Superhydrophobic Modification of Hydroxyapatite Decreased *in vitro Streptococcus mutans* Adhesion and Calcium Dissolution

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Objective: *To investigate the effects of superhydrophobic modification of hydroxyapatite on the adhesion of* Streptococcus mutans *and calcium dissolution.*

Methods: *The surface of hydroxyapatite (HAP) discs were modified with a self-assembling film of fatty acids (FAs),* $CH_3(CH_2)_{n-1}COOH$ *in ethanol, with different carbon chain lengths (n = 1, 2, … , 16) for 12 hrs to obtain superhydrophobicity. The water contact angle on the surface of the modified HAP discs was measured to determine the superhydrophobicity. The superhydrophobically modified HAP discs were coated with human saliva, incubated in a* S. mutans *suspension for 24 hrs and the amount of the bacterium bound to the HAP disc surface was measured as colony-forming units. The HAP discs modified with FAs with different carbon chain length were also examined for their resistance to acid by immersing in an artificial caries demineralisation solution for 12 hrs. The calcium dissolved in the demineralisation solution was measured by atomic absorption spectroscopy. Further, the resistance of superhydrophobically modified HAP discs to acid was examined by the same method at 1, 8, 24 and 48 hrs.*

Results: *The contact angle increased significantly with the increase of the FA carbon chain length, and the surfaces of the modified HAP discs became superhydrophobic (contact angle larger than 150 degrees) when the FA carbon chain length was 12 or more. The amount of bacteria that adhered to the superhydrophobically modified HAP discs was significantly less than the control group (*$P < 0.05$*). The calcium dissolved from the FA-modified HAP discs into the demineralisation solution was dramatically decreased when the FA carbon chain length was 12 or more. The superhydrophobically modified HAP discs had less calcium dissolved into the demineralisation solution compared with the control (non-modified) for up to 48 hrs (*$P < 0.01$*).*

Conclusion: *HAP discs were easily superhydrophobically modified by FAs. The superhydrophobic modification efficiently reduced both the oral bacterial adhesion to the surface of the modified HAP discs and calcium dissolving from the modified HAP discs. This is likely to be of significant interest in caries prevention.*

Key words: *acid erosion, bacterial adhesion, hydroxyapatite, superhydrophobicity*

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Dental caries is caused by acid produced by bacteria in dental plaque. Therefore, the key steps for caries prevention are to reduce dental plaque formation and to prevent acid infusion to the tooth surface[1,2]. Modification of tooth surfaces physically and chemically is a novel strategy to inhibit bacterial adhesion to the surfaces, to prevent dental plaque formation.

It is well known that bacterial adhesion to tooth surfaces is a prerequisite for the formation of dental plaque, also known as biofilm[3]. Bacterial adhesion is a complicated process depending on the physical and chemical properties of substrates and bacteria[4-7]. Many studies on substrate surface characteristics and *in vitro* biofilm formation have been carried out on powdered hydroxyapatite, restorative dental materials and prosthetic and implant dental materials. These studies explored the effects of surface hydrophobicity, free energy and roughness on bacterial retention[8-12]. Superhydrophobicity, a special wettability of a solid surface, occurs when the contact angle of water to the solid surface is larger than 150 degrees. Superhydrophobicity renders the surface extremely water repellent, and in some cases it causes water drops to roll off, removing particular contamination[13]. Therefore, superhydrophobic surfaces have been applied for self-cleaning and other purposes[14-16]. In the present study, it was hypothesised that superhydrophobicity may be applied for carries prevention.

In previous work[17], a nanostructured copper surface was transformed from superhydrophilicity to superhydrophobicity by the self-assembly method using fatty acids (FAs) with different carbon chain lengths. To test the present hypothesis, the same technique was used to transform the surface of hydroxyapatite (HAP) discs and examine the effects of superhydrophobic modification on *in vitro Streptococcus mutans* adhesion to and calcium dissolution from the HAP disc.

Methods

Morphological examination and modification of HAP disc surface

High-density hydroxyapatite discs (7 mm diameter, 1.7 mm thickness, Clarkson Chromatography Products, South Williamsport, PA, USA) were used in this study. To examine if the surface of the HAP disc was nanostructured, the surface of the HAP disc was examined by a field emission scanning electron microscopy (SEM) apparatus (JSM-6700F, JEOL, Tokyo, Japan). For surface modification, the HAP discs were immersed in the ethanol solutions of FAs, $CH_3(CH_2)_{n-1}COOH$, with different carbon chain lengths ($n = 1, 2, \ldots, 16$) at a concentration of 0.03 mol/l for 12 hrs, respectively. The samples were rinsed with adequate ethanol and pure water and dried with a flow of N_2 gas. Three specimens were used for each group.

Measurement of surface contact angle and confirmation of superhydrophobicity of the HAP disc surface

To characterise the surface wettability, the contact angle was measured using a SCA 20 system (DataPhysics Instruments, Filderstadt, Germany). Briefly, after completely dried by a flow of N_2 gas, a droplet of water was added to the surface of the HAP disc. The profile of the water drop was recorded and the contact angle was calculated. All data were collected at ambient humidity and temperature (approximately 25°C).

Saliva coating of HAP disc

Non-stimulated human whole saliva was collected from three volunteers without active periodontal disease or active caries. The whole saliva was then pooled and clarified by centrifugation at 3000 × g for 20 min at 4°C, sterilised by filtration (pore diameter, 0.2 µm), and stored at -20°C until used. The FA-modified HAP discs and control discs (without any modification) were sterilised in 70% ethanol solution for 20 min and air-dried for 30 min; the sterilisation steps were repeated 3 times. The discs were then immersed in the clarified whole saliva, rotated on a shaker for 60 min at 37°C, washed in distilled water to remove unbound and loosely bound substances, and air-dried for 30 min.

Bacterial adhesion to HAP disc

S. mutans (ATCC 25175) was grown on a mitis salivarius-bacitracin (MSB) agar plate in an atmosphere of 5% CO_2, 10% H_2 and 85% N_2 at 37°C for 48 hrs and used to inoculate a second culture which was grown with agitation for another 48 hrs in brain heart infusion (BHI) broth at the same conditions. The bacteria were harvested by bench-top centrifuge for 15 min at 600 × g at 10°C, washed twice with phosphate buffered saline (PBS) (pH 7.2) and resuspended in PBS. A final concentration of approximately 1×10^8 colony-forming units (CFU)/ml of bacteria was obtained according to the OD values ($\lambda = 540$ nm) measured by a spectrophotometer.

Bacterial adhesion to the HAP disc was evaluated as CFU by an indirect assay. Briefly, each saliva-coated disc was immersed in 1 ml of the bacterial suspension (approximately 1×10^8 CFU/ml) supplemented with 5 ml BHI growth medium and incubated for 24 hrs at

37°C in 5% CO_2, 10% H_2 and 85% N_2. In total, seven superhydrophobically modified and seven non-modified discs were evaluated for bacterial adhesion. The discs were washed with sterilised saline solution. Each disc was put into a tube containing 2 ml PBS and shaken on a vortex for 1 min to release the adhered bacteria from the disc's surface into the solution. Aliquots of 100 µl of the solution were plated onto MSB agar and incubated under anaerobic conditions (5% CO_2, 10% H_2 and 85% N_2) at 37°C for 48 hrs, and the colonies were counted.

Resistance to demineralisation

Resistance of the FA-modified HAP discs to demineralisation was evaluated as calcium dissolving in an acid solution. Briefly, different carbon chain length (n = 1, 2, 3, … , 16) FA-modified HAP discs were immersed in an artificial caries demineralisation solution (0.1 mol/l lactic acid, pH 4.4) in a volume of 25 ml for 12 hrs. The calcium that dissolved from the HAP discs into the demineralisation solution was measured by atomic absorption spectroscopy (AAS) (Avanta PM, GBC Scientific Equipment, Dandenong, Australia). In total, five discs were evaluated for each FA modification. According to the results of calcium dissolution from different carbon chain length FA-modified HAP discs, HAP discs modified by 12 carbon chain FA and the control discs were further examined for calcium dissolution at 1, 8, 24, and 48 hrs (n = 5 for each time point).

Statistical analysis

A paired *t* test was used for examination of differences in the adhesion of bacteria to control and modified HAP surfaces. An independent Student *t* test was used to evaluate the amount of calcium that dissolved into the demineralisation solution for the superhydrophobically modified HAP discs and the control discs.

Results

Effect of FA modification on the surface wettability of HAP discs

As shown in Figure 1a, an SEM image of the intact HAP disc showed that the surface was a typical nanostructure with feature sizes of about 100 to several hundreds of nanometers. The surfaces of the modified HA discs were superhydrophilic (contact angle about 0 degrees) when the FA carbon chain length was 5 or less than 5 carbons, as shown in Figure 1b. The contact angle increased significantly with the increase of the FA carbon chain length. The surfaces of the modified HAP discs became superhydrophobic (contact angle larger than 150 degrees) when the FA carbon chain length was 12 or more (Fig 1b). Therefore, the 12-carbon chain FA-modified HAP discs were used as superhydrophobic HAP discs for bacterial adhesion and calcium dissolution experiments.

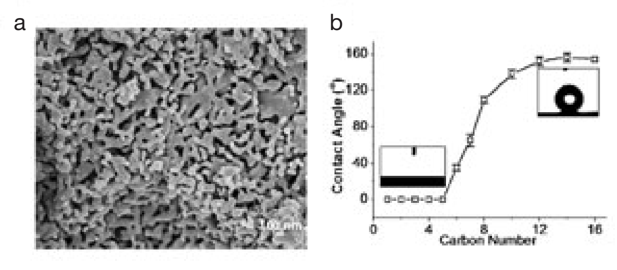

Fig 1 Nanostructure of HAP disc and superhydrophobic modification of HAP disc by FAs. a) SEM image of intact HAP disc. The surface showed typical nanostructures with feature sizes of about 100 to several hundreds of nanometers. b) Water contact angles on the surfaces of the modified HAP discs dependent on the FA carbon chain length. Superhydrophobicity (contact angle > 150 degrees) was formed when the FA carbon chain was 12 carbon atoms or more.

Reduction of S. mutans *adhesion to superhydrophobic HAP discs*

The amount of S. mutans adhering to the superhydrophobically modified HAP discs was examined. As shown in Figure 2, the amount adhering to the modified HAP discs ($0.845 \pm 0.169 \times 10^3$ CFU/ml) was significantly lower than that of the control ($1.698 \pm 0.335 \times 10^3$ CFU/ml $P < 0.05$).

Decrease of calcium dissolution from superhydrophobic HAP discs

As shown in Figure 3a, the calcium dissolved from the FA-modified HAP discs into the demineralisation solution was dramatically decreased when the FA carbon chain length was 12 or more. Further experiments showed that the 12-carbon chain FA-modified HAP discs had less calcium dissolved into the demineralisation solution up until 48 hrs, compared to the control (non-modified) ($P < 0.01$).

Discussion

In the present study, superhydrophobicity of the surfaces of HAP discs was acheived after modification with FAs that had carbon chain lengths of 12 or more. The adhes-

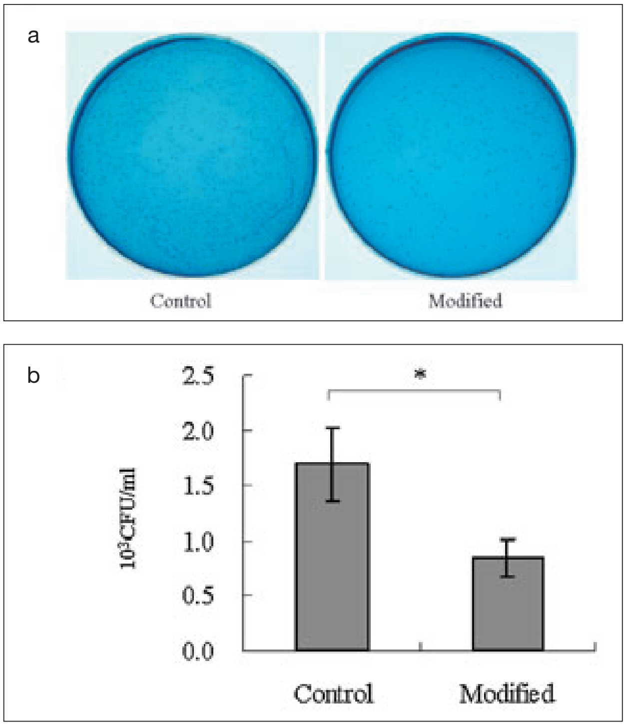

Fig 2 Decrease in the amount of bacteria adhering to the surface of superhydrophobic HAP discs. (a) Bacterial colonies on the agar plate. The bacteria that adhered to the control or superhydrophobically modified HAP discs were released and cultured on the agar plate as described in Methods. (b) Colony-forming units were counted. * $P < 0.05$ vs. control.

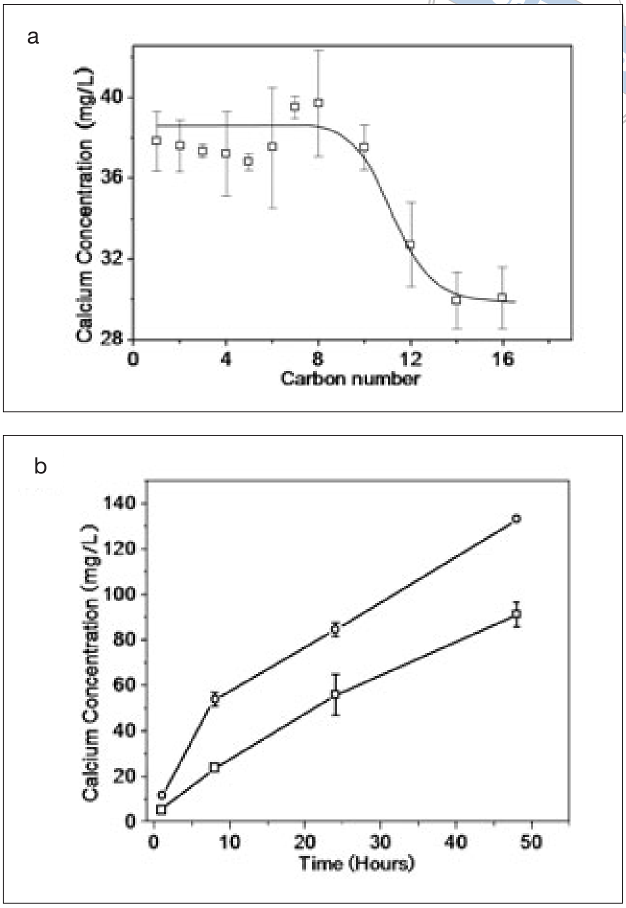

Fig 3 Decrease of calcium dissolved from superhydrophobic HAP discs. (a) Relationship between the Ca^{2+} concentration dissolved from the FA-modified HAP discs into the demineralisation solution and the carbon chain lengths of the FAs. Calcium dissolved from the FA-modified HAP discs dramatically decreased when the FA carbon chain length was 12 or more (the superhydrophobicity was formed, see Figure 1b). (b) Time course of calcium dissolution into the demineralisation solution for the superhydrophobically modified (□) and non-modified (○) HAP discs. Superhydrophobically modified HAP discs had less calcium dissolved into the demineralisation solution as compared with the non-modified HAP discs. $P < 0.01$ vs. control.

ion of *S. mutans* to and the calcium dissolved from the superhydrophobically modified HAP discs were decreased significantly. Modification of HAP discs with FAs increased the water contact angles on its surfaces. The contact angle became 150 degrees (superhydrophobicity formed) when the FA carbon chain length was 12 or more. This superhydrophobic modification may potentially be useful in caries prevention.

It has been well-recognised that micro- and nanostructures are important factors affecting surface wettability[18,19]. Studies have shown that the well-known self-cleaning properties of lotus leaf are due to the superhydrophobicity of its surface[20]. The superhydrophobicity is formed due to the fact that the surface of the lotus leaf is covered with micro- and nano-bumps, with air trapped inside the grooves between the bumps to decrease the solid-liquid interfacial areas. This nanostructure makes difficulty for water to retain on the surface of lotus leaf and results in the self-cleaning properties of lotus leaf. It is also known that the hydrophobicity of a surface can be increased to superhydrophobic levels by increasing the surface roughness[21,22]. Although the intact HAP surface was nanostructured (Fig 1a), similar to that of a natural tooth surface[23], its surface was hydrophilic (contact angle around 0 degrees) (Fig 1b), due to its intrinsic chemical composition with high free energy. Interestingly, it was found that modification of HAP discs with FAs could make its surface hydrophobic, the degree to which depended on the carbon chain lengths of the FAs as the contact angle increased from 0 to 120 degrees (Fig 1b); its surface could become superhydrophobic (contact angle > 150 degrees) when the FA chain length was 12 or more (Fig 1b). The mechanism underlying the superhydrophobicity of the modified HAP discs could be due to the carboxyl groups of FAs attaching to the nanostructure of the HAP disc surface with the carbon chains of the FAs exposed outside. This arrangement might increase the roughness of the HAP surface and trap much more air between the nanostructures and thereby decrease the solid–liquid interfacial area, like the lotus leaf does.

Although most studies on superhydrophobicity have mainly focused on a drop of liquid on a solid surface in air, it has also been proposed that superhydrophobicity may be useful for treatment of underwater systems, such as anti-biofouling of ship hulls and medical devices[24]. The present study also showed the potential of superhydrophobicity in caries prevention. Although it was questioned whether or not the advantages of a superhydrophobic surface would disappear once it was entirely immersed in water[25], studies showed some promising results where superhydrophobicity was still feasible and potentially thermodynamically stable in underwater conditions.

Liu et al provided evidence that a superhydrophobic surface can improve the anticorrosion properties of copper in seawater[26]. The present study explored the superhydrophobic properties of HAP surfaces with regard to oral bacteria adhesion and protection against acid infusion. It was found that the superhydrophobically modified HAP discs had lower amounts of calcium dissolved into the demineralisation solution and less bacterial adhesion compared with the control, supporting the idea that even in underwater conditions, the superhydrophobicity may still have some advantages in caries prevention. Besides, the oral environment is not a fully underwater condition since teeth will be exposed to air during speech. Therefore, superhydrophobicity may potentially be applied in caries prevention.

Decrease of bacterial adhesion to the superhydrophobically modified HAP discs may be due to a disturbance of the formation of saliva film on the surface of the discs. It is known that protein adsorption onto a solid surface is the first stage for biological adhesion. Gyo et al reported that significantly smaller amounts of biofilms are retained on a modified restorative material with highly hydrophobic surfaces than those without modification, when a very strong driving force was applied[27]. Superhydrophobic surfaces reduce the extent of protein adsorption due to the reduction in solid surface area at the liquid interface[28]. Koc et al even found that superhydrophobic modification of copper surfaces promotes the detachment of protein under flow conditions[29]. To mimic *in vivo* conditions, the superhydrophobically modified HAP discs were coated with human saliva before bacterial adhesion and found that significantly less *S. mutans* bound to the superhydrophobic surfaces of the HAP discs (Fig 2). Although the biofilms on the surfaces of the HAP discs were not examined, it was reasonable to suppose that the decrease in bacterial adhesion to the superhydrophobically modified HAP discs was, at least partially, due to the disturbance of the formation of saliva film on the surfaces and thus fewer bacteria could adhere to the discs. Reduction of bacterial adhesion is one of the key steps for caries prevention. Therefore, the decrease in bacterial adhesion to the superhydrophobically modified HAP discs may potentially be useful in caries prevention in the future.

Superhydrophobicity may also contribute to caries prevention by providing resistance to demineralisation. Interestingly, the present study was the first to find that superhydrophobically modified HAP discs showed less calcium dissolution (Fig 3). The calcium dissolved from the HAP discs into the demineralisation solution significantly decreased only when the FA chain length was 12 or more, and for up to 48 hrs (Fig 3b). Although the mechanism is not clear, it may be possible that the interfacial free energy, surface tension and electric potential of the HAP discs were greatly changed by the superhydrophobicity. Therefore, the kinetics of crystal growth and dissolution was affected and consequently reduced the dissolution rate of HAP in the acid solution[30]. The results also suggested that superhydrophobic modification by FAs may also contribute to caries prevention by providing resistance to acid erosion, another key process of caries development.

The present study also showed that superhydrophobic modification of the HAP surface, to a large extent, could be easily done with the FA system. Considering that the main inorganic component of the human tooth is hydroxyapatite, the FA system may be also hold promise for the modification of natural teeth. Further study is needed to test this speculation. Since FAs are derived from animal fat or vegetable oil, which are basic nutrients for humans, there should not be a big barrier for applying it *in vivo*.

In conclusion, superhydrophobic modification efficiently reduced both the oral bacterial adhesion to the surface of HAP discs and calcium dissolution from the HAP discs. This is likely to be of significant interest in caries prevention.

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