UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de São José dos Campos
Instituto de Ciência e Tecnologia

ALINE LINS DE LIMA

PRODUÇÃO DE MEMBRANAS ANTIMICROBIANAS DE FIBRAS NANOMÉTRICAS CONTENDO CINAMALDEÍDO A PARTIR DA TÉCNICA DE *SOLUTION BLOW SPINNING*

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Tese apresentada ao Instituto de Ciência e Tecnologia, Universidade Estadual Paulista (Unesp), Campus de São José dos Campos, como parte dos requisitos para a obtenção do título de DOUTOR, pelo Programa de Pós-Graduação em ODONTOLOGIA RESTAURADORA, Área de Prótese Dentária.

Orientador: Prof. Dr. Adj. Alexandre Luiz Souto Borges

Coorientador: Prof. Dr. Eliton Souto de Medeiros

São José dos Campos

2018

Instituto de Ciência e Tecnologia [internet]. Normalização de tese e dissertação [acesso em 2018]. Disponível em http://www.ict.unesp.br/biblioteca/normalizacao

Apresentação gráfica e normalização de acordo com as normas estabelecidas pelo Serviço de Normalização de Documentos da Seção Técnica de Referência e Atendimento ao Usuário e Documentação (STRAUD).

Lima, Aline Lins de
Produção de membranas antimicrobianas de fibras nanométricas contendo cinamaldeído a partir da técnica de Solution Blow Spinning / Aline Lins de Lima. - São José dos Campos : [s.n.], 2018.
39 f. : il.

Tese (Doutorado em Odontologia Restauradora) - Pós-Graduação em Odontologia Restauradora - Universidade Estadual Paulista (Unesp), Instituto de Ciência e Tecnologia, São José dos Campos, 2018.
Orientador: Alexandre Luiz Souto Borges
Coorientador: Eliton Souto de Medeiros

1. Candida. 2. Antifúngicos. 3. Materiais biocompatíveis. I. Borges, Alexandre Luiz Souto, orient. II. Medeiros, Eliton Souto de , coorient. III. Universidade Estadual Paulista (Unesp), Instituto de Ciência e Tecnologia, São José dos Campos. IV. Universidade Estadual Paulista 'Júlio de Mesquita Filho' - Unesp. V. Universidade Estadual Paulista (Unesp). VI. Título.

Ficha catalográfica elaborada pela Biblioteca Prof. Achille Bassi e Seção Técnica de Informática, ICMC/USP
com adaptações - STATI, STRAUD e DTI do ICT/UNESP.
Renata Aparecida Couto Martins CRB-8/8376

BANCA EXAMINADORA

Prof. Adj. Alexandre Luiz Souto Borges (Orientador)
Universidade Estadual Paulista “Júlio de Mesquita Filho” - Unesp
Instituto de Ciência e Tecnologia
Campus de São José dos Campos

Prof. Dra. Laís Regiane da Silva Concilio
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Campus de Taubaté

Prof. Dra. Eliandra de Sousa Trichês
Universidade Federal de São Paulo
Instituto de Ciência e Tecnologia da Unifesp
Campus de São José dos Campos

Prof. Dr. Estevão Tomomitsu Kimpara
Universidade Estadual Paulista “Júlio de Mesquita Filho” - Unesp
Instituto de Ciência e Tecnologia
Campus de São José dos Campos

Prof. Dr. Eduardo Bresciani
Universidade Estadual Paulista “Júlio de Mesquita Filho” - UNESP
Instituto de Ciência e Tecnologia
Campus de São José dos Campos

São José dos Campos, 08 de junho de 2018.

DEDICATÓRIA

*A **Deus** que sempre abençoou, iluminou e esteve comigo durante esta jornada.*

*Aos meus queridos pais, **Renato** e **Marinalva**, por todo o incentivo, amor e dedicação, que me ajudaram a superar todas as dificuldades, sempre me dando força, acalmando-me e fazendo-me seguir em frente para alcançar todos os meus sonhos. Sou-lhes imensamente grata!*

*Ao meu amado filho, **Luiz Fernando**, por sua maturidade e compreensão em aceitar nossa distância física e por todo seu amor e carinho.*

AGRADECIMENTOS

Ao Instituto de Ciência e Tecnologia na pessoa do diretor da Faculdade de Odontologia de São José dos Campos, ***Prof. Tit. Estevão Tomomitsu Kimpara****.*

Ao ***Prof. Tit. Estevão Tomomitsu Kimpara****, agora não mais como diretor, mas sim como pessoa e como meu primeiro orientador, que mesmo por um breve tempo de convivência, deu-me atenção e teve cuidado para comigo na minha primeira etapa na UNESP e quando pude retornar também acreditou e aceitou-me novamente em sua Instituição.*

Ao Programa de Pós-graduação em Odontologia Restauradora, em nome do coordenador do programa ***Prof. Adj. Alexandre Luiz Souto Borges.***

Ao nosso caro e estimado coordenador da especialidade de Prótese Dentária ***Prof. Tit. Marco Antônio*** **Bottino** que me aceitou novamente em seu curso e mais uma vez acreditou em meu potencial.

Aos ***Docentes do Programa de Pós-graduação em Odontologia Restauradora,*** *pelo aprendizado.*

Aos ***Colegas de Pós-Graduação*** *da Especialidade Prótese Dentária pela amizade e companheirismo nesta jornada, em especial a* ***Gabriela, Jean, Vinícius, Nathália, Ronaldo, Lígia, Hilton, Júlio, e Alexandre.***

Às colegas do Grupo de Estudos de Bioengenharia Aplicada à Odontologia.

A todos os colegas do Laboratório Multiusuário de Materiais e Biossistemas ***(LAMAB)*** *que me receberam muito bem e sempre me ajudaram da forma que puderam.*

Às colegas de república, ***Fernanda e Ana Elisa*** *pelo companheirismo, carinho e amizade.*

À CAPES pelo auxílio desta pesquisa e concessão de Bolsa Nacional e Bolsa Sanduíche.

Aos funcionários do Departamento de Materiais Odontológico e Prótese: ***Juliane, Marco Alfredo, Fernando Fontes e Lílian****.*

Ao Professor ***Tarcísio José de Arruda Paes Júnior*** *por sua disponibilidade em sempre ajudar.*

AGRADECIMENTOS ESPECIAIS

Ao meu orientador ***Prof. Alexandre Luiz Souto Borges*** *pela orientação, por confiar e acreditar em mim na realização desta pesquisa e durante todo o meu curso; por estar sempre disposto a ajudar e se preocupar em todos os momentos que precisei. Pela amizade, bom humor, disponibilidade, e por me receber tão bem no ICT- UNESP, bem como pelos ensinamentos clínicos e paciência comigo durante o curso. Professor "Alex", tenho uma grande estima por você e irei levá-lo para sempre no coração e como exemplo de mestre.*

Ao meu Coorientador ***Prof. Eliton Souto de Medeiros*** *por abrir as portas de seu laboratório sem nem saber quem eu era, dar-me todo suporte material e intelectual frente a um novo mundo que foi trabalhar junto à Engenharia. Agradeço ainda o carinho e amizade que fomos construindo.*

Ao Professor ***Alexander Bismarck*** *por me aceitar, por orientar-me em meu trabalho, por receber-me tão bem na Universidade de Viena durante o meu estágio de Doutorado sanduíche e permitir trabalhar com seu grupo Polymer and Composite Engineering (PaCE) no qual fiz grandes amigos.*

Às amigas Suzanne Alvim e Marta Fortea-Verdejo por todo carinho, atenção, paciência e amizade para comigo durante minha estadia em Viena.

Ao amigo ***Marcos Vestali*** *pelos auxílios prestados durante o desenvolvimento minha permanência em São José dos Campos.*

À ***Sarina Maciel*** *e* ***sua família*** *por me acolherem em sua casa e em suas vidas. Vocês são minha família paraibana em São José dos Campos.*

À ***Viviane Figueiredo*** *por ter me acolhido tão bem em São José dos Campos, por nossos momentos divertidos e por sua amizade.*

Ao ***Ronaldo Luís****, meu melhor amigo! Aquele que esteve comigo em todos os momentos, fossem de tristeza ou de alegria. Que compartilhou os mais difíceis momentos nessa minha caminhada. Te agradeço por sua paciência, amizade e carinho. Não foi fácil chegar até aqui, mas sem você teria sido ainda mais difícil.*

Às queridas e doces amigas que ganhei nessa minha trajetória ***Pollyanna, Gabriela Ramos, Karen, Jéssica, Tabata, Dayanna Campaneli e Cristiane Quishida*** *por todos os momentos passados juntas e pelo carinho de vocês.*

Ao Professor ***Ricardo Castro*** *e suas pupilas,* ***Ana Cláudia Macedo****,* ***Rebeca Rhuane****,* ***Rebeca Dantas****,* ***Tereza Vieira****,* ***Marianne Rangel*** *e* ***Taisa Mara****, que contribuíram de forma fundamental nesse trabalho*

À ***Professora Vanessa Macedo*** *por todos momentos de aprendizagem e também os momentos alegres que pudemos compartilhar. Você é uma pessoa muito especial.*

À ***Professora Flávia Rodrigues*** *por sua atenção, incentivo e paciência na minha formação desde o mestrado, o que acabou nos aproximando e me fez ter grande carinho e admiração por você.*

Vovó Iracema e vovô Luiz *por estarem sempre torcendo por mim.*

À ***Laura Elia*** *minha grande amiga e irmã, que me deu força em todos os momentos em que precisei, muito obrigada por toda sua amizade e carinho.*

À ***Denise Esquerra*** *não apenas por seu cuidado profissional, mas por toda paciência, incentivo, carinho e força me dada para que eu pudesse alcançar todos os passos e essa grande e desejada conquista.*

À ***titia Jacinta*** *que se fez presente o tempo inteiro nessa minha caminhada, torcendo por mim, preocupando-se sempre e me dando incentivo em todos os momentos.*

Aos demais familiares que torceram por mim durante esta etapa de minha vida.

Ao meu grande amigo ***Bruno Rafael****, que mesmo de longe me apoiou quando estava desmotivada e por me ajudar na formatação de meu trabalho.*

*A **Maria Helena e Fernando** pelo incentivo me dado ao cuidar, amar, dar carinho e contribuir na educação de meu filho querido enquanto estive distante.*

*À **Tatiana Rita por amizade,** por sempre se preocupar comigo e pela grande força me dada para finalização do meu trabalho.*

*A **Vinícius Dias e Kaline Nascimento** pelo companheirismo e pela imensa colaboração em meu trabalho de Tese.*

*À doce e querida **Natali**, quem apareceu na minha vida e foi um anjo da guarda em muitos momentos críticos na finalização desse curso. Natali meu muito obrigada, a levarei no coração sempre.*

*À Professora **Lucianne Dias**, **Lucas Ramos** e **Nádia Domingues** pela grande contribuição e dedicação a este trabalho.*

*Ao querido e estimado **Meyson Cássio** que foi uma pessoa determinante em todo o processo de execução desta tese, desde de maneira operacional, científica e principalmente emocional. **Meyson**, não tenho palavras para te agradecer tudo o que fez por mim, como também acredito que não há nenhuma maneira de retribuir toda sua dedicação e atenção que você dispensou a mim e ao meu trabalho.*

SUMÁRIO

Lima AL. Produção de membranas antimicrobianas de fibras nanométricas contendo cinamaldeído a partir da técnica de *Solution Blow Spinning* [tese]. São José dos Campos (SP): Universidade Estadual Paulista (Unesp), Instituto de Ciência e Tecnologia; 2018.

RESUMO

A estomatite protética é uma das afecções mais comuns e recorrentes em pacientes portadores de próteses totais. O fungo do gênero *Candida*, promotor dessa patologia, além de resistente se torna ainda mais complexo de ser combatido devido à dificuldade da ação de fármacos tópicos que só conseguem permanecer por um curto período no local da infecção, em virtude da dinâmica da cavidade bucal. O processo de *Solution Blow Spinning* permite a obtenção de fibras ultrafinas que podem ser aplicadas em vastas áreas, inclusive na bioengenharia. Uma das aplicabilidades das fibras ultrafinas é sua utilização para liberação controlada de fármacos de forma eficiente e duradoura. Dessa forma, o intuito do presente trabalho foi incorporar Cinamaldeído (CA), composto que possui propriedades antimicrobianas, a mantas de Poli(ácido lático) e Poli(etileno glicol) (PLA/PEG) e avaliá-las quanto à produção e caracterização por, Microscopia eletrônica de varredura (MEV), Mensuração do ângulo de contato, Termogravimetria (TGA), Calorimetria Exploratória Diferencial (DSC), Espectroscopia de infravermelho por transformada de Fourier (FTIR), Espectroscopia no ultravioleta visível (UV/vis), ensaios mecânicos e ação antifúngica. Para realização dos experimentos, foram fiadas as seguintes mantas: PLA, PLA/PEG e PLA/PEG 23,8% CA. As micrografias obtidas por MEV mostraram, que os diâmetros das fibras que não continham CA, apresentaram diâmetros semelhantes entre si, PLA (354±160 nm)^{a} e PLA/PEG (428±250nm)^{a}, sendo esses diâmetro menores dos que encontrados nas fibras de PLA/PEG 23,8% CA (749±370 nm)^{b} . O ângulo de contato e tensão superficial não puderam ser verificados em virtude da proporção de polímeros nas blendas que apresentaram alta afinidade pelos solventes utilizados no teste. No ensaio de TGA, a curva de PLA/PEG com acréscimo de 23,8% CA exibiu uma maior estabilidade térmica. No teste de DSC o ponto de transição vítrea das mantas contendo 23,8% CA foi o que apresentou menor valor. A liberação de CA foi satisfatória ocorrendo até o 6° dia. No teste de ensaios mecânicos, o acréscimo de CA às mantas aumentaram significativamente o Módulo elástico (24,94±4,45) e a Tensão máxima de ruptura (0,99±0,16 MPa) com relação às mantas puras de PLA/PEG (18,74±3.41 MPa) and (0,85±0.09 MPa), esse acréscimo ainda promoveu redução estatisticamente significante ($p< 0,05\%$) em mais de 50% nos biofilmes monotípicos de *C. albicans* e *C. krusei* e no multiespécie de *C. albicans*, *C. krusei* e *C. glabrata*. Mediante os resultados encontrados pode-se depreender que é possível se obter mantas de fibras ultrafinas de PLA/PEG contendo 23,8% de CA com propriedades antifúngicas e capacidade de liberação do agente antimicrobiano por cerca de 12 dias.

Palavras-chave: *Candida*. Antifúngicos. Materiais biocompatíveis.

Lima AL. Antifungal nanofibrous membranes of pla/peg/cinnamaldehyde developed by Solution Blow Spinning [doctorate thesis]. São José dos Campos (SP): São José dos Campos (SP): São Paulo State University (Unesp), Institute of Science and Technology; 2018.

ABSTRACT

Denture stomatitis is one of the most common and recurrent conditions in patients with total dentures. The fungal of the genus Candida, the causer of this pathology, besides being resistant, becomes even more complex to be combated due to the difficulty of the action of topical drugs that can only remain for a short time at the site of infection due to the dynamic of the oral cavity. The Solution Blow Spinning process allows the production of ultrafine fibers that can be applied in large areas, including bioengineering. One of the applications of ultrafine fibers is their use for controlled release of drugs in an efficient and long-lasting manner. Thus, the aim of the present work was to incorporate Cinnamaldehyde (CA), a compound that has antimicrobial properties, to Poly (lactic acid) and Poly (ethylene glycol) blankets (PLA / PEG) and to evaluate them for the production and characterization by Scanning Electron Microscopy (SEM), Contact Angle Measurement Thermogravimetric (TGA), Differential Scanning Calorimetry (DSC), Fourier transform infrared spectroscopy (FTIR), Ultraviolet–visible (UV/vis) spectroscopy, mechanical properties and antifungal action. Antifungal activity was verified against C. albicans, C. krusey and C. glabrata by broth microdilution test, disk diffusion and anti-biofilm activity, in both multi-species and mono-species biofilms. For the experiment, three types of meshes were spun: pure PLA, PLA/PEG and PLA / PEG 23.8% CA. The micrographs obtained by SEM showed that the fibers that did not contain CA had similar diameters to each other and smaller than the fibers containing PLA / PEG 23, 8% CA. The contact angle and surface tension could not be measured by virtue of the proportion of polymers in the blends which showed high affinity for the solvents used in the test. In the TGA assay, the PLA/PEG curve with 23.8% CA increase exhibited a higher thermal stability while in the DSC test the glass transition point of the meshes containing 23.8% CA it was the one with the lowest value. The release of CA was satisfactory occurring until the 6^{th} day. PLA membranes with fibres of diameter exhibited the lowest fibre diameter (354 ±160 nm)a followed by PLA/PEG (428±250nm)^{a} and PLA/PEG/CA (749±370 nm)^{b}. Addition of CA resulted in an increase in mechanical properties of the membranes from (24.94±4.85 MPa) the elastic modulus and (0.99±0.16 MPa) tensile strength in comparison to PLA/PEG (18.74±3.41 MPa) and (0,85±0.09 MPa). CA incorporation increased improved the thermal stability, with release of CA of 0.10 µg/mL over a 6 days period. The PLA/PEG CA membranes presented antifungal activities, showing reductions in more than 50% of the biofilm biomass, being statistically significant ($p<0.05\%$) to the control group. Fibrous membranes of PLA/PEG/CA ultrathin fibres were produced by SBS that exhibited antifungal properties and release over a 12-day period.

Keywords: Candida. Antifungal. Biocompatible materials.

1 INTRODUÇÃO

A Estomatite Protética (EP) é a patologia mais comumente encontrada em pacientes que utilizam próteses dentárias removíveis, total ou parcial. Apesar de geralmente ser assintomática, em alguns casos, pode ser observado leve sangramento, eritema, edema da área envolvida, sensação de queimação da mucosa, dor, presença de petéquias hemorrágicas e alteração do paladar. Normalmente acomete pacientes que relatam o uso contínuo da prótese e clinicamente é localizada na área recoberta pela prótese dentária (Neville et al., 2009).

Os micro-organismos do gênero *Candida*, são responsáveis por causar a estomatite protética e Mujica et al. (2008) também a cita como a mais prevalente dentre as lesões bucais, e que necessita ser diagnosticada e eficazmente tratada a fim de se propiciar uma melhor qualidade de vida aos usuários de próteses dentárias (Scalercio et al., 2007).

Outra variável que deve ser considerada é a capacidade de o fungo crescer nos interstícios microscópicos do material acrílico ocasionando o edema das camadas superficiais e infiltração inflamatória celular crônica da mucosa (Cawson et al.,1995). Necessitando-se de um agente que ao mesmo tempo que trate a lesão também a reduza os micro-organismos na superfície da prótese.

Apesar das terapias antifúngicas convencionais serem amplamente utilizadas no tratamento da estomatite protética, a infecção é, em alguns casos, recorrente o que sugere que o biofilme possa servir como reservatório protetor para os micro-organismos (Chandra et al., 2001), como o que poderia estar depositado sobre a superfície da prótese. Além disso, os micro-organismos utilizam diversos mecanismos para aumentar sua resistência aos agentes antimicrobianos e também aos procedimentos utilizados para a desinfecção das próteses, acarretando muitas vezes no fracasso das terapias antifúngicas convencionais.

Os devidos cuidados para se contornar a estomatite protética envolvem a utilização de agentes antifúngicos (tópicos e/ou sistêmicos), porém não totalmente eficaz sem que haja uma boa higiene oral, limpeza da prótese, descontinuidade do uso noturno e troca da dentadura (Webb et al., 1998; Pires et al., 2002).

Fibras ultrafinas, como por exemplo as nanofibras, podem ser potencialmente usadas em uma infinidade de aplicações que como a liberação controlada, curativo, vestuário de proteção, os sensores e as membranas de filtração dentre outras (Medeiros et al., 2009; Oliveira JE et al., 2013). A maioria das membranas de nanofibras hoje são produzidas por electrospinning e, recentemente, por Solution Blow Spinning (Medeiros et al., 2009).

As nanofibras tornam-se um material de revestimento promissor para preparar enxerto com eluição de fármaco, permitindo a liberação local destes em virtude de sua estrutura porosa e fibrosa, de sua grande área de superfície, proporção satisfatória entre comprimento e diâmetro, da morfologia das superfícies ajustáveis e desempenho flexível (Kim, Yoo, 2014).

O Óleo de Canela e o Cinamaldeído são substâncias bem documentadas na literatura como agentes antifúngicos e em especial contra o gênero Candida, que são os micro-organismos envolvidos mais frequentemente nas lesões de estomatite protética (Oliveira JA et al., 2014; Castro et al., 2013a, 2013b).

A técnica de Solution Blow Spinning tem sido utilizada com sucesso na entrega controlada de substâncias, tais como os óleos naturais com propriedades antimicrobianas (Bonan et al., 2015) e linalol (Souza et al., 2015).

Entre as vantagens da técnica de *Solution Blow Spinning* estão: baixo custo, facilidade de fabricação e dimensionamento de estruturas 3D com maior tamanho de manta; virtualmente qualquer superfície pode ser revestida, tal como as superfícies não condutoras; as taxas de produção mais rápidos; e nenhuma dependência em constante dieléctrica do solvente (Medeiros et al., 2009).

Santos et al. (2014) procedeu fiação de mantas junto ao agente antimicrobiano cetilpiridínio obtendo eficácia contra a *Candida albicans* fato que motiva, mais uma vez, a inserção do óleo de canela e do seu fitoconstituinte, o cinamaldeído, às nanofibras formadas pelas blendas de Poli (ácido lático) e Poli(etileno glicol) (PLA/PEG), uma vez que esses polímeros são biocompatíveis e reabsorvíveis pelo organismo, para testar a atividade antimicrobiana da mesma maneira.

O desenvolvimento de mantas de nanofibras impregnadas com substâncias antimicrobianas pode ser uma interessante alternativa em relação às formas convencionais para o tratamento de enfermidades, principalmente na cavidade

bucal. Em virtude da dinâmica bucal, constantemente exposta a fluidos e alimentos, tem-se dificuldade para a manutenção da concentração de fármacos em seus locais de aplicação (Santos et al., 2014). Desta forma, a confecção das nanofibras pela técnica de fiação por Solução por Sopro em Solução contendo agentes antimicrobianos pode possibilitar um maior controle na liberação desses agentes antimicrobianos ao longo do tempo.

2 ARTIGO

2.1 - Aline Lins de Lima, Lucas de Paula Ramos, Luciane Dias de Oliveira, Eliton Souto de Medeiros, Alexandre Luiz Souto Borges. Membranas nanofibrosa antifúngicas de pla/peg/cinamaldeído desenvolvidas por *Solution Blow Spinning* / Antifungal nanofibrous membranes of pla/peg/cinnamaldehyde developed by Solution Blow Spinning*

RESUMO

Objetivos: Os objetivos deste estudo foram desenvolver membranas nanofibrosas contendo cinamaldeído (CA), um fitoconstituinte de Cinnamomum zeylanicum, como material para aplicações antifúngicas. Métodos: Membranas nanofibrosas de poli (D, L lactide) (PDLLA) e poli (etilenoglicol) (PEG) (blends) contendo cinamaldeído (CA) foram produzidas pela técnica de Solution blow spinning. As membranas fibrosas produzidas foram caracterizadas morfologicamente por microscopia eletrônica de varredura, termogravimetria, calorimetria exploratória diferencial, molhabilidade por análise de ângulo de contato e tensão superficial, bem como espectroscopia de infravermelho por transformada de Fourier (FTIR). A liberação de CA foi determinada por espectroscopia no ultravioleta-visível (UV). Propriedades mecânicas também foram caracterizadas por tensão máxima de ruptura e módulo elástico. A atividade antifúngica foi verificada contra *C. albicans*, *C. krusey* e *C. glabrata* pelo teste de microdiluição em caldo, difusão em disco e atividade anti-biofilme, em biofilmes multi-espécies e mono-espécies. Resultados: As membranas de PLA exibiram o menor diâmetro de fibra (354 ± 160 nm)[a] seguido de PLA / PEG (428 ± 250 nm) e PLA / PEG / CA (749 ± 370 nm)[b]. A adição de CA resultou em um aumento nas propriedades mecânicas das membranas a partir de (24,94 ± 4,85 MPa) o módulo elástico e (0,99 ± 0,16 MPa) de resistência à tração em comparação a PLA / PEG (18,74 ± 3,41 MPa) e (0,85 ± 0,09 MPa). A incorporação de CA aumentou melhorou a estabilidade térmica, e permitiu uma liberação de CA de 0,10 µg / mL durante um período de 12 dias. As membranas de PLA / PEG / CA apresentaram atividade antifúngica, apresentando reduções em mais de 50% da biomassa do biofilme, sendo estatisticamente significante (p <0,05%). Conclusão: As membranas fibrosas das fibras ultrafinas de PLA / PEG / CA produzidas por SBS exibiram propriedades antifúngicas e liberação por um período de 6 dias.

Palavras-Chave: Candida ssp. Antifúngico. Biocompatibilidade de materiais. Fiação por Sopro em Solução.

*Artigo elaborado de acordo com as normas do Periódico The Journal of Evidence-Based Dental Practice (Print version ISSN: 1532-3382). Submetido em: 13/05/2018.

ABSTRACT

Objectives: The aims of this study were to development nanofibrous membranes containing cinnamaldehyde (CA), a phytoconstituent from Cinnamomum zeylanicum, as a material towards antifungal applications. Methods: Nanofibrous membranes of poly(D,L lactide) (PDLLA) and poly (ethylene glycol) (PEG) (blends) loaded with CA were prepared by solution blow spinning. The produced fibrous membranes were characterized morphologically by scanning electron microscopy, as well as thermally via differential scanning calorimetry and thermogravimetry, wettability by water-in-air contact angle analysis and surface tension, as well as Fourier transform infrared spectroscopy (FTIR). Release of CA was determined by ultraviolet-visible (UV) spectroscopy. Mechanical properties were also characterized in tension. Antifungal activity was verified against C. albicans, C. krusey and C. glabrata by broth microdilution test, disk diffusion and anti-biofilm activity, in both multi-species and mono-species biofilms. Results: PLA membranes exhibited the lowest fibre diameter (354 ±160 nm)a followed by PLA/PEG (428±250nm)a and PLA/PEG/CA (749±370 nm)b. Addition of CA resulted in an increase in mechanical properties of the membranes from (24.94±4.85 MPa) the elastic modulus and (0.99±0.16 MPa) tensile strength in comparison to PLA/PEG (18.74±3.41 MPa) and (0,85±0.09 MPa). CA incorporation increased improved the thermal stability, with release of CA of 0.10 µg/mL over a 6 days period. The PLA/PEG CA membranes presented antifungal activities, showing reductions in more than 50% of the biofilm biomass, being statistically significant (p<0.05%) to the control group. Conclusion: Fibrous membranes of PLA/PEG/CA ultrathin fibres were produced by SBS that exhibited antifungal properties and release over a 12-day period.

Keywords: Candida ss., Antifungal. Biocompatible materials. Solution Blow Spinning.

1. Introduction

Fungal oral infections are classified as opportunistic mycotic, caused by fungi of low virulence or commensal that can promote subcutaneous and *disseminate infections in immunocompromised patients. Candida ssp microorganisms are responsible for producing denture stomatitis, the most prevalent pathology among such infections [1,2]. Candida ssp present some virulence factors that contribute to their pathogenic potential such as adhesion to host surfaces, formation of hyphae and production of extracellular enzymes [3].*

Denture stomatitis is most commonly found in users with partially or totally removable prostheses. This pathology requires diagnosis and effective treatment. Clinically this is localized in the oral mucosa covered by the dental prostheses in the form of erythematous lesions, and usually affects patients who report continuous use.

Although generally asymptomatic, it may also cause a burning sensation and, rarely, dysphagia [4].

Bioengineering has tried to develop materials as biocompatible as possible and biodegradable materials in the production of membranes of nano and submicron fibres of poly(lactic acid) (PLA) [5], poly(ε-caprolactone) (PCL) [6], poly(vinyl alcohol) (PVA) [7] and poly(ethylene glycol) (PEG) [8]. Such membranes can act as matrices for the release of drugs [9], an improve their therapeutic efficacy and reduce local and systemic toxicity [10], and reduce frequency of drug administration [11,12]. Moreover, the ease of administration, faster action and give improved patient acceptance and consequent adherence to the treatment [13,14].

In view of the common recurrence of denture stomatitis when exposed to conventional treatments [15], the side effects found, as well as the potential development of resistance to classical medications, there has been a constant search for alternative solutions such as the use of phytotherapeutics using essential oils or antimicrobial agents as reported in the literature [16–19]. Electrospinning [20] and solution blow spinning [21] [22] are the most common fibre forming methods.

Solution blow spinning has been successfully used to incorporate phytoconstituents and natural oils with antimicrobial properties as copaiba [23], melaleuca [24] oils into polymer micro and nanofibers. Cinnamaldehyde, phytoconstituent extracted of Cinnamon, has also been used as an antimicrobial agent in many studies [25–27].

In this work, nanofibrous membranes of PLA/PEG were prepared with and without CA via solution blow spinning for oral use as a potential treatment against denture stomatitis.

2. Materials and Methods

2.1. Materials

Poly (D,L- lactide acid) (PDLLA 4060D, PDLLA), with an average molecular weight of Mw of 120,000 g/mol was purchased from Jamplas Inc. (MO, USA). Poly(ethylene glycol), PEG, with Mw~5000-7000 g/mol was purchased from Merck, UK. Chloroform was used as a solvent and obtained from Sigma-Aldrich (USA). The

polymer solutions were dissolved under vigorous stirring for 6 h until complete dissolution. In microbiology tests were utilized nystatin as a positive control (Sigma-Aldrich, São Paulo – SP, Brazil) and cinnamaldehyde purchased from Sigma-Aldrich (Germany).

2.2. Preparation of Spinning Solutions and Fibre Formation

PLA/PEG blends with different polymer weight ratios (75:25, 80:20, 90:10) were prepared at polymer concentrations of 10, 15, and 20 wt.% in chloroform. To produce ultrathin meshes, the solutions were added to a plastic syringe and connected a syringe pump (PHD ultra, harvard apparatus, UK). The following parameters were used for fibre spinning: flow rate of 108, 116 and 133 µL/min, compressed air pressure of 40, 50 and 60 psi and working distance (fibre formation to collection distance) of 20 cm. Further details regarding the SBS apparatus set-up can be found in [22]. Fibres were collected at room temperature on a rotatory collector at 600 rpm covered with aluminum foil.

2.3. Nanofibre Membrane Characterization

2.3.1. Fibre and membrane morphological assessment by Scanning Electron Microscopy (SEM)

Fibre morphology by was assessed by scanning electron microscopy (SEM) and inspection showed that the 75:25 PLA/PEG blend had the best spinnability displaying fewer beads and uniform smooth fibres .

Fibre diameters and membrane/fibre morphologies were assessed using a Inspect S 50, FEI (Brno, Czech Republic) scanning electron microscope (SEM). Membranes were coated with gold using a SC7620 sputter coater (Emitech, East Sussex, UK). Fibre diameters were determined by image analysis (ImageJ, National Institutes of Health) and the average diameter was determined from 100 measurements.

2.3.2. Contact angle analysis

Contact angles were measured on a DSA30 Krüss goniometer (Hamburg, Germany). Droplets of 20 µL were placed on the surface of 10 mm x 10 mm nanofiber membranes. Water and diiodomethane were used in this test, three samples were measured with one droplet in each one. All measurements were performed at 25°C.

2.3.3. Thermogravimetric Analysis (TGA)

Thermogravimetric analysis (TGA) was conducted using a TGA (TA Instruments, USA). A heating regime of 25ºC to 600 ºC at 10 ºC/min was applied under nitrogen at a flow rate of 25 mL/min. The samples mass were 6 mg.

2.3.4. Differential scanning calorimetry (DSC)

Differential scanning calorimetry analysis was performed on a Discover DSC (TA Instruments, USA) with a heating regime 0ºC to 200ºC at a rate of 10 ºC/min under nitrogen flow 50mL/min. The sample mass was 5-6 mg in all instances.

2.3.5. Fourier transform infrared spectroscopy (FTIR-ATR)

Attenuated total reflectance (ATR)-FTIR spectra were taken from the different fibre membranes using a Prestige-21 IR Affinity-1 IR (Japan) apparatus. A total of 64 scans were taken per sample with a wavelength range 400 to 4,000 cm^{-1} and resolution of 2 cm^{-1}.

2.3.6. Drug release study

Ultraviolet-visible (UV-Vis) tests were performed using a 1800 UV spectrophotometer (SHIMADZU, Japan) in the range of 200-400 nm; CA is partially soluble in water and its absorbance peak was determined at 268 nm. A standard curve was obtained with solution concentrations at 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 and 7.0

µg/mL. CA release was determined from triplicates of fibre membranes weighing 5 mg in separate vials containing 10 mL phosphate buffered saline (PBS). The samples were maintained at constant temperature and shaken at 100 rpm and 37 °C in an SP-222 incubator (SPLABOR, São Paulo, Brazil). A sample, PLA/PEG without CA, was used as control. Measurements were performed with 2 mL aliquots in quartz cuvettes and taken in the period 24h to 390h.

2.3.7. Mechanical Properties

Mechanical properties were measured to in order to know whether the addition of the antifungal agent would affect the mechanical properties of the material. Oar-shaped samples with a 20 mm gauge length, width 2 mm with sample thickness 300-400 µm were utilized for the strength test. The experiments were performed using on a universal tensile test machine (INSTRON, USA) at a speed of 2 mm/min and a 2 kN load cell and 15 samples were tested.

2.3.8. Microbiology test

For antifungal analysis, three different tests were performed, which are complementary. Initially, to verify the best fungicidal concentration for incorporation of CA in the PLA / PEG membrane, the broth microdilution test was performed. After defining the concentration of CA (23.8%), the diffusion test was performed on agar to define the thickness of the membrane that presented the highest halo of microbial inhibition. Finally, after definition of the membrane thickness, it was used for the anti-biofilm tests of Candida.

Broth Microdilution test

For the broth micro-dilution test, reference strains (ATCC - American Type Culture Collection) of C. albicans ATCC 60193; C. Krusei ATCC 34135; C. glabrata ATCC 90030 from the Laboratory experimental pharmacology and cell cultive, Graduate Program in Dentistry, Federal University of Paraiba. For the determination of Minimum inhibitory concentration (MIC) and Minimum fungicidal concentration (MFC)

it was used the protocol M27-A3 (2012) from the Clinical Laboratory Standards Institute Protocol (CLSI Standardized solutions of the Candida species were prepared in a spectrophotometer containing 2.5 x 103 CFU / mL. Subsequently, serial dilution of 1000 to 7.81 µg / mL of CA in RPMI 1640 (glutamine, no bicarbonate and red phenolic indicator) culture medium (Himedia) was diluted in 96-well plates with MOPS [3- (N-morpholino) propanesulfonic acid] (Sigma-Aldrich, St. Louis, USA). Candida inocula were then added to the plates and incubated at 37 ° C for 24 h. The MIC was determined by observing the well containing CA at a lower concentration which did not show turbidity. To determine CFM, MIC aliquots were seeded at Sabouraud dextrose agar (Himedia, Mumbai, India) at the above mentioned concentrations. The plates were incubated for 48 h / 37 ° C and it was verified which the lowest concentration of the substance promoted absence of colony growth. All tests were performed in triplicate.

Antimicrobial activity of PLA / PEG / CA

After the determination of the CFM, the membranes of PLA / PEG were prepared with the incorporation of the CFM multiplied 250 times, due to the dissipation characteristics of the antimicrobial agent to be smaller after its incorporation into the membrane. Sampes of disks shape were made with a diameter of 5 mm in 4 different thicknesses (0.35, 0.70, 1.75 and 2.80 mm), containing the concentration 31.20 mg of CA. The disks were sterilized with incidence of UV light for 20 min on both sides, then the antifungal tests were started on disk diffusion.

Disk diffusion assay

The assay was carried out using the strains of C. albicans (ATCC 18804), Candida glabrata (ATCC 90030) and Candida krusei (ATCC 6258) from the Microbiology and Immunology Laboratory of the Institute of Science and Technology (ICT / UNESP).

The strains were diluted in 0.9% saline solution and standardized in a spectrophotometer at a concentration of 1 × 106 CFU / mL. After 100 µl of the standard solutions were seeded on Sabouraud dextrose agar (Himedia, Mumbai,

India) and the membranes containing different thicknesses (0.35, 0.70, 1.75 and 2.80 mm) with 31.2 mg CA were applied on the surface of the agar containing the yeasts sown. As a control group only the membranes were used without incorporation of CA. The plates were incubated for 24 h / 37 ° C and by digital calliper (0.01 mm graduation) and a reflected light source, the diameters of the fungal growth inhibition halos were measured around the samples, using as value for analysis the average of two measures perpendicular to each other.

Biofilm assay

According to the antifungal results in the diffusion disk test, the membrane PLA / PEG CA with 2.8 mm thickness and incorporated with the concentration of 31.2 mg of CA (2.8 mm + 31.2 = 23.8% CA) for the anti-biofilm tests was selected.
The strains of C. albicans ATCC 60193, C. krusei (ATCC 34135) and C. glabrata ATCC 90030 from the Laboratory of Experimental Pharmacology and Cell Culture of the Federal University of Paraíba were used for biofilm formation. Sabouraud broth plus sucrose (2%) containing 2,5 × 105 CFU / mL, standardized in a spectrophotometer, were prepared. Then, in 24-well plates (TPP, Switzerland), the inoculums were added in the volume of 1000 µl / well together with the membranes of PLA / PEG CA. For the multi-species biofilm, the volume of each yeast was 333 µl / well. The plates were incubated for 48 h / 37 ° C and for biofilm measurement the biomass test was used applying 1000 µl of the solution of violet crystal to 0.4% in contact for 45 min. Subsequently, washes were performed to remove excess dye and 1000 µl of 95% ethanol was added to demonstrate the dye incorporated into the biofilm. To read the test, solutions from each well were transferred to a 96-well plate, sent to the microplate spectrophotometer for reading at 595 nm [29]. The inhibition of adherence was measured indirectly relative to the yeast growth group, which was assigned a value of 100% fungal adherence.

2.3.9. Statistical analysis

One-way Analysis of Variance and Tukey's post-tests were applied to fibre diameter and mechanical data, with type I error (α) set as 0.05.An unpaired Student-

T test was applied to evaluate biofilm reduction, using the statistical program Bioestat 5.0.

3. Results

The volume ratio of PLA/ PEG (75:25), the flow rate of 7.0 mL/h, protrusion of 3 mm, work distance of 20 cm and under an air pressure 40 psi was selected as the best fibres parameters and applied for analysis. Figure 1 shows the SEM analysis of the meshes. In all micrographics is possible to see fibres discontinuous defects suggested beads and beside each mesh it is possible to see the mean diameter of the fibres by histograms and normal curve. The values were obtained from 100 measurements from randomly selected fibres.

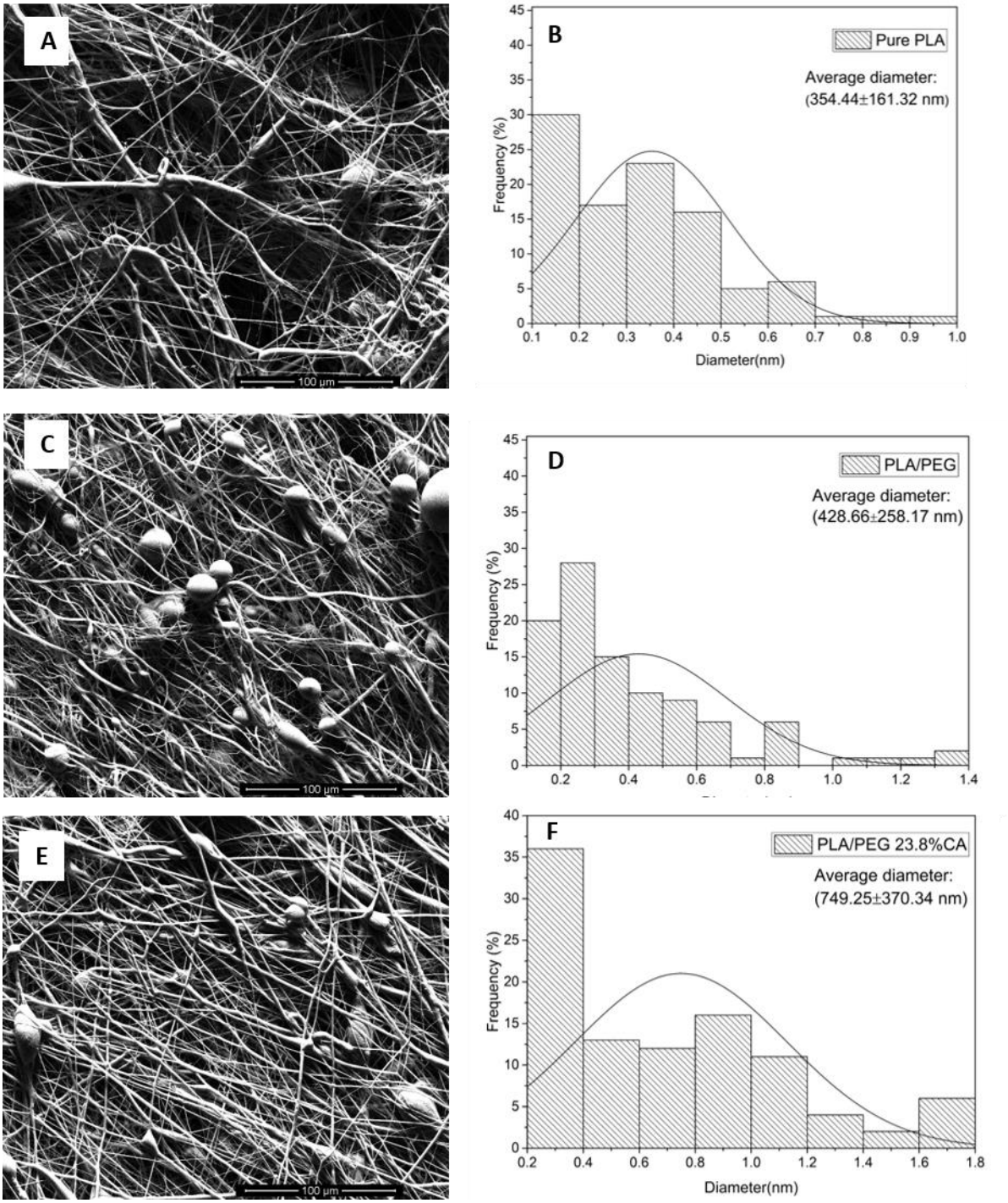

Figure 1 – SEM images of nanofibres on different diameter and morphology without and with cinnamaldehyde (CA). (A and B) PLA pure fibres, (C and D) PLA/PEG fibres, (E and F) PLA/PEG 23.8%CA.

Table 1 - Mechanical properties of the membranes and fibre diameters.

Sample	Tensile Strength (MPa)	Elastic Modulus (MPa)	Diameter (nm)
PLA	0.94 ± 0.06^{ab}	9.77 ± 1.40^{a}	354 ± 160^{a}
PLA/PEG	0.85 ± 0.09^{a}	18.74 ± 3.41^{b}	427 ± 250^{a}
PLA/PEG 23.8%CA	0.99 ± 0.16^{b}	24.94 ± 4.85^{c}	749 ± 370^{b}

**Different letters indicate statistically significant differences (5%).*

The contact angle of the distilled water and diiodomethane, in order to test the hydrophilicity and hydrophobicity of the membranes with CA, could not measurable because the drops were immediately absorbed by the meshes when in contact with them.

The thermogravimetric analyses (Figure 2) describes the thermal behaviour of meshes of PLA with Cinnamaldehyde with the heating of the temperature as a function of time.

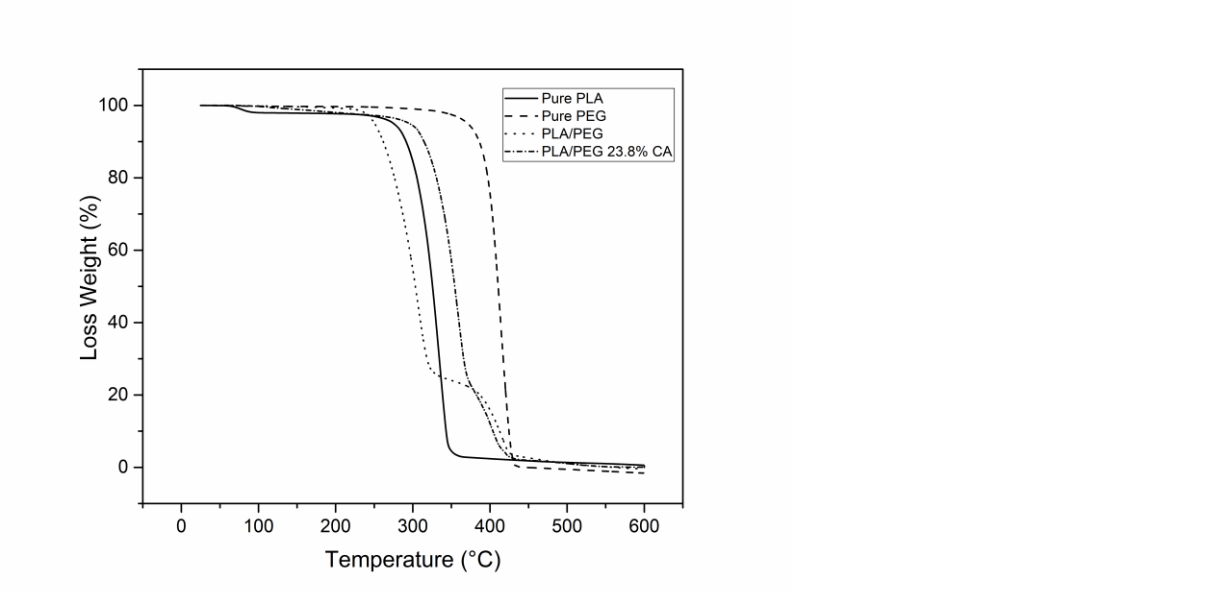

Figure 2. *TGA profiles for PDLLA, the PDLLA/PEG blend and PDLLA/PEG loaded with CA.*

The information found makes possible the thermal understanding of the material and the amount in % of organic material present in these meshes [24]. The onset loss of mass of pure PDLLA occurred at 255°C and with a loss of 3.22% in the pure PLA pure nanofibres that are related to the splitting of the main chains and to inter/intramolecular transesterification reactions. In the second stage of mass loss occurred at 350°C and with a loss of 93.34% that is related to pyrolysis the thermal

degradation of PLA chains. PEG pure: occurred at 337°C and with a loss of 2% in the PEG pure nanofibres that are related to the splitting of the main chains and to inter/intramolecular transesterification reactions. In the second stage of mass loss occurred at 435°C and with a loss of 99.63% that is related to pyrolysis the thermal degradation of PLA chains. PLA/PEG: had 3 stages from PLA and PEG curves then occurred at 335°C and with a loss of 1.62% in the PLA/PEG nanofibres to first stage to inter/intramolecular transesterification reactions. Another stage of mass loss occurred at 353°C and with a loss of 75% that is related to pyrolysis the thermal degradation of PLA and occurred 426°C and with a loss of 98.60% for pyrolysis from PEG chains, that showed that is really had 75:25 for blend ratio from PLA/PEG. PLA/PEG 23.8%CA: This sample to change a few degrees for an increase of the thermal stability to PLA/PEG blend.

The DSC analyzed, the thermal behavior of the studied meshes (PLA, PLA/PEG, and PLA/PEG 23.8%) and the heat flux (Figure 3) as a function of the temperature increase. Since PDLLA and PEG used are amorphous, only the glass transition temperature (Tg).

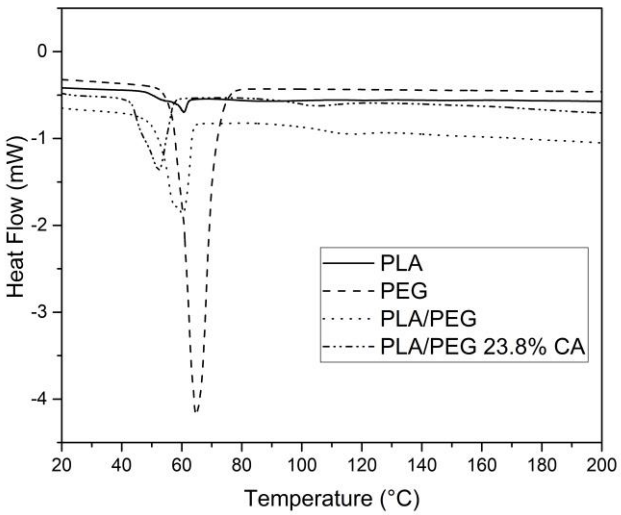

Figure 3 - DSC heating curves of PLA, PLA/PEG and PLA/PEG with CA (endotherm down).

The Fourier Transform Infrared analysis of nanofibres PLA, PEG, PLA/PEG is shown in Figure 4.

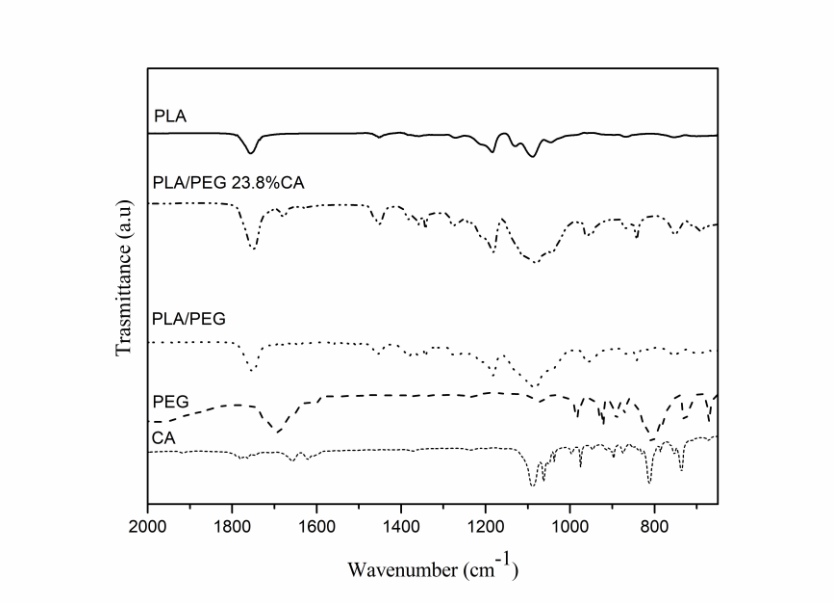

Figure 4 - *FTIR spectra of PLA, PLA/PEG and PLA/PEG with 23.8%CA.*

In vitro release kinetics of CA by membranes is shown in Figure 5.

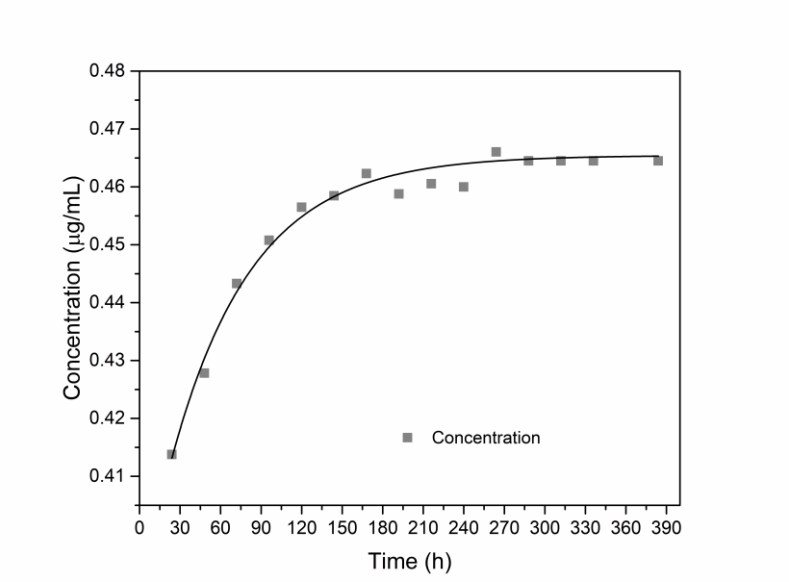

Figure 5 - *In vitro release kinetics of CA in PBS.*

Microbiology tests

Broth microdilution test

The broth micro-dilution test was performed on the MIC and the CFM of CA (125 µg / mL) on the yeasts C. albicans, C. kusei and C. glabrata.

Disk diffusion assay

The agar diffusion test showed that the PLA / PEG CA results with the membrane PLA / PEG CA with the concentration of 31.2 mg resulted in the formation of large inhibition halos, varying according to the thickness of the membrane used (Table 2).

The largest inhibition halos were obtained by the PLA / PEG CA mesh with 2.80 mm thickness and a concentration of 31.2 mg of cinnamaldehyde, with formation of halos with 29.8 mm for the strain of C. krusei, 33.2 mm for C. albicans and 35.5 mm for C. glabrata strain.

Control groups just PLA / PEG mesh application did not exhibit inhibition halo formation on the Candida strains evaluated.

Tabela 2– Agar diffusion test results.

Micro-organisms	Membrane thickness (mm)	Halo (mm)
C. glabrata	0.35 mm	21.8
C. glabrata	0.7 mm	26.85
C. glabrata	1.75 mm	28.04
C. glabrata	2.80 mm	35.54
C. albicans	0.35 mm	19.69
C. albicans	0.70 mm	19.69
C. albicans	1.75 mm	21.73
C. albicans	2.80 mm	33.27
C. krusei	0.35 mm	19.65
C. krusei	0.70 mm	23.35
C. krusei	1.75 mm	23.41
C. krusei	2.80 mm	29.81

Biofilm

Table 3 - Values of biofilms mono-species and multi-species percent reduction.

Biofilm	*Candida albicans*	*Candida krusei*	*Candida glabrata*	*Multi-species*
	56.64%	*79.14%*	*14.30%*	*61.08%*
	p=0.0002	*p=0.0005*	*p=0.1451*	*p<0.0001*

*$p \leq 0.05$

The results about fungal biomass assay shown that the meshes exhibited antifungal activities reducing more than 50%, exception to C. glabrata, of the microorganisms tested (Table 3).

The best activity of CA was against Candida krusei, with 79.14% reducing the biomass; C. albicans showed reductions of 56.64% and to C. glabrata just 14.3% its biomass had reduction, being the only one not to show reduction with statistical significance (p<0.05%). Multi-species biofilms also showed a significant reduction, having its biomass reduced in 61.08%.

4 Discussion

For the mechanical tests, the maximum tensile strength values showed only difference between PLA/PEG and PLA/PEG 23.8% CA membranes, however for the elastic modulus values, PLA meshes showed the lowest value and were followed by PLA/PEG and PLA/PEG 23.8%. Broz et al (2003) defenced that bending could change the properties the composite depend sensitively on the mechanical properties of the components as well as the blend microstructure and the interface between the phases [36]. The improvement of the mechanical properties of the mesh is important due the constant movement cause in prosthesis by the tongue and the chewing.

It was show beaded fibres that can be attributed to an instability in the pressure during the spinning process. The mean diameter of the fibres was influenced by CA addition as shown. Fibres had a significantly increased diameter with the addition of CA [25,27]. This was similar to other studies that added another second polymer to PLA and the second polymer and Copaiba oil together increase de diameter fibres [16]. According to the literature, three parameters are involved with increased diameter: the pressure, the flow rate injection and the solution viscosity [34].

Probability, the fact of PEG hydrophilicity and the affinity of PLA with diiodomethane could not allow the measure of contact angle. The fact that is a good property for the application of the experimental material that goal is release CA to a hydrophilic surface as human gum.

Other explanation for the difficult of contact angle measurement could be due to the increase of the surface hydrophilicity caused by the introduction of the plasticizer, PEG, which presents high hydrophilic characteristic, fact that do not allow necessary time for contact angle measurement during the test [28].

Thermal stability of nanofibers decreased with addition of PEG and increased with CA added to the blend. It is supposed the reason for this was the effect of plasticizer by PEG and probably the CA. cause a positive effect, as can see in better stability of the blend with 23.8% CA.

The PLA value glass transition temperature (Tg 60 °C) is according to other studies in literature[16,24] . PLA is the polymer in the highest proportion in the blend, whereby the proportion of PEG has only a small effect such as a shoulder in the curve because of the low PEG concentration in the fibres. The addition of PEG, which is a plasticizer, was able to promote a stable bond with PLA and allowed the insertion of CA between the polymer chains. PEG is a plasticizing agent and surfactant which could displace the peak of the blend if used in higher concentrations as in [24]. Tg peaks relative to fibres with CA (PLA / PEG 23.8%) showed that the phytoconstituent may have been mixed with PEG (since they have lower molar masses than PLA) and thus acting as a plasticizer by packaging the enveloped chains of the amorphous PLA, however having a Tg peak below 0 ° C would not change the curve, provided that it acted as an emulsifier between the PLA chains. Thus, in Figure 3 it can be seen that with the lowest CA concentration (1.23%) two Tg peaks (59 °C / 63 °C) appeared, and when increasing the concentration of CA (23.8%) the amount of emulsifying solution was higher which noted significant displacement of the peak to 52 °C, proving that the plastification of the polymer may have occurred due to the presence of CA.

The PLA results showed the active modes overlapped to give a broad asymmetric band at about 1754 cm^{-1}, that were described in the [29,30]. The ($C–H_3$) asymmetric deformation modes appeared at about 1452 cm^{-1}. Their stability infrequency reflected a pure vibrational mode. This region was characterized by a band at 1360 cm^{-1}.The1182 cm^{-1} band observed in PLA could be assigned to symmetric (C–O–C) stretching mode of ester groups. Asymmetric (C–O–C) modes were observed at 1086 cm^{-1}. Other bands were assigned as follows: the band near 1045 cm^{-1} corresponded to ($C–CH_3$) stretching and the band 868 cm^{-1} to (C–COO) stretching [30,31]. Regarding PEG nanofibres, spectra were observed that the strong band observed at 1110 cm^{-1} was assigned to the skeletal stretching mode, that could band association with the (C–O–C) asymmetric stretching mode. The doublet at 963 and 947 cm^{-1} has been much discussed in parallel with the confirmation of the ($C–H_2$) groups. This band had been assigned to the symmetric ($C–H_2$) rocking mode of the ($O–CH_2$; CH_2– O)

group in the gauche conformation. The weak band at 947 cm^{-1} is due to the hybridized mode of the (C–O–C) asymmetric stretching and the ($C–H_2$) symmetric rocking mode. Three strong bands are observed at ~1148, ~1062, and ~843 cm^{-1}. The band at 843 cm^{-1} has been assigned previously to the (C–H2) asymmetric rocking mode of the (C–H2) group in the gauche conformation. The band at 1148 cm^{-1} is primarily due to the ($C–H_2$) symmetric rocking mode, whereas the band at 1062 cm^{-1} is primarily due to the (C–O–C) asymmetric stretching mode coupled with the ($C–H_2$) symmetric rocking mode. The interaction of the characteristic bands of CA in the blends containing 1674 and 2812 cm^{-1} of (C=O) and (C-H) bonds, respectively, was not clearly perceived, since the same PEG and PLA/PEG bands may be superimposed [24].

The release occurred over 6 days, after which a plateau was observed, signifying complete release. In the present study is considered satisfactory, once the application of this product on the surface of the prosthesis in contact with the injured region is objectified, and a longer time than the one found could occur a relapse in the local and even formation of new types of biofilm aggravating or developing new lesions. In this way, the product obtained allowed what has proposed the technique of controlled release, which would be the delivery of the therapeutic agent in determined doses, at the treatment sites, controlling several problems, mainly as adverse reactions [21].

It was observed that the CA presented a curve with heterogenous release profile, initially superior in the first days and slower in the following days, fact justified by the release of the CA present on the surface of the fibres and later by the diffusional release, from the interior of the fibre, or even by the onset of degradation of the polymer [32,33].

The release profile may also have been favourable because of the high hydrophilicity of the meshes that can promote a release fast and controlled and affinity with the environments, which again endorses the promising use in wet circumstances such as the buccal cavity during the use. The release profile may also have been favourable because of the hydrophilicity of the meshes and affinity with the environments, which again endorses the promising use in wet circumstances such as the buccal cavity.

The study of the antifungal activity on biofilm growth models is important to create greater proximity to clinical situations. The present study showed activity of CA against Candida krusei in sequence to Candida albicans and multi- species biofilm. It was showed that have not reduction value to C. glabrata. The literature reported the higher capacity production of protein stress response possibly makes the species C. glabrata potentially more resistant [34], and consequently, this species shows the highest morbidity case amount

Candida non-albicans on immunocompromised populations [35]. The multi-species biofilm showed a significant statistical reduction.

5. Conclusion

Within methods applied and the obtained results in this study it was possible to conclude that:

When CA was added the diameter of fibres and number of beads was significantly increased. Thermal analyses (TGA and DSC) indicated better stability of the meshes. The release controlled of CA occurred until 12 days and the CA addition in PLA/PEG nanofibrous meshes (PLA/PEG CA 23.8% and 2.8 mm thickness) showing reductions in more than 50% of the biofilm biomass, being statistically significant (p<0.05%), provide effective action antifungal in yeast of Candida albicans and no albicans can potentially be used in treatment of fungal disease.

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3 CONSIDERAÇÕES GERAIS

A maioria dos fármacos utilizados para tratamento de infecções fúngicas bucais apresentam dificuldade de eficácia, por não conseguir se manter no local, devido os movimentos linguais e o ambiente bucal constantemente úmido em virtude da saliva. Outras limitações encontradas pelos antifúngicos são a resistência de várias espécies a estes bem como as reações adversas provocadas nos pacientes.

A busca por tratamentos alternativos, como o uso da fitoterapia, tem crescido a cada dia, e é frequente a utilização desse tipo de terapia no tratamento de inúmeras doenças.

A bioengenharia hoje consegue aliar a utilização de drogas já consagradas ou mesmo fitoterápicos para produzir estruturas tais como mantas de nanofibras que permitem a liberação controlada, reduzido efeitos adversos.

Neste trabalho, o sistema de fiação Solution Blow Spinning permitiu que se produzissem mantas de fibras ultrafinas contendo um fitoconstituinte do Óleo de Canela, o Cinamaldeído, que permitiu a redução de biofilme de espécies tais como: *Candida albicans, Candida krusei e Candida glabrata.*

Mediante o observado é interessante que mais estudos utilizando a técnica do Solution Blow Spinning, que é promissora por produzir fibras em maior quantidade sem grande demanda de tempo, pudesse buscar outros produtos naturais que possam ser adaptados e utilizados não só na cavidade bucal, mas como para tratamento para outras patologias causadas por infecções microbianas.

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