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Inhibition of multispecies biofilms by a fluoride-releasing dental prosthesis copolymer

Abstract

Objectives: This study aimed to develop a new mixed-species acidogenic biofilm model and use it to assess the antimicrobial properties of a novel fluoride-releasing copolymer.

Methods: Stubs composed of a copolymer of methyl methacrylate (MMA) and 2-hydroxyethyl methacrylate (HEMA) with polymethyl methacrylate (PMMA) were produced by chemically-activated free radical polymerization. A fluoride-releasing copolymer was developed by incorporating sodium fluoride in place of a portion of the PMMA. Samples were mounted in polysulfone Modified Robbins Devices (MRDs) and were optimized for single- and mixed-species biofilm formation by *Candida albicans*, *Lactobacillus casei* and *Streptococcus mutans*.

Results: Fluoride release was sustained for at least 48 h in flowing conditions. Fluoride did not affect the colonization and biofilm growth of any of the microorganisms in monocultures. However, in mixed-species biofilms, cell densities of all three species were reduced approximately ten-fold ($p < 0.05$) on the fluoridated material compared with the non-fluoridated copolymer.

Conclusions: These data demonstrate that intermicrobial interactions in mixed-species acidogenic biofilms are sensitive to fluoride, and that the inclusion of fluoride in a denture lining copolymer reduces the formation of polymicrobial biofilms.

Clinical Significance: The growth of acidogenic microorganisms on denture materials is associated with denture stomatitis and dental caries on surrounding teeth. A fluoride-releasing copolymer that inhibits acidogenic mixed-species biofilms, such as the material described in this study, has the potential to control these diseases by limiting biofilm growth.

Introduction

Dentures provide an artificial surface in the mouth that is susceptible to colonization by microbial biofilms. Denture plaque is somewhat similar in composition to dental plaque on tooth surfaces, although recent studies indicate that denture plaque has slightly lower species diversity and is enriched in *Actinobacteria* and *Bacilli* [1]. As denture plaque ages, proportions of acidogenic/aciduric microorganisms, including mutans streptococci, lactobacilli and *Candida albicans* tend to increase [2-6]. *C. albicans* is associated with denture stomatitis that presents in approximately 65% of edentulous individuals [7]. Furthermore, *Candida* species (especially *C. albicans*) are recognized as common colonizers of obturator prostheses, particularly in patients with dry mouth [8]. In addition to *C. albicans*, previous studies have indicated that bacteria including oral streptococci such as *S. mitis*, *S. oralis*, *S. sanguinis*, and *S. mutans* and lactobacilli (for example, *L. casei*) contribute to the development of oral appliance-related diseases including denture stomatitis and root caries [9-12]. There is some evidence that removable partial dentures (RPDs) also promote denture stomatitis [13]. Further, a strong link has been established between wearing an RPD and root caries [11].

Polymethyl methacrylate (PMMA) is a widely used denture base material for both partial and complete dentures [14]. This material is chosen for its good working characteristics, good esthetics, and low cost [15]. A number of previous studies have reported approaches to reduce biofilm formation on dentures by incorporating different materials into PMMA such as fillers, copolymers, and antimicrobials to minimize biofilm formation on the acrylic prosthesis [16]. Fluoride is particularly effective against cariogenic microorganisms, since it inhibits acid production as well as limiting the growth of certain microbes. In attempts to control dental caries, fluoride has been incorporated into a denture-coating material [17], and denture lining material [18]. However, these studies were focused on the physical and chemical properties of the materials and did not extend to assessing anti-caries activity or the abilities of the materials to control the growth of mixed-species acidogenic biofilms.

There is strong evidence that intermicrobial interactions play important roles in the development and stabilization of polymicrobial biofilms, including those that form in the human mouth [19-22]. Many different interkingdom interactions, for example between oral streptococci and *Candida* spp., have now been characterized in some detail [23]. Therefore, when modelling

oral biofilms it is important to include mixed species of microorganisms in order to replicate some of these interactions. Acidogenic microorganisms including mutans streptococci, lactobacilli and *C. albicans* are particularly abundant on dentures in patients with denture stomatitis [2]. In addition, there is evidence that increased numbers of *C. albicans* in denture biofilms are associated with increases in lactobacilli [1]. Therefore, this work aimed to develop a novel mixed-species model for culturing biofilms containing *C. albicans*, *L. casei* and *S. mutans* on acrylic materials under flowing conditions. This model could then be employed to assess the antimicrobial properties of a novel fluoride-releasing material consisting of a copolymer of methyl methacrylate (MMA) and 2-hydroxyethyl methacrylate (HEMA) with polymethyl methacrylate (PMMA). This material was originally developed for bonding orthodontic appliances [24], and for this study has been modified to contain higher levels of fluoride. This fluoride-enhanced material offers potential for clinical applications where control of acidogenic biofilms is important, for example denture liners, obturators and surgical coverplates.

Materials and Methods

Copolymer sample preparation

Cold cure acrylic resin (Wintercyl Rapid Repair; John Winter & Co, Halifax, UK) was used, and 40% of the MMA was replaced by HEMA (Sigma Aldrich, St. Louis, USA). For the fluoridated copolymer, 30% of the PMMA was replaced by an equal mass of NaF (Sigma Aldrich). The mixtures were transferred to custom-made plastic molds to produce either disc shapes for fluoride measurements under static conditions or stubs of dimensions to fit the Modified Robbins Device (MRD), and allowed to set by chemically-activated free radical polymerization for 48 h.

Fluoride release and recharge

Fluoride release from autoclaved samples was measured under static conditions or in some cases, from samples placed in the MRD under liquid flow. Fluoride was measured with an ion selective electrode (Orion Research, Thermo Scientific, MA, USA), after adding 500 μ l acetate buffer (TISAB III, Orion Research, MA, USA) to 5 ml of the storage medium. For static conditions, five flat disc shaped samples of each copolymer were incubated independently in deionized water. Storage water was changed 24 h prior to measurements being taken and fluoride release over 24 h was determined by measuring the fluoride content in the water. For measurements of fluoride release under flowing conditions, a single stub was placed in the MRD system, and water was introduced at a rate of 40 ml h⁻¹. Fluoride was measured in the effluent periodically up to 48 h, and the experiment was repeated three times.

For fluoride recharging, freshly prepared fluoridated copolymer specimens were washed daily and placed into fresh water in order to take out as much fluoride possible from the material. Before recharging, the daily fluoride release from all specimens was measured. Samples were sonicated in deionized water containing 0.2% w/v NaF for 3 min in an ultrasonic bath, rinsed with distilled water, dried and incubated at 37°C in 5 ml of fresh deionized water. Fluoride release was measured daily for one week. Samples were incubated in deionized water for one further week and the storage water changed 24 h prior to a second recharge cycle, to facilitate measurement of daily fluoride release.

Growth media and microbial culture

Microbial strains used in this study were *Candida albicans* VPSA1 [25], *L. casei* ATCC334 and *S. mutans* UA159. *C. albicans* was cultured aerobically in Yeast Extract-Peptone-Dextrose (YPD) medium (Sigma-Aldrich) at 37°C. *L. casei* was cultured in Rogosa medium (Sigma-Aldrich) in a candle jar at 37°C, and *S. mutans* was cultured in Tryptone Yeast Cystine (TYC) medium (Sigma-Aldrich) supplemented with 20% (w/v) sucrose in a candle jar at 37°C. Solidified media were prepared by adding 15 g L⁻¹ Bacto-Agar (Difco, Oxford, UK). Artificial saliva [26] was employed for culturing organisms in the MRD, and sucrose was added as described below.

Standardised inocula were prepared for the MRD by culturing *C. albicans*, *L. casei* or *S. mutans* to mid-exponential phase, harvesting and re-suspending in one tenth volume of the appropriate growth medium supplemented with 25% (v/v) glycerol (Sigma-Aldrich). Cells were stored at -80°C, and an aliquot was removed for measuring total viable counts (TVCs) by the method of Miles and Misra [27]. Briefly, serial ten-fold dilutions were prepared in PBS (pH 7.4, Sigma-Aldrich) and triplicate 20 µl aliquots were added to solidified media. *C. albicans* was incubated aerobically at 37°C for 24 h, while *L. casei* or *S. mutans* were incubated at 37°C in a candle jar for 48 h. Colonies were counted from dilutions that contained between 10-100 colonies. For enumeration of individual species in mixed cultures, selective media were used as follows: YPD, counted at 24 h for *C. albicans*; Rogosa Agar containing 21.6 µg ml⁻¹ natamycin (Sigma-Aldrich) for *L. casei*; and TYC Agar containing 21.6 µg ml⁻¹ natamycin and 0.1 unit ml⁻¹ bacitracin (Sigma-Aldrich) for *S. mutans*.

The Modified Robbins Device model biofilm system

Two 12-port polysulfone MRDs (Tyler Research, Alberta, Canada) were assembled with stubs of copolymer, sterilized by autoclaving for 15 min at 121°C, and arranged in parallel at 37°C (Figure 1). Fluoridated copolymer stubs were included in one device, and control stubs (non-fluoridated) in the other. To coat surfaces with human saliva, parafilm-stimulated saliva was collected from six different volunteers who had not consumed food or drink other than water in the previous hour and had not taken antibiotics in the previous three months. Dithiothreitol (Sigma-Aldrich, St. Louis, Mo.) was added to a final concentration of 2.5 mM and stirred gently on ice for 10 min. Samples were centrifuged at 15,000 g, 4°C for 30 min and filter sterilized. The MRD was conditioned by the addition of 20 ml saliva at a rate of 40 ml h⁻¹ and inoculated with 1 x 10⁵ *C. albicans* cells, 1 x 10⁷ *L. casei* cells, and/or 1 x 10⁷ *S. mutans* cells. Artificial saliva was then

flowed through the model at 40 ml h⁻¹. At time points up to 48 h, stubs were removed and microbial biofilms were recovered by scraping the surfaces into 1 ml PBS (pH 7.4) or were prepared for microscopy as detailed below. Measurements of pH were made using a Mettler Toledo S20 SevenEasy pH meter.

Quantification of microorganisms by qPCR

For enumeration of microorganisms by qPCR, DNA was extracted using the ZR Fungal/Bacterial DNA extraction kit (Zymo Research Co., CA, USA). The following previously published oligonucleotide primers and TaqMan probes were used to quantify bacterial biofilm species: Smut3368-F, Smut3481-R, and Smut3423T [28] (for *S. mutans*, targeting the *gtfB* gene) and L_case_IS, R_case_IS, and P_case_IS [29] (for *L. casei*, targeting the 16S-23S intergenic region). For *C. albicans* VPSA1, the region surrounding the 5.8S rRNA gene was amplified by PCR using primers CaFP1 5'-GGAACCGAGAAGCTGGTCAA-3' and CaRP1 5'-GTCATCTCATCGCACGGGAT-3'. The PCR product was cleaned using the EZ-10 Spin Column PCR Products Purification kit (NBS Biological, Huntingdon, UK), and sent for sequencing (Eurofins Genomics, Ebersberg, Germany). The sequence was deposited in GenBank (accession KJ39863). Based on this sequence, primers and TaqMan probe sequences for *C. albicans* were adapted from [30], and were: CaFP2 5'-GGAACCGAGAAGCTGGTCAA-3', CaRP2 5'-GTCATCTCATCGCACGGGAT-3', Ca-probe 5'-TGAAGATATACGTGGTAGACGTTAG-3'). The probe was modified with FAM-6 fluorescence reporter at the 5' end and MGBNFQ fluorescence quencher at the 3' end.

Standards for qPCR were constructed by amplifying qPCR target regions from each microorganism. The amplified products were cloned in pCR2.1 (Invitrogen) to generate plasmids pTOPO-*Ca*, pTOPO-*Lc* and pTOPO-*Sm*, respectively, and used to transform *E. coli* TOP10 cells (Invitrogen). Plasmids were extracted from *E. coli* using the Qiagen Plasmid Mini Kit, and concentrations were determined by staining with Quant-iTTM PicoGreen dsDNA Reagent and Kit (Invitrogen). Amplification and detection of DNA by qPCR were performed with an Opticon 2 DNA Engine (Bio-Rad Laboratories Ltd., Hertfordshire, UK) using optical grade 96-well plates (Eurogentec, Belgium). Quantitative PCR reactions were prepared, and contained the following reagents in each well: forward primer (stock concentration, 2.5 µM) 1.2 µL, reverse primer (2.5 µM) 1.2 µL, probe (2.5 µM) 0.6 µL, 2X Takara master mix 7.5 µL (Quantace, London, UK), H₂O

3.3 μL and template DNA 1.0 μL . The thermocycling conditions were as follows: initial denaturation at 95 °C for 2 min, denaturation at 95 °C for 5 sec, annealing/extension at 60 °C for 30 sec, plate read and cycle repeated 40 times. All the reactions were carried out in triplicate and the final analysis was based on the mean of the three reactions. The qPCR data analysis was carried out using Opticon Monitor 3 software (MJ Research). The quantity of template DNA in each sample well was calculated by reference to wells containing serial 10-fold dilutions of standards of known DNA concentration and corrections were made for target sequence copy number based on the assumption that individual cells contained 110 copies of *C. albicans* ITS2 [31], 5 copies of *L. casei* 16S-23S rRNA [32], or 1 copy of *S. mutans* *gtfB*.

Scanning electron microscopy (SEM)

For SEM, samples were fixed in 2% (v/v) glutaraldehyde at 4 °C for 16 h, rinsed with PBS (pH 7.4) and dehydrated through a series of ethanol washes as follows: 25% ethanol for 30 min, 50% ethanol for 30 min, 75% ethanol for 30 min, and two washes for 1 h in 100% ethanol. Samples were dried in a critical point dryer (Bal-tec, Reading, UK), mounted on aluminium stubs and sputter coated with gold at Electron Microscopy Research Services, Newcastle University. Biofilms were visualized using a Stereoscan 240 scanning electron microscope (Cambridge Instruments, Cambridge, UK).

Confocal scanning laser microscopy (CSLM)

For CSLM, the Live/Dead BacLight Bacterial Viability Kit (Life technologies Ltd, Paisley, UK) was used according to the manufacturer's instructions to stain single-species biofilms. Mixed-species biofilms were stained with 50 $\mu\text{g ml}^{-1}$ Alexa Fluor 488-conjugated Concanavalin A (ConA) (Life technologies Ltd, Paisley, UK), 5 $\mu\text{g ml}^{-1}$ propidium iodide (Sigma Aldrich, St. Louis, USA) and 25 $\mu\text{g ml}^{-1}$ 4',6-diamidino-2-phenylindole (DAPI) (Life technologies Ltd, Paisley, UK). Biofilms were examined and image stacks collected using a Leica TCS SP2 confocal laser scanning microscope with a x63 HCX PL APO oil immersion objective lens (numerical aperture 1.32) and an argon/neon laser for visualisation of SYTO 9 (excitation 488 nm, emission 530 nm), propidium iodide (excitation 530 nm, emission 630 nm), ConA (excitation 485 nm, emission 519 nm), and DAPI (excitation 358 nm, emission 461 nm). The collected image stacks of the biofilms were computationally rendered in three-dimensions using IMARIS Ver 7.3.1 software (Bitplane,

Zurich, Switzerland) on a MSI PC computer (MSI computer corp, City of Industry, CA) equipped with a Radeon HD5850 graphics card (AMD, Sunnyvale, CA).

Statistical analyses

In each case, means and standard deviations were based on three independent biological experiments. Student's unpaired two-sample T test was used for all statistical comparisons between means and $p < 0.05$ was considered statistically significant. Spearman's rank correlation coefficients were calculated to assess correlations between qPCR and TVCs. All statistical tests were performed in Sigmaplot (Systat Software Inc., San Jose, CA).

Results

Fluoride release from a fluoridated copolymer

In order to assess the stability of fluoride when introduced into the copolymer, fluoride release was monitored under static conditions for 6 months (Figure 2). The rate of fluoride release decayed approximately exponentially over this timeframe. High rates of release ($>250 \mu\text{g}/\text{cm}^2\cdot\text{day}$) were sustained for 6 days and fluoride release was still detectable up to 6 months, though at a much lower level. The release of fluoride from the copolymer was also determined under flowing conditions in the MRD biofilm model by analyzing the fluoride concentration in the effluent. Under these conditions, fluoride was released at $>90 \mu\text{g}/\text{cm}^2\cdot\text{h}$ for 2 days (Figure 2 inset).

To assess the capacity of the PMMA material to recharge with fluoride, fluoridated PMMA discs were first incubated in sterile distilled water for 30 days until the rate of release was $<10 \mu\text{g}/\text{cm}^2\cdot\text{day}$. They were then recharged as describe in the Methods. One day after recharging, the rate of fluoride release increased to $24 \mu\text{g}/\text{cm}^2\cdot\text{day}$ and release rates of $>10 \mu\text{g}/\text{cm}^2\cdot\text{day}$ were sustained for 7 days (Figure 3). After a further period of washout for one week, the material was recharged once again. After one day, fluoride release increased to $17 \mu\text{g}/\text{cm}^2\cdot\text{day}$. Subsequently, the rate of fluoride release reduced to $<10 \mu\text{g}/\text{cm}^2\cdot\text{day}$ (Figure 3).

Optimization of qPCR for enumeration of microbial cells in mixed species biofilms

To monitor the growth of microorganisms in biofilms, targeted TaqMan qPCR assays were developed for the specific enumeration of *C. albicans*, *S. mutans*, and *L. casei*. Initially, qPCR techniques were compared with total viable counts (TVCs) for enumeration of microorganisms in exponentially growing planktonic cultures. As shown in Figure 4, the qPCR assay for *C. albicans* correlated well with TVCs ($R^2 = 0.86$) between 10^4 and 10^8 input cells. Cell counts were slightly (approximately 1 order of magnitude) higher by qPCR than TVCs. There were also strong correlations between TVCs and qPCR quantification for *S. mutans* ($R^2 = 0.91$) and *L. casei* ($R^2 = 0.91$) (Figure 4B and 4C). For *S. mutans*, cell counts were <1 order of magnitude higher by qPCR than by TVCs, whereas in the case of *L. casei*, cell counts were 2-3 orders of magnitude higher by qPCR than by TVCs. The lower limit of *C. albicans* was around 10^3 - 10^4 cells, *S. mutans* could be detected down to 10^4 cells, and *L. casei* could be detected at $>10^3$ cells. Below these levels, the relationship between qPCR counts and TVCs appeared to break down (indicated by red squares in Figure 4).

Impact of fluoride release on single species biofilms

In monoculture, *C. albicans* and *L. casei* grew in artificial saliva with sucrose added three times per day to form diffuse biofilms on saliva-conditioned copolymer (Figure 5). *S. mutans* grew well in medium supplemented with 1% (w/v) sucrose and formed biofilms consisting of aggregated microcolonies (Figure 5). Extracellular material was observed in biofilms of each microorganism by SEM (Figure 5A and data not shown). Single species biofilms were also cultured in the MRD on fluoridated copolymer stubs. Total counts of each microorganism, determined by qPCR, were not significantly different on the two types of surface, indicating that fluoride did not reproducibly inhibit the growth of monospecies biofilms (Figure 5B).

Effects of fluoridated copolymer on mixed species biofilms

To assess the impact of fluoride release from copolymer on the composition and biomass of mixed-species biofilms, *C. albicans*, *L. casei* and *S. mutans* were co-cultured in parallel MRD chambers containing samples of fluoridated copolymer or non-fluoridated substrata. All three

species grew in biofilms formed in artificial saliva supplemented with 0.1% (w/v) sucrose and could be distinguished within microcolonies by SEM (Figure 6A). On non-fluoridated copolymer, numerous *C. albicans* yeast cells, hyphae and pseudohyphae were observed interacting with bacteria and producing large microbial aggregates. By contrast, biofilms on fluoridated surfaces were relatively thin, sparsely distributed, and microbial cells were often restricted to pits within the acrylic material (Figure 6B).

To distinguish between *C. albicans* and bacteria more clearly and to obtain three-dimensional images without dehydration artefacts, mixed-species biofilms were also imaged by CSLM. *C. albicans* hyphae could be clearly distinguished from bacterial cells (Figure 6C and D). Bacteria in particular were clearly more abundant on non-fluoridated surfaces than on fluoride-releasing copolymer. Quantification of cells by qPCR demonstrated significant reductions in all three microorganisms on fluoridated surfaces compared with non-fluoridated copolymer after 48 h (Student's T test, $p < 0.05$; Figure 6E). To determine whether the reduction in cell numbers on fluoridated surfaces was associated with the capacity of fluoride to inhibit acid production, the pH of the effluent from the MRD was measured during biofilm formation. After 24 h, the pH from mixed-species biofilms had reduced from $6.99 (\pm 0.03)$ to $4.38 (\pm 0.02)$ on non-fluoridated surfaces and to $4.47 (\pm 0.04)$ on fluoridated material. These values were significantly different (Student's T test, $p < 0.05$). By 48 h, the pH from biofilms on non-fluoridated copolymer was $4.32 (\pm 0.01)$, and the pH from fluoridated copolymer was $4.35 (\pm 0.05)$ ($p > 0.05$).

Discussion

Problems arising from plaque on removable prostheses, including denture stomatitis and root caries, are associated with an overgrowth of acidogenic and aciduric microorganisms [2]. Fluoride inhibits acid production and, at an appropriate concentration, can limit the growth of acidogenic microorganisms [33]. Potentially, the prolonged release of fluoride from an acrylic material could have long-term benefits in controlling the establishment of an acidogenic microbial community in the oral cavity. An ideal material needs to release fluoride at a relatively constant rate over a sustained period, and should have the capacity to be recharged periodically with fluoride. This material could be used to line prostheses in contact with caries-susceptible sites for

example overdenture abutments or to construct appliances such as surgical coverplates which are used short term but are difficult to clean.

We chose to incorporate fluoride in the form of NaF, since the release of NaF from materials is relatively stable across a range of pH values [34]. The addition of HEMA facilitates the sustained release of fluoride by promoting the diffusion of water [24]. In line with previous studies [17, 24], under static conditions fluoride was released at a relatively high rate initially, followed by a gradually decreasing rate of release. Even after six months, fluoride release was in the range of 12-15 $\mu\text{g}/\text{cm}^2\cdot\text{day}$. Although we did not measure the fluoride concentration in the immediate vicinity of the disc, it is likely that this sustained release of fluoride will have significant effects on dental plaque bacteria in the base of a biofilm formed on the resin. Dental plaque can retard the diffusion of fluoride and therefore the thickness of the plaque present will affect the local concentration of fluoride achieved [35]. As little as 10 mg L⁻¹ fluoride inhibits acid production by dental plaque [36, 37]. In the MRD biofilm model, which is a flowing system that will continually wash away dissolved fluoride, high levels of fluoride release were sustained for at least 48 h. Although fluoride will eventually be washed out of the copolymer, it was found that the material has the potential to be recharged. Here, recharging with fluoride was achieved using sonication for 3 min in a water bath. Further studies are needed to assess recharging characteristics under conditions that might be achieved in a home environment, for example by soaking for a defined period in a fluoride solution.

The Modified Robbins Device (MRD) was employed to culture biofilms since it is a well-established model system that can hold stubs of material flush against the wall of the vessel with minimal turbulent flow [38]. Initially, biofilms were cultured on non-fluoridated copolymer in artificial saliva medium, supplemented with 10% (w/v) sucrose three times daily to mimic meals. Although *C. albicans* and *L. casei* grew well in monoculture under these conditions, it was not possible to obtain reproducible biofilms of *S. mutans*. Therefore, for *S. mutans* monoculture experiments, 1% sucrose was introduced continuously. These observations indicate that *S. mutans* is highly dependent on sucrose for biofilm formation in monoculture under flowing conditions, as has been demonstrated previously in batch culture [39]. Interestingly, when mixed-species biofilms were cultured in medium containing 1% sucrose, the production of polysaccharide was so great that it blocked the tubing and interfered with the running of the system. Therefore, the

sucrose concentration was reduced to 0.1% in order to culture mixed-species biofilms. It appears that exopolysaccharide production occurs to a greater extent in mixed-species biofilms than in single-species *S. mutans* biofilms, possibly as a result of sucrose utilization by *C. albicans* [40]. It is not yet clear to what extent exopolysaccharide production is modulated by intermicrobial interactions between the three organisms, although it has been shown that *L. casei* induces down-regulation of *gtfB*, encoding glucosyltransferase B, in *S. mutans* [41].

When introducing chemically modified surfaces into a biofilm model, it is important that sterilization of surfaces does not affect the chemical or physical properties of the substratum. Using Fourier Transform Infrared (FTIR) spectroscopy, it was found that the sample stubs did not undergo chemical changes during autoclaving in the system (data not shown). There was a slight increase in the average roughness (R_a) of the fluoridated copolymer after autoclaving, measured by stylus profilometry ($R_a = 3.49 \pm 0.90 \mu\text{m}$ after autoclaving compared with $2.77 \pm 0.91 \mu\text{m}$ before autoclaving). Using SEM, small pits were seen in the autoclaved fluoridated copolymer (data not shown). Some of these pits appeared to harbor microorganisms (Figure 6B). Nevertheless, when biofilms were developed with single species of microorganisms, there were no significant differences in biofilm growth over 48 h on fluoridated or non-fluoridated copolymer.

Even though single species biofilms were apparently unaffected by fluoride release from the copolymer, biofilm formation by a mixed species community of oral acidogenic microorganisms was significantly reduced approximately 10-fold in the presence of fluoride. Therefore, it appears that fluoride interferes with a critical factor involved in intermicrobial interactions rather than inhibiting individual species directly. Recent research has shown that oral bacteria communicate with one another and with oral *Candida* spp., and that these interactions are critical for biofilm development [42-44]. It is possible that fluoride interferes with some aspect of cell-cell communication, and that this destabilizes mixed-species biofilms. Synergistic interactions between *C. albicans* and oral bacteria have been documented that appear to depend on physical cell-cell binding (coaggregation) and/or metabolic cooperation, and these processes may also be targets of the action of fluoride [45, 46]. In fact, it is likely that fluoride inhibits carbohydrate metabolism and acid generation with mixed-species biofilms since fluoride is well-known to inhibit sugar uptake and glycolysis [47, 48]. Although there was a significant reduction in acid production by biofilms on non-fluoridated versus fluoridated copolymer after 24 h, no significant

differences between the pH in the effluent from biofilms on the different surfaces were seen at 48 h. These data indicate that the inhibitory effects of fluoride on acidogenicity may be most important during the early stages of biofilm formation. However, it would be interesting to determine the pH in the local microenvironment within biofilms, for example by using pH-sensitive fluorescent dyes [49]. Even in the presence of fluoride, mixed-species biofilms acidified the medium to below the critical pH for enamel dissolution [50]. Within more complex microbial communities, it is likely that other species may offset the pH reductions by catabolizing lactic acid [51] or by producing ammonia from arginine or urea [52]. In these more complex communities, it has been shown that fluoride can shift the ecological balance and prevent the overgrowth of strongly acidogenic microorganisms such as *S. mutans* [53]. Therefore, we are now aiming to investigate the potential of the fluoridated copolymer to reduce the growth of acidogenic microorganisms within dental plaque communities that more closely resemble those found in the human mouth.

In this study, enumeration of microorganisms was achieved by qPCR. This approach will detect total DNA from each species, which is derived from live and dead cells. To ensure the validity of the qPCR approach when applied to biofilm samples, viable cells of each species were also enumerated on selective media (data not shown). Generally, qPCR tends to give higher counts than TVCs [54] and, in line with this, absolute counts of *L. casei* and *S. mutans* in samples from biofilms were consistently 2-3 orders of magnitude higher by qPCR than by TVCs. For *C. albicans* the two techniques gave similar cell counts when applied to biofilm cells (data not shown). It should be noted that both methods have limitations. Viable counts depend on efficient recovery of cells from biofilms and dispersal of aggregated microorganisms. Various hyphal and budding forms of *C. albicans* were observed by SEM and CSLM that were not well-separated, and would have resulted in an underestimation of cell numbers by TVCs. The qPCR method depends on disruption of thick fungal cell walls that are not easily lysed [55]. Therefore, qPCR may also have underestimated *C. albicans* cell numbers. Nevertheless, when the two methods were employed to assess growth reductions on fluoridated surfaces, both techniques gave similar trends. Importantly, statistically significant reductions in all three species within mixed biofilms on fluoride-releasing copolymer were observed by both qPCR and TVCs.

358 **Conclusions**

359 In conclusion, a novel fluoride-releasing copolymer has been developed that inhibits the
360 growth of mixed-species acidogenic oral biofilms in vitro. Biofilm inhibition was apparent for
361 mixed-species cultures but not for single-species biofilms, indicating that fluoride may interfere
362 with interspecies or interkingdom cell-cell interactions. Further work is required to elucidate the
363 mechanism by which fluoride inhibits mixed-species biofilm development. Ultimately, this study
364 demonstrates the potential for a fluoride-releasing copolymer to control mixed-species oral
365 biofilms and provides a foundation for future clinical investigations.

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Figure legends

Figure 1. Design of the MRD biofilm system. Biofilms are formed on copolymer pieces held within a plug and flush against the wall of the vessel. The samples were conditioned with natural saliva, and then inoculum was introduced. Artificial saliva and sucrose were added as required. The flow of liquid was controlled using clamps and a peristaltic pump.

Figure 2. Fluoride release from fluoridated copolymer material, Teflon moulds were used to prepare discs of 10 mm diameter and 1 mm thickness. Fluoride release under static incubation (A) gradually declined over the 6 month period. The release of fluoride was also monitored in flowing conditions, using samples held in the MRD for 48 h (B). In the latter case, fluoride was only released from the exposed surface of the stub. Bars show mean values and standard deviations from five discs of each sample (static model) or three independent experiments (MRD model).

Figure 3. Fluoride recharge and release. Samples were recharged by bathing for 3 min in 0.2% NaF in an ultrasonic bath at day 0 and day 14. The first measurement after recharging is marked with a dashed line. Bars represent means of five samples and standard deviations are shown.

Figure 4. Correlations between cell counts measured as TVC's or by qPCR. Dilutions of cells were prepared from mid-logarithmic cultures of *C. albicans*, *L. casei* or *S. mutans*. Cell numbers quantified by TVC's were compared with numbers from qPCR assays. At low cell numbers, the relationships between qPCR counts and TVCs appeared to break down (red squares highlighted within circles), indicating the lower limits of sensitivity of the qPCR assay. These data were not included for calculation of the trendline.

Figure 5. Single species biofilm formation by three microorganisms on non-fluoridated material after 48 h. (A) Biofilms of *C. albicans*, *L. casei* and *S. mutans* were visualized by SEM. Note that *C. albicans* and *L. casei* were cultured in artificial saliva pulsed three times daily with 10% sucrose, whereas *S. mutans* was cultured in artificial saliva supplemented with 1% sucrose throughout growth. In each case, extensive biofilms were observed and cell clusters, or microcolonies, could clearly be seen. (B) Cell numbers in biofilms developed in the MRD control samples (dark bars) or fluoridated copolymer (light bars) were enumerated by qPCR and expressed as genome equivalents (GE)/cm² of acrylic surface area. Bars represent means and standard deviations from three independent experiments. A lower cell density of *C. albicans* was used to inoculate the MRD biofilm model than *L. casei* or *S. mutans* and total counts of *C. albicans* were relatively low after 48 h (presented as an inset graph as there is a different scale on the y axis). Overall, there were no significant differences in cell counts of any species on control or fluoridated samples.

Figure 6. Mixed species biofilm formation after 48 h on non-fluoridated material after 48 h. (A) Biofilm microcolonies were visible across the surface of control copolymer samples (arrows). At high magnification (inset), *C. albicans* and bacteria were seen to be closely interacting within these biofilms. (B) On fluoridated samples, surface coverage was reduced, and in places growth was restricted to pits within the material (arrow). Biofilms on control (C) or fluoridated (D) copolymer were also visualized by CSLM. Samples were stained with propidium iodide (red) and DAPI (blue) to highlight bacterial cells (appear purple), and ConA (green), which labels fungal cell walls including *C. albicans*. (E) Cells were removed from surfaces at different times after inoculating the MRD biofilm model and each species in biofilms on control samples (dark bars) and fluoridated samples (light bars) enumerated by qPCR. Bars represent mean values and standard deviations from three independent experiments. In all cases, cell counts on fluoridated material 48 h after inoculation were significantly lower than on non-fluoridated copolymer, as indicated by an asterisk (Student's T-test, $p < 0.05$).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6