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NANOTUBOS DE NITRETO DE BORO E BROMETO DE ALQUIL TRIMETIL AMÔNIO COMO CARGA EM UM ADESIVO ORTODÔNTICO EXPERIMENTAL

Carolina Jung Ferreira

Orientador: Prof. Dr. Fabrício Mezzomo Collares

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CAROLINA JUNG FERREIRA

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Orientador: Prof. Dr. Fabrício Mezzomo Collares

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Resumo

O objetivo do presente estudo foi formular um adesivo ortodôntico experimental com a incorporação de nanotubos de nitreto de boro (BNNTs) e brometo de alquil trimetil amônio (ATAB) como carga, e caracterizar conforme suas propriedades. Foi formulada uma resina base, composta por 75% de Bis-GMA e 25% TEGDMA, 1% mol de CQ, EDAB, DPIH, 0,01% de BHT em peso, além de 5% de sílica coloidal. Foi adicionado à resina base 0,1% BNNT (G_{BNNT}); 0,1% ATAB (G_{ATAB}) e 0,2% BNNT/ATAB ($G_{BNNT/ATAB}$), e um grupo permaneceu sem adição de carga o qual foi usado como grupo controle (G_{Ctrl}). Os grupos foram avaliados através dos testes de resistência de união ao cisalhamento (n=15), ação antimicrobiana (n=3), grau de conversão (n=3), ângulo de contato e energia livre de superfície (n=5), amolecimento em solvente (n=5), deposição mineral (n=5) e citotoxicidade (n=3). Os dados foram analisados utilizando teste *t-student* pareado para amolecimento em solvente e ANOVA de uma via para os outros testes, havendo diferenças entre os grupos, foi aplicado o teste de comparações múltiplas de Tukey. O nível de significância foi de 5%. Todos os grupos alcançaram valores de conversão acima de 50% e viabilidade celular superior a 90%. Os adesivos G_{BNNT} (12,74±7,32) e G_{ATAB} (13,67±8,28) exibiram redução do amolecimento no solvente em comparação com o adesivo G_{Ctrl}. O valor médio da energia livre de superfície foi diminuído ($p<0,05$) no adesivo G_{BNNT} (44,86±3,41). Uma redução significativa no crescimento bacteriano foi observada com o adesivo $G_{BNNT/ATAB}$ ($p<0,05$). Todos os grupos foram semelhantes nas avaliações de resistência ao cisalhamento ($p>0,05$). Teste de deposição mineral revelaram deposição de fosfato nos grupos G_{BNNT}, G_{ATAB} e $G_{BNNT/ATAB}$ após 14 e 28 dias. A adição de 0,2% de BNNT/ATAB a um adesivo ortodôntico experimental é capaz de inibir o crescimento bacteriano e induzir deposição mineral sem afetar as propriedades do material.

Palavras-chave: Adesivo ortodôntico, nanotubos, cárie dentária.

Abstract

The aim of the present study was to formulate an experimental orthodontic adhesive with the incorporation of boron nitride nanotubes (BNNTs) and alkyl trimethyl ammonium bromide (ATAB) as a filler, and characterize according to its properties. A base resin composed of 75% Bis-GMA and 25% TEGDMA, 1 mol% of CQ, EDAB, DPIH, 0.01% BHT by weight, and 5% of colloidal silica was formulated. 0.1% BNNT was added to the base resin (G_{BNNT}); 0.1% ATAB (G_{ATAB}) and 0.2% BNNT/ATAB ($G_{BNNT/ATAB}$), and one group remained unfilled, which was used as the control group (G_{Ctrl}). The groups were evaluated through shear bond strength tests (n = 15), antimicrobial action (n = 3), degree of conversion (n = 3), contact angle and free surface energy (n = 5), softening in solvent (n = 5), mineral deposition (n = 5) and cytotoxicity (n = 3). The data were analyzed using paired t-student test for solvent softening and one-way ANOVA for the other tests. There were differences between groups, Tukey's multiple comparisons test was applied. The level of significance was 5%. All groups achieved conversion values above 50% and cell viability greater than 90%. G_{BNNT} (12.74 ± 7.32) and G_{ATAB} (13.67 ± 8.28) adhesives exhibited reduced softening in solvent compared to G_{Ctrl} adhesive. The mean free surface energy was decreased ($p < 0.05$) in G_{BNNT} adhesive (44.86 ± 3.41). A significant reduction in bacterial growth was observed with $G_{BNNT/ATAB}$ adhesive ($p < 0.05$). All groups were similar in the shear strength ratings (p>0,05). Mineral deposition test revealed phosphate deposition in the G_{BNNT}, G_{ATAB} and $G_{BNNT/ATAB}$ groups after 14 and 28 days. The addition of 0.2% of BNNT/ATAB to an experimental orthodontic adhesive is able to inhibit bacterial growth and induce mineral deposition without affecting the properties of the material.

Key words: Orthodontic adhesive, nanotubes, dental caries.

Sumário

1. Introdução

O uso de aparelho ortodôntico fixo atua como um fator retentivo de biofilme dificultando a higiene oral dos pacientes (1). A falta de uma adequada higiene oral gera alteração da flora microbiana oral (2), e consequente diminuição do pH salivar (1,2), desse modo, torna os pacientes suscetíveis ao acúmulo de biofilme ao redor dos *brackets* (3). O início da desmineralização ocorre quando há a adesão microbiana aos dentes e/ou aparelhos ortodônticos. Uma vez ocorrida a adesão, a proliferação bacteriana pode levar ao desenvolvimento de placa patogênica, que é a principal causa da desmineralização do esmalte (4), e formação de lesões de mancha branca (3).

Desse modo, as lesões de cáries progridem devido a um desequilíbrio entre fatores patogênicos: presença de bactérias cariogênicas, uma dieta rica em carboidratos, redução do fluxo salivar; e fatores protetores da doença cárie: o fluxo salivar, a escovação dentária, e o uso de fontes de flúor (5). Devido a esse desequilíbrio, há atualmente uma prevalência de 68,4% de lesões de mancha branca em pacientes submetidos a tratamento ortodôntico por mais de 12 meses (1). Essas condições podem contribuir para o desenvolvimento de lesões precoces de cárie em um período relativamente curto de tempo, e considerando que o tratamento ortodôntico pode levar em média de 2 a 3 anos (6), indica a necessidade de novos métodos preventivos para diminuir a ocorrência dessas lesões.

Abordagens preventivas, tais como instruções de higiene oral, regimes de flúor e controle dietético são geralmente indicadas para evitar um aumento do risco de cárie. No entanto, estratégias que não dependem da colaboração do paciente podem ser mais eficazes no controle da desmineralização (6). Uma alternativa para prevenir as lesões de mancha branca que não depende do paciente é com o uso de agentes antimicrobianos incorporados nos adesivos ortodônticos (5,7). Com este propósito, o flúor (8), a clorexidina (9), o glutaraldeído (6), os compostos quaternários de amônio (10), a 1,3,5-

triacriloilhexahidro-1,3,5-triazina (TAT) (11), as nanopartículas de prata (4), o dióxido de titânio (TiO_2) (12) têm sido adicionados a adesivos ortodônticos com o objetivo de inibir ou reduzir o crescimento bacteriano (7,13).

O flúor e a clorexidina são os aditivos preventivos mais comumente usados (4). Como a desmineralização do esmalte pode ser inibida pelo flúor, o aumento da atenção tem sido focado no desenvolvimento de adesivos ortodônticos que liberam flúor (8), porém estudos mostram que a duração da liberação do flúor é curta (6,8). A possibilidade de recarga de íons de flúor nos adesivos ortodônticos, através de protocolos de aplicações tópicas de flúor, tem sido demostrada como uma forma de reservatório de flúor (8). Entretanto, Ahn *et al.* (2011) demonstraram que um adesivo contendo flúor não mostrou capacidade significativamente melhor para atuar como um reservatório de flúor eficaz comparado a um adesivo ortodôntico não contendo flúor (8). A clorexidina também foi adicionada a adesivos ortodônticos, devido seu efeito bactericida (9) e por ser liberada em pequenas quantidades ao longo do tempo (6). Porém a clorexidina é um antibacteriano altamente solúvel, e assim, pode afetar as propriedades mecânicas devido à alta absorção de água (6).

Com o intuito de promover atividade antimicrobiana, diminuir a adesão bacteriana no esmalte e o acúmulo de biofilme, os compostos quaternários de amônio (CQA) estão sendo utilizados em resinas adesivas (14-16). Os sais de amônio quaternário exibem a capacidade de interagir com a membranas celulares bacterianas o qual resulta na destruição da membrana e à morte celular (17). A atividade antimicrobiana dos CQAs está diretamente relacionada ao comprimento de cadeia de hidrocarbonetos, quanto maior o número de átomos de carbono maior é sua tendência de adsorção na membrana celular bacteriana (17-19), e também pode estar associada ao contra-íon o qual é composto (18,19), Chen *et al.* (2010) mostrou que CQAs compostos por ânion de brometo são antimicrobianos mais potentes do que aqueles compostos por ânion de cloreto (19).

Também podemos classificar os CQAs em monômeros copolimerizáveis e não polimerizáveis, os CQAs copolimerizáveis possuem em sua fórmula uma dupla ligação de carbono o qual possuem a vantagem de serem incorporados a matriz resinosa (20), apesar dessa diferença os CQAs não polimerizáveis também possuem sua ação antimicrobiana descrita na literatura (19).

O brometo de alquil trimetil amônio (ATAB) é um composto quaternário de amônio considerado um surfactante (Figura 1), o qual é amplamente utilizado como desinfetantes, conservantes de produtos farmacêuticos e na indústria de alimentos (17). A ação antimicrobiana do ATAB é descrita na literatura desde 1985 (21) e pequenas concentrações são o suficiente para inibir o crescimento bacteriano (17), tanto de bactérias gram-positivas e gram-negativas, bem como contra leveduras e fungos (21). Porém, até o presente momento, não há estudos na Odontologia com o uso do brometo de alquil trimetil amônio.

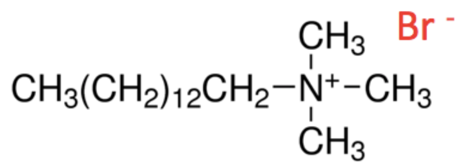

Figura 1. Estrutura molecular do brometo de alquil trimel amônio.

Duas estratégias principais para combater a cárie são possuir uma resina antimicrobiana e que promova a remineralização (22). Hoje podemos encontrar algumas marcas comerciais de adesivos ortodônticos com atividade antimicrobiana como: Transbond XT (3M/Unitek, Monróvia, CA, EUA), Fuji ORTHO LC (GC Corporation, Tóquio, Japão), Clearfil Protect Bond (CPB, Kuraray Medical Inc., Okayama, Japão) e iBond (iBond, Heraeus Kulzer GmbH). A Transbond XT e Fuji ORTHO LC contêm flúor em sua composição, já o Clearfil Protect Bond apresenta o monômero MDPB em sua composição juntamente com o flúor e o iBond contém glutaraldeído. Mas nenhum desses adesivos é capaz de induzir a deposição mineral no esmalte dentário (6). Portanto, seria ideal desenvolver resinas combinando esses dois benefícios (7). Além disso, adição de um agente antimicrobiano não deve afetar as propriedades do material (6,23).

Nanopartículas são consideradas partículas com tamanho menor que 100 nanômetros (24) e podem ser apresentadas em diversas formas tridimensionais como: bastões (25), fibras (26), cápsulas (27) e tubos (28). A redução da partícula para tamanho nanométrico diminui a rugosidade superficial dos adesivos (24), e assim, podem diminuir a fixação bacteriana nesses adesivos (4) além disso, são capazes de permear e atingir facilmente a membrana celular bacteriana (24). Com isso, nanopartículas estão sendo adicionadas como carga aos materiais a base de resina, com o intuito de conferir atividade antimicrobiana, melhorar as propriedades mecânicas e induzir a remineralização da cárie dentária de tamanho sub-micrométrico (28,29).

Os nanotubos de nitreto de boro (BNNTs) são nanopartículas tubulares formados por átomos de boro e nitrogênio (30). Os BNNTs possuem propriedades similares aos nanotubos de carbono, como baixa densidade e excelentes propriedades mecânicas (30). Isso é devido ao boro e nitrogênio serem adjacentes ao carbono na tabela periódica, e a ligação boro-nitrogênio possuir o mesmo número atômico que a ligação carbono-carbono (30).

BNNTs possuem características como: estabilidade química; hidrofobicidade; resistência à oxidação; isolamento térmico e elétrico (31-33); possuem a capacidade de ser carreadores de fármacos (32,34); e além disso, a biocompatibilidade dos BNNTs já foi mostrada anteriormente na literatura em células tais como osteoblastos (35), macrófagos (35) e fibroblastos (36), e não há relato na literatura sobre o efeito adverso do BNNT em células vivas (37).

O BNNT é considerado um material altamente hidrofóbico (32), podendo formar ângulos de contato de até 170 graus com a água (38). Em resina adesiva a incorporação de BNNT aumentou os ângulos de contato da água e do α-bromonaftaleno e, consequentemente, a energia livre de superfície diminuiu (39). Uma vez que a extensão da absorção de água dos adesivos é dependente da sua formulação (7), a alta hidrofobocidade dos BNNTs poderia diminuir a degradação de resinas adesivas ao longo do tempo, e, portanto, ser mais resistente a degradação hidrolítica (7,40). A baixa energia de superfície promovida pelos BNNTs (39) poderia diminuir a adesão bacteriana ao redor dos *brackets,* uma vez que há um aumento de deposição de bactérias cariogênicas em materiais com maior energia de superfície (41).

Os BNNTs também possuem a capacidade de induzir a formação de hidroxiapatita (39,42), o que poderia gerar uma ação remineralizante nas lesões de mancha branca. Estudos recentes avaliaram a incorporação de BNNTs a um adesivo experimental (36,39) e mostram que: o grau de conversão e a taxa de polimerização aumentaram após adicionar BNNT (36); a resistência a microtração se manteve estável após 6 meses em adesivos com adição de 0,1% de BNNT (36); o tamanho dos BNNTs variam de 5-10 micrometros (39); a microdureza e a resistência à degradação a solvente foi maior que o adesivo sem BNNT (39); e apresentou deposição mineral após 7 dias de imersão em SBF (*simulated body fluid*) (39).

Uma nova geração de adesivos com atividade antimicrobiana e com cargas bioativas são necessárias (43) para reduzir a alta prevalência de lesões de mancha branca, em pacientes com ortodôntica fixa (1), e implementar estratégias que não dependem da colaboração do paciente (6). Os adesivos ortodônticos ideais não devem apenas minimizar a adesão bacteriana (4,6,23), mas também, induzir a deposição mineral no substrato dentário (7), sem alterar a força de adesão (4,6,23).

2. Objetivo

Formular um adesivo ortodôntico experimental que possua ação antimicrobiana e remineralizante, sem afetar as propriedades mecânicas do material.

4. Artigo

Esta dissertação de mestrado se apresenta na forma de um manuscrito escrito nas normas do periódico *Clinical Oral Investigations* para o qual foi submetido.

Original paper

ANTIBACTERIAL AND REMINERALIZING FILLERS IN EXPERIMENTAL ORTHODONTIC ADHESIVES

Carolina Jung Ferreira[1.]

Vicente Castelo Branco Leitune[2]

Gabriela de Souza Balbinot[3]

Felipe Weidenbach Degrazia[4]

Salvatore Sauro[5]

Fabrício Mezzomo Collares[2*]

[1] DDS, MSc student, Dental Materials Laboratory, School of Dentistry, Federal University of Rio Grande do Sul, Rua Ramiro Barcelos, 2492/4º floor, Porto Alegre, RS, Brazil. Phone: + 55 51 3308 5198. carolinajungferreira@yahoo.com.br
[2] Adjunct Professor, Dental Materials Laboratory, School of Dentistry, Federal University of Rio Grande do Sul, Rua Ramiro Barcelos, 2492/4º floor, Porto Alegre, RS, Brazil. Phone: + 55 51 3308 5198. vicente.leitune@ufrgs.br
[3] DDS, MSc, PhD student, Dental Materials Laboratory, School of Dentistry, Federal University of Rio Grande do Sul, Rua Ramiro Barcelos, 2492/4º floor, Porto Alegre, RS, Brazil. Phone: + 55 51 3308 5198. gabi_balbinot@hotmail.com
[4] DDS, MSc, PhD, Dental Materials Laboratory, School of Dentistry, Federal University of Rio Grande do Sul, Rua Ramiro Barcelos, 2492/4º floor, Porto Alegre, RS, Brazil. Phone: + 55 51 3308 5198. fdegrazia@hotmail.com
[5] Dental Biomaterials, Preventive and Minimally Invasive Dentistry, Departamento de Odontologia - Facultad de Ciencias de la Salud, Universidad CEU-Cardenal Herrera, C/Del Pozo s/n, Alfara del Patriarca, Valencia, Spain. Tissue Engineering and Biophotonics Research Division, King's College London Dental Institute (KCLDI), Floor 17 Tower Wing, Guy's Hospital, Great Maze Pond, London SE1 9RT (UK). salvatore.sauro@uchceu.es
[2] Adjunct Professor, Dental Materials Laboratory, School of Dentistry, Federal University of Rio Grande do Sul, Rua Ramiro Barcelos, 2492/4º floor, Porto Alegre, RS, Brazil. Phone: + 55 51 3308 5198. fabricio.collares@ufrgs.br

* Corresponding author:

Fabrício Mezzomo Collares

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

Phone number: +55-51-33085198

fabricio.collares@ufrgs.br

Abstract

Objective: Develop and characterize an experimental orthodontic adhesive that incorporates boron nitride nanotubes (BNNT) and alkyl trimethyl ammonium bromide (ATAB) as a filler. **Methods:** The experimental orthodontic adhesive was formulated with methacrylate monomers and photoinitiators. The following agents were added into an adhesive: 0.1 wt% BNNT (G_{BNNT}); 0.1 wt% ATAB (G_{ATAB}); and 0.2 wt% BNNT with ATAB ($G_{BNNT/ATAB}$). In one group, the filler was not added (G_{Ctrl}). The degree of conversion, cytotoxicity, softening in solvent, contact angle and free surface energy, antibacterial activity, shear bond strength and mineral deposition were evaluated. The data were evaluated using a paired Student's t-test to compare KHN1 and KHN2 in terms of degradation in a solvent, and one-way ANOVAs were used to examine the other data. **Results:** All groups achieved degree of conversion values above 50% and cell viabilities higher than 90%. The G_{BNNT} (12.74 ±7.32) and G_{ATAB} (13.67 ±8.28) adhesives exhibited reduced softening in the solvent compared to the G_{Ctrl} adhesive. The mean free surface energy value was decreased ($p<0.05$) in the G_{BNNT} adhesive (44.86 ±3.41). A significant reduction in bacterial growth was observed with the $G_{BNNT/ATAB}$ adhesive ($p<0.05$). All groups were similar in shear bond strength evaluations. Mineral deposition tests revealed phosphate deposition in the G_{BNNT}, G_{ATAB} and $G_{BNNT/ATAB}$ groups after 14 and 28 days. **Conclusions:** The addition of 0.2% BNNT/ATAB to an experimental orthodontic adhesive is able to inhibit bacterial growth and induce mineral deposition without affecting the properties of the material. **Clinical relevance:** May be an alternative material to preventing white spot lesions.

Keywords: Adhesive; Orthodontic; Mineral deposition.

Introduction

Fixed orthodontic appliances act as biofilm retentive factors that make oral hygiene difficult for patients [1]. The lack of adequate oral hygiene results in alterations of the oral microbial flora [2] and a consequent decrease in salivary pH [1,2]. Thus, fixed orthodontic appliances make patients susceptible to the accumulation of bacterial plaque around the brackets, which leads to enamel demineralization in the form of white spot lesions [3]. Sundararaj [1] reported a prevalence of white spot lesions in patients with fixed orthodontic appliances of 68,4%, which indicates the need for new preventive methods to reduce the occurrence of these lesions [1].

Because new materials with antimicrobial activities and bioactive fillers are necessary [4], an alternative to preventing white lesions is the use of antimicrobial agents [5] to inhibit or reduce bacterial growth [6] and to induce the remineralization of enamel that is already affected [7]. Alkyl trimethyl ammonium bromide (ATAB) is a quaternary ammonium compound that is considered a surfactant and is widely used as a disinfectant and a preservative in pharmaceuticals and in the food industry [8]. The antimicrobial action of ATAB has been described in the literature since 1985 [9], and small concentrations are sufficient to inhibit bacterial growth [8], including the growth of both gram-positive and gram-negative bacteria. ATAB also effectively inhibits yeast and fungi growth [9,10]. In dentistry, quaternary ammonium compounds are used in adhesive resins to promote antimicrobial activity [4,10-12] and to decrease bacterial attachment in the enamel and biofilm accumulation [11]. However, to date, there are no dentistry studies of the use of alkyl trimethyl ammonium bromide.

The addition of an antimicrobial agent may decrease the properties and biocompatibilities of materials [10]. With the aim of improving the mechanical properties of materials and inducing mineral deposition, tubular nanoparticles are being used as fillers in resin-based materials [13]. Boron nitride nanotubes (BNNTs) are tubular nanoparticles with properties similar to those of graphene [14] that also have characteristics that include biocompatibility, chemical stability, superhydrophobicity, oxidation resistance, and thermal and electrical insulation [15-17]. BNNTs also have the ability to induce the formation of hydroxyapatite [18], which can elicit a remineralizing action in white spot lesions. Because BNNT functionalized nanoparticles may increase the mechanical resistances of materials [17], and they have the abilities to carry drugs [16] and the potential to confer antibacterial activities to materials [19], it is possible to functionalize particles of boron nitride nanotubes with alkyl trimethyl ammonium bromide for use as fillers in orthodontic adhesives.

The development of an experimental orthodontic adhesives with potential antimicrobial action could represent a possible alternative for the bonding of brackets to control the development of white spot lesions. The aim of this study is to develop and characterize an experimental orthodontic adhesive that incorporates boron nitride nanotubes and alkyl trimethyl ammonium bromide as a filler.

Materials and Methods

Experimental adhesive resin formulation

The experimental orthodontic adhesives were formulated with a mixture of 75 wt% bisphenol A glycidyl methacrylate (Bis-GMA) and 25 wt% triethylene glycol dimethacrylate (TEGDMA). To enable a photoinitiator system, 1 mol% each of camphorquinone (CQ), ethyl-4-dimethylamino benzoate (EDAB) and diphenyliodonium hexafluorophosphate (DPIH) were added, and 0.01 wt% butylated hydroxytoluene (BHT) was added as a polymerization inhibitor. All of these agents were from Sigma-Aldrich (St Louis, MO, USA). To adjust the viscosity, 5 wt% of fumed silica (AEROSIL 200; Evonik, Piscataway, NJ) [5] was added.

Boron nitride particles (BNNT, LLC, Newport News, VA, USA) and alkyl trimethyl ammonium bromide (Sigma-Aldrich, St Louis, MO, USA) were dissolved in 10 mL of absolute alcohol at a ratio of 1:1. The mixture was maintained under ultrasonic agitation for 3 hours and subsequently maintained for 7 days in desiccator at 37 °C for total evaporation of the solvent. The following agents were added to the experimental orthodontic adhesives: 0.1 wt% BNNT and 0.1 wt% alkyl trimethyl ammonium bromide (ATAB) ($G_{BNNT/ATAB}$); 0.1 wt% BNNT (G_{BNNT}); 0,1 wt% of ATAB (G_{ATAB}); and the addition of the filler was omitted in one group (G_{Ctrl}). Thus, there were a total of four experimental groups.

Degree of conversion

The degree of conversion (DC) was measured by Fourier transform infrared spectroscopy (FTIR) on a spectrometer (Vertex 70, Bruker Optics, Ettlingen, Germany) equipped with an attenuated total reflectance device (Platinum ATR-QL, Bruker Optics, Ettlingen, Germany) with a horizontal diamond crystal. The adhesives were inserted directly onto the crystal in a 6-mm diameter and 2-mm-thick polyvinylsiloxane matrix (ADSIL, VIGODENT; Rio de Janeiro, Brazil) (n=3). The samples were photoactivated for 20 seconds using LED equipment (Radii, SDI, Bayswater, Australia) with an irradiation of 1200 mW/cm^2 [6]. The absorbance spectra were obtained before and after sample polymerization. The DC was calculated as described in a previous study [7].

Cytotoxicity

For the cytotoxicity assay, human keratinocytes of the HaCaT lineage were used. The cells were cultivated in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin in 37 °C with 5% of CO_2. Eluates were prepared by immersing samples (3-mm diameter x 1-mm thickness) from each experimental group (n = 3) in 1 mL DMEM for 24 hours. The cells were later placed into 96-well plates at a concentration of $5x10^3$ and treated with 100 µl of an eluate. After 72 hours, the cells were fixed with 10% trichloroacetic acid (TCA), incubated at 4°C for one hour, washed six times with running water and dried at room temperature. Four percent sulforhodamine B (SRB, Sigma-Aldrich, St. Louis, USA) was added to color the cells, and the plate was incubated for 30 minutes at room temperature. The plates were then washed four times with 1% acetic acid to remove the excess unbound dye and dried at room temperature. Trizma solution was used to resuspend the cells, and the microplates were measured at 570 nm (Multiskan EX Microplate Reader, MTX Lab Systems, Vienna, USA) [20].

Softening in solvent

To evaluate the softening in solvent, five specimens from each group were analyzed. The specimens were made with a polyvinyl siloxane matrix with a 6.0-mm (± 0.5 mm) diameter and 1.0-mm (± 0.2 mm) thickness and photoactivated with a LED (Radii Cal., SDI Ltd., Australia) for 20 seconds on each side. Subsequently, the specimens were polished before Knoop microhardness (KHN) measurements were performed. Three indentations made with a 10-g load were created over 5 s at distances equal to 100 µm using a microdurometer (HMV 2, Shimadzu, Japan) before (KHN1) and after immersion in a solution of 50% ethanol and 50% water for 2 hours (KHN2). The percentage of variation in the Knoop hardness (ΔKNH%) was calculated for each specimen [13].

Contact angle and surface free energy

The contact angle analysis was performed by the sessile drop method. Forty discs of 6 mm in diameter and 1 mm in thickness were photoactivated for 20 s on each side with a LED (Radii Cal, SDI Ltda., Australia), embedded in acrylic resin and divided into

4 groups according to each concentration of the inorganic filler (n = 10). Subsequently, polishing was performed on a rotary electric polishing machine with a multiple polishing system to obtain a flat surface (Model 3v, Arotec, Cotia, SP, Brazil). The contact angle measurements were performed with an optical tensiometer (Theta, Attension Biolin Scientific, Stockholm, Sweden) by imaging the distributions of a polar distilled water liquid (n = 5) and non-polar alpha-bromonaphthalene liquid (n = 5) on the sample surface. Three replicates per liquid were performed for each specimen. The images were captured for 20 s, and the contact angles were measured after 10 s of contact of the droplet with the surface of the disc. The average between the right and left angles was calculated according to the point of the liquid-air-adhesive intersection. The SFE calculations were performed according to the OWRK/Fowkes method using the OneAttension program (Biolin Scientific, Stockholm, Sweden), and the results are reported in mN/m [13].

Antibacterial activity

For the antibacterial activity assay, three specimens per group with 6-mm (± 0.1 mm) diameters and 1.0-mm (± 0.01 mm) thicknesses were fixed in the lid of a 48-well plate and submitted to hydrogen peroxide sterilization. Each well of the test plate contained 900 µL of brain-heart infusion broth (BHI; Aldrich Chemical Co., St. Louis, Missouri, USA) with 1% sucrose and 100 µL of a suspension of *Streptococcus mutans (*NCTC 10449), which corresponded to 6.66 (±0.05) log CFU/mL. Additionally, 3 wells without the specimens containing 900 µL of BHI broth were inoculated with 100 µL of the bacterial suspension as controls. For the evaluation of antibacterial activity against biofilm formation on the resin surface, the specimens were removed from the lid, vortexed for 1 min in 1 mL of saline solution (0,9%) and diluted until a concentration of 10^{-6} was reached. For the planktonic bacteria viability evaluation, 100 µL from each well was diluted in 900 µL of saline solution until a 10^{-6} dilution was reached. Two 25-µL drops of each dilution were plated in BHI agar in Petri dishes, and the dishes were incubated for 48 hours at 37 °C. The numbers of colony forming units (CFUs) were counted using microscopy and transformed to log UFC/mL values.

Shear bond strength

Sixty pre-molar teeth were used to evaluate the shear bond strength (n=15). The teeth were fixed with acrylic resin using a metal matrix of 15 mm in height and 21 mm in diameter with the vestibular face perpendicular to the acrylic base. The labial surfaces of the teeth were preconditioned with 37% phosphoric acid for a period of 30 seconds, rinsed with water for 30 seconds and air-dried. Brackets (Roth Max, Morelli, Sorocaba, SP, Brazil) were fixed to the centers of the vestibular faces of the teeth using the experimental orthodontic adhesives (G_{Ctrl}, G_{BNNT}, G_{ATAB} and $G_{BNNT/ATAB}$). A 300-g force was applied to the surface of the brackets to standardize the adhesive thickness, and the excess adhesive was removed. The adhesive was photoactivated for 10 seconds on each face of the bracket; thus, the photoactivation totaled 40 seconds. The samples were subjected to the shear bond strength test in a universal test machine (Shimadzu EZ Test EZ-SX, Kyoto, Japan). Using a knife-edge chisel (0.1 mm) applied at 180° to the labial face of the tooth and positioned at the adhesive-enamel interface, a force was applied with the speed of 1 mm/min until the moment the bracket was detached, and the results were recorded in MPa. After the shear bond strength test, the residual adhesive on the tooth surface (Adhesive Remnant Index, ARI) was evaluated with a stereomicroscope (x10). The score 0 corresponds to no resin left on enamel, score 1 less than 50% of resin left on enamel, score 2 more than 50% of resin left on enamel and score 3 all the resin adhered to the enamel including the printing of the bracket mesh [21].

Mineral deposition

For the evaluation of mineral deposition, three disks of 6 mm (± 0.1 mm) in diameter and 1 mm (± 0.1 mm) in thickness were made from each adhesive. The surfaces of the specimens were evaluated before and after immersion in 10 mL of simulated body fluid (SBF) at 37 °C for 14 days more 28 days by means of Raman spectroscopy (Senterra, Bruker Inc., Karlshure, Germany) using a 785-nm laser. The standard area (2000×2000 µm) was analyzed (900 equidistant points) for each specimen. Changes in the intensities of the peaks located in the regions of the 960 cm^{-1} wavenumbers (characteristic of the bonds present in $PO4^{3}$ ions) were taken as references for formation of hydroxyapatite in the Raman spectra.

Statistical analysis

The normalities of the data were analyzed using the Shapiro-Wilk test. The KHN1 and KHN2 data regarding degradation in solvent were normally distributed and compared with a paired Student's t-test. One-way ANOVAs were applied to examine the contact angle, free surface energy, degree of conversion, cytotoxicity, antimicrobial test results, KHN1 and ΔKNH% degradation in the solvent and shear strength test results. When differences between the groups were identified, Tukey's multiple comparison tests were applied. All analyses were performed adopting a significance level of 5% using appropriate statistical software.

Results

The mean degree of conversion and softening in solvent values are presented in Table 1. The G_{ATAB} exhibited the highest (58.98 ±0.51) degree of conversion among the groups ($p<0.05$), the G_{Ctrl} (56.36 ±0.82) and $G_{BNNT/ATAB}$ (55.64 ±1.15) were similar ($p>0.05$), and the G_{BNNT} (52.64 ±0.40) exhibited the lowest degree of conversion

($p<0.05$). Regarding softening in the solvent, the initial microhardness values (KNH1) were similar for all groups ($p>0.05$). The values after immersion in ethanol were lower than the initial values in all groups ($p<0.05$). The percentage differences between KHN1 and KHN2 (ΔKHN%) were lower for the G_{BNNT} (12.74 ±7.32) and G_{ATAB} (13.67 ±8.28) compared with the G_{Ctrl} (34.29 ±9.18), and the $G_{BNNT/ATAB}$ exhibited a value similar to those of the other groups (20.43 ±10.98). The cell viabilities of the experimental orthodontic adhesives were not significantly different from that of the control group ($p>0.05$). All groups presented cell viabilities higher than 90% as illustrated in Figure 1. The SFE mean value for the G_{BNNT} decreased ($p<0.05$; Table 2). The results of the antibacterial activity tests are presented in Table 3. Compared with the other groups, a significant reduction in bacterial growth was observed in the $G_{BNNT/ATAB}$ group after 24h of incubation at 37 °C ($p<0.05$). The planktonic analysis did not revealed any significant differences. The results of the shear bond strength evaluations (Table 3) were similar for all of the experimental orthodontic adhesives ($p>0.05$). The ARI scores are presented in Figure 2; the G_{Ctrl} scores were mainly 3, the G_{BNNT} and $G_{BNNT/ATAB}$ scores were mainly 0, and the G_{ATAB} scores were mainly 1. The mineral deposition test revealed phosphate deposition in the G_{BNNT}, G_{ATAB} and $G_{BNNT/ATAB}$ groups after 14 and 28 days of immersion in SBF.

Discussion

The formation of white spot lesions around brackets is a common complication of fixed orthodontic treatments. In the present study, experimental orthodontic adhesives that included particles of boron nitride nanotubes and alkyl trimethyl ammonium bromide were formulated. These adhesives were produced with the objective of inhibiting or reducing bacterial growth and inducing the remineralization of already affected enamel without affecting the mechanical properties of the material.

The degree of conversion (DC) of an orthodontic adhesive influences the mechanical properties of the adhesive due to the cross-linking density that is determined during polymerization [22]. In this study, the G_{BNNT} adhesive presented with the lowest degree of conversion among all groups. According to our results, when added to an adhesive resin at levels above 0.1% wt, BNNT resulted in a decrease in the degree of conversion [23]. BNNTs originate from graphene and have a grayish color before polymerization [14]; thus, the availability of light in the resin matrix is decreased, which leads to a lower DC of the adhesive resin.

A low degree of conversion of adhesives may cause biological reactions because it enables the release of unconverted monomers. Quaternary ammonium compounds exhibit low cytotocicity [10]; however, ATAB is a unpolymerizable monomer, and it is may be cytotoxic to keratinocytes. Cytotoxicity was examined using keratinocytes because keratinocyte cells comprise most of the oral mucosa [24]. To date, there are no reports are available on the adverse effects of BNNT on living cells [23,25]. Despite the differences in the degrees of conversion found between the tested adhesives, no significant differences in cytotoxicity were found for any of the specimens compared to the control, and the cell viabilities of the keratinocytes were above 90% for all of the adhesives.

One method of studying the stability of a polymer network is through softening in solvent [26]. The softening in solvent after 2 hours of immersion in alcohol was lower in the groups that contained G_{BNNT} and G_{ATAB} than in the G_{Ctrl} group. BNNT has a high modulus of elasticity [18] and is considered to be a superhydrophobic material [16]; thus, this material reduces the degradation of adhesive resins over time and thus increases the resistance to hydrolytic degradation [27].

BNNTs may reach contact angles above 170 degrees with water [11]. The incorporation BNNTs into orthodontic adhesives produced adhesives with higher contact angles and lower free surface energies compared with the other experimental orthodontic adhesives. ATAB is considered to be a surfactant [8], and when added to adhesives with BNNT ($G_{BNNT/ATAB}$), ATAB increased the hydrophilicity of the material, which resulted in an increase in the surface free energy (59.41 ±4.19) compared to the G_{BNNT} adhesive (44.86 ±3.41). The low surface free energy of G_{BNNT} decreased the wetting of the orthodontic adhesive and thus may decreased bacterial fixation around the brackets [28].

Furthermore, BNNT particle size ranges from 5 to 10 nm according to previous studies [29]. The addition of nanoparticles to adhesives is capable of conferring antimicrobial activity due to the particle size and large free surface area [30]. The antimicrobial activity of ATAB has previously been described in the literature [8-10] and is related to the long alkyl chain length. However, the separate additions 0.1% BNNT and 0.1% ATAB were insufficient to ensure antimicrobial activity. In contrast, when 0.2% BNNT/ATAB was added, a decrease in bacterial adhesion to the specimens was observed. This observation can be explained by a synergistic effect of the two fillers and by the addition of a double-nano filler. The planktonic analysis revealed no significant differences of any of the groups compared with the broth without the specimens in terms of the growth of bacteria in the medium over 24 h. These data indicate that the specimens did not leach or did not leached enough to decrease the growth of bacteria in the medium. Despite the similar bacterial growths, lower bacterial adhesion was observed in the test specimens, which indicates that the novel adhesives did not leach but were rather antimicrobial by contact.

The shear bond strength data did not reveal any differences between the experimental groups and the control group. To regulate the viscosities of the materials, 5% wt silica was added to the experimental adhesives. When an orthodontic adhesive is viscous, some researchers have chosen to use a more fluid resin as a primer prior to the application of the orthodontic adhesive to improve the imbrication between the adhesive and the enamel [31]. The addition of BNNT may increase the mechanical strength of a materials [23], and in our study, we did not use any primer prior to the application of the orthodontic adhesive and did not observed any effects on shear strength. The use of a primer may be omitted for any type of orthodontic adhesive because no differences in the shear strengths of bonded brackets result from the use of a primer [31].

We need new materials with bioactive fillers [4] that are capable of inducing the deposition of phosphate, and this study found mineral deposition in all of the experimental groups after 14 and 28 days of immersion in SBF as observed based on the 960 cm^{-1} Raman peak (Figure 3). The immersion of specimens in SBF is a fast and reliable method for predicting the in vitro bioactivities of new biomaterials [32]. The mineral deposition capacity of BNNT has previously been demonstrated [18,29], already, the bioactivity of the ATAB was not yet described in the literature.

Conclusions

Based on the results of this study, the addition of 0.2% BNNT/ATAB to an experimental orthodontic adhesive is able to inhibit bacterial growth and induce mineral deposition without affecting the properties of the material.

Clinical relevance

A new materials with antimicrobial activity and with ability to induce mineral deposition may be an alternative to preventing white spot lesions.

Compliance with Ethical Standards

Conflict of Interest: Author CF declares that he has no conflict of interest. Author VL declares that he has no conflict of interest. Author GB declares that he has no conflict of interest. Author FD declares that he has no conflict of interest. Author SS declares that he has no conflict of interest. Author FC declares that he has no conflict of interest.

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Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent: For this type of study, formal consent is not required.

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Table 1. Mean and standard deviation of degree of conversion (DC) and microhardness value of the model adhesives before (KHN1), after immersion in solvent (KHN2) and the variation of microhardness values [Δ%].

	DC [%]	KHN1	KHN2	ΔKHN%
G_{Ctrl}	56.36 (±0.82)B	25.89 (±2.71) Aa	16.82 (±0.84) b	34.29 (±9.18) A
G_{BNNT}	52.64 (±0.40)C	23.92 (±1.55) Aa	20.82 (±1.41) b	12.74 (±7.32) B
G_{ATAB}	58.98 (±0.51)A	23.14 (±2.15) Aa	19.84 (±0.62) b	13.67 (±8.28) B
$G_{BNNT/ATAB}$	55.64 (±1.15)B	23.90 (±2.99) Aa	18.99 (±2.73) b	20.43 (±10.98) AB

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

Table 2. Outcomes obtained for the contact angle and surface free energy with means and standard deviation for each resin tested.

Groups	Contact Angle [θ]		SFE [mN/m]
	Water	α-Br	
G_{Ctrl}	52.48 (±11.72) B	12.39 (±5.39) C	58.54 (±6.10) A
G_{BNNT}	70.25 (±8.58) A	36.96 (±4.12) A	44.86 (±3.41) B
G_{ATAB}	51.81 (±7.28) B	23.29 (±3.65) B	57.46 (±3.89) A
$G_{BNNT/ATAB}$	46.85 (±5.87) B	37.80 (±2.69) A	59.41 (±4.19) A

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

Table 3. Mean and standard deviation of antibacterial activity for the biofilm, planktonic evaluation [log CFU/ml] and shear bond strength (n=15) of experimental orthodontic adhesives [MPa].

Groups	Biofilm evaluation [log CFU/mL]	Planktonic evaluation [log CFU/mL]	Shear bond strength [MPa]
G_{Ctrl}	5.94 (±0.26) A	8.21 (±0.07) A	12.37 (±3.01) A
G_{BNNT}	5.79 (±0.11) A	8.19 (±0.08) A	14.17 (±3.39) A
G_{ATAB}	5.78 (±0.16) A	8.19 (±0.06) A	13.62 (±1.64) A
$G_{BNNT/ATAB}$	5.14 (±0.10) B	8.22 (±0.08) A	13.22 (±3.05) A
Planktonic control	-	8.18 (±0.06) A	-

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

Figure 1. Epithelial cells viability (±standard deviation) [%]. Distinct capital letters indicate a significant difference ($p<0.05$).

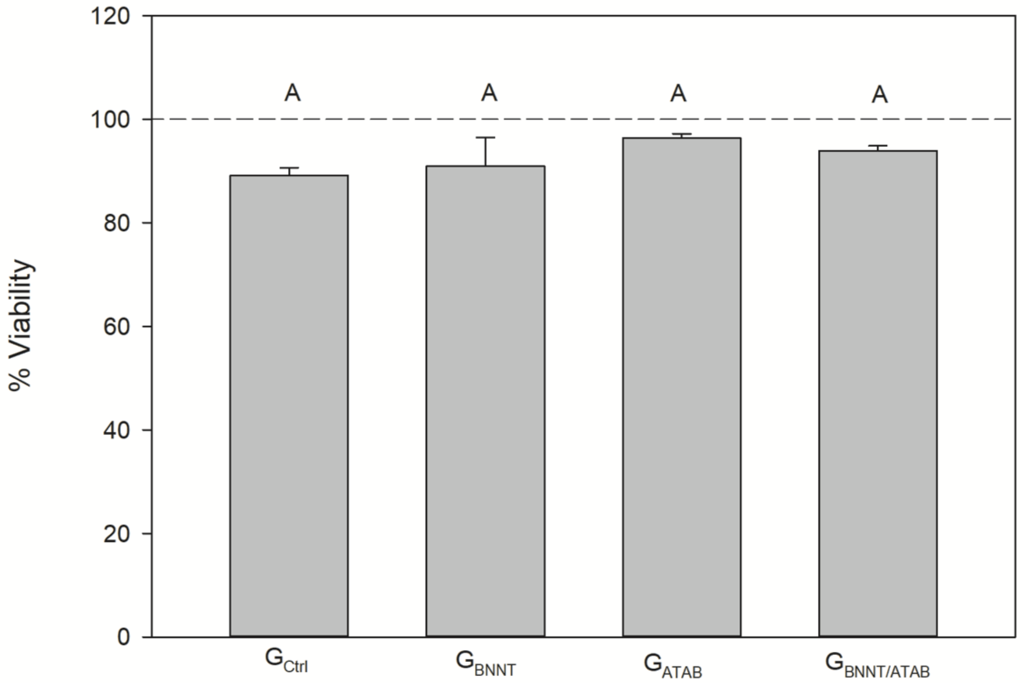


Figure 2. ARI scores for shear bond strength test.

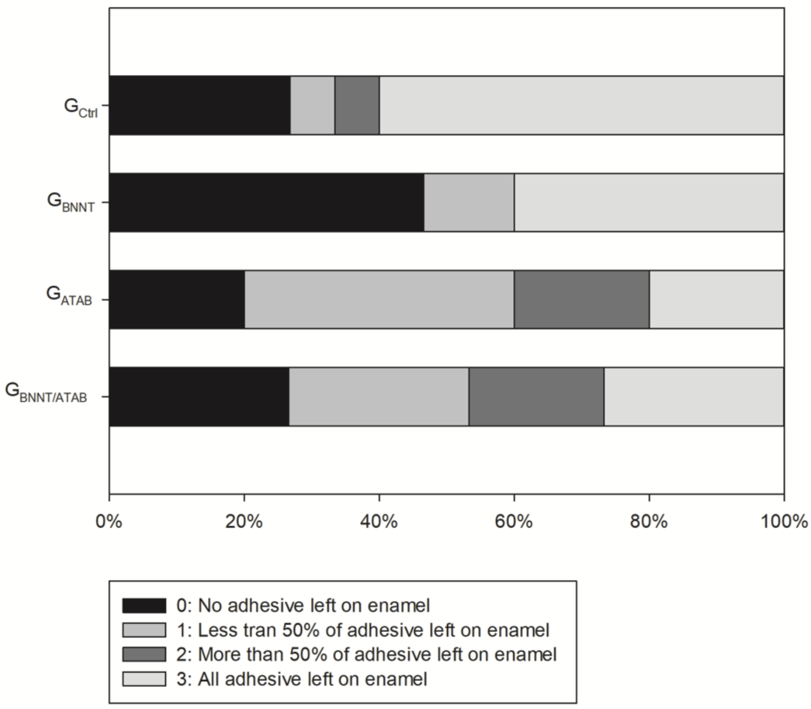
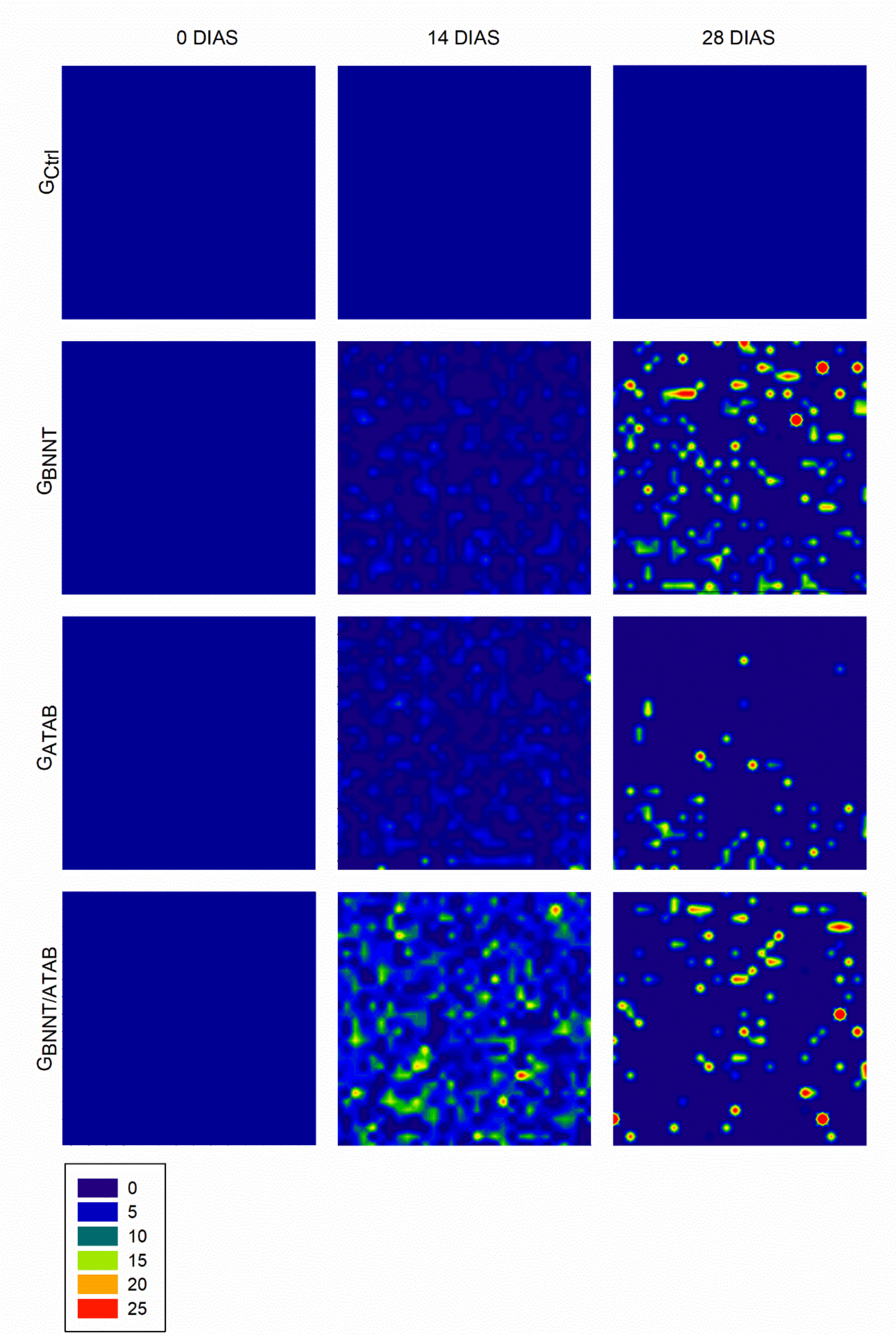


Figure 3. Image representative of mineral deposition test integrated for $960 cm^{-1}$ Raman peak.



5. Considerações Finais

A busca de materiais com capacidade de diminuir a prevalência de lesões de mancha branca ao redor de *brackets* tem sido o objetivo de diversos estudos (5-12). A falta de uma adequada higiene oral é a principal causa para a ocorrências dessas lesões (2), por esse motivo é necessário desenvolver estratégias que não dependem da colaboração do paciente (6). Sendo assim, nanotubos de nitreto de boro e brometo de alquil trimetil amônio foram utilizados como carga e incorporados em um adesivo ortodôntico experimental resultando em um adesivo com atividade antimicrobiana e remineralizante sem afetar as propriedades do material.

A adição de apenas BNNT no adesivo ortodôntico experimental houve aumento no ângulo de contato, diminuição da energia de superfície, diminuição do amolecimento em solvente e deposição de íons de fosfato, de acordo com a literatura (36,39). Apesar de que com seu tamanho nanométrico (39) poderia permear e atingir a membrana celular bacteriana (14), foi demonstrado nesse estudo que o BNNT não apresenta atividade antimicrobiana na concentração de 0,1%. Até o momento não havia estudos com o ATAB na Odontologia e com o presente estudo foi possível demonstrar a sua bioatividade. Apesar do ATAB ser um monômero não-polimerizável foi possível mostrar que ele não é citotóxico para as células epiteliais e que não foi liberado para o meio no ensaio antimicrobiano. A adição do ATAB a 0,1% não foi o suficiente para possuir ação antimicrobiana.

Apesar das limitações, o objetivo do presente estudo foi alcançado. Uma continuidade desse estudo seria interessante comparar o adesivo experimental contendo 0,2% BNNT/ATAB com um adesivo ortodôntico experimental, além de fazer um estudo longitudinal da atividade antimicrobiana e avaliar a dispersão das cargas de BNNT e ATAB no adesivo ortodôntico experimental. Também poderíamos avaliar o adesivo do presente estudo em um modelo *in situ* para simular o ambiente bucal, além de ser um método relativamente rápido e clinicamente relevante para estudar o comportamento de adesivos antibacterianos contra a desmineralização causada pela placa dental.

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