Tufts UNIVERSITY | School of Dental Medicine

Evaluation of antibacterial activity and shear bond strength of orthodontic adhesive containing chitosan nanoparticles

A Thesis

Presented to the faculty of Tufts University School of Dental Medicine in partial fulfillment of the requirements for the degree of

Master of Science in Dental Research

Submitted by

Reem Meshal Almeshal, B.D.S.

October 2021

Thesis Committee

Research Advisor:

Driss Zoukhri, Ph.D.

Professor

Department of Comprehensive Care

Tufts University School of Dental Medicine

Committee members:

Gerard Kugel DMD, MS, Ph.D.

Professor and Associate Dean of Research

Tufts University School of Dental Medicine

Ala Ali, B.D.S., MSc, DSc
Assistant Professor
Department of Prosthodontics and Operative Dentistry
Tufts University School of Dental Medicine

Sarah Pagni, Ph.D., M.P.H.
Assistant Professor
Department of Public Health and Community Service
Tufts University School of Dental Medicine

Abstract

Aim and Hypothesis: The study aimed to investigate the effect of orthodontic adhesive that contains various sizes of chitosan nanoparticles (CS-NPs) on *Streptococcus mutans* growth, enamel integrity, and shear bond strength. The research hypotheses were, first, that smaller-sized chitosan nanoparticles combined with an orthodontic adhesive will reduce white spot lesions when compared to larger-sized chitosan nanoparticles. Second, chitosan nanoparticles combined with an orthodontic adhesive will not affect the shear bond strength.

Methods: The antimicrobial activity was tested in two stages. In stage 1, disc agar diffusion test was used. Four 6-mm wells were created in the petri dishes and each was inoculated with 80 µl of broth (negative control), chlorhexidine (CHX, positive control), CS-NPs (20 nm), or CS-NPs (131 nm). In stage 2, a total of 24 teeth were randomly divided into three groups. In group 1 (control), the brackets were bonded with an adhesive with no treatment (Transbond XT), in group 2 the adhesive was blended with CS-NPs (20 nm), and in group 3 the adhesive was blended with CS-NPs (131 nm). Caries assessment and enamel integrity were measured using the International Caries Detection and Assessment System (ICDAS) and the profilometry machine. To measure the shear bond strength, the teeth were placed in an occlusal gingival direction and the occlusal load was directed parallel to the tooth surface. All readings were recorded by an Instron machine in MPa. One-way ANOVA was performed to compare the means of the zones of inhibition, shear bond strength, and enamel integrity, followed by post hoc Tukey's multiple comparison test.

Results: CS-NPs significantly reduced the growth of *S. mutans* when compared to the control ($p = 0.024$); however, no statistical significance was noted between the 2 sizes (20 nm and

131 nm, $p>0.99$). Furthermore, orthodontic adhesive that contained chitosan nanoparticles showed comparable shear bond strength to the control ($p = 0.466$). For carries assessment, the weighted kappa was (0.87) indicating an excellent agreement between the two investigators. The profilometry readings reported higher surface roughness values for the control group when compared to the CS-NPs groups ($p<0.001$). However, no statistical significant difference was found between the two CS-NPs groups ($p=0.72$).

Conclusion: CS-NPs exhibited bactericidal effect with no statistical difference between the two sizes. In addition, adhesive containing chitosan nanoparticles showed no effect on shear bond strength and exhibited lower surface roughness.

DEDICATION

I dedicate my work to my husband, Rami, for his endless devotion and sacrifice. You were a constant source of love and support throughout my journey. To my beautiful son, Hisham, you have made me stronger and more fulfilled than I could imagine.

A special feeling of gratitude to my loving parents, Meshal and Maha, for their unconditional love and for believing in me

To my brother, Mohammed, for his constant support and care

ACKNOWLEDGMENTS

I am greatly thankful to Dr Driss Zoukhri, my mentor and research advisor, for guiding me through my project, and for his tremendous support he has giving in the past two years.

Thank you to my supportive research committee, Dr Gerard Kugel for his time and effort, Dr Sarah Pagni and Dr. Ala Ali, for their continued support and patience during this project and throughout my graduate study.

Also, a special thanks to Cristiane Cornwell from Gavel lab, for her endless support. Dr. Robert White and Dr. James Vlahakis from the Department of Mechanical Engineering, School of Engineering, Tufts University, for their support and sharing their knowledge.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

CHX	Chlorhexidine
CLSM	Confocal Laser Scanning Microscopy
CS-NPs	Chitosan nanoparticles
DLS	Dynamic Light Scattering
DAD	Disc Agar Diffusion
GTR	Guided Tissue Regeneration
HA	Hydroxyapatite
ICDAS	International Caries Detection and Assessment System
MIC	Minimum inhibition concentration
SBS	Shear Bond Strength
Ra	Roughness average
ROS	Reactive Oxygen Species

EVALUATION OF ANTIBACTERIAL ACTIVITY AND SHEAR BOND STRENGTH OF ORTHODONTIC ADHESIVE CONTAINING CHITOSAN NANOPARTICLES

Introduction

I. WHITE SPOT LESIONS IN ORTHODONTICS

White spot lesions (WSLs) refer to a chalky white appearance observed during the early stages of enamel demineralization. It is a common problem following fixed orthodontic treatment[1] and affects patients' satisfaction and perception of the overall treatment.[2] A review done by Sundararaj has shown that 68.4% of orthodontic patients developed WSLs during their treatment.[3] Buschang et al. reported an increased incidence of WSLs in patients undergoing fixed orthodontics compared to those treated with aligners.[13] The total incidence of WSLs found in the traditional group was approximately 25.7%, compared to the aligner group, of whom only 1.2% had WSLs. A study conducted by Knosel found no difference between WSLs underneath the lingual brackets and WSLs adjacent to the bracket.[14] Many orthodontists have observed that, despite emphasizing the importance of basic preventive oral care, patients still seem to be affected by enamel demineralization.

Enamel decalcification often occurs at the 4th week of an "undisturbed" biofilm.[4] This is due to the accumulation of bacteria on teeth surfaces. Bacterial growth can be accelerated by many factors, including poor oral hygiene,[5] malocclusion, low salivary flow,[6] diet,[7,8] and mechanical objects such as orthodontic appliances.[9] The brackets and rings occasionally interfere with patients' oral hygiene and promote plaque accumulation, creating an optimal environment for the cariogenic bacteria. Studies have shown that patients treated with fixed orthodontics are more susceptible to WSLs.[10–12] The prolonged plaque accumulation and entrapment of cariogenic bacteria such as *S. mutans* increase the acid content, consequently dissolving the hydroxyapatite crystals in the enamel, which creates the white appearance.

Orthodontic adhesives appear to play a central role in WSLs formation. Sukontapatipark et al. suggested the negative effects of excess composite underneath the

brackets, such as the adherence of microorganisms on the material's rough surface. In addition, internal micro-gaps between the brackets and the enamel surface increases bacterial accumulation.[15]

II. PREVENTIVE MEASURES

Several studies have investigated different interventions to limit WSLs. Methods include emphasizing basic oral care, such as recommending a chlorhexidine combined with fluoride oral rinse[16] and scheduling checkup appointments to assess the patient's oral health. A randomized double blind clinical trial conducted by Blicks showed that the application of topical fluoride every 6 weeks during orthodontic treatment can reduce the incidence of WSLs.[17] Moreover, in their clinical study, Geiger et al. found a significant reduction in the incidence of WSLs when a 0.05% sodium fluoride rinse was used during orthodontic treatment.[18] Another study by Gorton et al. showed that the use of fluoride-releasing cement can significantly decrease the WSLs, compared to a non-fluoridated cement.[19] In contrast, Tufekci et al. concluded that no difference was found between the tested teeth and contralateral ones when primer filled with fluoride was used.[12] Lasers were also suggested as an alternative solution for WSLs.[20] The high temperature of the laser initiates enamel alterations and melting recrystallization of the hydroxyapatite, making the enamel surface less susceptible to dental caries.[21] Adel et al. compared laser irradiation and the use of casein phosphopeptides-amorphous calcium phosphate.[20] Their study found a significant reduction in enamel demineralization with the combination of both methods, compared to using each method separately. [20] Another randomized controled study by Mahmoudzadeh found that the use of a CO_2 laser significantly reduced the appearance of WSLs between baseline and 6 months post radiation.[22] Nevertheless, adverse effects were mentioned in several studies, for

example, histopathological changes such as pulpal vasodilation, periodontal cell necrosis, and minor bone resorption.[23]

III. NANOPARTICLES IN DENTISTRY

Nanoparticles have been used in various dental applications as a new strategy to improve clinical outcomes. Naslapur et al. reported the antibacterial effect of 10% silver oxide nanoparticles on *S. mutans*.[24] Diaz et al. reported high antimicrobial activity against *S. mutans* with silver nanoparticles at a concentration of 50 ppm, compared to the lower concentration of 10 ppm.[25] Nanoparticles were also incorporated into interim restorations. These restorations are commonly used in dental clinics as a way of preserving tooth structure.[26] Hojati et al. used flowable resin blended with zinc oxide nanoparticles on *S. mutans* and found a significant reduction in both direct contact and ageing test.[27] A study conducted by Figueroa et al. showed that combining cupper nanoparticles with orthodontic adhesives at a concentration of 0.01% can significantly enhance the antibacterial effect against *Staphylococcus aureus*, *Escherichia coli* and *S. mutans*.[28] Hydroxyapatite (HA) nanoparticles have been used in guided tissue regeneration (GTR), a technique that is used to repair periodontal defects by preventing the growth of soft tissue and aiding with osseous regeneration.[29] Another study revealed that incorporating silver nanoparticles into scaffolds can promote bone marrow stromal cell formation.[30] In 2016, Haghshenas et al. evaluated the effect of magnesium oxide nanoparticles on *S. mutans.* The percentage of biofilm formation of the magnesium oxide NPs was significantly lower compared to the control group.[31] However, some studies have addressed the potential cytotoxicity of some of the metallic nanoparticles.[32] Shrivastava et al. assessed the toxic effect of three metallic nanoparticles (TiO_2, ZnO and Al_2O_3) on male rats by measuring the reactive oxygen species (ROS) levels.[33] ROS are induced as a cellular response, and their buildup is used to indicate cellular

imbalance, which can eventually lead to DNA, RNA and proteins damage. The authors measured the level of dichlorofluorescein diacetate, a material used as an oxidative stress indicator, in blood and concluded that fluorescence increased significantly in erythrocytes as compared to the control.[33]

IV. CHITOSAN NANOPARTICLES

Recently, there has been a shift towards the use of organic substances, as they are more biocompatible with living cells. Chitin was discovered by Braconnot in 1811 during his work with fungi.[34] It is a positively charged polysaccharide, a natural and biocompatible material specifically found in the exoskeleton of crustaceans, insects,[35] and fungi.[36] Chitin is a polymer that is known as a promising organic component, and it has significant antibacterial properties. Based on the source of chitin and because of its biodegradability,[37] it can be utilized for different purposes, such as in medical imaging for cancer detection, for wound dressings,[38] in the food industry,[37] and in anti-inflammatory medications.[39] Despite having such varied applications, chitin has a major limitation, namely that it is insoluble in water, which restricts its application in the biomedical field. The insolubility of chitin was addressed by Rouget in 1859.[34] He discovered that the deacetylation of chitin results in a more soluble material, modified chitin, with the same antibacterial potential, which later became known as chitosan. The process of partial deacetylation involves the partial removal of the acetyl group from the molecular chain by mixing the chitin with KOH or NaOH at 100°C for several hours, forming an ammino group.[40]

V. CHITOSAN NANOPARTICLES IN DENTISTRY

Nanoparticles made from this chitosan polymer show a promising antibacterial effect against a wide variety of microorganisms.[41–43] Many dentistry researchers have tested its

effect on gram-negative and gram-positive bacteria and fungi.[44–46] Several studies have shown positive results when incorporating chitosan nanoparticles into toothpaste, mouthwash, adhesive resin, and root canal sealers.[47] A study conducted by Ganss et al. compared dentifrices combined with the following compositions: NaF, NaF/$SnCl_2$, AmF/NaF/$SnCl_2$, AmF/NaF/$SnCl_2$/chitosan, and AmF/SnF_2.[48] According to the profilometry readings, the group that used dentifrice that contained chitosan had the least enamel erosion of all the groups.[48] Moreover, Sulandjari et al. conducted a study with orthodontic patients, instructing them to use a dentifrice containing chitosan for one week. Sulandjari used disclosing agent to record the level of plaque adherence. The use of chitosan-containing dentifrice led to a recorded lower percentage of plaque adherence compared to non-chitosan-containing dentifrice. A double-blind study revealed that chewing chitosan-containing gum reduced the growth of *S. mutans* after one week, compared to a non-chitosan-containing gum.[49] In endodontics, many studies have discussed the elimination *Enterococcus faecalis (E. faecalis)*, a bacterium sometimes detected in infected root canal systems.[50] Kishen et al. demonstrated that chitosan nanoparticles significantly reduced the adherence and colonization of *E. faecalis* in the root canal system.[50] Moreover, a study conducted by Dasilva found that combining CS-NPs with a zinc-oxide eugenol sealer effectively inhibits the growth of *E. faecalis* after a 7-days period, according to the confocal microscopy readings.[51] Furthermore, a study by Perochena et al. investigated the influence of incorporating CS-NPs as a chelating agent. He reported a significant reduction in biofilm formation in the group containing chitosan.[52] In 2019, Sadony et al. studied the effect of chitosan in combination with a diode laser on *Escherichia coli* in the root canal system.[53] They found lower bacterial count in the group treated with the chitosan nanoparticles, as compared with the other groups. Another study conducted by Zeza in 2017 assessed the bone level in patients with mild peri-implantitis.[54] Baseline readings of the area of the implant

before and after a chitosan brush was applied were compared. The periapical radiographs revealed stabilization in bone levels, compared with the baseline readings in 73% of patients. Atai et al. examined the use of chitosan as an antifungal solution for patients with denture stomatitis.[55] After 2 weeks, the investigators collected palatal smears, and, compared to the nystatin solution, found that addition of chitosan led to a significant reduction in the erythematous zone. Chitosan nanoparticles have also been applied as a medicament for wound healing and in dressings. Marta et al. studied the effect of a topical gel used in post-operative care after 3rd molar extractions.[56] The gel consisted of 10 mL chitosan, 0.2 chlorohexidine, dexpanthenol, and allantoin. They found better wound healing by day 7 and day 14 than those who did not use the gel.[56] Moreover, in their study, Qasim et al. used CS-NPs membranes with and without HA for GTR. They found that adding CS-NPs was beneficial in terms of cellular response.[57]

VI. MODE OF ACTION

The exact mechanism of the antibacterial effect of chitosan nanoparticles is not fully understood. Disruption of cell membranes indicate that the electrostatic interaction between the positively charged nanoparticles and the negatively charged bacteria has resulted in the deterioration of the cell wall, leakage of essential components, and, consequently, cellular death was discussed in several studies.[58] The degree of acetylation is what differentiates chitosan from chitin. The degree of acetylation is below 50 % in chitosan, making it more soluble in acidic environments, and above 50% in chitin.[59] Properties such as solubility, bioactivity, and biodegradability in CS-NPs are all determined by degree of acetylation.[60] Furthermore, some studies have found that different degrees of chitosan solubility can be accomplished by manipulating the degree of polymerization or molecular weight and the degree of acetylation.[61] Some studies suggest that there is an association between the

molecular weight of the nanoparticles and their ability to perforate bacterial cell walls. A study conducted in 2011 compared chitosan nanoparticles with three different molecular weights while preserving a neutral pH. [62] The results showed higher bacterial cell wall penetration of nanoparticles with a lower molecular weight (> 95% of cells damaged) in live/dead staining as compared to those with a higher molecular weight (20–25% of cells damaged).[62] The effect of the concentration of nanoparticles on their antimicrobial potential has also been investigated. Aliasghari et al. have shown that a concentration of 5 mg/mlL of chitosan nanoparticles decreases the biofilm formation of *S. mutans* up to 92.5%.[63] In addition, Ikono et al. have shown that higher concentrations of chitosan nanoparticles with an average size range of 20–30 nm have greater antibacterial activity on both *S. mutans* and *Candida albicans*.[64]

Many studies have focused on the concentrations of nanoparticles, but only a limited number has compared the effect of different sizes. To be able to state the nano effect of any molecule, it is crucial to compare the effects of different sizes of the same molecule (e.g., nanoparticles and microparticles). Such a comparison can prove that the noted antibacterial effect is not due to the chemical structure of the molecule but rather to the nano-effect produced by the smaller particles. Moreover, studies have been conducted to assess whether chitosan nanoparticles can release their antibacterial effects when suspended in orthodontic adhesive, which is the proposed approach in this project, to utilize the antibacterial effect of chitosan nanoparticles to prevent accumulation bacteria that cause WSLs around orthodontic brackets.

VII. SHEAR BOND STRENGTH

Another important factor that must be considered is the shear bond strength of the orthodontic adhesive. Ideally, an orthodontic adhesive should be able to withstand the occlusal load during mastication as well as the forces exerted by the orthodontics wires. A review done by Reynolds suggests an acceptable range of bond strength of 60 kg/cm^2 (5.9 MPa) to 80 kg/cm^2 (7.9 MPa) that can be applied in clinical situations.[65] Failure to apply the optimal adhesive force can jeopardize the treatment outcome. Concerns associated with bracket debonding include delays in the treatment duration and enamel damage.[66] Many factors, such as adhesive type, characteristic of bracket base, duration of light cure, and techniques used during the bonding process, can influence the bond strength.[67–69]

In the current project, the shear bond strength was assessed after the addition of chitosan nanoparticles into the orthodontic adhesive to assess whether this bond would be affected. Enamel integrity after debonding can be measured by various techniques, such as scanning electron microscopy, confocal laser microscopy, or surface profilometry.[70]

Aims

This project aims to investigate the antibacterial activity and shear bond strength of orthodontic adhesive containing different sizes of chitosan nanoparticles.

Research objectives

1- To compare the antibacterial effect of two different sizes of chitosan nanoparticles mixed with an orthodontic adhesive on *S. mutans*.

2- To investigate whether the addition of chitosan nanoparticles affects the shear bond strength of the orthodontic adhesive.

Hypotheses

First, smaller-sized chitosan nanoparticles combined with an orthodontic adhesive will reduce white spot lesions to a greater degree than larger-sized chitosan nanoparticles. Second, chitosan nanoparticles combined with an orthodontic adhesive will have no effect on the shear bond strength of the adhesive when used in orthodontics patients.

Materials and methods

Preparation of chitosan nanoparticles. Chitosan nanoparticles were prepared using the ionic gelation method, as described by Calvo.[71] In brief, chitosan (low molecular weight, Sigma-Aldrich, St. Louis, MO) was dissolved in 1% acetic acid. Thereafter, 20 mL of chitosan solution was mixed with 20 mL of a crosslinker solution, sodium triphosphate (Fisher Scientific). The cross-linker solution helps to break the chitosan molecules down into nano-sized particles by interacting with the active amino groups of chitosan. Subsequently, the mixture was centrifuged for 4 minutes at 15,700 rpm to isolate the nanoparticles, which were then suspended in distilled water and kept at room temperature until use. Different-sized nanoparticles were generated by altering the ratio of the chitosan and sodium triphosphate solution, as indicated in some studies.

Characterization of chitosan nanoparticles. The size distribution of nanoparticles in each sample was confirmed with the use of a dynamic light scattering machine (DLS) (ZetaPALS Zeta Analyzer, Brookhaven Instruments Corporation) located at the Tufts University Science & Technology Center, Medford.

Bacterial strain growth condition and collection. *S. mutans* (ATCC© 25175™) was used in the study. The bacterial culture was started by impregnating a pellet with 0.5 mL of Brain Heart Infusion (BHI) broth. Thereafter, the bacterial culture was added into a tube that contained 5 mL of BHI broth. To isolate bacterial colonies, an agar plate was streaked using the quadrant streak method. This technique starts by sterilizing the inoculating loop, then inserting the loop into the culture broth, and streaking one quadrant of the plate. After that, the plate is rotated 90° clockwise while continuing the streaking of the agar until four

quadrants have been established. Next, the plate was aerobically incubated overnight at 37°C. After 24 hours of incubation, the bacterial growth was confirmed by visual inspection of the agar plate. A bacterial colony was then collected with the inoculation loop and added into 5 mL BHI broth, which was further incubated for 24 hours at 37°C to use for each experiment.

Preparations of composite adhesives containing chitosan nanoparticles. In a semi-dark room, 300 mg of composite resin (3M Unitek transbond XT) was blended with 15 µL of chitosan nanoparticles on a glass slab with a mixing spatula until a uniform thickness was observed.

Preparation of composite discs containing chitosan nanoparticles. To investigate the antibacterial effect delivery of CS-NPs from the adhesive, this experiment was conducted twice for each size of CS-NPs (20 nm and 131 nm). Three composite discs containing CS-NPs with different volumes (8, 10, and15 µL) were created. The discs were molded by mixing 300 mg of composite resin (3M Unitek transbond XT) in different concentrations. Thereafter, the composite was inserted between two glass slabs, light-cured for 20 seconds, and then carefully placed in empty sterilized well plates. After inoculating the nutrient agar plate with 100 µL of bacterial solution, the discs were added, one by one. The plate was incubated for 24 hours at 37°C. The zones of inhibition were measured with a ruler.

Tooth preparation. Twenty-four extracted human lower central incisors were collected from the Department of Oral and Maxillofacial Surgery at Tufts University. The teeth were inspected for any existing enamel alterations and then disinfected in a 10% bleach solution for 5 days. The roots were then resected with a precision saw (Isomet 1000, Buehler). The enamel surfaces were etched with 35% phosphoric acid (UltraEtch) for 15

seconds and washed for 10 seconds. Next, adhesive primer (3M Unitek transbond XT) was applied by loading the microbrush and swiping it on the enamel surface of the teeth. The primer was polymerized with a light cure for 30 seconds. After the orthodontic adhesive containing chitosan nanoparticles had been prepared as described above, the teeth were divided into three groups of eight:

A. Group A – control (regular adhesive)
B. Group B – adhesive with chitosan nanoparticles 20 nm
C. Group C – adhesive with chitosan nanoparticles 131 nm

The adhesive was then applied to the tooth surfaces with conventional orthodontic metal brackets (Forestadent, USA) and cured for 30 seconds.

Preparation of artificial saliva

To make a biomimetic model relevant to clinical practice, artificial saliva was prepared with a mixture of potassium chloride, magnesium chloride, sodium bicarbonate, potassium phosphate, and calcium chloride dissolved in distilled water with the quantities shown in Table 1. The solution was then placed on the magnetic stirrer for 10 minutes, autoclaved, and stored at 4°C**.**

Table1: Ingredients used for the artificial saliva preparation:

Name	**g/L**
Calcium Chloride: $CaCl_2 \cdot 2H_2O$	0.147
Magnesium Chloride: $MgCl_2 \cdot 6H2O$	0.041
Potassium Phosphate: KH_2PO_4	0.544
Potassium Chloride: KCl	2.237
Sodium Bicarbonate: $NaHCO_3$	0.025

Antibacterial assessment

Disc agar diffusion test

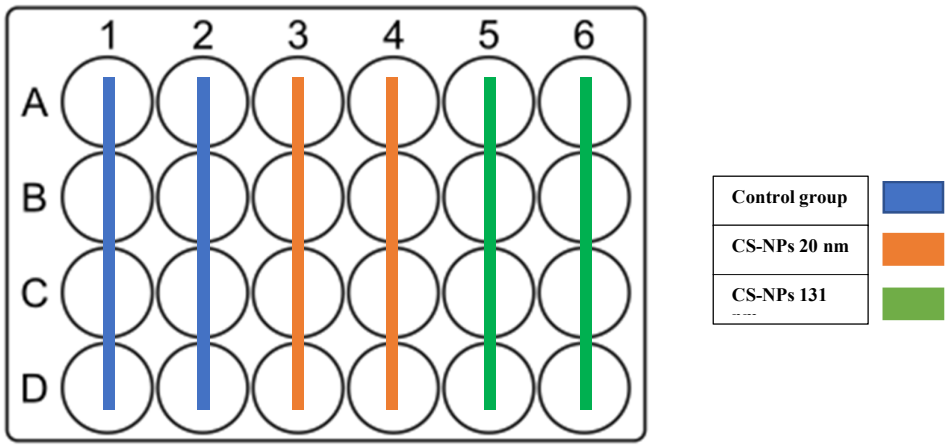
Three plates of BHI agar were prepared and then a tube with *S. mutans* culture was diluted as previously described. Thereafter, 100 μL of the bacterial culture was suspended on each plate, spread uniformly, and incubated for 30 minutes, after which four wells of 6 mm in diameter were made with a cork borer. The testing materials were distributed into the wells as follows:

D. Group A – negative control (broth)
E. Group B – positive control (0.12% CHX)
F. Group C – chitosan nanoparticles (20 nm)
G. Group D – chitosan nanoparticles (131 nm)

Eighty μL of the control and testing materials were added to each well, and the plates were incubated for 24 hours at 37°C, after which the zones of inhibition were measured with a ruler to evaluate the antibacterial effects. The test was repeated 3 times with new colonies.

White spot lesion assessment

The extracted teeth with bonded braces were placed in a 24-well plate, and 100 μL of the adjusted bacterial suspension and 1,000 μL of artificial saliva were added to each well. The 24-well plate was then incubated for 48 hours at 37°C. The medium was carefully exchanged every 48 hours for 5 weeks period.



A. Schematic of the 24-well plate and the placement of the teeth, according to the treatment material

After 5 weeks, the biofilm was removed by wiping the teeth with sterilized gauze and running a water syringe for 5 seconds. The shear bond strength was then evaluated using a universal testing machine (Instron 5566 Instron corp., Norwood, MA, USA) (Figure 11). The teeth were positioned so that the force was directly parallel to the gingival-occlusal direction, with an orthodontic wax below the tooth for better retention during the test, as shown in the photo. The crosshead speed was set to 1mm/min and run until the bracket separated from the enamel, and then the failure compression (MPa) force at break (N) was recorded.

Caries progression assessment

The WSLs were evaluated by visual assessment under a stereomicroscope (SZX16, Olympus) with a 4x magnification. An International Caries Detection and Assessment System (ICDAS) was used to categorize the caries seen on the extracted teeth by two investigators. The inter-rater assessment was analyzed using weighted Cohen's kappa.

Criteria for the full ICDAS system:

Score	ICDAS futures
0	Sound
1	First visual change in enamel (seen only after prolonged air drying)
2	Distinct visual change in enamel
3	Localized enamel breakdown (without clinical visual signs of dentinal breakdown)
4	Underlying dark shadow from dentine
5	Distinct cavity with visible dentine
6	Extensive distinct cavity with visible dentine

Evaluation of the surface roughness (Ra)

Surface profilometry (DekTak XT Profilometer) was used to scan the surface roughness of the enamel surfaces.[72] The profilometer parameters were: 3 mg of force, 2 μm stylus radius, 20s scanning time and 0.111 of resolution. Three measurements were taken mid-sample to average the roughness value per sample in μm.

Statistical analyses

The data were analyzed with the IBM SSPS Statistics 27 software. Descriptive statistics (mean, standard deviation, minimum and maximum) were calculated. Normality was confirmed with the Q-Q plots, and then Levene's test was performed to assess the homogeneity of variance, with the level of significance of 5%. One-way ANOVA was performed to compare the means of the zones of inhibition, shear bond strength, and surface roughness. Tukey's HSD was performed as a post-hoc test. Inter-rater examiner agreement was assessed using weighted kappa statistic measure for the ICDAS scoring.

Results

Characterization of chitosan nanoparticles. The size distribution of the nanoparticles in each sample was confirmed with the use of a dynamic light scattering machine (DLS) (ZetaPALS Zeta Potential Analyzer, Brookhaven Instruments Corporation). The DLS report indicated that the average particle size in the smaller group was (20 nm). For the larger group, the DLS report showed that the average particle size was (130 nm), as shown in (Figures 1,2).

Disc agar diffusion test. The antibacterial effect of the different-sized CS-NPs is shown in Tables 1 and 2 and Figure 3. There was a statistically significant difference between the chlorohexidine group and both the CS-NPs groups ($p = 0.025$). The chlorohexidine had a higher antibacterial effect on *S. mutans*. Moreover, a comparison of the CS-NPs groups revealed that the smaller-sized CS-NPs particles (20 nm) had a slightly higher growth inhibition than the larger size (131 nm). However, the difference was not statistically significant ($p > 0.99$).

Shear bond strength values. The results for the shear bond strength of the three groups of orthodontic adhesives are shown in Tables 3 and 4. There were no significant differences between the bonding force of the regular adhesive with no treatment and the adhesive containing CS-NPs ($p = 0.4$, Figure 6).

Carries progression assesement. The overall findings showed higher incidence of WSL in the control group when compared to the treatment groups. The kappa agreement was

(0.875) indicating an excellent agreement between the two evaluators according to Fleiss interpretation.

Surface profilometry findings:

Twenty-four enamel surfaces were analyzed for surface roughness (μm). The mean and SD for each group is shown in Tables 6 and 7. The control group revealed higher roughness values compared to the CS-NPs group ($p < 001$). For the treatment groups, no statistical significance was found between the smaller sized CS-NPs (20 nm) and the larger sized CS-NPs (131 nm) ($p=0.72$, Figure 7).

Discussion

The prevention of WSLs is a major interest, especially in the field of orthodontics.[73] According to a study conducted by Julien et al., multiple risk factors should be considered during treatment planning.[74] Therefore, a number of studies have considered combining nanoparticles with an orthodontic adhesive as a way to reduce dental plaque.[75] The material focused on in this study is chitosan nanoparticles. Chitosan is the second most abundant natural polysaccharide in nature after cellulose, and it has attracted attention mainly because of characteristics such as biocompatibility, biodegradability, and antibacterial properties. Many studies have found that chitosan nanoparticles significantly inhibit the growth of *S. mutans* and *lactobacillus*, which are commonly associated with dental caries.[41,76]

There are a limited number of studies that have investigated the possible correlation between the size of the chitosan nanoparticles and its antimicrobial efficiency. Therefore, the effects of a smaller and a larger chitosan particle size was compared. The disc agar diffusion test showed the antibacterial effect of chitosan nanoparticles on *S. mutans*. The smaller-sized nanoparticles (20 nm) showed higher growth inhibition compared to the larger ones. However, the difference between the CS-NPs groups was not statistically significant. For the smaller-sized CS-NPs, the findings in this study coincide with what Ikono et al. reported. They found that nanochitosan particles ranging in size between 20 and 30 nm decreased the biofilm mass of *S. mutans* and *Candida albicans* in an 18-hours incubation period. Moreover, Wassel et al. also found significant growth reduction of *S. mutans* with CS-NPs ranging in size between 10–40 nm. [77]

Several studies have tested the advantageous nature of a nanoparticles-based approach by combining materials such as silver nanoparticles,[25] silica nanoparticles,[78] and zinc oxide nanoparticles with dental adhesives.[27] Florez combined titanium dioxide (N_TiO_2) nanoparticles with adhesive resin, which resulted in higher growth reduction of *S. mutans*

than the control group according to the confocal laser scanning microscopy (CLSM) readings.[79] All biofilms in all the groups that contained N_TiO_2 were altered, depending on the concentration; the higher the concentration, the higher the bacterial reduction. However, there are also studies related to the cytotoxicity of these non-organic nanoparticles, including, for example, the findings of Xu et al.[80] They reported that human fibroblast cells were subjected to higher cytotoxicity when treated with 20 nm silica nanoparticles as compared to the 80 nm size. In addition, Komatsu conducted a study on Leydig cells found in the reproductive system of male mice. According to the transmission electron microscope (TEM) results, more stress signals were noticed when the mice were subjected to TiO_2NPs, as compared to diesel exhaust particles (DEP).[81]

To test the delivery of the CS-NPs when combined with dental adhesive, a method used by Sodagar et al. was implemented.[82] Composite discs were made with the DAD, and the results indicated that CS-NPs was effectively released from the discs and inhibited the growth of *S. mutans*. The zones of inhibition were significantly higher compared to the control. While the inhibition zones of the two sizes of CS-NPs (20 nm and 131 nm) are comparable, these findings are considered to be of clinical importance.

The results align with findings from several studies, including the work of Sevinc et al. Their study revealed that composite discs with zinc oxide NPs caused a decrease in biofilm thickness compared to the unmodified composite, according to the readings of both the scanning electron microscopy and the CLSM.[83]

When introducing an adhesive with an antibacterial characteristic, proper bond strength should be considered. Shear bond strength should withstand the forces exerted during orthodontic treatment. Failure to withstand the forces can lead to increased treatment time and, consequently, affect the treatment outcome.[84] In addition, the shear bond strength should not exceed a high force (40–60 MPa) that can affect the enamel surface after the

debonding of the brackets.[85] A study conducted by Hasan et al. found higher mechanical properties with calcium hydroxyapatite nanoparticles incorporated into dental resin than in the control group.[86] On the other hand, several studies revealed that adding calcium hydroxyapatite nanoparticles significantly reduced the shear bond force. Scribante et al. reported a significant difference in shear bond strength scores. The control group showed the highest shear bond strength compared to the treated groups, even though all scores were within an acceptable range.[87] In our study, no significant differences were found between the groups. The mean shear bond strength ranged between 10 to 19 MPa for the control group, 10 to 17 MPa for the smaller-sized CS-NPs (20 nm), and 8 to 17 MPa for the larger-sized CS-NPs (131 nm). The latter results are similar to those from a study conducted by Sorourhomayoun et al.[88] They tested the shear bond strength of orthodontic adhesive blended with chitosan nanoparticles with various concentrations and molecular weights. They found no significant differences in adhesive bond strength among the five groups.[88] In the same context, Farzanegan et al. tested the shear bond strength with different concentrations of CS-NPs and TiO_2-NPs. They reported no significant differences between the groups when concentrations of 1% CS-NPs and TiO_2-NPs were used.[89] However, when the concentration was increased to 1.5% for both nanoparticles, the shear bond strength decreased. The difference in the latter report may be caused by the agglomeration of CS-NPs and TiO_2-NPs.[90]

With the integration of antibacterial agents in dentistry, enamel integrity is considered an important aspect to value. After debonding the orthodontics brackets, the enamel surfaces should be free from any adhesive remanent or enamel cracks. Studies have shown that the thickness of the enamel can be reduced by up to 30 μm after debonding orthodontic brackets.[91] Mechanical removal of the remaining composite can cause microcracks on the enamel surface, therefore other factors should also be considered during the removal of

composite to minimize the enamel damage. Examples include etching time,[92] debonding technique,[93] type of bur used during adhesive removal.[94] A comparison was done by Vidor et al. between various types of finishing techniques after bracket removal.[95] Three groups were used in their study, group one was representing a 30-blade tungsten carbide bur, group 2, 30-blade tungsten carbide bur, followed by 4 polishing discs and group 3, 30-blade tungsten carbide bur in high speed followed by Enhance tips. Vidor concluded that a 30-blade tungsten carbide bur followed by a polishing discs provided least amount of enamel damage and better surface polish. [95] In our study, tungsten carbide bur 30 blades was used followed by polishing disc and pumice for adhesive removal. The ICDAS was used as a standardized method to visually assess the caries progression and the depth of the WSLs.[96] Many have concluded moderate to acceptable inter-rater agreement reliability.[97] In the present study, higher WSLs scores were reported in the control group when compared to both CS-NPs groups. Zhang et al. showed greater surface remineralization when using CS as pretreatment material on enamel surfaces.[98] A possible factor is the size of the CS-NPs. The nanosized of the CS enabled it to penetrate the cell wall leading to cellular leakage. This is in agreement with a study done by Robles et al.[99] Similar NPs sizes were used in their study. Silver NPs were coated with two organic materials (16.5, 23.3, 115.2 nm) for the non-coated group, (22.5, 44.1, 133.7 nm) for the bovine serum albumin coated group and (7.1, 17.4, 87.6 nm) for the CS coated group on *S. mutans*. They found an association between the size of the NPs and antibacterial effectiveness. The lower the size of the NPs, the higher the antibacterial activity is recorded.[99] Another explanation is the electrostatic theory. Where the positive charge of the amino group in CS-NPs binds with the negative charge of the bacterial cell membrane. This interaction alters the bacterial cell membrane leading to intracellular components discharge and eventually cell death. [100] Studies have found greater antibacterial results with gram-positive bacteria. This can be due to the vulnerability of the bacterial cell wall of the gram-

positive when compared to the gram-negative cell wall.[101] Moreover, others have found stronger antibacterial effect with the gram-negative bacteria owing to the higher interaction between lipopolysaccharides of the gram-negative cell wall with the CS-NPs. [100,102]

To further evaluate the caries progression, surface roughness of the enamel samples was measured. Enamel demineralization is characterized by white and chalky appearance of the tooth surface. The deeper the WSLs, the higher surface roughness and porosity and consequently the resulting increase in plaque retention and external stains.[103] In clinical settings, the WSLs are inspected through visual inspection and tactile sensation using a dental explorers.[104] Surface roughness measurement reported that CS-NPs groups had less amount of surface irregularities when compared to the control group. A possible explanation for the reduced roughness is hydrogen bond existence between the hydroxyapatite group located in the enamel prisms and the amino group found in CS-NPs.[105]

Limitations of the study include lack of simulation of oral flora such as testing biofilm, temperature fluctuation consideration, and the role of oral hygiene habits and its effects on caries progression. Another limitation includes the use of single concentration of CS-NPs in this study. Further research should be conducted comparing wider range of particle size and concentrations of the CS-NPs.

Conclusion

Within the study limitations, we conclude that:

1. The addition of CS-NPs into composite resin significantly inhibited the growth of *S. mutans* with no difference in performance between the nanoparticle sizes.

2. Incorporation of the CS-NPs in composite resin did not affect the mechanical properties of the adhesive.

References

1. Lucchese A, Gherlone E. Prevalence of white-spot lesions before and during orthodontic treatment with fixed appliances. *Eur J Orthod*. 2013;35(5):664-668. doi:10.1093/ejo/cjs070
2. Bailey DL, Adams GG, Tsao CE, et al. Regression of post-orthodontic lesions by a remineralizing cream. *J Dent Res*. 2009;88(12):1148-1153. doi:10.1177/0022034509347168
3. Sundararaj D, Venkatachalapathy S, Tandon A, Pereira A. Critical evaluation of incidence and prevalence of white spot lesions during fixed orthodontic appliance treatment: A meta-analysis. *J Int Soc Prev Community Dent*. 2015;5(6):433. doi:10.4103/2231-0762.167719
4. Øgaard B, Rølla G, Arends J. Orthodontic appliances and enamel demineralization. Part 1. Lesion development. *Am J Orthod Dentofac Orthop*. 1988;94(1):68-73. doi:10.1016/0889-5406(88)90453-2
5. Klaus K, Eichenauer J, Sprenger R, Ruf S. Oral microbiota carriage in patients with multibracket appliance in relation to the quality of oral hygiene. *Head Face Med*. 2016;12(1):1-7. doi:10.1186/s13005-016-0125-x
6. Karaoglanoglu S, Duymus ZY, Akgul N, Ozdabak N, Seven N, Ozabacygil F. Salivary clearance of sugar before and after insertion of fixed partial dentures. *Asian Biomed*. 2010;4(3):475-478. doi:10.2478/abm-2010-0059
7. Anderson CA, Curzon MEJ, Van Loveren C, Tatsi C, Duggal MS. Sucrose and dental caries: A review of the evidence. *Obes Rev*. 2009;10(SUPPL. 1):41-54. doi:10.1111/j.1467-789X.2008.00564.x
8. Mobley CC. Nutrition and dental caries. *Dent Clin North Am*. 2003;47(2):319-336. doi:10.1016/S0011-8532(02)00102-7
9. Buschang PH, Chastain D, Keylor CL, Crosby D, Julien KC. Incidence of white spot lesions among patients treated with clear aligners and traditional braces. 2019;89(3). doi:10.2319/073118-553.1
10. Mitchell L. Decalcification during orthodontic treatment with fixed appliances--an overview. *Br J Orthod*. 1992;19(3):199-205. doi:10.1179/bjo.19.3.199
11. Bjorn Ogaard DO. Prevalence of white spot lesions in 19 -year - olds: A study on untreated orthodontically treated persons 5 years after treatment. *Am J Orthod Dentofac Orthop*. 1989;96:423-427.
12. Tüfekçi E, Pennella DR, Mitchell JC, Best AM, Lindauer SJ. Efficacy of a fluoride-releasing orthodontic primer in reducing demineralization around brackets: An in-vivo study. *Am J Orthod Dentofac Orthop*. 2014;146(2):207-214. doi:10.1016/j.ajodo.2014.05.016
13. Buschang PH, Chastain D, Keylor CL, Crosby D, Julien KC. Incidence of white spot lesions among patients treated with clear aligners and traditional braces. *Angle Orthod*. 2019;89(3):359-364. doi:10.2319/073118-553.1
14. Knösel M, Klang E, Helms HJ, Wiechmann D. Occurrence and severity of enamel decalcification adjacent to bracket bases and sub-bracket lesions during orthodontic treatment with two different lingual appliances. *Eur J Orthod*. 2016;38(5):485-492. doi:10.1093/ejo/cjv069
15. Sukontapatipark W, El-agroudi MA, Selliseth NJ, Thunold K, Selvig KA. Bacterial colonization associated with fixed orthodontic appliances . A scanning electron microscopy study. 2001;23:475-484.
16. Dehghani M, Abtahi M, Sadeghian H, Shafaee H, Tanbakuchi B. Combined

chlorhexidine-sodiumfluoride mouthrinse for orthodontic patients: Clinical and microbiological study. *J Clin Exp Dent*. 2015;7(5):e569-e575. doi:10.4317/jced.51979
17. Stecksén-Blicks C, Renfors G, Oscarson ND, Bergstrand F, Twetman S. Caries-preventive effectiveness of a fluoride varnish: A randomized controlled trial in adolescents with fixed orthodontic appliances. *Caries Res*. 2007;41(6):455-459. doi:10.1159/000107932
18. Geiger AM, Gorelick L, Gwinnett AJ, Benson BJ. Reducing white spot lesions in orthodontic populations with fluoride rinsing. *Am J Orthod Dentofac Orthop*. 1992;101(5):403-407. doi:10.1016/0889-5406(92)70112-N
19. Gorton J, Featherstone JDB. In vivo inhibition of demineralization around orthodontic brackets. *Am J Orthod Dentofac Orthop*. 2003;123(1):10-14. doi:10.1067/mod.2003.47
20. Adel SM, Marzouk ES, El-Harouni N. Combined effect of Er,Cr:YSGG laser and casein phosphopeptide amorphous calcium phosphate on the prevention of enamel demineralization: An in-vitro study. *Angle Orthod*. 2020;90(3):369-375. doi:10.2319/032819-238
21. Poosti M, Ahrari F, Moosavi H, Najjaran H. The effect of fractional CO2 laser irradiation on remineralization of enamel white spot lesions. *Lasers Med Sci*. 2014;29(4):1349-1355. doi:10.1007/s10103-013-1290-9
22. Mahmoudzadeh M, Alijani S, Soufi LR, Farhadian M, Namdar F, Karami S. Effect of CO2 laser on the prevention of white spot lesions during fixed orthodontic treatment: A randomized clinical trial. *Turkish J Orthod*. 2019;32(3). doi:10.5152/TurkJOrthod.2019.18052
23. Mortazavi H, Baharvand M, Mokhber-Dezfuli M, et al. Lasers in Dentistry: Is It Really Safe? *Dent Hypotheses*. 2016;7(4):123-127. doi:10.4103/2155-8213.195967
24. Journal AS, Naslapur S, Malik SA, Sm L. Dental Health : Current Research The Effect of Silver Oxide Nanoparticles on the Antibacterial Property and Shear Bond Strength of Dental Composite. Published online 2019:1-10. doi:10.4172/2470-0886.1000138
25. Pérez-Díaz MA, Boegli L, James G, et al. Silver nanoparticles with antimicrobial activities against Streptococcus mutans and their cytotoxic effect. *Mater Sci Eng C*. 2015;55:360-366. doi:10.1016/j.msec.2015.05.036
26. Saber AM, El-Housseiny AA, Alamoudi NM. Atraumatic restorative treatment and interim therapeutic restoration: A review of the literature. *Dent J*. 2019;7(1):1-10. doi:10.3390/dj7010028
27. Tavassoli Hojati S, Alaghemand H, Hamze F, et al. Antibacterial, physical and mechanical properties of flowable resin composites containing zinc oxide nanoparticles. *Dent Mater*. 2013;29(5):495-505. doi:10.1016/j.dental.2013.03.011
28. Argueta-Figueroa L, Scougall-Vilchis RJ, Morales-Luckie RA, Olea-Mejía OF. An evaluation of the antibacterial properties and shear bond strength of copper nanoparticles as a nanofiller in orthodontic adhesive. *Aust Orthod J*. 2015;31(1):42-48. doi:10.21307/aoj-2020-139
29. Gottlow J, Dibart S. Guided Tissue Regeneration. *Pract Periodontal Plast Surg*. 2008;(1 998):45-48. doi:10.1002/9780470344637.ch9
30. Abdelaziz D, Hefnawy A, Al-Wakeel E, El-Fallal A, El-Sherbiny IM. New biodegradable nanoparticles-in-nanofibers based membranes for guided periodontal tissue and bone regeneration with enhanced antibacterial activity. *J Adv Res*. 2021;28:51-62. doi:10.1016/j.jare.2020.06.014
31. Haghshenas L, Amini A, Bashir Bahati A, Rahimi G. In vitro Antibacterial Biofilm effect of Magnesium Oxide Nanoparticles on Streptococcus mutans. *Micro Nano Biomed*. 2016;1(1). doi:10.15412/j.mnb.05010104

32. Xiaoli Feng1 Aijie Chen1 Yanli Zhang1 Jianfeng wang2 Longquan Shao1 Limin wei2. Application of dental nanomaterials: potential toxicity to the central nervous system. *Int J Nanomedicine*. 2015;10:5593-5594. doi:10.2147/IJN.S91856
33. Shrivastava R, Raza S, Yadav A, Kushwaha P, Flora SJS. Effects of sub-acute exposure to TiO2, ZnO and Al2O3 nanoparticles on oxidative stress and histological changes in mouse liver and brain. *Drug Chem Toxicol*. 2014;37(3):336-347. doi:10.3109/01480545.2013.866134
34. Crini G. *Historical Landmarks in the Discovery of Chitin*.; 2019. doi:10.1007/978-3-030-16538-3_1
35. Merzendorfer H, Zimoch L. Chitin metabolism in insects: Structure, function and regulation of chitin synthases and chitinases. *J Exp Biol*. 2003;206(24):4393-4412. doi:10.1242/jeb.00709
36. Pochanavanich P, Suntornsuk W. Fungal chitosan production and its characterization. *Lett Appl Microbiol*. 2002;35(1):17-21. doi:10.1046/j.1472-765X.2002.01118.x
37. Islam S, Bhuiyan MAR, Islam MN. Chitin and Chitosan: Structure, Properties and Applications in Biomedical Engineering. *J Polym Environ*. 2017;25(3):854-866. doi:10.1007/s10924-016-0865-5
38. Jayakumar R, Menon D, Manzoor K, Nair S V., Tamura H. Biomedical applications of chitin and chitosan based nanomaterials - A short review. *Carbohydr Polym*. 2010;82(2):227-232. doi:10.1016/j.carbpol.2010.04.074
39. Elieh Ali Komi D, Sharma L, Dela Cruz CS. Chitin and Its Effects on Inflammatory and Immune Responses. *Clin Rev Allergy Immunol*. 2018;54(2):213-223. doi:10.1007/s12016-017-8600-0
40. Antonino RSCMDQ, Fook BRPL, Lima VADO, et al. Preparation and characterization of chitosan obtained from shells of shrimp (Litopenaeus vannamei Boone). *Mar Drugs*. 2017;15(5):1-12. doi:10.3390/md15050141
41. Cha LE, Paz D, Resin A, Howard KA, Sutherland DS, Wejse PL. Antimicrobial Effect of Chitosan Nanoparticles on Streptococcus mutans Biofilms ▪ . 2011;77(11):3892-3895. doi:10.1128/AEM.02941-10
42. Divya K, Vijayan S, George TK, Jisha MS. Antimicrobial properties of chitosan nanoparticles: Mode of action and factors affecting activity. *Fibers Polym*. 2017;18(2):221-230. doi:10.1007/s12221-017-6690-1
43. Costa EM, Silva S, Pina C, Tavaria FK, Pintado MM. Anaerobe Evaluation and insights into chitosan antimicrobial activity against anaerobic oral pathogens. 2012;18. doi:10.1016/j.anaerobe.2012.04.009
44. Pham TT, Nguyen TH, Thi TV, et al. Investigation of chitosan nanoparticles loaded with Protocatechuic acid (PCA) for the resistance of Pyricularia oryzae Fungus against rice blast. *Polymers (Basel)*. 2019;11(1). doi:10.3390/POLYM11010177
45. Ing LY, Zin NM, Sarwar A, Katas H. Antifungal activity of chitosan nanoparticles and correlation with their physical properties. *Int J Biomater*. 2012;2012. doi:10.1155/2012/632698
46. Ke CL, Deng FS, Chuang CY, Lin CH. Antimicrobial actions and applications of Chitosan. *Polymers (Basel)*. 2021;13(6). doi:10.3390/polym13060904
47. Cicciù M, Fiorillo L, Cervino G. Chitosan use in dentistry: A systematic review of recent clinical studies. *Mar Drugs*. 2019;17(7):1-15. doi:10.3390/md17070417
48. Ganss C, Von Hinckeldey J, Tolle A, Schulze K, Klimek J, Schlueter N. Efficacy of the stannous ion and a biopolymer in toothpastes on enamel erosion/abrasion. *J Dent*. 2012;40(12):1036-1043. doi:10.1016/j.jdent.2012.08.005
49. Hayashi Y, Ohara N, Ganno T, et al. Chewing chitosan-containing gum effectively inhibits the growth of cariogenic bacteria. *Arch Oral Biol*. 2007;52(3):290-294.

doi:10.1016/j.archoralbio.2006.10.004
50. Kishen A, Shi Z, Shrestha A, Neoh KG. An Investigation on the Antibacterial and Antibiofilm Efficacy of Cationic Nanoparticulates for Root Canal Disinfection. *J Endod*. 2008;34(12):1515-1520. doi:10.1016/j.joen.2008.08.035
51. Dasilva L, Finer Y, Friedman S, Basrani B, Kishen A. Biofilm formation within the interface of bovine root dentin treated with conjugated chitosan and sealer containing chitosan nanoparticles. *J Endod*. 2013;39(2):249-253. doi:10.1016/j.joen.2012.11.008
52. del Carpio-Perochena A, Bramante CM, Duarte MAH, de Moura MR, Aouada FA, Kishen A. Chelating and antibacterial properties of chitosan nanoparticles on dentin. *Restor Dent Endod*. 2015;40(3):195. doi:10.5395/rde.2015.40.3.195
53. Sadony D. ANTIBACTERIAL EFFECT OF CHITOSAN NANOPARTICLES IN COMBINATION WITH DIODE LASER ON E.COLI BACTERIAL STRAIN. 2019;65:2543-2549.
54. Zeza B, Wohlfahrt C, Pilloni A. Chitosan brush for professional removal of plaque in mild peri-implantitis. *Minerva Stomatol*. 2017;66(4):163-168. doi:10.23736/S0026-4970.17.04040-7
55. Atai Z, Atai M, Amini J, Salehi N. In vivo study of antifungal effects of low-molecular-weight chitosan against Candida albicans. *J Oral Sci*. 2017;59(3):425-430. doi:10.2334/josnusd.16-0295
56. Madrazo-Jiménez M, Rodríguez-Caballero Á, Serrera-Figallo MÁ, et al. The effects of a topical gel containing chitosan, 0,2% chlorhexidine, allantoin and despanthenol on the wound healing process subsequent to impacted lower third molar extraction. *Med Oral Patol Oral Cir Bucal*. 2016;21(6):e696-e702. doi:10.4317/medoral.21281
57. Qasim SB, Delaine-Smith RM, Fey T, Rawlinson A, Rehman IU. Freeze gelated porous membranes for periodontal tissue regeneration. *Acta Biomater*. 2015;23:317-328. doi:10.1016/j.actbio.2015.05.001
58. Divya K, Vijayan S, George TK, Jisha MS. Antimicrobial Properties of Chitosan Nanoparticles : Mode of Action and Factors Affecting Activity. 2017;18(2):221-230. doi:10.1007/s12221-017-6690-1
59. Matica MA, Aachmann FL, Tøndervik A, Sletta H, Ostafe V. Chitosan as a wound dressing starting material: Antimicrobial properties and mode of action. *Int J Mol Sci*. 2019;20(23):1-34. doi:10.3390/ijms20235889
60. Kasaai MR. Various methods for determination of the degree of N-acetylation of chitin and chitosan: A review. *J Agric Food Chem*. 2009;57(5):1667-1676. doi:10.1021/jf803001m
61. Chatelet C, Damour O, Domard A. Influence of the degree of acetylation on some biological properties of chitosan films. *Biomaterials*. 2001;22(3):261-268. doi:10.1016/S0142-9612(00)00183-6
62. de Paz LEC, Resin A, Howard KA, Sutherland DS, Wejse PL. Antimicrobial effect of chitosan nanoparticles on Streptococcus mutans biofilms. *Appl Environ Microbiol*. 2011;77(11):3892-3895. doi:10.1128/AEM.02941-10
63. Aliasghari A, Khorasgani MR, Vaezifar S, Rahimi F, Younesi H, Khoroushi M. Evaluation of antibacterial efficiency of chitosan and chitosan nanoparticles on cariogenic streptococci: An in vitro study. *Iran J Microbiol*. 2016;8(2):93-100. doi:10.5281/zenodo.3342597
64. Ikono R, Vibriani A, Wibowo I, et al. Nanochitosan antimicrobial activity against Streptococcus mutans and Candida albicans dual-species biofilms. *BMC Res Notes*. 2019;12(1):1-7. doi:10.1186/s13104-019-4422-x
65. Reynolds IR. A Review of Direct Orthodontic Bonding. *Br J Orthod*. 1975;2(3):171-178. doi:10.1080/0301228x.1975.11743666

66. Bishara SE, Laffoon JF, VonWald L, Warren JJ. The effect of repeated bonding on the shear bond strength of different orthodontic adhesives. *Am J Orthod Dentofac Orthop*. 2002;121(5):521-525. doi:10.1067/mod.2002.123042
67. Hellak A, Ebeling J, Schauseil M, Stein S, Roggendorf M, Korbmacher-Steiner H. Shear Bond Strength of Three Orthodontic Bonding Systems on Enamel and Restorative Materials. *Biomed Res Int*. 2016;2016. doi:10.1155/2016/6307107
68. Bazargani F, Magnuson A, Löthgren H, Kowalczyk A. Orthodontic bonding with and without primer: A randomized controlled trial. *Eur J Orthod*. 2016;38(5):503-507. doi:10.1093/ejo/cjv075
69. Talic NF. Effect of fluoridated paste on the failure rate of precoated brackets bonded with self-etching primer: A prospective split-mouth study. *Am J Orthod Dentofac Orthop*. 2011;140(4):527-530. doi:10.1016/j.ajodo.2010.09.030
70. Paepegaey AM, Barker ML, Bartlett DW, et al. Measuring enamel erosion: A comparative study of contact profilometry, non-contact profilometry and confocal laser scanning microscopy. *Dent Mater*. 2013;29(12):1265-1272. doi:10.1016/j.dental.2013.09.015
71. Calvo P, Remunan-Lopez C, Vila-Jato JL, Alonso MJ. Chitosan and Chitosan/Ethylene Oxide-Propylene Oxide Block Copolymer Nanoparticles as Novel Carriers for Proteins and Vaccines. *Pharm Res*. 1997;14(10):1431.
72. Dehghan M, Ozorio JEV, Chanin S, Tantbirojn D, Versluis A, Garcia-Godoy F. Protocol for measurement of enamel loss from brushing with an anti-erosive toothpaste after an acidic episode. *Gen Dent*. 2017;65(4):63-68.
73. Lopatiene K, Borisovaite M, Lapenaite E. Prevention and Treatment of White Spot Lesions During and After Treatment with Fixed Orthodontic Appliances: a Systematic Literature Review. *J Oral Maxillofac Res*. 2016;7(2):1-11. doi:10.5037/jomr.2016.7201
74. Julien KC, Buschang PH, Campbell PM. Prevalence of white spot lesion formation during orthodontic treatment. *Angle Orthod*. 2013;83(4):641-647. doi:10.2319/071712-584.1
75. Song W, Ge S. Application of antimicrobial nanoparticles in dentistry. *Molecules*. 2019;24(6):1-15. doi:10.3390/molecules24061033
76. Neilands J, Sutherland D, Resin A, Wejse PL, Chávez De Paz LE. Chitosan nanoparticles affect the acid tolerance response in adhered cells of Streptococcus mutans. *Caries Res*. 2011;45(6):501-505. doi:10.1159/000331206
77. Wassel MO, Khattab MA. Antibacterial activity against Streptococcus mutans and inhibition of bacterial induced enamel demineralization of propolis, miswak, and chitosan nanoparticles based dental varnishes. *J Adv Res*. 2017;8(4):387-392. doi:10.1016/j.jare.2017.05.006
78. Cousins BG, Allison HE, Doherty PJ, et al. Effects of a nanoparticulate silica substrate on cell attachment of Candida albicans. *J Appl Microbiol*. 2007;102(3):757-765. doi:10.1111/j.1365-2672.2006.03124.x
79. Esteban Florez FL, Hiers RD, Larson P, et al. Antibacterial dental adhesive resins containing nitrogen-doped titanium dioxide nanoparticles. *Mater Sci Eng C*. 2018;93(August):931-943. doi:10.1016/j.msec.2018.08.060
80. Xu Z, Chou L, Sun J. Effects of SiO 2 nanoparticles on HFL-I activating ROS-mediated apoptosis via p53 pathway. *J Appl Toxicol*. 2012;32(5):358-364. doi:10.1002/jat.1710
81. Komatsu T, Tabata M, Kubo-Irie M, et al. The effects of nanoparticles on mouse testis Leydig cells in vitro. *Toxicol Vitr*. 2008;22(8):1825-1831. doi:10.1016/j.tiv.2008.08.009

82. Sodagar A, Akhavan A, Arab S, Bahador A, Pourhajibagher M, Soudi A. Evaluation of the Effect of Propolis Nanoparticles on Antimicrobial Properties and Shear Bond Strength of Orthodontic Composite Bonded to Bovine Enamel. *Front Dent*. 2019;13(5). doi:10.18502/fid.v16i2.1360
83. Sevinç BA, Hanley L. Antibacterial activity of dental composites containing zinc oxide nanoparticles. *J Biomed Mater Res - Part B Appl Biomater*. 2010;94(1):22-31. doi:10.1002/jbm.b.31620
84. Bakhadher W, Halawany H, Talic N, Abraham N, Jacob V. Factors Affecting the Shear Bond Strength of Orthodontic Brackets - a Review of In Vitro Studies. *Acta Medica (Hradec Kralove)*. 2015;58(2):43-48. doi:10.14712/18059694.2015.92
85. Giannini M, Soares CJ, De Carvalho RM. Ultimate tensile strength of tooth structures. *Dent Mater*. 2004;20(4):322-329. doi:10.1016/S0109-5641(03)00110-6
86. Hasan LA. Evaluation the properties of orthodontic adhesive incorporated with nano-hydroxyapatite particles. *Saudi Dent J*. 2021;10. doi:10.1016/j.sdentj.2021.01.001
87. Scribante A, Dermenaki Farahani MR, Marino G, et al. Biomimetic Effect of Nano-Hydroxyapatite in Demineralized Enamel before Orthodontic Bonding of Brackets and Attachments: Visual, Adhesion Strength, and Hardness in in Vitro Tests. *Biomed Res Int*. 2020;2020. doi:10.1155/2020/6747498
88. Sorourhomayoun S, Alaghehmand H, Mahjoub S, Khafri S, Author C, Ghasempour M. Shear bond strength of composite to primary enamel teeth treated with different concentrations and various molecular weights of chitosan. *Casp J Dent Res*. 2021;10(1):35-41.
89. Farzanegan F, Shafaee H, Darroudi M, Rangrazi A. Effect of the Incorporation of Chitosan and TiO 2 Nanoparticles on the Shear Bond Strength of an Orthodontic Adhesive: An In Vitro Study . *J Adv Oral Res*. 2021;12(2):261-266. doi:10.1177/23202068211015447
90. Zare Y. Study of nanoparticles aggregation/agglomeration in polymer particulate nanocomposites by mechanical properties. *Compos Part A Appl Sci Manuf*. 2016;84:158-164. doi:10.1016/j.compositesa.2016.01.020
91. Pus MD, Way DC. Enamel loss due to orthodontic bonding with filled and unfilled resins using various clean-up techniques. *Am J Orthod*. 1980;77(3):269-283. doi:10.1016/0002-9416(80)90082-2
92. Michael G. Buonocore. A SIMPLE METHOD OF INCREASING THE ADHESION OF ACRYLIC FILLING MATERIALS TO ENAMEL SURFACES. *J Med Chem*. 1987;30(10):849-853.
93. Retief DH, Denys FR. Finishing of enamel surfaces after debonding of orthodontic attachments. *Angle Orthod*. 1979;49(1):1-10. doi:10.1043/0003-3219(1979)049<0001:FOESAD>2.0.CO;2
94. Eliades T, Gioka C, Eliades G, Makou M. Enamel surface roughness following debonding using two resin grinding methods. *Eur J Orthod*. 2004;26(3):333-338. doi:10.1093/ejo/26.3.333
95. Vidor MM, Felix RP, Marchioro EM, Hahn L. Enamel surface evaluation after bracket debonding and different resin removal methods. *Dental Press J Orthod*. 2015;20(2):61-67. doi:10.1590/2176-9451.20.2.061-067.oar
96. Pitts NB, Stamm JW. International Consensus Workshop on Caries Clinical Trials (ICW-CCT) - Final consensus statements: Agreeing where the evidence leads. *J Dent Res*. 2004;83(SPEC. ISS. C):2002-2005. doi:10.1177/154405910408301S27
97. Jablonski-Momeni A, Stachniss V, Ricketts DN, Heinzel-Gutenbrunner M, Pieper K. Reproducibility and accuracy of the ICDAS-II for detection of occlusal caries in vitro. *Caries Res*. 2008;42(2):79-87. doi:10.1159/000113160

98. Zhang J, Lynch RJM, Watson TF, Banerjee A. Remineralisation of enamel white spot lesions pre-treated with chitosan in the presence of salivary pellicle. *J Dent*. 2018;72(December 2017):21-28. doi:10.1016/j.jdent.2018.02.004
99. Martínez-Robles ÁM, Loyola-Rodríguez JP, Zavala-Alonso NV, et al. Antimicrobial properties of biofunctionalized silver nanoparticles on clinical isolates of Streptococcus mutans and its serotypes. *Nanomaterials*. 2016;6(7). doi:10.3390/nano6070136
100. Chandrasekaran M, Kim KD, Chun SC. Antibacterial activity of chitosan nanoparticles: A review. *Processes*. 2020;8(9):1-21. doi:10.3390/PR8091173
101. Kyoon H, Young N, Ho S, Meyers SP. Antibacterial activity of chitosans and chitosan oligomers with different molecular weights. 2002;74:65-72.
102. Chung YC, Su YP, Chen CC, et al. Relationship between antibacterial activity of chitosan and surface characteristics of cell wall. *Acta Pharmacol Sin*. 2004;25(7):932-936.
103. Murphy TC, Willmot DR, Rodd HD. Management of postorthodontic demineralized white lesions with microabrasion: A quantitative assessment. *Am J Orthod Dentofac Orthop*. 2007;131(1):27-33. doi:10.1016/j.ajodo.2005.04.041
104. Ando M, Shaikh S, Eckert G. Determination of caries lesion activity: Reflection and roughness for characterization of caries progression. *Oper Dent*. 2018;43(3):301-306. doi:10.2341/16-236-L
105. Yamaguchi I, Tokuchi K, Fukuzaki H, et al. Preparation and microstructure analysis of chitosan/hydroxyapatite nanocomposites. *J Biomed Mater Res*. 2001;55(1):20-27. doi:10.1002/1097-4636(200104)55:1<20::AID-JBM30>3.0.CO;2-F

APPENDICES

- Appendix A: Tables (1-7)
- Appendix B: Figures (1-7)

Appendix A: Tables

Table 1: Descriptive statistics for the Disc Agar Diffusion method (mm)

Group	Mean ± SD	Minimum	Maximum
CHX	32.44 ± 7.49	26.00	40.67
CS-NPs 20nm	19.55 ± 2.50	17.00	22.00
CS-NPs 131nm	19.44 ± 2.52	17.67	22.33

Table 2: Tukey's multiple comparison test

Groups	comparison	P value
CHX	CS-NPs 20nm	0.038
	CS-NPs 131nm	0.037
CS-NPs 20nm	CHX	0.038
	CS-NPs 131nm	1.000
CS-NPs 131nm	CHX	0.037
	CS-NPs 20nm	1.000

Table 3: Shear bond strength values (MPa)

Groups	N	Mean ± SD (MPa)	Minimum	Maximum
Control (no treatment)	8	14.26 ± 2.93	10.11	19.24
ChNPs 20 nm	8	13.13 ± 1.91	10.89	17.01
ChNPs 131 nm	8	13.3 ± 2.69	8.09	17.90

Table 4: Tukey's multiple comparison test

Groups	comparison	P value
Control (no treatment)	CS-NPs 20nm	0.96
	CS-NPs 131nm	0.44
CS-NPs 20nm	Control	0.96
	CS-NPs 131nm	0.91
CS-NPs 131nm	Control	0.44
	CS-NPs 20nm	0.91

Table 5: Mean and SD of ICDAS used for caries assessment

Groups	N	Mean ± SD (Ra)
Control (no treatment)	8	62.62 ± 8.45
CS-NPs 20 nm	8	43.78 ± 9.51
CS-NPs 131 nm	8	47.32 9.23

Table 6: Surface roughness (Ra) and after combining the orthodontic adhesive with CS-NPs (µm)

Groups	n	Mean ± SD
Control (no treatment)	8	62.62 ± 8.45
CS-NPs 20nm	8	43.78 ± 9.51
CS-NPs 131nm	8	47.32 ± 9.23

Table 7: Tukey's multiple comparison test

Groups	comparison	P value
Control (no treatment)	CS-NPs 20nm	< 0.001
	CS-NPs 131nm	0.008
CS-NPs 20nm	Control	< 0.001
	CS-NPs 131nm	0.720
CS-NPs 131nm	Control	0.008
	CS-NPs 20nm	0.720

Appendix B: Figures

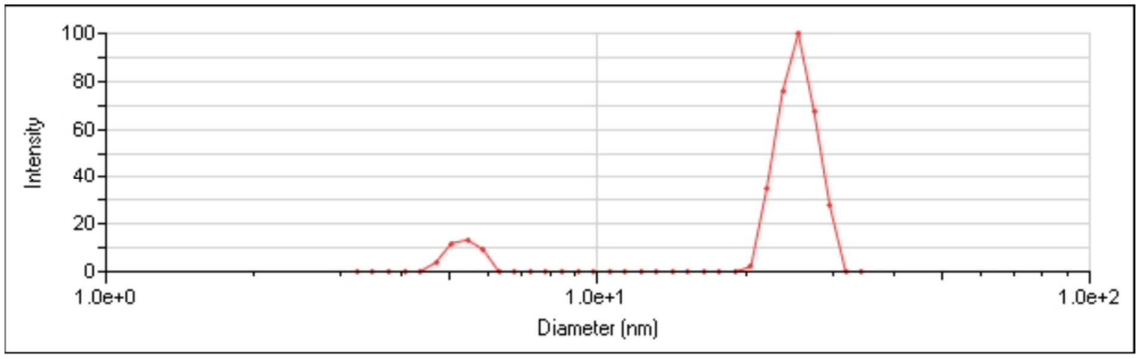


Figure 1: Size distribution of the smaller particles after suspension in distilled water. The peak represents the mean particle size (20 nm).

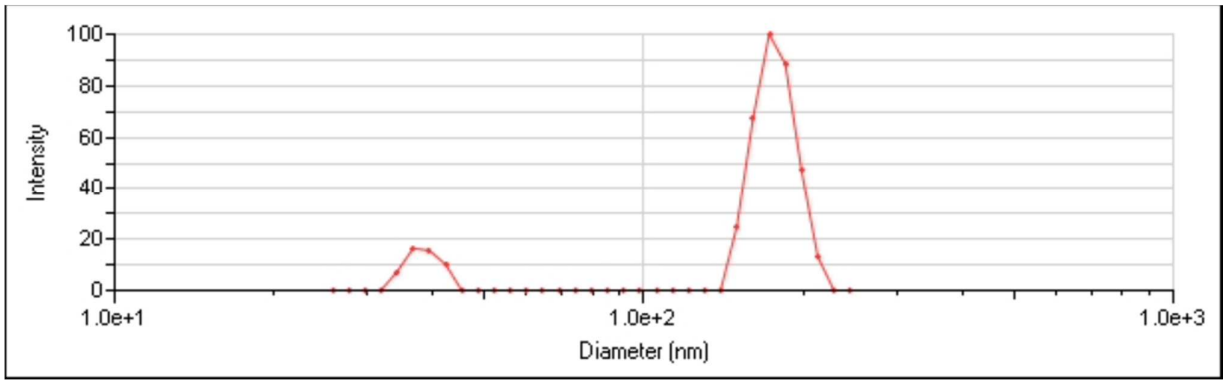


Figure 2: Size distribution of the larger particles after suspension in distilled water. The peak represents the mean particle size (131 nm).

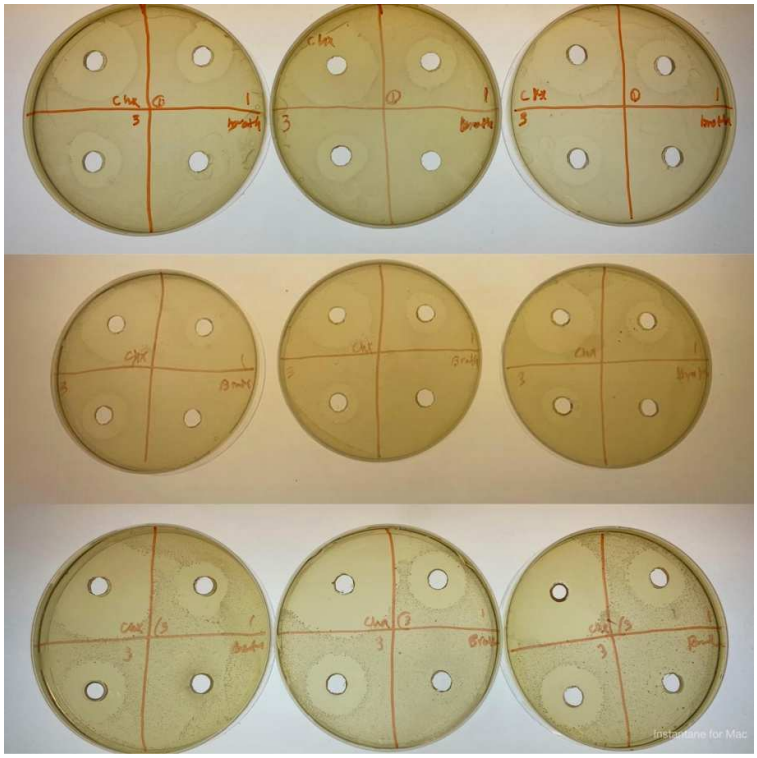


Figure 3: Disc agar diffusion for S. *mutans.* Zones of inhibition of the testing groups in each plate: upper left (CHX, positive control), upper right (CS-NPs 20nm), lower left (CS-NPs 131nm), lower right (Broth, negative control).

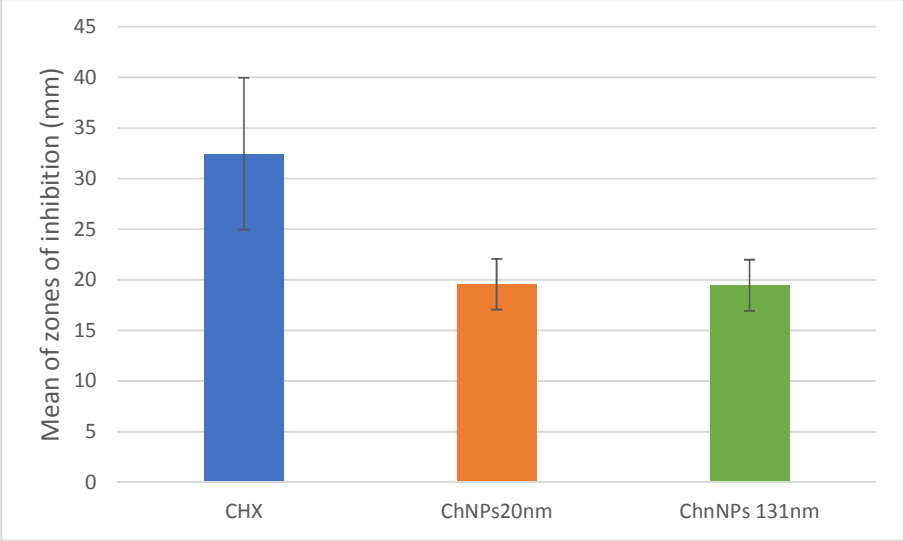
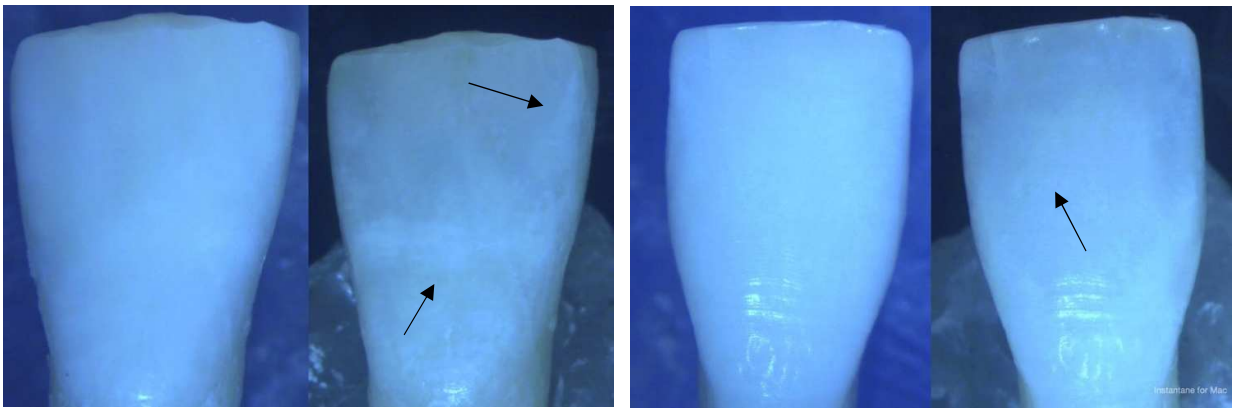


Figure 4: Antibacterial effect of various sizes of chitosan nanoparticles on *S.mutans.* The test was done in triplicate, the data was presented as means ± SD.



(A) (B)

Figure 5: Comparison of the induced white spot lesions (pointed by arrows) before and after between the control group (A) and treatment group (B).

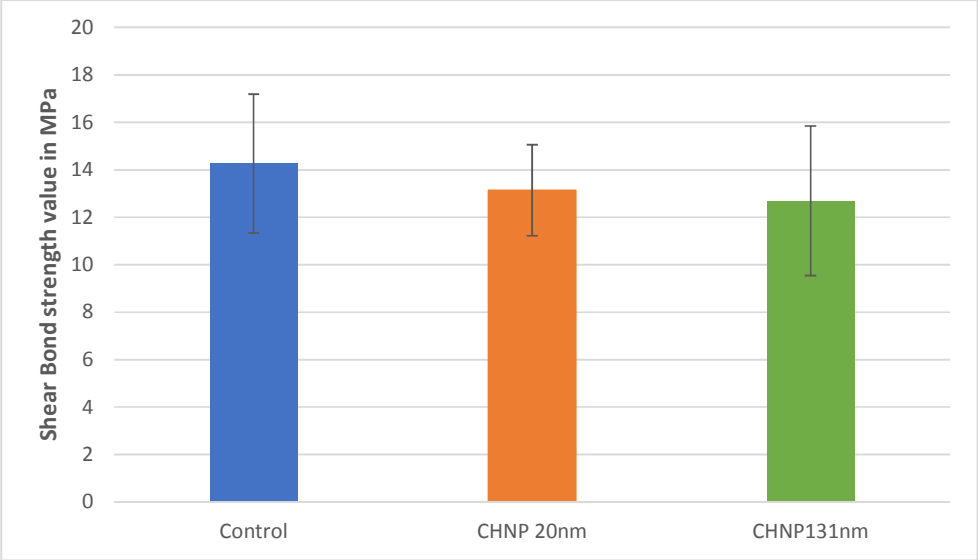


Figure 6: Shear bond strength values of three orthodontic adhesives bonded with CS-NPs. The data was presented as means ± SD (n=8).

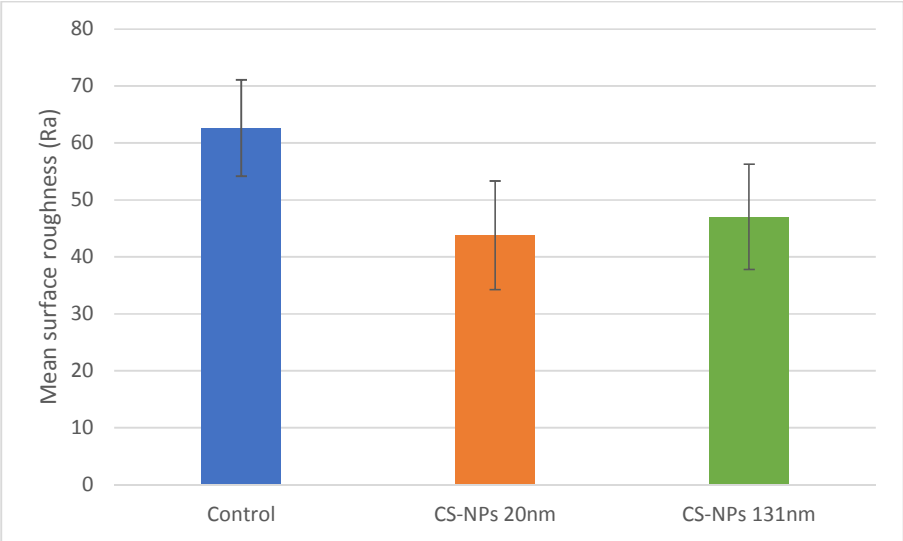


Figure 7: profilometry results for the mean surface roughness between the groups. The data was presented as means ± SD (n=8).