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Shear stress tolerance of *Streptococcus mutans* aggregates determined by microfluidic funnel device (μFFD)

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ABSTRACT

Dental caries are initiated by the attachment of *Streptococcus mutans* aggregates to the surface of teeth. Bacterial adhesion to the interproximal space, the space between adjacent teeth, has not been investigated due to the lack of devices that mimic the space. Herein, we describe a method for determining the effect of shear stress and sucrose on the attachment of *S. mutans* aggregates to the interproximal space using microfluidic funnel device (μFFD). Using μFFD, the shear stress tolerance of sucrose-independent and sucrose-dependent *S. mutans* aggregates (larger than 50 μm in diameter) trapped in the funnel was tested against various flow rates of saliva solution (5 to 50 μl/min). Sucrose-independent aggregates were completely removed from the funnel walls at a low flow rate (10 μl/min) within 7 min., while sucrose-dependent aggregates were removed from the walls only at higher flow rates (25 and 50 μl/min) within several minutes. These results suggest that sucrose-dependent aggregates are more tolerant of shear stress than sucrose-independent aggregates, and are more likely to remain in the region with the smallest shear stress in the teeth.

1. Introduction

Streptococcus mutans is commonly found in the human oral cavity and plays important roles in the process of tooth decay (Leme et al., 2006). Early colonization is usually initiated on the mucosal surfaces of the oral cavity (Gronroos et al., 1998). *S. mutans* grows slowly in a planktonic condition, and often forms aggregates with or without sucrose (Kreth et al., 2004). In the presence of sugar, aggregates of *S. mutans* on the tooth surface produce very sticky exopolysaccharides (EPS) (Kreth et al., 2004), which are major components of biofilm. These sucrose-dependent aggregates are thus considered to be more difficult to remove from the tooth surface than sucrose-independent aggregates, which do not produce EPS (Busscher and van der Mei, 2006; Hope et al., 2005; Hughes et al., 1988; Kolenbrander et al., 1989; Sutherland, 2001). In the absence of sugar, *S. mutans* adheres to the tooth surface by interacting with previously adherent bacteria through a process called co-aggregation (Rosentritt et al., 2008; Yankell et al., 2002). However, different adherence capabilities of sugar-independent and sugar-dependent aggregates have not been quantitatively analyzed.

In recent years, bacterial attachment capability has been quantified by the use of flow displacement systems (Busscher and van der Mei, 2006; Martines et al., 2004). However, most of these devices are suitable only for analyzing the adherence capabilities of bacteria attached to a plain surface, while *S. mutans* biofilms are more often found in the interproximal space, the space between adjacent teeth, than on the plain and smooth surfaces of teeth (Yankell et al., 2002). In spite of their medical importance, biofilms in the interproximal space have not been well characterized due to the lack of devices having geometric structures mimicking the space.

Microfluidic device (μFD) consists of one or more microchannels, and is frequently used to study the effects of local geometry on various microbial behaviors, such as bacterial social interaction, quorum sensing, and mechanical stress (Park et al., 2003a, 2003b; Weibel et al., 2007; Shumi et al., 2010). Recently, microfluidic funnel device (μFFD) has been used to study the topotactic behavior of motile bacteria by rectifying their motion along the funnel wall (Galajda et al., 2007, 2008). Most recently, μFFD has been used to separate cells based on size and deformability (Guo et al., 2012; McFaul et al., 2012). However, the microfluidic funnel device has not been used to study bacterial attachment under fluidic condition.

The present study describes a simple method for evaluating the shear stress tolerance of *S. mutans* aggregates attached to the microfabricated funnels that mimic the space between teeth. *S. mutans* aggregates with larger than 50 μm (diameter) were collected by the funnels with a gap of 32.5 μm in μFFD by flowing bacterial culture into it. Then, saliva was

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flown into bacterial aggregates attached to the walls of the funnels at various flow rates (5 to 50 μl/min) to evaluate the tolerance of aggregates to shear stress. Using this microfluidic method, the effects of sucrose and incubation time on the shear stress tolerance of *S. mutans* aggregates were evaluated.

2. Methods

2.1. Fabrication of a microfluidic funnel device

A mask with μFFD design was created using AutoCAD (Autodesk, Inc., San Rafael, CA, USA). The layout of the device is shown in Fig. 1(b). The device has a rectangle chamber (400 μm long × 265 μm wide × 60 μm deep) connected to an inlet and an outlet channels with the same dimensions (4 mm long × π 65 μm wide × 60 μm deep). The sides of the funnels in the chamber are 45 μm long and form a 60° angle (Galajda et al., 2007). Gaps (32.5 μm wide) are situated at the apexes of the funnels. μFFD (Fig. 1(a)) was made of PDMS (polydimethyl siloxane) (Dow-Corning) using soft lithography (Xia and Whitesides, 1998).

2.2. Simulation for velocity, pressure and shear stress in the device

The flow behavior inside μFFD was analyzed using FLUENT 3D lam, a computational fluid dynamics (CFD) software package (ANSYS Inc., Canonsburg, PA, USA) (Glatzel et al., 2008). Gambit 2.2.30 (ANSYS, Inc.) Pre-processing software (modeling tool) was used, and meshing was performed. 57,680 total cells were considered as a mesh. The analysis method assumed laminar flow, since low Reynolds numbers (≤10.66) were used for the simulation. All the data were stored in a data file. Post-processor-generated 2D x–y plots as well as 3D (x, y and z) contour and velocity field plots were recorded. The velocity profile, pressure drop, and wall shear stress were estimated assuming the physical properties of pure water.

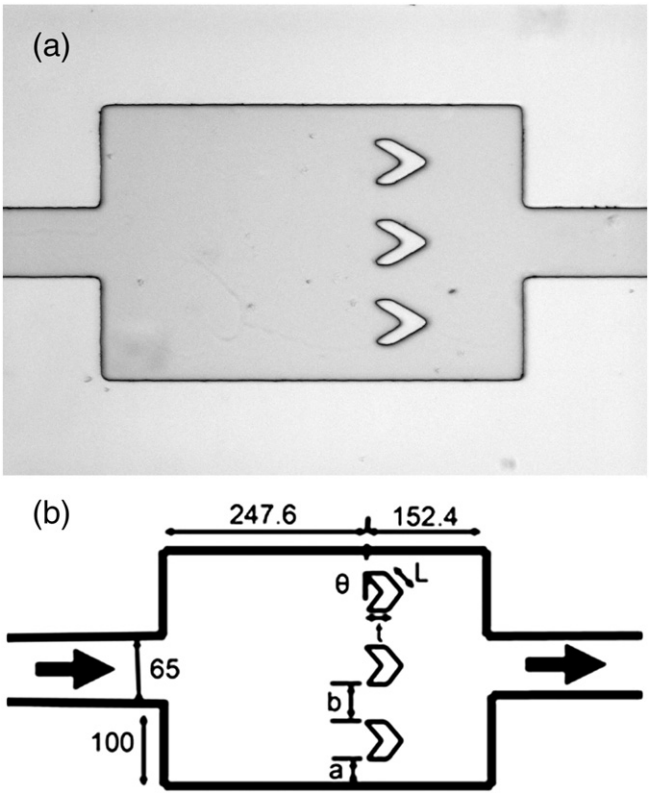

Fig. 1. An optical image (a) of μFFD and its design (b). In (b), both a (distance between the device wall and funnel wall) and b (a gap between the funnel walls) are 32.5 μm; L (length of the angled walls), 45 μm; t (thickness of the funnel walls), 30 μm; and θ (funnel wall angel), 60°.

2.3. Collection of saliva

Saliva was collected from several healthy volunteers 1 h after meals. Saliva secretion was stimulated by chewing parafilm as described elsewhere (Pecharki et al., 2005), and saliva was then collected in ice-chilled sterilized tubes before processing. The collected saliva was centrifuged at 12,000 *g* for 15 min at 4 °C to remove insoluble material or cell debris. The clarified saliva supernatant was filtered (pore diameter: 0.22 μm) and transferred to new tubes (Pecharki et al., 2005). Cell-free transferase activity was assessed by total plate count method (Scheie and Rolla, 1984). The saliva was stored at −80 °C.

2.4. Bacterial culture

The strain of bacteria used in this study was *S. mutans* ATCC 3065 (ATCC, Manassas, VA, USA). The glycerol stock of the strain was preserved at −80 °C. Bacterial culture was maintained on a brain heart infusion (BD, Franklin Lakes, NJ, USA) agar plate every week. Tryptic soy broth (TSB) (BD) with or without sucrose (100 μM) was used to grow cells and induce aggregates.

2.5. Estimation of adhesion capability of aggregates to the funnels

An overnight culture of *S. mutans* in TSB was diluted 100-fold into fresh TSB with or without sucrose (100 μM sucrose) and grown again at 37 °C until an optical density of 0.5 at 600 nm was reached. Prior to inoculating the culture into μFFD, the device was first washed with TSB at 1 μL/min for 5 min and then conditioned with saliva at the same flow rate to prepare the chamber for the attachment of the aggregates (Kreth et al., 2004). Then, the culture was inoculated into the chamber by gravitational force through the inlet channel. Usually, aggregates larger than 50 μm in diameter were collected due to the gap (32.5 μm) between the funnels and only these aggregates were then subjected to shear stress by flowing saliva at the rates of 5–50 μL/min for various periods (0–10 min) of time. The inlet channel was connected to a syringe pump (KDS 220) (KD Scientific Inc., Holliston, MA, USA) fitted with a 5-ml syringe (Hamilton Co., Reno, NV, USA) through a tygon tubing (Fisher Scientific) to flow saliva into the channel. Adherent aggregates on the walls of funnels were observed with an inverted microscope (Eclipse TE2000-U) (Nikon, Japan) equipped with a Pro 2.6 Jenoptik laser optic system (Jenoptik Korea Co., Pyeongtaek, Korea). The fraction of adherent cells was calculated by comparing the size of the aggregates before flowing saliva with that of the aggregates remaining on the funnels. All experiments were conducted at room temperature.

2.6. Imaging EPS from S. mutans aggregates

In order to stain EPS produced by *S. mutans* aggregates on the funnels, a microchamber was filled with phosphate-buffered saline (PBS) containing 20 μg/mL of tetramethylrhodamine-concanavalin A (TMR-ConA) (Invitrogen, Grand Island, NY, USA) and incubated for 30 min in the dark at room temperature (Shumi et al., 2010). Excess dye was washed out by flowing PBS into the chamber at 1 μL/min for 30 min. To stain the live cells of aggregates on the funnels, the chamber was filled with PBS containing 20 μM of SYTO 16 green fluorescence nucleic acid stain solution (Invitrogen) and incubated in the dark at room temperature. Excess stain was removed as described above. Fluorescent images of the biofilms were obtained using confocal laser scanning microscopy (CLSM) (LSM 510, Zeiss) with a 520 nm excitation and 560–620 nm emission filters at 200× magnifications. Fluorescence images were captured at least three times, and their mean fluorescence intensities were measured using Image J program