Microbial colonization on polymers: Effects of sample preparation and quaternary ammonium salts

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Abstract Controlled, reproducible testing methods are needed to evaluate the efficacy of antibacterial materials and accelerate efforts to produce materials and treatments that control bacterial growth. In this study, a reproducible method for photopolymerizing samples was selected and utilized to evaluate a novel cationic monomer containing a quaternary ammonium functionality. Using a model dimethacrylate resin system, several preparative methods were compared to identify a preferred protocol. The opposing substrate against which the samples were polymerized significantly affected the surface properties of the polymer and the initial colonization of *Streptococcus mutans*. Polymerizing the samples between untreated glass slides was selected as the preferred method. Using this method, bacterial colonization was evaluated on polymers containing this novel dimethacrylate monomer that was synthesized using a modified Menschutkin reaction and incorporated into the model resin system at various concentrations. At only 10 % mass fraction, the cationic monomer reduced initial bacterial colonization. Thus, a novel monomer with the potential to alter bacterial growth, as well as a reproducible method for fabricating samples, was identified. These fabrication protocols have utility in evaluating dental materials, including various antibacterial monomers, and are expected to translate to other applications where controlled bacterial attachment is desired.
Keywords Antibacterial; dental polymer; photopolymerization; quaternary ammonium salt; *Streptococcus mutans*; surface chemistry

INTRODUCTION[*]

Polymeric composite fillings have seen increased usage as tooth restorations; yet they often fail due to bacterial infiltration and recurrent tooth decay (secondary caries). *Streptococcus mutans (S. mutans)* is one bacteria species known to contribute significantly to both primary and secondary decay (Loesche, 1986). One approach to reduce recurrent caries is through the use of antimicrobial dental materials (Imazato, 2003). For instance, quaternary ammonium salts, which have been shown to have antibacterial properties (Gilbert, 2005), could be incorporated into dental polymers and composites to reduce microbial attachment.

Methods to quantify bacterial interactions with dental polymers and composites are essential for understanding the formation of caries and for evaluating novel antimicrobial materials. However, seemingly insignificant aspects of sample preparation, such as the material against which samples are polymerized, are often overlooked. The first objective of this study was to identify a straightforward method for preparing polymer samples, with the hypothesis that the preparative method affects surface properties and, in turn, bacterial colonization. After identifying a preferred method for sample preparation, the second objective was use that method to assess the effects of potential antimicrobial monomers on bacterial colonization.

[*] Certain commercial materials and equipment are identified in this article to specify the experimental procedure. In no instance does such identification imply recommendation or endorsement by NIST or that the material or equipment identified is necessarily the best available for the purpose.

METHODS

Variations in polymer fabrication

A series of dimethacrylate films was prepared using the same resin system but varying the fabrication protocol. The resin system consisted of an equal mass ratio of 2,2-bis[*p*-(2'-hydroxy-3'methacryloxypropoxy)phenylene]propane (BisGMA) and triethylene glycol dimethacrylate (TEGDMA) photo-activated using camphorquinone and ethyl 4-*N*-*N*-dimethylaminobenzoate. All polymer disks (4 mm in diameter, 1 mm in height) were photopolymerized by visible light irradiation in a Dentsply Triad 2000 (250 W, 120 V) for 1 min per side. Changes in the fabrication protocol were achieved by varying the opposing substrate used during polymerization. The resin solution was either left uncovered and placed in an argon-purged chamber (Fig. 1A) or covered with one of several opposing substrates in regular atmospheric conditions (Fig. 1B). The opposing substrates consisted of untreated glass slides (UNT), glass slides treated with *n*-octadecyltrimethoxysilane (OTMS), glass slides treated with (tridecafluoro-1,1,2,2-tetrahydrooctyl)-trimethoxysilane (PFCS), or a thin film of polyethylene terephthalate (PET).

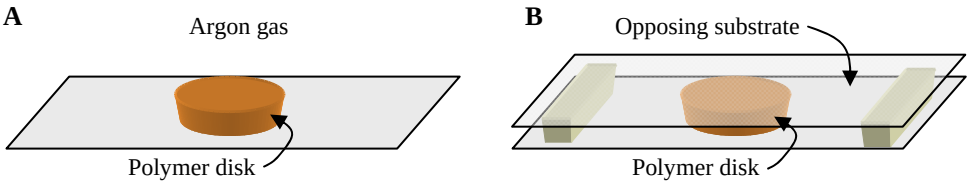


Figure 1. Schematic of fabrication protocol. (A) One set of disks was polymerized in an argon-purged chamber. (B) Other sets were polymerized while covered with different opposing substrates.

Surface characterization

The surface hydrophobicity of the opposing substrates and the resultant polymer disks was measured using a water contact angle goniometer in sessile drop mode. The surface functionalities of the polymers were determined using X-ray photoelectron spectroscopy (XPS).

Inoculation of polymers with *S. mutans*

Polymers were sterilized with 70 % (by volume) ethanol and stored overnight in phosphate buffered saline (PBS) in a 24-well plate. *S. mutans* Clarke (UA159) were cultured in brain heart infusion (BHI) broth at 37 °C. Overnight cultures were centrifuged, washed with PBS, centrifuged again, and resuspended in PBS for a final OD_{600} of 0.06. Polymers were inoculated with 1 mL of *S. mutans* (n = 3). After 4 h (37 °C, 5 % CO_2 by volume), samples were rinsed with PBS three times and fixed with formaldehyde (37 mg/mL in PBS), all without passing the samples through the air-liquid interface. Samples were stained with SYTOX green (1 µmol/L). Controls were *S. mutans* inoculated on clean glass coverslips due to reproducible colonization on glass.

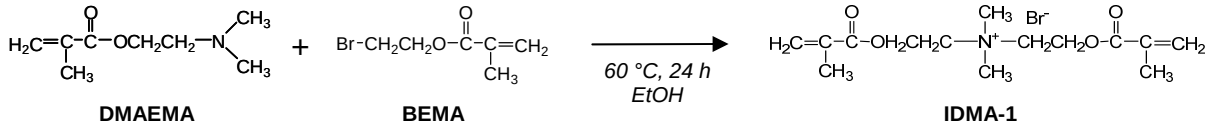
Quantitative image analysis

Samples were imaged using a Zeiss laser scanning confocal microscope (LSCM). Five images were captured for each of three samples using a 40×, 0.80 numerical aperture water immersion objective. Projection images were prepared using the manufacturer's software, and image analysis was performed using a custom macro in ImagePro Plus. The macro counted the objects in a given image and provided the following measurements: total objects per image, fraction of the surface covered per image, and the area of each object. An object was defined as a single fluorescence entity and, depending on the size, could be a single bacterium or a microcolony of multiple bacteria.

Synthesis of quaternary ammonium monomers

The synthetic scheme for preparation of bis(2-methacryloyloxy-ethyl) dimethyl-ammonium bromide (IDMA-1) is shown in Fig. 2. Briefly, 10 mmol of 2-(N,N-dimethylamino)ethyl

methacrylate (DMAEMA), 10 mmol of 2-bromoethyl methacrylate (BEMA), and 3 g ethanol were stirred and heated at 60 °C for 24 h. Solvent and residual reagents were removed, and a clear, viscous, liquid product was isolated in near quantitative yield. The structure was evaluated via Fourier transform infrared spectroscopy (FTIR) and proton-nuclear magnetic resonance spectroscopy (^{1}H-NMR) following standard protocols.



DMAEMA **BEMA** **IDMA-1**

Figure 2. Scheme for the synthesis of IDMA-1.

Evaluation of IDMA-1

Resins were formulated to include 0 %, 10 %, 20 %, and 30 % IDMA-1 (mass fraction) in activated BisGMA:TEGDMA resin. Samples were photopolymerized between untreated glass slides. The polymer disks were inoculated with *S. mutans*, stained, and imaged as described above.

RESULTS AND DISCUSSION

Effect of fabrication protocol

The opposing substrate significantly affected the surface properties of the resulting polymer disks. Substrates and sample molds are often treated with silane agents to facilitate release of the polymer. In the present experiment, some polymer disks were prepared with glass slides treated with release agents OTMS and PFCS. The silane-treated glass slides showed increased hydrophobicity as compared to the untreated glass slides, particularly the perfluorinated silane-treated slide. Polymer disks fabricated on silane-treated slides also exhibited increased hydrophobicity, due to either preferential orientation of polymer chain functionalities with free energy similar to the opposing substrate or transfer of the silane coating to the polymer. XPS results indicated that transfer of the silane to the polymer accounts for at least part of the increase in polymer hydrophobicity. For instance, there is no fluorine in the polymer itself, yet fluorine was detected on the surface of polymer disks prepared using PFCS-treated slides, consistent with a transfer of the perfluorinated silane to the polymer surface. Taken together, these results indicate that the opposing substrate affects the surface properties and even transfers to the polymer disk.

Analysis of *S. mutans* on these polymers revealed a significant effect of the opposing substrate on colonization pattern (Fig. 3), although no clear trend with respect to hydrophobicity was evident. Bacteria colonized the surface either individually or as microcolonies, depending on the opposing substrate used during fabrication. Thus, when studying colonization on these materials, the surface preparative method must be considered. The use of untreated glass slides (UNT) was identified as the preferred fabrication method, as it resulted in minimal chemical modification to the polymer surface and reproducible bacteria colonization in terms of surface coverage and colony size.

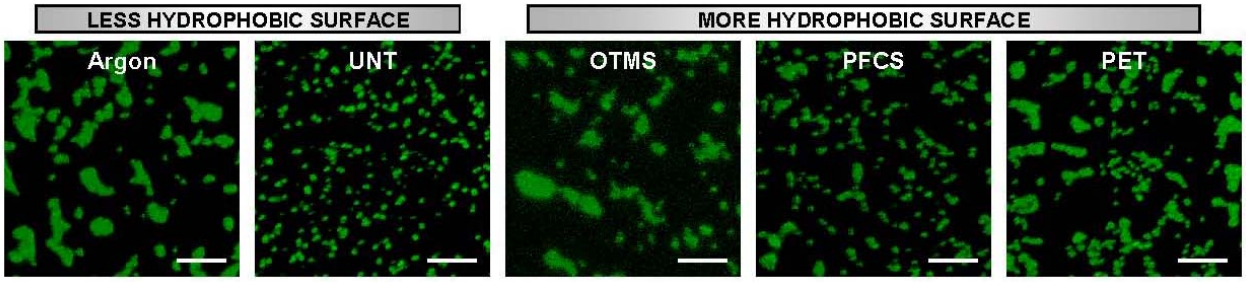


Figure 3. Confocal microscopy images of *S. mutans* on polymers prepared in an argon-purged chamber or in regular atmosphere with varying opposing substrates, as listed. Scale bars = 10 µm.

Efficacy of quaternary ammonium monomers

Utilizing untreated glass slides as the preferred opposing substrate, the cationic monomer IDMA-1, which contained a quaternary ammonium salt site, was incorporated into polymers and shown to reduce bacterial colonization. The classical Menschutkin reaction (addition reaction of tertiary amines with organo-halides) was successfully adapted for the synthesis of a free radical, cross-linking monomer that contained a quaternary ammonium group. The monomer structure was confirmed via FTIR and ^{1}H-NMR, and the monomer was soluble and easily miscible in common dental resin systems (such as the currently used BisGMA:TEGDMA system).

The presence of IDMA-1 in the BisGMA:TEGDMA polymers significantly altered *S. mutans* colonization at 4 h (Fig. 4). With 10 % (mass fraction) IDMA-1 in the formulation, the number of bacteria on the surface was reduced as compared to the control (without IDMA-1). As the mass fraction increased to 30 %, the bacteria morphology changed from individual bacteria to irregularly shaped microcolonies. These results confirm that IDMA-1 altered the bacterial colonization.

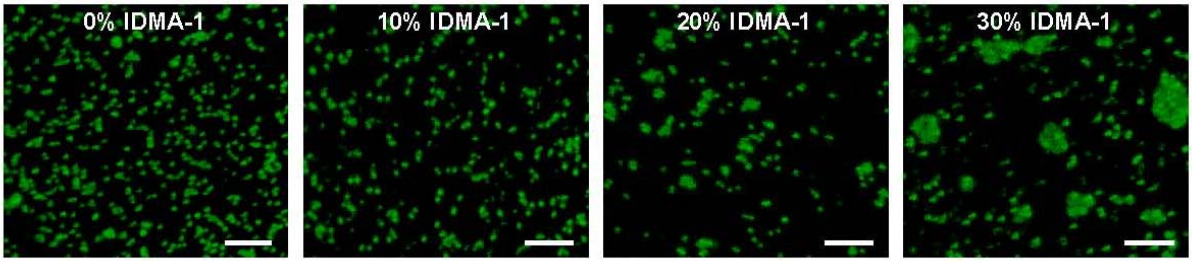


Figure 4. Confocal microscopy images of *S. mutans* on polymers containing up to 30 % (mass fraction) IDMA-1. Scale bars = 10 μm.

CONCLUSIONS

Variations in bacterial colonization patterns were induced on a series of polymeric samples prepared from the same material simply by changing the substrate upon which the samples were photopolymerized. Untreated glass slides were established as the preferred fabrication method since they had minimal material transfer to the polymer surface and gave consistent, reproducible bacterial colonization. Using this fabrication method, polymers containing quaternary ammonium salts were prepared and shown to reduce bacterial colonization. Future studies will evaluate the effects of IDMA-1 on biofilm formation at longer time points.

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REFERENCES

Gilbert, P. and Moore, L. E. (2005). Cationic antiseptics: diversity of action under a common epithet. *Journal of Applied Microbiology*, **99,** 703–715.

Imazato, S. (2003). Antibacterial properties of resin composites and dentin bonding systems. *Dental Materials*, **19**(6), 449-457.

Loesche, W. J. (1986). Role of *Streptococcus mutans* in human dental decay. *Microbiological Reviews*, **50**(4), 353-380.