J Clin Periodontol 2005; 32: 1151–1156 doi: 10.1111/j.1600-051X.2005.00836.x

Journal of Clinical Periodontology

Removal of *Streptococcus mutans* biofilm by bubbles

Michael R. Parini[1], Dennis L. Eggett[2] and William G. Pitt[1]

Departments of [1]Chemical Engineering and [2]Statistics, Brigham Young University, Provo, UT, USA

Parini MR, Eggett DL, Pitt WG. Removal of Streptococcus mutans *biofilm by bubbles. J Clin Periodontol 2005; 32: 1151–1156. doi: 10.1111/j.1600-051X.2005.00836.x.*

Abstract

Background: Air bubbles had been shown to remove particles and bacteria from surfaces, but they had not yet been studied regarding the removal of mature biofilm from a surface.

Methods: *Streptococcus mutans* were grown as a biofilm on glass coverslips and were exposed to a fluid stream with or without bubbles. Three parameters (stream velocity, gas fraction, and bubble size) were varied in the bubble stream to determine which conditions best remove the biofilm.

Results: At low velocities bubbles enhance biofilm removal compared with the liquid alone. Stream conditions that were shown to be the most effective in removing biofilm were large bubbles at low gas fractions.

Conclusions: These results suggest that flowing bubble streams may be a desirable feature to incorporate into oral hygiene products to remove accumulated biofilms such as dental plaque.

Key words: air–water interface; biofilm; biofilm removal; bubbles; *Streptococcus mutans*

Accepted for publication 12 July 2005

Powered toothbrushes often generate a stream of liquid and entrained air bubbles flowing across surfaces in the oral cavity. Bubbles entrained in liquid have been shown to effectively remove particles (Suarez et al. 1999a, b) and adherent bacteria (up to a monolayer thick) from surfaces (Pitt et al. 1993, Gomez-Suarez et al. 2001, Busscher et al. 2003). These previous studies have found that the efficiency of bacterial removal increases as the velocity of the bubble decreases, and thus predict that slower bubble streams might be more efficient in removing bacteria than fast-moving bubble streams.

On the other hand, recent reports of removal of mature biofilms (such as oral plaque) by streams of bubbles and liquid suggest that the flowing bubble stream is effective in removing biofilm (Yang et al. 2001, Adams et al. 2002, Pitt 2005). This paper presents a quantitative study of the use of bubbles to remove mature mono-species bacterial biofilms under different flow conditions. Specifically, the velocity of the bubble stream, the amount of gas in the stream, and the size of the bubbles were correlated with the efficacy of biofilm removal by bubbles. By determining the conditions in which a liquid stream containing air bubbles removes biofilm, additional options for oral health care can be explored.

Materials and Methods

Biofilm development

Biofilms of *Streptococcus mutans* (ATCC #700610) were grown on glass coverslips in a drip flow reactor for 16 h at 37°C as described previously (Heersink et al. 2003, Pitt 2005). The biofilms for these experiments were grown in a quiescent (non-flowing) solution of brain heart infusion supplemented with 2% sucrose (BHI-S). The depth of the BHI-S solution was 6 mm from the headspace to the glass, leaving 10 mm of headspace to pass CO_2 at about 2 ml/min. Individual coverslips used in this research had an average biofilm thickness of about $40 \pm 8.5\ \mu$m as measured by scanning laser confocal microscopy (LSM 1, Zeiss, Thornwood, NY, USA).

Bubble generator

To create a continuous stream of bubbles for removing biofilm, an apparatus was built which generated bubbles of a controlled size, gas fraction, and velocity. A 25-gauge needle was mounted an aluminium block tht had been machined to allow a pressurized air stream to mix with a pressurized artificial saliva stream in the hub of the needle. The beveled tip of the needle was removed using wire electrical discharge machining, so the fluid exited parallel to the needle axis. The velocity, gas fraction, and the size of the bubbles in the stream were controlled by adjusting the pressures of these two streams.

Measurement of bubble size, velocity, and gas fraction

To develop a correlation between the bubble size and the system pressures, bubbles generated at different pressure settings were measured just after leaving the tip of the needle using a CCD-IRIS/RGB video camera (DXC-151A, Sony Electronics, Park Ridge, NJ, USA)

connected to a 10-in laboratory telescope. The camera images were stored in a computer using image capturing software (Image-Pro® Plus, Media Cybernetics®, Silver Spring, MD, USA). The horizontal and vertical diameters of at least 30 bubbles were recorded for nine different pressure combinations of the two streams. The median horizontal bubble diameter was correlated with the flow parameters. Volumetric flow rates and gas fractions were measured by volumetric displacement. Total volumetric flow rate was converted into an exit velocity at the needle tip. These measurements were also correlated with the flow parameters.

Biofilm experiment chamber

The chamber used for performing experiments on the biofilm was a rectangular Plexiglas box into which the needle was inserted through a rubber septum in the bottom of the box (see Fig. 1). The coverslip covered with biofilm was clipped into a Plexiglas frame at a 45° angle such that the centre of the coverslip was 0.375 in above the needle.

Artificial saliva

A 1.5 mg/l solution of scleroglucan (Clearogel 11D, MMP Inc., So. Plainfield, NJ, USA) in water was used in these experiments to make artificial saliva (Vanderreijden et al. 1994), because its viscoelastic properties were similar to human saliva. This solution has a viscosity of 1.4 cP, which is slightly lower than the viscosity of whole human saliva at 1.9 cP (Christersson et al. 2000). The artificial saliva was used as fluid for the bubble generator and to fill the experiment chamber.

Measurement of biofilm removal

Forty biofilms were exposed under varying conditions of stream velocity, gas fraction, and bubble size. A three-factor two-level factorial design was implemented for the experiment. The experimental design was replicated three times and the experiments were performed in random order. Additional experiments were performed for selected values of the parameters to aid in the generation of a mathematical model for biofilm removal.

Each biofilm was exposed to flowing artificial saliva for 5 s by opening a shutter in front of the biofilm. The coverslip was then removed and placed biofilm-side-down on a transparent Petri dish and scanned on a flatbed scanner (C7710A, Hewlett-Packard, Omaha, NE, USA) (Pitt 2005). Scion Image software was used to measure the average grey scale value for the area that had been exposed to the bubble stream. Preliminary experiments showed that biofilms that are more densely populated return values closer to white when scanned against a black background. The grey scale value, V, measured by Scion Image for the exposed area was then compared with a value for undisturbed biofilm on the same sample, V_u, and a percentage of the amount of biofilm removed was calculated by

$$\%\ \text{Removal} = \frac{V - V_u}{V_b - V_u} \times 100\%$$

where V_b is the grey scale value of the black background.

Statistical criteria

The biofilm removal as a function of these three variables was analysed statistically using SAS software (SAS Institute, Cary, NC, USA) and fit to a linear model with cross-interactions terms. The model was refined by rejecting all terms that had a $p > 0.05$.

Results

Bubbles *versus* liquid

Before determining whether bubble velocity, size, or the fraction of gas in the stream was the most significant factor in removing biofilm, it was important to determine if the presence of bubbles in the liquid jet removed more biofilm than a stream without them.

Figure 2 illustrates that at low velocities the stream with bubbles removed more biofilm than the stream without bubbles. At a low velocity of about 3 m/s, the addition of bubbles to the liquid stream removed about twice the biofilm than without bubbles. For example, the liquid stream alone removed an average of $27 \pm 9\%$ of the biofilm in the affected area, while the jet with bubbles removed about $56 \pm 5\%$. As the velocities of the flow in the streams increased, the amount removed, both with or without bubbles, increased.

However, at higher velocities, the addition of bubbles did not increase removal. For example, at about 11 m/s, the average amount removed was $81 \pm 8\%$ and $61 \pm 5\%$, without and with bubbles, respectively. However, velocities around 10 m/s are more than an order of magnitude greater than those produced by commercially available toothbrushes, which are usually less than 1 m/s (Adams et al. 2002). In the range at which toothbrushes currently propel bubbles, bubbles assist in biofilm removal.

Biofilm removal with bubbles

The removal of biofilm is not a simple function with dependence upon one variable; rather it is dependent upon the stream velocity, the gas fraction, and the bubble size. As mentioned, the biofilm removal was analysed statistically using SAS software and fit to a linear model with cross-interactions terms. After rejecting all terms that had a $p > 0.05$, the remaining terms in the model were the stream velocity, the gas fraction, the bubble size, and the interactions between velocity and gas fraction, and between velocity and bubble size. The resulting mathematical model that best predicted biofilm removal was

$$R = A \cdot Vel + B \cdot Gas + C \cdot Size + D \cdot (Vel \cdot Gas) + E \cdot (Vel \cdot Size)$$

where R is the fraction of biofilm removed at the point of impact, *Vel* the velocity in m/s, *Gas* the gas fraction, *Size* the bubble diameter size (μm), and A, B, C, and D are constants with the following values: $A = 8.06495$, $B = -113.8$, $C = 0.3806$, $D = 12.85$, and $E = -0.04836$. This four-dimensional model has an overall regression coefficient R^2-value of 0.975. Figure 3 illustrates the predictability of this model by plotting the actual amount of removal *versus* the amount of removal predicted by the model. In general the data show a good correlation (near the $y = x$ line) with the exception of two outlying data points. For a comprehensive summary of the results, see Table 1.

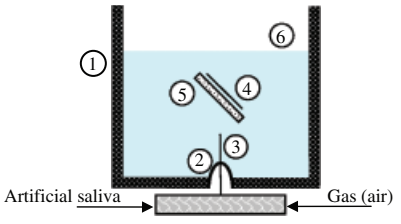

Fig. 1. Schematic of the experimental chamber. (1) Plexiglas chamber, (2) rubber septum, (3) 25-gauge needle, (4) biofilm on glass coverslip, (5) glass slide acting as a shutter, and (6) artificial saliva level.

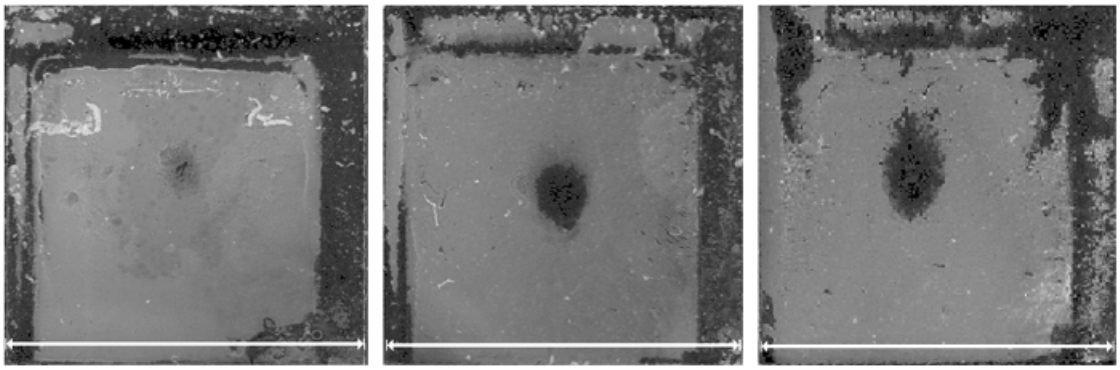

Fig. 2. Biofilm of *Streptococcus mutans* after exposed to a liquid jet stream. The black areas in the centre of each image are where the biofilm (which appears white) was removed. Left, biofilm exposed to a 3.3 m/s stream without bubbles. Centre, biofilm exposed to a stream of bubbles. The velocity of the stream was 3.3 m/s, the gas fraction was 0.29, and the average bubble diameter was 231 μm. Right, velocity of the stream was 7.3 m/s, gas fraction was 0.30, and the average bubble diameter was 246 μm. The scale bar represents 2.54 cm.

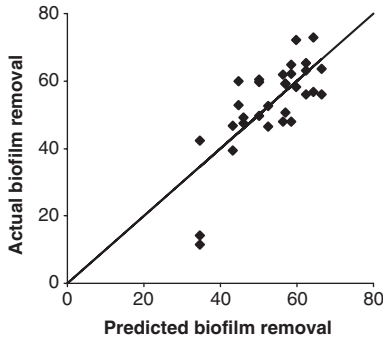

Fig. 3. Plot comparing actual biofilm removal data (individual points) to the predicted biofilm removal values from the mathematical model. The solid line is a guide to the eyes showing a $y = x$ line.

Table 1. Results of biofilm removal experiments

Velocity* (m/s)	Gas fraction*	Bubble size* (μm)	% Biofilm removal (average ± SD)
3.3 (<0.01–7.3)	0.29 (0.20–0.37)	231 (206–260)	56 ± 5
4.0 (1.2–6.8)	0.39 (0.32–0.46)	143 (129–166)	23 ± 17
4.3 (0.5–7.5)	0.41 (0.33–.048)	205 (184–229)	48 ± 1
5.7 (3.5–7.9)	0.43 (0.32–0.53)	139 (127–274)	43 ± 5
6.5 (3.6–9.4)	0.05 (0.001–0.25)	200 (180–222)	65 ± 11
6.7 (3.2–10.2)	0.48 (0.39–0.57)	205 (184–229)	50 ± 4
6.8 (3.0–10.6)	0.42 (0.35–0.50)	246 (217–274)	55 ± 10
6.8 (3.0–10.6)	0.45 (0.37–0.54)	135 (124–148)	57 ± 6
7.3 (5.5–9.1)	0.30 (0.18–0.42)	246 (217–274)	65 ± 10
10.1 (8.0–12.2)	0.55 (0.46–0.63)	257 (229–280)	61 ± 5
12.2 (9.8–14.6)	0.34 (0.20–0.48)	261 (222–293)	58 ± 9
3 (<0.1–7)	0 (0–0)	N/A	27 ± 9
6 (4–8)	0 (0–0)	N/A	77 ± 14
11 (9–13)	0 (0–0)	N/A	81 ± 8

*Values in parentheses are the 95% confidence interval.

Biofilm removal as a function of velocity

To show how the removal of biofilm is related to the velocity of the stream, the partial derivative of the fraction removed with respect to velocity ($\partial R/\partial Vel$) can be calculated:

$$\frac{\partial R}{\partial Vel} = A + D \cdot Gas + E \cdot Size$$
$$= 8.0635 + 12.8536 \cdot Gas - 0.0484 \cdot Size$$

This equation shows that velocity affects biofilm removal in a complex manner. For example, if the gas fraction is 0.30 and the bubble diameter is 246 μm or less, higher velocities will remove more biofilm ($\partial R/\partial Vel$ is positive). On the other hand, if the gas fraction is 0.30 but the bubble diameter is greater than 246 μm, the model predicts that removal decreases as velocity increases. Figure 2 shows the images of two biofilms after being exposed to bubble streams of 3.3 and 7.3 m/s. The gas fraction for these two biofilms is approximately 0.30. As the bubble diameter is not greater than 246 μm, the model predicts that the removal of biofilm should be greater at the higher velocity. Figure 2 shows that, in fact, more biofilm is removed from the sample exposed to the higher velocity stream.

Biofilm removal as a function of gas fraction

Figure 4 shows the difference between two biofilms that have been exposed to bubble streams of differing gas fractions. The image on the left is of a biofilm that has been exposed to a large gas fraction of 0.48, whereas the biofilm shown on the right was exposed to a stream with a gas fraction of 0.05. The difference in the amount of biofilm removed is very small. The partial derivative of biofilm removal with respect to gas fraction is

$$\frac{\partial R}{\partial Gas} = B + C \cdot Vel$$
$$= -113.8 + 0.3806 \cdot Vel$$

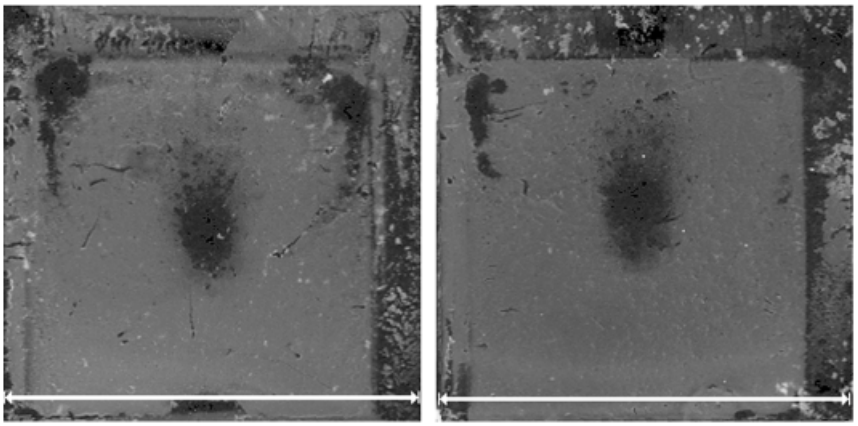

Fig. 4. Biofilms after exposure to bubble streams of different gas fractions. Left, velocity of the stream was 10.1 m/s, gas fraction was 0.55, and the bubble diameter was 257 μm. Right, velocity of the stream was 12.2 m/s, gas fraction was 0.34, and the bubble diameter was 261 μm. The white area is the biofilm and the black area in the centre is where the biofilm was removed. The scale bar represents 2.54 cm.

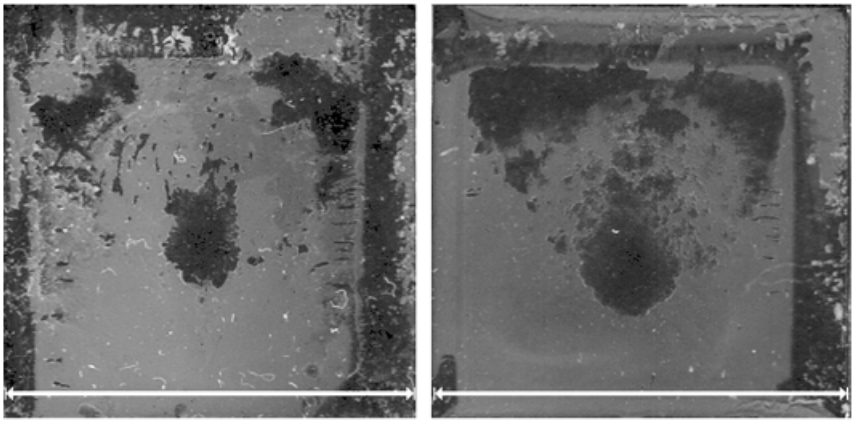

Fig. 5. Biofilms after exposure to bubble streams of different bubble sizes. Left, velocity of the stream was 6.8 m/s, gas fraction was 0.42, and the bubble diameter was 246 μm. Right, velocity of the stream was 6.8 m/s, gas fraction was 0.45, and the bubble diameter was 135 μm. The black spots in the top corners are where the biofilm had been removed by bubbles that had been trapped in the lip of the fixture clipping the coverslip in place. The white is the biofilm and the black area in the centre is where the biofilm was removed. The scale bar represents 2.54 cm.

Within the range of the velocities (2–12 m/s) studied, this equation suggests that an increase in gas fraction will reduce the amount of biofilm removed for velocities less than 8.85 m/s.

Biofilm removal as a function of bubble size

The partial derivative of the amount of biofilm removal with respect to average bubble diameter is

$$\frac{\partial R}{\partial Size} = C + E \cdot Vel$$
$$= 0.3807 - 0.04836 \cdot Vel$$

This equation shows that if the velocity is greater than 7.87 m/s, the amount of removal increases as bubble size decreases. Figure 5 displays the images of two biofilms that have been exposed to bubble streams with velocities less than 7.87 m/s. As predicted, there is greater biofilm removal on the coverslip that was exposed to the larger bubbles.

Discussion

Biofilm removal cannot be described by a simple relationship between velocity, gas fraction, and bubble size, but requires a more sophisticated mathematical model that includes the interactions between these three variables. This more complex model includes numerical coefficients that were determined by statistical regression of the data from these experiments. Because these data were obtained from biofilms grown in vitro, the coefficients of this model may differ for in vivo oral plaque, although it is believed that in general the removal of in vivo biofilm would follow the same trends with respect to velocity, gas fraction, and bubble size. The statistical results of this research show that bubble streams with velocities in the range of 2–9 m/s remove more biofilm when the bubbles are larger and the gas fraction is smaller (in other words, bigger and fewer bubbles). However, for bubble streams travelling at higher velocities, smaller bubbles and higher gas fractions (more bubbles) are predicted to remove more biofilm. It is important to note that although the statistical model predicts that these conditions are the optimum for removing biofilm, the difference in the amount of removal is only a few percent. Thus, variation of these parameters creates a statistically significant difference in removal, but the differences are small.

During the experiments, the removal of biofilm by streams without bubbles was observed. It is believed that the removal of the biofilm by liquid is caused by shear forces generated when the liquid impinges on the biofilm. In the situation of a liquid jet impinging on a surface, the velocity is highest near the point of impingement, and decreases as the fluid flows out radially along the flat surface. Thus, the velocity gradient and shear stress on the biofilm decrease radially from the impingement point. We hypothesize that there is a critical shear stress on the surface, above which biofilm is removed by fluid shear forces. The area encompassed by the boundary at this critical shear stress will be greater at faster flows, and smaller at slower flows. Within this boundary, the biofilm will be perturbed by shear forces. This area within the critical shear boundary will increase with fluid velocity. Our data show that a fluid stream with no bubbles at high velocity affects a larger area and removes a larger amount of biofilm than a lower velocity stream.

When bubbles are added to the flowing stream, additional removal mechanisms arise. Bubbles are capable of removing bacteria from a surface as the three-phase line (surface, liquid and gas) contacts the bacteria (Pitt et al. 1993, Gomez-Suarez et al. 2001). As a bubble contacts the surface, the three-phase boundary creates interfacial

forces that pull bacteria away from the substrate. In our apparatus, interfacial forces of the same nature are generated that pull biofilm from the surface. Thus, with bubbles in a rapidly moving stream there are two forces that can remove the bacteria: the fast-moving fluid forces and the shear stress forces created when bubbles contact the biofilm. Our data suggest that at velocities on the order of 3 m/s, the interfacial forces generated by the bubble contacts are more dominant than the shear forces generated by the fluid without bubbles, and thus more biofilm is removed in the presence of bubbles.

Visual observation of the bubbles exiting the needle indicates that they do not exit in a perfect linear stream, but their flow is turbulent and chaotic, thus creating a column of bubbles instead of a line of bubbles. We postulate that the area of contact of the bubble column with the biofilm is larger than the area encompassed by the boundary of critical sheer stress of liquid flow alone, and thus the stream with bubbles removes more biofilm than without bubbles. Our data indicate that at low flow velocities, the area of removal by the bubbles is much greater than the area of removal by flowing liquid without bubbles. At higher velocities, our data indicate that the percent of biofilm removed by the bubble stream is somewhat larger, but the percentage removed by the faster flowing liquid (without bubbles) is much larger than that removed by the bubble stream. It appears that the removal forces caused by the liquid flow alone may be greater than forces caused by a mixture of liquid and bubbles. Why might a liquid at high velocities produce move removal force than a gas–liquid mixture at the same velocity? We speculate that the decrease in removal is caused by a decrease in the fluid shear stresses on the surface. These shear stresses are proportional to the viscosity of the fluid, and introduction of a low viscosity gas into a liquid decreases the overall viscosity of the mixture (Perry 1997). In addition, the density and thus the momentum of the mixture decreases with addition of bubbles, and drag forces on a surface are proportional to fluid momentum.

Previous work has shown that a bubble moving at slower velocities removes more bacteria or particles than a bubble moving at higher velocities (Suarez et al. 1999b, Gomez-Suarez et al. 2001). The overall efficiency of a bubble removing a bacterium was described as the product of three efficiencies: bubble–bacterium collision, bubble–bacterium attachment, and the stability of the bubble–bacterium aggregate. By decreasing the velocity of the bubble, the liquid film surrounding the bacterium becomes thinner and increases the probability that the air–liquid interface of the bubble will come into contact with the bacterium. In so doing, the efficiency of the bubble–bacterium collision is increased. The major difference between the studies of Gomez-Suarez and our present study is that they reported that bacterium removal always increased as flow velocity decreased, whereas we have shown that, when the bubble size was small, biofilm removal increased as flow velocity increased. We attribute this divergence of observation to two factors. Our fully developed biofilm is different than a distribution of adherent bacteria on a surface. But perhaps, more importantly, the experiments of Gomez-Suarez were done with large bubbles (25×5 mm) at low velocities (0.001 m/s) compared with those in the experiments of this study (135–270 μm and 2–12 m/s). Both sets of data confirm the importance of both fluid dynamics and surface tension in removal of bacteria and biofilms from surfaces.

To take advantage of the capacity of bubbles to remove biofilm when teeth are brushed, a toothbrush that propels bubbles at the tooth surface is desired. A powered toothbrush that is able to rapidly propel bubbles towards the teeth would be able to clean beyond the reach of the bristles, i.e., be able to clean the proximal surfaces and sulci of the teeth. Thus, the ability to propel bubbles against teeth should be an important criterion for the powered toothbrush design.

Acknowledgements

Funding for this research was provided by Philips Oral Healthcare. There are no conflicts of interest in this research or publication, and this publication does not endorse any particular dental product. We appreciate the assistance of Eric Richardson with the experiments.

References

Adams, H., Winston, M. T., Heersink, J., Buckingham-Meyer, K. A., Costerton, J. W. & Stoodley, P. (2002) Development of a laboratory model to assess the removal of biofilm from interproximal spaces by powered tooth brushing. *American Journal of Dentistry* **15** (Spec No), 12B–17B.

Busscher, H. J., Rustema-Abbing, M., Bruinsma, G. M., de Jager, M., Gottenbos, B. & van der Mei, H. C. (2003) Non-contact removal of coadhering and non-coadhering bacterial pairs from pellicle surfaces by sonic brushing and de novo adhesion. *European Journal of Oral Sciences* **111**, 459–464.

Christersson, C. E., Lindh, L. & Arnebrant, T. (2000) Film-forming properties and viscosities of saliva substitutes and human whole saliva. *European Journal of Oral Sciences* **108**, 418–425.

Gomez-Suarez, C., Busscher, H. J. & van der Mei, H. C. (2001) Analysis of bacterial detachment from substratum surfaces by the passage of air–liquid interfaces. *Applied and Environmental Microbiology* **67**, 2531–2537.

Heersink, J., Costerton, W. J. & Stoodley, P. (2003) Influence of the Sonicare toothbrush on the structure and thickness of laboratory grown *Streptococcus mutans* biofilms. *American Journal of Dentistry* **16**, 79–83.

Perry, R. H. (1997) *Chemical Engineers' Handbook*, 7th edition. New York: McGraw-Hill.

Pitt, W. G. (2005) Removal of oral biofilm by sonic phenomena. *American Journal of Dentistry* (in press).

Pitt, W. G., Mcbride, M. O., Barton, A. J. & Sagers, R. D. (1993) Air–water-interface displaces adsorbed bacteria. *Biomaterials* **14**, 605–608.

Suarez, C. G., Noordmans, J., Van der Mei, H. C. & Busscher, H. J. (1999a) Detachment of colloidal particles from collector surfaces with different electrostatic charge and hydrophobicity by attachment to air bubbles in a parallel plate flow chamber. *Physical Chemistry Chemical Physics* **1**, 4423–4427.

Suarez, C. G., Noordmans, J., Van der Mei, H. C. & Busscher, H. J. (1999b) Removal of colloidal particles from quartz collector surfaces as stimulated by the passage of liquid–air interfaces. *Langmuir* **15**, 5123–5127.

Vanderreijden, W. A., Veerman, E. C. I. & Amerongen, A. V. N. (1994) Rheological properties of commercially available polysaccharides with potential use in saliva substitutes. *Biorheology* **31**, 631–642.

Yang, J., Bos, R., Belder, G. F. & Busscher, H. J. (2001) Co-adhesion and removal of adhering bacteria from salivary pellicles by three different modes of brushing. *European Journal of Oral Sciences* **109**, 325–329.

Address:
William G. Pitt
350 Clyde Building
Brigham Young University
Provo, UT 84602,
USA
E-mail: pitt@byu.edu

Clinical Relevance

Scientific rationale for study: Powered toothbrushes generate dynamic bubbles in the mouth, but the specific role of bubbles in oral bacteria removal had not been studied. This research examined the relative effect of fluid dynamics and bubble dynamics on bacterial removal from a simulated oral surface.

Principal findings: Bubbles are very effective at removing bacteria from simulated oral surfaces. Velocity, gas fraction, and bubble size all contribute to the amount of biofilm removal.

Practical implications: The practical implications are that designers of oral hygiene equipment should consider including a method of propelling bubbles in their equipment to enhance biofilm removal.