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Novel multifunctional nanocomposite for root caries restorations to inhibit periodontitis-related pathogens

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ABSTRACT

Objectives: The objectives of this study were to: (1) develop a novel multifunctional composite with nanoparticles of silver (NAg), 2-methacryloyloxyethyl phosphorylcholine (MPC), dimethylaminohexadecyl methacrylate (DMAHDM) and nanoparticles of amorphous calcium phosphate (NACP); and (2) investigate biofilm-inhibition via the multifunctional nanocomposite against three species of periodontal pathogens for the first time.

Methods: The multifunctional nanocomposite was fabricated by incorporating NAg, MPC, DMAHDM and NACP into the resin consisting of pyromellitic glycerol dimethacrylate (PMDGDM) and ethoxylated bisphenol A dimethacrylate (EBPADMA). Three species *(Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans and Fusobacterium nucleatum)* were tested for metabolic activity (MTT), live/dead staining, polysaccharide production and colony-forming units (CFU) of biofilms grown on resins.

Results: Incorporation of 0.08% to 0.12% NAg, 3% MPC, 3% DMAHDM and 30% NACP did not compromise the mechanical properties of the composite ($p > 0.1$). The multifunctional nanocomposite reduced protein adsorption to nearly 1/10 of that of a commercial control ($p < 0.05$). For all three species, the biofilm CFU was reduced by about 5 and 1 orders of magnitude via the nanocomposite containing NAg + MPC + DMAHDM, compared to commercial control and the composite with MPC + DMAHDM, respectively.

Conclusions: The novel multifunctional nanocomposite achieved the greatest reduction in metabolic activity, polysaccharide and biofilm growth of three periodontal pathogens.

Clinical significance: The strongly-antibacterial, multifunctional composite is promising for treating root lesions, alleviating periodontitis and protecting the periodontal tissues.

1. Introduction

The incidence of root caries has been increasing as the population is aging and seniors usually have gingival recession and reduced saliva flow [1,2]. Compared with crown caries, root caries progresses much more rapidly since the cementum has a higher solubility than enamel [3]. Root caries is difficult to detect in the early stage due to its obscure location, and filling therapy is necessary when a cavity is formed in the root area. The root caries cavity can be treated with a Class V restoration. However, the subgingival margins of Class V restorations are often difficult to clean, and provide pockets for periodontal bacterial plaque accumulation [4]. This in turn will aggravate the development of periodontitis and enhance the loss of the tooth's attachment [5]. Unfortunately, currently-available resin-based Class V restorative

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materials usually lack the ability to inhibit biofilm growth, and instead, they tend to accumulate more biofilms and plaque [6–8].

It has been demonstrated that bacterial plaque is the initial factor of periodontal disease [9], which is caused by biofilms including species of *Porphyromonas gingivalis* (*P. gingivalis*), *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*), *Fusobacterium nucleatum* (*F. nucleatum*) [10]. *P. gingivalis* is the predominant bacteria in periodontal disease, especially in the diseased region or active region of chronic periodontitis [11]. Besides, its presence is related to the recurrence or aggravation of periodontitis after periodontal treatment [11]. *A. actinomycetemcomitans* is in connection with aggressive periodontitis, with its main pathogenic effect being the reduction of the host resistance, thus causing bone absorption and tissue destruction [12]. *F. nucleatum* is a Gram-negative anaerobe and is correlated with an increased probing depth and progressive periodontal ligament reduction in periodontitis [13]. Therefore, these three periodontitis-related species were selected in the present study.

Composites are widely used in dental fillings due to their excellent esthetics ability and direct-filling performance [14–18]. However, most currently-available composites are challenged with plaque accumulation, which could result in secondary caries and periodontal inflammation [6,19]. Hence, efforts were made to develop antibacterial resin composites. Quaternary ammonium methacrylates (QAMs) were effective in contact-killing of bacteria and were copolymerized in resins to achieve antibacterial activities [20–23]. With the increase of the alkyl chain length (CL) in its chemical structure, the antibacterial capability of QAMs could be increased. A previous study showed that a composite containing dimethylaminohexadecyl methacrylate (DMAHDM) with a CL of 16 demonstrated the strongest antibacterial properties among all the groups tested [24].

A second approach to reduce caries occurrence is protein-repellency. Salivary proteins coating on the surface of resin provides the medium for bacterial adhesion to the composite [25,26]. Thus, it is important to repel the salivary proteins from the composite surface. 2-methacryloyloxyethyl phosphorylcholine (MPC) is a common biopolymer with phospholipid polar groups [27,28]. Previous studies showed that resins containing MPC had favorable protein-repellent properties, which enhanced the inhibition efficacy of the resins against cariogenic biofilms [29,30].

A third approach for suppressing dental caries is the use of silver (Ag) nanoparticles. Ag has shown to possess a long-lasting antimicrobial effect [31–33] and has less bacterial resistance than antibiotics [34]. Our previous studies incorporated nanoparticles of silver (NAg) into dental composites, which effectively inhibited *Streptococcus mutans (S. mutans)* growth [35].

In the fourth approach, bioactive composites were developed to contain nanoparticles of amorphous calcium phosphate (NACP), which exhibited long-lasting calcium (Ca) and phosphate (P) ion releases [36–38]. The resins containing NACP possessed acid-neutralization ability which could raise an acidic pH to a nearly neutral pH, thus protecting the tooth structures from acid challenges. In addition, the released ions promoted tooth remineralization, resulting in the mineral regeneration in the tooth lesions [36–38].

To date, there has been no report on developing a dental composite for Class V restorations that combined all the four aforementioned approaches, with the purpose of inhibiting periodontitis-related pathogens. Our pilot study [39] incorporated DMAHDM, MPC and NACP into a resin to achieve a good antibacterial property against periodontal bacteria. However, that material contained no NAg [39]. Furthermore, that study showed that the composite with DMAHDM + MPC had a relatively weak antibacterial function against certain periodontal bacterial species, for example, *F. nucleatum* [39]. In addition, DMAHDM and MPC were both copolymerized in resin. Hence, the composite was only effective in killing the bacteria that were in contact with the composite surface. The composite was not able to kill the pathogens suspended in the periodontal pocket way from the composite surface. In contrast, NAg was reported to be effective in inhibiting *F. nucleatum* [40], and NAg could be capable of long-distance killing of bacteria, due to the Ag ion release to inhibit bacteria away from the composite surface [41]. Therefore, it would be highly desirable to combine NAg with DMAHDM, MPC and NACP in a new multifunctional composite for Class V restorations to inhibit periodontitis-related bacteria.

Accordingly, the objectives of the present study were to: (1) develop a novel multifunctional nanocomposite for Class V restorations with incorporations of NAg, DMAHDM, MPC and NACP; and investigate its inhibition effects on biofilms of three periodontal pathogens (*P. gingivalis, A. actinomycetemcomitans,* and *F. nucleatum*) for the first time. It was hypothesized that: (1) the nanocomposite containing NAg, DMAHDM, MPC and NACP would not compromise the mechanical properties compared to control composite without bioactive agents; (2) NAg, DMAHDM and MPC together would be much more effective than each agent alone, and would achieve the maximal reduction in biofilms of the three periodontal pathogens; (3) different periodontal pathogens would exhibit different biofilm properties with different killing efficacy when growing on the new nanocomposite.

2. Materials and methods

2.1. Preparation of nanocomposite containing NAg, MPC, DMAHDM and NACP

Ethoxylated bisphenol A dimethacrylate (EBPADMA, Esstech, Essington, PA, USA) and pyromellitic dianhydride glycerol dimethacrylate (PMDGDM, Esstech) were mixed at a mass ratio of 1:1 to form the EBPADMA-PMDGDM resin [42]. This resin was then render light-curable with 0.8% ethyl 4-N,N-dimethylaminobenzoate and 0.2% camphorquinone [42]. This resin matrix was referred as EBPM.

Silver 2-ethylhexanoate (Strem, Newburyport, MA, USA) was dissolved into 2-(tert-butylamino)ethyl meth-acrylate (TBAEMA, Sigma-Aldrich, St. Louis, MO, USA). This was achieved by gentle stirring, using silver 2-ethylhexanoate/(Silver 2-ethylhexanoate + TBAEMA) mass fractions of 0%, 8%, 12% and 16%, respectively [43]. TBAEMA could promote the dissolution of the Ag-salt in the resin monomers [35,41]. Subsequently, different Ag salt amounts were added to the EBPM resin to form the EBPM-NAg resin, yielding 0%, 0.08%, 0.12% and 0.16% mass fractions of the Ag salt in the final composite, respectively. These mass fractions were selected following previous studies [35,41]. Mass fractions of greater than 0.16% were not used to avoid negatively affecting the mechanical properties of the resin.

MPC was obtained commercially (Sigma-Aldrich), which was synthesized according to a classical method described previously [30]. The MPC powder was added into the EBPM-NAg resin monomers, yielding a 3% MPC mass fraction in the final composite. This mass fraction was selected following a previous study [30]. This mass fraction was shown to produce a strong protein-repellency without adversely affect the mechanical properties of the resin [30].

DMAHDM was synthesized using a modified Menschutkin reaction [44,45]. Briefly, 10 mmol of 1-bromohexadecane (BHD, TCI America, Portland, OR, USA) and 10 mmol of 2-(dimethylamino)ethyl methacrylate (DMAEMA, Sigma-Aldrich) were mixed with 3 g of ethanol in a 20 mL scintillation vial. The vial was constantly stirred at 70 °C for 24 h. Subsequently, the solvent was removed via evaporation, yielding DMAHDM as a colorless, clear, and viscous liquid [44]. DMAHDM was then incorporated into the EBPM-NAg-MPC resin at a DMAHDM mass fraction of 3% in the final nanocomposite. This mass fraction was selected following previous studies [30,44]. The 3% DMAHDM could produce a potent antibacterial activity without compromising the mechanical properties of the composite [30,44].

NACP were synthesized using a spray-drying technique [37]. Briefly, dicalcium phosphate anhydrous and calcium carbonate were dissolved in acetic acid to produce calcium (Ca) and phosphate (P) concentrations of 8 mmol/L and 5.333 mmol/L, respectively. The

solution was sprayed into a heated chamber of the spray-drying machine. An electrostatic precipitator was employed to collect the dried particles. This produced NACP with a mean particle size of 116 nm [37]. In addition, barium boroaluminosilicate glass particles with a median size of 1.4 μm (Caulk/Dentsply, Milford, DE, USA) were used as a co-filler for mechanical reinforcement. The glass particles were silanized using 4% 3-methacryloxypropyltrimethoxysilane and 2% npropylamine [37]. The fillers were mixed with the EBPM-NAg-MPC-DMAHDM resin to achieve a NACP mass fraction of 30% and a glass filler mass fraction of 35% in the final composite, yielding a cohesive composite paste. This mass fraction was selected following a previous study [46]. The 30% NACP could provide strong acid-neutralization and Ca and P ion release capabilities, thereby promoting tooth remineralization [46].

The mixed composite paste was placed into molds of 2 × 2 x 25 mm for mechanical testing, and molds of 9 mm in diameter and 2 mm in thickness for protein and biofilm experiments. Each sample was covered in Mylar strips and light-cured (Triad 2000, Dentsply, York, PA) for 1 min on each open side of the mold [38]. Five composites were fabricated:

(1) 35% EBPM + 30% NACP + 35% glass (referred to as EN control);
(2) 29% EBPM + 30% NACP + 3% MPC + 3% DMAHDM + 35% glass (referred to as ENMD);
(3) 28.92% EBPM + 30% NACP + 3% MPC + 3% DMAHDM + 0.08% NAg + 35% glass (referred to as ENMD-0.08NAg);
(4) 28.88% EBPM + 30% NACP + 3% MPC + 3% DMAHDM + 0.12% NAg + 35% glass (referred to as ENMD-0.12NAg);
(5) 28.84% EBPM + 30% NACP + 3% MPC + 3% DMAHDM + 0.16% NAg + 35% glass (referred to as ENMD-0.16NAg).

For mechanical testing, two commercial nanocomposites, Renamel Microfill (Cosmedent, Chicago, IL, USA) and Heliomolar (Ivoclar, Amherst, NY, USA), were used as controls. They are referred to as Renamel control and Heliomolar control, respectively. They were selected because both of them are indicated for Class V restorations for root caries according to the manufacturers. For biofilm experiments, Renamel was used as a control. All specimens were light-cured in the same manner as described above.

2.2. Mechanical testing and dentin shear bond strength testing

The composite specimens were stored at 37 °C for 24 h, and then fractured in three-point flexure with a 10-mm span at a crosshead-speed of 1 mm/min on a computer-controlled Universal Testing Machine (5500R, MTS, Cary, NC, USA) [46]. Flexural strength (S) was calculated as: $S = 3PmaxL/(2bh^2)$, where P is the fracture load, L is span, b is specimen width and h is thickness. Elastic modulus (E) was calculated as: $E = (P/d)(L^3/[4bh^3])$, where load P divided by displacement d is the slope in the linear elastic region [46].

To investigate whether adding NACP, MPC, DMAHDM and NAg into the resin would affect the bonding strength between dentin and composite, dentin shear bond strength test was performed. Scotchbond Multi-Purpose (SBMP, 3 M, St. Paul, MN, USA) was used to bond with the composites as described above. As a widely used commercial dental bonding system, the SBMP primer contained 35–45% 2-hydroxyethyl methacrylate (HEMA, Esstech), 10–20% copolymer of acrylic and itaconic acids, and 40–50% water. SBMP adhesive contained 60–70% bisphenol A glycidyl dimethacrylate (BisGMA, Esstech) and 30–40% HEMA.

The use of extracted human teeth was approved by the University of Maryland Baltimore (UMB) and West China Hospital of Stomatology Institutional Review Board. Extracted healthy human third molars were obtained from the dental clinic. The collected teeth were disinfected in a 0.005% promodyne solution for 4 h, and then stored at 4 °C in distilled water. The cleaned molars were subsequently sawed to remove the crowns (Isomet, Buehler, Lake Bluff, IL, USA), then ground perpendicularly to the longitudinal axis on SiC paper (320 grit) until occlusal enamel was completely removed [22]. The dentin surface was acid-etched with 37% phosphoric acid gel for 15 s and rinsed with running distilled water [22,41]. SBMP primer was applied, and the solvent was removed using an air stream. Then SBMP adhesive was applied and light-cured for 10 s (Optilux-VCL401, Demetron, Danbury, CT). A stainless-steel iris, having a central opening with a diameter of 4 mm and a thickness of 1.5 mm, was held against the SBMP adhesive-treated dentin surface. The central opening was filled with a composite and then light-cured for 60 s [41]. The composites tested were Renamel control, Helimolar control, EN control, EMD, EMD-0.08Ag, EMD-0.12Ag and EMD-0.16Ag. The bonded samples were stored in distilled water at 37 °C for 24 h. Dentin shear bond strength, S_D, was measured following previous studies [22,41]. Briefly, a chisel was held parallel to the dentin-composite interface and loaded with a Universal Testing Machine (MTS, Eden Prairie, MN, USA) at 0.5 mm/min until the dentin-composite bond failed. S_D was calculated as: $S_D = 4P/(\pi d^2)$, where P is load at failure, and d is the diameter of the composite [22,41]. Ten teeth were tested for each composite (n = 10).

2.3. Protein adsorption on composites

The composite ENMD-0.16NAg was not included in the following tests because it had significantly lower mechanical properties than the other composites ($p < 0.05$). ENMD-0.12NAg was selected as the experimental group for the following tests because it had good mechanical properties, while 0.12% NAg would produce stronger antibacterial effect than 0.08% NAg. Therefore, the following four groups were tested for protein adsorption and biofilm experiments:

(1) Renamel control;
(2) EN control;
(3) ENMD;
(4) ENMD-0.12NAg.

The cured composite disks were immersed in deionized water at 37 °C for 24 h. A micro bicinchoninic acid (BCA) method was used to determine protein adsorption [47]. Each disk was immersed in phosphate buffered saline (PBS) for 2 h, and then immersed in 4.5 g/L bovine serum albumin (BSA, Sigma-Aldrich) solution at 37 °C for another 2 h. The disks were rinsed with PBS, and then immersed in 1% sodium dodecylsulfate (SDS) in PBS and sonicated for 20 min to detach the BSA adsorbed on the disk. Following previous studies, a protein analysis kit (micro BCA, Fisher Scientific, Pittsburgh, PA, USA) was employed to measure BSA concentration in the SDS solution. 200 μL of BCA reagent and 25 μL of SDS solution were added into a 96-well plate and incubated at 60 °C for 30 min. The absorbance at 562 nm was recorded by a microplate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA, USA). Standard curves were prepared with the BSA standard [29].

2.4. Bacteria culture and biofilm formation on composites

The use of bacteria was approved by University of Maryland Baltimore Institutional Review Board. *A. actinomycetemcomitans ATCC 43717, F. nucleatum ATCC* 25586 and *P. gingivalis ATCC33277* were obtained from ATCC (Manassas, VA, USA). Each species was grown in a tryptic soy broth (TSB, Sigma-Aldrich) supplemented with 1 mg/L of menadione, 5 mg/L of hemin, 0.5 g/L of L-cysteine hydrochloride, 5 g/L of yeast extract at 37 °C anaerobically (5% H_2, 5% CO_2, 90% N_2) [48]. The inoculum was adjusted to 10^7 colony-forming unit counts (CFU/mL), based on the OD_{600nm} standard curve versus CFU/mL for each bacterial species [48].

The composite disks were immersed in 200 mL of deionized water and stirred with a magnetic bar at 100 rpm for 1 h to remove any uncured monomers, following a previous study [49]. Subsequently, the

disks were sterilized with ethylene oxide (AnproleneAN 74i, Andersen, HawRiver, NC, USA) and de-gassed for 1 week, following manufacturer's instructions.

The human saliva collection protocol was approved by the University of Maryland Baltimore Institutional Review Board. Saliva was collected from 12 healthy adult donors with natural dentition, without periodontal disease or active caries, and without using antibiotics during the last 3 months. The donors did not brush their teeth for 24 h and did not intake any food or water for 2 h prior to the donation. The twelve donors each donated an equal volume of saliva, which was combined to form the saliva sample. The saliva was centrifuged at 3000 rpm for 20 min. The supernatant was taken and filter-sterilized through sterile 0.22 μm filters (VWR International, Radnor, PA, USA) to remove the bacteria and obtain sterile saliva [50]. Then the composite disks were immersed in the sterile saliva at 37 °C for 2 h to coat a salivary pellicle on the composite. The salivary pellicle coating on the resin facilitated bacteria attachment and biofilm formation on the specimens [50]. Each bacteria species was individually cultured to form single-species biofilms on the specimens to determine the antibacterial efficacy against each species, following a previous study [50].

The salivary pellicle-coated composite disks were transferred to a new 24-well plate, and then each bacterial species was inoculated at a concentration of 10^7 CFU/mL in 1.5 mL of culture medium into each well onto a composite disk. After 24 h of culture, the composite disks with adherent biofilms were transferred to new 24-well plates filled with fresh culture medium, and incubated for another 24 h. This totaled 2 days of culture which was previously shown to be suitable to form relatively mature biofilms on resins [51].

2.5. MTT metabolic assay of biofilms

MTT assay is a method of detecting cell survival and growth. This assay relies on the enzymatic reduction of a yellow tetrazolium salt, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), to form a purple formazan crystal in metabolically active cells [43]. Each group contained eighteen nanocomposite disks for the metabolic assay, with six disks for each bacterial species. Composite disks with 2-day biofilms were transferred into new 24-well plates. One mL of MTT dye (0.5 mg/mL MTT in PBS) was dropped into each well and incubated at 37 °C in 5% CO_2 for 1 h. These disks were then transferred to new 24-well plates filled with 1 mL of dimethyl sulfoxide (DMSO) in each well to solubilize the formazan crystals [43]. The plates were gently mixed and incubated for 20 min at room temperature in a dark room. After that, 200 μL of the DMSO solution was transferred to a 96-well plate after mixing. The absorbance at $OD_{540\ nm}$ was measured using a microplate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA, USA). Higher absorbance indicates a higher metabolic activity in the biofilm on the composite [43].

2.6. Live/dead bacteria staining of biofilms

Composite disks with 2-day biofilms were rinsed with cysteine peptone water (CPW) to remove any non-adherent bacteria. These disks were stained with Live/dead bacterial kit (Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions. Briefly, 2.5 μM propidium iodide and 2.5 μM SYTO 9 was mixed and used to stain the composite disks for 15 min. Dead bacteria were stained by propidium iodide to emit a red fluorescence, and live bacteria were stained with SYTO 9 to produce a green fluorescence [43]. Biofilms were imaged using an inverted epifluorescence microscope (TE2000-S, Nikon, Melville, NY, USA). Three disks were examined for each group with each bacterial species, requiring a total of 36 disks for live/dead staining. Five randomly chosen view fields were taken for each disk, yielding a total of 15 images for each composite with each bacterial species.

2.7. Biofilm polysaccharide production measurement

Six composite disks were made for each bacterial species, using a total of eighteen disks for each group for the polysaccharide production measurement. Polysaccharides in the extracellular polymeric substance (EPS) were measured using a phenol-sulfuric acid method [39]. Each disk with 2-day biofilm was stored in a vial with 2 mL CPW, with the collection of biofilm via sonication/vortexing (Fisher, Pittsburg, PA, USA). The precipitate was collected by centrifugation, then rinsed with PBS and resuspended in 1 mL of deionized water. The supernatant was discarded by decantation. Subsequently, 1 mL of 6% phenol solution was added into the vial, followed by 5 mL of sulfuric acid (95–97%). After the vial was incubated for 30 min, 100 μL of the solution was transferred to a 96-well plate. The amount of polysaccharide was measured at 490 nm with the microplate reader. Six glucose concentrations of 0, 5, 10, 20, 50 and 100 mg/L were used as standard in the conversion of OD readings [39].

2.8. Biofilm colony-forming unit (CFU) counts

Six composite disks were made for each bacterial species, requiring eighteen disks for each group. Disks with 2-day biofilms were transferred into tubes filled with 2 mL of CPW, and the biofilms were collected by sonication/vortexing. Tryptic soy blood agar plates (supplemented with 5% sheep blood, 1 mg/L menadione, 5 mg/L hemin, 0.5 g/L L-cysteine hydrochloride, 5 g/L yeast extract) were used, following ATCC's instructions. Biofilm suspensions were serially-diluted and spread onto agar plates, and then incubated at 37 °C anaerobically for 48 h for CFU analysis. The number of colonies was counted by a colony counter (Reichert, NY, USA), which was used, along with the dilution factor, to calculate CFU counts [41].

2.9. Statistical analyses

All data were checked by Kolmogorov-Smirnov and verified for normal distribution. One-way and two-way analyses of variance (ANOVA) were performed to detect the significant differences. Tukey's multiple comparison test was used to compare the data at a p value of 0.05. Statistical analyses were performed by SPSS 19.0 software (SPSS, Chicago, IL, USA) at an alpha of 0.05.

3. Results

The mechanical properties of the composites are plotted in Fig. 1: (A) Flexural strength and (B) elastic modulus (mean ± SD; n = 6). ENMD-0.16NAg had significantly lower flexural strength and elastic modulus than other groups ($p < 0.05$). All other composites had similar flexural strengths and elastic modulus ($p > 0.1$), suggesting that ENMD-0.08NAg and ENMD-0.12NAg could potentially be used in restorations where Heliomolar and Renamel are used, with the additional benefits of antibacterial properties. Dentin shear bond strengths are plotted in Fig. 1C (mean ± sd; n = 10). All composites had similar bond strengths ($p > 0.1$), indicating that adding NACP, MPC, DMAHDM, NAg into the resin did not affect the bonding strength between dentin and the composite. Based on these results, ENMD-0.12NAg was selected for the following experiments since a previous study showed that increasing the level of NAg could achieve a stronger antibacterial effect [35].

Protein adsorption on composites is plotted in Fig. 2 (mean ± SD; n = 6). Renamel control and EN control had the greatest amounts of protein adsorption ($p > 0.1$). ENMD and ENMD-0.12NAg substantially decreased the protein adsorption, which was nearly 1/10 that of Renamel and EN controls ($p < 0.05$).

The metabolic activity results of 2-day biofilms are shown in Fig. 3 for: (A) *P. gingivalis*, (B) *A. actinomycetemcomitans*, and (C) *F. nucleatum* (mean ± SD; n = 6). Renamel and EN controls had similar values

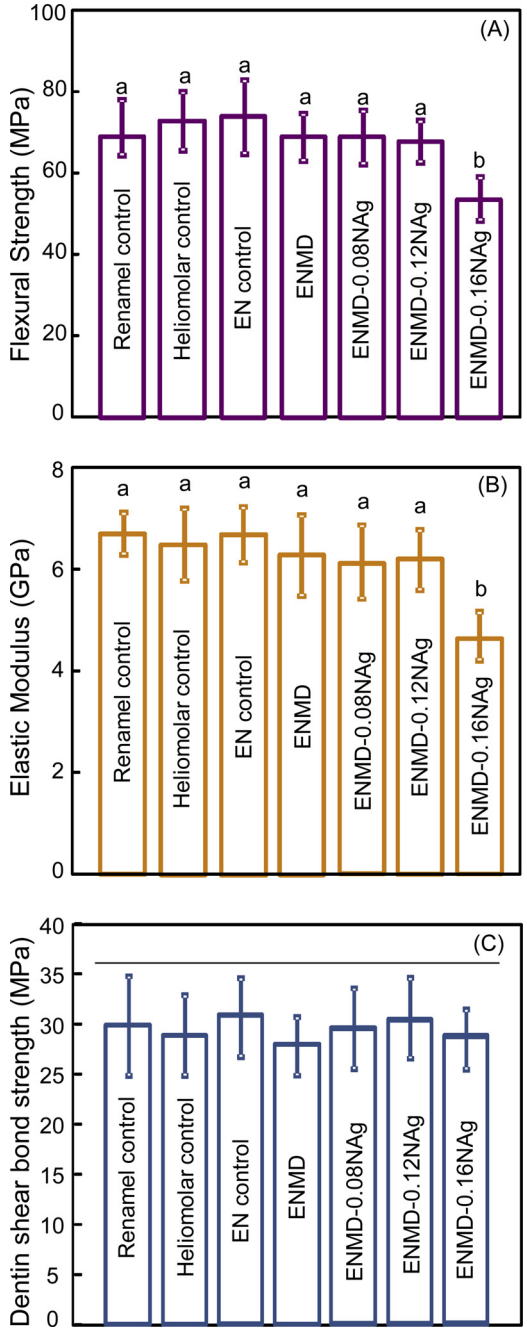

Fig. 1. Mechanical properties of nanocomposites and dentin shear bond strength: (A) Flexural strength, (B) elastic modulus (mean ± SD; n = 6), and (C) dentin shear bond strength tested after storage in water for 24 h (mean ± SD; n = 10). ENMD-0.08NAg and ENMD-0.12NAg had flexural strength and modulus similar to Renamel and EN composite controls. All composites had similar dentin shear bond strengths ($p > 0.1$) Dissimilar letters in (A) and (B) indicate values that are significantly different from each other ($p < 0.05$). Horizontal line in (C) indicates values that are not significantly different from each other ($p > 0.1$).

($p > 0.1$). ENMD and ENMD-0.12NAg yielded much lower metabolic activity of biofilms than Renamel and EN controls ($p < 0.05$). In addition, the incorporation of NAg in ENMD-0.12NAg helped reduce the metabolic activity of biofilms for all three species of bacteria, compared to ENMD without NAg ($p < 0.05$).

Fig. 4 shows representative live/dead images of 2-day biofilms on composites. The three species (*P. gingivalis, A. actinomycetemcomitans* and *F. nucleatum*) is labeled on the top. The four composites (Renamel control, EN control, ENMD, ENMD-0.12NAg) are labeled on the left side. Renamel and EN had full coverage of primarily live bacteria. In contrast, ENMD and ENMD-0.12NAg had much less biofilms, and the bacteria were mainly compromised.

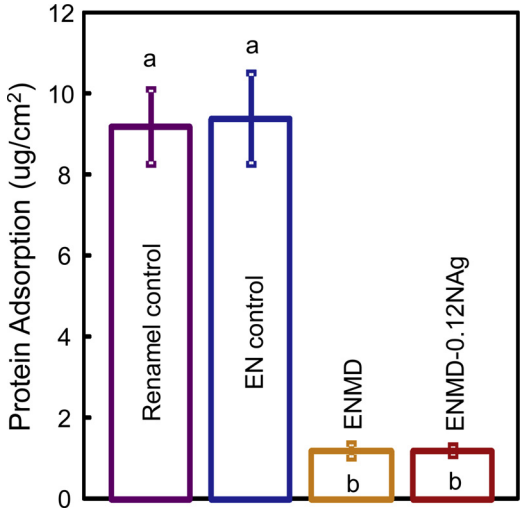

Fig. 2. Protein adsorption onto nanocomposite surfaces (mean ± SD; n = 6). The ENMD and ENMD-0.12NAg nanocomposites showed much less protein adsorption, which were nearly 1/10 those of Renamel and EN composites ($p < 0.05$). Dissimilar letters indicate significantly different values ($p < 0.05$).

Polysaccharide production by 2-day biofilms on composites are plotted in Fig. 5 for: (A) *P. gingivalis*, (B) *A. actinomycetemcomitans*, and (C) *F. nucleatum* (mean ± SD; n = 6). Polysaccharide on Renamel and EN were similar ($p > 0.1$). The polysaccharide amount on ENMD was much less than that on controls ($p < 0.05$). ENMD-0.12NAg had the least polysaccharide production by the biofilms on the composite ($p < 0.05$).

The 2-day biofilm CFU counts are plotted in Fig. 6 for: (A) *P. gingivalis*, (B) *A. actinomycetemcomitans*, and (C) *F. nucleatum* (mean ± SD; n = 6). Different species had different CFU counts on the composites, some having close to 10^{10} CFU/disk, and some with close to 10^4 CFU/disk. For each species, Renamel and EN had similar CFU values ($p > 0.1$). In contrast, the CFU counts of the three species on ENMD decreased significantly ($p < 0.05$), while ENMD-0.12NAg had the lowest CFU ($p < 0.05$). For *P. gingivalis* and *A. actinomycetemcomitans*, ENMD reduced the CFU counts by nearly four orders of magnitude, while ENMD-0.12NAg reduced the CFU counts by about five orders of magnitude. For *F. nucleatum*, ENMD and ENMD-0.12NAg reduced the CFU counts by nearly three and five orders of magnitude, respectively.

4. Discussion

The present study developed a novel multifunctional nanocomposite containing NAg, MPC, DMAHDM and NACP for root caries restorations to inhibit periodontitis-related pathogens for the first time. The hypotheses were proven that adding NAg, MPC, DMAHDM and NACP did not compromise the mechanical properties; NAg + MPC + DMAHDM had an excellent anti-biofilm potency against periodontal pathogens, which was more potent than MPC + DMAHDM; the three species of periodontal pathogens exhibited different biofilm properties and inhibition efficacy on the new nanocomposite. Therefore, the new composite containing 3% MPC + 3% DMAHDM + 0.12% NAg (ENMD-0.12 NAg) was shown to be promising for Class V restorations to inhibit periodontal biofilms, manifested by the reduction of biofilm CFU by nearly 5 orders of magnitude for the three periodontitis-related pathogens.

Periodontal disease is prevalent worldwide and represents a severe public oral health problem [52]. Nearly 45.9% of the population 30

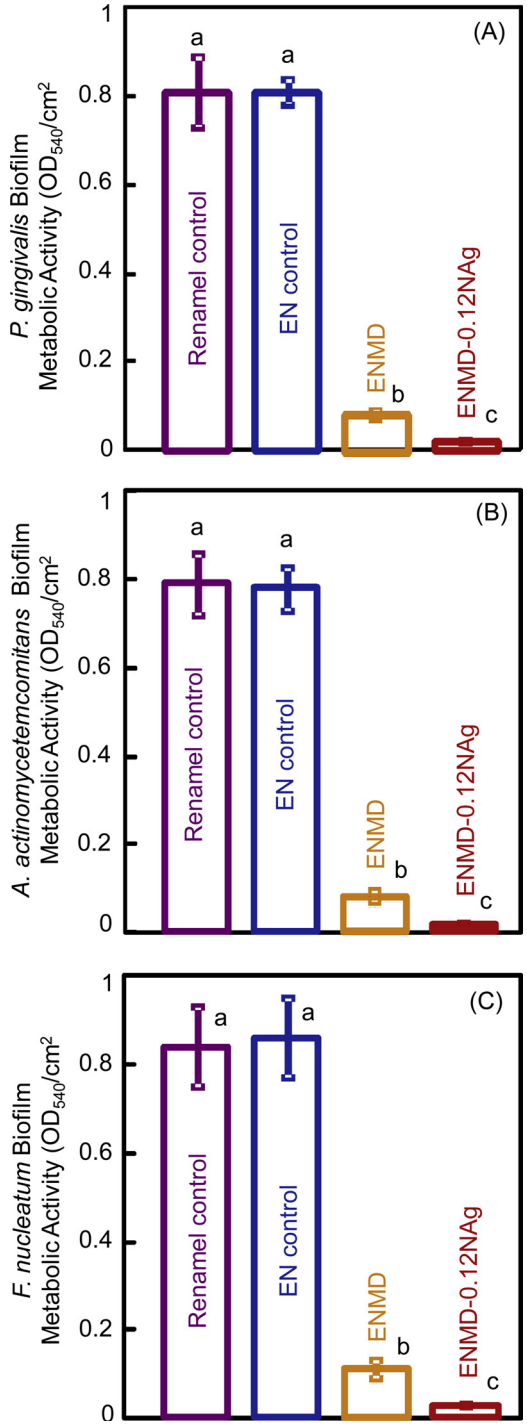

Fig. 3. Metabolic activity of 2-day biofilms on composites of the three species of periodontal pathogens measured via the MTT assay: (A) *P. gingivalis*, (B) *A. actinomycetemcomitans*, and (C) *F. nucleatum* (mean ± SD; n = 6). Dissimilar letters indicate values that are significantly different from each other ($p < 0.05$).

years of age and older in the USA suffer from periodontal disease [1]. The increasing prevalence of root caries is highly related to the increasing prevalence of periodontal disease [53]. Class V restorations often have subgingival margins, which are difficult to clean with pockets for periodontal pathogen growth. This could initiate a vicious cycle, with biofilms resulting in more gingival recession, which in turn results in more root exposure and more root caries. Hence, it would be highly desirable to develop a multifunctional bioactive composite for Class V restorations that has the capability to inhibit periodontitis-related bacteria to prevent periodontitis and protect the periodontal attachment.

In the present study, *P. gingivalis, A. actinomycetemcomitans* and *F. nucleatum* were used to grow biofilms on the composites. Three points should be noted. First, *P. gingivalis* and *A. actinomycetemcomitans* are commonly detected in gingivitis [10]. Gingivitis is reversible and does not affect the underlying supporting structures of the teeth. However, if gingivitis is not treated well, it can advance to periodontitis, which will lead to bone resorption and eventually result in tooth loss [54]. Second, *P. gingivalis* and *A. actinomycetemcomitans* are also frequently detected in subgingival plaque of periodontitis and peri-implantitis sites [54]. They produce virulence factors in periodontal pockets, resulting in the loss of the periapical bone and alveolar bone [54]. Third, *F. nucleatum* is usually found in advanced periodontitis, and plays synergistic effects with *P. gingivalis* on the virulence [13]. However, currently-available composites for Class V restorations (such as those used as commercial controls in the present study) do not inhibit periodontal pathogens.

DMAHDM has a wide antimicrobial spectrum, and its antibacterial mechanism is contact-inhibition [55]. The positively-charged quaternary amine N^+ sites contact the negatively-charged cell membrane of the bacteria, resulting in membrane disruption and cytoplasmic leakage [55]. Since the antibacterial monomer is copolymerized with the resin matrix by forming a covalent bond with the polymer network [24], DMAHDM is immobilized in the composite and would not be released or lost over time. This feature ensures that DMAHDM would constantly fulfill its antibacterial capability, but also limits its antibacterial range to close to its surface only. Therefore, DMAHDM can effectively inhibit the bacteria grown on the composite; but it is unable to kill the bacteria suspended in the periodontal pockets away from the composite surface.

MPC is hydrophilic with free water in the hydrated MPC polymer [27], which could help detach proteins [28]. The MPC polymer has been demonstrated to resist protein adsorption and bacterial adhesion [30]. The present study showed that nanocomposites with MPC had protein adsorption only 1/10 that of Renamel and EN. Nanocomposite covered with salivary proteins affects the contact-killing efficacy of DMAHDM. Hence, incorporation of MPC can repel proteins, thereby exposing the composite surface to increase the contact-inhibition efficacy of DMAHDM. Indeed, the composite with DMAHDM and MPC together (the ENMD composite) achieved much greater antibacterial effect than either agent lone. Furthermore, MPC is also immobilized in the resin and would not be released or lost over time, thereby providing a long-term protein-repellent function.

The antibacterial action of Ag is believed to be that the released Ag ions would interact with the vital enzymes of the bacteria, leading to enzyme inactivation [41]. Then, the DNA in the bacteria would lose its replication ability, thus causing cell death [56]. A previous study showed that adding NAg into an adhesive greatly reduced the biofilm viability and metabolic activity [41]. The biofilm CFU counts for *mutans streptococci*, total streptococci, and total microorganisms on bonding agents containing NAg were reduced by more than an order of magnitude, compared to those of controls [57]. In addition, NAg was also shown to have strong antibacterial activities against a number of periodontal pathogens, including *A. actinomycetem-comitans, F. nucleatum, P. gingivalis, P. denticola, C. gracilis, P. intermedia, B. forsythus, C. rectus,* and *E. corrodens* [58]. The first advantage of NAg is the small particle size of 2.6 nm [59], yielding a high surface area to mass ratio. This enabled a small quantity of NAg to be sufficient for the composite to be strongly antibacterial, thus avoiding the need to use a high mass fraction of NAg and compromising the nanocomposite's mechanical properties and esthetics [59]. The second advantage is that Ag is known to have superior biocompatibility and low toxicity to humans, with silverware being used in dining for centuries [60]. The third advantage is that the NAg-containing resins have long-lasting antibacterial effects. For example, one study indicated that a resin containing NAg showed

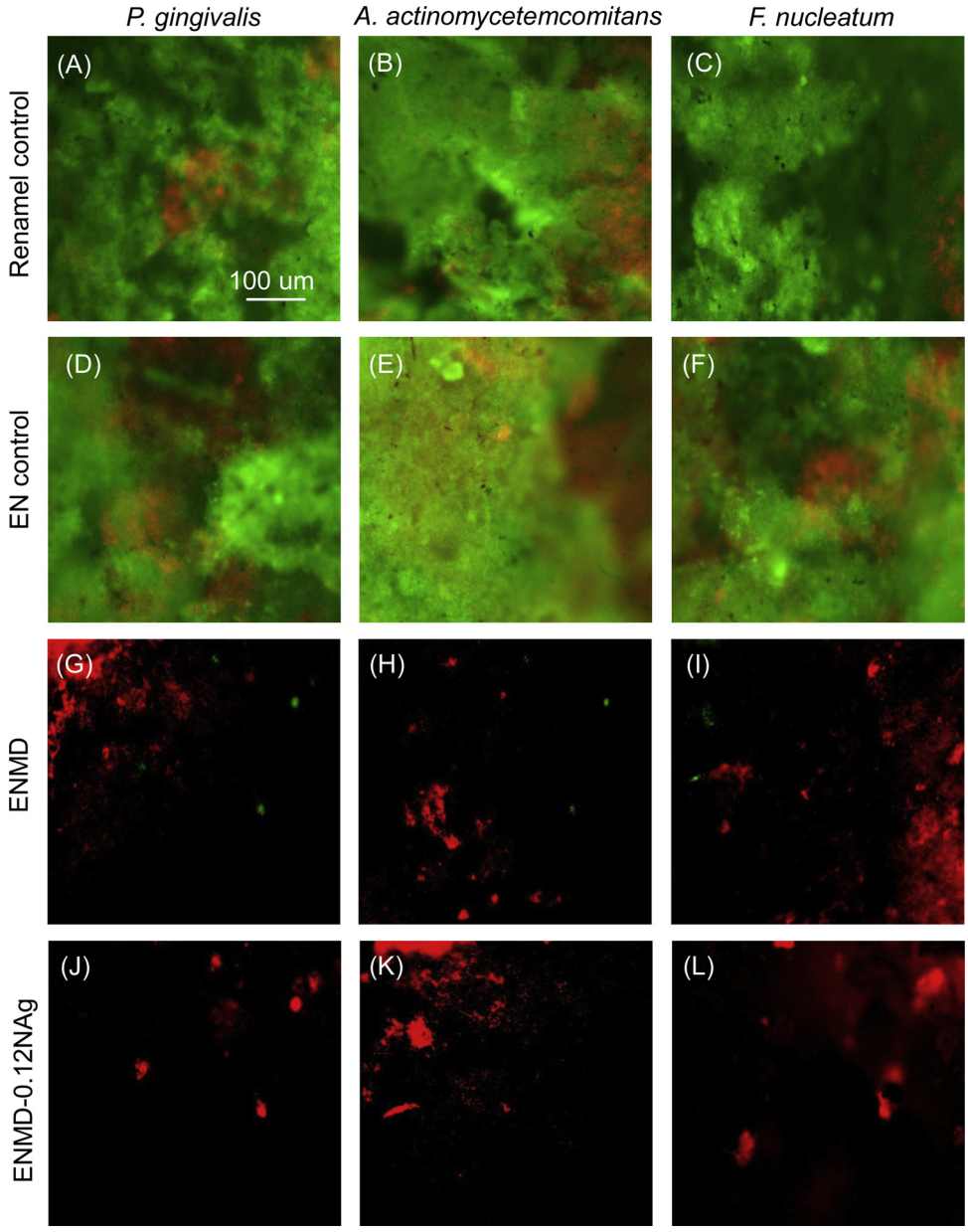

Fig. 4. Typical live/dead staining images of 2-day biofilms of the three species of periodontal pathogens on the four nanocomposites: (A–C) Renamel control, (D–F) EN control, (G–I) ENMD nanocomposite, (J–L) ENMD-0.12NAg nanocomposite. All images had the same magnification as shown in (A). Live bacteria were stained green. Compromised bacteria were stained red. The species names are listed on the top (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

antibacterial activity even after 12 months of water-ageing [43]. The fourth advantage is that Ag has been shown to cause less bacterial resistance than antibiotics, which alleviates the drug-resistance concern [34]. The fifth advantage is that NAg is capable of long-distance killing of bacteria due to the release and diffusion of the Ag ions. This is expected to be able to inhibit the suspended bacteria in the periodontal pockets away from the composite surface, a subject that will be investigated in the next study. In the present study, ENMD-0.16NAg had lower flexure strength and elastic modulus, likely due to a darker greyish color of the paste at 0.16% NAg negatively affecting the photopolymerization efficacy. Therefore, the 0.12% silver concentration was used in the composite to produce the most potent antibacterial effect while also obtaining the best mechanical properties. In the present study, the combined use of MPC, DMAHDM and NAg indeed achieved the reduction in biofilm CFU by nearly 5 orders of magnitude, which is much greater than those achieved by previous antibacterial dental composites. For example, in a previous study, the composite containing NAg and NACP reduced the total microorganism, total streptococci, and *mutans streptococci* CFU by one order of magnitude [35]. In another study, the biofilm CFU counts on an adhesive resin containing QAMD + NAg were two orders of magnitude lower than that on a commercial control resin [41]. In a separate study, the composite with double agents of DMAHDM and MPC reduced the biofilm CFU by 3 orders of magnitude [30]. In contrast, the present study with triple agents of MPC, DMAHDM and NAg together reduced biofilm CFU by nearly 5 orders of magnitude, indicating the significant merit and advantage of applying the triple agents of MPC, DMAHDM and NAg in other dental materials as well.

Besides biofilm CFU tests, MTT conversion has been used to quantify the cellular metabolism of biofilms [37]. In addition, polysaccharide is synthesized by live bacteria and therefore has a close relationship with the bacterial viability [37]. In the present study, ENMD-

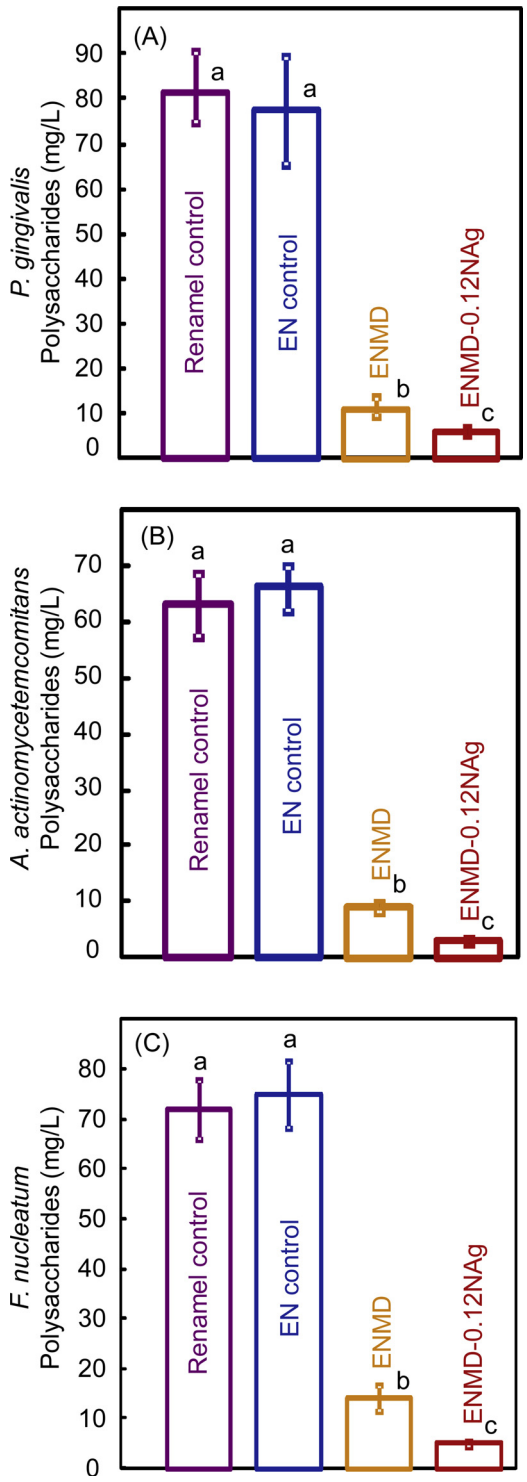

Fig. 5. Polysaccharide production of 2-day periodontal biofilms grown on nanocomposites: (A) *P. gingivalis*, (B) *A. actinomycetemcomitans*, and (C) *F. nucleatum* (mean ± SD; n = 6). Bars with dissimilar letters indicate significantly different values ($p < 0.05$).

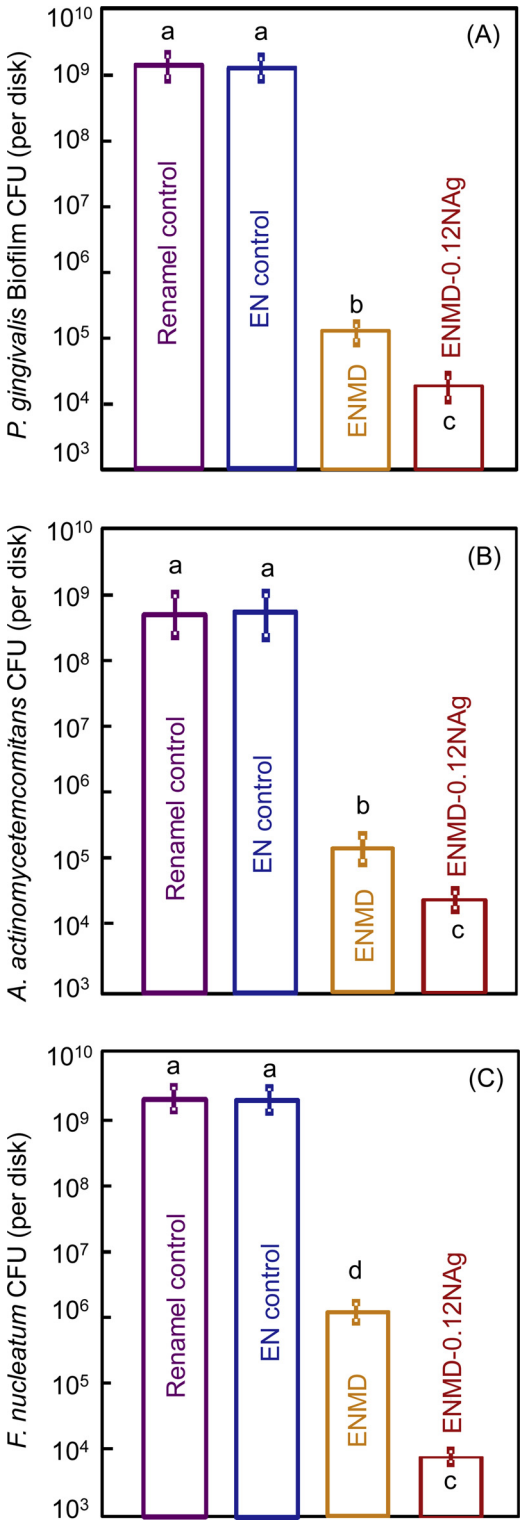

Fig. 6. Colony-forming unit (CFU) counts of 2-day biofilms of three periodontal pathogens grown on nanocomposites: (A) *P. gingivalis*, (B) *A. actinomycetemcomitans*, and (C) *F. nucleatum* (mean ± SD; n = 6). Note the log scale for the y axis. Bars with dissimilar letters are significantly different from each other ($p < 0.05$).

0.12NAg exhibited significantly lower metabolic activity and polysaccharide amount for all three biofilms when compared with ENMD. This demonstrated that incorporating NAg enhanced the antibacterial capability of the ENMD nanocomposite. Another point worth noting is that the three bacterial species showed different extent of inhibition efficacy by the same composites. ENMD reduced the biofilm CFU of *P. gingivalis* and *A. actinomycetemcomitans* by nearly 4 orders of magnitude, and reduced the CFU of *F. nucleatum* by nearly 3 orders of magnitude. This was consistent with a previous study showing that *F. nucleatum* was less susceptible to ENMD than *P. gingivalis* and *A. actinomycetemcomitans* [33]. In contrast, ENMD-0.12NAg showed stronger antibacterial capability than ENMD, especially against *F. nucleatum*. All

of the three pathogens were reduced via ENMD-0.12NAg by nearly 5 orders of magnitude (some by slightly > 5 log, others by slightly < 5 log). This was also consistent with previous study showing that the susceptibility of *F. nucleatum* depended on the different kinds of antibacterial agents, and was more sensitive to nano-silver than *P. gingivalis*, and *A. actinomycetemcomitans* [34]. ENMD-0.12NAg showed more superior antibacterial function against *F. nucleatum* than ENMD alone, suggesting that ENMD-0.12NAg has a great potential to be used in the patients with advanced periodontitis.

Besides the antibacterial agents, NACP were also incorporated in the new multifunctional composite. While having little antibacterial activity, the NACP composite was "smart" and could greatly increase the Ca and P ion release at a cariogenic low pH, when such ions were most needed to inhibit caries [36]. Indeed, a previous study showed that an NACP nanocomposite successfully remineralized enamel lesions, and achieved an enamel lesion remineralization efficacy that was 4-fold greater than that of a commercial fluoride-releasing control [61]. In addition, a previous study showed that NACP composite could be recharged repeatedly with Ca and P ions, which ensured that it could continuously release Ca and P ions to provide long-term remineralization [62]. In Class V restorations, the Ca and P ions from the composite are expected to help remineralize tooth roots, reduce root sensitivity, neutralize acids, and protect root structures. Further studies are needed to investigate these issues.

5. Conclusions

The present study developed a novel multifunctional nanocomposite for Class V restorations containing a combination of NAg, MPC, DMAHDM and NACP to combat periodontal biofilms. Flexural strength and elastic modulus of the new bioactive ENMD-0.12NAg nanocomposite matched those of commercial control composites without antibacterial functions. The combination of NAg + MPC + DMAHDM achieved much greater reductions than previous antibacterial composites in metabolic activity and polysaccharide production of biofilms, with CFU reducing by nearly 5 orders of magnitude for the first time. In addition, while DMAHDM killed biofilms on the composite surface, the NAg in the composite had long-distance antibacterial functions via the release of silver ions, which could kill the suspended pathogens in the periodontal pocket. Therefore, the novel ENMD-0.12NAg nanocomposite has great potential for Class V restorations to treat root caries, inhibit periodontal pathogens and protect periodontal tissues.

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