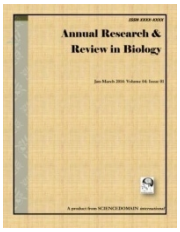

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Anti-bacterial Effects of Polymethylmethacrylate with *In situ* Generated Silver Nanoparticles on Primary Colonizers of Human Dental Plaque and Cariogenic Bacteria

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Authors' contributions

This work was carried out in collaboration between all authors. Author AB designed the study, performed the statistical analysis, wrote the protocol and the first draft of the manuscript. Author SOM performed the microbiological testing. Authors BP and RG managed the analyses of the study. Authors AB and AS managed the literature searches and revised the final draft of the manuscript. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: Polymethylmethacrylates (PMMAs) remain the most popular material for use in orthodontics, particularly in developing countries, because of their low cost and ease of use. Biofilm formation of cariogenic bacteria on these surfaces may contribute to tooth decay, gingivitis, and periodontitis. Nanoparticle-sized silver (NanoAg) inhibits the growth of microorganisms, but anticariogenic effects of NanoAg *in situ* in PMMA (NanoAg-IS-

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PMMA) in orthodontics have not been explored. Therefore, the primary purpose of the current study was to evaluate the antimicrobial activity of NanoAg-IS-PMMA against primary colonizers of human dental plaque and cariogenic bacteria in planktonic and biofilm cultures, grown as mono-species biofilms and mixed-species biofilms.
Study Design: *In vitro.*
Place and Duration of Study: Department of Medical Microbiology, Department of Orthodontics, Pediatrics Infectious Disease Research Center; Tehran, Iran. August, 2012–May, 2013.
Methodology: Chemical-cure orthodontic resin was used to synthesize NanoAg-IS-PMMA using silver benzoate. Antibacterial effects of NanoAg-IS-PMMA were assessed against *Streptococcus sobrinus, S. sanguinis, S. mitis,* and *Actinomyces naeslundii* as well as cocultures of the four species by inhibition of bacterial adherence and growth inhibition of planktonic and biofilm bacterial cells on NanoAg-IS-PMMA.
Results: NanoAg-IS-PMMA reduced bacterial adhesion by 36.2%–88.1%, depending on the bacterial type. Planktonic cultures of the microorganisms exposed to NanoAg-IS-PMMA showed a significant decrease (99.99%) in a time-dependent manner, over 28 days. Exposure to NanoAg-IS-PMMA inhibited biofilm formation of all test bacteria and their cocultures by 77.6%–99.96%, compared to PMMA. In addition, NanoAg-IS-PMMA retained some inhibitory effects for the third generation of biofilm formation.
Conclusion: Our data supports the finding that NanoAg-IS-PMMA effectively inhibited adherence of primary colonizers of human dental plaque and cariogenic bacteria to NanoAg-IS-PMMA surfaces, in addition to rendering strong anticariogenic effects in the planktonic and subsequent mono-species biofilms and mixed-species biofilms formation. This demonstrated that NanoAg-IS-PMMA has the potential to minimize colonization of cariogenic bacteria and biofilm formation on orthodontic appliances.

Keywords: Polymethylmethacrylate; orthodontics; silver nanoparticles; cariogenic bacteria; antibacterial agents.

1. INTRODUCTION

Polymethylmethacrylates (PMMAs) remain the most popular material for use in dentures and baseplates of orthodontic appliances, particularly in developing countries, because of their low cost and ease of use [1]. In addition, PMMAs are used for individual impression trays and early orthopedic appliances for patients with a cleft lip and palate and as splints for orthognathic surgery. At present, PMMA resins are commonly used in routine orthodontic treatments for the construction of removable and auxiliary fixed appliances as well as Hawley retainers [2]. However, the long-term existence of removable orthodontic acrylic appliances with their porous surfaces in the mouth may lead to biofilm formation, with a negative effect on oral microbiota and consequently result in tooth decay, gingivitis, and periodontitis [3-7]. This is a challenge resulting from the higher number of plaque-retentive sites and impaired mechanical plaque removal that is most often observed with orthodontic appliances [8]. Insertion of acrylic appliances may also affect the metabolic activity and pathogenicity of the biofilm [9].

Mechanical cleaning of the appliances is helpful in reducing the formation of biofilms and plaques, particularly with the adjunctive use of antimicrobial solutions [10,11]. However, such measures usually rely on patient conformance, and may not be favorable in pediatric and handicapped individuals. Consequently, an additive that forcefully enhances the inhibitory

effects of orthodontic acrylic appliances, while maintaining its biocompatibility is highly desirable [12].

Silver in a nanoparticle form (NanoAg) is an effective microbicidal agent due to its high surface area-to-volume ratios; the nanoparticles attach to the outer membrane and affect permeability as well as induce structural changes in the cell, ultimately leading to cell death. In addition, NanoAg does not trigger bacterial resistance [13-15]. The literature reports various studies related to the inhibitory effect of very low concentrations of NanoAg on oral bacterial growth, without any side effects [16-18]. PMMA incorporated with NanoAg has been reported to reduce adherence and inhibit growth of microorganisms [19,20]. PMMAs incorporated with NanoAg are also reported to be noncytotoxic and nongenotoxic [20]. However, the incorporation of NanoAg in PMMAs has rendered problems, often due to an incapability to homogenously disperse NanoAg; this can be detrimental to the device's mechanical properties and necessitates the use of harsh chemicals and complex processes for synthesis, which is unsuitable for dental and medical applications [21-24].

Recently, Fan et al. [25], developed a broad-spectrum antimicrobial PMMA with well-dispersed NanoAg, without the use of harsh chemicals or multistep processes by synthesizing NanoAg *In situ* in PMMA (referred to as NanoAg-IS-PMMA). In a pilot study, Fan et al. [25] demonstrated that NanoAg-IS-PMMA inhibited the planktonic growth of *Streptococcus mutans*, the primary causative agent in the formation of dental cavities in humans. Caries area biofilm of *poly-microbial* infections in the complex oral cavity and mixed-species biofilms are generally more stable than mono-species biofilms. Cell-to-cell communications have been shown to play a key role in biofilm formation as well as in the resistance of biofilm community members against antimicrobials. It has been shown that *S. mutans* and *S. sobrinus* are two of the most important odontopathogens involved in the initiation and progression of dental caries. The coexistence of *S. mutans* and *S. sobrinus* in dental biofilms is correlated with the presence of a higher number of caries than when *S. mutans* is detected alone. Oral viridans *Streptococci sp.*, including *S. mitis* and *S. sanguinis*, are primary colonizers of human dental biofilms [26,27]. *Actinomyces naeslundii*, often found in high numbers from the earliest stages in dental plaque, has the potential to invade dentinal tubules and is associated with root caries [28].

Nonmutans viridans *Streptococci* are considered beneficial for human oral health. It has been suggested that colonization byviridans *Streptococci* may exclude more pathogens and protect against caries or periodontitis. For example, a negative association between *S. sanguinis* and mutans *Streptococci* (i.e. *S. mutans* and *S. sobrinus*) has been demonstrated in caries or the outcome of having caries [29,30]. *S. mitis* release rhamnolipidlike biosurfactants, which inhibitsthe adhesion of cariogenic mutans *Streptococci* strain [31]. Evidence suggested that high levels of viridans *Streptococci* have been associated with low numbers of *Tannerella forsythensis* or *Aggregatibacter actinomycetemcomitans*, and the absence of periodontal disease [32]. Because *S. sobrinus* is more cariogenic and also more resistant tochlorhexidinecompared to *S. mutans*, it is used as the internal control in this study [33]. A four-species biofilm of *S. sobrinus*, *S. sanguinis*, *S. mitis*, and *A. naeslundii* was also used to evaluate the antimicrobial activity of NanoAg-IS-PMMA and unmodified PMMA. These four coaggregating bacteria are amongst the first colonizers of human dental plaque [26,27,34], which has lead to their application as a model of multi-species dental biofilms [35].

To the best of our knowledge, no comprehensive data are available in the orthodontic literature regarding the microbicidal effects of orthodontic acrylic materials containing

NanoAg against primary colonizers of human dental plaque and cariogenic bacteria on the processes of attachment, planktonic growth, and biofilm formation. Thus, the primary objective of the current study was to investigate the adhesion inhibition and antimicrobial activity of the NanoAg-IS-PMMA against primary colonizers of human dental plaque and cariogenic bacteria in planktonic and biofilm cultures, grown as a single species (mono-species biofilms and coculture (mixed-species biofilms).

2. MATERIALS AND METHODS

2.1 Preparation of Nano Ag *In situ* in PMMA

The NanoAg-IS-PMMA was prepared using the Fan method [25]. In brief, 0.5% w/w of the total monomer of silver benzoate (AgBz) (Sigma-Aldrich, Germany) was dissolved in dimethyl-aminoethyl methacrylate (DMAEMA; 2%, w/w of total monomer; Sigma-Aldrich, Germany). PMMA powder and liquid monomer (both purchased from Selecta Plus, Dentsply, UK) were mixed in the ratio of 3 g of powder to 1 ml (1.18 g by weight) of liquid monomer according to the manufacturer's instructions. This mixture was then blended with a liquid orthodontic monomer (Dentsply, UK) and subsequently mixed with PMMA powder under laminar flow as described in the manufacturer's guidelines. Based on *in vitro* release data, the adherence and growth inhibition assay were only done with chemically cured resins made with 0% (negative control) and 0.5% AgBz.

The prepared NanoAg-IS-PMMA blend was then poured into a mold (5.0 mm in diameter and 2.0 mm in height) between two glasses and allowed to chemically self-cure for 24 h. The resulting acrylic discs, with a total surface area of 0.55 cm^2, were polished using a commercial system (superfine grit discs, Sof-Lex disc system, 3 M ESPE, USA). Select a Plus Orthodontic Resin without NanoAg was used as the control. Prior to the tests, the discs were sterilized with25 kGy of ^{60}Co irradiation, adhering to ISO 11135:1994 for medical devices.

2.2 Test Microorganisms and Growth Conditions

Lyophilized *S. sobrinus*, *S. sanguinis*, *S. mitis*, and *Actinomyces naeslundii* (ATCC cultures 33478, 10557, 49456and 12104, respectively, obtained from Iran-Rayen Biotechnology Co., Ltd., Tehran, Iran) were rehydrated in a brain heart infusion (BHI) broth (Merck, Darmstadt, Germany) and incubated in an anaerobic atmosphere at 37ºC for 48 h. To examine the antimicrobial efficacy of the NanoAg-IS-PMMA, the tested bacterial suspension of approximately 10^8 CFU/mL was prepared using a spectrophotometer. The optical density of the *A. naeslundii* culture was measured at 600 nm (OD_{600}); an OD=1.2 corresponds to approximately 10^8 cells/mL as determined by serial tenfold dilutions and anaerobic culturing on BHI agar plates for colony-forming units (CFU) counts. For *S. sobrinus*, *S. sanguinis* and *S. mitis*, OD_{600} of 0.9–1 corresponds to 10^8 cells/mL [36-39].

2.3 Bacterial Adherence Test

Adherence of test microorganisms was determined by an "Adhesion Test" using the Acosta-Torres method [40], with some modifications. In brief, sterilized disk specimens were placed in 24-well sterile culture plates (Nunc, USA), and 500 µL of bacterial suspensions adjusted to 5×10^5 CFU/mL were added. Following a 24 h incubation period at 37ºC, nonadherent bacteria were removed from the acrylic disks by three washings with distilled water for 1 min,

with shaking. Adherent bacteria on the discs were dislodged by ultrasonication (5 min) in a 150 W ultrasonic bath (Branson Ultrasonics Co., Shanghai, China) that works at a frequency of 50 Hz, followed by rapid vortex mixing (Scientific Industries, Bohemia, NY, USA) at the highest power for 1 min. This method is validated as being efficient in removing biomaterial-adherent bacteria [20]. Finally, a microbial cell viability assay based on luminescent ATP measurement (Bac Titer-Glo, Promega, Fitchburg, WI) was performed to determine the number of viable cells adhering to acrylic disks, according to the manufacturer's recommendations. In brief, 20 µL of each suspension was mixed with 30 µL of BacTiter-Glo reagent in 1.5 mL sterile Eppendorf tubes, and luminescence was recorded after 5 min in a luminometer (Turner Biosystems, Sunnyvale, CA) at an emission wavelength of 749 nm. Relative light unit (RLU) was measured in 10-s integration periods. In this study two controls were simultaneously assessed. In the first, PMMAdisks were dipped in bacterial suspensions and incubated, while in the second, NanoAg-IS-PMMA disks were incubated without inoculum. All assays were performed in triplicate, and the results were presented as percentage inhibition using the following formula: %I = 100 − (Log RLU sample/Log RLU control) × 100 [40].

2.4 Kinetic Measurement of Planktonic Bacterial Cell Growth Inhibition

For long-term antibacterial activity, NanoAg-IS-PMMA disks were incubated at 37ºC in 1mL of sterile deionized (DI) water for 4 weeks. The long-term potential of specimens, both PMMA disks and NanoAg-IS-PMMA disks, in inhibiting planktonic bacterial cell growth was evaluated at regular intervals (1, 3, 7, 14, 21 and 28 days) using a 24-well plate model as previously demonstrated, with some modifications [41]. In brief, on Days 1, 3, 7, 14, 21 and 28, the acrylic discs were taken from DI water and were placed in a fresh 24-well plate. Bacterial density was adjusted to 10^8 CFU/mL using a spectrophotometer. The exact density (CFU/mL) of each suspension was verified on BHI agar plates, and then 0.5 mL of each of *S. sobrinus*, *S. sanguinis*, *S. mitis* as well as *A. naeslundii*, and mixed cultures of the four species was added to wells containing the acrylic discs [41]. Plates were incubated in an anaerobic chamber (5% CO_2, 10% H_2, and 85% N_2) at 37ºC for 48h and bacterial growth and concentration was assessed. To determine the CFU/mL, the cells were serially diluted with phosphate-buffered solution (PBS) in micro-titer plates, and enumerated using the drop plate method [42], in which 20 µL drops of each dilution were placed onto media. BHI agar plates were used to determine CFU/mL of species, and selective media plates were used for specific identification of individual species in the case of mixed cultures. Mitis Salivarius-sobrinus (MS-SOB) agar, MM10-sucrose (MM10-S) agar, mitis salivarius agar with tellurite (MSAT) and selective medium containing mupirocin and metronidazole (SMMM) (QUELAB, UK) are useful for the detection of *S. sobrinus*, *S. sanguinis*, *S. mitis*, and *A. naeslundii*, respectively [43,44].

2.5 Biofilm Inhibition of NanoAg-IS-PMMA and PMMA

The biofilm inhibition potential of NanoAg-IS-PMMA and PMMA disks were evaluated using a biofilm model previously demonstrated for testing prospective antiplaque agents [45]. In brief, bacterial biofilms were generated on NanoAg-IS-PMMA discs using 24-well plates. BHI broth containing 0.5% glucose was used for biofilm cultivation. Each well was inoculated with one milliliter (approximately 10^8 CFU/mL) of the adjusted bacterial inoculum. Biofilms were grown at 37ºC with agitation at 100 rpm for 168 h; media were changed every 24h. The substrates were removed at 24 h intervals with sterile forceps, placed into a fresh 24-well plate and gently washed with sterile PBS four times to remove the planktonic cells and

loosely adherent bacteria. The adherent bacteria on the discs were dislodged and were detected as described above for the bacterial adherence test. The CFU/mL of bacteria in the vortexed solutions was determined as described above for the susceptibility tests of planktonic bacterial cultures. The CFU was normalized by the number determined for PMMA control suspension and is expressed relative to the surface area of the sample (CFUs/mm^2). For all experiments, the negative controls were acrylic discs without NanoAg to which no bacterial suspension was added. All tests were performed in triplicate.

2.6 Four-species Biofilm Inhibition on Acrylic Discs

Inoculum for the cultivation of four-species biofilms was prepared based on the procedure reported by Takenaka et al. [35], with some modification. In brief, separate liquid cultures of *S. sobrinus*, *S. sanguinis*, *S. mitis* and *A. naeslundii* were grown in BHI for 48 h at 37ºC under anaerobic conditions. The CFU/mL of all test bacteria was adjusted to 10^8, using a spectrophotometer as mentioned above, prior to preparing the inoculum and 150 µL from each bacterial culture was used to inoculate the acrylic disc in each well of 24-well plates. Next, the coculture of four-species was allowed to grow for 168 h at 37ºC, under anaerobic conditions. The medium was replaced every 24 h and viable counts were recorded as described above for the susceptibility tests of planktonic bacterial cultures. All tests were performed in triplicate [46].

2.7 Biofilm Inhibition on Discs Aged by Previous Biofilm Growth

Tests were performed using the method described by Sevinç and Hanley with some modifications [46]. Biofilms were generated on the same discs as3 cycles of 3 days of growth each. The same methodology explained above was used for biofilm generation on discs, except that the discs were separately sonicated in PBS for 5 minutes between each cycle to remove the planktonic and loosely adherent bacteria. The CFU/mL of bacteria in the vortexed solutions was determined as described above for the susceptibility tests of planktonic bacterial cultures, on Day 3 of biofilm growth for each cycle. The experiment was repeated four times under identical conditions.

2.8 Statistical Analysis

Since bacterial colony forming unit (CFU)/mL and CFU/mm^2 did not exhibit a normal distribution, the data were converted to logarithms to confer homogeneity among the groups and then submitted to variance analysis with repeated measures. Data for inhibition assays were evaluated for statistical significance as described by Sevinç et al. [46], using a multistep process. In brief, normalization of the data was assessed using Q–Q plots. If the data did not follow normal distribution, as in the cases of kinetic measurement of bacterial growth and biofilm inhibition potential of acrylic disks, then nonparametric tests (Mann–Whitney U Test and Wilcoxon Test) were used. For independent but abnormally distributed cases, the Kruskal–Wallis test for paired comparisons was used to compare the groups. If test results were smaller than 0.05, then the Mann–Whitney U test was used for paired comparisons. Independent but abnormally distributed groups, the comparison was performed by the Wilcoxon Signed-Rank test.

3. RESULTS

3.1 Microbial Adhesion

In *A. naeslundii*, the mean percentage of logarithmic value of the adherence levels in the NanoAg-IS-PMMA group dropped significantly (P=0.002) to 5.2% after 4 weeks, in comparison to the control/unmodified PMMA group, indicating its higher sensitivity to NanoAg.

Our data demonstrated that NanoAg-IS-PMMA was two-fold more potent in inhibiting the adherence of *S. sobrinus* than that of *S. sanguinis* or of *S. mitis*. The highest adherence on test acrylic disks was observed in unmodified PMMA with *S. sobrinus,* which showed RLU of $16.4\pm0.7 \times 10^3$. For all test bacteria and mixed cultures of the four species, there were clear differences between PMMA and NanoAg-IS-PMMA, with the later presenting significantly higher inhibition percentages (P = 0.05) Table 1.

3.2 Time Kinetics of Planktonic Bacterial Cells Growth Inhibition

Throughout the planktonic bacterial cell growth inhibition analysis, cultures exposed to NanoAg-IS-PMMA were compared to growth of test microorganisms in unmodified PMMA, showing approximately a $1.2 \times 10^3 – 4.5 \times 10^6$-fold decrease over a 28 day test period Table 1. Most notably, at all-time points of the planktonic bacterial cell growth inhibition assay, NanoAg-IS-PMMA demonstrated a statistically significant (P = 0.05) higher inhibitory activity than unmodified PMMA, which did not inhibit the growth of any test culture Figs.1A and 1B.

The antibacterial activity of NanoAg-IS-PMMA against all test bacteria and mixed cultures of the four species was time-dependent, such that increasing the duration of NanoAg-PMMA exposure by1 day enhanced the inhibitory effects of NanoAg-PMMA on these microorganisms. As shown in Fig. 1B, exposure to NanoAg-IS-PMMA for 1 day had a marked antibacterial effect on tested bacteria and cocultures of the four species, with a reduction in viability by 99.99%, compared to unmodified PMMA (P=0.05). On the other hand, NanoAg-IS-PMMA reduced the viability of tested bacteria and mixed cultures of the four species by 96.02%–97.9%, suggesting a bacteriostatic effect on these microorganisms, during an incubation period of 1–28 days Fig. 1B. Although the time points between Days 1 and 28 showed a progressive decrease in the viability of the test microorganisms treated with NanoAg-IS-PMMA, these reductions were not significant Fig. 1B.

3.3 Biofilm Inhibition Potential of Acrylic Disks

Fig. 1C shows the CFU/mm^2 of tested microorganisms and their coculture following biofilm growth for 168 h on NanoAg-IS-PMMA as effects of NanoAg, expressed relative to the maximal CFU/mm^2 recovered from the corresponding acrylic disks without NanoAg (control disks). CFU/mm^2 on the control disks had a maximum of about 6.3×10^3 CFU/mm^2 to 2.7×10^6 CFU/mm^2, depending on the microorganism type. This maximum value was used as the 100% value for each of the tested bacteria as well as mixed cultures of the four species in this study. The results obtained show that NanoAg-IS-PMMA caused a significant reduction in the number of viable test microorganisms and mixed organism cultures on the disc surface over a 168h period. Biofilm inhibition analysis demonstrated that NanoAg-IS-PMMA inhibited the biofilm of all test bacteria *and* mixed organism cultures by 77.6%–99.9% compared to PMMA. As shown in Table 1, biofilm formation by *S. sobrinus* showed the highest susceptibility to NanoAg-IS-PMMA, which reduced bacterial viability by 99.96% (P =

0.0011). However, the NanoAg-IS-PMMA demonstrated smaller but significant antibiofilm activity on mixed organism cultures (P = 0.024) Table 1. By the end of our experiment, it was evident that the number of viable cells of test bacteria and mixed organism cultures on the NanoAg-IS-PMMA discs was lower compared with the control discs. The NanoAg-IS-PMMA gives the highest long-term suppression of bacterial regrowth, without an increase in bacterial number, after 168 h (P = 0.005).

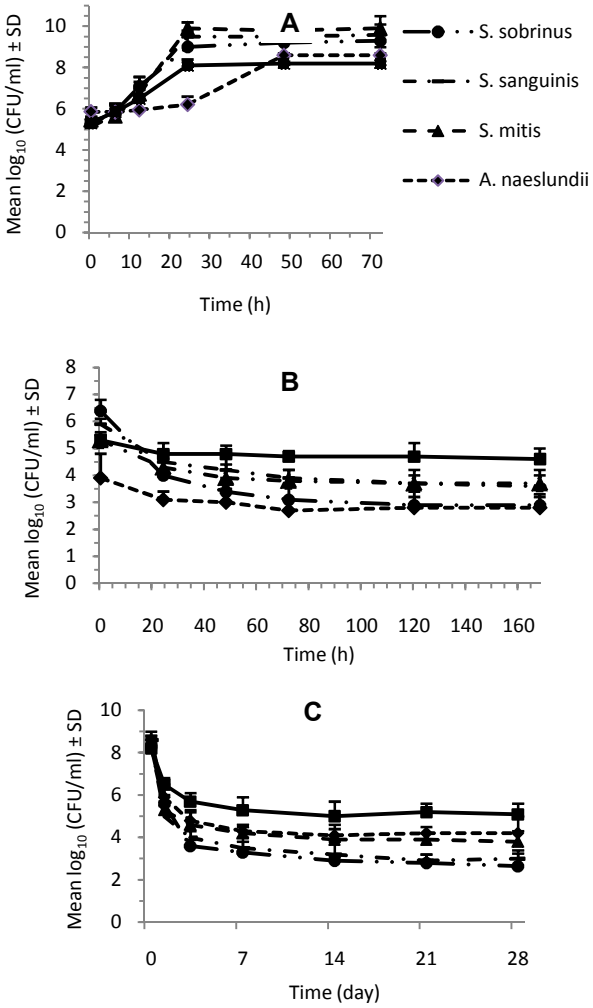

Fig. 1. Time kinetics of planktonic growth and biofilm bacterial cells growth inhibition: A) growth of test microorganisms in untreated PMMA, B) planktonic bacterial cells growth inhibition of NanoAg-IS-PMMA. C) biofilm bacterial cells growth inhibition of NanoAg-IS-PMMA. Bar errors show standard deviations

3.4 Biofilm Inhibition on Acrylic Disks Aged by Previous Biofilm Growth

The ability of the acrylic disks to maintain their antimicrobial properties after several cycles of biofilm formation was evaluated by scraping off biofilms and reusing the acrylic disks for new biofilm growth. As shown in Table 2, for all the test bacteria *and* mixed organism cultures, there was no statistically significant difference between the first and second cycle of growth on NanoAg-IS-PMMA discs (P = 0.05).

Table 1. Antimicrobial effects of NanoAg-IS-PMMA: Luminescence assay results of adherent microorganisms on NanoAg-IS-PMMA as well as planktonic 28 days *in vitro* antimicrobial activity and biofilm formation at 168 h on NanoAg-IS-PMMA; luminescence assay data for adherence is shown on a linear scale and the data of planktonic and biofilm assays are shown on a log scale

Microorganisms	Antimicrobial Effects of NanoAg-IS-PMMA								
	Luminescence assay results of adherent microorganisms			planktonic 28 days *In vitro* antimicrobial activity			Biofilm of 168 h *In vitro* antimicrobial activity		
	Relative Light Unite ×10^3*		Adhesion inhibition (%)	CFU/ml** After 28days		Reduction (%)	CFU/ml** After 48h		Reduction (%)
	NanoAg-IS-PMMA	PMMA		NanoAg-IS-PMMA	PMMA		NanoAg- IS-PMMA	PMMA	
S. sobrinus	1.9±0.3	16.4±0.7	88.1	2.65±0.6	9.3±0.5	99.99	2.93±0.6	6.43±1.4	99.96
S. sanguinis	6.5±0.5	12.9±1.1	48.4	3.06±0.4	9.6±0.7	99.99	3.65±0.4	5.09±0.5	96.3
S. mitis	8.3±1.3	13.1±0.6	36.2	3.82±0.5	8.4±1.1	99.99	3.72±0.7	5.33±0.7	97.54
A. naeslundii	0.67±0.2	12.7±1.1	94.7	4.21±0.8	8.6±0.6	99.99	2.85±0.3	3.79±0.9	88.4
Mixed organisms	7.7±0.5	14.1±1.1	45.3	5.1±0.5	8.2±0.4	99.99	4.61±0.5	5.26±0.6	77.6

Table 2. Viable cell counts after Day 3 of growth of test bacteria and mixed organism culture of the four species, biofilm growth on composite discs after the first, second, and third cycle of subsequent biofilm growth on the same discs.

Acrylic disks	Microorganisms (CFU/mm^2)*														
	S. sobrinus			*S. sanguinis*			*S. mitis*			*A. naeslundii*			Mixed organisms		
	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd
NanoAg-IS-PMMA	2.93±0.5	2.79±0.7	2.99 ±0.4	3.65±0.3	3.84±0.6	3.91 ±0.4	3.72±0.5	3.95±0.7	4.25 ±0.5	2.85±0.7	2.96±0.6	3.11 ±0.4	4.61±0.4	4.70±0.5	5.13 ±0.7
Unmodified PMMA	6.43±0.8	6.51±0.5	6.67 ±0.5	5.09±0.9	5.18±0.4	5.41±0.6	5.33±0.3	4.95±0.7	5.05 ±0.6	3.79±0.4	3.93±0.3	4.12 ±0.5	5.26±0.6	5.35±0.5	5.13 ±0.4

** Logarithmic scale*

Although the third cycle showed an increase in biofilm growth for *S. mitis and* mixed organisms *on* NanoAg-IS-PMMA compared to the second cycle, these increases were not significant (*P*=0.068 and *P*=0.193, respectively). However, following the third cycle of biofilm growth for *S. sobrinus*, *S. sanguinis,* and *A. naeslundii*, biofilm growth in unmodified groups (PMMA) was significantly greater than that on the NanoAg-IS-PMMA group (*P*=0.05).

4. DISCUSSION

Our results demonstrated that the highest and lowest adherence to PMMA disks was observed with *S. sobrinus* and *A. naeslundii,* respectively. It should be noted that *S. sobrinus* has a well-recognized adherence ability, due to its capability to rapidly synthesize exopolysaccharides [47]. Our study showed that NanoAg-IS-PMMA is significantly effective in inhibiting the adherence of all test bacteria and mixed cultures of the four species. These findings are in accordance with the findings of another study demonstrating a lower adherence of microorganisms on PMMA-discs containing silver nanoparticles [40]. On the other hand, for all test bacteria and mixed organisms, there were significant differences in adherence inhibition between NanoAg-IS-PMMA and PMMA, which can translate into meaningful clinical applications.

In our current NanoAg-IS-PMMA time-dependent study, a rapid and wide spectrum inhibition of planktonic bacterial cell growth of the tested bacteria was observed. Our data are consistent with a report in which NanoAg-IS-PMMA showed excellent antimicrobial properties against planktonic bacterial cell growth of *Pseudomonas aeruginosa, Acinetobacter bumannii, Proteus mirabilis, S. mutans* and *Staphylococcus aureus* [25,41]. An advantage of a quick bactericidal effect may be that it permits a trial to proceed without bacterial interference, and reduces the likelihood for resistance development. Our study demonstrated that the inhibition of planktonic bacterial cell growth of all the test bacteria and cocultures of the four species by NanoAg-IS-PMMA was time-dependent, such that increasing the length of NanoAg-IS-PMMA exposure from 1 to 3 days enhanced the growth inhibitory effects on these microorganisms. Oei et al. [41] detected a 0.4–4.2 µg/ml release of Ag ions from NanoAg-IS-PMMA containing 1% w/w AgBz up to 28 days, which seemed to plateau after Day 7. In this study, the NanoAg-IS-PMMA showed similar results of antimicrobial efficacy for all tested bacteria (28 days), thus corroborating Oei's time course study using a similar type of NanoAg-IS-PMMA; however, the inconsistency in inhibition between these and our current results again suggests that the effect is genus-dependent.

The initial phase, right after the usage of orthodontic materials, is important and prone to colonization, and so the appropriate ability of NanoAg-IS-PMMA to eliminate the surrounding planktonic bacteria helps prevent plaque formation and dental caries. Our results showed that the NanoAg-IS-PMMA exhibit a high antimicrobial efficacy; despite the low content of silver nanoparticles (0.5% w/w) in NanoAg-IS-PMMA, all test bacteria and mixed cultures of the four species are inhibited over 99.9% within 28 days. This low amount of NanoAg does not have any adverse effect on the mechanical properties of the polymer [19]. The NanoAg-IS-PMMA surfaces show excellent antibacterial ability throughout the 28-day period, but it should be noted that the efficiency diminishes gradually with time as a result of the release of Ag overtime. Initially, abundant Ag is released that diminishes gradually over the immersion time. The initial flood of Ag may stem from the NanoAg on the surface of the NanoAg-PMMA, which oxidize on reacting with water.

Results from this study indicate that the minimum inhibitory concentration (MIC) of NanoAg-IS-PMMA was 250–1500-fold higher for tested bacteria growing in the biofilm compared with

cells in suspension. However, in the present study, a complete elimination of the biofilm did not occur because biofilm structures represent an anchor that renders protection to the microorganisms. Our biofilm inhibition data seems consistent with several studies that have reported the increase in biofilm resistance to antimicrobial agents compared with their planktonic counterparts [48,49]. This makes biofilms of cariogenic bacteria particularly disturbing in oral health, while their presence frequently creates dental caries [50]. Mechanisms proposed to increase the biofilm resistance to antimicrobial agents compared with their planktonic counterparts can be divided into three categories: modulation of the environment, transport limitation, and modulation of the environment [51].

In the present study, we observed thatthe mixed culture in general showed lower inhibition by the NanoAg-IS-PMMAthan the individual bacterial strains. Cooperation (mainly coaggregation and metabolic interactions) is the main bacterial interaction observed in mixed-species communities. Interactions among species within a mixed-species can be antagonistic or synergistic. The latter can result in the promotion of biofilm formation by coaggregation, metabolic cooperation, where one species utilizes a metabolite produced by a neighboring species, and increased resistance to antimicrobials or host immune responses compared to the mono-species biofilms [52]. Knowledge of the physiological behavior of mixed-species biofilm communities formed by cariogenic bacteria on abiotic surfaces in orthodontics could provide the information necessary to prevent their formation, and thereby reduce the contamination of orthodontic appliances and reduce dental caries.

This study demonstrated that the NanoAg-IS-PMMA provides the highest long-term suppression of bacterial regrowth, without an increase in bacterial number after 168 h, which is due to the high amount of Ag ions diffusing through the medium, enough to continue killing microorganisms. Therefore, the NanoAg-IS-PMMA formulation could be a beneficial approach for inhibition of plaques. It was quite interesting to discover that NanoAg-IS-PMMA tested here demonstrated a persistent consequence, while PMMA alone could not produce antibacterial effects against cariogenic microorganisms for an extended duration (168 h). Our result showed that NanoAg-IS-PMMA discs were not as effective for multiple cycles of biofilm growth as their first time use, but biofilm formation on NanoAg-IS-PMMA discs was still significantly less than unmodified acryl. For *S. sobrinus*, *S. sanguinis*, and *A. naeslundii*, biofilm growth in NanoAg-IS-PMMA was significantly lower than that on PMMA, given the role of NanoAg in the antimicrobial mechanism of NanoAg-IS-PMMA. The NanoAg-IS-PMMA loses its antimicrobial effects after the third cycle of subsequent biofilm growth on the same discs, particularly when challenged with a mixed-species suspension. The simultaneous existence of mixed-species inside the biofilm structure seemed to influence the resistance pattern of the strains. The ability to retain antimicrobial activity following repeated microbial challenge is important in clinical situations, where orthodontic appliances may remain in place for several weeks.

Considering the robust antimicrobial activity of NanoAg-IS-PMMA in comparison with PMMA observed in our study and those by others, combined with the non-detrimental effects of NanoAg-IS-PMMA on the mechanical properties of acrylic [25,41], it appears clinically advantageous to use NanoAg-IS-PMMA and to benefit from their antimicrobial properties. Although in vitro experiments are a good indicator of how NanoAg-IS-PMMA can work in practice, clinical efficacy and effectiveness require suitable clinical studies, including randomized control clinical trials. However, additional studies are needed to assess the appropriate concentrations for the final products. We acknowledge that further evaluation of the antibacterial activity of NanoAg-IS-PMMA against additional oral biota and the

composition of the oral flora is necessary, since NanoAg does not differentiate between cariogenic and oral beneficial bacteria.

5. CONCLUSION

The NanoAg-IS-PMMA efficiently inhibited adherence of test cariogenic bacteria to disk surfaces as well as showed appropriate antimicrobial activity in the planktonic phase and subsequent mono-species biofilm and mixed-species biofilm formation. This proves that NanoAg-IS-PMMA has the potential to minimize colonization of cariogenic bacteria and biofilm formation on orthodontic appliances, and this novel acrylic resin formulation could be developed as a denture and orthodontic appliances base.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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