Chemical, Mechanical and Biological Properties of an Adhesive Resin with Alkyl Trimethyl Ammonium Bromide-loaded Halloysite Nanotubes

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Purpose: The aim of this study was to evaluate the chemomechanical properties, antibacterial activity, and cytotoxicity of an experimental adhesive resin containing halloysite nanotubes (HNT), doped with alkyl trimethyl ammonium bromide (ATAB).

Materials and Methods: A filler of HNT doped with ATAB was obtained (ATAB:HNT) and incorporated (5 wt%) into a resin blend made of bisphenol A glycerolate dimethacrylate, 2-hydroxyethyl methacrylate and a photoinitiator/co-initiator system (GATAB:HNT). The same resin blend without ATAB:HNT was used as control (Ctrl). The ATAB:HNT filler was assessed by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The two tested adhesives were evaluated for degree of conversion (DC) in vitro and in situ, softening in alcohol, dentin microtensile bond strength (μTBS), antibacterial activity, and cytotoxicity (n = 5).

Results: SEM showed that the nanotubes had a characteristic tubular-needle morphology, while the TEM analysis confirmed the presence of ATAB inside the lumens of HNT. The incorporation of ATAB:HNT induced no reduction ($p > 0.05$) of the DC either in situ or in vitro. No difference was encountered after the softening challenge test ($p > 0.05$) and no difference was found in μTBS between the two adhesives, both at 24 h ($p > 0.05$) and after 6 months of storage in distilled water ($p > 0.05$). However, ATAB:HNT reduced *Streptococcus mutans* viability ($p < 0.05$) without a cytotoxic effect on pulp cells ($p > 0.05$).

Conclusions: GATAB:HNT adhesive demonstrated appropriate polymerization without significant differences in softening after solvent immersion, while concomitantly maintaining reliable bond strength after 6 months of water aging. Moreover, the ATAB:HNT filler can provide antibacterial activity to the adhesive resin without affecting pulp cell viability.

Keywords: dentin bonding agents, anti-bacterial agents, cytotoxicity, drug delivery system.

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Dentin hybridization is the process of micromechanical interlocking achieved via a complex combination of biological and chemical reactions such as etching of the dental substrate, infiltration of resin monomers, and polymerization reactions.[35] Such processes create a resin-infiltrated layer known as the hybrid layer, thus permitting minimally invasive restorative procedures. In comparison, amalgam restorations require specific cavity preparations in order to ensure adequate retention. However, due to the heterogeneity of dentin (with approx. 50 vol% mineral phase, 30 vol% organic phase, and 20 vol% water), as well as the greater amount of extrinsic water (eg, intratubular fluid),[35] it is not easy for the adhesive-dentin interface created with several simplified adhesives to maintain reliable long-lasting performance.[31] This remains the "Achilles' heel" of composite restorations.[19] Such failure at the composite-dentin interface may be responsible for bacterial penetration and recurrent caries, increasing the risk for replacement need of the composite restorations.[39] Furthermore, the restoration's interface can be prone to failure due to biofilm accumulation, along with bacterial adhesion on the restorative materials and bacterial acid production in situ.[16,48] All these factors, in addition to a loss of physicochemical stability of the resin-based materials over time, increase the risk for bacterial colonization and early need for restoration replacement.[37]

To overcome this issue, restorative materials with improved antibacterial activity have been proposed. Quaternary ammonium compounds (QACs) have been extensively studied and have shown antimicrobial activity in vitro[3,20,21,30,53] and in situ.[13,40,41] The QAC alkyl trimethyl ammonium bromide (ATAB, CnH38BrN) is largely used as a disinfectant known as cetrimide and it presents long alkyl chain, usually with a carbon chain of 10, 12, 14, or 16 elements.[51] This QAC has been studied mainly as an anionic surfactant[24,51] and antimicrobial agent.[17,50] ATAB with fourteen carbons presented broad-antimicrobial activity, and it showed high antibacterial efficacy against *Enterococcus faecalis* when used as an endodontic irrigant,[30] as well as when applied in a commercial resin sealer (AH Plus, Dentsply Sirona; Konstanz, Germany).[2] Recently, ATAB was also incorporated into an experimental resin sealer to provide activity against *E. faecalis*. In this context, halloysite nanotubes (HNT, $Al_2Si_2O_5(OH)_4 2H_2O$) were used as carriers for ATAB.[34]

The use of nanotubes as nanofillers has drawn much attention in modern research due to their low density and ability to carry and release drugs. HNT are natural nanoclays that have been used in powder form or ceramic raw materials, as catalysts, drug carriers,[33] and filler for polymers.[5] In the polymer industry, HNT have demonstrated interesting characteristics, not only in improving mechanical properties, but also in assisting the polymerization reaction of monomers (eg, hydroxyethyl methacrylate) via their electrophilic behavior and electron exchange.[46] Recently, HNT were modified by ATAB in the attempt to increase the hydrophobicity of this nanoclay, creating a new inorganic micelle for industrial and biological uses.[7] Among biological applications, HNT were highlighted for drug loading, mainly of cationic agents. These agents could electrostatically interact with HNT's polyanionic surfaces and could be entrapped in HNT's lumen and interlamellar spaces.[27] Therefore, the use of HNT for sustained delivery of drugs has been encouraged.[28] In dentistry, these nanoclays were initially used to carry antimicrobial agents such as triclosan[10,14,15] and doxycycline.[18] Subsequently, they were doped with ATAB and incorporated into an experimental resin sealer, showing antibacterial activity and maintaining high pulp cell viability without changing its chemical and mechanical properties.[34]

The aim of this study was to evaluate the chemical and mechanical properties, antibacterial activity, and cytotoxicity of an experimental adhesive resin containing halloysite nanotubes (HNT) doped with alkyl trimethyl ammonium bromide (ATAB). The first hypothesis of this study was that the incorporation of ATAB:HNT would not affect the chemical and mechanical properties of the experimental adhesive resin. The second hypothesis was that the incorporation of ATAB:HNT would improve the antibacterial properties without affecting the biocompatibility of the experimental adhesive resin prepared in this study.

MATERIALS AND METHODS

Monomers, photoinitiators, HNT, and ATAB were all purchased from Sigma Aldrich (St Louis, MO, USA) and used without any further purification. An LED light-curing unit (Radii Cal, SDI; Bayswater, Australia) with a light intensity of 1200 mW/cm^2 was used in all tests in this study. A schematic representation of the protocol design employed in this study is depicted in Fig 1.

Filler Formulation and Morphologic Characterization

The experimental filler was created by mixing HNT and ATAB at a 1:1 ratio in an absolute ethanol solution. This solution was kept under constant magnetic stirring to allow complete solvent evaporation, and the resultant powder was stored at 25°C for no longer than three days in a vacuum-pump desiccator.[34] Both fillers (ATAB-loaded HNT; no-ATAB-HNT [control]) were analyzed employing both SEM and TEM. For SEM (EVO, Zeiss; Oberkochen, Germany), the samples were gold-sputter coated (15-25 nm) (SCD 050, Baltec; Vaduz, Liechtenstein). An acceleration voltage of 15 kV was used (magnification up to 30,000X). For TEM analysis, the filler was analyzed (JEM 1200 EXII, JEOL; Tokyo, Japan) using a dispersion of the powder (5%) in isopropyl alcohol sonicated for 15 min. A 10-µl drop was dispensed on a carbon-coated copper grid (Electron Microscopy Sciences; Hatfield, PA, USA) which was then placed in a desiccator for 24 h. The analysis was performed at an acceleration voltage of 80 kV (magnification 100,000X and 500,000X).

Formulation of the Experimental Adhesive Resins

A resin co-monomer blend was prepared using 66.7 wt% bisphenol A glycerolate dimethacrylate (bis-GMA) and 33.3 wt% 2-hydroxyethyl methacrylate (HEMA) and used as a control adhesive (Ctrl).[22,43] Moreover, a photoiniciator/

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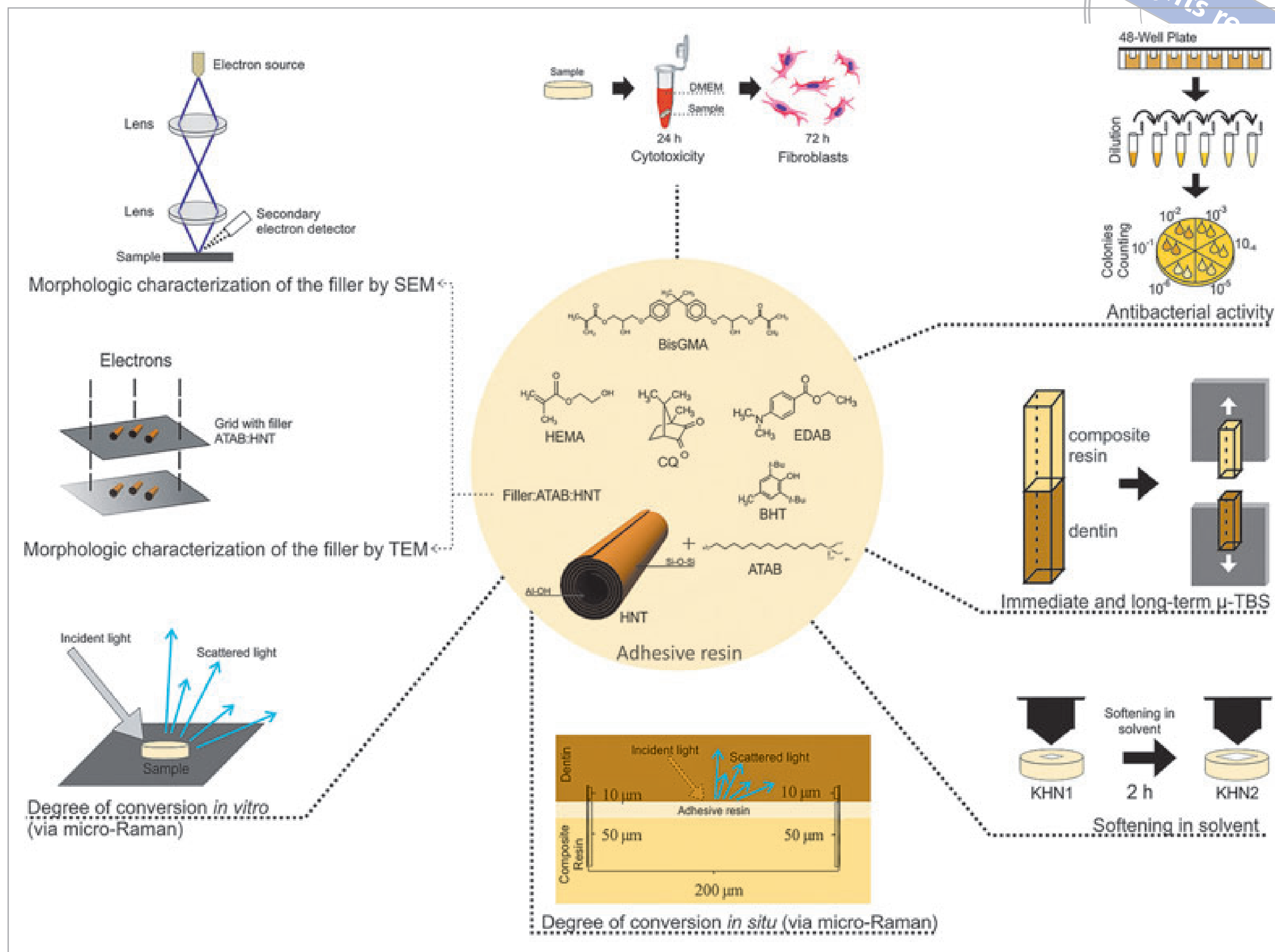

Fig 1 Schematic of materials and methods used in this study with the chemical structures of bis-GMA, HEMA and ATAB used to formulate the experimental adhesive resins.

co-initiator system of camphorquinone (1 mol%) and ethyl 4-dimethylaminobenzoate (1 mol%) was added to the blend. Butylated hydroxytoluene was also incorporated (0.01 wt%) as stabilizer for shelf life. The experimental filler previously prepared (ATAB:HNT) was incorporated at 5 wt% within the resin co-monomer blend to generate the experimental adhesive resin (GATAB:HNT). It was stirred for 5 min using a magnetic agitator and finally sonicated for 180 s.[22]

Degree of Conversion In Vitro and In Situ

The degree of conversion (DC) was assessed through micro-Raman spectroscopy (Senterra, Bruker Optics; Ettlingen, Germany). The adhesives tested in this study were placed uncured (n = 5) in a polyvinylsiloxane matrix (diameter: 4 mm; thickness: 1 mm) and analyzed before and after photoactivation (20 s). A 785-nm-wavelength laser was employed, with 3 s and 5 co-additions, in the range 1800-440 cm^{-1}. The results were elaborated using Opus 7.5 software (Opus 7.5, Bruker Optics). The DC was calculated based on the intensity of the C=C stretching vibrations at 1640 cm^{-1} (aliphatic chain) and the symmetric ring stretching at 1610 cm^{-1} (aromatic chain) using the following equation:

$$DC(\%) = 100 \times \left(\frac{\text{peak height of cured aliphatic C = C)/(peak height of cured aromatic C = C}}{\text{peak height of uncured aliphatic C = C)/(peak height of uncured aromatic C = C}} \right)$$

In addition, for the in situ evaluation of the DC at the adhesive-dentin interface, 10 freshly extracted bovine teeth were used. The superficial dentin of the buccal surface of these teeth (n = 5/group) was ground flat (600-grit SiC paper) under continuous irrigation for 30 s, in order to create a standard smear layer. Dentin was etched using 37% phosphoric acid for 15 s and rinsed with distilled water for 30 s.

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The surface was gently dried with filter paper, a commercial primer (Scotchbond Multi-Purpose Primer, 3M Oral Care; St Paul, MN, USA) was gently rubbed on dentin for 20 s, after which the solvent was evaporated for 20 s. The adhesives were applied on dentin and light cured for 20 s. Two 2-mm layers of composite resin (Filtek Z350 XT, 3M Oral Care) were placed onto dentin and each layer was light cured for 20 s. The specimens were stored in distilled water at 37°C for 24 h and sectioned into sticks with 0.5 mm^2 cross-sectional areas. The 5 teeth in each group were analyzed using three sticks per tooth with two lines per stick (60 points per line with 1 µm distance between each point; a total of 10 points on dentin and 50 points on adhesive). On each stick, the lines were separated from each other by 200 µm (Fig 1). Micro-Raman spectroscopy was performed as previously described in the DC assessment in vitro, as was the DC calculation.

Softening in Solvent

Five specimens (n = 5) 1 mm thick and 4 mm in diameter were prepared for each adhesive tested and light cured for 20 s on both sides. These were then kept moist at 37°C for 24 h. The specimens were embedded in self-cure acrylic resin and polished using an ascending series of SiC abrasive papers (600-, 1200-, and 2000-grit) under continuous water irrigation. The specimens were rinsed with distilled water and then immersed in an ultrasonic water bath for 2 min. These were stored in the dark for 24 h, and then submitted to Knoop hardness testing to attain the initial hardness number (KHN1) using a hardness testing machine (HMV2, Shimadzu; Tokyo, Japan). The embedded specimens were immersed in a solution of 70:30 ethanol:water for 2 h and then evaluated again to obtain the final Knoop hardness number (KHN2). The difference between KHN1 and KHN2 was calculated for each sample and expressed in percentage of Knoop hardness variation (ΔKHN%).

Microtensile Bond Strength (µTBS) Test

Twenty bovine (n = 20) teeth per group were prepared as described before for in situ degree of conversion. After preparation and restoration of the specimens, these were cut into 1-mm sticks. Half of the sticks obtained from each tooth were tested for immediate µTBS, while the remaining specimens were tested after immersion in distilled water for 6 months. Specimens were fixed with cyanoacrylate glue onto metallic jigs and submitted to µTBS testing (EZ-SX Series, Shimadzu; Kyoto, Japan) at a crosshead speed of 1 mm/min. The fracture pattern was evaluated using a stereomicroscope (HMV2, Shimadzu) at 10X magnification and classified as adhesive, mixed, or cohesive in dentin or composite resin.

Antibacterial Activity Evaluation

The antibacterial activity assay was performed using *Streptococcus mutans* (UA 159, ATCC 700610) and five specimens per group (n = 5). The specimens were prepared with the experimental adhesives as described above, but without the polishing steps. After 24 h at 37°C in distilled water, the specimens were attached to Teflon matrices inside the lid of a 48-well plate and then sterilized using hydrogen peroxide plasma at 58% for 48 min at 56°C.[10,11] Subsequently, the wells of a sterile 48-well plate were filled with 900 µl of brain-heart infusion broth (BHI) containing 1 wt% sucrose and 100 µl of a suspension of *S. mutans* (at 10^7 CFU/ml after 24 h culturing at 37°C in a microaerophilic environment with 5% CO_2) in BHI and 1 wt% sucrose. The lid containing the specimens was positioned on the base of the plate and kept in contact with BHI and *S. mutans* at 37°C for 24 h in a microaerophilic environment with 5% CO_2. After this period, the specimens were detached from the lid and placed in Eppendorf tubes containing 1 ml of sterile saline solution (0.9%) to be vortexed and the solution to be diluted to a 10-6 ml saline solution. Two 25-µl drops from each Eppendorf tube were plated on BHI agar in petri dishes. The plates were incubated at 37°C for 48 h in the microaerophilic environment. The number of colonies was visually counted and transformed into CFU/ml using the following equatin:

$$\left(\frac{\text{Average number of colonies x dilution factor}}{\text{Volume of culture plate}} \right)$$

Cytotoxicity Evaluation

Pulp cells were obtained from an extracted third human molar after receiving the patient's informed consent (local ethics Committee no. 1.739.340). Cells were grown in Dulbecco's modified Eagle's medium (DMEM, Thermo Scientific; Waltham, MA, USA), supplemented with 10% fetal bovine serum (Thermo Scientific, 12657FBS), 5 mM Hepes (Thermo Scientific, 15630080), 3.7 g sodium bicarbonate (Sigma-Aldrich, S5761), 100 U/ml penicillin and 100 mg/ml streptomycin (Thermo Scientific, 15240062). The flasks were kept in an incubator at 37°C and 5% CO_2. Cell growth was monitored daily in an inverted-phase microscope, and culture medium was changed every 2 or 3 days. Confluent cultures in 75-cm^2 flasks were used to evaluate the cytotoxic effect of the experimental adhesive resins using the sulforhodamine B sodium salt (SRB) method.

The specimens (n = 5) were prepared as described for the softening in solvent test, but without the polishing procedures. After 24 h at 37°C in distilled water, the specimens were sterilized with hydrogen peroxide plasma. On the first day of the assay, the specimens were placed in 1 ml of DMEM and the cells were seeded at 5x10^3 cells per well in 96-well plates. The Eppendorf tubes and the plates were kept at 37°C. Twenty-four hours later, the cells were placed in contact with the eluates (DMEM with possible leached materials from samples) by adding 100 µl of eluate per well. The eluates were tested in quintuplicate. Five wells were used as test controls since they were filled with 100 µl of DMEM without eluate. Seventy-two hours later, the cells were fixed with 50% trichloroacetic acid and washed with running water. After drying at room temperature, the fixed cells were stained with SRB (Sigma-Aldrich,

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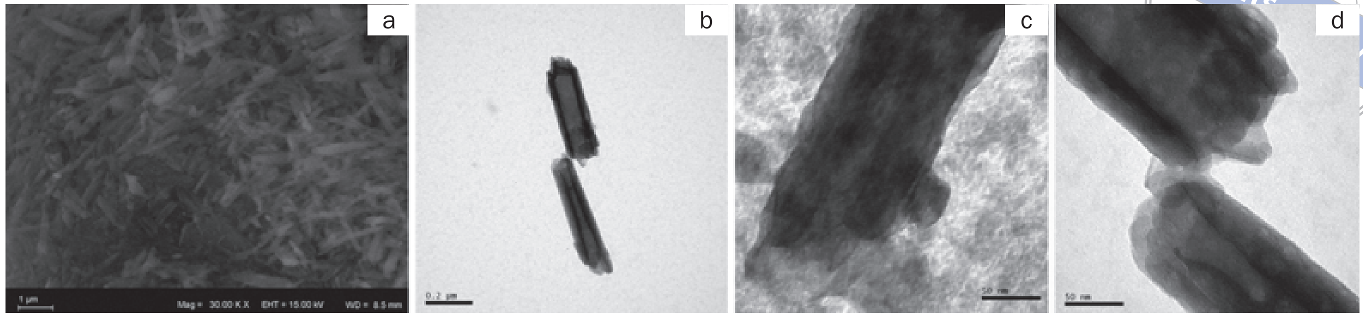

Fig 2 a. SEM images of ATAB:HNT at 30,000X (original magnification). b. TEM images of ATAB:HNT at 100,000X (original magnification). c. TEM images at 500,000X (original magnification) show no bright spots inside the unloaded-HNT. d. Bright spots inside the HNT for ATAB:HNT filler.

Table 1 Means and standard deviations of degree of conversion (DC), initial Knoop hardness number (KHN1), final Knoop hardness number (KHN2), variation of Knoop hardness (ΔKHN%), immediate and long-term microtensile bond strength (μTBS), antibacterial activity regarding biofilm formation and human cell viability

Groups	DC (%)	KHN1	KHN2	DKHN (%)	μTBS (MPa) 24-h test	μTBS (MPa) 6-month test	Biofilm formation (log CFU/ml)	Viability of cells (%)
Ctrl	80.3 (±3.3)A	21.7 (±0.6)Aa	8.1 (±0.3)b	60.5 (±2.1)A	62.1 (±6.3)Aa	59.2 (±5.5)Aa	4.3 (±0.5)A	92.1 (±2.9)A
GATAB:HNT	81.9 (±3.4)A	18.4 (±0)Aa	8.0 (±0.4)b	62.3 (±3.2)A	61.4 (±5.2)Aa	57.1 (±9.5)Aa	2.9 (±0.5)B	96.0 (±3.3)A

Different capital letters indicate statistically significant differences in the same column (p>0.05). Different lowercase letters indicate statistically significant differences in the same line for the same test (p<0.05).

3520-42-1) at 0.4% and washed with acetic acid at 1%. After drying again, 100 µl of Trizma solution at 10 mM was added and the plates were incubated for 1 h at room temperature. The absorbance of the wells' contents was analyzed through a spectrometer microplate reader at 560 nm. The results were normalized against the absorbance found for wells without eluate (negative control) and expressed in percentage of cell viability.

Statistical Analysis

SEM, TEM, and in situ DC were descriptively analyzed. A paired t-test was used to compare KHN1 and KHN2 for the same group, and immediate and aged μTBS from the same group. Ctrl and GATAB:HNT were compared in all other analyses using the t-test. Statistical significance was set at 0.05.

RESULTS

SEM showed that the HNT fillers were characterized by irregular nano-tubular morphology with different sizes and widths (Fig 2a). However, TEM showed the presence of bright globular areas, which represented the accumulation of ATAB inside the lumen of the HNT filler (Figs 2b and 2d), whereas the presence of these bright globular areas were absent in the images of undoped HNT (Fig 2c). The results of in vitro DC in vitro are shown in Table 1. Ctrl adhesive could achieve a DC of 80.3% (±3.2), while the experimental GATAB:HNT adhesive had a DC of 81.9% (±3.4) ($p > 0.05$). The descriptive analysis of in situDC (Fig 3) showed that on dentin, the DC of the tested adhesives was lower than that observed in the bulk adhesive layer for both groups. However, the addition of ATAB:HNT induced no reduction of DC compared to Ctrl. The softening in solvent test (Table 1) showed that there was no difference between the two tested groups for KHN1 ($p > 0.05$) and that both groups had decreased Knoop hardness values after aging in the solvent ($p < 0.05$), with no significant difference for ΔKHN% between the two tested materials ($p > 0.05$). Ctrl presented 62.1 (±6.3) MPa for immediate μTBS and GATAB:HNT showed 61.4 (±5.2) MPa ($p > 0.05$). Both groups showed no difference at immediate and after 6-month immersion in distilled water (aged μTBS $p > 0.05$); in the paired analysis, there was no difference between GATAB:HNT and trl groups after aging (6-month immersion in distilled water; $p > 0.05$). Mixed and adhesive failure patterns were predominant for both groups in the immediate and 6-month aging analysis (Fig 4). The antibacterial test (Table 1) showed that the addition of ATAB:HNT in the adhesive resin decreased *S. mutans* viability, with fewer CFU/ml observed for

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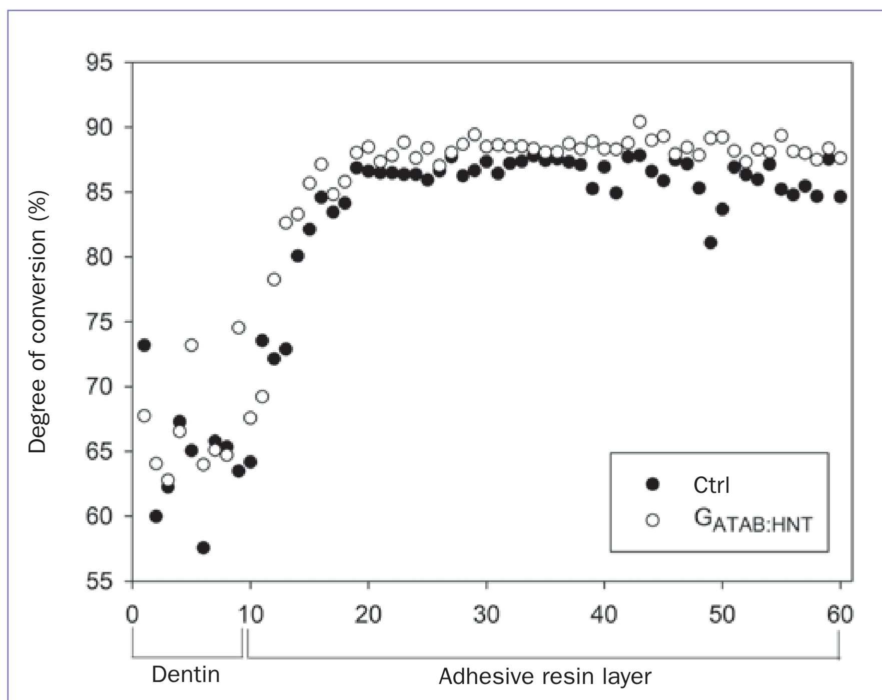

Fig 3 In situ degree of conversion of the experimental adhesive resins.

GATAB:HNT ($p < 0.05$). The cytotoxicity assay (Table 1) indicated that both groups had mean values higher than 90% of human pulp-cell viability, without a difference between GATAB:HNT and Ctrl ($p > 0.05$).

DISCUSSION

The incorporation of antibacterial agents in dental adhesives may improve the longevity of restorations by decreasing recurrent caries.[1] In this study, a nanoclay (HNT) filler was used to carry a quaternary ammonium compound (ATAB), which was then incorporated into an experimental adhesive resin. The results of this study showed that the presence of ATAB:HNT decreased the *S. mutans* biofilm on the adhesive surface without affecting the biocompatibility and the mechanical and chemical properties of the experimental adhesive. Therefore, the two hypotheses postulated in this study must be accepted.

It was interesting to observe a high DC in both tested materials. Such a high conversion of C=C into C-C bonds may be related to consistent mechanical and chemical properties of the polymer contained in the resin system.[9] Indeed, for adhesive resins, proper polymerization is desired in order to reduce water-induced degradation and increase the bon stability over time.[12,31] In this study, micro-Raman spectroscopy was used to determine the DC of the experimental adhesive resins both in vitro and in situ. In vitro, the tested materials showed high values recorded by micro-Raman spectroscopy (> 80%). The incorporation of 5 wt% of ATAB:HNT had no negative effect on the DC of the experimental adhesive resin, corroborating the results shown in a previous study, where 10 wt% of ATAB:HNT was incorporated in an experimental resin sealer.[34] Preservation of the DC may have been favored by the fact that HNT can influence the polymerization kinetics of methacrylates by increasing their polymerization rate.[4] HNTs present aluminol groups at the inner surface, which are composed of aluminum atoms with an electron-deficient orbit that is able to interact with carbonyl groups in methacrylates.[11] A previous study showed that with 5 wt% of HNT loaded with triclosan, the polymerization reaction started sooner, and a higher DC was achieved with up to 20 wt% of this filler compared to an unfilled resin.[15]

Furthermore, the in situ evaluation of the DC confirmed adequate polymerization of the adhesive resins. Micro-Raman spectroscopy allows analysis of the adhesive-dentin specimens by directly mapping the bonding interface. The DC within the adhesive layer for both adhesive resins was higher than within the hybrid layer. Such a difference might be due to intrinsic factors of dentin, such as the water content of collagen and dentin tubules, and to the hydrophilic composition of the primer, which prevented a proper polymerization reaction.[52] In the descriptive analysis of in situDC, despite the great variability of data mainly for dentin, it was possible to observe that the addition of ATAB:HNT

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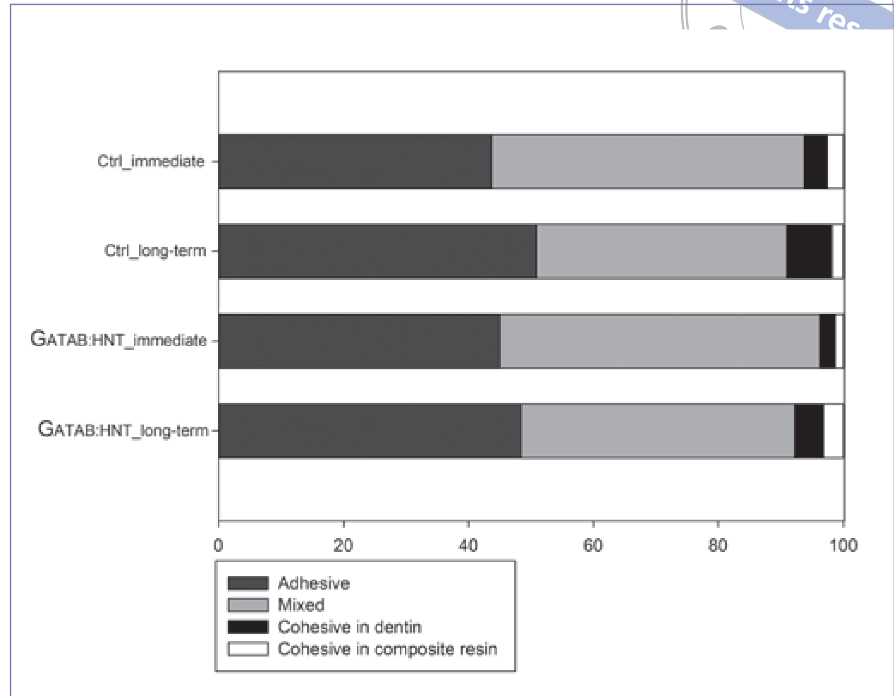

Fig 4 Failure pattern analysis after μTBS testing performed after 24-h or 6-month aging in distilled water.

did not jeopardize the DC at any level of the adhesive-dentin interface. This may be a promising result, since poorly cured adhesives are more prone to hydrolysis over time due to their higher permeability and sorption, leading to collagen cleavage and polymer degradation.[12,31] In addition, low DC is often associated with the leaching of monomers and a higher cytotoxic effect on pulp cells.[23]

Despite the fact that reliable DC was achieved with the tested resins, it is not improbable to obtain polymers with good DC and poor crosslinking density. A recent study has demonstrated that the addition of an organic salt similar to a QAC without co-polymerizable groups could increase the DC and decrease the KHN1. Moreover, there was an increase of ΔKHN, along with alteration of the thermogravimetric profile of the resin.[32] This situation occurs due to an increase in mobility of monomers via plasticization, thus augmenting the DC, and decreasing crosslinking density.[42] Therefore, the experimental adhesive resins used in this study were also evaluated through the softening in solvent test after 2-h immersion in an alcoholic solution. We demonstrated that there was no difference in KHN1 for either group; however, both materials exhibited a decrease in hardness after the solvent challenge. There is a strong possibility that the alcoholic solution was initially adsorbed from the surface of the polymer, causing polymer chain relaxation and/or degradation, thereby reducing the KHN2 compared to KHN1.[45] As ATAB is a non-copolymerized QAC, it could jeopardize the crosslinking density. However, there was no difference for ΔKHN between Ctrl and GATAB:HNT, confirming the results observed in another study in which 10 wt% of ATAB:HNT (with double the amount of ATAB compared to HNT in the filler composition) was used. In this latter case, the ΔKHN was not significantly affected.[34] In the present research, the addition of 5 wt% ATAB:HNT had no effect on the softening in solvent test.

The experimental adhesive resins created in this study were tested for μTBS to evaluate their adhesion performance on dentin. The results showed no difference between groups for immediate μTBS. The evaluation of μTBS after aging (6-month immersion in water) was also essential to better understand the adhesives' performance and correlate them to in vivo outcomes.[49] After prolonged aging, it is possible to achieve polymer hydrolytic degradation and plasticization followed by hydrolytic degradation of the collagen fibrils.[6] Nevertheless, GATAB:HNT and trl groups showed no change after 6-month aging. With respect to failure pattern analysis, mixed and adhesive failures were extensive for Ctrl and GATAB:HNT for immediate and aging analyses. trl has a chemical composition as a conventional 3-step etch-and-rinse adhesive,[38] suggesting that GATAB:HNT presented similar behavior to this gold-standard adhesive.[49]

The QAC selected for this study does not present carbonyl groups which could chemically interact with aluminol groups on the inner surface of HNT. In this way, ATAB was probably carried in HNT lumens and adsorbed on HNT outer surfaces

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instead of chemically bonding to this nanoclay. There were distinguishable bright spots of ATAB inside the lumens of HNT (Fig 2), similar to those presented in a recent study.[34] QACs have the ability to disrupt a bacterial membrane by disturbing its electrical balance via contact between the positive species from QACs and negative species of the bacteria's wall and membrane.[30] Besides the increase of osmotic pressure of the cells, QACs are able to penetrate into the cell, causing bacterial death.[8] Despite being effective against *E. faecalis*,[34] ATAB showed antibacterial activity against *S. mutans* biofilm on dentin at 0.2% in solution.[44] In this study, the biofilm formation of *S. mutans* on the polymer surface was evaluated when in contact with trl and GATAB:HNT. Ctrl presented higher CFU/ml compared to GATAB:HNT. This result indicates that ATAB inside the nanotubes could diffuse through the resin onto the surface and decrease the viability of the biofilm. Thus, this filler might help stabilize the adhesive-dentin interface due to its antibacterial properties, since *S. mutans* can degrade resins in the intraoral environment.[26] The protection mechanism of ATAB:HNT on the interface should be further investigated since QACs may also decrease host-derived metalloproteinase activity.[47] It is not clear how long such an effect may last. However, drugs encapsulated by HNT can be released 30 to 100 times more slowly compared to situations where antibacterial agents are dispersed in a monomeric blend.[29]

To evaluate possible cytotoxic effects on human cells, SRB staining of proteins of viable cells was performed. The cell viability of each group (Ctrl and GATAB:HNT) was compared with the viability of cells without contact with eluates from samples (negative control). Both groups had values over 90%, which is above the cut-off of 70% stipulated by ISO 10993-5 25. When ATAB:HNT was added to an experimental resin sealer, a high percentage of pulp cell viability was also observed.[34] Despite the positive results, this study has some limitations. Future studies by our group will focus on the evaluation of the bioactivity of GATAB:HNT and remineralization process on caries-affected dentin. However, it is already well known that HNT is a nanoclay and contains high amounts of silicon, which was reported as the main factor inducing mineral deposition on resins once immersed in simulated body fluid.[14,15] Moreover, in future work, the present authors will also perform an assessment of the ability of the materials used here to inhibit proteases such as MMP2 and MMP-9, as well as analyze long-term antibacterial activity and the drug-release profile in situ and in vivo. Based on the results obtained in this study, the set of reliable properties achieved by GATAB:HNT supports the use of ATAB:HNT as a novel filler for adhesive resins.

CONCLUSIONS

The GATAB:HNT adhesive successfully maintained reliable and stable bond strength after 6-month aging in distilled water. Moreover, the ATAB:HNT filler can provide antibacterial activity for the adhesive without affecting the viability of pulp cells.

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Clinical relevance: The mechanical and chemical properties exhibited by the adhesive containing ATAB:HNT (GATAB:HNT), in addition to its antibacterial effect and lack of cytotoxicity, may make it a promising material for long-lasting restorations.