A high-sensitive and non-radioisotopic fluorescence dye method for evaluating bacterial adhesion to denture materials

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INTRODUCTION

Dentures are an excellent dental prosthesis that can recover oral form and function; however, inadequate care of dentures causes biofilm formation on the denture surface (denture plaque), resulting in various oral diseases, such as periodontitis and caries of the remaining teeth1,2 and stomatitis3,4. In addition, denture plaque is related to aspiration pneumonia5,6. Since denture wearers are generally elderly, denture plaque control is essential for preventing aspiration pneumonia, as well as caries, periodontitis and stomatitis. Under these circumstances, new denture materials decreasing or prohibiting the formation of oral bacteria, except Streptococcus mutans8,19, Staphylococcus aureus, Enterococcus hirae, Escherichia coli and Morganella morganii20. However, to our knowledge, there has been no report about the application of this method to the quantification of adhesion to denture materials of oral bacteria, except S. mutans21. In addition, the fluorescence intensity derived from resazurin depends on bacterial endogenous metabolic activity, which is usually low and unstable, and may result in decreased sensitivity and accuracy of this method.

Therefore, the present study aimed to establish a sensitive and accurate resazurin method for the quantification of adhesion to denture materials of oral bacteria, Streptococcus, Actinomyces and Veillonella, which have been detected frequently from denture plaque7,10,23,26. First, we attempted to improve the sensitivity of the method by adding bacterial metabolic substrates to the reaction mixture in order to activate bacterial metabolic activity. Second, we developed a new experimental device, which was carefully designed for quantification of bacteria adhered to denture materials, pretreated with or without saliva. Third, we applied the method to evaluate and compare bacterial adhesion to the surfaces of conventional denture materials.
MATERIALS AND METHODS

Bacterial strains

*Streptococcus mutans* (Sm) NCTC 10449, *Streptococcus sanguinis* (Ss) ATCC 10556, *Actinomyces naeslundii* (An) ATCC 12104, *Actinomyces oris* (Ao) WVU 627 and *Veillonella atypica* (Va) ATCC 17744 were used in the present study. These bacteria were maintained on blood agar plates under anaerobic conditions.

**Growth conditions**

Culture medium for *Streptococcus* contained 1.7% tryptone (Difco Laboratories, Detroit, MI, USA), 0.3% yeast extracts (Difco Laboratories), 0.5% NaCl, 50 mM potassium phosphate buffer (PPB) (pH 7.0) and 0.5% glucose. Culture medium for *Actinomyces* contained 1.7% tryptone, 0.3% yeast extracts, 0.5% NaCl and 0.1% ammonium bicarbonate, 50 mM PPB (pH 7.0), 0.5% glucose. Culture medium for *Veillonella* contained 0.5% tryptone, 0.3% yeast extracts and 1.8% sodium lactate. Glucose, sodium lactate and PPB were added through potassium phosphate buffer (PPB) (pH 7.0) and 0.5% yeast extracts (Difco Laboratories), 0.5% NaCl, 50 mM tryptone (Difco Laboratories, Detroit, MI, USA), 0.3% glucose. Culture medium for *Streptococcus* contained 1.7% tryptone, 0.3% yeast extracts, 0.5% NaCl and 0.1% ammonium bicarbonate, 50 mM PPB (pH 7.0), 0.5% glucose. Culture medium for *Veillonella* contained 0.5% tryptone, 0.3% yeast extracts and 1.8% sodium lactate. Glucose, sodium lactate and PPB were added through a sterile membrane filter (pore size 0.22 μm; Millipore Corp., Bedford, MA, USA) after autoclaving.

This culture medium was kept in an anaerobic chamber (ANB-180L; Hirasawa Works, Tokyo, Japan: 80% N2, 10% H2 and 10% CO2) for at least 3 days before use to remove oxygen. Each strain was pre-cultured in the medium overnight at 37ºC, transferred (1% inoculum size) to the new medium and incubated at 37ºC until the logarithmic growth phase (optical density (OD)=0.9–1.0 at 660 nm) in the anaerobic chamber. Bacterial cells were harvested, washed and centrifuged (21,000×g for 7 min at 4ºC) 3 times with KCl buffer (1 mM KCl, 1 mM CaCl2, and 0.1 mM MgCl2). The cells were re-suspended in KCl buffer (OD=2.0 at 660 nm).

**Saliva preparation**

Human whole saliva was collected during stimulation, by chewing paraffin film (Parafilm; American Can Co., Greenwich, CT, USA) at least 1 h after a meal, from one healthy woman (30 years old) without caries and periodontal disease, and not taking prescription medication. Sampled saliva was centrifuged (10,000×g for 7 min at 4ºC) to remove any insoluble material or cell debris, and then filtered through a membrane filter (pore size 0.22 μm; Millipore Corp.). The collected saliva was used immediately for the following experiments.

**Estimation of bacterial amounts by resazurin**

Bacterial amounts were estimated by resazurin (7-hydroxy-3H-phenoxazin-3-one 10-oxide), which is readily reduced to fluorescent resolfin (7-hydroxy-3H-phenoxazin-3-one) by bacterial oxidation-reduction metabolic reactions. Bacterial cell suspensions were diluted serially with KCl buffer and adjusted by OD at 660 nm; 0–0.01 for Sm, 0–0.03 for Ss, 0–0.05 for An, Ao and Va. The cell suspensions were mixed with 1% resazurin (alamarBlue; AbD Serotec, Kidlington, UK), incubated at 37ºC for 3 h and then centrifuged (12,000 rpm for 5 min at 4ºC) for cell removal. The fluorescence intensity of the supernatants was measured with a fluorescence spectrophotometer (excitation wavelength, 560 nm; emission wavelength, 590 nm; model 650-40; Hitachi, Tokyo, Japan). In order to evaluate the effects of bacterial metabolic substrates on sensitivity, 0.5% glucose for Sm, Ss, An and Ao, or 1.8% sodium lactate for Va was added to the reaction mixture. The reaction curves of the amount of bacteria (expressed as OD at 660 nm) vs the fluorescence intensity were used as standard curves to calculate the amounts of bacteria.

**Preparation of test denture materials**

Polymethyl methacrylate (PMMA) (Acron MC; GC Corporation, Tokyo, Japan) was used as a representative denture material throughout the present study. Plates of PMMA (approximately 50×50×10 mm) were prepared according to the manufacturer’s instructions and polished using waterproof abrasive papers (320, 600, 1000 and 1200; Plane-5-Grip, Fineplane Hermes Schleif scheiben; Scandia Corporation, Hagen, Germany) and denture abrasives (Pika Top Past D, Pika Past G; Topradental Corporation, Souja, Japan) with a horizontal polishing machine (Scandmatic Universal; Scandia Corporation). The test plates were soaked in deionized water for 1 day to remove residual monomer of acrylic resin. In addition to PMMA, 2 autopolymerizing resins (UNIFAST III; GC Corporation, Tokyo, Japan [UNI for short], and REBASE II, Normal; Tokuyama Dental Corporation, Tokyo, Japan [REBASE for short]) and a silicon series soft liner (SOFRILINER MS; Tokuyama Dental Corporation, Tokyo, Japan [SOFT in short]) were prepared as test plates, according to the manufacturers’ instructions.

The surface roughness of test plates was measured by a surface profilometer (Surfcom 480A; Tokyo Seimitsu, Tokyo, Japan) with an evaluation length of 4.0 mm, a stylus speed of 0.6 mm/s and a cutoff value of 0.8 mm. The surface roughness was given as parameter Ra, the arithmetical mean of the absolute values of the profile departures within the length evaluated. The Ra values of PMMA, UNI, REBASE and SOFT were 0.0720, 0.1367, 0.1682 and 0.3569, respectively.

**Measurement of bacterial adhesion to denture material surfaces using an experimental device**

Test material plates were prepared and washed twice with distilled water for 5 min in an ultrasonic bath and dried completely. Then, a silicon plate with 16 holes (68 mm) was tightly attached to the test plate. In addition, a plastic lid with 16 holes (68 mm) was fixed tightly to the silicon plate, resulting in an experimental device having wells with a test plate at the bottom (Fig. 1, left). Each well was filled with 200 μL filter-sterilized saliva or KCl buffer overnight with gentle oscillation at 4ºC, and washed three times with KCl buffer.

Five hundred microliters of bacterial cell suspension (OD at 660 nm=2.0) or KCl buffer were added to wells. After incubation at 37ºC for 2 h with gentle oscillation, the cell suspension was removed from the wells, which
Fig. 1. Schematic diagrams of an experimental device for quantification of bacterial adhesion.

A silicon plate with punched holes (Φ 8 mm) was attached to the surface of the test plate of materials, and the silicon plate was covered by a lid (a plastic plate with punched holes of Φ 8 mm). Each well was filled with bacterial cell suspension (a) and incubated for 2 h. After removal of cell suspension, the well was washed twice with KCl buffer. The silicon plate and lid were replaced with larger punched holes (Φ 10 mm) (b), and the well was filled with resazurin and incubated (c).

were washed twice with KCl buffer. Then, to avoid contamination by bacteria adhered to the side wall surfaces of wells, the silicon plate and plastic lid with holes (Φ 8 mm) on the test plate were replaced with a new silicon plate and plastic lid with larger holes (Φ 10 mm) (Fig. 1a and 1b).

The wells were filled with 500 μL of 1% resazurin, 1% resazurin containing 0.5% glucose (for Sm, Ss, An and Ao) or 1% resazurin containing 1.8% sodium lactate (for Va), and incubated at 37°C for 30 min (Sm, Ss, An and Ao) or 1 h (Va). The reaction mixtures were collected into Eppendorf tubes from the wells and centrifuged (12,000 rpm for 5 min at 4°C). The fluorescence intensity of the supernatants was measured with a fluorescence spectrophotometer as described above. Amounts of bacteria were calculated from the standard curve of each bacterium.

Statistical analysis
Statistical analyses were performed using Student’s t-test to compare differences between two groups. When more than three groups were compared, Tukey’s test was adopted.

RESULTS

Reaction of resazurin with bacteria and effects of metabolic substrates
Resazurin was reacted with all the bacterial cell suspensions used in the present study, and the fluorescence intensity increased in proportion to time for 3 h (data not shown). The fluorescence intensity for 3 h also increased in proportion to the amounts of bacteria (R=0.984–0.998) (Fig. 2, open squares), although the fluorescence intensity was low in general. The addition of metabolic substrates, glucose (for Sm, An and Ao) and sodium lactate (for Va), to the reaction mixtures increased the fluorescence intensity markedly, with the result that the inclination of bacterial amounts vs fluorescence intensity increased 110, 63.5, 6.5 and 2.3 times for Sm, Ss, Ao, An and Va, respectively. The relationship between bacterial amounts and fluorescence intensity was proportional (R=0.966–0.998) (Fig. 2, closed circles).

Establishment of a resazurin method for evaluating bacterial adhesion using a new experimental device
The amount of bacteria adhered to material surfaces was measured using a new experimental device (Fig. 1). The amount of Sm adhered to PMMA is shown in Fig. 3. The fluorescence intensity obtained from incubation with resazurin only was too low to show a significant difference in bacterial amounts between pretreated with KCl buffer and saliva; however, the addition of glucose (a metabolic substrate for Sm) to resazurin solution increased the fluorescence intensity so that a significant difference between the two groups was detected. In the following experiments, resazurin solution containing metabolic substrates was used.

Bacterial adhesion to PMMA and the effects of saliva pretreatment on bacterial adhesion in all the species are shown in Fig. 4. In the amount of bacterial adhesion to PMMA pretreated with KCl buffer, Ao was the highest, followed by Va, An, Ss and Sm. Pretreatment of PMMA with saliva, mimicking the condition in the oral cavity, decreased bacterial adhesion by 42, 6.9 and 38% for Ss, An and Va, respectively, while it increased by 445 and 107% for Sm and Ao, respectively. The adhesion of Ao was the highest, followed by An, Va, Sm and Ss.

Comparison of bacterial adhesion to various denture materials
Bacterial adhesion to 4 denture materials, PMMA, UNI, REBASE and SOFT, was evaluated using the established resazurin method (Fig. 5); for example, using Sm, when the fluorescence intensity of KCl buffer-pretreated PMMA was regarded as 100%, KCl buffer-pretreated UNI, REBASE and SOFT were 101, 53 and 113%, respectively. In the saliva-pretreatment group, PMMA, UNI, REBASE and SOFT were 172, 35, 50 and 49%, respectively. PMMA in the saliva-treated groups showed the highest adhesion (p<0.05).

Bacterial adhesion seems to vary among denture materials and bacterial species (Fig. 5); however, in saliva-pretreated groups, mimicking the condition in the oral cavity, adhesion profiles of Sm, An and Va were basically similar, with the observation that UNI had the lowest bacterial adhesion. In contrast, in the adhesion profiles of Ss and Ao, UNI had the highest values.
Fig. 2. Fluorescence intensity of resazurin by bacterial cells in the presence and absence of metabolic substrates (glucose or sodium lactate). Bacterial amounts are expressed as OD at 660 nm of cell suspensions. ●, in the presence of glucose or sodium lactate; □, in the absence of glucose or sodium lactate. Vertical bars, standard deviations from three independent experiments.

Fig. 3. Effect of glucose on fluorescence intensity of resazurin by *Streptococcus mutans* cells adhered to PMMA. PMMA surfaces were treated with KCl buffer or saliva. Vertical bars, standard deviations from three independent experiments. *, significant difference ($p<0.05$).

Fig. 4. Amounts of oral bacteria adhered to PMMA. Amounts of adhered bacteria are expressed as OD at 660 nm, assuming that the adhered bacteria are resuspended with 500 μL KCl buffer. The values were calculated from the standard curves of bacterial amounts vs fluorescence intensity. Gray boxes, KCl buffer-treated PMMA surface; black boxes, saliva-treated PMMA surface. Vertical bars, standard deviations from three independent experiments. Significant difference, * $p<0.05$ and ** $p<0.01$ by $t$-test.
DISCUSSION

Quantification of bacterial adhesion to denture materials by the improved resazurin method using a new experimental device

Bacterial quantification using resazurin was improved by adding bacterial metabolic substrates to the reaction mixture (Fig. 2). In the previous resazurin method, bacterial metabolic activity was derived from endogenous energy sources, such as intracellular polysaccharides and the intracellular amino acid pool; however, this endogenous metabolic activity is usually low and unstable, and results in a low intensity of fluorescence. In the present study, glucose can be metabolized by *Streptococcus*<sup>29</sup> and *Actinomyces*<sup>30</sup>, and lactate can be metabolized by *Veillonella*<sup>31</sup>, leading to the activation of bacterial metabolic reactions, including reduction-oxidation reactions, and the subsequent increase of the fluorescence intensity derived from reduced resazurin.

In addition, an experimental device and procedure were carefully designed for the quantification of bacterial adhesion to material surfaces (Fig. 1). Especially in the experimental procedure, the silicone plate was replaced by a new silicone plate with larger holes after incubation of bacterial cell suspensions (Fig. 1). This process was essential to avoid contamination by bacteria adhered to the side walls of wells in the silicone plate and to measure the metabolic activity only derived from bacteria adhered to the test plate. Using this experimental device, bacterial adhesion to material surfaces was successfully measured, and the addition of bacterial metabolic substrates to the reaction mixture was required to increase the sensitivity (Fig. 3).

Bacterial adhesion to various denture materials

The amount of bacteria adhered to PMMA differed among bacterial species (Fig. 4), suggesting different binding forces among bacterial species. In addition, bacterial adhesion to PMMA was modified by saliva pretreatment, which increased the adhesion to PMMA of Sm and Ao (Sm: *p*<0.01), while Ss and An were decreased (Ss: *p*<0.05) (Fig. 4). It is known that the main early colonizers of the saliva-coated enamel surface are *Streptococcus* and *Actinomyces* species such as Ss and An<sup>32</sup>, showing a discrepancy from the present study. The specific mechanism of bacterial adhesion to a saliva-pretreated PMMA surface might be different from that to a saliva-coated enamel surface, since the components of the pellicle formed on the denture surface made of PMMA are different from those of the acquired pellicle formed on enamel<sup>33</sup>. It is reported that cystatin and proline-rich protein, usually found in an acquired pellicle formed on enamel<sup>34,35</sup>, are not contained in the pellicle formed on the denture surface<sup>36</sup>. Further study...
is required for the surface alteration of materials by saliva pretreatment.

Bacterial adhesion differed among denture materials (Fig. 5). It is reported that the surface roughness of dental materials above an Ra value of 0.2 increased the bacterial colonization of their surfaces\(^3\). Although the specimens in the present study were polished for an Ra value below 0.2, except for SOFT (Ra=0.3569), bacterial adhesion to SOFT was not always the highest. In addition, there was no association between Ra values and bacterial adhesion in the present study. The previous study indicated that the adherence of microorganisms (Streptococcus, black-pigmented Bacteroides, and Candida albicans) to PMMA polished at Ra=0.09, 0.22 and 1.12 and pretreated by saliva was not always associated with surface roughness\(^12\). It is possible that various factors, such as hydrophobicity and electrostatic statement, as well as surface roughness, are involved in bacterial adhesion.\(^38,39\)

There were statistical differences in bacterial adherence, especially in saliva pretreatment groups (Fig. 5). Saliva pretreatment increased the adhesion of Sm and Va to PMMA, while it increased that of Va to UNI. Other bacterial species also showed various increases and decreases. Most of the increases and decreases seemed to correspond to those without saliva pretreatment (Fig. 5), suggesting that saliva pretreatment has little effect on the quality of saliva pretreatment (Fig. 5), suggesting that not only material surfaces but also bacterial surfaces to the reaction mixture. The improved sensitivity by adding bacterial metabolic substrates to the reaction mixture. The improved resazurin method using a new experimental device enabled the measurement of a minute amount of oral bacteria, such as Streptococcus, Actinomyces and Veillonella, adhered to denture material surfaces. Moreover, adherence to material surfaces differed among bacterial species and materials. This bacterial adhesion not only to other denture materials but also to other materials, irrespective of newly prepared or deteriorated by long-term use, in dental, medical and industrial fields.

**CONCLUSION**

The present study showed that the resazurin method had improved sensitivity by adding bacterial metabolic substrates to the reaction mixture. The improved resazurin method using a new experimental device enabled the measurement of a minute amount of oral bacteria, such as Streptococcus, Actinomyces and Veillonella, adhered to denture material surfaces. Moreover, adherence to material surfaces differed among bacterial species and materials. This bacterial quantitation method can be used to evaluate bacterial adhesion not only to other denture materials but also to other materials, irrespective of newly prepared or deteriorated by long-term use, in dental, medical and industrial fields.

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