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Quantitative analyses of *Streptococcus mutans* biofilms with quartz crystal microbalance, microjet impingement and confocal microscopy

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Abstract

Microbial biofilm formation can be influenced by many physiological and genetic factors. The conventional microtiter plate assay provides useful but limited information about biofilm formation. With the fast expansion of the biofilm research field, there are urgent needs for more informative techniques to quantify the major parameters of a biofilm, such as adhesive strength and total biomass. It would be even more ideal if these measurements could be conducted in a real-time, non-invasive manner. In this study, we used quartz crystal microbalance (QCM) and microjet impingement (MJI) to measure total biomass and adhesive strength, respectively, of *S. mutans* biofilms formed under different sucrose concentrations. In conjunction with confocal laser scanning microscopy (CLSM) and the COMSTAT software, we show that sucrose concentration affects the biofilm strength, total biomass, and architecture in both qualitative and quantitative manners. Our data correlate well with previous observations about the effect of sucrose on the adherence of *S. mutans* to the tooth surface, and demonstrate that QCM is a useful tool for studying the kinetics of biofilm formation in real time and that MJI is a sensitive, easy-to-use device to measure the adhesive strength of a biofilm.

INTRODUCTION

Bacteria in nature often exist in surface-bound structures called biofilms (Hall-Stoodley *et al.*, 2004). A typical biofilm comprises microcolonies encased in a matrix of exopolysaccharide interspersed by void areas or water channels (Stoodley *et al.*, 2002). Biofilm formation proceeds in sequential steps: initial surface attachment, cell growth and microcolony formation, and maturation into a three-dimensional biofilm. Each step may be influenced by physiological as well as genetic factors, and affects the final property of the biofilm. The property of a biofilm can be described by a number of physical parameters such as cell mass, adhesive strength and architecture. These parameters not only affect the effectiveness of mechanical removal of

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biofilms from surfaces but also determine the physiological states of the constituent cells. For example, it is well known that cells growing in biofilms develop increased resistance to antibiotics as compared with their planktonic counterparts (Costerton *et al.*, 1999), and that a biofilm with changed architecture due to genetic mutations may render the cells more susceptible to antibiotic treatments (Hazlett *et al.*, 1998, 1999).

Since biofilm formation plays an important role in areas from environmental industry to medicine, understanding the mechanisms of biofilm formation has become the focus of the biofilm research community. A variety of methods have been developed to characterize biofilm properties. The most widely used is the microtiter plate assay for measuring the amount of biofilm formation (O'Toole & Kolter, 1998). Although not a means of direct measurement, this method has been used by a number of laboratories for screening mutants that affect biofilm formation (O'Toole & Kolter, 1998; Yoshida & Kuramitsu, 2002; Friedman & Kolter, 2004). Although semi-quantitative and easy to perform, the microtiter plate assay does not provide information on the kinetics of biofilm formation or the architecture of the biofilm. Confocal laser scanning microscopy (CLSM) is a powerful tool with which to analyze biofilm structure in its native state and, combined with a time series program, it can be used to monitor biofilm growth in real time (Lawrence *et al.*, 1991). However, until recently, quantitative analysis of CLSM data from biofilms has not been common. An easily accessible computer program, COMSTAT, performs quantitative analyses of biofilm images acquired from CLSM and gives reports on features such as biovolume, surface coverage, film thickness, and surface roughness, among others (Heydorn *et al.*, 2000). COMSTAT has been used to compare biofilms formed by wild-type and mutant *Pseudomonas aeruginosa* in a number of studies (Heydorn *et al.*, 2002; Klausen *et al.*, 2003; Stapper *et al.*, 2004), which has helped in the construction of models for biofilm formation by *P. aeruginosa*. However, since CLSM images are snap-shots of a particular confocal field and tremendous variations exist within the same biofilm among different fields, multiple data sets and sophisticated statistical analyses have to be performed to obtain reliable data (Heydorn *et al.*, 2000, 2002). In addition, the requirement for an expensive confocal microscope limits its application.

Quartz crystal microbalance (QCM) is a sensitive gravimetric tool that has been used to a limited extent in biofilm studies. Originally developed for use in the field of metal corrosion, QCM was first used to monitor biofilm formation in *Pseudomonas cepacia* by Nivens *et al.* (1993). In recent years, more studies on biofilms have been conducted with QCM. For example, Rodahl *et al.* (1997) studied the effect of biofilm formation on energy dissipation of QCM and Otto *et al.* used QCM to study surface sensing and attachment in *Escherichia coli* (Otto *et al.*, 1999; Otto & Silhavy, 2002; Otto & Hermansson, 2004). Although QCM is an easy-to-use, inexpensive device, for some reason it has not been used widely in laboratories studying biofilms.

As compared with measuring cell mass, quantifying adhesive strength is an even less developed area. In recent years, sophisticated techniques such as atomic force microscopy, microcantilever, spinning disk device, and micromanipulation have been developed to measure the adhesive strength of monolayer eukaryotic cells on substrates or bacterial electrostatic interactions (Yamamoto *et al.*, 1998; Cargill *et al.*, 1999; Camesano & Logan, 2000; Zreiqat *et al.*, 2002; Poppele & Hozalski, 2003). Owing to high technical demands, these techniques have not found wide application in the laboratories. As the field of biofilm research expands, there are urgent needs for easy-to-use tools that can measure biofilms quantitatively.

In this study, we applied the QCM and MJT systems to quantitatively measure the biomass and adhesiveness, respectively, of biofilms formed by *Streptococcus mutans* under different sucrose concentrations. This bacterium is considered to be the major pathogen causing dental caries and sucrose is well known to play an important role in the adherence of *S. mutans* cells

to the tooth surface (Hamada & Slade, 1980). QCM is a gravimetric tool that can measure mass changes on the crystal surface at nanogram levels, and MJI is based on shear force created by a microjet stream that is required to detach biofilm cells from the surface (for details, see Material and Methods). In conjunction with CLSM and COMSTAT, we demonstrate that QCM is a useful tool for monitoring the kinetics of cell mass accumulation during the process of biofilm formation, and that MJI is a sensitive and easy-to-use device to quantify the adhesive strength of a biofilm.

MATERIALS AND METHODS

Bacterial strains, media and biofilm formation

Streptococcus mutans strain UA140 (Qi *et al.*, 2001) was used for this study. Cells were routinely cultured in brain heart infusion (BHI, Difco) or BHI agar at 37 °C under anaerobic conditions. For biofilm formation, cells were grown anaerobically in BHI broth overnight, and diluted 1:100 (final cell density of approximately 10⁶ colony-forming units/ml) into BHI with different concentrations of sucrose compensated for by different concentrations of glucose to equalize the total concentrations of carbon source. This cell suspension was inoculated into a slide chamber (for imaging), a 12-well plate (for biofilm strength analysis), or into a flow chamber (for cell mass measurements). Biofilms were grown typically as a static culture for 16 h at 37 °C anaerobically unless otherwise specified.

Confocal laser scanning fluorescence microscopy

For microscopy the Lab-Tek[®] II Chamber Slide[™] System (Nalge Nunc International; Naperville, IL, USA) was used. The objective slide was replaced by a thin cover-slide for proper CLSM microscopy. Biofilm cells were stained with CellTracker[™] Orange CMTMR (5-(and-6)-(((4-chloro-methyl)-benzoyl)amino)tetramethylrhodamine) according to the manufacturer's instructions (Molecular Probes; Eugene, OR, USA). CLSM was performed using LSM 5 PASCAL with LSM 5 PASCAL software (Carl Zeiss). The microscope was equipped with detectors and filter sets for monitoring red fluorescence (excitation wavelength 540–580 nm (560 CWL), dichroic mirror wavelength: 595 nm (LP), barrier wavelength 600–660 nm (630 CWL)). Images were obtained with a 10 × 0.3 Plan-Neofluar and a 40 × 1.4 Plan-Neofluar oil objective. Images were processed further for display using COREL DRAW.

Image analysis with COMSTAT

CLSM images were analyzed by the computer program COMSTAT (Heydorn *et al.*, 2000). The program uses CLSM files converted into .tif files for further calculation. The pictures in the .tif format are converted from gray-scale into a black-and-white picture that could be analyzed by the COMSTAT program. The conversion into black and white requires setting a threshold by the user. This includes a manual adjustment of the threshold value by comparing the original gray-scale picture with the black-and-white picture, and the best value is chosen to give the most accurate conversion of the gray-scale to the black-and-white picture. This threshold value is fixed and then used for all image stacks. Two independent experiments on different days were performed with different sucrose concentrations in Lab-Tek[®] II Chamber Slides[™]. For each sucrose concentration, seven image stacks were acquired randomly. Calculations of the results were performed with data sets from 14 image stacks per sucrose concentration (seven image stacks from each independent chamber) acquired from two independent experiments. A paired Student's t-test was used for statistical analysis.

Quartz crystal microbalance measurement

QCM was used for real-time measurement of mass accumulation during biofilm formation. QCM is an ultra-sensitive mass sensor where a piezoelectric AT-cut quartz crystal is

sandwiched between a pair of electrodes. When a high-frequency AC electric field is applied across the quartz crystal thickness, the crystal oscillates in a mechanically resonant shear mode. As material attachment or detachment occurs on the electrode, the resistance to the frequency signal is modified, hence altering the output frequency signal. This alteration in the frequency signal is directly proportional to the change in mass on the surface. For the Maxtek Research Quartz Crystal Microbalance (RQCM) with gold-plated 5 MHz AT-cut quartz crystals (Maxtek Inc., Santa Fe Springs, CA, USA) that we used in this study, the relationship between changes in the observed frequency and mass on the surface of the electrode of rigid films is described by Sauerbrey's equation:

$$\Delta f = -C_f \times \Delta m$$

where Δf is the observed change in frequency (Hz), C_f is the sensitivity factor of the crystal (0.056 Hz/ng per cm² for a 5 MHz crystal at 20 °C), and Δm is the mass change per unit area (g/cm²). Modification of this equation to be applicable to liquid media and viscoelastic films (such as biofilms) has been the subject of ongoing research relating additional measurements such as impedance power dissipation of the crystal and cyclic voltammetry readings to characteristics of elastic films (Rodahl *et al.*, 1997; Etchenique & Calvo, 1999; Voinova *et al.*, 2002). In this study, we used the currently available Sauerbrey's equation for rigid film to convert delta frequency to mass.

The QCM apparatus features a flow cell (approximately 0.1 ml) where the quartz crystal is located with only one side of the electrode exposed to the liquid media. The quartz crystals were coated with sterile saliva and allowed to dry to promote initial attachment of *S. mutans*. Biofilms were grown at 37 °C in BHI with sucrose concentrations of 0%, 0.1%, and 1% (v/v) compensated for with 1%, 0.9%, and 0% glucose, respectively, to make the final concentrations of the carbon source equal. Before inoculation of the flow cell, the quartz crystal was equilibrated in air at 37 °C for a minimum of 2 h to warm up the QCM.

Overnight cultures of *S. mutans* were diluted 1:100 into fresh media and injected into the flow cell and incubated at 37 °C for 2.5 h as a static culture for cells to attach. After the initial attachment, unattached cells were washed away by supplying sterilized medium at 0.2 ml/min using a peristaltic pump. This flow rate for medium supply was continued for 10 h to allow biofilm growth. Throughout this period, the QCM software acquired data including frequency, mass, and resistance, every minute.

The microjet impingement system

Biofilm adhesion strength was quantified by the microjet impingement method (Bundy *et al.*, 2001; Hallab *et al.*, 2001; Giliberti *et al.*, 2002). Jet impingement involves directing an axisymmetric column of fluid perpendicular to a flat plane (cell culture surface) where the fluid is redirected in the horizontal plane. The cells cultured on the horizontal substrate are exposed to a range of shear stresses, depending on their proximity to the fluid source. Previous studies presented computational models describing shear stress distribution along the horizontal plane for a range of Reynold's numbers (Re) and jet heights above the plane (Deshpande, 1983). From the stagnation point at the center of the fluid column, the shear stress rises sharply as a function of radial distance, reaching a maximum, and further increases in radial distance results in decreases in shear stress. Correlation curves relating non-dimensionalized distance to the fluid source and non-dimensionalized shear stress have been developed to calculate the shear stress along the horizontal plane, with the input of impingement geometry (nozzle size, nozzle height) and fluid flow rate (Deshpande, 1983). Jet impingement has been adapted for cell adhesion strength studies by impinging liquid jets onto cells cultured onto flat substrates (Bundy *et al.*, 2001; Hallab *et al.*, 2001; Giliberti *et al.*, 2002). Circular lesions are created in

each impinging region where cells are detached. The shear stress experienced at the perimeter of each lesion is believed to represent the threshold shear stress required to detach the biofilm from the substrate.

In the current assay, the fluid jet was delivered from a 0.20 mm ID nozzle oriented perpendicular to the culture surface (Fig. 4) at a height of 0.40 mm. The jet of phosphate-buffered saline (PBS) was directed towards the biofilm for 5 s at a flow rate of 0.077 ml/s. Biofilms were stained with 1% methylene blue then imaged using a Leica microscope fitted with a digital camera. Bioquant image analysis software was used to determine the area of each circular lesion, which was later used to calculate the radius of each lesion.

Non-dimensionalized shear stress versus radial distance calibration curves for the laminar flow regimen found in previous studies (Deshpande, 1983) were used to determine the threshold shear stress for each lesion.

RESULTS

Image analysis of *S. mutans* biofilms formed under different sucrose concentrations

The effect of sucrose on biofilm formation of *S. mutans* is a well-known phenomenon (Yamashita *et al.*, 1993). To analyze the effect of sucrose on the biofilm thickness, architecture and strength, we grew *S. mutans* biofilms in the presence of 0%, 0.1% and 1% sucrose, and labeled the biofilm cells with a cell tracker dye. Biofilms were grown in two parallel slide chambers at 37 °C as static cultures. For imaging the biofilms, a 10 × 0.3 Plan-Neofluar objective was first used to get an overview of the entire field, and then a 40 × 1.4 Plan-Neofluar oil objective was used to take seven snap-shots randomly from different positions in the confocal field of each chamber. A representative image of the biofilm from each sucrose concentration is shown in Fig. 1a–c. It is apparent that, without sucrose, *S. mutans* attached to the surface mostly as unstructured cell layers or small aggregates, and formed a thin biofilm (Fig. 1a). In contrast, with 1% sucrose, a thick, confluent biofilm was formed, which was composed of dense microcolonies with small, water-channel-like void areas (Fig. 1c). In the presence of 0.1% sucrose, an intermediate biofilm was formed, which comprised distinct but sparsely distributed microcolonies and large areas occupied by unstructured or single-cell chains (Fig. 1b). It is conspicuous to the naked eye that these biofilms are quantitatively different.

To see whether cells in the biofilms formed under different sucrose concentrations adhered differently to the surface or to each other, a mild shear stress was created by washing the biofilms with PBS in an orbital shaker at 100 revolutions per minute three times at 10 min for each period. A new set of images was taken after this wash, and the representative ones are presented in Fig. 1d and e. As shown in Fig. 1d, the biofilm formed without sucrose could not withstand this mild shear stress, since no cells remained attached after washing. In contrast, the majority of cells and microcolonies in biofilms grown with 0.1% and 1% sucrose remained attached after washing (Fig. 1e and f), indicating that sucrose is required for the formation of stable biofilms by *S. mutans*. It is worth pointing out that the difference in apparent cell mass attached to the chamber slide surface observed with biofilms formed under different sucrose concentrations is not due to difference in cell growth, since the total cell mass (planktonic plus sessile) was very similar in each of the three cultures (0%, 0.1%, and 1% sucrose; data not shown).

Quantitative analyses of biofilms formed under different sucrose concentrations using COMSTAT

The CLSM analysis of washed and unwashed biofilms described above provided a qualitative means to assess the quantity (mass and thickness) and quality (adhesive strength) of different biofilms; however, it cannot determine the extent of these differences. To quantify the biofilms formed under different sucrose concentrations, we used the COMSTAT software to calculate the biovolume and average film thickness from a total of 14 CLSM images taken randomly as described in Materials and Methods. In the absence of shear stress (Fig. 1a–c), the biofilm grown in the absence of sucrose had an average biovolume of $2.1 \pm 0.39 \mu\text{m}^3/\mu\text{m}^2$ (Fig. 2a). This value increased to 6.51 ± 1.79 with 0.1% sucrose and to 16.71 ± 2.27 with 1% sucrose. A similar result was obtained when film thickness was calculated. Statistical analysis indicated that the difference in biovolume and film thickness between each sucrose concentration was significant ($P < 0.001$). In the presence of shear stress (Fig. 1d–f), the biovolume for the biofilm formed without sucrose reduced to below $0.01 \mu\text{m}^3/\mu\text{m}^2$ (Fig. 2b). In contrast, in the presence of 0.1% and 1% sucrose, both biomass and average thickness decreased only 20% and 1%, respectively. This reduction was not significant ($P > 0.05$). These results correlated well with the CLSM observations described above.

Real-time monitoring of the kinetics of biofilm formation by using QCM

Although COMSTAT analyses provided numerical values to the CLSM images, the accuracy of these values was a cause for concern, because these values were obtained from a limited number of snap-shots, not from the entire biofilm. In addition, depending on the set-up value of the threshold, data could easily be biased. To directly measure biofilm cell mass in an unbiased environment, we used the QCM system (see Material and Methods). *Streptococcus mutans* biofilms were grown on the crystal surface with a constant supply of medium containing 0%, 0.1% or 1% sucrose, respectively, and delta frequency was recorded over a 10 h period. On the basis of Sauerbrey's equation, delta frequency (Δf) relates directly to the changes of mass on the surface of the quartz crystal. As mass attachment increases on the surface, delta frequency decreases. Fig. 3a depicts the Δf trends of biofilms grown with 0%, 0.1% or 1% sucrose. After the initial 2.5 h attachment period, an exponential decrease of delta frequency was observed for the 0.1% and 1% sucrose concentrations (intermediate and thick lines). However, for the 0% sucrose, the delta frequency increased continuously after the start of pump flow, indicating a steady detachment of cells from the surface.

For better visualization of the biofilm growth curve, the Sauerbrey equation was used to translate the delta frequency to mass per unit area ($\mu\text{g}/\text{cm}^2$). As shown in Fig. 3b, after the initial 2.5 h static culture period, both biofilms with 0.1% and 1% sucrose showed exponential growth, which is reminiscent of a typical growth curve of a planktonic batch culture (dashed and continuous lines). For the 0.1% sucrose biofilm, even a stationary phase was detected (dashed line). Using data from the exponential growth phase, a doubling time of 91 ± 4 min and 110 ± 18 min was obtained for biofilms grown with 0.1% and 1% sucrose, respectively. Both growth rates are lower than cells grown in planktonic culture in BHI without additional sucrose under static conditions (approximately 70 min, data not shown). As expected, biomass of the biofilm formed with 0% sucrose continued decreasing after the start of pump flow, reaching the basal level at the end of the experiment (dotted line).

Quantification of biofilm strength by using the microjet impingement system

The CLSM images taken before and after washing gave us a visual estimation of a biofilm's ability to resist shear stress. The amount and rate of mass accumulation on the crystal surface at a given flow rate as measured in the QCM assay also served as an indication of the adhesive strength of a biofilm. However, none of these assays could provide a numerical value to the biofilm strength so that different biofilms could be compared. To measure biofilm strength

more accurately and objectively, we introduced the MJI system. MJI uses a jet stream to detach cells from the surface, and the cleared zone from the detachment is reversely proportional to the attachment strength. MJI is used frequently for attachment strength determination of eukaryotic cell adhesion, e.g. fibroblasts or osteoblastic cells (Bundy *et al.*, 2001).

Streptococcus mutans biofilms were grown in a polystyrene 12-well cell culture plate with 0.1%, 0.5% and 1% sucrose (biofilms with 0% sucrose fell outside the measurable range). To detach cells, we used a needle of size 0.20 mm and a distance of 0.40 mm to the surface at a flow rate of 0.077 ml/s to impinge on the biofilms for 5 s. The jet produced cleared zones that varied in diameter, depending on the sucrose concentration (Fig. 4a and c). The cleared zone was then measured, and the shear stress was calculated using standard curves and equations as described by Deshpande (1983). As shown in Fig. 4b, to detach biofilm cells grown with 0.1% sucrose, a shear stress of 202 ± 39 dynes/cm² was required; this shear stress increased to 3984 ± 694 dynes/cm² with 0.5% sucrose and 6422 ± 575 dynes/cm² with 1% sucrose. The difference in shear stress between 0.1% and 0.5% sucrose was about 20-fold but between 0.5% and 1% sucrose was only 1.6-fold. This suggests a non-linear relationship between the sucrose concentration and the shear stress that is required to detach biofilm cells. When the size of the cleared zone was plotted together with the shear stress, both as a function of sucrose concentration, a near inverse correlation was obtained (Fig. 4c). This suggests that, under conditions where no accurate shear stress measurement is required, the size of a cleared zone may be used as an estimation of biofilm strength.

DISCUSSION

In this study, we qualitatively and quantitatively characterized *S. mutans* biofilms, formed under different sucrose concentrations, by using a combination of three methods: CLSM in conjunction with COMSTAT, QCM, and MJI. With CLSM, a clear difference in biofilm architecture was observed between biofilms formed under 0.1% and 1% sucrose. While the biofilm formed with 0.1% sucrose has large but loose-structured microcolonies interspersed with large void areas (Fig. 1b and e), the biofilm formed with 1% sucrose has smaller but dense microcolonies interconnected with much smaller channel-like structures (Fig. 1c and f and insert). Despite the clear difference in architecture from the CLSM image, the COMSTAT software was unable to distinguish any difference between the two biofilms in resistance to mild stress created by slow rotation of liquid (Fig. 2). This problem was overcome by using the MJI analysis. With MJI, the biofilm formed with 1% sucrose showed a >30-fold higher resistance to shear-stress-induced detachment than the one formed with 0.1% sucrose (6422 versus 202 dynes/cm²). It is fair to say that this is the first time the architecture of a biofilm has been quantified in terms of its adhesive strength. Moreover, MJI allowed even more detailed analysis of the effect of sucrose on biofilm adhesiveness, which could not be detected or measured by other methods. For example, a nonlinear relationship between sucrose concentration and biofilm strength was detected. From 0.1% to 0.5% sucrose, biofilm strength increased 20-fold, suggesting a rapid increase in glucan synthesis by *S. mutans* cells in response to increase in sucrose concentration. However, further increase of sucrose concentration to 1% resulted in only a <2-fold increase in adhesiveness, indicating a near linear relationship. Taken together, it is obvious that the combination of CLSM with MJI allows us much better understanding of the biofilm properties than any other method alone.

While the combination of CLSM and MJI provides a quantitative measurement of biofilm architecture and strength, no information could be provided with respect to the kinetics of biofilm formation. This need was met by introducing the QCM system in this study to examine whether different sucrose concentrations affect the kinetics of biofilm formation and the terminal biomass of the biofilm under certain shear stress. By using a flow cell set-up to create shear stress, we demonstrated that *S. mutans* could not form a biofilm under a flow rate of 0.2 ml/min without sucrose, although surface attachment could happen in static culture. With

sucrose, a stable biofilm was formed following a course reminiscent of a typical growth curve (Fig. 3). Sucrose concentrations affected both the biofilm growth rate and the total biomass. In the presence of 0.1% sucrose, the biofilm cell mass showed a doubling time of 91 min at the initial stage, reached a stationary phase about 4 h after flow started, and achieved a final cell mass of approximately $7 \mu\text{g}/\text{cm}^2$. In comparison, at 1% sucrose, the biofilm cell mass doubled every 110 min during most of the experimental period, reached a stationary phase at approximately 10 h after flow started, and achieved a final biomass of approximately $23 \mu\text{g}/\text{cm}^2$. These results clearly demonstrate that the QCM not only measures the kinetics of biofilm formation in real time but can also provide some clues to the biofilm strength if the biofilm growth curve is carefully examined or the terminal biomass is compared. For example, the biofilm formed under 0.1% sucrose reached a stationary phase 6 h earlier with a terminal biomass 70% less than that formed under 1% sucrose. This may indicate that with 0.1% sucrose, the biofilm is less “sticky”. Thus, when biofilm gets thicker, the force that holds the cells together could not withstand the shear force exerted by the flow. As a result, some cells are sloughed off, allowing others to continue growing. This process is repeated until the end of the experiment. With 1% sucrose, more glucans are synthesized, which makes the biofilm cells more “sticky”, allowing their continued growth on the surface despite constant shear stress.

It is important to note that, although the CLSM and the MJI analyzed biofilms grown under static conditions whereas the QCM measured biofilm grown with a flow cell set-up, the characteristics of biofilms grown under the same sucrose concentration but in the two different systems (static versus flow) are very similar. Without sucrose, although cells could attach to the surface in static culture, the attached cells could not withstand a mild stress introduced by either rotation of the wash solution (with static system) or the flow of the medium (with the flow system). With 0.1% sucrose, the biofilm formed in static culture is 30 times less resistant to shear stress introduced by the microjet than the biofilm formed under 1% sucrose, while under a flow rate of 0.2 ml/min, the biofilm under 0.1% sucrose accumulated 70% less cell mass than the biofilm under 1% sucrose. Since the concentration of total carbon source was made the same in the growth media containing different concentrations of sucrose (see Materials and Methods), the total cell mass (planktonic plus biofilm) in cultures with difference sucrose concentrations was similar (data not shown). Therefore, these differences in biofilm architecture, adhesive strength, and total accumulated biomass provide clear evidence that sucrose quantitatively and qualitatively affects the biofilm properties of *S. mutans*. This may provide an explanation for the positive correlation between the amount of sugar intake and the probability of caries development.

In conclusion, we describe for the first time the use of QCM and MJI in conjunction with CLSM and COMSTAT to analyze biofilms of *S. mutans* formed under different sucrose concentrations. Combination of these methods allows for more in-depth analysis of biofilm formation with regard to the kinetics, biomass, adhesive strength and architecture. Although the three systems used in this study were not integrated, so two culture set-ups (static and flow) had to be used, the results obtained were consistent and comparable. We are currently constructing a see-through flow cell with an integrated crystal chip, which allows biofilm growth to be measured by QCM in real time, its architecture to be analyzed by CLSM *in situ*, and its adhesiveness measured by MJI at any designated time point.

Finally, it is worth noting that the experiments described in this study were also conducted using different *S. mutans* strains and different surfaces such as hydroxyapatite (HA) disks and glass slides. Different strains of *S. mutans* showed similar biofilm architectures under the respective sucrose concentrations. Different surfaces showed a similar course of response to microjet treatments; however, the values of shear stress required to detach biofilms are different. The order of adhesiveness of *S. mutans* to the different surfaces is polystyrene > HA

> glass. This finding is also consistent with previous observations (Yoshida & Kuramitsu, 2002).

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Fig. 1. CLSM images of *S. mutans* biofilms grown in a Lab-Tek® II Chamber Slide™. Pictures were taken with an overall magnification of 400×. Cells were stained with CellTracker™ Orange CMTMR and grown anaerobically overnight at 37 °C in BHI with no sucrose (a, d), 0.1% sucrose (b, e), and 1% sucrose (c, f). (a–c) Before washing; (d–f): after washing. Insert: a single microcolony. Scale bar represents 50 μm.

Fig. 2. COMSTAT analysis of *S. mutans* biofilm structures grown with indicated sucrose concentrations. (a) Before washing with PBS; (b) after washing with PBS. Values are means with standard deviations of data from 14 image stacks per sucrose concentration taken from two independent chambers (seven image stacks from each independent chamber). Gray bar: biovolume ($\mu\text{m}^3/\mu\text{m}^2$). Black bar: average thickness (μm).

Fig. 3. QCM data plot showing delta frequency (Hz) (a) and mass ($\mu\text{g}/\text{cm}^2$) (b) change over time of *S. mutans* biofilms grown in the presence of 0% (dotted line), 0.1% (dashed line), and 1% (continuous line) sucrose. Arrows indicate the time points at which inoculation and pump flow started. The experiments were repeated three times with similar results. Shown here are representative graphs out of the three independent experiments.

Fig. 4.

Detachment of *S. mutans* biofilms by MJ. (a) Set-up of the MJ system and pictures of lesions caused by the treatment with the microjet. (b) Shear stress required for detaching cells from biofilms grown in the presence of 0.1%, 0.5% and 1% sucrose. Values and standard deviations are calculated from at least three independent experiments. (c) Plot of shear stress and lesion diameters of MJ treatments as a function of sucrose concentration. Solid line: shear force. Dashed line: diameter of the cleared zone.