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Effects of single species versus multispecies periodontal biofilms on the antibacterial efficacy of a novel bioactive Class-V nanocomposite

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ABSTRACT

Objective. The objectives of this studywere to: (1) develop a novel bioactive nanocomposite for Class V restorations with subgingival margins to inhibit periodontal pathogens; and (2) investigate if the bioactive nanocomposite could inhibit multi-species periodontal biofilms with a potency as strong as that against single species biofilms.

Methods. Nanocomposite was fabricated using dimethylaminohexadecyl methacrylate (DMAHDM), 2-methacryloyloxyethyl phosphorylcholine (MPC) and nanoparticles of amorphous calcium phosphate (NACP). Biofilms with 1, 3, 6 and 9 species of periodontal pathogens were grown on the composites and tested for live/dead staining, colony-forming units (CFU), metabolic activity, and biofilm matrix polysaccharide production.

Results. The bioactive composite reduced protein adsorption by an order of magnitude ($p < 0.05$) and greatly reduced biofilm viability. It decreased the biofilm CFU by more than 3 orders of magnitude for all four types of periodontal biofilms, compared to control composite. With increasing the biofilm species from 1 to 9, the antibacterial efficacy of DMAHDM composite decreased; the CFU reduction folds decreased from 947 folds to 44 folds. In contrast, the MPC + DMAHDM composite maintained a CFU reduction folds of greater than 3000, showing a similar antibacterial potency from 1 to 9 species in the biofilms ($p > 0.1$).

Conclusion. Dual agents MPC + DMAHDM achieved the greatest inhibition in biofilm, without decreasing its antibacterial potency when the biofilm species was increased from 1 to 9. A single agent became less effective when the biofilm species was increased from 1 to 9.

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Significance. The multifunctional MPC + DMAHDM composite is promising for root caries treatment and Class V restorations with subgingival margins to effectively inhibit multispecies periodontal biofilms, combat periodontitis and protect the periodontium.

1. Introduction

The world population is aging, and it is estimated that by 2050, the number of people 65 years of age and older will reach 1.5 billion [1]. In the past 20 years, the focus on prevention of oral disease has led to an increase in the number and percentage of older adults who are retaining their teeth over their lifetime [2]. However, increased retention of teeth signifies higher risks of tooth root caries due to gingival recession and reduced saliva flow in seniors [3]. Periodontitis is accompanied by gingival recession, with exposure of the root surface to the oral environment, which is susceptible to the development of root caries [1]. Root caries can be treated with a Class V restoration, which is often accompanied with subgingival margins. This would hinder cleaning and provide pockets for bacterial growth, which in turn could enhance the loss of the tooth's attachment and the development of periodontitis. There is well-established evidence of the role of dental microbial biofilms in the etiology of periodontal diseases [4]. However, current restorations for root caries have no antibacterial function. Instead, resin-based Class V restorations may even accumulate more biofilms and plaques than metal-based but un-esthetic materials [5], which could aggravate the progress of periodontitis.

Periodontitis is an infectious disease involving a complex interaction between the oral microorganisms in a subgingival plaque and the host immune response [6]. Although multispecies species biofilms dominate in vivo, single species biofilms also exist in a variety of infections and on the surfaces of medical implants [7]. Researchers have performed experiments on single species biofilms, probably due to the experimental limitations associated with more complex communities [8]. However, single species models are not representative of natural biofilms where multispecies communities are by far the most predominant [9]. The eradication of multispecies biofilms is more difficult to achieve than single species, as multispecies biofilms are more highly resistant to antimicrobial agents than single species [10]. Furthermore, the biofilm composition may influence the outcome of periodontitis treatments [11] and the killing efficacy of antibacterial agents [10,12]. Therefore, new antibacterial restorative materials should be tested against multispecies biofilms.

Resin composites are widely-used dental filling materials due to their esthetics and direct-filling capabilities [13,14]. Nevertheless, composite restorations are challenged with accumulation of biofilms [5] and marginal leakage [15,16] which could result in secondary caries. Therefore, efforts were made to develop antibacterial composites [17–19]. Quaternary ammonium methacrylates (QAMs) exhibited excellent antibacterial properties [20,21]. QAMs have polymerizable groups which can be immobilized in the polymer backbone to provide long-term antibacterial activity. Recently, a new antibacterial monomer dimethylaminohexadecyl methacrylate (DMAHDM) were synthesized and incorporate into dental resins, achieving strong antibacterial functions [22]. In a previous study, composite with DMAHDM showed different killing efficacies against six single species periodontal biofilms [23]. However, Class V restorations against multispecies periodontal biofilms with different species compositions have not been reported.

The adsorption of salivary proteins onto the resin composite is a prerequisite for bacterial adhesion, which is the initial step for biofilm formation. Therefore, repelling protein adsorption could reduce bacterial adhesion to the resin. A protein-repellent agent, 2-methacryloyloxyethyl phosphorylcholine (MPC), is a methacrylate with phospholipid polar groups and is a common biopolymer [24]. MPC can copolymerized with acrylic resin through covalent bonding, and the strong C—C bonding offered durable resistance to protein adsorption [24]. In previous studies, composites containing MPC showed strong protein-repellent properties and inhibition against cariogenic bacteria [25]. To date, there has been no report on the effects of MPC and DMAHDM on periodontal biofilms containing different numbers of bacterial species.

The objectives of this study were to develop a bioactive composite for root caries restorations and investigate the inhibition efficacy on periodontal biofilms with different compositions (single species, three-species, six-species and nine-species biofilms) for the first time. Fig. 1 illustrates the anti-biofilm strategy. It was hypothesized that: (1) the addition of DMAHDM and MPC into the composite would substantially reduce biofilm growth; (2) periodontal biofilms with increasing number of species would be increasingly more difficult to kill, compared to single species biofilm; (3) incorporating dual agents of DMAHDM + MPC in the composite would be more effective than a single agent in inhibiting biofilms, and would maintain a high potency even when the number of species in the biofilms increases.

2. Materials and methods

2.1. *Fabrication of composites*

The resin matrix consisted of ethoxylated bisphenol A dimethacrylate (EBPADMA, Sigma-Aldrich, St. Louis, MO, USA) and pyromellitic dianhydride glycerol dimethacrylate (PMGDM, Esstech, Essington, PA, USA) at 1:1 mass ratio. The EBPADMA-PMGDM resin was rendered light-curable with 0.2% camphorquinone and 0.8% ethyl 4-*N*,*N*-dimethylaminobenzoate [26]; this resin matrix was referred to as EBPM.

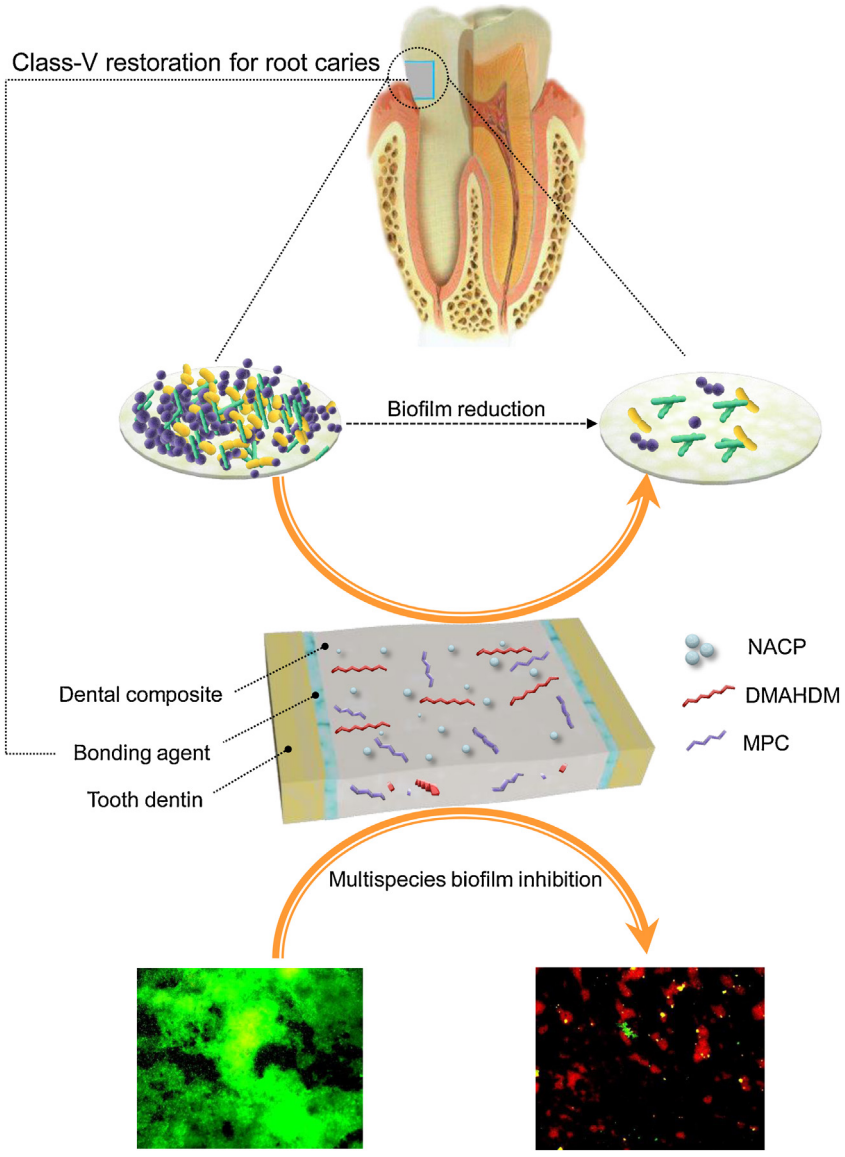

Fig. 1 – Antibacterial strategy using dual agents in dental composite. Dimethylaminohexadecyl methacrylate (DMAHDM) can inactivate periodontal pathogens by contact without leaching from resins. Methacryloyloxyethyl phosphorylcholine (MPC) can detach proteins, thereby hampering bacterial attachment. DMAHDM and MPC are both non-voltile, chemically stable and can sustain long-term antibacterial acitivity.

DMAHDM was synthesized using a modified Menschutkin reaction in which a tertiary amine group was reacted with an organohalide [27]. Briefly, 10 mmol of 2-(dimethylamino)ethyl methacrylate (DMAEMA, Sigma-Aldrich) and 10 mmol of 1-bromohexadecane (BHD, TCI America, Portland, OR, USA) were combined with 3 g of ethanol in a 20 mL scintillation vial. The vial was stirred at 70 °C for 24 h. The solvent was then removed via evaporation, yielding DMAHDM as a clear, colorless, and viscous liquid. DMAHDM was mixed with the resin at a DMAHDM mass fraction of 10%. The 10% DMAHDM mass fraction followed a previous study [26]. MPC powder was commercially obtained and incorporated into the resin at a MPC mass fraction of 10%. MPC mass fractions greater than 10% were not included due to mechanical property loss as measured in a previous study [26].

In addition, 20% by mass of nanoparticles of amorphous calcium phosphate (NACP), $Ca_3(PO_4)_2$, were incorporated into the resin. The reason for including NACP was for calcium (Ca) and phosphate (P) ion release which could remineralize tooth lesions as shown previously [28]. NACP were synthesized using a spray-drying technique [29]. Briefly, calcium carbonate and dicalcium phosphate anhydrous were dissolved in acetic acid to produce Ca and P concentrations of 8 and 5.333 mmol/L, respectively. This solution was sprayed into a heated chamber of the spray-drying machine. The dried particles were collected by an electrostatic precipitator. This yielded NACP with a mean particle size of 116 nm. As a co-filler for mechanical reinforcement, barium boroaluminosilicate glass particles (1.4 μm median particle size, Caulk/Dentsply, Milford, DE, USA) was silanized with 4% 3-methacryloxypropyltrimethoxysilane

and 2% *n*-propylamine. The NACP and glass filler mass fractions were 20% and 50%, respectively, with a total filler level of 70%, which yielded a cohesive composite paste. Since the resin mass fraction in the composite was 30%, the DMAHDM and MPC mass fractions in the final composite were both at 3%. The composite with 0% DMAHDM and 0% MPC served as a control. In addition, a commercial composite (Heliomolar, Ivoclar, Amherst, NY) served as a comparative control. Therefore, five composites were tested:

(1) Commercial control: heliomolar composite;
(2) Experimental control: 30% EBPM + 20% NACP + 50% glass;
(3) MPC composite: 27% EBPM + 3% MPC + 20% NACP + 50% glass;
(4) DMAHDM composite: 27% EBPM + 3% DMAHDM + 20% NACP + 50% glass;
(5) MPC + DMAHDM composite: 24% EBPM + 3% DMAHDM + 3% MPC + 20% NACP + 50% glass.

For each composite, disk specimens were made using molds with a diameter of 9 mm and thickness of 2 mm. The composite was light-cured (Triad 2000, Dentsply, York, PA) for 1 min on each open side between Mylar Strips [23,26]. The disks were immersed in 200 mL of water and magnetically-stirred with a bar at 100 rpm for 1 h to remove any uncured monomers [23,26]. The disks were then sterilized with ethylene oxide (AnproleneAN 74i, Andersen, Haw River, NC, USA) and de-gassed for 7 days, following the manufacturer's instructions.

2.2. Atomic force microscopy

An atomic force microscope (AFM, 5500SPM, Agilent, Santa Clara, CA, USA) was used at a high resolution with a sharp silicon tip in the tapping mode. The surface topography of the composite was obtained over an area measuring 20 μm × 20 μm and 5 μm × 5 μm. The surface roughness of the samples was provided by a software (SPIWIN 2.0, Seiko, Tokyo, Japan), and the average surface roughness R_a data of the composites were measured. Six disks were tested for each composite.

2.3. Charge density of composites

The surface density of quaternary ammonium groups present on the polymer surfaces was quantified using a fluorescein dye method as described previously [27,30]. Composite disks of each group were placed in a 48-well plate. Fluorescein sodium salt (200 μL of 10 mg/mL) was added into each well, and samples were kept in the dark for 10 min at room temperature. After removing the fluorescein solution and rinsing extensively with distilled water, each specimen was transferred into a new well, and 200 μL of 0.1% of cetyltrimethylammonium chloride (CTMAC) was added. Samples were vibrated for 20 min to desorb the bound dye. Sample absorbance was read at 501 nm using a plate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA) [27,30]. The fluorescein concentration was calculated using Beers Law and an extinction coefficient of 77 $mM^{-1}\,cm^{-1}$ [27,30]. Using a ratio of 1:1 for fluorescein molecules to the accessible quaternary ammonium groups, the surface charge density was calculated as the total molecules of charge per exposed surface area (the summation of top, bottom and side areas, measured independently for each polymer disk due to slight variations in disk sizes) [27]. Six disks were tested for each composite.

2.4. Protein absorption assessment

Six disks were fabricated for each composite for protein absorption assessment. A micro bicinchoninic acid (BCA) method was used to determine the protein adsorption [31]. Briefly, each disk was immersed in phosphate buffered saline (PBS) for 2 h prior to in 4.5 g/L bovine serum albumin (BSA, Sigma-Aldrich) solution at 37 °C for 2 h. The disks were then rinsed with PBS, immersed in 1% sodium dodecylsulfate (SDS) in PBS and sonicated for 20 min to detach the BSA from the disk [31]. A protein analysis kit (micro BCA, Fisher Scientific, Pittsburgh, PA, USA) was used to determine BSA concentration in the SDS solution [31]. 25 μL of SDS solution and 200 μL of BCA reagent were mixed into the wells of a 96-well plate and incubated at 60 °C for 30 min. The absorbance at 562 nm was measured via the microplate reader (SpectraMax M5). Standard curves were prepared using the BSA standard [31].

2.5. Bacterial strains and culture conditions

The use of bacteria was approved by University of Maryland Institutional Review Board. All species were obtained from the American Type Culture Collection (ATCC, Manassas, VA): *Porphyromonas gingivalis* (*P. gingivalis*, ATCC33277), *Streptococcus gordonii* (*S. gordonii*, ATCC10558), *Fusobacterium nucleatum* (*F. nucleatum*, ATCC 25586), *Actinomyces naeslundii* (*A. naeslundii*, ATCC12104), *Prevotella intemedia* (*P. intermedia*, ATCC 25611), *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*, ATCC 43717), *Prevotella nigrensens* (*P. nigrensens* ATCC 25261), *Tannerella forsythia* (*T. forsythia*, ATCC 43037), and *Parvimonas micra* (*P. micra*, ATCC 33270). They were selected for the experiments because *S. gordonii* and *A. naeslundii* are regarded as predominant initial colonizers, which can recognize receptors in the salivary pellicle [8]. *P. gingivalis, P. intemedia, P. nigrensens, P. micra, A. actinomycetemcomitans* and *T. forsythia* act as late colonizers and are highly associated with the occurrence and progression of periodontitis [6,8]. *F. nucleatum* are important 'bridge' organisms in the succession of genera in naturally developing dental biofilm since they can co-aggregate with initial, early and late colonizers [8].

Each species (except *S. gordonii* and *A. actinomycetemcomitans*) was grown in a tryptic soy broth (TSB, Sigma) supplemented with yeast extract (5 g/L), L-cysteine hydrochloride (0.5 g/L), hemin (5 mg/L) and menadione (1 mg/L) at 37 °C anaerobically (90% N_2, 5% CO_2, 5% H_2) [23]. *S. gordonii* and *A. actinomycetemcomitans* were grown in culture aerobically (95% air, 5% CO_2) in freshly prepared BHI agar plates and supplemented with 1% yeast extract for 16 h at 37 °C [23]. In the following experiments, the microbial concentration was adjusted to 10^8 CFU/mL according to the McFarland turbidity standards by measuring the absorbance of the inoculum with a spectrophotometer at 660 nm [32].

2.6. Biofilm formation on composites

Saliva was collected from healthy adult donors having natural dentition without active caries or periopathology, and without the use of antibiotics within the last 3 months. The collected saliva was centrifuged at 3000 rpm for 20 min to remove cellular debris. The supernatant was filter-sterilized through sterile 0.22 μm filters (VWR International, Radnor, PA, USA). The protocol was approved by the University of Maryland Baltimore Institutional Review Board (HP-00050407).

Prior to biofilm formation, all composite disks were precoated with sterile saliva to form a salivary pellicle on the composite to facilitate bacterial attachment. This was achieved by immersing the disk in saliva in a 24-well plate for 2 h at 37 °C [23]. The following four types of biofilms were grown on the pellicle-coated composite disks in static 24-well microtiter plates [32–34].

(1) Single species biofilm: *P. gingivalis*.
(2) Three-species biofilm: *P. gingivalis*, *S. gordonii* and *F. nucleatum*.
(3) Six-species biofilm: *P. gingivalis*, *S. gordonii*, *F. nucleatum*, *A. naeslundii*, *P. intermedia* and *A. actinomycetemcomitans*.
(4) Nine-species biofilm: *P. gingivalis*, *S. gordonii*, *F. nucleatum*, *A. naeslundii*, *P. intermedia*, *A. actinomycetemcomitans*, *P. nigrensens*, *T. forsythia* and *P. micra*.

P. gingivalis was chosen to form a single species biofilm, since most periodontal diseases are associated with the presence or overgrowth of this anaerobic species. For multispecies biofilms, the natural addition of species in oral biofilms provided a paradigm for biofilms [35]. Therefore, *S. gordonii*, *F. nucleatum* and *P. gingivalis* were chosen to form a three-species biofilm mainly because of their roles in aggregation and colonization succession [8]. Moreover, another pioneer species, *A. naeslundii*, and two species of periodontal pathogens *P. intermedia* and *A. actinomycetemcomitans,* were added into the aforementioned three-species biofilm to form a six-species biofilm. To further increase the complexity of the periodontal biofilm, three key pathogens *P. nigrensens*, *T. forsythia* and *P. micra* were further incorporated into the aforementioned biofilm to form the nine-species biofilm.

For consistency, the initial inoculum was adjusted to approximately 10^7 CFU/mL for all four types of biofilms, following previous studies [23]. For single species, the bacterial inoculum in TSB-supplemented broth was prepared to contain 10^7 CFU/mL of *P. gingivalis*.

For the three-species biofilm, a mixed bacterial suspension in TSB-supplemented broth was prepared to contain 10^3 CFU/mL of *S. gordonii*, 5×10^6 CFU/mL of *P. gingivalis,* and 5×10^6 CFU/mL of *F. nucleatum*.

For the six-species biofilm, a mixed bacterial suspension in TSB-supplemented broth was prepared to contain 10^3 CFU/mL of *S. gordonii*, 10^5 CFU/mL of *A. naeslundii*, and 2.5×10^6 CFU/mL each of *P. gingivalis*, *F. nucleatum*, *P. intermedia* and *A. actinomycetemcomitans*.

For the nine-species biofilm, a mixed bacterial suspension in TSB-supplemented broth was prepared to contain 10^3 CFU/mL of *S. gordonii*, 10^5 CFU/mL of *A. naeslundii*, and 1.5×10^6 CFU/mL each of *P. gingivalis*, *F. nucleatum*, *P. intermedia*, *A. actinomycetemcomitans*, *P. nigrensens*, *T. forsythia* and *P. micra*.

The salivary pellicle-coated composite disks were transferred to a new 24-well plate, and one of the four types of bacterial suspensions was inoculated at the aforementioned concentration in 1.5 mL medium, and was added into each well. All biofilms were grown in TSB-supplemented broth anaerobically at 37 °C for 96 h, and the medium was changed every 24 h. This totaled 4 days of culture, which was shown to form relatively mature periodontal biofilms on resins [33].

2.7. Live/dead bacteria imaging

Composite disks with 4-day biofilms were washed with cysteine peptone water (CPW) to remove the non-adherent bacteria. Live/dead bacterial kit (Molecular Probes, Eugene, OR) was used following the manufacturer's instructions. A mixture of 2.5 μM SYTO 9 and 2.5 μM propidium iodide was used to stain each sample for 15 min in the dark room. Live bacteria were stained with SYTO 9 to emit a green fluorescence. Bacteria with compromised membranes were stained with propidium iodide to emit a red fluorescence. Biofilms were examined with an inverted epifluorescence microscope (TE2000-S, Nikon, Melville, NY). The area of green staining (live bacteria) was computed with NIS Elements imaging software (Nikon). The area fraction of live bacteria = green staining area/total area of the image [22]. Six specimens were evaluated for each composite. Three randomly-chosen fields of view were photographed from each resin disk, yielding a total of 18 images for each composite.

2.8. Colony-forming unit (CFU) counts

For CFU counts, twenty-four disks were made for each composite, with six disks for each type of biofilm. Four types of biofilms were formed by culturing for 4 day as described above. Disks were transferred into vials with 2 mL CPW, and the biofilms were harvested by scraping and sonication/vortexing (Fisher, Pittsburg, PA). Tryptic soy blood agar plates (supplemented with 5 g/L yeast extract, 0.5 g/L L-cysteine hydrochloride, 5 mg/L hemin, 1 mg/L menadione, 5% sheep blood) were used. Biofilm suspensions were serially diluted, spread onto agar plate and incubated at 37 °C anaerobically for 72 h [34]. Then, the number of colonies was counted by a colony counter (Reichert, NY), which was used with the dilution factor to calculate the CFU counts.

2.9. XTT metabolic assay

Twenty-four disks of each composite were made for the metabolic assay, with six disks for each type of biofilms. Following a described study [46], the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide (XTT) assay was conducted to measure the metabolic activity of biofilms. Disks with biofilms were washed via CPW, followed by transferring into new 24-well plates with 1 mL of XTT working regent (0.5 mg/mL XTT and 1 μM menadione). The solutions were mixed gently, covered with aluminum foil and then cultured for 2 h at 37 °C. The absorbance at OD_{492nm}

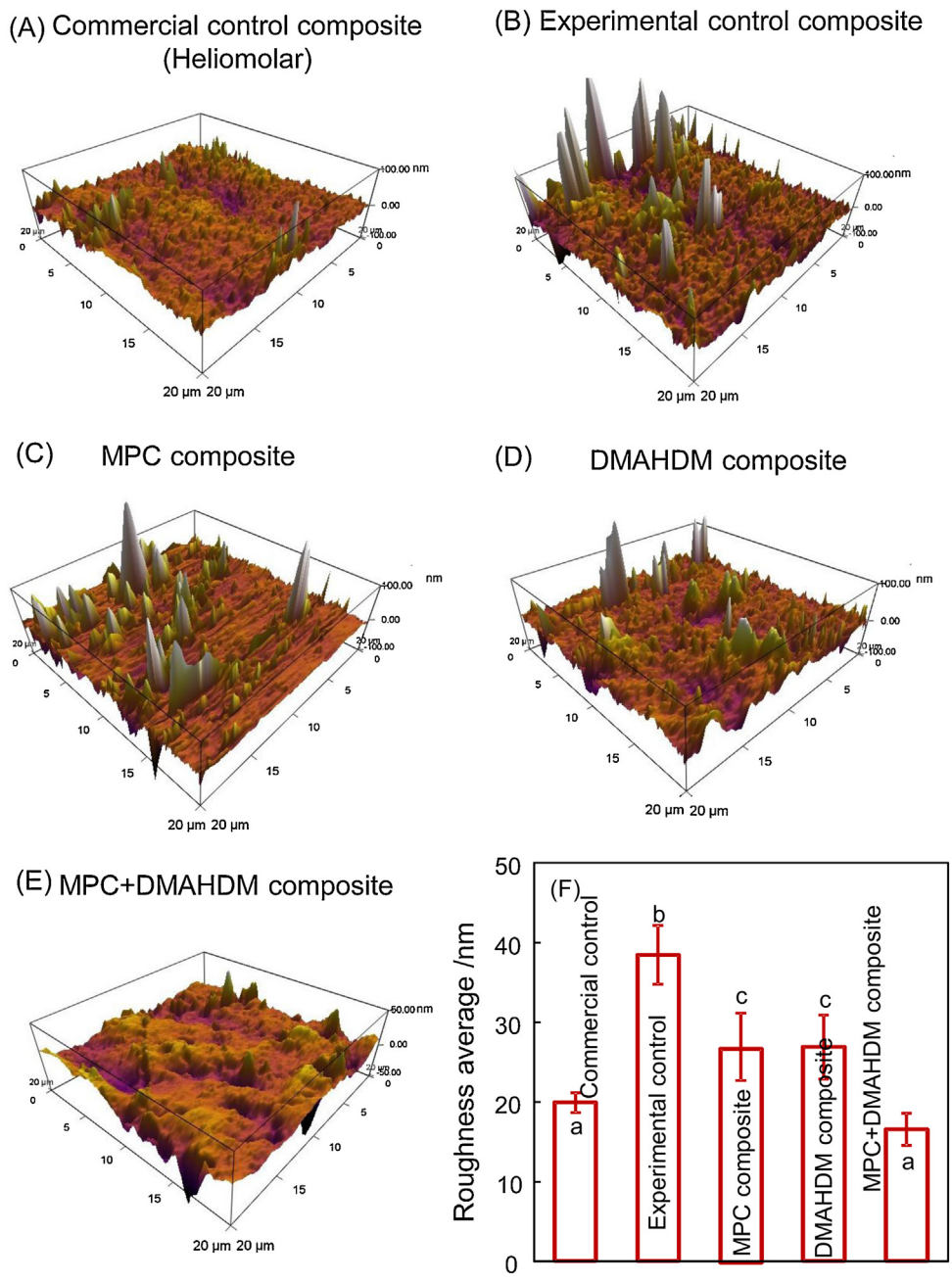

Fig. 2 – Typical AFM image for suface properties of: (A) commercial control composite, (B) experimental control composite, (C) MPC composite, (D) DMAHDM composite, and (E) MPC + DMAHDM composite. (F) The average roughness of nanocomposite containing different mass fractions of MPC and DMAHDM (mean ± sd; n = 6). Values with dissimilar letters are significantly different from each other ($p < 0.05$).

was measured via the microplate reader (SpectraMax M5) [36]. The conversion of the XTT substrate to a soluble and colored formazan product correlates with the cell viability. A higher absorbance is related to a higher metabolic activity in the biofilm on the composite disks [36].

2.10. *Measurement of polysaccharide production by biofilms*

For polysaccharide production, twenty-four disks of each composite were made with six disks for each type of biofilm. The water-insoluble polysaccharide in the extracellular polymeric substance (EPS) of the biofilms was determined using a phenol-sulfuric acid method [26]. Each disk with biofilm was immersed in a vial with 2 mL CPW, and the biofilm was collected by sonication/votexing. Centrifugation yielded a precipitate, which was rinsed with PBS and resuspended in 1 mL of de-ionized water. Then, 1 mL of a 6% phenol solution was added to the vial, followed by 5 mL of 95–97% sulfuric acid [26]. The vial was incubated for 30 min. Then, 100 µL of the solution was transferred into a 96-well plate. The amount of polysaccharide in biofilms was determined by measuring the

absorbance at OD_{490nm} with the microplate reader. Five glucose concentrations of 0, 5, 10, 20, 50 and 100 mg/mL were used as standard in the conversion of the OD readings to the polysaccharide concentrations [26].

2.11. *Statistical analysis*

All data were checked for normal distribution with the Kolmogorov–Smirnov test. One-way analysis of variance (ANOVA) was performed to evaluate differences in surface roughness, charge density and protein absorption. Two-way ANOVA was performed to assess differences in biofilm types and various composites. Post-hoc multiple comparisons were performed using Tukey's honestly significant difference test. Statistical analyses were performed by SPSS 19.0 (SPSS, Chicago, IL) at alpha of 0.05.

3. Results

Representative AFM images of composite surfaces are shown in Fig. 2A–E to compare the surface roughness. Fig. 2F plots the average roughness R_a for the composites (mean ± sd; n = 6). The experimental control composite had a significantly greater R_a than commercial composite control ($p < 0.05$). Adding 3% DMAHDM or 3% MPC in the experimental control composite decreased the R_a ($p < 0.05$) The R_a of MPC + DMAHDM composite was significantly lower than that of other composites containing EBPM ($p < 0.05$). Additionally, MPC + DMAHDM composite had a similar R_a to commercial composite control ($p > 0.1$).

The composite surface charge density is plotted in Fig. 3A. Fluorescein binding to the cationic quaternary groups revealed significant increases in quaternary ammonium sites on the surface of composite with DMAHDM incorporation ($p < 0.05$). MPC incorporation did not influence the surface charge density ($p > 0.1$). The charge density value of DMAHDM composite was approximately 3 times than that of experimental control and commercial control ($p < 0.05$). Control samples without DMAHDM had slightly nonspecific interactions with fluorescein with a small amount of absorption of fluorescein.

The protein adsorption results are plotted in Fig. 3B (mean ± sd; n = 6). DMAHDM incorporation in composite had no effect on protein adsorption ($p > 0.1$). Adding 3% MPC in composite decreased the protein adsorption by approximately an order of magnitude, compared to that without MPC and the commercial control ($p < 0.05$).

Representative live/dead images of biofilms on composites are shown in Fig. 4: (A–E) *P. gingivalis* single species biofilm, (F–J) three-species biofilm, (K–O) six-species biofilm, and (P–T) nine-species biofilm. The composites are: (A, F, K, P) commercial control Heliomolar, (B, G, L, Q) experimental control, (C, H, M, R) DMAHDM composite, (D, I, N, S) MPC composite, and (E, J, O, T) MPC + DMAHDM composite. The control composites were nearly completely covered by live bacteria. In contrast, composites with 3% DMAHDM had substantial amounts of dead bacteria. MPC composite had much less bacterial adhesion. MPC + DMAHDM composite had much less bacterial adhesion, and the bacteria were mostly dead. Remarkably, with the bacterial species in the biofilm increasing from single species to nine species, there was an increasing trend of more bacteria, both dead and alive, on the composites. In Fig. 4U and Fig. 4V. For quantification of live bacteria coverage for *P. gingivalis* single-species biofilm and nine-species biofilm, values with dissimilar letters are significantly different from each other.

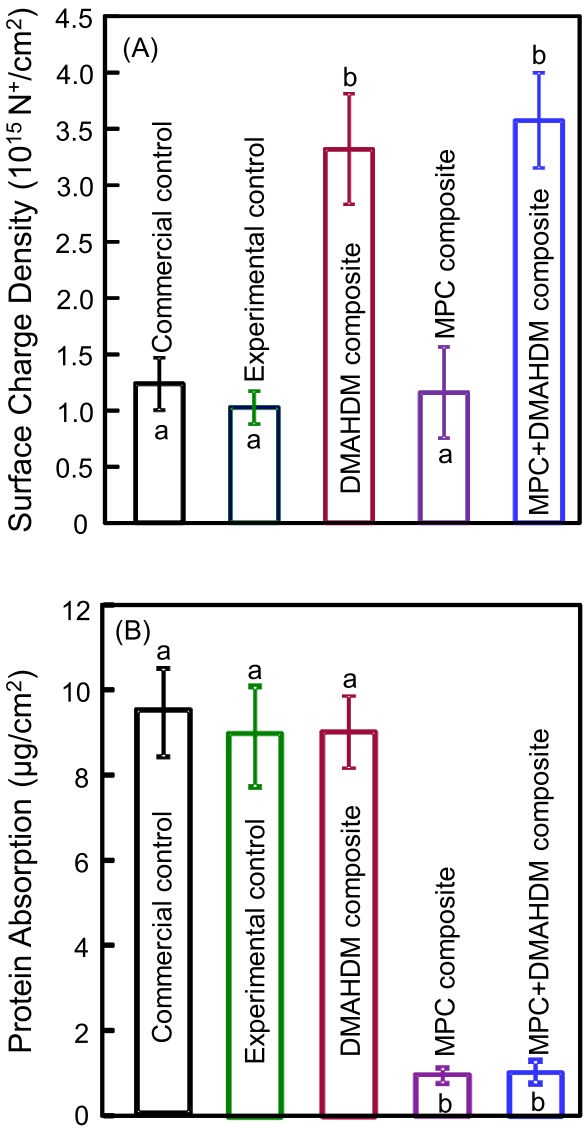

Fig. 3 – Composite surface charge density and protein adsorption (mean ± sd; n = 6). Surface charge density statistically significantly increased on the surfaces of the composite with DMAHDM ($p < 0.05$).The charge density of DMAHDM composite was approximately 3 times that of experimental control and commercial control ($p < 0.05$). MPC + DMAHDM composite had much less protein which was approximately 1/10 that of control ($p < 0.05$). Bars with dissimilar letters are significantly different from each other ($p < 0.05$).

Fig. 5 plots the CFU counts of periodontal biofilms on composites for: (A) single species biofilms, (B) three-species biofilms, (C) six-species biofilm, and (D) nine-species biofilm (mean ± sd; n = 6). Two-way ANOVA showed significant effects

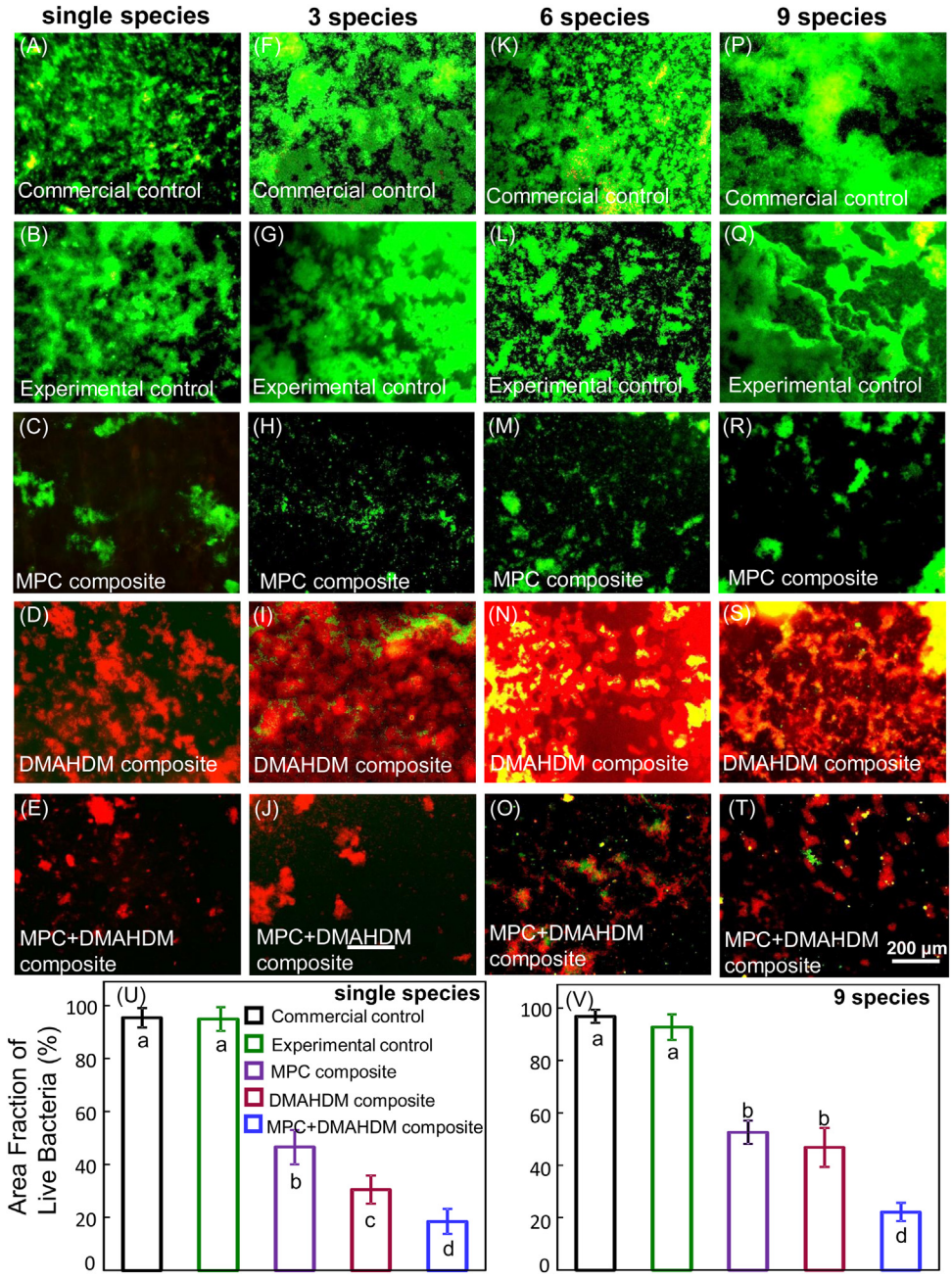

Fig. 4 – Representative live/dead images of: (A–E) single species biofilms, (F–J) three species biofilms, (K–O) six-species biofilms, and (P–T) nine-species biofilms on: (A, F, K, P) commercial control composite, (B, G, L, Q) experimntal control composite, (C, H, M, R) MPC composite, (D, I, N, S) DMAHDM composite, and (E, J, O, T) MPC + DMAHDM composite. (U,V) area fraction of green staining of live bacteria coverage on composite surface for (U) single-species biofilm and (V) nine-species biofilm (mean ± sd; n = 6) All images had the same scale bar as shown in (T). Live bacteria were stained green. Bacteria with compromised membranes were stained red. Live and dead bacteria together or on the top of each other yielded yellow/orange colors. Nanocomposite without DMAHDM had primarily live bacteria. MPC + DMAHDM composite had much less bacteria, and the biofilms consisted of primarily dead bacteria. Dissimilar letters in (U, V) indicate values that are significantly different from each other ($p < 0.05$) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

of composite type and biofilm type ($p < 0.05$), with a significant interaction between the two variables ($p < 0.05$). The two controls had similar CFU ($p > 0.1$). Adding DMAHDM or MPC alone decreased the CFU ($p < 0.05$). MPC + DMAHDM composite reduced the CFU greater than that with DMAHDM composite or MPC composite. Compared with the biofilm CFU on experimental control, the folds of biofilm CFU reduction on DMAHDM composite for 1-, 3-, 6-, 9-species were 947, 243, 67 and 44 folds, respectively. At each number of species, the folds of reduction = biofilm CFU on experimental control compos-

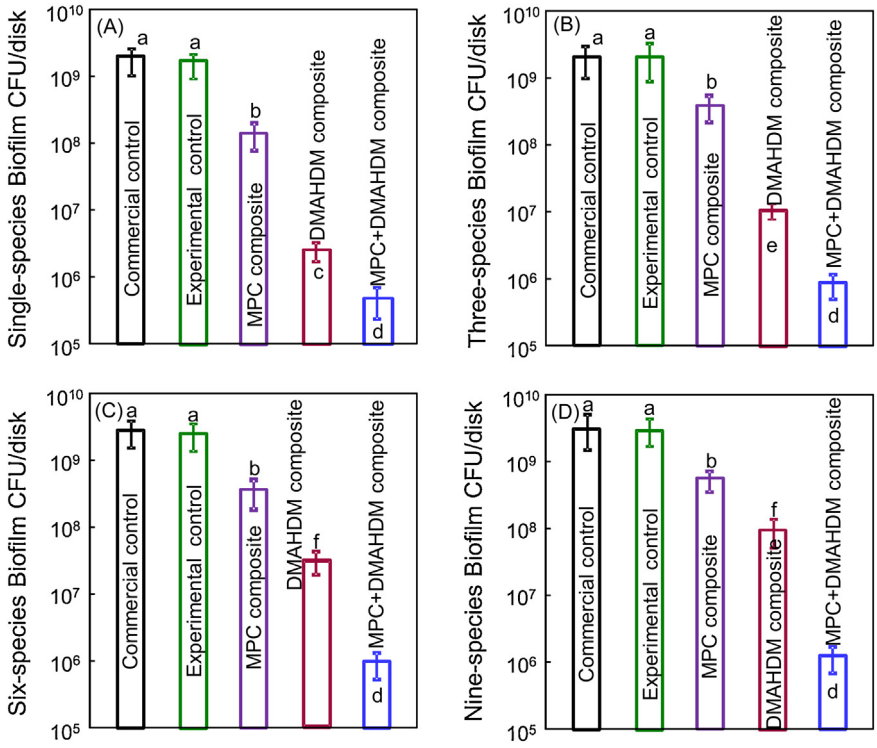

Fig. 5 – Periodontal biofilm CFU on different composites: (A) single species biofilms, (B) three-species biofilms, (C) six-species biofilms, and (D) nine-species biofilms (mean ± sd; n = 6). In each plot, values with dissimilar letters are significantly different from each other ($p < 0.05$). Note log scale in y axis.

ite/biofilm CFU on DMAHDM composite. This demonstrated that the killing efficacy of DMAHDM composite decreased with increasing the number of species in the periodontal biofilm.

In contrast, the folds of reduction in biofilm CFU on MPC + DMAHDM composite were all above 3000 for 1-, 3-, 6-, 9-species and were not significantly different from each other ($p > 0.1$). The MPC + DMAHDM composite had more than 3 log reductions in CFU for all four types of biofilms.

The metabolic activity of periodontal biofilms on composites are plotted in Fig. 6: (A) single species biofilms, (B) three-species biofilms, (C) six-species biofilm, and (D) nine-species biofilm (mean ± sd; n = 6). The commercial control and experimental control had similar metabolic activity ($p > 0.1$). DMAHDM or MPC alone greatly reduced the metabolic activity ($p < 0.05$). MPC + DMAHDM composite had the least metabolic activity ($p < 0.05$). DMAHDM composite exhibited a lower killing power with when the number of species in the biofilms was increased from 1 to 9. However, MPC + DMAHDM composite showed a similarly strong killing efficacy against biofilms with 1 to 9 species.

The polysaccharide production results by biofilms on composites are plotted in Fig. 7: (A) single species, (B) three species, (C) six species, and (D) nine species (mean ± sd; n = 6). Polymicrobial biofilms synthesized more polysaccharides than single species biofilms ($p < 0.05$). DMAHDM or MPC alone significantly reduced the polysaccharide production by biofilms ($p < 0.05$). MPC + DMAHDM composite had the least polysaccharide production for all four types of biofilms ($p < 0.05$).

4. Discussion

In the present study, the effects of the number of species (from 1 to 9) in the periodontal biofilm on the inhibition efficacy of bioactive nanocomposite for Class-V restorations were investigated for the first time. The hypotheses were proven that DMAHDM and MPC incorporation into the composite greatly reduced the biofilm growth, CFU, metabolic activity and polysaccharide production. The periodontal biofilms with increasing number of species from 1 to 9 became increasingly more difficult to kill, compared to single species biofilm, when using DMAHDM or MPC alone. However, with dual agents of MPC + DMAHDM in the composite, a high efficacy of antibacterial function was achieved that was independent of the number of species in the biofilms. The biofilm CFU was reduced by 3000–4000 folds, and the folds of reduction were not significantly different against biofilms with the number of species increasing from 1 to 9.

Imazato developed 12-methacryloyloxydodecylpyridinium bromide (MDPB) which was copolymerized in dental resins to reduce bacterial growth [21]. The mechanism was suggested to be that, QAMs could result in bacteria lysis by binding to cell membrane to cause cytoplasmic leakage [37]. The N^+ charge density of QAMs was important because when negatively-charged bacterial cell contacts the positively-charged QAM resin, the electric balance of cell membrane could be disturbed, leading to bacterial destruction [37]. A previous study [38] developed antimicrobial polymeric brushes, showing that a high density of 5×10^{15} N^+/cm^2 cationic surface had a strong

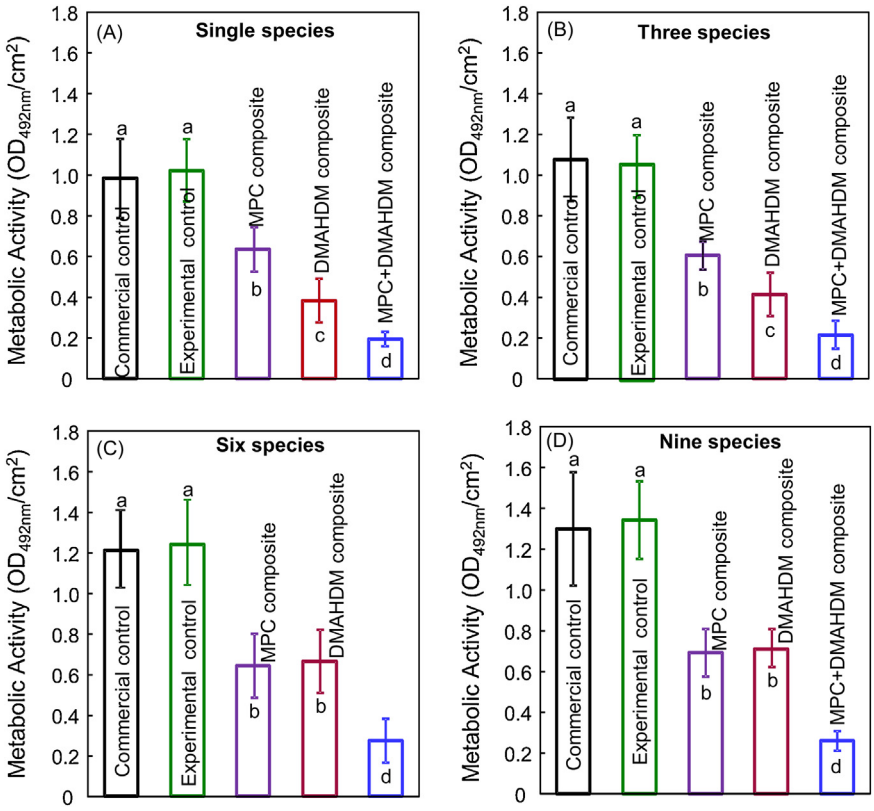

Fig. 6 – XTT metabolic activities of periodontal biofilm on composites: (A) single species biofilms, (B) three-species biofilms, (C) six-species biofilms, and (D) nine-species biofilms (mean ± sd; n = 6). In each plot, values with dissimilar letters are significantly different from each other ($p < 0.05$).

kill capacity at almost 10^8 cells/cm^2. Furthermore, another study indicated that immobilized QAMs were antibacterially active only when the positive-charge density was above a critical threshold (10^{15} N$^+$/cm^2) [39]. In the present study, the composite with 3% DMAHDM had a surface charge density of about 3.5×10^{15} N$^+$/cm^2, which was above a critical threshold, to provide the antibacterial potency.

The main drawback of QAM-containing composites is that the adsorption of salivary proteins on composite surfaces could decrease the efficacy of "contact-inhibition". MPC is a methacrylate with phospholipid polar groups in the side chain, and MPC polymers are highly hydrophilic and can reduce protein adsorption and bacterial adhesion [31,40]. A previous study [25] showed that incorporating MPC into dental composite reduced protein adsorption by approximate an order of magnitude. The protein-repellent mechanism was suggested to be that in the hydrated MPC polymer, there is an abundance of free water but no bound water [40]. The bound water would lead to protein adsorption, while free water around phosphorylcholine groups could detach proteins, thereby repelling protein adsorption [41]. In the present study, SDS as an ionic surfactant was applied to elute the protein attached onto the surface of the resin disks. It should be noted that SDS at a high concentration could destroy the non-covalent bonds such as ionic bonds and hydrogen bonds in proteins, and could even change the conformation of proteins. However, as indicated by the manufacturer, the BCA protein kit could tolerate the SDS up to a high concentration of 5%. Therefore, the relatively low concentration of 1% in the present study was expected to have little effect on the adsorbed protein values.

In vitro single species biofilm models are widely used in research [23,42]. However, clinically, the subgingival biofilm is an aggregation of multispecies bacteria. Therefore, to investigate antibacterial dental materials for inhibition of oral biofilms including periodontal biofilms, it is important to develop multispecies biofilm models. Multispecies biofilms are more challenging to eradicate than single species biofilms and planktonic bacteria [43]. Socransky et al. classified five main microbial complexes in the subgingival biofilm [44]. The early attached dominant species of bacteria are streptococci and members of the yellow and purple complexes, such as *Actinomyces* spp., which soon develop a polymicrobial community [45]. *A. naeslundii* represents 27% of the pioneer strains and is able to adapt to extreme fluctuating conditions, which is necessary for a pioneer bacteria strain to attach to dental surfaces [46]. *S. gordonii* was included because streptococci accounts for 60–90% of initial colonizers of tooth surfaces [47]. Members of the red complexes, such as *P. gingivalis* and *T. forsythia*, were verified to be closely associated with periodontitis [48]. Other studies confirmed the involvement of red complex and several members in the orange complex, such as *P. intermedia*, *P. nigrences*, *P. micra*, *F. nucleatum*, and *A. actinomycetemcomitans*, with the etiology of different periodontal conditions [49]. Therefore, in the present study, these nine

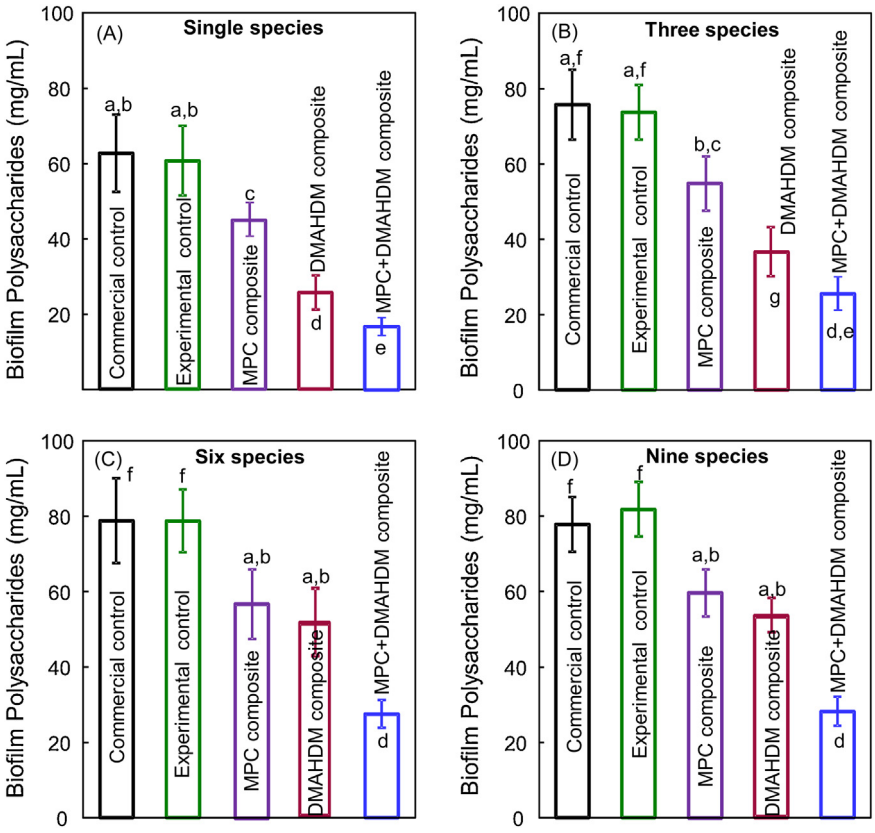

Fig. 7 – Polysaccharide production by periodontal biofilm on composites: (A) single species biofilms, (B) three-species biofilms, (C) six-species biofilms, and (D) nine-species biofilms (mean ± sd; n = 6). In each plot, values with dissimilar letters are significantly different from each other ($p < 0.05$).

species of bacteria were selected and combined to form multispecies biofilms on composites.

Regarding the selection of a QAM, it was shown that when the alkyl chain length of the QAM was increased, its hydrophobicity was increased which enhanced its propensity to penetrate the hydrophobic bacterial membrane [30]. Previous studies showed DMAHDM with an alkyl chain length of 16 exhibited a potent anti-biofilm function against cariogenic and periodontal pathogens [22,26]. However, the present study showed that DMAHDM alone exhibited a decreasing killing efficacy with increasing the number of species in the periodontal biofilms. Since DMAHDM was shown to exhibit no risk of inducing bacterial drug-resistance [50], this lower killing efficacy was mainly attributed to the increased complexity of multispecies biofilms when the number of species was increased from 1 to 9. The self-protective effects of biofilms have been demonstrated to be enhanced in a synergistic manner when multiple species are present [51]. One mechanism involves changes in the composition of the EPS matrix in multispecies biofilm [51]. ESP can be regulated by external stimuli since most glycoproteins are located in the outer membrane of Gram-negative bacteria. In the present study, the EPS level was expressed by measuring the polysaccharide level. The higher polysaccharide levels in multispecies biofilms was probably due to the coaggregation between bacterial interactions, which would be closely relate to the decreased anti-biofilm efficacy of DMAHDM. For example, *F. nucleatum* could possess the unique ability to coaggregate with all of the early and late colonizers such as *S. gordonni*, A. naeslundii, *P. gingivalis* and *P. micra*. This consequently could lead to an enhanced biofilm formation and an increased resistance to antimicrobial agents.

The second mechanism whereby species within a mixed biofilm cooperate to survive in hostile environments involves one member providing conditions that promote survival of other members [52]. The survival of anaerobic periodontal pathogens was enabled when they co-existed with aerobes or facultative anaerobes, since these bacteria could consume the oxygen and thus provide anaerobic conditions within the deeper layers of the biofilm in which anaerobic bacteria could multiply [53]. *P. gingivalis* cold not survive in an aerated environment above 6% O_2 when grown as a monoculture; however, when grown as a co-culture with *F. nucleatum*, *P. gingivalis* could survive O_2 levels of up to 20% [54].

The third mechanism that bacteria within a mixed biofilm cooperate to survive under challenging conditions is speculated to involve one member inducing transient changes in resistance in the proximal neighbors [52]. For example, periodontal pathogens such as *P. gingivalis* and *A. actinomycetemcomitans* survived various antibacterial agents (taurolidine or minocycline) more successfully in a twelve-species biofilm than as a single species biofilm [10]. In addition, single species biofilm of *S. sanguinis* had a higher susceptibility to a novel modified titanium surface compared with

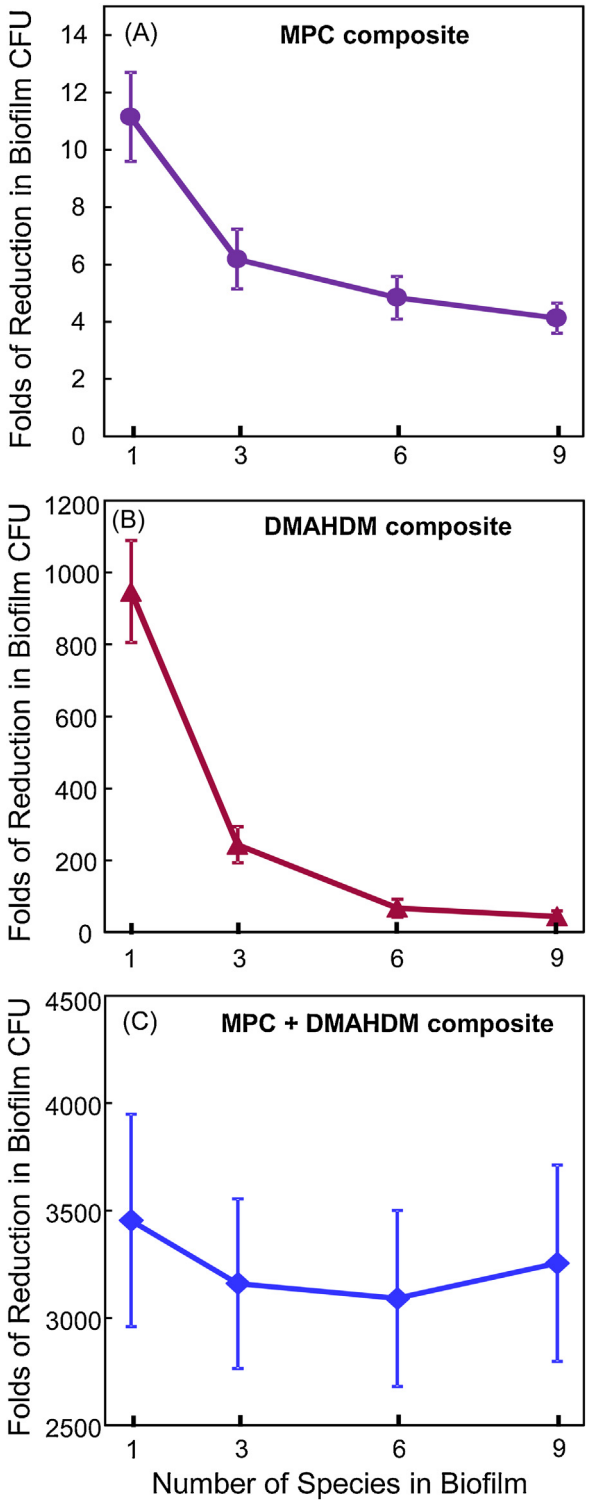

Fig. 8 – Folds of biofilm CFU reduction on: (A) MPC composite, (B) DMAHDM composite, and (C) MPC + DMAHDM composite (mean ± sd; n = 6). For example, in (A), the folds of reduction = biofilm CFU on control composite/CFU on MPC composite. A folds of reduction = 10 means that the biofilm CFU on MPC composite was 1/10 that on control composite. Using a single agent, MPC composite and DMAHDM composite each exhibited a decrease in antibacterial potency with increasing the number of species in the biofilm ($p < 0.05$). However, in sharp contract, dual agents MPC + DMAHDM showed no decrease in antibacterial potency, with statistically similar folds of reduction when the number of species in biofilms was increased from 1 to 9 ($p > 0.1$).

multispecies dental plaque [12]. *S. gordonii* has been shown to expresses several genes that are required to recruit *P. gingivalis* into the mixed biofilm [55]. The genes involved in the synthesis of extracellular polymers in *S. gordonii* likely function as co-aggregation receptors for a surface adhesin expressed by *P. gingivalis* [55]. Similar synergistic interactions could be also found between *P. gingivalis* and *T. denticola*, since isobutyric acid produced by *P. gingivalis* stimulated the growth of *T. denticola* [56].

These previous studies showed results consistent with the present study, exhibited by Fig. 8 to more clearly demonstrate the decreased inhibition efficacy when a single antibacterial agent was used in (A) and (B). For both DMAHDM composite and MPC composite, the folds of reduction in biofilm CFU (the ability to reduce the biofilm CFU) decreased with increasing the number of species in the biofilm. Using the experimental composite as the baseline, the DMADDM composite reduced the single species biofilm CFU by 947 folds. In sharp comparison, it reduced the nine-species biofilm CFU by only 44 folds. However, the inhibition efficacy was dramatically increased when dual agents (MPC + DMAHDM) were used in the composite. Fig. 8C shows that the MPC + DMAHDM composite achieved nearly the same high efficacy as the number of species was increased from 1 to 9 in the biofilm. This was likely due to the synergistic effects between MPC and DMAHDM. MPC with protein-repellency substantially reduced the initial bacteria adhesion and colonization due to the lack of sufficient protein receptors on the resin surface. This in turn likely reduced the biofilm matrix EPS level and the interactions between various species. In a biofilm, the bacterial cells are surrounded by EPS, which is mainly composed of polysaccharides, proteins and extracellular DNA. Previous studies measured the polysaccharide amount which could be more easily determined, instead of measuring the EPS amount [23,26]. In the present study, the polysaccharide was evaluated as a supplement for the CFU and XTT tests. The polysaccharide synthesis for all four types of biofilms was substantially reduced via MPC. EPS with polysaccharide protects pathogens from the antibacterial agents, and contributes to the virulence and pathogenicity of the pathogens. Therefore, the reduction in ESP via MPC could reduce and destroy the protection that the bacteria have, thereby reducing the toxicity of the periodontal pathogens. In addition, such reductions in biofilm matrix EPS and interactions between various species by MPC would be expected to render the DMAHDM much more effective. The multispecies biofilm EPS and the interactions between various species provided the “fortress” against DMAHDM. However, MPC substantially reduced and weakened this “fortress”, thereby exposing the bacteria to DMAHDM with an increased sensitivity. As a result, the MPC + DMAHDM composite was much more effective in inhibiting multispecies biofilms, and achieved a great folds of biofilm reduction that did not decrease with increasing the number of species from 1 to 9 in biofilms. It should be noted that the biofilm CFU counts

and XTT test only demonstrated that the novel bioactive nanocomposite for Class V restorations could substantially decrease the biofilm mass. These tests did not evaluate the virulent factors of the biofilms; biofilm virulence is an important subject and requires further studies.

In spite of the promising results of MPC + DMAHDM composite against multispecies periodontal biofilms, it is not clear how the antibacterial monomers in dental composites can regulate the bacterial composition in a multispecies biofilm. Fluorescence in situ hybridization (FISH) is a valuable technique to visualize and quantify the localization of different microbial species within the biofilm. Our previous study developed a bioactive adhesive material containing QAM and investigated the proportion change of bacterial species in a three-species biofilm (*S. mutans, S. gordonii,* and *S. sanguinis*) [57]. The QAM resin changed the proportion of bacteria in the biofilm, and shifted it from a cariogenic state toward a healthy non-cariogenic state. The proportional shift in bacterial species was attributed to the dual pressure of both the competition among bacteria and the antibacterial challenge [57]. However, that model was limited to only three species. It is unrealistic to make all the species visible simultaneously by FISH in a nine-species biofilm, because the high selectivity and specificity of species-specific probes are difficult to design and achieve. On the other hand, the fluorescence reporting group had limited color which could not distinguish all nine species in the present study. Furthermore, a typical confocal laser scanning microscope can differentiate three to five different fluorescent wavelengths, but microscopes with nine different channels are rare and very expensive [58]. Regarding PCR-based multiple species cell counting, the high specificity of primers and probes in PCR enables it to distinguish target pathogens from the numerous closely-related species in the oral cavity [59]. This method was used in our previous study to verify that a DMAHDM-containing nanocomposite could modulate the biofilm species composition from a cariogenic state toward a non-cariogenic tendency by reducing *S. mutans* in a three-species biofilm model [60]. However, it would be difficult to use PCR to perform accurate quantification on nine species biofilms, due to the following reasons. First, it is difficult for the specificity of primers and probes to guarantee specific recognition to all target microorganisms in a nice-species biofilm. Second, the use of these primers in PCR assays aiming for reproducible quantification is not feasible, because there might be different numbers of 16S rRNA sequences per cell. Third, it is impossible to simultaneously maintain the amplification efficiency for all microorganisms in the biofilm above 90%. Thus, subsequent calculation would calibrate numerous complicated factors and might result in incorrect conclusion. Fourth, it is difficult to isolate specific nucleotide sequences from the genes of closely related species. For example, streptococci group (including *S. mitis*, *S. oralis* and *S. gordonii*) or prevotella group (including *P. intermedia* and *P. nigrensens*) have long been considered difficult [59]. Fifth, the genome information was not available for the target species, and although the strain was available at ATCC, its sequence was unknown from ATCC. Further studies are needed to investigate not only the proportion shift of key pathogens in a subgingival biofilm, but also the microbial modulation by DMAHDM under conditions mimicking the complex biofilm compositions in vivo.

The novel composite of the present study is expected to exhibit strong anti-biofilm properties, with a long-term durability. First, a previous study showed that the MPC-polymerized surface was resistant to mechanical stresses caused by brushing [61]. In the present study, MPC was mixed into the composite, and copolymerized and immobilized in the EBPM resin. MPC was dispersed in the composite and not limited to the surface only. Therefore, the protein-repellent effect is expected to be durable and will not be lost by wear. Second, DMAHDM was also copolymerized with the resin matrix and would not be lost over time. Previous studies indicated that dental composites containing quaternary ammonium monomers exhibited excellent antibacterial functions (with more than 3 log reduction in biofilm CFU) against various types of dental biofilms [23,62]. Third, our previous study investigated the effects of water-aging for 180 days on the mechanical properties and durability of antibacterial and protein-repellent activities of MPC + DMAHDM composite [63]. An oral plaque microcosm biofilm model was employed to evaluate oral biofilm viability vs. water-aging time. The resistance to protein adhesion and the bacteria-eradicating ability were not reduced after water-aging for 180 days, compared to that at 1 day. This demonstrated the durability and long-term benefits because of the covalent bonding of MPC and DMAHDM in the polymeric matrix [63]. Consistent results were also found in a recent study on dental bonding agent containing MPC + DMAHDM [64]. Fourth, the lack of significant QAM leach-out minimizes the cytotoxicity. Our previous study tested uncured monomers and cured resin eluents with human gingival fibroblasts and odontoblast-like cells, showing minimal and acceptable cytotoxicity similar to that of clinically-used dental monomers [22]. Therefore, the composite containing DMAHDM and MPC is expected to have long-term durability and excellent cytocompatibility.

Besides the capability to kill periodontal pathogens, the MPC + DMAHDM composites also contained 20% NACP. The reason for incorporating NACP was to have Ca and P ion release and remineralization of root dentin in order to strengthen the tooth structure [27]. NACP was shown to effectively remineralize enamel [65] and dentin [66], and greatly increased the hardness of tooth structures that were pre-demineralized [67,68]. Further studies are needed to investigate the effects of the new composite on eradicating periodontal multispecies biofilms in Class-V restorations in clinically-relevant experiments, as well as the protection of the periodontium and the strengthening of tooth roots.

5. Conclusion

The present study developed a novel bioactive composite for Class-V restorations containing DMAHDM and MPC, and investigated the effects of the number of species (from 1 to 9) in the periodontal biofilm on the inhibition efficacy of the composite for the first time. Moreover, the effect of dual agents (MPC + DMAHDM) versus single agent on the inhibition efficacy was investigated as a function of the number of species

in the biofilm for the first time. DMAHDM composite was more effective in inhibiting single species biofilm; the inhibition efficacy decreased with increasing the number of species in the biofilms. Adding MPC into the DMAHDM composite increased the efficacy against multi-species biofilms, achieving nearly the same high efficacy against biofilms with 1–9 species. The novel nanocomposite containing dual agents with potent antibiofilm and protein-repellent functions is promising for Class V restorations to treat root caries, inhibit periodontal pathogens, and protect the periodontal tissues.

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