UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de Araçatuba

Programa de Pós-graduação em Ciência Odontológica
Área de Saúde Bucal da Criança

KELLY LIMI AIDA

EFEITO MICROBIOLÓGICO E CITOTÓXICO DE SISTEMAS NANOESTRUTURADOS BIOADESIVOS CONTENDO FRAGMENTOS DE PEPTÍDEOS

Araçatuba
2017

KELLY LIMI AIDA

EFEITO MICROBIOLÓGICO E CITOTÓXICO DE SISTEMAS NANOESTRUTURADOS BIOADESIVOS CONTENDO FRAGMENTOS DE PEPTÍDEOS

Tese apresentada à Faculdade de Odontologia do Campus de Araçatuba – FOA, Universidade Estadual Paulista "Júlio de Mesquita Filho" – UNESP, como parte dos requisitos para obtenção do título de Doutor em Ciência Odontológica, na Área de concentração Saúde Bucal da Criança.

Orientador: Prof. Adj. Robson F. Cunha
Co-orientadoras: Profa. Dra. Cristiane Duque
Profa. Adj. Sandra M. H.C.A. Aguiar

Araçatuba
2017

Catalogação-na-Publicação

Diretoria Técnica de Biblioteca e Documentação – FOA / UNESP

A288e

Aida, Kelly Limi.

Efeito microbiológico e citotóxico de sistemas nanoestruturados bioadesivos contendo fragmentos de peptídeos / Kelly Limi Aida. - Araçatuba, 2017

46 f. : il.

Tese (Doutorado) – Universidade Estadual Paulista, Faculdade de Odontologia de Araçatuba

Orientador: Prof. Robson Frederico Cunha

Coorientadora: Profa. Cristiane Duque

Coorientadora: Profa. Sandra Maria Herondina Ávila de Aguiar

1. Peptídeos catiônicos antimicrobianos 2. Cárie dentária 3. *Streptococcus mutans* I. Título

Black D27

CDD 617.645

Dados Curriculares

Kelly Limi Aida

Nascimento	05.01.1987 – Assaí-Pr
Filiação	Lauro Akikazu Aida Cleusa Hiromi Ueno Aida
2006-2011	Curso de Graduação em Odontologia pela Universidade Estadual de Londrina
2012-2014	Curso de Mestrado em Odontologia pela Universidade Estadual de Londrina
2013-2014	Curso de Especialização em Estatística com Ênfase em Pesquisa Quantitativa pela Universidade Estadual de Londrina
Associações	CROPR – Conselho Regional de Odontologia do Paraná CROSP – Conselho Regional de Odontologia de São Paulo SBPqO – Sociedade Brasileira de Pesquisa Odontológica

Dedicatória

Com todo amor e carinho, dedico este trabalho à maior preciosidade da minha vida, minha *FAMÍLIA*.

AGRADECIMENTOS

“Deus é o dono de tudo. Devo a Ele a oportunidade que tive de chegar aonde cheguei. Muitas pessoas têm essa capacidade, mas não têm essa oportunidade. Ele a deu para mim, não sei por quê. Sei que não posso desperdiçá-la”. (*Ayrton Senna)*

Senhor Deus,

Obrigada por sempre me dar força, coragem, esperança e persistência para alcançar meus objetivos.

Aos meus queridos Pais,

Lauro Akikazu Aida e Cleusa Hiromi Ueno Aida, obrigada pelo carinho, pelo apoio, pelos ensinamentos, pelos conselhos e pelos sacrifícios que fizeram para me dar uma vida melhor. Mesmo com a distância, meu amor e admiração por vocês cresce a cada dia.

Ao meu Marido,

Yudi Yamada, obrigada pelo companheirismo, pela compreensão, pelo carinho, pela atenção, pela preocupação e pelo apoio durante este período. O seu amor é a fonte de alimento para meu coração. Com sua presença, os meus dias são mais alegres, enfrentar as dificuldades parece ser mais fácil, sem você nada mais tem sentido em minha vida. Com a Graça de Deus, viveremos juntos eternamente. Te amo muito!

À toda Família,

Obrigada pela força, pelo apoio e pelos momentos de descontração. Vocês são tudo para mim, obrigada por fazerem parte da minha vida.

Ao meu orientador,

Prof. Dr. Robson Frederico Cunha, obrigada por me aceitar como sua aluna de doutorado. Sem sua ajuda talvez não pudesse estar concluindo este curso. Obrigada pela oportunidade de estagiar na Bebê-Clínica. Enfim, obrigada por transmitir toda sua experiência e conhecimento.

À minha co-orientadora,

Prof. Dra. Cristiane Duque, foi uma honra conhecer uma pessoa tão competente, esforçada, trabalhadora, generosa, humilde, sonhadora e encantadora. Admiro muito suas qualidades. Muito obrigada pela dedicação, pelo desempenho, pela persistência, pela competência, e especialmente, pela disposição de sempre me ajudar e lutar pelo nosso bem. Muito obrigada pela oportunidade de vivenciar na pesquisa laboratorial. Graças a você, levo uma grande bagagem de conhecimento e aprendizado do doutorado.

Aos Professores do Programa de Pós-Graduação,

Sandra M. H C. Avila de Aguiar, Juliano Pelim Pessan, Célio Percinoto e Alberto Carlos Botazzo Delbem, obrigada pelos ensinamentos, pela vivência e pela colaboração com a minha formação docente.

Aos colaboradores da Faculdade de Ciências Farmacêuticas da Unesp de Araraquara,

Pós-doutoranda Giovana Maria Fioramonti Calixto, e ao Professor Marlus Chorilli, obrigada pela contribuição com toda a parte da caracterização dos sistemas. A ajuda de vocês foi essencial para a conclusão deste trabalho.

Aos colaboradores do Instituto de Química da Unesp de Araraquara,

Norival Alves Santos-Filho, Professor Eduardo Maffud Cilli, obrigada pelo auxílio com a síntese dos peptídeos. A contribuição de vocês foi fundamental para o prosseguimento no trabalho.

À Professora Denise M. P. Spolidorio,

Obrigada por todas as vezes que solicitamos seu laboratório para realizar o ensaio de citotoxicidade e obrigada pelo auxílio com o crescimento e doação das células.

Às amigas do laboratório de Microbiologia,

Paula Fernanda Kreling, Loiane Massunari, Karina Sampaio Caiaffa, Juliana de Carvalho, obrigada por toda ajuda, pela amizade que construímos, pelas confidências e pelo conhecimento que me passaram, levarei a amizade de vocês para sempre.

Aos demais amigos de Laboratório,

Natália, Remberto, Jesse, Vanessa, Laís, Carla Mendes, Liliana, Guilherme, Laís, Luhana, Renan, Mayra, Nayara, Marcelle, Carla Favretto, Marjully, José Antônio, Isabel, Jorge, Jackeline, Jaqueline, Mariana e Thayse, obrigada pela amizade e pelos momentos de descontração, vocês tornaram esta caminhada mais leve e feliz.

Aos Funcionários,

Mário, Ricardo e Luizinho, agradeço a colaboração para o bom funcionamento do laboratório e da clínica.

À Faculdade de Odontologia de Araçatuba,

Obrigada pela oportunidade de oferecer todo o apoio para realizar o doutorado.

À Capes,

Obrigada pela bolsa de estudos.

À FAPESP,

Obrigada pelo auxílio financeiro (Processo 2012/19235-5).

Por vezes sentimos que aquilo que fazemos não é senão uma gota de água no mar. Mas o mar seria menor se lhe faltasse uma gota.

Madre Teresa de Calcutá

Aida, KL. **Efeito microbiológico e citotóxico de sistemas nanoestruturados bioadesivos contendo fragmentos de peptídeos**. 2017. 83f. Tese (Doutorado em Ciência Odontológica), Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista, Araçatuba, 2017.

Resumo Geral

O uso de agentes antimicrobianos naturais que reduzam a adesão e proliferação de S. *mutans* no biofilme poderia ser uma estratégia interessante para o controle da cárie dentária. No entanto, a estabilidade química e física de alguns desses agentes, como os peptídeos catiônicos antimicrobianos e fragmentos de peptídeos, pode ser comprometida por fatores externos, como temperatura e pH, reduzindo sua ação antimicrobiana. Com isso, os objetivos deste estudo foram desenvolver e caracterizar sistemas de liberação de fármaco nanoestruturados bioadesivos para a incorporação dos fragmentos peptídicos D1-23 e P1025 e avaliar seu efeito citotóxico e atividade contra biofilme de *S. mutans*. A primeira formulação (F1), composta de ácido oleico, polyoxypropylene-(5)-polyoxyethylene-(20)-cetyl alcohol (PPCA), Carbopol® 974P e Carbopol® 971P, foi analisada por microscopia de luz polarizada (MLP), reologia e bioadesão in vitro. A concentração inibitória mínima (CIM) e concentração bactericida mínima (CBM) de D1-23 foram determinadas contra *S. mutans* para posterior avaliação da atividade sobre biofilme formado após 4h e 24h de tratamento. A segunda formulação (F2) foi selecionada a partir de três diferentes concentrações de ácido oleico, PPCA e Carbopol® 974P. Cada formulação foi analisada por MLP, espalhamento de raios x a baixo ângulo (SAXS), reologia e bioadesão. CIM e CBM de P1025 sobre *S. mutans* e seu efeito quando incorporado ou não em F2 sobre biofilme de *S. mutans* em formação foram analisados. A citotoxicidade em células epiteliais HaCat foi avaliada para os dois sistemas líquido cristalino (SLC) usando testes de MTT. Análise descritiva foi realizada para os dados dos ensaios de caracterização e para os ensaios microbiológicos e citotóxicos os dados foram submetidos aos testes de ANOVA/Tukey ou Kruskall-Wallis/Mann-Whitney U ($p<0.05$). Os resultados indicaram que F1 apresentou características de SLC com alta viscosidade e bioadesão. CIM e CBM de D1-23 foram de 15,60 e 31,25µg/mL, respectivamente. D1-23 incorporado em F1 apresentou melhores resultados contra biofilme de S. *mutans* que quando em solução, após 24h de tratamento. F2 apresentou melhores propriedades reológicas e força bioadesiva comparada aos demais sistemas, caracterizando um SLC. P1025 teve somente efeito inibitório sobre *S. mutans* (CIM=0.25 mg/mL). O efeito antibiofilme de P1025 incorporado em F2 foi observado após 24h de tratamento, principalmente quando aplicado na fase de adesão. Ambos os SLC contendo D1-23 e P1025 não apresentaram toxicidade sobre as células epiteliais, nas condições de tempo e concentrações avaliadas. A incorporação de peptídeos em SLC bioadesivos

nanoestruturados aumenta suas propriedades antimicrobianas, podendo ser uma interessante estratégia para a prevenção da cárie dentária.

Palavras-chave: Peptídeos catiônicos antimicrobianos. Cárie dentária. *Streptococcus mutans*.

Aida, KL. Microbiological and citotoxicity effect of nanostructured bioadhesive system containing peptide fragments. 2017. 83f. Tese (Doutorado em Ciência Odontológica), Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista, Araçatuba, 2017.

General abstract

The use of natural antimicrobial agents for reducing the adhesion and proliferation of *S. mutans* in the biofilm could be an interesting strategy for the control of dental caries. However, the chemical and physical stability of some natural antimicrobials, such as cationic antimicrobial peptides and peptide fragments, can be compromised by external factors such as temperature and pH, reducing their antimicrobial action. Thus, the objectives of this study were to develop and characterize nanostructured bioadhesive drug delivery systems for the incorporation of D1-23 and P1025 peptide fragments and to evaluate their citotoxicy and activity against *S. mutans* biofilm. The first formulation (F1) was composed of oleic acid, polyoxypropylene- (5) -polyoxyethylene- (20) - cetyl alcohol (PPCA), Carbopol® 974P and Carbopol® 971P and analyzed by polarized light microscopy (PLM), rheology and in vitro bioadhesion. Minimum inhibitory concentration (MIC) and minimal bacterial concentration (MBC) of D1-23 were determined against *S. mutans* for further evaluation of activity against *S. mutans* biofilm after 4h and 24h of treatment. The second formulation was selected from three different concentrations of oleic acid, PPCA and Carbopol® 974P. Each formulation was analyzed by PLM, small-angle x-ray scattering (SAXS), rheology and bioadhesion. MIC and MBC of P1025 were determined against *S. mutans*. Thus, P1025 was incorporated in the best formulation (F2). The effect of P1025 incorporated or not into F2 on *S. mutans* biofilm formation was analyzed. Cytotoxicity in HaCat epithelial cells for both formulations was evaluated using MTT assays. Descriptive analysis was performed for the characterization assays and data from microbiological and cytotoxic assays were submitted to ANOVA / Tukey or Kruskall-Wallis / Mann-Whitney U ($p<0.05$). The results indicated that F1 presented characteristics of liquid-crystalline type system (LCS) with high viscosity and bioadhesion. The MIC and MBC of D1-23 were 15.60 and 31.25μg / mL, respectively. D1-23 incorporated in F1 showed better results than D1-23 in solution against *S. mutans* biofilm after 24h. F2 had better rheological properties and bioadhesive strength compared to other systems analyzed and characteristics of LCS. P1025 had only inhibitory effect against *S. mutans* (MIC=0.25mg/mL). The antibiofilm effect of P1025 incorporated into F2 was observed after 24h of treatment, mainly when applied in surface-bound salivary phase. Both LCS had no toxicity on epithelial cells, considering time and concentrations tested. The incorporation of peptides in nanostructured bioadhesive LCS increased their antimicrobial properties and could be an interesting strategy for caries prevention.

Keywords: Antimicrobial cationic peptides. Dental caries. *Streptococcus mutans.*

LISTA DE FIGURAS

CAPÍTULO 1

CAPÍTULO 2

LISTA DE TABELAS

CAPÍTULO 1

CAPÍTULO 2

LISTA DE ABREVIATURAS

°C – grau Celsius

µg/mL – micrograma por mililitro

µL – microlitro

µm - micrômetro

Å – angstrom

Ag I/II – antígeno I/II

AMP – peptídeo antimicrobiano

AO – ácido oleico

C971 - Carbopol® 971P

C974 - Carbopol® 974P

CAMP – peptídeo catiônico antimicrobiano

CFU/mL – unidades formadoras de colônias por mililitro

CH3COOH – ácido acético

CHX – diacetato de clorexidina

cm – centímetro

CO2 – dióxido de carbono

D1-23 – fragmento peptídico da β-defensina 3

F – formulação

g/mol – massa molar

G’ – módulo de armazenamento

G’’ – módulo de perda

h – hora

hBD - β-defensinas

HIV – vírus da imunodeficiência humana

HNP – peptídeo neutrofílico humano

HPLC – cromatografia líquida de alta eficiência

Hz – hertz

kDa – kilodalton

KH2PO4 – fosfato de potássio monobásico

L – litro

LCS – sistema líquido cristalino

MBC – concentração bactericida mínima

mg/mL – miligrama por mililitro

MIC – concentração inibitória mínima

min – minuto

mm – milímetro

mm/s – milímetro por segundo

mN – milinewton

MS – estreptococos mutans

Na2HPO4 – fosfato dissódico

NaCl – cloreto de potássio

nm – nanômetro

OD – densidade óptica

OVS – sistema viscoso opaco

p – nível de significância

P1025 – peptídeo análogo a antígeno I/II de S. *mutans*

Pa – pascal

pH – potencial hidrogeniônico

PLM – microscopia de luz polarizada

PPCA - polyoxypropylene-(5)-polyoxyethylene-(20)-cetyl alcohol

PPG-5-CETETH-20 - polyoxypropylene-(5)-polyoxyethylene-(20)-cetyl alcohol

PRO - polyoxypropylene-(5)-polyoxyethylene-(20)-cetyl alcohol

PS – separação de fases

rpm – rotação por minuto

s – segundo

S. *mutans* - *Streptococcus mutans*

SA I/II – antígeno I/II de S. *mutans*

SAXS – espalhamento de raios X a baixo ângulo

SLC – sistema líquido cristalino

SM – *streptoccus mutans*

TLS – sistema líquido transparente

TrLS – sistema líquido translúcido

TrVS – sistema viscoso translúcido

TTO – óleo de melaleuca

TVS – sistema viscoso transparente

W – água

μm – micrômetro

μmol/L – micromolar por litro

k – índice de consistência

γ – taxa de cisalhamento

η – índice de fluxo

τ – tensão de cisalhamento

SUMÁRIO

1 INTRODUÇÃO GERAL

A cárie dentária é a doença bucal mais comum no mundo, atingindo todas as faixas etárias. No Brasil, em crianças muito jovens é denominada de cárie precoce da infância e apresenta-se como um grave problema de saúde pública, com prevalência de 43% em crianças de cinco anos de idade (SBBRASIL, 2003). É conceituada atualmente como uma doença biofilme-dependente associada à ingestão frequente de carboidratos da dieta. A fermentação desses carboidratos pelos microrganismos do biofilme leva à produção de ácidos o que perturba a homeostase do biofilme e pode causar a dissolução dos tecidos minerais do dente (MARSH, 2009).

Streptococcus mutans (*S. mutans*) tem sido considerado o principal agente etiológico da cárie dentária, devido à sua capacidade de metabolizar uma ampla variedade de carboidratos e produzir grandes quantidades de ácidos, e ao mesmo tempo, tolerar altas concentrações de açúcares e ácidos (HAMADA & SLADE, 1980). *S. mutans* é uma bactéria Gram-positiva anaeróbia facultativa que participa de duas fases da formação do biofilme dental. Na fase de adesão, proteínas da superfície de *S. mutans* denominadas adesinas ou antígeno I/II (Ag I/II ou SA I/II) interagem com proteínas salivares adsorvidas na superfície do dente, permitindo a adesão bacteriana. Na fase de proliferação, *S. mutans* e outras bactérias, principalmente aquelas resistentes aos ácidos, acumulam-se por agregação com a mesma ou outras espécies e produzem uma matriz polissacarídica por degradação dos açúcares da dieta (SMITH, 2003). Embora o biofilme dental seja composto por múltiplas espécies bacterianas, agentes antimicrobianos que interfiram na adesão e proliferação de S. *mutans* poderiam ser utilizados como estratégias para auxiliar na prevenção da cárie dentária.

Substâncias naturais com propriedades antimicrobianas têm sido estudadas como agentes tópicos para aplicação na cavidade bucal, a fim de reduzir patógenos com menor risco de desenvolver resistência bacteriana (MARSH, 2012). Os peptídeos catiônicos antimicrobianos são sintetizados pelas mucosas e apresentam a função de barreira física contra a entrada de organismos estranhos. Entre esses peptídeos, estão as β-defensinas que foram introduzidas como futuros agentes antimicrobianos devido ao seu rápido início de ação e atividade de amplo espectro contra bactérias Gram-positivas, Gram-negativas, fungos e vírus, aliados a níveis potencialmente baixos de resistência induzida (GORR & AGDOLHOSSEINI, 2011; MCCORMICK & WEINBERG, 2010; WIESNER & VILCINSKAS, 2010).

As defensinas são peptídeos pequenos, de 15 a 45 aminoácidos, que dependendo do padrão de pareamento de seus resíduos de cisteína, são subdivididas em duas principais

subfamílias: α e β-defensinas. Foram identificadas seis α-defensinas em humanos, sendo que quatro são produzidas pelos neutrófilos e denominadas de peptídeo neutrofílico humano (HNP-1 a 4) e as outras duas são produzidas por células de Paneth nas criptas intestinais. As β-defensinas (hBDs) são produzidas por células epiteliais de diversos órgãos como olhos, pele, pulmão, rim, pâncreas, mucosa nasal e oral e embora tenham sido encontradas quase 40 regiões gênicas potenciais para hBDs, as mais bem caracterizadas são hBD 1 a 4. As α e β-defensinas apresentam função imunomoduladora, modificando a migração e maturação celular, induzindo citocinas e a liberação de histamina e prostaglandina A2 de mastócitos. (McCORMICK & WEINBERG, 2010; WIESNER & VILCINSKAS, 2010; ABIKO, NISHIMURA & KAKU, 2003).

A hBD-3 mostrou ação contra importantes colonizadores primários e secundários de biofilme relacionados à cárie dentária, como *Streptococcus mutans* e *Lactobacillus casei*, com doses inibitórias mínimas variando de 1,25 a 200 µg/mL (OUHARA et al., 2005). Contudo, a grande sequência de aminoácidos (45 resíduos) e a presença de ligações de cisteína na estrutura tornam a síntese de hBD-3 muito onerosa. Adicionalmente, os peptídeos em sua forma nativa tendem a ser facilmente degradados por fatores externos, tais como temperatura e pH, e apresentarem uma redução na sua atividade na presença de sal, soro e proteinases (GORDON, ROMANOWSKI & MCDEROTT, 2005). Por conseguinte, modificações na sequência ou estrutura de peptídeos naturais poderiam gerar um novo peptídeo sintético ou fragmento de peptídeo que teria a mesma ou superior ação de largo espectro contra agentes patogênicos bacterianos, baixa toxicidade para o hospedeiro e um tamanho molecular mínimo para produção de baixo custo (ZHANG & FALLA, 2009). Recentemente, fragmentos ou análogos de peptídeos foram sintetizados e suas funções imunológicas e microbiológicas estudadas. Reynolds et al. (2010) avaliaram a atividade bactericida de fragmentos peptídicos de um derivado de β-defensina 3 e descobriram que a metade N-terminal de 23 aminoácidos (D1-23) de Defb14-1Cv (ortólogo de rato da β-defensina 3 humana) é um potente agente antimicrobiano. Kreling et al. (2016) verificaram que D1-23 apresentou baixa toxicidade sobre células epiteliais e melhor atividade contra *S. mutans*, em condições planctônicas e em biofilme, quando comparado a outros fragmentos de peptídeos.

Ensaios *in vitro* avaliando a capacidade de adesão de *S. mutans* à superfície dentária identificaram que a aglutinina salivar presente na saliva atua como receptor do antígeno I/II (Ag I/II) de *S. mutans*, e, portanto, medeia a ligação entre esse antígeno e a película adquirida (LEE et al., 1989; CARLÉN, 1995; YOUNSON & KELLY, 2004). Evidências de que Ag I/II facilita a adesão bacteriana têm sido obtidas por estudos com linhagens de *S. mutans*

deficientes desse antígeno, que demonstraram diminuição na adesão sobre a superfície de hidroxiapatita. Este mediador compreende a glicoproteína 440 kDa e componentes de baixo peso molecular (YOUNSON et al., 2004). Agregados entre a glicoproteína 440 kDa de *S. mutans* também podem exercer papel na defesa humana, uma vez que podem ser eliminados, por exemplo, pela deglutição. Por outro lado, em condições favoráveis de crescimento, esses agregados podem aderir às superfícies dos dentes, iniciando a formação do biofilme dental.

Estudo realizado por Kelly et al. (1999) identificaram os resíduos 1025-1044 na região C-terminal de Ag I/II como o epítopo de adesão desse antígeno. Nesse estudo, foi demonstrado que o peptídeo sintético (P1025) correspondente a esses resíduos pode inibir a ligação *in vitro* entre a adesina de *S. mutans* e a aglutinina salivar. Em estudos *in vivo*, observou-se que o peptídeo P1025 reduziu também a recolonização de *S. mutans* (KELLY *et* al., 1999). Outro estudo realizado por Li et al. (2009) demonstraram, in vitro, que dentifrício contendo peptídeo P1025 diminuiu a aderência de *S. mutans* à superfície de hidroxiapatita coberta por saliva. Adicionalmente, um estudo clínico comprovou que o efeito do dentifrício contendo o peptídeo diminuiu o índice de biofilme dental após 1 mês de tratamento.

Embora a etiologia da doença cárie seja multifatorial, a maioria dos seus fatores causadores é controlável, como a dieta, hábitos de higiene bucal, entre outros. Entretanto, a susceptibilidade do hospedeiro ao desenvolvimento da doença, representada pelas respostas imaturas ou deficientes do sistema imune, considerando principalmente as crianças, poderia ser contornada utilizando-se peptídeos antimicrobianos como medida terapêutica direta para a redução da microbiota cariogênica, minimizando a possibilidade de resistência bacteriana, por seu caráter natural ou ainda como medida indireta, modulando a resposta imunológica, que favoreceria o melhor desempenho do organismo contra os patógenos. Apesar de todas as vantagens que os peptídeos antimicrobianos demonstram, existem algumas limitações para o seu uso terapêutico. Uma limitação da maioria dos peptídeos, em condições não fisiológicas, é que sua atividade antimicrobiana é significantemente reduzida em fluidos biológicos como o plasma, saliva ou soro. Assim, altas concentrações seriam necessárias para manter sua ação, o que aumentaria sua toxicidade. Aliando essa desvantagem ao fato dos peptídeos apresentarem uma rápida excreção por via renal, é difícil ou impossível administrá-los rotineiramente por via oral ou parenteral. (BATONI et al., 2011).

A eficácia dos agentes antimicrobianos no tratamento de infecções orais não está unicamente relacionada com a atividade antimicrobiana *in vitro*. Para ser adequadamente eficaz, é necessário conseguir um suprimento adequado do fármaco por um período prolongado. No entanto, as formulações tópicas normalmente utilizadas (enxaguatórios,

pulverizações, géis e pastilhas) são frequentemente caracterizadas por um tempo de retenção de fármaco limitado na cavidade bucal e tendem a ser rapidamente deslocadas, diluídas ou removidas, o que pode alterar a eficácia do medicamento. Dessa maneira, são necessárias numerosas e repetidas administrações dos medicamentos para atingir e manter níveis eficazes do fármaco (GAJDZIOK et al., 2010; LOFTSSON et al., 1999).

Os lipossomas, os cristais líquidos, as microemulsões e as nanopartículas representam uma estratégia interessante para a administração local de peptídeos (na cavidade bucal), porque estes sistemas podem promover liberação controlada de fármacos, protegerem os princípios ativos da degradação térmica ou fotodegradação, além de aumentarem a eficácia dos fármacos (GUTERRES, ALVES & POHLMANN, 2007; KANG, CHO & YOO, 2009). O sistema de cristais líquidos ou líquido-cristalinos (SLC) tem recebido considerável atenção devido ao seu excelente potencial como veículos de fármacos. Entre estes sistemas, as mesofases reversas cúbicas e hexagonais são as mais importantes e têm sido amplamente investigadas quanto à sua capacidade de manter a liberação de uma gama de bioativos a partir de fármacos de baixo peso molecular para proteínas, peptídeos e ácidos nucleicos (GUO et al., 2010).

Os SLC apresentam propriedades tanto de sólidos quanto de líquidos. Possuem ordem estrutural, rigidez e ligações definidas como os sólidos e mobilidade, regiões desordenadas e fluidas como os líquidos (CHORILLI, et al., 2009). Em se tratando da aplicação bucal de peptídeos, uma estratégia interessante a ser adotada é a utilização de polímeros mucoadesivos nas formulações. O termo mucoadesivo é comumente usado para substâncias que se ligam à camada de mucina das membranas biológicas, sendo essenciais para permanência íntima e prolongada da formulação no local de ação (PATEL, LIU & BROWN, 2011). Os polímeros mucoadesivos podem ser empregados objetivando manter uma alta concentração do peptídeo no local de ação por um longo período, além de protegê-lo da degradação ambiental, pelo fato de terem grande afinidade pela mucosa bucal (VEUILLEZ et al., 2005). Também, eles podem competir com enzimas proteolíticas, o que é muito importante para fármacos propensos à degradação enzimática, como fármacos peptídicos (SALAMAT-MILLER, CHITTCHANG & JOHNSTON, 2005). Na literatura, poucos são os trabalhos que avaliaram os sistemas de liberação controlada contendo fragmentos de peptídeos visando à prevenção da cárie dentária (BERNEGOSSI et al., 2015). Calixto et al. (2016) avaliaram um sistema líquido cristalino desenvolvido a partir de polyoxypropylene-(5)-polyoxyethylene-(20)-cetyl alcohol (PPCA), óleo de melaleuca (tea tree oil – TTO) e policarbophil contendo P1025 e observaram boas propriedades reológicas e capacidade mucoadesiva, porém a ação antimicrobiana de P1025

contida no sistema não foi avaliada. Assim, os objetivos deste estudo foram desenvolver e caracterizar sistemas de liberação nanoestruturados para a incorporação dos fragmentos peptídicos D1-23 ou P1025 e avaliar sua atividade contra biofilme de *S. mutans* e citotoxicidade.

REFERÊNCIAS

1. Abiko Y, Nishimura M, Kaku T. Defensins in saliva and the salivary glands. Med Electron Micros. 2003;36(4);247-252.
2. Batoni G, Maisetta G, Brancatisano FL, Esin S, Campa M. Use of antimicrobial peptides against microbial biofilms: advantages and limits. Curr Med Chem. 2011;18(2):256-279.
3. Benergossi J, Calixto G, Fonseca-Santos B, Aida KL, de Cássia Negrini T, Duque C, Gremião MP, Chorilli M.Highlights in peptide nanoparticle carriers intended to oral diseases. Curr Top Med Chem. 2015;15(4):345-355.
4. Calixto GM, Garcia MH, Cilli EM, Chiavacci LA, Chorilli M.Design and Characterization of a Novel P1025 Peptide-Loaded Liquid Crystalline System for the Treatment of Dental Caries.Molecules. 2016 Jan 28;21(2):158.
5. Carlén A, Olsson J. Monoclonal antibodies against a high-molecular-weight agglutinin block adherence to experimental pellicles on hydroxyapatite and aggregation of *Streptococcus mutans*, J Dent Res. 1995;74(4):1040-1047.
6. Chorilli M, Prestes PS, Rigon RB, Leonardi GR, Chiavacci LA, Scarpa MV. Development of liquid-crystalline systems using silicon glycol copolymer and polyether functional siloxane. Quím Nova. 2009;32(4):1036-1040.
7. Filoche S, Wong L, Sissons CH. Oral Biofilm: emerging concepts in microbiol ecology. J Dent Res. 2010;89(1):8-18.
8. Gajdziok J, Bajerová M, Chalupová Z, Rabisková M. Oxycellulose as mucoadhesive polymer in buccal tablets. Drug Dev Ind Pharm. 2010;36(9):1115–1130.
9. Gordon YJ, Romanowski EG, McDermott AM. A review of antimicrobial peptides and their therapeutic potential as anti-infective drugs. Cur Eye Res. 2005;30(7):505-515.
10. Gorr, SU, Abdolhosseini, M. Antimicrobial peptides and periodontal disease. J Clin Periodontol. 2011;38:126-141.
11. Guo C, Wang J, Cao F, Lee RJ, Zhai G. Lyotropic liquid crystal systems in drug delivery. Drug Discov Today. 2010;15(23-24):1032-1040.
12. Guterres SS, Alves MP, Pohlmann AR. Polymeric nanoparticles, nanospheres and nanocapsules, for cutaneous application. Drug Target Insights 2007;2:147-157.

13. Hamada S, Slade HD. Biology immunology, and cariogenicity of *Streptococcus mutans.* Microbiol Rev. 1980;44(2): 331-384.
14. Kang ML, Cho CS, Yoo HS. Application of chitosan microspheres for nasal delivery of vaccines. Biotechnol Adv. 2009;27(6):857–886.
15. Kelly CG, Younson JS, Hikmat BY, Todryk SM, Czisch M, Haris PI, Flindall IR, Newby C, Mallet AI, Ma JK, Lehner T. A synthetic peptide adhesion epitope as a novel antimicrobial agent. Nat Biotechnol. 1999;17(1):42-47.
16. Kreling PF, Aida KL, Massunari L, Caiaffa KS, Percinoto C, Bedran TB, Spolidorio DM, Abuna GF, Cilli EM, Duque C. Cytotoxicity and the effect of cationic peptide fragments against cariogenic bacteria under planktonic and biofilm conditions. Biofouling. 2016;32(9):995-1006.
17. Lee SF, Progulske-Fox A, Erdos GW, Piacentini DA, Ayakawa GY, Crowley PJ, Bleiweis AS. Construction and characterization of isogenic mutants of *Streptococcus mutans* deficient in major surface protein antigen P1 (I/Il). Infec Immun. 1989;57(11):3306-3313.
18. Li MY, Wang J, Lai GY. Effect of a dentifrice containing the peptide of streptococcal antigen I/II on the adherence of mutans streptococcus. Arch Oral Biol. 2009;54(11):1068-1073.
19. Loftsson T, Leeves N, Bjornsdottir B, Duffy L, Masson M. Effect of cyclodextrins and polymers on triclosan availability and sub- stantivity in toothpastes in vivo. J Pharm Sci. 1999;88(12):1254–1258.
20. Marsh PD. Dental plaque as a biofilm: the significance of pH in health and caries. Compend Contin Educ Dent. 2009;30 (2): 76-8, 80, 83-7; quiz 88, 90.
21. Marsh, PD. Contemporary perspective on plaque control. Br Dent J. 2012;212(12): 601-606.
22. McCormick TS, Weinberg A. Epithelial cell-derived antimicrobial peptides are multifunctional agents that bridge innate and adaptive immunity. Periodontol 2000. 2010;54(1):195–206.
23. Ouhara K, Komatsuzawa H, Yamada S, Shiba H, Fujiwara T, Ohara M, Sayama K, Hashimoto K, Kurihara H, Sugai M. Susceptibilities of periodontopathogenic and cariogenic bacteria to antibacterial peptides. {beta}-defensins and LL37, produced by human epithelial cells. J Antimicrob Chemother. 2005;55(6):888-896.
24. Patel VF, Liu F, Brown MB. Advances in oral transmucosal drug delivery. J Control Release. 2011;153(2):106–116.

25. Reynolds NL, De Cecco M, Taylor K, Stanton C, Kilanowski F, Kalapothakis J, Seo E, Uhrin D, Campopiano D, Govan J, Macmillan D, Barran P, Dorin JR. Peptide fragments of a beta-defensin derivative with potent bactericidal activity. Antimicrob Agents Chemother. 2010;54(5):1922-9.
26. Salamat-Miller N, Chittchang M, Johnston TP. The use of mucoadhesive polymers in buccal drug delivery. Adv Drug Deliv Rev. 2005;57(11):1666– 1691.
27. SBBRASIL. Ministério da Saúde. Departamento de Atenção Básica. Projeto SB Brasil 2003: condições de saúde bucal da população brasileira 2002-2003: resultados principais. Brasília: Ministério da Saúde, abr. 2010. http://bvsms.saude.gov.br/bvs/publicacoes/projeto_sb2004.pdf. Acesso: 16/01/2016.
28. Smith DJ. Caries vaccines for the twenty-first century. J Dent Educ. 2003;67(10): 1130-1139.
29. Veuillez F, Kalia YN, Jacques Y, Deshusse J, Buri P. Factors and strategies for improving buccal absorption of peptides. Eur J Pharmac and Biopharm. 2001;51(2):93-109.
30. Wiesner J, Vilcinskas A. Antimicrobial peptides – the ancient arm of the human immune system. Virulence. 2010;1(5):440-464.
31. Younson J, Kelly C. The rational design of an anti-caries peptide against *Streptococcus mutans*. Mol Divers. 2004; 8(2):121–126.
32. Zhang L, Falla TJ. Host defense peptides for use as potential therapeytics. Cur Opin Investig Drugs. 2009;10(2):164-171.

2 CAPÍTULO 1 - Antimicrobial Peptide-loaded Liquid Crystalline Precursor Bioadhesive System for the Prevention of Dental Caries*

ABSTRACT

Objective: Anti-caries agents must interfere with *Streptococcus mutans* (SM) adhesion and proliferation in dental biofilm, without causing host toxicity and bacterial resistance. Natural substances, including cationic antimicrobial peptides (CAMP) and their fragments, such as β-defensin 3 peptide fragment (D1-23), have been widely studied. However, the chemical and physical stability of CAMP may be compromised by external factors, such as temperature and pH, reducing the period of antimicrobial activity. In order to overcome this disadvantage, this study developed and characterized a drug delivery system and evaluated the cytotoxicity and SM anti-biofilm effect of a D1-23-loaded liquid crystalline bioadhesive system (LCS).

Design: LCS was composed by oleic acid, polyoxypropylene-(5)-polyoxyethylene-(20)-cetyl alcohol, Carbopol® 974P and Carbopol® 971P. LCS was analyzed by polarized light microscopy (PLM), rheology (viscoelasticity and flow properties) and in vitro bioadhesion. Epithelial cells viability was evaluated. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) against SM was determined to D1-23 for further evaluation of the effect SM anti-biofilm after 4 h and 24 h of exposure to treatment.

Results: PLM, rheology and *in vitro* bioadhesion tests showed that LCS diluted with artificial saliva became a liquid crystalline system with high viscosity and bioadhesion values. D1-23-loaded LCS system presented better antimicrobial activity when compared to D1-23 in solution against SM biofilm after 24 h. Finally, LCS containing or not D1-23 system did not present toxicity on human epithelial cell line.

Conclusions: D1-23-loaded LCS is a promising drug delivery system for the prevention of dental caries.

Keywords: antimicrobial peptides, drug delivery system, dental caries, biofilm, cytotoxicity.

Hightlights

- LCS is a promising drug delivery system for D1-23 to prevent dental caries
- D1-23-loaded LCS is effective against SM biofilm after 24h of treament
- D1-23-loaded LCS is non-toxic on human epithelial cell line

* According to guidelines for authors of Archives of Oral Biology (Anexo C).

1 INTRODUCTION

Dental caries is the most common oral disease worldwide. The development of this disease depends on the bacterial colonization of the tooth surface and biofilm formation (Fejerskov, 1997). *Streptococcus mutans* has been considered as the primary etiological agent of dental caries, due its ability to metabolize a wide variety of carbohydrates and produce large amounts of acid, while also tolerating extreme concentrations of sugars and acids (Hamada & Slade, 1980). *S. mutans* is a versatile bacterium, participating in the two phases of biofilm formation. In the first phase, bacterial surface proteins, such as adhesins, interact with salivary proteins adsorbed on the tooth surface. In the second phase, *S. mutans,* and other bacteria that are resistant to acid, accumulate by aggregation with the same and other species and produce a polysaccharide matrix by degradation of dietary sugars (Smith, 2003). Although dental biofilm is composed by multiple bacterial species, antimicrobial agents, which could interfere with S. *mutans* adhesion and proliferation in dental biofilm, would be useful for controlling dental caries.

Natural substances with antimicrobial properties have been studied as topical agents for the oral cavity, in order to reduce pathogens without causing bacterial resistance (Marsh, 2012). Cationic antimicrobial peptides (CAMP), such as β-defensins, have been introduced as future antimicrobial agents due to their rapid onset killing and broad-spectrum activity against Gram-positive and Gram-negative bacteria, fungi and viruses, allied with potentially low levels of induced resistance (Gorr & Agdolhosseini, 2011).

β-defensins (hBDs) are produced by epithelial cells in various organs, such as: eyes, skin, lung, kidney, pancreas, and nasal and oral mucosa. In addition to their antimicrobial activity, β-defensins have an immunomodulatory function by modifying cell migration and maturation, inducing cytokines, releasing histamine and prostaglandin A2 (Abiko, Nishimura, & Kaku, 2003; McCormick & Weinberg, 2010; Wiesner & Vilcinskas, 2010). hBD-3 has shown action against important primary and secondary colonizers of caries-related biofilm, such as *Streptococcus mutans* and *Lactobacillus casei*, with minimal inhibitory doses ranging from 1.25 to 200 μg/mL (Ouhara et al., 2005). However, the large amino acid sequence (45 residues) and the presence of cysteine linkages on the structure make the synthesis of hBD-3 very expensive. Additionally, native peptides tend to be easily degraded by external factors, such as temperature and pH, with a reduced activity when in the presence of salt, serum and proteinases (Gordon, Romanowski, & McDerott, 2005). Therefore, modifications on the sequence or structure of natural peptides could generate a new synthetic CAMP that would

have the same or superior broad-spectrum action against bacterial pathogens, low toxicity to the host, and a minimal molecular size for low cost production (Zhang & Falla, 2009).

Recently, fragments or analogues of CAMP have been synthesized and their immunological and microbiological functions studied. Reynolds et al. (2010) evaluated the bactericidal activity of peptide fragments of a β-defensin 3 derivative and found that the 23-amino acid N-terminal half (D1-23) of Defb14-1C^{v} (mouse orthologue of human β-defensin 3) is a potent antimicrobial, while the C-terminal half is not. In order to maintain chemical and physical stability and provide controlled release, drug delivery systems could be an attractive strategy for the administration of peptides (Bhardwaj & Kumar, 2012).

Several studies have evaluated the incorporation of peptides in drug delivery systems in the treatment of cardiovascular diseases (Lestini et al., 2002), AIDS (Avachat & Parpani, 2015) and diabetes (Okawara, Hashimoto, Todo, Sugibayashi & Tokudome, 2014). Liquid crystalline systems (LCS) are a drug delivery system that can be used for incorporating peptides (Bernegossi et al., 2015; Calixto, Garcia, Cilli, Chiavacci, & Chorilli, 2016). LCS can promote controlled release of drugs; protect active ingredients from thermal degradation and photodegradation, while improving the effectiveness of these peptides (Guterres, Alves, & Pohlmann, 2007). The bioadhesive property of these systems can maintain a high concentration of the peptide at the site of action for a long period, while also protecting it from environmental degradation. Therefore, the objective of this study was to characterize a liquid crystalline precursor bioadhesive system and evaluate the cytotoxicity and effect against *S. mutans* biofilm when incorporating D1-23 (β-defensin 3 peptide fragment) in LCS.

2 MATERIALS AND METHODS

2.1 Preparation of the Liquid Crystalline Precursor Bioadhesive System (LCS or F)

Firstly, the binary polymeric dispersion were prepared dispersing 2.5% (w/w) Carbopol® 974P (C974) and 2.5% (w/w) Carbopol® 971P (C971) in water and homogenized at 2000 rpm in a mechanical stirrer for approximately 10 min at room temperature (25°C ± 0.5°C). Following complete dissolution, the pH of the binary polymeric dispersion was adjusted to 6.0 with triethanolamine and manual agitation (Marques, Loebenberg, & Almukainzi, 2011).

Then, LCS (or F from formulation) was prepared weighing and mixing 40% (w/w) polyoxypropylene-(5)-polyoxyethylene-(20)-cetyl alcohol as the surfactant, 50% (w/w) oleic acid as the oil phase, and 10% (w/w) binary polymeric dispersion containing 2.5% C974 and

2.5% C971 as the aqueous phase at room temperature (25°C ± 0.5°C). Thus, the final polymeric concentration in the F is 0.25% (w/w) for each polymer.

In order to evaluate the in situ lyotropic behavior, F was diluted with artificial saliva in a 1:10 (w/w) ratio to generate F10, a 1:30 (w/w) ratio to generate F30, a 1:50 ratio to generate F50 and a 1:100 ratio to generate F100. A volume of 1 L of artificial saliva was prepared by mixing 8.0 g of NaCl, 0.19 g of KH2PO4, and 2.38 g Na2HPO4. After complete mixing, the pH was measured as 6.8 (Marques, Loebenberg, & Almukainzi, 2011).

2.2 Polarized Light Microscopy (PLM)

A small amount of F, F10, F30, F50 and F100 was applied on a glass slide and covered with a coverslip. The samples were analyzed using a polarized light microscope (Jenamed, Carl Zeiss, Oberkochen, Germany), evaluating the homogeneity of the dispersion and observing the presence of anisotropy or isotropy at a magnification of 20x and at room temperature (Salmazi et al., 2015).

2.3 *Rheological Analysis*

The rheological measurements were performed at 37 ± 0.1^{o}C in triplicate using a controlled-stress AR2000 rheometer (TA Instruments, New Castle, DE, USA) with parallel plate geometry (40 mm diameter) and a sample gap of 200 μm for F100 and with cone plate geometry (40mm diameter) and sample gap of 52 μm for F. The samples of the formulations were carefully applied to the lower plate to minimize sample shearing and were allowed to equilibrate for 3 min prior to analysis (Calixto, Yoshii, Rocha, Stringhetti Ferreira Cury, & Chorilli, 2015).

2.3.1 Determination of Flow Properties

The flow properties were determined using a controlled shear rate procedure ranging from 0.01 to 100 s−1 and back. Each stage lasted for 120 s with an interval of 10 s between the curves. The consistency and flow indexes were determined from the power law described in (1) for a quantitative analysis of flow behavior:

$$\tau = k \cdot \gamma^{\eta}, \quad (1)$$

where "τ" is the shear stress, "γ" is the shear rate, "k" is the consistency index, and "η" is the flow index (Calixto, Yoshii, Rocha, Stringhetti Ferreira Cury, & Chorilli, 2015).

2.3.2 Oscillatory Analyses

The oscillatory analyses were started by the conduction of a stress sweep in order to determine the viscoelastic region of the formulations. The stress sweep was carried out at a constant frequency of 1Hz over a stress range of 0.1–10 Pa. A constant shear stress of 1 Pa was selected to perform the frequency sweep over a range of 0.1–10 Hz, which was within the previously determined linear viscoelastic region for all formulations. Thus, the storage (G') and loss (G") module were recorded (Calixto, Yoshii, Rocha, Stringhetti Ferreira Cury, & Chorilli, 2015).

2.4 In Vitro Bioadhesion Strength

2.4.1 Preparation of Discs

Sound bovine permanent central incisors (Ethics Committee on the Use of Animals – FOA/UNESP, Protocol 2014/00618) were collected and scaled to remove periodontal tissue and other debris. Teeth with enamel cracks, hypoplasia, and calculus in the middle third of the crown or other morphological alterations were excluded. Enamel blocks were cut transversally from the middle third of the buccal surface of each tooth using a water-cooled, double-faced diamond disc (KG Sorensen, Barueri, SP, Brazil). The specimens were then rounded using a high-speed, water-cooled cylindrical diamond bur (1095; KG Sorensen) to obtain specimens with a diameter of 1 cm containing enamel. Teeth surfaces were polished with wet 200-grit silicon carbide paper (T469-SF- Norton; Saint-Gobain Abrasivos Ltda., Jundiaí, SP, Brazil) to normalize the surface (Soares et al., 2011).

2.4.2 Bioadhesion Measurement (tensile strength method)

A TA-XTplus texture analyzer (Stable Micro Systems, Surrey, UK) was used for the tensile strength measurements. The teeth model was fastened to the upper movable probe with double side tape, and the formulation sample was located on the lower platform. Before the test, the tooth was immersed in artificial saliva. The upper probe was lowered until it made contact with the sample and it was kept in contact without any force applied for 60 s. The probe was then raised at 0.5 mm/s, and the force needed for detachment was registered. The tensile work (g.s), which is proportional to the area under the force–time curve, was used to describe the bioadhesive characteristics. Seven replicates were analyzed at 37 ± 0.5°C (Calixto, Yoshii, Rocha, Stringhetti Ferreira Cury, & Chorilli, 2015).

2.5 Preparation of Peptide

D1-23 - Defb14-1C^{V} (D1-23) (FLPKTLRKFFARIRGGRAAVLNA) (Reynolds et al., 2010) (MW: 2603,88) were synthesized at the Institute of Chemistry - UNESP (Araraquara, SP, Brazil). Solid phase peptide synthesis was performed manually using Fmoc (9-fluorenylmethyloxycarbonyl) protocols on the Rink-amide resin (Merrifield, 1963; Kaiser, Colescott, Bossinger, & Cook, 1970). The molecular masses of the peptides were determined using mass spectrometry, with a positive ion-mode electrospray ionization apparatus (Bruker, Germany) in agreement with corresponding calculated values (Reynolds et al., 2010). Purification of synthesized peptides was performed in a semi-preparative HPLC Beckman System Gold on a reverse phase C18 column (2.1 × 25 cm). Final purity levels of peptides were determined at least 95% on a Shimadzu chromatography equipped with an analytical C18 reverse phase column (0.46 × 25 cm) (Crusca et al., 2011). The positive control group was chlorhexidine diacetate (CHX), purchased from Sigma Aldrich (St. Louis, MO, USA).

2.6 Incorporation of Agents into LCS

Peptide fragment D1-23 and CHX were incorporated into the aqueous phase of formulation (F) at the concentration of 1 mg/mL based on a previous study showing that about 4% of peptide is released from LCS (Hu, Hong, & Yuan et al., 2004). Then, the groups of the study were D1-23 and CHX solutions, LCS (F), LCS containing D1-23 (F+D1-23) and LCS containing CHX (F+CHX). LCS was diluted tenfold before incorporating CHX and D1-23 to reduce the viscosity for biofilm assays (Gawande, Leung & Madhyastha, 2014).

2.7 Microbiological Evaluation

2.7.1 Determination of MIC and MBC

MIC (minimal inhibitory concentration) and MBC (minimal bactericidal concentration) of D1-23 and CHX solutions were determined using the microdilution broth method, in 96-well microtiter plates, following the criteria previously described by the National Committee for Clinical Laboratory Standards (NCCLS) (2002b) for bacteria and according to Mor, Hani, & Nicolas (1994) with some modifications. S. *mutans* (UA 159) cell suspensions at the mid-log phase (OD_{550}=0.5) were harvested by centrifugation (Hanil Combi centrifuge, 514R) for 10 min, at 3000x g, the supernatant was discarded and the pellet re-suspended in 2x concentrated Mueller-Hinton broth (Difco Laboratories) and diluted 1000x in Mueller-Hinton broth. The final concentration of S. *mutans* suspension in the wells was 5-10x10^{5}CFU/mL. Initially, the antimicrobial solution were serially diluted in sterile distilled

water in order to obtain concentrations ranging from 0.006 to 2 mg/mL and after 50 µL microbial suspensions (correctly adjusted for the concentrations described above) were inoculated in each well. The plates were incubated at 37°C for 24 h in a 5% CO_2 atmosphere. Afterwards, 15 µl of 0.01% resazurin (Sigma-Aldrich) was applied in each well and incubated for 4 h to promote an oxidation-reduction reaction and to determine cell viability by visually detecting any color change. The wells corresponding to MIC (the last blue well) and at least three previous wells were then homogenized, serially diluted and plated on Mueller-Hinton agar to determine the MBC. The plates were incubated at 37°C for 24 h in a 5% CO_2 atmosphere. The colonies were counted and the number of viable bacteria was determined in CFU/mL. The MBC was considered when the peptide fragments killed (99%) the tested microbial culture. Chlorhexidine diacetate (CHX) was used as a positive control. The negative control was the bacterial cultures without antimicrobial agents in Mueller-Hinton broth. All experiments were performed in duplicate.

2.7.2 Biomass Biofilm Assays

Briefly, 200 µL of 5-10x10^5 cells of *S. mutans* (UA 159) in Brain Heart Infusion (Difco Laboratories) added sucrose 1%, were distributed per well in polystyrene U-bottom cell culture plates (TPP, Switzerland) in triplicate and incubated at 37°C for 48 h. After incubation, the wells were washed by immersion in 0,9% saline. Then, 150 µL of each treatment (at 1 mg/mL): D1-23 and CHX solutions, LCS (F), LCS containing D1-23 (F+D1-23) and LCS containing CHX (F+CHX) were added and incubated for 4 h and 24 h at 37°C and 5% CO2. Negative controls were LCS without antimicrobial agents (F) and 0.9% NaCl solution. After incubation, the plates were washed by immersion in distilled water three times to remove non-adhered cells. After a brief drying, 150 µL of aqueous 1% crystalline violet was added to each well, and the plates were incubated at room temperature for 30 min. Next the crystalline violet solution was removed and the plates washed again 3 times. The plates were inverted on paper towels and remained for 2 h at room temperature to dry. The crystalline violet dye (stained biofilm) was then solubilized by incubation with 200 µL of ethanol per well for 30 min. Then, 100 µL of the dye in ethanol was transferred to wells of a new microplate submitted to reading absorbance at 500 nm in a spectrophotometer reader (Eon Microplate, BioTek Instruments, USA) to quantify the biomass of the biofilm (Mattos-Graner, Napimoga, Fukushima, Duncan, & Smith, 2004).

2.8 Cytotoxicity Evaluation

These assays were conducted based on the methodology described by Bedran, Mayer, Spolidorio, & Grenier (2014) in duplicate. Skin epidermal cells from the HaCat lineage were cultured in Dulbecco's modified Eagle's medium – DMEM (Gibco BRL, Carlsbad, CA, USA) plus 10% fetal calf serum and 100 µg/mL of penicillin G/streptomycin. The cell lineage was grown until reaching a subconfluent density at 37^{o}C in 5% CO2 atmosphere. The epithelial cells were harvested following a trypsin treatment (5 min) (TrypLETM Express; Life Technologies Inc.) at 37°C. Proteases were then inactivated by adding 0.3 mg/mL of trypsin inhibitor, and the cells were harvested by centrifugation (500xg for 5 min), suspended in fresh medium, seeded in a 96-well microplate (200 µL/well, 1x10^{6} cells/mL) and incubated overnight at 37°C in a 5% CO2 atmosphere to allow for cell adhesion before stimulation. The cells were then stimulated with D1-23 and CHX solutions at concentrations of 0.001 to 1 mg/mL and 10 µL of LCS containing or not containing D1-23 and CHX at 1 mg/mL (F, F+D1-23, F+CHX) for 24 h at 37°C in a 5% CO2 atmosphere. A colorimetric MTT cell viability assay (Roche Diagnostics, Mannheim, Germany), using 3-[4,5-diethylthiazol- 2-yl]-2,5-diphenyltetrazolium bromide as the substrate, was used to determine the effect of cationic peptide fragments and the controls on cell viability. After exposure of agents, the extracts were aspirated and replaced by 90 µL of DMEM plus 10 µL of MTT solution (5 mg/mL sterile PBS; Sigma Aldrich). After that, the culture medium with the MTT solution was aspirated and replaced with 100 µL of acidified isopropanol solution (0.04 NHCl). Two 50 µL aliquots of each well were transferred to 96-well plates (Costar Corp., Cambridge, MA, USA). Cell viability was evaluated using spectrophotometry as being proportional to the absorbance measured with a 570 nm wavelength and an ELISA microplate reader (model 3550-UV, Bio-Rad Laboratories, Hercules, CA, USA). The means were calculated for the groups and transformed into percentages, which represented the inhibitory effect of the mitochondrial activity of the cells by the extracts. The negative control (DMEM) was defined as having 100% cell metabolism.

2.9 Statistical Analyses

The characterization of the liquid crystalline system was presented using descriptive analysis. For the anti-biofilm assay, the mean absorbance values obtained from the control group (bacterial growth without antimicrobial agents) were calculated and considered as 100% of cell viability and the percentage values of biofilm biomass reduction for the tested

groups was calculated based in this criteria. Kruskal-Wallis and Mann-Whitney tests were applied to compare the percentage of biofilm biomass reduction for the groups, at different times. Data from cytotoxicity were submitted to ANOVA and pos-hoc Tukey tests in order to compare the effect of treatments on epithelial cells, considering each concentration separately. All tests were applied considering $p<0.05$.

3 RESULTS

The results of photomicrographs of the formulations after adding 10, 30, 50 and 100% artificial saliva are shown in Figure 1. These photomicrographs demonstrate that the addition of saliva caused the isotropic systems to change to anisotropic systems. The addition of 30% saliva caused the "Maltese crosses" to appear, indicating the formation of a lamellar liquid crystalline system. The hexagonal liquid crystalline system formation occurred after the addition of 50% saliva based on the appearance of ribbed structures. The addition of 100% saliva to the formulation caused a change to an isotropic system, however, due to its high viscosity, indicated by a cubic liquid crystalline system - LCS (F).

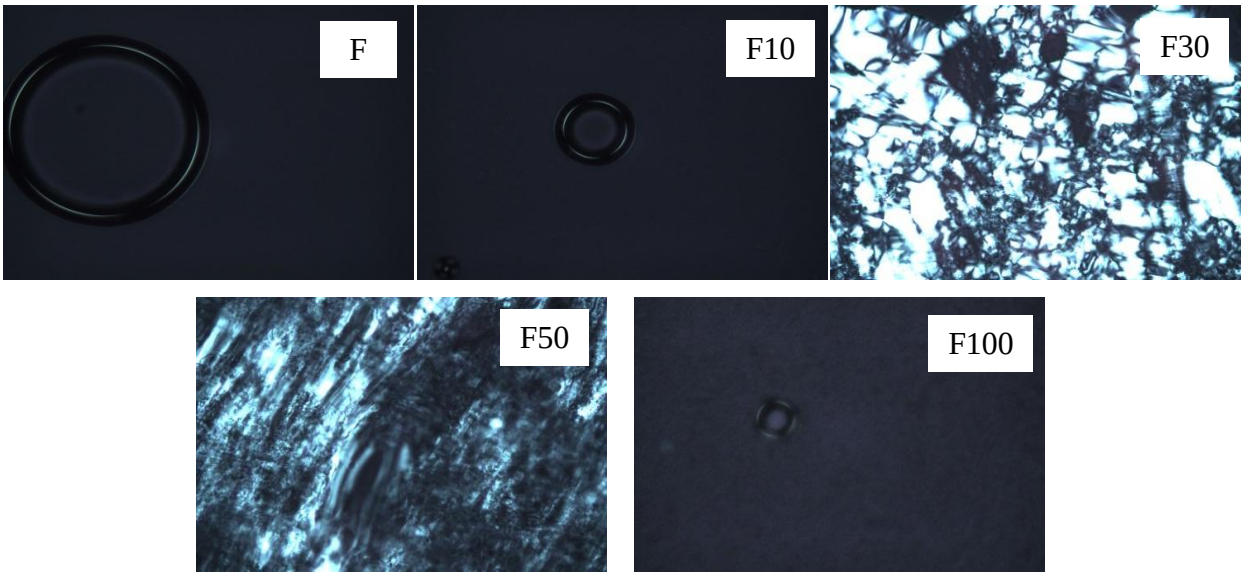

Figure 1- Photomicrographs representing the effect of artificial saliva in the structure of F by LPM at 20x magnification (Fn, n = 10, 30, 50 and 100% of added artificial saliva).

The rheological characteristics analyzed in F and F100 determined the flow properties and oscillatory analyzes seen in Figures 2 and 3, respectively. Figure 2 shows that F has a low viscosity, and when 100% saliva was added, F100 exhibited a high viscosity. In addition, it also observed that F100 is a thixotropic system, since the upward curve does not overlaps the

downward curve indicating that F100 is more structured, because it need a more time to return for its initial organization.

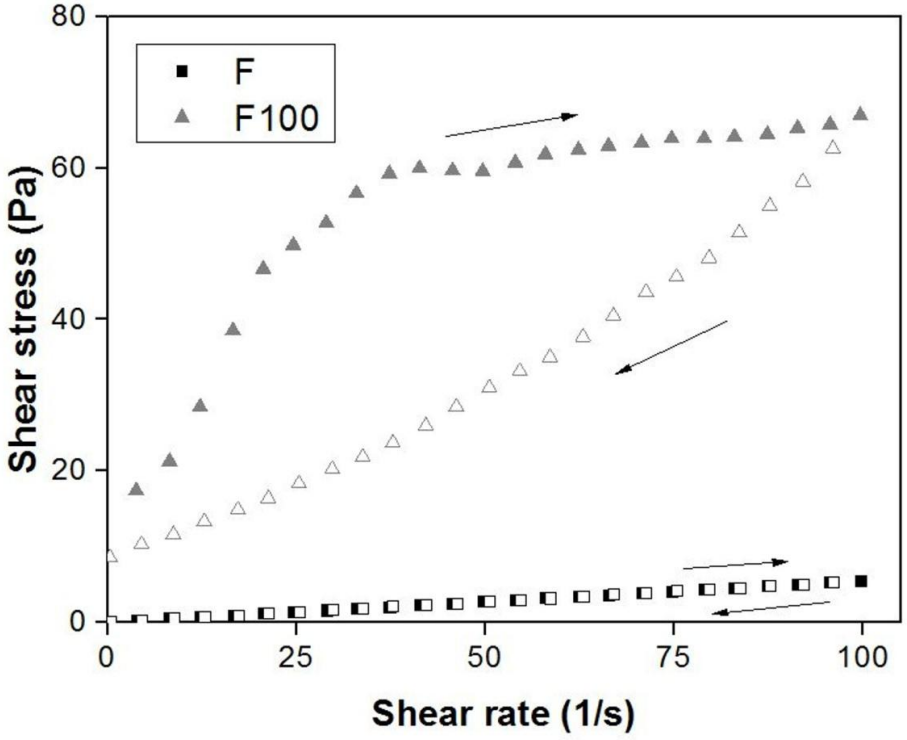

Figure 2 – Rheograms of formulations F and F100. (Ascendent curve - filled symbol and descendent curve - empty symbols).

The frequency sweep test showed changes in the oscillatory properties of the formulations after adding 100% saliva, because F100 presented G' greater than F. Moreover, F100 possess a very elastic character since its G' is much larger than G", indicating that the incorporation of saliva increased the rigidity of the system. Table 1 shows the results of the bioadhesion test, indicating that the incorporation of 100% saliva into the F-system increased the bioadhesion strength and the work of bioadhesion on the surface of the bovine tooth, at five times larger than F.

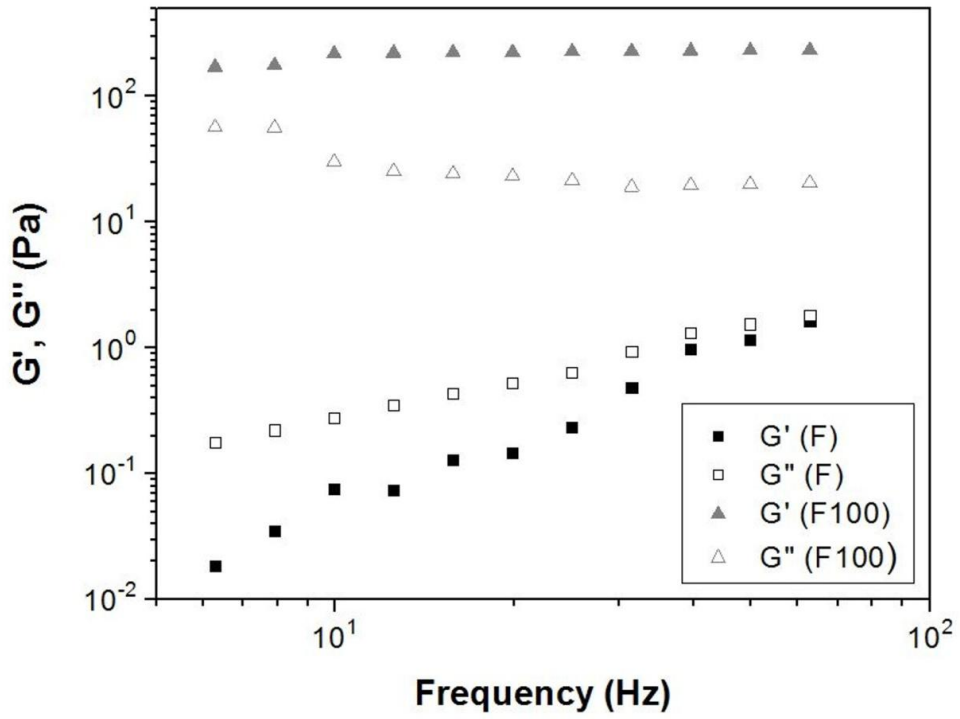

Figure 3 - Variation of the storage module (G' – filled symbol) and loss module (G" – empty symbol) as a function of the frequency to F and F100.

Table 1– Results of in vitro bioadhesion strength in F and F100. Values represent means ± standard deviations.

Formulations	Peak bioadhesive strength (mN)	Work of the bioadhesive strength (mN.s)
F	2.10 ± 0.350	2.00 ± 0.001
F100	11.00 ± 1.200	11.5 ± 0.500

Table 2 presents values of MIC and MBC for the antimicrobial agent in solution, demonstrating that CHX was more effective in solution when compared to D1-23. Considering the biofilm assays, the percentage of S. *mutans* reduction in the biofilm after 4 h and 24 h with the appropriated treatments are shown in Figure 4. After 4 h of exposure, there was no statistical difference in the biofilm reduction comparing CHX and D1-23 incorporated in F and both agents in solution. After 24 h, no difference was observed comparing CHX in solution or incorporated into F, but higher biofilm reduction was noted for D1-23 in solution compared to D1-23 incorporated into F. Overall, biofilm reduction was higher in 24 h when

compared to 4 h, suggesting a cumulative effect of antimicrobial agents when incorporated into F.

Table 2 – Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of D1-23 and CHX solution against planktonic S. *mutans*.

	MIC (µg/mL)	**MBC (µg/mL)**
D1-23	15.60	31.25
CHX	0.30	0.60

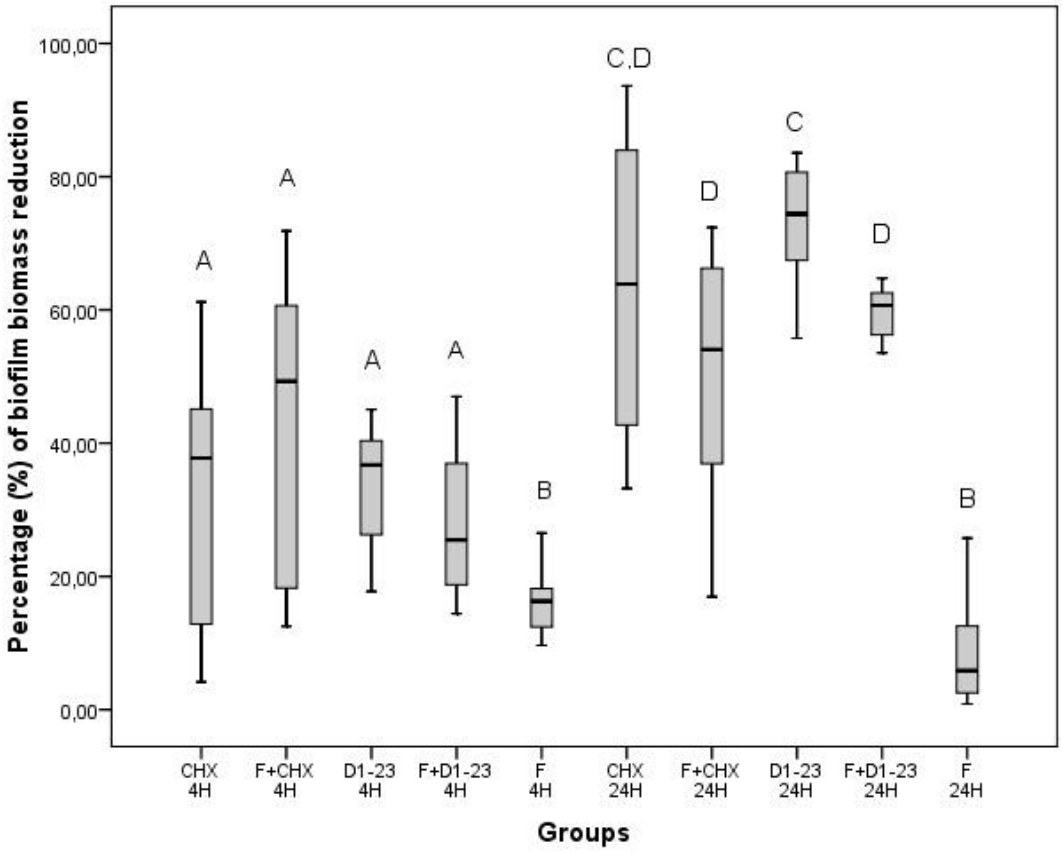

Figure 4 – Boxplot of the percentage of biofilm biomass reduction after 4h and 24h of the treatments.

[a] Different letters show statistical difference among the groups, according to Kruskal-Wallis/Mann-Whitney tests ($p<0.05$).

F – Liquid crystalline formulation; D1-23 – peptide D1-23; F+D1-23 – formulation + peptide D1-23; CHX – chlorhexidine; F+CHX – formulation + chlorhexidine.

Figure 5 shows the cytotoxicity effects of the antimicrobial agents on epithelial cells. CHX and D1-23 solutions at 1 mg/mL were very cytotoxic, but when these agents were incorporated in F at 1 mg/mL concentration, cytotoxicity was not observed.

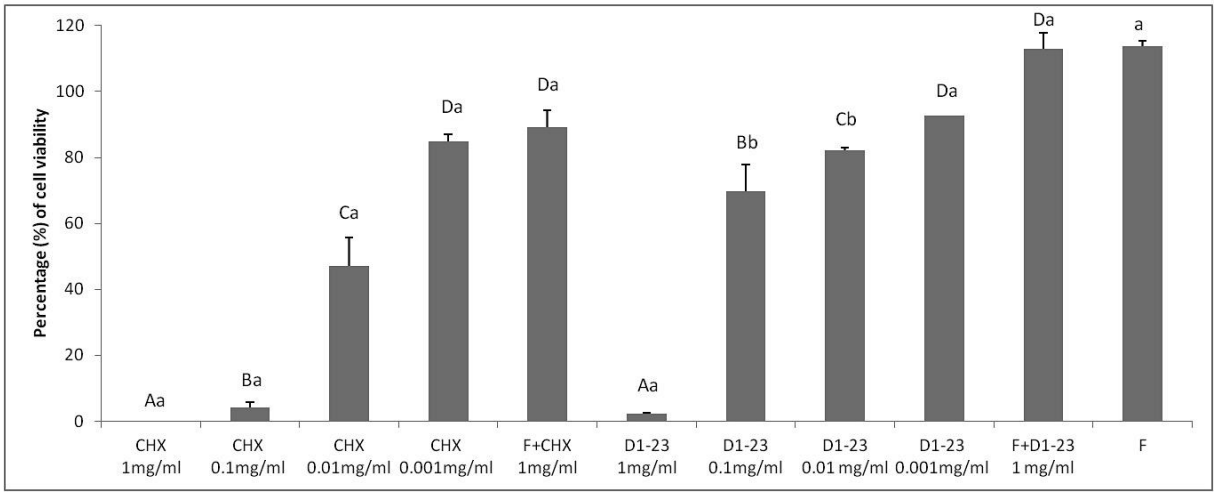

Figure 5 – Epithelial cell viability after 24 h of the treatments.

[A] Different upper case letters show statistical difference among the concentrations of the same agent, according to ANOVA/Tukey tests ($p<0.05$).

[a] Different lower case letters show statistical difference among CHX and D1-23 or F+CHX and F+D1-23, according to t Student test ($p<0.05$).

4 DISCUSSION

This is the first *in vitro* study investigating the applicability of LCS containing an AMP fragment on tooth surfaces aimed to prevent dental caries. All of these characterization results indicated that, in contact with the saliva, F (LCS) present a more structured system, with greater viscosity, and better bioadhesive power on the tooth surface, which may increase the action of the peptide and improve the performance of the treatment.

Formulations composed of surfactant, an aqueous phase and an oil phase in different proportions can generate microemulsions, emulsions, liquid crystallines or phase separation (Lawrence & Rees, 2000). The identification of the liquid crystalline phase is fundamental in the development of drug delivery systems because the mesophase structure may influence drug release. This study observed that the addition of saliva, i.e., the increase of concentration of aqueous phase of system caused isotropic systems to convert to anisotropic systems. The structures observed in the anisotropic field, such as streaks and “Maltese crosses”, are referred to as hexagonal and lamellar, respectively, whereas the microemulsions and cubic mesophases

are isotropic, visualized as dark field (Calixto, 2013). “Maltese crosses” starting to appear from the addition of 30% saliva indicate the formation of a lamellar liquid crystalline system. The hexagonal liquid crystalline system formation occurred after the addition of 50% saliva with the appearance of ribbed structures. The addition of 100% saliva in formulation caused a change to an isotropic system, indicated by a cubic liquid crystalline system. The rigidity and high viscosity of the cubic phase retards diffusion and can provide a slow sustained release of the incorporated drug (Shah, Sadhale, & Chilukuri, 2001).

The continuous rheological analysis is a widely used method for characterizing delivery systems, because it evaluates the facility that the material flows from a bottle, pumping a product in an industrial process, the spreading of a cream or lotion on skin and passage of product through the hole of a syringe (Pestana, 2009). In the ascending curve, the flow behavior is classified into two types: Newtonian or non-Newtonian. The non-Newtonian flow can be further classified into pseudoplastic, dilatant or plastic. In the descending curve, the material are classified as thixotropic or reopetic. Flow is classified as Newtonian when the shear stress and rate are constant, or its viscosity is constant (Scharamm, 2006). The pseudoplastic flow occurs in materials that undergo a decrease in viscosity when the shear rate goes from low levels to higher levels. The increase of the shear rate guides rigid particles in the flow direction, creating shear thinning (Savic et al., 2011). F was determined to have a Newtonian flow and low viscosity. When 100% saliva was added to F, F100 exhibited a non-Newtonian pseudoplastic behavior ($n<1$) and high viscosity. In addition, F100 was observed to have a thixotropic system, because the descending curve did not overlap the ascending curve, indicating that the system is more structured for F100 when compared to F.

The oscillatory analysis determined the viscoelastic properties of the formulations, providing information on the structural nature of the system, leading directly to the performance of the formulation. This information is obtained through analysis of the elastic module (G') and viscous module (G") obtained in this assay. The elastic module, G', is termed the storage module, representing both the energy stored during deformation when the tension increases and the energy released when the tension is relaxed. The G" viscous module is the viscous element that cannot store energy because the applied tension is dissipated in the form of irreversible deformation (Pènzes, 2004). In this present study, changes were observed in the oscillatory properties of the formulations after addition of 100% saliva by using the frequency sweep test. Figure 3 plots the G' storage module and the G" loss module versus frequency. The F organization degree increases when 100% saliva was incorporated, the F100 value G' module was higher than the F value G' module. Furthermore, the F100 G' storage

module was greater than the G" loss module, while the F value G' module was less than the G" module, indicating that the saliva incorporation increased the rigidity of the system.

The main advantage of using bioadhesive systems for drug delivery is to prolong the drug residence time at the site of application, which allows for an enhanced contact of the formulation with the biological barrier, reducing the frequency of application of the product and thus increasing patient compliance to therapy (Smart et al., 2003). In our study, we observed that when 100% saliva was added, the formulation exhibited the best bioadhesive properties (Figure 3). This characteristic obtained from the formulation is due to the use of Carbopol® as a bioadhesive agent. Carbopol is a bioadhesive polymer derived from the polyacrylic acid commonly used in bioadhesive pharmaceutical hydrogels, owing to their hydrophilic nature and reticulated structure, which makes them interesting for controlling drug release (Calixto, Yoshii, Stringhetti Ferreira Cury, & Chorilli, 2015).

Drugs can be evaluated in various methods such as antimicrobial properties and cytotoxicity. The present study investigated the toxicity in epithelial cells and anti-biofilm activity against *S. mutans* of a cationic antimicrobial peptide fragment (D1-23) incorporated into a controlled drug delivery system, liquid-crystalline system (LCS or F in this study). AMPs production is a major component of the innate immunity against infection that provides protection against bacteria, fungi, yeast and viruses (Pinheiro da Silva & Machado, 2012). These molecules have been shown to be promising agents in controlling microbial growth due to their low concentration and selective antimicrobial activity, as well as low rates of antimicrobial resistance induction (da Silva et al., 2012). *S. mutans* have been considered to be associated with dental caries (Tanzer, Livingston, & Thompson, 2001; Gupta et al., 2013; Nyvad, Crielaard, Mira, Takahashi, & Beighton, 2013) based on their acid production and acid tolerance (Becker et al., 2002; Takahashi & Nyvad, 2011). The peptide fragment (D1-23) was chosen based on a prior study (Kreling et al., 2016) testing the antimicrobial activity of various peptide fragments, including: defensins, cathelicidins, histatins and mucins. The D1-23 fragment showed the best results against *S. mutans* when compared to the other peptide fragments. In this present study, we determined MIC and MBC values for this peptide fragment, showing that this peptide has an antimicrobial activity against S. *mutans* with low values of MIC/MBC. Several studies have demonstrated the antimicrobial activity of defensins against *S. mutans* (Maisetta et al., 2003; Joly, Maze, McCray, & Guthmiller, 2004; Nishimura et al., 2004). The mechanism of action of defensins against bacteria is through cell rupture and inhibition of lipopolysaccharide production (da Silva et al., 2012).

Despite all of the advantages that AMPs have demonstrated, including activity at low concentrations and activity across a wide spectrum, there are some limits to their therapeutic application. One limitation is related to the high cationic activity of peptides. The inhibitory action of AMPs seems to be significantly reduced in biological fluids such as plasma, serum or saliva (da Silva et al., 2012), while their chemical and physical stability may be compromised by external factors, requiring special attention to their efficacy and security (Dailey, Wittmar, & Kissel, 2005). Therefore, LCS has been used to load peptides. LCS has been studied more intensively as a carrier of bioactive molecules in Medicine (Chen, Ma, & Gui, 2014), including applications for pulmonary drug delivery (Patil, Mahadik, & Paradkar, 2015), diabetes treatment (Okawara, Hashimoto, Todo, Sugibayashi, & Tokudome, 2014), and even AIDS treatment (Avachat & Parpani, 2015). There are some reports in Dentistry of using the drug delivery systems as the carrier of oral substances; with the majority of them being studied as an alternative for periodontal treatment (Shah, Sadhale, & Chilukuri, 2001; Horev et al., 2015). However, a few studies analyzed this specific system for oral application (Souza, Watanabe, Borheti-Cardoso, De Abreu Fantini, & Lara, 2014). Souza et al. (2014) evaluated a liquid crystalline system as a carrier for an antiseptic poly(hexamethylene biguanide) hydrochloride (PHMB). They observed that this system improved residence time and modulated drug release, indicating the use of this drug delivery system for PHMB.

With regards to cytotoxicity, the D1-23 and CHX solutions at 1mg/mL were toxic for epithelial cells; however, both agents did not affect cell metabolism at low concentrations. D1-23 showed toxicity against two epithelial cells lines at concentrations higher than 0.2mM (Kreling et al.; 2016). In this study, even at high concentrations, when both antimicrobial agents were incorporated into LCS, they did not have a cytotoxic effect on epithelial cells. This occurs because LCS was not cytotoxic and low concentrations of peptide and CHX are released from LCS. Any cytotoxic effect was observed when these LCS were applied on macrophage cultures (Oliveira et al., 2015).

Our main objective was to evaluate whether the liquid crystalline system would work as a carrier, and if it could slowly release the antimicrobial agent and consequently have a cumulative effect. The current results indicate that the peptide fragment D1-23 in solution and in LCS reduced the *S. mutans* biofilm growth in both times of exposures (after 4 h and 24 h); however, we should emphasize that the concentration selected for the D1-23 and CHX solutions was 1 mg/mL, regardless of the minimal bactericidal concentration. MIC/MBC assays of the tested agents showed that chlorhexidine had greater effect at lower concentrations when compared to D1-23. This means that the bactericidal concentration of

chlorhexidine used in the biofilm assays was much higher than for D1-23. Even so, D1-23 presented similar results to CHX in both times of exposure. The concentration of both agents incorporated into LCS was also 1 mg/mL. Data from literature reports that the release of agents from this liquid crystalline system is around 4% at 24 h (Hu, Hong, & Yuan, 2004). Although data from release of antimicrobial agents was not available in this present study, the peptide and CHX release from LCS was probably higher than 4%, since the formulation was diluted previously to incorporation of antimicrobial agents for biofilm assays to reduce viscosity. Even so, D1-23 at 1mg/mL into LCS presented similar antibiofilm activity to D1-23 in solution after 4 h. D1-23 into LCS increased the residence time of the antimicrobial agent but did not potentiate the antimicrobial effect. We believe that the antimicrobial agents interact with the LCS promoting a cumulative effect of D1-23.

Other studies are necessary to confirm the effectiveness of liquid crystalline systems for oral applications, perhaps using different methodologies promoting more time of contact between the antimicrobial agent and the site of action (tooth surface).

5 CONCLUSIONS

The present study characterized a liquid crystalline system with bioadhesive properties. Fragment of hBD-3, D1-23, incorporated in this LCS did not promote cytotoxic effect on epithelial cells. In solution, D1-23 had similar effect on *S. mutans* biofilm compared to CHX, however when this peptide was incorporated into LCS, promoted a cumulative antibiofilm effect, indicating that this drug delivery system might be a promising carrier to cationic antimicrobial peptide and could be used for the prevention of dental caries.

CONFLICTING OF INTEREST

The authors declare that there is no conflict of interest.

ACKNOWLEDGMENTS

This study was funded by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) - grants # 2012/19235-5 and # 2013/12285-0, Brazil.

REFERENCES

1. Abiko, Y., Nishimura M., & Kaku T. (2003). Defensins in saliva and the salivary glands. *Medical Electron Microscopy,* 36(4), 247-252.

2. Avachat, A.M., & Parpani, S.S. (2015). Formulation and development of bicontinuous nanostructured liquid crystalline particles of efavirenz. *Colloids and Surfaces. B, Biointerfaces,* 126, 87-97.

3. Becker, M.R., Paster, B.J., Leys, E.J., Moeschberger, M.L., Kenyon, S.G., Galvin, J.L., et al. (2002). Molecular analysis of bacterial species associated with childhood caries. *Journal of Clinical Microbiology* , 40(3), 1001– 1009.

4. Bedran, T.B., Mayer, M.P., Spolidorio, D.P., & Grenier, D. (2014). Synergistic anti-inflammatory activity of the antimicrobial peptides human beta-defensin-3 (hBD-3) and cathelicidin (LL-37) in a three-dimensional co-culture model of gingival epithelial cells and fibroblasts. *PLoS One,* 9(9), e106766.

5. Bernegossi, J., Calixto, G.M., Sanches, P.R., Fontana, C.R., Cilli, E.M., Garrido, S.S., et al. (2015). Peptide KSL-W-loaded mucoadhesive liquid crystalline vehicle as an alternative treatment for multispecies oral biofilm. *Molecules,* 21(1), E37.

6. Bhardwaj, V., & Kumar, M.N.V.R. (2012). Fundamentals and Applications of Controlled Release Drug Delivery Advances in Delivery Science and Technology. In: Springer, *Drug delivery system to fight cancer* (pp. 493-516).

7. Calixto, G., Yoshii, A.C., Rocha, E., Stringhetti Ferreira Cury, B., & Chorilli, M. (2015). Polyacrylic acid polymers hydrogels intended to topical drug delivery: preparation and characterization. *Pharmaceutical Development and Technology,* 20, 490-496.

8. Calixto, G.M., Garcia, M.H., Cilli, E.M., Chiavacci, L.A., & Chorilli, M. (2016). Design and characterization of a novel P1025 peptide-loaded liquid crystalline system for the treatment of dental caries. *Molecules,* 21(2), E158.

9. Calixto GMF. Desenvolvimento e caracterização de sistemas nanoestruturados bioadesivos contendo peptídeo análogo à adesina do *Streptococcus mutans*. Dissertação de Mestrado. Universidade Estadual Paulista "Júlio de Mesquita Filho", BR, 2013.

10. Chen, Y., Ma, P. & Gui S. (2014). Cubic and hexagonal liquid crystallines as drug delivery systems. *Biomed Research International,* 2014, 815981.

11. Crusca, E., Rezende, A.A., Marchetto, R., Mendes-Giannini, M.J., Fontes, W., Castro, M.S., et al. (2011). Influence of N-terminus modifications on the biological activity, membrane interaction, and secondary structure of the antimicrobial peptide Hylin-a1. *Biopolymers,* 96(1), 41-48.

12. da Silva, B.R., de Freitas, V.A., Nascimento-Neto, L.G., Carneiro, V.A., Arruda. F.V., de Aguiar, A.S., et al. (2012). Antimicrobial peptide control of pathogenic microorganisms of the oral cavity: a review of the literature. *Peptides,* 36, 315-321.

13. Dailey, L.A., Wittmar, M., & Kissel, T. (2005). The role of branched polyesters and their modifications in the development of modern drug delivery vehicles. *Journal of Controlled Release*, 101(1-3), 137-149.

14. Fejerskov, O. (1997). Concepts of dental caries and their consequences for understanding the disease. *Community Dentistry and Oral Epidemiology,* 25(1), 5-12.

15. Gawande, P.V., Leung, K.P., & Madhyastha, S. (2014). Antibiofilm and antimicrobial efficacy of DispersinB®-KSL-W peptide-based wound gel against chronic wound infection associated bacteria. *Current Microbiology*, 68(5), 635-41.

16. Gordon, Y.J., Romanowski, E.G., & McDermott, A.M. (2005). A review of antimicrobial peptides and their therapeutic potential as anti-infective drugs. *Current Eye Research,* 30(7), 505-15.

17. Gorr, S.U., & Abdolhosseini, M. (2011). Antimicrobial peptides and periodontal disease. *Journal of Clinical Periodontology,* 38, 126-141.

18. Gupta, P., Gupta, N., Pawar, A.P., Birajdar, S.S., Natt, A.S., & Singh, H.P. (2013). Role of sugar and sugar substitutes in dental caries: a review. *ISRN Dentistry,* 2013, 519421.

19. Guterres, S.S., Alves, M.P., & Pohlmann, A.R. (2007). Polymeric nanoparticles, nanospheres and nanocapsules, for cutaneous application. *Drug Target Insights*, 2, 147-157.

20. Hamada, S., & Slade, H.D. (1980). Biology, immunology, and cariogenicity of *Streptococcus mutans*. *Microbiological Reviews,* 44(2), 331-384.

21. Horev, B., Klein, M.I., Hwang, G., Li, Y., Kim, D., Koo, H., et al. (2015). pH-activated nanoparticles for controlled topical delivery of farnesol to disrupt oral biofilm virulence. *ACS Nano* ,9(3), 2390-2404.

22. Hu, F.Q., Hong, Y., & Yuan, H. (2004). Preparation and characterization of solid lipid nanoparticles containing peptide. *International Journal of Pharmaceutics*, 273(1-2), 29-35.

23. Joly, S., Maze, C., McCray, P.B. Jr., & Guthmiller, J.M. (2004). Human β-defensin 2 and 3 demonstrate strain-selective activity against oral microorganisms. *Journal of Clinical Microbiology*, 42(3):1024-1029.

24. Kaiser, E., Colescott, R.L., Bossinger, C.D., & Cook PI. (1970). Color test for detection of free terminal amino groups in the solid phase synthesis of peptides. *Analytical Biochemistry,* 34(2), 595-598.

25. Kreling, P.F., Aida, K.L., Massunari, L., Caiaffa, K.S., Percinoto, C., Bedran, T.B., et al. (2016). Cytotoxicity and the effect of cationic peptide fragments against cariogenic bacteria under planktonic and biofilm conditions. *Biofouling*, 32(9), 995-1006.

26. Lawrence, M.J., & Rees, D.G. (2000). Microemulsions-based as novel drug delivery systems. *Advanced Drug Delivery Reviews,* 45(1), 89-121.

27. Lestini, B.J., Sagnella, S.M., Xu, Z., Shive, M.S., Richter, N.J., Jayaseharan, J., et al. (2002). Surface modification of liposomes for selective cell targeting in cardiovascular drug delivery. *Journal of Controlled Release,* 78(1-3), 235-247.

28. Maisetta, G., Batoni, G., Esin, S., Luperini, F., Pardini, M., Bottai, D., et al. (2003). Activity of human β-defensin 3 alone or combined with other antimicrobial agents against oral bacteria. *Antimicrobial Agents and Chemotherarpy,* 47(10), 3349-3351.

29. Marques, M.R.C., Loebenberg, R., & Almukainzi, M. (2011). Simulated Biological Fluids with Possible Application in Dissolution Testing. *Dissolution Technologies*, 18(3), 15–28.

30. Marsh, P.D. (2012). Contemporary perspective on plaque control. *British Dental Journal,* 212(12), 601-606.

31. Mattos-Graner, R.O., Napimoga, M.H., Fukushima, K., Duncan, M.J., & Smith, D.J. (2004). Comparative analysis of Gtf isozyme production and diversity in isolates of *Streptococcus mutans* with different biofilm growth phenotypes. *Journal of Clinical Microbiology,* 42(10), 4586-4592.

32. Mccormick, T.S., & Weinberg, A. (2010). Epithelial cell-derived antimicrobial peptides are multifunctional agents that bridge innate and adaptive immunity. *Periodontology 2000*, 54(1), 195–206.

33. Merrifield, R.B. (1963). Solid Phase Peptide Synthesis. I. The synthesis of a tetrapeptide. *Journal of the American Chemical Society,* 85(14), 2149-2154.

34. Mor, A., Hani, K. & Nicolas, P.J. (1994). The vertebrate peptide antibiotics dermaseptins have overlapping structural features but target specific microorganisms. *The Journal of Biological Chemistry*, 269(50), 31635–31641.

35. Nishimura, E., Eto, A., Kato, M., Hashizume, S., Imai, S., Nisizawa, T., et al. (2004). Oral streptococci exhibit diverse susceptibility to human β-defensin - 2: antimicrobial effects of hBD-2 on ral streptococci. *Current Microbiology*, 48(2), 85-87.

36. Nyvad, B., Crielaard, W., Mira, A., Takahashi, N., & Beighton, D. (2013). Dental caries from a molecular microbiological perspective. *Caries Research,* 47(2), 89-102.

37. Okawara, M., Hashimoto, F., Todo, H., Sugibayashi, K., & Tokudome, Y. (2014). Effect of liquid crystallines with cyclodextrin on the bioavailability of a poorly water-soluble compound, diosgenin, afeter its oral administration to rats. *International Journal of Pharmaceutics*, 472(1-2), 257-261.

38. Oliveira, M.B., Calixto, G., Graminha, M., Cerecetto, H., González, M., & Chorilli, M. (2015). Development, characterization, and in vitro biological performance of fluconazole-loaded microemulsions for the topical treatment of cutaneous leishmaniasis. *Biomed Research International*, 396894.

39. Ouhara, K., Komatsuzawa, H., Yamada, S., Shiba, H., Fujiwara, T., Ohara, et al. (2005). Susceptibilities of periodontopathogenic and cariogenic bacteria to antibacterial peptides, {beta}-defensins and LL37, produced by human epithelial cells. *The Journal of Antimicrobial Chemotherapy*, 55(6):888-896.

40. Patil, S.S., Mahadik, K.R. & Paradkar, A.R. (2015). Liquid crystalline phase as a probe for crystalline engineering of lactose: carrier for pulmonary drug delivery. *European Journal of Pharmaceutical Sciences*, 68, 43-50.

41. Pènzes, T., Csóka, I. & Erős, I. (2004). Rheological analysis of the structural properties effecting the percutaneous absorption and stability in pharmaceutical organogels. *Rheologica Acta*, 43(5), 457–463.

42. Pestana, K.C. Microemulsões biocompatíveis de anfotericina b para administração oral. estudo estrutural, liberação in vitro e farmacocinética pré-clínica. PhD Thesis, Universidade Estadual Paulista "Júlio de Mesquita Filho", BR, 2009.

43. Pinheiro da Silva, F., & Machado, M.C. (2012). Antimicrobial peptides: clinical relevance and therapeutic implications. *Peptides*, 36(2), 308-314.

44. Reynolds, N.L., De Cecco, M., Taylor. K., Stanton, C., Kilanowski, F., Kalapothakis, J., et al. (2010). Peptide fragments of a β-defensin derivative with potent bactericidal activity. *Antimicrobial Agents and Chemotherapy*, 54(5), 1922-1929.

45. Salmazi, R., Calixto, G.M.F., Bernegossi, J., Ramos, M.A., Bauab, T.M., & Chorilli, M. (2015). A curcumin-loaded liquid crystalline precursor mucoadhesive system for the treatment of vaginal candidiasis. *International Journal of Nanomedicine*,10, 4815-4824.

46. Savic, S., Lukic, M., Jaksic, I., Reichl, S., Tamburic, S., & Müller-Goymann, C. (2011). An alkyl polyglucoside-mixed emulsifier as stabilizer of emulsion systems: The influence of colloidal structure on emulsions skin hydration potential. *Journal of Colloid and Interface Sciences*, 358(1), 182-191.

47. Schramm, G. (2006). *Reologia e Reometria. Fundamentos Teóricos e Práticos*. In São Paulo, Artiliber.

48. Shah, J.C., Sadhale, Y., & Chilukuri, D.M. (2001). Cubic phase gels as drug delivery systems. *Advanced Drug Delivery Reviews*, 47(2), 229-250.

49. Smart, J.D., Riley, R.G., Tsibouklis, J., Young, S.A., Hampson, F.C., Davis, J.A., et al. (2003). The retention of C-labelled poly(acrylic acids) on gastric and oesophageal mucosa: an in vitro study. *European Journal of Pharmaceutical Sciences*, 20(1), 89-90.

50. Smith, D.J. (2003). Caries vaccines for the twenty-first century. *Journal of Dental Education*, 67(10), 1130-1139.

51. Soares, D.G.S., Ribeiro, A.P.D., Sacono, N.T., Coldebella, C.R., Hebling, J., & Costa, C.A. (2011). Transenamel and transdentinal cytotoxicity of carbamide peroxide bleaching gels on odontoblast-like MDPC-23 cells. *International Endodontic Journal*, 44(2), 116-25.

52. Souza, C., Watanabe, E., Borgheti-Cardoso, L.N., De Abreu Fantini, M.C., & Lara, M.G. (2014). Mucoadhesive system formed by liquid crystallines for buccal administration of poly(hexamethylene biguanide) hydrochloride. *Journal of Pharmaceutical Sciences,* 103(12), 3914-3923.

53. Takahashi, N., & Nyvad, B. (2011). The role of bacteria in the caries process ecological perspectives. *Journal of Dental Research*, 90(3), 294–303.

54. Tanzer, J.M., Livingston, J., & Thompson, A.M. (2001). The microbiology of primary dental caries in humans. *Journal of Dental Education,* 65(10), 1028-1037.

55. Wiesner, J., & Vilcinskas, A. (2010). Antimicrobial peptides – the ancient arm of the human immune system. *Virulence*, 1(5), 440-464.

56. Zhang, L., & Falla, T.J. (2009). Host defense peptides for use as potential therapeutics. *Current Opinion in Investigational Drugs*, 10(2), 164-171.

3 CAPÍTULO 2 - Nanostructured Bioadhesive Liquid Crystalline System as Carrier for Peptide P1025 for Prevention of Dental Caries*

ABSTRACT

Objectives: *Streptococcus mutans* adhesion to dental structure is mediated for several bacterial surface proteins, such as adhesins antigen I/II and salivary receptors. The objectives of this study were to develop and characterize a nanostructured bioadhesive liquid crystalline system for incorporation of P1025, a peptide analogue of antigen I/II and to evaluate its cytotoxicity and effect against *S. mutans* biofilm.

Design: Formulations were composed by polyoxypropylene-(5)-polyoxyethylene-(20)-cetyl alcohol, oleic acid and Carbopol® 974P in 3 different concentrations (F1-C, F2-C and F3-C). Each phase was identified by polarized light microscopy (PLM), small-angle X-ray scattering (SAXS), rheological analysis and in vitro bioadhesion. Then, P1025 and control (chlorhexidine – CHX) were incorporated in aqueous phase of the best formulation (F) and determined their anti-biofilm effect and toxicity on epithelial cells.

Results: Structural characterization by PLM and SAXS showed that F1-C and F2-C were hexagonal and lamellar liquid crystalline systems and F3-C presented characteristic of microemulsion. F1-C presented time-dependent thixotropic behavior and elastic characteristic, and the highest bioadhesive strength compared to other formulations. F1-C was chosen for the incorporation of P1025 and CHX. The best anti-biofilm effects were observed for F+1025 and F+CHX, without statistical difference between them, when applied in the surface-bound salivary phase. CHX and P1025 solutions, at the concentrations tested, were not cytotoxic. F increased their toxicity with the increase of the time of exposure.

Conclusions: P1025-loaded nanostructured liquid crystalline system presented low toxicity on epithelial cells and had effect against *S. mutans* biofilm and seems to be an interesting drug delivery strategy for prevention of dental caries.

Keywords: antimicrobial peptides, drug delivery system, dental caries, biofilm, cytotoxicity.

Highlights

- Liquid crystalline system (LCS) is an interesting drug delivery system for P1025.
- P1025-loaded LCS presented antimicrobial effect against S. *mutans* and is a promising anticaries agent.
- P1025-loaded LCS presented low toxicity on epithelial cells.

* According to guidelines for authors of Archives of Oral Biology (Anexo C).

1 INTRODUCTION

Dental caries is still one of the most widely spread disease in the world, affecting people of all age throughout their lifetime (Lagerweij & Loveren, 2015). It is considered a biofilm-dependent disease because the pathogenic microbiota, which colonize and proliferate in dental biofilms, is the most important factor for the onset and progression of the disease (Marsh, 2006; Filoche, Wong & Sissons, 2010). *Streptococcus mutans (S. mutans)* is closely associated with the etiology of dental caries (Alam et al., 2000; Hamada & Slade, 1980). Virulence factors including adhesins antigen I/II (Ag I/II), glucosyltransferases and glucan-binding protein improve the ability of *S. mutans* to adhere and accumulate in dental biofilm (Smith, 2003; Smith, Lehner & Beverley, 1984; Russell, Bergmeier, Zanders & Lehner, 1980; Lee, Progulske-Fox & Bleiweis, 1988).

Numerous studies have demonstrated the importance of immune response against Ag I/II in the human protection against *S. mutans* colonization (Robinette et al., 2014). In vitro studies with Ag I/II knockout S. *mutans* strains showed decreased adhesion to hydroxyapatite in enamel surface, suggesting that Ag I/II facilitates the adhesion of the bacteria (Younson & Kelly, 2004). Besides, downregulation of Ag I/II in biofilm cells is critical for the initial biofilm formation but not for established biofilms (Sanui & Gregory, 2009). These findings may provide useful information regarding the importance of Ag I/II as tool for new strategies to control biofilm-mediated infections (Pecharki, Peterson, Assev & Scheie., 2005). In vitro assays of S. *mutans* adhesion activity to dental surfaces have identified that the salivary agglutinin present in human saliva is an Ag I/II receptor, mediating the binding between bacteria and teeth (Carlén & Olsson, 1995).

Kelly et al. (1999) identified amino acid residues 1025-1044 in the C-terminal region of Ag I/II as the adhesion epitope of this antigen. The synthetic peptide P1025 corresponding to these residues was able to inhibit in vitro binding between S. *mutans* adhesin and salivary agglutinin. In vivo study, P1025 reduced the recolonizing of S. *mutans* in dental biofilm (Kelly et al., 1999). Another study by Li, Wang & Lai. (2009) demonstrated that a dentifrice containing P1025 decreases the adhesion of S. *mutans* hydroxyapatite surfaces covered by saliva. The efficacy of antimicrobial agents in the treatment of oral infections is not solely related to antimicrobial activity, but also depends on a proper drug supply for an extended period to be adequately effective. However, topical formulations commonly employed (mouthwash, sprays, gels, and lozenge) are often characterized by a limited drug retention time in the oral cavity, and they tend to be rapidly dislodged, diluted, or removed, which can alter the efficacy of the antimicrobial agent. Therefore, numerous and repeated

administrations are required to achieve and maintain effective levels of the drug (Gajdziok, Bajerová, Chalupová & Rabisková, 2010; Loftsson, Leeves, Bjornsdottir, Duffy & Masson, 1999). Then, a development of a slow-release oral delivery device with an increased retention time to the mucosa would be advantageous (Souza, Watanabe, Borgheti-Cardoso, de Abreu Fantini & Lara, 2014).

Liquid crystalline system (LCS) has received considerable attention because of their excellent potential as drug vehicles. Among these systems, reversed cubic and hexagonal mesophases have been investigated for their ability to maintain the drug release of a wide range of biomolecules, including peptides (Guo, Wang, Cao, Lee & Zhai, 2010). LCS has double behavior, with mechanical properties of a crystalline solid and an isotropic form of a liquid. The structure of cubic phase of LCS is unique and consists of two continuous but non-intersecting water channels separated by a lipid bilayer. The cubic phases are used as the carriers for hydrophilic, lipophilic or amphiphilic drugs. The hexagonal phase is composed of cylindrical micelles packed in a hexagonal pattern. In contrast to the cubic phase, the water channels in the hexagonal phase are closed. Cubic and hexagonal phases provide a slow drug release matrix and protect peptides, proteins, and nucleic acids from chemical and physical degradation. As drug carriers, cubic phase liquid crystals have the ability to provide sustained drug release. The nanostructure of these lipid- based liquid crystalline systems had a significant impact on oral absorption of hydrophilic drugs. (Chen, Ma & Gui., 2014). An LCS is a suitable carrier of active molecules in topical formulations since it shelters the active molecule, sustains drug release, has low cost, can be produced on an industrial scale, and is a transparent system (Chorilli et al., 2011). They also may compete with proteolytic enzymes, important feature for administration of drugs prone to enzymatic degradation, such as peptides (Salamat-Miller, Chittchag & Johnston, 2005). Thereby, the objectives of this study were to develop and characterize a nanostructured bioadhesive liquid crystalline system for incorporation of P1025, a peptide analogue of antigen I/II and to evaluate its cytotoxicity and effect against formation of *S. mutans* biofilm.

2 MATERIALS AND METHODS

2.1 Construction of the Pseudoternary Phase Diagram and Preparation of the Liquid Crystal Precursor Bioadhesive System

The ternary phase diagram was constructed at 25 ± 0.5 ºC by weighing and mixing different amounts of polyoxypropylene-(5)-polyoxyethylene-(20)-cetyl alcohol (PPG-5-CETETH-20) as the surfactant, oleic acid as the oil phase, polymeric dispersion containing

5% (w/w) Carbopol® 974P and water as aqueous phase. The final polymeric concentration in each formulation was 0.5 % (w/w). The polymeric dispersion was prepared dispersing 5% (w/w) Carbopol® 974P (C974) in water and homogenized at 2000 rpm in a mechanical stirrer for approximately 10 min at 25°C ± 0.5°C. Following complete dissolution, the pH was adjusted to 6.0 with triethanolamine (Synth) and manual agitation. After, all formulations were visually classified as a transparent liquid system (TLS), translucent liquid system (TrLS), transparent viscous system (TVS), translucent viscous system (TrVS), opaque viscous system (OVS) or phase separation (PS). Then, the different regions in the phase diagram could be delineated. From these data, the formulations were selected for physicochemical characterization (Calixto, Garcia, Cilli, Chiavacci & Chorilli, 2016).

2.2 Structural Features of the Formulations

2.2.1 Polarized Light Microscopy (PLM)

The PLM analyses were performed by placing a small amount of each formulation on a glass slide and covering it with a coverslip. The samples were analyzed using a polarized light microscope (Jenamed Carl Zeiss, Oberkochen, Germany), evaluating the homogeneity of the dispersion and observing the presence of anisotropy or isotropy at a magnification of 20x and at room temperature (Salmazi et al., 2015).

2.2.2 Small-Angle X-Ray Scattering (SAXS)

The structural arrangements of the LCSs were analyzed by SAXS using the Brazilian Synchrotron Light Laboratory instrument (LNLS, Campinas, Brazil) equipped with a type Si (111) monochromator with a wavelength of 1.608 Å that yields a horizontally focused beam. A vertical Pilatus 300K SAXS detector located at 858.45 mm from the sample and a multichannel analyzer 13 were employed to record the intensity of the scattering I(q) from 0.1 to 3.8 Å1 at 25 °C. The scattering particles in the system without sample were subtracted from the total intensity of the sample, as a function of the module of the scattering vector q, q = 4sin/, where is the wavelength and 2 is the scattering angle. The intensities of all samples were measured in relative units, but a quantitative comparison of the measurements was standardized under the same experimental conditions (Calixto, Garcia, Cilli, Chiavacci & Chorilli, 2016).

2.2.3 Rheological Analysis

The rheological measurements were performed at 37 ± 0.1°C in triplicate using a controlled-stress AR2000 rheometer (TA Instruments, New Castle, DE, USA) with parallel plate geometry (40 mm diameter) and a sample gap of 200 μm. The samples of the formulations were carefully applied to the lower plate to minimize sample shearing and were allowed to equilibrate for 3 min prior to analysis (Calixto, Yoshii, Rocha e Silva, Stringhetti Ferreira Cury & Chorilli, 2015).

2.2.3.1 Determination of Flow Properties

The flow properties were determined using a controlled shear rate procedure ranging from 0.01 to 100 s^{-1} and back. Each stage lasted for 120 s with an interval of 10 s between the curves. The consistency and flow indexes were determined from the power law described in (1) for a quantitative analysis of flow behavior:

$$\tau = k \cdot \gamma^{\eta}, \quad (1)$$

where "τ" is the shear stress, "γ" is the shear rate, "k" is the consistency index, and "η" is the flow index (Calixto, Yoshii, Rocha e Silva, Stringhetti Ferreira Cury & Chorilli, 2015).

.

2.2.3.2 Oscillatory Analyses

The oscillatory analyses were started by the conduction of a stress sweep in order to determine the viscoelastic region of the formulations. The stress sweep was carried out at a constant frequency of 1 Hz over a stress range of 0.1–10 Pa. A constant shear stress of 1 Pa was selected to perform the frequency sweep over a range of 0.1–10 Hz, which was within the previously determined linear viscoelastic region for all formulations. Thus, the storage (G') and loss (G") module were recorded (Calixto, Yoshii, Rocha e Silva, Stringhetti Ferreira Cury & Chorilli, 2015).

2.2.4 In Vitro Bioadhesion Strength

2.2.4.1 Preparation of Discs

Sound bovine permanent central incisors (Ethics Committee on the Use of Animals – FOA/UNESP, Protocol 2014/00618) were collected and scaled to remove periodontal tissue and other debris. Teeth with enamel cracks, hypoplasia, and calculus in the middle third of the crown or other morphological alterations were excluded. Enamel blocks were cut transversally from the middle third of the buccal surface of each tooth using a water-cooled, double-faced diamond disc (KG Sorensen, Barueri, SP, Brazil). The specimens were then rounded using a high-speed, water-cooled cylindrical diamond bur (1095; KG Sorensen) to

obtain specimens with a diameter of 1 cm containing enamel. Teeth surfaces were polished with wet 200-grit silicon carbide paper (T469-SF- Norton; Saint-Gobain Abrasivos Ltda., Jundiaí, SP, Brazil) to normalize the surface (Soares et al., 2011).

2.2.4.2 Bioadhesion Measurement (tensile strength method)

A TA-XTplus texture analyzer (Stable Micro Systems, Surrey, UK) was used for the tensile strength measurements. The teeth model was fastened to the upper movable probe with double side tape, and the formulation sample was located on the lower platform. Before the test, the tooth was immersed in artificial saliva. The upper probe was lowered until it made contact with the sample and it was kept in contact without any force applied for 60 s. The probe was then raised at 0.5 mm/s, and the force needed for detachment was registered. The tensile work (g.s), which is proportional to the area under the force–time curve, was used to describe the bioadhesive characteristics. Seven replicates were analyzed at 37 ± 0.5°C (Calixto, Yoshii, Rocha e Silva, Stringhetti Ferreira Cury & Chorilli, 2015).

.

2.3 Preparation of the Peptide P1025

The peptide P1025 with sequence Ac-QLKTADLPAGRDETTSFVLV-NH2 (MW: 2202,5) was synthesized and purified according to a recently reported method (Santos, Oyafuso, Kiill, Gremião & Chorilli, 2013). The peptide was solubilized in sterile deionized water with 0.1% acetic acid (CH3COOH) at a concentration of 1000 µg/mL prior to use and stored in a freezer at -20°C until use. The molecular weight of the peptide P1025 is 2202.5 g/mol.

2.4 Incorporation of Agents into LCS

The positive control group was chlorhexidine diacetate (CHX), purchased from Sigma Aldrich (St. Louis, MO, USA). Peptide P1025 and CHX were incorporated into the aqueous phase of formulation (F) at the concentration of 1 mg/mL based on a previous study (Hu, Hong, & Yuan, 2004). Then, the groups of the study were P1025 and CHX solutions, LCS (F), LCS containing P1025 (F+P1025) and LCS containing CHX (F+CHX). LCS was diluted tenfold before incorporating CHX and P1025 to reduce the viscosity for biofilm assays (Gawande, Leung & Madhyastha, 2014).

2.5 Microbiological Evaluation

2.5.1 Biomass Biofilm Assays

The effect of treatments on biofilm formation was studied following the methodology described in Ahn et al. (2008) with some modifications in biofilm growth in the presence of saliva proposed by Castillo (2016). Firstly, stimulated saliva was collected from healthy volunteers with good oral and general health in a sterile tube, after approval of Human Research Ethics Committee of Araçatuba Dental School, Universidade Estadual Paulista, Brazil (#CAAE 13079213.4.0000.5420). Volunteers were asked not to eat or drink for at least 2h prior to collection. The saliva was centrifuged at 4000xg for 10 min at 4^{o} C. to remove cell debris. After that, the supernatant was mixed with the buffered solution (0.05M KCl, 0.02M KPO_4, 0.02M $CaCl_2$; 0.02 M $MgCl_2$) in the proportion of 1:1 and added 0.1M PMSF (phenylmethylsulfonyl fluoride, Sigma). Clarified saliva was used after filter sterilization through a 0.22µm filter bottle (Corning) (Castillo, 2016). For the following experiments, each treatment (LCS containing P1025 (F+P1025), LCS containing CHX (F+CHX), P1025 and CHX solutions, all of them at 1mg/mL) was diluted 1:3 in saliva.

Overnight cultures of *S. mutans* UA159 were transferred to prewarmed BHI containing 1% glucose and grown at 37^{o} C in a 5% CO_2 to the mid-exponential phase (OD=0.5). The cultures were then diluted 1:10 and centrifuged at 8000xg for 5 minutes, washed in 0.9% saline solution and resuspended in prewarmed buffered tryptone-yeast-extract broth (TYE) 1.25x at pH 7.0 containing 0.5% sucrose (cell suspension at $1\text{-}5x10^{5}$ CFU/mL). Biofilm formation assays were done using polystyrene U-bottom cell culture plates (TPP, Switzerland) following two different ways: 1) salivary preparation with each treatment was added to each well with the cell culture (salivary fluid-phase) or 2) wells were first coated with salivary preparations before being inoculated with cell suspension (salivary surface-bound phase). For the experiments with fluid-phase salivary preparation, 120 µL of the cell suspension was inoculated into the wells concomitantly with 10µL of each treatment + 30 µL of saliva, maintaining the proportion of 25% of saliva, as proposed by Castillo (2016). For the experiments with surface-bound salivary preparations, each well was conditioned with each treatment diluted in saliva (1:3) and incubated at 37^{o} C for 1h previously to inoculation of cell suspensions into the wells. All plates were incubated at 37^{o}C in a 5% CO2 atmosphere for 24h. After that, the plates were washed by immersion in sterile distilled water to remove non-adhered cells. After a brief drying, 150 µL of aqueous 1% crystalline violet was added to each well, and the plates were incubated at room temperature for 30 min. Next, the crystalline violet solution was removed and the plates washed again. The plates were inverted on paper

towels and remained for 2 h at room temperature to dry. The crystalline violet dye (stained biofilm) was then solubilized by incubation with 200 µL of ethanol per well for 30 min. Then, 100 µL of the dye in ethanol was transferred to wells of a new microplate submitted to reading absorbance at 575 nm in a spectrophotometer reader (Eon Microplate, BioTek Instruments, USA) to quantify the biomass of the biofilm (Mattos-Graner, Napimoga, Fukushima, Duncan & Smith, 2004).

2.6 Cytotoxicity Evaluation

These assays were conducted based on the methodology described by Bedran, Mayer, Spolidorio, & Grenier (2014) in duplicate. Epithelial cells from HaCat line were cultured in Dulbecco's modified Eagle's medium – DMEM (Gibco BRL, Carlsbad, CA, USA) plus 10% fetal calf serum and 100 µg/mL of penicillin G/streptomycin. The cell lineage was grown until reaching a subconfluent density at 37^{o}C in 5% CO2 atmosphere. The epithelial cells were harvested following a trypsin treatment (5 min) (TrypLETM Express; Life Technologies Inc.) at 37°C. Proteases were then inactivated by adding 0.3 mg/mL of trypsin inhibitor, and the cells were harvested by centrifugation (500xg for 5 min), suspended in fresh medium, seeded in a 96-well microplate (200 µL/well, 1x10^{6} cells/mL) and incubated overnight at 37°C in a 5% CO2 atmosphere to allow for cell adhesion before stimulation. All treatments (at 1mg/mL) were diluted at 1:150 in DMEM, previously their application on cell cultures. This dilution was based in the microbiological data and cytotoxicity assays previously performed with P1025 (concentrations above 62.5 µg/mL were toxic to HaCat cells). The cells were then stimulated with F, P1025, F+P1025, F+CHX and CHX for 5, 30 and 60 min at 37°C in a 5% CO2 atmosphere. After these times of exposure, culture medium was removed and new medium was replaced to the wells and cells were again incubated for 24h. After that, a colorimetric MTT cell viability assay (Roche Diagnostics, Mannheim, Germany), using 3-[4,5-diethylthiazol- 2-yl]-2,5-diphenyltetrazolium bromide as the substrate, was used to determine the effect of the treatments on cell viability. After 24h, the extracts were aspirated and replaced by 90 µL of DMEM plus 10 µL of MTT solution (5 mg/mL sterile PBS; Sigma Aldrich). After that, the culture medium with the MTT solution was aspirated and replaced with 100 µL of acidified isopropanol solution (0.04 NHCl). Two 50 µL aliquots of each well were transferred to 96-well plates (Costar Corp., Cambridge, MA, USA). Cell viability was evaluated using spectrophotometry as being proportional to the absorbance measured with a 570 nm wavelength and an ELISA microplate reader (model 3550-UV, Bio-Rad Laboratories, Hercules, CA, USA). The means were calculated for the groups and transformed into

percentages, which represented the inhibitory effect of the mitochondrial activity of the cells by the extracts. The negative control (DMEM) was defined as having 100% cell metabolism.

2.7 Statistical Analyses

The characterizations of the formulations are presented using descriptive analysis. The results for the quantification of biofilm biomass (in optical density – Abs 550nm) were analyzed according to the different phases of biofilm: surface-bound salivary phase and salivary fluid-phase and submitted to Kruskal-Wallis and Mann-Whitney tests, comparing the different groups of treatments. Data from cytotoxicity were analyzed by ANOVA/Tukey tests. All tests were analyzed using SPSS version 17.1, considering $p<0.05$.

3 **RESULTS**

The ternary phase diagram illustrated in Figure 1 shows that the formulations begins to structure from 40 % of surfactant and the viscosity of the formulations increases from 40% surfactant with water addition. Then, three points named F1-C, F2-C and F3-C of the diagram were selected because they are located in a region of viscosity transition, which may promote higher retention and a longer drug release. Table 1 indicates the composition (%) of the three selected systems. The results of PLM are shown in Figure 2. These photomicrographs demonstrate that F3-C is a microemulsion because is a TLS and isotropic visualized as dark field, while the formulation F2-C and F1-C are lamellar and hexagonal liquid crystalline mesophases since they are anisotropic samples identified respectively by malt crosses and striations.

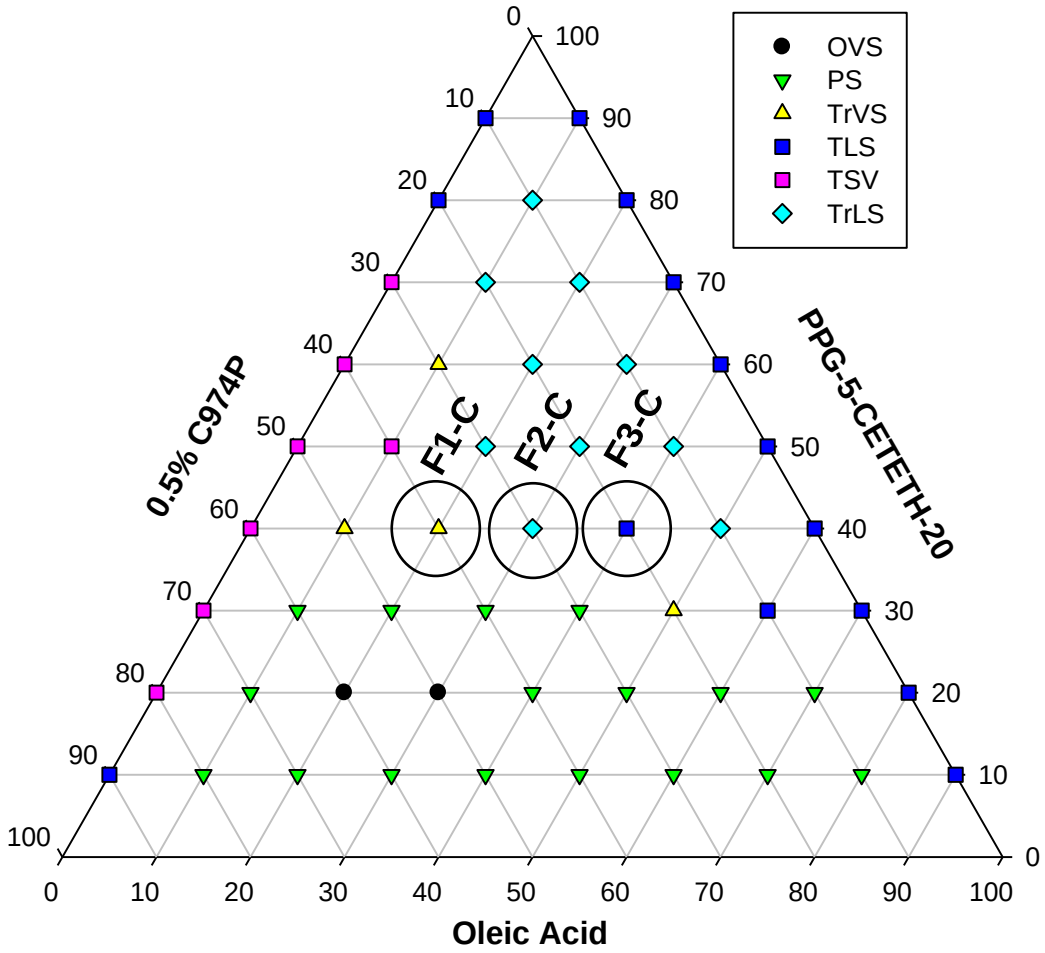

Figure 1 - Ternary phase diagram of polyoxypropylene-(5)-polyoxyethylene-(20)-cetyl alcohol (PPG-5-CETETH-20), oleic acid and 0.5% C974P dispersion. F1-C, F2-C and F3-C were the selected formulations for the structural characterization.

Table 1 – Composition (%) of the selected formulations for the characterization.

Sistemas	% OA	% PRO	% W	% C974
F1-C	20	40	30	10
F2-C	30	40	20	10
F3-C	40	40	10	10

OA: oleic acid; PRO: PPG-5-CETETH-20; W: water, C974: Polymer dispersion of Carbopol® C974P.

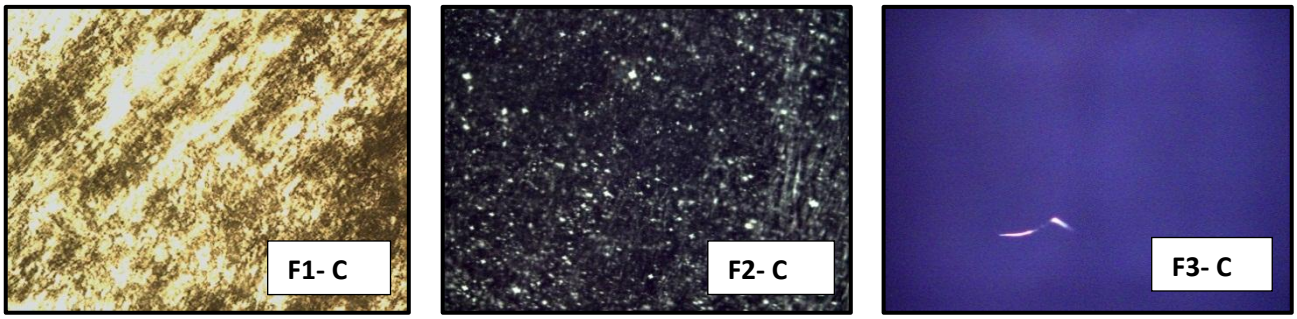

Figure 2 - Polarized light microscopy photomicrographs of the formulations F1-C, F2-C and F3-C.

In order to confirm the results obtained by polarized light microscopy, SAXS measurements were presented in Figure 3. F3-C presents a broad and wide peak characteristic of microemulsion, confirming PLM results. F1-C and F2-C indicate that these systems are lamellar phase LC. PLM showed that only F2-C is a lamellar mesophase; thus, F1-C might be in a transition phase from lamellar to hexagonal mesophase.

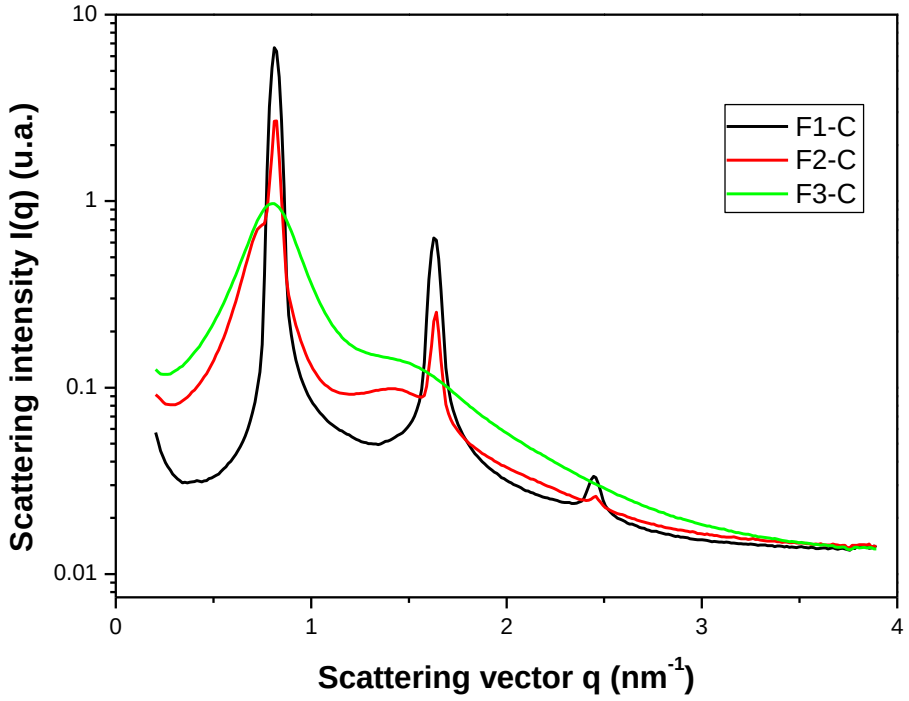

Figure 3 - SAXS results obtained for the formulations F1-C, F2-C and F3-C.

Table 2 shows the distance (d) between lamellae using the equation d = 2p/qmax, where d is correlation distance and qmax is the value of the scattering vector q at the

maximum intensity I(qmax) to calculate the spacing between the lamellae. It was observed that d increases with the water amount (7.75 to 7.85 nm) suggesting the polarization of the polar head of the surfactant by the addition of water leading to the increase of the curvature and the volume of the polar region, generating hexagonal structures because of the greater packaging.

Table 2 - Values of qmax (Å) and distance (d) between lamellae.

Formulations	q_{max1}	q_{max2}	q_{max3}	**d (nm)**
F1-C	0,81	1,62	2,43	7,85
F2-C	0,83	1,62	2.45	7,75
F3-C	na*	na	na	na

*not applicable.

The rheological characteristics analyzed in F1-C, F2-C e F3-C determined the flow properties and oscillatory analysis and these properties are shown in Figures 4 and 5, respectively. Figure 4 shows that both F1-C and F2-C exhibit non-Newtonian pseudoplastic behavior, whereas F3-C exhibits Newtonian behavior. Regarding the analysis of the downward curve, F1-C did not overlap its upward curve, forming an area of hysteresis, thus classified as time-dependent thixotropic. In the Figure 5, F1-C obtained storage module (G') values higher than loss module (G"), a characteristic of elastic formulations. The other formulations presented the values of the loss modulus (G") higher than the storage modulus (G'), throughout the studied frequency range, that indicates a predominantly viscous behavior characteristic of poorly organized systems. Table 3 shows the results of the bioadhesion test, indicating that F1-C had higher biadhesion strength compared to F2-C and F3-C.

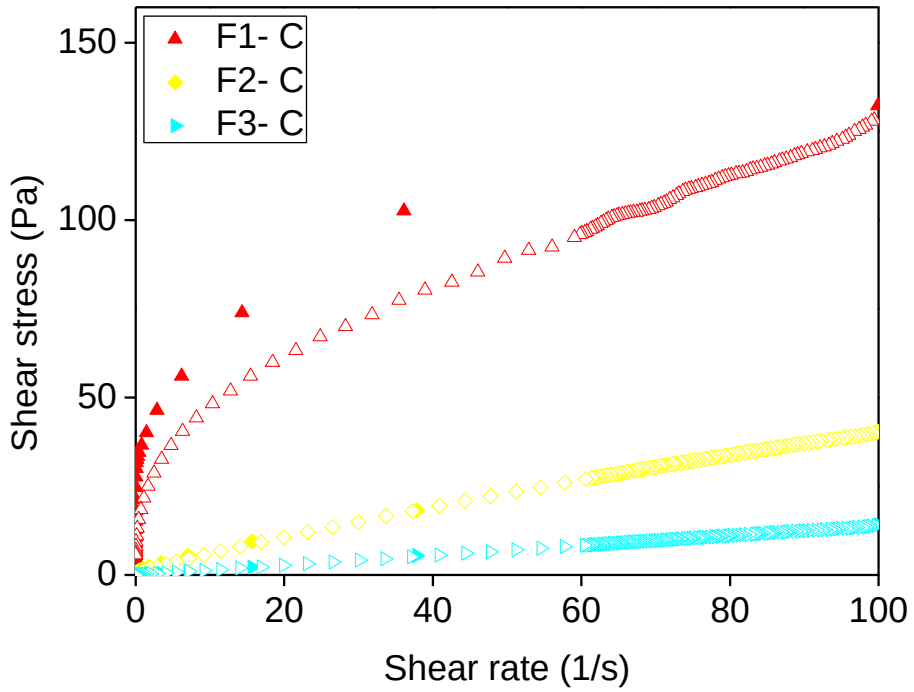

Figure 4 – Flow properties of formulations F1-C, F2-C and F3-C. (Ascendent curve - filled symbol and descendent curve - empty symbols).

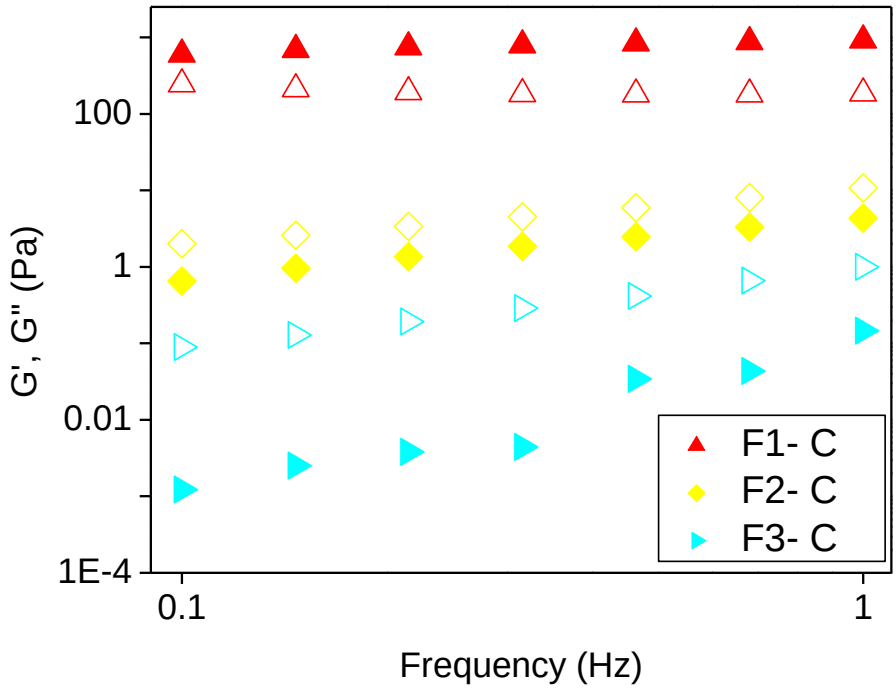

Figure 5 - Variation of the storage module (G' - filled symbol) and loss module (G"- empty symbol) as a function of the frequency to F1-C, F2-C and F3-C.

Table 3 – Work of the bioadhesive strength (mN.s) of the formulations. Values represent the mean ± standard deviation at 37 ° C.

Formulations	Bioadhesive work (mN.s)
F1-C	26.29 ± 3.15
F2-C	12.84 ± 2.14
F3-C	11.23 ± 0.70

The results for the quantification of biofilm biomass in the two different phases of biofilm: surface-bound salivary phase and salivary fluid-phase are shown in Figures 6 and 7, respectively. All treatments had effect on biofilm formation when applied in the surface-bound salivary phase. The best anti-biofilm effects were observed for F+1025 and F+CHX, without statistical difference between them. When applied in salivary fluid-phase, P1025 had no effect on biofilm formation. There was no statistical difference among the F groups (F, F+P1025 and F+CHX). CHX solution had the best effect on biofilm formation.

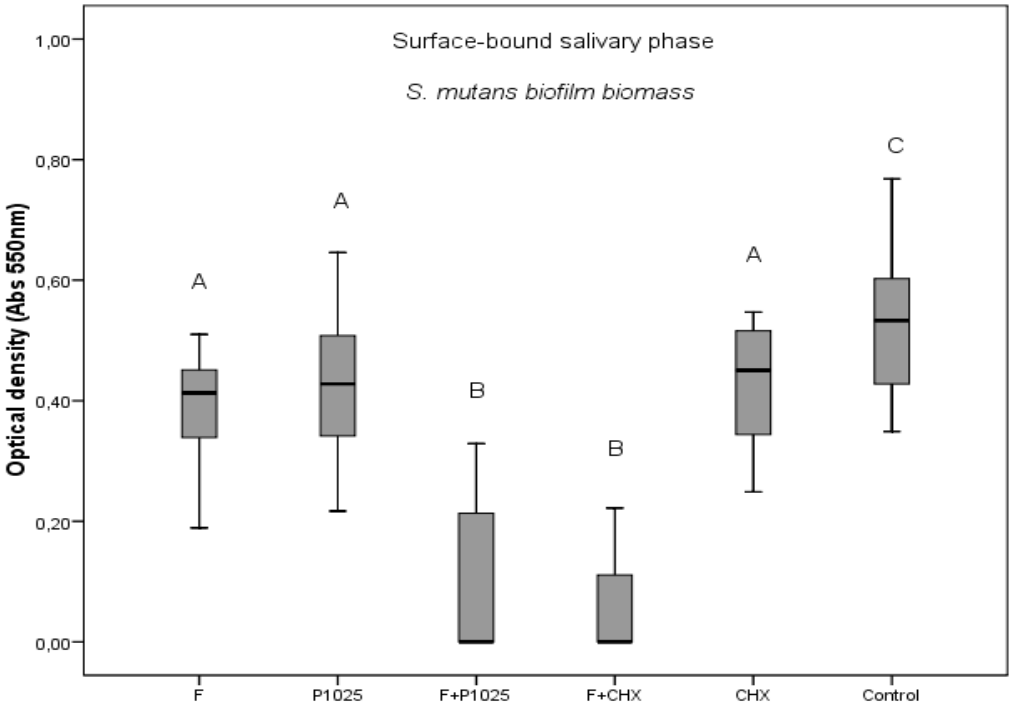

Figure 6 – Quantification of S. *mutans* biofilm biomass after 24h of the treatments applications in the surface-bound salivary phase.

[A] Different upper case letters show statistical difference among the groups, according to Kruskal-Wallis/Mann-Whitney tests ($p<0.05$)

F – Liquid crystalline formulation; P1025 – peptide 1025; F+P1025 – formulation + peptide 1025; CHX – chlorhexidine; F+CHX – formulation + chlorhexidine; Control – bacterial culture without treatments.

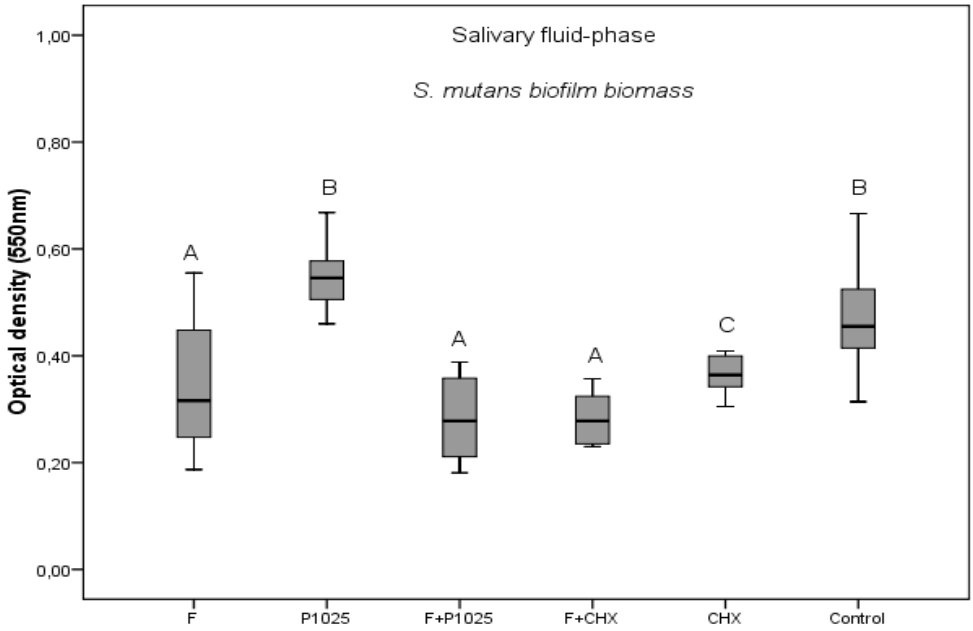

Figure 7 – Quantification of S. *mutans* biofilm biomass after 24h of the treatments applications in the salivary fluid-phase.

[A] Different upper case letters show statistical difference among the groups, according to Kruskal-Wallis/Mann-Whitney tests ($p<0.05$)

Figure 8 shows the effect of the treatments on epithelial cells, according the time of exposure. Considering the conditions of this study, CHX and P1025 solutions were not cytotoxic at any time evaluated. F increased its toxicity with the increase of the time of exposure. There was no statistical difference among the times of exposure for F+P1025. After 30 and 60 min, the toxicity of F+CHX statistically increased when compared with 5 min. Overall, percentage of cell viability was 70% or higher for F, F+P1025 and F+CHX, independent of the time of exposure, showing low cytotoxicity for the treatments.

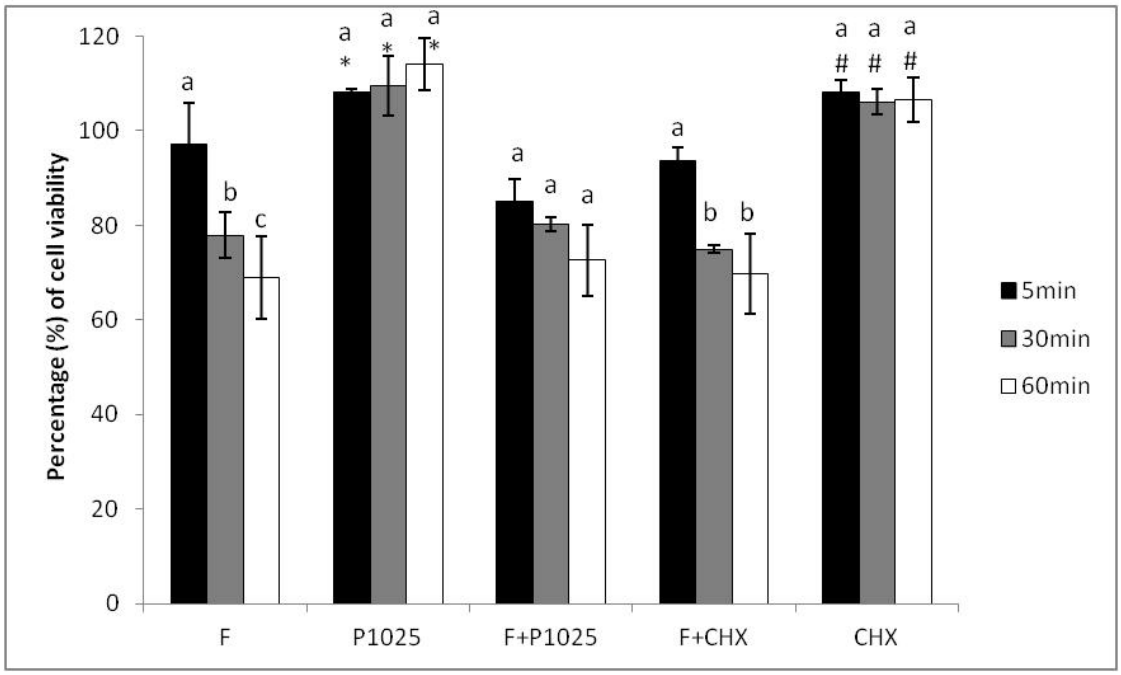

Figure 8 – Epithelial cell viability after 5, 30 and 60 min of the treatments and growth for 24h.

[a] Different letters show statistical difference among the times of exposure (5, 30 or 60min), considering each group separately, according to ANOVA/Tukey tests.

[*] Statistical difference between P1025 and the other groups (F, F+P1025, F+CHX), except for CHX, considering each time of exposure separately, according to ANOVA/Tukey tests.

[#] Statistical difference between CHX and the other groups (F, F+P1025, F+CHX), except for P1025, considering each time of exposure separately, according to ANOVA/Tukey tests.

4 DISCUSSION

The controlled release system is an efficient alternative for carrying peptides (Guterres, Alves & Pohlmann, 2007). This study analyzed three formulations with the same components, varying only the concentration of each component. The selected formulations were structurally analyzed by polarized light microscopy, SAXS, rheological and bioadhesive strength. Thus, the formulation with the best characteristics of a liquid crystalline system was selected for later incorporation of the peptide P1025 and testing the activity on biofilm of *S. mutans*, the main etiological agent of dental caries (Alam et al., 2000; Hamada & Slave, 1980).

Structural analysis of formulations tested in this study showed that F1-C formulation composed of 20% oleic acid, 40% PPG-5-CETETH-20, 30% water and 10% C974 at 5% presented the best characteristics of a nanostructured bioadhesive liquid crystalline system. The three points were selected in the ternary phase diagram because they are in a region of viscosity transition, which may promote higher retention and a longer drug release. Besides, in this region, formulations have low concentration of surfactant in order to decrease the potential toxicity of the formulations (Klein, 2007). Polarized light microscopy is a widely

used tool for primary identification of microemulsions and mesophases of liquid-crystalline systems. In order to confirm the results obtained by PLM, SAXS measurements were performed to provide more precise results about the nanostructure of the formulations. The formulations F1-C and F2-C showed well-defined peaks, characteristic of liquid crystalline mesophases. However, the liquid crystalline mesophase was identified correlating the position of the peaks with the first peak. Thus, lamellar mesophases follow the relation 1 ÷ 2 ÷ 3 and hexagonal mesophases 1 ÷ 1.73 ÷ 2 ÷ 7. Finally, the values were 1.41 ÷ 1.73 ÷ 2.82 ÷ 3 for cubic mesophases. Both formulations F1-C and F2-C exhibited three well-defined Bragg peaks with 2:3 peak ratio indicating that they were lamellar phase liquid-crystalline systems. PLM showed that only F2-C was a lamellar mesophase and F1-C might be in a transition phase from lamellar to hexagonal mesophase. Furthermore, in F1-C, the distance between lamellae increases with the water amount (7.75 to 7.85 nm) suggesting that the polarization of the polar head of the surfactant by the addition of water leads to the increase of the curvature and the volume of the polar region, generating hexagonal structures because of the greater packaging. Therefore, these results seem compatible with the proposed objective since the formulations chosen in the diagram were able to behave as a precursor system of liquid crystal, because when increasing amounts of water were added, the formation of liquid crystal systems occurred.

F1-C and F2-C exhibit non-Newtonian pseudoplastic behavior, but analyzing the downward curve, the F1-C did not overlap its upward curve, forming an area of hysteresis, thus classified as time-dependent thixotropic. From the rheograms, Equation 1 below was used to calculate the value of n to characterize the flow behavior and k to characterize the viscosity of the material. $\tau = k \cdot \gamma^n$ (Equation 1). Where: τ is the shear rate, k is the consistency index, γ is the shear stress, and n is the flow behavior. In this model, n> 1 represents a dilatant fluid, n <1 represents a pseudoplastic fluid and n = 1 represents a Newtonian fluid. In addition, the degree of pseudoplasticity can be measured by the flow behavior (n), which increases with decreasing pseudoplasticity (Formariz et al., 2008). The dynamic viscosity of the formulations can be evaluated with the consistency index (k), which increases with the viscosity of the formulation. In general, as the amount of aqueous phase increases in the systems, the degree of pseudoplasticity becomes larger as well as the index of consistency. Thus, the formulations F1-C had the highest degree of pseudoplasticity (n = 0.30, k = 34.17), followed by F2-C (n = 0.76, k = 1.18) and F3-C (n=1; k = 0.15). The microemulsions presented Newtonian behavior, since they did not have any type of organized structure that can be unstructured by the shear stress (Longo, 2006). The pseudoplasticity is

due to the liquid-crystalline structure, which causes higher resistance to flow than the microemulsions, which are colloidal systems (Carvalho, 2009). Such property is desirable for formulations developed for oral administration, for example, during application at high shear rates, the interlacing of the polymer chains and subsequent thinning of the flow will occur, thereby facilitating administration of the formulation. However, upon withdrawal of this tension, the formulation will recover its initial viscosity, remaining longer in the oral environment.

In relation to the thixotropic character, F1-C shows thixotropy that is directly related to the interaction between the components of the formulation. In virtue of the existence of interactions forces among the more structured regions, the structuring can be destroyed with the increase of the shear rate and easily recovered when that speed decreases. Thus, the formulations with the highest degree of thixotropy also have a higher degree of initial structuring. This increase in the area of hysteresis is closely related to the increase in the microstructure of the liquid-crystalline networks, proving that the area of hysteresis is strongly influenced by the presence of liquid crystals (Chorilli, 2007).

The oscillatory rheological analysis evaluates the viscoelastic properties of the formulations, which provides information about the structural nature of the system, which directly implies in the performance of the formulation. This information is obtained by analysis of the elastic modulus (G') and the viscous modulus (G''). The elastic modulus, G' is called the storage modulus, representing both the energy stored during deformation when the voltage increases, and the energy released when the voltage is relaxed. However, the viscous modulus G'' is the viscous element that can not store energy, because the applied stress dissipates in the form of irreversible deformation (Pènzes, 2004). Thus, in order to study the temporal evolution of the storage modulus (G') and loss (G'') as a function of the frequency applied, the oscillatory analysis was carried out in the formulations. The formulations F2-C and F3-C presented the values of the loss modulus (G'') higher than the storage modulus (G'), throughout the studied frequency range, that indicates a predominantly viscous behavior, characteristic of poorly organized systems, unlike F1-C. The results also showed that the higher of the aqueous phase ratio in all systems, the higher elastic character of the formulations. This affirmation can be explained by the formation of a liquid crystal with the addition of aqueous phase. The lamellar phase generally appears as viscous liquid and the hexagonal phase has the gel-like viscosity (Gabboun, Najib, Ibrahin & Assaf, 2001; Hyde, 2001). The rheological data corroborate with the SAXS analysis, showing that when the

aqueous phase increases, the systems structure becomes more ordered indicated by the increase in lamellae spacing and elasticity.

The main advantage of bioadhesive systems as drug delivery system is the maintenance of the drug at the application site for longer periods, which allows enhanced contact of the formulation with the biological barrier, reducing the frequency of product application and increasing patient compliance (Smart et al., 2003). The bioadhesion results show that with the increase of the aqueous phase in the systems significantly increased the bioadhesion of the formulations, also due the formation of liquid crystals. The highest values of bioadhesion for the liquid-crystalline systems can be explained by their rheological properties. The increase of their viscosity and elastic characteristics contributes to the increase in the residence time of the formulation. Furthermore, synthetic polymers derived from polyacrylic acid are negatively charged and also bioadhesive. In this case, bioadhesion results are due to physicochemical processes, such as hydrophobic interactions, hydrogen and van der Waals interactions, which are controlled by pH and ionic composition (Woodley, 2001). In addition, their chains are flexible as enough to diffuse in the mucus layer and penetrate to form a network. Most of the polyacrylic acid derivatives are not soluble in water, but form viscous gels when hydrated (Carvalho, 2009), increasing the ability to adhere to the tooth surfaces. There is no standard formula available for bioadhesive drug delivery systems for dental application. However, the liquid crystalline system formed by the Carbopol® polymer as aqueous phase has proved to be suitable, because with the contact with water and consequent hydration, there is an increase on bioadhesion strength, pseudoplasticity and elasticity showing a promising platform for the prevention of dental caries.

In this present study, P1025, a peptide that corresponds to residues 1025-1044 in the C-terminal region of SA I/II, was chosen to be incorporated in LCS. When applied in the salivary surface-bound phase, P1025 incorporated or not in LCS showed inhibitory effect on the biofilm formation. It is know that synthetic peptide P1025 is able to bind to salivary agglutinins and interfere with *S. mutans* adhesion mediated by SA I/II, as confirmed by surface plasmon resonance (Kelly et al., 1999). The same effect was not observed when P1025 was applied in the salivary fluid-phase. It is possible that P1025 attached to salivary agglutinins will be not so easily degraded by salivary proteases as in the fluid-phase. Data from literature reports that the release of agents from liquid crystalline system is around 4% at 24 h (Hu, Hong, & Yuan, 2004). Although data from release of antimicrobial agents were not available in this present study, the peptide and CHX release from LCS was probably higher than 4%, since the formulation was diluted previously to incorporation of antimicrobial agents

for biofilm assays to reduce viscosity. Besides, there was an increase in the anti-biofilm activity of F+P1025 compared to P1025 or F+CHX compared to CHX within 24h, showing a cumulative effect of these agents when incorporated into F. This result may suggest that P1025 and CHX were released slowly within 24h, since mean of bacterial reduction was around 20% for P1025/CHX and 80% for F+P1025/F+CHX. Lower bacterial reduction (around 40%) was observed for both F containing P1025 or CHX when applied in salivary fluid-phase, probably related to dilution in saliva. The additional antimicrobial effect of F (around 27%) was observed for the biofilm assays, adding with the effects of the agents incorporated into them. The high concentration of oil in LCS limits water access, which is one of the main causes of antimicrobial action (Oliveira et al., 2007).

There are few studies evaluating the inhibitory activity of P1025 on pathogenic microorganisms related to dental caries etiology. Kelly et al. (1999) demonstrated that topical application of P1025 might selectively prevent recolonization of teeth surface by S. *mutans*. The relatively long-term resistance to colonization cannot be explained by persistence of the peptide in the oral cavity. The authors suggested that the peptide competitively inhibits initial adhesion of the bacterium to dental structure, but another bacteria competing by the same ecological niche could prevent subsequent colonization. Another study developed by Li, Wang & Lai (2009) evaluated the influence of P1025 in solution and two different dentifrices containing P1025 on adherence of mutans streptococcus (MS) and observed an inhibitory effect on MS adherence in both hydroxyapatite (in vitro) and dental biofilm (in vivo). The most significant inhibitory in vitro activity was shown at 50 μmol/L. The clinical efficacy of the dentifrices was confirmed by the significant reduction in the plaque scores and MS counts from subjects after 1 month-treatment with both dentifrices containing P1025 in comparison with control dentifrice.

No study evaluated the toxicity of P1025 against eukaryotic cells. In this study, P1025 had toxic effect against epithelial cells at the concentrations above 62.5 μg/mL. The toxicity of P1025 was increased when incorporated into LCS; however, percentage of cell viability was maintained around 70% until 1h of exposure. Any cytotoxic effect was observed when LCS was applied on macrophage cultures (Oliveira et al., 2015).

Considering the high capacity of pathogenic bacteria in developing resistance to current antimicrobial agents, topical application of P1025 in LCS could provide a rational adjunctive approach for the prevention of dental caries. However, more studies are necessary to prove this efficiency, mainly in vivo studies.

5 CONCLUSIONS

The formulation composed of 20% oleic acid, 40% Procetyl, 30% water and 10% C974P at 5% presented the best characteristics of a liquid-crystalline bioadhesive nanostructured system. P1025-load nanostructured liquid crystalline system presented low toxicity on epithelial cells and effect on biofilm of *S. mutans* and seems to be an interesting drug delivery strategy for prevention of dental caries.

CONFLICTING OF INTEREST

The authors declare that there is no conflict of interest.

ACKNOWLEDGMENTS

This study was funded by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) - grants # 2012/19235-5 and # 2013/12285-0, Brazil.

REFERENCES

1. Ahn SJ, Ahn SJ, Wen ZT, Brady LJ, Burne RA. (2008). Characteristics of biofilm formation by *Streptococcus mutans* in the presence of saliva. *Infection and Immunity*. 76(9),4259-4268.

2. Alam, S., Brailsford, S.R., Adams, S., Allison, C., Sheehy, E., Zoitopoulos, L., et al. (2000). Genotypic heterogeneity of *Streptococcus oralis* and distinct aciduric subpopulations in human dental plaque. *Applied and Environmental Microbiology*, 66(8), 3330-3336.

3. Calixto, G., Yoshii, A.C., Rocha e Silva, H., Stringhetti Ferreira Cury, B., & Chorilli, M. (2015). Polyacrylic acid polymers hydrogels intended to topical drug delivery: preparation and characterization. *Pharmaceutical Development Technology*, 20(4), 490-496.

4. Calixto, G.M., Garcia, M.H., Cilli, E.M., Chiavacci, L.A., & Chorilli, M. (2016). Design and characterization of a novel P1025 peptide-loaded liquid crystalline system for the treatment of dental caries. *Molecules*, 21(2), 158.

5. Carlén, A., & Olsson, J. (1995). Monoclonal antibodies against a high-molecular-weight agglutinin block adherence to experimental pellicles on hydroxyapatite and aggregation of *Streptococcus mutans*, *Journal of Dental Research*, 74(4), 1040-1047.

6. Castillo MCP. Função de DNA extracelular e de ácido lipoteicóico nas propriedades estruturais e funcionais da matriz extracelular de biofilmes cariogênicos. Dissertação de mestrado. Universidade Estadual Paulista “Júlio de Mesquita Filho”, BR, 2016.

7. Carvalho, F.C. Desenvolvimento e caracterização de sistemas nanoestruturados para potencial administração nasal de zidovudina. 2009. 143f. Dissertação de Mestrado .Universidade Estadual Paulista "Júlio de Mesquita Filho", BR, 2009.

8. Chen, Y., Ma, P., & Gui, S. (2014). Cubic and hexagonal liquid crystals as drug delivery systems. *BioMed Research International*, 2014, 815981.

9. Chorilli, M., Prestes, P.S., Rigon, R.B., Leonardi, G.R., Chiavacci, L.A., Sarmento, V.H.V., et al. (2011) Structural characterization and in vivo evaluation of retinyl palmitate in non-ionic lamellar liquid crystalline system. *Colloids and Surfaces. B, Biointerfaces*, 85, 182−188.

10. Chorilli, M. Desenvolvimento e caracterização físico-química de sistemas nanoestruturados contendo palmitato de retinol: controle microbiológico, avaliação da segurança e eficácia no tratamento do envelhecimento cutâneo. Dissertação de Mestrado. Universidade Estadual Paulista "Júlio de Mesquita Filho, BR, 2007.

11. Clinical and Laboratory Standard Institute (CLSI). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard, 9th ed., Wayne, PA, CLSI document, 2012, M7-A9.

12. Filoche, S., Wong, L., & Sissons, C.H. (2010). Oral Biofilm: emerging concepts in microbiol ecology. *Journal of Dental Research*, 89(1), 8-18.

13. Formariz, T.P., Chiavacci, L.A., Sarmento, V.H., Franzini, C.M., Silva, A.A. Jr, Scarpa, M.V., et al. (2008). Structural changes of biocompatible neutral microemulsions stabilized by mixed surfactant containing soya phosphatidylcholine and their relationship with doxorubicin release. *Colloids and Surfaces. B, Biointerfaces*, 63(2), 287-295.

14. Gabboun, N.H., Najib, N.M., Ibrahim, H.G., & Assaf, S. (2001). Release of salicylic acid and diclofenac acid salts from isotropic and anisotropic nonionic surfactant systems across rat skin. *International Journal of Pharmaceutics*, 212(1), 73-80.

15. Gajdziok, J., Bajerová, M., Chalupová, Z., & Rabisková, M. (2010). Oxycellulose as mucoadhesive polymer in buccal tablets. *Drug Development and Industrial Pharmacy*, 36(9), 1115–1130.

16. Gawande, P.V., Leung, K.P., & Madhyastha, S. (2014). Antibiofilm and antimicrobial efficacy of DispersinB®-KSL-W peptide-based wound gel against chronic wound infection associated bacteria. *Current Microbiology*, 68(5), 635-641.

17. Guo, C., Wang, J., Cao, F., Lee, R.J., & Zhai, G. (2010). Lyotropic liquid crystal systems in drug delivery. *Drug Discovery Today*, 15(23-24), 1032-1040.

18. Guterres, S.S., Alves, M.P. & Pohlmann, A.R. (2007). Polymeric nanoparticles, nanospheres and nanocapsules, for cutaneous application. *Drug Target Insights*, 2,147-157.

19. Hahnel, S., Mühlbauer, G., Hoffmann, J., Ionescu, A., Bürgers, R., Rosentritt, M., et al. (2012). *Streptococcus mutans* and *Streptococcus sobrinus* biofilm formation and metabolic activity on dental materials. *Acta Odontologica Scandinavica*, 70(2), 114-21.

20. Hamada, S., & Slade, H.D. (1980). Biology immunology, and cariogenicity of *Streptococcus mutans. Microbiological Reviews*, 44(2), 331-384.

21. Hu, F.Q., Hong, Y., & Yuan, H. (2004). Preparation and characterization of solid lipid nanoparticles containing peptide. *International Journal of Pharmaceutics*, 273(1-2), 29-35.

22. Hyde, S.T. Handbook of Applied Surface and Colloid Chemistry. New York: John Wiley & Sons, 2001. cap. 16. p. 299-332.

23. Kelly, C.G., Younson, J.S., Hikmat, B.Y., Todryk, S.M., Czisch, M., Haris, P.I., et al. (1999). A synthetic peptide adhesion epitope as a novel antimicrobial agent. *Nature Biotechnology*, 17(1), 42-47.

24. Klein, T. Desenvolvimento de sistemas nanoestruturados estabilizados com álcool cetilico etoxilado e propoxilado contendo fluconazol potencialmente ativo contra esporotricose. 2007. 114f. Dissertação de Mestrado. Universidade Estadual Paulista "Júlio de Mesquita Filho", BR, 2007.

25. Lagerweij, M.D., & van Loveren, C. (2015). Declining caries trends: are we satisfied?. *Current Oral Health Reports*, 2(4), 212-217.

26. Lee, S.F., Progulske-Fox, A., & Bleiweis, A.S. (1988). Molecular cloning and expression of a *Streptococcus mutans* major surface protein antigen, P1 (I/II), in Escherichia coli. *Infection and Immunity*, 56(8), 2114–2119.

27. Li, M.Y., Wang, J., & Lai, G.Y. (2009). Effect of a dentifrice containing the peptide of streptococcal antigen I/II on the adherence of mutans streptococcus. *Archives of Oral Biology*, 54(11), 1068-1073.

28. Loftsson, T., Leeves, N., Bjornsdottir, B., Duffy, L., & Masson, M. (1999). Effect of cyclodextrins and polymers on triclosan availability and substantivity in toothpastes in vivo. *Journal of Pharmaceutical Sciences*, 88(12), 1254–1258.

29. Longo, D.P. Obtenção, caracterização e estudos de liberação *in vitro* e permeação *in vivo* de sistemas microestruturados contendo cafeína**.** Dissertação de Mestrado. Universidade Estadual Paulista "Júlio de Mesquita Filho", BR, 2006.

30. Marsh, P.D. (2006). Dental plaque as a biofilm and a microbial community - implications for health and disease. *BMC Oral Health*, 6:S14.

31. Mattos-Graner, R.O., Napimoga, M.H., Fukushima, K., Duncan, M.J., & Smith, D.J. (2004). Comparative analysis of Gtf isozyme production and diversity in isolates of *Streptococcus mutans* with different biofilm growth phenotypes. *Journal of Clinical Microbiology*, 42(10), 4586-4592.

32. Oliveira, T.A.; Paixão, F.G.; Prestes, O.S.; Pires-de-Campos, M.S.M.; Polacow, M.L.O.; Chorilli, M.; et al. (2007). Avaliação da atividade antimicrobiana de sistemas nanoestruturados. *Acta Farmacológica Bonaerense*, 26:878-882, 2007.

33. Oliveira, M.B., Calixto, G., Graminha, M., Cerecetto, H., González, M., & Chorilli, M. (2015). Development, characterization, and in vitro biological performance of fluconazole-loaded microemulsions for the topical treatment of cutaneous leishmaniasis. *Biomed Research International*, 396894.

34. Pecharki, D., Petersen, F.C., Assev, S., & Scheie, A.A. (2005). Involvement of antigen I/II surface proteins in *Streptococcus mutans* and *Streptococcus intermedius* biofilm formation. *Oral Microbiology and Immunology*, 20(6), 366–371.

35. Pènzes, T. Rheological analysis of the structural properties effecting the percutaneous absorption and stability in pharmaceutical organogels. Rheol Acta. 2004**;** 43(5):457–463.

36. Robinette, R.A., Heim, K.P., Oli, M.W., Crowley, P.J., McArthur, W.P., & Brady. L.J. (2014). Alterations in immunodominance of *Streptococcus mutans* Ag I/II: Lessons learned from immunomodulatory antibodies. *Vaccine*, 32(3), 375-382.

37. Russell, M.W., Bergmeier, L.A., Zanders, E.D., & Lehner, T. (1980). Protein antigens of *Streptococcus mutans*: purification and properties of a double antigen and its protease-resistant component. *Infection and Immunity*, 28(2), 486-493.

38. Salamat-Miller, N., Chittchang, M., & Johnston, T.P. (2005). The use of mucoadhesive polymers in buccal drug delivery. *Advanced Drug Delivery Reviews*, 57(11), 1666– 1691.

39. Salmazi, R., Calixto, G., Bernegossi, J., Ramos, M.A., Bauab, T.M., & Chorilli, M.A. (2015). Curcumin-loaded liquid crystalline precursor mucoadhesive system for the treatment of vaginal candidiasis. *International Journal of Nanomedicine*, 10, 4815-4824.

40. Santos, F.K., Oyafuso, M.H., Kiill, C.P., Gremião, M.P.D., & Chorilli, M. (2013). Nanotechnology-based drug delivery systems for treatment of hyperproliferative skin diseases-a review. *Current Nanoscience*, 9(1):159–167.

41. Sanui, T., & Gregory, R.L. (2009). Analysis of *Streptococcus mutans* biofilm proteins recognized by salivary immunoglobulin A. *Oral Microbiology and Immunology*, 24(5), 361–368.

42. Smart, J.D., Riley, R.G., Tsibouklis, J., Young, S.A., Hampson, F.C., Davis, J.A., et al. (2003). The retention of 14C-labelled poly(acrylic acids) on gastric and oesophageal mucosa: an in vitro study. *European Journal of Pharmaceutical Sciences*, 20(1), 83-90.

43. Smith, R., Lehner, T., & Beverley, P.C.L. (1984). Characterization of monoclonal antibodies to *Streptococcus mutans* antigenic determinants III, I, II, and III and their serotype specificities. *Infection and Immunity*, 46(1), 168-175.

44. Smith, D.J. (2003). Caries vaccines for the twenty-first century. Journal *of Dental Education*, 67(10), 1130-1139.

45. Soares, D.G.S., Ribeiro, A.P.D., Sacono, N.T., Coldebella, C.R., Hebling, J., & Costa, C.A. (2011). Transenamel and transdentinal cytotoxicity of carbamide peroxide bleaching gels on odontoblast-like MDPC-23 cells. *International Endodontic Journal*, 44(2), 116-125.

46. Souza, C., Watanabe, E., Borgheti-Cardoso, L.N., De Abreu Fantini, M.C., & Lara, M.G. (2014). Mucoadhesive system formed by liquid crystals for buccal administration of poly(hexamethylene biguanide) hydrochloride. *Journal of Pharmaceutical Sciences*, 103(12), 3914-3923.

47. Woodley, J. (2001). Bioadhesion, new possibilities for drug administration?. *Clinical Pharmacokinetics*, 40(2), 77-84.

48. Younson, J., & Kelly, C. (2004). The rational design of an anti-caries peptide against *Streptococcus mutans*. *Molecular Diversity*, 8(2), 121–126.

GLOSSÁRIO

Anisotropia: propriedade que caracteriza as substâncias que possuem propriedades ópticas diferentes dependendo da direção considerada; é capaz de alterar o plano da luz polarizada.

Carbopol: polímero sintético carregado negativamente e derivado do ácido poliacrílico; altamente reticulado que produz géis muito viscosos chamados fluidos viscoelásticos, que se comportam como sólidos elásticos em baixa tensão, porém começa a fluir quando essa tensão aumenta.

Cristal líquido: pertencem a um estado de matéria com propriedades entre um sólido cristalino e um líquido isotrópico, são viscosas e transparentes.

Cromatografia Líquida de Alta eficiência de fase reversa: técnica cromatográfica que separa componentes de uma mistura pela diferença de hidrofobicidade entre cada um deles.

Espalhamento de raios-X a baixo ângulo (SAXS): analisa distâncias interplanares com dimensões nanométricas, possibilitando a caracterização dos sistemas pela definição de tamanho médio e distância entre os objetos espalhadores. É utilizada para detectar estruturas cristalinas, gotículas, micelas ou mesmo avaliar a estrutura de objetos espalhadores desordenados.

Fluxo dilatante: aumento da viscosidade com o aumento da taxa de cisalhamento.

Fluxo não-Newtoniano: valores de tensão e taxa de cisalhamento não são constantes.

Fluxo Newtoniano: valores de tensão e taxa de cisalhamento são constantes, ou seja, a viscosidade desse tipo de fluxo é constante.

Fluxo plástico: necessitam de uma força externa maior que supere sua força de reticulação interna para fluir, sofrem diminuição da viscosidade quando aumenta a taxa de cisalhamento.

Fluxo pseudoplástico: materiais que sofrem diminuição na viscosidade quando a taxa de cisalhamento aumenta.

Fluxo reopético: são caracterizados pelo aumento da viscosidade durante o cisalhamento, e quando esse cisalhamento for diminuído, o material recuperará sua forma original, ou seja, de baixa viscosidade.

Fluxo tixotrópico: capacidade de um material se reestruturar, ou seja, recuperar sua estrutura inicial quando o taxa de cisalhamento for diminuída. Ocorre diminuição da viscosidade durante o aumento da taxa de cisalhamento, e quando essa taxa é diminuída, a viscosidade volta a aumentar.

Histerese: tendência de um sistema conservar suas propriedades na ausência de um estímulo que as gerou, ou ainda, é a capacidade de preservar uma deformação efetuada por um estímulo.

Isotropia: propriedade que caracteriza as substâncias que possuem as mesmas propriedades ópticas independentemente da direção considerada; não é capaz de desviar o plano de luz polarizada.

Liotrópico: formam cristais líquidos pela adição de solventes e depende de sua concentração.

Lipossoma: pequenas vesículas esféricas formadas por bicamadas concêntricas de fosfolipídios que se organizam espontaneamente ou por ultrassons em meio aquoso em que o componente da solução usado (íons, moléculas) pode preencher a cavidade do interior do lipossoma. Possui flexibilidade estrutural seja no tamanho, composição e fluidez da bicamada lipídica, como na sua capacidade de incorporar uma variedade de compostos tanto hidrofílicos como hidrofóbicos.

Mesofase líquido cristalina cúbica: micelas empacotadas em arranjo cúbico.

Mesofase líquido cristalina hexagonal: micelas empacotadas em arranjo hexagonal e são separadas por uma região contínua de água.

Mesofase líquido cristalina lamelar: bicamadas alternadas de moléculas ordenadas de tensoativo e solvente, de modo que as cadeias hidrofóbicas do tensoativo são o centro da lamela e sua parte hidrofílica está em contato com a camada de solvente.

Microemulsão: é termodinamicamente estável, isotrópicos, fluidas e transparentes de dois líquidos imiscíveis (óleo/água), estabilizados por um filme interfacial de tensoativos.

polyoxypropylene-(5)-polyoxyethylene-(20)-cetyl alcohol: O Procetyl™ AWS é um surfactante líquido não iônico solúvel em álcool e água. É útil como um agente emoliente, solubilizante, umectante, plastificante, emulsificador e umidificante.

Reologia: estuda o fluxo e a deformação dos materiais sob aplicação de forças.

Surfactante ou tensoativo: substâncias que diminuem a tensão superficial ou influenciam a superfície de contato entre dois líquidos

Taxa de cisalhamento: variação de velocidade de fluxo com a variação da altura (distância da superfície que provoca o cisalhamento).

Tensão de cisalhamento: definida como sendo a força que, aplicada a uma área da interface entre a superfície móvel e o líquido abaixo, provoca um fluxo na primeira camada de líquido e esta, na segunda, etc.

ANEXOS

ANEXO A

PARECER DE APROVAÇÃO DO COMITÊ DE ÉTICA

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"

CAMPUS ARAÇATUBA
FACULDADE DE ODONTOLOGIA
FACULDADE DE MEDICINA VETERINÁRIA

CEUA - Comissão de Ética no Uso de Animais
CEUA - Ethics Committee on the Use of Animals

CERTIFICADO

Certificamos que o Projeto de Pesquisa intitulado **"Efeito da incorporação de fragmentos de peptídeos antimicrobianos em sistemas de nanopartículas como agentes anticárie"**, Processo FOA nº 2014-00618, sob responsabilidade de Cristiane Duque apresenta um protocolo experimental de acordo com os Princípios Éticos da Experimentação Animal e sua execução foi aprovada pela CEUA em 26 de junho de 2014.

VALIDADE DESTE CERTIFICADO: 27 de Julho de 2015.
DATA DA SUBMISSÃO DO RELATÓRIO FINAL: até 27 de Agosto de 2015.

CERTIFICATE

We certify that the study entitled **"Effect of incorporation of antimicrobial peptides fragments in nanoparticles systems as anticaries agents"**, Protocol FOA nº 2014-00618, under the supervision of Cristiane Duque presents an experimental protocol in accordance with the Ethical Principles of Animal Experimentation and its implementation was approved by CEUA on June 26, 2014.

VALIDITY OF THIS CERTIFICATE: July 27, 2015.
DATE OF SUBMISSION OF THE FINAL REPORT: August 27, 2015.

Prof. Dr. Edilson Ervolino
Coordenador da CEUA
CEUA Coordinator

CEUA - Comissão de Ética no Uso de Animais
Faculdade de Odontologia de Araçatuba
Faculdade de Medicina Veterinária de Araçatuba
Rua José Bonifácio, 1193 – Vila Mendonça - CEP: 16015-050 – ARAÇATUBA – SP
Fone (18) 3636-3234 Email CEUA: ceua@foa.unesp.br

ANEXO B

APROVAÇÃO DO RELATÓRIO FINAL PELO COMITÊ DE ÉTICA

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
CAMPUS ARAÇATUBA
FACULDADE DE ODONTOLOGIA
FACULDADE DE MEDICINA VETERINÁRIA
CEUA - Comissão de Ética no Uso de Animais
CEUA - Ethics Committee on the Use of Animals

CERTIFICADO

Certificamos que o Relatório Final do trabalho intitulado **"Efeito da incorporação de fragmentos de peptideos antimicrobianos em sistemas de nanoparticulas como agentes anticárie"**, Processo FOA nº 2014-00618, sob responsabilidade de Cristiane Duque e colaboração de Kelly Limi Aida, Giovana Maria Fioramonti Calixto, Marlus Chorilli, Paula Fernanda Kreling, Karina Sampaio Caiaffa, Telma Blanca Lombardo Bedran e Denise Madalena Palomari Spolidorio foi aprovado pela CEUA em 09 de Dezembro de 2015.

CERTIFICATE

We certify that the study entitled **"Effect of incorporation of antimicrobial peptides fragments in nanoparticles systems as anticaries agents"**, Protocol FOA nº 2014-00618, under the supervision of Cristiane Duque and collaboration of Kelly Limi Aida, Giovana Maria Fioramonti Calixto, Marlus Chorilli, Paula Fernanda Kreling, Karina Sampaio Caiaffa, Telma Blanca Lombardo Bedran and Denise Madalena Palomari Spolidorio had its the Final Report approved by the CEUA on December 09, 2015.

Profa. Adj. Maria Cristina Rosifini Alves Rezende
Vice-Coordenadora da CEUA
CEUA Vice-Coordinator

CEUA - Comissão de Ética no Uso de Animais
Faculdade de Odontologia de Araçatuba
Faculdade de Medicina Veterinária de Araçatuba
Rua José Bonifácio, 1193 – Vila Mendonça - CEP: 16015-050 – ARAÇATUBA – SP
Fone (18) 3636-3234 Email CEUA: ceua@foa.unesp.br

ANEXO C

ARCHIVES OF ORAL BIOLOGY - GUIDE FOR AUTHORS

Article structure

Manuscript Structure

Follow this order when typing manuscripts: Title, Authors, Affiliations, Abstract, Keywords, Main text (Introduction, Materials & Methods, Results, Discussion for an original paper), Acknowledgments, Appendix, References, Figure Captions and then Tables. Do not import the Figures or Tables into your text. The corresponding author should be identified with an asterisk and footnote. All other footnotes (except for table footnotes) should be identified with superscript Arabic numbers.

Introduction

This should be a succinct statement of the problem investigated within the context of a brief review of the relevant literature. Literature directly relevant to any inferences or argument presented in the Discussion should in general be reserved for that section. The introduction may conclude with the reason for doing the work but should not state what was done nor the findings.

Materials and Methods

Enough detail must be given here so that another worker can repeat the procedures exactly. Where the materials and methods were exactly as in a previous paper, it is not necessary to repeat all the details but sufficient information must be given for the reader to comprehend what was done without having to consult the earlier work. Authors are requested to make plain that the conditions of animal and human experimentation are as outlined in the "Ethics" and "Studies on Animals" sections above

Results or Findings

These should be given clearly and concisely. Care should be taken to avoid drawing inferences that belong to the Discussion. Data may be presented in various forms such as histograms or tables but, in view of pressure on space, presentation of the same data in more than one form is unacceptable.

Discussion

This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions

The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Essential title page information

• ***Title.*** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.

• ***Author names and affiliations.*** Please clearly indicate the given name(s) and family name(s) of each author and check that all names are accurately spelled. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in

front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.

• ***Corresponding author.*** Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. **Ensure that the e-mail address is given and that contact details are kept up to date by the corresponding author.**

• ***Present/permanent address.*** If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes. As titles frequently stand alone in indexes, bibliographic journals etc., and indexing of papers is, to an increasing extent, becoming computerized from key words in the titles, it is important that titles should be as concise and informative as possible. Thus the animal species to which the observations refer should always be given and it is desirable to indicate the type of method on which the observations are based, e.g. chemical, bacteriological, electron-microscopic, histochemical, etc. A "running title" of not more than 40 letters and spaces must also be supplied. A keyword index must be supplied for each paper.

Structured abstract

The paper should be prefaced by an abstract aimed at giving the entire paper in miniature. Abstracts should be no longer than 250 words and should be structured as per the guidelines published in the Journal of the American Medical Association (JAMA 1995; 273: 27-34). In brief, the abstract should be divided into the following sections: (1) Objective; (2) Design - if clinical, to include setting, selection of patients, details on the intervention, outcome measures, etc.; if laboratory research, to include details on methods; (3) Results; (4) Conclusions.

Highlights

Highlights are mandatory for this journal. They consist of a short collection of bullet points that convey the core findings of the article and should be submitted in a separate editable file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point). You can view example Highlights on our information site.

Keywords

Immediately after the abstract, provide a maximum of 6 keywords, using British spelling and avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Abbreviations

As Archives of Oral Biology is a journal with a multidisciplinary readership, abbreviations, except those universally understood such as mm, g, min. u.v., w/v and those listed below, should be avoided if possible. Examples of abbreviations which may be used without definition: ADP, AMP, ATP, DEAE-cellulose, DNA, RNA, EDTA, EMG tris. Other abbreviations used to improve legibility should be listed as a footnote on the title page. Chemical symbols may be used for elements, groups and simple compounds, but excessive use should be avoided. Abbreviations other than the above should not be used in titles.

Acknowledgements

Collate acknowledgements in a separate section at the end of the article before the references

and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Formatting of funding sources

List funding sources in this standard way to facilitate compliance to funder's requirements:
Funding: This work was supported by the National Institutes of Health [grant numbers xxxx, yyyy]; the Bill & Melinda Gates Foundation, Seattle, WA [grant number zzzz]; and the United States Institutes of Peace [grant number aaaa]. It is not necessary to include detailed descriptions on the program or type of grants and awards. When funding is from a block grant or other resources available to a university, college, or other research institution, submit the name of the institute or organization that provided the funding.
If no funding has been provided for the research, please include the following sentence: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Bacterial nomenclature

Organisms should be referred to by their scientific names according to the binomial system. When first mentioned the name should be spelt in full and in italics. Afterwards the genus should be abbreviated to its initial letter, e.g. 'S. aureus' not 'Staph. aureus'. If abbreviation is likely to cause confusion or render the intended meaning unclear, the names of microbes should be spelt in full. Only those names which were included in the Approved List of Bacterial Names, Int J Syst Bacteriol 1980; 30: 225-420 and those which have been validly published in the Int J Syst Bacteriol since 1 January 1980 have standing in nomenclature. If there is good reason to use a name that does not have standing in nomenclature, the names should be enclosed in quotation marks and an appropriate statement concerning the nomenclatural status of the name should be made in the text (for an example see Int J Syst Bacteriol 1980; 30: 547-556). When the genus alone is used as a noun or adjective, use lower case Roman not italic, e.g.'organisms were staphylococci' and 'streptococcal infection'. If the genus is specifically referred to use italics e.g. 'organisms of the genus Staphylococcus'. For genus in plural, use lower case roman e.g. 'salmonellae'; plurals may be anglicized e.g.'salmonellas'. For trivial names, use lower case Roman e.g. 'meningococcus'

Artwork

Image manipulation

Whilst it is accepted that authors sometimes need to manipulate images for clarity, manipulation for purposes of deception or fraud will be seen as scientific ethical abuse and will be dealt with accordingly. For graphical images, this journal is applying the following policy: no specific feature within an image may be enhanced, obscured, moved, removed, or introduced. Adjustments of brightness, contrast, or color balance are acceptable if and as long as they do not obscure or eliminate any information present in the original. Nonlinear adjustments (e.g. changes to gamma settings) must be disclosed in the figure legend.

Electronic artwork

General points

- Make sure you use uniform lettering and sizing of your original artwork.
- Embed the used fonts if the application provides that option.

- Aim to use the following fonts in your illustrations: Arial, Courier, Times New Roman, Symbol, or use fonts that look similar.
- Number the illustrations according to their sequence in the text.
- Use a logical naming convention for your artwork files.
- Provide captions to illustrations separately.
- Size the illustrations close to the desired dimensions of the published version.
- Submit each illustration as a separate file.

A detailed guide on electronic artwork is available.

You are urged to visit this site; some excerpts from the detailed information are given here.

Formats

If your electronic artwork is created in a Microsoft Office application (Word, PowerPoint, Excel) then please supply ‘as is’ in the native document format. Regardless of the application used other than Microsoft Office, when your electronic artwork is finalized, please 'Save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):

EPS (or PDF): Vector drawings, embed all used font.

TIFF (or JPEG): Color or grayscale photographs (halftones), keep to a minimum of 300 dpi.

TIFF (or JPEG): Bitmapped (pure black & white pixels) line drawings, keep to a minimum of 1000 dpi.

TIFF (or JPEG): Combinations bitmapped line/half-tone (color or grayscale), keep to a minimum of 500 dpi.

Please do not:

• Supply files that are optimized for screen use (e.g., GIF, BMP, PICT, WPG); these typically have a low number of pixels and limited set of colors;

• Supply files that are too low in resolution;

• Submit graphics that are disproportionately large for the content.

Illustration services

Elsevier's WebShop offers Illustration Services to authors preparing to submit a manuscript but concerned about the quality of the images accompanying their article. Elsevier's expert illustrators can produce scientific, technical and medical-style images, as well as a full range of charts, tables and graphs. Image 'polishing' is also available, where our illustrators take your image(s) and improve them to a professional standard. Please visit the website to find out more.

Tables

Please submit tables as editable text and not as images. Tables can be placed either next to the relevant text in the article, or on separate page(s) at the end. Number tables consecutively in accordance with their appearance in the text and place any table notes below the table body. Be sparing in the use of tables and ensure that the data presented in them do not duplicate results described elsewhere in the article. Please avoid using vertical rules.

Data references

This journal encourages you to cite underlying or relevant datasets in your manuscript by citing them in your text and including a data reference in your Reference List. Data references

should include the following elements: author name(s), dataset title, data repository, version (where available), year, and global persistent identifier. Add [dataset] immediately before the reference so we can properly identify it as a data reference. The [dataset] identifier will not appear in your published article.

Reference management software

Most Elsevier journals have their reference template available in many of the most popular reference management software products. These include all products that support Citation Style Language styles, such as Mendeley and Zotero, as well as EndNote. Using the word processor plug-ins from these products, authors only need to select the appropriate journal template when preparing their article, after which citations and bibliographies will be automatically formatted in the journal's style. If no template is yet available for this journal, please follow the format of the sample references and citations as shown in this Guide. Users of Mendeley Desktop can easily install the reference style for this journal by clicking the following link:

http://open.mendeley.com/use-citation-style/archives-of-oral-biology

When preparing your manuscript, you will then be able to select this style using the Mendeley plug-ins for Microsoft Word or LibreOffice.

Reference style

Text: Citations in the text should follow the referencing style used by the American Psychological Association. You are referred to the Publication Manual of the American Psychological Association, Sixth Edition, ISBN 978-1-4338-0561-5, copies of which may be ordered online or APA Order Dept., P.O.B. 2710, Hyattsville, MD 20784, USA or APA, 3 Henrietta Street, London, WC3E 8LU, UK.

List: references should be arranged first alphabetically and then further sorted chronologically if necessary. More than one reference from the same author(s) in the same year must be identified by the letters 'a', 'b', 'c', etc., placed after the year of publication.

Examples:

Reference to a journal publication:

Van der Geer, J., Hanraads, J. A. J., & Lupton, R. A. (2010). The art of writing a scientific article. *Journal of Scientific Communications, 163,* 51–59.

Reference to a book:

Strunk, W., Jr., & White, E. B. (2000). *The elements of style.* (4th ed.). New York: Longman, (Chapter 4).

Reference to a chapter in an edited book:

Mettam, G. R., & Adams, L. B. (2009). How to prepare an electronic version of your article. In B. S. Jones, & R. Z. Smith (Eds.), *Introduction to the electronic age* (pp. 281–304). New York: E-Publishing Inc.

Reference to a website:

Cancer Research UK. Cancer statistics reports for the UK. (2003). http://www.cancerresearchuk.org/aboutcancer/statistics/cancerstatsreport/ Accessed 13.03.03.

Reference to a dataset:

[dataset] Oguro, M., Imahiro, S., Saito, S., Nakashizuka, T. (2015). *Mortality data for Japanese oak wilt disease and surrounding forest compositions*. Mendeley Data, v1. http://dx.doi.org/10.17632/xwj98nb39r.1.