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DIANA ARAÚJO CUNHA

PROPRIEDADES FÍSICO-QUÍMICAS E ANTIMICROBIANAS DE COMPÓSITO RESINOSO MODIFICADO PELA INCORPORAÇÃO DE NANOTUBOS DE HALOISITA E TRICLOSAN

FORTALEZA
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Dissertação de Mestrado apresentada ao Programa de Pós-Graduação em Odontologia da Faculdade de Farmácia, Odontologia e Enfermagem da Universidade Federal do Ceará, como requisito parcial para a obtenção do Título de Mestre em Odontologia. Área de Concentração: Clínica Odontológica.

Orientador: Prof. Dr. Vicente de Paulo Aragão Saboia.
Coorientador: Prof. Dr. Salvatore Sauro.

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A Deus.

Aos meus pais, Vitória e Vinicio.

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RESUMO

Um das razões para substituições de restaurações de resina composta é a ocorrência de cáries secundárias. Assim, compósitos resinosos incorporados com agentes antimicrobianos foram sendo desenvolvidos ao longo do tempo. O Triclosan (TCN) é um agente antibacteriano utilizado em diversos produtos como dentifrícios e enxaguatórios bucais, entretanto necessita de um agente carreador. Os nanotubos de haloisita (HNT) são aluminossilicatos naturais que podem ser utilizados como um agente de reforço, o que melhora as propriedades mecânicas, como na forma de reservatórios biologicamente seguro. Portanto, o presente estudo avaliou in vitro o comportamento físico-químico e microbiológico de uma resina experimental incorporada com 8% de HNT/TCN (HNT/TCN 8%), uma resina experimental sem HNT/TCN (HNT/TCN-0%) e outra resina comercial nanoparticulada. Foram realizados os testes de grau de conversão (GC), módulo de elasticidade (ME), resistência à flexão (RF), rugosidade (RU), tensão de polimerização (TP), análise dinâmico-mecânica (ADM), termogravimetria (TGA) e ensaio microbiológico (EM). Os dados das propriedades mecânicas foram submetidos à análise de variância um critério (ANOVA), assim como para análise de efeito antimicrobiano, e pós-teste de Tukey. O nível de significância foi fixado em 5% e o programa utilizado foi SigmaStat (CA, EUA). Os testes ADM e TGA foram analisados de um modo descritivo para caracterização. O GC e RU dos compósitos não sofreram alteração pela incorporação de nanotubos, já o ME da resina experimental incorporada se apresentou numericamente maior quando comparado aos outros grupos, assim como a tensão de polimerização. A RF da resina HNT/TCN 8% e da Z350XT obtiveram estatisticamente maiores valores quando comparados a HNT/TCN-0%. No ADM, HNT/TCN 8% apresentou melhor relação entre carga e matriz orgânica que a HNT/TCN-0%, além de uma maior reticulação da rede polimérica em relação às outras resinas. Já as curvas de TGA apresetaram temperatura máxima de 422ºC para a resina HNT / TCN-0%, 418ºC para a resina HNT / TCN-8% e 409ºC para resina comercial. Numericamente, o ensaio microbiológico demonstrou que a resina HNT/TCN-8% apresentou menor quantidade de unidades formadoras de colônias em relação aos outros grupos. A incorporação de HNT/TCN parece demonstrar efeitos benéficos nas propriedades físico-quimicas.

Palavras-chave: Triclosan. Nanotubos. Streptococcus mutans

ABSTRACT

One of the reasons for replacements of composite resin restorations is the occurrence of secondary caries. Thus, composite resin incorporated with antimicrobial agents were developed over time. Triclosan (TCN) is an antibacterial agent used in several products as dentifrices and mouthwashes, however it needs a carrier agent. Halloysite nanotubes (HNT) are natural aluminosilicates that can be used as a reinforcing agent, improving mechanical properties, such in the form of biologically safe reservoirs. Therefore, the present study evaluated in vitro the physicochemical and microbiological behavior of an experimental resin incorporated with 8% HNT/TCN (HNT/TCN-8%), an experimental resin without HNT/TCN (HNT/TCN-0%) and another nanoparticulate commercial resin. Degree of Conversion (GC), flexural modulus (FM), flexural strength (FS), roughness (RU), polymerization stress (PE), dynamic-mechanical analysis (ADM) thermogravimetry (TGA) microbiological (MS). The data of the mechanical properties were submitted to analysis of variance one way (Anova) as well as for analysis of antimicrobial effect, followed with post-test of Tukey. The level of significance was set at 5% and the program used was sigmastat (CA, USA). The DMA and TGA tests were analyzed in a descriptive way for characterization. The GC and RU of the composites were not altered by the incorporation of nanotubes, whereas the ME of the incorporated experimental resin was numerically higher when compared to the other groups, as well as the polymerization stress. RF of 8% HNT/TCN and Z350XT resin obtained statistically higher values when compared to HNT/TCN-0%. In ADM, HNT/TCN 8% showed a better load-to-matrix ratio than HNT/TCN-0%, in addition to a greater crosslinking of the polymer network than the other resins. The TGA curves showed a maximum temperature of 422ºc for HNT/TCN-0%, 418ºc for HNT/TCN-8% and 409ºc for comercial composite. Numerically, the microbiological assay showed that the HNT/TCN-8% resin presented smaller amount of colony-forming units in relation to the other groups. The incorporation showed a trend of improvement in the physical-chemical and antimicrobial properties of composites resins. The incorporation showed improvements in some physical-chemical and mechanical properties of composites resin, besides a tendency of antimicrobial activity against *Streptococcus mutans*.

Keywords: Triclosan. Nanotubes. *Streptococcus mutans*.

SUMÁRIO

1 INTRODUÇÃO

Uma das razões para substituições de restaurações de resina composta é a recorrência de cáries (BERNADO et al., 2007; DEMARCO et al., 2012; MURDOCH; MCLEAN, 2003). A cárie ao redor de restauração é causada pela microbiota bacteriana e a formação de um biofilme, provavelmente facilitada por fendas formadas na interface dente-restauração que podem ocorrer devido à carga durante a mastigação (KHVOSTENKO et al., 2016) e/ou pela tensão gerada pela contração de polimerização do compósito (WANG et al., 2016). Assim, se houver a formação do biofilme, a interrupção do crescimento acontece ou de forma mecânica (escovação), ou biológica, uso de drogas que são capazes de desorganizar a matriz do biofilme (BJARNSHOLT et al., 2013). As cepas de *Streptococcus mutans* foram identificadas como uma das espécies bacterianas presentes sob a restauração em casos de cáries ao redor de restaurações (WJ, 1986).

Os materiais dentários evoluíram com o advento da pesquisa nanotecnológica com foco na produção e aplicação de nanopartículas com características estruturais de alta qualidade para melhorar as propriedades químicas e físicas desses materiais (SAURO et al., 2012). As resinas compostas foram bastante modificadas nos últimos anos para que seu uso pudesse ser cada vez mais abrangente (PADOVANI et al., 2015). A maioria das resinas compostas comerciais contém um elevado teor de partículas inorgânicas, em torno de 60-80 % em peso, para apresentem um bom desempenho, especialmente alta resistência ao desgaste e baixa tensão de contração de polimerização. Assim, a incorporação de nanopartículas e nanofibras nos compósitos permite que ocupem espaços vazios presentes entre as micropartículas, o que proporciona um reforço (WANG et al., 2016).

Tais resultados sugerem que o desenvolvimento de compósitos restauradores com propriedades antimicrobianas pode ser considerado como alternativa para tratamentos minimamente invasivos (KHVOSTENKO et al., 2016). Várias incorporações de agentes antimicrobianos foram realizadas e testadas em compósitos resinosos. As nanopartículas de prata foram estudadas por possuir efetiva ação contra biofilme formado (DE LIMA; SEABRA; DURÁN, 2012), mesmo utilizado em baixas concentrações (0.5–1.0%). Já as nanopartículas à base de cobre comprovaram o seu potencial em causar modificações nas membranas celulares, síntese proteica e replicação de DNA dos microorganismos (ALLAKER, 2012). Outro agente estudado, as nanopartículas de quitosana, apresenta efeito antibacteriano por meio da sua estrutura química que aumenta a permeabilidade da membrana bacteriana e inibe a transcrição do RNA mensageiro (FRIEDMAN et al., 2013).

Entre os agentes antibacterianos, o Triclosan (TCN) é utilizado em diversos produtos como dentifrícios e enxaguatórios bucais. Estudos comprovam a eficácia dessa substância contra microorganismos gram-positivos, como o Streptococcus mutans, um dos principais agentes patogênicos, juntamente com *Staphylococcus aureus, Lactobacillus spp., e Actinomyces spp.,* envolvidos no processo de cárie dentária. Devido à sua eficácia antimicrobiana, baixo peso molecular e fácil processamento, o uso do TCN tem aumentado constantemente nos últimos 30 anos (DAVIES, 2007; JONES et al., 2000; KAFFASHI; DAVOODI; OLIAEI, 2016).

No entanto, o TCN necessita de um agente carreador que possa incorporá-lo no compósito para que ele possa realizar sua liberação química, expondo suas propriedades antimicrobianas. Alguns materiais podem servir como carreadores, como as nanopartículas lipídicas sólidas que agem como veículos eficazes para quimioterapêuticos hidrofóbicos (HOLPUCH et al., 2010). Nos hidrogéis, uma malha de cadeias de polímero hidrófilas dispersas em água capazes de interagir com as glicoproteínas da saliva, a distribuição das drogas acontece química e fisicamente (BAHRAM et al., 2014). Além desse material, existem os nanotubos de carbono que proporcionam estabilidade coloidal em meios biológicos, o que facilita a entrega de moléculas e promove a liberação sustentada de agentes biológicos, o que pode o desempenho de materiais restauradores e endodônticos (PADOVANI et al., 2015).

Os nanotubos de haloisita (HNT) são provenientes de um mineral que ocorre naturalmente, de fácil purificação, biocompatíveis e seguro, por isso foram inicialmente investigados (CAVALLARO et al., 2018). Além disso, esses materiais se tornaram mais rentáveis que os nanotubos de carbono (LVOV et al., 2016). Eles são aluminossilicatos naturais com uma estrutura tubular oca com diâmetro externo de 40-60 nm, diâmetro interno de 10-15 nm e comprimento de 700-1000 nm (LVOV et al., 2016) e também podem ser usados como um agente de reforço para melhorar as propriedades mecânicas dos compósitos (FEITOSA et al., 2015), tais como resistência à tração, resistência à flexão, módulo de armazenamento (GUIMARAES et al., 2010), microdureza e resistência de união (BOTTINO et al., 2013).

Além disso, eles podem atuar como reservatórios biologicamente seguros para o encapsulamento e liberação controlada de uma variedade de drogas terapêuticas (ALKATHEERI et al., 2015; MASSARO et al., 2016; PALASUK et al., 2017; YENDLURI et al., 2017), e moléculas bioativas (GHADERI-GHAHFARROKHI; HADDADI-ASL; ZARGARIAN, 2018), DNA, proteínas e inibidores da matriz de metaloproteinase (Alkatheeri

et al., 2015; BOTTINO et al., 2013; LIU et al., 2014). Por conseguinte, muitas substâncias foram carregadas nestes nanotubos por imersão em uma solução saturada de fármaco, como a doxorrubicina (ZHANG; LI, 2018), doxiciclina nifedipina dexametasona (ZHANG et al., 2015) e curcumina. O fármaco liberado pelos HNT pode durar de 30 a 100 vezes mais do que o que foi liberado sozinho ou por outros nanocarreadores (LIU et al., 2014).

Neste estudo, uma resina composta experimental (HNT/TCN-8%) com 8% de nanotubos haloisita/triclosan foi avaliada, uma vez que foi demonstrado que a incorporação de 5% em peso de HNTs favoreceu as propriedades físico-químicas, como dureza knoop, resistência à flexão (FEITOSA et al., 2014) e taxa máxima de polimerização (DEGRAZIA et al., 2017). No entanto, caso seja realizada uma incorporação superior a 10% em peso, algumas propriedades físico-químicas podem ser negativamente afetadas, como a resistência à flexão (FEITOSA et al., 2014) e a taxa máxima de polimerização (DEGRAZIA et al., 2017) possivelmente devido a aglomerações desses nanotubos (CHEN et al., 2012). A concentração de 8% foi testada para obter efeito antibacteriano sem diminuir as propriedades físico-químicas. Uma resina comercial nanoparticulada foi adicionada ao nosso estudo como grupo controle, suas propriedades físico-químicas foram testadas e resultados satisfatórios em testes laboratoriais (MONTEIRO; SPOHR, 2015; CHAVES et al., 2015; KHOSRAVI et al., 2016; NAIR, 2017).

2 PROPOSIÇÃO

O presente trabalho teve como objetivos:

2.1 Objetivo Geral

Avaliar *in vitro* o efeito da incorporação de 8% de nanotubos de haloisita carreados com triclosan por uma resina composta experimental através da realização de testes que possam fornecer dados sobre suas propriedades físico-químicas e microbiológicas.

2.2 Objetivos específicos

- Avaliar *in vitro* as propriedades físico-químicas através de grau de conversão, módulo de elasticidade, resistência a flexão, análise dinâmico mecânica, termogravimetria e tensão de polimerização de uma resina experimental incorporada com 8 % nanotubos de haloisita/triclosan, comparando com uma resina composta experimental controle e outra resina composta nanoparticulada comercial.

- Avaliar o potencial antimicrobiano de uma resina composta experimental incorporada com 8 % nanotubos de haloisita/triclosan comparando com uma resina composta experimental controle e outra resina composta nanoparticulada comercial através de contagem de unidade formadora de colônias após crescimento de biofilme maduro de cinco dias.

3 CAPÍTULO

Esta dissertação está baseada no Artigo 46 do Regimento Interno do Programa de Pós-Graduação em Odontologia da Universidade Federal do Ceará que regulamenta o formato alternativo para dissertações de Mestrado e teses de Doutorado, e permite a inserção de artigos científicos de autoria ou coautoria do candidato. Assim sendo, esta dissertação é composta de um artigo científico que será submetido ao periódico *Dental Materials*, conforme descrito abaixo:

Capítulo 1

PHYSICOCHEMICAL AND MICROBIOLOGICAL ASSESSMENT OF A EXPERIMENTAL RESIN DOPED WITH TRICLOSAN-LOADED HALLOYSITE NANOTUBES

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PHYSICOCHEMICAL AND MICROBIOLOGICAL ASSESSMENT OF A EXPERIMENTAL RESIN DOPED WITH TRICLOSAN-LOADED HALLOYSITE NANOTUBES

ABSTRACT

Objectives. To evaluate *in vitro* the effect of the incorporation of triclosan-encapsulated aluminosilicate-(halloysite) nanotubes (8% w/w) in experimental resin on the physical-chemical and microbiological properties.

Methods. The behaviour of resin-composite doped with halloysite / triclosan nanotubes (8% W / W) (HNT/TCN-8%), an experimental resin-composite without nanotubes (control) (HNT/TCN-0%) and a commercial nanofiller resin-composite were analyzed. Initially, the degree of conversion (DC) using micro-Raman spectroscopy, flexural strength (FS) and flexural modulus (FM) in a 3-point bending, dynamic thermomechanical analysis (DMA), thermogravimetric analysis (TGA), roughness (RU), polymerization stress measurements (PS) were evaluated. The microbiological (M) assay was obtained by calculating the colony forming units (CFU / mL) of the 5-day biofilm. Data was submitted to one-way ANOVA/Tukey test, except for DMA and TGA.

Results. The DC and FM showed no statistical difference among HNT/TCN-8% and the other two resin-composite ($p > 0.05$). The FS of the HNT/TCN-8% and the commercial resin-composite (data of results) was higher ($p < 0.05$) than the control resin-composite. The HNT/TCN-8 % resin-composite showed greater crosslinking in the polymer network, in addition to higher PS and it lost less mass in actuality to the other two groups.

Significance. The incorporation of TCN-loaded aluminosilicate-(halloysite) nanotubes into experimental resin-composite showed improvements in some physical-chemical and mechanical properties of resin-composites. Thus, being able to contribute to an alternative for therapeutic minimally invasive treatments.

KEYWORDS: Triclosan, Nanotubes, Resin-composites, *Streptococcus mutans.*

1. INTRODUCTION

The resin-composites have been widely modified in recent years so that their use could be more comprehensive [1]. Most commercial resin-composite s contain a high content of inorganic particles, amount 60-80 % by weight, to achieve good performance, especially high wear resistance and low shrinkage of polymerization. The incorporation of nanoparticles and nanofibers may lead to improved reinforcement [2] and bioactive properties [3].

Recurrent caries is one of the reasons for resin-composite restoration replacement restorations [4][5][6]. Caries lesions adjacent to restorations present causal relation with bacterial microbiota and the formation of a biofilm probably facilitated by gaps formed at the tooth-restoration interface or increased roughness of resin-composite [7]. *Streptococcus mutans* is one of the bacterial species present under restoration in cases of caries lesions adjacent to restorations [8]. In this way, restorative materials with bioactive properties such as antimicrobial or remineralization characteristics are needed [9]

Triclosan (TCN) is a well-known antibacterial agent used in a wide range ofproducts from toothpaste to mouthwashes. Studies demonstrate the efficacy of this substance against gram-positive microorganisms, such as *Streptococcus mutans*[10] [11], *Staphylococcus aureus* [12], *Lactobacillus spp*. [13], and *Actinomyces spp.* [14], involved in the dental caries process. Due to its antimicrobial efficacy, low molecular weight and easy processing, the use of TCN has increased steadily in the last 30 years [12]. However, neat TCN incorporation in resin-based materials could lead to a high licheability decresing the antimicrobial activity. To overcome this issue, TCN was already carriedin nanotubes elsewhere [3][11].

Halloysite nanotubes (HNT) are natural aluminosilicates with a hollow tubular [3] used as a reinforcing agent to improve the mechanical properties of resin-composites [15], such as tensile strength, flexural strength, storage modulus [16] microhardness and bond strength [17]. HNT is a green nanomaterial, biocompatible, presenting low cytotoxicity [18]. Also, it acts as biologically safe reservoirs for the encapsulation and controlled release of a variety of therapeutic drugs [15][19], bioactive molecules [20] and matrixmetalloproteinase inhibitors [21] [22]. The drug released by halloysite may last 30 to 100 times more than, when alone or with other nanocarriers [23].

Therefore, dental materials have evolved with the advent of nanotechnological research focusing on the production and application of nanoparticles with high-quality

structural characteristics to improve the chemical and physical properties of these material [24]. In this study, experimental resin-composite HNT/TCN-8% material was tested once the incorporation of 5 % wt of HNTs has improved Knoop hardness, flexural strength [15] and maximum polymerisation rate [11]. However, if incorporation of more than 10% by weight is performed, it allowed the decrease of both flexural strength [15] and maximum polymerisation rate [11], possibly due to the agglomerations of these nanotubes [25]. The concentration of 8% was tested to obtain antibacterial effect without decreasing the physicochemical properties.

The aim of the present study was to evaluate *in vitro* the effect of the incorporation of 8% HNT / TCN in experimental resin-composite by conducting tests that can provide data on its physicochemical and microbiological properties A commercial nanofilled resin-compositethat obtained physicochemical properties satisfactory results in laboratory tests [26][27][28][29] was added to our study as a control group.

2. MATERIAL AND METHODS

2.1. Material

Halloysite nanotube ($Al_2Si_2O_5(OH)_4.2H_2O$) with a diameter of 30-70 nm and length of 1–3 μm (Sigma-Aldrich, St. Louis, MO, USA) were silanisatedusing 5 wt.% of 3-metacryloxypropyltrimetoxysilane and 95 wt.% acetone at 110 °C for 24 h. Subsequently, the treated nanoparticles were mixed [1:1 ratio] with 2,4,4-Trichloro-2-hydroxydiphenyl ether (Triclosan, Fagron, Rotterdam, SH, Netherlands) with continue agitation for 1 h, as described in a previous study [3]. The mixture was then dispersed in 95 wt% pure ethanol (0.03 mg/mL^{-1}) and sonicated for 1 h. Subsequently, the HNT-TCN was desiccated for 10 days at 30°C to ensure complete evaporation of the residual solvents. TCN-HNT was finally characterised using a Transmission Electron Microscope (TEM) JEM 120 Exll (JEOL, Tokyo, Japan) at 80 kV at a magnification X 300,000.

An experimental resin-composite blend was created by mixing 75 wt% Bis-GMA (2,2-bis-[4-(hydroxyl-3-methacryloxy-propyloxy)phenyl]propane) and 25 wt% triethylene glycol dimethacrylate (TEGDMA) (Sigma–Aldrich, St. Louis, MO, USA) for 30 min with continuous sonication. Camphorquinone (CQ), ethyl4-dimethylaminobenzoate (EDAB), and diphenyliodoniumhexafluorophosphate (DPIHFP; Milwaukee, MI, USA) were added at 1 mol % to obtain a light-curable resin-based material. Incorporation of 8 % by weight of TCN-loaded aluminosilicate-(halloysite) nanotubes (HNT/TCN-8 %) were done (stirred for 1

h under continuous sonication) into the resin-composite. The control experimental resin-composite was formulated with the same organic matrix and filler, however without nanotubes (HNT/TCN-0%) (Table 1).

A commercial nanofilled resin-composite was the control group of this study. According to the manufacturer (3M ESPE), this material has nanoclusters of zirconia (4–11 nm) and silica (20 nm) nanoparticles, whereas the microfilled resin-composite contains discrete silica/zirconia microparticles (0.6 mm). The organic matrix presents Bis-GMA, Bis-EMA, UDMA, TEGDMA (Table 1).

2.2 Degree of Conversion (DC)

The degree of conversion (DC) of each experimental resin-based material was evaluated using Xplora micro-Raman spectroscopy (Horiba, Paris, France). Three specimens for each group were analyzed at a standardized room temperature of 23 ± 1 ºC and 60 ± 1% relative humidity. DC was calculated as described in a previous study based on the intensity of the C=C stretching vibrations (peak height) at 1635 cm^{-1} and using the symmetric ring stretching at 1608 cm^{-1} from the polymerized and non-polymerized specimens. A support holded a 1200 mW/cm^2 irradiance light-curing system (Bluephase, Ivoclar Vivadent, Schaan, Liechtenstein), at a standardised 2-mm distance from the surface, to polimerise the samples for 40 s. An Xplora micro-Raman coupled software registered the Raman spectra data in the range of 1590–1670 cm^{-1} using the 638 nm laser emission wavelength, with 5 s acquisition time and 10 accumulations [30][11].

2.3 Flexural strength and flexural modulus

The flexural strength (FS) and flexural modulus (FM) (n=5) were evaluated according to ISO 4049/2000 [31]. Samples of 25 x 2 x 2 mm were prepared using a Teflon split mould. A polyester strip and a glass slide covered the resin-composite, and the light tip guide was placed over the centre of the mould, to polimerise the specimens for 40 s. Subsequently, the adjacent sections on either side were irradiated until the full length of the sample was polymerised. After irradiation, the specimens were removed from the moulds and carefully finished with 140 and 320 grit abrasive paper, and stored in water at 37 °C for 24 h. The samples were positioned in a 3-point bending apparatus on 2 parallel supports separated by 20 mm, and loaded until fracture with a 500 Kgf load cell at a cross-head speed of 0.05 mm/min. The flexural strength (MPa) was calculated using the following formula:

$$\sigma = \frac{3L \times F_{max}}{2w \times h^2} \quad (1)$$

, whereas L is the distance between the parallel supports (mm); F_{max} is the load at fracture (N); w is the width (mm), and h is the height (mm)].

The flexural modulus (GPa) was calculated using the following formula:

$$E_f = \frac{F \times L^3}{4wh^3 d} \quad (2)$$

, whereas F is the load recorded (N); L is the distance between the parallel supports (mm); w is the width (mm), h is the height (mm) and d is the deflection, in millimetres.

2.4 Dynamic thermomechanical analysis (DMA)

Three specimens (8 mm x 2 mm x 2 mm) were prepared for each group (Table 1) and the tip of the light guide was placed over the centre of the mould and then light-cured for 40 s using a light-emitting as described. A DMA system (Mettler Toledo), equipped with a single bending cantilever, was used to determine the mechanical properties in clamped mode. The viscoelastic properties were characterised by applying a sinusoidal deformation force to the material under dynamic conditions: temperature, time, frequency, stress, or a combination of these parameters. The storage modulus (E´), glass transition temperature (Tg) and tangent delta (TAN-δ) of the tested materials were evaluated at different temperatures under cyclic stress (frequency of 2.0 Hz and amplitude of 10 μm) and from 50 to 800 ºC at the heating rate of 2 ºC min^{-1}. The TAN-δ value represents the damping properties of the material, serving as an indicator of all types of molecular motions and phase transitions.

2.5 Thermogravimetric analysis (TGA)

Three specimens (8 x 2 x 2 mm) were prepared for each group (Table 1) and the tip of the light guide was placed over the center of the mould and the curing light was activated during 40 s using a light-emitting as described. A thermogravimetric analysis determined the thermal degradation and the weight percentage of fillers resin-composites. A thermal program from 30 to 193 ºC at the heating rate of 2ºC min^{-1} in nitrogen by cooling room temperature determined the weight changes as a function of time and temperature.Thermogravimetric analysis was performed on a Pyris 1 TGA (SDTA851 - Mettler Toledo) thermal analyzer using about 10 mg of each sample.

2.6 Roughness

The resin-composite specimens were made (n = 12) measuring 4 × 4 × 2 mm. The specimens were light-cured for 40 s using a light-emitting as described. Subsequently, the adjacent sections on either side were irradiated until the full length of the sample was polymerised. Then, the specimens were removed from the moulds and any flash was carefully removed by gently abrading it with 600 and 1200 grit abrasive paper. The mean surface roughness was measured by a profilometer (Hommel Tester, T1000) with a tracing length of 1.5 mm and 0.08 mm/s cut-off. Tracing was performed in triplicate for each sample and the mean was calculated.

2.7 Polymerization stress measurements (PS)

Poly (methyl methacrylate) rods, 5 mm in diameter and 13 or 28 mm in length, had one of their flat surfaces sandblasted with 250 μm alumina. On the shorter rod, to allow for the highest possible light transmission during photoactivation, the opposite surface was polished with silicone carbide sandpaper (600, 1200, and 2000 grit) and felt disks with 1μm alumina paste (Alumina 3, ATM, Altenkirchen, Germany). The sandblasted surfaces received a layer of methyl methacrylate (JET Acrilico Auto Polimerizante, Artigos Odontologicos Classico, Sao Paulo, Brazil) ,followed by two thin layers of unfilled resin (Scotchbond Multi-purpose Plus, bottle 3, 3M ESPE).

The resin-composite was light-cured with 1200 W/cm^2 (Bluephase, Ivoclar Vivadent, Schaan, Liechtenstein) for 40 s. The rods were attached to the opposing clamps of a universal testing machine (Instron 5565, Canton, MA, USA) with the treated surfaces, facing each other with a 1-mm gap. The resin-composite was inserted into the gap and shaped into a cylinder following the perimeter of the rods. An extensometer (0.1 μm resolution), attached to the rods (Instron 2630-101, Bucks, UK) for the purpose of monitoring the specimen height, provided the feedback to the testing machine to keep the height constant. Therefore, the force registered by the load cell, was necessary to counteract the polymerisation shrinkage to maintain the specimen's initial height. A hollow stainless steel fixture with a lateral slot attached the short rod to the testing machine, allowingthe tip of the light guide to be positioned in contact with the polished surface of the rod. Force development was monitored for 10 min from the beginning of the photoactivation and the nominal stress was calculated by dividing the maximum force value by the cross-section of the rod. Five specimens were tested for each resin-composite.

2.8 Microbiology assay

Streptococcus mutans (S. mutans) UA159 (ATTCC) was obtained from single colonies isolated on blood agar plates, inoculated in Tryptone yeast-extract broth containing 1% glucose (w/v) and incubated for 18 h at 37 ºC under micro-aerophilic conditions in partial atmosphere of 5% CO_2.

To analyze the antimicrobial effect, blocks (4 x 4 x 2 mm) of each group were produced (Table 1). Materials were dispensed in a silicone mould, covered with a polyester tape and then submitted to digital pressure during 2 s to better accommodate the material and curing light was activated 40 s. Samples were sterilized by exposure to Plasma Hydrogen Peroxide before starting biofilm formation. Mono-species *S. mutans* biofilms were formed on blocks placed in bath cultures at 37 ºC in 5 % CO_2 up to 5 days in 24-well polystyrene plates. The biofilms grew in tryptone yeast-extract broth containing 1 % sucrose (w/w), and kept untroubled for 24 h to allow an initial biofilm formation. During the biofilm formation period, once daily the discs were dip-washed three times in a plate containing NaCl 0.89 % solution to remove the loosely bound biofilm and they were transferred to new 24-well plates with sterile medium. The blocks of each experimental group were removed after 5 days of initial biofilm formation and transferred to pre-weighed microtubes containing 1 mL of NaCl 0.89 % solution. Biofilms were then dispersed with 3 pulses of 15 s with 15 s of interval at a 7-W output (Branson Sonifier 150; Branson Ultrassonics, Danbury, CT). An aliquot (0.05 mL) of the homogenised biofilm was serially diluted (10^{-1} - 10^{-7}) and plated onto blood agar plates. Plates were then incubated at 37 ºC, 5% CO_2 for 48 h, before enumerating viable microorganisms. Results were expressed as 28 colony forming units (CFU)/mL and transformed in $\log_{10}$ CFU to reduce variance heterogeneity [32].

To determine the biofilm dry weight, 200 µL aliquots of the initial biofilm suspension were transferred to pre-weighed tubes and dehydrated with ethanol solutions (99 %). The tubes were centrifuged, and the supernatants were discarded before the pellet was dried into a desiccator (P_2O_5) for 24 h and weighted (± 0.00001 mg). The dry weight of the biofilm was determined by calculating the weight in the tube (initial weight − final weight) and in the original suspension (dry weight in 1 mL = dry weight in 200 µL × 5) [33].

2.9 Statistical analysis

Physicochemical properties data was submitted to analysis of variance with one

factor (One way-ANOVA), followed by Tukey test. For analyzing antimicrobial effects analysis of variance with one factor (One way-ANOVA). Significance level was set at 5%. The program used to perform the analyses was SigmaStat (CA, USA). The DMA and TGA tests were done in a descriptive mode to characterize as resin-composites.

3. RESULTS

TEM images show that TCL was sucessfully desposited inside and outside of the HNTs. Means and standard deviations of physicochemical experiments are presented in Table 2, 3 and 4. The degree of conversion (DC) showed no statistical difference between experimental groups and commercial resin-composite ($p = 0.879$). The flexural strength of HNT/TCN-8% resin-compositeand Z350XT were higher than HNT/TCN-0% ($p = 0.005$); however, the flexural modulus (E_f) had no statistical difference among groups. Also, the roughness presented no significant differences among all groups ($p = 0.844$). There was difference in the Maximum polymerization stress among the groups and the addition of HNT/TCN-8% resin-composite, resulted in a significant increase. According to DMA (Fig.1), there were differences between the values of elastic modulus, TAN δ at Tg and Tg between the three resin-composite. According to the TGA curves in the nitrogen atmosphere (Fig. 2), the initiation of the degradation can be vary depending on the resin, HNT / TCN-0% resin-composites can distinguish 422 ° C, 418 ° C for HNT / TCN-8% composite resin and 409 ° C for Z350XT, indicating weight loss after degradation thermal behavior of composite resin was 25 7; 24.5; 30%, respectively (table 4).

The results of the microbiological test are in Fig. 3 and 4. There was no statistical significant differences ($p = 0.511$) between the experimental groups for CFUs and ($p = 0.557$) for dry weight.

4. DISCUSSION

The results of this study demonstrated there was no difference in DC% after incorporation of HNTs (Table 2). Our results agreed with those obtained by a previous study *in situ* [11]. The organic matrix structure and characteristic of fillers exert a direct influence on the surface roughness, degree of conversion, finishing, and polishing procedures and may influence the surface quality of resin-composite [34]. Achieve increased degree of polymer conversion could lead to increased long-term stability of resin-based dental materials, especially with relatively short light curing periods [24]. It is known that during the light

curing process of HNT / TCN-8% there is an increase in intermolecular interactions due to the presence of nanotubes [25], as well as the interaction between the C=O and Al-O-H groups present on the inner and outer surface of the HNTs [36]. In general, a higher percentage value of the cross-link conversion degree monomers may result in a higher density of the polymer network [37].

The dynamic mechanical analysis is an evaluation performed in the resin-composite to obtain the glass transition temperature which allows a more in-depth knowledge of the formed network, network homogeneity and crosslinking density [38]. It is suggested that the addition of nanotubes can cause a formation of intermolecular interactions (as a hydrogen bond occurs between hydroxyl groups) between an outer surface of HNTs and Bis-GMA molecule by hydroxyl groups [25]. The HNT / TCN-8% material showed the highest dynamic elastic modulus (E´) (Table 4). The E´ can reveal the behaviour of the material in storing the elastic energy associated with recoverable elastic deformation. The maximum E´ suggests that the resin-composite may relieve excess energy accumulated during tooth function [39].

It has been reported that the most sensitive way to evaluate the characterization parameters in the interface variation of the resin-composite structure is TAN δ at Tg. In fact, low values of TAN δ at Tg suggest a better interfacial adhesion between the organic matrix and the filler [37]. In the present study, the glass transition temperature of the resin-composite, determined by peak TAN δ curves, showed that the HNT / TCN-8% resin-composite presented a lower TAN δ at Tg when purchased with the HNT / TCN-0% resin-composite, suggesting that the nanotubes provide a better interaction between the fillers and the organic matrix (Fig. 1).

The strength, modulus, and impact resistance of polymers can be increased when they are mechanically reinforced by HNTs, even at low filler weight (5 wt %) [23]. According to the ISO 4049/2000 standard [31], the flexural strengths of universal resin-based restorative materials should be higher than 80 MPa. The Flexural strengths in Table 3 showed that FS of the commercial resin-composite and of HNT/TCN-8% were statistically higher than that obtained for the HNT/TCN-0%, which did not meet the projected standard value in the ISO 4049/2000. High flexural strengths protect restoration materials against breaks and they also protect the tooth structure in posterior restorations [40]. Although the FS did not revel any statistical difference between HNT/TCN-8% and commercial resin-composite, as this study is about the characterization of an experimental material, our aim was to achieve properties that were higher or in the same level of the current gold standard commercial material. Even with

no statistical difference compared to the commercial resin-composite, the highest FS obtained by HNT/TCN-8% seems to occur due the addition of HNTs in the resin-composite materials.

Another important physical property that influences the stress development is the flexural modulus (FM), which is also associated with the composition of the material. In this study, FM showed no statistical difference among groups. However, there was a tendency of increase this value when resin-composite was dopped with HNT / TCN compared to the other tested groups (Table 3). Correlation of FM and polymerization stress values is a valid but simplified approach [41]. The limitations of this *in vitro* test were the light needed to pass through the lower acrylic rod, as well as the 1 mm thick material until reaching the upper rod, to make the bonding between the materials and the rods. During the polymerization process, resin-composites s incorporating 5% or more of HNTs presents trend to agglomerate [25], which may have influenced the passage of light in the experimental resin-composite, generating a low degree of polymerization in depth. In addition, a conversion across a polymer sample may not be uniform [42].

Although the flexural modulus and the polymerisation stress increase exponentially with respect to the advance of the polymer conversion [43], in this study the high standard deviation of polimerisation stress test found in the HNT / TCN-8% resin-composite may be related to the bond between the rods and the experimental resin-composite and thus the compliance of the test. Tensilometer test configurations have been also presented some limitations when representing the cavity conditions[44]. However, it was previously reported that it is a valid methodology for smaller cavities, where the volume of the resin-composite is restricted, representing a minimally invasive restorative procedure [45].

Surface irregularities provide increased colonization and subsequent plaque growth, since bacteria on such surfaces are more protected against shear forces. However, it has been reported that a material incapable of attaining and/or maintaining at Ra below 0.2 μm would be susceptible to an increase in plaque accumulation and higher risk of caries and periodontal inflammation [48]. The surface roughness of the restoration appears optically acceptable when its Ra of surfing is less than 0.1 μm [49]. Incorporation of HNTs did not affect the roughness and thus, it is reasonable to suggest that this experimental material could clinically be used once Ra were six times smaller than the values related to the increase of plaque accumulation [48] (Table 3).

To test the antibacterial effect, it was carried out a test with 5 days of biofilm growth. According to Degrazia and collaborators [11], the resin-composite doped with

HNT/TCN showed antibacterial effect up to 72h and usually it can be evaluated after 24h of biofilm growth. It is suggested that the antibacterial effect can be obtained by direct contact of *S. mutans* with the inhibitory agent present in the resin-composite (TCN in the present work). Corroborating this idea, Feitosa and collaborators [22] demonstrated inhibition of *S. mutans* when in direct contact with doxycycline-encapsulated nanotube-modified dentin adhesive after 24 h. In the presente study there was no statistical difference between the CFUs values of a 5 day mature biofilm formed when the 3 resin-composite s were used (P = 0.511) (Fig 1 and Fig 2). It seems that the decrease in the inhibitory antibacterial effect overtime might be related to the non-release of such agent from the resin-composite to reach the whole thickness of the plaque, particularly the outer layers of bacteria. The absence in degradation of the material seems to be considered as advantage since it can help to maintain the bond strength over time [50]. Due to the curious low CFU of the HNT/TCN-8%, in the present work, we believe that the daily removal of the biofilm can reduce its thickness and allow the direct contact with the agent with the bacteria and promoting a more effective antibacterial action.

Future studies are suggested to improve the development of antimicrobial materials and the understanding of the relationship between their formulations, morphology and properties, to promote the longevity (shelf-life and ageing) of resin-based materials restorations. We suggest that triclosan incorporation should be tested in daily biofilm analysis rather than in a 5-day approach. Studies including its rheological properties can enhance the range of its application and future both patients' and clinicians' satisfaction. **5. CONCLUSION**

It can be concluded that:

- Incorporation of 8% wt seems to be a satisfactory formulation of halloysite nanotube for achieving a high mechanical property performance of an experimental resin;
- 8 %-halloysite nanotube experimental resin doped with triclosan showed to be a suitable restorative material for minimally invasive treatments, such as the current commercial gold standard resin-composite;
- The experimental material used in this study seemed to be safe and promising for sealants, cements, and other resin-based materials' formulations;
- Incorporation of triclosan seems to be test-dependent, since it showed no response in mature biofilms as used in this study.

In this way, the development of resin-composites with enhanced mechanical and antimicrobial properties should be stimulated, as it would translate into restorations with longer-term.

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TABLES & FIGURES

Table 1. Composition of the resin-composite used in this study.

		Manufacturer/ Lot No.	Composition
HNT/TCN-0%	Experimental resin-composite	-----	**Organic matrix:** Bis-GMA, TEGDMA. **Filler type:** Silica filler **Filler content:** 78 wt%
HNT/TCN-8%	Experimental resin-composite	-----	**Organic matrix:** Bis-GMA, TEGDMA. **Filler type:** Silica filler and halloysite nanotubes. **Filler content:** 78 wt%
Filtek Z-350XT (Shade A1D)	Filtek – 3M	3M ESPE (St Paul, MN, USA)/ N702257	**Organic matrix:** Bis-GMA, Bis-EMA, UDMA, TEGDMA **Filler type**: Silica and zirconia nanofillers, agglomerated zirconia-silica nanoclusters **Filler content:** 82 wt%

Table 2. Results of chemical proprieties, Degree of Conversion (DC).

	DC (%)
HNT/TCN-0%	75.9 (5.4)
HNT/TCN-8%	78.5 (2.2)
Z350XT (Filtek-3M)	72.5 (10.6)

*No letters in a column represent absence of significant difference (p>0.05).

Table 3. Results of physical proprieties, Flexural Modulus (E) and Flexural Strength (FS), Roughness (Ra), Maximum polymerization stress (Mps).

	E (Gpa)	**FS (Mpa)**	**Ra**	**PS (Mpa)**
HNT/TCN-0%	6.8(0.9)	75.92(10.1)^{B}	0.03(0.002)	3.6(0.3)^{B}
HNT/TCN-8%	7.5(0.2)	107.23(6.6)^{A}	0.03(0.01)	5.4(0.9)^{A}
Z350XT	6.8(0.4)	101.45(18.4)^{A}	0.03(0.008)	3.6(0.3)^{B}

*Different capital letters in column indicate statistical difference (p<0.05). No letters in a column represent absence of significant difference (p>0.05).

Table 4. Results of physical proprieties, Dynamic elastic modulus E´ (GPa), Glass Transition Temperature (DMA), TAN-δ and Thermogravimetric analysis (TGA).

	Dynamic elastic Modulus - E´ (GPa)	**Tg (ºC)**	**Tanδ (x10^3) at Tg**	**TGA weight loss (%)**
HNT/TCN- 0%	1.8	97	160	25.7
HNT/TCN- 8%	2.7	155	120	24.5
Z350XT	2.3	109	90	30

The DMA and TGA tests were analyzed in a descriptive mode to characterize the resin-composites s.

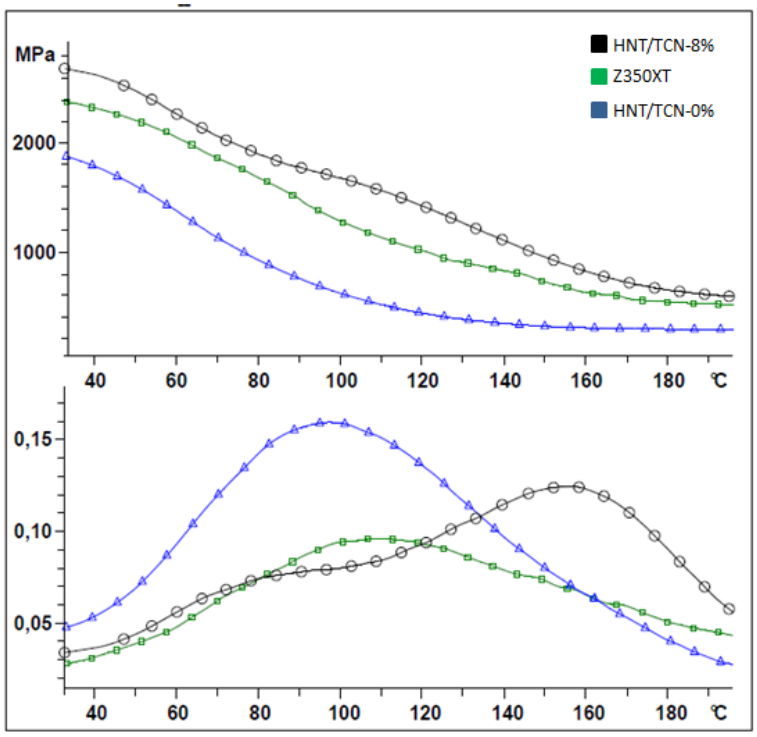


Figure 1. Results of Dynamic thermomechanical analysis.

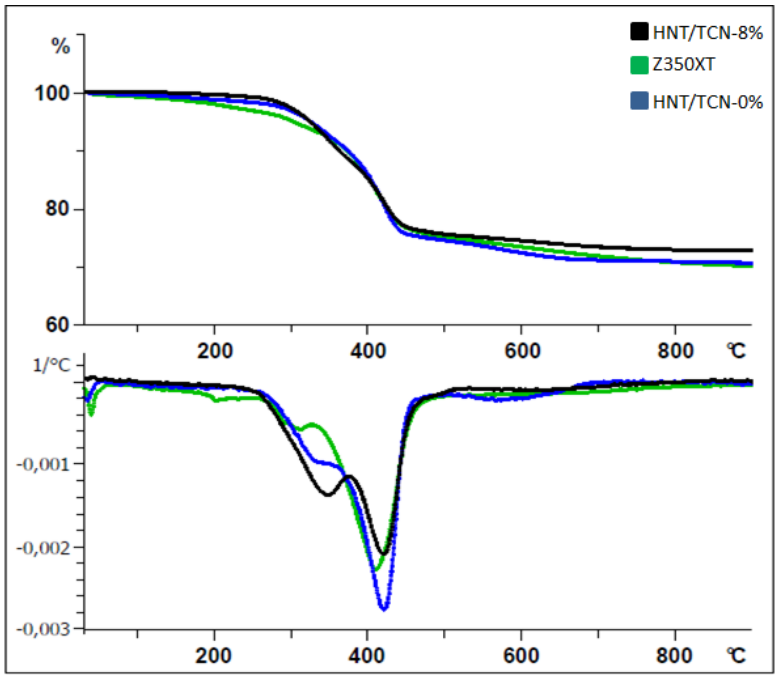


Figure 2. Results of Thermogravimetric analysis.

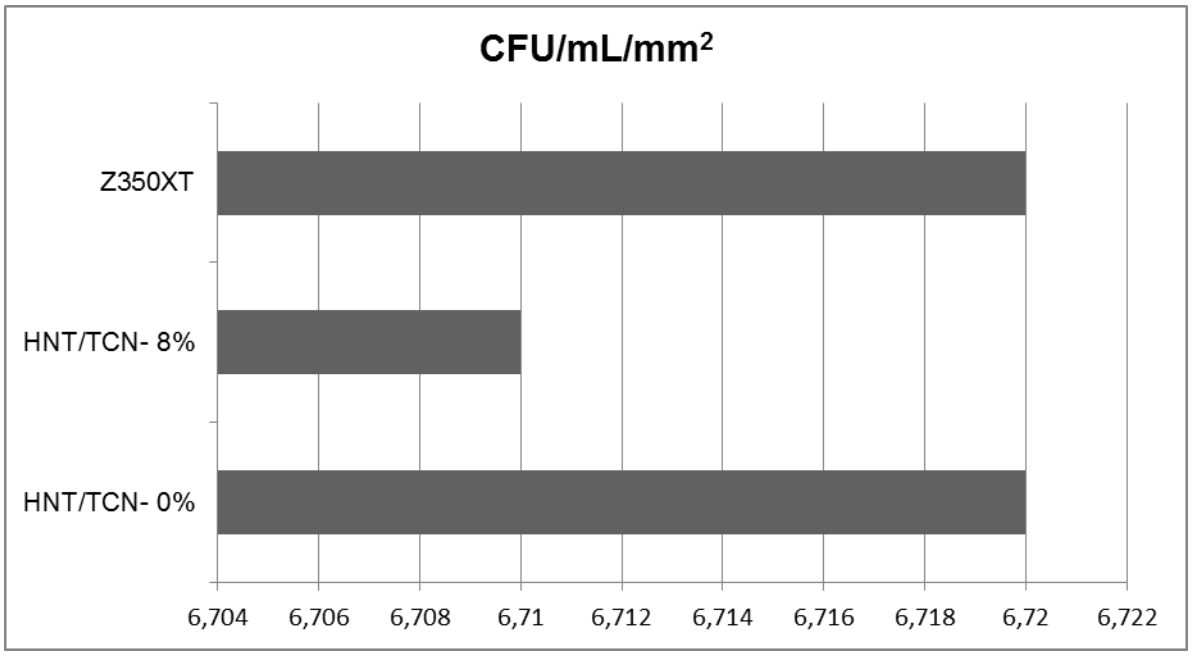


Figure 3. Results of microbiological tests, as colony forming units after 5 days (CFU)/mL/mm^2. This test showed no statistical difference between experimental groups and comercial resin-composite (p=0.511).

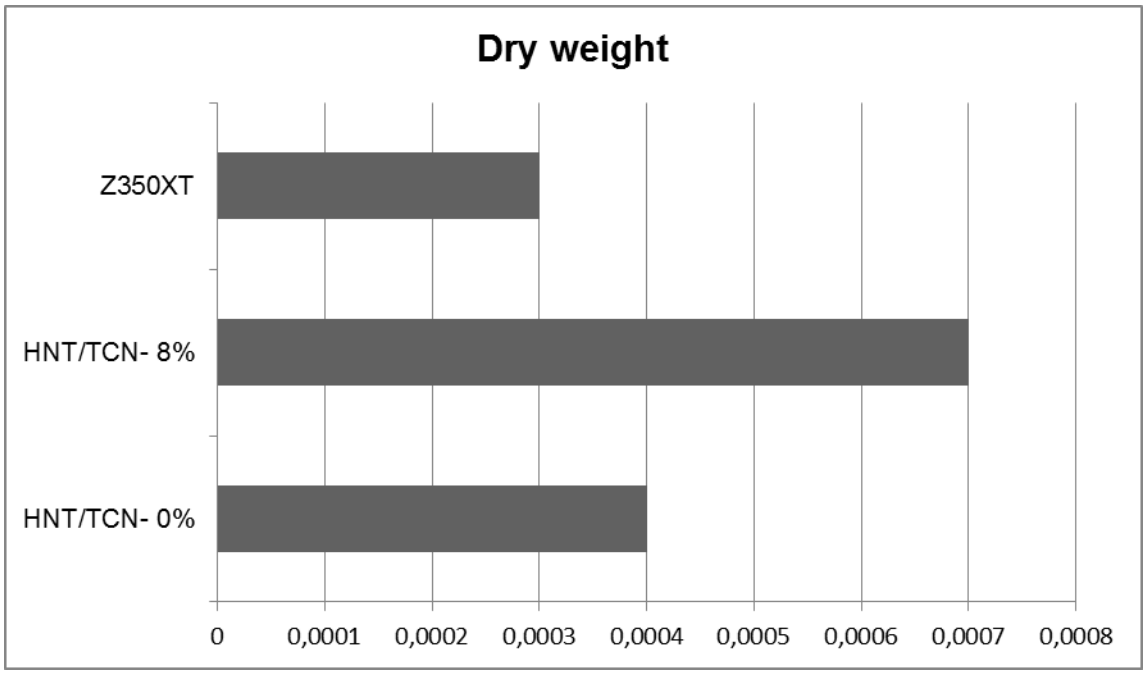
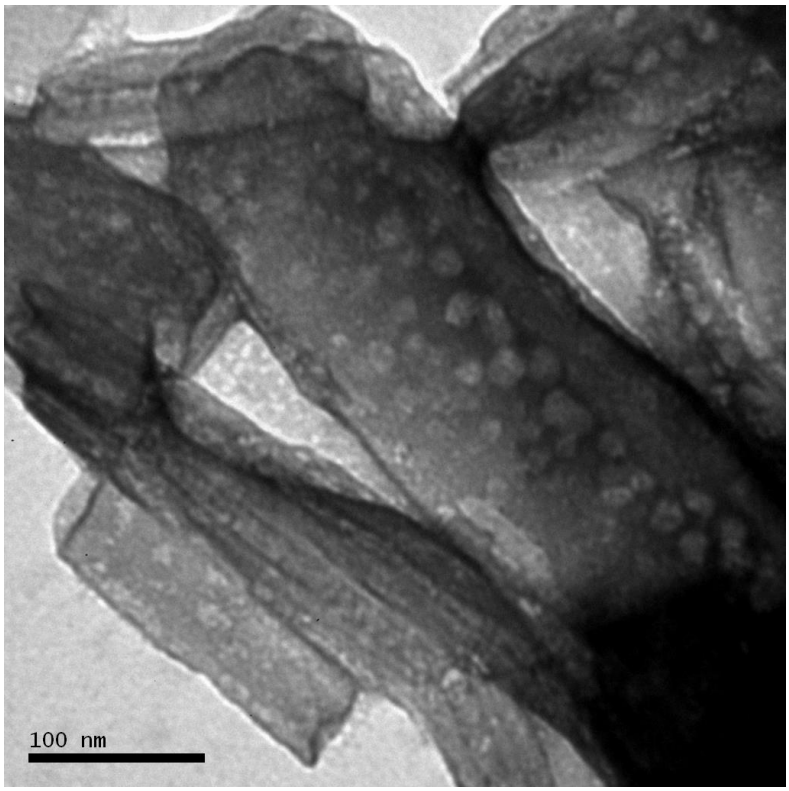


Figure 4. Results of the biofilm dry weight after 5 days. This test showed no statistical difference between experimental groups and comercial resin-composite (p=0.557).

Figure 01. TEM image that show the presence ofTCN nanoparticles with diameter of 5–10 nm.



4 CONCLUSÃO

Após investigar o comportamento do reforço de nanotubos de haloisita/triclosan em resina composta experimental pode-se observar que esta incorporação gerou uma tendência de melhoria nas propriedades físico-químicas e biológicas deste compósito, visto que:

- Quanto ao grau de conversão, o triclosan não promoveu prejuízos à polimerização,
- Os nanotubos permitiram um aumento numérico nos valores do modulo de elasticidade, grau de conversão e estatístico nos valores de resistência flexural, tan-δ, temperatura de transição vítrea e módulo elástico dinâmico,
- Numericamente, houve uma tendência de descréscimo na quantidade de unidades formadoras de colônias de *S.mutans*.

Assim, pesquisas futuras devem ser realizadas para sugerir tratamentos com maior longevidade usando este material experimental.

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