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Triclosan-loaded chitosan as antibacterial agent for adhesive resin

Ana Helena Schauenberg Machado[a], Isadora Martini Garcia[a], Amanda de Souza da Motta[b], Vicente Castelo Branco Leitune[a], Fabrício Mezzomo Collares[a,*]

[a] Dental Materials Laboratory, School of Dentistry, Federal University of Rio Grande do Sul, Rua Ramiro Barcelos, 2492, Rio Branco, 90035-003, Porto Alegre, RS, Brazil
[b] Department of Microbiology, Institute of Basic Health Sciences, Federal University of Rio Grande do Sul, Rua Sarmento Leite, 500, Centro, 90050-170, Porto Alegre, RS, Brazil

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ABSTRACT

Objectives: The aim of this study was to formulate and to evaluate the immediate and long-term physical, chemical and antibacterial properties of an experimental adhesive resin with chitosan or triclosan-loaded chitosan.

Materials and methods: Chitosan, triclosan and triclosan-loaded chitosan were evaluated for scanning electron microscopy (SEM) and micro-Raman spectroscopy. An experimental adhesive resin was formulated with methacrylate monomers and photoinitiators. Chitosan or triclosan-loaded chitosan were added at 2 ($G_{Q2\%}$ and $G_{QT2\%}$) or 5 ($G_{Q5\%}$ and $G_{QT5\%}$) wt.% in the base resin. The base resin was used as control (G_{CTRL}). The adhesives were evaluated for degree of conversion (DC), Knoop hardness (KHN), softening in solvent (ΔKHN), immediate and long-term microtensile bond-strength (μ-TBS) and antibacterial activity.

Results: SEM indicated triclosan sticks and chitosan porosity. Triclosan-loaded chitosan presented structures on chitosan. Micro-Raman indicated no chemical interaction between chitosan and triclosan. There was no difference among groups for DC ($p > 0.05$). Initial KHN ranged from 17.36 (± 1.56) to 20.38 (± 1.72), with higher value for $G_{QT5\%}$ compared to G_{CTRL} ($p < 0.05$). G_{CTRL} presented the lowest ΔKHN% ($p < 0.05$). There were no differences in the immediate or long-term μ-TBS ($p > 0.05$). G_{CTRL} and $G_{Q2\%}$ decreased the μ-TBS after storage ($p < 0.05$). Chitosan groups showed higher biofilm formation ($p < 0.05$). Triclosan-loaded chitosan groups presented lower biofilm formation ($p < 0.05$). There was no activity against planktonic bacteria regardless the time of evaluation ($p > 0.05$).

Conclusion: Triclosan-loaded chitosan at 5 wt.% addition in an experimental adhesive resin showed reliable properties, with the highest antibacterial activity immediately and after six months, and induced dentin/adhesive interface stability over time.

Clinical significance: Triclosan-loaded chitosan groups showed antibacterial activity immediately and over time and induced dentin/adhesive interface stability, may positively affecting long-lasting marginal sealing.

1. Introduction

The biofilm formation at tooth/restoration interface may lead to recurrent caries, which is the main reason for restoration replacement at long term [1]. Replacement of failed restorations has been half of all restorations placed (160 million procedures per year) in U.S. [2]. The commercially available adhesive resins are mainly composed by monomers as bisphenol A glycerolate dimethacrylate (Bis-GMA) and 2-hydroxyethyl methacrylate (HEMA), which do not present antibacterial activity [3]. In order to reduce the bacterial colonization, antibacterial agents have been incorporated in comonomer blends [4–6]. Antibacterial-agent-releasing is only dispersed in resin network and it is detached over time with no kinetics control of release [7]. In addition, its release may negatively affect physical and chemical properties of polymers and increase cytotoxicity [8]. To overcome these disadvantages, non-antibacterial-agent-releasing [6,9,10] and drug delivery systems with antibacterial compounds [11–13] have been proposed.

Triclosan (2,4,4-trichloro-2-hydroxy-diphenyl ether) is an antibacterial agent widely used in oral hygiene products, such as mouth rinses [14] and tooth pastes [15], cosmetics [16] and in polymers to prevent degradation process and colonization over time [17]. At low

* Corresponding author.
E-mail addresses: anahelena.13@hotmail.com (A.H.S. Machado), isadora.garcia@ufrgs.br (I.M. Garcia), amanda.motta@ufrgs.br (A.d.S.d. Motta), vicente.leitune@ufrgs.br (V.C.B. Leitune), fabricio.collares@ufrgs.br (F.M. Collares).

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concentration, triclosan is bacteriostatic due to deleterious effects to bacterial enzymes responsible for the composition of fatty acid from cells wall and membrane [18]. At high concentration, triclosan disrupts bacteria membrane, leading it to death [18]. Due to its broad antimicrobial spectrum, it has been used to develop antibacterial restorative materials against oral biofilms since 1995 [19]. It is used in a commercial resin sealant [20] and it was previously carried in halloysite nanotubes for composite resins [21–23]. In addition, it was encapsulated by nanocapsules for addition in an adhesive resin [11], indicating reliable physical and chemical properties without cytotoxic effect [11,20]. However, at 120 h, almost 20% of triclosan was released from the adhesive resin network even in nanocapsules [11] and the antibacterial effect at longer periods is unknown. Carrier systems with higher drug retention may reduce triclosan leaching and maintain its chemical structure available to interact with bacteria over time.

Chitosan is a cationic molecule synthesized from alkaline deacetylation of chitin, which mainly composes the exoskeletal of arthropods (as crustaceans and insects), diatoms, algae and some fungal wall [24]. It is the second most abundant biopolymer in nature, only behind cellulose, and it presents low-cost and non-cytotoxicity [25]. This polysaccharide shows reactive amino (NH_2) groups, which may confer antibacterial property by electrostatic attraction with negative charges of bacteria wall and membrane [24,25]. Due to its chemical composition and porosity, chitosan has also been explored as a new green adsorbent [26] and as carrier for drug delivery systems [27,28]. There are no reports on the use of chitosan as drug carrier in resins for dentistry. The aim of this study was to formulate and to evaluate the immediate and long-term physical, chemical and antibacterial properties of an experimental adhesive resin with chitosan or triclosan-loaded chitosan. The null hypothesis to be tested is that the addition of chitosan or triclosan-loaded chitosan does not influence the adhesive resins properties.

2. Materials and methods

Monomers, initiators, chitosan, solvents and broth culture media were purchased from Aldrich Chemical Company (St. Louis, Missouri, USA). All components were weighed using an analytical balance (AUW220D, Shimadzu, Kyoto, Kyoto, Japan). A light-emitting diode (Radii Cal, SDI, Australia) at 1200 mW/cm^2 was used for specimens photo-activation.

2.1. Triclosan-loaded chitosan preparation

Low molecular weight ≥75% (deacetylated) chitosan flakes were mixed with 2,4,4-trichloro-2-hydroxy-diphenyl ether (triclosan) powder (Fagron, Rotterdam, SH, Netherlands) at ratio of 1:1 by weight in absolute ethanol. The mixture was magnetic stirred until complete solvent vaporization and subsequently dried in a desiccator for 7 days.

2.2. Adhesive resins formulation

The experimental adhesive resins were formulated by mixing 66.66 wt.% of bisphenol A glycerolate dimethacrylate (Bis-GMA) and 33.33 wt.% of 2-hydroxyethyl methacrylate (HEMA). Camphorquinone (CQ) and ethyl 4-dimethylaminobenzoate (EDAB) at 1 mol% were added as photoinitiator system.

Pure chitosan was added at 2 ($G_{Q2\%}$) or 5 ($G_{Q5\%}$) wt.% in the base resin. Triclosan-loaded chitosan was added at 2 ($G_{QT2\%}$) or 5 ($G_{QT5\%}$) wt.% in the base resin. One group remained without chitosan or triclosan-loaded chitosan as control group (G_{CTRL}). The description of each group is shown in Table 1. Chemical structure of monomers, chitosan and triclosan are presented in Fig. 1.

Table 1
Name of each experimental adhesive resin group and description according to each formulation.

Group	Description
G_{CTRL}	Base resin (BisGMA, HEMA, CQ and EDAB)
$G_{Q2\%}$	Base resin (BisGMA, HEMA, CQ and EDAB); Chitosan at 2 wt.%.
$Q_{Q5\%}$	Base resin (BisGMA, HEMA, CQ and EDAB); Chitosan at 5 wt.%.
$G_{QT2\%}$	Base resin (BisGMA, HEMA, CQ and EDAB); Triclosan-loaded chitosan at 2 wt.%.
$G_{QT5\%}$	Base resin (BisGMA, HEMA, CQ and EDAB); Triclosan-loaded chitosan at 5 wt.%.

2.3. Scanning electron microscopy of chitosan, triclosan and triclosan-loaded chitosan

Chitosan, triclosan, triclosan-loaded chitosan were morphologically evaluated by scanning electron microscopy (SEM) at 10 kV (EVO MA10, Zeiss, Oberkochaen, Germany). The powders were fixed in carbon conductive adhesive tapes in metallic stubs to be gold-sputter coated (15–25 nm, SCD 050, Baltec, Vaduz, Liechtenstein).

2.4. Micro-Raman spectroscopy characterization of chitosan, triclosan and triclosan-loaded chitosan

Chitosan, triclosan or triclosan-loaded chitosan were analyzed by micro-Raman spectroscopy (SENTERRA, Bruker OptikGmbH, Ettlingen, Baden-Württemberg, Germany) using a 100 mW diode laser with a 785 nm wavelength and a spectral resolution of ~ 3.5 cm^{-1}. The spectra were obtained in 413-1775 cm^{-1} range with 5 coadditions and 5 s.

2.5. Degree of conversion

The experimental adhesive resins were evaluated for degree of conversion (DC) with three specimens per group (n = 3, 1.0 mm thickness × 4.0 mm diameter) by micro-Raman spectroscopy (SENTERRA, Bruker OptikGmbH, Ettlingen, Baden-Württemberg, Germany) using a 100 mW diode laser with a 785 nm wavelength and a spectral resolution of ~ 3.5 cm^{-1}. Raman spectrum of each uncured adhesive resin was collected to compare with the polymerized specimens after 20 s of photoactivation. The spectra were acquired at three different points in each specimen. The phenyl C═C peak at 1610 cm^{-1} was used as reference, while the aliphatic C═C peak at 1640 cm^{-1} was used as the carbon double bond converted during polymerization process [29].

2.6. Softening in solvent

The softening in solvent of experimental adhesive resins was evaluated with five specimens per group (n = 5, 1.0 mm thickness × 4.0 mm diameter) photoactivated for 30 s on each side. The specimens were embedded in an acrylic resin to be polished (Model 3v, Arotec, Cotia, SP, Brazil) with silicon carbide sandpapers (1000, 1200 and 2000-grit) and a felt disc saturated with alumina suspension of 0.5 μm. After 24 h, five indentations (10 g for 10 s) were performed on each specimen using a microhardness tester (HMV 2; Shimadzu, Tokyo, Japan) to obtain the initial Knoop hardness number (KHN1). The specimens were immersed in a solution of ethanol and water (70:30) for 2 h and washed with distilled water to be evaluated again to obtain the final Knoop hardness number (KHN2). The percentage difference between KHN1 and KHN2 was calculated (ΔKHN%) for each group [30].

2.7. Microtensile bond-strength

The experimental adhesive resins were evaluated for microtensile

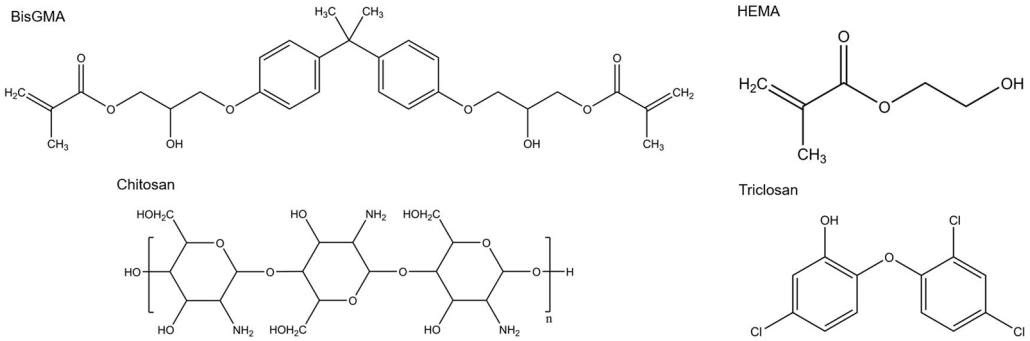

Fig. 1. Chemical structures of BisGMA, HEMA, chitosan and triclosan used in the study to formulate the experimental adhesive resins.

bond strength (μTBS) with 120 bovine teeth (n = 12) stored in distilled water at 4 °C up to 1 month. The superficial dentin of buccal surface of each tooth was ground flat (600-grit) under running water for 30 s. The dentin of each tooth was etched with 37% phosphoric acid for 15 s and rinsed off with distilled water for 30 s A commercial primer (Primer Scotch Bond Multi-Purpose, 3 M ESPE, St. Paul, MN, USA) was actively applied for 20 s, and the solvent was evaporated for 5 s. The experimental adhesive resins were applied and light-cured for 20 s. Two layers, with 2.00 mm each one, of composite resin (Filtek Z350 XT- 3 M ESPE, St. Paul, MN, USA) were used to dentin restoration. Composite increments were placed onto dentin to be photoactivated during 20 s each one. The restored teeth were stored in distilled water at 37 °C for 24 h and sectioned into sticks of 0.5 mm^2 of cross-sectional area. Sixty teeth were tested at 24 h and sixty were restored, cut and stored for six months before testing. The sticks were fixed by a cyanoacrylate resin in a metallic device to be submitted to a universal testing machine (EZ-SX Series, Shimadzu, Kyoto, Japan) at 1 mm/min. The values in newtons (N) were divided for the cross-sectional area of each stick to obtain the results in megapascals (MPa).

2.8. Evaluation of antibacterial activity against biofilm formation and planktonic bacteria

For the antibacterial activity assays, three specimens per group (n = 3, 1.0 mm thickness × 4.0 mm diameter) were photoactivated for 30 s on each side. The specimens were attached on the lid of a test plate and the assembly was submitted to hydrogen peroxide plasma (58%) sterilization for 48 min at 56 °C. In each well of a 48-well plate, it was added 900 μL of brain-heart infusion broth (BHI) with 1 wt.% sucrose and 100 μL of a suspension of an overnight broth culture of *Streptococcus mutans* (NCTC 10449) at 10^6 CFU/mL. The 48-well plate was incubated with the ensemble (lid and specimens) at 37 °C for 24 h. Three wells with broth and *Streptococcus mutans* without specimens were used as negative control. For the evaluation of antibacterial activity against biofilm formation on resins surfaces, the specimens were removed from the lid and vortexed in 1 mL of saline solution (0.9%) to be diluted until 10^{-6} dilution. Two 25-μL drops of each dilution were platted in BHI-agar Petri dishes and incubated at 37 °C for 48 h. For the evaluation of antibacterial activity against planktonic bacteria, 100 μL of each well from the analysis of antibacterial activity against biofilm formation were vortexed in 900 μL of saline solution (0.9%) to be diluted until 10^{-6} dilution and platted in brain-heart infusion agar Petri dishes as previously described. The number of colony forming units (CFUs) was visually counted and transformed to log CFU/mL. The dilution and the platting were performed using automatic graduated pipettes.

2.9. Statistical analysis

Data distribution was evaluated by Shapiro-Wilk test. One-way ANOVA and Tukey post hoc test were used to compare groups for DC, KHN1 and ΔKHN. Paired *t*-test was used to compare KHN1 and KHN2 in each group. Two-way ANOVA and Tukey post hoc test were used to compare groups and different times for antibacterial activity assays and μ-TBS. All tests were performed at a level of 0.05 of significance.

3. Results

SEM images are shown in Fig. 2. It is observed agglomerates of oriented sticks of triclosan (a and b) and the disorganized and porous structure of chitosan (c and d). The analysis of triclosan-loaded chitosan powder shows structures adsorbed in chitosan particles (e and f), suggestive of triclosan on chitosan walls.

The images of micro-Raman spectroscopy analyses are presented in Fig. 3. The evaluation of chitosan indicated peaks mainly of symmetrical stretches of C—O—C bond in the 850-900 cm^{-1} attributed to glyosidic bonds. Antisymmetric stretches of the C—O—C bond were identified in the 1150-1060 cm^{-1} region. Peak at 1270 cm^{-1} indicates the bond of C—OH stretch. Peak at 1370 cm^{-1} indicates symmetrical CH_3. Also, peak related to symmetric bond of CH_2 is shown at 1460 cm^{-1} [31]. The highest and narrowest Raman peak of triclosan is in 710 cm^{-1} and it is related to inelastic light scattering attributed to C—C stretching vibration of benzene rings of triclosan [32]. It was not observed different peaks by micro-Raman analysis of triclosan-loaded chitosan powder.

Table 2 shows the results of DC, KHN1, KHN2, ΔKH immediate and long-term μ-TBS. The values of DC ranged from 84.12 (± 1.04)% to 86.61 (± 2.59)% without statistically significant difference among groups ($p > 0.05$). KHN1 ranged from 17.36 (± 1.56) to 20.38 (± 1.72), with higher value for $G_{QT5\%}$ compared to G_{CTRL} ($p < 0.05$). All groups decreased the KHN1 after storage in solvent ($p < 0.05$) and the G_{CTRL} indicated the lowest ΔKHN% ($p < 0.05$) with no statistically significant difference among chitosan and triclosan-loaded chitosan groups ($p > 0.05$). The values of immediate μ-TBS ranged from 35.1 (± 12.7) MPa to 45.4 (± 12.7) MPa without statistically significant difference among groups ($p > 0.05$). G_{CTRL} and $G_{Q2\%}$ decreased the μ-TBS after six months in water ($p < 0.05$) and there was no statistically significant difference among groups for long-term μ-TBS ($p > 0.05$).

Table 3 presents the results of direct contact inhibition test against biofilm formation on adhesive surfaces and planktonic bacteria inhibition test. Chitosan groups indicated higher biofilm formation than G_{CTRL} ($p < 0.05$) without statistically significant difference between $G_{Q2\%}$ and $G_{Q5\%}$ ($p > 0.05$). Triclosan-loaded chitosan groups presented lower biofilm formation than other groups ($p < 0.05$). With higher triclosan-loaded chitosan addition in the adhesive resin, lower the biofilm formation ($p < 0.05$). There were no statistically significant differences for immediate and long-term evaluations in each group ($p > 0.05$). After six months of water storage, the differences among groups for biofilm formation was the same than the results observed in the immediate analysis, with lower biofilm values for triclosan-loaded chitosan groups ($p < 0.05$). In the planktonic bacteria inhibition test, it was observed no statistically significant differences

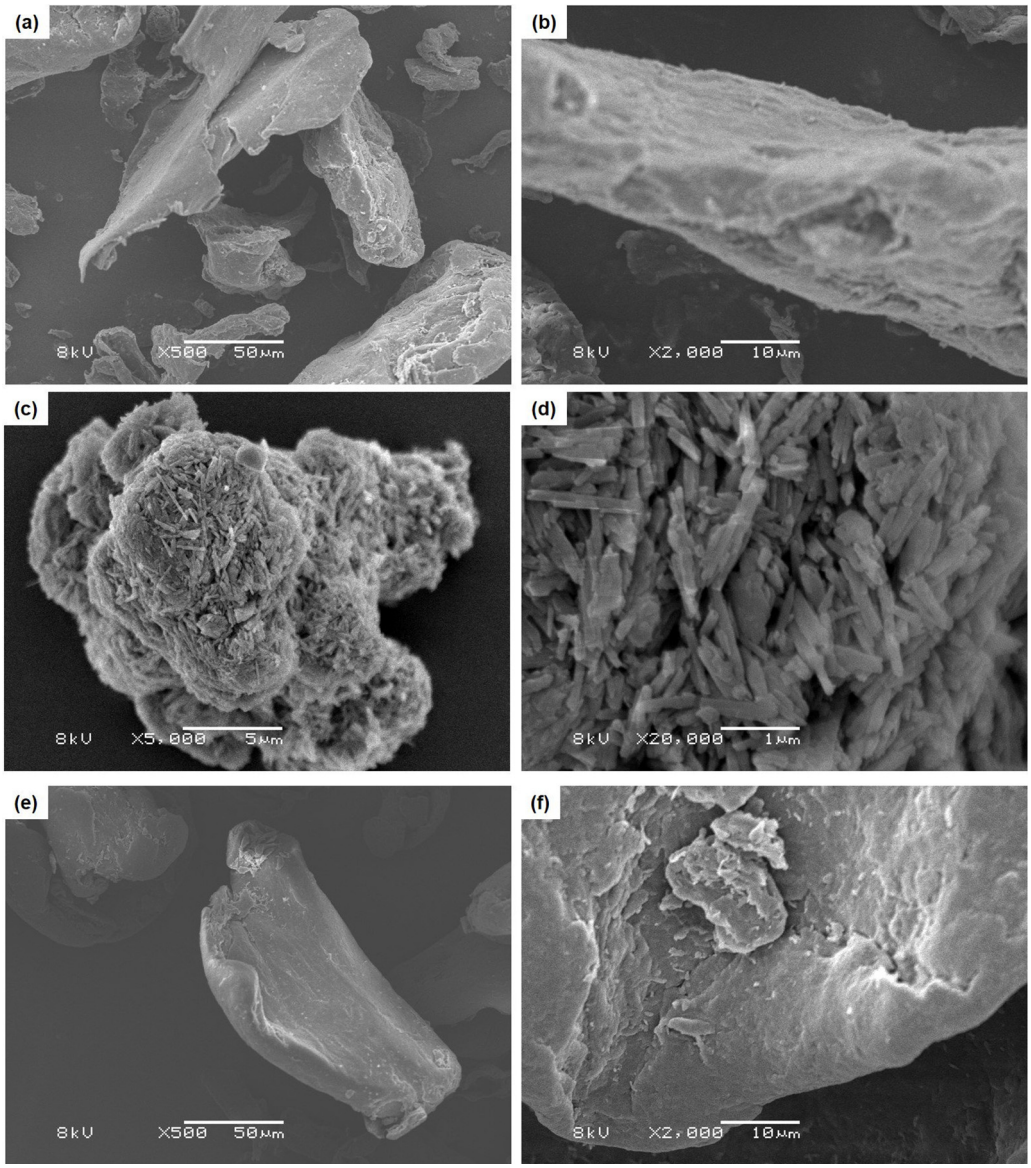

Fig. 2. SEM images of chitosan at 500× (a) and 2000× (b), triclosan at 5000 x (c) and 20,000× (d), triclosan-loaded chitosan at 500×(e) and 2000× (f).

among groups in the immediate ($p > 0.05$) and in the long-term ($p > 0.05$) analyses, without statistically significant differences for immediate and long-term evaluations in each group ($p > 0.05$).

4. Discussion

The development of antibacterial adhesive resins is the current trend to decrease bacterial adhesion and biofilm formation at tooth/restoration interface [33,34]. The antibacterial activity of dental materials is important unless physical and chemical properties are not in

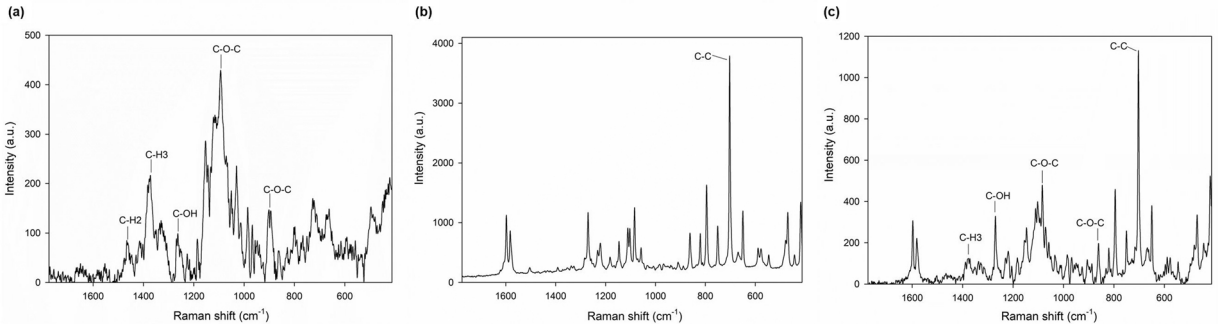

Fig. 3. Images of micro-Raman spectroscopy analyses. The image (a) shows chitosan spectrum, image (b) shows triclosan and image (c) is from triclosan-loaded chitosan.

Table 2
Mean and standard deviation values of degree of conversion (DC%) after 20 s of photoactivation, initial Knoop hardness number (KHN1), final Knoop hardness number (KHN2), percentage of microhardness variation (ΔKHN%) and microtensile bond-strength (μ-TBS) for experimental adhesive resins.

Group	DC (%)	KHN1	KHN2	ΔKHN%	μ-TBS (MPa)	
					24 h	6 months
G_{CTRL}	85.78 (± 2.30)^{A}	17.36 (± 1.56)^{Ba}	9.86 (± 2.72)^{b}	43.96 (± 10.41)^{B}	45.4 (± 12.7)^{Aa}	34.1 (± 10.0)^{Ab}
$G_{Q2\%}$	86.61 (± 2.59)^{A}	17.74 (± 0.85)^{ABa}	5.95 (± 1.06)^{b}	66.57 (± 4.87)^{A}	36.2 (± 10.2)^{Aa}	27.0 (± 5.7)^{Ab}
$G_{Q5\%}$	85.26 (± 1.27)^{A}	19.48 (± 1.93)^{ABa}	7.06 (± 0.74)^{b}	63.77 (± 1.11)^{A}	37.2 (± 10.9)^{Aa}	30.5 (± 9.3)^{Aa}
$G_{QT2\%}$	84.45 (± 1.19)^{A}	20.38 (± 1.72)^{Aa}	7.54 (± 1.76)^{b}	63.08 (± 7.52)^{A}	40.2 (± 10.2)^{Aa}	34.1 (± 10.3)^{Aa}
$G_{QT5\%}$	84.12 (± 1.04)^{A}	20.00 (± 0.95)^{ABa}	5.57 (± 0.65)^{b}	72.10 (± 3.52)^{A}	35.1 (± 12.7)^{Aa}	35.0 (± 14.5)^{Aa}

Different capital letter indicates statistical difference in the same column ($p < 0.05$). Different small letter indicates statistical difference in the same row ($p < 0.05$) for the same test.

detriment over time [33]. In the present study, experimental adhesive resins were formulated with chitosan or triclosan-loaded chitosan and the physical, chemical and antibacterial properties were evaluated. The incorporation of triclosan-loaded chitosan at 2 or 5 wt.% presented immediate and long-term antibacterial activity against biofilm formation on polymerized adhesive resins and stability of adhesive/dentin interface over time. Thus, the null hypothesis must be rejected.

Surface morphology and chemical bonds of chitosan, triclosan and triclosan-loaded chitosan powders were analyzed respectively by SEM and micro-Raman spectroscopy. The low weight chitosan used was commercially presented as a thin light-yellow powder and showed high surface porosity with a disorganized three-dimensional network architecture as previously reported [35]. Triclosan is also commercially presented as a powder, but white and crystalline. SEM analysis indicated oriented sticks of triclosan mainly agglomerated. In the triclosan-loaded chitosan analysis, it was observed aggregates of triclosan distributed on chitosan surface, as previously reported for chitosan loaded with silver nanoparticles [35]. Chitosan is an abundant material in nature and it has unique properties, such as non-toxicity, high porosity and low-cost, leading to its use as a green adsorbent for dyestuffs and heavy metals uptake from contaminated water [26]. The porosity and irregular surface of chitosan explain the adsorption of triclosan as observed in SEM analyses.

Chitosan is an heteropolysaccharide derived from the deacetylation of chitin, consisting mainly of several *N*-acetyl-D-glucosamine and D-glucosamine units, one NH_2 group and two hydroxyl (OH) groups per glycosidic unit [25]. Micro-Raman spectroscopy indicated peaks related to its structure, as C—O—C bond from glyosidic bonds, C—OH bond, CH_3 and CH_2. Triclosan is a trichlorinated diphenyl ether molecule with one OH group and Micro-Raman indicated the strong peak related to C—C stretching vibration of its benzene rings [32]. As expected, micro-Raman analysis did not indicate chemical bond for chitosan and triclosan when both were mixed. Thus, all triclosan structures were available for interaction with bacteria in broth, may positive affecting the antibacterial activity for triclosan-loaded chitosan groups.

The experimental adhesive resins were evaluated for the DC, determined as the conversion of unsaturated carbon-carbon double bonds in saturated bonds of monomers [36]. Polymers with high DC generally show reliable mechanical properties and are less susceptible to degradation [37]. Also, lower residual monomers in the organic matrix are expected due to high DC, leading to less monomers leaching and cytotoxic effects [38]. The addition of chitosan or triclosan-loaded chitosan in the organic matrix could affect the DC by altering the chain mobility [39] or decreasing the light transmission through the adhesive resin [40]. Despite the lower degree of functionality of chitosan and triclosan-loaded chitosan groups due to lower methacrylate compounds compared to G_{CTRL}, there was no difference for DC among all experimental adhesive resins. The addition of 2 or 5 wt.% of chitosan or triclosan-loaded chitosan showed acceptable DC, as the values were not different for G_{CTRL}, a resin with similar composition to the adhesive of 3-step etch-and-rinse adhesive system or the so-called gold standard [41].

Despite the DC showed high values irrespective of adhesive resin formulation, the microhardness assessment and the softening in solvent evaluation assist the comprehension about the polymer network [30,42]. The incorporation of triclosan-loaded chitosan at 2 wt.% increased the KHN1 compared to G_{CTRL}. Triclosan showed entanglement with a comonomer blend (BisGMA and triethyleneglycol dimethacrylate (TEGDMA)) by secondary hydrogen bonding and aromatic–aromatic interactions (π-π stacking) by non-covalent forces, increasing polymer strength and toughness [43]. Some triclosan dispersed in the resin matrix could lead to the slight but significant higher KHN1 observed for $G_{QT2\%}$ compared to G_{CTRL}. On the other hand, the group with the highest triclosan-loaded chitosan addition ($G_{QT5\%}$) did not differ for G_{CTRL}. The higher amount of chitosan in $G_{QT5\%}$ may explain this result, since chitosan presents weak mechanical structure and it does not confer higher resistance to the organic matrix [35]. Despite the difference observed for $G_{QT2\%}$ in KHN1, all groups softened after immersion in solvent and presented lower KHN2 than KHN1. It suggests that the intermolecular forces among adhesive resins chains were prone to solvent uptake, increasing intermolecular distances and leading to solvent diffusion in the polymer network [42] for all formulated materials.

Table 3
Mean and standard deviation values of direct contact inhibition assay in colony forming units per milliliter (CFU/mL) and planktonic bacteria inhibition assay in CFU/mL with log transformation.

Group	Direct contact inhibition assay		Plancktonic bacteria inhibition assay	
	Immediate	6 months	Immediate	6 months
G_{CTRL}	4.90 (± 0.11)^{Ba}	4.87 (± 0.12)^{Ba}	8.21 (± 0.10)^{Aa}	8.22 (± 0.09)^{Aa}
$G_{Q2\%}$	5.07 (± 0.08)^{Aa}	5.15 (± 0.09)^{Aa}	8.29 (± 0.12)^{Aa}	8.28 (± 0.12)^{Aa}
$G_{Q5\%}$	5.11 (± 0.09)^{Aa}	5.14 (± 0.09)^{Aa}	8.30 (± 0.08)^{Aa}	8.30 (± 0.08)^{Aa}
$G_{QT2\%}$	3.71 (± 0.10)^{Ca}	3.77 (± 0.13)^{Ca}	8.22 (± 0.14)^{Aa}	8.22 (± 0.13)^{Aa}
$G_{QT5\%}$	3.03 (± 0.19)^{Da}	2.99 (± 0.11)^{Da}	8.23 (± 0.10)^{Aa}	8.24 (± 0.15)^{Aa}
Negative control	–	–	8.23 (± 0.13)^{Aa}	8.23 (± 0.13)^{Aa}

Different capital letter indicates statistical difference in the same column ($p < 0.05$). Different small letter indicates statistical difference in the same row ($p < 0.05$) for the same test.

In the softening in solvent analysis, G_{CTRL} presented lower ΔKHN% than chitosan and triclosan-loaded chitosan groups. Chitosan is a rigid and semi-crystalline polymer not soluble in ethanol nor in water [44]. The attraction of polymer chains is more overtaken by the attraction between polymer and solvent in materials with lower crosslinks, leading to higher ΔKHN% [42,45]. Thus, it is possible that the higher values found for chitosan groups were due to differences in the cross-linking density, with more linear polymers formulated with chitosan incorporation [30,37,42,46]. Despite the higher KHN1 found for $G_{QT2\%}$, all groups presented higher ΔKHN% than G_{CTRL}. Previous studies also demonstrated that higher triclosan incorporation increased the softening by ethanol [23,47]. While triclosan is weakly soluble in water (0.001 g of triclosan / 100 g of water) [48] and physiological saline solution (0.0005 g of triclosan / 100 mL of saline solution) due to its non-polar property [32], it presents high solubility in organic solvents as ethanol (2 g / 100 mL of ethanol:water (50:50) solution) [32]. Therefore, there was higher ethanol uptake for triclosan-loaded chitosan groups in softening in solvent-test compared to G_{CTRL}. However, the storage of the experimental materials in different media, as water or physiological saline solution, may modify the outcome.

To access the effectiveness of adhesive system at hybrid layers, long-term μ-TBS tests are indicated due to association with *in vivo* data [49]. The addition of chitosan or triclosan-loaded chitosan at any concentration did not influence the bond-strength of dentin/adhesive interface immediately. After 6 months of water storage, $G_{QT2\%}$, $G_{QT5\%}$ and $G_{Q5\%}$ preserved dentin/adhesive against degradation, while G_{CTRL} and $G_{Q2\%}$ showed lower μ-TBS between immediate and long-term assessments. Chitosan is not soluble in water [44], providing better results between immediate and longitudinal test for $G_{Q5\%}$ than $G_{Q2\%}$. Triclosan is a highly hydrophobic structure, which turns it resistant against water degradation [32,48], leading to interface stability for triclosan-loaded chitosan groups compared to G_{CTRL}. There was no statistically significant difference among groups in the 6 months analysis. However, the results found for G_{CTRL} and $G_{Q2\%}$ could be jeopardized with higher storage time. Despite the higher softening in solvent for all chitosan and triclosan-loaded chitosan groups, the higher amount of pure chitosan ($G_{Q5\%}$) and any concentration of triclosan-loaded chitosan ($G_{QT2\%}$ and $G_{QT5\%}$) addition induced interface stability over time.

The antibacterial activity was evaluated against *Streptococcus mutans* by a direct contact inhibition assay against biofilm formation on adhesive resins surfaces and against planktonic bacteria in broth. Chitosan groups indicated higher biofilm formation than other groups and the addition of triclosan-loaded chitosan decreased the biofilm formation compared to G_{CTRL}. Also, $G_{QT5\%}$ showed higher antibacterial activity against biofilm formation than $G_{QT2\%}$. The antimicrobial activity of chitosan depends on its type, molecular weight, degree of deacetylation, viscosity and concentration [24]. Further, extrinsic factors such as the temperature and the pH of medium highly influence the results [24]. Although chitosan is normally insoluble in water, alkaline media and most organic solvents, an acid environment at pH ≤ 6.00 leads to it higher solubility [50]. Also, in acid medium, NH_2 groups become protonated and form cationic amine groups (NH_3^+) [24,50], increasing the electrostatic attraction for chitosan and negative charges of prokaryotic cells walls and membranes. Previous study dissolved chitosan in acetic acid to be added in a simplified commercial etch-and-rinse adhesive system and it demonstrated antibacterial activity [51]. In the present study, the powder of chitosan was used without further treatments, not changing the NH_2 groups, leading to no antibacterial effect. $G_{Q2\%}$ and $G_{Q5\%}$ presented higher biofilm formation probably due to the high porosity of chitosan and increased surface roughness of polymerized adhesive resins [52]. Antibacterial tests with biofilm maturation for longer periods could decrease the pH environment and increase the charged groups on chitosan, leading to lower biofilm formation for chitosan groups. On the other hand, triclosan addition decreased biofilm formation at any tested concentration, corroborating to previous studies [11,19,22,23,48,53].

Triclosan is a broad-spectrum antibacterial agent with affinity for prokaryotic membrane [54] due to its non-polarity and high solubility in oleic acids (40 g of triclosan / 100 g of oleic acid), which composes the phospholipid cell membranes [43]. In this way, with higher triclosan concentration, higher the membrane disruption and the leakage of cytoplasmic constituents [55]. Triclosan previously demonstrated ability to disorganize biofilm of *Streptococcus mutans*, decreasing its biovolume, thickness and roughness coefficient [53]. Besides that, triclosan modifies the expression of *Streptococcus mutans* genes related to bacteria virulence after only 4 h of contact of bacteria in broth and a dental resin with triclosan methacrylate monomer [53]. The genes *vicR* and *gtfD*, which are important, respectively, for vital functions [56] and regulation of soluble glucan synthesis of *Streptococcus mutans* [57], have previously shown reduced expression profile in this short period [53]. As a consequence, the virulence of *Streptococcus mutans* decreases, despite the biofilm disorganization and bacterial death [53], leading to the antibacterial effect against biofilm formation as observed in the present study for triclosan-loaded chitosan groups.

In the antibacterial activity evaluation against planktonic bacteria, there was no difference among the experimental adhesive resins. Chitosan is an insoluble linear mucopolysaccharide [24] and its porosity is related to the non-release of triclosan for broth, positive affecting the antibacterial property over time. The antibacterial property against biofilm formation was maintained after six months of water storage, indicating that $G_{QT2\%}$ and $G_{QT5\%}$ are promising non-antibacterial-agent-releasing for adhesive resins. In this way, triclosan properties as resistance to hydrolysis and stability in acids associated with the possibility of antibacterial activity of chitosan in acid environment may lead to higher antibacterial effect of triclosan-loaded chitosan groups for patients with high caries risk. The storage of the experimental adhesive resins in different pH media may be further assessed.

To the best of our knowledge, this is the study with the longest time of antibacterial activity evaluation of an adhesive resin against biofilm formation and planktonic bacteria, with major investigations being performed only by disk diffusion tests [58,59]. The adhesive resins with chitosan did not provide antibacterial activity and $G_{Q2\%}$ decreased the μ-TBS after six months. However, triclosan-loaded chitosan groups showed antibacterial activity immediately and over time and induced dentin/adhesive interface stability, may positive affecting long-lasting marginal sealing.

5. Conclusion

Triclosan-loaded chitosan at 5 wt.% addition in an experimental adhesive resin showed reliable properties, with the highest antibacterial activity immediately and after six months, and induced dentin/adhesive interface stability over time.

Declaration of interest

The authors declared no conflict of interest.

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