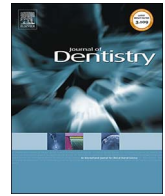




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Polymerisation, antibacterial and bioactivity properties of experimental orthodontic adhesives containing triclosan-loaded halloysite nanotubes

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ABSTRACT

Objective: To evaluate the immediate enamel bond strength, *in situ* degree of conversion and the polymerisation rate of three experimental orthodontic adhesives containing triclosan-loaded halloysite nanotubes. The antibacterial and bioactivity properties of such experimental materials were also assessed.

Materials and methods: Three experimental orthodontic adhesives were formulated by incorporating triclosan-loaded halloysite nanotubes (TCN-HNT) at different concentrations (5 wt%, 10 wt% and 20 wt%) into a resin blend (Control). The maximum polymerisation rate of the tested adhesives was evaluated through FTIR, while Raman was used to analyse the *in situ* degree of conversion (DC) at the bracket/enamel interface. The shear bond strength (SBS) of the enamel-bonded specimens was assessed at 24 h. The antibacterial properties of the experimental materials against *S. Mutans* were evaluated up to 72 h, while, their bioactivity was evaluated after 14 days of artificial saliva (AS) storage through SEM-EDS and Raman spectromicroscopy.

Results: Incorporation of TCN-HNT increased the polymerisation properties without interfering with the immediate bonding properties of the experimental adhesives. All experimental adhesives containing TCN-HNT inhibited bacterial growth at 24 h, and induced mineral deposition after 14 days of AS storage. At 72 h, only the experimental system containing 20% TCN-HNT maintained such a capability.

Conclusions: Adhesives doped with TCN-HNT present improved polymerisation properties and suitable bonding performance. However, only the adhesives containing TCN-HNT > 10% might promote long-term antibacterial activity and reliable mineral deposition.

Clinical significance: The use of adhesives containing triclosan-loaded halloysite represents a promising “smart” approach to bond orthodontic brackets and bands; these might prevent enamel demineralisation and induce enamel remineralisation during the treatment.

1. Introduction

Enamel demineralisation is one of the main causes responsible for the formation of white spot lesions (WSL) during fixed orthodontic treatments, in particular in those patients with limited oral hygiene

compliance [1]. Wide gaps are often observed at the adhesive–enamel interface around brackets [2]; this represents the most common site for demineralisation to occur due to accumulation of a biofilm rich in cariogenic species such as *Streptococcus mutans* [3,4].

It has been advocated that the use of ion-releasing materials such as

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glass ionomer cements may reduce the risk of enamel demineralisation and prevent excessive bacterial growth [5]. Based on this information, several therapeutic agents such as fluoride [6], chlorhexidine [7] and nano-silver [8] have been incorporated into experimental orthodontic bonding materials to overcome such a clinical issue. However, it seems that although their early effectiveness, the release of such active principles tends to reduce overtime. Moreover, some of these agents may induce tooth discoloration; for instance, the release of silver ions or chlorhexidine may often cause anaesthetics appearance when incorporated into orthodontic cements [9].

It has been reported that the use of triclosan nanoparticles may represent an alternative to conventional antibacterial agents due to its small particle size, as well as to the little amount needed to produce antibacterial effects [10]. Several researchers have been inspired to create innovative composites containing such inorganic nanoparticles due to their large surface area and to their high surface reactivity [11,12].

Nano-compounds based on mesoporous aluminosilicate clay (i.e. halloysite nanotubes – HNTs) have been incorporated into dental adhesives as nano-carriers for antibacterial agents [13]. Moreover, these nanotubes have an inner diameter of 30–70 nm, which could be infiltrated by resin monomers and increase the mechanical properties of resin-based materials due to their great elastic modulus (140 GPa) [14].

We hypothesised that triclosan-loaded HNTs might be used as a promising nano-filler for innovative therapeutic orthodontic adhesives to induce mineral deposition on the enamel around brackets.

Thus, the first objective of this study was to evaluate the enamel bond strength, and the polymerisation properties (i.e. *in situ* degree of conversion and polymerisation rate) of experimental orthodontic adhesives containing different concentrations of triclosan-loaded halloysite nanotubes. The antibacterial and bioactivity properties of such experimental materials doped with triclosan-loaded HNTs were also assessed.

The first hypothesis tested in this study was that the incorporation of triclosan-loaded HNTs within the composition of experimental orthodontics adhesives would not interfere with their immediate bonding performance to acid-etched enamel and polymerisation properties compared to the control experimental adhesive containing no TCN-HNT.

The second hypothesis was that the incorporation of different amount of triclosan-loaded HNTs would enhance the antibacterial, and bioactivity properties of such materials compared to the control experimental adhesive containing no TCN-HNT.

2. Materials and methods

2.1. Preparation of TCN-loaded HNTs (TCN-HNT)

Halloysite nanotube (HNT) – $\text{Al}_2\text{Si}_2\text{O}_5(\text{OH})_4 \cdot 2\text{H}_2\text{O}$ with a diameter of 30–70 nm and length of 1–3 μm (Sigma-Aldrich, St. Louis, MO, USA) were submitted to a silanisation process using a solution containing 5 wt.% of 3-metacryloxypropyltrimetoxysilane and 95 wt.% acetone at 110 °C for 24 h. Subsequently, the treated nanoparticle were mixed [1:1 ratio] with 2,4,4-Trichloro-2-hydroxydiphenyl ether (TCN – Triclosan, Fagron, Rotterdam, SH, Netherlands) under continues agitation for 1 h as described in a previous study [15]. The mixture was then dispersed in 95 wt.% pure ethanol (0.03 mg ml⁻¹) and sonicated for 1 h. Subsequently, the TCN-HNT nanotubes were desiccated for 10 days at 30 °C to ensure complete evaporation of residual solvents [15]. TCN-HNT was finally characterised using a Transmission Electron Microscope (TEM) JEM 120 Exll (JEOL, Tokyo, Japan) at 80 kV at a magnification X 300,000.

2.2. Formulation of experimental adhesive – incorporation of TCN-HNT

A monomer resin blend was created by mixing 75 wt.% bisphenol-

A-glycol dimethacrylate (BisGMA – Sigma-Aldrich Co., St. Louis, MO, USA) and 25 wt.% triethylene glycol dimethacrylate (TEGDMA- Sigma-Aldrich) and used as a control adhesive containing no TCN-HNT. Moreover, a photoinitiator (CQ: Camphorquinone, 0.5 mol%) and two co-initiators (EDAB: ethyl 4-dimethylaminobenzoate; DPIHFP: diphenyliodonium hexafluorophosphate) (Aldrich Chemical Co., Milwaukee, WI, USA) were also added at a concentration of 1 mol%.

The TCN-HNT filler was added at concentrations of 5, 10 and 20 (wt.%) into the resin blend in order to create three experimental adhesives. All formulations were mixed and maintained in an ultrasonic bath for 1 h. The four experimental resin adhesives were finally stored in a dark chamber.

2.3. Polymerisation rate evaluation (FTIR-ART)

Polymerisation rate of the experimental adhesives was evaluated using Fourier transform infrared spectroscopy (FTIR) using a spectrometer equipped with an attenuated total reflection (ATR) (Vertex 70; Bruker Optics, Ettlingen, Germany). Three specimens for each group were analysed by directly applying the tested materials (3 μL) onto the diamond crystal. These were light activated for 40 s using a light emitting diode unit (Radii Cal, SDI, Bayswater, VIC, Australia) with an irradiation value of 1200 mW/cm² at a standardised distance of 2 mm. Analysis was performed at a controlled room temperature of 23 \pm 1 °C and 60 \pm 1% relative humidity.

The IR-Solution software was used to standardise the assessment parameters such as scanning range (4000–800 cm⁻¹), resolution (4 cm⁻¹), and scanning time (40 s). This setup allowed the acquisition of each single scan, every 0.7 s during photo-activation procedure. The maximum polymerization rate [Rp. (s⁻¹)] was also evaluated as described in a previous study [16] based on the intensity of the C=C stretching vibrations at 1635 cm⁻¹ (peak height) as a function of time, and using the symmetric ring stretching at 1608 cm⁻¹ from the polymerised and non-polymerised samples as an internal standard.

The data was plotted and a sigmoidal curve fitting method was applied with the linear regression method using Sigma Plot 12.0 for Windows (Systat Software Inc, San Jose, CA, USA).

2.4. Bonding procedures and bond strength evaluation

The buccal surface of 60 anterior bovine teeth were polished using a 600 grid SiC paper for 5 s in order to remove the aprismatic enamel only; this was checked by using a stereo microscope (\times 80). All specimens were then individually placed in cylinder-shape moulds and embedded in a self-curing polymethyl-methacrylate resin (Esschem Linwood, Pennsylvania, PA, USA), with buccal surface perpendicular to the horizontal plane; fifteen enamel specimens were used for each adhesive group (Table 1). The enamel surface of each specimen was first etched for 30 s with a 37% phosphoric acid gel (Acid Gel, Villevie, Joinville, SC, Brazil) and rinsed with water for the same time. After air-drying for 5 s, orthodontic brackets were positioned on the enamel surface and kept under constant pressure (276 g); resin excess around the bracket was removed with a sharp dental probe.

Light-curing was performed for 40 s [17] using a light emitting diode unit (Radii Cal, SDI).

Specimens bonded to their brackets (Morelli Ltd, Sorocaba, SP, Brazil) with 11.18 mm² area, were stored in distilled water at 37 °C for 24 h. Subsequently, these were positioned in a Universal Testing Machine (Shimadzu EZ-SX, São Paulo, SP, Brazil) positioning the incisal edge of the bracket base parallel to a sharp chisel blade. They were stressed to failure using a 500 N load cell with a crosshead speed of 1.0 mm/min. The maximum force required for bracket's debonding was recorded in N, which was used to calculate the bond strength in MPa (N/mm²). Following debonding, the amount of adhesive on each enamel surface was with analysed using a stereomicroscope (\times 10) and the adhesive remnant index (ARI) calculated as described in a previous

Table 1

Antimicrobial effect in \log_{10} CFU/mL at different incubation time, degree of conversion in situ (DC%), maximum polymerisation rate [Rp. (s-1)] and shear bond strength (MPa) of the adhesive with different concentrations of TCN-HNT.

Groups	Log ₁₀ CFU/mL			DC in situ (%)	Max. polymerisation rate	Shear bond strength (MPa)
	24 h	48 h	72 h			
0%	9.19 ± 0.15 ^{B,b}	8.21 ± 0.29 ^{B,a}	7.97 ± 0.36 ^{B,a}	76.85 ± 1.09 ^A	12.23 ± 4.42 ^{AB}	17.77 ± 4.70 ^A
5%	6.99 ± 1.07 ^{A,a}	8.10 ± 0.38 ^{B,b}	7.54 ± 0.12 ^{AB,b}	75.69 ± 1.87 ^A	14.46 ± 3.31 ^A	17.23 ± 4.91 ^A
10%	6.02 ± 0.87 ^{A,a}	6.95 ± 0.41 ^{A,b}	7.31 ± 0.09 ^{AB,b}	76.72 ± 3.03 ^A	9.11 ± 0.24 ^{AB}	13.51 ± 2.93 ^A
20%	5.42 ± 1.44 ^{A,a}	7.07 ± 0.35 ^{A,b}	6.84 ± 0.41 ^{A,b}	74.05 ± 2.42 ^A	7.70 ± 0.83 ^B	13.80 ± 2.22 ^A

Different uppercase letters in the same column means statistically significant difference ($p < 0.05$).

Different lowercase letters in the same row means statistically significant difference ($p < 0.05$).

study [18].

2.5. In situ degree of conversion – micro-Raman assessment (DC)

Three teeth for each group were prepared and bonded as previously describe. These were subsequently sectioned in two halves after 24 h of storage in distilled water at 37 °C. The specimens were then submitted to the *in situ* analysis of the DC along the bracket-enamel interface. This analysis was accomplished using micro-Raman spectroscopy using the Senterra equipment (Bruker Optik GmbH, Ettlingen, Germany) for five times (3 s) by a 100-mW diode laser with 785-nm wavelength. Firstly, it was collected the spectra of each un-polymerised adhesive at three different individual points, and subsequently, the spectra of the polymerised adhesive was obtained at three different sites along the bracket-enamel interface. The aliphatic and aromatic peaks at 1635 cm^{-1} and 1608 cm^{-1} were considered, respectively. The average value of the measurements from the same group was used to calculate the ratio of double bond content after polymerisation.

2.6. Mineral deposition – bioactivity assay

The same specimens prepared for the *in situ* DC assay were analysed again through micro-Raman spectroscopy using the same parameters as aforementioned. The specimens were then immersed for 14 days in artificial saliva (AS) [19]. The specimens were analysed through eight scanning lines (20 μm distance) running perpendicularly along the adhesive-enamel interface for an area of 100 $\mu\text{m} \times 160 \mu\text{m}$. The aromatic peak at 1608 cm^{-1} and the phosphate peak at 962 cm^{-1} were integrated and the mean value per group was plotted in graphs to evaluate mineral deposition at the bracket-enamel interface. Scanning Electron Microscopy (SEM) with Energy Dispersive Spectroscopy (EDS) was also performed to characterise the content of the minerals deposited along the interface after 14 days of AS storage.

2.7. Microbiology assay

Thirty-six specimens (4 mm × 1 mm) from each experimental group (n = 9) were fixed on 6 × 6 × 8 mm teflon matrices and attached to the lid of a 48-well plate. This device was similarly used by previous studies [20,21] to allow bacteria grown on the surface of tested samples. The plate was sterilised using ethylene oxide. Each well was filled with 500 μL of brain-heart infusion (BHI) broth supplemented with 0.5% of sucrose and 50 μL of inoculum, which was prepared adjusting *Streptococcus mutans* (UA159) to the optical absorbance of 0.3. The cover was placed with specimens in contact to the inoculum, immersed in BHI broth and incubated at 37 °C for 24 h.

Afterward, three disks for each group were transferred to a micro-tube containing 900 μL of sterile saline solution (0.9% NaCl) and the biofilms were harvested. The bacterial suspensions were serially diluted (100 μL) in sterile saline solution. Two aliquots of 25 μL were placed onto a BHI agar, incubated anaerobically at 37 °C for 48 h, followed by evaluation of the biofilm colony-forming unit (CFU). The other discs were incubated into a new 48-well plate with fresh medium and incubated at 37 °C for 24 h, completing 48 h of incubation, and sequentially up to 72 h (n = 3). At these periods, the described above protocol of serial dilution and plate onto agar was repeated.

2.8. Statistical analysis

The normality of data was evaluated using Shapiro-Wilk test ($p > 0.05$ for all tests). Homogeneity of variance was calculated using the Brown-Forsythe test. For all tests the variances were homoscedastic ($p > 0.05$). Two-way ANOVA and Tukey's post hoc were used to assess differences in the microbiology assay (\log_{10} CFU/mL) in the concentrations of TCN-HNT and different incubation periods. One-way ANOVA and Tukey's post-hoc were used for the statistical analysis of the data obtained during the DC, maximum polymerisation rate and shear bond strength tests. ARI score values were evaluated using the Kruskal-Wallis and Tukey's post hoc tests.

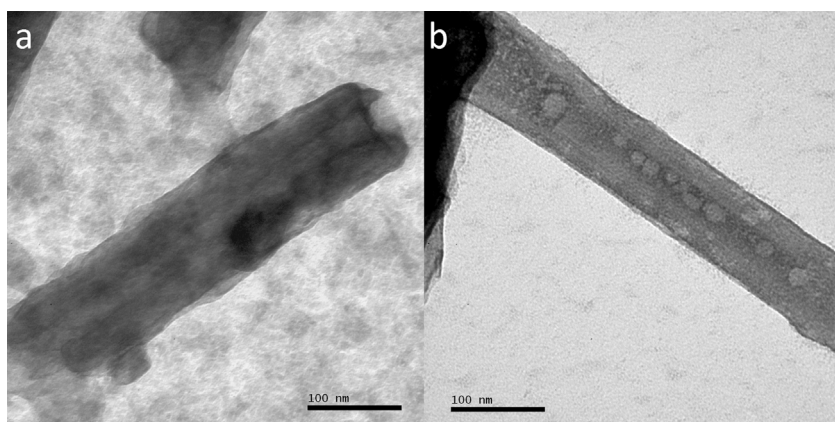


Fig. 1. (a) TEM image of a nanotube with its inner-surface of 40–50 nm diameter range and outer-surface length of 90 nm diameter. (b) TEM image shows the presence of TCN nanoparticles inside nanotubes. It is also possible to observe empty dark spaces, which may permit the mobility of triclosan through the tube.

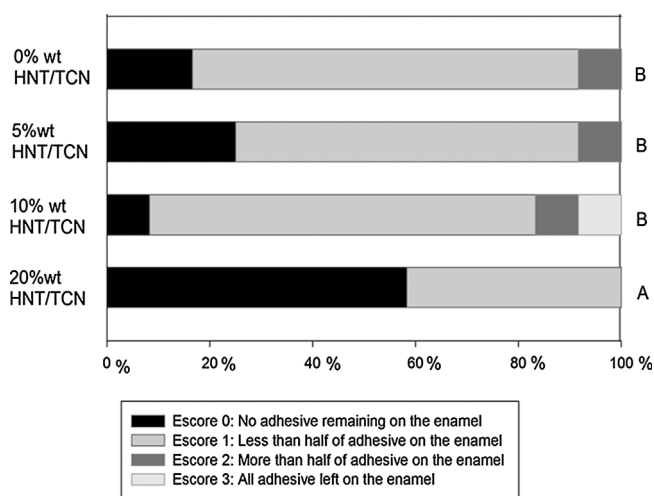


Fig. 2. ARI score of the adhesive with different concentrations of TCN-HNT. Different letters mean statistical difference between groups ($p < 0.05$).

3. Results

The TEM analysis showed the overall morphology and confirmed the nano-dimension of halloysite nanotubes (Fig. 1A) as well as the successful functionalisation of TCH inside lumen of the halloysite nanotubes (Fig. 1B).

The results of *in situ* DC are depicted in Table 1 and these varied from 74.05 to 76.72. There was no significant difference in DC between the experimental groups ($P > 0.05$) of TCN-HNT compared to the control resin blend containing no TCN-HNT nanotubes. The R_p (s^{-1}) reduced significantly with TCN-HNT concentration $> 10\%$ compared to the control resin blend containing 0% and the experimental 5% TCN-HNT (Table 1).

Shear bond strength mean and standard deviation are expressed in MPa and presented in Table 1. The adhesive containing no TCN-HNT and that with 5% TCN-HNT showed the highest results, although no significant difference was observed between the groups tested in this study ($P > 0.05$). The ARI score is presented in Fig. 2. It was observed a significant decrease of adhesive remaining on enamel ($P < 0.05$) in specimens bonded with the adhesive containing 20% TCN-HNT compared to all the other groups. While, no significant difference ($P > 0.05$) was found between the experimental resins containing 0, 5 and 10% TCN-HNT.

SEM-EDS analysis showed mineral deposition in all adhesives containing 5%, 10% and 20% of TCN-HNT after 14 days of saliva immersion (Fig. 3). However, the greatest mineral precipitation at the adhesive-enamel interface was observed when using the experimental resins containing 10% and 20% TCN-HNT (Fig. 4).

The results of the microbiology analysis (\log_{10} CFU/mL) are shown in Table 1. At 24 h, adhesives with 5%, 10% and 20% of TCN-HNT differed from the resin containing 0% TCN-HNT ($P < 0.05$). It was found that the greater the concentration of TCN-HNT within the adhesive composition the lower CFU counts. Indeed, adhesives with 10% and 20% of TCN-HNT presented lower CFU counts ($P < 0.05$) at 48 h compared to all the other groups. After 72 h of incubation, only the adhesive with 20% of TCN-HNT maintained its antimicrobial activity. During the period of incubation a decreased of antibacterial activity was found after 48 and 72 h for all experimental groups compared to 24 h ($p < 0.05$).

4. Discussion

It is well-know that the prevalence of white spot lesions formation in enamel may increase during orthodontic treatment [22]. This is the reason why we believe that the use of antibacterial/remineralising

bonding materials may prevent, or at least reduce, enamel demineralisation during fixed orthodontic treatments.

It has been demonstrated that halloysite nanotubes can be used as functional fillers for resin-based materials due to several advantages. These include relatively low production cost, natural availability and their suitability as drug carrier [11].

This study showed that triclosan-loaded halloysite nanotubes could be successfully incorporated within the composition of experimental resin-based adhesives. The bond strength results obtained in this study showed no substantial alteration of the immediate bonding performance after incorporation of different amounts of TCN-HNT into the experimental adhesives compared to the experimental adhesive containing no TCN-HNT. Moreover, TCN-HNT incorporation induced an increase of the degree of conversion (DC) of the experimental adhesives compared to the one containing no TCN-HNT. However, similar degree of conversion (DC) was observed among all the experimental orthodontic adhesives containing the TCN-HNT at different concentrations. Conversely, the highest polymerisation rate between all the experimental orthodontic adhesives containing TCN-HNT was observed with the system containing 5 wt% TCN-HNT. The lower polymerisation rate observed in the experimental adhesives containing TCN-HNT > 5 wt% compared to the control resin blend may have been caused by some sort of interference with the scattering of the light during light-curing procedures; it is well-known that the higher the filler amount the lower the diffusion of the light through the resin bulk [13,14]. However, high cross-linking density leads to increase mechanical stability, lowering the degradation of polymeric matrix [23,24].

Such a situation was also evident during the bond strength test. Indeed, the incorporation of TCN-HNT > 5 wt% caused a reduction of the bonding performance of the tested experimental adhesives. Although such a reduction was not significant, we hypothesised that it was caused by an excessive presence of nanoparticles that may have deposited within the porosities of the acid-etched dentine and interfered with the diffusion of the resin monomer and with a proper hybrid layer formation. Indeed, in the specimens bonded with the adhesive containing 20 wt% TCN-HNT, it was observed a higher failure in adhesive mode compared to other groups in this study (Fig. 2). However, our results are in accordance with the results of a recent meta-analysis [25], where it was affirmed that the incorporation of antimicrobial agents into an orthodontic adhesive has no influence on bond strength to enamel. It was also demonstrated that higher SBS may lead to sound enamel loss during bracket debonding [26]. However, the bond strength results obtained with 10 and 20 wt% of TCN-HNT were higher than those considered clinically acceptable [27,28]; this may also reduce the risk for critical enamel loss during bracket debonding [29].

Therefore, the hypothesis that the incorporation of triclosan-loaded HNTs within the composition experimental orthodontics adhesives would not interfere with their immediate bonding performance and the polymerisation properties of experimental orthodontics adhesives must be partially accepted, as the incorporation of TCN-HNT at a concentration higher than 5% interfered with the polymerisation rate of some adhesives.

The HNT filler used in this study is characterised by multi-walled inorganic nanotubes, whose structure is tubular with aluminols in its inner part and siloxanes and silanols/aluminols groups in the outer portion of nanotube [30]. Brackets and archwires usually create numerous retention sites hampering tooth cleaning and increasing potential to develop white spot lesions on enamel around them [31]. Because of this unique composition, the adhesives containing HNTs showed bioactive mineral precipitation on their surfaces after 14 days of immersion in AS. This bio-reactivity may be attributed due exchange of hydrogen ions $[H^+]$ with the buffer solution and the SiO_2 layer, which attracts Ca^{2+} and PO_4^{3-} [32]. This may serve as a source of ions to prevent demineralisation of enamel when low pH occurs due to plaque accumulation during orthodontic treatment.

Moreover, the HNT can also serve as a reservoir for sustained

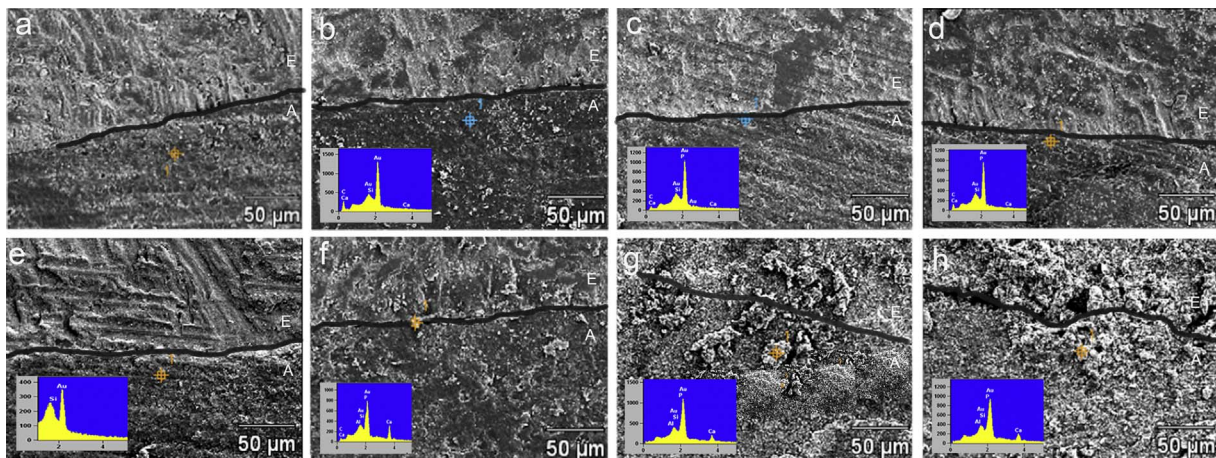


Fig. 3. SEM images with EDS scales of mineral deposition. In images a, b, c and d, no mineral deposition was observed prior artificial saliva. e) No mineral deposition after saliva immersion in control group. In images f, g and h, mineral deposition was observed in adhesives containing respectively 5%, 10% and 20% of TCN-HNT after 14 days of saliva immersion signalled by an orange target and confirmed by EDS scales. Dark lines highlight the interface between adhesive (A) and enamel (E). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

release of active substances such as antibacterial agents [33,34]. In this regard, the neutral charge of triclosan allows its localisation inside and outside of nanotubes [35], as shown in Fig. 1. The relatively hydrophobicity of the lumen of HNT favoured the penetration of hydrophobic molecules of triclosan into the nanotubes [11,33]. With a concentration of triclosan of 20% it was possible to obtain an inhibition effect on the growth of *S. mutans* up to 72 h. The limitation of this study was the antibacterial effect that could not be evaluated due to bacteria death after 72 h. Usually, the antimicrobial effect of materials is assessed at 24 h [36]. Despite the challenged behaviour of this supersaturated model of *S. mutans*, in this study we evaluated antibacterial action up to 72 h to test the persisting effect of TCN-HNT. Although a decreased of antibacterial activity after 72 h compared to 24 h of incubation for the experimental groups, the prolonged diffusion of triclosan from the nanotubes may have diffused over time into bacterial membrane, improving the antimicrobial capability of the experimental adhesive containing 20% of TCN-HNT [37]. Thus, the second hypothesis of this study must be accepted as different amounts of TCN-HNT incorporated to orthodontic adhesives had different effect on the antibacterial and bioactivity properties of the experimental adhesive tested in this study.

5. Conclusion

The present findings confirm that triclosan-doped halloysite nanotubes can be successfully incorporated into resin-based materials up to 20 wt.%. Such therapeutic adhesives may promote long-term antimicrobial activity and mineral deposition, with acceptable degree of conversion *in situ* and shear bond strength. Thus, the use of orthodontic adhesives containing triclosan-doped halloysite nanotubes may represent a promising approach to prevent *in vivo* demineralisation and therapeutic enamel remineralisation during orthodontic treatments. However, such hypothesis must be confirmed by further *in vivo* and clinical trials studies.

Authors contributions

Felipe Weidenbach Degrazia: ROLE: contributed to conception, design, data acquisition, analysis, and data interpretation, drafted, gave final approval and agrees to be accountable for all aspects of work ensuring integrity and accuracy.

Bruna Genari: ROLE: contributed to conception, data acquisition, critically revised the manuscript and agrees to be accountable for all aspects of work ensuring integrity and accuracy.

Vicente Castelo Branco Leitune: ROLE: contributed to conception,

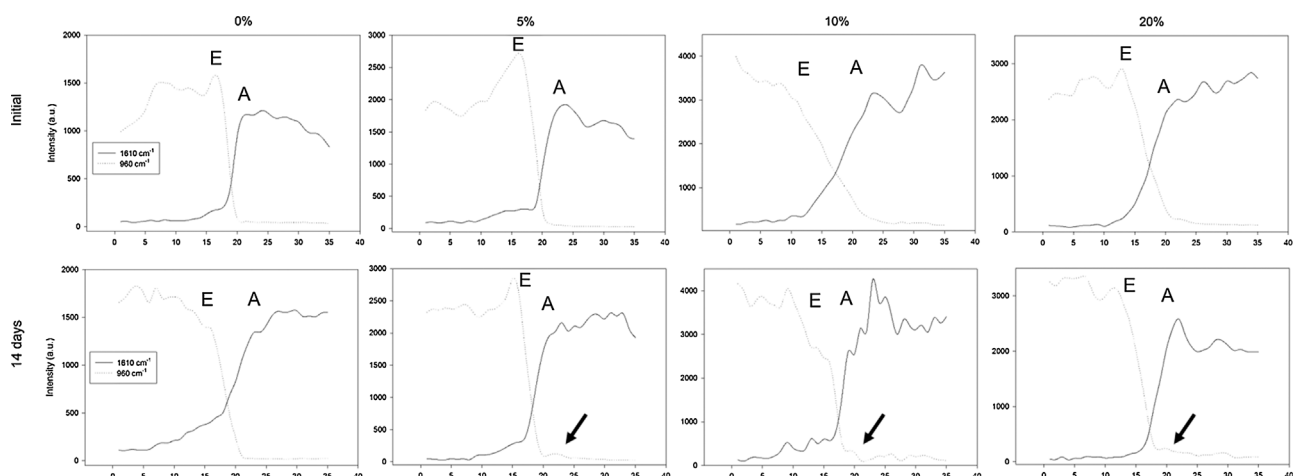


Fig. 4. Analyses of interface between adhesive and enamel surface with different TCN-HNT concentrations, initially and after 14 days. Arrows indicate mineral deposition along the interface with adhesive containing 5%, 10% and 20% of TCN-HNT after 14 days of immersion in artificial saliva. Peaks of 960 cm^{-1} and 1610 cm^{-1} represent calcium and methacrylate chemical groups, respectively. Crossing lines represent interface, E represents enamel side and A represents adhesive side.

design, data interpretation, critically revised the manuscript and agrees to be accountable for all aspects of work ensuring integrity and accuracy.

Rodrigo Alex Arthur: ROLE: specimen preparation and data acquisition, revised the manuscript and agrees to be accountable for all aspects of work ensuring integrity and accuracy.

Santiago Arias Luxan: ROLE: contributed data interpretation, statistical analysis, revised the manuscript and agrees to be accountable for all aspects of work ensuring integrity and accuracy.

Susana Maria Werner Samuel: ROLE: Specimen preparation and data acquisition, revised the manuscript and agrees to be accountable for all aspects of work ensuring integrity and accuracy.

Fábricio Mezzomo Collares: ROLE: contributed to conception, design, and data interpretation, drafted, critically revised the manuscript, gave final approval and agrees to be accountable for all aspects of work ensuring integrity and accuracy.

Salvatore Sauro: ROLE: contributed to conception, design, data acquisition, analysis, and data interpretation, drafted, critically revised the manuscript, gave final approval and agrees to be accountable for all aspects of work ensuring integrity and accuracy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.jdent.2017.11.002>.

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