Nanoscale characterization of effect of L-arginine on Streptococcus mutans biofilm adhesion by atomic force microscopy

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Received 27 November 2013
Accepted 18 April 2014

INTRODUCTION

Dental caries is the most prevalent and costly oral infectious disease worldwide (Jeon et al., 2011). One of the main aetiologic factors of caries is the pathology of the dental plaque biofilm (Marsh, 2004). The bacterial biofilm consists of both acidogenic and arginolytic strains (Hicks et al., 2003). Microbial species in the acidogenic genera, specifically Streptococcus mutans (Loesche, 1986), have been proposed as specific agents of the acid production that is principal to the dental caries process. These cariogenic organisms metabolize dietary fermentable carbohydrates to form organic acids as end products (Loesche, 1986). Acid production causes a local drop in pH and it is the proximity of these acidogenic bacteria to the tooth surface and the contact of the acid with the surface that eventually causes the breakdown of enamel or demineralization.

Enamel, the outer layer of the tooth, is composed of calcium and phosphate mainly in the form of hydroxylapatite (HA) (Hicks et al., 2003). Dental caries is a dynamic process. The cariogenic organisms generate acids that diffuse into enamel, dissolving mineral crystals in their path. As the pH of the plaque increases (as a result of the saliva’s buffering capacity or ammonia production from arginolytic and/or ureolytic plaque bacteria), the environment becomes supersaturated in calcium and phosphate leading to tooth remineralization. This process of demineralization and remineralization occurs frequently throughout the day. Cavities form when the tooth environment stays longer under demineralizing (net mineral loss) versus remineralizing (net mineral gain) conditions (Hicks et al., 2003).

Therefore, the aetiology of this disease is very dependent on the ecology of the plaque microbiome, salivary composition...
and flow rate, and frequency of carbohydrates consumed (Zero et al., 2009). Plaque is derived from salivary planktonic bacteria that form multicellular communities via cell–cell and cell–surface interactions. These bacteria colonize on oral surfaces, by binding to surface-specific binding sites on proteins. There have been over 1000 bacteria species detected within the oral environment, many of which remain unculturable (Paster et al., 2001). Of these species, both healthy and potentially pathogenic strains exist. When the microbiome is balanced, the oral environment can maintain its healthy existence. However, when conditions change to favour the growth of cariogenic organisms, there is an increased risk of cavity formation (Marsh, 1994).

Like other cariogenic bacteria, S. mutans not only metabolizes carbohydrates but has evolved multiple mechanisms to survive under acidic conditions (Cross et al., 2007). S. mutans also has the ability to synthesize extracellular glucans from sucrose using glucosyltransferases, which is a major virulence factor (Yamashita et al., 1993). The insoluble glucans synthesized by glucosyltransferases provide specific binding sites for bacterial colonization on the tooth surface and to each other, modulating the formation of tightly adherent biofilms (Schilling & Bowen, 1992; Xiao & Koo, 2010). In addition, the secreted extracellular polysaccharides (EPSs) provide stability, structural integrity, and resistance to bacteria from antimicrobials and other chemicals (Paes Leme et al., 2006).

Several technologies have been developed that specifically target and minimize the acid-producing effects of S. mutans (He et al., 2010; Lang et al., 2010; Sullivan et al., 2011). These approaches have been proven to enhance the remineralization process resulting in the arrest and reversal of caries lesions. Other anti-cavity approaches to arrest caries initiation and progression, by promoting a more alkaline-generating microbiome, have also been suggested recently (Morou-Bermudez et al., 2011; Nascimento et al., 2009). When plaque pH is elevated above the pH which maintains the supersaturated conditions of calcium and phosphate, remineralization will be favoured. In fact, populations who are able to generate higher ammonia concentrations have been shown to be at lower risk of cavity formation (Nascimento et al., 2013). Thus, shifting the oral microbiome to a healthier, less cariogenic population is an evolving concept being studied to manage the caries process (Morou-Bermudez et al., 2011; Nascimento et al., 2009).

One such approach to promote a less cariogenic microbiome is through the use of the amino acid L-arginine (Arg), which is known for its anti-caries effect (Van Wyckhuysie et al., 1995). A recent publication demonstrated that the use of a dentifrice containing Arg and an insoluble calcium compound and fluoride provides superior efficacy in preventing enamel demineralization, enhancing remineralization and producing a more basic plaque biofilm than a placebo control containing no Arg (Cantore et al., 2013). Arginolytic organisms in the oral cavity catabolize Arg through an internal cellular pathway called the arginine deiminase system (Burne & Marquis, 2000). When Arg is utilized, the pathway yields ammonia as an end product. Ammonia, the final metabolic product, neutralizes organic acids produced within the oral cavity (Burne & Marquis, 2000).

Although the biochemical characteristics of S. mutans have been studied (Cross et al., 2007), the biofilm extracellular matrix organization has not been investigated quantitatively under physiological conditions at high resolution in the presence and absence of Arg. In this study, we used atomic force microscopy (AFM) (Binnig et al., 1986) nanomechanical characterization of S. mutans biofilm extracellular matrix under physiological buffer conditions. S. mutans bacterial biofilms grown in growth media containing Arg and sucrose were studied. The findings provide nanomechanical evidence for the effect of Arg on the adhesive behaviour and structural properties of EPSs in S. mutans biofilms. Our results suggest that S. mutans biofilms grown in the presence of Arg could influence the production and/or composition of extracellular membrane glucans and thereby affect their adhesion properties. High-resolution imaging of biofilm surfaces can reveal structural information on bacterial cells embedded within the surrounding extracellular matrix. A dense extracellular matrix was observed in biofilms without Arg compared to those grown in the presence of Arg. Our results suggest that the presence of Arg in the oral cavity could influence the adhesion properties of S. mutans to the tooth surface.

**METHODS**

**Growth of in vitro biofilms.** Overnight cultures (OD$_{600}$=1) of S. mutans strain UA140 (Qi et al., 2001) grown at 37 °C in a 5% CO$_2$ atmosphere were diluted 1:100 in fresh Todd–Hewitt (TH; Difco) medium containing 1% (w/v) sucrose, 1% mannose and 1% glucose as the supplementary carbon and energy source. Additionally, Arg or glycine (both purchased from Sigma-Aldrich) was added at different concentrations to the medium. No Arg or glycine was added to control samples. Having small quantities of Arg available during biofilm growth is a realistic representation of the natural conditions. The quantities of Arg used in our test are substantially greater than the low levels present in the salivary secretions and the TH medium.

Diluted culture (500 μl) was added to each well of six-well polystyrene microtitre plates in which sterile coverslips had been placed. After overnight S. mutans biofilm formation, the plates were rinsed with PBS three times to remove planktonic and loosely bound cells. Biofilm adhesion was measured via AFM under PBS (pH 7.2) at room temperature (20 °C). The pH in the different biofilms was determined using standard pH microelectrodes (Fischer Scientific) following previously described methods (Revsbech, 2005).

**AFM.** All force measurements in fluid were conducted using a Nanoscope V Catalyst (Bruker) mounted on an inverted optical Zeiss Microscope. AFM measurements were collected in contact mode using sharpened silicon nitride cantilevers (MLCT, Bruker) with experimentally determined spring constants of 0.01 N m$^{-1}$ and a nominal tip radius of 20 nm as defined by the manufacturer (Bruker).
Before obtaining force measurements, the surface attachment and stability of the bacterial biofilm were tested by imaging the surfaces at low forces (~200 pN). Force–distance measurements were collected on S. mutans biofilms by lowering the cantilever tip towards the cell, pressing against the cell surface and retracting the tip from the cell as shown schematically in Fig. 1. The resulting curves, generated from the cantilever displacement, were analysed to reveal the force magnitude and relative cell surface adhesion by monitoring the rupture events revealed in the associated tip retraction traces from the cell surface as shown in Fig. 1(c, d). About 100 force–distance curves were obtained at ten different biofilm regions for each growth condition, with a trigger force of 5 nN, using a z ramp size of 18 μm and 1 Hz at 1024 × 1024 samples per line. The adhesion forces and rupture lengths were calculated from force–distance curves using SPIP software. The reproducibility of subsequent force curves (magnitude of adhesion force and rupture lengths taken at the same point) suggests that the ruptures occurred due to adhesion between the biofilm and AFM probe. As a control, we repeat-measured control bare substrates to check for tip contamination. Further, the order in which adhesion was measured was randomized. On alternating the sequence of samples between control, Arg and glycine, similar results were observed for biofilm adhesion.

AFM imaging. Biofilm samples were briefly rinsed with PBS and water and imaged under ambient conditions using tapping-mode AFM imaging. To obtain structural characteristics of biofilm surfaces, the height, deflection and phase images were simultaneously acquired as they yield complementary information. Images were recorded at 1024 samples per line at 1 Hz using TESP (Bruker) probes. Image processing was carried out using SPIP software.

RESULTS

Influence of Arg on adhesive properties of S. mutans biofilms measured by AFM

We used AFM to measure the adhesion properties of S. mutans biofilm surfaces at the nanoscale. S. mutans biofilms were immobilized by growing them on glass coverslips as described above. Force–distance curves were measured on the bacterial biofilm by lowering the tip and pressing it against the cell surface with a maximum force of ~5 nN. Upon tip retraction, a sequence of rupture events occurred at distances of up to 18 μm, presumably arising from the breakage of multiple adhesions between the AFM tip and the biofilm surface (Fig. 1). A typical cycle of bond formation and subsequent rupture at varying distances away from the biofilm surface is shown in Fig. 1(d).

The observed adhesion forces of S. mutans biofilm grown in media containing different concentrations of Arg are shown in Fig. 2. In the absence of Arg, the adhesion forces were broadly dispersed with a mean ± SD adhesion force of 3.5 ± 0.6 nN (N=230) (Fig. 2a). As the amount of Arg was increased, the frequency of rupture events shifted to lower adhesion forces. Adhesion forces for S. mutans biofilms grown under 0.5 mg ml⁻¹, 1 mg ml⁻¹ and 5 mg ml⁻¹ Arg were 3.1 ± 2.4 nN (N=217), 1.4 ± 0.7 nN (N=208) and 1.3 ± 0.6 nN (N=169), respectively. In the presence of 1 mg Arg ml⁻¹ and above, the adhesion forces were significantly reduced (Student’s t-test; P<0.05) and principally clustered at less than 2 nN.

Changes in rupture tip–cell retraction lengths of S. mutans biofilms in the presence of Arg

The corresponding tip–cell distances before rupture for biofilms grown under varying Arg conditions, measured from the AFM force–distance curves, are shown in Fig. 3. As the concentration of Arg increased in the growth media, the distance that the tip retracts before contact is lost (i.e. rupture length) decreased. Rupture lengths for S. mutans biofilms grown under 0 mg ml⁻¹, 0.5 mg ml⁻¹, 1 mg ml⁻¹ and 5 mg ml⁻¹ Arg were measured. The histogram of
rupture lengths displayed a bimodal population (Fig. 3) with two main peaks. When no Arg is present, about 50% of the ruptures occur at distances 6000–8000 nm from the biofilm surfaces (peak 2) while another shorter population (peak 1) (15%) is observed closer (<2000 nm) to the biofilm surface. The mean ± SD rupture length values (N=185) for peak 1 and peak 2 for control biofilms were 1400 ± 800 nm and 6500 ± 100 nm, respectively. However, longer rupture length populations (peak 2) show a gradual and significant reduction upon treatment with Arg. Mean rupture lengths for 0.5 mg Arg ml⁻¹ (N=147) were 1500 ± 100 nm (peak 1) and 5900 ± 200 nm (peak 2); for 1 mg ml⁻¹ (N=147) and 5 mg ml⁻¹ (N=138) the rupture lengths were 1600 ± 100 nm (peak 1) and 4800 ± 200 nm (peak 2) and 1500 ± 200 nm (peak 1) and 5700 ± 200 nm (peak 2), respectively (P<0.05 compared to control biofilms).

To test whether adhesion forces are influenced by the presence of another amino acid, we grew the biofilm in the presence of glycine. Glycine did not impede the adhesive interactions and rupture lengths of biofilms. S. mutans biofilms cultured in growth media containing glycine showed adhesion forces and rupture lengths comparable to controls (no Arg). Mean ± SD adhesion forces for 1 mg ml⁻¹ and 5 mg ml⁻¹ glycine were 3.7 ± 1.4 nN (N=98) and 3.8 ± 1.8 nN (N=86), respectively, similar to control biofilms. For 1 mg ml⁻¹ and 5 mg ml⁻¹ glycine, rupture lengths were 1900 ± 100 nm (peak 1) and 5800 ± 200 nm (peak 2), and 1500 ± 500 nm (peak 1) and 5700 ± 200 nm (peak 2), respectively (P>0.05 compared to control biofilms).

Biofilm viability
The topography of the biofilms was similar in the presence or absence of Arg. Experiments were also conducted to determine if there was any reduction in the number of colony forming units as a result of Arg being present in the growth media. No evidence of growth inhibition was observed.

Ultrastructure of S. mutans biofilms imaged using AFM
To evaluate further the quantitative changes in surface adhesion forces and rupture lengths for S. mutans biofilms observed in the presence of Arg, we used AFM tapping-mode imaging under ambient conditions to observe the ultrastructure and distribution of the EPS materials of these biofilms. Individual S. mutans cells showed characteristic elongated shapes with length, width and height dimensions of approximately 1 µm, 0.5 µm and 0.4 µm, respectively, as reported previously (Cross et al., 2006). S.
*mutans* strain UA140 cells showed two typical parallel bands in their nanoscale surface topology (Fig. 4). Fig. 4(a) shows a topographical image of *S. mutans* control biofilms. AFM images reveal the presence of EPS materials around the bacterial cells that form a mesh-like network of surrounding extracellular matrix (Fig. S1, available in the online Supplementary Material). Interestingly, AFM images obtained for *S. mutans* biofilms grown in the presence and absence of Arg showed differences in the abundance and localization of the extracellular matrix, as shown in representative AFM amplitude images (Fig. 4b, e). There was also a notable decrease in the surface roughness of biofilms grown in the presence of Arg. In the presence of Arg, the extracellular matrix is less dense compared to control biofilms. The observed changes in the biofilm matrix were more clearly visualized in the phase images (Fig. 4c, f). The biofilm EPS shows characteristic phase contrast (Fig. 4c, f), suggesting heterogeneous density and/or viscoelastic properties of the matrix compared to bacterial structures. To quantify the quality of a surface topography, the biofilm surface roughness was measured as: $R_a$ (arithmetic mean value), $R_q$ (quadratic mean) and $R_{\text{max}}$ (the maximum roughness height). Roughness values obtained from AFM topographic images were 89.1 nm, 67.3 nm and 515 nm, respectively, for biofilms grown in the absence of Arg, whereas 1 mg Arg ml$^{-1}$ biofilms showed decreased roughness values of 52 nm, 42.8 nm and 297 nm, respectively.

**DISCUSSION**

The composition and structure of the *S. mutans* extracellular matrix vary as a function of environmental conditions (Thurnheer et al., 2006); it mainly consists of polysaccharides, proteins, lipids and nucleic acids (Vu et al., 2009). It has been suggested that EPS materials may play a major role in the organization and cohesion of bacterial biofilm (Jorand et al., 1998). Bacterial adhesion is of interest to understanding bacterial infection mechanisms including dental caries. Recently, AFM has evolved rapidly into a tool that can quantitatively probe biomolecules as well as bacterial and biofilm surface interactions (Cross et al., 2006; Dufrene, 2008; Pelling et al., 2005; Sharma et al., 2010). AFM-based force spectroscopy has been used
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Fig. 4. AFM images showing differences in ultrastructure of S. mutans biofilms grown without (a, b, c) and with 1 mg L-arginine ml$^{-1}$ (Arg) (d, e, f). (a, d) Topography, (b, e) amplitude and (c, f) phase images. The structure of an individual bacterial cell is shown in inset in (a). The secreted EPS around the bacterial cells is marked with asterisks (∗) in amplitude images. The corresponding phase images (c, f) show enhanced contrast in regions of extracellular matrix marked with arrows. In the presence of Arg, the extracellular matrix is less abundant compared to control biofilms.

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successfully to measure and quantify biopolymers such as DNA, proteins and polysaccharides (Marszalek et al., 1999; Yakovenko et al., 2008). One early study used AFM to measure the adhesive forces between the cantilever tip and E. coli mutants (Razatos et al., 1998). Various efforts subsequently were made to measure adhesive forces of bacteria (Arce et al., 2009; Dufrene, 2003; Dupres et al., 2005; Harimawan et al., 2011) and adhesion forces of S. mutans were measured to reveal its role in dental caries (Busscher et al., 2007; Liu et al., 2013; Loskill et al., 2013; Mei et al., 2011). In this study, we used AFM to characterize the adhesive properties of S. mutans biofilms by probing the adhesive forces between the biofilm and the AFM tip as well as measuring the lengths of the rupture events that occur at varying distances from the biofilm surface as the AFM tip is pulled away from the surface.

The significant reduction in biofilm adhesion forces observed in the presence of Arg suggests potential changes in the S. mutans biofilm surface resulting in reduced hydrogen-bonding interactions. Such an effect may occur due to less glucan being produced; this was shown to affect the adherence properties of S. mutans (Cross et al., 2007). Since Arg is not metabolized by non-arginolytic bacteria strains, it may itself inhibit interactions between glucan sites. The biofilms showed no significant change in pH at time zero and after 24 h of incubation (Table S1) for cultures grown in 1 mg ml$^{-1}$ Arg or less. Hence, any effect on pH is unlikely to result in the observed reduction in adhesion forces of the Arg-treated biofilms. Additionally, we found no evidence of a reduction in S. mutans biofilm viability due to the presence of Arg (Fig. S2). Robust biofilms were observed up to 5 mg Arg ml$^{-1}$ concentrations.

Additionally, the reduction in the measured rupture lengths from AFM force–distance curves could result from changes in the density and adhesiveness of the extracellular matrix surrounding the bacterial cells within the biofilm. All these results are consistent with reduced glucan production or fewer hydrogen bond interactions within the biofilm extracellular matrix. Furthermore, no significant changes in biofilm properties were observed in the presence of glycine. The effect of Arg was not specific to S. mutans biofilms. Studies were also conducted on Streptococcus sanguinis biofilms grown in the presence and absence of Arg (data not shown). Although the bacterial extracellular surface was not as adhesive as in S. mutans biofilms, in the presence of Arg there was a significant reduction in biofilm adhesion forces. S. sanguinis is an arginolytic bacterium that metabolizes Arg to produce ammonia. No such pathway exists to utilize Arg for S. mutans (Liu et al., 2013).

AFM is also a powerful technique to probe biological surface structure and properties at nanometre resolution (Cross et al., 2007; Dufrene, 2008; Sharma et al., 2003, 2011) and can be used for high-resolution studies of EPS and biofilm surfaces. By employing high-resolution AFM images, we were able to detect density variations in S. mutans biofilm EPS. Our results indicate a change in the characteristics of the extracellular matrix of S. mutans biofilm formed in the presence of Arg and provide structural evidence for the changes in nanomechanical profiles (adhesion and EPS unbinding events) of the biofilms reported in this study.

Within the human oral environment, the fate of any bacterium/biofilm is complex and depends on several associated factors. The physical properties, net charge, and hydrophobicity of the substrate surface can influence the composition of adherent bacteria and the efficiency of adsorption (Li et al., 2010; Mei et al., 2011). Within the oral environment, the tooth surface is spontaneously covered by salivary secretion, which influences the maturation/conditioning of tooth enamel. The presence of saliva results in the deposition and incorporation of calcium, phosphates and fluoride into the maturing hydroxylapatite, due to the saturation of saliva with these ions. Additionally, the tooth is coated with the acquired enamel pellicle (Lee et al., 2013) which comprises glycoproteins and mucins, and can include cystatins,
histatins, lysozyme, amylase, lactoferrin, lactoperoxidase, sialic acid, albumin, carbonic anhydrate, secretory IgA and bacteria-derived glucosyltransferases and fructosyltransferases from the surrounding saliva (Garcia-Godoy & Hicks, 2008; Tabak, 2006). The pellicle protects the porous HA from demineralization of calcium and phosphates by plaque-generated acids. Many of the proteins, as well as water-insoluble glucan, adsorb to the tooth surface via primary colonizing bacteria that initiate plaque development. The results of this pilot study on S. mutans biofilms grown on unconditioned glass substrates, while encouraging, should be considered preliminary. Further studies are necessary to assess the extent and influence of Arg in preventing or reducing S. mutans biofilm adhesiveness on more physiologically relevant substrates such as HA and saliva-treated HA surfaces that better mimic oral microenvironments. Such an approach would use HA functionalized AFM probes with or without saliva coating to test the effectiveness of Arg in reducing changes in nanomechanical profiles (adhesion and EPS unbinding events) of the initial-stage S. mutans biofilms.

In summary, our study provides further evidence that AFM can be used to measure and quantify the interactions between cell surface materials present on bacterial membranes. We were able to measure changes in the adhesion force magnitude and rupture length signatures of S. mutans biofilms grown in the presence of Arg. The interactions are thought to originate between glucan polymers and membrane-bound proteins. Our results suggest that the presence of Arg in the oral cavity could modulate the adhesion properties of S. mutans to the tooth surface and may possess anti-cariogenic potential.

ACKNOWLEDGEMENTS

The authors would like to acknowledge financial support from Colgate-Palmolive Company, Piscataway, NJ, USA, and International Center for Materials Nanoarchitectonics Satellite (MANA), National Institute for Materials Science (NIMS), Tsukuba, Japan. We acknowledge the use of the Scanning Probe Microscope facility at the Nano and Pico Characterization Laboratory at the CNSI at UCLA.

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Edited by: R. Palmer