin composite surface with lower surface roughness has less bacterial adhesion than a rougher specimen surface using AMS. Surface hydrophobicity is also another factor that will influence biofilm formation⁽¹⁸⁾. However, in the present study no distinct differences were identified in biofilm formation despite the differences in surface roughness and hydrophobicity, which were determined by contact angles, between HA and Ti. To clarify the mechanism of biofilm formation, the conditions of AMS employed in the present study, such as flow rate and sucrose concentration etc., will be investigated further.

Pore formation was observed on HA. This was due to the pH decrease during biofilm formation. Ti could not be influenced by the decrease of pH. This was confirmed by acid etching of HA

and Ti. Pores were also formed by acid etching of enamel but not Ti. It is suggested that the formation of such pores is related with caries formation. The details will be investigated further.

In conclusion, biofilm formation on HA and Ti could be monitored using AMS. Study of the mechanism of biofilm formation and/or the difference in biofilm components is needed using AMS.

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