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JACQUELINE DE SANTIAGO NOJOSA

CARACTERIZAÇÃO DE SELANTES RESINOSOS CONTENDO MICROPARTÍCULAS POLIMÉRICAS CARREGADAS COM CLOREXIDINA

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Dissertação apresentada ao Programa de Pós-Graduação em Odontologia da Universidade Federal do Ceará, como requisito parcial à obtenção do título de Mestre em Odontologia. Área de concentração: Clínica Odontológica.

Orientadora: Profª Drª Monica Yamauti.
Coorientadores: Prof. Dr. Francisco Fábio Oliveira de Sousa e Prof. Dr. Juliano Sartori Mendonça.

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BANCA EXAMINADORA

Monica Yamauti

Profª Drª Monica Yamauti (Orientadora)
Universidade Federal do Ceará (UFC)

Prof. Dr. Sérgio Lima Santiago
Universidade Federal do Ceará (UFC)

Profª. Drª. Paula Borges Jacques
Universidade de Fortaleza (UNIFOR)

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“Ninguém pode voltar atrás e fazer um novo começo, mas qualquer um pode recomeçar e fazer um novo fim”.

Chico Xavier

RESUMO

Os selantes representam uma medida muito utilizada na prevenção de cárie e são indicados para pacientes de alto risco à cárie, especialmente, quando há presença de dentes com fissuras profundas, estreitas e retentivas. A clorexidina (CLX) é um agente antimicrobiano de amplo espectro, que poderia trazer benefícios aos selantes. Este estudo será apresentado em dois capítulos, cujos objetivos foram: Capítulo 1) Desenvolver e caracterizar micropartículas poliméricas carregadas com CLX; e Capítulo 2) Avaliar o efeito da incorporação de CLX, em suas formas livre e microencapsulada, nas propriedades físico-químicas de selantes resinosos. Material e métodos: 1) Micropartículas poliméricas de Poli(ácido láctico-co-glicólico) (PLGA) contendo diacetato (DA) ou digluconato de CLX (DG) foram preparadas, utilizando a técnica de secagem por pulverização, e caracterizadas em termos de estabilidade, rendimento de produção, tamanho da partícula, morfologia, eficácia de encapsulação (EE), carga do fármaco (CF), liberação cumulativa e resposta antimicrobiana. 2) Dois tipos de CLX (DA ou DG), nas respectivas formas livre a 1 e 2%, ou microencapsulada (PDA OU PDG) a 5 e 10%, foram incorporados ao selante resinoso com flúor, Bioseal® (BI), e ao selante experimental (EX) sem flúor. Após a obtenção das formulações dos selantes, realizaram-se testes de grau de conversão dos monômeros, avaliação da liberação de CLX em meios de dissolução e liberação de fluoreto. A análise estatística foi realizada por meio da análise de variância a dois critérios, seguida pelo teste de Bonferroni. Em todos os testes, utilizou-se o nível de confiança de 95% ($p<0,05$). Resultados: 1) A estabilidade do fármaco em solução ocorreu por 50 dias. O maior rendimento de produção ocorreu nas partículas contendo DA (mais de 71,56%). O tamanho das partículas variou de 1,01 a 3,07 µm, apresentando micropartículas homogêneas, esféricas e sem agregação. O processo de microencapsulação demonstrou EE de 4,42 a 36,67% e CF de 0,26 a 4,07% entre as formulações. Observou-se um padrão de liberação controlada e, após a análise microbiológica, *in vitro*, as micropartículas com DA e DG inibiram *Streptococcus mutans*. 2) No grau de conversão, a média dos valores obtidos variaram de 69,74% no grupo BI-PDG10 a 77,69% no EX-DG2. Nos selantes contendo DG e DA livre, a liberação de CLX foi discreta a partir de 6 h, exceto para os grupos EX-DG1 e EX-DA2. No selante experimental, a maior liberação ocorreu no grupo EX-PDA5 (22,91%), enquanto EX-PDG5 (8,00%) e EX-PDG10 (8,99%) apresentaram as menores liberações ($p<0,001$). No teste de liberação de fluoreto, BI-DA1 apresentou a maior liberação quando comparado aos outros grupos com CLX livre ($p<0,01$). Nos selantes

contendo micropartículas carregadas com CLX, BI-PDA5 apresentou a maior liberação de fluoreto ($p<0,01$). Conclusão: A caracterização de micropartículas carregadas com CLX resultou em diferentes perfis de liberação. A adição de CLX livre ou microencapsulada nos selantes afetou o grau de conversão, dependendo do tipo de CLX incorporada e do selante. Grupos com CLX livre liberaram maior quantidade de fármaco no início do estudo. Selantes contendo micropartículas apresentaram liberação lenta e gradual. A liberação de fluoreto foi maior nos grupos com micropartículas comparado ao controle.

Palavras-chave: selantes de fossas e fissuras, clorexidina, flúor e polímeros.

ABSTRACT

Sealants represent a highly used measure in the prevention of caries and are indicated for patients at high risk for dental caries, especially when there is presence of teeth with deep, narrow and retentive fissures. Chlorhexidine (CHX) is a broad-spectrum antimicrobial agent that can bring benefits to sealants. This study will be presented in two chapters, whose objectives were: Chapter 1) To develop and characterize polymeric microparticles loaded with CHX; and Chapter 2) To evaluate the effect of incorporation of CHX, in free and microencapsulated forms, in the physicochemical properties of resin sealants. Material and methods: 1) Polymer microparticles of Poly(lactide-co-glycolide acid) (PLGA) loaded with diacetate (DA) and CHX digluconate (DG) were prepared using the spray drying technique and characterized in terms of stability, production yield, particle size, morphology, efficiency encapsulation (EE), drug loading (DL), cumulative release and antimicrobial response. 2) Two types of CHX (DA or DG), in respective free form 1 and 2%, or microencapsulated form 5 and 10% were added to the resin sealant with fluoride, Bioseal® (BI), or to the experimental sealant (EX) without fluoride. After obtaining the sealants formulations, tests were conducted to evaluate degree of conversion of the monomers, CHX release in dissolution media and fluoride release. Statistical analysis was performed by analysis of variance (ANOVA) Two-way, followed by Bonferroni *post-hoc* test. In all tests the level of confidence was 95% ($p < 0.05$). Results: 1) The stability of drug in solution was detected for 50 days. The highest yield of production was observed with DA (over 71.56%). The particle size ranged from 1.01 to 3.07 μm, presenting homogeneous, spherical and non-aggregated microparticles. The microencapsulation process showed EE in the range of 4.42 to 36.67%, and DL between 0.26 and 4.07% amoung the formulations. It was observed a controlled release pattern. After the microbiological analysis, in vitro, DA and DG microparticles inhibited *Streptococcus mutans*. 2) In degree of conversion test, the mean values varied from the lowest DC for BI-PDG10 (69.74%) to the highest DC for EX-DG2 (77.69%). In the sealants containing free CHX (either DG or DA), the release was discrete from the first 6 h, except for EX-DG1 and EX-DA2 groups. In the experimental sealant, the highest release was detected in EX-PDA5 (22.91%), while EX-PDG5 (8.00%) and EX-PDG10 (8.99%) presented the lowest releases ($p<0.001$). In the fluoride release test, BI-DA1 showed the highest release when compared to the other sealants that contained free CHX ($p<0.01$). In the sealants containing CHX loaded PLGA-microparticles, the highest fluoride release was detected from BI-PDA5 ($p<0.01$). Conclusion: The characterization of microparticles loaded with CHX resulted in different

release profiles. Addition of free or microencapsulated CHX into sealants affected the DC, depending on the type of CHX and sealant. Resin sealants containing free CHX released a large amount at the beginning of the study. Sealants containing microparticles showed a slow and gradual release. The fluoride release was higher in sealants that contained microparticles compared to the control.

Keywords: pit and fissure sealants, chlorhexidine, fluorine and polymers.

SUMÁRIO

1 INTRODUÇÃO GERAL

Os selantes representam uma das medidas mais utilizadas na prevenção de cárie e são indicados para pacientes de alto risco à cárie (WELBURY *et al.*, 2004; AHOVUO-SALORANTA *et al.*, 2008; FRENCKEN, 2014), especialmente, quando há presença de dentes com fissuras profundas, estreitas e retentivas (BERGER *et al.*,2010). Nesses casos, há limitação da ação protetora exercida pela saliva e pelo flúor, dificultando a autolimpeza e a remineralização dentária (BUONOCORE, 1971; SILVERSTONE, 1984; SALAMA; AL-HAMMAD, 2002; FEATHERSTONE, 2006), favorecendo a retenção de biofilme e a proliferação bacteriana (YENGOPAL *et al.*, 2009).

As propriedades requeridas para um selante incluem biocompatibilidade, anticariogenicidade, retenção, boa integridade marginal, resistência à abrasão e ao desgaste, e rentabilidade (PEREZ-LAJARIN *et al*., 2003). Os materiais mais utilizados no selamento de fóssulas e fissuras são selantes resinosos e ionoméricos (FRENCKEN, 2014). Os selantes resinosos atuam através da formação de uma zona microrretentiva, após o condicionamento ácido do substrato dentário e a formação de microporos que facilitam a penetração do material selador (FEIGAL, 2002). Devido à sua elevada taxa de retenção, os selantes resinosos são os materiais de escolha preferenciais para o selamento de fóssulas e fissuras (KLOUKOS *et al.*, 2013).

Outro fator que pode contribuir na prevenção de cárie é a adição de fluoretos aos materiais, como ocorre em alguns selantes resinosos e ionoméricos. Os cimentos de ionômero de vidro (CIVs) foram introduzidos como materiais alternativos para selamento dentário. Diferentemente dos selantes resinosos, a adesão dos CIVs ocorre por meio de trocas iônicas, produzindo uma retençao inferior (FRENCKEN; HOLMGREN, 1999). Por apresentarem uma taxa de retenção relativamente baixa (RAADAL *et al*., 1996; PAPACCHINI, 2005; BASEGGIO *et al.*, 2010; BHAT, 2013), os CIVs são mais indicados por serem um veículo de liberação de fluoreto do que como um selante de fissura tradicional (WELBURY *et al*., 2004). Os selantes resinosos fluoretados conseguem associar a boa retenção - inerente ao material - com a liberação de um agente que atua inibindo a desmineralização e favorecendo a remineralização (FEATHERSTONE, 2006). Alguns selantes fluoretados comerciais podem conter um

sal de fluoreto solúvel, como o fluoreto de sódio (NaF) (NATIONAL INSTITUTE OF DENTAL RESEARCH, 1985).

Um agente antimicrobiano extensivamente estudado - que poderia favorecer o efeito anticárie - é a clorexidina (CLX). Essa substância tem sido utilizada como referência para a avaliação da eficácia de outros agentes antimicrobianos. Sua estrutura é composta por dois anéis clorofenólicos e dois grupos biguanida, interligados simetricamente por cadeias hexametilênicas (DENTON, 1991). A molécula base é praticamente insolúvel em água, motivo pelo qual se buscou melhorar as propriedades de solubilidade através da conversão da mesma em formas salinas, tais como acetato, cloridrato e gluconato de clorexidina (ZAMANY *et al*., 2003; ZEHNDER, 2006). Estudos sugerem que um dos cátions da CLX se fixa à película adquirida sobre o dente e o outro fica livre para interagir com as bactérias que estão tentando colonizar as superfícies bucais (JENKINS *et al.,* 1988). Dessa forma, o efeito antimicrobiano da CLX ocorre pela atração e adsorção das moléculas catiônicas da CLX à superfície celular aniônica dos microrganismos. Essa interação promove a alteração da permeabilidade da membrana celular, resultando na perda dos componentes intracelulares e no desequilíbrio osmótico da célula (HENNESSEY, 1973; DELANY *et al.*, 1982; GOMES *et al.*, 2006). Essa característica é altamente relevante para a eficácia da CLX, que se constitui como um agente antimicrobiano de amplo espectro, bastante efetivo contra microrganismos gram-positivos e gram-negativos, fungos, leveduras e vírus, bem como possui elevada eficácia contra cepas de *Streptococcus mutans* (WADE; ADDY, 1989; MISTRY *et al*., 2014).

Um dos primeiros estudos a incorporar CLX às resinas compostas avaliou a eficácia antimicrobiana da CLX em várias concentrações, associada à resina, e testou as propriedades mecânicas desse material. No teste de difusão em ágar com bactérias das espécies *Streptococcus viridans*, *Streptococcus pyogenes*, *Streptococcus mutans*, *Escheria coli* e *Lactobacillius acidophilus*, demonstrou-se que, no geral, a adição de acetato e gluconato de CLX aos materiais restauradores aumentaram sensivelmente a atividade antimicrobiana. Nos testes de tração, compressão e resistência de união, as propriedades mecânicas não foram alteradas significativamente (JEDRYCHOWSKY *et al*., 1983).

Um estudo *in vitro* avaliou os efeitos antibacterianos de uma resina 4-META/MMA-TBB (4-metacriloxietil trimelitato anidro / metil metacrilato tri-n-butil-

borano), contendo digluconato de CLX em concentrações de 0,0 (controle) a 3,0%. Este estudo sugeriu que a incorporação de 1,0 a 1,5% de digluconato de CLX à resina 4-META é o ideal em termos de efeitos antibacterianos e de resistência de união da resina à estrutura dentária (KWON *et al.*, 2010). Outros estudos também avaliaram a incorporação de CLX em materiais resinosos, apresentando um potencial de inibição bacteriana (LEUNG *et al.*, 2005; CHENG *et al.*, 2012).

Uma das questões bastante controversas quando se avalia a associação de agentes antibacterianos na prevenção de cárie é a viabilidade da combinação da CLX ao fluoreto, tanto nas formas de solução como em gel (ANUSAVICE, 2005). Na forma de solução, demonstrou-se que CLX, frequentemente, precipita quando são misturadas soluções de CLX e fluoreto (BARKVOLL *et al*., 1988). No entanto, evidências sugerem que as ações dos íons de CLX, carregados positivamente, e dos íons fluoreto, carregados negativamente, não necessariamente se anulam (ANUSAVICE, 2005). De fato, vernizes que contêm CLX e fluoreto podem contribuir para a diminuição do desafio cariogênico em longo prazo (VAN LOVEREN *et al.*, 1996.; TWETMAN; PETERSON, 1997).

Além disso, a adequada atuação dos agentes anticariogênicos depende da sua liberação em níveis terapêuticos ao longo do tempo (FEATHERSTONE, 2006). Para a manutenção desses agentes no local de atuação, os protocolos clínicos propostos dependem, frequentemente, da colaboração dos pacientes. Estes devem estar comprometidos com o tratamento para não interferir nos resultados clínicos. Para tentar superar essa problemática e potencializar os benefícios dos fármacos, dispositivos de liberação controlada e sustentada de fármacos poderiam ser utilizados (MIRTH, 1980).

Micropartículas poliméricas são dispositivos de liberação controlada, desenvolvidos e aprimorados para o tratamento de diversas condições patológicas, onde um efeito prolongado do fármaco é desejado ou um alvo terapêutico específico é almejado. Para a produção dessas partículas, polímeros - como o Poli(ácido láctico-co-glicólico) (PLGA) - vêm sendo utilizados em sistemas de liberação de fármacos. Sua favorável biodegradação e sua biocompatibilidade o tornam um material padrão-ouro na liberação controlada de fármacos, sendo uma das principais alternativas no desenvolvimento de sistemas de liberação (PRIOR *et al.*, 2000; BLANCO-PRIETO *et al.*, 2002; GRAVES *et al.*, 2004; SCHNIEDERS *et al.*, 2006). Para obtenção desses sistemas, a técnica de secagem por atomização é a mais amplamente utilizada para

formação de micropartículas secas (JIN; CHEN, 2011). As micropartículas são pequenas partículas sólidas e esféricas com tamanho que varia entre 1 e 500 μm (MAGILL, 1990; RANADE; HOLLINGER, 2004), podendo ser produzidas pelo processo de microencapsulação. O mecanismo de liberação do fármaco a partir das micropartículas pode ser influenciado pelas propriedades do polímero utilizado e do fármaco encapsulado. O PLGA é um polímero sintético que apresenta uma degradação por hidrólise, resultando em decréscimo da massa molecular e redução das propriedades mecânicas do polímero (GÖPFERICH, 1997).

Geralmente, utilizam-se dispositivos poliméricos incorporados com flúor (MIRTH *et al*., 1982), clorexidina (MIRTH *et al*., 1989;. YUE *et al*., 2004), amoxicilina (SOUSA *et al.*, 2010) ou outros agentes antimicrobianos (FRIEDMAN; STEINBERG, 1990). Suas propriedades farmacotécnicas também foram atestadas na formulação de sistemas de liberação de fármacos para aplicação em periodontia e endodontia (SOUSA *et al.*, 2010; DE SOUSA *et al.*, 2012).

Em Odontologia, o tratamento *in situ* poderia ser de grande utilidade (COVENTRY *et al.*, 2000) e o desenvolvimento de formulações biodegradáveis, que atuem através da liberação controlada, seria importante no controle das infecções da cavidade oral. Além disso, a proteção do fármaco frente aos processos fisiológicos degradativos e à bioabsorção também seria vantajosa, visto que a ocorrência desses fenômenos acarretaria na perda da atividade farmacológica.

A incorporação de componentes dotados de propriedades antimicrobianas em selantes poderia ser uma estratégia eficaz para modificar a cariogenicidade do biofilme e inibir o desenvolvimento das lesões de cárie. Por sua vez, o tratamento preventivo-restaurador poderia se tornar mais eficaz através do controle da infecção por meio da liberação controlada de um agente antimicrobiano *in situ*. Adicionalmente, uma combinação de flúor e da liberação controlada de clorexidina poderia apresentar resultados terapêuticos promissores.

2 PROPOSIÇÃO

Essa dissertação de mestrado será apresentada em capítulos, tendo como objetivos:

Capítulo 1: Desenvolver e caracterizar micropartículas de Poli(ácido láctico-co-glicólico) (PLGA) carregadas com clorexidina, visando sua incorporação em produtos odontológicos como um sistema de liberação controlada.

Capítulo 2: Avaliar o efeito da incorporação de clorexidina (digluconato ou diacetato), na forma livre ou carregada em micropartículas de PLGA, nas propriedades físico-químicas de selantes resinosos.

3 CAPÍTULOS

3.1 Capítulo 1

Development and characterization of chlorhexidine loaded-PLGA microparticles for dental applications

Short title: Characterization of chlorhexidine loaded-PLGA microparticles

J S Nojosa[1], C A A Alencar[1], A P M Pires[2], R S Araújo[2], L K A Rodrigues[1], M Yamauti[1*] and F F O Sousa[3]**

[1] Graduate Program of Dentistry, Faculty of Pharmacy, Dentistry and Nursing, Federal University of Ceara, Rua Monsenhor Furtado, s/n, 60430-355, Fortaleza, Brazil.

[2] Faculty of Pharmaceutical Sciences, Federal University of Amapa, Rod. Juscelino Kubitschek, km 02, Macapa, Amapa, 68902-280, Brazil.

[3] Department of Chemistry and Environment, Federal Institute of Education, Science and Technology of Ceara, Av. Treze de Maio, 2081, 60040-531, Fortaleza, Brazil.

E-mail: *myamauti@gmail.com; **fabio@unifap.br

Abstract. The aim of this study was to develop and characterize the incorporation of two types of chlorhexidine (CHX) into Poly(lactide-co-glycolide acid) (PLGA) biodegradable microparticles to be used in dental products as a controlled release system. Polymeric microparticles of CHX diacetate (CDA) and digluconate (CDG) were prepared using spray-drying technique and characterized in terms of stability, yield of production, particle size, morphology, encapsulation efficacy (EE), drug loading (DL), cumulative release and anti-microbial response. The drug in solution was stable for 50 days. The yield of production showed the best result for CDA, starting from 71.56%. Particle size ranged from 1.01 to 3.07 μm. Morphological analysis showed homogenous, spherical and non-aggregated microparticles. The microencapsulation process led to an EE in the range of 4.42 - 36.67% and DL between 0.26 and 4.07% among the formulations. It was observed a controlled release pattern among formulations. The CDA and CDG obtained from the microparticles were able to inhibit *Streptococcus mutans* in vitro. The characterization of microparticles loaded with CHX resulted in different release profiles, which suggest these systems feasible for different clinical procedures and applications.

1. Introduction

The great diversity of oral pathogens and the different susceptibility profiles to antimicrobial agents difficult the individual prescription in dental treatments [1]. Most oral diseases carry a bacterial component; however, systemic antimicrobials do not show a great role in dental treatment, making the treatment *in situ* very useful [2].

Polymeric microparticles have been studied for the treatment of various pathological conditions, where the prolonged effect or therapeutic targeting is desired. Poly(lactide-co-glycolide acid) (PLGA) is one of the main polymers used in the development of release systems, mostly due to its superior biocompatibility [3-6]. The development of biodegradable formulations to control the release of antimicrobial drugs would be important for controlling the infections in the oral cavity. Additionally, the protection of the drug from the physiological degradation and bioadsorption would be also advantageous, since those phenomena could result in loss of pharmacological activity [7].

The association of the polymeric microparticles to antimicrobial agents in dental materials may prevent bacterial activity, biofilm formation, demineralisation and dental caries formation [8-10]. Therefore, the choice of the dental product is also important, since the release ability of anti-caries substances from each type of material can be determinant in the development of dental injury [11].

Chlorhexidine (CHX) is an antimicrobial agent extensively studied [12]. It has been incorporated into various materials, such as experimental polymers [13, 14], cements [15, 16], primers [17], dental adhesives [18], nanocomposites [19], and varnish and chip for periodontal treatment [20, 21]. Others studies showed the feasibility of CHX incorporation, without compromising the physical and chemical properties of the material [13, 16, 17]. Additionally this association presented a potential bacterial inhibition [14, 18, 19, 22].

The present study aimed to develop and characterize the incorporation of two types of CHX into PLGA biodegradable microparticles to be used in dental products as a controlled release system.

2. Materials and methods

2.1. Materials

Poly(lactide-co-glycolide acid) (PLGA), Resomer® RG502H, was purchased from Sigma-Aldrich (Taufkirchen, Germany). Chlorhexidine digluconate (CDG) was purchased from Fagron (São Paulo, Brazil). Chlorhexidine diacetate (CDA) was donated from Evonik® (Barcelona, Spain). All the other reagents were analytical standard type and used as received.

2.2. Analytical validation

2.2.1. Standard curve of CHX concentration

Validation was performed to guarantee linearity, precision and accuracy in the measurements. Concentrations of 1, 2, 4, 5, 10 and 20 µg/ mL were assayed using a UV-visible spectrophotometer (Evolution 60S, Thermo scientific, Ohio, USA).

2.2.2. Chlorhexidine stability in solution

A stability experiment was performed to evaluate the degradation kinetics in aqueous medium of these molecules, after storage under different conditions. Concentrations of 1 and 10 µg/mL CHX were measured after storage in room temperature (25 °C), incubator (37 °C) and freezer (4 °C). Solutions were measured at pre-set time intervals along 50 days.

2.3. Preparation of microparticles

Microparticles were prepared by a spray-drying technique. Two different formulations were tested for each drug (CDG and CDA) with different polymer:drug proportions. Formulations were prepared in the ratio PLGA:drug of 16:1 (DA_1 and DG_1) and 8:1 (DA_2 and DG_2). A blank formulation (PLGA only) was obtained and used as a reference.

Due to differences in solubility among the drugs and polymer, an emulsification process was proposed [23]. Briefly, PLGA was dissolved in dichloromethane (DCM) at 4% (w/v). CDA or CDG were dissolved in 1 mL of Milli-Q water in another vial. The solutions were mixed in a disperser system (Ultra Turrax T-10 Basic, IKA Works, Campinas, Brazil).

The emulsions were immediately spray dried in a laboratory spray drier (Mini Spray Drier Buchi 290, Buchi, Flawil, Switzerland). After the procedure, microparticles were collected, weighted to determine the yield of production and stored

into clean sealed glass vials. For further studies, they were preserved at 4 °C. The storage temperature was based on previous testing.

2.4. Characterization of the formulations

2.4.1. Yield of production

The yield of production of the microparticles was determined according to the ratio between the obtained mass and the theoretical mass of the polymer and drug used.

2.4.2. Particle size and morphology

After the formulations were obtained, microparticles size and polydispersity were determined by light scattering (Zetasizer Nano ZS, Malvern, Worcestershire, United Kingdom) in an aqueous solution of Tween® 20 (0.1%). Measurements were made in triplicate.

The morphology of the microparticles was examined by scanning electronic microscopy (FE-SEM ULTRA PLUS, Carl Zeiss, Baden-Württemberg, Germany). Samples of dried microparticles were placed on double-sided carbon adhesive stickers and measured with no prior specimen preparation.

2.4.3. Encapsulation efficacy (EE) and Drug loading (DL)

The amount of CHX entrapped within the microparticles was determined using the solvent-separation method. Thus, 10 mg of microparticles accurately weighted were added to 0.5 mL of DCM and stirred vigorously, followed by the addition of 1 mL of Milli-Q water. The samples were then centrifuged for 10 min at 10.000 rpm (NT810, Novatecnica, São Paulo, Brazil) to separate the two phases. The procedure was repeated until the complete extraction of the drug.

After obtaining the data, EE and DL were obtained by equations (1) and (2) respectively:

$$\text{EE (w/w \%)} = \frac{M_{ENC}}{M_0} \quad (1)$$

$$\text{DL (w/w \%)} = \frac{M_{ENC}}{M_{MS}} \quad (2)$$

where M_{ENC} is the real content of drug in the microparticles, M_0 is the theoretical amount of drug in the formulation and M_{MS} is the mass (mg) of microparticles used in the assay.

2.4.4. Stability of the microparticles

A stability study of the microparticles was carried out to evaluate the drug integrity after storage under different conditions. Microparticles of CHX were storage in room temperature (25 °C), incubator (37 °C) and freezer (4 °C). The amount of CHX was determined using the solvent-separation method after 15 and 60 days.

2.5. Release experiments

The release study of the encapsulated forms of CDA and CDG was performed and some physiological conditions of the oral cavity were reproduced (pH 6, 37 °C). The medium was renovated along the required treatment conditions up to 120 days. Dried CDA and CDG microparticles (n=3) were placed into vials in direct contact with the release medium (1.0 mL of Milli-Q water) and were stored inside an incubator (BOD-Biochemical Oxygen Demand-TE-391, Tecnal, Piracicaba, Brazil) at 37 ºC to constant stirring. Salivary flow was simulated and the release medium was collected (0.8 mL) with a micropipette (Eppendorf AG, Hamburgo, Alemanha). The medium was changed every hour during the initial 6 hours. After this period, collection of the aliquots was spaced for every 6 hours and just after, for every 24 hours, in every case to avoid medium saturation. The collected aliquots were measured immediately and replaced with an equal amount of fresh release medium.

2.6. Antimicrobial activity

To guarantee the efficacy of the proposed formulations, it was important to evaluate the microbiological response after the technical procedures involved in their production. Collected solutions used in the release experiments were tested by diffusion disk technique in plates.

Streptococcus mutans UA 159 and culture medium Brain Heart Infusion (BHI) agar (Difco Lab. Detroit, Michigan, USA) were used. Lyophilized inocula was rehydrated in sterile BHI medium, inoculated in BHI agar plates and incubated at 37 °C for 24 h in a 5% CO_2 atmosphere (Forma Direct Heat CO_2 Incubator IR 230, Thermo

Fisher Scientific Inc, Massachusetts, USA). After that, pure cultures were collected with a disposable sterilized point and placed into BHI medium. This procedure was repeated 4 times to guarantee proper bacteria response. Cultures were adjusted to a 0.5 McFarland turbidity scale to obtain test inoculants.

Approximately 15 mL of BHI agar were disposed in sterilized plates and allowed to dry in a laminar airflow cabin (BioFlux II 120 A1, Filtracom, SP, Brazil). After that, bacteria were inoculated in the agar surface. Sterilized paper disks (6 mm) were hydrated with 10 µL of CDA or CDG solutions and distributed equally along the plates. The dishes were kept under the same conditions aforementioned. The inhibition zones obtained were examined to evaluate the microbiological response of the encapsulated drug. Negative (Milli-Q water) and positive (CHX at 0.3 µg/mL) control groups were used to check the bacterial viability and also the sensitivity, respectively.

3. Results

3.1. Analytical validation

From the stability study in solution it was verified that drug levels were stable for 50 days, despite the concentration (1 or 10 µg/mL), storage condition (room temperature, incubator and freezer) and molecule studied (CDA or CDG) (Figure 1).

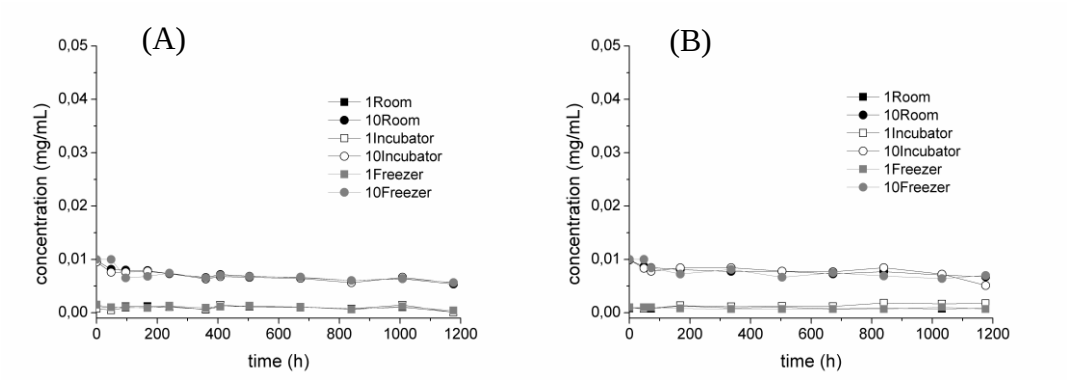

Figure 1. Stability in solution of CDA (A) and CDG (B); where 1 and 10 refer to the concentration in mg/mL, after storage under different conditions: room (25 °C), incubator (37 °C) and freezer (4 °C) up to 50 days.

3.2. *Characterization of the formulations*

The formulations obtained for both drugs are listed in table 1. The yield of production of the formulations showed the best result for CDA (over 71.56%) whereas CDG ranged between 34.81 and 64.33%.

Microparticles size ranged between 1.01 and 3.07 μm (table 1). The polydispersity index was averaged in 0.219 and 0.392 for CDA and CDG microparticles, respectively. The microparticles containing CDA showed a more uniform pattern for both compositions compared to CDG. Blank microparticles exhibited a uniform pattern.

Table 1. Main characteristics of CHX loaded - PLGA microparticles.

Encapsulated drug	**Ratio PLGA: drug**	**Production yield (%)**	**Particle size (μm)**	**Encapsulation efficiency (%)**	**Drug loading (%)**
Blank	[B]	18.91	1.01	NA	NA
Chlorhexidine diacetate (CDA)	[DA_1] 16 : 1	71.56	1.6	10.63	0.62
	[DA_2] 8 : 1	71.84	1.5	36.67	4.07
Chlorhexidine digluconate (CDG)	[DG_1] 16 : 1	34.81	3.07	4.42	0.26
	[DG_2] 8 : 1	64.33	1.04	21.99	2.44

The formulation process resulted in microparticles with EE in the range of 4.42 - 36.67%, depending on the drug and on the polymer:drug ratio used. CDA microparticles showed higher EE compared to CDG. DL varied between 0.26 and 4.07% among the formulations (table 1). The incorporation of CDA also increased the DL compared to CDG. The increment in both drugs concentration (DA_2 and DG_2) in the formulation resulted in a superior EE and DL.

The surface morphology of the microparticles was examined by scanning electron microscopy (SEM). It was observed uniform, spherical and non-aggregated microparticles (Figure 2).

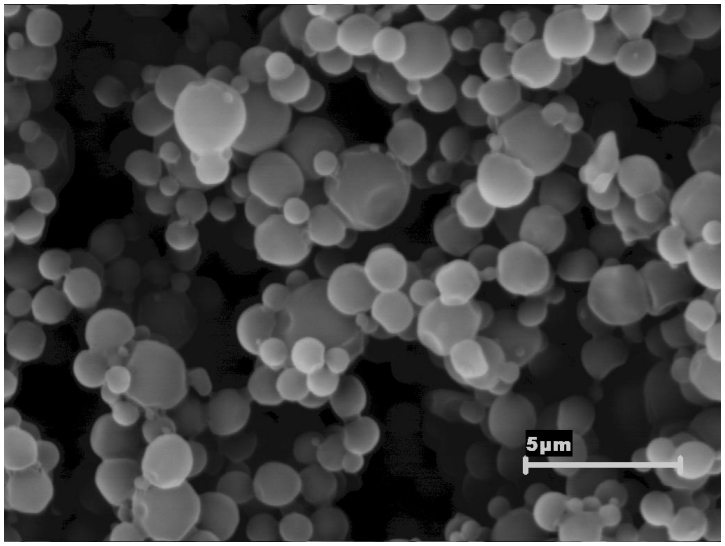

Figure 2. SEM image of PLGA blank microparticles.

Concerning the stability study of the microparticles, DG ones were more susceptible to heat than those containing DA, resulting in a decrease of their content for 60 days under different conditions. Moreover, DA_1 maintained its loading unchanged for 15 days, and DA_2 remained intact for 60 days.

3.3. Release study

The formulations containing higher amount of drugs (DA_2 and DG_2) presented the highest cumulative release (%) and drug released (µg), in agreement to the entrapment levels aforementioned. The DA_1 formulation presented a two-step burst effect release: in the first 24 h it reached over 25% remaining constant up to 21 days, and then has increased the release speed. The formulation DA_2 presented a burst effect reaching approximately 60% of its content released over the first 24 hours. After that, its release slowed and remained controlled over the studied period (Figures 3 and 4).

Both DG formulations presented a prominent burst effect along the first 48 h, reaching over 85% and 75% in the formulations DG_1 and DG_2, respectively. The formulation named DG_2 showed the highest amount of drug released during the experimental period, reaching 100% when the study was concluded (Figure 3).

In any case, the DA formulations presented a more controlled release pattern over the experimental period (Figure 4) as evidenced by the drug amount release at each interval.

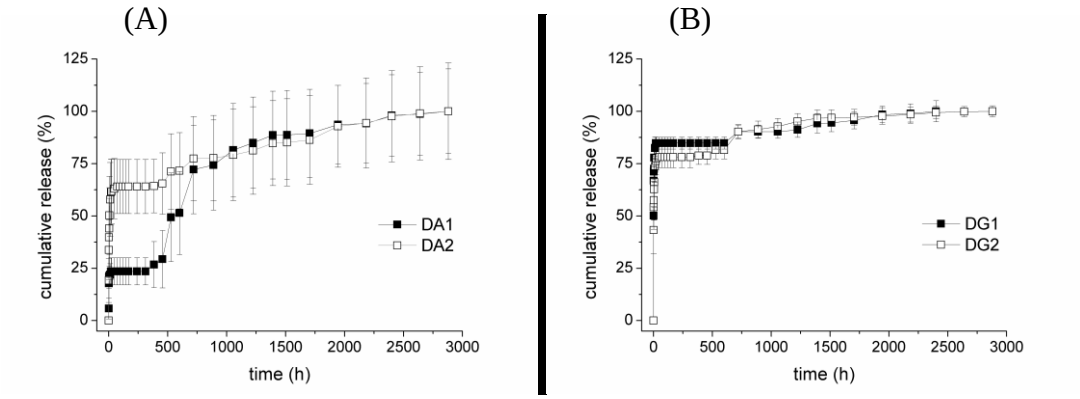

Figure 3. Release profiles of CDA (A) and CDG (B) from PLGA microparticles (see after Table 1).

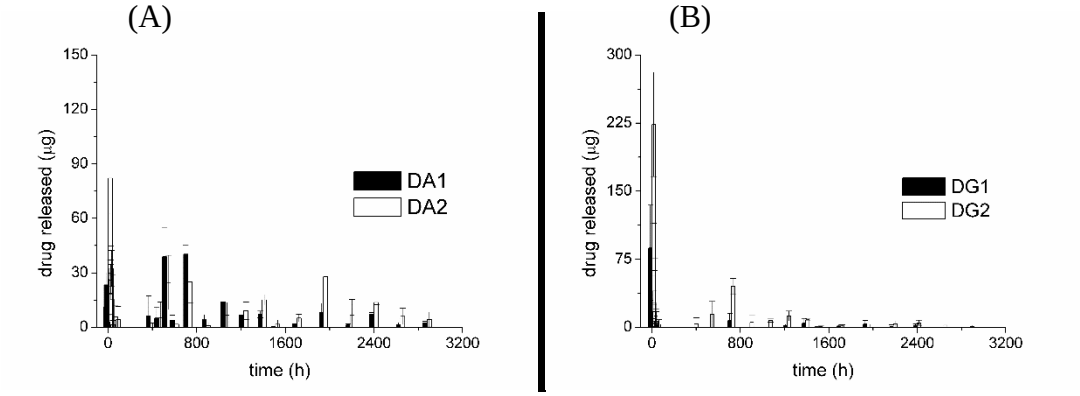

Figure 4. Drug released over time of CDA (A) and CDG (B) from PLGA microparticles.

3.4. *Antimicrobial activity of the encapsulated CDA and CDG*

As it can be visualized in Figure 5, the colonies of *Streptococcus mutans* were homogeneous and the halos' diameters evidenced their susceptibility.

The assayed samples inhibited *Streptococcus mutans in vitro*, depicting the pharmacological activity of CHX (DA and DG) after the encapsulation process. The formulations DA_2 and DG_2 were responsible for the largest inhibition zones (Figure 5) in agreement to their respective EE and DL.

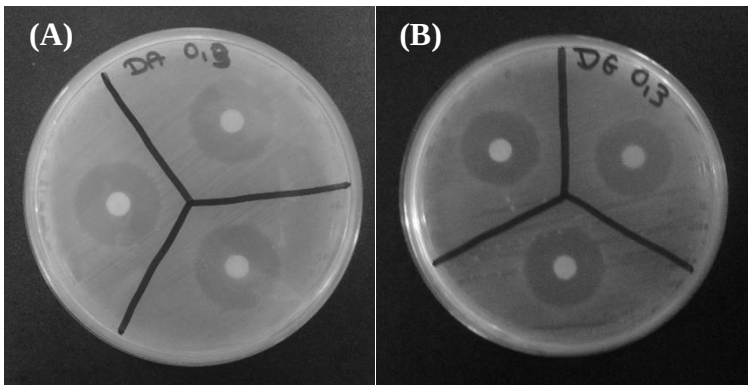

Figure 5. Inhibition zones obtained from the release of CDA (A) and CDG (B).

4. Discussion

The association between CHX and polymeric materials has not been extensively studied, especially when it concerns the physical and/or chemical properties of the molecules and the resulting effect on the release ability from the material. Some studies, related to dental materials, pointed good results after CHX incorporation [13, 16, 17]. However drugs are released more quickly when uncoated [23, 24, 25]. Moreover, PLGA has been extensively used in several applications concerning the controlled release of drugs [23, 26, 27]. Accordingly, the production of CHX-loaded PLGA microparticles aimed to obtain a long-acting drug delivery system, which could be applied in dental therapeutics.

Two types of CHX (CDA and CDG) were used in the present study. CDG is extensively used in dentistry in a large variety of applications and procedures [28, 29]. In contrast, CDA has not received much attention from the professionals and it is not explored commercially. Due to the fact that CDG is only available in solution [30], the incorporation in dental materials becomes problematic. As a result, the production of CDG-loaded microparticles is undoubtedly a great purpose.

As a requirement to the drug delivery development, in this study the analytical validation of the methodology applied in the quantifications presented an acceptable correlation, and demonstrated the stability of CHX in solution under different conditions. In addition, microencapsulated CHX remained mainly stable overtime under the same environmental conditions, similarly to another study [31]. The chosen encapsulation technique – spray drying – generated uniform and reproducible

populations of microparticles. Therefore, these microparticles are feasible to dental clinical applications.

The release study showed different profiles for each CHX and also between the individual formulations. Period and amount of released drug are crucial for the choice of CHX formulation for clinical use. After the burst effect, the release of the formulation DA_2 became gradually sustained. The controlled release of CDA over time favours procedures which require a delayed release of the drug. Thus, CDA microparticles could be used in the pure form or incorporated into dental materials. In contrast, CDG microparticles may be used in clinical procedures that require a high initial concentration of the drug, such as intracanal dressing, periodontal chemical treatment, biofilm control and temporary procedures.

Inhibition of *Streptococcus mutans* was remarkable. DA_2 and DG_2 formulations presented the best microbiological response in the corresponding assay. The results demonstrated the potential of these systems in controlling the release of CHX, which could be very promising in dental applications where antimicrobial activity is required.

5. Conclusion

The development and characterization of CHX-loaded PLGA microparticles was achieved, resulting in systems with different behaviours. CDA showed a more sustainable and uniform release throughout the study and could be used in procedures where long term release of the drug is required. However, CDG microparticles may be indicated in clinical procedures that demand a high initial concentration of the drug. The different release profiles, the chemical and biological integrity of the drug make these systems feasible for different clinical procedures and applications.

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3.2 Capítulo 2

Title: Physicochemical properties of a controlled release chlorhexidine-loaded resin dental sealant

Authors: Jacqueline S. Nojosa,[1] Carolina A. A. Alencar,[1] Juliano S. Mendonça,[1] Lidiany K. A. Rodrigues,[1] Francisco F. O. Sousa[2] and Monica Yamauti[1*]

Affiliations:

[1] Graduate Program of Dentistry, Faculty of Pharmacy, Dentistry and Nursing, Federal University of Ceara, Rua Monsenhor Furtado, 1273, 60430-355, Fortaleza, Ceara, Brazil.

[2] Graduate Program of Pharmacy, Department of Biological & Health Sciences, Federal University of Amapa, Rod. Juscelino Kubitschek, km 02, Macapa, Amapa, 68902-280, Brazil.

*Corresponding author:

Av. Presidente Antonio Carlos, 6627, Pampulha, Belo Horizonte, MG, 31270-902, Brazil.

E-mail: myamauti@gmail.com

Tel: +553191929924.

Abstract

This study aimed to evaluate the effect of incorporation of chlorhexidine (digluconate or diacetate), in its free form or loaded into Poly(lactide-co-glycolide acid) microparticles, on the physicochemical properties of resin sealants. For the degree of conversion (DC), sealants formulations were prepared, and samples were analyzed by FTIR Spectrometry. For the release experiments, samples from each formulation were stored individually, medium aliquots were collected and quantified chlorhexidine and fluoride releases at pre-defined times by means of UV-visible spectrophotometry and fluoride-ion-specific potentiometry, respectively. The commercial sealant containing 2%w/w chlorhexidine diacetate in the free form ($p<0.05$) and 10%w/w chlorhexidine digluconate loaded microparticles presented the lowest DC ($p<0.01$). Experimental resin containing chlorhexidine digluconate at 2%w/w in free form presented the highest DC ($p<0.05$). Concerning the chlorhexidine release, experimental resin containing 5%w/w of microparticles loaded with chlorhexidine diacetate presented the highest cumulative release ($p<0.01$), while the same resin containing 5 and 10%w/w of microparticles loaded with chlorhexidine digluconate presented the lowest ratios ($p<0.001$). The groups containing microparticles loaded with chlorhexidine diacetate at 1% and 5%w/w showed the highest fluoride release levels ($p<0.001$). DC, chlorhexidine and fluoride releases varied among sealant formulations containing chlorhexidine in free and microencapsulated forms, contributing in clinical uses to prevent caries.

Keywords: Pit and fissure sealants, microparticles, chlorhexidine, fluoride.

INTRODUCTION

The high risk of caries in pits and fissures[1,2] allowed the development of materials capable of acting as a physical barrier, preventing the accumulation of substrates and cariogenic biofilm. The most recommended materials are resin sealants, because they have adequate viscosity, retention, resistance, marginal sealing, durability and aesthetics.[3,4] Sealants may have fluoride in its composition, as a viable alternative to prevent the occurrence of caries. Fluoride release for enamel inhibits demineralization[5,6] and enhances dental remineralization.[6,7,8,9]

The association between fluoride and chlorhexidine could contribute to the anticaries effect in the treatment of high caries risk individuals.[10,11] Chlorhexidine shows antimicrobial activity against bacterial found in the oral cavity[12,13] and can reduce cariogenic bacterial activity. In dentistry, the most commonly forms of chlorhexidine used are digluconate and diacetate. Some studies incorporated chlorhexidine particles as fillers into resin composites, which resulted in chlorhexidine release and reduced bacteria growth.[14,15,16] Chlorhexidine particles were also incorporated into glass ionomer cements,[17] thus combining its antimicrobial activity to the fluoride ions effect.[18] Other studies have also evaluated the incorporation of chlorhexidine into resin materials, presenting a potential for bacterial inhibition.[15,19,20]

However, appropriate action of the anticariogenic agents depends on their release at therapeutic levels over time.[21] The controlled drug delivery systems could contribute to a prolonged effect of the drug in the specific therapeutic site. These systems can be produced from Poly(lactide-co-glycolide acid) (PLGA), which is considered a gold standard material in controlled drug delivery due to its incomparable biocompatibility and biodegradation.[22,23,24] Commonly, polymeric devices are incorporated with fluoride,[25] chlorhexidine[26,27] or other antimicrobial agents.[28]

The control of cariogenic activity through the controlled release of chlorhexidine incorporated in resin sealant could be an effective strategy in preventive-restorative treatments. Additionally, the combination of fluoride and chlorhexidine microparticles could provide promising therapeutic results. Therefore, the aim of this study was to evaluate the effect of incorporation of chlorhexidine (digluconate or diacetate), in the free form or loaded into PLGA-microparticles forms, in the physicochemical properties of commercial and experimental sealants. The null hypotheses tested were: *(i)* incorporation of chlorhexidine in free form or loaded into PLGA-microparticles forms have no effect on sealants degree of conversion; *(ii)* the incorporation of free chlorhexidine or PLGA-microparticles loaded with chlorhexidine into commercial and experimental sealants will not be able to release chlorhexidine; *(iii)* commercial sealant incorporated with free chlorhexidine or PLGA-microparticles loaded with chlorhexidine will not be able to release fluoride ion.

MATERIAL AND METHODS

Chlorhexidine microparticles

The materials and chemicals used in this study are described in Table 1.

Microparticles were prepared with Poly(lactide-co-glycolide acid) (PLGA, Sigma-Aldrich, Germany) by means of spray-drying technique.[29] Two different formulations of microparticles were prepared using chlorhexidine digluconate (DG) (Fagron, São Paulo, Brazil) and chlorhexidine diacetate (DA) (Evonik®, Barcelona, Spain), resulting in drug loading (DL) of 2.44% (w/w) for DG and 4.07% (w/w) for DA (unpublished data). A blank formulation (PLGA only) was obtained and used as control.

PLGA microparticles loaded either with DG or DA were examined in terms of yield of production, particle size, morphology, encapsulation efficacy and drug loading (unpublished data).

Preparation of sealants formulations

The sealants formulations were prepared by incorporating DG or DA into light-cured commercial (BI) (Bioseal®, Biodinâmica, Curitiba, Brasil) and experimental sealants (EX) (Biodinâmica, Curitiba, Brasil). The difference among the sealants was the presence of fluoride (F) in the commercial product (Table 1). Free forms of DG and DA were added to commercial and experimental materials to obtain sealants formulations containing 1.0 and 2.0% (v/w) of chlorhexidine (CHX).

The amount of microparticles to be incorporated into the sealants was determined as 5.0 and 10.0% (w/w), in order to adjust to their drug loading. Thus, PLGA-microparticles loaded either with DG or DA (respectively, PDG or PDA) were mixed into the sealants to obtain formulations with 5.0 and 10.0% (w/w). Each sealant formulation was manually manipulated on a glass plate for 1 minute at reduced ambient light. These procedures were made isothermally at 23 °C and relative humidity of 45%. Except for the controls group (BI-C and EX-C, no drug added), each formulation was mixed as described above to prepare the sealants of experimental groups (Table 2).

Characterization of the formulations

Degree of conversion (DC)

Five samples of each group were produced and analyzed in both uncured and cured states. For a methodological issue, DC was evaluated in the groups with the highest CHX concentration: BI-DA2, BI-DG2, BI-PDA10, BI-PDG10, EX-DA2, EX-

DG2, EX-PDA10 and EX-PDG10. These samples were analyzed using Fourier Transform Infrared Spectrometry (Vertex 70 FT-IR Spectrometer, Billerica, MA, USA) equipped with the horizontal zinc selenide crystal (ZnSe) element of an Attenuated Total Reflectance (ATR) attachment (Miracle ATR, PIKE Technologies, Madison, WI, USA). Spectra were obtained in the range of 1750 and 1550 cm^{-1} (8 cm^{-1} resolution), 64 scans and in the transmittance mode. For the analysis of uncured sealants, each sample containing about 5 μL was placed on the ATR ZnSe crystal top-plate. Samples in cured states were prepared with dimensions of 1 cm diameter and 0.3 mm thick. The sealant was inserted into the mold and covered with a polystyrene matrix strip. The samples were individually light-cured for 20 seconds with an intensity of 1200 mW/cm^2cured using a blue LED light source (Elipar^{TM} FreeLight 2, 3M ESPE, Neuss, Germany). The distance between the tip of the optical fiber and the sample was 5 mm. The software OPUS was used to monitor the assay, which was performed at 25 °C and relative humidity of 70%.

The DC was calculated using standard methods[30] that evaluated changes in the ratios of aliphatic (1636 cm^{-1}) to aromatic C=C absorption peaks (1608 cm^{-1}) in the uncured and cured states obtained from the infrared spectra, according to the following equation: DC=100-[(*R* cured)/(*R* uncured) x 100], where *R*=ratio of peak height at 1636 cm^{-1} and 1608 cm^{-1}.

Release studies of sealants containing free and microencapsulated chlorhexidine

Individual cylindrical polystyrene matrices with one free surface (2.0 mm diameter x 4.0 mm thick) were used to enclose the sealant formulations, which were used in triplicate. The matrix was filled with each sealant formulation, a mylar strip was placed on the top of it and a glass slide was placed to perform the light-curing process.

The material was light-cured with LED (Elipar^{TM} FreeLight 2, 3M ESPE, Neuss, Germany) for 20 seconds at 1200 mW/cm^2. Samples were stored in individual vials containing 1.0 mL of Milli-Q water, and stored inside an incubator (BOD-Biochemical Oxygen Demand-TE-391, Tecnal, Piracicaba, Brazil) at 37 ºC.

The medium was collected in pre-determined times (up to 6 months) and immediately quantified in a spectrophotometer (Amershan Biosciences, Ultrospec Pro 1100, Cambridge, England), renewing with an equivalent amount of fresh release medium, in order to simulate the saliva clearance. The absorbance of blank microparticles was used to eliminate the polymer influences.

Fluoride (F) release studies

The concentration of F released from sealants formulations was measured using the same aliquots (1 mL) used for the quantification of CHX in the first two months. The amount of F was determined electrochemically using a–fluoride-ion-specific electrode (Orion 96-09, Thermo Electron Corporation, Waltham, Massachusetts, USA). Calibration of measuring device was performed by standard F solutions according to pre-defined curve of F concentration. A linear relationship between the logarithm of F concentration and relative voltage was established for each reference solution by means of a fluoride-ion-specific electrode and a digital voltmeter.[31] The amount of F was measured by determining the potential difference (ddp) for each solution at 25º C.

Statistical analysis

Statistical analyses were performed with GraphPad Prism® 5 program and data were expressed as Mean (SD). Test of normality Shapiro-Wilk was realized in degree of conversion (n=5) and data was submitted to Levene's test. The DC and the

release of CHX and F data were analyzed using Two-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* test. In all tests the confidence level was 95% ($p<0.05$).

RESULTS

Degree of conversion

The results of the DC analysis of commercial and experimental sealants are presented in Table 3. The statistical analyses showed that sealant ($F=13.64$; $p=0.0007$) and type of CHX ($F=5.615$; $p=0.0011$) significantly affected the DC ($p<0.001$). The interactions between variables were not significant ($F=2.175$; $p=0.0893$). In the commercial sealant, DC of groups with free CHX and PLGA-microparticles of CHX were similar to each other ($p>0.05$). BI-DA2 ($p<0.05$) and BI-PDG10 ($p<0.01$) showed lower DC than BI-C. The experimental sealant (EX-DG2) showed the highest DC, differencing significantly from the others ($p<0.05$). The comparison among the commercial and experimental sealants demonstrated a difference when DG2 was incorporated in the sealants. BI-DG2 presented no difference in DC compared to respective control, while EX-DG2 showed higher DC than control.

Chlorhexidine release

CHX release profiles of commercial and experimental sealants are shown in Figures 1 and 2. Mean CHX release values were affected by CHX type ($F=1131$; $p<0.0001$) and time ($F=593.4$; $p<0.0001$). The interactions between variables were significant ($F=44.06$; $p<0.0001$). The groups containing the free form of CHX (either DG or DA) in the commercial and experimental sealants presented a discrete release from the first 6 h, except from the EX-DG1 and EX-DA2, which started releasing the

drug at 576 h (24 days) and at 24 h, respectively (Fig. 1b and 2b). Groups containing the free form CHX exhibited lower release amounts compared to groups containing microparticles after 6 months (Figures 1 and 2).

Sealants containing PLGA-microparticles with CHX exhibited a late and gradual release after 576 h (24 days), except for BI-PDA10 and EX-PDA5, groups that presented a discrete release after 24 h (Figs. 1b and 2b). Cumulative CHX release from sealants containing PLGA-microparticles of DG or DA showed differences when compared to control groups ($p<0.001$) (Fig. 1a e 2a). In the commercial sealant, materials containing PLGA-microparticles had no significant difference between each other ($p>0.05$) (Fig. 1a). In the experimental sealants containing PLGA-microparticles, the highest release was detected in the EX-PDA5 group (22.91%) ($p<0.01$) (Fig. 2a). In contrast, the experimental sealant group containing 5% of PLGA-microparticles loaded with DA (EX-PDA5) showed higher release than EX-PDA10 ($p<0.01$) (Fig. 2a). Globally, the groups containing PLGA-microparticles of CHX exhibited superior release rates than groups with free CHX.

Fluoride release

The F release profile of the tested commercial sealant is shown in Figure 5. The F release from sealants was measured in all the groups, but no F was detected in the experimental sealant, as expected. Mean F release values were affected by CHX type ($F=24.63$; $p<0.0001$) and evaluation time ($F=105.5$; $p<0.0001$). The interactions between variables were significant ($F=3.879$; $p<0.0001$). All the groups of commercial sealant started the F release at 6 h. The F cumulative release of BI-C (60.24 µg) was used as reference for the other groups. BI-DA1 (131.6 µg) was different from the control and showed the highest release among the groups containing free CHX

($p<0.001$). The others sealants containing free CHX presented no difference to the control ($p>0.05$) (Fig. 3).

In the groups containing PLGA-microparticles loaded with CHX, the F release from all groups were significant different from the control ($p<0.001$). BI-PDA5 (193.6 µg) showed the highest release among the sealants containing PLGA-microparticles ($p<0.001$). The other groups did not present any difference between each other: BI-PDG5 (148.0 µg), BI-PDG10 (142.1 µg) and BI-PDA10 (144.9 µg) ($p>0.05$) (Fig. 3).

DISCUSSION

In this study, the monomer conversion analysis in FT-IR showed that incorporating CHX affected DC in BI-DA2, BI-PDG10 and EX-DG2 groups compared to the respective controls. These results indicated a reduction in the conversion of monomers in commercial sealant incorporated with DA 2%, while the addition of DG 2% in the experimental sealant contributed for increase of DC. Previous investigator reported no change in DC of an adhesive when adding 0.01 to 0.2% concentrations of DA.[32] Similarly, several studies have shown that mechanical properties of polymers[16,33,34] and bonding resins[35,36] were not affected after incorporating CHX, however this fact seems to be dependent on the CHX concentration.[32] Conversely, another study showed that the incorporation of 1-5% of CHX in an adhesive reduced the DC of experimental hydrophilic adhesives.[36] The incorporation of CHX into methacrylates could hinder the polymerization process resulting in a high level of residual monomers,[34,37] which is in agreement with the present results of commercial sealant incorporated with DA 2%. In contrast, the presence of DG 2% in experimental sealant could have contributed to increase of DC due to possibly stabilization of free radicals in the polymerization reaction.[15,36]

The DC of the commercial and experimental sealants containing PLGA-microparticles showed no difference compared to the respective controls, except for BI-PDG10. Probably the association between PLGA-microparticles loaded with DG and F caused a decrease in DC of BI-PDG10. Then, the first null hypothesis that incorporation of CHX in free form or loaded in PLGA-microparticles forms has no effect on sealant´s degree of conversion must be partially rejected.

Concerning the CHX release, the present results demonstrated different cumulative release profiles between the commercial and experimental sealants incorporated with free and microencapsulated CHX. The incorporation of free CHX to various methacrylate polymers has shown high drug release rates.[15,32,38] The incorporation of free CHX into adhesives may serve as a CHX reservoir within the polymer matrix, gradually leached out from polymer to the medium.[32] In the present study sealants loaded with high content of free DG (BI-DG2 and EX-DG2) started releasing discretely along the first 216 h, suggesting that the drug release was CHX-type and concentration-dependent, mainly due to the higher solubility of this drug,[39] increasing the diffusion and releasing process.[40] The release of DG and DA from the sealants containing its free form steadied after 24 h and 216 h, respectively, for experimental and commercial sealants, possibly due to the presence of non-entrapped drug.[16,35] This initial release profile was reported in other studies[17,20] that showed an initial rapid release of CHX in the first week, followed by a plateau.

The drug release from groups containing PLGA-microparticles loaded with CHX started after 576 h (24 days), except for BI-PDA10 and EX-PDA5 which started releasing discretely after 24 h. Sealants loaded with PLGA-microparticles showed a delayed and gradual drug release over 6 months, which depicts a controlled release system. The microencapsulation of CHX benefited the drug release.[41] Even if groups of

microencapsulated DG and DA had much smaller drug loading (5 to 10 times) than the groups of free CHX, they presented much higher release rates (unpublished data). This result suggests that PLGA-microparticles protected the CHX from the interaction with sealant components and inactivation, keeping its bioavailability.

For the commercial sealant, the cumulative release in the groups containing microencapsulated CHX was similar in the end of study. It could be hypothesized that F release to the dissolution medium favored the CHX release from the commercial sealant. CHX and F have opposite charges ions,[42] which induce ionic attraction between the species in aqueous solution. As F was not incorporated in the microparticles, its release occurred faster and in a more favorable way than CHX. Thus, increasing the F concentration in the medium where the devices were placed, inducing the CHX release from the commercial sealant, independent of the tested group.

For instance, the experimental sealants loaded with DA presented a higher release rate than groups with DG. The drug loading results could justify the different profiles. Microparticles with DG presented half drug loading amount than that of DA microparticles (unpublished data). As the experimental sealant does not have F in its composition, release of DG and DA was not influenced by F in the leaching medium and was driven basically by the type and the amount of encapsulated drug. Thus, the second null hypothesis that the release of CHX from sealants incorporated in PLGA-microparticles has no difference to the release behavior from sealants containing free CHX has to be rejected.

Fluoride release from sealants containing free and microencapsulated CHX was quantified over two-months as described by Hoszek.[43] Different amounts of F were detected within the commercial groups, once F was not present in experimental sealant. Thus, the third null hypothesis that commercial sealant containing free CHX and

microencapsulated CHX will not be able to release F must be rejected. The F release was increased in the commercial sealant formulations containing 1% of free DA and 5% of microencapsulated DA, while increasing DA proportion decreased the anion release. The microparticles with high proportion of CHX may have carried a certain amount of non-entrapped superficial drug, which could have interacted with F in solid state impeding its release from inside of the formulations. Probably, the association between DA and F allowed the formation of CHX difluoride (CHXDF) after chemical changes, transforming DA in an insoluble salt[44] and preventing F release.

The sealants presenting free DG showed lower F release rates than sealants containing free DA. The decrease in F release when associated to DG was reported in a previous study regarding the compatibility between DG and sodium monofluorophosphate (MFP). It was observed a slight decrease in F concentration in 0.8% MFP solution after increasing DG concentration from 0.0125 wt% to 2.0 wt%.[45] It could be speculated that the association between DG and F might result in the precipitation of CHX fluoride salt with low solubility, leaving the F less available.[43] For instance, when F is free in the leaching medium, it would increase the CHX release from the microparticles. It was observed in the sealants containing PLGA-microparticles that the beginning of CHX release coincided with the increase of F release after 576 h.

The benefits of pit and fissure sealants have been demonstrated in previous studies.[46,47,48] The additive effect of CHX could contribute in specific clinical uses to prevent biofilm formation and caries development. The incorporation of free and microencapsulated CHX exhibited high DC levels of commercial and experimental sealants. Although some significant differences have been observed in the groups, the high DC results may suggest favorable dental clinical applications. In addition, the

incorporation of CHX showed different profiles in commercial and experimental sealants. The inclusion of microencapsulated CHX to the sealant increased the drug release and also incremented the F release, contributing to the anti-cariogenic effect of the sealant. The best results were found for BI-PDA5 and BI-PDA10 groups. BI-PDA10 was not functionally affected by the incorporation of microencapsulated CHX (according to the DC test), while BI-PDA5 presented the highest CHX and F release rates. Therefore, complementary studies are needed to evaluate other properties, paired with antimicrobial activity to feasible these new formulations of resin sealants for caries prevention.

CONCLUSIONS

- The addition of free or microencapsulated forms of chlorhexidine significantly affected the degree of conversion of resin sealants, depending on the type and form of chlorhexidine incorporated and the sealant.

- Sealants with chlorhexidine in free form or loaded in PLGA-microparticles were able to release chlorhexidine, presenting significant differences among their release profiles.

- Resin sealants containing free chlorhexidine started releasing discretely at the beginning and ceased after some days. Sealants containing microencapsulated chlorhexidine showed a delayed and controlled chlorhexidine release over 6 months.

- Commercial sealant (Bioseal) containing chlorhexidine in free or PLGA-microparticles forms was able to release fluoride in a controlled regimen. The sealants with PLGA-microparticles released greater amounts of fluoride in comparison to the control and sealants loaded free chlorhexidine.

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Table 1. Materials and chemicals used in the study

Material (Abbreviation) /Manufacturer	Batch number	Basic Formulation
Bioseal Sealant /Biodinâmica, Curitiba, Brazil	549 13	Bisphenol A glycidyl methacrylate (35.60%), dimethacrylate groups, BHT, silicon dioxide, sodium fluoride, calcium fluoride and catalyst.
Experimental Sealant /Biodinâmica, Curitiba, Brazil		Bisphenol A glycidyl methacrylate (35.60%), dimethacrylate groups, BHT and silicon dioxide.
Chlorhexidine Digluconate (DG) /Panreac, Barcelona, Spain	9418600021	----------
Chlorhexidine Diacetate (DA) /Evonik®, Barcelona, Spain	8320017837	----------
Poly (D-L lactide - co - glycolide acid) (PLGA 50:50) Resomer® RG502H /Sigma-Aldrich, Taufkirchen, Germany	STBC263V	----------
Methylene Chloride (Dichloromethane) P.A. /ACS, Rio de Janeiro, Brazil	1200542	----------

Table 2. Description of the experimental groups

Groups	Material	Free chlorhexidine and microencapsulated forms
BI-C	Bioseal Sealant	Control
BI-DG1		Containing 1% (v/w) of chlorhexidine digluconate
BI-DG2		Containing 2% (v/w) of chlorhexidine digluconate
BI-DA1		Containing 1% (w/w) of chlorhexidine diacetate
BI-DA2		Containing 2% (w/w) of chlorhexidine diacetate
BI-PDG5		Containing 5% (w/w) of microparticles of chlorhexidine digluconate
BI-PDG10		Containing 10% (w/w) of microparticles of chlorhexidine digluconate
BI-PDA5		Containing 5% (w/w) of microparticles of chlorhexidine diacetate
BI-PDA10		Containing 10% (w/w) of microparticles of chlorhexidine diacetate
EX-C	Experimental Sealant	Control
EX-DG1		Containing 1% (v/w) of chlorhexidine digluconate
EX-DG2		Containing 2% (v/w) of chlorhexidine digluconate
EX-DA1		Containing 1% (w/w) of chlorhexidine diacetate
EX-DA2		Containing 2% (w/w) of chlorhexidine diacetate
EX-PDG5		Containing 5% (w/w) of microparticles of chlorhexidine digluconate
EX-PDG10		Containing 10% (w/w) of microparticles of chlorhexidine digluconate
EX-PDA5		Containing 5% (w/w) of microparticles of chlorhexidine diacetate
EX-PDA10		Containing 10% (w/w) of microparticles of chlorhexidine diacetate

Table 3. Mean (S.D.) of degree of conversion (%) results from commercial and experimental sealants

Free chlorhexidine and microparticulated forms	Bioseal	Experimental
Control	74.67 (2.06) ^{A1}	73.74 (2.73) ^{B1}
Chlorhexidine digluconate 2%	72.90 (1.13) ^{AB1}	77.69 (2.07) ^{A2}
Chlorhexidine diacetate 2%	70.56 (3.62) ^{B1}	72.55 (3.09) ^{B1}
Microparticles of chlorhexidine digluconate 10%	69.74 (1.23) ^{B1}	73.00 (1.40) ^{B1}
Microparticles of chlorhexidine diacetate 10%	71.58 (2.13) ^{AB1}	74.26 (1.76) ^{B1}

For each vertical column, values with identical letters indicate no significant difference using Bonferroni *post hoc* pair-wise comparisons tests ($p>0.05$). For each horizontal row, values with identical numbers indicate no significant difference between the groups using Bonferroni post hoc test ($p>0.05$)

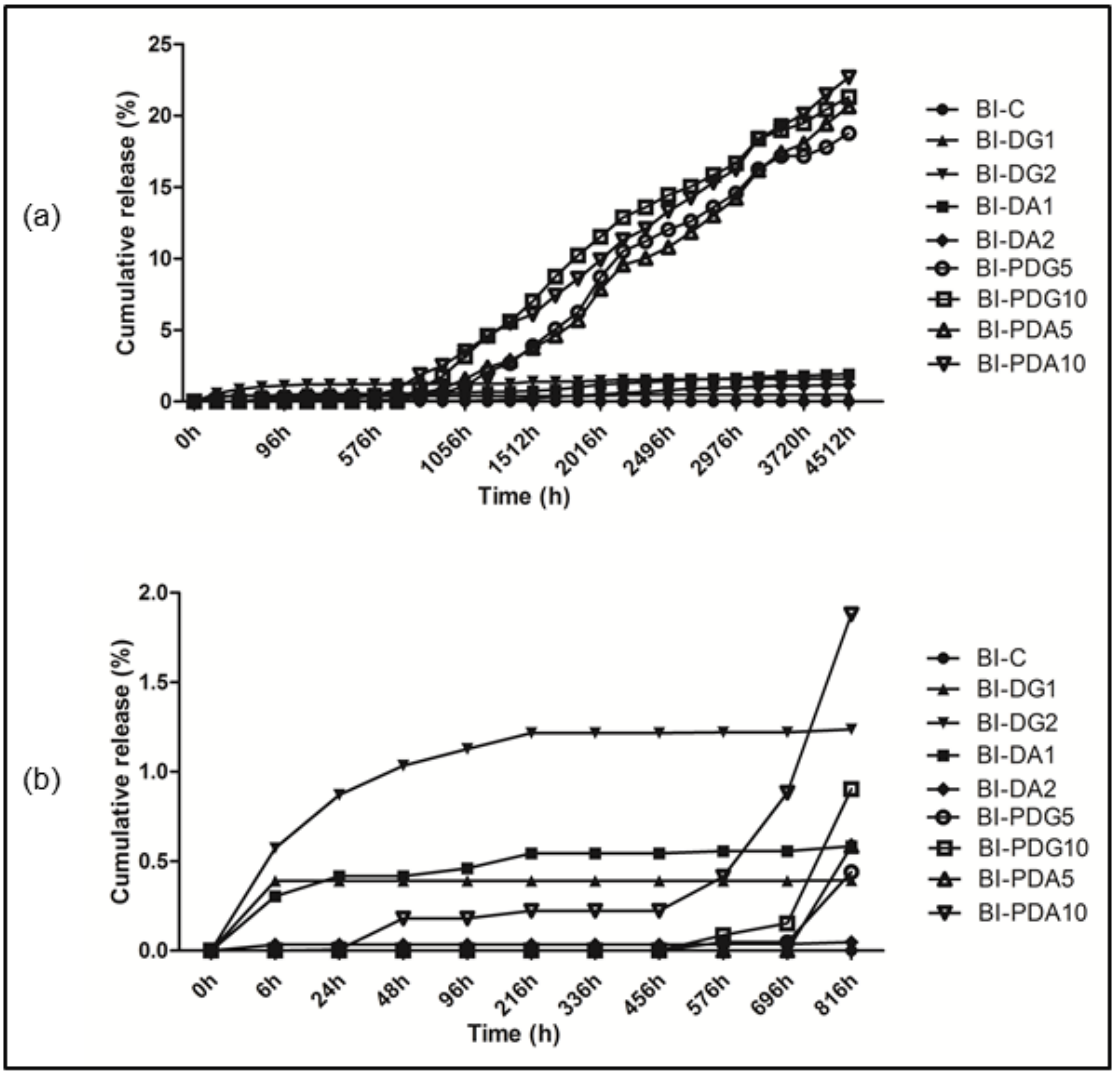

Fig. 1. Cumulative chlorhexidine release from commercial sealant in aqueous medium. a) Cumulative release (%) during the entire evaluation period (4512 h). b) Cumulative release (%) at the first 816 h.

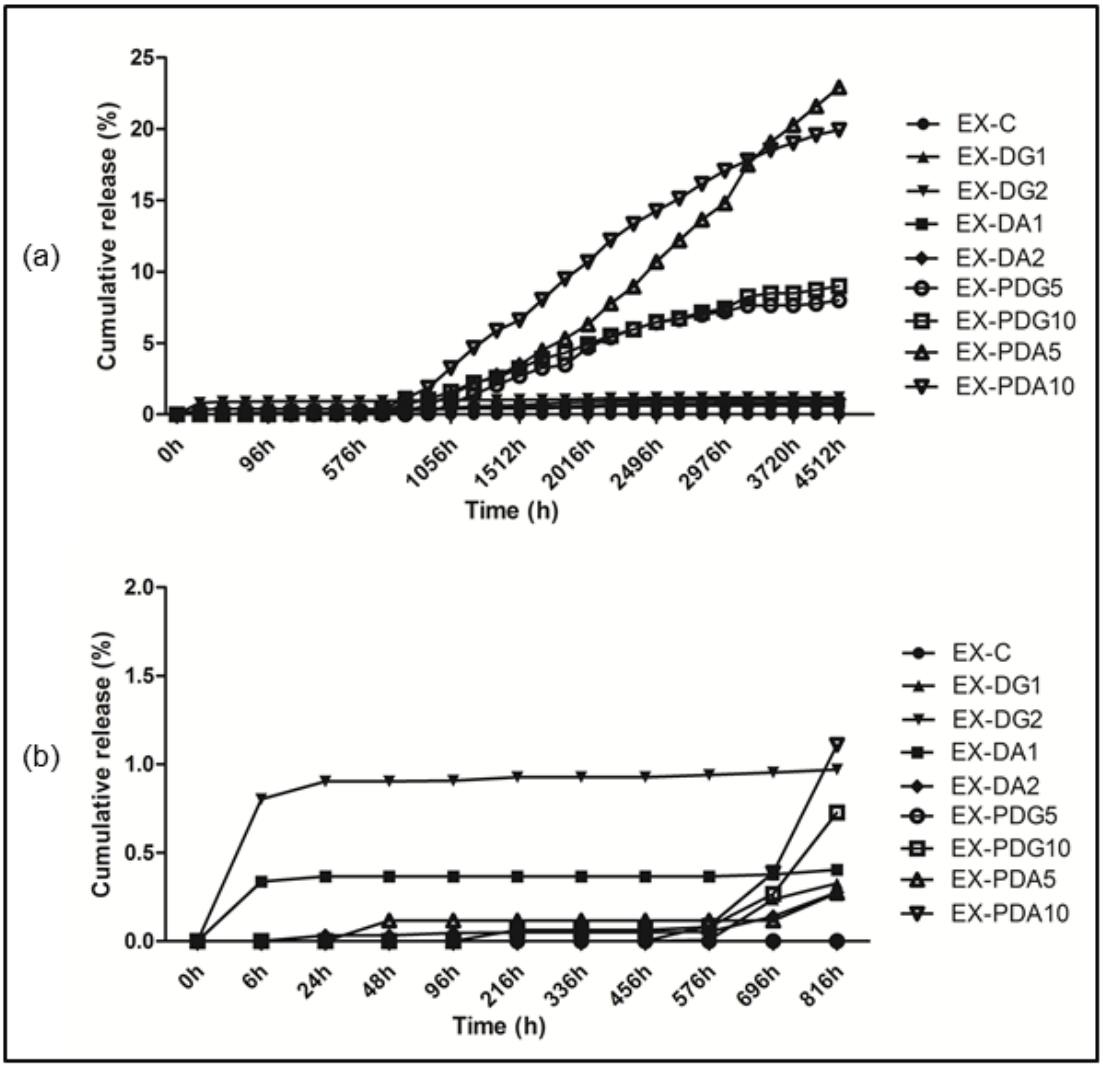

Fig. 2. Cumulative chlorhexidine release from experimental sealant in aqueous medium. a) Cumulative release (%) during the entire evaluation period (4512 h). b) Cumulative release (%) at the first 816 h.

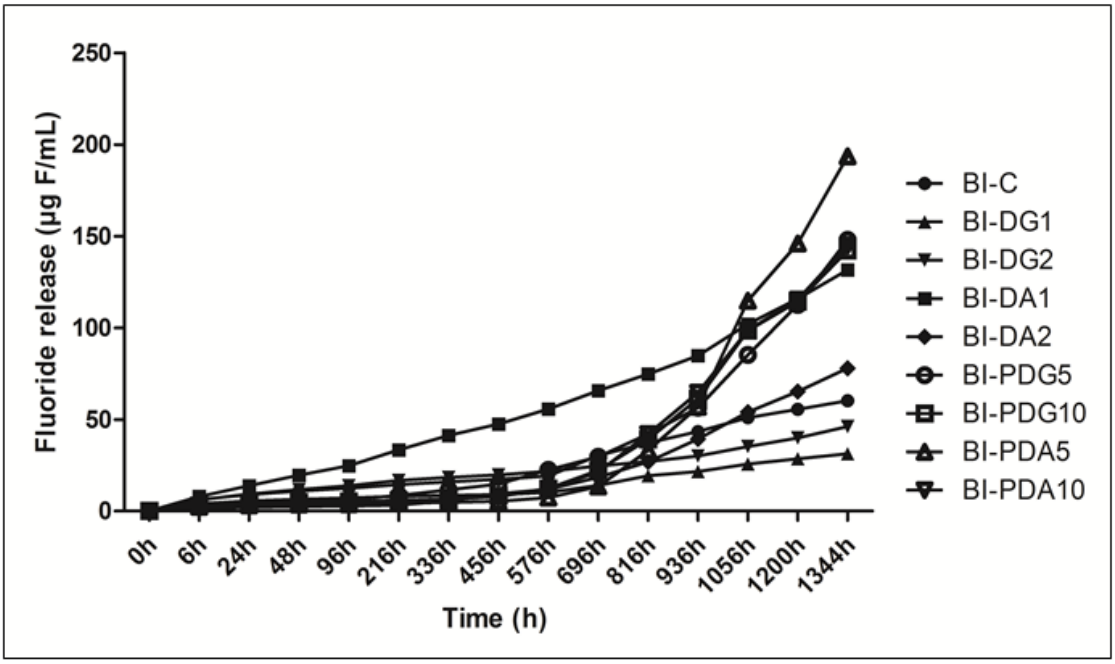

Fig. 3. Fluoride release (µg F/mL) from commercial sealant in aqueous medium.

FIGURE LEGENDS

Fig. 1. Cumulative chlorhexidine release from commercial sealant in aqueous medium. a) Cumulative release (%) during the entire evaluation period (4512 h). b) Cumulative release (%) at the first 816 h.

Fig. 2. Cumulative chlorhexidine release from experimental sealant in aqueous medium. a) Cumulative release (%) during the entire evaluation period (4512 h). b) Cumulative release (%) at the first 816 h.

Fig. 3 Fluoride release (µg F/mL) from commercial sealant in aqueous medium.

4 CONCLUSÃO GERAL

A caracterização de micropartículas poliméricas carregadas com clorexidina (digluconato e diacetato) resultou em diferentes perfis de liberação, tornando esses sistemas viáveis para diversos procedimentos e aplicações odontológicas.

A incorporação de clorexidina, nas formas livres e microencapsuladas, afetou o grau de conversão dos selantes comercial e experimental, dependendo do tipo e da forma de clorexidina incorporada e do tipo de selante.

Grupos incorporados com clorexidina livre e micropartículas apresentaram perfis de liberação diferentes. Grupos incorporados com clorexidina livre apresentaram uma grande liberação inicial, enquanto os grupos contendo micropartículas poliméricas com clorexidina tiveram uma liberação retardada e gradual de clorexidina durante o período de avaliação.

O teste de liberação de fluoreto foi afetado significativamente pela incorporação de clorexidina nas formas livre e microencapsulada. Os grupos com micropartículas de clorexidina liberaram maior quantidade de fluoreto quando comparado ao controle.

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ANEXOS

ANEXO A – NORMAS DO PERIÓDICO BIOMEDICAL MATERIALS

How to prepare your article

Please read these guidelines carefully and familiarize yourself with the style and editorial policies of your chosen journal by examining the online version and taking a look at the Featured Articles. It is important to check that your research fits well into the scope of your chosen journal before you submit it. You are also advised to read the IOP ethical policy. If you have any queries, please contact us.

Your article should normally consist of the following:

- a title page with title of article, name(s) of author(s) and address(es) of establishment(s) where the work was carried out
- an abstract
- the text
- figures
- a list of references

The following sections give a brief overview of the main elements or structure of an article. Read them first.

You can find more detail in our LaTeX and Word guidelines which are presented in the style of a typical article.

Title page

Title of article

This should be concise but informative.

Authors and addresses

For multiple-authored articles list the names of all the authors first, followed by the full postal addresses, using superscript numeric identifiers to link an author with an address, where necessary (see LaTeX and Word guidelines). If an author's present address is different from the address at which the work was carried out, this should be given as a footnote to the page. You can also include e-mail addresses, telephone numbers and fax numbers on the title page.

Short title

This is used at the top of odd-numbered pages in the printed journal and should not exceed 80 characters. You do not need to provide short titles for Fast Track Communications, Rapid Communications or Topical Reviews.

Classification numbers

Many of our journals use the Physics and Astronomy Classification Scheme (PACS), published by the American Institute of Physics to help with the refereeing process. We therefore ask you

to supply a list of appropriate classification numbers. You do not need to supply classification numbers when submitting to *Physics Education*. When submitting to *Inverse Problems* and *Nonlinearity* you should include suitable classification numbers from either the Physics and Astronomy scheme or the Mathematics Subject Classification (MSC), but MSC is preferred. You should include a list of keywords when submitting to *Measurement Science and Technology*, *Physics in Medicine and Biology* and *Physiological Measurement*.

Abstract

Your abstract should give readers concise information about the content of your article. It should be informative and not only indicate the general scope of the article but also state the main results obtained and conclusions drawn. For *Journal of Neural Engineering*, the abstract should consist of the following elements: problem addressed; methodology; results, significance and potential impact. As the abstract is not part of the text it should be complete in itself; no table numbers, figure numbers, references or displayed mathematical expressions should be included. It should be suitable for direct inclusion in abstracting services and should not normally exceed 200 words. If the article is not in English, an English version of the abstract must also be supplied.

When readers are searching for information online, an abstract of an article is likely to be the first thing they see. Consequently your abstract needs to be concise but convey as much information as possible about the content of your article.

Text

Research papers and review articles can be divided into numbered sections and subsections.

You should use tables only to improve conciseness or where the information cannot be given satisfactorily in other ways such as by histograms or graphs. Tables should be numbered serially and referred to in the text by number (table 1, etc). Each table should have an explanatory caption which should be as concise as possible.

If your article consists of a very large amount of tabular material such as long lists of crystallographic results, computer programs and spectrographic results we would not normally publish these in full. Instead these may be published online as supplementary data files.

In terms of general style, conciseness in writing helps the reader, but clarity is most important. Short sentences and paragraphs make reading easier. You should aim for consistency within your article in matters such as hyphenation and spelling.

All acronyms and abbreviations should be clearly explained when they first appear in the text, and all units used should be consistent throughout the article.

If English is not your first language, you should ask an English speaking colleague to read through your article or at least apply a UK English spellchecker to your article.

Mathematics

Detailed information on the presentation of mathematics, formulae and equations is provided in our LaTeX and Word guidelines.

Acknowledgments

All authors and co-authors are required to disclose any potential conflict of interest when submitting their article (e.g. employment, consulting fees, research contracts, stock ownership, patent licenses, honoraria, advisory affiliations, etc). This information should be included in an acknowledgments section at the end of the manuscript (before the references section). All sources of financial support for the project must also be disclosed in the acknowledgments section. The name of the funding agency and the grant number should be given.

References

It is vitally important to fully acknowledge all relevant work and we advise that you also consult our ethical policy for general guidance on compiling your reference list.

A complete reference should provide your reader with enough information to locate the article concerned and should consist of: name(s) and initials, date published, title of journal or book, volume number, editors (if any) and, for books, town of publication and publisher (in parentheses), and finally the page numbers. Where there are up to ten authors, all authors' names should be given in the reference list. Where there are more than ten authors, only the first name should appear followed by *et al*.

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Material which is really a footnote to the text should not be included in the reference list, which should contain only references to bibliographic data.

Copies of cited publications not yet available publicly should be submitted for the benefit of the referees. Unpublished results and lectures should be cited for exceptional reasons only.

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Journal specific notes:

- It can be helpful to include first and last page numbers, particularly for review articles and for journals such as *Reports on Progress in Physics*; final page numbers are a requirement for submissions to *Physics in Medicine and Biology* and *Physiological Measurement*.
- *Inverse Problems*, *Journal of Neural Engineering*, *Measurement Science and Technology*, *Physical Biology*, *Physics in Medicine and Biology* and *Physiological Measurement* require titles of articles in journals in their reference lists.

You can use either of the referencing systems, alphabetical (Harvard) or numerical (Vancouver), described below, except for *Physics in Medicine and Biology* and *Physiological Measurement*, which insist on the Harvard system.

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Alphabetical system (Harvard)

In the Harvard alphabetical system the name of the author appears in the text together with the year of publication, e.g. (Smith 2001) or Smith (2001) (as appropriate). Where there are only two authors both names should be given in the text (Smith and Jones 2001) or Smith and Jones (2001); however, if there are more than two authors only the first name should appear followed by *et al*, (Smith *et al* 2001) or Smith *et al* (2001). If you refer to different works by one author or group of authors in the same year they should be differentiated by including a, b, etc after the date (e.g. 2001a). If you refer to different pages of the same article, the page number may be given in the text, e.g. Smith (2001, p 39). The reference list at the end of your article using this system should be in alphabetical order.

Numerical system (Vancouver)

In the numerical system you should number your references sequentially through the text. The numbers should be given in square brackets and one number can be used to refer to several instances of the same reference. The reference list at the end of the article lists the references in numerical order, not alphabetically.

Figures

Carefully chosen and well-prepared figures, such as diagrams and photos, can greatly enhance your article. We encourage you to prepare figures that are clear, easy to read and of the best possible quality. Characters should appear as they would be set in the main body of the article. We will normally use figures as submitted; it is therefore your responsibility to ensure that they are legible and technically correct.

Note: If you are intending to use previously published figures, you must obtain written permission from the copyright holder before using them in your article.

To get the best possible results in print and online, please consider the following points when preparing your figure files:

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please consider identifying curves by methods other than colour, for example: by letters (upper case Roman), by the symbols used for the data points (e.g.*) or by the type of line (e.g. --, full curve; - - - , broken curve; - · - · -, chain curve).

- When producing figures using colours, light colours such as yellow, light green, light blue, light grey, etc should be avoided because they generally reproduce poorly during the black and white printing process.
- Wherever possible electronic figures should be tightly cropped to minimize superfluous white space surrounding them. This reduces file sizes and helps the alignment of figures on the printed page.

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All of our journals encourage authors to submit supplementary data attachments to enhance the online versions of published research articles. Supplementary data enhancements typically consist of video clips, animations or supplementary data such as data files, tables of extra information or extra figures. They can add to the reader's understanding and present results in attractive ways that go beyond what can be presented in the print version of the journal.

The printed journal remains the archival version, and supplementary data items are supplements which enhance a reader's understanding of the article but are not essential to that understanding. For electronic-only journals, supplementary data attachments may be used to convey essential information.

Length of submissions

Most journals have guidelines for the maximum recommended length of each different type of article, as detailed in the scope available from the journal's homepage. It is important that you follow these guidelines when preparing your submission.

The length of an article can be calculated by allowing 600 words per page in a B5-sized journal or 900 words per page in an A4-sized journal. Diagrams and tables usually occupy the equivalent of 200-300 words each, and you should allow for this in your total.

ANEXO B – NORMAS DO PERIÓDICO JOURNAL OF BIOMEDICAL MATERIALS RESEARCH PART A

Aims and Scope

The Journal of Biomedical Materials Research Part A is an international, interdisciplinary, English-language publication of original contributions concerning studies of the preparation, performance, and evaluation of biomaterials; the chemical, physical, toxicological, and mechanical behavior of materials in physiological environments; and the response of blood and tissues to biomaterials. The Journal publishes peer-reviewed articles on all relevant biomaterial topics including the science and technology of alloys, polymers, ceramics, and reprocessed animal and human tissues in surgery, dentistry, artificial organs, and other medical devices. The Journal also publishes articles in interdisciplinary areas such as tissue engineering and controlled release technology where biomaterials play a significant role in the performance of the medical device.

The Journal of Biomedical Materials Research is the official journal of the Society for Biomaterials (USA), the Japanese Society for Biomaterials, the Australasian Society for Biomaterials, and the Korean Society for Biomaterials.

Articles are welcomed from all scientists. Membership in the Society for Biomaterials is not a prerequisite for submission.

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3. Gilmor ML, Rouse ST, Heilman CJ, Nash NR, Levey AI. Receptor fusion proteins and analysis. In: Ariano MA, editor. Receptor localization. New York: Wiley-Liss; 1998. p 75-90. Please note that journal title abbreviations should conform to the practices of Chemical Abstracts.

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