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MARY ANNE SAMPAIO DE MELO

ESTUDOS DO EFEITO ANTICÁRIE DE MATERIAIS ODONTOLÓGICOS BENEFICIADOS POR NANOTECNOLOGIA

FORTALEZA

2012

MARY ANNE SAMPAIO DE MELO

ESTUDOS DO EFEITO ANTICÁRIE DE MATERIAIS ODONTOLÓGICOS BENEFICIADOS POR NANOTECNOLOGIA

Tese apresentada ao Programa de Pós-Graduação em Odontologia da Faculdade de Farmácia, Odontologia e Enfermagem da Universidade Federal do Ceará, como requisito parcial para obtenção do Título de Doutor em Odontologia.

Área de Concentração: Clínica Odontológica

Orientadora: Profa. Dra. Lidiany Karla Azevedo Rodrigues

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RESUMO

A aplicação da nanotecnologia na odontologia pode promover a otimização de características estéticas e mecânicas de materiais odontológicos bem como propiciar uma ação anticárie pelo menor acúmulo de biofilme oral sobre estes materiais e liberação de compostos antimicrobianos ou remineralizantes. Objetivos: 1) revisar a literatura concernente ao uso de nanopartículas em materiais odontológicos com implicações diretas ou indiretas no controle de lesões de cárie (capítulo 1); 2) investigar *in vitro* a liberação de flúor e o potencial inibitório de cimento ortodôntico nanoparticulado fluoretado na desmineralização do esmalte dental submetido a modelo microbiológico de desmineralização (capítulo 2); 3) estudar o efeito *in situ* de resina composta fluoretada contendo nanopartículas na liberação de flúor e na capacidade inibitória da desmineralização em esmalte (capítulo 3); 4) avaliar o efeito da incorporação de nanopartículas de prata (NAg) e fosfato de cálcio amorfo (NACP) em sistema adesivo comercial na resistência de união à dentina humana bem como o efeito antimicrobiano em modelo microcosmo de biofilme oral (capítulo 4); 5) avaliar o efeito da incorporação de monômero associado a quaternário de amônio (QADM), NAg e NACP em sistema adesivo experimental na resistência de união à dentina humana bem como o efeito antimicrobiano em modelo microcosmo de biofilme oral (capítulo 5) e 6) estudar o efeito *in situ* de resina composta contendo NACP no biofilme oral e em lesões de cáries em esmalte (capítulo 6). Metodologia: foram realizados: uma revisão de literatura, 3 estudos *in vitro* com indução de cárie por modelo microbiológico e 2 estudos *in situ* relacionados aos objetivos citados. Resultados: o desenvolvimento de materiais nanométricos anticárie ainda necessita de um melhor conhecimento dos mecanismos de ação, segurança e eficácia das nanopartículas (capítulo 1). A liberação de flúor e o potencial inibitório na desmineralização do esmalte de cimento ortodôntico nanoparticulado fluoretado foi inferior ao apresentado por cimento de ionômero de vidro modificado por resina (capítulo 2). Resina composta nanoparticulada apresentou pequena atividade anticárie *in situ* sem alteração significante de sua lisura de superfície (capítulo 3). A inclusão de NAg e NACP em sistema adesivo apresentou significativo efeito antimicrobiano *in vitro* sem diminuição da resistência de união quando comparado ao controle (capítulo 4). A atividade metabólica, produção de ácido lático e número de unidades formadoras de colônias foram menores em biofilmes formados sobre sistemas adesivos experimentais contendo QADM, NAg e NACP sem diminuição da resistência de união quando comparado ao controle (capítulo 5). Compósito com NACP liberou mais cálcio e fósforo em biofilme cariogênico formado *in situ* e propiciou menores lesões de cárie em esmalte ao redor das restaurações que o compósito controle (capítulo 6). Conclusão: a presença de nanopartículas de carga na composição básica de materiais odontológicos não garante por si só uma atividade anticárie efetiva. A incorporação de QADM, NAg e NACP em materiais odontológicos podem ter efeito anticárie, no entanto, mais estudos *in vitro*, *in situ* e clínicos precisam ser executados para a comprovação de sua eficácia e segurança possibilitando que o uso destes materiais se torne uma realidade na odontologia preventiva e restauradora.

Palavras-chave: Cárie dentária, nanotecnologia, biofilme dentário, materiais dentários.

ABSTRACT

The application of nanotechnology in dentistry can promote the optimization of aesthetic and mechanical characteristics of dental materials as well as provide an anticaries action due to the lowest oral biofilm accumulation on these materials and release of antimicrobial or remineralizing compounds. Objectives: 1) to review the literature concerning the use of nanoparticles in dental materials with direct or indirect implications for the control of caries lesions (chapter 1), 2) to investigate *in vitro* the fluoride release and the inhibitory potential of nanoparticulated fluoridated orthodontic cement on demineralization of enamel subjected to microbiological demineralization model (chapter 2), 3) to study the *in situ* effect of nanoparticle composite containing fluoride on fluoride release and the inhibition of enamel demineralization (chapter 3) 4) to evaluate the effect of incorporation of silver nanoparticles (NAg) and amorphous calcium phosphate (NACP) in commercial adhesive system on bond strength to human dentin and the antimicrobial effect in model oral biofilm microcosm (chapter 4), 5) to evaluate the effect of incorporation monomer associated with quaternary ammonium (QADM), NAg and NACP in experimental adhesive system on bond strength to human dentin and the antimicrobial effect in model oral biofilm microcosm (chapter 5) and 6) to study the *in situ* effect of composite resin containing NACP on the oral biofilm and enamel caries lesions (chapter 6). Methodology: a literature review, 3 *in vitro* studies with microbiological caries model and 2 *in situ* studies were performed according to the cited objectives. Results: developing anticaries nanomaterials still need a better understanding of the action mechanisms, safety and efficacy of nanoparticles (chapter 1). Fluoride release and the enamel demineralization inhibition of the nanoparticulated fluoridated orthodontic cement were lower than that shown by resin-modified glass ionomer cement (chapter 2). Nanoparticle-containing composite showed little in situ anticaries activity with no significant alteration of its surface roughness (chapter 3). The inclusion of NAG and NACP in adhesive system showed significant *in vitro* antimicrobial effect without decreasing bond strength when compared to control (chapter 4). Metabolic activity, lactic acid production and number of colony forming units were lower in biofilms formed on experimental adhesive systems containing QADM, Nag and NACP without decreased bond strength when compared to control (chapter 5). NACP composite released more calcium and phosphorus in cariogenic biofilm formed *in situ* and caused shallow caries lesions on enamel around the restorations that the control composite (chapter 6). Conclusion: The presence of nanoparticles fillers as basic composition of dental materials does not assure effective anticaries activity. Incorporating QADM, Nag and NACP in dental materials can have anticaries effect, however, more *in vitro, in situ* and clinical studies need to be performed to prove its efficacy and safety allowing the use of these materials becomes a reality in preventive and restorative dentistry.

Key words: Dental caries, nanotechnology, oral biofilm, dental materials.

SUMÁRIO

1. INTRODUÇÃO GERAL

A nanotecnologia ganhou importância substancial nos anos 90 com o conhecimento de que propriedades dos materiais (óticas, mecânicas, magnéticas, catalíticas, etc) dependem fortemente do tamanho das partículas do material.[1] Nanomateriais são por definição os materiais com componentes estruturados com dimensões nanométricas (~100 nm), tais como nanofios, nanotubos, nanopartículas, materiais nanocristalinos e grãos nanométricos.[2] O grande diferencial desses materiais é potencializar propriedades físicas e químicas em concentrações extremamente reduzidas e delegar características antes não apresentadas por um dado produto. Esta extensão de propriedades se deve basicamente ao fato de tais estruturas possuírem dimensões nanométricas que resultam em uma área de superfície elevada, maior grau de dispersão e exibição de fenômenos físicos, químicos e/ou biológicos novos e modificados, dependentes do tamanho da estrutura.[3,4]

Existe uma infinidade de áreas onde a nanotecnologia pode oferecer uma contribuição significativa, algumas das quais já possuem produtos sendo comercializados.[5] A Odontologia tem procurado não se excluir da corrida por esta tecnologia, e a área de materiais dentários tem sido favorecida por esse segmento científico estabelecido como um dos mais inovadores da atualidade. Diversas áreas da saúde, incluindo-se a odontologia, consistem nos setores com premissa de serem mais impactados pelas aplicações da nanotecnologia no Brasil entre 2011-2015.[6]

Na Odontologia restauradora, os materiais restauradores resinosos tem recebido essa tecnologia pela introdução de nanopartículas e outros nano-objetos que podem ser dissolvidos em um meio macroscópico (matriz) e polimerizados no sistema resinoso, alterando as propriedades deste meio macroscópico no qual estão inseridos.[7]

Atualmente no Brasil, já são comercializados nanomateriais odontológicos de uso clínico que apresentam ganhos e melhorias nos seus desempenhos relacionados as suas propriedades mecânicas e estéticas.[8,9] Cimentos ortodônticos têm sido introduzidos no mercado com sua composição alterada por incorporação de nanopartículas. Avanços no comportamento óptico das restaurações, capacidade de manutenção do polimento e de propriedades físicas, tais como resistência às forças mastigatórias e ao desgaste têm sido observados nestes materiais.[10]

Apesar de todo o aprimoramento das características mecânicas e ópticas dos materiais resinosos nanoparticulados, os mesmos continuam a falhar em virtude do desenvolvimento de uma das mais prevalentes doenças humanas induzidas por bactérias, a cárie dental. Esta é uma

doença biofilme-açúcar-dependente que, quando não tratada, progride de forma lenta, porém bastante invasiva destruindo tecidos mineralizados e formando cavitações nos dentes.[11] A pequena eficácia dos materiais nanoparticulados frente à doença cárie permanece como principal desafio a ser superado no aprimoramento desses materiais.[12]

Estudos sugerem que a incorporação de partículas de vidro fluoretado ou compostos puros como trifluoreto de itérbio (YbF_3), trifluoreto de ítrio (YF_3) e fluoreto de estrôncio (SrF_2) em materiais restauradores diretos ou cimentos ortodônticos pode funcionar como uma abordagem auxiliar na prevenção e progressão de lesões de cárie.[13-15] Como a cinética de liberação de flúor é bastante complexa, diversos fatores como o tipo de partícula fluoretada, o tamanho da partícula, o processo de silanização e a porosidade da matriz, podem influenciar no processo de liberação de flúor. Desta forma, a presença de nanopartículas nesses materiais pode significar uma promoção ao processo inibitório de cárie, uma vez que resinas com partículas de tamanho reduzido podem apresentar padrões diferenciados de difusão de íons[16] e menor acúmulo de biofilme, em virtude de uma maior lisura de superfície promovida por esses materiais. Um aumento na liberação de íons fluoreto e consequente captação desses íons pelos tecidos adjacentes às restaurações seriam presumidamente relevantes para a prevenção da cárie ao redor destes materiais. A investigação destas possíveis propriedades foi executada em estudos experimentais com produtos fluoretados contendo nanopartículas já comercializados constituindo-se dois capítulos desta tese.

Sistemas adesivos e resinas compostas têm sido largamente utilizadas em restaurações diretas de preparos cavitários realizados por consequência da doença cárie[17], entretanto estima-se que aproximadamente 50% das restaurações adesivas confeccionadas necessitem ser refeitas, sendo a cárie secundária uma das causas mais frequente de insucesso.[18] Simultaneamente, a percepção de que a nanotecnologia representa um novo patamar, com imensos benefícios ainda não adequadamente explorados, levou à investigação da inclusão de agentes antimicrobianos/remineralizantes beneficiados pela nanotecnologia em sistemas resinosos, especificamente sistemas adesivos dentinários experimentais. Tais sistemas apresentam a premissa que podem contribuir para a inibição do surgimento e progressão de lesões de cárie.

O uso da nanotecnologia vem modificando substancialmente a aplicabilidade e forma de utilização de diversos agentes.[19] O uso da prata em dimensões nanométricas tem progressivamente aumentado na área médica em virtude de suas propriedades antibacterianas. O aumento das superfícies de contato das nanopartículas quando comparado àquelas das usuais dimensões micrométricas e o fato das reações químicas ocorrerem nas superfícies,

explica a sua maior reatividade em comparação a partículas maiores.[20] Tal condição se mostra fundamental na incorporação de prata em materiais resinosos uma vez que possibilita a redução na concentração necessária para uma eficaz atividade antibacteriana e permite, ao mesmo tempo, a obtenção de um material sem comprometimento das características estéticas, sempre almejadas e enaltecidas em resinas compostas.[21] A redução da concentração de prata quando esta se apresenta na forma de nanopartículas contribui positivamente para a diminuição dos riscos de sua toxidade, haja vista que a concentração efetiva para atuação contra microrganismos é de 0,1 μg/L e a concentração tóxica a seres humanos é de 10 mg/L.[22] Em recentes estudos, resinas compostas contendo 0,01% (0,1 μg/L) de nanopartículas de prata obtiveram uma redução de cerca de 50% da carga bacteriana sobre resinas compostas.[23,24]

Além da incorporação de nanopartículas de prata em polímeros resinosos como agente antimicrobiano, uma importante classe de materiais vem sendo testada com esse mesmo propósito: compostos quaternários de amônio.[25,26] Os cátions quaternários de amônio são íons poliatômicos carregados positivamente e com a estrutura NR_4^+ complexada à cadeia monomérica resinosa. O agente antibacteriano é co-polimerizado com outros monômeros presentes no sistema adesivo e permanece imobilizado na matriz polimérica, exercendo ação somente contra bactérias que entram em contato com o polímero.[27] Recentemente, um composto de amônio quaternário foi associado a um monômero dimetacrilato (QADM) formando um monômero mais reativo do que aqueles que apresentam somente um grupo metacrilato, por exibir diversos grupos funcionais de amônio quaternário livres e com parâmetros de solubilidade semelhantes aos das resinas atualmente em uso.[28] A atividade antibacteriana do QADM tem se demonstrado promissora em estudos *in vitro*[23,24] que utilizaram culturas planctônicas de bactérias orais ou modelos de biofilme, onde a concentração de 10% mostrou uma redução microbiológica satisfatória sem comprometimento da viabilidade celular de fibroblastos ou alteração enzimática de células teciduais humanas.[28]

Além dos materiais resinosos antibacterianos, outra abordagem para redução de lesões de cárie recorrentes ao redor de restaurações adesivas é a incorporação de agentes remineralizantes, tais como o fosfato de cálcio amorfo (ACP). Estudos prévios mostraram a possibilidade de aumento do conteúdo mineral do tecido dentário ao redor da restauração pela liberação de cálcio e fosfato do material restaurador e precipitação na forma de hidroxiapatita dentro da lesão de cárie.[29,30] Com vistas a obter a capacidade de inibição do processo de desmineralização, partículas de fosfato de cálcio foram desenvolvidas e incorporadas em resinas compostas. Estes materiais apresentaram resultados promitentes ao exibir em níveis

supersaturados de íons de cálcio (Ca) e fosfato (PO_4) em soluções aquosas.[31] Entretanto, o tamanho das partículas de Ca e PO_4 destes materiais variava de 1 a 55 µm (micropartículas) e os mesmos demonstraram resistência mecânica reduzida, o que os tornou inadequados para uso clínico. Por conseguinte, mais recentemente estudos *in vitro* mostraram o aprimoramento desses materiais restauradores com incorporação de partículas de reforço e liberadoras de íons cálcio e fosfato em tamanho nanométrico, a exemplo, fosfato de cálcio amorfo (NACP). Estes nanomateriais podem promover a remineralização sem detrimento das características mecânicas de resistência flexural, apresentando valores semelhantes aos de resinas compostas microparticuladas.[32-36] Adicionalmente, observou-se que estes materiais apresentam atividade antimicrobiana através da inibição da aderência e crescimento de *Streptococcus mutans*,[37] característica que somada às propriedades supracitadas torna ainda mais promissora a utilização deste material.

Tendo em vista o potencial da nanotecnologia em trazer inúmeros benefícios para materiais restauradores, principalmente no que se refere às propriedades anticárie, esta tese objetivou revisar a literatura sobre o atual desenvolvimento de materiais restauradores beneficiados pela nanotecnologia; investigar a possível ação anticárie de materiais fluoretados contendo nanopartículas atualmente comercializados bem como avaliar a ação antimicrobiana ou remineralizante de materiais dentários experimentais contendo agentes em nanoescala através de estudos investigativos laboratoriais (*in vitro*) e pré-clinicos (*in situ*).

2- PROPOSIÇÃO

Essa tese de doutorado será apresentada em capítulos, tendo como objetivos:

Capítulo 1: Revisar a literatura concernente ao uso de nanopartículas em materiais odontológicos com implicações diretas ou indiretas no controle de lesões de cárie;

Capítulo 2: Investigar *in vitro* a liberação de flúor e o potencial inibitório de cimento ortodôntico nanoparticulado fluoretado na desmineralização do esmalte dental, ao redor de braquetes ortodônticos, submetido a modelo microbiológico de desmineralização com biofilme mono-espécie;

Capítulo 3: Estudar o efeito *in situ* de resina composta fluoretada contendo nanopartículas de carga na liberação de flúor e na capacidade inibitória da desmineralização em esmalte. Verificar-se ainda o efeito do biofilme cariogênico na rugosidade da superfície de resinas compostas nanoparticuladas;

Capítulo 4: Avaliar o efeito da incorporação de nanopartículas de prata (NAg) e fosfato de cálcio amorfo (NACP) em um sistema adesivo comercial na resistência de união à dentina humana. Avaliar-se também o efeito antimicrobiano obtido com esta incorporação em modelo microcosmo de biofilme oral;

Capítulo 5: Avaliar o efeito da incorporação de monômero associado a quaternário de amônio (QADM), NAg e NACP em sistema adesivo experimental na resistência de união à dentina humana. Verificar-se também o efeito antimicrobiano obtido após esta incorporação em modelo microcosmo de biofilme oral;

Capítulo 6: Estudar o efeito *in situ* de resina composta contendo NACP no biofilme oral e em lesões de cáries em esmalte ao redor das restaurações.

3- CAPÍTULOS

REGIMENTO INTERNO

Esta tese 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 co-autoria do candidato. Por se tratar de pesquisas envolvendo seres humanos, os projetos de pesquisa dos trabalhos referente aos capítulos 3 e 6 foram submetidos à apreciação do Comitê de Ética em Pesquisa da Universidade Federal do Ceará, tendo sido aprovados (Anexo A e B). Assim sendo, esta tese é composta de seis capítulos contendo artigos a serem submetidos para publicação em revistas científicas, conforme descrito abaixo:

Capítulo 1

"*Current perspectives of nano-restorative materials for caries management*" Mary A.S. Melo, Sarah F.F. Guedes, Hockin H. K. Xu, Lidiany K.A. Rodrigues. Este artigo será submetido à publicação no periódico "Journal of Biomaterials and Nanobiotechnology".

Capítulo 2

"*Caries inhibition around orthodontic brackets by nanofilled fluoride-releasing composite*" Mary A. S. Melo, Weslanny A. Morais; Vanara F. Passos; Juliana P. M. Lima; Lidiany K. A.Rodrigues. Este artigo será submetido à publicação no periódico "Brazilian Oral Research".

Capítulo 3

"*In situ randomised trial investigating anticaries properties of fluoride-releasing nanohybrid composite resin"* Mary A. S. Melo, Bruna M. Codes; Vanara F. Passos; Juliana P. M. Lima; Lidiany K. A. Rodrigues. Este artigo será submetido à publicação no periódico "Operative Dentistry".

Capítulo 4

"*Antibacterial dental adhesive containing silver and amorphous calcium phosphate nanoparticles"* Mary A. S. Melo; Lei Cheng; Ke Zang, Michael D. Weir; Lidiany K. A. Rodrigues; Hockin H. K. Xu. Este artigo foi submetido à publicação no periódico "Dental Materials".

Capítulo 5

"*Dental bonding agents containing antibacterial agents and calcium phosphate nanoparticles"* Mary A. S. Melo; Lei Cheng; Michael D. Weir; Ru-ching Hsia; Lidiany K. A. Rodrigues; Hockin H. K. Xu. Este artigo foi submetido à publicação no periódico "Journal of Biomedical Materials Research Part B: Applied Biomaterials".

Capítulo 6

"*Anticaries effects of novel nanocomposites with Ca and PO4 ion release: A randomized human in situ trial"* Mary A. S. Melo; Michael D. Weir; Lidiany K. A. Rodrigues; Hockin H. K. Xu. Este artigo foi submetido à publicação no periódico "Dental Materials".

3.1 CAPÍTULO 1

Current perspectives of nanotechnology-based restorative materials for caries management

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Key words: nanoparticles, dental materials, nanotechnology, dental caries

ABSTRACT

Nanotechnology has been applied to dental materials as an innovative concept to the development of materials with better properties and anticaries potential. This review is current progress and future applications of functional nanoparticles for dental restoratives with direct or indirect implications to caries management. The literature was searched from January 2000 to September 2012 for review and original research papers relating "nanotechnology", "dental materials", "antimicrobial", "remineralizing", and "dental caries" using Medline, Web of Sciences, Scopus and manual tracing of references cited in key papers otherwise not elicited. The main approaches related to anticaries effects included silver, zinc oxide, calcium-phosphate, calcium fluoride, quaternary ammonium polyethylenimine and nano-hydroxyapatite/nano-fluorohydroxyapatite incorporated in restorative materials such as composite resins, glass ionomer and adhesive systems. Existing literature reveals that there is strong evidence that nanomaterials present great potential to decrease biofilm accumulation, inhibit the demineralization process being a promising approach for remineralizing tooth structure. Although the literature findings are at the initial phase of evidence and more investigations are necessary, these results are encouraging and open the doors to future clinical studies that will allow the therapeutic value of nanotechnology-based restorative materials to be established.

INTRODUCTION

Dental caries remains the most common and widespread oral infectious disease, resulting in the destruction of tooth structure by acidic attack from cariogenic bacteria found in dental biofilm.[1] This process still represents a significative burden, especially for individuals at high risk for its development. According to contemporary preventive and minimal intervention dentistry, significant progress has been made in reducing and controlling dental caries using fluoride therapies.[2] Fluoride can inhibit demineralization in early carious lesions and promote remineralization of hard dental tissues. Water fluoridation, fluoride-containing dentifrices and fluoride-releasing materials in association with other therapies such as placement of sealants and improved oral hygiene have been used as preventive approaches. However, prevention and management of early and secondary caries lesions are still challenges for dental caries research.[3]

Nanotechnology has contributed to the development of several areas in dentistry bringing tangible benefits, which are moving from the bench into clinical practice. This is especially evident in applications in a wide range of dental materials: composites, bonding systems, imprint materials, ceramics, coatings for dental implants and bioceramics.[4] In the preventive dentistry field, nanotechnology is promising to yield novel strategies to be applied to restorative materials to control and manage oral biofilms and remineralization of caries lesions.[5]

In this review, we focus on recent advances in the applications of novel functionalized nano-structured components added to dental materials with anticaries capabilities. To identify suitable literature, an electronic search was performed using Medline, Web of Sciences, Scopus and manual tracing of references cited in key papers considering the keywords "nanotechnology", "dental materials", "antimicrobial", "remineralizing", and "dental caries" from January 2000 to September 2012. Approaches by addition of agents such as silver, zinc oxide, calcium-phosphate, calcium fluoride, quaternary ammonium polyethylenimine and nano-hydroxyapatite/nano-fluorohydroxyapatite incorporated in restorative materials such as composite resins, glass ionomer and adhesive systems were reviewed. Dental studies pertinent to key aspects of this review, and those that focus on nanoparticle additions in anticaries dental materials commercially available were selected.

WHAT DOES "NANO" MEAN?

The word "nano" is one of the most attractive prefixes in the contemporary materials science due to tangible benefits provided by the micro-to-nano shift. To explain these phenomena related to nanoscale, there is a specific field named nanoscience.[6] The production of functional materials and structures in the nanoscale using various physical and chemical methods and the application of nanoscience discoveries is known as nanotechnology.[7] This field focuses on the control and manipulation on nanoscale, lengths of 1–100 nm range, that have novel properties and functions because of their small and/or intermediate size.[8]

The intense interest in using nanotechnology in restorative dental materials is based on two main factors that cause the properties of nanomaterials to significantly differ from other materials. One is related to the optical properties of materials containing these particles. Nanoparticles have dimensions well below the wavelength of visible light (400–800 nm); they cannot scatter that particular light resulting in the inability to detect the particles by naked eye.[9] The other factor is due to the high ratio of surface area to volume. For example, one particle of 30 nm has 5% of its atoms on its own surface, other particle with 10-nm size has 20%, and another particle with 3-nm size has around 50%.[10] This is particularly relevant to molecular scale interaction between these nanoparticles and the matrix of materials. Recently, nanotechnology is being extensively used in studies of emerging functionalized nanoparticles incorporated in dental materials that may be translated into strategies to prevent oral biofilm acumulation and dental caries lesions (Table 1).

Nanotechnology in current restorative materials

Considering the commercially available direct restorative materials, composite resins were by far the most nanotechnology-benefited materials, although glass ionomer and adhesive systems also have gained some benefits. Composite resins have been predominantly selected as restorative material for cavitated carious lesions but are also used for other applications in dentistry, including cavity liners, pit and fissure sealants, cores and buildups, indirect restorations, cements for crowns or orthodontic devices, provisional restorations, endodontic sealers, and root canal posts.[11]

A dental nanocomposite can be defined as a material that contains one or more nano-sized components in a polymer matrix.[12] Nanoparticles are available in two forms: a single unit particle (nanomer) and a group of nanoparticles (nanocluster). There are currently two types of dental nanocomposites available, which are the nanofills and the nanohybrids. Nanofills contain nanometer-sized particles (1–100 nm) throughout the resin matrix, with no

other large primary particles being included. Nanohybrids consist of larger particles ranging from 0.4 to 5 μm mixed with nanoparticles.[9]

Table 1. Nanotechnology-based strategies for combating oral biofilms and dental caries.

Nanotechnology-based agent	Action	Benefiting restorative material	Reference
Nanosilver (NAg)	Antimicrobial	Composite resin; Dental primer; Dental adhesive	Cheng et al. 2012[39] Cheng et al. 2012[40] Zheng et al. 2012[41]
Zinc oxide (ZnO) nanoparticles	Antimicrobial	Composite resin	Sevinc and Hanley, 2010[49]
Quaternary ammonium polyethylenimine (QAS-PEI)	Antimicrobial	Composite resin; Glass ionomer cement;	Beyth et al. 2006;2010[54,55] Beyth et al. 2012[53]
Calcium fluoride (CaF_2) nanoparticles	Remineralizing	Composite resin	Xu et al. 2008[70];2010[71]
Calcium Phosphate ($Ca\text{-}PO_4$) Nanoparticles	Remineralizing	Composite resin; Dental adhesive; Glass ionomer cement	Xu et al. 2007[60] Weir et al, 2012[62]
Nano-hydroxyapatite (nano-HA) or nano-fluorohydroxyapatite (nano-FHA)	Remineralizing	Resin-modified glass ionomer cement	Lee et al. 2012[78] Lin et al. 2011[81]

Advantages expressed by these materials include superior hardness, flexural strength, modulus of elasticity, wear resistance, polishability, translucency, reduction in filling shrinkage, and excellent handling properties. They have been attributed to the reduction in filler size of inorganic components.[13-16] The dispersed phase or the filler particles provide strength and reinforcement to the matrix. Fillers include quartz, alumina silicate, pyrolytic silica, lithium aluminum silicates, etc. The fillers vary in particle size depending on the manufacturing process. Oxide nanoparticles have been the main type of nanomaterials included with this purpose.[9,17]

In the clinical management of the caries process, the use of nano restorative materials may be related to less biofilm accumulation promoted by a decrease in the surface roughness of these materials, which is one of the most significant factors for initial bacterial adhesion.[18,19] This is especially important to composite resins since previous studies have reported more biofilm accumulation over composites than over other restorative materials, which may lead to the development of gingival inflammation and/or secondary caries.[20-22] The nanotechnology strategies for inhibition of biofilm formation on restorative materials still warrant further investigation.

Another class of materials benefited from nanotechnology is resin-modified glass ionomer cements. A material was recently introduced in the dental market, which contains fluoroaluminosilicate glass and nanofiller "clusters", with the aim to improve its mechanical properties. It has been investigated if the nanoionomer shows a performance similar to ionomeric materials in regard to fluoride-releasing properties, and to composite classes with regard to esthetics and resistance,[23,24] Nanoionomers showed be suitable for bonding since they fulfil the bond streght ranges for clinical acceptability, but they are inferior to a orthodontic cements.[24]

As it is known, nanotechnology-based approach for caries management is still in an initial phase of evolution, where much needs to be understood about the behavior of the materials in nanoscale. The anticaries and remineralizing effects of nano ionomer has been investigated.[25,26] Similar to the dental composites, a continuous decrease in particle dimensions results in a large interface between filler and organic matrix. This changes not only alter the morphology of the material but also operate as a influencing factors, modifying the mobility of the macromolecules at the particle surfaces and ion kinetics.[25] It is possible that the increased surface area to volume ratio of the nano-sized filler has the capacity to alter the fluoride-releasing pattern of the material. Neelakantan et al.[27] demonstrated a higher release of fluoride from the nano ionomer than other restorative materials. However, Mitra et al.[26] showed that the fluoride ion release behavior of nano ionomer in a long-term evaluation was similar to typical conventional and resin-modified glass ionomer. More consistent evidence is necessary to support the nano ionomers.

The adoption of nanotechnology has noticeably improved many of the properties of composite resins. Nanotechnology may improve and transform dental materials, and give a potential for a protective and promoting behavior, besides improving optical and mechanical properties bring advantageous changes in properties, either within the material itself or in the material-tooth complex. This possibility is extremely relevant to the role of dental materials in controlling oral biofilms and combating dental caries.

NANOTECHNOLOGY-BASED STRATEGIES FOR CARIES MANAGEMENT

Silver nanoparticles

The broad antimicrobial effect of silver ions or salts is well known and it has been used in different fields in medicine for years. More recently, nanoparticles of silver (NAg) have been used in a wide range of antimicrobial applications.[28]

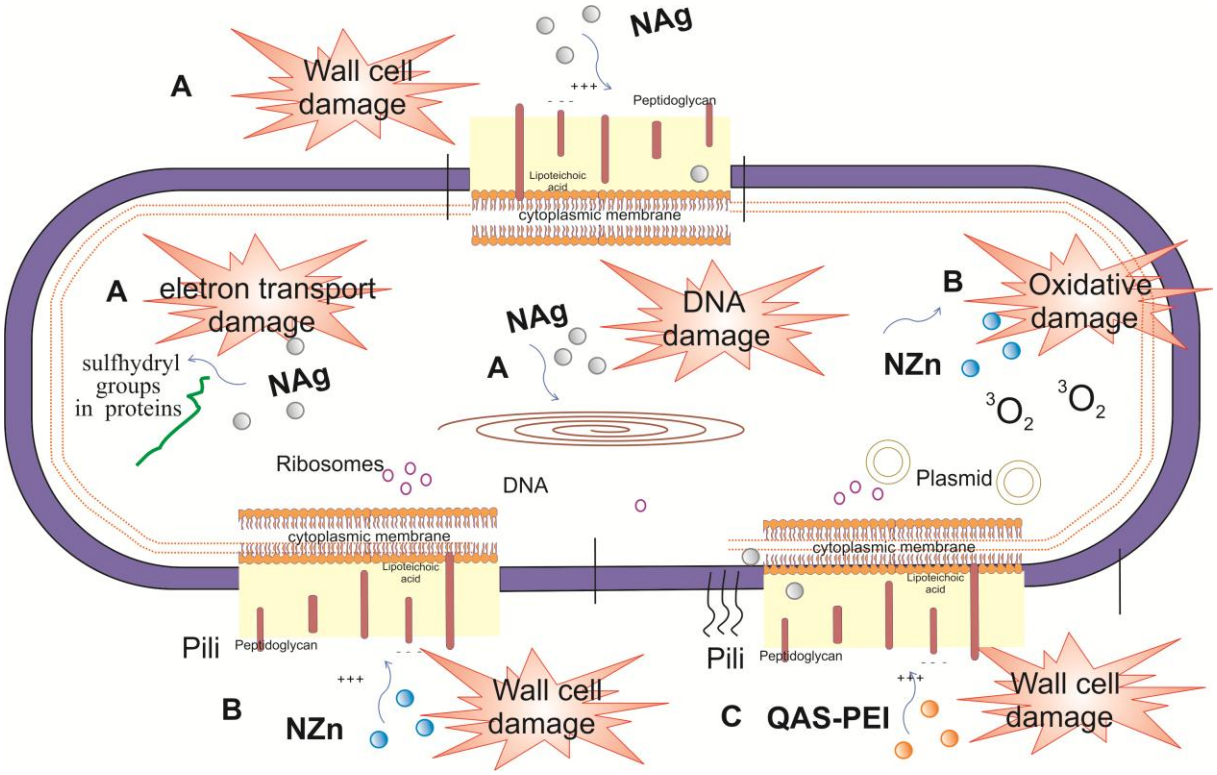

Figure 1. Schematic of a bacterial cell showing the targeted components by nanoparticles of: A- Nanosilver; B- Zinc Oxide and C- Quaternary ammonium polyethylenimine nanoparticles.

The mode of action of NAg has not been clearly elucidated, but observations from recent studies suggest that the antimicrobial action of silver is proportional to the amount of released bioactive silver ions (Ag+) and its interaction with bacterial cell membranes.[29] The incremental higher surface area to volume ratio of nanoparticles potentially results in high reactivity and stronger antibacterial activity.[30] A larger surface area of the NAg allows a larger amount of atoms to interact with their surroundings. Several studies suggested that bactericidal effect of NAg is size-dependent, with smaller NAg being more potent.[31,32] It is believed that silver ions provide the bactericidal effect by interactions with peptidoglycan cell wall and the cytoplasmatic membrane, which causes its lysis[8]. In addition, silver ions affect the nucleic acid structures and prevent the bacterial DNA replication[9]. Furthermore, silver ions interact with the exposed sulfhydryl groups in bacterial proteins, especially with the enzymes involved in vital cellular processes such as the electron transport chain (Figure 1).[33,34]

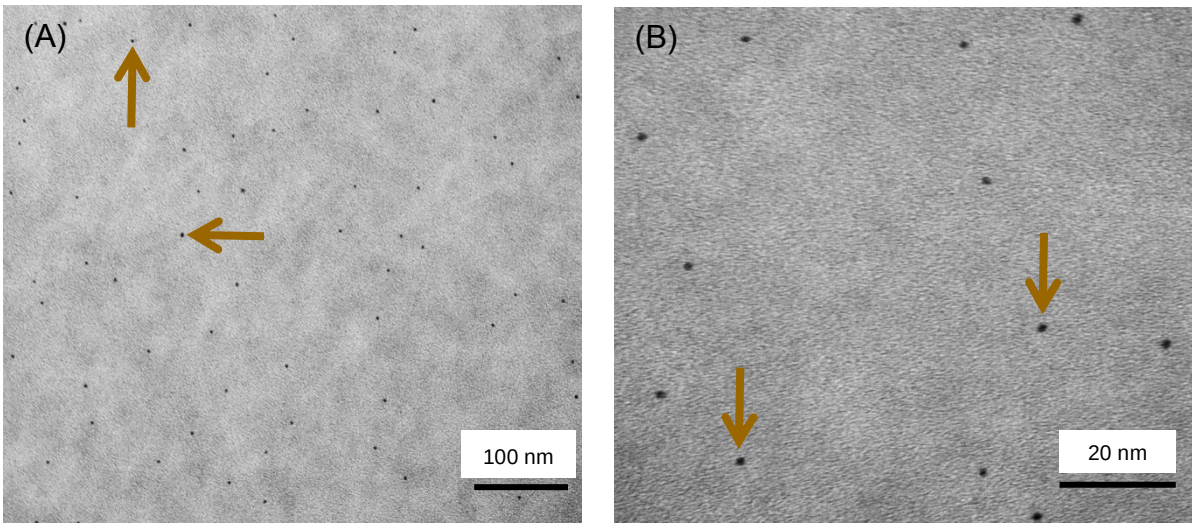

Figure 2. Representative transmission electron microscopy (TEM) micrographs of the size and dispersion of silver nanoparticles (NAg) in a resin matrix: (A) lower and (B) higher magnifications (Adapted from ref # 39, with permission).

NAg have been incorporated in restorative materials to combat the cariogenic bacteria colonization in the marginal gaps and on their surfaces. NAg exhibit antibacterial effects against a large number of bacterial species[35] including cariogenic bacteria such as *Streptococcus. mutans* and lactobacilli.[36,37]

NAg-containing composite adhesive for bonding orthodontics brackets have antibacterial effects on *S. mutans.*[38] Similarly, inhibitory effects on the growth of *S. mutans* were found when NAg was incorporated in a dental composite. Cheng et al.[39,40] carried out *in vitro* studies on the effects of nanocomposite containing NAg of 2.7-nm particle size well-dispersed in the resin matrix (Figure 2). Their results demonstrated a high antibacterial efficacy against oral biofilms, without significantly compromising the composite color or mechanical properties.

Modified adhesives by the addition of NAg also achieved great antibacterial effects on oral biofilms, without altering the dentin bond strength.[41] Besides, the small size of NAg could allow them to flow with the primer into dentinal tubules to kill residual bacteria inside the tubules.[42]

There is an ongoing debate regarding the role of released Ag ions from nanosilver and its toxicity. To address this issue, NAg at 0.08 to 0.10% mass fraction concentrations have been used in experimental investigations.[41,42] These concentrations are very low compared to the concentration of 10%, capable of inducing citotoxity to human cells.

Durner et al.[43] suggested that NAg may influence the degree of monomer conversion in dental materials and lead to an increase in elutable residual monomers from the hardened composite. Some monomers or compounds eluted from composites are known to cause allergic reactions or may metabolized to reactive oxygen species.[43] Further understanding of the complex interactions between biofilm and NAg as well between silver nanoparticles and the polymerization process, may hold the key to more effective application of this antibacterial agent in restorative materials.

Zinc oxide (ZnO) nanoparticles

Metals have been used as antimicrobial agents for decades in dentistry. Similar to silver, zinc oxide has shown antibacterial effects against several types of bacteria, including *S. mutans.*[44] ZnO particles in nano-size have been found to be more effective than conventional particles against both Gram negative and Gram positive bacteria.[45] Xie et al.[46] suggested that the antibacterial mechanism of ZnO nanoparticles is most likely due to modification of the cell membrane activity and oxidative stress since they generate active oxygen species such as H_2O_2 which inhibit growth of planktonic microbes (Figure 1). The authors described, via analysis of ZnO nanoparticle-modulated stress gene expression, that the transcription levels of oxidative and general stress genes were significantly increased, up to 3- to 52-fold in a gram (-) bacteria. On the other hand, these nanoparticles have selective toxicity to bacteria with minimal effects on human cells.[47]

Another potential antimicrobial mechanism of ZnO nanoparticles is the leaching of Zn^{2+} into the growth media. Zinc ions can inhibit biofilm formation by inhibiting the active transport and metabolism of sugars as well as disrupting enzyme systems by displacing magnesium ions essential for enzymatic activity of the of dental biofilms.[48] Hernández-Sierra et al.[49] recently investigated the antibacterial effect of zinc oxide nanoparticles on *S. mutans* strains. These particles were able to inhibit bacteria grown. However, a higher concentration than nanosilver was required to be effective. The antibacterial activity of dental composites containing ZnO nanoparticles were investigated by Sevinc and Hanley[50], which achieved moderate reduction in bacterial counts and biofilm growth when a 10% ZnO-nanoparticles-containing composite was used and compared to a commercial composite. However, a lower antibacterial efficacy was observed when compared to nanosilver-containing composite. Future research should address this issue to increase the antimicrobial potency of this agent.

Quaternary ammonium polyethylenimine (QAS-PEI) nanoparticles

Quaternary ammonium salts (QAS) are antibacterial agents and used widely in paint, water treatment, textile, and food industries.[51] Polymers containing quaternary ammonium salts have been incorporated into dental materials with antibacterial activities.[52,53] The advantage of QAS-containing composites is that the antibacterial agent is copolymerized with the resin by forming a covalent bonding with the polymer network, and therefore is immobilized in the composite and not released or lost over time. This method imparts a durable and permanent antibacterial capability to the composite.[39] The detailed antimicrobial mechanism of QAS is yet to be established; however, it appears that QAS materials can cause bacteria lysis by binding itself to the cell membrane and causing cytoplasmic leakage[54] (Figure 1) due to the high activity of polycationic agents that causes the absorption of positively charged polymers onto negatively charged cell surfaces of the bacteria.

In an attempt to further improve the antibacterial activity of this agent and reduce the effects of its incorporation on mechanical properties, quaternary ammonium polyethylenimine nanoparticles were incorporated in restorative materials.[55] Beyth et al.[56] showed the incorporation of QAS-PEI nanoparticles in dental composite resin at 1%. This compound exhibited an immediate and strong antibacterial effect against *S. mutans* and maintained this activity over 1 month without leaching out and with no alteration of the original mechanical properties of the composite resin. Comparable improvement was reached when QAS-PEI nanoparticles were incorporated in conventional glass ionomer and tested on *S. mutans* and *Lactobacillus casei*.[54] Future studies should focus on long-term antibacterial and mechanical durability.

Calcium phosphate (Ca-PO_4) nanoparticles

The comprehensive caries-prevention approaches should promote release of fluoride and other agents affecting the de/remineralization balance as well as antimicrobial strategies.[57] Founded on nanotechnology, several calcium phosphate nanoparticles of the more soluble calcium phosphate phases, such as monocalcium phosphate monohydrate (MCPM), dicalcium phosphate anhydrous (DCPA), tetracalcium phosphate (TTCP) and amorphous calcium phosphate (ACP) were developed.[58,59] The small size and high solubility of the nano calcium phosphate compounds made them good candidates to be anti-demineralizing and remineralizing agents. It has been demonstrated that calcium phosphate nanoparticles have the advantage of better ion-release profiles due to the small size to release

Ca and P ions in higher concentration than micro-sized particles.[60] ACP is easily transformed into crystalline phases such as octacalcium phosphate and apatite due to microcrystalline growth.[61] The presence of ACP nanoparticles (NACP) (Figure 3) in dental composite resins is an approach to continuously release calcium and phosphate ions into the oral environment. These ions can diffuse out of the interior of the pre-saturated resin to create a high local concentration at the surface thus stimulating precipitation and deposition into tooth structures as apatite mineral (Figure 4).

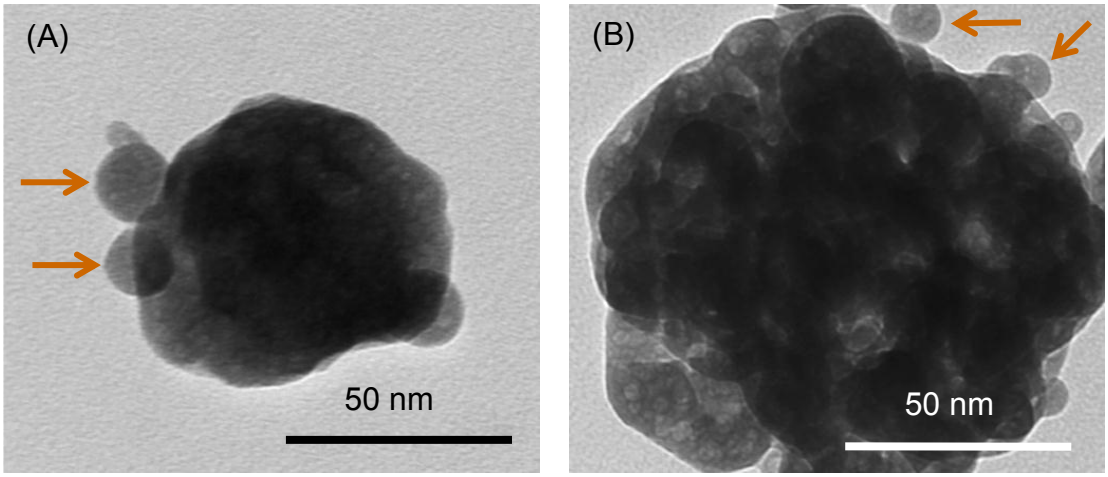

Figure 3. TEM micrographs of the spray-dried nanoparticles. (A) Small ACP nanoparticles, (B) ACP cluster (Adapted from ref # 74, with permission).

Studies have showed that the enrichment of nanocomposite resin with reinforcement fillers and calcium and phosphate nanoparticles can promote the remineralization without loss of the mechanical characteristics of flexural strength, presenting similar values to microfill composite resins.[62,63] Recently, Weir et al.[64] showed that a nanocomposite was able to possess the combination of load-bearing and remineralization capabilities with inclusion of NACP and glass particles. The NACP nanocomposite released calcium and phosphate ions into the environment around the enamel leading to remineralization of the lesion, while the glass particles increased the strength of the composite. This material was capable of promoting a higher enamel remineralization (4-fold) than a fluoride-releasing composite.

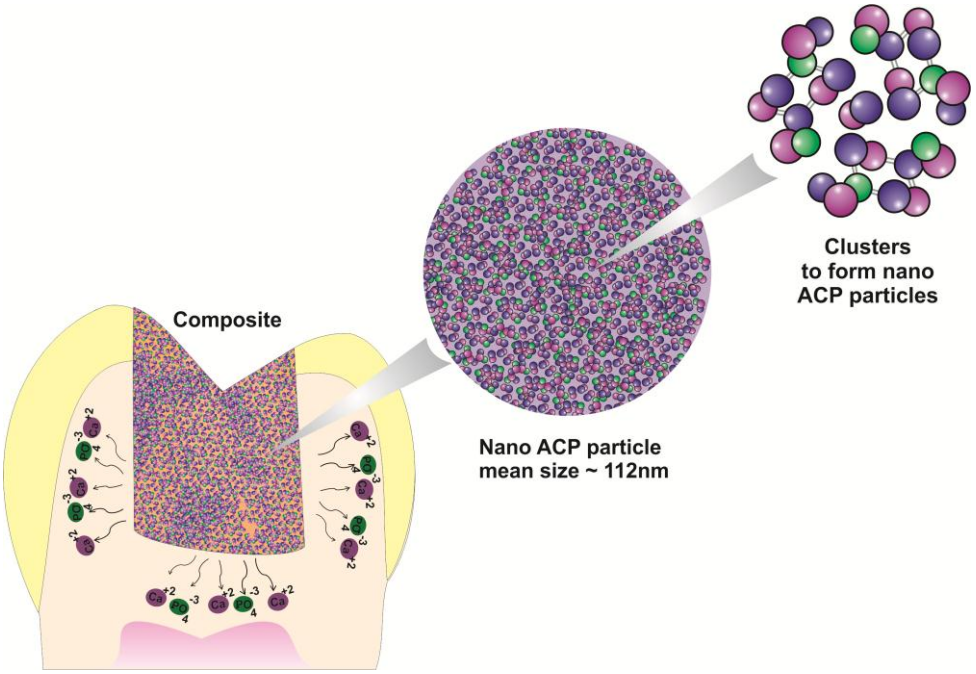

Figure 4. Schematic illustration of the suggested ion-diffusion process from NACP-containing nanocomposite to surround dental tissue and NACP particles details composition.

A human *in situ* caries model also was used[65] to study the ability of NACP-containing nanocomposite to prevent demineralization in enamel at the restoration-enamel margins. NACP-containing nanocomposite produced lower enamel mineral loss compared to the control composite. Oral biofilm exposed to NACP had higher calcium and phosphorus concentrations than plaque exposed to the control composite. These findings suggest that the incorporation of Ca-P nanoparticles in restorative materials may lead to a biopromoting and bioprotective features to resin composite.[39,40]

Calcium-fluoride nanoparticles

There is clear evidence of the caries-inhibiting effect of fluoride, and extensive studies have been undertaken to understand and further improve the performance of F-releasing restorative materials.[66-69] Current dental materials with high fluoride release generally have lower mechanical properties.[63] Existent composite resin releases only a small amount of fluoride and have low fluoride-recharge capability.[67]

The nanotechnology-based development of dental composites that have high fluoride release, while maintaining the strength and wear resistance, were explored by incorporation of calcium fluoride (CaF_2) nanoparticles.[70] Xu et al.[71,72] demonstrated that the cumulative fluoride release increased with nano-CaF_2 content, and the composite containing 20-30%

CaF_2 nanoparticles were able to match the fluoride release rates of traditional and resin-modified glass ionomer materials. The authors credited this process to the higher surface area of nano-CaF_2 which was nearly 20-fold that of traditional CaF_2 (Figure 5). Recent studies carried out by this research group showed higher fluoride release from the CaF_2 nanoparticles at cariogenic low pH, when these ions would be most needed to inhibit caries; that was achieved without compromising long-term mechanical properties.[73-75]

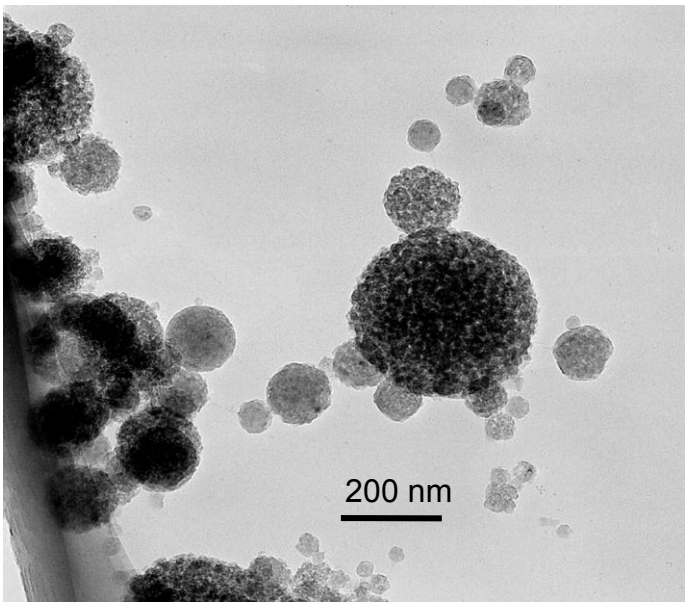

Figure 5. TEM micrograph of the new CaF_2 nanopowder, with median particle size of 56 nm (Adpted from ref # 73, with permission).

Nano-hydroxyapatite (nano-HA) /nano-fluorohydroxyapatite (nano-FHA)

Synthetic hydroxyapatite (HA) is a biologically compatible material, and it is considered a logical mineral compound to substitute the natural mineral constituent of dentin.[76] Its applicability in biomaterials by the addition of HA powders to restorative dental materials for remineralization effects and improvement of mechanical properties has been extensively investigated due to its excellent biocompatibility and bioactivity.[77-79] In order to fabricate restorative materials which imitate human hard tissues, nano-HA were incorporated in resin-modified glass ionomer cement.[80] The addition of 10% nano-HA (60-100nm) to glass ionomer cement resulted in an increased resistance to demineralization and acceptable bonding strength, compared to micro-hydroxyapatite added to glass ionomer cement. However, the setting time of nano-HA containing glass ionomer cement exceeded the maximum setting time.[81] Moshaverinia et al.[82,83] demonstrated that nano-HA/nano-FHA-

containing glass-ionomer cements exhibited higher compressive, diametral tensile, and biaxial flexural strength compared to the control. In addition, Lin et al.[84] suggested that the presence of fluoride in the fluoride-substituted apatite has the potential to increase the amount of fluoride release from the glass-ionomer cements.

Several studies proposed bioactive glass nanoparticles for application in the remineralization of human dentin and the potential as a filler component in mineralizing restorative materials.[85] The high remineralization rate promoted by nano-sized particles (20 nm) in relation to micro-sized-bioactive glass particles highlights the importance of nanotechnology approaches with potential for future clinical applications.[86]

CONCLUSION

Nanotechnology has been expanded in many fields in dentistry. One of its goals in cariology is to improve the research and development of restorative materials capable of controlling secondary caries lesions around the restorations. Different up-to-date strategies have been presented in this review, with the focus on the use of cutting-edge nanotechnology incorporated in restorative materials. To better develop anticaries materials, a knowledge base of modes of action, safety and development of new properties remain to be achieved. Most of the articles covered in this review were across a range of disciplines and focused primarily on *in vitro* testing, with their results demonstrating a promising future for these materials as anticaries tactics. However, to confirm these results *in vivo*, further clinical trials are still needed.

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3.2 CAPÍTULO 2

Caries inhibition around orthodontic brackets by fluoride-releasing composite containing nanoparticles

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ABSTRACT

Fluoride-containing materials have been suggested to control enamel demineralization around orthodontic brackets during the treatment with fixed appliances. The improvement of their properties has been made through innovations such as the application of nanotechnology by incorporation of nanofillers. *Objective:* This *in vitro* study evaluated the capacity of fluoride releasing and enamel demineralization inhibition of fluoride-releasing nanofilled cement around orthodontic brackets using an artificial caries biofilm model. *Materials and methods* Forty bovine enamel discs were selected by evaluating surface microhardness and randomized into 4 groups (n=10): non-fluoride-releasing microfilled composite (MC), fluoride-releasing microfilled composite (FM), resin-modified glass-ionomer cement (RMGI) and fluoride-releasing nano-filled composite (FN). After brackets bonding in each disc, the specimens were subjected to a cariogenic challenge through a *Streptococcus mutans* biofilm model. After the experimental period, the biofilm formed around the brackets was collected for fluoride analysis and the mineral loss around the brackets was determined by cross-sectional microhardness measurement at 20 and 70 μm from the bracket margin. Additionally, samples of each group were subjected to energy-dispersive X-ray spectroscopy (EDX) analysis and were examined under a scanning electron microscopy (SEM). ANOVA followed by Tukey test were applied for fluoride concentration and mineral loss data, respectively. *Results* At both distances, only RMGI statistically differed from the other groups presenting the lowest demineralization. Similar condition was found to fluoride concentration and EDX/SEM analysis. *Conclusions* Under the cariogenic exposure condition of this study, the fluoride-releasing nano-filled material had similar performance to fluoride-releasing micro-filled materials.

Clinical relevance The presence of nanofillers in the fluoride–releasing materials studied did not promote further benefits against caries lesion development around brackets and presented inferior demineralization inhibition than the resin-modified glass ionomer material.

Key words: Nanotechnology, biofilm, fluoride releasing materials, orthodontic brackets, EDX-element analysis.

INTRODUCTION

One of the main problems related to orthodontic treatment is the development of demineralized areas around brackets during fixed orthodontic therapy,[1] which is reported to occur in short time and with high rates among patients.[2] The fixed orthodontic appliance components as brackets, arch wires, O-ring, and metal ligature ties do create additional retentive sites, encouraging biofilm accumulation.[3] Microbiological changes after brackets placement towards increased levels of cariogenic species such as *Streptococcus mutans*[4], which may result clinically in a higher enamel demineralization adjacent to orthodontic appliances. Over time, this condition associated with patient's caries risk factors may result in active white spot lesions, and, if untreated, cavitated caries lesions can occur.[5]

Adjunctive preventive strategies to avoid the increase of caries risk for patients with fixed orthodontic treatment involve the use of fluoride, a well-documented anticaries+ agent, able to promote remineralization of early caries, and to inhibit demineralization[6,7]. One of these preventive methods is focused on development of various fluoride-releasing materials to be used as orthodontic bonding agents.[8] This is particularly interesting since these materials with sustained release of fluoride ions will be applied adjacent to the brackets, an area with great association to enamel demineralization. This way, materials for the adhesive fixation of the brackets also were formulated to release fluoride, typically by the inclusion of fluoride-containing compounds such as fluoroaluminosilicate glasses, yttrium fluoride, stannous fluoride or organic amine fluorides.[9]

Nowadays, there are numerous available fluoride-containing orthodontic cements in the dental market including glass-ionomer cements (GICs), resin modified glass-ionomer cements, (RMGICs), polyacid-modified composites (compomer) and composites.[10,11] The glass-ionomer materials are highlighted by their anticariogenic properties, due to their capacity to release and store fluoride.[12] However, the disadvantage of glass ionomer material for orthodontic use is its low bond strength to dental substrate leading to more bracket debond failure.[12] The inclusion of filler in composite for bonding orthodontic brackets has been done in order to increase the shear bond strength to the enamel structure without reducing the low flowability, well-known characteristic of these materials.[13]

The application of nanotechnology has changed the size-scale in dental research materials. Dental materials approaching nanoscale dimensions exhibit unusual properties with numerous applications.[14] The nano-sized material particles can be dispersed in higher filler concentrations and polymerized into the resin system to increase filler loading of composites.

It have led to the optimization of individual material characteristics and claim impart distinct advantages, such as superior polishability, better mechanical and wear properties.[15] Additionally, the distribution of nanoparticles results in a smoother, satisfactory consistency and adequate flowability of the material.[16] Due to the benefits from the nanofiller incorporation, the manufacturers of the recently commercially available nanofilled resin-based orthodontic bonding agent claims that it is a material with ideal flowability able to show significantly improved surface smoothness and high immediate shear bond strength values of metal brackets bonded to bovine enamel. All those feature combinated with fluoride release capacity.

The ability to release fluoride is a quite complex process and different factors such as the fluoridated particle type and size, different matrices, setting mechanisms as well as silane treatment may influence the process of ion migration.[17,18] It is possible that the adoption of nanofiller loading might have implications on fluoride releasing mechanism of bonding orthodontic brackets due to specific features promoted by presence of nanofiller. Numerous studies on the amount of fluoride provided by fluoride-releasing materials and its effect on enamel demineralization have been published[19,20] but an insight into fluoridated composite resins containing nanofillers is still lacking. The purpose of this study was to investigate the concentrations of fluoride found in a *S. mutans* cariogenic biofilm grown around orthodontic brackets bonded with fluoridated composite containing nanofillers and to compare it to a fluoride-releasing microfilled composite, a non-fluoride-releasing microfilled composite and a resin-modified glass-ionomer cement. Another aim was to evaluate the extent of enamel demineralization around the brackets after these materials had been used.

MATERIALS AND METHODS

Four commercially available materials, including a non-fluoride-releasing micro-filled composite (MC) that was chosen to be the negative control group for fluoride release, a fluoride-releasing micro-filled composite (FM), a resin-modified glass-ionomer cement (RMGI) as a positive control group, and a fluoride-releasing nano-filled composite (FN) were used in the experiment (Table 1).

Specimen preparation

Forty polished enamel discs (diameter 5.0 mm and thick 2.0 mm) were prepared from the labial surface of 90 freshly extracted, sound bovine mandibular incisors. The discs were

prepared using a water-cooled diamond core drill (trephine) and a bench-type drilling machine (Schulz S/A, Joinville, SC, Brazil). The samples were serially flattened with water-cooled abrasive discs (320, 600, and 1200 grit Al_2O_3 papers; Buehler, Lake Bluff, IL, USA) and polished with felt paper and diamond spray (1 µm; Buehler) mounted in a polishing machine (Arotec SA, Cotia, SP, Brazil).

The surface Knoop microhardness number (KHN) was determined by performing 5 indentations (FM 100, Future Tech, Tokyo, Japan) in the center of the enamel surface, with parameters set at 50 g, 5 s, for selection and randomization distribution purposes. Enamel discs presenting mean microhardness values (302.4 ± 22.1 KHN) were randomly assigned in each group (n=10) according to a computer generated randomization list.

A bracket clamp (Ref. 75.01.022; Morelli®, Sorocaba, SP, Brazil) was used to hold and keep the standard edgewise incisor metal brackets (Morelli®; slot 0.018-in) in position on the most central area of the enamel disc surface after using the cements according to the respective manufacturer's instructions for each material (Table 1). The bonding procedures were performated according the manufacturer's instructions. Then, the bonding materials were applied to the bracket base and pushed against the enamel surface. In order to standardize bonding pressure and cement thickness, a 453.6-g Gillmore needle was held vertically on the bracket while excess adhesive around the bracket base was removed with a clinical probe and the material was then light-activated for 20 sec from the mesial and the distal sides with a LED (Optilight LD Max (Gnatus, Ribeirão Preto, SP, Brazil) with a power density of 600 mW/cm^2 at a fixed distance and angle to the surface.

S. mutans **microbial biofilm model**

The enamel-bracket set, which provide minimal bonding materials beneath the brackets and similarly mimics the clinical situation, were prepared for this study.[21] The sets were fixed in the lids of glass container vessels with plastic rods, immersed in sterile distilled water and sterilized prior to inoculation for biofilm growth as described by Zanin et al.[22] *S. mutans* strain UA159 was first grown in an overnight culture of Brain Heart Infusion medium (BHI Difco, Sparks, MD, USA) in a 10% CO_2 atmosphere (Thermo Fisher Scientific Inc., Waltham, MA, USA) to prepare the inoculum to grow mono-specie biofilms. Each enamel disc was placed into an individual container, which was filled with 5 ml of BHI, supplemented with 5% fresh prepared sucrose (w/v).[23] Inoculation of each BHI-containing recipient was performed only once on the first day. Every 24 h, the medium was replaced with

pre-warmed fresh BHI with 5% sucrose during 5 days. The inspection for contamination of the cultures in the media was verified every day using Gram staining and plating samples onto a new fresh BHI agar media.

Table 1. Commercial materials used in this study.

Group/ Material	Type	Composition *	Batch#	Manufacturer
MC/ Natural Ortho	Non-fluoride-releasing microfilled cement	BisGMA, Glass borosilicate, UDMA, Silic, Barium glass, PEGMA, DMAEMA, camphorquinone, Hidroxitoluene and Pigments	10111434	Nova DFL, Rio de Janeiro - RJ - Brazil
FM/ Orthodontic Fill Magic	Fluoride-releasing microfilled cement	BisGMA, Methacrylic acid esters. fluorsilicate glass.	016/10	Vigodent AS, Rio de Janeiro - RJ - Brazil
RMGI/ Vitremer	Resin-modified glass-ionomer cement	Polyacrylic-itaconic acid with pendant methacrylate, water, HEMA, fluoraluminosilicate glass.	0707500147	3M ESPE, Dental Products, Saint Paul, MS, USA
FN/ Orthocem	Fluoride-releasing nanofilled cement	Methacrylate monomers such as BisGMA, TEGDMA, phosphate methacrylates, stabilizers, fluorsilicate glass, camphorquinone, co-initiator and silicium dioxide nanoparticles.	040410	Dentscare ltda, Joinville – SC, Brazil

HEMA, Hydroxyethyl methacrylate; Bis-GMA, bisphenol glycidyl methacrylate; UDMA, urethane dimethacrylate; TEGDMA, tetraethyleneglycol dimethacrylate; PEGMA, Poly(ethylene glycol) dimethacrylate; DMAEMA, Dimethylaminoethyl Methacrylate

* Based on information provided by the manufacturers.

Concentrations of acid-soluble fluoride (F) in the biofilm

After experimental period, the biofilms grown around the brackets were removed for fluoride analysis. The fluoride content in the plaque samples was measured according to the method described by Cury et al. [24] Breafly, the samples were placed in pre-weighed tubes and 0.5 M HCl was added (0.50 ml/10 mg of wet biofilm). After extraction for 3 h at room temperature via agitation, the samples were centrifuged and TISAB II solution (containing 20 g NaOH/L) was added to supernatant in order to maintain pH 5.0 and to eliminate the interference effect of complexion ions.[25] The amount of acid-soluble F was determined

electrochemically using a fluoride-sensitive electrode (model 96–09; Orion Research, Cambridge, MA, USA) and an ion analyzer Orion EA-940. The standard solutions were used to plot the calibration graph. All determinations were performed in triplicate and the fluoride concentration, expressed as micrograms per milligram of biofilm.

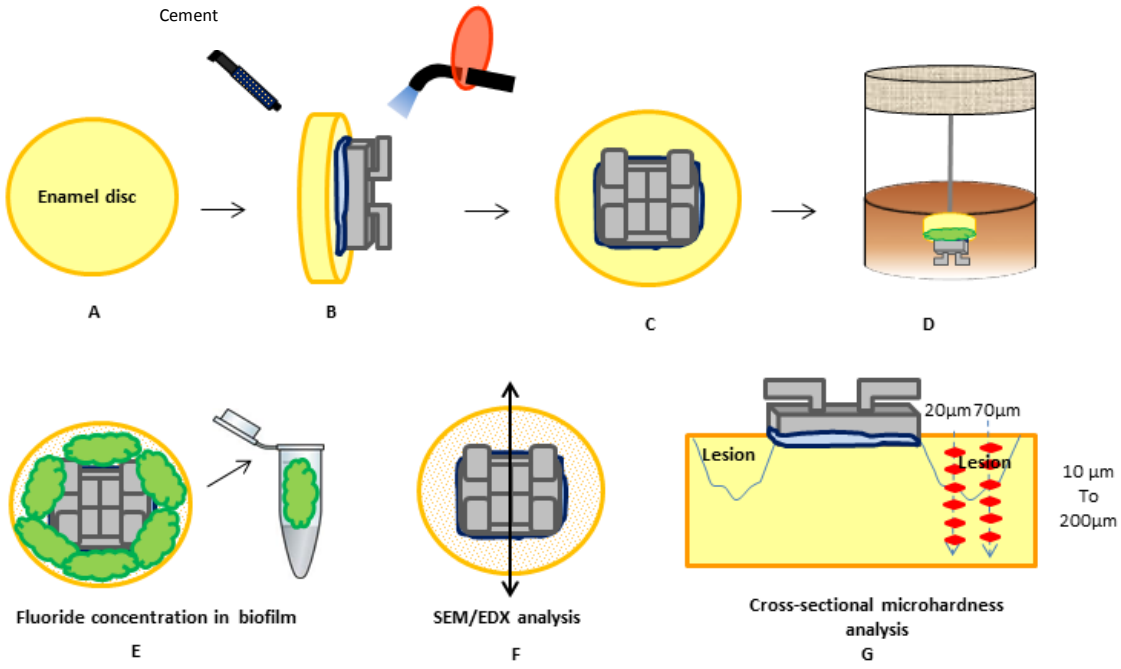

Figure 1. Schematic diagram illustrating the set up for this study. A-Enamel disc samples (5mm diameter); B-Bonding procedures. C—Brackets bonded on central area. D-Fixation of the slabs in the device (5-day microbial model). E-Biofilm removal for fluoride analysis. F-Demineralized enamel area around brackets submitted to SEM/EDX analysis G-Transversal cut to cross-sectional microhardness analysis.

Cross-section microhardness analysis (CSMH)

Enamel discs were longitudinally sectioned through the center of the bracket. One of the remaining halves of each slab was randomly selected to be analyzed. It was embedded in acrylic resin, the cut surface being exposed, for subsequent flattening and polishing, respectively. Microhardness was measured using a Knoop indenter with 25 g load for 5 s. Two lanes of 15 indentations each were made in duplicate (each side of bracket), which the lanes were 20 and 70 µm distant from the bracket margin.[26] The distance between the lanes was established to minimize interactions between neighboring marks. The first ten

indentations were spaced 10-µm from the previous one and the last ones at 20-µm intervals. Integrated demineralization (ΔS) was calculated according to Sousa et al.[26]

SEM/EDX analysis

In order to investigate alterations in chemical composition (element quantities) contained in the enamel sample, SEM/EDX analysis was performed for all groups. The demineralized samples were rinsed, dried in silica gel for 2 h, and mounted on a holder using double-sided adhesive carbon tape previously the analysis. Five points in each surface were selected around the bracket. Normalized high-resolution spectra of the main elements' concentration in weight % were performed and later calculated by an energy-dispersive X-ray spectrometer (EDX), using the backscattered electron detector attached to a scanning electron microscope (TESCAN Model VEGA II\XMU, Brno, Czech Republic) operating at 30 kV and working distance (WD) of 20 mm. Data acquisition and analysis were performed using Quantax 800 software (Bruker AXS, Karlsruhe, Baden-Württemberg, Germany).

Statistical Analysis

Statistical analyses were performed with SPSS for 13.0 Windows (SPSS Inc. Chicago, IL, USA). The normality and homogeneity were checked for each response variable. For fluoride concentration and elemental concentration, one-way ANOVA and Tukey's post-hoc tests were applied. For ΔS and CSMH per depth data were transformed as suggested by the software. Tests were performed at a significance level of $p<0.05$.

RESULTS

Concentrations of acid-soluble fluoride (F) in biofilm

Group RMGI (Vitremer) exhibited significantly higher fluoride amounts in biofilm compared to the other tested materials ($p<0.05$), as expected. The FN group released a more fluoride compared to the FM group but afterward, both materials exhibited similar fluoride concentrations, which were not significantly different ($p>0.05$) (Table 2). Group MC expressed the lowest value.

Cross-sectional microhardness (CSMH)

Integrated demineralization (ΔS) related to RMGI group was statistically lower ($p<0.05$) in relation to all groups. The findings show that there was a trend to lower demineralization of enamel around brackets fixed by the FN group comparing to FM and MC

groups, but the difference among them was not statistically significant (Table 2). The microhardness data at each depth from the enamel surface are expressed in Fig. 2.

Table 2. Mean and standard deviations of fluoride concentration (µg/g wet biofilm) and demineralization at studied distances for each tested material.

Group	F µg/g wet biofilm	ΔS at distance from bracket base	
		20 µm	70 µm
MC (n= 10)	0.27 (0.08) [b]	5320.44 (1241.06) [a]	4661.94 (1615.80) [a]
FM (n=9)	0.91 (0.56) [b]	5204.19 (1761.33) [a]	4573.67 (1221.77) [a]
RMGI (n=8)	10.50 (4.02) [a]	876.55 (440.80) [b]	1392.37 (916.74) [b]
FN (n=10)	1.48 (1.24) [b]	4017.63 (1872.54) [a]	4228.56 (1990.69) [a]

Non-fluoride-releasing microfilled composite (MC), fluoride-releasing microfilled composite (FM), resin-modified glass-ionomer cement (RMGI) and fluoride-releasing nano-filled composite (FN).
The same superscripts indicate no statistically significant difference between the indicated groups ($p > 0.05$).

SEM/EDX analysis

In the EDX analysis, the main elements observed in the enamel were calcium (Ca), phosphorus (P), oxygen (O) and fluorine (F). Minor quantities of other elements: sodium (Na), magnesium (Mg), chlorate (Cl), silica (Si) and carbon (C) were also observed in the present study. Elemental mappings showed calcium and phosphorus primarily detected while fluoride and oxygen were identified in reduced amounts. Means and standard deviation of percentage of elemental concentration in weight (wt%) detected in the samples are summarized in Fig 3. A quantitative element analysis revealed no statistically significant differences in Ca, P and O content among the groups ($p > 0.05$); exception to F, which presented a significantly higher amount in RMGI group.

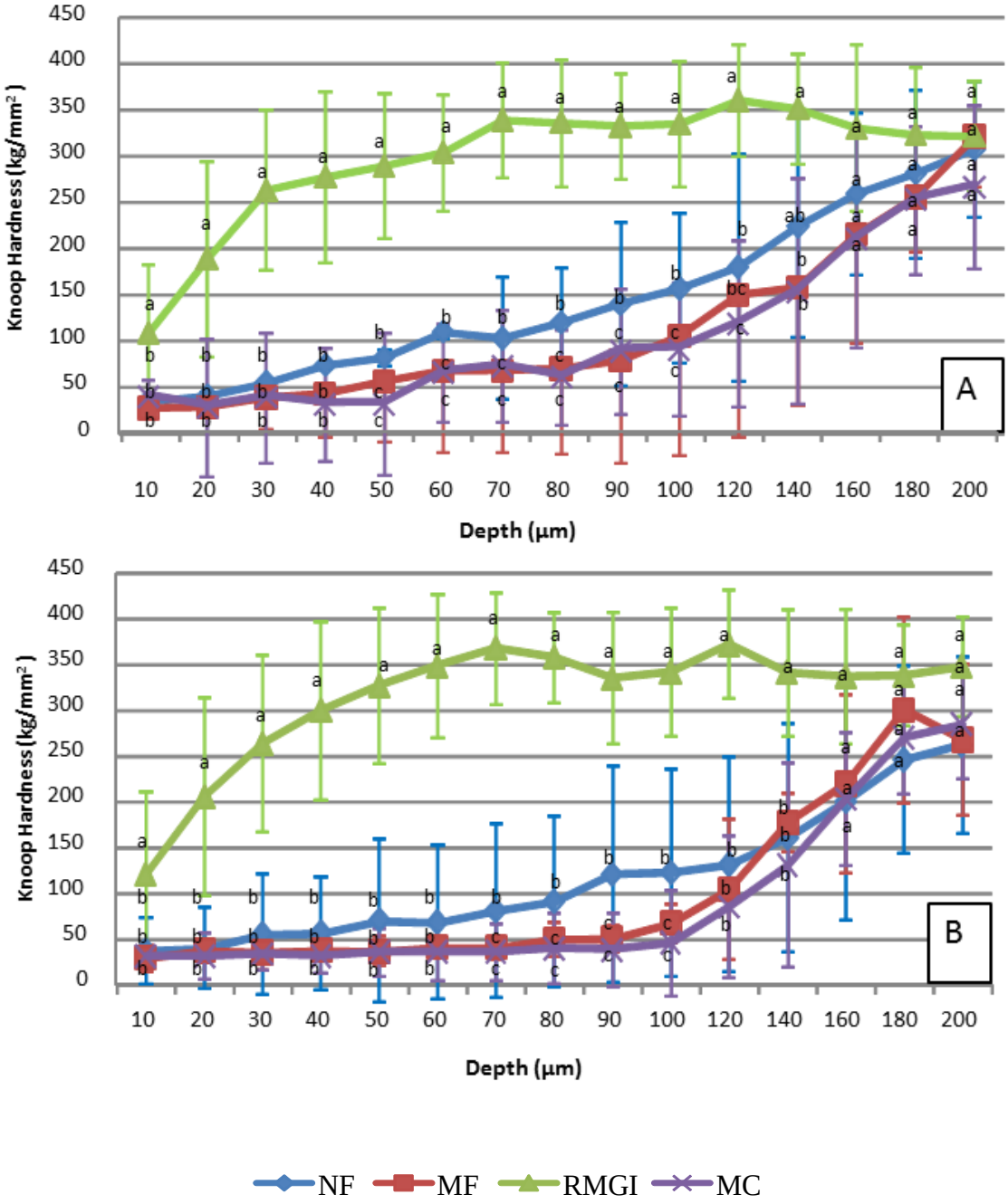

Figure 2. Mean ± standard deviation of the values of enamel Knoop hardness according to the materials and depth, at 20 µm (A) and at 70 µm (B) from the edge of the bracket base. Small letters indicates statistically significant difference between groups at each distance ($p < 0.05$).

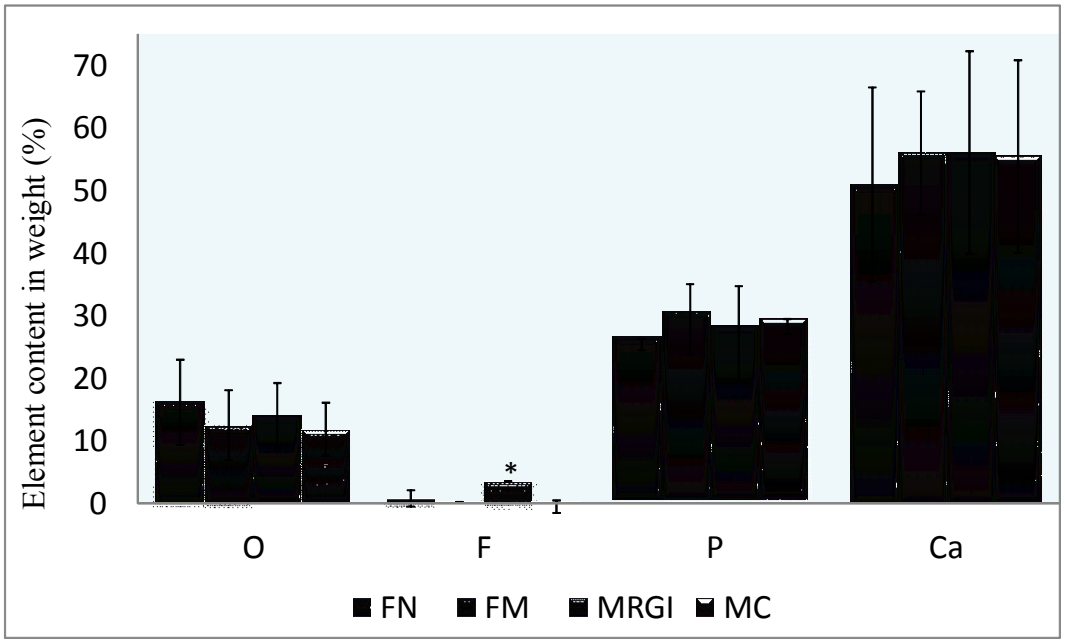

Fig. 3 Means and standard deviation of percentage of elemental concentration in weight (wt%) of the main elements found in enamel around the brackets.

* Statistical significant difference.

DISCUSSION

The incorporation of nanoparticles in resin material may allow the production of materials for the adhesive fixation of brackets with flowability and higher mechanical properties than for microfilled materials. Also this process could alter characteristics of the ion-releasing materials[17,27] The *in vitro* study mainly showed that the fluoride-releasing composite containing nanoparticles for bonding orthodontics brackets exert similar performance of the microfilled composite in relation to fluoride-release and anticaries effect. This showed that nanofiller incorporation did not improve the fluoride release and consequently did no alter the anticaries potential of this material.

Enamel demineralization *in vitro* was inhibited to a certain degree in our study. Among the tested materials, the control group RMGI exhibited significantly higher performance in all performed analysis compared to the other tested materials. In agreement with other studies,[28,29] our results have demonstrated that resin-modified glass ionomer materials showed a fluoride releasing capacity sufficient to promote lower enamel mineral loss over the tested composite resin materials.[30] This was an expected outcome since the total amount of fluoride released differs significantly between the resin-modified glass-ionomer

cements and the composite resins. It has been suggested that this superior performance of RMGI is attributed to some factors as the acid–base setting reaction between the fluoride-containing aluminosilicate glass powder base and the polyacid liquid, which results in the liberation of fluoride ions.[29] In poly salt matrix, fluoride ions can be bound in strong complexes with the metal ions, especially aluminium and the pore liquid, in which the fluoride ions are loosely bound and free to move.[31]

In our results KHN for 50-100µm and 70-120µm in depth at 20 and 70µ- distance, respectivately, showed significant difference for FN group expressing a trend to better performance of floride-releasing nanofilled composite than microfilled materials. Also the ΔS and fluoride concentration data from FN group supported this, however, they were not significantly different. Only few previous studies investigated fluoride release from nanomaterials. In a previous study, Kusgöz et al.[32] compared fluoride release of a nanofilled resin based fissure sealant and an unfilled resin based fissure sealant. The authors observed that the nanofilled sealant released a bit more fluoride than the unfilled sealant, but these results were not significantly different. These finding are consistent with results of the present study, showing that there is no significantly influence ion fluoride release but it was able to show higher values than microfilled materials. It can be hypothetized that nanoparticles present in resin matrix of commercial materials can be related to this situation, since the studied floride-releasing nanofilled composite presents 100% of its reinforcement particles (silicon dioxide) in nanoscale.

On the other hand, Xu et al.[33] showed that a nanocomposite containing calcium fluoride (CaF_2) had a higher fluoride release capacity when compared to the control. This fact may be related to the nano-CaF_2 surface area, which presented a surface area nearly 20-fold-higher surface area than a traditional 1-µm CaF_2. This clearly suggests that the size of particles from fluoride source itself presents more relevance for an anticaries effect than the simple presence of nanoparticles in resin matrix.

Fluoride release from composite resin materials is based on the diffusion process where initial leaching of fluoride from glass particles in the surface layer of the material may account for this mechanism. Fluoride ions diffuse through fluid and plaque from the composite to the adjacent enamel surface.[34] The SEM/EDX analysis explored the possibility of different ion diffusion rates from fluoride-containing nanomaterials to the enamel adjacent to bonded bracket and consequently alteration in chemical composition of enamel around the tested materials. The amounts (wt%) of fluoride found for both fluoride releasing composite resins, regardless of the presence of nanoparticles, were very low or undetectible. Similar

results were found with fluoride releasing composites resin by Gjorgievska et al.[35] in a study that aimed to determine the extent to which ions released from fluoride-containing dental restorative materials migrated through the enamel. In relation to the calcium and phosphorus content in the surface enamel around the brackets, the values were similar in all groups showing that no material was able to promote a more significant diffusion of ions in the enamel surface.

Overall, our results showed that under the conditions of this investigation, only RMGI was capable of decreasing demineralization around orthodontic brackets and the inclusion of nanofillers fluoridated composite resin did not provide greater inhibition of demineralization.

Future research should focus in applying the nanotechnology to preventive dentistry in attempt to improve fluoride-releasing capacity of dental materials. Until then, oral hygiene and other regular fluoride sources should be suggested for the orthodontic patient.

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CONFLICT OF INTEREST STATEMENT

None declared.

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3.3 **CAPÍTULO 3**

In situ randomised trial investigating anticaries properties of fluoride-releasing nanohybrid composite resin

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ABSTRACT

The current study investigated the anticaries action of a fluoride-releasing nano-hybrid composite resin and the effect of cariogenic biofilm on material roughness on cariogenic biofilm through a split mouth *in situ* study. A fluoride-releasing nanohybrid composite resin (FCR) and non-fluoride-releasing nanohybrid composite resin (CR) were tested. Forty restored bovine enamel slabs and 20 specimens made with each of the studied materials were included in intra-oral palatal appliances that were used for 20 adult volunteers. For the cariogenic challenge, each volunteer dropped a 20% sucrose solution onto all slabs 8x/day during 14 days. In the course of the experimental period, all subjects used non-fluoride-containing dentifrice. The biofilm formed over the slabs was analyzed to determine amounts of fluoride (F), calcium (Ca) and inorganic phosphate (Pi) ions. Demineralization (ΔS) was determined in enamel by cross-sectional microhardness at 30 and 80-μm from the restoration margin. Surface roughness of material samples was also determined. In order to verify the differences between the groups, t test was used. No statistically significant difference was found in concentrations of Pi and Ca of the biofilm grown neither on the restorations nor on the specimens. The F concentration in the whole biofilm related to the group FCR was higher than the group CR. At 30-μm distance, the ΔS was 2.579±1.582 and 1.705±1.292, respectively, for the groups FCR and CR showing significant lower enamel demineralization (p=0.039); no statistical difference was found at 80 μm. Significant difference was found between baseline and post challenge surfaces roughness for both groups (p = 0.03 and 0.016, respectively for CR and FCR but this difference was not find between the ΔRa of these materials (p=0.49). Concluding, under the cariogenic exposure condition of this study, the fluoride-releasing composite can have a slight anticaries action without damages for its surface smoothness.

Key words: Dental caries, biofilm, nanotechnology, *in situ* study, composite resin.

INTRODUCTION

Dental composites have become the most popular and widely used direct restorative material in today's clinical dentistry due to their esthetic quality and good physical properties.[1] However, bulk fracture and secondary caries around restorations are the main reasons for failure and still remain as challenges to be overcome in this material.[2,3]

There are several strategies involved to combat dental caries and incorporation of fluoride compounds in restorative materials has been one of tactics to reduce secondary caries found in the commercially available products, and its incorporation becomes a desirable attribute for a dental material.[4] An extensive scientific literature has shown that glass-ionomer cements have the highest anticaries effect among fluoride-releasing materials.[5-7] However, relative low mechanical properties suggest that glass ionomer materials are inadequate for stress bearing areas.[5] Fluoride compounds are also added into composite resin attempting to get a significant anticaries effect, associated with suitable physical strength in the same material, however, current composite resin releases only a small amount of fluoride.[8]

At the same time, dental composites are constantly changing, and a lot of effort has been focused in their improvement.[9] Since the properties of composite resin are considerably influenced by the filler size, nanotechnology has driven the re-design of dental composite resins by incorporation of nanofillers.[10] The addition of nanoparticles in the set of microfiller has claimed to combine acceptable mechanical strength with optimal polishing/optical properties.[11,12] The benefits from the combining different average sizes of particles leaded to nanohybrid composite resin, a category of material widely used in last years.[13]

These differentiated characteristics of nanotechnology-based composites may be reflected on alterations in oral biofilm over the restorations, since surface roughness has a great impact on plaque accumulation.[14] Also, the performance in relation to fluoride release may be differentiated in nanofillers-containing composite resin, since ion-exchange process for the different particle size fractions of resins influences the kinetics release (diffusion rates) of the ions immobilized in the polymeric matrix.[15,16] Nonetheless, little is known as regards to the anticaries properties of fluoride-releasing nanohybrid composite resins. Thus, in the present study an *in situ* model was used to evaluate the anticaries effect of fluoride-releasing nanohybrid composite resin.

MATERIALS AND METHODS

The study protocol was approved by the Research and Ethics Committee of the local Institution (protocol # 027/2011) and volunteers signed an informed. Twenty healthy adults (14 females and 6 males), mean age 26.4 years, satisfied the following criteria: normal salivary flow, good general and oral health with no active caries or periodontal diseases, ability to complete the experimental protocol. Volunteers who had used antibiotics in the last three months, as well those under orthodontic treatment were excluded from the study sample.

A split-mouth *in situ* design was conducted in one phase during which, 20 volunteers wore palatal devices containing 4 bovine enamel slabs restored with F-containing nanohybrid composite resin with (FCR) or without fluoride (CR), and 2 specimens made using each studied material (Fig.1A).

Eighty enamel slabs (5 x 5 x 2 mm) were prepared according to a previous study.[17] Size-standardized cavities were prepared in the enamel slabs with approximately 2.4 ± 1 mm, and 1.5 mm deep were prepared with water-cooled high-speed turbine and a # 2294 cylindrical diamond tip (KG Sorensen, São Paulo, SP, Brazil). Slabs were randomly divided according to a computer-generated list, into 2 groups of 40 slabs each. The cavities were restored with one of the following materials: Tetric-N-Ceram composite resin (shade A3 Ivoclar Vivadent AG, Schaan, Liechtenstein)/Single Bond (3M ESPE Dental Products, St. Paul, MN, USA), and EvoluX)/Single Bond (shade A3, Dentsply, Milford, DE, USA) (Table 1), according to the manufacturers' recommendations. Following, the composite resin was inserted in 2 increments and light activated for 20 s using a Light Emitting Diode Optilight LD Max (Gnatus, Ribeirão Preto, SP, Brazil) with an output of 600 mW/cm^2 (Fig.1A). All restored slabs were stored in 100% humidity for 24 hours then polished using the set of aluminum oxide discs (Sof-lex disk system, 3M Dental Products, St. Paul, Minn., USA). To prepare the composite resin specimens, a squared silicone index mold was used. The unpolymerized resin was carefully packed inside the mold in 2 increments; the surface was covered with a polyester matrix tape (TDV, Pomerode, SC, Brazil) and then light-activated per 20 s.

Before and after the *in situ* cariogenic challenge, the average surface roughness (Ra-µm) of the composite specimens was measured using, a surface profilometer Hommel Tester T1000 (Hommelwerke GmbH, Germany). The stylus traversed the surface of the specimen at a constant speed of 0.5 mm/s, force of 4 mN, with a 0.25-mm cutoff value and 1.5-mm tracing

length. The difference between the Ra final and Ra initial was calculated and expressed as delta Ra (ΔRa) (Fig.1C).

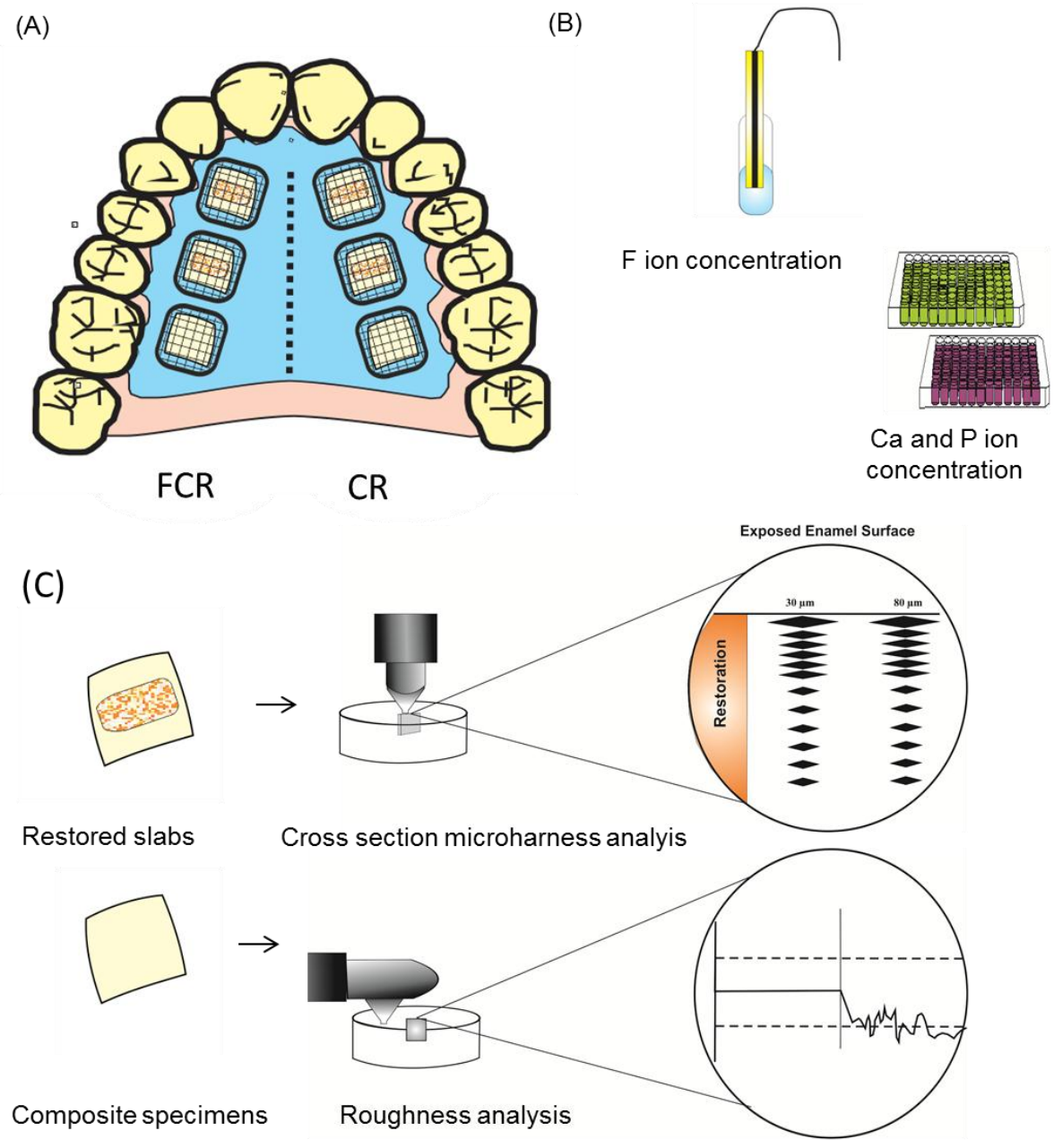

Figure 1. Illustration of the experimental design (A) and performed analyses (B and C) in this study.

Table 1. Characteristics of composite resin used in this study.

Restorative material	Formulation*	Classification	Manufacturer
FCR Tetric-N-Ceram, shade A3, lot: L55479	Urethane dimethacrylate, Bis-GMA; Ethoxylated Bis-EMA; Triethyleneglycol dimethacrylate, Barium glass, ytterbium trifluoride, mixed oxide, silicon dioxide, Prepolymers, Additives, stabilizers, catalysts, pigments; filler level: 63.5wt%	Nanohybrid composite resin; particle sizes in the micro- (< 1 μm) or nano range (< 100 nm, mean 40 nm)	Ivoclar Vivadent, Schaan, Liechtenstein
CR EvoluX, shade A3, lot: L234534B	Urethane dimethacrylate, Bis-GMA; Ethoxylated Bis-EMA; Triethyleneglycol dimethacrylate, barium aluminum borosilicate glass; silica nanofillers; filler: 75-77 wt%	Nanohybrid composite resin; particle sizes in the micro- (0.02 to 3.0 μm) or nano range (4-7 to 10-20 nm)	Dentsply; York, PA, USA

* Based on information provided by the manufacturers.

During 14 days, volunteers wore removable acrylic custom-made palatal devices, each containing 4 restored enamel slabs and 2 composite-made specimens. The slabs and specimens restored/made with the different materials were positioned in opposite sides of the appliance (Fig.1A). Each slab/specimen was covered with a plastic mesh with 1-mm space to protect the biofilms.[18] Having slabs with 2 different treatments on 2 sides of the same device enabled them to experience the same oral environment without cross-over effect.[19] Seven days prior to and 14 days during the experimental period, volunteers brushed their teeth with non-fluoridated toothpaste and consumed fluoridated water (0.6-0.8 ppm). Eight times per day at predetermined times, volunteers removed the appliance from the mouth and dripped one drop of a 20% sucrose solution on each slab/specimen and after a 5-min waiting time, which was standardized for diffusion of sucrose in the biofilm, the device was placed back into mouth.

On day 14, biofilms on slabs/specimens were collected for analyses (Fig. 1B). Calcium (Ca), inorganic phosphorus (Pi), and fluoride (F) were measured in the dental biofilm according to Tenuta et al.[20] (Fig. 1B). Samples were treated with 0.5 M of HCl to extract acid-soluble whole-biofilm calcium and phosphate ions agitated at 30 rpm for 3 h and centrifuged. The supernatant was collected for calcium and phosphate ion measurement via a spectrophotometric method. For F measurement, TISAB II solution (containing 20 g NaOH/L) was added to supernatant. The amount of acid-soluble F was determined using an ion-selective electrode Orion 96-09 and an ion analyser Orion EA-940.[21]

For cross-section microhardness testing (CSMH), restored enamel slabs were longitudinally sectioned through the center of the restoration. One of the remaining halves was embedded in acrylic resin and subsequent flattened and polished. Microhardness was perfomed using a Knoop indenter with 25 g load for 5 s (Future Tech FM-ARS, Tokyo, Japan). The ΔS (integrated demineralization) was obtained at 30 and 80 µm from the preparation margin (Fig.1C). Two lanes of 15 indentations each were made in duplicate (each side of restoration); the first 10 indentations were spaced 10-µm from the previous one and the last ones at 20-µm interval according a previous study.[22] The mean depths higher than 120 µm were used as measure of the integrated hardness profile of inner sound enamel and ΔS was calculated, for details see previous publication.[17]

The assumptions of equality of variances and normal distribution of errors were checked for all the response variables tested. Results of ΔS were transformed using Box-Cox power transformation to the linear equation.[23] Data of F, Ca, Pi, ΔS and Ra were submitted to unpaired t-test and ΔRa data were analised by paired t-test. The significance level of all tests was set at 5%. Statistical appraisal was computed with SPSS for Windows XP 17.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Table 2 shows the results for all variables studied. No significant effects of calcium and phosphate concentration in whole biofilm were found for any of the biofilm location under study. Fluoride concentrations on biofilm were significantly higher for fluoride releasing nanohybrid composite resin in both locations: over the resin (p=0.033) and over the restored slab (p=0.031).

Regarding mineral loss (ΔS) in enamel, statistically significant difference between FCR and CR (p=0.039) was found only at 30-µm from restoration margin; no statistical difference was found at 80 µm (table 2). The microhardness data at each depth from the dental surface are illustrated in figure 2.

The mean Ra values for nanohybrid composite resins tested after cariogenic challenge are reported in table 2. The differences on surface roughness for materials tested before and after cariogenic challenge and ΔRa were analyzed. Significant difference was found between baseline and post challenge surfaces roughness for both nanohybrid composite resins (p=0.030 and 0.016, respectively for CR and FCR). No statistically significant differences were found between the ΔRa of tested materials (p=0.490).

Table 2. Mean ± Standard Deviation of Ca, Pi and F, concentrations in whole plaque like biofilm, demineralization (ΔS) and surface roughness Ra (µm) for studied conditions.

Restorative material	Biofilm location	Ca, µmol/g Wet biofilm	Pi, µmol/g Wet biofilm	µg F / mg wet weight biofilm	Distance from restoration margin (Δ S)		Δ Ra
					30 µm	80 µm	
CR	Over slabs	75 (43.2)^{a}	19.9 (12.2)^{a}	0.3 (0.2)^{a}	2579.8 (1582.4)^{a}	655.6 (392.9)^{a}	--
FCR	Over slabs	75.3 (50.6)^{a}	19.7 (13.8)^{a}	0.5 (0.2)^{b}	1705.7 (1292.1)^{b}	749.3 (598.1)^{a}	--
CR	Over composite	80.8 (53.7)^{A}	17.9 (12.6)^{A}	0.3 (0.2)^{A}	--	--	0.05 (0.03)^{a}
FCR	Over composite	77.2 (28.0)^{A}	18.6 (13.2)^{A}	0.5 (0.1)^{B}	--	--	0.04 (0.03)^{a}

Data are expressed as mean ± standard deviation (n = 20). Mean values represented with the small superscript letters within the columns are related to biofilm over the slabs and same small letters are not statistically different ($p > 0.05$). Supercript capital letter within the columns are related to biofilm over the resin specimens and the same capital letters are not statistically different ($p > 0.05$)

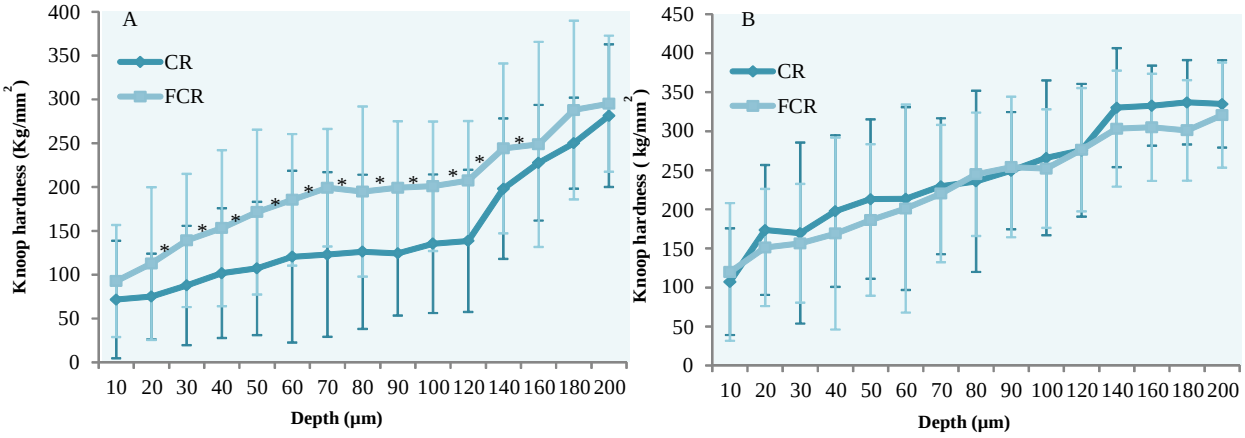

Figure 2. Graphs showing the depth profile of microharness from evaluated groups, at the distance 30 and 80 µm. (*) Means values statistically diferent by unpaired t test.

DISCUSSION

Fluoride release is one of the many desired characteristics of a restorative material since fluoride ions help to inhibit demineralization and promote remineralization.[4] Currently, in commercially available fluoride-releasing composites, the incorporation of inorganic fluoride has resulted in increased fluoride release, however fluoride ions from these materials

are only slightly released and this phenomenum depends on exchange reactions.[24] These reactions causes fluoride release from the particles followed by a diffusion gradient driven movement into the environment.[25] Nanohybrid composite resins present different oxide particles mixed to nano-sized particles and larger fillers compounding the disperse phase. It is known that the size, shape and amount of the particles, which reinforce the composite might affect its properties, whereas smaller particle size results in less interparticle space and higher filler loading, which may significantly influence dynamical loading on fluoride release kinetics.[16]

Laboratory investigations are crucial for an early assessment of dental materials, but to better understand the behavior of materials under fundamental aspects of the caries process, *in situ* models should be considered. Simulating a more realistic clinical condition, variables related to the environment such as components of saliva, acquired pellicle, pH, ion concentration and temperature may be conceived on the complexity of fluoride release process. A number of those factors might alter the F-diffusion from the restorative materials in the oral cavity leading to the more rational outcome.[26]

The findings of this study demonstrated that even a small amount of fluoride leaching from composites might provide some cariostatic effect. The fluoride-releasing nanohybrid resin was able to improve enamel resistance to demineralization closer to the restoration margens in relation to the control. The clinical significance of this improvement is yet to be fully confirmed since other factors such as fluoride dentrifice may be considerated to a final outcome. Altought, fluoride-releasing composites have demonstrated recurrent caries inhibition at enamel margins when exanimated *in vitro*.[27] Currently, only few studies with confliting results determined the demineralization behavior of enamel around fluoride-releasing composites using an *in situ* model.[19,26,28] Kielbassa et al.[29] evaluated the effects of composite containing fluoride on initial secondary caries formation and showed no caries protective effect on surrounding enamel. In other study, Dijkman et al.[30] observed that fluoridated composites were able to reduce the enamel demineralization significantly just when a higher fluoride content (26%) was incorporated in this material.

The tested nanohybrid fluoride-releasing composite presented similar performance in relation to caries inhibition to microfilled composite resin evaluated by *in vitro* study,[27] which may suggest that nanofiller incorporation did not improve the anticaries effect of this material. This could be attributed to type and particle size of leachable fluoridated glass fillers. Weir et al.[31] highlighted that the nano size of the fluoride source can play an important role in the performance of fluoride-releasing composite. This study confirms a previous one[32], which

found high fluoride release when nanoparticles of calcium fluoride (CaF_2) were incorporated in a composite, yielding long-term mechanical properties that were comparable to those of a commercial composite with little F releasing.

Another factor is related to the amount of mixed nanoparticles that are unknown in commercial nanohybrid material evaluated. Thus, the full effects of nanofillers on fluoride diffusion might not exist or be undetectable due to low nanofillers load; since commercial composites are mainly a combination of components that are not nano-sized (fillers in the nanocomposites are composed of micrometer sized fillers, nanofillers, clusters of nanofillers or prepolymerized resin filler). To understand the relationship between nanofiller size and fluoride-releasing nanocomposites containing uniform sized nanofillers should be considerated in further studies.

Some limitations of this study were the compositional variability of commercially available materials and the absence of occlusal forces on materials. It is known that, under cycling loading, there are some microcracks on materials that are potential paths for the fluoride ions difusion. Future studies should consider using well-known filler composition, filler particle size and size distribution to better understand their influence in the fluoride release process.

Surface roughness is another key property for caries management and is directly related to the plaque accumulation on restorative materials.[33,34] The presence of fluoride filler particles can be another point that should be considered. According to previous reports[35,36], the release of fluoride increases the roughness of the finished materials. Results for the tested nanohybrid composite resins showed similar ΔRa for both tested materials. It can be suggested that fluoride release not interfered significantly in surface roughness of tested nanohybrid composite mainly due to an improvement in surface smoothness expected when the nanofillers are incorporated to matrix. An explanation to this phenomena can be related to the exposure of particles, such as silicon dioxide and barium fluorosilicate, which are extremely small and irregular, improving the quality of roughness of that surface and lowering the contact angles for exposure with water.[36] Also the ΔRa values were close to the critical surface roughness (Ra=0.2 μm)[37] previously determined as the limit for sufficient surface smoothness of composite restorations, which leads to suggest a negative effect on the surface roughness mainly due to the exposition to acid production by the bacterial biofilm, during the experimental days.[38]

The efforts to improve the performance of restorative composite by nanofiller inclusion are mainly focused on the reduction of polymerization shrinkage as well as on the

improvement of polishability, and mechanical properties. In this context the use of new nanofillers in nanohybrid commercial fluorated materials may not be associated to anticaries properties. This material has a limited anticaries action with no alterations in superficial roughness.

Despite intensive studies and development in composites, the major challenge in relation to secondary caries still remains. Future fluoride-releasing composite resin materials may be further improved by nanotechnology with respect to their anticaries effect. It can be suggested for further studies the investigation of applicability of new trends from materials science to accomplish the caries around composite restorations such as nanoparticles capable of high levels of F release or the addition of fluoridated monomers.

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CONFLICT OF INTEREST STATEMENT

None declared.

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3.4 CAPÍTULO 4

Antibacterial dental adhesive containing silver and amorphous calcium phosphate nanoparticles

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Short title: Antibacterial adhesive with silver and calcium phosphate nanoparticles

Key words: Antibacterial dental adhesive, dentin bond strength, silver nanoparticles, calcium phosphate nanoparticles, human saliva microcosm biofilm, caries inhibition.

ABSTRACT

Antibacterial bonding agents are promising to inhibit residual bacteria in the cavity preparations. Our objectives were to incorporate nanoparticles of silver (NAg) and nanoparticles of amorphous calcium phosphate (NACP) into a bonding agent, and to investigate the effects on dentin bond strength and dental plaque microcosm biofilm response. NAg were incorporated into the primer and adhesive at 0.1% by mass. NACP concentration were 10%, 20%, 30% and 40%. A microcosm biofilm model was used on composite disks with primer covering the adhesive on the top. Biofilm metabolic activity, colony-forming unit (CFU) and lactic acid production were measured. The addition of studied agents did not alter the bond strength (26 to 34 MPa; $p > 0.1$). NAg and NACP-incorporated-groups greatly reduced the biofilm viability and metabolic activity; CFU for total microorganisms, total streptococci, and mutans streptococci in an order of magnitude and lactic acid production reduction to ¼; all related to their respective controls. In conclusion, biofilm viability and acid production were greatly reduced on bonding agents containing NAg and NACP, without compromising dentin bond strength. The incorporation of remineralizing agent NACP and an antibacterial agent NAg may have wide applicability to other adhesive systems.

INTRODUCTION

Extensive studies have been undertaken to improve dental composites with advances in filler compositions and resin chemistry [1-5]. The property enhancements have enabled composites to be increasingly used as esthetic filling materials [6-8]. Indeed, recent data showed that composite restorations represented the highest percentage among three categories: 77.3 million restorations (46.6%) were composites, 52.5 million (31.6%) were amalgams, and 36.2 million (21.8%) were crowns, totaling 166 million restorations placed in the USA in 2005 [9]. However, a major shortcoming of composites is that they accumulate more biofilms and plaques *in vivo,* compared to other restorative materials [10]. The acid production by biofilms can lead to secondary caries especially at the tooth-restoration margins [11-15]. Recurrent caries was the main reason for restoration failure, and replacement of failed restorations accounts for 50-70% of all restorative dentistry [11]. Replacement dentistry costs $5 billion in the USA annually [12].

To address this problem, novel antibacterial dental composites were developed [16-20]. Quaternary ammonium monomers, such as 12-methacryloyloxydodecylpyridinium bromide (MDPB), were copolymerized in resins to yield antibacterial activities [16,21,22]. The MDPB composite decreased the viability of *Streptococcus mutans (S. mutans)* and plaque accumulation, and reduced the progression of root caries [19]. Another class of antibacterial composites incorporated silver (Ag) particles [23,24]. For example, a composite containing Ag inhibited *S. mutans* growth and had a long-lasting antibacterial activity [24]. Ag nanoparticles were highly effective for antibacterial applications [25,26]. Dental resins containing Ag nanoparticles were recently reported [20,27]. One study incorporated nanoparticles of silver (NAg) into a composite containing nanoparticles of amorphous calcium phosphate (NACP), yielding a potent antibacterial activity while using a low NAg filler level [28].

The development of composites containing calcium phosphate (CaP) particles with remineralization capabilities represents an important approach to inhibiting secondary caries [29-32]. These composites could release supersaturating levels of calcium (Ca) and phosphate (PO_4) ions and remineralize enamel and dentin lesions [30,32]. Traditional CaP composites contained CaP particles of several microns in sizes [29,30,32]. Recently, CaP nanoparticles of sizes of about 100 nm were synthesized via a spray-drying technique and filled into dental composites [31,33]. These nanocomposites achieved Ca and PO_4 release similar to those of traditional CaP composites, while possessing much better mechanical properties [31,33].

Besides composites, it is also important to develop adhesives capable of inhibiting secondary caries. Adhesives bond the composite to the tooth structure and infiltrate into dentinal tubules to form an interlocked interface [34,35]. Vast efforts have been made to enhance adhesive bonding to the tooth structure [36-38]. Clinically, residual bacteria could exist in the prepared tooth cavity. In addition, microleakage could allow bacteria to invade the tooth-restoration interfaces. Therefore, it is desirable for the adhesive to be antibacterial to inhibit recurrent caries [16,18,39,40]. However, commercial adhesives in the cured state are usually not antibacterial. Hence, antibacterial agents such as MDPB [16,39], methacryloxylethyl cetyl dimethyl ammonium chloride [40], and cetylpyridinium chloride [18] were used to develop antibacterial adhesives. In addition, dentin primer directly contacts the tooth structure and could kill residual bacterial if rendered antibacterial [21,22,41]. However, a literature search revealed only a small number of publications on antibacterial adhesives and primers [16,18,21,22,39,40]. There has been no report on adhesive and primer containing NAg and NACP.

The objective of this study was to incorporate NAg and NACP into a bonding system, and to investigate the antibacterial properties using a dental plaque microcosm biofilm model. The hypotheses were: (1) Incorporating NACP into the adhesive at filler mass fractions of 10-40% would not decrease the shear bond strength to human dentin, compared to control without NACP; (2) Incorporating NAg into both primer and adhesive would not decrease the dentin shear bond strength; (3) The antibacterial and calcium phosphate-containing bonding system would greatly decrease the viability and lactic acid production of microcosm biofilms.

MATERIALS AND METHODS

NAg incorporation into primer and adhesive

Scotchbond Multi-Purpose (3M, St. Paul, MN, USA), referred to as "SBMP", was used as the parent bonding system to test the effect of incorporation of NACP and NAg. The purpose was to investigate a model system, and then the method of incorporating NACP and NAg could be applied to other bonding agents. According to the manufacturer, SBMP etchant contained 37% phosphoric acid. SBMP primer single bottle contained 35-45% 2-Hydroxyethylmethacrylate (HEMA), 10-20% copolymer of acrylic and itaconic acids, and 40-50% water. SBMP adhesive contained 60-70% BisGMA and 30-40% HEMA.

Silver 2-ethylhexanoate powder (Strem, New Buryport, MA, USA) was dissolved in 2-(tert-butylamino) ethyl methacrylate (TBAEMA, Sigma) at 0.08 g of silver salt per 1 g of

TBAEMA, following previous studies [27,28]. TBAEMA was used because it improves the solubility by forming Ag-N coordination bonds with Ag ions, thereby facilitating the Ag salt to dissolve in the resin solution. TBAEMA was selected since it contains reactive methacrylate groups and can be chemically incorporated into a resin upon photopolymerization. This method produced NAg with a mean particle size of 2.7 nm that were well dispersed in the resin [27,28]. To incorporate NAg into the primer, the aforementioned Ag-TBAEMA solution was mixed with the SBMP primer at a silver 2-ethylhexanoate/(primer + silver 2-ethylhexanoate) of 0.1% by mass; this mass fraction was selected based on previous studies [27,28]. To incorporate NAg into the adhesive, the Ag-TBAEMA was mixed with the SBMP adhesive at 0.1% mass fraction.

Addition of NACP into adhesive

Nanoparticles of ACP ($Ca_3[PO_4]_2$) were synthesized using a spray-drying technique as described previously [31,42]. Briefly, calcium carbonate ($CaCO_3$, Fisher, Fair Lawn, NJ, USA) and dicalcium phosphate anhydrous ($CaHPO_4$, Baker Chemical, Phillipsburg, NJ, USA) were dissolved into an acetic acid solution to obtain final Ca and PO_4 ionic concentrations of 8 mmol/L and 5.333 mmol/L, respectively. The Ca/P molar ratio for the solution was 1.5, the same as that for ACP. The solution was sprayed into the heated chamber of the spray-drying apparatus. The dried particles were collected via an electrostatic precipitator (AirQuality, Minneapolis, MN, USA), yielding NACP with a mean particle size of 116 nm [43].

The NACP were mixed with the adhesive containing 0.1% silver 2-ethylhexanoate. The NACP mass fractions in the adhesive were: 0%, 10%, 20%, 30%, and 40%, following previous studies on NACP nanocomposites [43,44].

Hence, six bonding agents were tested:

[1]. SBMP primer, SBMP adhesive (termed "SBMP control").

[2]. Primer + 0.1% NAg, adhesive + 0.1% NAg (termed “P&A + NAg”. P = primer, A = adhesive).

[3]. Primer + 0.1% NAg, adhesive + 0.1% NAg + 10% NACP (termed “P&A+NAg, A + 10NACP”).

[4]. Primer + 0.1% NAg, adhesive + 0.1% NAg + 20% NACP (termed “P&A+NAg, A + 20NACP”).

[5]. Primer+ 0.1% NAg, adhesive + 0.1% NAg + 30% NACP (termed “P&A+NAg, A + 30NACP”).

[6]. Primer + 0.1% NAg, adhesive + 0.1% NAg + 40% NACP (termed "P&A+NAg, A + 40NACP").

Dentin shear bond strength testing and SEM examination

Extracted caries-free human third molars were cleaned and stored in 0.01% thymol solution. The tips of the molar crowns were cut off via a diamond saw (Isomet, Buehler, Lake Bluff, IL, USA) to yield flat mid-coronal dentin surfaces. Following a previous study [45], the tooth was embedded in a polycarbonate holder (Bosworth, Skokie, IL, USA) and ground perpendicular to the longitudinal axis using 320-grit SiC paper until there was no occlusal enamel left. The bonding procedures are shown in Fig. 1A. Briefly, the dentin surface was etched with 37% phosphoric acid gel for 15 s and rinsed with water for 15 s [45]. The primer was applied with a brush-tipped applicator and rubbed in for 15 s, and the solvent was removed with a stream of air. The adhesive was then applied and photo-activated for 10 s (Optilux VCL 401, Demetron Kerr**,** Danbury**,** CT, USA). Then, a stainless-steel iris with a central opening (diameter = 4 mm, thickness = 1.5 mm) was held against the adhesive-treated dentin surface. The central opening was filled with a composite (TPH, Caulk/Dentsply, Milford, DE, USA) and photo-activated for 60 s.

The bonded specimens were stored in distilled water at 37 °C for 24 h [45]. Then, the dentin shear bond strength, S_D, was measured as schematically shown in Fig. 1B. The chisel was connected with a computer-controlled Universal Testing Machine (MTS, Eden Prairie, MN), held parallel to the composite-dentin interface, and loaded at 0.5 mm/min until the bond failed. S_D was calculated as: $S_D = 4P/(\pi d^2)$, where P is the load at failure, and d is the diameter of the composite. Ten teeth were tested for each group (n = 10).

For scanning electron microscopy (SEM) examination, the bonded tooth was cut through the center parallel to the longitudinal axis via a diamond saw (Isomet) with copious water. The sectioned surface was polished with increasingly finer SiC paper up to 4000 grit. According to a previous study [22], the polished surface was treated with 50% phosphoric acid for 30 s, then with 5% NaOCl for 10 min. After being thoroughly rinsed with water for 10 min, the specimens were air dried and then sputter-coated with gold. Three specimens were prepared for each group. The specimens were then examined in an SEM (Quanta 200, FEI, Hillsboro, OR, USA).

Specimen fabrication for biofilm experiments

Following previous studies [21,40], layered disk specimens were made as shown in Fig. 3A. A polyethylene mold (inner diameter = 9 mm, thickness = 2 mm) was situated on a glass slide. A primer was applied into the mold to cover the glass. After drying with a stream of air, an adhesive was applied and activated for 20 s with Optilux. A composite (TPH) was placed on the adhesive to completely fill the mold, and light activated for 1 min. The bonded specimens were immersed in water and agitated for 1 h to remove any uncured monomer [21]. The disks were then dried and sterilized with ethylene oxide (Anprolene AN 74i, Andersen, Haw River, NC, USA).

Dental plaque microcosm model and live/dead assay

Saliva is ideal for growing dental plaque microcosm biofilms *in vitro* which maintain much of the complexity and heterogeneity of dental plaque *in vivo* [46]. University of Maryland approved the dental plaque microcosm model. Saliva was collected from a healthy adult donor having natural dentition without active caries or periopathology, and without the use of antibiotics within the last 3 months [47]. The donor did not brush teeth for 24 h and abstained from food/drink intake for at least 2 h prior to donating saliva. Stimulated saliva was collected during parafilm chewing and kept on ice. The saliva was diluted in sterile glycerol to a concentration of 30%, and stored at -80ºC [47].

The saliva-glycerol stock was added, with 1:50 final dilution, to a growth medium as inoculum. The growth medium contained mucin (type II, porcine, gastric) at a concentration of 2.5 g/L; bacteriological peptone, 2.0 g/L; tryptone, 2.0 g/L; yeast extract, 1.0 g/L; NaCl, 0.35 g/L, KCl, 0.2 g/L; $CaCl_2$, 0.2 g/L; cysteine hydrochloride, 0.1 g/L; haemin, 0.001 g/L; vitamin K1, 0.0002 g/L, at pH 7 [48]. The inoculum was cultured in an incubator (5% CO_2, 37 °C) for 24 h. Each disk was placed into a well of 24-well plates, with the primer on the top. Then, 1.5 mL of inoculum was added to each well, and incubated for 8 h. The disks were transferred to new 24-well plates with fresh medium and incubated. After 16 h, the disks were transferred to new 24-well plates with fresh medium and incubated for 24 h. This totaled 2 d of incubation, which was shown in a previous study to be sufficient to form microcosm biofilms [47].

Disks with 2-day biofilms were washed three times with phosphate buffered saline (PBS), and then stained using a live/dead bacterial kit (Molecular Probes, Eugene, OR, USA). Live bacteria were stained with Syto 9 to produce a green fluorescence, and bacteria with compromised membranes were stained with propidium iodide to produce a red fluorescence.

Specimens were examined with an epifluorescence microscope (TE2000-S, Nikon, Melville, NY, USA).

MTT assay of metabolic activity

MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay was used to measure the metabolic activity of biofilms [28,49]. MTT is a colorimetric assay that measures the enzymatic reduction of MTT, a yellow tetrazole, to formazan. Each disk with the 2-day biofilm was transferred to a new 24-well plate. One mL of MTT dye (0.5 mg/mL MTT in PBS) was added to each well and incubated for 1 h. During this process, metabolically active bacteria reduced the MTT to purple formazan. After 1 h, the disks were transferred to a new 24-well plate, 1 mL of dimethyl sulfoxide (DMSO) was added to solubilize the formazan crystals, and the plate was incubated for 20 min in the dark. After mixing via pipetting, 200 μL of the DMSO solution from each well was transferred to a 96-well plate and the absorbance at 540 nm (optical density OD540) was measured via a microplate reader (SpectraMax M5, Molecular Devices, Sunnvale, CA, USA). A higher absorbance is related to a higher formazan concentration, which indicates a higher metabolic activity in the biofilm adherent on the disk.

Lactic acid production and colony-forming unit (CFU) counts

Disks with 2-day biofilms were rinsed with cysteine peptone water (CPW) to remove loose bacteria, and transferred to 24-well plates containing buffered peptone water (BPW) plus 0.2% sucrose. The samples were incubated for 3 h to allow the biofilms to produce acid. The BPW solutions were then stored for lactate analysis. Lactate concentrations were determined using an enzymatic (lactate dehydrogenase) method [47]. The microplate reader was used to measure the absorbance at 340 nm (optical density OD_{340}) for the collected BPW solutions. Standard curves were prepared using a lactic acid standard (Supelco, Bellefonte, PA).

Disks with biofilms were transferred into tubes with 2 mL CPW, and the biofilms were harvested by sonication and vortexing via a vortex mixer (Fisher, Pittsburgh, PA, USA). Three types of agar plates were used. First, tryptic soy blood agar culture plates were used to determine total microorganisms [47]. Second, mitis salivarius agar (MSA) culture plates, containing 15% sucrose, were used to determine total streptococci [50]. This is because MSA contains selective agents crystal violet, potassium tellurite and trypan blue, which inhibit most gram-negative bacilli and gram-positive bacteria except streptococci, thus enabling

streptococci to grow [50]. Third, cariogenic mutans streptococci are known to be resistant to bacitracin, and this property was used to isolate mutans streptococci from the oral microflora. The MSA agar plates with 0.2 units of bacitracin per mL were used to determine mutans streptococci.

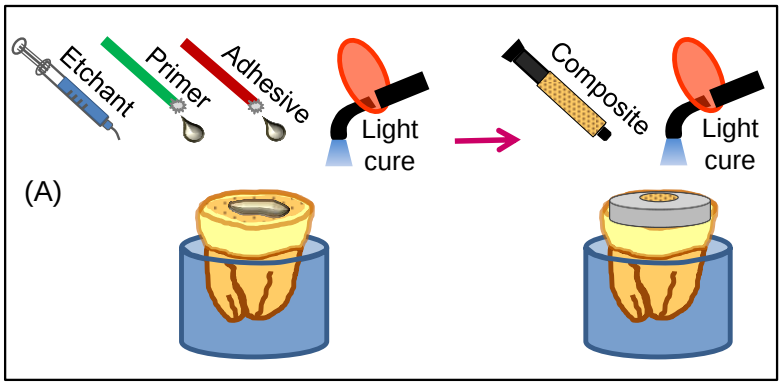
One-way analysis of variance (ANOVA) was performed to detect the significant effects of the variables Tukey's multiple comparison was used to compare the data at a p value of 0.05.

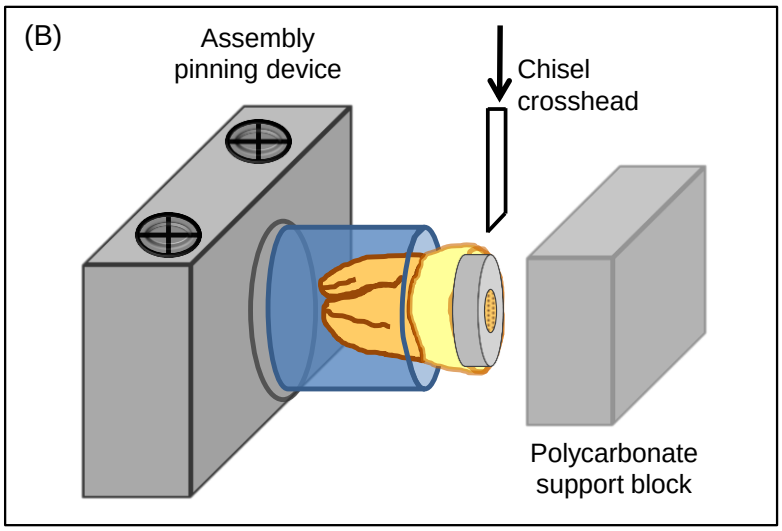
RESULTS

Figure 1 shows (A) schematic of the bonding procedures, (B) schematic of the bond test, and (C) human dentin shear bond strength data (mean ± sd; n = 10). Adding 0.1% NAg into primer and adhesive yielded a bond strength of (30.7 ± 8.3) MPa, similar to (30.2 ± 5.0) MPa for the control ($p > 0.1$). Further adding 10% NACP into the adhesive slightly increased the bond strength to (34.3 ± 7.7) MPa ($p > 0.1$). While 40% NACP slightly decreased the bond strength, all the six groups had bond strengths that were not significantly different ($p > 0.1$).

Typical SEM images of the dentin-adhesive interfaces are shown in Fig. 2 for (A) SBMP control, (B) P&A+NAg, A+20NACP, and (C) P&A+NAg, A+40NACP. Numerous resin tags "T" from well-filled dentinal tubules were visible in all the samples. The resin tags were slightly shorter at 40% NACP than the other groups. "HL" refers to the hybrid layer between the adhesive and the underlying mineralized dentin. At a higher magnification, the NACP nanoparticles were visible in (D) with 20% NACP. Arrows in (D) indicate examples of NACP nanoparticles infiltrated into the dentinal tubules. This feature became more visible at higher magnifications in (E) and (F), where arrows indicate NACP, which infiltrated into not only the straight and smooth tubules (E), but also the bent and irregularly-shaped tubules (F).

Figure 3 shows (A) a schematical representation of biofilm culture on the layered specimen, (B-G) live/dead staining images for the six groups. Biofilms adherent on the control disks were primarily alive with continuous green staining. In sharp contrast, biofilms adherent on A&P+NAg had substantial areas with red, yellow and orange staining, indicating that the biofilm viability was substantially compromised. Adding NACP from 10% to 40% mass fraction did not appear to significantly alter the biofilm appearance. All the disks modified with NAg and NACP were covered mainly with red and orange staining, with small areas of green staining.





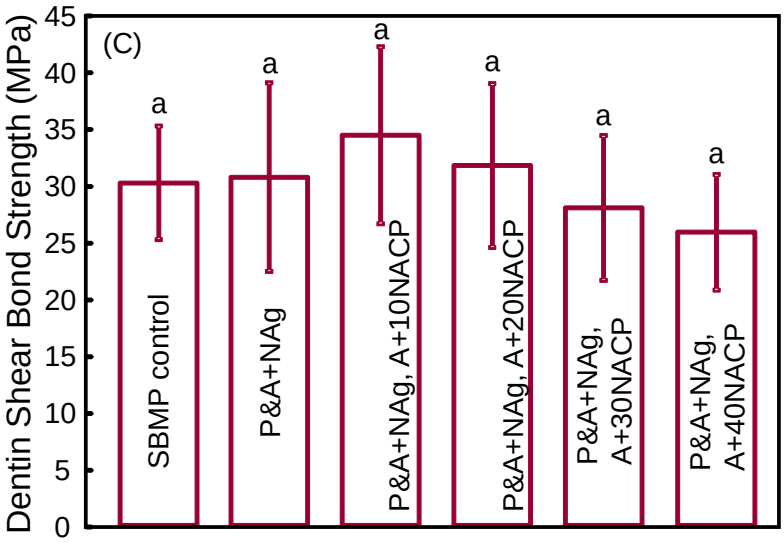


Figure 1. Dentin shear bond testing. (A) Schematic of specimen preparation, (B) schematic of testing, (C) human dentin shear bond strengths. Each value is mean ± sd (n = 10). The same letter at the bars indicates that all the six groups had bond strengths that were not significantly different ($p > 0.1$).

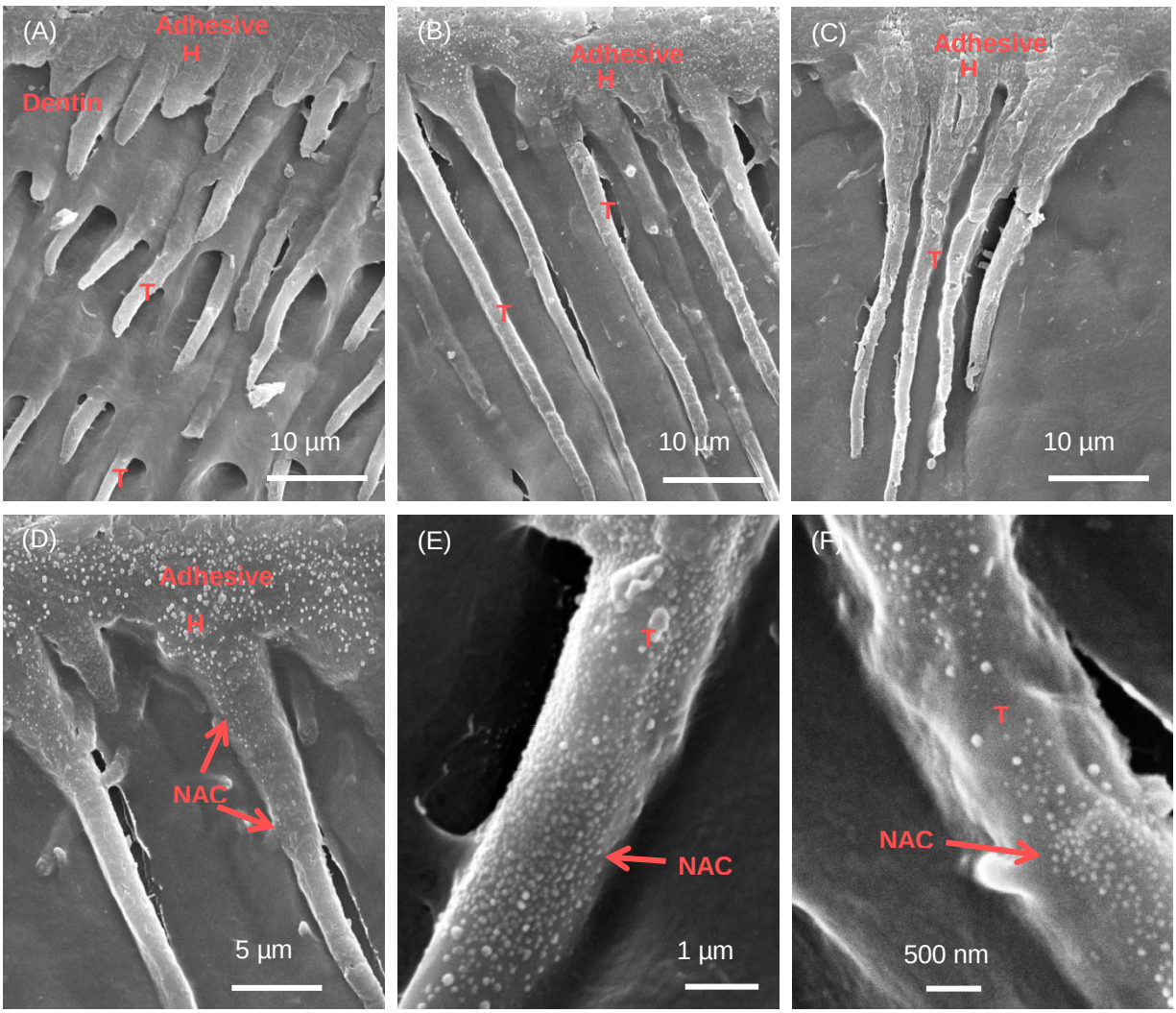


Figure 2. SEM micrographs of dentin-adhesive interfaces. (A) SBMP control, (B) P&A+NAg, A+20NACP, (C) P&A+NAg, A+40NACP. (D) P&A+NAg, A+20NACP at a higher magnification, and (E, F) at even higher magnifications. Adhesives filled the dentinal tubules and formed resin tags "T" for all six groups. "HL" indicates the hybrid layer between the adhesive and the underlying mineralized dentin. High magnification SEM in (D-F) revealed numerous NACP nanoparticles in the adhesive layer, in the hybrid zone, and inside the dentinal tubules. Arrows in (D-F) indicate NACP in the dentinal tubules. NACP were not only able to infiltrate with the adhesive into straight tubules (E), but also into bent and irregularly-shaped tubules (F).

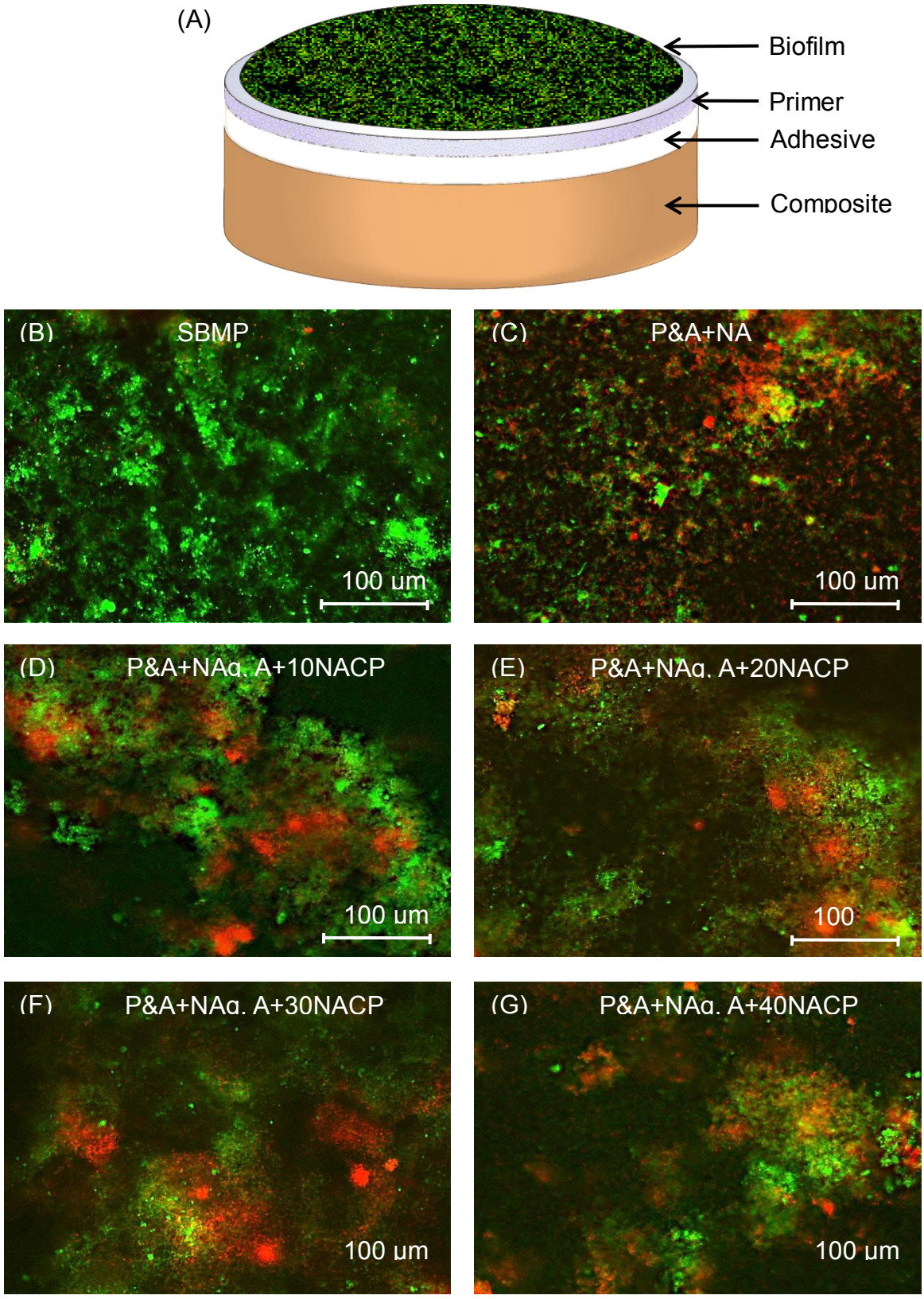


Figure 3. Dental plaque microcosm biofilm testing and live/dead assay. (A) Schematic of biofilm on the layered specimen, (B-G) live/dead images for the six groups. Live bacteria were stained green, and dead bacteria were stained red. Live and dead bacteria in the

proximity of each other produced yellow/orange colors. SBMP control disks were covered with live biofilms. A&P+NAg, with or without NACP, had mostly dead bacteria on the specimens. Therefore, the modified bonding agents with NAg and NACP possessed a potent antibacterial effect.

The MTT metabolic results are plotted in Figure 4. Control disks had biofilms with a high metabolic activity. Incorporation of 0.1% of NAg decreased the metabolic activity by more than half ($p < 0.05$). Adding NACP to the adhesive did not further significantly decrease the metabolic activity, although there was a decreasing tend at 30% and 40% NACP ($p > 0.1$). Figure 5 plots biofilm CFU counts per disk for: (A) Total microorganisms, (B) total streptococci, and (C) mutans streptococci. NAg greatly reduced the CFU compared to that of the control ($p < 0.05$). Specimens with NAg and NACP reduced the CFU by an order of magnitude, compared to the control. Specimens with 40% NACP slightly reduced the CFU, compared to P&A+NAg without NACP; however, this decrease was not statistically significant ($p > 0.1$).

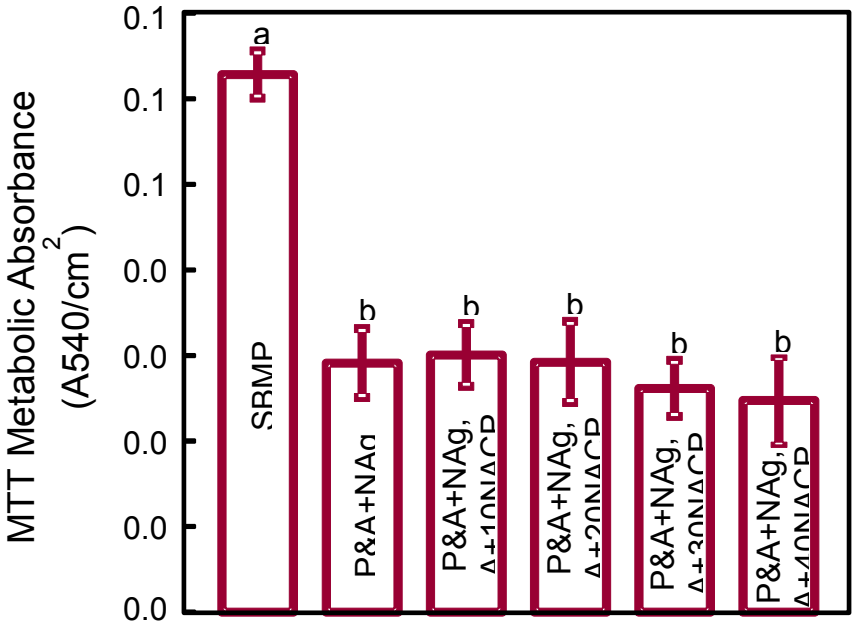


Figure 4. MTT assay on metabolic activity of biofilms on specimens of the six groups (mean ± sd; n = 6). Control disks had biofilms with a relatively high metabolic activity, indicating no antibacterial effect. However, all the bonding agents modified with NAg and NACP decreased the metabolic activity by more than half. Values with dissimilar letters are significantly different ($p < 0.05$).

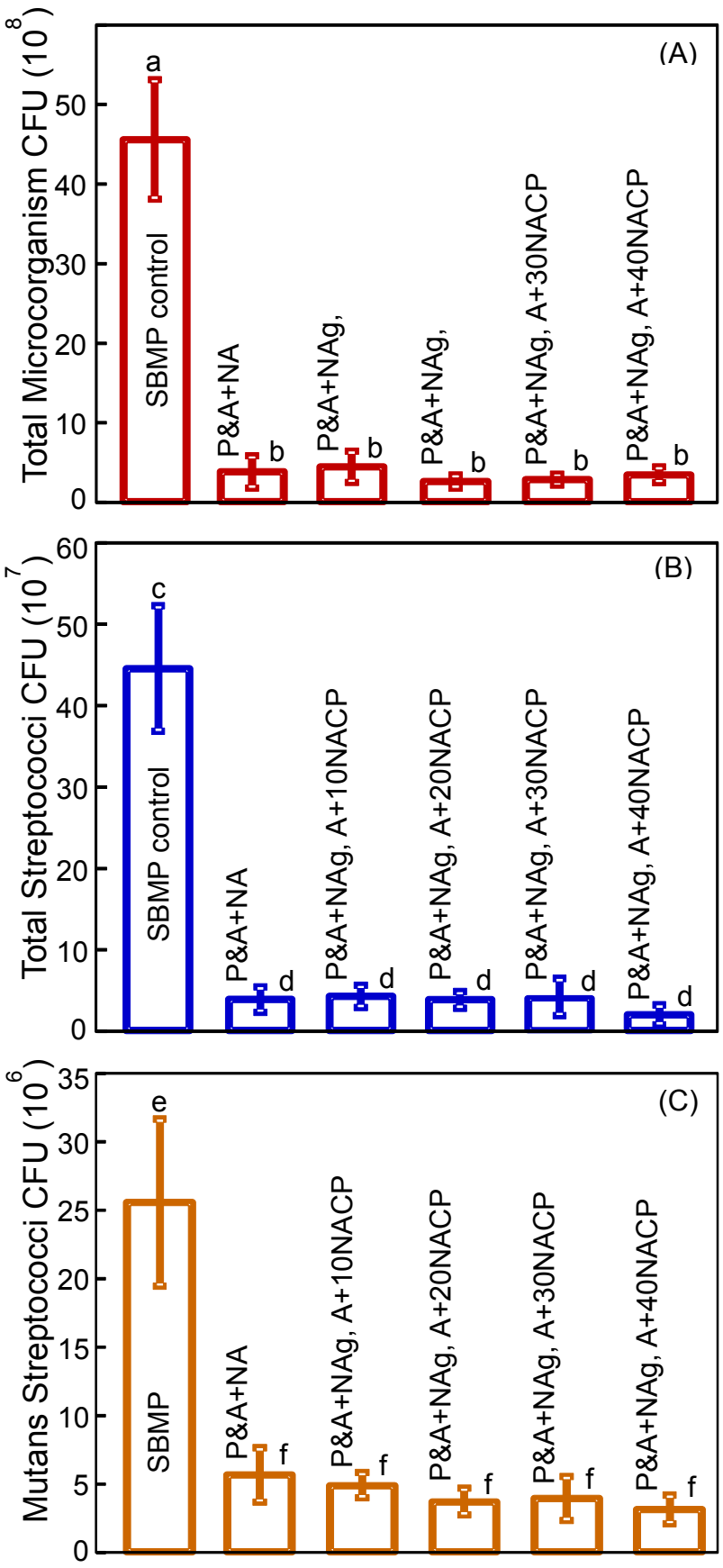


Figure 5. Microcosm biofilm CFU per disk for: (A) total microorganisms, (B) total streptococci, and (C) mutans streptococci (mean ± sd; n = 6). In each plot, values with dissimilar letters are significantly different ($p < 0.05$). The modified bonding systems reduced

the CFU counts by about an order of magnitude, compared to the commercial control. Therefore, the bonding agents containing NAg and NACP were strongly antibacterial.

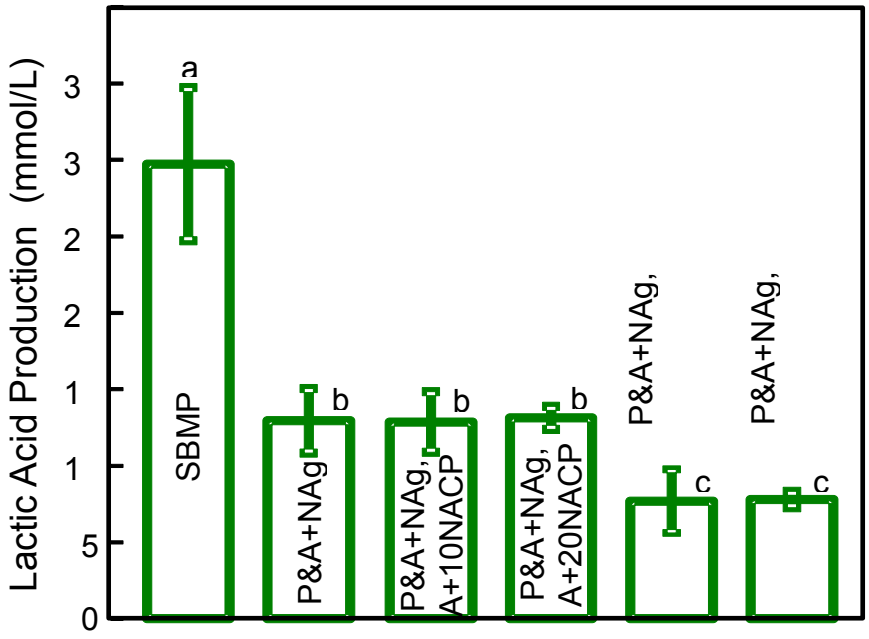


Figure 6. Lactic acid production by biofilms for the six groups (mean ± sd; n = 6). Acid production by biofilms on disks with NAg plus 30% and 40% NACP were approximately 1/4 of that on the control, indicating a potent antibacterial effect of specimens containing NAg and NACP. Values with dissimilar letters are significantly different ($p < 0.05$).

Fig. 6 plots the lactic acid production by biofilms. Biofilms on control disks produced the most acid, indicating that the un-modified commercial bonding agent was not antibacterial. Incorporation of NAg dramatically decreased the acid production, to less than half of that of the control ($p < 0.05$). Adding 30% and 40% NACP slightly and significantly ($p < 0.05$) decreased the acid production, compared to P&A+NAg without NACP. Lactic acid production by biofilms on the disks with 30% and 40% NACP were about 1/4 of the acid production for the control.

DISCUSSION

NACP are a promising type of therapeutic nano-filler for dental resins, shown recently for composites to release high levels of Ca and PO_4 ions with remineralization potential, while the composite maintained good mechanical properties [43,44]. NAg are another type of promising fillers for dental resins, shown in recent studies with NAg of 2-3 nm sizes being

well-dispersed in the resin matrix [27,28]. Due to the small size and high surface area of the NAg, a strong antibacterial activity could be achieved at a low filler level, without adversely affecting the composite color and mechanical properties [27,28]. In the present study, NACP and NAg were incorporated into a bonding system, with the rationale to achieve the double benefits of remineralization and antibacterial properties. The present study demonstrated that it was feasible to incorporate NACP and NAg into the bonding agent, yielding much lower biofilm viability and acid production, without significantly compromising dentin bond strength. The incorporation of 0.1% NAg into both primer and adhesive had no adverse effect on the bond strength, and had no noticeable difference in color and esthetics compared to the commercial control without NAg. The incorporation of 10%-30% NACP also had no adverse effect on bond strength. A previous study showed that a composite with 20% NACP released Ca and PO_4 ions to levels comparable to traditional CaP composites shown to effectively remineralize tooth lesions [43]. Another study showed that a composite with 30% NACP neutralized an acid attack, and raised the solution pH from a cariogenic pH of 4 to a safe pH of 6.5 [44]. Therefore, the incorporation of 0.1% NAg plus 20% or 30% NACP into the bonding agent is expected to acquire antibacterial, acid neutralization and remineralization benefits, which require further study. The method of incorporating dual remineralizing and antibacterial agents may be applicable to a wide range of dental bonding systems to inhibit secondary caries.

Traditional CaP composites were filled with CaP particles of about 1-55 μm in sizes [29,30,32]. These composites released Ca and PO_4 ions and remineralized tooth lesions [30,32]. The spray-drying technique produced a new generation of CaP nanoparticles having sizes of around 100 nm with a high specific surface area. The resulting nanocomposites could have substantial releases of Ca and PO_4 ions, while possessing mechanical properties nearly two-fold those of traditional CaP composites [31,33]. The NACP nanocomposite was smart and greatly increased the ion release at a low pH, when such ions are most needed to combat caries [43]. The NACP nanocomposite was moderately antibacterial against planktonic *S. mutans*, likely because NACP was alkaline [44]. This is consistent with the present study which showed that the specimens containing 30% and 40% NACP caused a moderate decrease in MTT activity, CFU and lactic acid, although in most cases such a decrease was not statistically significant. Therefore, the main purpose of incorporating NACP was Ca and PO_4 release and remineralization, not antibacterial activity. One reason of using NACP, and not traditional CaP fillers, was that NACP could release more Ca and PO_4 ions at lower filler levels [43]. This is important because a low filler level could be used for adhesives so that the

adhesive could maintain a low viscosity and the ability to flow into dentinal tubules. Another reason of using NACP was that particles of about 100 nm in sizes could infiltrate into dentinal tubules more easily than traditional CaP particles of several microns to tens of microns in sizes.

Besides Ca and PO_4 ions, there is a need for a potent antibacterial agent to be present in the bonding agent. Caries is a dietary carbohydrate-modified bacterial infectious disease caused by acid production by biofilms [13,14]. More than half of tooth cavity restorations placed by the dentists are replacements, with secondary caries as the main reason for failure [11,12,15]. An antibacterial bonding agent at the tooth-restoration interface is beneficial because there are often residual bacteria in the prepared tooth cavity [22,39,40]. There has been an increased interest in less removal of tooth structure and the minimal intervention dentistry [51], which could leave behind more carious tissues with active bacteria in the tooth cavity. Furthermore, the atraumatic restorative treatment (ART) method has received increasing attention, which usually does not remove the carious tissues completely [52]. While the NACP could help remineralize the demineralized tooth structure, the NAg primer and adhesive could kill the residual bacteria. In addition, while a complete sealing of the tooth-restoration interface is highly desirable, it is difficult to achieve, as many studies showed microgaps at the interfaces which could allow for bacteria invasion [53,54]. Residual bacteria and new invading bacteria along the tooth-restoration margins could harm the pulp and cause recurrent caries. The primer directly contacts the dentin, and therefore could be an important vehicle to deliver antimicrobial agents such as NAg to kill bacteria in the tooth cavity including those in the dentinal tubules. Furthermore, microgaps were observed between the adhesive resin and the primed dentin, or between the adhesive resin and the hybrid layer [53,54]. This would suggest that a large portion of the marginal gap is surrounded by the cured adhesive resin, hence the invading bacteria would mostly come into contact with the adhesive surface [39]. Therefore, it is desirable to not only have an antibacterial primer, but also an antibacterial adhesive. Therefore, the present study incorporated NAg into both the adhesive and the primer. This study showed that the commercial adhesive without modification was not antibacterial, consistent with previous results showing that the cured commercial adhesives had normal bacteria growth with no antibacterial activity [39,40,55]. In contrast, the NAg primer-adhesive system greatly decreased the biofilm viability, reducing the CFU to nearly 1/10, and acid production to 1/4, of those of the commercial control.

Ag has a strong antibacterial activity [26], low toxicity and good biocompatibility with human cells [56], and a long-term antibacterial effect due to sustained silver ion release [25].

Composites containing Ag inhibited oral bacteria such as *S. mutans* [20,23,24,27]. Recently, Ag nanoparticles with a high surface area were incorporated into resins to reduce the Ag particle concentration necessary for efficacy, without compromising the composite color and mechanical properties [27]. Evenly-dispersed NAg in a composite had a 40% reduction in bacteria coverage [28]. Regarding the antibacterial mechanism, Ag ions could inactivate the vital enzymes of bacteria, thus causing DNA in the bacteria to lose its replication ability, which leads to cell death [26]. Regarding the durability, Ag-containing composites showed long-term antibacterial effects and inhibited *S. mutans* growth for more than 6 months [23]. The Ag salt was dissolved in the TBAEMA monomer in the present study, which was mixed with the resin and photo-cured, thus forming the NAg *in situ*. This method yielded well-dispersed NAg in the resin without agglomeration [27,28]. This method avoided the need to mix pre-fabricated nanoparticles with the adhesive, which could cause agglomeration. The potent antibacterial effects of the present study suggest that the NAg may have a wide applicability to other bonding agents.

The dentin shear bond strengths of the six groups in the present study ranged from 26 MPa with 40% NACP, to 34 MPa with 10% NACP. A previous study used the same bonding and testing procedures, and measured a dentin shear bond strength of about 20 MPa for a resin containing 40% by mass of ACP [45]. Their ACP particle sizes were 3 and 58 μm [45]. While the difference in polymer composition may have influenced the bond strength, their ACP particles being much larger than the NACP likely also contributed to lower bond strength than that of the present study. Regarding the test method, a recent article reviewed 100 studies published between 2007 and 2009 [57]. They found that most studies used bonding areas between 3 and 4 mm in diameter, consistent with the 4 mm in the present study. The most frequently used crosshead speeds were 0.5 and 1 mm/min [57]; the present study used 0.5 mm/min. Regarding sample size, previous studies used n as low as 5 and as high as 25, with the majority using n = 10 [57], which is the same as that of the present study. Previous studies tested commercial bonding agents and reported dentin shear bond strengths of 23 to 34 MPa for SBMP [58,59]. Hence the bond strengths of the present study are consistent with the previous reports. Regarding the scatter in the shear bond strength, the biological, chemical, microstructural variations and anisotropy in the extracted human teeth likely contributed to the standard deviation in the data. The standard deviation/mean in the present study ranged from 16% to 27%, which is consistent with previous studies [45,57-59]. The bonding agents appeared to have infiltrated and wetted the dentin surface well, manifested by the formation of long resin tags from well-filled dentinal tubules. These features are believed to result in strong

and long-lasting bonds to dentin [37,60]. SEM examinations revealed the prevalence of resin tags in the present study, hence the dentin bonds are expected to be durable. The successful infiltration of numerous nanoparticles into dentinal tubules is expected to exert therapeutic and remineralizing effects. Further study is needed to investigate the long-term bonding properties as well as antibacterial and remineralizing effects using bonding agents containing NAg and NACP.

CONCLUSION

The present study showed that the incorporation of 0.1% NAg and 10%-40% NACP did not compromise the dentin shear bond strength. Groups with NACP and NAg reduced the metabolic activity of dental plaque microcosm biofilms by more than half. CFU counts for total microorganisms, total streptococci, and mutans streptococci were reduced by an order of magnitude. Lactic acid production by biofilms on disks containing NACP and NAg were reduced to 1/4 of that on control. The antibacterial and CaP nanoparticle-containing bonding agents with high bond strength have the potential to inhibit residual bacteria in the tooth cavity, hinder invading bacteria along the tooth-restoration interface, and remineralize tooth lesions. The method of incorporating NACP and NAg may be applicable to other dental bonding agents.

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3.5 CAPÍTULO 5

Dental bonding systems containing antibacterial agents and calcium phosphate nanoparticles

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Short title: Antibacterial and CaP nanoparticle-containing adhesives

Key words: Antibacterial primer and adhesive, dentin bond strength, silver nanoparticles, calcium phosphate nanoparticles, human saliva microcosm biofilm, caries inhibition.

ABSTRACT

Recurrent caries remains the main reason for dental restoration failure, and replacement of failed restorations accounts for 50-70% of all restorations performed. Antibacterial bonding agents could help inhibit biofilms and their acids at tooth-restoration margins, and calcium phosphate (CaP) ions could remineralize tooth lesions. The objectives of this study were to: (1) incorporate nanoparticles of silver (NAg), quaternary ammonium dimethacrylate (QADM), and nanoparticles of amorphous calcium phosphate (NACP) into bonding agent; and (2) investigate their effects on dentin bond strength and microcosm biofilms. An experimental primer was made with pyromellitic glycerol dimethacrylate (PMGDM) and 2-hydroxyethyl methacrylate (HEMA). An adhesive was made with bisphenol glycerolate dimethacrylate (BisGMA) and triethylene glycol dimethacrylate (TEGDMA). NAg was incorporated into primer at 0.1% by mass. The adhesive contained 0.1% NAg and 10% QADM, and 0%, 10%, 20% 30%, or 40% of NACP. Incorporating NAg into primer and NAg-QADM-NACP into adhesive did not adversely affect the dentin shear bond strength ($p > 0.1$). SEM showed numerous resin tags, and TEM revealed the incorporation of NAg and NACP into dentinal tubules. Viability of human saliva microcosm biofilms adherent on the layered primer/adhesive/composite disks was substantially reduced via addition of NAg and QADM. Metabolic activity, lactic acid, and colony-forming units of biofilms were much lower on the new bonding agents, than those on the control ($p < 0.05$). In conclusion, novel dental bonding agents containing NAg, QADM and NACP were developed with the potential to kill residual bacteria in the tooth cavity and inhibit the invading bacteria along the tooth-restoration margins, with NACP to remineralize tooth lesions. The novel method of combining antibacterial agents (NAg and QADM) with remineralizing agent (NACP) may have wide applicability to other adhesives for caries inhibition.

INTRODUCTION

Composite resins are being increasingly used as esthetic dental restorative materials.[1-5] Their popularity is a result of significant improvements in properties and performance.[6-13] Nonetheless, composites tend to accumulate more biofilms *in vivo*,[14,15] which could produce acids and cause dental caries.[16,17] Recurrent caries is the main reason for restoration failure, and replacement of the failed restorations accounts for 50-70% of all operative work.[18-20] To reduce secondary (recurrent) caries, novel antibacterial resins containing quaternary ammonium salts (QAS) were developed.[21-26] Resins containing 12-methacryloyloxydodecylpyridinium bromide (MDPB) significantly reduced the bacteria growth.[21,22,27] Other resins employed antibacterial agents such as methacryloxylethyl cetyl dimethyl ammonium chloride (DMAE-CB) and cetylpyridinium chloride (CPC).[23,24,28,29]

Another method to combat dental caries involved the development of antibacterial resins by incorporating silver (Ag) filler particles.[30-32] Nanoparticles of Ag (NAg) were demonstrated to be effective for antibacterial applications.[26,32,33] A different method for caries-inhibition employed calcium phosphate (CaP) particle-filled composites, which released Ca and P ions and remineralized tooth lesions.[34-36] Recently, CaP nanoparticles were synthesized via a spray-drying technique.[37] The new nanocomposites containing nanoparticles of amorphous calcium phosphate (NACP) released Ca and P ions similar to traditional CaP composites, while possessing much higher mechanical properties for load-bearing tooth restorations.[38,39]

Besides composites, it is also important to develop caries-inhibiting adhesives. Adhesive bond the composite to the tooth structure and infiltrates into dentinal tubules.[40-42] Extensive studies were performed to improve and characterize enamel and dentin bonding.[43-46] Residual bacteria could exist in the prepared tooth cavity, and microleakage could allow bacteria to invade the tooth-restoration interfaces.[21,28] Therefore, antibacterial adhesives were developed using MDPB and other antibacterial agents.[27-29] It is also beneficial to render the primer antibacterial because it directly contacts tooth structure.[47,48] Recently, quaternary ammonium dimethacrylate (QADM) was combined with NAg in a commercial primer (SBMP, 3M).[49] However, QADM and NAg have not been incorporated into any other adhesive systems. It remains to be investigated (1) whether QADM and NAg could be incorporated into another bonding system to also impart potent antibacterial functions; (2) whether NACP (as a remineralizer with Ca and P ions) can be incorporated into the antibacterial adhesive without compromising the dentin bond strength; (3) whether the NACP

and NAg nanoparticles could flow into the dentinal tubules with the adhesive. The rationale was that while QADM and NAg could eradicate residual bacteria in the tooth cavity and dentinal tubules, NACP could remineralize the remnants of lesions in the tooth cavity, thus yielding a unique combination of antibacterial/remineralizing capabilities.

Accordingly, the objectives of this study were to incorporate NAg, QADM, and NACP into an experimental bonding system and to investigate the effects on anti-biofilm and dentin bonding properties. It was hypothesized that: (1) The new experimental bonding agent containing NAg and QADM would greatly reduce the biofilm viability, metabolic activity, and lactic acid production in a dental plaque microcosm model; (2) Incorporation of NACP into the experimental bonding agent containing NAg and QADM would not decrease the dentin bond strength; (3) NAg and NACP could flow with the bonding agent into the dentinal tubules.

MATERIALS AND METHODS

Synthesis of experimental bonding agent containing NAg, QADM and NACP

The experimental primer contained pyromellitic glycerol dimethacrylate (PMGDM) (Hampford, Stratford, CT, USA) and 2-hydroxyethyl methacrylate (HEMA) (Esstech, Essington, PA) at a mass ratio 3.3/1, with 50% acetone solvent (all mass fractions).[50] The photo-initiator for the primer and adhesive was 1% phenyl bis(2,4,6-trimethylbenzoyl) phosphine oxide (BAPO) (Irgacure819, Ciba Chemicals, Japan). The experimental adhesive consisted of bisphenol glycerolate dimethacrylate (BisGMA) and triethylene glycol dimethacrylate (TEGDMA) (Esstech) at 70/30 mass ratio.[51]

Silver 2-ethylhexanoate salt (Strem, New Buryport, MA, USA) of 0.1 g was dissolved in 1 g of 2-(tert-butylamino)ethyl methacrylate (TBAEMA) (Sigma-Aldrich, St. Louis, MO, USA).[26,32] TBAEMA was used because it improves the solubility by forming Ag-N coordination bonds with Ag ions, thereby facilitating the Ag salt to dissolve in the resin solution. TBAEMA was selected since it contains reactive methacrylate groups and therefore can be chemically incorporated into a dental resin upon photopolymerization.[32] This method produced NAg *in situ* in the resin matrix with particle sizes of less than 10 nm.[26,32] The present study used a silver 2-ethylhexanoate/(primer + silver 2-ethylhexanoate) mass fraction of 0.1%, because this imparted a strong antibacterial effect, without adversely affecting dentin bond strength or primer color in preliminary studies. The adhesive contained a silver 2 ethylhexanoate/ (adhesive + silver 2-ethylhexanoate) of 0.1%.

In addition, QADM and NACP were incorporated into the adhesive. Bis (2-methacryloyloxy-ethyl) dimethylammonium bromide, a quaternary ammonium dimethacrylate (QADM), was recently synthesized.[25] The synthesis of QADM was performed using a modified Menschutkin reaction, where a tertiary amine group was reacted with an organo-halide. A benefit of this reaction is that the reaction products are generated at virtually quantitative amounts and require minimal purification.[25] Briefly, 10 mmol of 2-(*N*,*N*-dimethylamino)ethyl methacrylate (Sigma-Aldrich) and 10 mmol of 2-bromoethyl methacrylate (Monomer-Polymer Labs, Trevose, PA) were combined in ethanol and stirred at 60 °C for 24 h.[25,26] This yielded QADM as a clear, colorless, and viscous liquid, which was mixed with the adhesive at QADM/(adhesive + QADM) mass fraction of 10%, following a previous study.[49]

A spray-drying technique was used to make NACP.[37,38,52] Amorphous calcium phosphate (ACP, $Ca_3[PO_4]_2$) is important because it is a precursor that can convert to apatite, similar to the minerals in tooth enamel and dentin. A spraying solution was prepared by adding 1.5125 g of acetic acid glacial (J.T. Baker, Phillipsburg, NJ, USA) into 500 mL of distilled water. Then, 0.8 g of calcium carbonate ($CaCO_3$, Fisher, Fair Lawn, NJ, USA) and 5.094 g of DCPA (Baker) were dissolved into the acetic acid solution. The final Ca and PO_4 ionic concentrations were 8 mmol/L and 5.333 mmol/L, respectively. This yielded a Ca/P molar ratio of 1.5, the same as that for ACP. This solution was sprayed through a nozzle into a heated chamber. The water and volatile acid were evaporated into the dry, heated air flow and expelled into an exhaust-hood. The dried particles were collected by an electrostatic precipitator.[38] This method produced NACP with a mean particle size of 112 nm.[38] The NACP were mixed with the adhesive at NACP/(NACP + adhesive) mass fractions of 0%, 10%, 20%, 30%, and 40%, following previous studies.[38,39]

Therefore, seven primer and adhesive systems were formulated:

[1]. Experimental primer control, and adhesive control (termed "P control, A control").

[2]. P+NAg, A control (Primer had 0.1% NAg, while adhesive had no NAg).

[3]. P+NAg, A+NAg+QADM (Both P and A had 0.1% NAg. A also had 10% QADM).

[4]. P+NAg, A+NAg+QADM+10NACP (10NACP means 10% NACP by mass).

[5]. P+NAg, A+NAg+QADM+20NACP.

[6]. P+NAg, A+NAg+QADM+30NACP.

[7]. P+NAg, A+NAg+QADM+40NACP.

Dentin bond strength and SEM and TEM examinations

The use of extracted human teeth was approved by the University of Maryland. Caries-free third-molars were ground to remove the occlusal enamel.[49,53] Flat mid-coronal dentin surfaces of caries-free molars were prepared by cutting off the tips of crowns with a diamond saw (Isomet, Buehler, Lake Bluff, IL, USA). Each tooth was embedded in a poly-carbonate holder (Bosworth, Skokie, IL, USA) and ground perpendicularly to the longitudinal axis on 320-grit silicon carbide paper until occlusal enamel was removed. Dentin was etched with 37% phosphoric acid for 15 s.[49,53] A primer was applied, then an adhesive was applied and light-cured for 10 s (Optilux VCL401, Demetron**,** Danbury**,** CT, USA). A stainless-steel iris (inner diameter = 4 mm, thickness = 1.5 mm) was held against the adhesive-treated dentin, and the opening was filled with a composite (TPH, Caulk/Dentsply, Milford, DE, USA) and activated for 60 s. The specimens were stored in water at 37 °C for 24 h.[49,53] To measure the dentin shear bond strength, a chisel was aligned parallel to the composite-dentin interface and loaded (MTS, Eden Prairie, MN, USA) at 0.5 mm/min until the composite-dentin bond failed. Dentin shear bond strength $S_D = 4P/(\pi d^2)$, where P is load-at-failure, and d is composite diameter.[49,53]

To examine the dentin-adhesive interface, the bonded teeth were cut longitudinally. The sections were treated with 50% phosphoric acid and 10% NaOCl,[49] then gold-coated and examined via scanning electron microscopy (SEM, Quanta 200-FEI, Hillsboro, OR, USA). For transmission electron microscopy (TEM), thin sections with an approximate thickness of 120 μm were cut and fixed with 2% paraformaldehyde and 2.5% glutaraldehyde following a previous study.[54] Samples were embedded in resin epoxy (Spurr's, Electron Microscopy Sciences, PA, USA). Ultra-thin sections with approximate thickness of 100 nm were cut using a diamond knife (Diatome, Bienne, Switzerland) with an ultra-microtome (EM-UC7, Leica, Germany). The non-demineralized sections were examined in TEM (Tecnai-T12, Hillsboro, OR, USA).

Dental plaque microcosm biofilm model and live/dead assay

The microcosm biofilm model was approved by the University of Maryland. Human saliva was shown to be ideal for growing plaque microcosm biofilms *in vitro*, with the advantage of maintaining much of the complexity and heterogeneity of the dental plaque *in vivo*.[55] The saliva for biofilm inoculums was collected from a healthy adult donor having natural dentition without active caries or periopathology, and without the use of antibiotics

within the last 3 months, following a previous study.[49] The donor did not brush teeth for 24 h and abstained from food/drink intake for at least 2 h prior to donating saliva. Stimulated saliva was collected during parafilm chewing and kept on ice. Saliva was diluted in sterile glycerol to a concentration of 70% saliva and 30% glycerol, and stored at -80 ºC.[49]

Layered disks were fabricated as schematically shown in Fig. 3A, following previous studies.[47,49] A polyethylene mold with an inner diameter of 9 mm and a thickness of 2 mm was situated on a glass slide. A primer was first applied into the mold to cover the glass slide. After drying with a stream of air, the adhesive was applied and cured for 20 s with Optilux. Then, a composite (TPH) was placed on the adhesive to fill the disk mold and light-cured for 1 min. The cured disks were immersed in sterile water and agitated for 1 h to remove any uncured monomer, following a previous study.[47] The disks were then dried and sterilized with ethylene oxide (Anprolene AN 74i, Andersen, Haw River, NC, USA).

The saliva-glycerol stock was added, with 1:50 final dilution, to a growth medium as inoculum. The growth medium contained mucin (type II, porcine, gastric) at a concentration of 2.5 g/L; bacteriological peptone, 2.0 g/L; tryptone, 2.0 g/L; yeast extract, 1.0 g/L; NaCl, 0.35 g/L, KCl, 0.2 g/L; $CaCl_2$, 0.2 g/L; cysteine hydrochloride, 0.1 g/L; haemin, 0.001 g/L; and vitamin K1, 0.0002 g/L, at pH 7.[56] The inoculum was cultured at 37 °C in an incubator containing 5% CO_2 for 24 h. Each disk was placed into a well of 24-well plates, with the primer surface on the top. To each well was added 1.5 mL of inoculum, which was incubated for 8 h. The disks were then transferred to new 24-well plates with fresh medium and incubated. After 16 h, the disks were transferred to new 24-well plates with fresh medium and incubated for 24 h. This constructed a 2-day incubation, which was shown to form plaque microcosm biofilms.[49] The disks with 2-day biofilms were washed with phosphate buffered saline (PBS), and stained using a live/dead bacterial viability kit (Molecular Probes, Eugene, OR). Live bacteria were stained with Syto 9 to produce a green fluorescence, and bacteria with compromised membranes were stained with propidium iodide to produce a red fluorescence. The stained disks were examined using an epifluorescence microscope (TE2000-S, Nikon, Melville, NY, USA).[26]

Metabolic activity of microcosm biofilms

The MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay is a colorimetric assay that measures the enzymatic reduction of MTT, a yellow tetrazole, to formazan.[26] Each disk with the 2-d biofilm was transferred to a new 24-well plate, then 1 mL of MTT dye (0.5 mg/mL MTT in PBS) was added to each well and incubated at 37 °C in 5%

CO_2 for 1 h. During this process, metabolically active bacteria reduced the MTT to purple formazan. After 1 h, the disks were transferred to a new 24-well plate, 1 mL of dimethyl sulfoxide (DMSO) was added to solubilize the formazan crystals, and the plate was incubated for 20 min with gentle mixing at room temperature in the dark. After mixing via pipetting, 200 μL of the DMSO solution from each well was transferred to a 96-well plate, and the absorbance at 540 nm (optical density OD540) was measured via a microplate reader (SpectraMax M5, Molecular Devices, Sunnvale, CA). A higher absorbance is related to a higher formazan concentration, which indicates a higher metabolic activity in the biofilm on the disk.[26]

Lactic acid production and colony-forming units (CFU) of microcosm biofilms

To measure lactic acid production, disks with 2-day biofilms were transferred to 24-well plates containing buffered-peptone water (BPW) plus 0.2% sucrose, and incubated for 3 h to allow the biofilms to produce acid. The lactate concentrations in the BPW solutions were determined using an enzymatic (lactate dehydrogenase) method, following previous studies.[26,49] The microplate reader (SpectraMax M5) was used to measure the absorbance at 340 nm (optical density OD_{340}) for the collected BPW solutions. Standard curves were prepared using a lactic acid standard (Supelco, Bellefonte, PA, USA).

For colony-forming units (CFU), disks with 2-day biofilms were transferred into tubes with 2 mL CPW, and the biofilms were harvested by sonication and vortexing via a vortex mixer (Fisher, Pittsburgh, PA, USA). Four types of agar plates were used. First, tryptic soy blood agar culture plates were used to determine total microorganisms.[49] Second, mitis salivarius agar (MSA) culture plates, containing 15% sucrose, were used to determine total streptococci.[57] This is because MSA contains selective agents crystal violet, potassium tellurite and trypan blue, which inhibit most gram-negative bacilli and most gram-positive bacteria except streptococci, thus enabling streptococci to grow.[57] Third, cariogenic mutans streptococci are known to be resistant to bacitracin, and this property is often used to isolate mutans streptococci from the highly heterogeneous oral microflora. Hence, MSA agar culture plates plus 0.2 units of bacitracin per mL were used to determine mutans streptococci.[58] Forth, Rogosa SL culture plates were used to determine lactobacilli. Rogosa SL plates contained high levels of sodium acetate and ammonium citrate at a low pH which would inhibit most microorganisms but allow the growth of lactobacilli.[59]

One-way analysis of variance (ANOVA) was performed to detect the significant effects of the variables. Tukey's multiple comparison was used to compare the data at a *p* value of 0.05.

RESULTS

Dentin shear bond strengths (Fig. 1A) (mean±sd; n=10) were not significantly different between control and those containing NAg, QADM and NACP (p>0.1). SEM examinations of dentin-adhesive interfaces revealed numerous resin tags from well-filled dentinal tubules. An example is shown for control (Fig. 1B); other groups had similar features. TEM (Fig. 2A) revealed that NACP were successfully incorporated into dentinal tubules, with an example shown for P+NAg, A+NAg+QADM+30NACP. NAg were also incorporated into dentinal tubules (2B).

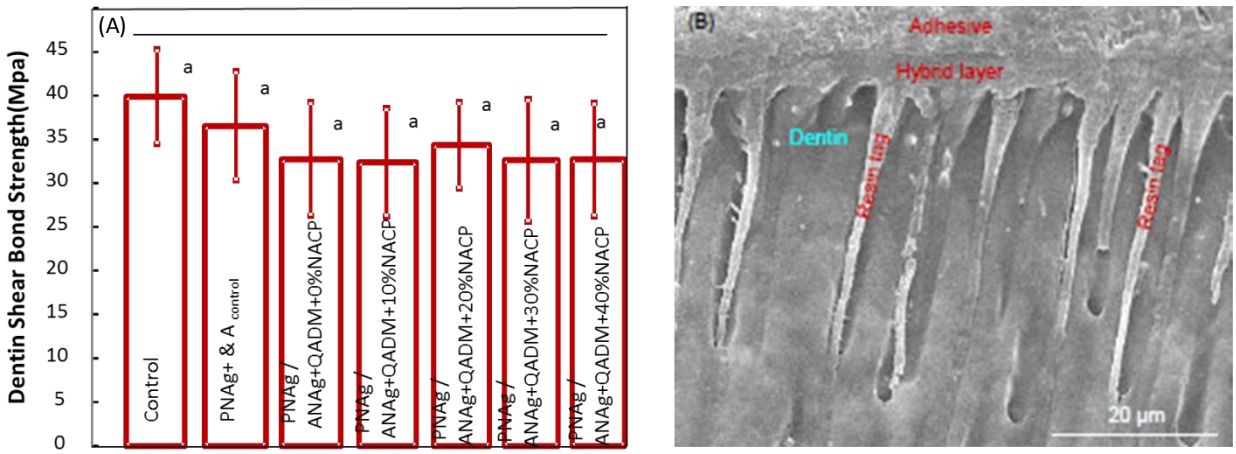

Figure 1. Dentin shear bond testing. (A) Dentin shear bond strength using extracted human molars. Each value is mean ± sd (n = 10). P = primer. A = adhesive. P+NAg had 0.1% NAg (by mass) in primer. A+NAg+QADM had 0.1% NAg + 10% QADM in adhesive. Adding NAg into the primer, and adding NAg, QADM and NACP into the adhesive did not decrease the dentin bond strength. Horizontal line indicates statistically similar values (p > 0.1). (B) SEM examination of dentin-adhesive interfaces revealed numerous resin tags from well-filled dentinal tubules, with an example shown for the control.

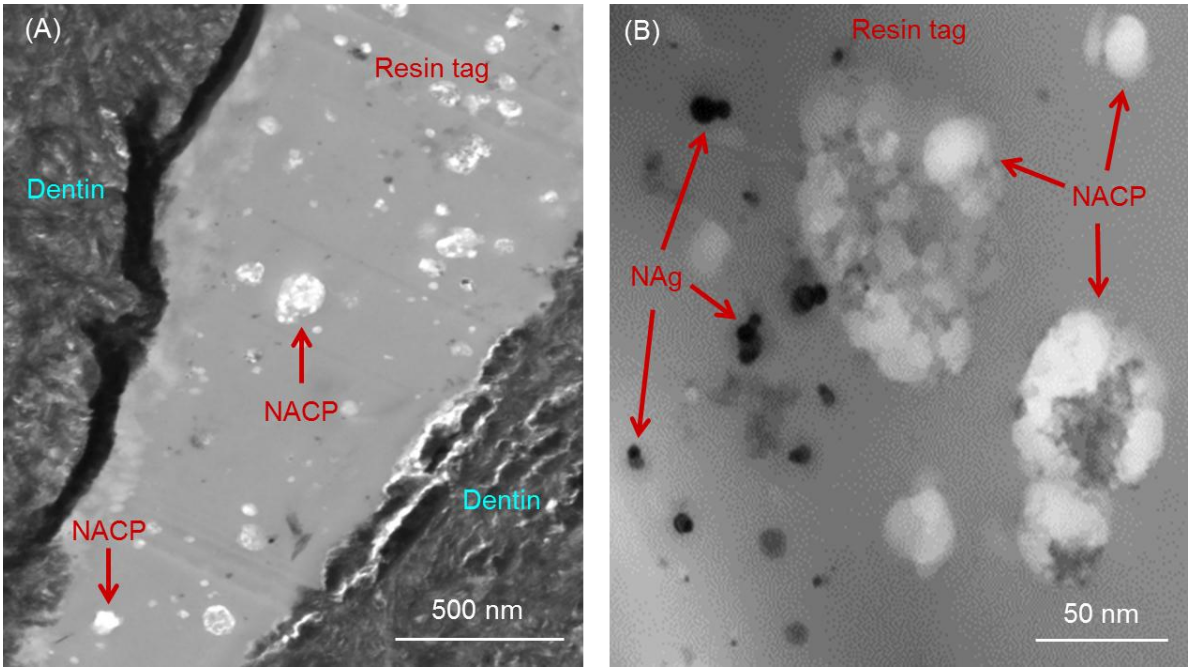

Figure 2. High magnification TEM examination of dentin-adhesive interfaces. (A) Typical resin tag for the primer and adhesive group of P+NAg, A+NAg+QADM+30NACP, shown as an example. Arrows indicate NACP that successfully flowed with the adhesive into dentinal tubules. (B) Higher magnification TEM revealed NAg as well as NACP in the resin tags in the dentinal tubules. The NAg appeared as black dots in TEM images with sizes of less than 10 nm, similar to those observed in previous study.[49]

Biofilms were grown on layered specimens (Fig. 3A). Live/dead staining showed primarily live bacteria (green) on control (3B). Disks containing NAg and NAg+QADM had many dead bacteria (red/orange staining in 3C-3D). Adding 10-40% NACP did not noticeably alter the biofilms (examples in 3E-3F). Incorporating NAg and QADM greatly decreased biofilm metabolic activity (Fig.4). Lactic acid from biofilms (Fig. 5) was also greatly-reduced via NAg-QADM. Compared to control, biofilm CFU counts were reduced by 3-4 folds for total microorganisms, total streptococci, mutans streptococci, and lactobacilli (Fig. 6A-D). The new bonding agents greatly reduced the CFU compared to that of control ($p<0.05$).

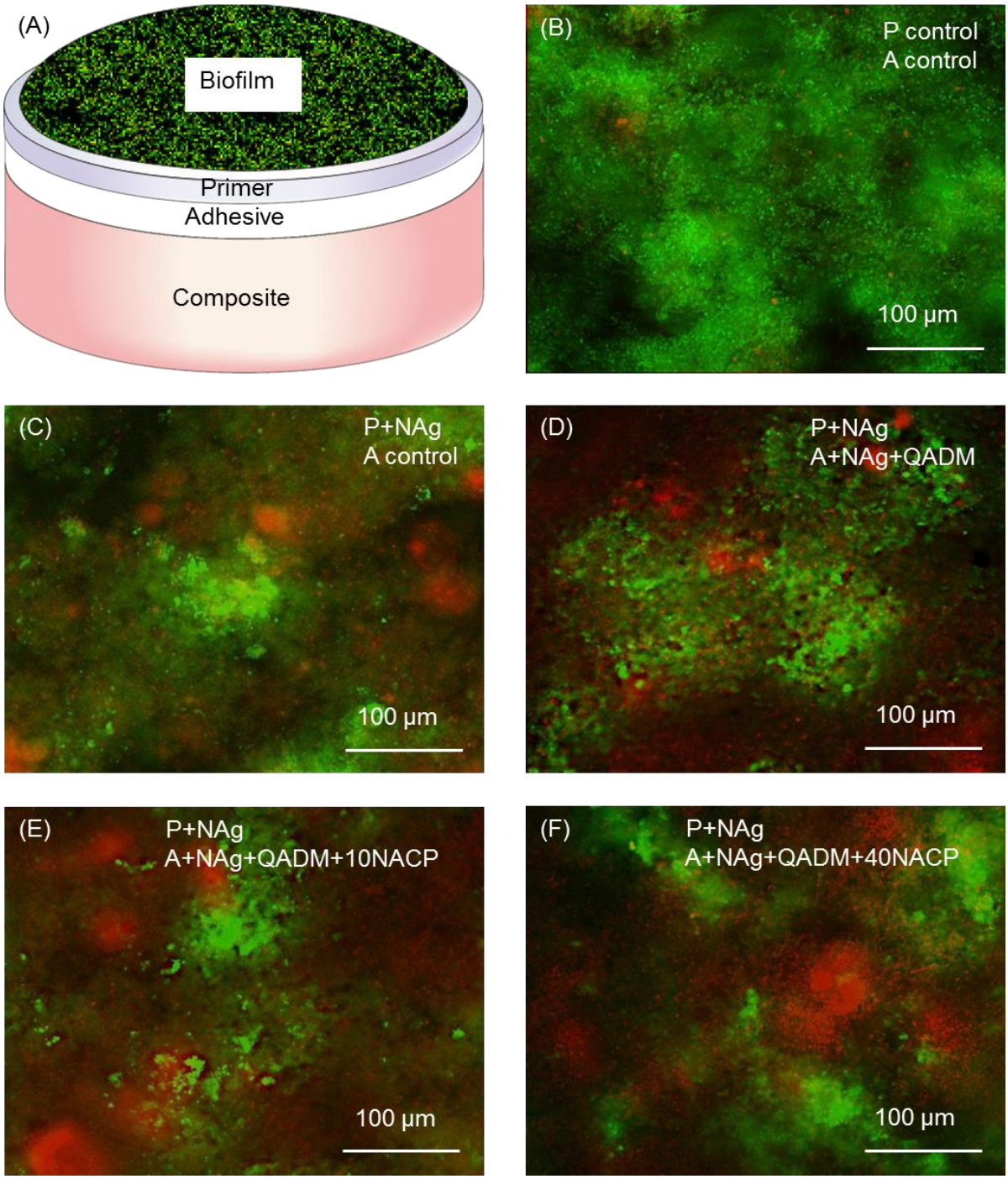

Figure 3. Human saliva microcosm biofilm live/dead assay. (A) Schematic of biofilm on cured disk with three layers: Primer, adhesive, and composite. (B-F) Representative live/dead images. The live bacteria were stained green, and the compromised bacteria were stained red. The live and dead bacteria in close proximity to each other produced orange/yellow colors. The biofilms were primarily alive on the control. In contrast, disks containing NAg and NAg+QADM had large amounts of dead bacteria. Incorporation of NACP into adhesive from 10% to 40% did not noticeably change the biofilm features.

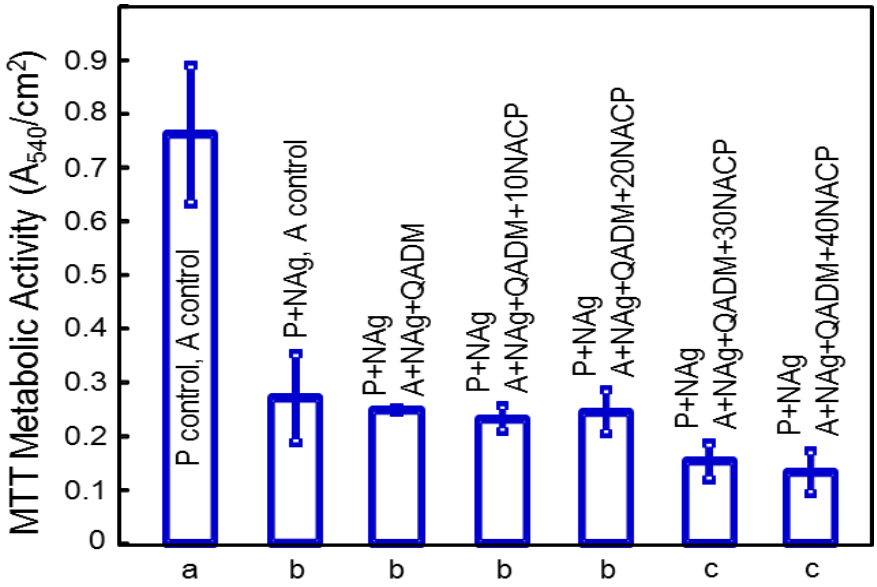

Figure 4. MTT metabolic activity of biofilms (mean ± sd; n = 6). The control disks had adherent biofilms with a relatively high metabolic activity. The modified bonding agents with QADM and NAg had much lower metabolic activity. The addition of NACP of 10% to 20% into the adhesive did not significantly change the metabolic activity, while 30% and 40% NACP significantly ($p < 0.05$) reduced the metabolic activity. Values with dissimilar letters at the bottom are significantly different from each other ($p < 0.05$).

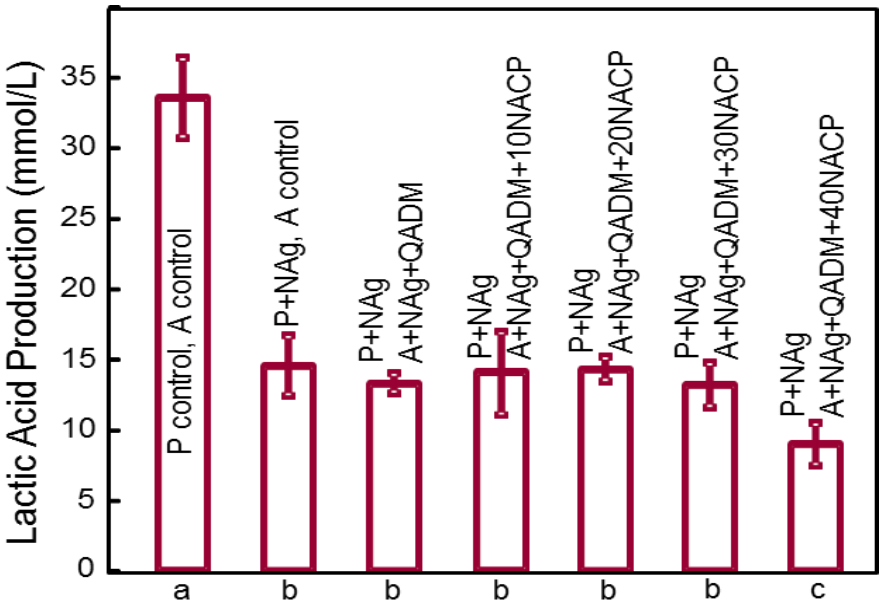

Figure 5. Microcosm biofilm lactic acid production (mean ± sd; n = 6). Incorporation of NAg and QADM into the bonding agent greatly lowered the lactic acid production, compared to control. NACP incorporation into the adhesive had little effect, except for the 40% NACP which significantly lowered the lactic acid production. Values with dissimilar letters at the bottom are significantly different from each other ($p < 0.05$).

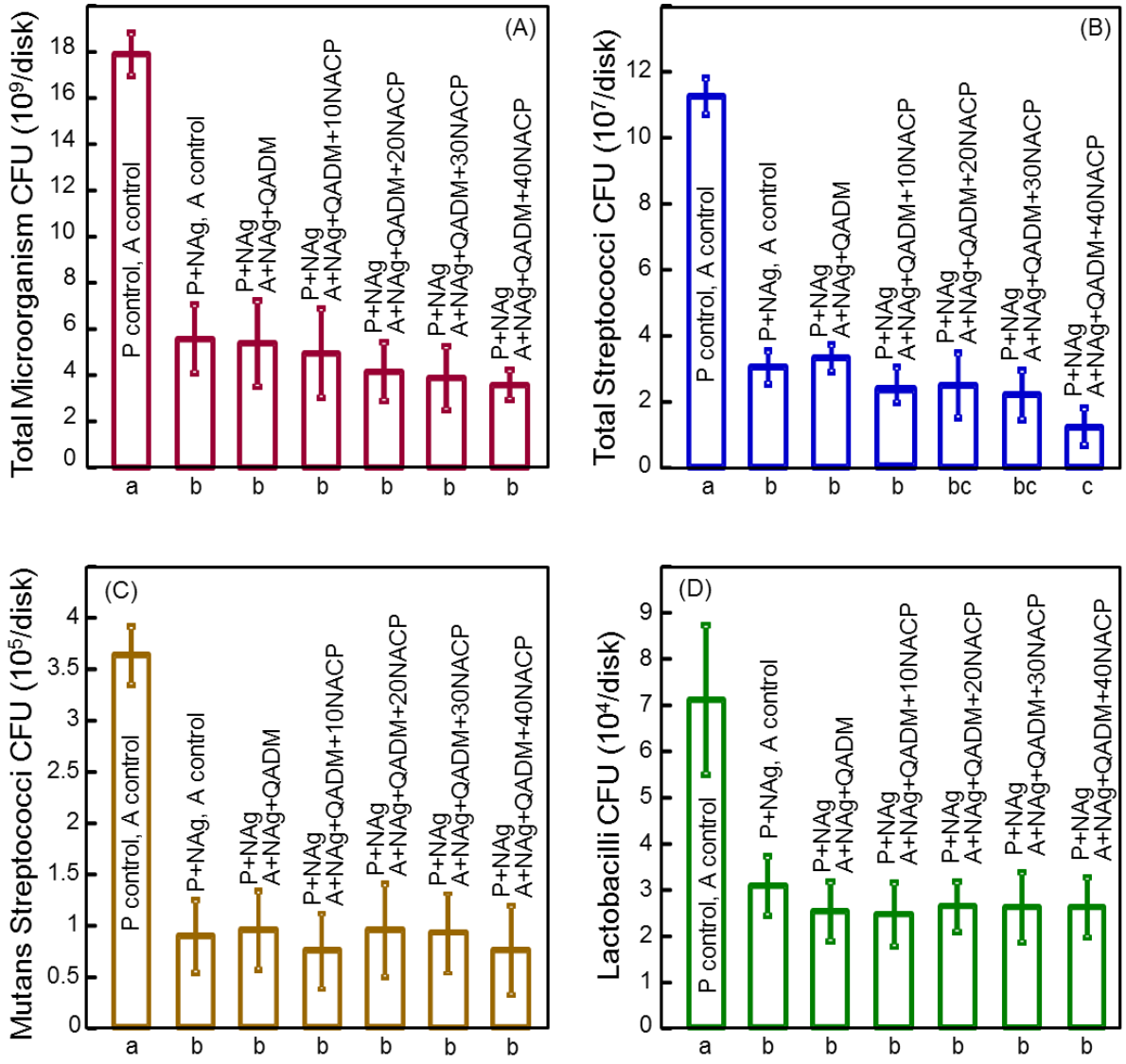

Figure 6. Colony-forming unit (CFU) counts for biofilms on the layered disks (mean ± sd; n = 6). (A) Total microorganisms, (B) total streptococci, (C) mutans streptococci, and (D) lactobacilli. The CFU counts for biofilms adherent on the new bonding agents were reduced to about 20-30% of the CFU of biofilms on the control. Hence, the new bonding agents had strong anti-biofilm effects. In each plot, values with dissimilar letters at the bottom are significantly different from each other ($p < 0.05$).

DISCUSSION

This study incorporated NAg into an experimental primer, and QADM and NACP into an experimental adhesive. A previous study incorporated NAg and QADM into a commercial bonding agent (SBMP, 3M),[49] but it was unclear if the NAg and QADM could be similarly applied to other bonding systems to achieve potent antibacterial activities without compromising the dentin bond strength. The present study used a primer comprising of PMGDM and HEMA, and an adhesive comprising of BisGMA and TEGDMA, which are an experimental bonding system and not commercially available. The results showed that QADM and NAg were successfully incorporated into this experimental bonding system, exhibiting strong antibacterial effects, which suggest that QADM and NAg are promising for applications into other bonding agents. While several previous studies used single-species bacteria models,[25,32,48] the present study used a dental plaque microcosm model. Dental plaque is a complicated ecosystem with about 1,000 bacterial species,[17] hence microcosm models could maintain much of the complexity and heterogeneity *in vivo*.[55] The new bonding agent reduced the biofilm viability and CFU to about 1/3 of those of the control. CFU counts of total microorganisms, total streptococci, mutans streptococci, and lactobacilli were all greatly reduced by the new bonding agents.

The results showed that using the layered disks, antibacterial primer P+NAg had strong antibacterial effects. Biofilms were inoculated on the primer layer, hence the adhesive underneath did not directly contact the biofilms. It should be noted that clinically, both antibacterial primers and adhesives are needed for caries inhibition. This is because, first, residual bacteria in tooth cavity can lead to caries and pulp damage. Therefore, antibacterial primer with direct contact to dentin could disinfect the prepared tooth cavity and eradicate residual bacteria. Second, although a complete sealing of the tooth-restoration interface is highly desirable, it is difficult to achieve. Indeed, previous studies revealed microgaps between the adhesive and the primed dentin, or between the adhesive and the hybrid layer.[60,61] This would suggest that a large portion of the marginal gap is surrounded by the adhesive resin, hence the invading bacteria would mostly come into contact with the adhesive surface.[27] Therefore, antibacterial adhesives are highly beneficial to combat the invading bacteria along the tooth-restoration margins due to bacterial leakage, thereby to protect the pulp and inhibit recurrent caries.

The results of the present study showed that incorporating 10% to 40% of NACP fillers into the BisGMA-TEGDMA adhesive did not adversely affect the dentin bond strength.

The purpose of NACP incorporation was for the adhesive to obtain CaP ion release and remineralization capabilities. Previous studies showed that CaP-containing resins remineralized enamel and dentin lesions *in vitro*.[35,36] Recent studies showed that NACP-containing composites released high levels of Ca and P ions.[38] Furthermore, NACP nanocomposite rapidly neutralized a cariogenic acid challenge and raised the pH from 4 to above 6.[39] While the present study focused on the effects of NACP-containing adhesive on dentin bond strength and NACP incorporation into dentinal tubules, further study should measure the mineral content of enamel and dentin around NACP adhesive under biofilms to investigate the caries-inhibition efficacy. In addition, the results of the present showed that the incorporation of NACP had little effect on the antibacterial effects. This is because CaP materials are not known to have significant antibacterial activities. In Fig. 4, a slight reduction in biofilm metabolic activity was observed at 30% and 40% NACP. In Fig. 5, there was a small reduction in lactic acid production at 40% NACP. These small reductions in bacteria activity may be due to the alkaline property of NACP leading to an increase in local pH.[39] However, any antibacterial function from NACP appears to be minor, hence the incorporation of antibacterial agents such as QADM and NAg are needed in adhesives to achieve potent anti-biofilm capabilities.

Quaternary ammonium monomers (QAMs) have been incorporated into dental resins to obtain antibacterial functions.[21-29,47,62] The QAM is copolymerized with the resin by forming a covalent bonding with the polymer network, and therefore is immobilized in the resin yielding a contact-inhibition effect against bacteria that attach to the surface.[21,22,47] Extensive studies have been performed on MDPB-containing dental composites and bonding agents, which showed strong antibacterial effects.[21,22,27,47] MDPB was effective against various oral bacteria, including facultative and obligate anaerobe in coronal lesions, and actinomyces and *Candida albicans* isolated from root caries.[63-65] In other studies, a QAM chloride was incorporated to develop an antibacterial bonding agent.[28] In addition, QAM bromides and chlorides were synthesized to develop antibacterial glass ionomer cements.[62] Recently, QADM was synthesized and incorporated into composites which hindered *S. mutan* growth.[26] Furthermore, the microcosm biofilm viability was greatly reduced when QADM was incorporated into a commercial primer.[49]

Besides quaternary ammonium monomers, silver (Ag) is another effective antimicrobial agent.[66,67] It was suggested that the Ag ions could inactivate the bacterial enzymes, causing the DNA to lose its replication ability, which leads to cell death.[67] Ag has good biocompatibility and low toxicity to human cells, has long-term antibacterial effects,[66]

and causes less bacterial resistance than antibiotics.[68] Compared to traditional Ag particles of several micrometers in size, an advantage of NAg is their high surface area, so that a low filler level of NAg in resin is sufficient for the resin to be strongly-antibacterial, without compromising the resin color or mechanical properties.[26,32,49] In the present study, Ag salt was dissolved in TBAEMA which was then mixed with resin. Ag ions agglomerated to form nanoparticles that became part of the resin upon photo-polymerization.[26,32,49] An advantage of this method was that it reduced the Ag salt to NAg *in situ* in the resin, avoiding the mixing of preformed Ag nanoparticles which could form large agglomerates. Another advantage was that TBAEMA contained reactive methacrylate functionality, and could be chemically-incorporated into resin upon photo-polymerization. In the present study, NAg were incorporated into the experimental primer and adhesive, yielding potent antibacterial effects. TEM examination revealed successful incorporation of NAg and NACP into dentinal tubules. Further study should investigate the effect of NAg in the tubules on the killing of residual bacteria inside the tubules, and the effect of NACP in bonding agent on the remineralization of residual lesions in the tooth cavity.

SUMMARY

The present study investigated an experimental primer comprising of PMGDM and HEMA, and an adhesive comprising of BisGMA-TEGDMA, with the incorporation of NAg, QADM and NACP. The purpose was to develop a new antibacterial primer using NAg, and a new antibacterial adhesive using NAg and QADM, with NACP for remineralization capability. The combination of antibacterial agents (NAg and QADM) with remineralizing agent (NACP) did not compromise the dentin bond strength. TEM examination revealed successful incorporation of NAg and NACP into the dentinal tubules at the dentin-adhesive interface. Human saliva microcosm biofilm viability, metabolic activity, lactic acid, and CFU were greatly reduced by the new bonding agents. Therefore, the new formulations have the potential to kill residual bacteria in the tooth cavity, and inhibit the invading bacteria along the tooth-restoration margins, with NACP for Ca and P ions to remineralize tooth lesions. The novel combination of antibacterial and remineralizing agents is promising for incorporation into a wide range of dental adhesives to inhibit caries.

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3.6 CAPÍTULO 6

Anticaries effects of novel nanocomposites with Ca and PO_4 ion release: A randomized human *in situ* trial

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ABSTRACT

Objectives. Caries at the restoration margins remains the main reason for failure of restorative procedures. Although calcium phosphate (CaP) composites are promising for caries inhibition, there has been no report of CaP composite to inhibit caries *in situ*. The objectives of this study were to investigate the caries-inhibition effect of nanocomposite containing nanoparticles of amorphous calcium phosphate (NACP) in a human *in situ* model, and to determine colony-forming units (CFU) and Ca and P ion concentrations of biofilms on the composite restorations.

Methods. NACP were synthesized via a spray-drying technique with a mean particle size of 116 nm. Two composites were fabricated: NACP nanocomposite, and control composite filled with glass particles. Twenty-five volunteers wore palatal devices containing bovine enamel slabs with cavities restored with NACP or control composite. After 14 days, the adherent biofilms were collected for analyses. Transverse microradiography determined the enamel mineral profiles at the margins, and the enamel mineral loss ΔZ was measured.

Results. NACP nanocomposite released Ca and P ions and the release significantly increased at cariogenic low pH ($p<0.05$). Biofilms on NACP nanocomposite contained higher Ca ($p=0.007$) and P ions ($p=0.005$) than those of control (n=25). There was no significant difference in biofilm CFU between the two composites ($p>0.1$). Microradiographs showed typical subsurface lesions in enamel next to control composite, but much less lesion around NACP nanocomposite. Enamel mineral loss ΔZ (mean ± sd; n = 25) around NACP nanocomposite was 13.8 ± 9.3 μm, much less than 33.5 ± 19.0 μm of the control ($p = 0.001$).

Significance. Novel NACP nanocomposite substantially reduced caries formation in a human *in situ* model. Enamel mineral loss at the margins around NACP nanocomposite was less than half of the mineral loss around control composite. Therefore, the Ca and P ion-releasing NACP nanocomposite is promising for caries-inhibiting around restorations.

INTRODUCTION

Dental composites consisting of fillers in a resin matrix are increasingly used because of their esthetics and enhanced performance [1-7]. Extensive efforts have been performed to improve the resin compositions, filler reinforcement, and polymerization properties [8-15]. However, secondary caries remains a main challenge facing composite restorations [16-18]. Caries at the restoration margins is a frequent reason for replacement of existing restorations [19]. The replacement of the failed restorations accounts for 50% to 70% of all restorations performed [20]. Replacement dentistry costs $5 billion annually in the U.S. [21]. To inhibit caries around the restorations, calcium phosphate (CaP)-resin composite materials were developed, which were shown to be effective in remineralizing tooth lesions *in vitro* [22-25]. However, to date, the caries-inhibition efficacy of CaP composite in a human *in situ* model has not been reported.

Traditional CaP composites were developed by filling micrometer-sized CaP particles into dental resins [22,23]. For example, amorphous calcium phosphate (ACP, $Ca_3[PO_4]_2$) particles with a mean size of 55 μm were filled into resins [23]. ACP is a precursor that forms initially and then transforms to apatite. Hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$, the prototype of minerals in teeth/bones, is the final stable product in the precipitation of calcium and phosphate ions in neutral and basic solutions [26]. Traditional ACP composite released supersaturating levels of calcium phosphate ions and remineralized enamel lesions *in vitro* [23]. However, traditional calcium phosphate composites were mechanically weak and not suitable for bulk restoratives [22,23]. Recently, novel nanocomposites were developed with nanoparticles of ACP (NACP) having a mean particle size of 116 nm [27]. The NACP nanocomposite released calcium and phosphate ions similar to traditional CaP composites, but with a 2-fold increase in flexural strength and elastic modulus for load-bearing restorations [27]. The flexural strength and elastic modulus of the NACP nanocomposite matched those of a commercial composite control [27]. Furthermore, NACP nanocomposite effectively neutralized a lactic acid challenge, while commercial control restoratives failed to neutralize the acid [28]. However, the caries inhibition capability of the NACP nanocomposite in teeth has not been investigated.

Therefore, the objective of this study was to investigate the effects of NACP nanocomposite on caries inhibition *in situ* in the oral environment. A randomized split-mouth design was conducted with 25 volunteers wearing palatal devices for a 14-day period. After that, the samples were removed, the biofilms were analyzed for Ca and P ion concentrations,

and mineral content in enamel was measured via transverse microradiography. It was hypothesized that: (1) NACP nanocomposite will increase the Ca and P ion concentrations in biofilms *in situ*; (2) NACP nanocomposite will inhibit enamel caries at the enamel-composite interface to yield much less mineral loss, compared to control composite without NACP fillers.

MATERIALS AND METHODS

NACP nanocomposite fabrication

A spray-drying technique was developed to synthesize calcium phosphate and calcium fluoride nanoparticles [29,30]. To make NACP, a solution was prepared using 1.5125 g of acetic acid (Baker, Phillipsburg, NJ), 0.8 g of calcium carbonate (Fisher, Fair Lawn, NJ, USA) and 5.094 g of dicalcium phosphate-anhydrous with water. The calcium and phosphate concentrations in the solution were 8 and 5.333 mmol/L, respectively. The solution was sprayed through a nozzle into a heated chamber. The water and volatile acid were evaporated and the dried particles were collected by an electrostatic precipitator (AirQuality, Minneapolis, MN, USA). This method yielded NACP with a mean particle size of 116 nm as measured in a previous study [27].

A monomer consisting of Bis-GMA (bisphenol-glycidyl dimethacrylate) and TEGDMA (triethylene glycol dimethacrylate) at 1:1 ratio (all by mass) was rendered light-curable with 0.2% camphorquinone and 0.8% ethyl 4-N,N-dimethylaminobenzoate. As reinforcement co-fillers, barium-boroaluminosilicate glass particles with a median diameter of 1.4 μm (Caulk/ Dentsply, Milford, DE, USA) were silanized with 4% 3 methacryloxypropyltrimethoxysilane and 2% n-propylamine [31]. The fillers were mixed with the resin at a total filler mass fraction of 60% to form a cohesive paste. Two composites were fabricated with fillers of: (1) 40% NACP + 20% glass (NACP nanocomposite); and (2) 0% NACP + 60% glass (control composite).

Calcium (Ca) and phosphate (P) ion release measurement

Ca and P ion release from the NACP nanocomposite was measured. The composite paste was placed into rectangular molds of 2 x 2 x 12 mm following previous studies [29,31]. The specimens were photo-cured (Triad 2000, Dentsply, York, PA, USA) in the air for 1 min on each side, and then incubated at 37 ºC in a humidor for 1 d. A sodium chloride solution (133 mmol/L) was buffered to 3 pH values: pH 4 with 50 mmol/L lactic acid, pH 5.5 with 50

mmol/L acetic acid, and pH 7 with 50 mmol/L HEPES. Following previous studies [29,31], 3 specimens of approximately 2 x 2 x 12 mm were immersed in 50 mL of solution at each pH, yielding a specimen volume/solution of 2.9 mm^3/mL. This compared to a specimen volume per solution of approximately 3.0 mm^3/mL according to a previous study [32]. At 1, 2, 3, 5, 7, 10, 14, 21, and 28 d, aliquots of 0.5 mL were removed and replaced by fresh solution. Ca and P ion concentrations at each time period were measured via a spectrophotometric method (DMS-80 UV-visible, Varian, Palo Alto, CA, USA) using known standards and calibration curves [22,23,32].

In situ tooth demineralization model

The *in situ* model of the present study was approved by the Research and Ethics Committee of the Federal University Institution (Protocol # 028/2011) and conformed to the Resolution # 196/96 of the National Health Council concerning the Human Research Code of Ethics. This *in situ* model was similar to those reported in previous studies which successfully enabled the investigation of enamel and dentin caries formation [33-36]. All participants signed written informed consent before being accepted into the study. Twenty-five volunteers were selected who fulfilled the following inclusion criteria: Normal salivary flow rate, stimulated-saliva buffering capacity, good general and oral health with no active caries lesions or periodontal treatment needs, ability to comply with the experimental protocol, not having used antibiotics during the 3 months prior to the study, and not using fixed or removable orthodontic devices [34-36].

Freshly-extracted bovine teeth were used following previous studies [37-40]. One hundred bovine incisors were used to prepare one hundred enamel slabs (5 x 5 x 2 mm) using a diamond saw (Buehler, Lake Bluff, IL, USA), as shown schematically in Fig. 1A. Following a previous study [41], circular cavities with an approximate diameter of 2 mm and a depth of 1.5 mm were prepared (Fig. 1A). The 100 enamel slabs with cavities were randomly divided according to a computer-generated randomization list, into 2 groups of 50 slabs each: one group was filled with the NACP nanocomposite, and the other group was filled with the control composite. To focus on the effects of composite without interference from adhesive, no adhesive was used. Each composite paste was placed into the cavity and light-activated for 20 sec using a light emitting diode Optilight LD Max (Gnatus, Ribeirão Preto, SP, Brazil) with an output of 600 mW/cm^2. All the restored slabs were polished using aluminum oxide disc (Sof-lex disk system, M Dental, St. Paul, MN). As shown in Fig. 1A, an enamel surface area of approximately 5 x 1.5 mm adjacent to the restoration was covered with an acid-

resistant varnish to act as a control for mineral loss analysis. The filled enamel slabs were stored in a humidor at a relative humidity of 100% at 37 ºC for 24 hours and then used in the *in situ* model.

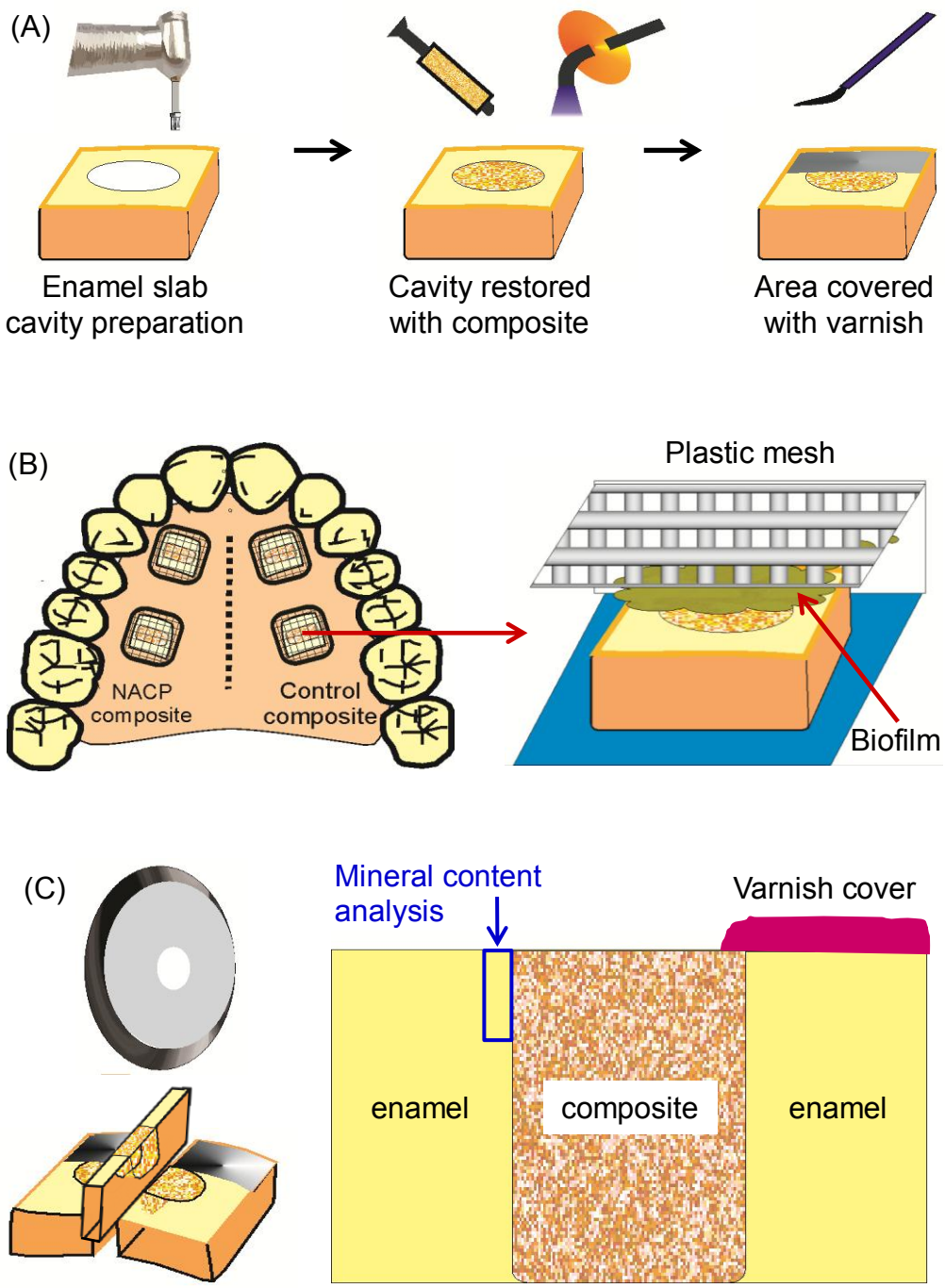

Figure 1. Schematical drawing of *in situ* experiment. (A) One hundred bovine enamel slabs of 5x5x2 mm were obtained. A cavity of 2 mm in diameter and 1.5 mm in depth was prepared. Varnish was applied on one side. (B) 25 volunteers wore palatal devices each

containing 4 slabs: 2 filled with NACP nanocomposite on one side, and 2 filled with control composite on the other side. A plastic mesh with 1 mm space protected the biofilm. (C) Enamel slabs were cut to obtain sections of 110-130 µm thickness. Contact microradiographs were obtained to measure the enamel mineral content at the margins.

For 14 days, the 25 volunteers wore removable acrylic custom-made palatal devices, each containing 4 enamel slabs: 2 slabs were restored with the NACP nanocomposite on one side, and 2 slabs were restored with the control composite on the other side (Fig. 1B). Having slabs with 2 different treatments on the two opposite sides of the same device enabled them to experience the same oral environment for a fair comparison between the two materials. This method was supported by the absence of cross-contamination shown in previous studies [38,42]. Each slab was covered with a plastic mesh with a 1 mm space, to allow biofilm accumulation and to protect the biofilms from mechanical disturbances [36,41]. As a single-blind test, the volunteers did not know which was control composite or NACP nanocomposite. Seven days prior to the experiment and during the 14 d experiment, the volunteers brushed their teeth with non-fluoridated toothpaste. The purpose of this was to determine the effect of NACP nanocomposite on caries formation without interference from fluoridated toothpastes. In order to provide a cariogenic challenge, 8 times daily at predetermined times, the volunteers removed the appliance, dripped one drop of a 20% sucrose solution on each slab, and then placed the appliance back into the mouth, following previous studies [36,41].

Biofilm analyses

On day 14, 12 h after the last application of the sucrose solution, the biofilm formed on each specimen was collected with sterilized plastic curettes. The biofilm was weighed in pre-weighed microcentrifuge tubes and agitated for 2 min in a Disrupter Genie Cell Disruptor (Precision Solutions, Rice Lake, WI, USA). An aliquot of 50 µl of the sonicated suspension was diluted in 0.9% NaCl and serial decimal dilutions were inoculated in triplicate by the drop-counting technique in the following culture media [36,41]: mitis salivarius agar containing 20% sucrose, to determine total streptococci; mitis salivarius agar plus 0.2 bacitracin/mL, to determine mutans streptococci; and Rogosa agar supplemented with 0.13% glacial acetic acid, to determine lactobacilli. The plates were incubated in 10% CO_2 at 37 ºC for 48 h. The colony-forming units (CFU) were counted and the results were expressed as CFU/mg of dental biofilm with wet weight [36,41].

In addition, the Ca and P ion concentrations in the biofilms were also measured. The biofilms were treated with 0.5 M of HCl to extract the acid-soluble whole-biofilm Ca and P ions. "Phosphate (P) ions" and "inorganic phosphorus (Pi)" are interchangeable in the context of the present study. Samples were agitated at 30 rpm for 3 h and centrifuged. The supernatant was collected for Ca and P ion measurement via a spectrophotometric method [33].

Transverse microradiography (TMR) to measure mineral content

For TMR, specimen sections were cut to a thickness of approximately 200 µm, as shown schematically in Fig. 1C. The sections were serially-polished with water-cooled abrasive (320, 600, and 1200-grit Al_2O_3 papers, Buehler) to a thickness of 110-130 µm, following a previous study [43]. Contact microradiographs of enamel sections were produced on holographic film (Integraf, Kirkland, WA, USA) exposed to 30-min Cu-Kα radiation (Faxitron-43855A, Hewlett Packard, McMinnville, OR, USA). Mineral profiles were determined by quantitative analysis of microradiographs using NIS Elements-3.2 (Nikon, NY, USA). Digital imaging captured the gray levels of a rectangular area (50 × 600 µm) of the radiographic image using an intensity resolution of 256 gray levels and a spatial resolution of 1.25 µm/pixel [23]. Following previous studies [34], the mineral loss (ΔZ) was obtained in the enamel region next to the enamel-composite, at 4 distances from the enamel-composite interface: 0-50, 50-100, 100-150, 150-200 µm, respectively. In addition, mineral loss ΔZ was also calculated for the enamel region of 0-250 µm as a whole [34]. This was done by analyzing the grayscale intensity of the rectangular area of 250 × 600 µm of enamel next to the composite to obtain the average ΔZ for this entire region. Net ΔZ was obtained as $\Delta Z_{exposed} - \Delta Z_{VarnishCovered}$ for the same enamel section, where $\Delta Z_{exposed}$ was the enamel mineral loss on the side without varnish (Fig. 1C). As shown in the results section, $\Delta Z_{VarnishCovered}$ was nearly 0, which indicates sound enamel without demineralization [38]. The unit of the integrated mineral loss ΔZ is µm, because 100 µm · vol% = 1 µm, following previous studies [22,23,44].

Statistical analysis

Sample size was determined based on previous data [41], where the required number of participants was determined to be 20, in anticipation of a dropout rate of 10%. This was based on an adequate power of 80% and a defined significance level of 5% ($p < 0.05$). Accordingly, to ensure that this group size was available at the end of the study, 25 volunteers were recruited to compensate for possible dropouts (However, all 25 volunteers completed

this study). One-way and two-way analyses-of-variance (ANOVA) were performed to detect the significant effects of the variables. Tukey's multiple comparison procedures were performed at a *p* value of 0.05.

RESULTS

The Ca and P ion release from NACP nanocomposite specimens is plotted in Fig. 2 (mean ± sd; n = 5). The solution pH significantly affected the Ca and P ion release ($p < 0.05$). Compared to pH 7, the ion release was slightly higher at pH 5.5, but substantially higher at pH 4.

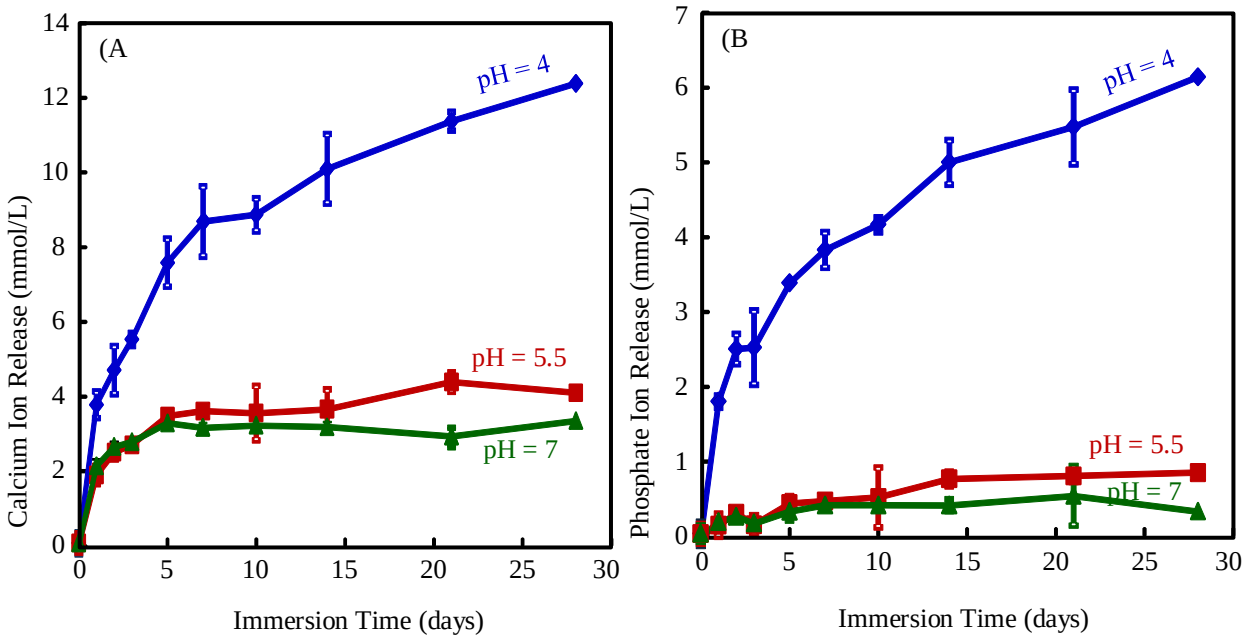

Figure 2. Calcium (A) and phosphate (B) ion releases from NACP nanocomposite immersed in solutions of pH 4, pH 5.5, and pH 7 (mean ± sd; n = 5). Ion releases at pH 4 were higher than those at pH 5.5 and 7 ($p < 0.05$). The NACP nanocomposite was "smart" and greatly increased the ion release at cariogenic pH 4, when these ions were most needed to inhibit caries.

The CFU results of biofilms collected from specimens *in situ* are plotted in Fig. 3A-C (mean ± sd; n = 25). Biofilms on NACP nanocomposite and control composite restorations had statistically similar total streptococci, mutans streptococci, and lactobacilli ($p > 0.1$). The Ca and P ion concentrations in biofilms are plotted in Fig. 3D and 3E (mean ± sd; n = 25). The biofilms adherent on NACP nanocomposite restorations had significantly higher calcium ($p = 0.007$) and phosphate ($p = 0.005$) ion concentrations than those on the control composite.

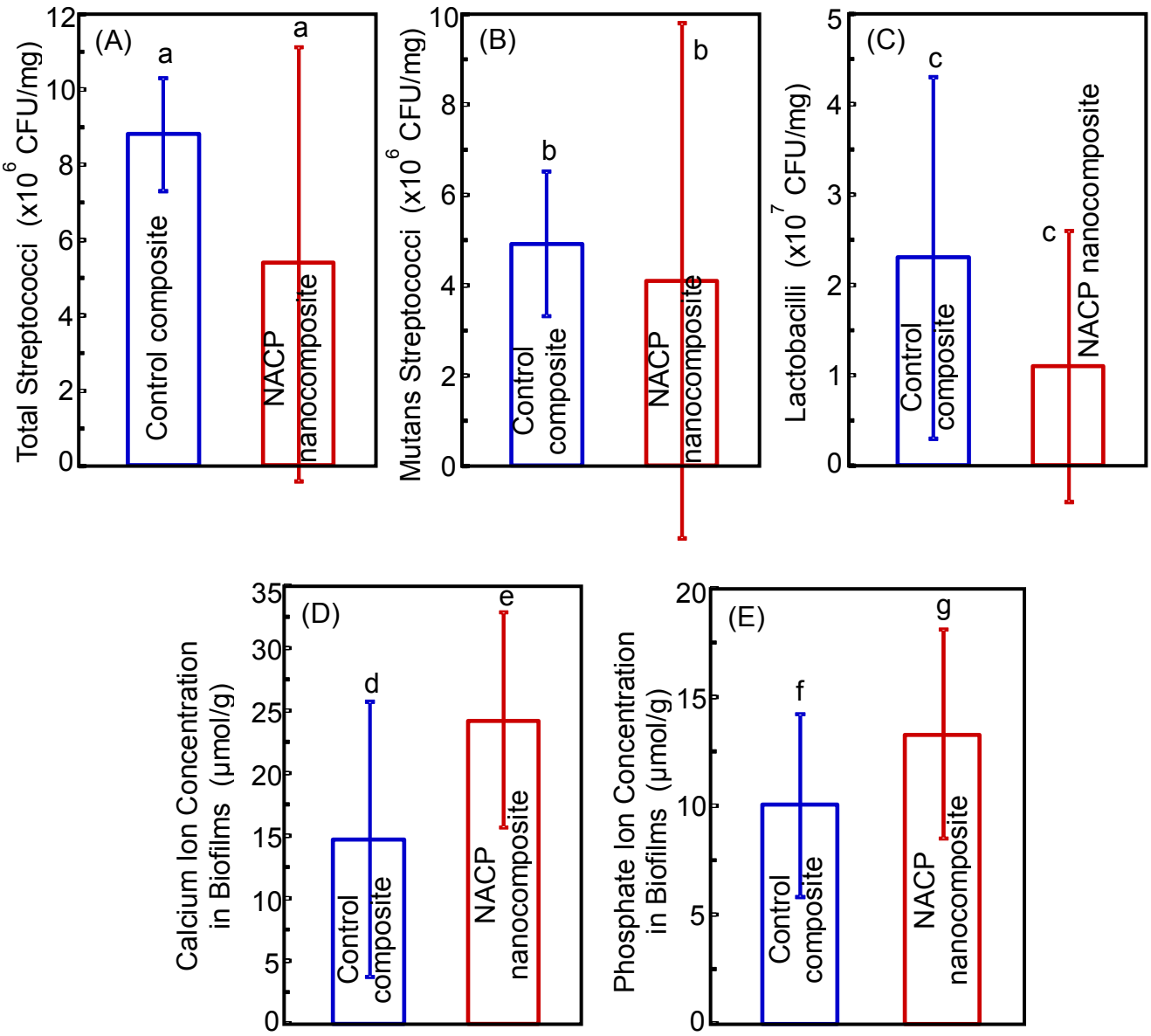

Figure 3. Analyses of oral biofilms harvested from the volunteers: (A) Total streptococci, (B) mutans streptococci, (C) lactobacilli, (D and E) Ca and P ion concentrations (mean ± sd; n = 25). In each plot, dissimilar letters indicate values significantly different from each other ($p < 0.05$). Biofilm CFU counts were not significantly different on the two composites ($p > 0.1$).

Figure 4 shows microradiographs for the exposed enamel without varnish. A representative enamel lesion around the control composite is shown in (A). In contrast, there was much less enamel lesion around the NACP nanocomposite, with an example shown in (B). Representative mineral profiles are shown in (C) and (D), having much larger lesion depth and mineral loss in enamel around the control composite than those around the NACP

nanocomposite.The biofilms on NACP nanocomposite contained significantly more Ca and P ions than those on control composite ($p < 0.05$)

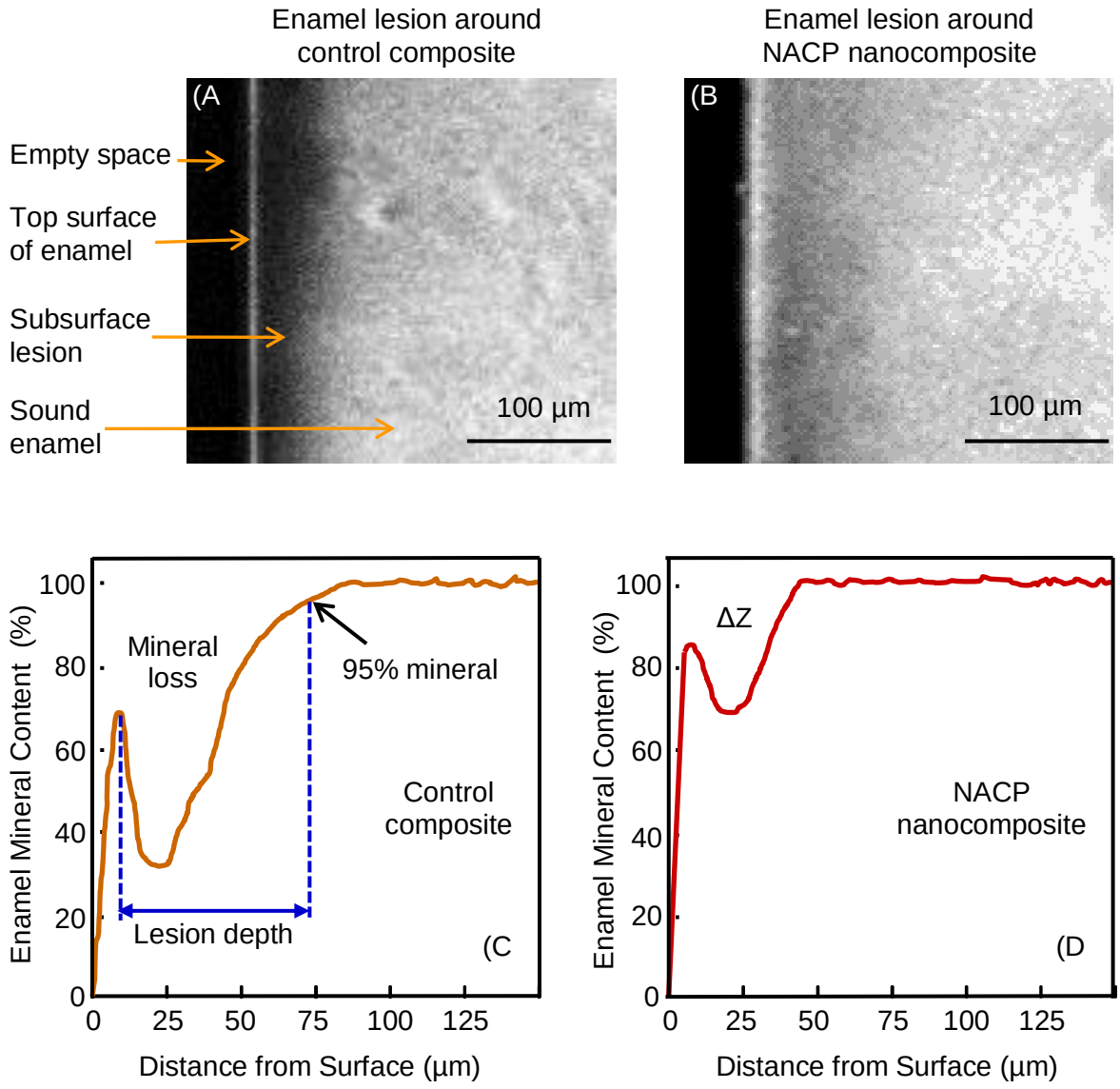

Figure 4. Transverse microradiography analysis. Representative images showed subsurface enamel lesions around (A) control composite, and (B) NACP nanocomposite. (C) The lesion depth was defined as the distance from the first bump on the mineral profile to the location which reached a mineral content of 95% of sound enamel, following a previous study [22,23,53]. (D) Exposed enamel (no varnish cover) under biofilms *in situ* had much less lesion around NACP nanocomposite than that around control composite in (C).

The varnish-covered enamel had no mineral loss, with $\Delta Z_{VarnishCovered} = 1.6 \pm 1.9$ µm around control composite, and 1.0 ± 0.8 µm around NACP nanocomposite ($p > 0.1$). For the exposed enamel, net ΔZ values were measured at 4 different distances away from the

composite-enamel interface, and the results are plotted in Fig. 5. The values at the x-axis indicate the distance of the enamel region away from the enamel-composite interface. At each distance, the mineral loss (mean ± sd; n = 25) was much less around the NACP nanocomposite than those around the control composite ($p < 0.05$)

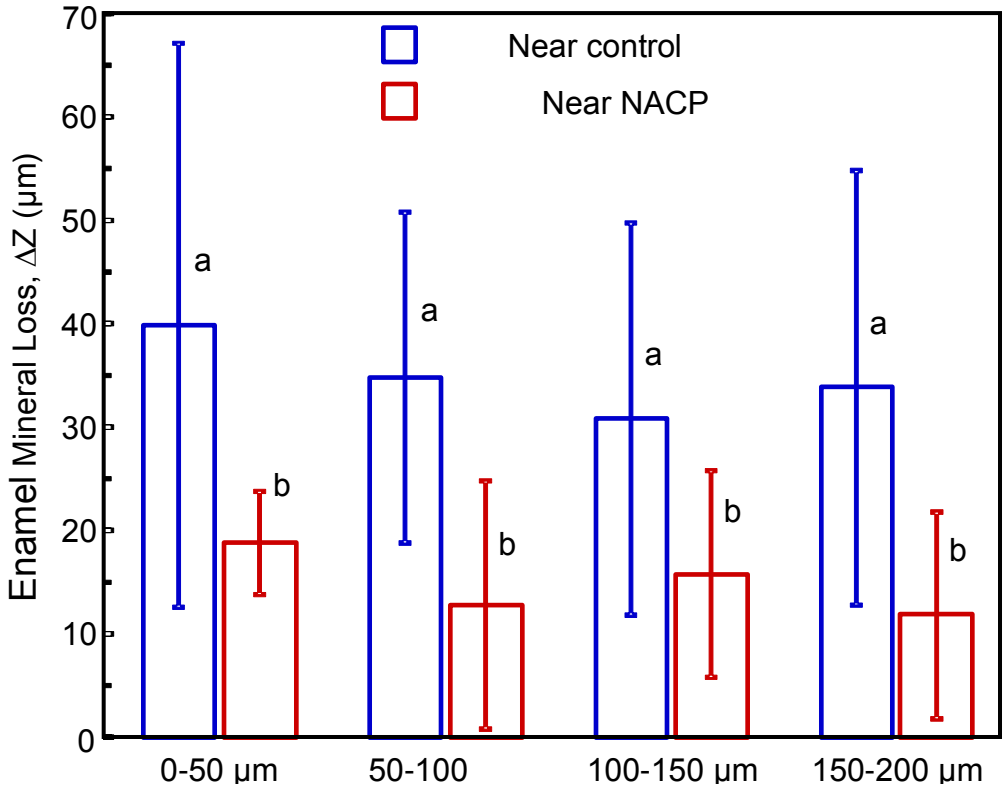

Figure 5. Enamel mineral loss around NACP nanocomposite and control composite versus the distance from the enamel-composite interface. The numbers at the x-axis refer to the location in enamel from the interface at four different distances. For each composite, the distance did not significantly change ΔZ ($p > 0.1$). At each distance, enamel mineral loss was much less around NACP nanocomposite than control composite (mean ± sd; n = 25). Values with dissimilar letters are significantly different from each other ($p < 0.05$).

Because the distance from the composite-enamel interface did not significantly affect the enamel mineral loss in Fig. 5, the enamel region of 0-250 μm was measured as a single area to obtain the average value for each enamel section, and the results are plotted in Fig. 6. The value at the x-axis indicates the enamel region from the enamel-composite interface. The enamel lesion depth around the NACP nanocomposite was significantly less than that around the control composite ($p = 0.001$). The enamel mineral loss (mean ± sd; n = 25) $\Delta Z_{NACPnanocomposite}$ = 13.8 ± 9.3 μm, much less than $\Delta Z_{ControlComposite}$ = 33.5 ± 19.0 μm (p = 0.001).

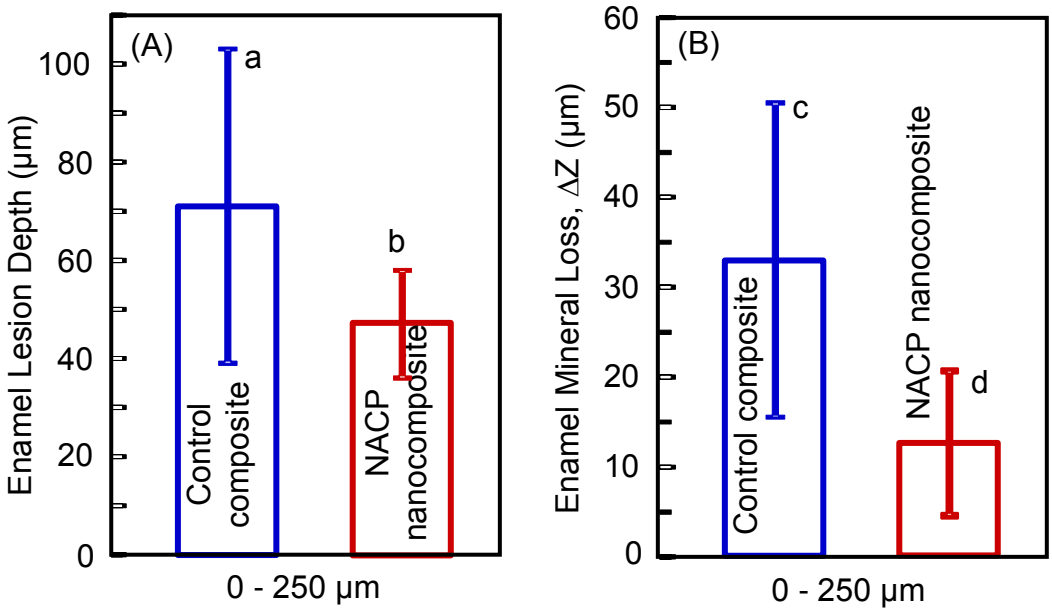

Figure 6. The enamel region of 0-250 µm from the enamel-composite interface was analyzed to obtain the average lesion depth and mineral loss in this region. (A) Enamel lesion depth, and (B) mineral loss (mean ± sd; n = 25). Enamel mineral loss around the NACP nanocomposite was reduced to nearly 1/3 of the mineral loss around the control composite ($p < 0.05$).

DISCUSSION

This study represents the first report on calcium phosphate composite behavior in a human *in situ* model which demonstrated significant enrichment of biofilms with calcium phosphate ions and effective inhibition of enamel lesions. Previous CaP composite studies focused on measurement of ion release and remineralization *in vitro* [23,44]. Traditional CaP composites had particles sizes of 1-55 µm [23,44]. In contrast, NACP consisted of individual particles with an average size of 37 nm, and clusters where several individual particles were connected to each other yielding an apparent size of 225 nm [45]. The mean particle size for NACP was 116 nm. As shown in recent studies, nanoparticles had high surface areas and released high levels of ions at relatively low filler levels, thereby making room in the resin for reinforcement glass fillers [27]. This method resulted in mechanical properties of the nanocomposite being 2-3 folds those of traditional CaP composites [46]. Recorrent caries shortens restoration longevity and requires replacement of failed restorations, which increases the subsequent restoration size and the complexity of the procedure. Hence, the NACP nanocomposite that greatly inhibited enamel caries *in situ* is promising to reduce the occurrence of secondary caries.

The human *in situ* model allowed salivary flow and encompassed the complexity of different volunteers [33-36]. Furthermore, the *in situ* model enabled biofilm formation with the heterogeneity and complication of biofilms and plaques *in vivo*. While single species biofilms were often used for *in vitro* investigations, dental plaque is a complicated ecosystem with about 1,000 bacterial species [47]. In addition, different individuals may have different biofilm compositions and dietary habits. Hence, the present study investigated *in situ* caries-inhibition via NACP nanocomposite using 25 volunteers. There are several previous *in situ* studies on fluoride treatments. For example, the effect of fluoride-releasing glass ionomers on bovine dentin caries was investigated in 16 human volunteers [38]. Calcium, phosphate and fluoride ion concentrations in biofilms were significantly affected by exposure to sucrose *in situ* [33]. Glass ionomer increased the fluoride level in biofilms, thereby decreasing caries progression in 14 volunteers [34]. An encapsulated resin-modified glass ionomer provided protection against secondary caries in 20 volunteers [41]. In the present study, CFU counts for total streptococci, mutans streptococci and lactobacilli were not significantly decreased in biofilms on the NACP nanocomposite, compared to control composite. This is expected because calcium phosphate composites are not known to have antibacterial activity. The purpose of using calcium phosphate composite was the release of Ca and P ions to combat dental caries. However, future study is needed to investigate NACP nanocomposite containing antibacterial agents to inhibit biofilm growth and caries formation in an *in situ* model [48,49]. The relatively large scatter in the CFU data in Fig. 3 was likely related to the different volunteers with different biofilm compositions and dietary habits. The CFU mean values and standard deviations in the present study are similar to those reported in previous studies [39,41].

Biofilms on NACP nanocomposite restorations *in situ* had Ca and P ion concentrations that were significantly higher than those on the control composite. This provided a large ion reservoir in biofilms and plaques that could be released during acid attacks. Furthermore, the NACP nanocomposite was "smart" and greatly increased the ion release at a cariogenic pH 4, when these ions would be most needed to inhibit caries. Previous studies demonstrated that the released ions were able to diffuse deep into the tooth structure, and these mineral ions were transferred into the body of lesions to restore the mineral lost due to acid attacks [23]. Tooth caries is a dietary carbohydrate-modified bacterial infectious disease, in which acidogenic bacteria ferment carbohydrates and produce organic acids [47,50]. Following a sucrose rinse, the plaque pH drops into the cariogenic area of 5.5 to 4, and then increases back to above 5.5 after the bacteria have completed their metabolization and the saliva has buffered

the acid. Therefore, the triggered Ca and P ion release at local cariogenic pH conditions is beneficial to inhibit caries. This is manifested by the much smaller enamel lesions around the NACP nanocomposite than those around the control composite.

The present study focused on caries formation at the restoration-enamel margins, hence the mineral content was measured for the enamel region up to a distance of 250 μm from the restoration-enamel interface. In enamel regions at 4 different distances of 0-50, 50-100, 100-150 and 150-200 μm from the interface, the NACP nanocomposite imparted the same protection to enamel against mineral loss. Previous studies quantified the caries-inhibiting effect of CaP composites using remineralization, R [44]. To illustrate the calculation of R, assume that the mineral loss in the tooth structure around a CaP composite is ΔZ_{CaP}, and the mineral loss around a non-releasing control restoration is $\Delta Z_{Control.}$ Remineralization is defined as: $R = (\Delta Z_{Control} - \Delta Z_{CaP})/\Delta Z_{Control}$ [44]. For example, if a CaP composite has no caries-inhibition capability, hence $\Delta Z_{CaP} = \Delta Z_{Control}$, then R = 0%. On the other hand, if a CaP composite completely inhibits the caries formation, yielding $\Delta Z_{CaP} = 0$, then R = 100%. In the present study, the average ΔZ in the 0-250 μm enamel region, $\Delta Z_{NACPnanocomposite}$ = 13.8 μm, and $\Delta Z_{ControlComposite}$ = 33.5 μm. This resulted in a remineralization R = 59%. This extent of remineralization was higher than the R values of 13-38% for traditional CaP restoratives previously measured using the same quantitative microradiographic method [44]. It would be interesting to investigate if R can be further increased by incorporating antibacterial agents into the NACP nanocomposite to combine the remineralizing and antibacterial capabilities to synergistically inhibit caries *in situ*.

The caries-inhibition results of this study, together with the previously-reported acid neutralization and good mechanical properties [27,28], indicate that the NACP nanocomposite is promising for dental restorations to inhibit secondary caries. For example, NACP nanocomposite is promising for high-caries-risk patients, for those with dry mouth such as senior patients, and in cavities where complete removal of caries tissues is contra-indicated. There is an increasing interest in less removal of tooth structure and minimal-intervention dentistry [51], which could leave behind more carious tissues in the prepared tooth cavity. Furthermore, atraumatic restorative treatments (ART) do not remove caries completely [52]. The NACP nanocomposite could be useful to release Ca and P ions and remineralize the remaining tooth lesions in the cavity. Many developing countries have a prevalence of dental caries. Even in the USA, people of certain ethnicity and poverty levels have a high incidence of untreated caries. NACP nanocomposite with an effective caries-inhibition efficacy could

help address these problems and potentially have a vast international market. In addition, NACP nanoparticles could be incorporated into adhesives, inlay and crown cements, and orthodontic bracket cements with caries-inhibiting benefits. Further studies are needed to investigate these applications.

CONCLUSION

Novel NACP nanocomposite was demonstrated to be effective in inhibiting secondary caries in a human *in situ* model. A split-mouth design was investigated in which 25 volunteers wore removable palatal devices for 14 d. The NACP nanocomposite significantly increased the Ca and P ion concentrations in the biofilms *in situ*. The release of Ca and P ions from the NACP nanocomposite was significantly increased at cariogenic low pH, when these ions were most needed to inhibit caries. Transverse microradiography of enamel section showed typical subsurface enamel lesions at the margins next to the control composite. However, the enamel lesions were greatly reduced around the NACP nanocomposite. In view of recurrent caries at the tooth-restoration margins as the main factor for restoration failure, the NACP nanocomposite with an effective caries-inhibiting capability is promising for a wide range of dental restorations.

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4 CONCLUSÃO GERAL

Com base nos resultados desta tese, conclui-se que:

I. As atuais estratégias beneficiadas pela nanotecnologia para o desenvolvimento de materiais restauradores com atividade anticárie se mostram possíveis e promissoras, entretanto se encontram em sua maioria em fase investigativa inicial representada por estudos laboratoriais, sendo necessário promover a busca por evidências na forma de ensaios clínicos e outros estudos para validação e condução de sua eficácia e segurança clínica.

II. Os cimentos ortodônticos nanoparticulados fluoretados mostraram uma tendência a apresentar valores maiores que os produtos microparticulados, entretanto não significante, sendo o controle (ionômero de vidro) o único material a expressar uma proteção adicional contra cárie secundária.

III. Resinas composta fluoretada contendo nanopartículas apresentam discreta ação anticárie sem diferenciação em rugosidade de superfície de resinas não-fluoretadas. Isto ressalta que os atuais materiais nanohíbridos não podem apresentar um desempenho diferenciando em relação à ação anticárie.

IV. Sistemas adesivos contendo nanopartículas de prata e fosfato de cálcio amorfo ACP ($Ca_3[PO_4]_2$) apresentam satisfatória ação antimicrobiana em biofilme orais, sem significativa alteração na resistência de união.

V. Sistemas adesivos experimentais contendo monômero associados a quaternário de amônio, nanopartículas de prata e fosfato de cálcio amorfo ACP ($Ca_3[PO_4]_2$) apresentam significante ação antimicrobiana em biofilme orais, sendo esta abordagem promissora na sua aplicabilidade em diferentes sistemas adesivos.

VI. Resina composta contendo nanopartículas de fosfato de cálcio amorfo apresenta significante ação anticárie. Isto ressalta uma promitente aplicação desse material como estratégia complementar na prevenção e combate à cárie dentária.

Os materiais restauradores nanoparticulados atualmente comercializados ainda não apresentam uma expressiva ação anticárie. O desenvolvimento de novos materiais restauradores com ação antibacteriana e/ou remineralizante beneficiados por nanotecnologia vem a contribuir para este conjunto de estratégias e representam perspectivas favoráveis para o combate á cárie.

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APÊNDICES

APÊNDICE A – *Curriculum vitae resumido*

Curriculum Vitae

CONTATO E INFORMAÇÕES ADICIONAIS

Nome: Mary Anne Sampaio de Melo
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Possui graduação em Odontologia pela Universidade de Fortaleza-UNIFOR (1996-2000), aperfeiçoamento em Odontologia Estética pela Academia Cearense Odontologia (2001), especialização em Odontologia Restauradora (2003-2004) e mestrado em Odontologia (2007-2009) pela Universidade Federal do Ceará (UFC). Atualmente é aluna do programa de pós-graduação (2009) em nível de doutorado em Odontologia área de Concentração - Clínica Odontológica na Universidade Federal do Ceará. Realizou estágio de Doutorado Sanduíche no Laboratório/Divisão de Biomateriais e Engenharia Tecidual - Universidade de Maryland, Baltimore/USA sendo bolsista CAPES/Fulbright - Programa de Doutorado Sanduíche 2011-2012. Tem experiência na área de Odontologia, com ênfase em Dentística e Cariologia, atuando principalmente nos seguintes temas: cárie, modelos de biofilme oral, uso de fluoretos/ laser na prevenção de erosão dentária.

ATUAÇÃO em PESQUISA CIENTÍFICA

Principais áreas de pesquisa são: abordagens para inibição do processo de formação de cárie e prevenção da erosão dentária. Participação em estudos de laboratório utilizando modelos de biofilme dentário e sua aplicabilidade em testes da ação antimicrobiana de substância e materiais dentários. Participação em projetos de pesquisa para a prevenção da

erosão e cárie dentária com envolvimento em atividades de preparo e submissão de propostas dos projetos á aprovação do comitê de ética, execução de estudo *in vitro*, ensaios *in situ* e clínico bem como elaboração de relatórios e artigos científicos.

Agosto de 2011 - Junho de 2012 - Pesquisador Assistente Visitante – Laboratório de Biomateriais e Divisão de Engenharia de Tecidos - Universidade de Maryland, Baltimore / EUA-Fulbright Scholarship - Programa de Doutorado Sanduíche 2011-2012 - Orientador: Prof Hockin Xu

Março de 2007 – Outubro de 2012 – Pesquisador/Colaborador do Programa de Pós-graduação em Odontologia- Laboratório de Pesquisa do Programa de Pós-graduação em Odontologia - Orientadora: Prof Lidiany Karla Azevedo Rodrigues.

ATUAÇÃO CLÍNICA

2001-2011- Possui 10 anos de experiência profissional na área odontológica realizando procedimentos de diagnóstico, prevenção e tratamento de doenças dentárias ou trauma. Clínico com enfoque em Odontologia Estética.

ORGANIZAÇÕES CIENTÍFICAS AFILIADAS:

SPBqO- Sociedade Brasileira de Pesquisa Odontológica
IADR- International Association of Dental Research
AADR- American Association of Dental Research
ORCA- European Organization for Caries Research

RESUMOS PUBLICADOS EM ANAIS DE CONGRESSOS (últimos 5 anos):

Apresentados em sessão oral:

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11. Lima J.P.M., **Melo M.A.S**., Codes B.M., Albuquerque filho F.B., Nogueira N.A.P., Zanin I.C.J., and Rodrigues L.K.A. Singlet Oxygen Produced in Photodynamic Therapy Performed against S. mutans. J Dent Res 90 (Spec Iss A):2652, 2011 (www.dentalresearch.org). In: 89th IADR/AADR Annual Meeting, San Diego, EUA.

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3. **Melo, MAS**; De-paula, DM; Lima JPM; Borges FMC; Steiner-oliveira C; Nobre-dos-santos M; Zanin ICJ; Barros EB; Rodrigues LKA. *In vitro* photodynamic antimicrobial chemotherapy in dentine contaminated by cariogenic bacteria. Laser Physics 2010, v. 20, p.1-10.

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REVISOR DE PERIÓDICOS:

2011-presente- Malaysian Journal of Medical Sciences

Endereço eletrônico: http://www.mjms.usm.my/

2011-presente- Journal of Dentistry and Oral Hygiene

Endereço eletrônico: http://www.academicjournals.org/jdoh/Advert.htm

2012-presente- International Journal of Biochemistry and Biotechnology

Endereço eletrônico: http://www.internationalscholarsjournals.com/journal/ijbb

2012-presente- Photomedicine and Laser Surgery

Endereço eletrônico: http://www.liebertpub.com/pho

Produção científica durante o doutorado

CO-ORIENTAÇÃO DE TRABALHOS DE CONCLUSAO DE CURSO (TTC):

2009-Diego Martins de Paula (graduando em Odontologia). Avaliação *in vitro* das alterações térmicas em dentes humanos durante a Quimioterapia Antimicrobiana Fotodinâmica Realizada com Fontes de Luz Vermelha.

2010-Diego da Costa Góes (graduando em Odontologia). Efeito da clorexidina na resistência de união de um sistema self-etching a dentina artificialmente desmineralizada.

2011-Sarah Florindo de Figueiredo Guedes (graduanda em Odontologia). Efeito concentração de ácido fosfórico em esmalte desmineralizado irradiados por ER: YAG laser.

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ANEXOS

ANEXO A- Aprovação no comitê de ética em pesquisa do estudo *in situ*- *capítulo 3*

Universidade Federal do Ceará
Comitê de Ética em Pesquisa

Of. Nº 038/11 Fortaleza, 25 de março de 2011
Protocolo COMEPE nº 027/11
Pesquisador responsável: Mary Anne Sampaio de Melo
Título do Projeto: "Efeito da incorporação de nanoparticulas na liberação de flúor e ação anticárie de resinas compostas: estudo in situ"

Levamos ao conhecimento de V.Sª. que o Comitê de Ética em Pesquisa da Universidade Federal do Ceará – COMEPE, dentro das normas que regulamentam a pesquisa em seres humanos, do Conselho Nacional de Saúde – Ministério da Saúde, Resolução nº 196 de 10 de outubro de 1996 e complementares, aprovou o protocolo e o TCLE do projeto supracitado na reunião do dia 24 de março de 2011.

Outrossim, informamos, que o pesquisador deverá se comprometer a enviar o relatório final do referido projeto.

Atenciosamente,

Dra. Miriam Parente Monteiro
Coordenadora Adjunta do Comitê
de Ética em Pesquisa
COMEPE/UFC

ANEXOS

ANEXO B- Aprovação no comitê de ética em pesquisa do estudo *in situ*- *capítulo 6*

Universidade Federal do Ceará
Comitê de Ética em Pesquisa

Of. Nº 039/11 Fortaleza, 25 de março de 2011
Protocolo COMEPE nº 028/11
Pesquisador responsável: Mary Anne Sampaio de Melo
Título do Projeto: "Avaliação do efeito anticárie de resinas compostas nanoparticuladas liberadoras de íons Ca e PO_4: estudo in situ"

Levamos ao conhecimento de V.Sª. que o Comitê de Ética em Pesquisa da Universidade Federal do Ceará – COMEPE, dentro das normas que regulamentam a pesquisa em seres humanos, do Conselho Nacional de Saúde – Ministério da Saúde, Resolução nº 196 de 10 de outubro de 1996 e complementares, aprovou o protocolo e o TCLE do projeto supracitado na reunião do dia 24 de março de 2011.

Outrossim, informamos, que o pesquisador deverá se comprometer a enviar o relatório final do referido projeto.

Atenciosamente,

Miriam Parente Monteiro

Dra. Miriam Parente Monteiro
Coordenadora Adjunta do Comitê de Ética em Pesquisa
COMEPE/UFC