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ABSTRACT

Physiochemical assessment of the parasite-biomaterial interface is essential in the development of new biomaterials. The purpose of this study was to develop a method to evaluate pH at the bacteria-dental cement interface and to demonstrate physiochemical interaction at the interface. The experimental apparatus with a well (4.0 mm in diameter and 2.0 mm deep) was made of polymethyl methacrylate with dental cement or polymethyl methacrylate (control) at the bottom. Three representative dental cements (glass-ionomer, zinc phosphate, and zinc oxide-eugenol cements) were used. Each specimen was immersed in 2 mM potassium phosphate buffer for 10 min, 24 hrs, 1 wk, or 4 wks. The well was packed with *Streptococcus mutans* NCTC 10449, and a miniature pH electrode was placed at the interface between bacterial cells and dental cement. The pH was monitored after the addition of 1% glucose, and the fluoride contained in the cells was quantified. Glass-ionomer cement inhibited the bacteria-induced pH fall significantly compared with polymethyl methacrylate (control) at the interface (10 min, 5.16 ± 0.19 vs. 4.50 ± 0.07 ; 24 hrs, 5.20 ± 0.07 vs. 4.59 ± 0.11 ; 1 wk, 5.34 ± 0.14 vs. 4.57 ± 0.11 ; and 4 wks, 4.95 ± 0.27 vs. 4.40 ± 0.14), probably due to the fluoride released from the cement. This method could be useful for the assessment of pH at the parasite-biomaterial interface.

KEY WORDS: pH, ISFET pH electrode, *Streptococcus*, glass-ionomer cement, fluoride, interface.

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Evaluation of pH at the Bacteria–Dental Cement Interface

INTRODUCTION

Dental caries is mainly caused by demineralization through acid production from sugar metabolism of acidogenic bacteria in the biofilm on tooth surfaces. The lost part of the tooth is filled or crowned with biomaterials to recover the form and function of the tooth. During and after caries treatment, the interface between the sound tooth structure and restoration materials provides a niche for bacteria to adhere, and the risk of secondary caries may increase. Therefore, biomaterials for restoring the teeth are desired to regulate bacterial activity, such as bacteriostatic and anti-bacterial adhesion properties, and thus a method to assess these properties of biomaterials is essential.

The observation that the pH of dental plaque decreases within a few min after sugar intake (Igarashi *et al.*, 1981, 1990; Takahashi-Abbe *et al.*, 2001) suggests that the short-term measurement of pH at the parasite-biomaterial interface is particularly needed to evaluate the inhibitory effect on microbial acid production. However, most previous studies on dental cements, including glass-ionomer cement (GIC) and zinc oxide-eugenol cement (ZOE), showed bacterial growth-inhibitory effects by incubating target bacteria in the presence of dental cements in liquid media or on agar plates for 6 hrs to 48 hrs (Boeckh *et al.*, 2002; Vermeersch *et al.*, 2005; Marczuk-Kolada *et al.*, 2006). Persson *et al.* (2005) reported that the pH fall in dental plaque formed on an ion-releasing resin, which releases hydroxyl, fluoride, and calcium ions, was inhibited *in vivo*, when compared with the pH fall on an enamel surface. However, it was not distinguished whether the inhibition was due to the suppression of bacterial growth (plaque formation), bacterial acid production, or the alteration of microbial composition by ions released from the resin. In addition, the pH at the parasite-biomaterial interface was not directly measured, due to the methodological limitation of the microtouch pH-electrode method, which may not keep the plaque structure intact by inserting the electrode into the plaque.

An ion-sensitive field-effect transistor (ISFET) pH electrode, invented by Esashi and Matsuo (1978), is very small and highly sensitive due to low internal electric resistance, and is not cytotoxic (Esashi and Matsuo, 1978). Thus, the ISFET pH electrode enabled us to measure *in vivo* acid production by human dental plaque (Igarashi *et al.*, 1981; Chida *et al.*, 1986), and artificial fissure plaque (Igarashi *et al.*, 1989, 1990). In addition, the ISFET pH electrode is small enough to assess pH at the interface between parasites and biomaterials, and is suitable for the development of a new apparatus to assess pH at the interface *in vitro*.

The present study therefore aimed to develop a new method to evaluate pH at the bacteria-dental cement interface, using an ISFET pH electrode to verify

Table. Dental Cements Used in This Study

Dental Cements	Product Name	Manufacturer	Main Components (Batch No.)	
			Powder	Liquid
Glass-ionomer cement (GIC)	FUJI IX GP®	GC, Tokyo, Japan	Fluoroaluminosilicate glass, Polyacrylic acid, (1002091)	Polyacrylic acid, Polybasic carboxylic acid (0911111)
Zinc phosphate cement (ZPC)	Elite cement 100®	GC, Tokyo, Japan	Zinc oxide (0911021)	Phosphoric acid (0910021)
Zinc oxide-eugenol cement (ZOE)	Eugedain®	Showa Yakuhin Kako, Tokyo, Japan	Zinc oxide, Benzoic acid, Magnesium stearate (9128QA)	Clove oil, Olive oil, Rosin (9125NA)

the hypothesis that conventional dental cements for filling restoration, such as GIC and ZOE, have an inhibitory effect on pH fall by bacterial acid production from sugar metabolism, compared with zinc phosphate cement (ZPC). In addition, the amount of fluoride released from dental cement was quantified during the pH fall by bacterial acid production.

MATERIALS & METHODS

Bacterial Strain and Growth Conditions

Streptococcus mutans NCTC 10449 was pre-cultured on TYG media containing 1.7% tryptone (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), 0.3% yeast extract (Becton, Dickinson and Company), 0.5% NaCl, and 0.5% glucose at pH 7.0 and 37°C under anaerobic conditions (80% N₂, 10% H₂, and 10% CO₂). The pre-cultures were then transferred to new TYG media (inoculum size, 5%) and incubated under the same conditions. When the cells reached an exponential growth phase (about 0.5 of optical density at 660 nm), they were harvested by centrifugation (21,000 × g for 15 min at 4°C), washed with 2 mM potassium phosphate buffer (PPB, pH 7.0), and suspended in the same buffer. Bacterial cells were incubated in air at 37°C for 1 hr to exhaust intracellularly accumulated polysaccharide, and washed with 2 mM PPB (pH 7.0). The cell suspension was distributed into 1.5 mL tubes and centrifuged (16,000 × g for 7 min at 4°C). The pellets were stored at 4°C until used.

Materials

Three representative dental cements, GIC, ZPC, and ZOE, were used in the present study (Table). Test disks of the studied materials were prepared with a polymethyl methacrylate plate (8.0 mm in diameter, 2.0 mm thick) as a mold according to the manufacturer's instructions. After the cement hardened, each specimen was immersed in 8 mL of 2 mM PPB at pH 7.0 in polyethylene tubes. Samples were artificially aged by being stored in a thermostatic chamber at 37°C for 10 min, 24 hrs, 1 wk, or 4 wks.

Experimental Apparatus and Measurement of pH

An experimental apparatus with a well (4.0 mm in diameter and 2.0 mm deep) for pH monitoring was made of polymethyl methacrylate with a specimen of dental cement at the bottom (Fig. 1). An ISFET pH electrode (H⁺ ion-sensitive area, 2.0 mm long, 1.0

mm wide, and 0.2 mm thick; model PH-60T1; Nihon Kodens, Tokyo, Japan) was placed on the cement. Cells of *S. mutans*, stored at 4°C, were packed into the well by means of a spatula and a syringe and kept at 37°C for 10 min. Then, a 500-μL quantity of 1% glucose or de-ionized water was added to the cells. The pH was monitored continuously with a pH meter (ISFET mV/pH METER, BAS, Tokyo, Japan) attached to a chart recorder (LR 4220, Yokogawa Electric Corporation, Tokyo, Japan) in an incubator (Merck KGaA, Darmstadt, Germany) at 37°C for 90 min. A polymethyl methacrylate plate (No. 99997, Sanplatec, Osaka, Japan) was used as the control material, since this material is stable physically, chemically, and biologically, and is not cytotoxic under the experimental conditions in the present study.

Measurement of Fluoride Concentration

After measurement of pH fall, the remaining glucose or de-ionized water was absorbed by filter paper, and bacterial cells were collected with a spatula and pipetted with 500 μL of 2 mM PPB into polyethylene tubes. A mixed solution of sodium acetate buffer and perchloric acid (pH 5.0) was added to the bacterial cell suspension at a final concentration of 1 M. The cell suspensions were kept at 4°C overnight for complete destruction of bacterial cells. The cell extracts were collected by centrifugation, and the fluoride concentration in the extracts was measured by a fluoride-ion-specific electrode (Model 9409 BN, Orion, Cambridge, MA, USA) and a comparison electrode (Model 900100, Orion). This measurement was based on the method of Hallsworth *et al.* (1976). The concentration of fluoride in the PPB used for cement immersion was also measured. It was confirmed that the concentration of fluoride in PPB before experiment was less than the detection level (< 0.53 μM).

Statistical Analysis

The differences in pH between control and dental cements at 90 min after glucose addition were evaluated by Dunnett's test. The differences in fluoride amounts in the *S. mutans* cells with glucose and without glucose were evaluated by paired t test. The concentrations of fluoride in the PPB used for cement immersion at different immersion times were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison. A p value of < 0.05 was considered significant.

Figure 1. Schematic drawing of the experimental apparatus used in this study.

Figure 2. The pH fall curves at the interface between the cell mass of *S. mutans* NCTC 10449 and different biomaterials (3 dental cements and polymethyl methacrylate) after addition of 1% glucose. The dental cements were immersed in the buffer for 10 min, 24 hrs, 1 wk, and 4 wks. PMMA, polymethyl methacrylate; GIC, glass-ionomer cement; ZPC, zinc phosphate cement; ZOE, zinc oxide-eugenol cement. The data are the means of 3 independent experiments. Vertical bars indicate standard deviations. * $p < 0.05$, ** $p < 0.01$ (significantly different from the control at 90 min after glucose addition).

RESULTS

pH Fall at the Interface

The pH fall by bacterial glucose fermentation in the presence of the materials after the addition of 1% glucose at the interface between *S. mutans* and materials is shown in Fig. 2. GIC inhibited the rate of pH fall by bacterial glucose fermentation at the

interface around pH 5.5, whereas ZPC and ZOE had no inhibitory effects. The pH at the interface with GIC at 90 min was significantly higher than that with polymethyl methacrylate, regardless of the immersion time of dental cements (Fig. 2). The pH at the interface without glucose addition at 90 min was between 6.50 and 6.98 (data not shown).

Concentration of Fluoride

The amounts of fluoride contained in *S. mutans* cells at the interface with GIC after 90 min are shown in Fig. 3A. Fluoride was detected in *S. mutans* cells, regardless of the addition of glucose. However, the amounts of fluoride in *S. mutans* cells after glucose addition were significantly higher than in those without glucose addition, except at 4 wks. The amounts of fluoride in the cells on polymethyl methacrylate, ZPC, and ZOE were less than the detection level (< 0.27 nmol/well).

The fluoride contents of the immersion fluid of GIC are shown in Fig. 3B. As the immersion time was prolonged, the amounts of fluoride in the PPB increased gradually, although the increase was not significant after 24 hrs. The fluoride contents in the PPB used for cement immersion of ZPC and ZOE were less than the detection level (< 0.53 μ M).

DISCUSSION

The pH at the parasite-material interface was successfully monitored by the experimental apparatus with an ISFET pH electrode. In this apparatus, the pH was stable for about 10 to 20 min after glucose addition, because it took time for glucose to diffuse into *S. mutans* cells and also for *S. mutans* to produce acids from glucose (Dawes and Dibdin, 1986; Hata and Mayanagi, 2003). When the acids penetrated the interface between *S. mutans* and cements or polymethyl methacrylate, the pH started to decrease. In contrast, the cement components were expected to dif-

fuse into the interface gradually, affecting bacterial glucose metabolism and organic acid production. In addition, bacterial acidification probably influenced the diffusion of cement components from the corresponding cement. The pH at the interface can be determined due to these interactions between bacteria and dental cement.

The pH at the *S. mutans* cells–GIC interface at 90 min after glucose addition was around 5.0–5.5, significantly higher than that at the *S. mutans* cells–polymethyl methacrylate interface, regardless of immersion time. Seppä *et al.* (1992) reported that the pH in the fluid phase after sucrose fermentation by *S. mutans* cells layered on the surfaces of GIC discs for 24 hrs was higher than that on other materials, such as composite resin discs. It was also reported that the GIC eluate ceased the pH fall around pH 4.8–5.0, although *S. mutans* cells were suspended (Nakajo *et al.*, 2009). The pH change at the *S. mutans* cells–GIC interface in the present study is consistent with the results of those studies.

Nakajo *et al.* (2009) further indicated that fluoride released from GIC seemed to be the most probable reason for the inhibitory effect on acid production. The result in the present study, that a large amount of fluoride was detected only in the *S. mutans* cells at the interface with GIC, also supports a significant inhibitory effect of fluoride on bacterial acid production. Fluoride is known to have inhibitory effects on bacterial acid production by various mechanisms, such as inhibition of the glycolytic enzyme, enolase (Hüther *et al.*, 1990; Kaufmann and Bartholmes, 1992), and the proton-translocating ATPase (Bender *et al.*, 1985; Sturr and Marquis, 1990). In addition, inhibition is more effective at acidic pH, since hydrogen fluoride (HF, unionized fluoride) is more formed at acidic pH and penetrates bacterial cells efficiently, resulting in the enhancement of fluoride inhibition (Gutknecht and Walter, 1981).

The amounts of fluoride after bacterial fermentation were larger than those without bacterial fermentation in the present study. Al-Naimi *et al.* (2008) and Kantovitz *et al.* (2009) reported that acidic conditions led to higher fluoride release from GIC than neutral pH conditions. Moreover, the initial pH fall curves of GIC were similar to those of the other cements and polymethyl methacrylate, but the GIC curves ceased to fall gradually around pH 5.5. These findings suggest that, in addition to the effective inhibition by fluoride at acidic pH as stated above, larger amounts of fluoride are released from GIC as environmental pH becomes acidic, resulting in higher inhibitory effects on bacterial acid production. These characteristics of GIC seem to be favorable as a “smart” biomaterial inhibiting or decreasing the secondary caries activity adjacent to a restoration (Persson *et al.*, 2005; Moreau and Xu, 2010).

The acidity of GIC itself might contribute to antimicrobial activity (Hiraishi *et al.*, 2003; Vermeersch *et al.*, 2005). However, when GIC was evaluated in the experimental apparatus under the conditions of the present study, the initial pH at the bacteria–dental cement interface was shown to be around neutral (Fig. 2), probably because the acidity of GIC was neutralized during immersion in buffer solution. Similar phenomena may occur under the assumption that GIC is used as a filling material, in which GIC can be covered with oral biofilm and exposed to continuous salivary flow.

Large amounts of fluoride were released from GIC to PPB with 10 min of cement immersion, and the fluoride release rate slowed gradually and continued for 4 wks. This release curve is similar to those obtained in previous studies (De Moor *et al.*, 1996; Shaw *et al.*, 1998; Lee *et al.*, 2000). However, it was evident that the GIC immersed in PPB for 4 wks still had the ability to release fluoride and had an inhibitory effect on the pH fall.

Figure 3. (A) The amounts of fluoride detected in the *S. mutans* NCTC 10449 cells at the interface with glass-ionomer cement at 90 min after the addition of glucose or de-ionized water and (B) the amounts of fluoride released into the immersion buffer from glass-ionomer cement. The data are the means of 3 and 6 independent experiments in (A) and (B), respectively. Vertical bars indicate standard deviations. * $p < 0.05$, ** $p < 0.01$.

These results indicated that GIC inhibited the pH fall at the interface after cement immersion for a long time, suggesting that the inhibitory effect is mainly due to slow but steady release of fluoride from GIC, especially at acidic conditions, as discussed above.

The pH fall curves of ZPC and ZOE were similar to that of polymethyl methacrylate in the present study, suggesting that these cements had little inhibitory effect on bacterial acid production. Previous studies reported that ZPC and ZOE had bacterial growth-inhibitory effects (Coogan and Creaven, 1993; Boeckh *et al.*, 2002; Lewinstein *et al.*, 2005). The strong acidity of ZPC can be attributed to antibacterial activity (Lewinstein *et al.*, 2005), but the acidity of ZPC can be neutralized under our experimental conditions, and thus antibacterial activity is probably decreased. ZOE is known to disrupt the bacterial cell membrane and inhibit bacterial growth (Devi *et al.*, 2010). Although acid production activity was not inhibited under our experimental conditions, prolonged exposure of bacterial cells to ZOE might inhibit acid production.

The present study suggests that this method is useful for the assessment of the inhibitory effects of biomaterials on bacterial acid production. The advantages of this method are that the pH

at the parasite-biomaterial interface can be measured directly, considering physiochemical interactions between bacteria and materials. This method will be able to evaluate the bacteriostatic activity of dental biomaterials, especially filling materials and replacement materials, which are exposed to the oral cavity where oral biofilm is easily formed, and will also contribute to the development of new biomaterials for caries prevention.

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