Antibacterial Effect of Experimental Orthodontic Elastomeric Ligature Coated with Zinc Oxide Particles

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Abstract. Objectives. This study aimed to evaluate antibacterial activity of elastomeric ligatures coated with Zinc oxide particles against \textit{Streptococcus mutans}. Methods. ZnO particles grafted with (3-Aminopropyl)trimethoxysilane (APTMS) were prepared in situ. The ATR-FTIR spectrum was used to analyze the APTMS grafted on ZnO surfaces. Two concentrations of ZnO-APTMS, i.e., 5 and 10 wt\%, were coated on orthodontic elastomeric ligatures by the dip coating method. Antibacterial property of the ZnO-APTMS coated elastomeric ligatures against \textit{S. mutans} ATCC25175 were investigated by the agar diffusion test. The effect of ligature aging on antibacterial property was evaluated by the direct contact test, in which the growth of bacteria was determined by the turbidity after exposed to the samples that had been immersed in distilled water for 0, 3, 7, 14, 28 days. The drop plate test was also performed to determine the inhibitory and the bactericidal effects. Results. The analysis of ATR-FTIR spectrum confirmed that APTMS was successfully grafted on ZnO surfaces. The agar diffusion test could not demonstrate the antimicrobial effects of the ZnO-coated elastomeric ligatures. However, results from the direct contact and the drop plate tests showed the inhibitory effects on bacterial growth compared to the positive controls ($p < 0.05$). The inhibitory effect of the ZnO-coated elastomeric ligatures was observed even after they had been immersed in distilled water for 28 days. Conclusions. The surface coating elastomeric ligatures with 5 and 10 wt\% ZnO-APTMS exhibited antibacterial activity against cariogenic bacteria, \textit{S. mutans}. The bacterial inhibitory effect was prolonged until 28-day.

Introduction

Nowadays, orthodontic treatment has become increasingly popular among the adolescent and adult population. The presence of fixed appliances on tooth surfaces makes the cleaning process difficult. Increased dental biofilm accumulation can result in white spot lesion (WSL) or enamel decalcification due to acid production from biofilm microorganisms.[1, 2] Development of WSL during fixed appliances can occur rapidly and almost 50\% of orthodontic patients develop at least one WSL during the orthodontic treatment.[3, 4] Ligation material is one of the variables that affects bacterial adhesion during orthodontic treatment.
Traditionally, there are three approaches to connect orthodontic arch wire with the bracket, i.e., ligation with stainless steel wires, elastomeric ligatures, and self-ligating brackets.[5] In addition to the ease and practicality, elastomeric ligatures have become one of the promising ligatures because of their smooth continuous force and consistent long-lasting seating of the arch wire.[6] Unfortunately, the studies by Forsberg et al. and Garcez et al. have shown the adherence of the biofilms on various types of ligations, and also found that elastomeric ligatures had the highest amount of cariogenic bacteria, Streptococcus mutans, accumulated in biofilms.[5, 6] Elastomeric ligatures (O-ring) derived from polyurethane (PU) was initially incorporated with fluoride to overcome the accumulation of microorganisms. The release of fluoride aims to enhance not only the antibacterial activity but also the remineralization process.[7] However, Wilson and Gregory reported that fluoride incorporated elastomeric ligatures exhibited temporary reduction of S. mutans within 1 week only.[7]

Metal ions and metal oxides are inorganic materials that have been investigated for their use as antimicrobial and antiviral agents in various fields, including medicine.[8] Compared to other metal oxides, such as copper oxide (CuO) and titanium dioxide (TiO2), zinc oxide (ZnO) is a preferred alternative because of its cost-effective, free of surface water, and easy synthesis process.[9] The antimicrobial activity of ZnO nanoparticles microparticles (NPs/MPs) is relied on Zn²⁺ ion release that causes the destabilization of microbial membranes and the cell wall. The adsorption and generation of reactive oxygen species (ROS) resulting in the disruption of the cell wall and cell membrane of the microorganisms.[10-12] Moreover, Zinc is proven to be effective against cariogenic bacteria. At lower concentrations, Zinc can inhibit S. mutans indicating that it was safe to be used in oral health products.[13]

Various methods have been used to incorporate metal oxides to PU, such as in situ polymerization, blending, and coating. Nirlama et al. compared antibacterial effects of silver nanoparticles incorporated into PU nanofiber by two different approaches. They showed that the silver-coated PU had greater antibacterial efficacy than the PU which added silver solution before fabrication. They suggested that the silver ions coating on the PU were released to the medium more easily than the those that embedded in PU fiber.[14]

Taken together, we established the hypothesis that the antibacterial activity could be expected from elastomeric ligatures contains ZnO and therefore, focused on the fabrication of PU based elastomeric ligatures surface functionalized with ZnO.

Materials and methods

**Materials.** Utechllan UDS-70AU DPS300 thermoplastic polyurethane was purchased from Covestro, Germany. Zinc oxide powder (ZnO) was purchased from QRëC, New Zealand. (3-Aminopropyl) trimethoxysilane (APTMS) was purchased from Sigma-Aldrich, Singapore. Ethanol and toluene were purchased from RCI Labscan, Thailand. All chemicals were the medical grade and used without any purification.

**Preparation of APTMS grafted ZnO.** ZnO powder (5 g, 61.4 mmol) was dispersed in toluene (25 mL) in a round-bottom flask and stirred by using magnetic stirrer for 10 minutes. After that, the ZnO suspension was sonicated for 15 min. APTMS (0.5 g, 2.80 mmol) was gradually added into the suspension at room temperature under vigorous stirring for 24 h. The suspension obtained was filtered and washed several times with ethanol to obtain APTMS grafted ZnO (ZnO-APTMS). The powder obtained was dried in a vacuum oven at 60 °C for 4 h.
Characterization. The chemical structures of the samples were investigated by a Bruker ALPHA attenuated total reflectance Fourier transform infrared spectrophotometer (ATR-FTIR) in frequency ranging from 4000 to 550 cm\(^{-1}\). The ATR-FTIR spectrum was analyzed by using OPUS7.0 software.

Fabrication of Elastomeric ligature. An Utechllan UDS-70AU DPS300 thermoplastic polyurethane was fabricated as an elastomeric ligature by using an Nigata, MD50S-IV injection molding machine at 200 °C. The elastomeric ligatures obtained without surface modification were named as normal ligature (NL).

Surface modification of elastomeric ligature with ZnO. The surface coating of elastomeric ligatures with ZnO-APTMS was prepared by dip coating method. ZnO-APTMS powder (5%, 41.67 mg/mL) was dispersed in ethanol (99%) and sonicated for 10 minutes. Experimental elastomeric ligature was dipped in ZnO-APTMS suspension for 5 minutes and rinsed with ethanol (99%). This process was repeated for 3 times. The coated elastomeric ligature obtained was dried at 80°C 30 minutes to obtain 5% ZnO elastomeric ligature (5ZL). Similarly, 10% ZnO elastomeric ligature (10ZL) was prepared by using ZnO-APTMS powder (10%, 83.33 mg/mL). After fabrication, all specimens were sterilized under UV light for 15 minutes per side.

Antibacterial activity testing of elastomeric ligature

Bacterial cultivation. In this study, a cariogenic bacterium S. mutans ATCC 25175 was maintained routinely in Brain Heart Infusion (BHI) agar and incubated in a 5% CO\(_2\) incubator for 48 h at 37 °C. Prior to the experiment, bacterial colonies were cultured in BHI broth and incubated at 37°C for 24 h in a 5% CO\(_2\) incubator.

Antibacterial activity test by agar diffusion technique. The in vitro antibacterial activity of the ligature samples, i.e., NL, 5ZL, and 10ZL, against S. mutans was determined using the agar diffusion technique. The 24-h S. mutans culture was adjusted with BHI broth to achieve a turbidity equivalent to a 0.5 McFarland standard (10\(^8\) cells/mL). The BHI agar plates were each inoculated with 100 µl of the standardized S. mutans culture, then the bacteria were spread uniformly over the entire agar surface using a sterile cotton swab. The NL, 5ZL, and 10ZL rings were placed on the plate which was then incubated in a 5% CO\(_2\) incubator for 48 h at 37 °C. After incubation, the antibacterial activity was determined by observing the inhibition zone around the samples.

Antibacterial activity test by direct contact test and turbidimetric determination. Another in vitro study, the direct contact test and turbidimetric determination were carried out to further confirm the antibacterial activity of the ligature samples against S. mutans. A total of 90 O-rings per group of the ligature specimens (NL, 5ZL, and 10ZL) were immersed separately in sterilized distilled water (100 µl per sample), and incubated at 37 °C. After immersing for 0, 3, 7, 14, and 28 days, two O-rings and the supernatant obtained from each group were tested for the antibacterial activity. Briefly, the separated O-ring samples and the corresponded supernatant were transferred in wells of a flat-bottom 96-well polystyrene plate. Prior to the experiment, the 24-h grown S. mutans ATCC 25175 in BHI broth adjusted to 0.5 McFarland standard was diluted 100 times to achieve a cell density of 1.0 × 10\(^6\) cells/mL. Bacterial suspension (100 µl) was added into each well containing the specimens or their supernatant, then incubated under an atmosphere of 5% CO\(_2\) at 37 °C for 24 h. After 24-h incubation, the optical density (OD) at 590 nm was measured by using a BioTek Instruments absorbance microplate reader (San Diego, USA). The OD obtained from the wells of the samples and the supernatant was compared with the positive control containing bacterial suspension without samples and the negative control containing only BHI broth (n = 9). The antimicrobial activity was indicated
as no bacterial growth in which the OD was comparable to the negative control. If this was the case, the drop plate test was further performed to elucidate whether the tested samples exhibited the inhibitory or the bactericidal effect. In doing so, the broth sample (20 µl) was dropped onto the brain heart infusion agar (BHIA), and incubated in 5% CO2 at 37 °C for 48 h. After incubation, the inhibitory activity was indicated by the presence of colony growth, whereas the bactericidal activity was indicated by the absence of bacterial growth.

**Statistical Analysis.** Statistical analysis was performed by using PASW statistics 18.0 (SPSS/IBM, Chicago, IL, USA). Shapiro-Wilk Test was used to check the distribution of data. Non-parametric Kruskal-Wallis and Mann-Whitney U tests with Bonferroni adjustment were used to compare the difference of antibacterial effects of ligature samples. The test was interpreted for the level of significance at \( p < 0.05 \).

**Results**

**Preparation of ZnO-APTMS.** The surface modification of ZnO with APTMS was represented in Fig. 1. ZnO exhibited the absorption peaks at 3439 cm\(^{-1}\) and 865 cm\(^{-1}\) corresponding to O-H stretching and characteristic peak of ZnO [15], respectively [Fig. 2a]. ZnO-APTMS showed the new absorption peaks at 3360 cm\(^{-1}\) (N-H stretching), 2933 cm\(^{-1}\), 2887 cm\(^{-1}\) (C-H stretching), and 1113 cm\(^{-1}\) (Si-O stretching) as shown in Fig. 2b.

![Fig. 1. Surface modification of elastomeric ligature with ZnO. (a) The surface modification of ZnO with APTMS, (b) Coated surface of elastomeric ligature with ZnO-APTMS](image)

**Fig. 2. Absorption peak of ATR-FTIR spectra. (a) ZnO and (b) ZnO-APTMS.**

**Antibacterial activity of elastomeric ligature.** The antibacterial properties of elastomeric ligature NL, 5ZL, and 10ZL were screened by the agar diffusion method. There was no clear zone around all
elastomeric ligatures indicating no growth inhibition. However, using direct contact method where the planktonic bacteria were exposed to the elastomeric ligature coated with ZnO, the inhibitory effects were observed by the reduction of the bacterial turbidity. As shown in Fig. 3, the elastomeric ligature coated with ZnO, 5ZL and 10ZL, could inhibit growth of S. mutans, whereas the NL and the supernatant from ligature samples showed turbidity of bacterial growth compared to the positive controls.

**Fig. 3.** Direct contact method for antibacterial testing of the elastomeric ligatures immersed in distilled water for 7 days. (A) the sample before bacterial inoculation, (B) after inoculation with *S. mutans* and incubated for 24 h.

The pairwise comparisons of intragroup of each time point were shown in Fig. 4. At the beginning of the experiment (T0), as shown in Fig. 4A, all elastomeric ligatures reduced bacterial growth, whereas supernatant water groups did not do so. At T3, T7, T14, all ligatures as well as the supernatant showed significant reduction of bacterial growth except NL-w at T3, as shown in Fig. 4B, C, and D, respectively. At T28 (Fig. 4E), NL, 5ZL, 10 ZL and its supernatant showed significant growth reduction, whereas the supernatant water, NL-w, 5ZL-w did not show significant reduction.

**Fig. 4.** Bacterial growth (median OD 560 nm) after 24-h incubation with supernatant water or ligatures at various time points (*n* = 9): A) T0, B) T3, C) T7, D) T14, E) T28
The percentage of bacterial growth (OD values) were calculated compared to the positive control. The groups of 5ZL and 10ZL showed less than 5% bacterial growth within T3 and showed the killing effect until T28 (Fig. 5), whereas NL group showed 50-60% bacterial growth from T3 to T28. Regarding the supernatant groups, they all showed 50% bacterial growth at T7 until T14, but 10% increase at T28. Additionally, the drop plate method confirmed the growth of *S. mutans* from various conditions even in groups of 5ZL and 10ZL that showed OD values comparable to the negative control (0.003-0.01).

![Graph](image)

**Fig. 5.** Antibacterial activity of elastomeric ligature after immersion in distilled water at various duration.

**Discussion**

Enamel decalcification on the buccal surfaces of teeth causes serious complications during treatment with fixed appliances and compromises the therapeutic and aesthetic advantages of orthodontic treatment. Several studies reported the greater *S. mutans* accumulation in biofilms resulting in the white spot lesion or enamel decalcification of the elastomeric ligatures compared to the stainless-steel ligature.[5, 6] To overcome this disadvantage, efforts have been made to incorporate antimicrobial compounds in elastomeric ligature. Due to its low toxicity, good biocompatibility and antibacterial activity, ZnO has come to our interest as a promising additive in elastomeric ligatures.[16] However, ZnO particles have high value of surface to volume ratio and high surface energy leading to aggregation that limits its application, especially in polymer-ZnO hybrid material.[17] Therefore, ZnO particle surface has to be modified to improve the stability and dispersity in the solution and/or polymer hybrid.[18] To modify the surface of ZnO, APTMS has been added to substitute the hydrogen atom on ZnO surface through the surface ligand exchange with trimethoxysilane groups.[19] Various concentration of ZnO ranging from 0.2 to 10 wt% was incorporated into PU for antibacterial properties.[13] In this study, the surface coating of elastomeric ligatures with ZnO-APTMS was prepared using a dip coating method, yielding 5 wt% ZnO elastomeric ligature (5ZL) and 10 wt% ZnO elastomeric ligature (10ZL). To confirm the surface modification of ZnO-APTMS, ATR-FTIR technique was applied to observe the absorption peak of the silane functional groups. The ATR-FTIR spectra indicated that the APTMS was successfully grafted on ZnO surface. To improve the interaction between the PU and ZnO, silane coupling agent containing the amine groups was functionalized on ZnO. PU based elastomeric ligatures can be fabricated by simply immersing in surface modified ZnO.
To evaluate antibacterial activity of the normal ligature (NL), 5% ZnO elastomeric ligature (5ZL) and 10% ZnO elastomeric ligature (10ZL), we initially used the agar diffusion technique as previously reported by other investigators for modified PU based material like orthodontic elastomeric ligatures.[14, 20] However, we could not observe any inhibition zone from our elastomeric ligatures. Our findings by the agar diffusion test was similar to the previous studies as there were no inhibition zones around the resin specimens containing ZnO-NPs[21, 22]. It is noted that the principle of this method is based on water-soluble components released from the test samples. Therefore, it is not practical to assess the antibacterial effectiveness of less water-soluble compounds like zinc oxide.[21, 22] Therefore, we used the direct contact technique which the bacterial suspension was directly exposed to the test materials. Results from this method indicated the inhibitory effect of ZnO-coated ligature (5ZL and 10ZL) as well as non-coated ligature (NL) after 24-h exposure. The drop plate test indicated the bacteriostatic property of the test ligatures. According to the previous studies showing the dose-dependent antibacterial properties of ZnO against S. mutans, the MIC using ZnO-NPs have ranged from 0.390 to 500 ± 306.18 µg/mL and the MBC using ZnO-NPs ranged from 3.125 to 500 µg/mL.[10-13] We suggested that the concentration of ZnO released from the ligature might not reach the bactericidal concentration. We also found that the normal ligatures could reduce S. mutans growth but lesser than the ZnO-coated ligatures, which might be due to bacterial toxicity of the PU material.

Moreover, the results of this study showed the sustaining antibacterial effect of ZnO-coated elastomeric ligatures. After 28-day immersed in distilled water in static environment, the treated ZnO-coated the elastomeric ligatures still inhibited S. mutans growth. Previous in vitro and in vivo studies of fluoride-releasing elastomer have indicated that fluoride released from elastomer was reduced after 1 week and the effectiveness to reduce salivary S. mutans was also significantly decreased.[7, 23]

We also investigated the antibacterial effect of the supernatant obtained from the distilled water that had been treated with NL, 5ZL, and 10ZL. The antibacterial activity in the supernatant groups is primarily due to the soluble zinc ion species from ZnO.[24] After 3 days of immersion in distilled water, the supernatant groups showed a reduction in bacterial growth. In addition, the inhibitory effect was lower than the ligatures themselves and NL, NL-w, 5ZL-w, and 10ZL-w did not show significant different effects. These results indicated the reduction of bacterial growth of supernatant groups might be due to the bacterial toxicity of PU material. In addition, the small release of Zn$^2+$ in water could be the reasons for insignificant difference of bacterial reduction among supernatant groups. According to Pasquet et al., the dissolution process of ZnO in aqueous suspension was depend on types of the solvent. They found that very low Zn$^2+$ released in the supernatant of ZnO dispersion in pure water.[24] Regarding the ligatures groups, they showed a high antibacterial activity because ZnO particles on the surface of ligatures can directly contact with bacteria cells. Moreover, the study of Pasquet et al. found that type of liquid medium affected the solubility of ZnO. The presence of proteins enhances the solubility of ZnO particles by the binding of their peptides to zinc, creating a complex of high solubility.[24, 25] In our study, the use of BHI broth containing various animal proteins might increase the solubility of zinc species. However, the oral environment is dynamic due to the constant changes in temperature, pH, salivary flow, as well as external changes, i.e., diets, oral hygiene. Therefore, antimicrobial efficiency of ZnO-coated ligatures should be considered for further in vivo study.

Taken together, our study showed the promising potential of ZnO-coated ligatures to prevent accumulation S. mutans inducing dental caries in orthodontic treatment. Further studies on mechanical properties of ZnO-coated PU elastomeric ligature should be investigated. In addition, cytotoxicity of
the ZnO-coated ligatures should be evaluated, although selective toxicity to bacteria and minimal effect on human cells of ZnO particles have been reported.

Conclusions
The surface coating of elastomeric ligatures with 5% and 10% ZnO-APTMS exhibited antibacterial activity against cariogenic bacteria, *Streptococcus mutans*. The inhibitory effect of the ZnO-coated ligatures was still observed following immersion in distilled water for 28 days in the static *in vitro* condition.

References


