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Anti-microbial Activity of Acrylic Resins with *In-Situ* Generated Nanosilver on Cariogenic Planktonic and Biofilm Bacteria

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Abstract

Polymethylmethacrylate (PMMA) widely used in prosthodontics and orthodontics, but there is a problem with acrylic appliances-centered dental caries, inflammation of gingival and periodontal disease. With cariogenic organisms such as Streptococcus mutans, Streptococcus sobrinus, Lactobacillus casei and Lactobacillus acidophilus, there is a required for an anti-microbial delivery system with long-term anti-microbial activity. Thus, the main purpose of this work is to explore the effects of the increase in silver nanoparticles (NanoAg) concentration as well as the addition of initiator and accelerator to NanoAg in-situ in PMMA on antibacterial properties. Chemical-cure acrylic resins were used to synthesize NanoAg in-situ in PMMA using silver benzoate, benzoyl peroxide and dimethyl-p-toluidine (NanoAg-IS-PMMA-BD). Antibacterial effectiveness of NanoAg-IS-PMMA-BD was assessed against the cariogenic bacteria and their co-cultures by adherence inhibition as well as planktonic and biofilm bacterial cells growth inhibition. NanoAg-IS-PMMA-BD reduced bacterial adherence by 61.3-99.9% ($P<0.05$) depending on the microorganism type. Planktonic growth inhibition showed 5-7 log (99.9%; $P<0.05$) decrease in time-dependent manner over a 28 day period. NanoAg-IS-PMMA-BD inhibited the biofilm of all test bacteria and co-cultures by 3-5 log (99.9%; $P<0.05$), compared to PMMA. NanoAg-IS-PMMA-BD maintained anti-microbial effects after the third generation of biofilm formation. The data presented here are novel in that they prove that NanoAg-IS-PMMA-BD effectively inhibited adherence of cariogenic bacteria as well as strong anti-microbial activity in the planktonic phase and subsequent biofilm formation. This showed NanoAg-IS-PMMA-BD has the potential to minimize cariogenic microorganism's colonization on denture and baseplates of orthodontic appliances.

Keywords: Antibacterial effects, cariogenic bacteria, polymethylmethacrylate, silver nanoparticles.

Introduction

Acrylic resins, such as polymethylmethacrylates (PMMAs), widely used as denture base material in prosthodontics and baseplates of orthodontics appliances particularly in developing countries, because of their low price and simplicity of use. Additionally, PMMAs are used for impression trays and orthopedic appliances for patients with cleft lip and as orthognathic splints. Currently, PMMA resins are commonly used in routine orthodontic treatments for construction of orthognathic appliances and Hawley retainers[1]. Regrettably, regarding the long term existence of removable orthodontic acrylic appliances in mouth and their surfaces porosities may have a negative effect on oral microbiota and promote the biofilm formation[2]. It is challenging which results from higher number of plaque retentive situates and impaired mechanical plaque removal that most often seen with orthodontic appliances[3].

Insertion of acrylic appliances also may affect the pathogenicity of the biofilm forming bacteria[4]. The activities of microorganisms such as *Lactobacillus casei*, *Lactobacillus acidophilus*, *Streptococcus sobrinus* and *Streptococcus mutans* in biofilm on orthodontic appliances may contribute to dental caries, gingival inflammation and periodontal disease[4,5]. Mechanical cleaning of orthognathic appliances is useful in reducing accumulation of plaque and biofilm formation, mainly with the adjunctive use of anti-microbial solutions[6]. However, such measures rely generally on consistency of patients, and may not be most favorable in pediatric and handicapped individuals. consequently, an additive that robustly enhances the inhibitory effects of orthodontic acrylic appliances, while maintaining its biocompatibility, is highly enviable[7].

Silver in nanoparticulate form (NanoAg) is an effective microbiocidal agent as a result of their high surface area-to-volume ratios which attach to the microbial cell surface and affect cell permeability as well as induce major changes in the cell-ultimately leading to microbial death. Additionally, nanoparticles of silver does not cause resistant microbial species to develop[8-11]. Various studies have been explored that NanoAg inhibits growth of oral bacteria without side effects[9-14]. PMMA incorporated with NanoAg has been reported to reduce adherence and inhibited growth of microorganisms[15, 16]. The

PMMA incorporated with NanoAg are also reported to be noncytotoxic and nongenotoxic[17]. However, the PMMA incorporated with NanoAg has been problematic often due to homogenous dispersion failure of NanoAg, which is spiteful to the appliance's mechanical strength, and the need for complex processes and harsh chemicals required for synthesis, which is incompatible for dental and medical applications[18-21]. Resently, Fan *et al*.[22] have introduced a broad spectrum, anti-microbial acryl with well dispersed NanoAg and without multistep or harsh chemicals processes by synthesizing NanoAg *in situ* in PMMA (referred to as NanoAg-IS-PMMA). In A pilot study Fan *et al*.[22]showed that NanoAg-IS-PMMA inhibited *S. mutans* growth. Oei *et al*.[23] demonstrated that NanoAg-IS-PMMA inhibited the growth of *Acinetobacter baumannii* and *methicillin-resistant staphylococcus aureus.*

However, we have reported the NanoAg-IS-PMMA had significant antibacterial properties on cariogenic bacteria,[24] the objective of this work is to investigate the effects of addition of the initiator and accelerator in NanoAg-IS-PMMA on *in vitro* adhesion inhibition and anti-microbial activity against four cariogenic bacteria in planktonic and biofilm cultures grown as a single species and in co-culture.

Material and Methods

Preparation of NanoAg In Situ in PMMA containing initiator and accelerator: The NanoAg *in situ* in PMMA containing benzoyl peroxide (BP) and dimethyl-*p*-toluidine (DMPT) (referred to as NanoAg-IS-PMMA-BD) was prepared using the Fan method[22]. Briefly, 1% w/w of total monomer (Sigma- Aldrich, Germany) of silver benzoate (AgBz; Sigma-Aldrich, Germany) was mixed in dimethyl-aminoethyl methacrylate (2%, w/w, of total monomer; Sigma-Aldrich, Germany). This was then blended with monomer (Dentsply, UK) and extra benzoyl peroxide (BP1% w/w; Sigma-Aldrich, Germany) and dimethyl para toluidine (1% w/w; Sigma-Aldrich, Germany). From Oei *et al*.[23] results, NanoAg-IS-PMMA-BD with 1AgBz:1BP:1DMPT, which had similar hardness to controls as well as had the most effective antibacterial action out to 28 days, was chosen for this study. After mixing with PMMA powder (Selecta Plus; Dentsply, UK) as recommended in the manufacturer's guidelines under laminar flow, the prepared NanoAg-IS-PMMA-BD blend was then cascaded into a mold (2.0 mm in height and 5.0 mm in diameter) between two glasses and allowed to chemically self-cure for 24 h. The acrylic discs were polished using superfine-grit discs (3M ESPE, USA). The total surface area of each disc was 0.55(0.015)cm^2. Selecta Plus resin was used as the control. Prior to teststing, the acrylic discs were sterilized by 25 kGy of ^{60}Co irradiation according to ISO 11135:1994 for medical devices.

Test Microorganisms and Growth Conditions: Lyophilized *Streptococcus mutans*, *Streptococcus sobrinus*, *Lactobacillus casei* and *Lactobacillus acidophilus* (ATCC cultures 25175, 33478, 393T and 4356 in that order, obtained from Rayen Biotechnology Co., Ltd., Tehran, Iran) were rehydrated in brain heart infusion (BHI) broth (Merck, Germany), and incubated in an anaerobic atmosphere for 48 h at 37 °C. To examine the anti-adherence and anti-microbial efficacy of the NanoAg-IS-PMMA-BD, the test bacterial suspension of approximately 10^5-10^8 colony forming units (CFU)/mL was prepared using a spectrophotometer[23,24]. The optical density of the *L. acidophilus* culture was measured at 600 nm (OD_{600}); an OD = 1 corresponds to approximately 10^8 cells/mL as determined by serial tenfold dilutions and anaerobic culturing on BHI agar plates for CFU counts. For the *L. casei* OD_{600} of 0.8 for a 1/100 dilution correspond to 10^8 cells/mL. For the *S. mutans* and the *S. sobrinus* OD of 0.9-1correspond to10^8 CFU/mL[25].

Bacterial adherence test: Adherence of test microorganisms was determined by "Adhesion Test" using the Acosta-Torres method[17] with some modifications. Briefly, sterilized acrylic disk were located into 24-well culture plates (Nunc, USA) and 0.5ml bacterial suspensions adjusted to 5×10^5 CFU/mL was added. After 24 h incubation period at 37 °C, nonadherent bacterial cells were evacuated from acrylic discs by 3 washings with phosphate buffered saline (PBS) for 1 min under shaking. Adherent bacterial cells on the acrylic discs were detached by ultrasonication (50 Hz for 5 min) [14, 26]. Finally, a bacterial cell viability test based on luminescent ATP assay (Bac Titer-Glo, Promega, Fitchburg, WI) was performed according to the manufacturer's guidelines. Relative light unit (RLU) was measured in integration periods. In this study, adherence inhibition percentages were determined using the following formula: %I = 100 - (Log RLU sample/Log RLU control) × 100. All assays were performed in triplicate.

Kinetic measurement of planktonic bacterial cells growth inhibition: For long-term antibacterial activity, NanoAg-IS-PMMA-BD discs was incubated at 35°C in 1 mL of sterile deionized (DI) water for 4 weeks. The long-term potential of specimens to inhibition of planktonic bacterial cells growth was evaluated at certain intervals using 24-well plates model previously demonstrated with some modifications[20]. Briefly, on days 1, 3, 7, 14, 21 and 28, the acrylic discs were taken from DI water and were located into a fresh 24-well sterile plate. Then 0.5 mL of adjusted overnight culture of each *S. mutans, S. sobrinus, L. casei* as well as *L. acidophilus* and co-cultures of the four species suspension to 10^8 CFU/mL was added to wells containing acrylic discs[20]. Plates were incubated in an anaerobic condition (85% N2, 10% H2 and 5% CO2) at 37°C for 48 h. The CFU/mL was assessed on a culture, before the exposure and after the interval time of exposure to acrylic discs. To determine the CFU/mL, the cells were then diluted serially with phosphate buffered solution (PBS) in microtiter plates, and enumerated using the drop-plate method in which 50 µL drops of each dilution were placed onto media. BHI agar was used to determine the bacterial CFU/mL of species. Individual species were identified in the case of co-cultures using selective culture media. Mitis Salivarius- sobrinus (MS-SOB) agar, Mitis

Salivarius-Mutans valinomycin (MS-MUTV) agar, L. casei (LC) agar and De Man Rogosa Sharpe agar containing maltose (MRSM) was used for detection of S. sobrinus, S. mutans, L. casei and L. acidophilus, respectively. The inoculated plates were incubated anaerobically in an anaerobic chamber at 37°C for 48 h before CFU counting to determine the inhibitory effect of NanoAg-IS-PMMA-BD discs[27]. All tests were performed in triplicate.

Biofilm inhibition of NanoAg-IS-PMMA-BD: The biofilm inhibition potential of NanoAg-IS-PMMA-BD was evaluated using a biofilm formation model previously described for evaluating prospective anti-plaque agents[28]. Briefly, biofilms were procreated on NanoAg-IS-PMMA-BD discs using 24-well plates. BHI broth containing 0.5% glucose was used for biofilm cultivation. Each well was inoculated with one milliliter (~10^8 CFU/mL) adjusted bacterial inoculum. Biofilms were generated at 37 °C with agitation at 100 rpm for 168 h; media were refreshed every 24 hours. After 24 h intervals the discs were removed and were located into a fresh 24-well sterile plate and washed with PBS to remove the planktonic cells and loosely adherent bacteria. The adherent bacterial cells on the discs were displaced as described above for the test achieved in bacterial adherence test. The CFU/mL of bacterial cells in the vortexed solutions was determined as mentioned above for the test achieved in susceptibility tests of planktonic bacteria cultures. The number of CFUs was normalized by the number detected for the PMMA without NanoAg (as control group) suspension and is revealed relative to the surface area of the discs (CFUs/cm^2). All tests were achieved in triplicate.

Four-species biofilm inhibition on NanoAg-IS-PMMA-BD: Co-culture for the cultivation of four-species biofilm formation was prepared based on the procedure described by Takenaka *et al*.[29] Briefly, separate liquid cultures of *L. casei*, *L. acidophilus*, *S. mutans* and *S. sobrinus* were grown in BHI agar at 37°C for 24 h in anaerobic chamber. The CFU/mL of all suspensions were adjusted to 10^8, using a spectrophotometer as described above, and 150 µL from each suspensions was used to inoculate the acrylic disc in each well. Next, four-species biofilms were allowed to grow for 168 h at 37 °C in anaerobic chamber. Media were changed every 24 hours. Viable counts were recorded as described above for the test performed in susceptibility tests of planktonic bacteria cultures. All tests were performed in triplicate.

Biofilm inhibition on discs aged by previous biofilm growth: Experiments were run using the method described by Sevinç and Hanley[30] with some modifications. Biofilms were done on the same discs for three generations of three days of growth each. The similar procedure explained above was used to biofilms formation on discs, except that the discs were independently sonicated in PBS for 5 min between each round to discard the loosely adherent bacteria and planktonic cells. The CFU/mL of bacteria in the vortexed solutions was determined as mentioned above for the test done in susceptibility tests of planktonic bacteria cultures at the triennial day of biofilm formation for each cycle. The experiment was performed three times under the same conditions.

Statistical Analysis: Data for inhibition assays were evaluated for statistical significance as described by Sevinç and Hanley,[30] using multistep process. Briefly, normalization of our data was assessed using Q-Q plots. If the results were not distributed normally, as in the cases of kinetic measurement of bacterial growth and biofilm inhibition potential of acrylic discs, then nonparametric tests were done. In independent abnormal distributed cases, the Kruskal-Wallis test for paired comparisons was done. If the data were smaller than 0.05, then the Mann-Whitney test was used to compare the groups. Comparison of abnormally distributed but dependent groups was evaluated by the Wilcoxon Signed Ranks test.

Results and Discussion

Microbial Adhesion: As shown in table 1, the highest adherence inhibition was seen in *S. sobrinus*, which was reduced 99.9% with NanoAg-IS-PMMA-BD when compared to unmodified PMMA (control) indicating its higher sensitivity to NanoAg. Our data demonstrated that NanoAg-IS-PMMA-BD was 30% more potent in inhibiting the adherence of *S. sobrinus* than that in *L. acidophilus.* Highest adherence on test acrylic surface was observed in unmodified PMMA with *S. mutans,* which showed Light Relative Unite (LRU) 16.6(1.4) $\times 10^3$. For all test cariogenic bacteria and co-cultures there were clear differences between PMMA and NanoAg-IS-PMMA-BD, with the last-mentioned displaying significantly higher inhibition effects (table 1).

Time kinetics of planktonic bacterial cells growth inhibition: Figure 1 shows that NanoAg-IS-PMMA-BD reduced the viability of planktonic bacterial cells and co-cultures within hours of exposure ($P < 0.05$). Throughout planktonic bacterial cells growth inhibition analysis, cultures exposed to NanoAg-IS-PMMA-BD were compared to growth of test microorganisms in unmodified PMMA showing about 5-8 log decrease over a 28 days test period (table 1). Most notably, at all-time points of the planktonic bacterial cells growth inhibition assay, NanoAg-IS-PMMA-BD showed higher inhibitory activity than unmodified PMMA ($P < 0.05$), which did not inhibit the growth of any test culture. For NanoAg-IS-PMMA-BD, the antibacterial activity against all test bacteria and co-cultures was time-dependent, such that extending the length of NanoAg-PMMA exposure to 1 day enhanced the inhibitory effects of NanoAg-PMMA on these microorganisms ($P < 0.05$). As shown in Figures 1, exposure to NanoAg-IS-PMMA-BD for 1 day had a marked antibacterial effect on test bacteria and co-cultures, with a reduction in culture viability by 99.99%, compared to unmodified PMMA. On the other hand, NanoAg-IS-PMMA-BD reduced test bacteria and co-cultures viability by 1.4-1.8 log, suggesting a bacteriostatic effect on these

microorganisms, during 1-28 days of incubation period (figure 1). Although period between 1 day and 28 day time points showed reductions in the viability of test microorganisms treated with NanoAg-IS-PMMA-BD, these reductions were not significant (figure 1) ($P > 0.05$).

Table-1

Anti-microbial Effects of NanoAg-IS-PMMA: luminescence assay results of adherent microorganisms onto NanoAg-IS-PMMA as well as planktonic 28 days *in vitro* anti-microbial activity and biofilm of 168 h *in vitro* anti-microbial activity of NanoAg-IS-PMMA

Microorganisms	Anti-microbial Effects of NanoAg-IS-PMMA								
	Luminescence assay results of adherent microorganisms			planktonic 28 days *in vitro* anti-microbial activity			Biofilm of 168h *in vitro* anti-microbial activity		
	Light Relative Unite $\times 10^3$a ±SD		Adhesion inhibition (%)	CFU/cm^2* After 28 days±SD		Reduction (%) (*P*=0.000)	CFU/cm^2* After 48h±SD		Reduction (%) (*P*=0.000)
	NanoAg-IS-PMMA-BD	PMMA		NanoAg-IS-PMMA-BD	PMMA		NanoAg-IS-PMMA-BD	PMMA	
S. mutans	2.1±0.3	16.6±1.4	87.4 (*P*=0.000)	1.12±0.4	9.13±0.4	99.99	1.12±0.2	6.23±0.4	99.9
S. sobrinus	0.4±0.2	14.7±1.2	99.9 (*P*=0.000)	1.06±0.2	8.57±0.7	99.99	0.71±0.3	5.89±0.7	99.9
L. acidophilus	3.5±0.4	11.5±0.9	69.6 (*P*=0.007)	2.71±0.5	9.21±0.6	99.99	2.72±0.3	5.38±0.6	99.9
L. casei	1.8±0.5	7.1±0.8	74.9 (*P*=0.002)	2.83±0.3	8.88±0.7	99.99	0.91±0.2	4.31±0.4	99.9
Co-culture	5.1±0.7	13.3±1.4	61.3 (*P*=0.004)	3.35±0.4	8.38±0.4	99.99	1.63±0.4	5.09±0.5	99.9

[a] logarithmic scale

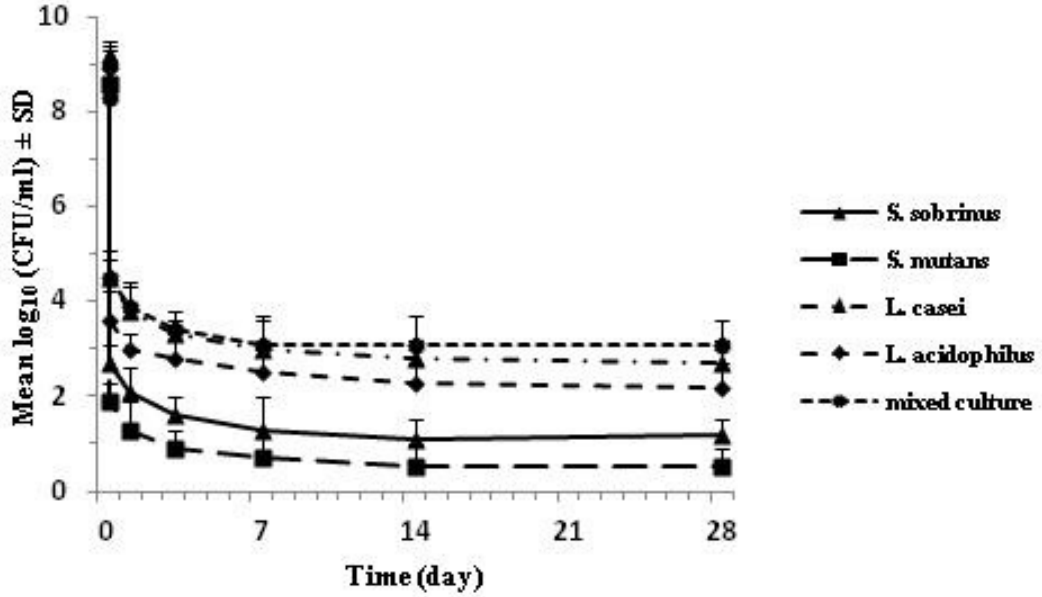

Figure-1

Time kinetics *in vitro* anti-microbial activity: Planktonic bacterial cells growth inhibition assay against *Streptococcus mutans*, *Streptococcus sobrinus*, *Lactobacillus acidophilus* and *Lactobacillus casei* by NanoAg-IS-PMMA-BD (resins made with 1benzoyl peroxide:1dimethyl-*p*-toluidine and 1% w/w silver benzoate). All NanoAg-IS-PMMA-BD groups had greater than 99.9% bacterial inhibit ion. No inhibition was observed for controls without NanoAg. Bar errors show standard deviations

Biofilm inhibition potential of NanoAg-IS-PMMA-BD: Figure 2 summarizes the number of CFU/cm^2 of test bacteria and co-cultures after 168 h biofilm growth on NanoAg-IS-PMMA-BD as a function of NanoAg as expressed relative to the maximal number of CFU/cm^2 recovered from the corresponding control acryl discs without NanoAg. The number of CFU/cm^2 on the acryl discs without NanoAg had a maximum of about 1.2×10^5 CFU/cm^2 to 1.6×10^6 CFU/cm^2, which was dependent of the types of test bacteria (table 1). This maximum was used as the 100% level for each test bacteria as well as co-cultures in this study.

The results obtained show that NanoAg-IS-PMMA-BD caused decrease in the quantity of viable test microorganism and co-cultures on the discs over a 168 h period ($P < 0.05$). Biofilm inhibition analysis demonstrated that NanoAg-IS-PMMA-BD inhibited the biofilm of all test bacteria and co-cultures by 99.9% compared to PMMA. As shown in table 1, biofilm of *S. sobrinus* and *S. mutans* showed the highest susceptibility to NanoAg-IS-PMMA-BD, which reduced bacterial viability by 5.3 and 5.1 log, respectively (both had $P= 0.000$). However, the NanoAg-IS-PMMA-BD showed smaller, but significant anti-biofilme effects on co-cultures (table 1). Our study demonstrated lower number of viable cells of test bacteria and co-cultures on the NanoAg-IS-PMMA-BD discs compared with the PMMA discs without NanoAg by the end of the testing ($P < 0.05$). The NanoAg-IS-PMMA-BD gives the long term suppression of microorganism regrowth, without an increase in number after 168 h.

Biofilm inhibition on acrylic discs aged by previous biofilm growth: Experiments were also done to find out whether the acrylic discs continuo anti-microbial effects after several runs of biofilm formation, assessed by discard biofilms and reusing the discs for fresh biofilm formation. As shown in table 2, for all test bacteria and co-cultures while there was no significant difference between the first and second run of biofilm formation on NanoAg-IS-PMMA-BD ($P <0.05$). Although third cycle showed increase in the biofilm growth of the co-cultures on NanoAg-IS-PMMA-BD compared to second cycle, these increases were not significant ($P=0.934$). However, after the third run of biofilm formation, for all test bacteria and co-cultures, biofilm growth in unmodified PMMA (control groups) were statistically significant greater than that on the NanoAg-IS-PMMA-BD group ($p=0.000$).

Our results showed that highest and lowest adherence to unmodified PMMA (control) discs was observed with *S. mutans* and *L. casei,* respectively. Considering that *S. mutans* has identified adherence ability, due to its fructosyltransferases and glucosyltransferases, and a capacity to fast synthesize exopolysaccharides [31]. The results of bacterial adherence inhibition in this study are consistent with our recent report in which NanoAg *in situ* in PMMA showed significantly effective in inhibiting the adherence of all test cariogenic bacteria and their co-culture[29]. However, NanoAg-IS-PMMA-BD has a higher anti-adherence activity than NanoAg-IS-PMMA against some microorganisms. Specifically, the increase in silver benzoate (AgBz) concentration and addition of the benzoyl peroxide and dimethyl-*p*-toluidine in NanoAg-IS-PMMA increased anti-adherence activity from 31.7-90.8% inhibition to 61.3-99.9% with the *Adhesion Test* even at 28 days. It is possible that the activities were influenced by the NanoAg dispersing and consistent adding into the resin.

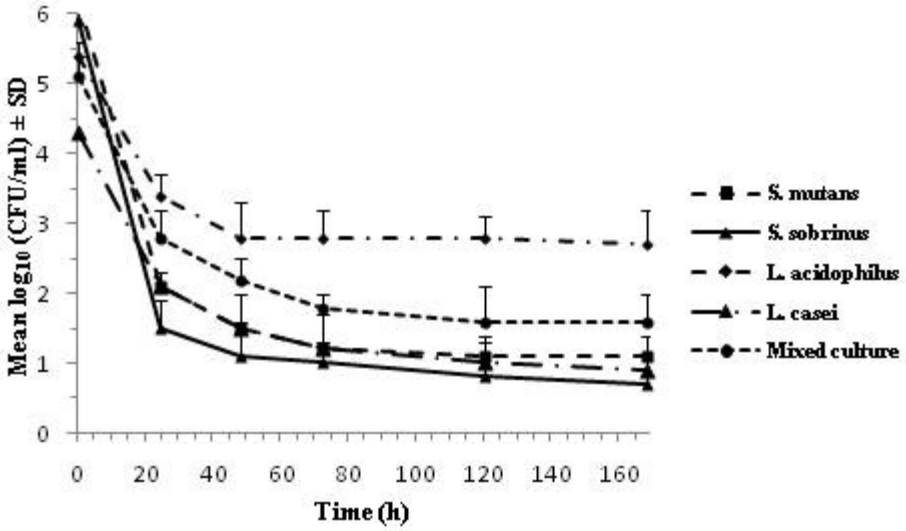

Figure-2

***In vitro* anti-biofilm activity: Biofilm bacterial cells growth inhibition assay against *Streptococcus mutans*, *Streptococcus sobrinus*, *Lactobacillus acidophilus* and *Lactobacillus casei* by NanoAg-IS-PMMA-BD (resins made with 1benzoyl peroxide:1dimethyl-*p*-toluidine and 1% w/w silver benzoate), NanoAg-IS-PMMA-BD had greater than 99.9% bacterial inhibition. No inhibition was observed for controls without NanoAg. Bar errors show standard deviations**

Table-2
Viable cell counts after the third day of test bacteria and co-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. Discs were cleaned between growth cycles to remove loosely adsorbed species (see text)

Acrylic disks	Microorganisms (CFU/cm^2)[a]±SD														
	S. mutans			*S. sobrinus*			*L. acidophilus*			*L. casei*			Co-culture		
	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd
NanoAg-IS-PMMA-BD	1.72 ±0.6	1.85 ±0.8	1.99 ±0.4	1.31 ±0.5	1.23 ±0.7	1.76 ±0.3	2.42 ±0.5	2.65 ±0.4	2.80 ±0.7	1.71±0.6	1.79±0.7	1.83 ±0.5	3.16±0.7	2.99±0.5	3.38 ±0.5
Unmodified PMMA	6.23 ±0.6	6.45 ±0.3	6.38 ±0.7	5.89 ±0.5	5.40±0.4	5.52±0.5	5.38±0.7	5.25±0.9	5.55 ±0.6	4.31±0.4	3.88±0.5	4.26 ±0.3	5.09±0.8	4.95±0.4	5.13 ±0.5

[a] logarithmic scale

Many studies have demonstrated the addition of NanoAg to PMMA has been ambiguous often due to failure to disperse consistently NanoAg, which is adverse to the appliance's strengths, and the need for complex multistep and harsh chemicals processes required for synthesis, which is improper for dental applications[18-21]. However, Kassaee *et al*.[16] showed an adequately good distribution of NanoAg in acryl, with little aggregation after addition of NanoAg to PMMA, which expedite a high release of Ag+ ions and therfore high anti-bacterial effect against *Escherichia coli* and demonstrated rather improved device's strengths. Recently, Fan *et al*.[22] used the novel approach to generate NanoAg *in situ* in PMMA (NanoAg-IS-PMMA-BD). The NanoAg-IS-PMMA-BD were eliminated the issue of non-homogenous NanoAg dispersion as well as the need for omplex multistep and harsh chemicals processes. This NanoAg-IS-PMMA-BD showed excellent anti-microbial properties against planktonic bacterial cells growth of *Acinetobacter bumannii, Pseudomonas aeruginosa, Proteus mirabilis, staphylococcus aureus* and *Streptococcus mutans*[22, 23].

In this NanoAg-IS-PMMA-BD time course study, it exhibited a rapid and wide spectrum inhibition of planktonic bacterial cells growth of the test cariogenic bacteria. An advantage of an expeditious microbicidal activity may be that it allows treatment to continue without microbial interference and decreases the possibility for resistance to develop. The initial phase after usage of denture and orthodontics appliances is important and susceptible to colonization, and so the robust ability of NanoAg-IS-PMMA-BD to kill colonizing planktonic bacteria helps to stop dental caries, gingival inflammation and periodontal disease. Similar to our previous reports,[29] This study showed inhibition of planktonic bacterial cells growth of NanoAg-IS-PMMA-BD against all test bacteria and co-cultures of the four species was time-dependent, such that extending the length of NanoAg-IS-PMMA-BD exposure from 6 h to 1 day enhanced the inhibitory effects of NanoAg-IS-PMMA-BD on these microorganisms. Oei *et al*.[23] showed that 4.2 µg/ml releases of Ag^+ ions were observed form NanoAg-IS-PMMA-BD over 28 days, however it appears to plateau when day seven. In this study the NanoAg-IS-PMMA-BD showed similar longevity of anti-microbial efficacy for all test bacteria (28 days) to a previous Oei's time course study[23] using a similar type of resins, but the variability in inhibition between these and current results again suggests this effect is genus dependent.

Results of this study demonstrated that the NanoAg-IS-PMMA-BD exhibit a high anti-microbial efficacy, even if the content of silver nanoparticles in NanoAg-IS-PMMA-BD is as low as 1% w/w all test bacteria and co-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[15]. The NanoAg-IS-PMMA-BD surfaces show glorious anti-bacterial activity throughout the twenty eight day period, however it ought to be noted that the effectiveness diminishes continuously with time as a result of the Ag^+ ions release profile with time. Initially, an outsized quantity of Ag^+ ions is released however it diminishes continuously with immersion time. The large initial Ag^+ ions release might stem from the NanoAg on the surface of the NanoAg-PMMA that oxidize once reacting with water. This study demonstrated that the NanoAg-IS-PMMA-BD had the highest long-term suppression of bacterial regrowth, without an increase in number after 168 h, which is due to the fact that the amount of Ag^+ ions diffusing through continues to be high sufficient to continue killing microorganisms. Therefore, NanoAg-IS-PMMA-BD formulation may well be a beneficial approach for biofilm inhibition.

Results from this study demonstrate that the impact of NanoAg-IS-PMMA-BD was a minimum of a pair of fold higher for test microorganisms growing in planktonic culture compared with biofilm forming cells. However, in the present study, complete elimination of biofilm did not occur, since biofilm structures serve associate anchor structure and protection for bacteria. Our biofilm inhibition data seems consistent with our recent report in which NanoAg-IS-PMMA containing showed significantly efficient in inhibiting biofilm formation of all test cariogenic bacteria and their co-culture[29]. However, NanoAg-IS-PMMA-BD has a higher anti-biofilm activity than NanoAg-IS-PMMA against some microorganisms. For instance, unlike NanoAg-IS-PMMA, NanoAg-IS-PMMA-BD inhibited the growth of *L. acidophilus* and co-cultures of the four species by 99.9%. In this work, we have used much higher concentrations of AgBz (1% w/w against 0.5%), and developed Ag^+ ions release system using benzoyl peroxide (BP) and dimethyl-*p*-toluidine (DMPT). As a result of Ag^+ ions are reduced to create NanoAg via the resin's polymerization procedure, the increase in AgBz concentration is associated with Ag^+ ions release, and increased

anti-microbial activity. It has been shown that reduction of Ag^+ ions to NanoAg by means of the resin's polymerization procedure hindered with the curing of the material and decreased device's strengths[23]. Oei *et al*.[23] demonstrated that the addition of BP and DMPT increased the mechanical properties of NanoAg-IS-PMMA similar to unmodified PMMAs (controls).

In our study, the results of biofilm inhibition assay are consistent with several studies that have reported of increase of biofilm resistance to anti-microbials compared with their planktonic analogues[32, 33]. This makes biofilms of cariogenic bacteria significantly perturbing in tooth health, whereas their presence usually creates tooth decay[34]. Mechanisms proposed to increase of biofilm resistance to anti-microbials compared with their planktonic analogues will be divided into 3 categories: transport limitation, modulation of the environment and new genes expression[35]. The NanoAg at first killing the microorganisms and therefore the recovery in cell number is then due to the dead microorganisms forming a layer through that the Ag^+ ions should diffuse. However, the exopolymers matrix excludes and/or deters the access of anti- microbials to microorganisms at intervals a biofilm[36]. Some cells may differentiate into a new constitution once attached to different surfaces, which provides them enhanced resistance. These resistant bacteria survive, multiply, and lead to a rise within the viable cell count of the bacterial biofilm. The limitation of nutrients within the biofilm ends up in a reduced rate of growth of bacteria and leads to a gradient of anti-microbials sensitivity from the higher layers to the innermost layers of biofilm, wherever the metabolism and targets of microorganisms could also be altered[37]. Change in gene expression in biofilm forming bacteria could then influence the susceptibility of bacteria to biocide[38].

Our result showed that NanoAg-IS-PMMA-BD used for multiple runs of biofilm formation weren't as effective as their initial time use; however biofilm growth on them was still considerably less than unmodified PMMA. For all test bacteria and their co-cultures, biofilm growth in NanoAg-IS-PMMA-BD were significantly lower than that on the PMMA, given the role of NanoAg in the anti-microbial mechanism of NanoAg-IS-PMMA-BD. The ability to retain anti-microbial activity following repeat microbial challenge is important in clinical situations where orthodontic appliances may remain in place for several weeks. Based on results of the present study, the strong anti-microbial activity of NanoAg-IS-PMMA-BD in comparison PMMA and regarding other studies which have shown considering non-detrimental effects of NanoAg-IS-PMMA-BD on mechanical properties of acrylic [22, 23], it seems clinically advantageous to use NanoAg-IS-PMMA-BD and benefit their anti-microbial properties. Although *in vitro* experimentation gives a good indication of how NanoAg-IS-PMMA-BD can work in practice, clinical efficacy and effectiveness require suitable clinical studies, including randomized controlled trials. However, further studies are needed to assess the optimal concentrations for final products.

Conclusion

In conclusion, the NanoAg-IS-PMMA-BD effectively inhibited adherence of cariogenic bacteria to disk surfaces as well as showed strong anti-microbial activity in the planktonic phase and subsequent biofilm formation. This demonstrated NanoAg-IS-PMMA-BD has the potential to minimize cariogenic microorganism's colonization on dentures and baseplates of orthodontic appliances and this new acrylic resin formulation may well be developed as an orthodontic appliances base.

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