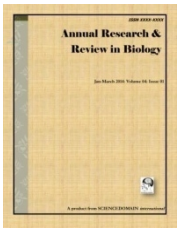

Annual Research & Review in Biology
4(17): 2695-2708, 2014

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Photocatalytic Effects of Acrylic Resins Incorporated with Nano-titanium Dioxide on Planktonic and Biofilm Growth of Four Cariogenic Bacteria

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Authors' contributions

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

Original Research Article

Received 25th August 2013
Accepted 13th November 2013
Published 6th May 2014

ABSTRACT

Aims: The activities of cariogenic bacteria in biofilm on acrylic baseplates of removable orthodontic appliances and partial denture contribute to dental caries, inflammation of gingiva and periodontal disease. This *in vitro* study evaluates the

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photocatalytic antimicrobial activity of acrylic specimens (AS) containing NanoTiO_2 under ultraviolet type A (UVA) illumination against four cariogenic bacteria.
Study Design: An *in vitro* study
Place and Duration of Study: Department of Microbiology and Department of Orthodontics, Tehran University of Medical Sciences (TUMS); Department of Chemistry, Tarbiat Modares University, between June 2011 and March 2012.
Methodology: Chemical-cure acrylic resins, Polymethylmethacrylates (PMMAs), were used to synthesize acrylic specimens containing NanoTiO_2 (NanoT-AS). Antibacterial activity of NanoT-AS were assessed against *Streptococcus sobrinus*, *Streptococcus mutans*, *Lactobacillus acidophilus* as well as *Lactobacillus casei* and co-cultures of the four species by adherence inhibition as well as planktonic and biofilm bacterial cells growth inhibition on NanoT-AS under UVA illumination (NanoTiO_2+/UVA+).
Results: Exposure to NanoTiO_2+/UVA+ reduced bacterial adherence by 43.8-96.5% depending on the microorganism type. NanoTiO_2+/UVA+ showed 1.7-6.0 log decrease planktonic cultures in time-dependent manner over a 4h period (P<0.05). NanoT-AS inhibited significantly biofilm formation of all test bacteria and co-cultures by 44.2-93.1%, compared to unmodified PMMA (control) under UVA illumination. Some inhibitory activity of NanoTiO_2+/UVA+ could be maintained even after the third generation of biofilm growth.
Conclusion: The data presented here are novel in that they prove that NanoTiO_2+/UVA+ effectively inhibited adherence of cariogenic bacteria to NanoT-AS surfaces as well as strong antimicrobial activity in the planktonic phase and subsequent biofilm formation. This data shows NanoTiO_2+/UVA+ has the potential to minimize cariogenic microorganism's adherence and colonization on acrylic specimens and this novel acrylic resin formulation could be developed as a denture and orthodontic appliances base.

Keywords: Cariogenic Bacteria; Orthodontics, Photocatalystic effects; Polymethylmethacrylate; Titanium dioxide Nanoparticles.

1. INTRODUCTION

The importance of acrylic resins, polymethylmethacrylate (PMMA), in dentistry is evident, since being widely used as denture base material in prosthodontics, provisional prosthesis and acrylic baseplates of removable orthodontic appliances such as retainers and functional appliances [1]. However one of the major problems that patients and dentists commonly faced using these acrylic based appliances (ABA) is their potential for plaque accumulation due to their porous surface and food retentive configuration, which in turn increase cariogenic activity of oral flora and may contribute to dental caries, inflammation of gingiva and periodontal disease [2-4].

Mechanical cleaning of ABA is helpful in reducing biofilm and accumulation of microbial plaque, particularly with the adjunctive use of antimicrobial solutions [5,6]. However, such measures rely generally on conformity of patients, and may not be optimal in children and handicapped individuals. Therefore, an additive that robustly enhances the inhibitory effects of ABA, while maintaining its biocompatibility, is highly desirable [7]. A recent method to overcome this dilemma is to manufacture self-cleaning acrylic resins (AR) containing antiseptics by incorporating antiseptic agents such as quaternary ammonium compound (QAC) into AR [8,9]. However, it was demonstrated that bacterial biofilm formation on QAC incorporating into AR correlated with resistance to QAC. Incorporating more appropriate antimicrobial additives to acrylic resin, utilization of some nanoparticles has been suggested.

Silver in nanoparticulate form (NanoAg) *in situ* in PMMA (NanoAg-IS-PMMA) have been reported to inhibit the cariogenic bacteria in planktonic and biofilm cultures [10,11]. The effect of NanoAg addition to the dental material is important from cosmetic viewpoint. Applying NanoAg makes the material turns black and this is problematic.

Nanoparticles of Titanium dioxide (NanoTiO_2) are good examples since their antibacterial properties have been shown in various biomaterials [9,10]. In addition to antibacterial effects, TiO_2 offers white color and high stability as well as low cost and less toxicity [12-14]. Thus, among orthodontic materials TiO_2 would be appropriate substance to be incorporated to dental materials Choi et al. [15] and Shah et al. [16] studied wires and brackets coated with NanoTiO_2 and their antimicrobial effects on planktonic growth of *streptococcus mutans* and *Lactobacillus acidophilus*, respectively [17]. However no previous study has investigated the antimicrobial effects of PMMA incorporated with these particles. It is hypothesized that by incorporation of NanoTiO_2 into acrylic resins, PMMA, we can take the advantages of their photocatalistic and self-sterilizing properties for acrylic appliances.

Accordingly, this study was conducted with the purpose of evaluating the anti-adherent and antimicrobial activity of PMMA acrylic resins containing NanoTiO_2 against *S. mutans*, *S. sobrinus*, *L. acidophilus* and *L. casei* as the most important cariogenic oral bacteria in planktonic and biofilm cultures grown as a single species and in co-culture.

2. MATERIALS AND METHODS

2.1 Preparation of Acrylic Specimens (AS) Incorporated with NanoTiO_2

PMMA specimens (Selecta Plus; Dentsply, UK) incorporated with the 1% colloidal 21 nm diameter NanoTiO_2 (Degussa, Germany) (referred to as NanoT-AS) was prepared using the Sodagar method [10]. The total surface area of each NanoT-AS slide was 4.00±0.35 cm^2. Prior to the tests, AS were sterilized by 25 kGy of ^{60}Co irradiation according to ISO 11135:1994 for medical devices [18].

2.2 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., Tehran, Iran) were rehydrated in brain heart infusion (BHI) broth (Merck, Darmstadt, Germany), and incubated in an anaerobic atmosphere at 37°C for 48h [19]. To examine the antimicrobial activity of the NanoT-AS slides, the test bacterial suspension of approximately 10^8 CFU/mL was prepared using a spectrophotometer. 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 colony forming units (CFU) counts. For the *L. casei* OD_{600} of 0.8 and for the *S. mutans* and the *S. sobrinus* OD_{600} of 0.9-1 for a 1/100 dilution is equivalent to10^8 cells/mL.

2.3 Experimental Conditions

Samples to be lit were placed in a noncommercial chamber equipped with a 15W BLB lamp (Philips Electronics, Seoul, Korea), emitting radiation over 350-410 nm. Distance between the lamp and the samples in an anaerobic cabinet were set up to obtain 1.0 mW/cm^2 of

ultraviolet type A (UVA) light incident on the samples [16]. UVA light intensity was measured by the UVA radiometer (Konica Minolta). In all experiments, four conditions were test: (1) presence of NanoTiO$_2$ (i.e. NanoT-AR) and UVA light (NanoTiO$_2$+/UVA+), (2) no NanoTiO$_2$ (i.e. PMMA) and presence of UVA light (NanoTiO$_2$-/UVA+), (3) presence of NanoTiO$_2$ under dark condition (NanoTiO$_2$+/UVA-), (4) no NanoTiO$_2$ under dark condition (NanoTiO$_2$-/UVA-) [16,20].

2.4 Bacterial Adherence Test

Adherence of test microorganisms and their co-cultures was determined by "solid-liquid system" using the Polo method [20] with some modifications. Briefly, one mL of adjusted fresh suspension of test bacteria and their co-cultures to 5×10^5 CFU/mL was placed on NanoT-AS and on unmodified PMMA (control). Adherent bacteria on the specimens were determined based on luminescent ATP measurement (Bac Titer-Glo, Promega, Fitchburg, WI) according to the manufacturer's instructions after 15, 30, 60, 120, 180 and 240min of illumination [21].

2.5 Susceptibility Tests of Planktonic Bacteria Cultures on NanoT-AS

The planktonic inhibition potential of NanoT-AS were evaluated using the Polo method [20]. Briefly, one mL of fresh suspension of test bacteria and their co-cultures adjusted to 10^8 CFU/mL were added to "solid-liquid system" as mentioned the bacterial adherence test. The system was incubated in an anaerobic chamber (5% CO_2, 10% H_2, and 85% N_2) at 37 °C. The CFU/mL was assessed on a culture, after 15, 30, 60, 120, 180 and 240min of illumination. The CFU/mL enumerated using the drop-plate method [22].

2.6 Biofilm Inhibition on NanoT-AS

For the biofilm inhibition tests, the specimens were placed in falcon tubes containing BHI broth medium and the test bacteria hen their co-cultures suspensions adjusted to 5×10^5 CFU/mL was added [23]. The samples were incubated at 37°C for 24h under dark conditions to allow bacterial biofilm formation. The CFU/ mL were assessed after 15, 30, 60, 120, 180 and 240min of illumination as described above for bacterial adherence test. The CFU/mL was normalized by the number determined for the PMMA control suspension and was expressed relative to the surface area of the sample (CFUs/cm^2).

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 [24]. Biofilms were generated on the same NanoT-AS discs for 3 cycles of 3 days of growth each. The same methodology explained above was used to biofilms generation on discs, except that discs were separately sonicated in PBS for 5 minutes between each cycle to remove the planktonic and loosely adherent bacteria. As described above, the CFU/mL of bacteria in the vortexed solutions was determined for the test performed in susceptibility tests of planktonic bacteria cultures at the third day of biofilm growth for each cycle. The experiment was repeated three times under identical conditions.

2.8 Statistical Analysis

All tests were performed in triplicate and data were evaluated for statistical significance as described by Sevinç and Hanley [24].

3. RESULTS

3.1 Microbial Adhesion

After 4h irradiation we observed, only a mild (0.5-1.1 LRU) non-significant reduction in viable cells in NanoTiO_2-/UVA+ (Table 1).

As shown in Table 2, the highest adherence inhibition was seen in *S. mutans*, which was reduced 96.5% with NanoT-AS after 4h of UVA illumination, when compared to NanoTiO_2-/UVA+ indicating its higher sensitivity to NanoTiO_2.

As shown in Fig.1, UVA illumination for 15min had a marked anti-adherence effect on test bacteria and their co-culture, with a reduction in culture viability by 37.3-89.1%, compared to unmodified PMMA. On the other hand, NanoT-AS reduced test bacteria and their co-culture viability by 12.8-81.2%, was suggesting a bacteriostatic effect on these microorganisms, during 15min-4h of UVA illumination period (Fig.1). Our data demonstrated that NanoT-AS was two-fold more potent in inhibiting the adherence of *S. mutans* than that in *L. casei* and *L. acidophilus* under UVA illumination.

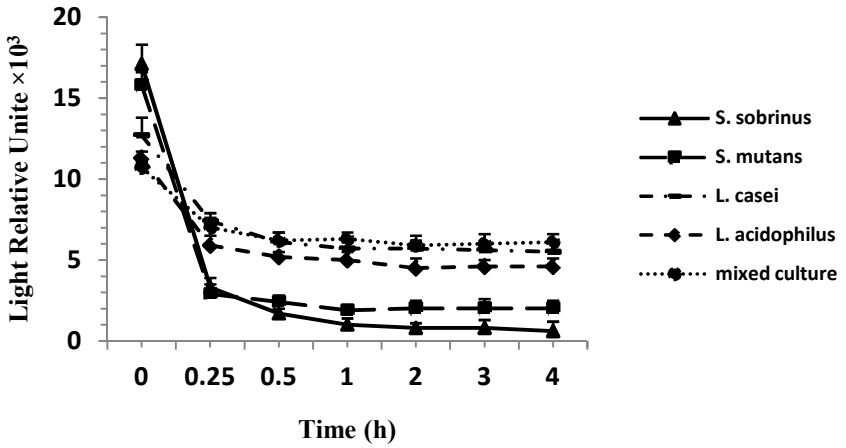

Fig. 1. CFU/ cm^2 values from the tests in solid-liquid system with photocatalytic NanoT-AS under UVA illumination and test bacteria and mixed cultures of the four species in the initial adhesion step. Each value corresponds to the mean of three analytical replicates for all experimental condition. Bar errors show standard deviations

Table 1. *In vitro* antimicrobial effects of PMMA under dark condition ($NanoTiO_2$-/UVA-) and UV-A illumination ($NanoTiO_2$-/UVA+): luminescence assay results of adherent microorganisms onto PMMA as well as anti-planktonic and anti-biofilm formation of PMMA after 4h

Microorganisms	Antimicrobial effects of $NanoTiO_2$-/UVA+								
	Luminescence assay results of adherent microorganisms			Planktonic 4h *in vitro* antimicrobial activity			Biofilm of 4h *in vitro* antimicrobial activity		
	Light Relative Unite $\times 10^3$		Adhesion inhibition (%)	CFU/ml* After 4h		Reduction (%)	CFU/cm^2* After 4h		Reduction (%)
	$NanoTiO_2$-/UVA+	$NanoTiO_2$-/UVA-		$NanoTiO_2$-/UVA+	$NanoTiO_2$-/UVA-		$NanoTiO_2$-/UVA+	$NanoTiO_2$-/UVA-	
S. mutans	17.1±0.9	18.2±0.8	6.1(*P*=0.295)	8.93±0.8	9.04±0.5	22.3(*P*=0.283)	5.81±0.5	5.83±0.7	4.5(*P*=0.530)
S. sobrinus	15.8±0.7	16.7±0.5	5.3(*P*=0.341)	8.63±1.3	8.73±0.7	18.7(*P*=0.359)	5.03±0.9	5.05±0.6	4.1(*P*=0.684)
L. acidophilus	12.8±1.1	13.4±0.7	3.7(*P*=0.785)	7.89±0.7	7.96±0.9	14.8(*P*=0.437)	4.38±0.6	4.39±1.1	2.2(*P*=0.831)
L. casei	11.5±0.9	11.8±0.7	4.9(*P*=0.567)	8.31±0.4	8.37±0.8	12.9(*P*=0.512)	4.01±0.5	4.02±0.6	2.5(*P*=0.758)
Co-culture	10.7±0.6	11.2±0.9	4.4(*P*=0.632)	7.63±0.9	7.69±0.6	12.1(*P*=0.563)	3.88±0.4	3.89±0.7	1.8(*P*=0.962)

**; logarithmic scale*

Table 2. *In vitro* antimicrobial effects of PMMA and NanoT-AS under UV-A illumination ($NanoTiO_2$-/UVA+ and $NanoTiO_2$+/UVA+, respectively): luminescence assay results of adherent microorganisms onto PMMA and NanoT-AS as well as anti-planktonic and anti-biofilm formation of PMMA and NanoT-AS after 4h

Microorganisms	Antimicrobial Effects of NanoT-AS under UV-A illumination								
	Luminescence assay results of adherent microorganisms			Planktonic 4h *in vitro* antimicrobial activity			Biofilm of 4h *in vitro* antimicrobial activity		
	Light Relative Unite $\times 10^3$		Adhesion inhibition (%)	CFU/ml* After 4h		Reduction (%)	CFU/cm^2* After 4h		Reduction (%)
	$NanoTiO_2$ +/UVA+	$NanoTiO_2$-/UVA+		$NanoTiO_2$ +/UVA+	$NanoTiO_2$ -/UVA+		$NanoTiO_2$ +/UVA+	$NanoTiO_2$ -/UVA+	
S. mutans	0.6±0.2	17.1±0.9	96.5(*P*=0.000)	2.82±0.4	8.93±0.8	>99.99(*P*=0.000)	4.80±0.5	5.83±0.7	93.1(*P*=0.000)
S. sobrinus	2±0.1	15.8±0.7	87.3(*P*=0.000)	3.11±0.3	8.63±1.3	>99.99(*P*=0.000)	4.42±0.5	5.05±0.6	76.3(*P*=0.000)
L. acidophilus	5.5±0.7	12.8±1.1	57.2(*P*=0.000)	4.52±0.3	7.89±0.7	>99.99(*P*=0.004)	4.12±0.7	4.39±1.1	45.1(*P*=0.003)
L. casei	4.9±0.4	11.3±0.9	56.6(*P*=0.006)	4.28±0.5	8.31±0.4	>99.99(*P*=0.002)	3.66±0.4	4.02±0.6	55.6(*P*=0.006)
Co-culture	6.1±0.5	10.7±0.6	43.8 (*P*=0.014)	5.90±0.6	7.63±0.9	>99.99(*P*=0.007)	3.71±0.4	3.89±0.7	44.2(*P*=0.009)

**; logarithmic scale*

3.2 Time Kinetics of Planktonic Bacterial Cells Growth Inhibition

Fig. 2 illustrates, cultures exposed to NanoTiO$_2$+/UVA+ showing about 2-6 log decrease over a 4h test (Table 2). Nonsignificant decreases of CFU/mL values were detected in presence of NanoTiO$_2$+/UVA- (0.06-0.10 log reduction after 4h; P> 0.05). A 0.06-0.11 log decrease of CFU/mL was also verified in samples exposed to NanoTiO$_2$-/UVA+ after 4h (P> 0.05). Photoactivated NanoT-AS induced a 6 log reduction of CFU/mL values of *S. mutans* in 4h, compared with those found in samples without TiO$_2$. The *S. sobrinus* results exhibited the same trend as those of *S. mutans*. Significant CFU/mL reduction of 5 log units (P=0.000) was observed after 4h only in NanoTiO$_2$+/UVA+. In the case of *L. acidophilus*, *L. casei* and their co-culture, 3, 4 and 2 log reduction of CFU/mL values was observed after 4h in NanoTiO$_2$+/UVA+, respectively, compared with NanoTiO$_2$-/UVA+.

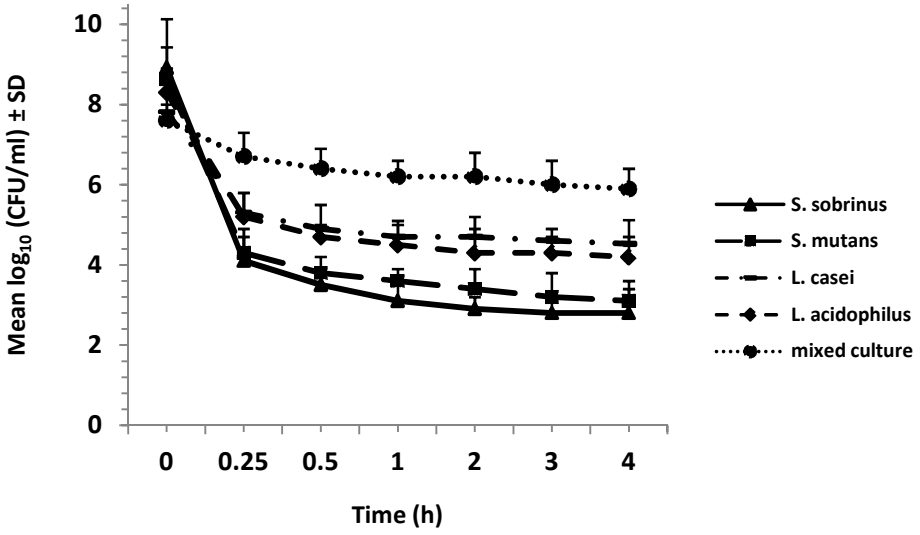

Fig. 2. Time kinetics of planktonic bacterial cells growth inhibition, Mean log$_{10}$ (CFU/mL) ± SD values obtained in the tests with NanoT-AS under UVA illumination and planktonic cells of test bacteria and mixed cultures of the four species. Each value corresponds to the mean of three replicates. Bar errors show standard deviations

For NanoT-AS, the antibacterial activity against all test bacteria and their co-culture was time-dependent. As shown in Fig. 2, exposure to NanoTiO$_2$+/UVA+ for 15min had a marked antibacterial effect on test bacteria and their co-culture, with a reduction in culture viability by 99.99%, compared to NanoTiO$_2$-/UVA+. On the other hand, NanoT-AS reduced test bacteria and their co-culture viability by 3.8-62.8% under UVA illumination, was suggesting a bacteriostatic effect on these microorganisms, during 15min-4h of UVA illumination period (Fig. 2). Although period between 15min and 4h time points showed reductions in the viability of test microorganisms treated with NanoT-AS. These reductions were not significant (Fig. 1).

3.3 Biofilm Inhibition Potential of NanoT-AS

As shown in Table 2 $NanoTiO_2$+/UVA+ inhibited the biofilm formation of all test bacteria and their co-culture by 44.2-93.1% compared to $NanoTiO_2$-/UVA+. Biofilm of *S. mutans* showed the highest susceptibility to $NanoTiO_2$+/UVA+, which reduced bacterial viability by 93.1%, (*P*=0.000). The $NanoTiO_2$+/UVA+ showed smaller, but significant anti-biofilm effects on co-culture (Fig.3). $NanoTiO_2$+/UVA+ induced in 4h a one log reduction of CFU/cm^2 values of *S. mutans*, compared with $NanoTiO_2$-/UVA+. No significant decreases of CFU/cm^2 values were detected in presence of $NanoTiO_2$+/UVA- (0.022 log reduction after 4h; *P*=0.530). A 0.08 log decrease of CFU/cm^2 was also verified in samples exposed to $NanoTiO_2$-/UVA+ after 4h (*P*=0.09). The *S. sobrinus* results exhibited the same trend as those of *S. mutans*. Significant CFU/cm^2 reduction of 0.63 log units (*P*=0.000) was observed after 4h only in the sample with photoactivated NanoT-AS. In the case of *L. acidophilus*, *L. casei* and co-culture of test bacteria, 0.27, 0.32 and 0.18 log reduction of CFU/cm^2 values was observed after 4h only in presence of $NanoTiO_2$+/UVA+, respectively, compared with $NanoTiO_2$-/UVA+.

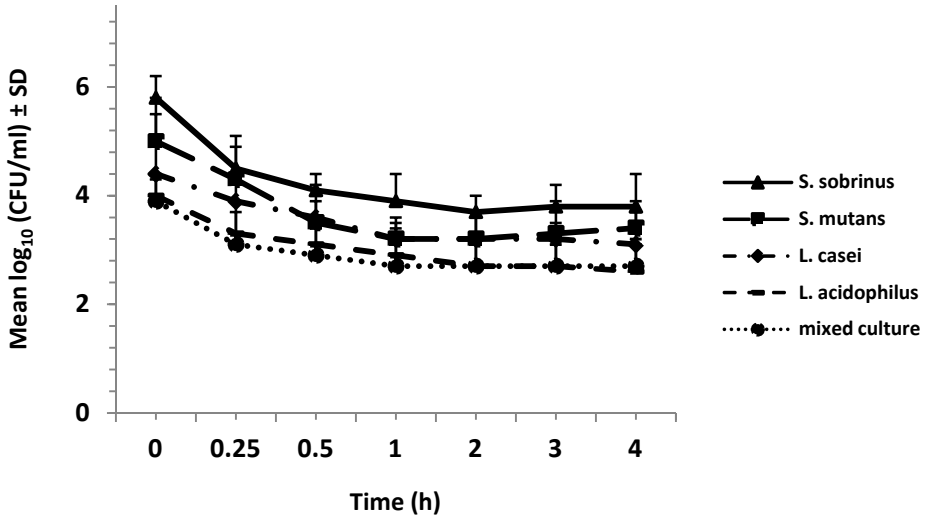

Fig. 3. CFU/ cm^2 values obtained in the tests with photocatalytic NanoT-AS under UVA illumination and test bacteria and mixed cultures of the four species in biofilm 24-h old. Each value corresponds to the mean of three analytical replicates for all experimental condition. Bar errors show standard deviations.

3.4 Biofilm Inhibition on Acrylic Slides Aged by Previous Biofilm Growth

Experiments were also run to find out whether the acrylic slides maintained antimicrobial properties after several cycles of biofilm growth, evaluated by scrapping off biofilms and reusing the acrylic slides for new biofilm growth. As shown in Table 3, for all test bacteria and their co-culture there was statistically significant difference between the first and second cycle of growth on $NanoTiO_2$+/UVA+ (*P*<0.05). Although third cycle showed increase in the biofilm growth of the *L. acidophilus* and co-culture on $NanoTiO_2$+/UVA+ compared to second cycle, these increases were not significant (*P*=0.246 and *P*=0.314, respectively). However, after the third cycle of biofilm growth, for all test bacteria and their co-culture, biofilm growth in $NanoTiO_2$-/UVA+ were significantly greater than that on $NanoTiO_2$+/UVA+ (*P*=0.000).

Table 3. Viable cell counts after the third day of test bacteria and mixed organisms culture of the four species biofilm growth on the same slides after the first, second, and third cycle of subsequent. Slides were cleaned between growth cycles to remove loosely adsorbed species (see text)

Acrylic slids	Microorganisms (CFU/cm^2)*														
	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**
NanoTS-AS	4.80±0.5	4.72±0.6	4.83±1.2	4.42±0.5	4.53±0.5	4.67±0.5	4.12±0.7	4.15±0.4	4.45±0.7	3.66±1.4	3.72±0.3	3.79±0.1	3.71±0.4	3.79±1.1	3.95±0.7
Unmodified PMMA	5.83±0.7	5.80±0.4	5.89±0.7	5.05±0.6	5.14±0.5	5.36±0.8	4.39±1.1	4.43±0.7	5.55±0.6	4.02±0.6	4.19±0.4	4.28±0.8	3.89±0.7	4.00±0.9	4.13±0.5

**a logarithmic scale*

4. DISCUSSION

Our results showed that the highest and lowest adherence to NanoTiO$_2$ under dark condition (NanoTiO$_2$-/UVA-) was observed with *S. mutans* and *L. casei,* respectively. *S. mutans* has a recognized adherence ability, due to its fructosyltransferases and glucosyltransferases, and a capability to rapidly synthesize exopolysaccharides [25]. A recent study showed that *L. casei* was unable to produce exopolysaccharides in the adherence of dental biofilm [26]. Our study showed NanoT-AS under UVA illumination (NanoTiO$_2$+/UVA+) is significantly effective in inhibiting the adherence of all test cariogenic bacteria and their co-culture. This agrees with the findings of another study that found lower adherence of *L. acidophilus* on orthodontic brackets covering with NanoTiO$_2$ [16]. These adherence inhibition properties are very meaningful to clinical applications. The initial phase after usage of materials for orthodontics is important and prone to colonization, and so the strong ability of NanoT-AS to inhibit surrounding adherent bacteria helps prevent plaque formation and dental caries. We also observed that NanoTiO$_2$+/UVA- and NanoTiO$_2$-/UVA+ treatment, compared with control groups NanoTiO$_2$-/UVA- , did not reduce in all test cariogenic bacteria and their co-culture attachment significantly. These results are in agreement with study by polo et al. [20], who have reported the photocatalytic inactivation of *Pseudomonas aeruginosa* in analogous conditions.

In our study NanoTiO$_2$+/UVA+ easily killed planktonic cells of all test cariogenic bacteria and their co-culture. This efficiency on NanoTiO$_2$+/UVA+ is significantly higher than that due to NanoTiO$_2$+/UVA- and NanoTiO$_2$-/UVA+ treatment, as reported by polo et al. [20] in analogous experiments with *P. aeruginosa*. The varied and broad spectrum *in vitro* activity of NanoTiO$_2$ has been reported before [27-28]. Our data showed that UVA alone had no significant antimicrobial activity. Comparing the results of NanoTiO$_2$+/UVA+ with NanoTiO$_2$+/UVA-, it can be concluded that although NanoTiO$_2$ could lead to reduce no significantly bacterial colonies in dark, utilizing UVA would enhance its antimicrobial activity. This agrees with the findings of another study that found UVA would enhance NanoTiO$_2$ antimicrobial activity [29]. According to Fujishima et al. [30] the intensity of UVA was directly related to the photocatalytic activity of NanoTiO$_2$ since higher intensity leads to formation of more free radicals of super oxide and hydroxyl group ($OH^{\cdot}$) which have high level of energy and are so active that react with organic materials (such as bacterial cell-wall), break the chemical bonds and decompose them to CO_2 and water.

According to another point of view about our results, the maximum antimicrobial activities of NanoT-AS was observed in 15-30 min under UVA illumination, then it trend reaches a plateau, since the last follow-up (after 4h) demonstrated the least CFU. Choi et al. [15] claimed that the maximum antimicrobial activity of orthodontic wires coated with NanoTiO$_2$ was observed in 20-30 minutes exposure of UVA and after that a reduction ensued. The same pattern was reported by Watts et al. [31] while a two-step decay dynamics of the photokilling process was reported by Sunada et al. [32]. The latter pattern consist a very low rate bactericidal step followed by a higher-rate one. The solar UVA intensity is about 4 mW/cm^2 in sunny days and drops about 10 times in cloudy days [33]. In order to take the advantage of UVA, the patients should be advised to keep their acrylic orthodontic appliances which contain NanoTiO$_2$ for about 5 min in outdoors under sun exposure in sunny days or at least 15 min under UVA lamps with the intensity of 1mW/cm^2.

In biofilm systems of our study, the NanoTiO$_2$+/UVA+ had biocidal and antifouling effects compared with NanoTiO$_2$+/UVA- and NanoTiO$_2$-/UVA after 4h of treatment. In this case there was a significant decrease, in cell concentration (0.18-1.02 log) compared with UVA

alone. The results of biofilm inhibition analysis presented here consist with some other cases reported in analogous studies [20]. Results from this study indicate that the minimum inhibitory concentrations (MICs) of NanoT-AS was 6-12 folds higher for test bacteria growing in biofilm compared with cells in suspension. However, in the present study, complete elimination of biofilm did not occur, since biofilm structures represent an anchor structure and protection for microorganisms. Our biofilm inhibition data seems consistent with several studies that have reported of increase of biofilm resistance to antimicrobial agents compared with their planktonic counterparts [34-35]. This makes biofilms of cariogenic bacteria particularly disturbing in tooth health, while their presence often creates dental caries [36]. Mechanisms proposed to increase of biofilm resistance to antimicrobial agents compared with their planktonic counterparts can be divided into three categories: transport limitation, modulation of the environment and new genes expression [37].

It was particularly interesting to find that NanoT-AS demonstrated a persistent effect, when PMMA were not capable of producing antibacterial effects against cariogenic microorganisms. Our result showed that NanoT-AS used for multiple cycles of biofilm growth were not as effective as their first time use, but biofilm growth on them was still significantly less than unmodified acryl ($NanoTiO_2$-/UVA+). It is due to the fact that the amount of $NanoTiO_2$ diffusing through is still high sufficient to continue killing bacteria. The ability to retain antimicrobial activity following repeat microbial challenge is important in clinical situations where acrylic appliances may remain in place for long time.

Based on results of the present study, the strong bacterial adherence inhibition and anti-biofilm activity of NanoT-AS in comparison with PMMA and regarding our previous study which have shown considering non-detrimental effects of 1% NanoT-AS on mechanical properties of poly (methyl methacrylate) acrylic resins [11,17,38], it seems clinically advantageous to use $NanoTiO_2$+/UVA+ and benefit their antimicrobial properties. Although in vitro experimentation gives a good indication of how NanoT-AS can work in practice, clinical efficacy and effectiveness require suitable clinical studies, including randomized controlled clinical trials. However, further studies are needed to evaluate the proper concentrations for final products.

5. CONCLUSION

The $NanoTiO_2$+/UVA+ effectively inhibited adherence of cariogenic bacteria to acrylic surfaces as well as showed strong antimicrobial activity in the planktonic phase and subsequent biofilm formation. This demonstrated $NanoTiO_2$+/UVA+ has the potential to minimize cariogenic microorganism's colonization on acrylic specimens and this novel acrylic resin formulation could have opportunity to be developed as a denture and orthodontic appliances base.

ACKNOWLEDGEMENTS

This study was supported by grant from Tehran University of Medical Sciences (TUMS) (grant No: 8778). We are greatly thankful to Dr. J. Kharazi for statistical analysis.

COMPETING INTERESTS

Authors have declared that no competing interests exist

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