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DESENVOLVIMENTO DE LIPOSSOMAS NANOMÉTRICOS PARA ARMAZENAMENTO E LIBERAÇÃO CONTROLADA DE PEPTÍDEOS ANTIMICROBIANOS

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DESENVOLVIMENTO DE LIPOSSOMAS NANOMÉTRICOS PARA ARMAZENAMENTO E LIBERAÇÃO CONTROLADA DE PEPTÍDEOS ANTIMICROBIANOS

Tese submetida ao Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos como requisito para a obtenção do grau de Doutora em Ciência e Tecnologia de Alimentos.

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"Todas as vitórias ocultam uma abdicação"

Simone de Beauvoir

RESUMO

Os compostos antimicrobianos naturais são um tema de grande interesse devido ao aumento da demanda por alimentos seguros e de alta qualidade. A utilização de lipossomas é uma alternativa interessante para proteger antimicrobianos nos alimentos, além de fornecer compostos naturais de liberação controlada. Os lipossomas revestidos com polissacarídeos apresentam melhor estabilidade, representando uma alternativa aos lipossomas convencionais. Inicialmente, os nanolipossomas que encapsulam a nisina foram preparados com fosfatidilcolina de soja (PC) e pectina ou ácido poligalacturônico. Os lipossomas desenvolvidos apresentaram alta eficiência de encapsulação, baixo índice de polidispersão e foram estáveis durante 21 dias a 7 °C e 25 °C. A atividade antimicrobiana foi observada contra cinco cepas diferentes de *Listeria* em placas de ágar de leite, com uma melhor eficiência contra *L. innocua* 6a. Em um segundo momento, as características estruturais dos lipossomas foram estudadas por dispersão de raios-X de pequeno ângulo (SAXS) e as amostras foram submetidas a ciclos de temperatura (20-60 °C). Para isso, os lipossomas foram desenvolvidos contendo pectina ou ácido poligalacturônico pelos métodos de hidratação de filme e evaporação em fase reversa, para encapsular nisina. A análise de SAXS confirmou a presença de estruturas lamelares em todas as amostras. Além disso, parte da estrutura multilamelar tornou-se cúbica, provavelmente devido à presença de nisina nos lipossomas. A adição de polissacarídeos mostrou diferenças entre as fases cúbicas formadas. Em última análise, a mistura de lisozima e nisina foi encapsulada em lipossomas contendo polissacarídeos. O diâmetro médio dos lipossomas foi de 85,6 e variou para 77,3 e 79,9 nm com a incorporação de pectina ou ácido poligalacturônico, respectivamente. O potencial zeta dos lipossomas com polissacarídeos foi de cerca de -30 mV, mostrando alta eficiência de encapsulação. A atividade antimicrobiana foi avaliada a 37 °C, mostrando que a PC-pectina reduziu a população de *L. monocytogenes* em 2 log UFC/mL e 5 log UFC/mL em leite integral e desnatado, respectivamente. Em refrigeração, a PC-pectina reduziu a população de *L. monocytogenes* para quase zero por até 25 dias em leite desnatado. Portanto, pode dizer-se que os lipossomas que contêm polissacarídeos podem ser uma tecnologia promissora para o encapsulamento da lisozima e nisina. Além disso, a existência de estrutura cúbica nos lipossomas pode proporcionar liberação controlada de antimicrobianos.

Palavras chave: nisina, lisozima, lipossomas, pectina, ácido poligalacturônico, SAXS.

ABSTRACT

Natural antimicrobial compounds are a topic of utmost interest due to the increased demand for safe and high-quality foods. The use of liposomes is an interesting alternative to protect antimicrobials in food, also providing controlled release natural compounds. Polysaccharides coated liposomes present better stability, representing an alternative to conventional liposomes. Initially, nanoliposomes encapsulating nisin were prepared with soy phosphatidylcholine (PC) and pectin or polygalacturonic acid. The liposomes developed presented high encapsulation efficiency, low polydispersity index, and were stable for 21 days at 7°C and 25°C. The antimicrobial activity was observed against five different strains of Listeria in milk-agar plates, with a better efficiency against L. innocua 6a. In a second moment, structural characteristics of liposomes were studied by small angle X-ray scattering (SAXS) and the samples were submitted to temperature cycles (20-60°C). For this, liposomes were developed containing pectin or polygalacturonic acid by the thin-film hydration method and reverse phase evaporation method for nisin encapsulation. The analysis of SAXS confirmed the presence of lamellar structures in all the samples. In addition, part of the multilamellar structure became cubic, probably due to the presence of nisin in the liposomes. The addition of polysaccharides showed differences between the cubic phases formed. Ultimately, the mixture of lysozyme and nisin were encapsulated in liposomes containing polysaccharides. The mean diameter of the liposomes was 85.6 and varied to 77.3 and 79.9 nm with the incorporation of pectin or polygalacturonic acid, respectively. The zeta potential of liposomes with polysaccharides were around -30 mV, showing high encapsulation efficiency. The antimicrobial activity was assessed at 37 °C, showing that PC-pectin reduced the population of L. monocytogenes to 2 log CFU/mL and 5 log CFU/mL in whole and skim milk, respectively. At under refrigeration, PC-pectin reduced the population of L. monocytogenes to almost zero for up to 25 days in skim milk. Therefore, it can say that the liposomes containing polysaccharides can be a promising technology for the encapsulation of lysozyme and nisin. In addition, the existence of cubic structure in the liposomes can provide controlled release of antimicrobials.

Keywords: nisin; lysozyme; liposomes; pectin; polygalacturonic acid; SAXS.

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1 INTRODUÇÃO

O constante avanço tecnológico tem inspirado o desenvolvimento de novas tecnologias para a conservação de alimentos e metodologias de processamento, tornando a segurança de alimentos uma questão de grande relevância. A utilização de biomoléculas com atividade antimicrobiana pode controlar a multiplicação de micro-organismos indesejáveis, estendendo a vida útil e mantendo a qualidade e segurança dos alimentos, além de não causar qualquer efeito tóxico ou indesejável para os consumidores (Gyawali e Ibrahim, 2014; Tajkarimi, Ibrahim e Cliver, 2010). O uso de substâncias naturais para a conservação de alimentos atende à crescente demanda por produtos mais saudáveis e seguros, incluindo alimentos minimamente processados e livres de aditivos sintéticos. Muitos agentes antimicrobianos naturais, entre eles, peptídeos antimicrobianos (bacteriocinas, lactoferrina), substâncias derivadas de plantas (polifenóis, isotiocianatos) e enzimas (lisozima, lisostafina) têm sido utilizados para controlar micro-organismos patogênicos através da incorporação na matriz alimentar ou em filmes e revestimentos. No entanto, esses compostos promissores podem ter seu efeito prejudicado devido a interações indesejáveis e inativação na matriz alimentar (Fathi, Martín e McClements, 2014; Meira et al., 2014; Rhim et al., 2013).

Neste contexto, a nanotecnologia pode ser utilizada para melhorar a estabilidade antimicrobiana em alimentos, geralmente mostrando vantagens em comparação com os antimicrobianos em sua forma livre. Nanoestruturas utilizadas como veículos para proteger compostos bioativos, podem ser adequadas no controle de bactérias patogênicas, mantendo sua viabilidade durante exposição à de diferentes estresses ambientais, melhorando assim a estabilidade e a eficácia (Brandelli, 2012). Com isso, novas perspectivas para a aplicação de compostos antimicrobianos fundamentam-se na nanotecnologia, entre elas o desenvolvimento de nanopartículas para encapsulação destes compostos.

Dentre as nanopartículas mais empregadas, as nanovesículas lipossômicas têm recebido atenção especial. É crescente a aplicação de lipossomas na área alimentícia, devido às inúmeras vantagens que essas estruturas podem fornecer ao serem usados como sistemas encapsulantes de substâncias bioativas, podendo ser utilizados para liberação controlada de componentes funcionais, tais como: proteínas, enzimas, vitaminas, entre outros componentes que podem ser utilizados com o intuito de alterar o sabor ou o aroma dos alimentos (Taylor et al., 2005).

Segundo Fathi, Martín e McClements (2014) o material de encapsulação deve possuir qualidade alimentar, ser biodegradável e estável em sistemas alimentares durante processamento, armazenamento e consumo. Sistemas de entrega à base de polissacarídeos são adequados para muitas aplicações na indústria, sendo considerados biocompatíveis e biodegradáveis, possuindo elevado potencial para serem modificados a fim de obter as propriedades requeridas.

Algumas modificações têm sido propostas para estabilizar lipossomas, visando a ação mais eficiente dessas estruturas e aumento da retenção do material encapsulado. O aumento no interesse em produtos naturais alternativos e a descoberta de polissacarídeos na superfície das células estimula o estudo sobre as interações entre lipossomas e diferentes tipos de polissacarídeos (Silva et al., 2014). A pectina é um polissacarídeo linear aniônico que pode formar complexos com outros polímeros (Canteri et al., 2012; Voragen et al., 2009). Devido a características eletrostáticas e de formação de géis, a pectina associada a outros polímeros tem sido avaliada como material de revestimento, com o intuito de conferir uma liberação sustentada e/ou direcionada do composto bioativo. No entanto, essas estratégias ainda não resultaram na obtenção de sistemas totalmente eficazes (Camilo, 2007).

Neste contexto, peptídeos antimicrobianos apresentam grande potencial de uso em substituição aos conservantes químicos comumente utilizados. Aliado a isso, o desenvolvimento de novas tecnologias para liberação controlada destes compostos, com a utilização de polissacarídeos, como a pectina ou o ácido poligalacturônico, se torna um tema promissor de estudo.

1.1 Objetivos

1.1.1 Objetivo geral

Desenvolver lipossomas contendo pectina ou ácido poligalacturônico como sistemas para encapsulação de peptídeos antimicrobianos.

1.1.2 Objetivos específicos

- Desenvolver lipossomas contendo pectina ou ácido poligalacturônico para encapsular nisina, pelo método de hidratação de filme.
- Caracterizar os lipossomas com nisina através de seu tamanho e polidispersividade, morfologia, eficiência de encapsulação.
- Avaliar o efeito antimicrobiano de lipossomas contendo nisina contra diferentes cepas de *Listeria* spp mediante ensaios de atividade em ágar leite como sistema modelo.
- Caracterizar a estrutura dos lipossomas de nisina produzidos pelo método de hidratação de filme e evaporação em fase reversa, através da técnica SAXS.
- Desenvolver lipossomas contendo pectina ou ácido poligalacturônico para encapsular a mistura de nisina e lisozima pelo método de hidratação de filme.
- Caracterizar os lipossomas com nisina e lisozima através de seu tamanho e polidispersividade, morfologia, eficiência de encapsulação.
- Avaliar a atividade antimicrobiana de lipossomas contendo a mistura de nisina e lisozima mediante curvas de crescimento microbiano usando leite como sistema modelo.

2 REVISÃO BIBLIOGRÁFICA

2.1 Nanopartículas

Nanopartículas (NP) são partículas coloidais, possuindo diâmetros que variam entre 1 e 1000 nm, em que os compostos podem ser encapsulados, adsorvidos ou dispersos. Uma grande variedade de nanopartículas compostas por diferentes materiais, incluindo lipídeos, polímeros naturais e sintéticos, e materiais inorgânicos vem sendo desenvolvidas, resultando em sistemas de liberação que variam nas suas propriedades físico-químicas e permitindo, assim, uma variedade de aplicações (Cushen et al., 2012; Mora-Huertas, Fessi e Elaissari, 2010; Letchford e Burt, 2007; Peters et al., 2011).

De uma forma geral, as nanopartículas podem ser obtidas em diversas morfologias como nanoesferas, nanocápsulas e nanolipossomas, como mostradas na Figura 1 (Gu et al., 2015; Mora-Huertas, Fessi e Elaissari, 2010; Quintanar-Guerrero et al., 1998; Rawat et al., 2006). Utilizando compostos orgânicos para formar dispersões nanométricas, substâncias insolúveis podem ser preparadas para se comportarem como moléculas verdadeiramente dissolvidas, abrindo um enorme potencial para produtos inovadores e competitivos. O desenvolvimento de aditivos alimentares sensíveis, tais como vitaminas e antioxidantes, em uma camada protetora pode prevenir a deterioração durante a produção e armazenamento. Outra aplicação é o mascaramento de propriedades organolépticas indesejadas dos compostos benéficos por meio da nanoencapsulação (Peters et al., 2011).

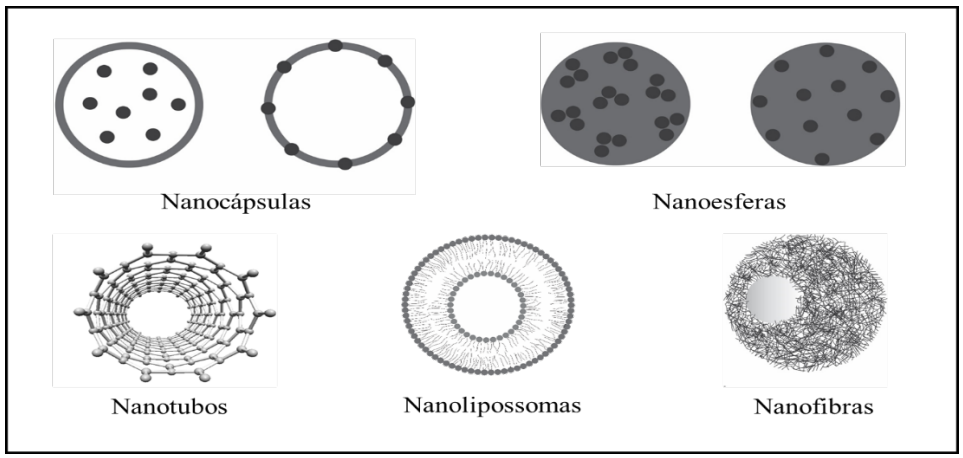

Fig. 1 - Diferentes tipos de nanoestruturas (Lopes e Brandelli, 2017).

O uso de nanocarreadores pode modular a liberação de agentes antimicrobianos, protegendo-os contra condições adversas, melhorando sua estabilidade, diminuindo assim a quantidade necessária para obter o efeito antimicrobiano. Os sistemas de nanoencapsulação têm excelentes propriedades de liberação controlada. Além disso, a encapsulação de compostos antimicrobianos reduz o custo, porque uma menor quantidade do ingrediente ativo é necessária (Blanco-Padilla et al., 2014).

O tipo de sistema nanoestruturado está frequentemente associado com as características da substância antimicrobiana a ser encapsulada e do material utilizado, além da natureza do alimento ao qual será destinado. Um resumo das principais propriedades de algumas nanoestruturas disponíveis para encapsulação de antimicrobianos estão apresentadas na Tabela 1.

Tabela 1. Comparação de diferentes nanoestruturas.

Nanoestruturas	Características	Vantagens
Nanolipossomas	Vesículas contendo uma ou mais bicamadas de fosfolipídios, com núcleo aquoso	Produzido utilizando ingredientes naturais em escala industrial, podendo encapsular compostos com diferentes solubilidades
Nanopartículas metálicas	Composto de metais como cobre, prata, zinco, paládio, ou titânio, podendo ser utilizadas para produzir nanoestruturas com tamanho forma e porosidade variável	Pode ser facilmente incorporado em diversos materiais, entre eles produtos têxteis e plásticos
Nanofibras	Nanoestruturas com diâmetros pequenos e grandes áreas de superfície, que promove a melhoria de propriedades físicas, químicas e biológicas	Produzido a partir de diferentes materiais orgânicos e inorgânicos
Nanotubos	Os nanotubos são divididos em tubos de carbono de parede única ou duplos	Podem ser de composição orgânica ou inorgânica
Nanopartículas poliméricas	Dependendo do método de preparação, nanoesferas ou nanocápsulas podem ser obtidas, onde os compostos são dissolvidos, aprisionados, encapsulados ou ligados à matriz das nanopartículas	Preparo relativamente fácil, podendo formar complexos com polissacarídeos, lipídeos e outros polímeros

Fonte: Lopes e Brandelli, 2017.

2.2 Lipossomas: Estrutura e propriedades

Nanopartículas a base de lipídeos (composta por fosfolipídios, triacilglicerídeos, ácidos graxos, entre outros) são as mais utilizadas, uma vez que podem ser produzidas a partir de ingredientes naturais, podendo encapsular compostos com diferentes solubilidades e ser aplicados em nível industrial (Peters et al., 2011).

Lipossomas são vesículas de bicamada concêntricas, onde o volume aquoso está inteiramente fechado através de uma bicamada lipídica composta principalmente de fosfolipídios, sendo este o nome dado a lipídeos que possuem um resíduo de ácido fosfórico em sua estrutura. Tal molécula possui duas caudas hidrofóbicas ou apolares, compostas de hidrocarbonetos, e um grupo hidrofílico chamado de cabeça polar (Rawat et al., 2006). A Figura 2 mostra uma molécula de fosfatidilcolina, um tipo de fosfolipídio cujo grupo polar é a colina.

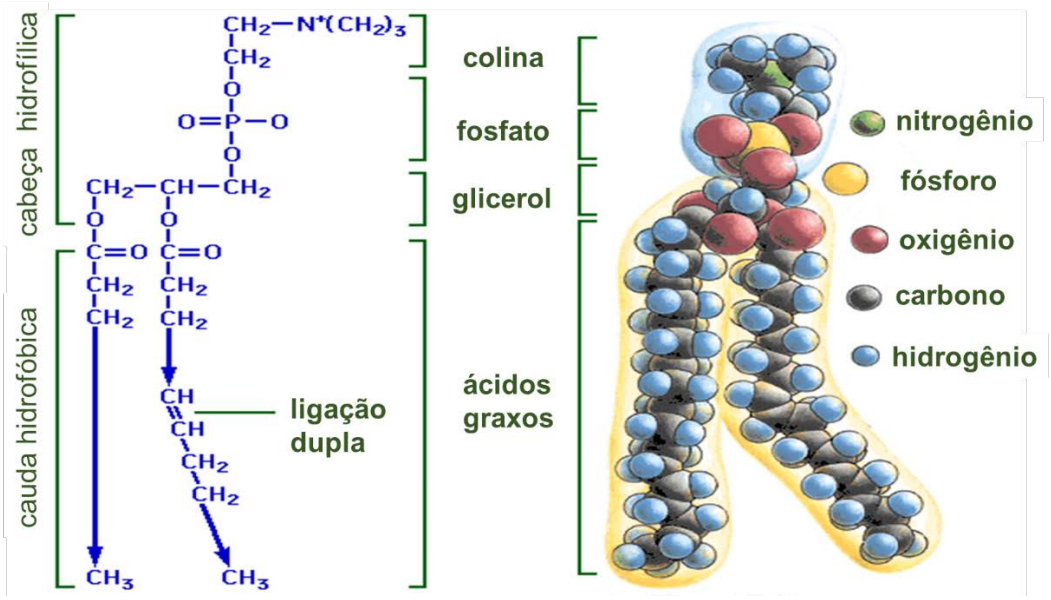

Fig. 2: Estrutura geral de um fosfolipídio (http://www.madsci.org/posts/archives/2006-12/1164999854.Bc.r.html).

As moléculas de fosfolipídios são insolúveis em água, porém, quando em ambientes aquosos, dependendo da concentração e da temperatura, formam dispersões e se ordenam em agregados, onde a parte hidrofílica fica em contato com a água, enquanto a parte hidrofóbica se localiza no interior da estrutura, formando as bicamadas lipídicas. Em solução aquosa, acima de uma determinada concentração, dependendo da temperatura, tais bicamadas lipídicas curvam-se sobre si mesmas dando origem aos lipossomas, onde os fosfolipídios encapsulam

parte do meio aquoso onde estão inseridos. A caracterização dos lipossomas é realizada pelo tamanho, carga da superfície e número de bicamadas (Rawat et al., 2006), podendo ser classificado nas categorias estruturais mostradas na Tabela 2 e representadas na Figura 3.

Tabela 2. Classificação dos lipossomas baseada em parâmetros estruturais.

Tipo de lipossoma	Diâmetro médio
Vesículas multilamelares (MLV)	Maior que 0,5 µm
Vesículas oligolamelares (OLV)	0,1-1,0 µm
Vesículas unilamelares (UV)	Não especificado
Vesículas unilamelares pequenas (SUV)	20-100 nm
Vesículas unilamelares grandes (LUV)	Maior que 100 nm
Vesículas unilamelares gigantes (GUV)	Maior que 1,0 µm
Vesículas multivesiculares (MVV)	Maior que 1,0 µm

Fonte: adaptado de Toniazzo, 2013.

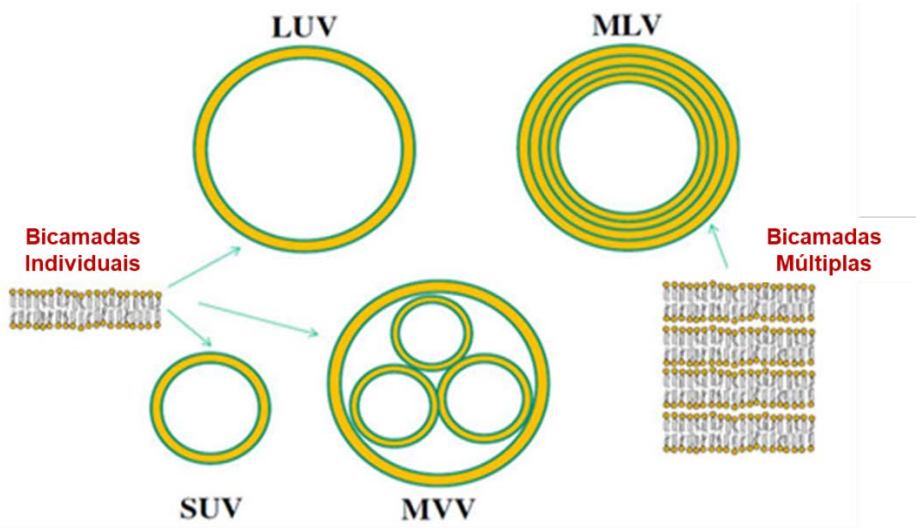

Fig. 3: Diferentes tipos de lipossomas formados a partir de bicamadas lipídicas. LUV: vesículas unilamelares grandes; MLV: vesículas multilamelares; SUV: vesículas unilamelares pequenas; MVV: vesículas multivesiculares. Fonte: (McClements, 2015).

Nanolipossomas são definidos como vesículas de bicamada lipídica (vesículas <30 ou 30-100 nm), que possuem e mantém o tamanho nanométrico durante a aplicação e armazenamento. A característica comum de moléculas formadoras de bicamadas está na sua anfifilicidade, isto é, possuem regiões polares e não polares definidas, que podem reter, entregar e controlar a liberação de materiais solúveis em água e lipídeos, como apresentado na Figura 4 (Bouwmeester et al., 2009; Mozafari et al., 2006).

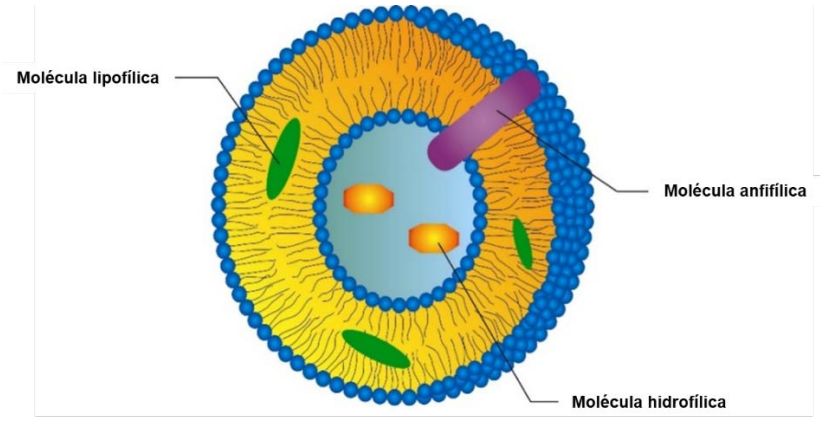

Fig. 4: Lipossoma formado por fosfolipídios em solução aquosa. Fonte: Brandelli, Lopes e Boelter, 2017.

A aparência das suspensões de lipossomas depende da sua estrutura, determinando o seu comportamento de dispersão de luz. Suspensões que possuem lipossomas pequenos se apresentam opticamente transparentes, devido ao fato de não dispersarem a luz fortemente, enquanto que as suspensões que contêm lipossomas maiores podem apresentar turbidez (Khlebtsov, 2001). As características elétricas dos lipossomas dependem do tipo e da concentração de fosfolipídios dentro da formulação, apresentando para a fosfatidilcolina grupos catiônicos e aniônicos ligados na sua cabeça hidrofílica. O grau de ionização dos grupos de cabeça varia de acordo com o pH, onde a carga da fosfatidilcolina pode ser negativa em valores altos de pH e positiva em pH baixo (McClements, 2015; Singh et al., 2012).

Alterações de pH podem reduzir as interações eletrostáticas e promover a agregação dos lipossomas, afetando a sua estabilidade. Para isso, alternativas tem sido utilizadas, como o revestimento da superfície das vesículas com certos polímeros, tais como polietileno glicol (PEG) e quitosana, ou através da utilização de ingredientes catiônicos/aniônicos na estrutura dos lipossomas. Assim, se torna desejável maximizar as forças de repulsão entre as vesículas para evitar a agregação e sedimentação das mesmas durante o armazenamento, aumentando sua

estabilidade. Em geral, as partículas com valores de potencial zeta maiores que +30 mV ou menores que -30 mV possuem maior estabilidade eletrostática (Mozafari et al., 2008).

2.3 Métodos de preparo

A formação de lipossomas e nanolipossomas não é um processo espontâneo. As vesículas lipídicas são formadas quando os fosfolipídios são colocados em água e, consequentemente, forma uma camada dupla ou uma série de bicamadas, cada uma separada por moléculas de água, uma vez que a energia adequada é fornecida (por exemplo, sob a forma de sonicação, homogeneização, aquecimento, etc.) (Mozafari et al., 2008). A escolha correta do método de preparação depende dos seguintes parâmetros (Gomez-Hens e Fernandez-Romero, 2006; Mozafari et al., 2008):

- Características físico-químicas do lipossoma e do material a ser encapsulado;
- Natureza do meio em que as vesículas estão dispersas;
- Concentração da substância encapsulada e sua potencial toxicidade;
- Processos adicionais envolvidos durante a aplicação das vesículas;
- Tamanho ótimo, polidispersividade e durabilidade das vesículas para a aplicação que se destina;
- Reprodutibilidade do lote, possibilidade de produção em larga escala de produtos seguros e eficientes.

Existem diversos tipos de métodos de preparo de lipossomas relatados na literatura, incluindo o método de hidratação do filme lipídico, evaporação em fase reversa, técnica de injeção de solvente e método de remoção de detergente. Além disso, algumas técnicas têm sido utilizadas para reduzir o tamanho dos lipossomas, como por exemplo, sonicação, extrusão de alta pressão e microfluidização. Alguns dos métodos mais importantes estão descritos a seguir (Huang et al., 2014; Patil e Jadhav, 2014):

Método de hidratação do filme lipídico: esse método baseia-se na solubilização de lipídeos em um solvente orgânico, seguida da etapa de evaporação desse solvente, resultando na formação de um filme lipídico seco no fundo de um balão de vidro. Esse filme é posteriormente hidratado, através da adição de um meio aquoso, sendo submetido ao processo de agitação, com a formação dos lipossomas (Figura 5).

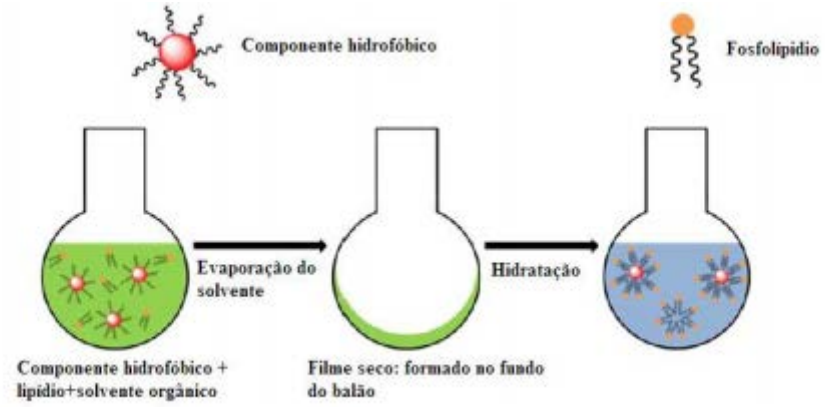

Fig. 5: Esquema do método de hidratação do filme lipídico (Toniazzo, 2013).

Evaporação em fase reversa: nesse método ocorre a mistura do fosfolipídio em um solvente orgânico, sendo posteriormente adicionado uma solução aquosa contendo o composto bioativo. A mistura é homogeneizada através de sonicação, formando uma emulsão água-em-óleo. Em seguida, o solvente orgânico é evaporado, resultando na formação de um gel, que é submetido à agitação para a formação da dispersão dos lipossomas (Figura 6).

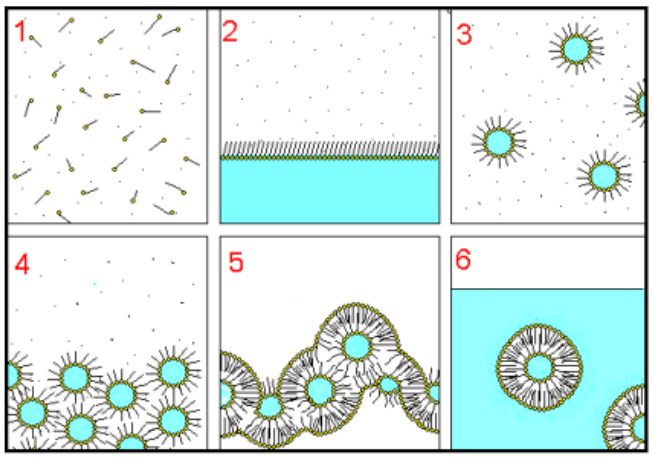

Fig. 6: Esquema para o processo de preparação de lipossomas por evaporação em fase reversa. 1: mistura do fosfolipídio em solvente orgânico; 2: adição de solução aquosa contendo o composto bioativo; 3: sonicação; 4: formação de emulsão água-em-óleo. 5: formação de gel; 6: formação dos lipossomas (Mertins, 2004).

Pró-lipossomas: esse método se baseia no preparo de uma mistura de lipídios secos, com posterior hidratação desta mistura para a formação dos lipossomas do tipo MLV. Tais partículas secas podem ser produzidas por atomização, resultando em estruturas amorfas, muito mais

fácies de hidratar quando comparado com outros métodos convencionais de formação de lipossomas.

Método da injeção de etanol: nesse método uma solução de etanol contendo fosfolipídios é injetada em água pura, ou em uma solução aquosa. Durante esse processo, as fases aquosa e orgânica entram em contato, levando a diluição imediata do etanol na fase aquosa. Assim, se obtém a precipitação das moléculas, formando fragmentos de bicamadas lipídicas. Durante o processo ocorre agitação do sistema, onde as bicamadas lipídicas se organizam, levando a formação dos lipossomas (Figura 7).

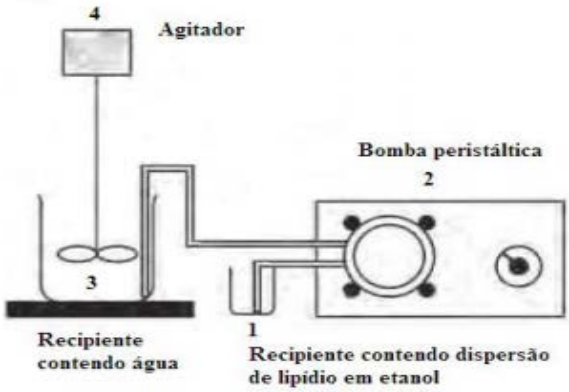

Fig. 7: Esquema da produção de lipossomas pelo método de injeção de etanol (Toniazzo, 2013).

Método de remoção de detergente: o método se baseia na solubilização de lipídios em detergente, formando micelas mistas de detergente/lipídio. A formação dos lipossomas ocorre quando o detergente é removido da solução, utilizando para isso técnicas como diálise. Esse método pode ser utilizado para formar uma grande variedade de tipos de lipossomas, possuindo a capacidade de produção de lipossomas com distribuição de tamanho homogênea.

De maneira complementar, a Tabela 3 apresenta as vantagens e desvantagens dos métodos de produção de lipossomas descritos no texto.

Tabela 3. Vantagens e desvantagens dos métodos convencionais de produção de lipossomas.

Método de preparo	Vantagens	Desvantagens
Pró-lipossomas	Componente bioativo previamente incorporado nas partículas de pró-lipossomas; alta eficiência de encapsulação; técnica simples e prática; facilidade para produção em grande escala	Formação de lipossomas com distribuição de diâmetros heterogêneos; somente formação de MLV
Hidratação do filme lipídico seco	Processo simples	Longo período na etapa de remoção do solvente; produção em pequena escala; baixa eficiência de encapsulação
Injeção de etanol	Processo relativamente simples	Resíduo do solvente orgânico
Remoção de Detergente	Projeto simples; controle do tamanho das partículas, dispersão homogênea	Resíduo de detergente; longo tempo de processo; baixo rendimento
Evaporação em fase reversa	Alta eficiência de encapsulação	Resíduo de solvente orgânico

Fonte: adaptado de Maherani et al. (2011).

2.4 Métodos de caracterização

O comportamento de lipossomas, tanto em relação à estabilidade de armazenamento, como em meios biológicos, é determinado por fatores como tamanho de partícula, carga de superfície, concentração de amostra e pelas propriedades da solução. Desta forma, é fundamental a determinação e controle de tais parâmetros para assegurar a eficiência e a estabilidade das formulações dos lipossomas (Lopes, 2014). Alguns métodos relevantes utilizados para caracterização de lipossomas são descritos abaixo.

2.4.1 Espalhamento de luz dinâmico (DLS)

A técnica de espalhamento de luz dinâmico (Dynamic Light Scattering, DLS) é amplamente utilizada para medir o tamanho e a distribuição do tamanho de partículas em suspensão. O espalhamento da luz está relacionado ao movimento Browniano das partículas, de modo que a intensidade da luz espalhada por estas, forma um padrão de movimento. Este movimento em suspensão faz com que a luz laser incidida nas partículas seja espalhada em diferentes intensidades (Lopes, 2014).

O princípio deste método consiste em relacionar a velocidade de difusão das partículas provocada pelos seus movimentos Brownianos com o tamanho das mesmas. A intensidade de dispersão da luz detectada está diretamente relacionada com a velocidade de difusão das partículas e consequentemente, com o tamanho destas. Partículas menores se movimentam mais rapidamente e causam rápidas modificações no espalhamento da luz. Por outro lado, partículas de maior diâmetro, as quais possuem menores coeficientes de difusão, resultam em menores flutuações na intensidade do espalhamento da luz (Delgado, 2013; Lopes, 2014).

Outro parâmetro calculado através dessa técnica é o índice de polidispersão (PDI), que consiste em uma análise da função de autocorrelação da intensidade de dispersão de luz. Para isso, um único tamanho de partícula é assumido e um ajuste exponencial simples é aplicado à função de autocorrelação. Assim, os valores de PDI são utilizados para descrever a largura da distribuição de tamanho das partículas (Delgado, 2013).

2.4.2 Potencial Zeta

A maioria das partículas dispersas em meio aquoso, tende a adquirir uma carga elétrica superficial, devido à natureza química dos constituintes da partícula e do próprio meio de dispersão. A presença dessas cargas afeta a distribuição de íons que estão em uma região muito próxima da partícula (interface entre a superfície da partícula e o meio de suspensão), promovendo o acúmulo de íons de carga oposta à carga da superfície, na região que cerca a partícula. Esses íons acabam formando uma “camada elétrica” que reveste a partícula e a mesma é dividida em duas partes (Figura 8): uma camada interna, onde os íons estão fortemente ligados à partícula, e uma camada externa, onde os íons difundem e estão fracamente ligados (Mertins, 2008).

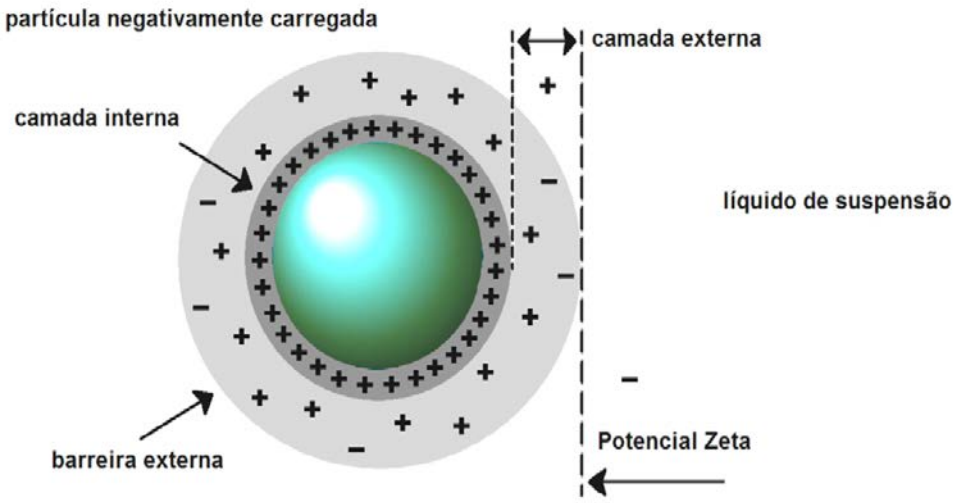

Fig. 8: Representação esquemática do potencial zeta (Mertins, 2008).

As medições do potencial zeta são comumente utilizadas para avaliar as interações repulsivas entre as partículas e prever a estabilidade dos sistemas coloidais. Valores de potencial zeta elevados (negativa ou positivamente) indicam que haverá repulsão entre as partículas, reduzindo assim a tendência de agregação das mesmas. Dispersões coloidais com valores de potencial zeta em torno de ± 30 mV são geralmente consideradas estáveis (Laouini et al., 2012).

O método utilizado para determinar o potencial zeta dos lipossomas consiste na incidência de um feixe de luz e aplicação de um campo elétrico de força conhecida através da amostra. Com isso, as partículas carregadas se deslocam com velocidades distintas induzindo deslocamentos da frequência do feixe de luz incidente, gerando um espectro de frequências, que são então utilizadas para os cálculos das velocidades, as quais são convertidas para valores de mobilidades eletroforéticas (Lopes, 2014).

2.4.3 Eficiência de encapsulação (EE)

A caracterização dos lipossomas através da determinação da eficiência de encapsulação é de extrema importância, uma vez que a eficácia de lipossomas *in vivo*, bem como suas propriedades físico-químicas depende do total de composto ativo incorporado às nanoestruturas (Lopes, 2014).

A obtenção de valores elevados de EE é importante, podendo ser alcançada pela escolha correta do método de encapsulação. Diferentes métodos são utilizados na quantificação dos teores de material encapsulado, entre eles: espectrofotometria, espectroscopia de fluorescência, métodos enzimáticos, técnicas eletroquímicas e cromatografia líquida de alta eficiência

(Edwards e Baeumner, 2006; Pinilla e Brandelli, 2016). Segundo Laouini et al. (2012), essas técnicas podem ser empregadas também para monitorar a estabilidade de armazenamento de lipossomas em relação à retenção/liberação do material encapsulado ao longo do tempo.

2.4.4 Espalhamento de raios-x a baixo ângulo (SAXS)

Os fosfolipídios, componentes das bicamadas dos lipossomas, são moléculas anfifílicas que, quando dispersos em água, podem formar diferentes fases. Dependendo da temperatura, geometria molecular dos lipídeos e as condições da mistura lipídeo/água, essas moléculas podem se organizar de diferentes formas, podendo ser classificados principalmente em fase lamelar, fase cúbica e fase hexagonal, de acordo com suas diferentes estruturas internas (Koynova e Tenchov, 2013).

As fases lamelares são classificadas em lamelar cristalina (Lc), lamelar gel (L_β) e lamelar fluida (L_α). Em determinadas temperaturas ocorrem transições de fases dos lipídeos, sendo que a principal transição ocorre quando a membrana lipídica passa de uma fase lamelar ordenada rígida (L_β) para uma fase lamelar fluida (L_α), momento no qual há um elevado grau de liberdade das moléculas. Com o aumento da temperatura, outras transições de fase podem ocorrer a partir da fase lamelar fluida. Essas transições são relativas à transição de uma fase lamelar para fases não lamelares. A sequência das fases não lamelares geralmente se inicia com a fase cúbica invertida (Q_{II}), onde três fases distintas podem ser identificadas: Pn3m, Ia3d e Im3m, passando para a fase hexagonal invertida (H_{II}), como ilustrado na Figura 9. Entre essas fases, a cúbica e a hexagonal receberam atenção considerável devido às suas estruturas internas altamente ordenadas, capazes de sustentar a liberação lenta de compostos bioativos com diferentes tamanhos e polaridades (Chen, Ma e Gui, 2014; Guo et al., 2010; Koynova e Tenchov, 2013).

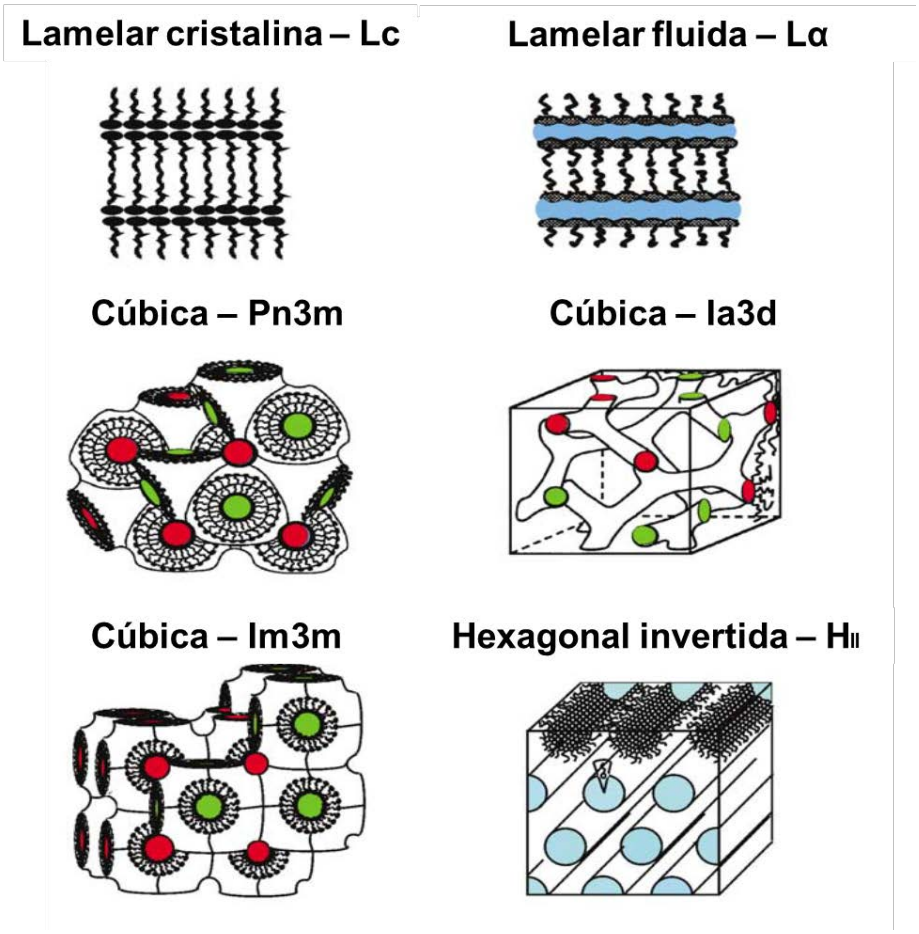

Fig. 9: Representação esquemática das fases lamelar, cúbica e hexagonal (Caffrey, 2003).

A determinação das transições de fase da membrana lipídica é importante, uma vez que esses parâmetros influenciam a estabilidade dos lipossomas nos sistemas biológicos. Diferentes métodos físicos são empregados na investigação das transições de fase dos fosfolipídios, entre eles, o emprego da técnica de espalhamento de raios-x, que visa compreender os mecanismos de associação do composto encapsulado aos lipossomas, bem como para o entendimento da estabilidade desses nanossistemas (Lopes, 2014).

Os raios-x, utilizados nos estudos de análise estrutural, podem ser obtidos a partir de fontes de luz síncroton. Essa fonte de luz emite a radiação por elétrons ou pósitrons em velocidade próxima à velocidade da luz, através de um anel circular. Quando acelerados radialmente, os elétrons emitem a radiação síncroton, durante a passagem por campos magnéticos colocados em alguns pontos da trajetória do anel de armazenamento. A obtenção de padrões de difração com elevado grau de informação é permitida pela radiação síncronton, que possui fluxo de algumas ordens de grandeza maior (aproximadamente 10^{12} fótons/s) que as demais fontes (Dong e Boyd, 2011).

O espalhamento de raios-x a baixo ângulo (SAXS) consiste em uma técnica fundamental para o estudo da organização de sistemas vesiculares em nível molecular. A interação da radiação com a estrutura eletrônica e o seu subsequente espalhamento oferece informações detalhadas de aspectos estruturais que dificilmente poderiam ser obtidas por outras técnicas (Mertins, 2008). A análise do SAXS permite a obtenção de informações referentes às mudanças estruturais dos lipossomas, desencadeadas após um estímulo específico (pH, temperatura, luz, etc.), o qual pode estar relacionado à desestruturação dos lipossomas com consequente liberação do material encapsulado (Dong e Boyd, 2011).

As análises de SAXS são utilizadas para medir a intensidade de raios-X dispersos em ângulos, que pode ser expresso pelo comprimento do vector de espalhamento *q*:

$$q = (\frac{4\pi}{\lambda}) sin \frac{\theta}{2}$$

onde θ é o ângulo de incidência e λ é o comprimento de onda do feixe de raios-X incidente (Lindblom e Rilfors, 1989). O ordenamento das bicamadas fornece razões de reflexão de Bragg, mostrando valores típicos para o espaçamento das reflexões dos raios-X característicos das mesofases (Tabela 4), permitindo a identificação das fases formadas nos lipossomas.

Tabela 4. Mesofases e índices de picos correspondentes observados utilizando SAXS.

Mesofases	Razões características para os diferentes espaçamentos
Lamelar	1:2:3:4:5:6:7...
Hexagonal	1:√3:2:√7:3:√12:√13...
Cúbica (Im3m)	1:√2:2:√6:√8:√10...
Cúbica (Pn3m)	1:√2:√3:2:√6:√8:3...
Cúbica (Ia3d)	1:√3:2:√10:√11...

Fonte: Lindblom e Rilfors, 1989.

2.5 Aplicação dos lipossomas em alimentos

A crescente aplicação de lipossomas na área alimentícia é devida às vantagens que os lipossomas podem fornecer ao serem usados como sistemas para encapsulação de substâncias bioativas, como a proteção de tais substâncias contra alterações químicas e enzimáticas, bem como variação da temperatura e da força iônica (Mozafari et al., 2008). A maioria das técnicas de encapsulação aplicadas na indústria de alimentos são baseadas em matrizes de biopolímeros

como amidos, gomas, proteínas, dextrinas e alginatos. A utilização de lipossomas para a liberação controlada de componentes funcionais, tais como peptídeos, enzimas e vitaminas têm motivado diferentes pesquisas (Taylor et al., 2005; Malheiros, Daroit e Brandelli, 2010).

Um dos primeiros relatos da aplicação de lipossomas em alimentos foi na fabricação de queijos (Law e King, 1985), com o objetivo de diminuir o tempo e custo de maturação. Para isso, foram adicionadas proteinases encapsuladas em lipossomas na mistura do queijo, demonstrando que a atividade e a estabilidade das enzimas foram melhores com a encapsulação, além de melhorar o sabor do queijo e diminuir os custos de produção.

Lee et al. (2000) encapsularam a enzima bromelina, utilizada no amaciamento de carnes, avaliando que a estabilidade e a biodisponibilidade da enzima aumentaram significativamente com a encapsulação em lipossomas. Rao et al. (1995) utilizaram a encapsulação de β-galactosidase em lipossomas, com o objetivo de auxiliar a digestão de alimentos lácteos, demonstrando que os lipossomas podem estabilizar a enzima durante o armazenamento.

Toniazzo et al. (2014) estudaram a encapsulação de β-caroteno em lipossomas para a aplicação em iogurte. Os lipossomas foram capazes de proteger o β-caroteno da degradação por um período de até 95 dias e, quando aplicados nos iogurtes, mostraram que a textura não foi afetada, sugerindo que parte dos corantes artificiais poderia ser substituída pelos lipossomas encapsulando β-caroteno.

Lipossomas foram estudados como transportadores de vitamina C e E, com o objetivo de serem incorporados em suco de laranja. A combinação de formulações de lipossomas e vitaminas não alterou as características organolépticas do suco de laranja, além disso, mostrou estabilidade microbiológica após a pasteurização e armazenamento a 4°C, por 37 dias (Marsanasco et al., 2011).

Peptídeos antimicrobianos têm sido extensivamente estudados como potenciais bioconservantes, porém, sua atividade antimicrobiana pode ter seu efeito diminuído devido à degradação proteolítica e a interação do peptídeo antimicrobiano com os componentes alimentares. A encapsulação de bacteriocinas em lipossomas pode ser uma alternativa para sobrepor esse problema (Maherani et al., 2011).

Malheiros, Daroit e Brandelli (2012) investigaram a eficácia da nisina livre e encapsulada em nanolipossomas para o controle de *Listeria monocytogenes* em queijo minas frescal. Para isso a nisina comercial foi encapsulada em lipossomas de lecitina de soja parcialmente purificada. Os resultados mostraram efeito bactericida com 0,25 mg/mL de nisina livre; efeito bacteriostático para nisina encapsulada em lipossomas e com 0,1 mg/mL de nisina livre.

O peptídeo antimicrobiano P34 foi encapsulado em lipossomas, preparados com fosfatidilcolina de soja parcialmente purificada. Os resultados apresentaram alta eficiência de encapsulação e estabilidade durante o armazenamento, além de apresentar ação contra a bactéria patogênica *L. monocytogenes*. O estudo sugere que a encapsulação deste peptídeo pode ter potencial para uso como conservante alimentar (Malheiros et al., 2011).

A encapsulação de pediocina em nanolipossomas foi avaliada por Mello et al. (2013). Os lipossomas obtidos apresentaram elevada eficiência de encapsulação e boa estabilidade, demonstrando potencial para aplicação em alimentos. A aplicação de bacteriocinas produzidas por *Lactobacillus sakei* subsp. *sakei* 2a para a conservação de alimentos foi estudada por Malheiros, Cuccovia e Franco (2016). A encapsulação em lipossomas foi eficaz e retardou o crescimento de *L. monocytogenes* por 5 dias em leite de cabra UHT armazenado a 7 °C.

2.6 Biopolímeros

A utilização de biopolímeros em sistemas de liberação controlada têm recebido muita atenção, devido a sua biocompatibilidade e biodegradabilidade, possuindo a vantagem de serem incorporados numa vasta gama de produtos comerciais (Hu et al., 2015; Khaksar et al., 2014).

A quitosana é um biopolímero muito empregado no revestimento de nanopartículas. A caracterização de nanovesículas contendo fosfatidilcolina de soja e o polissacarídeo quitosana foi realizada por Mertins et al. (2006), onde as nanovesículas foram preparadas através do método de evaporação em fase reversa. O raio hidrodinâmico das nanovesículas variou entre 106 e 287 nm, dependendo do conteúdo de quitosana e o tamanho de filtro utilizado. A obtenção de vesículas esféricas em escala manométrica foi alcançada com o uso de filtros 0,45 μm.

Lipossomas revestidos com quitosana representam uma alternativa aos lipossomas convencionais, pois apresentam melhor estabilidade e bioadesividade. Gonçalves et al. (2012) desenvolveram e avaliaram estabilidade físico-química de melatonina encapsulada em lipossomas com quitosana. Os resultados demonstraram que a encapsulação em lipossomas é viável e com boa eficiência. A quitosana melhorou a estabilidade dos lipossomas encapsulando melatonina, além de fornecer nanoestruturas melhor organizadas, devido a redução da espessura da bicamada.

Hasan et al. (2016) encapsularam curcumina em nanolipossomas contendo quitosana, mostrando que a adição de quitosana aumentou significativamente a estabilidade dos lipossomas. Silva et al. (2014) desenvolveram nanolipossomas revestidos com o biopolímero

quitosana ou sulfato de condroitina, demonstrando que a formulação com quitosana foi mais estável e eficiente na inibição de *L. monocytogenes* quando comparado com a formulação que utilizava o sulfato de condroitina.

Muitos relatos da literatura apontam para os benefícios que a adição do biopolímero quitosana pode fornecer ao ser adicionado como revestimento de nanopartículas, estimulando a busca por outros polímeros que possam estabilizar lipossomas.

2.6.1 Pectina

Propriedades únicas da pectina, como a flexibilidade, não toxicidade e baixo custo, torna esse polímero um candidato ideal para muitas práticas em diferentes áreas da ciência (Khazaei, Khazaei e Rahmati 2015). A pectina é um biopolímero aniônico extraído de diferentes fontes, principalmente de frutos cítricos ou maçã. A estrutura da pectina é heterogênea e possui uma ampla gama de massa molecular (50.000 a 150.000). A pectina é um polissacarídeo linear que consiste de unidades de ácido galacturônico com pequenas quantidades de açúcares neutros, onde os monômeros estão unidos por ligações α 1,4. Além disso, sugere-se que as moléculas de pectina possuem regiões galacturônicas longas e regulares (*smooth*), e regiões ricas em ramnose (*hairy*) que transportam açúcares como cadeias laterais (Figura 10), formadas por moléculas de arabinose, galactose e xilose, ligados covalentemente aos carbonos 2 e 3 dos resíduos de ácido galacturônico ou ao carbono 4 dos resíduos de ramnose. Os grupos carboxílicos dos ácidos galacturônicos podem ser parcialmente esterificados com grupamentos metil e parcialmente ou completamente neutralizados por íons sódio, potássio ou amônio (Kjøniksen, Hiorth e Nyström 2005; Monsoor, Kalapathy e Proctor 2001).

Pectinas são geralmente classificadas de acordo com seu grau de metoxilação (DM), definido como a porcentagem de resíduos de ácido galacturônico que são metilesterificados, sendo esse um parâmetro chave na determinação da funcionalidade da pectina. Pectinas com DM superior a 50% são classificadas como pectinas de alto teor de éster metílico (HM), enquanto que pectinas com DM inferior a 50% são classificadas como pectinas de baixo teor de éster metílico (LM) (Ventura e Bianco-Peled, 2015).

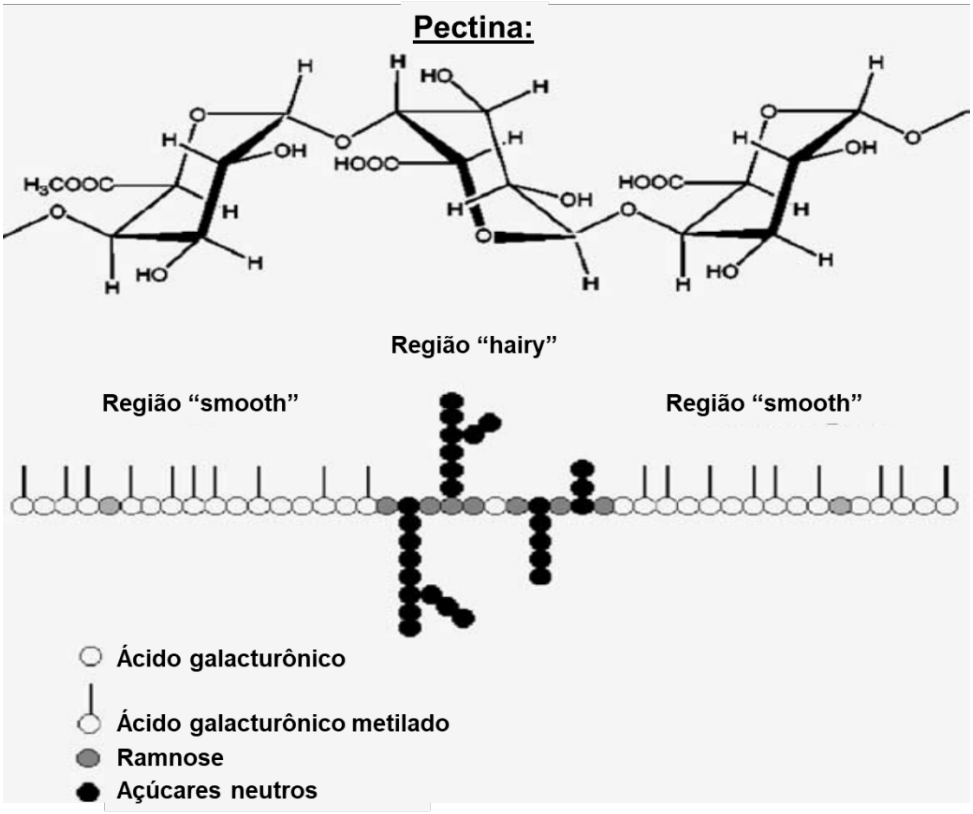

Fig. 10: Estrutura química da pectina (Kjøniksen, Hiorth and Nyström 2005).

O conteúdo de metoxila pode variar com a origem da pectina, sendo que a variação que ocorre em frutas está condicionada ao seu desenvolvimento e amadurecimento. As pectinas fazem parte de um grupo de substâncias, denominadas de substâncias pécticas, onde estão incluídos o ácido péctico, ácido pectínico e protopectina, apresentando-se como um polissacarídeo complexo de alto peso molecular (Kashyap et al., 2001).

O ácido péctico é considerado o componente mais simples das substâncias pécticas, sendo composto de unidades de ácido galacturônico combinados por ligações α- glicosídicas (Figura 11a). Ácidos pectínicos possuem estruturas similares aos ácidos pécticos, diferenciando-se por apresentar algumas unidades de ácidos galacturônicos esterificados (Figura 11b). A combinação da pectina com a celulose e hemicelulose, as quais também se apresentam como componentes do material estrutural da parede celular dos vegetais através de ligações covalentes, origina a protopectina. A protopectina, por sua vez, pode ser facilmente atacada por ácidos diluídos, liberando a pectina (Kashyap et al., 2001).

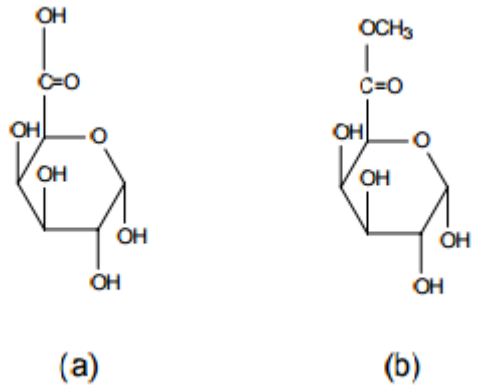

Fig. 11: Estruturas de unidades do ácido galacturônico (a) e ácido galacturônico esterificado (b) (Bobbio, 1989).

Na literatura é possível encontrar trabalhos com a utilização de diferentes biopolímeros, entre eles a pectina, para o desenvolvimento de nanopartículas. Essas nanopartículas são desenvolvidas com o objetivo de encapsular compostos antimicrobianos e substâncias ativas. O uso de três tipos de pectina (ácido péctico, pectina de alto e baixo grau de esterificação) foi testado por Krivorotova et al. (2016) para a formação de nanopartículas carregadas com nisina. O estudo mostrou que o grau de metoxilação da pectina influenciou a eficiência de carregamento da nisina e o tamanho das nanopartículas formadas. As nanopartículas contendo nisina apresentaram atividade antimicrobiana dependente do biopolímero utilizado, sendo considerado o ácido péctico a formulação mais adequada para aplicação na indústria como sistema antimicrobiano.

A mistura de alginato com pectina de alta metoxilação para a produção de micropartículas foi realizada por Khaksar et al. (2014), visando a liberação controlada de nisina. Os resultados mostraram que o teor de nisina teve influência significativa na eficiência de encapsulação e no tamanho das micropartículas, apresentando valores de 47-54% e 57-131 µm, respectivamente. Além disso, os estudos de liberação *in vitro* indicaram que o teor de nisina nas micropartículas influenciou sua taxa de liberação, apresentando-se mais elevada quando utilizada concentrações mais baixas de nisina nas micropartículas.

Nanopartículas preparadas com pectina e zeína (Hu et al., 2015) foram utilizadas para a encapsulação de curcumina, apresentando elevada eficiência de encapsulação (>86%), além do tamanho de partícula relativamente pequeno (250 nm) e índice de polidispersividade de 0,24. Através da análise de infravermelho foi possível concluir que a curcumina encapsulada interagiu com a zeína através de interações hidrofóbicas. O estudo sugere que essas

nanopartículas podem ser aplicadas em alimentos e bebidas funcionais, bem como suplementos dietéticos e produtos farmacêuticos.

Burapapadh, Takeuchi e Sriamornsak (2015) desenvolveram um método simples de produção de nanopartículas com pectina para a encapsulação de itraconazol, um antifúngico utilizado na indústria farmacêutica. Através do método de homogeneização mecânica foi possível produzir nanopartículas com pectina que apresentaram elevada porcentagem de fármaco dissolvido (60-80%) e mantiveram boas propriedades de dissolução, mesmo após o armazenamento por 1 ano. Os resultados sugerem que as nanopartículas de pectina preparadas a partir de nanoemulsões poderiam ser utilizadas como transportador eficaz no melhoramento da dissolução de itraconazol.

2.7 Antimicrobianos naturais

2.7.1 Nisina

O antimicrobiano nisina é uma bacteriocina produzida por *Lactococus lactis*, pertencente à família de lantibióticos, na qual contém grupos de lantionina e metil lantionina. A nisina é um peptídeo antimicrobiano catiônico e anfifílico, que possui ponto isoelétrico acima de 8,5 e tem sido utilizado como conservante em alimentos, por ser eficiente em baixo pH e a altas temperaturas (Meira et al., 2015; Salmieri et al., 2014).

A nisina é um polipeptídeo pequeno, com 34 aminoácidos, que apresenta as variantes A, Z, M, Q, que são derivados de *Lactococcus lactis*, enquanto os tipos U e U2 são obtidos a partir de *Streptococcus uberis*, e nisina P é produzida por *Streptococcus suis* e *Streptococcus gallolyticus* subsp. *pasteurianus* (Qi et al., 2012; Wu et al., 2014). As variantes A e Z diferem por um único aminoácido na posição 27, sendo histidina em nisina A, e asparagina em nisina Z. Dentre esses tipos, a nisina A é considerado o mais ativo contra patógenos (Prombutara et al., 2012).

Diferentes tipos de concentrados de nisina são comercialmente produzidos e adicionados a alimentos para controlar as bactérias patogênicas em produtos como leite, queijos pasteurizados, molhos e molhos para salada, apresentando características ideais para um aditivo alimentício, na medida em que não apresenta efeitos sobre a microbiota normal do intestino, é atóxica, não afeta a cor ou sabor dos alimentos e apresenta estabilidade térmica (Gálvez et al., 2007). O Nisaplin™ é um produto comercial, estabelecido no mercado, composto por 2,5%

nisina A, 77,5% NaCl e leite desidratado isento de gordura (12% de proteína e 6% de carboidratos) e é normalmente utilizado como conservante na indústria de alimentos (Guiga et al., 2010).

A nisina tem recebido atenção particular devido a sua grande atividade antimicrobiana contra uma ampla variedade de bactérias Gram-positivas, entre elas patógenos de origem alimentar, como *Listeria monocytogenes, Staphylococcus aureus*, bem como a inibição eficaz de esporos de *Bacillus* e *Clostridium*. Esse peptídeo é considerado um aditivo alimentar (E234) seguro para o uso em alimentos, sendo aprovado pela Food and Agriculture Organization/World Health Organization (FAO/WHO). Também faz parte da lista do FDA e é um aditivo geralmente reconhecido como seguro (GRAS) (Ibarguren et al., 2014; Salmieri et al., 2014).

A ingestão diária permitida pela FAO/WHO é de até 33.000 unidades de nisina por 70 kg de peso corporal, sendo que esse valor pode variar dependendo de cada país. Austrália, Grã-Bretanha e França não estipulam limite máximo de ingestão para a nisina, enquanto que nos EUA, o limite máximo é de 10.000 UI/g; na Rússia 8000 UI/g, e para a Argentina, Itália e México, o limite é de 500 UI/g, para os queijos e alimentos processados (Chen e Hoover, 2003). No Brasil, a nisina tem o seu uso permitido pela Legislação Brasileira (DETEN/MS nº 29, de 22 de janeiro de 1996) com a função de conservador para queijos pasteurizados no limite máximo de 12,5 mg/kg (500 UI/g).

A introdução direta de agentes antimicrobianos em produtos alimentares, entre eles a utilização de nisina em sua forma livre (não encapsulada), está associada com a perda de atividade devido à degradação e possível interação entre os diferentes componentes dos alimentos. Atualmente, alguns novos métodos de encapsulamento foram desenvolvidos com o objetivo de superar essas limitações, podendo melhorar a atividade antimicrobiana e a estabilidade desses conservantes em sistemas complexos (Ibarguren et al., 2014; Imran et al., 2015). Prombutara et al. (2012) encapsularam nisina em nanopartículas lipídicas sólidas, produzidas por homogeneização a alta pressão, com o objetivo de proteger e prolongar a atividade biológica da nisina. As nanopartículas apresentaram liberação controlada da nisina, inibindo a multiplicação de *Listeria monocytogenes* DMST 2871 e *Lactobacillus plantarum* TISTR 850 durante 15-20 dias, enquanto que a nisina livre mostrou inibição por apenas três dias com as mesmas bactérias.

Malheiros, Daroit e Brandelli (2012) investigaram a eficácia da nisina livre e encapsulada no controle de *Listeria monocytogenes* em queijo minas frescal. Nisina comercial foi encapsulada em lipossomas de lecitina de soja parcialmente purificada, apresentando efeito bactericida com 0,25 mg/mL de nisina livre e efeito bacteriostático com nisina encapsulada em

lipossomas e com 0,1 mg/mL de nisina livre. Silva et al., (2014) avaliaram a atividade antimicrobiana da nisina encapsulada em nanovesículas contendo polissacarídeos. A nisina foi encapsulada em lipossomas de fosfatidilcolina adicionados de quitosana ou de sulfato de condroitina, através do método de hidratação de filme e testados quanto a atividade antimicrobiana contra *Listeria* spp. A nisina encapsulada apresentou eficácia semelhante a nisina livre na inibição de *Listeria* spp. isolada de carcaça bovina, e maior eficiência na inibição de *Listeria monocytogenes*. A formulação contendo quitosana mostrou-se mais estável e eficiente na inibição de *L. monocytogenes*, quando comparada com outras nanovesículas testadas.

Nanocápsulas de quitosana e carragenina foram preparadas por Chopra et al. (2014) utilizando o método de complexação iônica, para a liberação controlada e sustentada da nisina. Dentre as formulações testadas, a que apresentou os melhores parâmetros foi a que continha 10,0 mg/mL de quitosana, 0,75 mg/mL de carragenina e 1,0 mg/mL de surfactante. As nanocápsulas apresentaram atividade antimicrobiana contra diferentes micro-organismos, entre eles *Micrococcus luteus* MTCC 1809, *Pseudomonas aeruginosa* MTCC 424, *Salmonella enterica* MTCC 1253 e *Enterobactor aerogenes* MTCC2823, e quando incorporadas em suco de tomate fresco demonstrou ação por até seis meses.

Filmes nanocompósitos de poli (ácido lático) contendo nanocristais de celulose (PLA-CNC) foram preparados pelo método de revestimento por adsorção, usando a nisina como agente antimicrobiano. Esses filmes foram introduzidos em embalagens que continham presunto cozido e estocados por 14 dias a 4 °C, mostrando-se estável neste período e apresentando inibição eficiente contra *L. monocytogenes* (Salmieri et al. 2014).

2.7.2 Lisozima

Lisozima é um antimicrobiano natural que pode ser derivado de ovos, plantas, bactérias e secreções animais (Were et al., 2003). Segundo Bugatti, Sorrentino e Gorrasi (2017), a União Européia classifica a lisozima como uma molécula com atividade bacteriostática, bacteriolítica e bactericida, e a Food and Drug Administration considera essa proteína uma substância GRAS.

A lisozima é uma molécula alcalina, com ponto isoelétrico de 11.35, e sua estrutura química é caracterizada por uma única cadeia polipeptídica com massa molecular de 14,6 kDa. A atividade antimicrobiana desta enzima está relacionada com a capacidade de hidrolisar as ligações β1,4- glicosídicas entre o ácido *N*-acetilmurâmico e a *N*-acetilglicosamina. Tais ligações estão presentes nos peptidoglicanos, que compreendem 90% da parede celular das

bactérias Gram-positivas, tornando-as muito suscetíveis à atividade antimicrobiana da lisozima (Bugatti, Sorrentino e Gorrasi, 2017; Liu et al., 2013). As bactérias Gram-negativas são menos propensas a ação da lisozima, pois são protegidas pela membrana externa, se tornando relativamente resistente às atividades antimicrobianas da lisozima (Bermudez e Forciniti, 2004). No entanto, a lisozima de ovo de galinha quando desnaturada, por meio de aquecimento e mudanças de pH, podem tornar-se efetivas contra bactérias Gram-negativas, sugerindo uma ação antimicrobiana independente da função catalítica, podendo ser atribuída à ligação da membrana e subsequente perturbação das funções da membrana (Ibrahim et al., 1996).

Devido à sua atividade antibacteriana, a lisozima tem recebido atenção considerável na indústria de alimentos (Venkataramani, Truntzer e Coleman, 2013). Comercialmente, a lisozima tem sido utilizada para inibir o crescimento de *Clostridium tyrobutyricum* em queijo (Chung e Hancock, 2000; Were et al., 2003) com o objetivo de reduzir a deformação do alimento devido à formação de gases fermentativos, resultantes do crescimento microbiano. Sua utilização também foi estudada na produção de vinho, com o objetivo de prevenir a fermentação heterolática durante o envelhecimento do produto, visto que a lisozima é capaz de controlar o crescimento de bactérias ácido láticas, responsáveis por essa fermentação (Lasanta et al., 2010).

Além disso, estudos estão sendo realizados visando aumentar a capacidade lítica da lisozima, promovendo a ação contra bactérias Gram-negativas. O efeito sinérgico da lisozima com outros conservantes, tem resultado em uma significante melhora no seu espectro de ação. Em muitos casos, a combinação de lisozima com alguns conservantes naturais tem tido um desempenho melhor em relação à enzima sozinha. Combinações de lisozima e nisina foram testadas em sistemas alimentares, reduzindo e controlando bactérias Gram-positivas, além de inibir *S.* Typhimurium e *E. coli* O157: H7 (Gill e Holley, 2000; Gill e Holley, 2000b).

Usualmente é realizada a introdução direta de antimicrobianos nos produtos alimentares, contudo, a interação dos compostos antimicrobianos com os componentes alimentares pode reduzir a eficácia contra patógenos, sendo muitas vezes necessárias grandes concentrações antimicrobianas (Were et al., 2004). Desta forma, a ação da lisozima pode ser reforçada através de revestimentos protetores para a liberação controlada (Liu et al., 2013), podendo aumentar a atividade antimicrobiana e a estabilidade em sistemas alimentares complexos.

Nanopartículas encapsulando lisozima foram revestidas com ácido poli-γ-glutâmico e quitosana, através do método de ionização, promovendo a liberação da lisozima *in vitro* de forma lenta. Além disso, as nanopartículas revestidas apresentaram boa atividade antimicrobiana contra *B. subtilis* e *E. coli* (Liu et al., 2013). A avaliação das propriedades da

lisozima encapsulada através da secagem por pulverização, bem como o efeito da complexação, com diferentes concentrações de pectina, foi avaliada por Amara et al. (2016). Os resultados revelaram que a secagem por pulverização apresentou um efeito significativo sobre a estrutura, a atividade e a mobilidade da lisozima, principalmente em concentrações mais elevadas de pectina (acima de 0,5 g/L).

2.8 Doenças transmitidas por alimentos

Doenças graves podem ser transmitidas pela ingestão de alimentos contaminados. Segundo a CDC (Center for Diases Control and Prevention), em 2015 foram notificados 20.107 casos de doenças transmitidas por alimentos, ocorrendo 4.531 hospitalizações e 77 mortes confirmadas por essa causa, incluindo infecções bacterianas e por parasitas. O agente de maior incidência foi a *Salmonella* sp, apresentando 7.728 casos, seguido do *Campylobacter* que apresentou 6.309 casos. *Listeria* é o agente patogênico com maior porcentagem de hospitalizações (96%), sendo que 12,9% dos casos levaram a óbito, demonstrando elevado percentual de mortes por hospitalização, quando comparado com *Salmonella* (0,4%) e *Campylobacter* (0,2%).

O gênero *Listeria* consiste em um grupo de bactérias Gram-positivas, anaeróbios facultativos, que não formam esporos e são móveis a temperatura de 20 a 25 °C. *Listeria* spp são isolados a partir de uma diversidade de fonte ambientais, incluindo solo, água, efluentes, fezes de humanos e animais, bem como uma grande variedade de alimentos. O gênero *Listeria* inclui atualmente dezessete espécies: *L. monocytogenes, L. ivanovii, L. innocua, L. welshimeri, L. seeligeri, L. grayi, L. marthii, L. rocourtiae, L. fleischmannii, L. weihenstephanensis, L. floridensis, L. aquatica, L. cornellensis, L. riparia, L. grandensis*, *L.booriae*, e *L. newyorkensis*. A doença infecciosa causada por essas bactérias é conhecida como listeriose, sendo que a *L. monocytogenes* pode causar sérias infecções localizadas e generalizadas em humanos, como meningite, encefalite e septicemia (Jarvis et al., 2016; Vázquez-Boland et al., 2001; Weller et al., 2015).

Entre estas, a espécie *Listeria monocytogenes* pode multiplicar-se em temperaturas que variam de -0,4 °C a 45 °C, com intervalo de pH 4,0 a pH 9,6 (pH ótimo de 6,0-8,0), sob condições aeróbicas e anaeróbicas. Além disso, *L. monocytogenes* é capaz de aderir em superfícies de contato com alimentos, como aço inoxidável e poliestireno, podendo persistir em instalações de processamento de alimentos durante meses ou anos como biofilmes. Quando

protegido em biofilmes, pode tolerar altas concentrações de muitos agentes, como desinfetantes, sanitizantes e antimicrobianos, o que pode resultar na contaminação de alimentos (Valimaa, Tilsala-Timisjarvi e Virtanen 2015).

Esse micro-organismo foi reconhecido pela primeira vez como patógeno de origem alimentar em 1981, em um surto ligado a repolho contaminado no Canadá (Schlech et al., 1983). Estima-se que mais de 99% dos casos de listeriose devido ao consumo de alimentos contaminados, particularmente alimentos prontos para consumo, tal como carnes, laticínios e produtos de pesca (Allen et al., 2016). Alimentos prontos para consumo podem ter uma vida útil longa, sendo armazenados a baixas temperaturas e sob embalagens a vácuo ou em atmosfera modificada. Esta capacidade de crescer em condições adversas, faz com que esse micro-organismo seja uma grande preocupação para a indústria de alimentos e necessita de controle ao longo da cadeia alimentar (Valimaa, Tilsala-Timisjarvi e Virtanen 2015). Além disso, a presença de *L. monocytogenes* em alimentos tem consequências econômicas importantes, tais como a retirada de produtos do mercado consumidor e diminuição nas vendas de produtos (Auvolat e Besse 2016). Desta forma, a investigação de novas tecnologias que aumentem e/ou promovam a liberação controlada de antimicrobianos naturais, como a nisina e a lisozima, tem recebido grande ênfase, pois possuem potencial de uso pela indústria de alimentos, podendo assim combater a *L. monocytogenes* em diferentes categorias de alimentos.

3 ARTIGOS CIENTÍFICOS

Os resultados obtidos no decorrer deste trabalho de pesquisa estão apresentados em forma de artigos científicos, seguindo as normas do Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos (PPGCTA-UFRGS). Cada artigo traz os materiais e métodos utilizados em seu desenvolvimento, juntamente com os resultados, as discussões e conclusões pertinentes a cada etapa do mesmo.

Artigo 1: Pectin and polygalacturonic acid-coated liposomes as novel delivery system for nisin: Preparation, characterization and release behavior.
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Artigo 2: Structural characterization of nisin loaded liposomes containing pectin and the polygalacturonic acid.
Formatado para submissão na revista: Materials Science and Engineering C

Artigo 3: Antimicrobial activity of Lysozyme-Nisin co-encapsulated in long circulatory liposomes coated with polysaccharides.
Formatado para submissão na revista: Food Hydrocolloids

3.1 Artigo 1

Pectin and polygalacturonic acid-coated liposomes as novel delivery system for nisin: preparation, characterization and release behavior

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Abstract

Liposome systems can increase the stability of encapsulated compounds, such as the bacteriocin nisin, improving its benefits as antibacterial agent. In this study, nanoliposomes encapsulating nisin were prepared with soy phosphatidylcholine (PC) and pectin or polygalacturonic acid. The efficiency of encapsulation was 87.9% to PC-pectin and 84.0% to PC-polygalacturonic acid nanolipossomes. The liposomes presented a well-defined spherical shape, with sizes ranging from 94 to 160 nm, showing minimal differences during storage at 7°C or 25°C for 28 days. The values for polydispersity index were around 0.2-0.3, and the zeta potential of polysaccharide-coated liposomes was in the range from -17 mV to -32 mV during the time of storage, indicating high physical stability. The antimicrobial activity was observed against five different strains of *Listeria* in milk-agar plates, with a better efficiency against *L. innocua* 6a. The in vitro release studies showed that the nisin release rate of PC-pectin and PC-polygalacturonic acid liposomes was lower when compared with PC liposomes, evidencing sustained-releasing potential for food applications.

Keywords: nisin; liposomes; pectin; polygalacturonic acid.

1. Introduction

Nisin is a natural antimicrobial substance that has received particular attention because of its broad antibacterial activity against Gram-positive bacteria. It is a heat-stable peptide produced by *Lactococcus lactis* subsp. lactis and has Generally Recognized As Safe (GRAS) approval by the US Food and Drug Administration (Chopra, Kaur, Bernela, & Thakur, 2014; Salmieri et al., 2014). However, its antimicrobial efficacy in food matrices may be reduced by the susceptibility to proteolytic degradation and interactions with food components (Aasen, Markussen, Moretro, Katla, Axelsson, & Naterstad, 2003; Sant'Anna, Malheiros, & Brandelli, 2011).

Novel delivery systems may overcome this problem by encapsulation of nisin in nanoformulations, improving controlled and sustained release (Xiao & Zhong, 2011; Brandelli, 2012). Liposomes are spherical vesicles, having an aqueous core enclosed by one or more phospholipid bilayers. Due to the presence of both lipid and aqueous phases in the structure, they can be utilized in the entrapment and delivery of either amphiphilic, water-soluble, or lipid-soluble materials (Mozafari, Johnson, Hatziantoniou, & Demetzos, 2008; Imran, Revol-Junelles, Paris, Guedon, Linder, & Desobory, 2015), representing an interesting technology for the encapsulation and delivery of bioactive agents.

Nanoliposomes composed of phospholipids and polysaccharides can be alternative for the controlled release of bacteriocins, such as nisin. This bacteriocin was encapsulated into liposomes coated with chitosan or chondroitin sulfate by the thin-film hydration method, demonstrating similar efficiency as free nisin in inhibiting *Listeria* spp. isolated from food, and greater efficiency in inhibiting *L. monocytogenes* ATCC 7644 (Silva, Boelter, Silveira, & Brandelli, 2014). However, the use of polysaccharides other than chitosan in liposome formulations has been poorly described, stimulating the investigation on liposomes with different types of polysaccharides.

Biopolymers, such as polysaccharides, are regularly used in the pharmaceutical, cosmetic and food industries. Pectin is an example of natural polymer that has received considerable attention in different research areas. Pectin is an anionic polysaccharide, comprising of a linear backbone composed by galacturonic acid units linked by α-1→4 bonds. The carboxylic groups of the polygalacturonic acid may be partially esterified by methyl groups, presenting a pKa of approximately 2.9, close to the pKa value of the monomeric galacturonic acid (Souza, Gebara, Ribeiro, Chaves, Gigante, & Grosso, 2012; Ventura & Bianco-Peled, 2015). The advantage of using pectin in the development of nanoparticles is due to its biocompatibility, absence of toxicity and biodegradability, thus allowing incorporation into a wide range of commercial products (Da Silva et al., 2014; Hu, Huang, Gao, Huang, Xiao, & McClements, 2015). Thus, the aim of the present research was to develop nanoliposomes containing pectin or polygalacturonic acid by the thin-film hydration method for nisin encapsulation, and to determine the physicochemical characteristics of the nanostructures, their stability and antimicrobial activity.

2. Materials and methods

2.1. Materials

The pectin and polygalacturonic acid used in this study were from Sigma (St. Louis, MO, USA): pectin from apple (degree of esterification 70-75%) and polygalacturonic acid (≥90%, enzymatic). Polygalacturonic acid was dissolved in 10 mM sodium phosphate buffer pH 7.0 and pectin was dissolved in ultrapure water, in concentrations of 0.5, 1.0 and 1.5 mg/mL. Nisaplin® (25 mg of nisin/g of Nisaplin®) was provided by Danisco (Vargem Grande, Brazil). Before each experiment, nisin was prepared as described by Pinilla & Brandelli (2016). The stock solution was diluted in 10 mM phosphate buffer pH 7.0, filter-sterilized through 0.22 μm membranes (Millipore; Billerica, MA, USA), stored into a sterile tube at 4 °C. The lipid used

in the experiments was Phospholipon 90G®, a purified soybean phosphatidylcholine (PC, ≥ 94%), provided by Lipoid (Ludwigshafen, Germany).

2.2. Nanoliposome production by film hydration

Nisin was encapsulated into PC nanoliposomes by the thin-film hydration method adapted from Silva et al. (2014). The lipid was dissolved in chloroform in a round-bottom flask, and the organic solvent was removed by rotary evaporation until formation of a thin lipid film. The flask was stored overnight in a desiccator for elimination of all traces of chloroform. The film was dispersed by adding 5 mL solution of nisin (0.5 mg/mL) in 10 mM phosphate buffer pH 7.0 and vortexed at 60 °C for 1 min. To assist the formation of the liposomes, the solution was heated and stirred for 5 cycles of heating to 60 ºC for 2 min, followed by 1 min under vigorous agitation. After this process 5 mL of the polysaccharide solution (0.5, 1.0 or 1.5 mg/mL) was added and the final concentration of nisin in solution was 0.25 mg/mL. The mixture was subjected to ultrasound for size reduction using a probe-type sonicator (Unique OF S500, frequency 20 kHz, power 250 W), for 5 cycles of 1 min with intervals of the same time under ice bath, and then sterilized by filtration through 0.22 μm membranes (Millipore, Billerica, MA, USA). The resulting nanoliposomes were immediately submitted to characterization tests.

2.3. Nanoliposome characterization

The mean diameter and polydispersity index (PDI) of nanoliposomes were determined by dynamic light scattering (DLS) performed in a Brookhaven Instruments standard setup (BI-200 M goniometer, BI-9000AT digital correlator) with a He-Ne laser of 35 mV at λ of 632.8 nm as the light source (Pecora & Berne, 2000). The zeta potential of liposomes containing pectin or polygalacturonic acid were carried out after dilution of the formulations in 10 mM sodium phosphate buffer pH 7.0 using a Brookhaven Zeta PALS equipment (Silva et al., 2014). For

determination of zeta potential and DLS, measurements were performed immediately after of the nanoliposomes preparation and were monitored for 28 days for filtered liposomes stored at 7 °C and at room temperature (25 °C).

The encapsulation efficiency (EE) was determined by HPLC (Pinilla & Brandelli, 2016). Encapsulated nisin was separated from unencapsulated nisin by ultrafiltration (Ultracel YM-10 Membrane, Millipore) at 10,000 g for 20 min. The amount of free nisin was determined in the filtrate by HPLC as described below. The EE was calculated using the following equation:

$$\%EE = \frac{\text{Nisin (used in preparation - in filtrate)}}{\text{Nisin used in preparation}} \times 100$$

For HPLC analysis, samples were filtered through 0.22 μm nylon membrane and loaded 100 μL onto the XBridge® C18 (150 x 4.6 mm, 5 μm) column (Waters, Milford, MA, USA). The column was eluted using mobile phases A (0.1% v/v TFA in water) and B (0.1% v/v TFA acid in acetonitrile) following this sequence: 100% A for 5 min, a linear gradient 0-50% B over 45 min, a linear gradient to 100% B over 5 min and 100% B for 7 min, at 1 mL/min flow rate. Mobile phases were filtered through 0.22 μm nylon filters and degassed by sonication prior to use. The UV detector was used at 215 nm and the column temperature was at 35 °C. The concentration of nisin was determined by means of a calibration curve standard. HPLC analysis and data acquisition were operated on the Empower® 2 software (Waters, Milford, MA, USA).

2.4. Transmission electron microscopy

The morphology of liposomes was evaluated by transmission electron microscopy. The sample was deposited on a Formvar-coated 300 mesh copper grids and left for 1 min, and stained with 2.5% (v/w) uranyl acetate solution for 30 seconds. All samples were observed by JEM-1200 Transmission Electron Microscope (JEOL, Tokyo, Japan) operated at 100 kV.

2.5. Fourier transform infrared spectroscopy (FTIR)

FTIR spectra of pectin, polygalacturonic acid, nisin, phosphatidylcholine and nanoliposomes, were obtained using a Shimadzu 8300 FTIR spectrophotometer (Shimadzu, Kyoto, Japan). The liposomal samples were freeze-dried prior analysis. The analysis was carried out using the KBr disk method. The scanning range used was 4000 to 400 cm-1 with 64 scans and resolution was set at 4 cm^{-1}.

2.6. Antimicrobial activity assay

Antimicrobial activity was determined by agar diffusion assay as described by Motta & Brandelli (2002). Serial dilutions of encapsulated nisin were made, and aliquots of 10 μL were applied onto BHI agar plates previously inoculated with a swab submerged in a suspension of L. monocytogenes ATCC 7644 (107 CFU/mL), incubated at 37 °C for 24 h. Antimicrobial activity was defined as the reciprocal of the last dilution giving an inhibition zone, and expressed as activity units (AU) per mL (Kimura, Sashihara, Matsusaki, Sonomoto, & Ishizaki, 1998).

An aliquot was withdrawn for test immediately after preparation of liposomes to analyze the stability over time. Samples of each treatment were stored at 7 and 25 °C, and the antimicrobial activity of each sample was evaluated at days 1, 7 and 14.

2.7. Antimicrobial activity in milk-agar plates

Milk-agar plates were used for simulate a model system, through the efficacy test of the liposomes against different strains of *Listeria* (*L. monocytogenes* ATCC 7644, *Listeria* sp. str1, *L. innocua* 6a, *L. monocytogenes* 4b isolated from bovine carcass, and *Listeria* sp. str2 isolated from cheese). The antimicrobial activity was detected by agar diffusion assay. Aliquots of the liposomes were applied onto milk-agar plates previously inoculated with a swab submerged in

a suspension of each indicator microorganism. Plates were incubated at 37 °C for 24 h, and the activity was determined by the diameter of the inhibition zone formed in the plate (Silva et al., 2014).

2.8. In vitro release assay

The in vitro nisin release from PC nanoliposomes containing pectin or polygalacturonic acid was measured in phosphate buffered saline pH 7.4 (Wang, Li, & Kong, 2013). Nanoliposomes were placed into dialysis bags (cutoff 12,000 Da), immediately after preparation. The experiments were performed in a shaker at 100 rpm and 37 °C with 25 mL dissolution solution. After a fixed time interval, 2.0 mL of the solution was removed and the absorbance at 220 nm was measured, and 2.0 mL of buffer solution was added to the release medium to maintain a constant solution volume. The nisin that was released could be calculated using the follow equation:

$$\%CR = (Cn/Ct) \times 100$$

where Cn is the concentration of the solution of the nth sample and Ct is the total amount of nisin in the suspension.

2.9. Statistical analysis

The experiments were performed in triplicate and means were compared using Tukey's test at a level of 95% of significance ($P<0.05$) by Statistica 7.0 software (Statsoft, Tulsa, OK, USA).

3. Results and discussion

3.1. Physicochemical characteristics of nanoliposomes

Nanoliposomes were successfully prepared in this study using biodegradable and biocompatible materials approved by the US Food and Drug Administration (FDA).

Phosphatidylcholine was used to produce nanoliposomes coated with pectin or polygalacturonic acid for encapsulation of nisin. For development of liposomes, three different concentrations of pectin or polygalacturonic acid were initially tested (0.5, 1.0 and 1.5 mg/mL). The results of particle size, polydispersity index (PDI) and zeta potential are summarized in Table 1. The control liposomes encapsulating nisin had a mean diameter of 105.8 ± 1.0 nm, the PDI was 0.271 ± 0.01, and zeta potential was -18.0 ± 4.9 mV.

Table 1. Physical characteristics of polygalacturonic acid and pectin-covered nanoliposomes encapsulating nisin.

Polysaccharide	PC-polygalacturonic acid			PC-pectin		
(mg/mL)	Size (nm)	PDI	ζ (mV)	Size (nm)	PDI	ζ (mV)
0.5	116.2±11.3^{b}	0.428±0.037^{a}	-7.0±3.2^{b}	122.6±5.7^{a}	0.529±0.014^{a}	-17.3±9.0^{a}
1.0	160.9±11.1^{a}	0.139±0.025^{b}	-17.1±5.0^{a}	108.1±1.3^{a}	0.274±0.010^{c}	-25.4±5.5^{a}
1.5	94.5±3.4^{b}	0.461±0.024^{a}	-24.2±11.6^{a}	121.3±11.5^{a}	0.454±0.048^{b}	-24.1±12.2^{a}

*Values are the means ± standard deviations of three independent experiments. Different superscript letters within the same column indicate significant differences at $P<0.05$.

The liposomes prepared with pectin showed similar results for the mean diameter and zeta potential for the three different concentrations tested. However, the PDI of the samples with concentrations of 0.5 and 1.5 mg/mL were above of 0.4, indicating an elevated polydispersity of liposomes. The sample with concentration of 1.0 mg/mL pectin showed polydispersity around 0.2, being this value appropriate for systems prepared from biological materials (Malheiros, Sant'Anna, Micheletto, Silveira, & Brandelli, 2011). The PDI values of liposomes prepared with polygalacturonic acid presented similar behavior, with the concentration of 1.0 mg/mL showing the PDI values under 0.2, indicating a narrow size distribution. Some significant differences ($P>0.05$) in the size and zeta potential were observed

among samples, but the PDI was determinant in the choice of polysaccharide concentration to continue the study. Thus, the concentration of 1.0 mg/mL of polysaccharide was selected as the best condition for the development of nisin nanoliposomes coated with pectin or polygalacturonic acid.

Despite specific studies with pectic polysaccharides have not been developed, the polysaccharide chitosan has been considered as a good candidate for stabilization of PC liposomes. In previous studies, the mean diameter of PC-chitosan nanovesicles varied from 174 to 285 when the chitosan concentration was increased from 0.1 to 1 mg/mL (Mertins, Sebben, Pohlmann, & Silveira, 2005). Nisin was encapsulated into PC liposomes containing the cationic polysaccharide chitosan and chondroitin sulfate, showing that mean particle size of PC liposomes was 145 nm and varied to 210 and 134 nm with the incorporation of chitosan and chondroitin sulfate, respectively. Besides, the polydispersity index was under 0.2 for all formulations tested (Silva et al., 2014).

The encapsulation efficiency of nisin into PC nanoliposomes without incorporation of polysaccharides was 86.9%, similar to that reported in previous studies (Taylor, Gaysinsky, Davidson, Bruce, & Weiss, 2008; Malheiros, Micheletto, Silveira, & Brandelli, 2010). The values for PC nanoliposomes with incorporation of pectin and polygalacturonic acid were 87.9% and 84.0%, respectively, showing a high encapsulation efficiency. Pectin-based nanoparticles had a loading efficiency for hydrophobic azole drugs, reaching values of 80-88%, HMP-based nanoparticles gave the highest loading efficiency compared with to LMP-based (Burapapadh, Takeuchi, & Sriamornsak, 2015). According to Krivorotova et al. (2016) the loading efficiency depends on the degree of pectin esterification, showing higher values for high methoxyl pectin and pectic acid at pH 7.0, the same as that used in the present study, suggesting that not only electrostatic interactions, but also hydrophobic interactions contribute to the complex formation at neutral pH values.

The nanoliposomes incorporating nisin were analyzed by transmission electron microscopy and their morphological characteristics can be observed in Fig. 1. All samples showed predominantly spherical structures, as often observed for nanoliposomes (Malheiros et al., 2010) and similar to that observed by Khaksar et al. (2014) and Krivorotova et al. (2016), which prepared nisin-loaded pectin particles. Fig. 1a and 1b show a dense coating suggesting that liposomes were covered with polygalacturonic acid and pectin, respectively. In addition, the histograms of size distribution (Fig. 2) showed that the preparations had a monomodal size distribution profile and 90% (D0.9) of the nanoliposomes presented diameters smaller than 200 nm.

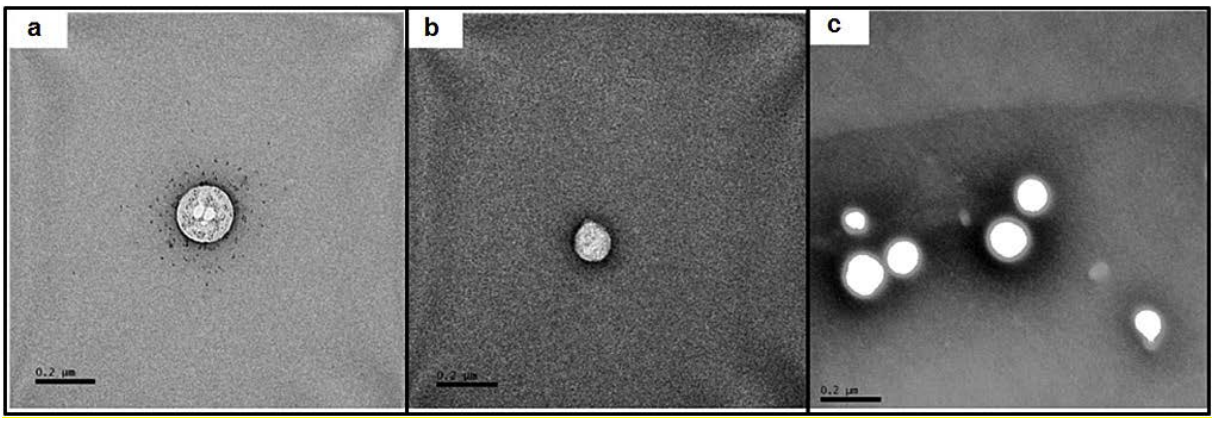

Fig. 1. Transmission electron microscopy images of nanoliposomes encapsulating nisin. (a) Polygalacturonic acid-coated PC liposomes, (b) pectin-coated PC liposomes and (c) control PC liposomes. Bar ¼ 200 nm.

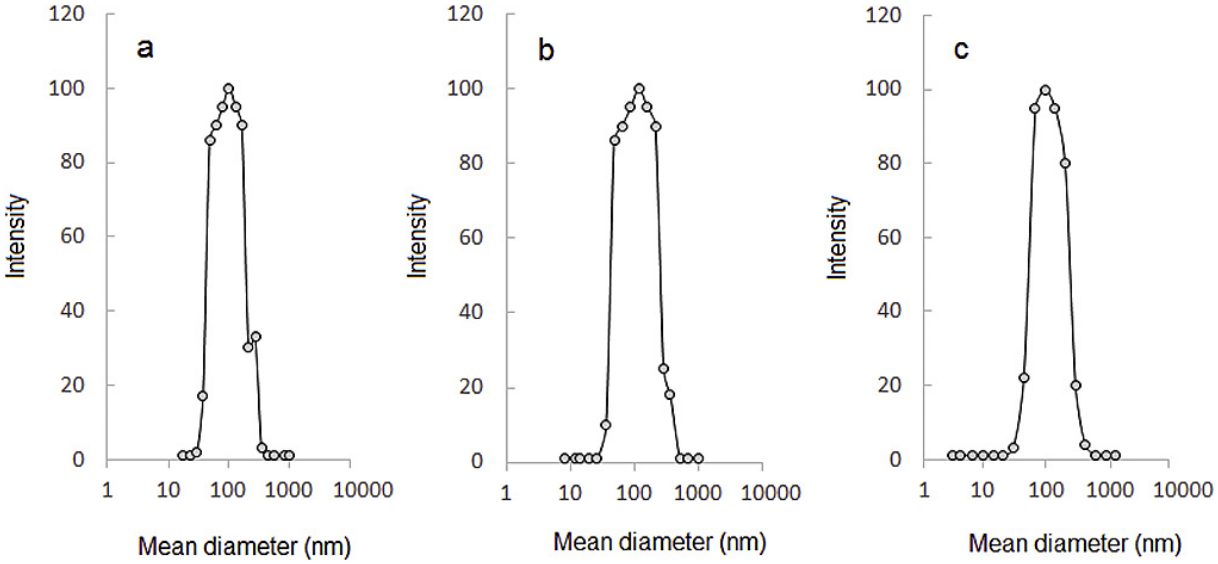

Fig. 2. Histograms of size distribution obtained by laser dynamic light scattering of (a) Polygalacturonic acid-coated PC liposomes, (b) pectin-coated PC liposomes and (c) control PC liposomes.

The correlogram also provides useful information to analyze different aspects of nanostructures such as the size and PDI. For the smaller and hence faster diffusing particles, the measured correlation curve decays to baseline within shorter times, while the larger and slower diffusing particles require more time before correlation in the signal is entirely lost. The more prolonged the decay becomes, the greater the sample polydispersity (Schätzel, 1987). The monomodal distribution of the nanoliposomes was confirmed by the correlogram analysis (Fig. 3). The liposomes showed a typical correlogram from samples containing large nanoparticles (>100 nm) in which the correlation of the signal takes time to decay (Agut, Taton, Brület, Sandre, & Lecommandoux, 2011). The correlogram of control PC-liposomes indicated more disperse particles because the correlation of the signal decays more gradually. This could be explained because the moving particles are constantly forming new patterns and the rate at which these intensity fluctuations occur will depend on the size of the particles (Pecora & Berne, 2000).

In addition, the intercepts of the correlation functions in Fig. 3 revealed a higher value for PC-polygalacturonic acid liposomes. The intercept of the correlation function is affected if the particle absorbs at the laser wavelength, thereby reducing the number of scattered photons compared to non-absorbing particles. The size of this loss correlates with the number of absorbed photons and therefore, with the absorption cross section of the particles and their number concentration (Geiβler, Gollwitzer, Sikora, Minelli, Krumrey & Resch-Genger, 2015).

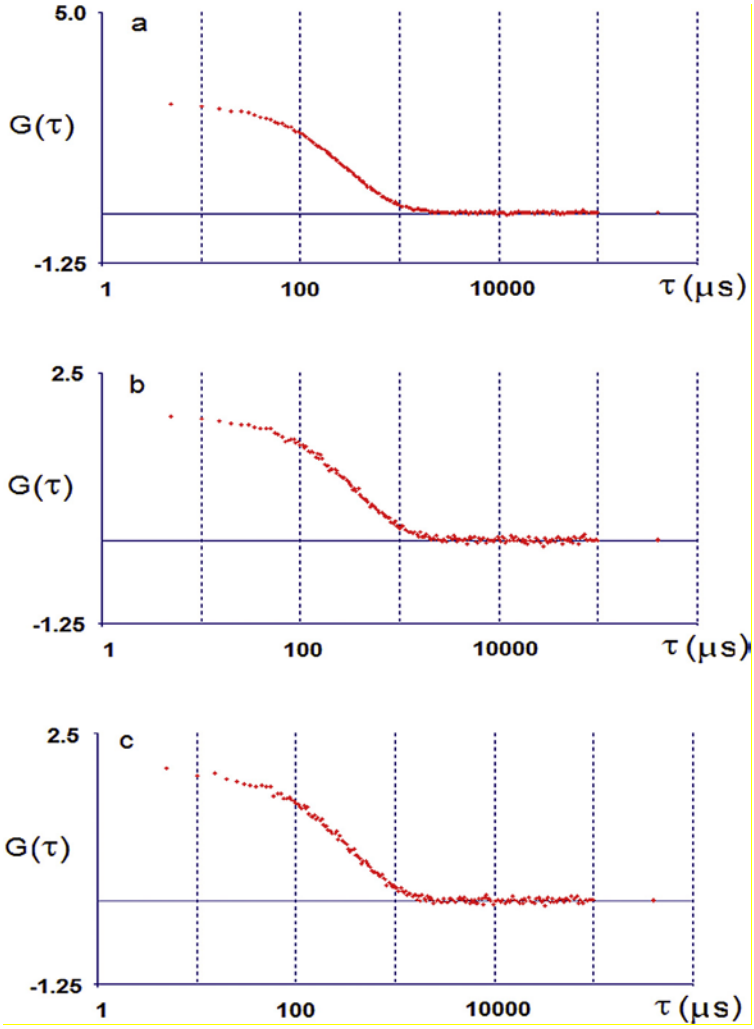

Fig. 3. Graphic representation showing the fluctuation in the intensity of scattered light as a function of time. Correlograms of (a) Polygalacturonic acid-coated PC liposomes, (b) pectin-coated PC liposomes and (c) control PC liposomes.

3.2. Fourier transform infrared spectroscopy (FTIR)

Fourier transform infrared measurements were employed to give information about the interactions of nanoliposomes with pectin and polygalacturonic acid. The FTIR peak assignments for the different spectra are presented in Fig. 4 for nisin, polygalacturonic acid and pectin, each of them separately and with different types of formulation.

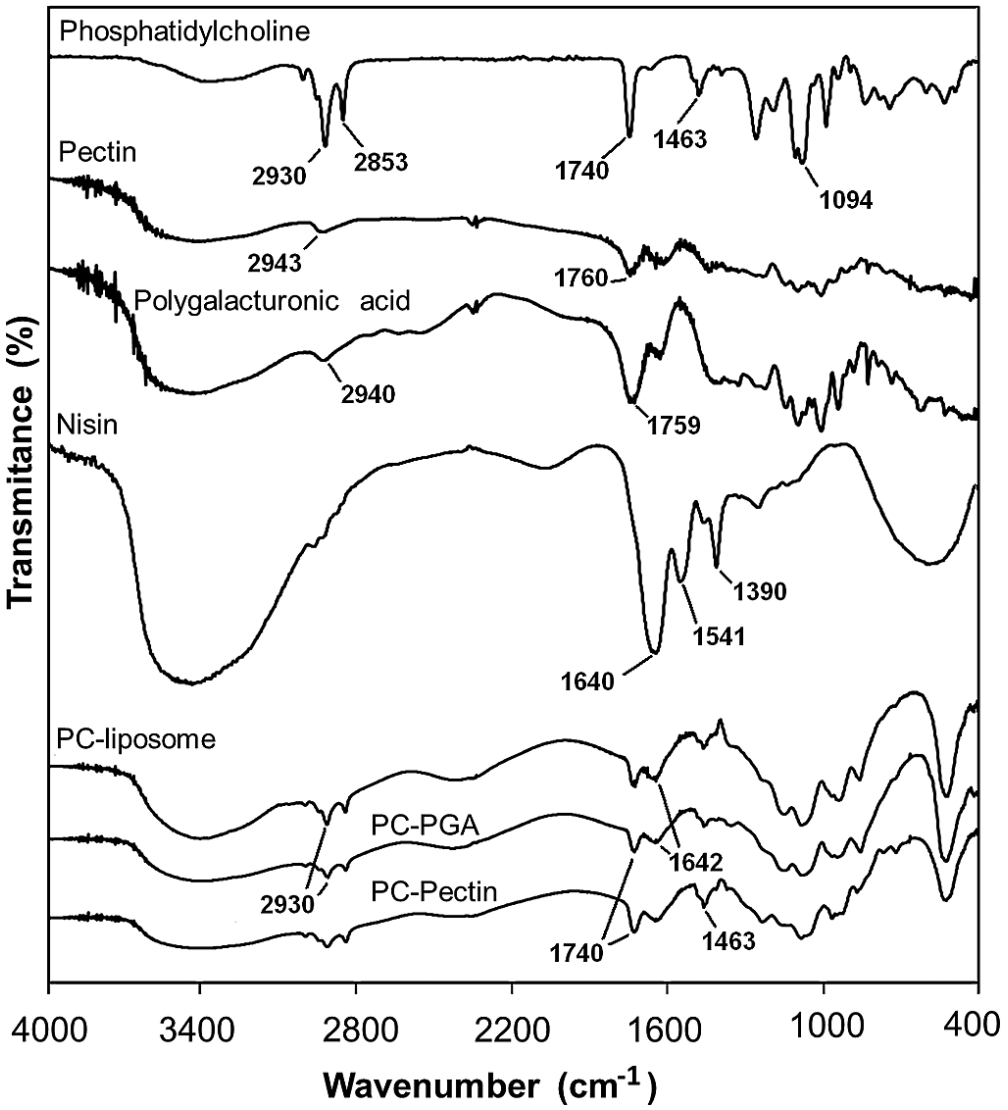

Fig. 4. FTIR spectra of pure polygalacturonic acid, pectin, nisin, phosphatidylcholine (PC), and nanoliposomes prepared with pectin and polygalacturonic acid (PGA).

Polysaccharides display a broad area of absorption above 3200 cm^{-1} due to stretching vibrations of O-H groups along the polysaccharide backbone (Burapapadh et al., 2015). The peak of absorption at 2943 cm^{-1} was due to C-H stretching vibration from pectin samples. In the case of polysaccharides, the 1000-1250 cm^{-1} interval is not very useful to differentiate carbohydrates and their derivatives. Thus, the bands in the 1000-2000 cm^{-1} region may be used to identify galacturonic acid, because are independent of pectin source (Monsoor, Kalapathy, & Proctor, 2001). The strong carbonyl absorption bands at 1675 and 1740 cm^{-1} were from free (COO-) and esterified (COO-R) carbonyl groups, respectively. For nisin, the peak at 1637 cm^{-1} is attributed to bending of primary amines and the peak at 1390 cm^{-1} is due to C-N stretching of amide group (Maquelin et al., 2002).

In FTIR spectra of nanoliposomes (Fig. 4), it was observed that most of the peaks appear at almost the same wavenumbers. However, some differences in 1000-1250 cm^{-1} and 1600-1750 cm^{-1} regions may indicate the presence of pectin and polygalacturonic acid on the surface of nanoliposomes prepared with polysaccharides. In addition, the peaks around 1520-1400 cm^{-1} attributed to amide groups of nisin most likely caused a shifting in the wavenumbers in liposomes with pectin and polygalacturonic acid. These subtle alterations may suggests that intermolecular interactions between amino groups of the peptide and the polysaccharides exist.

3.3. Stability of nanoliposomes during storage

The physicochemical characteristics of liposomes containing pectin or polygalacturonic acid stored for 28 days under refrigeration and at room temperature are depicted in Fig. 5 and 6. These parameters are mainly dependent on the chemical properties of nisin and liposome constituents, as well as the possible interactions among them. The different formulations evaluated showed different particle sizes, but an average diameter within the nanometrical scale, ranging from 90 to 160 nm (Fig. 5a and 5c). The PC-polygalacturonic liposomes

decreased mean diameter with the first 7 days, remaining stable over the 28 days. In general, the values for the PDI were around 0.2 and 0.3 indicating that the liposomes were physically stable during storage (Fig. 5). The PDI values of PC-pectin liposomes increased during storage and were higher than 0.4 after 20 days (Fig. 5b and 5d), indicating degradation and instability over time for this liposome formulation. Despite no significant alteration of size and PDI was observed for PC liposomes until 21 days, the system collapsed after this time at 25 °C and no further measurements were possible (Fig. 5 c,d). This result indicates that negatively charged polysaccharides may be interesting coatings for nisin-loaded liposomes, increasing their stability during storage at room temperature probably by providing increased electrostatic repulsion between the nanoparticles.

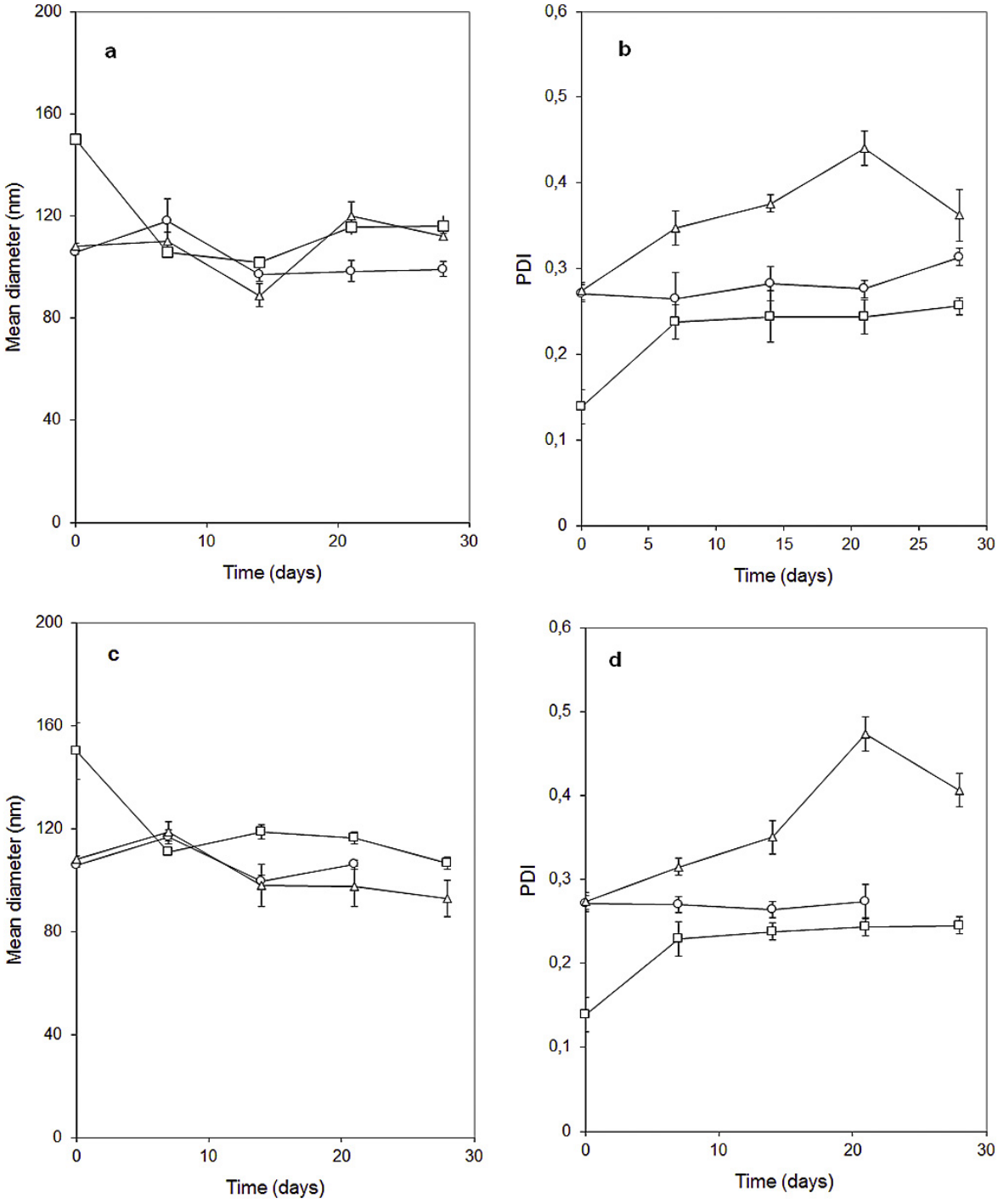

Fig. 5. Size and polydispersity index (PDI) of nanoliposomes containing nisin, during storage at 7 °C (a,b) and 25 °C (c,d). (□) Polygalacturonic acid-coated PC liposomes, (△) pectin-coated PC liposomes and (○) control PC liposomes. Values are the means ± standard deviations of three independent experiments.

Although pectin-coated liposomes were not previously studied, the physical stability of liposome-encapsulated nisin has been demonstrated. Soy lecithin liposomes kept at 4 °C were still intact after 6 weeks according to physical stability study by zeta-sizer. Nisin entrapped in soy lecithin liposomes, showed that average size of liposomes ranged from 151 to 181 nm, without or with nisin respectively (Imran et al., 2015). In addition, the stability of chitosan-coated liposomes encapsulating melatonin was investigated (Gonçalves, Mertins, Pohlmann, Silveira, & Guterres, 2012). The mean particle diameter, ranging from 220 to 250 nm, was stable over 90 days at 22 °C, whereas PDI values remained around 0.4 within the same period, reinforcing the hypothesis that the polysaccharide could be acting as a liposome stabilizer in the suspensions.

The zeta potential analysis measures the surface charge of particles and could be useful to estimate the influence of the polysaccharide incorporation on the stability of liposomes (Silva et al., 2014). In this study, zeta potential was negative to PC-polygalacturonic acid and PC-pectin liposomes, and remained constant over the period, indicating the physical stability of nanoliposomes at both 7 °C and 25 °C (Fig. 6). The incorporation of polysaccharide molecules seem to have formed an outer coating, and the net charge on the nanoliposomes showed to be negative. Otherwise, the charge of PC nanoliposomes significantly changed after 14 days at 7 °C and after 7 days at 25 °C (Fig. 6). Thus, a rearrangement during storage may have occurred, leading to the change of negative charge to positive charge from the possible exposition of nisin on the surface of liposomes without a polysaccharide coating. According to Imran et al. (2015), the introduction of nisin, as a cationic peptide, in liposomes significantly changes net surface charge of liposomes, neutralizing and reducing the electrostatic repulsion between liposomes.

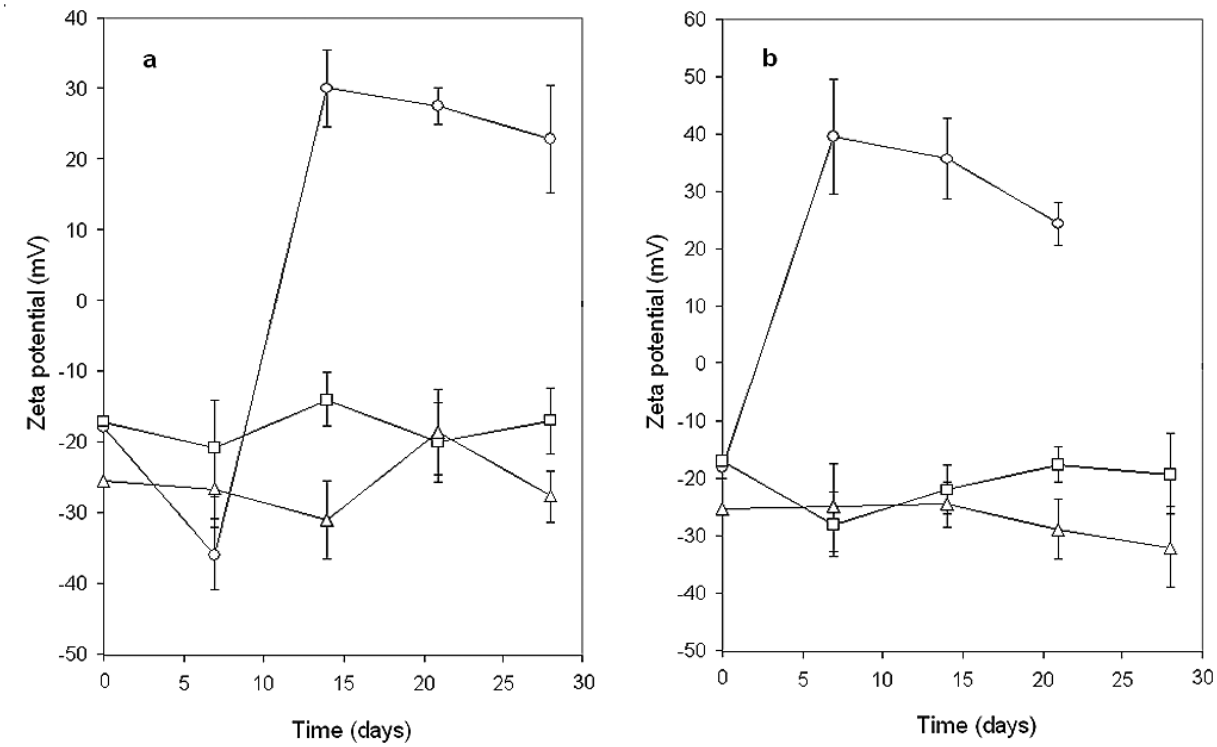

Fig. 6. Zeta potential (λ) of nanoliposomes containing nisin, during storage at 7 °C and 25 °C. (□) Polygalacturonic acid-coated PC liposomes, (△) pectin-coated PC liposomes and (○) control PC liposomes. Values are the means ± standard deviations of three independent experiments.

In a recent study, Krivorotova et al. (2016) demonstrated that zeta potential measurements exhibited the interaction between negatively charged pectin and cationic peptide. Nisin binding caused the reduction of the negative surface charge and led to the formation of nisin-loaded pectin particles. The pectin is an anionic polysaccharide composed of α-(1→4)-linked D-galacturonic acid units, some of which are naturally methyl-esterified (Ventura & Bianco-Peled, 2015). On the other hand, nisin is a cationic peptide that contains three lysine and one histidine positively charged residues. Besides, the N-terminal end of the peptide chain has hydrophobic residues that can contribute to nisin-pectin interaction by hydrophobic forces (Krivorotova et al., 2016), leading to formation nisin-loaded pectin nanoparticles.

3.4. Antimicrobial activity

The antimicrobial activities of nanoliposomes prepared with pectin or polygalacturonic acid were evaluated during storage at 7 °C and 25 °C. The activity against *L. monocytogenes* ATCC 7644 was monitored to understand the behavior of the liposomes containing biopolymers, considering the potential application of nanoformulated nisin in foods.

Samples of PC-pectin and PC-polygalacturonic acid liposomes showed initial activity of 800 AU/mL, whereas samples of PC liposomes had an initial activity of 400 AU/mL. It was observed that the liposomes were losing their antimicrobial activity after 14 days, but this decrease was lower for those stored at 7 °C. After 7 days, PC nanoliposomes maintained its initial activity, while the liposomes containing PC-polygalacturonic acid and PC-pectin showed 400 AU/mL and 200 AU/mL residual activity, respectively, for both temperatures. However, at the end of 14 days, the antimicrobial activity of PC and PC-polygalacturonic acid nanoliposomes was 200 AU/mL, while PC-pectin showed no antimicrobial activity. Thus, PC-polygalacturonic acid nanoliposomes maintained a higher antimicrobial activity after 14 days when compared with PC-pectin. The loss of antimicrobial activity could be associated with the establishment of strong interactions between cationic nisin and negatively charged polysaccharides during storage (Krivorotova et al., 2016). In this case, an increased nisin concentration could be encapsulated to warrant extended antimicrobial activity.

To simulate a food system, the antimicrobial activity of the nanoliposomes was tested against five different strains of *Listeria* in milk-agar plates, and then the diameter of the inhibition zones was measured (Table 2). All formulations showed a similar behavior, showing larger inhibition zones against *L. innocua* 6a. In a similar approach, Silva et al. (2014) observed the inhibition of PC liposomes containing chitosan and chondroitin sulfate against different strains of *Listeria*, showing more effective inhibition against *L. monocytogenes* ATCC 7644. Nisin can be used to control *Listeria* ssp. in food, but food-related environmental stresses may

have significant influence on its effectiveness. Cell wall modifications and alterations in membrane fluidity of *L. monocytogenes* may occur under salt stress or acid stress at low temperature, leading to increased resistance to bacteriocins, which can be variable depending on the strain (Bergholz, et al. 2013). Thus, alternative strategies for nisin delivery can be helpful to allow an effective antimicrobial action on target bacteria.

Table 2. Antimicrobial activity of phosphatidylcholine (PC) nanoliposomes encapsulating nisin in milk-agar against different strains of *Listeria*.

	Inhibition zone (mm)			
Indicator strain	Free nisin	PC	PC-PGA	PC-Pectin
L. monocytogenes ATCC 7644	10.9 ± 0.2^{aC}	11.0 ± 0.7^{aC}	0 ± 0^{bC}	$11.3\pm0,2^{Ad}$
L. monocytogenes 4b	17.5 ± 1.0^{bA}	19.6 ± 1.2^{bB}	27.4 ± 1.3^{aA}	$26.7\pm0.4^{aA,B}$
Listeria sp. str1	14.2 ± 1.4^{cB}	19.9 ± 2.3^{bB}	20.9 ± 1.6^{bB}	26.2 ± 1.0^{Ab}
L. innocua 6a	14.5 ± 1.0^{cB}	24.0 ± 0.5^{bA}	26.2 ± 1.1^{bA}	29.1 ± 1.1^{aA}
Listeria sp. str2	14.1 ± 0.6^{bB}	$21.7\pm0.4^{aA,B}$	23.2 ± 0.6^{aB}	23.5 ± 1.4^{aC}

Values are the means ± standard deviations (n=3). Different letters (a-d and A-B) represent significant differences among the rows and columns, respectively ($P<0.05$). PGA = polygalacturonic acid.

Nisin has been used as natural preservative in the food industry, but the antimicrobial activity can be reduced when it is directly applied in food, due to its interaction with food components. Encapsulation of nisin may reduce the interaction with food matrix components and overcome the problems related to the direct application in foods (Sant'Anna et al., 2011; Khaksar et al., 2014). A diversity of nanostructures have been proposed to delivery nisin, used to control spoilage and pathogenic microorganisms by incorporation into the food matrix or in

films and coatings (Prombutara, Kulwatthanasal, Supaka, Sramala, & Chareonpornwattana, 2012; Salmieri et al., 2014; Boelter & Brandelli, 2016). Thus, polysaccharide-coated nanoliposomes encapsulating nisin may be an interesting strategy to combat *Listeria* in food.

3.5. In vitro release assay

The in vitro release studies from PC, PC-pectin and PC-polygalacturonic acid nanoliposomes were performed using a dialysis membrane system. The release kinetics of nisin from the nanoliposomes is shown in Fig 7.

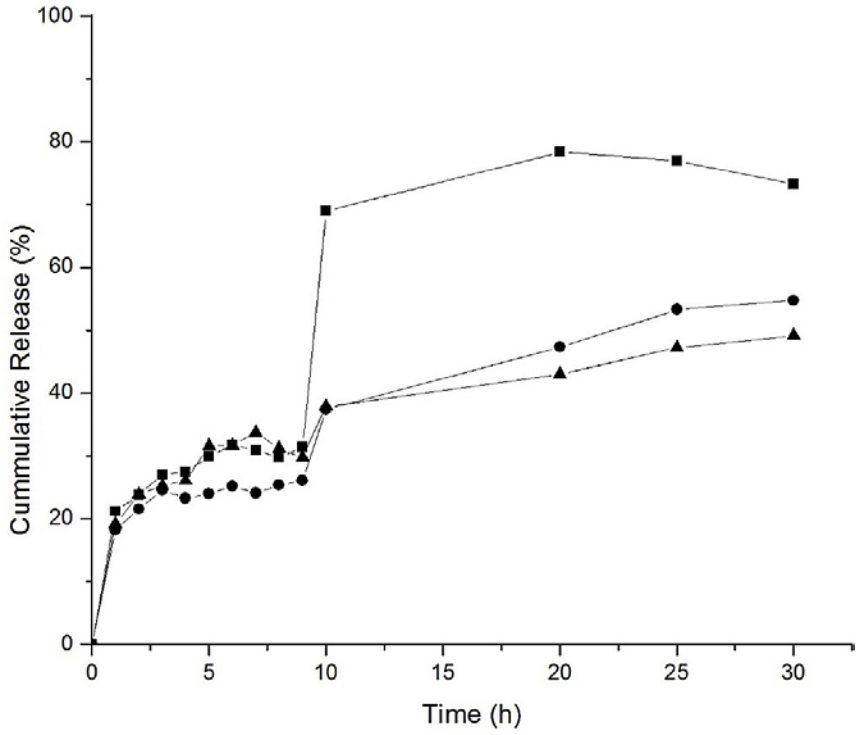

Fig. 7. Release rate of nisin encapsulated in (▲) polygalacturonic acid-coated PC liposomes, (●) pectin-coated PC liposomes and (■) control PC liposomes. Values are the mean of three independent experiments.

The initial nisin release rate from PC-pectin and PC-polygalacturonic acid nanoliposomes was lower and followed a more sustained release over 30 h as compared with PC nanoliposomes. The initial higher release rate from nanoliposomes can be attributed to nisin

located at the outer edge of the phospholipid bilayers (Silva et al. 2014), followed by a slower release phase that may be due to the diffusion of nisin from the inner core of nanoliposomes. The in vitro release profile of nisin from alginate-pectin particles can be described as an initial rapid release phase, followed by a slower release phase until the nisin content is constant, reaching about 65-75% cumulative release (Khaksar et al. 2014). A sharp increase of nisin release was observed at 10 h for PC nanoliposomes, reaching 70% cumulative release, whereas for PC-pectin and PC-polygalacturonic acid this value was 37% (Fig. 7). These results reveal an important difference in nisin release pattern for the formulations containing polysaccharide moieties, since interaction of nisin with the negatively charged polysaccharides probably warrants a slower release rate from the nanoliposomes.

The nanoliposomes containing pectin or polygalacturonic developed in this study presented high encapsulation efficiency, low polydispersity index, and were stable for 21 days at 7 °C and 25 °C, evidencing their technological applicability. In this sense, nanoliposomes composed with polysaccharides can be an alternative for the controlled release of antimicrobial peptides such as nisin.

Acknowledgments

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3.2 Artigo 2

Structural characterization of nisin loaded liposomes containing pectin and polygalacturonic acid

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Abstract

Polysaccharides coated liposomes present better stability, representing an alternative to conventional liposomes. In this work liposomes were developed containing pectin or polygalacturonic acid by the thin-film hydration method and reverse phase evaporation method for nisin encapsulation. Liposomes free from polysaccharides and nisin prepared in the same way were studied as reference. Structural characteristics of liposomes were studied by small angle X-ray scattering (SAXS) and the samples were submitted to temperature cycles (20-60°C). The relation between the Bragg peaks in the SAXS spectrum confirmed the presence of lamellar structures in all the samples. The temperature affected the multilamellar structures in the suspensions. As the temperature increased, the relative scattering intensity increased due to the presence of multilamellar structures. In addition, part of the multilamellar structure became cubic, probably due to the presence of nisin in the liposomes. The addition of polysaccharides showed differences between the cubic phases formed, influencing the aqueous encapsulated volume and evidencing sustained-releasing potential.

Keywords: liposomes; nisin; polysaccharides; SAXS.

1. Introduction

Liposomes are nanovectors recognized as efficient platforms for delivering therapeutic agents, besides having great potential for food applications because of their safety, non-toxicity and biocompatibility [1,2,3]. Moreover, liposomes are colloidal structures prominent to act as vehicle for both hydrophobic and hydrophilic compounds, and are produced by employing natural ingredients as raw material. In order to control the mechanism of action of nanovectors, the structure analysis are of fundamental importance, meeting the requirements of appropriate size and shape, with specific properties during sustained release [4,5,6].

During the last years, several studies on stabilization of liposomes for application in different environments have been developed, with the objective of controlling the release of encapsulated compounds. In the context of surface modification of liposomes aiming the production of structures with specific characteristics, the use of natural polymers is considered to be promising [3,7]. Therefore, the natural polysaccharide pectin can be a good candidate for liposome stabilization with phosphatidylcholine as the lipidic component of the bilayer membrane.

Pectin is a linear anionic polysaccharide, consisting mainly of D-galacturonic acid units with varying degrees of methylesterified carboxyl groups. According to its degree of methyl esterification, pectin can be classified as high methoxyl (HM) pectin or low methoxyl (LM) pectin. Studies have shown that pectin can be used to form nanoparticles suitable for delivery of bioactive ingredients [8,9,10,11]. Recently, we have applied pectin and polygalacturonic acid in liposome surface, evaluating the physicochemical characteristics [1]. The modified liposomes were stable for 21 days at 7°C and 25°C, evidencing their technological applicability.

A deeper evaluation of the pectin and the polygalacturonic acid influence on the structure of liposomes for nisin encapsulation is presented in this study, analyzing the data obtained from Small-Angle X-Ray Scattering (SAXS) technique. This technique has been used

to study the structure of liposomes [5,7,12,13]. Accordingly, the main objective of the current study is to characterize and evaluate at the molecular level the nanostructure of liposomes containing pectin or polygalacturonic acid by the thin-film hydration method and reverse phase evaporation method for nisin encapsulation.

2. Materials and methods

2.1. Materials

The nisin used in this study was obtained from the commercial preparation Nisaplin®, provided by Danisco (Vargem Grande, Brazil). Nisin was prepared by dissolving Nisaplin in 10 mM phosphate buffer pH 7.0, filter-sterilized through 0.22 μm membranes (Millipore; Billerica, MA, USA) and stored into a sterile tube at 4°C, before each experiment. Nisin amount: 25 mg of nisin/g of Nisaplin®.

The polysaccharides used in the experiments were pectin from apple (degree of esterification 70-75%) and polygalacturonic acid (≥90% enzymatic), both from Sigma (St. Louis, MO, USA). Pectin was dissolved in ultrapure water and polygalacturonic acid was dissolved in 10 mM sodium phosphate buffer pH 7.0, in concentration of 1.0 mg/mL.

The lipid used in liposome production was Phospholipon 90G®, a purified soybean phosphatidylcholine (PC, ≥ 94%), provided by Lipoid (Ludwigshafen, Germany).

2.2. Liposome production by film hydration

The method of preparing liposomes was described by Lopes, Pinilla and Brandelli [1]. Phospholipon 90G® was dissolved in chloroform (6 mg/mL) in a round-bottom flask, forming a mixture. The solvent was then removed under reduced pressure at 40 °C using a rotary evaporator until a thin film was formed on the walls. The dry lipid film was hydrated adding 2 mL solution of nisin diluted in 3 mL phosphate buffer (10 mM, pH 7.0) containing nisin (0.5

mg/mL) and vortexed at 60°C to formation of nisin loaded liposomes. The empty liposomes were dissolved in 5 mL phosphate buffer (10 mM, pH 7.0) and prepared by the same procedure described above. To assist the formation of liposomes, the solution was heated and stirred several times and after this process 5 mL of water (MilliQ) or 5 mL of the polysaccharide solution (1.0 mg/mL) was added. Then, the mixture (10 mL) was processed by ultrasound to size reduction using a probe-type sonicator (Unique OF S500, frequency 20 kHz, power 250 W), for 5 cycles of 1 min with intervals of the same time. After preparation, the liposomes were sterilized by filtration through 0.22 μm membranes.

2.3. Liposome production by reversed-phase

Liposomes were prepared by the reversed-phase evaporation through the method adapted from Malheiros et al [14]. The Phospholipon 90G® was dissolved in chloroform (6 mg/mL) and 500 μL of nisin solution (0.5 mg/mL) were dropped into the solution to form a water in oil emulsion, which was sonicated for 3 minutes using a bath sonicator (Unique USC 700, Indaiatuba, Brazil) yielding a homogeneous opalescent dispersion of reverse micelles. The empty samples were prepared by the same procedure described above using instead of nisin solution, 500 μL of phosphate buffer (10 mM, pH 7.0). Then, the organic solvent was evaporated in an evaporator at 35°C under vacuum, giving a highly viscous organogel [13].

The organogel was reverted to nisin loaded liposomes and empty liposomes after addition of 5 mL of water (MilliQ) or 5 mL of the polysaccharide solution (1.0 mg/mL) under shaking. Samples were sterilized by filtration through 0.22 μm membranes and submitted to analysis.

2.4. Small Angle X-ray Scattering (SAXS)

SAXS studies were carried out on the bending magnetic beamline SAS of the Laboratório Nacional de Luz Síncrotron (LNLS, Campinas, Brazil). The liquid samples of liposomes were placed in a stainless steel sample holder closed by two mica windows of thickness 20 μm. The wavelength of the incident beam was 1.605 Å and a linear detector (Princeton Instruments) was used at 43.5 cm from the sample. The intensities were corrected taking into account the detector response and the dark current signals, as well as the sample transmission and the background scattering [13].

The SAXS measurements were performed with nisin loaded liposomes and empty liposomes added or not pectin and polygalacturonic acid and the samples were submitted to temperature cycles. The cycle amplitudes varied in the range 20-60°C. The characteristic lengths *d* of the lamellae arrangements in the liposomes were determined through the Bragg relation:

$$q = n\frac{2\pi}{d} \quad (1)$$

with *q*, the wave vector, corresponding to the highest-intensity Bragg reflection [15]. Miller indices for crystal planes (*hkl*) where considered in order to identify the presence of characteristic sets of reflections of lipidic mesophases. Hence, the corresponding ratio of reciprocal *d*-spacings was ascribed to the specific space group of the respective cubic phase [16]. For each cubic phase, the lattice parameter was calculated using the peak position of the highest-intensity reflection [17] applying the standard crystallographic relationship between *d*-spacings and lattice parameter for cubic phase:

$$d_{cubic} = d_{hkl}\sqrt{h^2 + k^2 + l^2} \quad (2)$$

where d_{cubic} is the lattice parameter of a cubic phase, and d_{hkl} is *d* of the highest-intensity reflection of the given cubic phase.

3. Results and discussion

3.1 Thin-film hydration method

SAXS is a fundamental technique for the study of vesicular systems at the molecular level and can provide structural information on biomolecules in solution [5,18]. The SAXS analyses were performed to obtain detailed information on nisin-loaded liposomes structure and compared with those of the corresponding empty liposomes, obtained by thin-film hydration method, as shown in Figure 1. It was possible to identify the relation (1:2) between the Bragg peaks in the SAXS results in all samples of liposomes, denoting the lamellar structure and thus confirming the presence of bilayers. Multilamellar structures, i.e. stacks of lipid bilayers, lead to well-defined diffraction peaks of first and second order, denoting a rigid structure of molecular organization that rises with the superposition of the bilayers, whereas unilamellar structures result in broad curves of low intensity. Unilamellar structures usually present soft structure, where the shape changes constantly due to diffusional processes in aqueous suspension, producing an incoherent scattering that provides the broad curves. However, by means of SAXS even small quantities of multilamellar structures can be detected in samples where the unilamellar and multilamellar structures are present [5,15,19].

Liposomes showed differences in the SAXS profile as a function of presence of nisin in the formulations. Nisin liposomes (Figure 1A, 1B and 1C) showed low intensities, with broader peaks, if compared to those of the empty liposomes (Figure 1D, 1E and 1F). It is also evidenced that temperature affects the structures in the suspensions. In general, as temperature increases, the relative scattering intensity increases. In addition, pectin and polygalacturonic acid no effect in the presence of multilamellar structures. Mertins et al [13] evaluated the addition of chitosan to liposomes by small angle X-ray scattering demonstrating that the polysaccharide influences the preparation method by increasing the presence of multilamellar structures. In another study was evaluated solutions of pectin and chitosan by SAXS patterns, revealing rigid rod

conformation in chitosan solutions, whereas pectin formed a self-avoiding chain. Furthermore, no dramatic changes occurred in the pectin conformation at temperatures of 60 °C and 25 °C, suggesting that the scattering from pectin solutions do not vary significantly between the two temperatures [20].

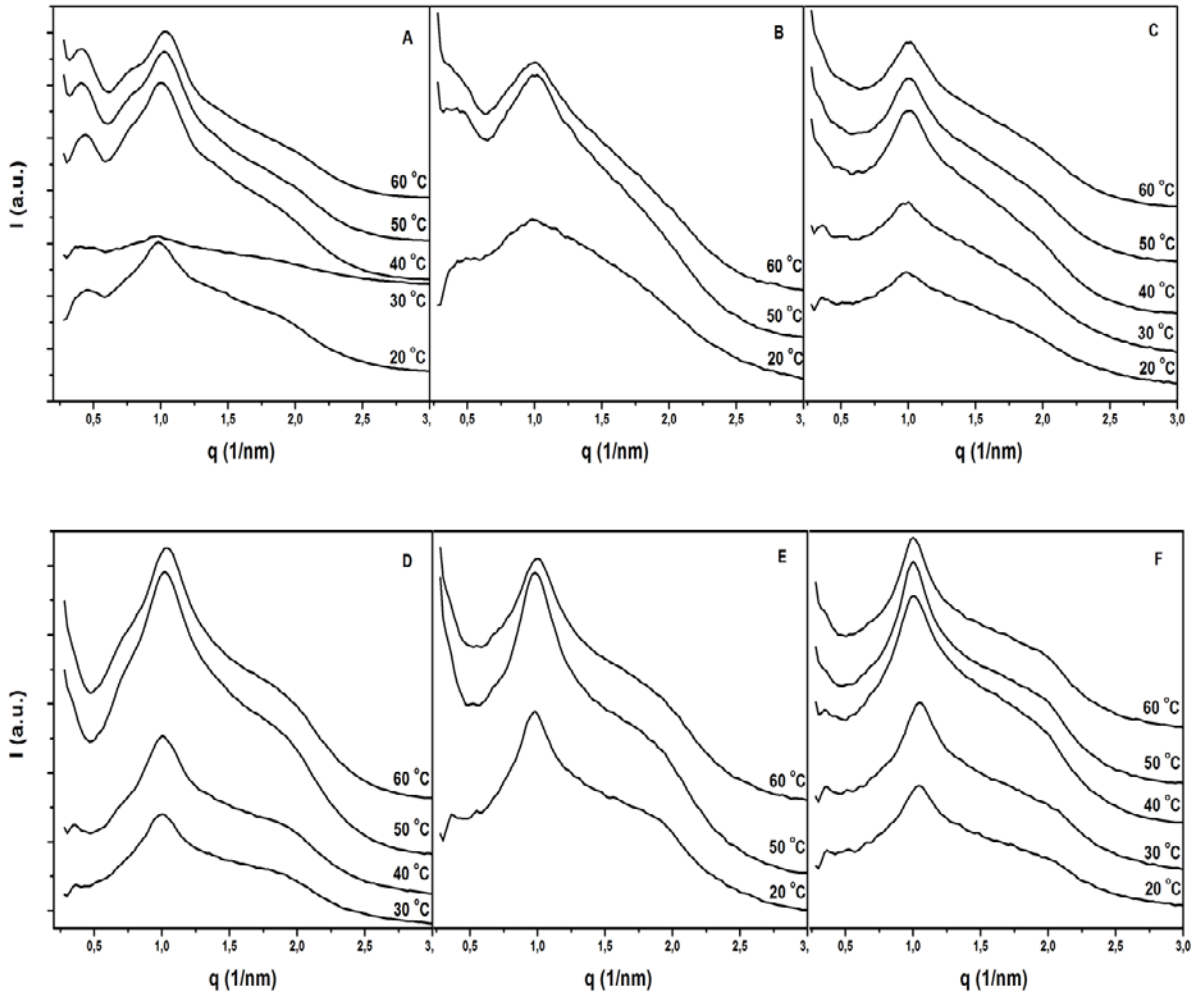

Figure 1: SAXS results of intensity (I) as a function of wave vector (q) of nisin loaded liposomes (A), nisin-loaded liposomes coated with polygalacturonic acid (B), nisin-loaded liposomes coated with pectin (C), empty liposomes (D), empty liposomes coated with polygalacturonic acid (E) and empty liposomes coated with pectin (F), produced by thin-film hydration method and measured at different temperatures.

Analyzing the data from SAXS of samples obtained at different temperatures, it was observed through the relation between the peaks that besides the existence of a lamellar structure, the existence of a cubic structure was also observed (Figure 2). Depending of their chemical structure and of external variables such as water content, temperature, pressure and aqueous phase composition, lipids self-assemble into a variety of different phases. These lipid phases are mutually related and transform into each other via phase transitions driven by temperature, pressure, lipid composition and water [21]. Besides the phase lamellar, the lipids are classified in cubic and hexagonal phase according to their different internal structures. Among them, cubic and hexagonal phase are most important and have attracting more attention because of their highly ordered internal structures and physicochemical properties, which offers the potential as a slow release matrix for active pharmaceutical ingredients with various sizes and polarities [22,23].

The most commonly found inverse bicontinuous cubic phases in lipid/water mixtures are the structures Ia3d, Pn3m and Im3d, in which the ordering of the inverse bicontinuous cubic phases with increasing water content follows the pattern Ia3d → Pn3m → Im3d [24]. In figure 2a, which shows the spectra of nisin loaded liposomes, the system is predominantly in an Ia3d phase, in which it remains during the temperature change (20 at 60 °C). The same behavior occurred in spectra of nisin-loaded liposomes coated with polygalacturonic acid (figure 2b). In the spectra of nisin-loaded liposomes coated with pectin the formation of cubic phases is different (figure 2c). The phase behavior below 40 °C is predominantly Im3m, however, as increase temperature, occurs the transition into cubic phase Pn3m. Although the ordering of the phases follows the pattern Ia3d → Pn3m → Im3d, the experimental evidence collected indicates that this is not the case, because, as the temperature increased, was observed a phase transition Im3m → Pn3m. Similar behavior was observed by Templer et al [24] in a study of homologous series of 2:1 (mol:mol) saturated fatty acid/phosphatidylcholine mixtures in water.

Temperature and water content are primary variables, responsible for the lipid behavior. With the increase in water content, occurs the hydration of lipid molecules, so-called excess water limit. The transitions into inverted bicontinuous cubic phases require water content well above the excess water limit for their development [21]. Thus, the cubic phase formed spontaneously in excess water, as show in the figure 2. Besides that, negatively charged phospholipids with pectin may have facilitated the transformation into cubic phase through the increasing the electrostatic repulsion between the lipid bilayers and reducing the energy required for dissipation of the lamellar phase prior to its conversion into cubic phase.

The ability of membrane to adopt cubic or hexagonal structures depends strongly on the type of lipids and their molecular shape. The phosphatidylcholine favors planar bilayers and stabilize the lamellar phase, without inducing a negative or positive membrane curvature [25]. According to Koynova and Tenchov [21], the phosphatidylcholine membrane are bilayer-forming lipids unable to transform into the non-lamellar phase even at very high temperatures, and the formation of inverted cubic phases is observed in phosphatidylcholine mixtures with other lipids, which alone also do not form a cubic phase. However, in this study the presence of cubic phases was observed using only phosphatidylcholine, without mixing with other lipids. This fact can be explained by the presence of nisin in the formulation, which may have facilitated the transformation into cubic phase. As can be seen in figure 2, only the samples containing the nisin peptide showed the formation of cubic phases, indicating that this may be the reason for the cubic phases in the liposomes produced by thin-film hydration method.

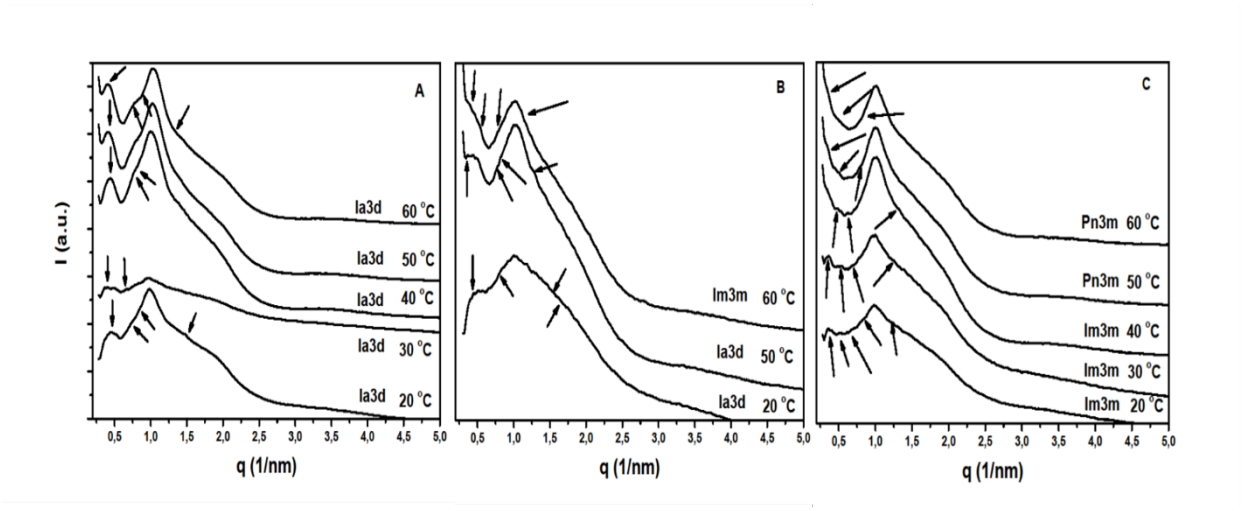

Figure 2: Cubic phases of the liposomes (arrows) produced by thin-film hydration method at different temperatures. SAXS results of intensity (*I*) as a function of wave vector (*q*) of nisin loaded liposomes (A), nisin-loaded liposomes coated with polygalacturonic acid (B), nisin-loaded liposomes coated with pectin (C).

Antimicrobial peptides, such as nisin, can be characterized as short (12–50 amino acids), positively charged, and amphipathic peptides. The antimicrobial peptides can influence the structure of phospholipid bilayer, through the changes in the curvature stress and the formation of non-lamellar phases. Among the peptides that induce a negative membrane curvature include, for example, the nisin [25]. Nisin promotes the formation of non-lamellar inverted phases by the increase of the spontaneous radius of curvature of the lipid plane, producing a change in lipid layer curvature [26]. Although phosphatidylcholine does not influence the formation of inverted cubic phases, the nisin seems to induce the formation by the perturb the structure of membranes formed of phosphatidylcholine, whereas the samples without the addition of nisin did not present the formation of cubic phases.

The data obtained by analysis of the SAXS corresponding to the lamellar thickness in each sample is given in Table 1. Analyzing the data, it was observed higher bilayer thicknesses *d* in nisin liposomes than in nisin liposomes-PA or nisin liposomes-PT, probably because of the

presence of polysaccharides, which leads to a different arrangement of the lipids in the membrane. The higher bilayer thickness was observed to samples at temperatures of 20, 30 and 40 °C. As increased temperature (50 to 60 °C), the lamellar phase imbibed water in the samples of nisin/empty liposomes-PA and nisin/empty liposomes-PT. Of the cubic phases, group Ia3d appears at the lowest water volume fractions, corresponding to nisin liposomes and nisin liposomes-PA. At a temperature of 60 °C, nisin liposome-PA showed phase transition Ia3d → Im3d, and increased water volume. The higher bilayer thicknesses *d* was in nisin liposomes-PT, that showed the phase Im3m and Pn3m.

Table 1: Repeated distances formed by the phospholipid bilayers *d* (nm) obtained by small angle X-ray scattering, produced by thin-film hydration method at different temperatures.

Samples	Phase	Temperature (°C)				
		20	30	40	50	60
Nisin liposomes	Lamellar	6.40	6.54	6.27	6.13	6.08
	Cubic	14.13	16.72	14.50	15.48	14.86
Nisin liposomes-PA	Lamellar	6.39	ND	ND	6.18	6.30
	Cubic	12.76	ND	ND	15.21	17.09
Nisin liposomes-PT	Lamellar	6.36	6.30	6.22	6.27	6.15
	Cubic	17.43	17.66	13.25	17.30	17.00
Empty liposomes	Lamellar	ND	6.27	6.25	6.16	6.05
	Cubic	-	-	-	-	-
Empty liposomes-PA	Lamellar	6.42	ND	ND	6.41	6.29
	Cubic	-	-	-	-	-
Empty liposomes- PT	Lamellar	6.03	5.97	6.25	6.27	6.27
	Cubic	-	-	-	-	-

ND= not determined.

The SAXS spectra showed differences between the cubic phases formed in nisin liposomes-PA and nisin liposomes-PT. This difference may be due to the structures of

polysaccharides. Pectin is an anionic polysaccharide consisting of D-galacturonic acid units with very small quantities of neutral sugars. The average molecular weight of pectin varies from 50,000 to 150,000. The pectin molecules have long and regular galacturonic regions (smooth), and rhamnose-rich regions (hairy) carrying sugars as side chains, such as galactose, arabinose and xylose [20,27]. Thus, pectin side chains carrying sugars may have contributed to the formation of cubic phase groups Im3m and Pn3m.

3.2 Reverse phase evaporation method

In order to gain more insight about the nisin-loaded liposomes structure and compare with those of the corresponding empty liposomes, obtained by reverse phase evaporation method, SAXS analysis was performed and the results shown in the Figure 3. The relation (1:2) between the Bragg peaks in the SAXS spectrum confirmed the presence of lamellar structures in all the samples. The temperature appears to affect in the amount of multilamellar structures in the suspensions, so that as the temperature increases, the definition of the peaks increases due to the presence of multilamellar structures. In addition, the liposomes produced by the reverse phase method appear to have been affected by the presence of polymers. It can be inferred that pectin and polygalacturonic acid tends to increase the fraction of multillamelar structures in liposomes suspensions.

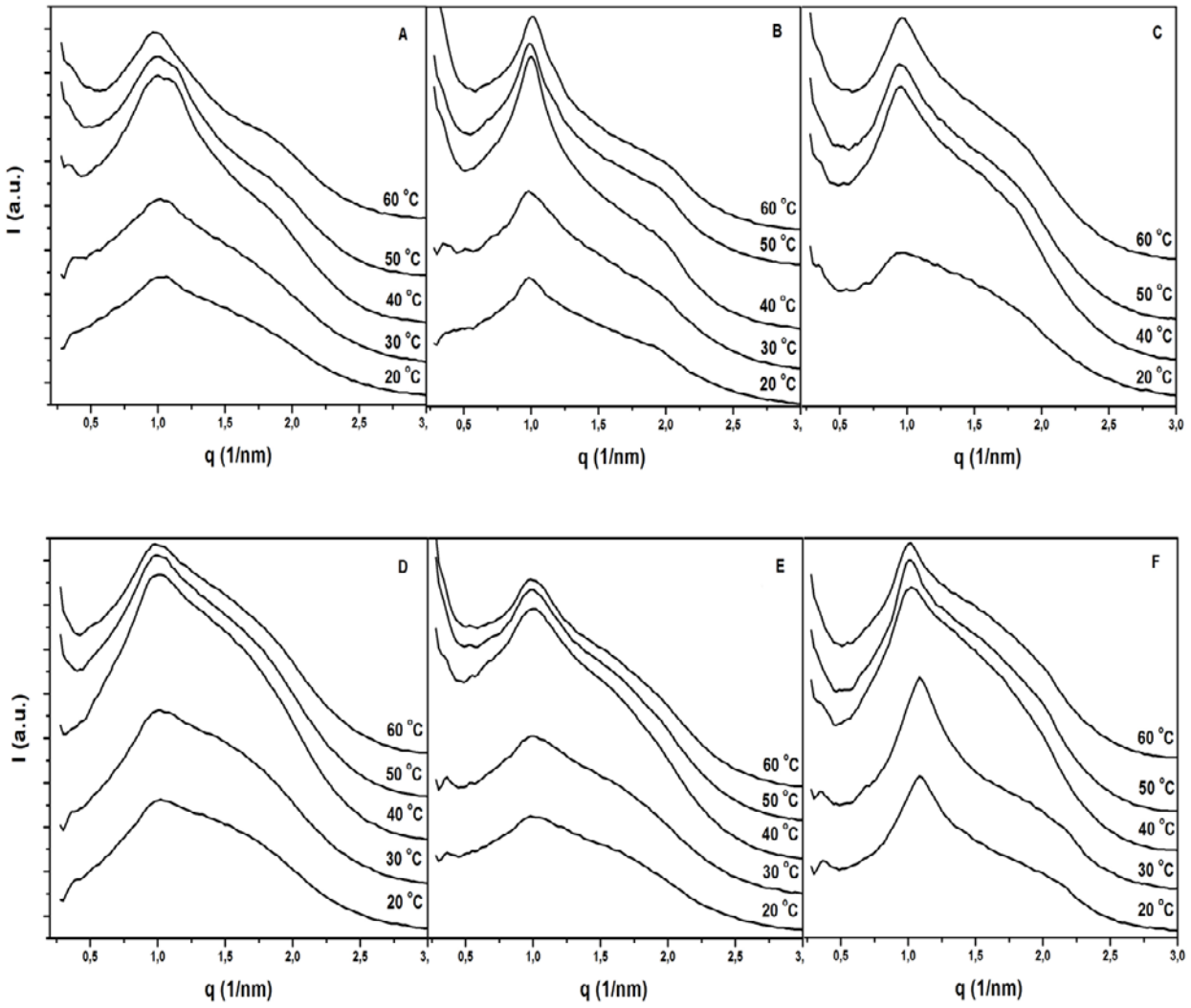

Figure 3: SAXS results of intensity (*I*) as a function of wave vector (*q*) of nisin loaded liposomes (A), nisin-loaded liposomes coated with polygalacturonic acid (B), nisin-loaded liposomes coated with pectin (C), empty liposomes (D), empty liposomes coated with polygalacturonic acid (E) and empty liposomes coated with pectin (F), produced by reverse phase evaporation method and measured at different temperatures.

The lamellar thickness *d* obtained by analysis of the SAXS profiles given in Table 2 around 6.0 nm is corresponding to two molecules of phosphatidylcholine. This value is found through the analysis of the first Bragg peak [15]. Until temperature of 50 °C, was found higher

bilayer thicknesses *d* in nisin liposomes-PA and nisin liposomes-PT, compared to nisin liposomes. Gonçalves et al [7] studied adding chitosan in liposomes and observed higher bilayer thicknesses d in liposomes without addition of chitosan. According to the study, the addition of chitosan leads to a different arrangement of the lipids in the membrane, decreasing the bilayer thickness due to the interaction of the phosphatidylcholine with the polymer. Therefore, the interaction of the phosphatidylcholine occurred only with polygalacturonic acid at a temperature of 60 °C, in nisin-containing liposomes. The same behavior was observed in empty liposomes-PA, when compared to empty liposomes, where the interaction occurred at 60 °C. In empty liposomes-PT, the behavior was different, suggesting that the interaction of the polymer with the phosphatidylcholine occurred from 20 °C to 60 °C (table 2).

Table 2: Repeated distances formed by the phospholipid bilayers *d* (nm) obtained by small angle X-ray scattering, produced by reverse phase evaporation method at different temperatures.

Samples	Phase	Temperature (°C)				
		20	30	40	50	60
Nisin liposomes	Lamellar	6.02	6.14	6.02	6.21	6.41
	Cubic	-	16.77	18.41	18.33	18.50
Nisin liposomes-PA	Lamellar	6.40	6.39	6.29	6.32	6.20
	Cubic	14.79	16.83	-	17.42	17.23
Nisin liposomes-PT	Lamellar	6.55	ND	6.58	6.68	6.59
	Cubic	18.38	ND	17.86	16.29	-
Empty liposomes	Lamellar	6.15	6.17	6.22	6.27	6.39
	Cubic	-	-	-	-	-
Empty liposomes-PA	Lamellar	6.40	6.30	6.23	6.31	6.33
	Cubic	-	-	-	-	-
Empty liposomes- PT	Lamellar	5.79	5.80	6.15	6.22	6.19
	Cubic	-	-	-	-	-

ND= not determined.

The addition of nisin in liposomes changed the peaks shape, showing besides the presence of a lamellar structure, also the presence of non-lamellar phase, such as cubic structure. Thus, the promotion of non-lamellar phase by nisin indicates that the association of the peptide with the bilayer modifies the amphiphilic balance, due to the penetration of the peptide in the bilayer. The insertion of nisin in the bilayer can lead to an increase of the hydrophobic contribution, favoring non-lamellar inverted phases [26]. Furthermore, factors such as excess water and negative charge present in the liposomes probably contributed to the formation of cubic structures [21].

In the spectra of nisin loaded liposomes, the formation of the cubic phase was at 30 °C and remained during the temperature change (30 at 60 °C) in an Pn3m phase, as shown in Figure 4a. In the spectra of nisin-loaded liposomes coated with polymers the formation of cubic phases is different (figure 4b and 4c). At a temperature of 20 °C both polymers showed Ia3d phase. At 30 °C liposomes coated with polygalacturonic acid showed the transition into cubic phase Im3m, and at 50 °C into cubic phase Pn3m, indicating that as the temperature increased, occurred a phase transition Ia3d → Im3m → Pn3m. The phase behavior of the liposomes coated with pectin is predominantly Pn3m from 40 °C, following the order pattern of the phases Ia3d → Pn3m.

In relation to the cubic phases, with increasing water content follows the pattern Ia3d → Pn3m → Im3d, which is defined by the space filling properties of the underlying minimal surfaces [24]. However, the thickness values of the bilayer *d* of liposomes produced by reverse phase evaporation method did not follow the pattern, showing different volumes of water in the same phase. For example, in the nisin liposomes at 30 °C the water volume was 16.77 and increased to 18.41, but remained in Pn3m phase, as can be seen in Table 2.

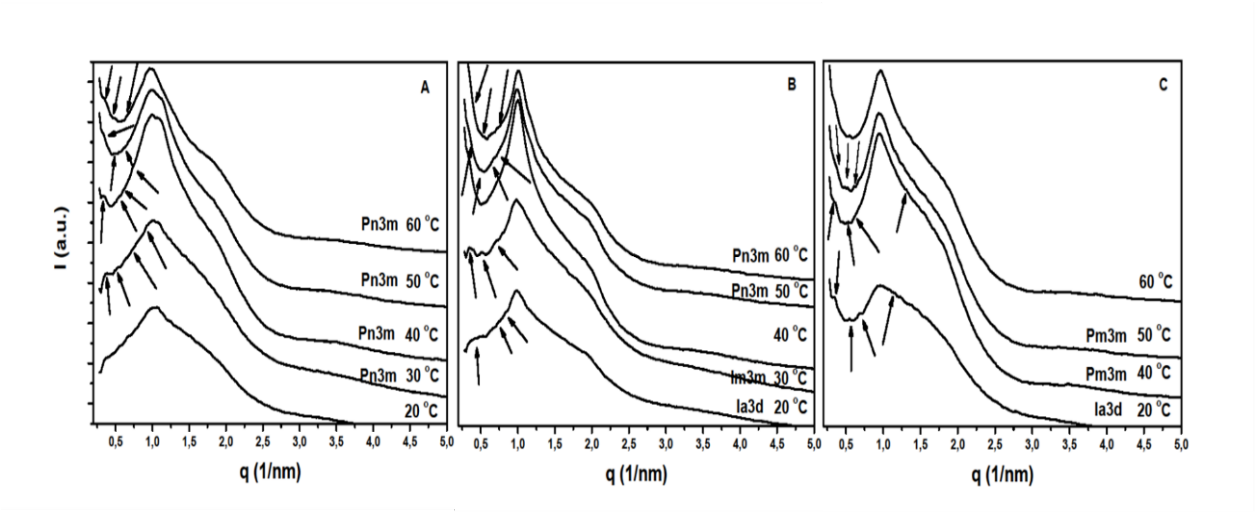

Figure 4: Cubic phases of the liposomes (arrows) produced by reverse phase evaporation method at different temperatures. SAXS results of intensity (*I*) as a function of wave vector (*q*) of nisin loaded liposomes (A), nisin-loaded liposomes coated with polygalacturonic acid (B), nisin-loaded liposomes coated with pectin (C).

In general, SAXS spectra of the solutions containing polymers showed differences between the cubic phases formed, compared to nisin liposomes. Thus, the presence of pectin or polygalacturonic acid in the liposomes may influence the aqueous encapsulated volume incorporating hydrophilic substances and to entrap amounts of hydrophobic compounds inside their apolar area, depending on delivery purposes in which can be designed.

4. Conclusions

The data from SAXS of samples obtained at different temperatures suggests the existence of lamellar structure. In addition, probably between the bilayer stacks, the presence of nisin may have led to structures containing cubic phases, thus, part of the multilamellar structure became cubic. The presence of pectin or polygalacturonic acid in the liposomes showed differences between the cubic phases formed. In this sense, the cubic phases can provide controlled release of antimicrobial peptides such as nisin.

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3.3 Artigo 3

Antimicrobial activity of lysozyme-nisin co-encapsulated in long circulatory liposomes coated with polysaccharides

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Abstract

Natural antimicrobials are an innovative alternative for food safety and its encapsulation could improve its control release and stability. In the present work, the mixture of lysozyme and nisin were encapsulated in phosphatidylcholine (PC) liposomes and coated with pectin or polygalacturonic acid were characterized. The mean diameter of the PC liposomes content the antimicrobials was 85.6 and varied to 77.3 and 79.9 nm with the incorporation of pectin or polygalacturonic acid, respectively. The formulations showed high encapsulation efficiency, zeta potential around -30 mV for liposomes with polysaccharides and also, revealed spherical structures when observed by transmission electron microscopy. The formulation containing pectin as coating was more efficient in inhibiting *Listeria* spp. when compared to the other liposomes tested *in vitro*. The antimicrobial activity was assessed at 37 °C using milk as model, showing that PC-pectin liposomes reduced the population of *L. monocytogenes* to 2 log CFU/mL in whole milk and 5 log CFU/mL in skim milk. Under refrigeration temperature, PC-pectin liposomes reduced the population of *L. monocytogenes* to almost zero for up to 25 days in skim milk, indicating that liposomes containing polysaccharides can be a promising technology for the controlled release of lysozyme and nisin in foods.

Keywords: Lysozyme; nisin; liposomes; pectin; polygalacturonic acid.

1. Introduction

The use of natural substances for food preservation meets the current tendency of consumers for healthier and safe products, minimally processed and additive-free foods (Brandelli, Lopes, & Boelter, 2015). To meet this demand, bioconservation emerges as a promising alternative, in which natural antimicrobial compounds are used in food in order to control the growth of pathogenic and spoilage microorganisms (Lopes & Brandelli, 2017).

Natural antimicrobial agents such as lysozyme and nisin have been used to control spoilage and pathogenic microorganisms (Were et al., 2003). The enzyme lysozyme is a naturally occurring antimicrobial that can be derived from eggs, plants, bacteria, and animal secretions (Were et al., 2004). Lysozyme is a small globular protein, with molecular weight of 14.3 kDa, strongly basic, bearing a net positive charge over a broad pH range. It is a safe food preservative and regarding the antibacterial specificity of lysozymes, Gram-positive bacteria are more susceptible than Gram-negative bacteria (Liu et al., 2013; Were et al., 2003). Nisin is a 3.5 kDa positively charged antimicrobial produced from *Lactococcus lactis* strains and is effective at inhibiting the growth of Gram-positive bacteria such as *Listeria monocytogenes* (Salmieri et al., 2014).

The antimicrobial agents are highly sensitive to the production process and /or storage conditions. Thereby, the antimicrobial efficacy of both lysozyme and nisin may be reduced because of undesirable interactions with food components, and, thus, large antimicrobial concentrations are often required. Nanotechnology may overcome this problem by encapsulation of antimicrobials in liposomes, protecting and enhancing their efficacy and stability in food applications (Lopes & Brandelli, 2017; Were et al., 2003). Liposomes are vesicular structures composed of one or more lipid bilayers that have the capacity to load and

release in controlled form, lipophilic and hydrophilic compounds, but their hydrodynamic properties, encapsulation efficiency and stability might be affected, depending on the nature and concentration of the loading material (Tan et al., 2013).

The use of polysaccharides in nanoliposomes can be an alternative for the controlled release of antimicrobials, as showed in our previous work, where were developed pectin and polygalacturonic acid coated nanoliposomes content nisin, with high encapsulation efficiency, low polydispersity index, and good stability for 21 days at 7 °C and 25 °C, evidencing their technological applicability (Lopes, Pinilla, & Brandelli, 2017). However, the use of nisin and lysozyme combinations encapsulated in liposomes containing polysaccharides has not been described.

Polysaccharides are natural polymers used as safe and low cost food ingredients that have attracted attention for delivery of bioactive food components (Fathi, Martin, & McClements, 2014). Pectin is a complex anionic polysaccharide mainly composed of a linear chain of galacturonic acid units joined by α-1.4 links (Krivorotova et al., 2016; Souza et al., 2012). The great advantage of using pectin is based on properties such as biodegradability, biocompatibility and generally consider as nontoxic substance, besides can be incorporated into a wide range of commercial products (Hu et al., 2015).

The aim of this research was to produce and characterize nanoliposomes coated with pectin or polygalacturonic acid by the thin-film hydration method for lysozyme and nisin encapsulation, in order to determine its antimicrobial activity and its potential as delivery systems in the food industries.

2. Materials and methods

2.1. Antimicrobial agents, polysaccharides, and culture media

Lysozyme provided by Sigma (St. Louis, MO, USA), contained ≥40,000 units/mg of protein and was dissolved in 10 mM Tris-HCl, pH 7.0, to a stock 2 mg/mL concentration. Nisaplin® (25 mg of nisin/g of Nisaplin®) was purchased from Danisco (Vargem Grande, Brazil), and before each experiment, was prepared as described by Pinilla and Brandelli (2016). The stock solution was diluted in 10 mM phosphate buffer pH 7.0 to reach the working concentrations of 0.16 mg/mL, later filter-sterilized through 0.22 μm membranes (Sartorius, Göt, Germany) and stored into a sterile tube at 4 °C. The levels of nisin are expressed as levels of pure nisin (μg/mL or μg/g) and should be multiplied by 40 to convert these units to International Units (IU/mL or IU/g).

The polysaccharides used in the experiments were polygalacturonic acid (≥90%, enzymatic) and pectin from apple (degree of esterification 70-75%), both from Sigma (St. Louis, MO, USA). Polygalacturonic acid was dissolved in 10 mM sodium phosphate buffer pH 7.0 and pectin was dissolved in ultrapure water, in a concentration of 1 mg/mL.

The indicator organisms for antimicrobial activity assays were *Salmonella* Enteritidis ATCC 13076 and different strains of *Listeria sp.* (*L. monocytogenes* ATCC 7644, *Listeria* sp. str1, *L. innocua* 6a, *L. monocytogenes* 4b isolated from bovine carcass, and *Listeria* sp. str2 isolated from cheese). Strains were maintained on Brain Heart Infusion (BHI, Oxoid, Basingstoke, UK) agar plates at 4 °C, and subcultured periodically. Before each experiment, strains were grown in BHI medium at 37 °C for 18-24 h.

2.2. Production of liposomes by film hydration

Nanoliposomes were prepared using Phospholipon 90G®, a purified soybean phosphatidylcholine (PC, ≥94%), provided by Lipoid (Ludwigshafen, Germany). Lysozyme and nisin were encapsulated into PC nanoliposomes by the thin-film hydration method (Lopes, Pinilla, & Brandelli, 2017). Briefly, the lipid was dissolved in chloroform and the organic

solvent was removed until formation of a thin lipid film. The film was dispersed by adding 5.0 mL solution of lysozyme or adding of 5.0 mL lysozyme-nisin mixture (2.5 mL of lysozyme plus 2.5 mL nisin). To assist the formation of the liposomes, the solution was heated and stirred, followed by under vigorous agitation. After this process, 5.0 mL of the pectin or polygalacturonic acid solution (1.0 mg/mL) was added and the final concentration of nisin and lysozyme in solution was 0.08 mg/mL and 2.0 mg/mL, respectively. Control liposomes (without the use of polysaccharides) were prepared with addition of 5.0 mL 10 mM phosphate buffer pH 7.0. Then, the mixture (10.0 mL) was subjected to ultrasound for size reduction using a probe-type sonicator (Unique OF S500, frequency 20 kHz, power 250 W). Shortly after preparation, the liposomes were filtered using a 0.22 μm membrane (Sartorius, Göt, Germany).

2.3. Light scattering analysis and zeta-potential measurements

The liposome size and polydispersity index (PDI) were determined by Dynamic Light Scattering (DLS) in a Brookhaven Instruments standard setup (BI-200M goniometer, BI-9000AT digital correlator) (Teixeira, Santos, Silveira, & Brandelli, 2008). The zeta potential of liposomes was carried out after dilution ratio 1:100 of the formulations in 10 mM sodium phosphate buffer pH 7.0 using a Zetasizernano-ZS ZEN 3600 instrument (Malvern Instruments, Herrenberg, Germany). The measurements of zeta potential and DLS were performed immediately after of the liposomes preparation.

2.4 Entrapment efficiency

The encapsulation efficiency (EE) was determined by HPLC (Shimadzu HPLC, Kyoto, Japan) equipped with quaternary pumps (LC-20AD) and a diode array detector (DAD) (SPD-M20A). The liposomes were separated from the solution by ultrafiltration using a 30-kDa membrane (Ultracel® 30K; Millipore, Billerica, MA, USA). The amount of lysozyme and nisin

were determined in the filtrate by HPLC and the EE was calculated separately for lysozyme and nisin using the following equation:

$$\%EE = \frac{[L/N]s - [L/N]filtrate}{[L/N]s} \times 100$$

where $[L/N]_S$ is the concentration of lysozyme or nisin in the solution used for encapsulation and $[L/N]_{filtrate}$ is the concentration of lysozyme or nisin in the filtrate.

For lysozyme determination, the samples were separated by HPLC method using a C18 Column, according with Guarino et al. (2011), with some modifications. Was used 0.1% of formic acid in water (v/v) as eluent A, and 0.1% of formic acid in acetonitrile (v/v) as eluent B. Initially a linear gradient from 10% to 60% of phase B within 15 min was used, followed by an isocratic step consisting in 90% of phase B for 5 min. Finally, the column was re-equilibrated at the initial conditions. The analyze was accomplished at a constant flow of 0,7 mL/min and the Column was thermostated at 25 °C.

For nisin determination the samples were filtered through 0.22 μm nylon membrane and separated by HPLC method using a C18 Column with an injection volume of 100 μL. The column was eluted using mobile phases A (0.1% v/v TFA in water) and B (0.1% v/v TFA acid in acetonitrile) in the following manner: 100% A for 5 min, a linear gradient 0-50% B over 45 min, a linear gradient to 100% B over 5 min and 100% B for 7 min, at 1 mL/min flow rate. The UV detector was used at 215 nm and the column temperature was at 35°C (Pinilla & Brandelli, 2016). The amount of nisin was calculated by means of a calibration curve of nisin A standard (Sigma, St. Louis, MO, USA).

2.5. Transmission electron microscopy

The morphology of liposomes was visualized using a transmission electron microscope (JEM-1200, Jeol Ltd., Tokyo, Japan). The samples were deposited on a Formvar-coated 300 mesh copper grids and left for 1 min, and stained with uranyl acetate solution 2.5% for 30 seconds.

2.6. Fourier transform infrared spectroscopy (FTIR)

The interaction between lysozyme and nisin with the phosphatidylcholine of the lipid membranes was evaluated by FTIR, using a Shimadzu 8300 FTIR spectrophotometer (Shimadzu, Kyoto, Japan). The samples of liposomes were freeze-dried prior analysis. All of samples were prepared using the KBr disk method. The scanning range used was 4000 to 400 cm^{-1} with 64 scans and the resolution was set at 4 cm^{-1}.

2.7. Antimicrobial activity assay

Antimicrobial activity was tested for 14 days, and determined by Tetrazolium/formazan test (TTC) adapted from Moussa et al. (2013). Briefly, 500 μL of liposomes (with pectin or polygalacturonic acid, and without the use of polysaccharides) was poured in 10 mL BHI medium containing 20 μL of 10^8 cell/mL challenge microorganisms (*S.* Enteritidis ATCC 13076 or *L. monocytogenes* ATCC 7644). Controls were inoculated with bacterial strains, and all flasks were incubated with shaking at 37 °C at for 3 h. Then, 1.0 mL from each flask containing the treated and the control cultures was added to tubes containing 100 μL of TTC solution (5mg/mL), and incubated again at 37 °C until the red formazan is observed, which indicates the presence of viable bacterial cells. The resulted formazan was centrifuged at 4000*g* for 5 min and the pellets obtained were resuspended in ethanol 50% and centrifuged again at the same conditions. The red formazan solution obtained at the end was measured by

spectrophotometer at 480 nm and de percentage of activity was calculated using the valor of absorbance (Abs) according to the following equation:

$$\% \text{ Activity} = \frac{\text{Abs control} - \text{Abs samples}}{\text{Abs control}} \times 100$$

2.8. Inhibitory effect of free and encapsulated lysozyme/nisin in milk

The inhibitory effect in milk were evaluated as described elsewhere (Pinilla & Brandelli, 2016). For this, overnight cultures were diluted in saline solution (8.5 g/L NaCl) to approximately 6 log CFU/mL. Therefore, 100 µL of bacterial each suspension was added in 10 mL of whole and skim UHT as a food model. Then, 1.0 mL of liposome-encapsulated lysozyme-nisin, 1.0 mL of free lysozyme-nisin, 500 µL of free lysozyme or 500 µL of nisin free were added to different tubes. The volume was adjusted to 1.0 mL with phosphate buffer in the samples of free lysozyme and nisin. Controls were inoculated only with bacterial strains. In addition, liposomes containing pectin or polygalacturonic acid were also tested. Two different methodologies were analyzed, in the first one, tubes contend *L. monocytogenes* ATCC 7644 and *Salmonella* Enteritidis were incubated for 2, 4, 6, 8 and 10 h at 37 °C; The second approach was at refrigeration temperature (7±1 °C), using the *L. monocytogenes* ATCC 7644 as control, and the mixture of strains of *Listeria* sp. str1, *L. innocua* 6a, *L. monocytogenes* 4b isolated from bovine carcass, and *Listeria* sp. str2 isolated from cheese, for obtain a cocktail of bacteria. Counts were estimated at 0, 5, 10, 15, 20 and 25 days. The quantification of viable cells was performed by the drop culture method (Naghili et al., 2013). BHI plates were incubated at 37 °C and colonies were enumerated after 24 h.

2.9. Statistical analysis

The measurements were made in triplicate and compared using Tukey's test at a level of 95% of significance ($P< 0.05$) by Statistica 7.0 software (Statsoft Inc., Tulsa, OK, USA).

3. Results and discussion

3.1. Encapsulation of lysozyme

Initially, liposomes were developed with the incorporation of the antimicrobial enzyme lysozyme, and were successfully prepared using phosphatidylcholine to produce liposomes coated with pectin or polygalacturonic acid. The characteristics of liposomes can be observed in Table 1. The incorporation of polysaccharides to the formulations caused an increase in the particle size, which was more accentuated in the case of PC-pectin liposomes. The mean diameter of PC liposomes containing lysozyme was close to the value found in our previous study (Lopes, Pinilla, & Brandelli, 2017), which obtained values between 88 to 160 nm to encapsulation of nisin in liposomes with pectin or polygalacturonic acid. The polydispersity index was around 0.2, with the exception of PC-pectin liposomes, which showed lower polydispersity index (0.102±0.1), indicating a narrow size distribution.

Table 1. Characterization of PC liposomes containing lysozyme.

	PC	PC-pectin	PC-polygalacturonic acid
Particle size (nm)	91.9±7.5	116.2±21.7	97.3±2.8
Polydispersity	0.219±0.01	0.102±0.10	0.206±0.17
Zeta potential (mV)	-18.0±6.43	-31.30±7.37	-27.99±7.06
EE (%)	86.83±2.34	76.60±3.41	86.68±1.89

Although encapsulation of lysozyme in liposomes has been investigated (Wu et al., 2016), the incorporation of polysaccharides into the liposomes are not previously described in the literature. Wu et al. (2016) optimized the preparation of lysozyme nanoliposomes, by

reverse-phase evaporation method and showed particle size of 245.6±5.2 nm, demonstrating particle size larger in comparison with lysozyme-PC liposomes produced in this study.

The results indicate that the incorporation of polysaccharides may be interesting coatings for lysozyme-loaded liposomes. Lysozyme binding to the lipid bilayer is a multistep process thought to involve both electrostatic and hydrophobic interactions (Al Kayal et al., 2012). Furthermore, the addition of pectin which has a high proportion of ionized carboxylic acid groups in the backbone make the macromolecule to be negatively charged contributing to electrostatic attractive interactions with cationic lysozyme at neutral pH (Amara et al., 2016).

The zeta potential is associated with particle stability in suspension since it indicates electrostatic repulsion between particles. The analysis measures the surface charge of particles, besides estimate the influence of the polysaccharide incorporation on the stability of liposomes (Malheiros et al., 2011; Silva et al., 2014). In this study, zeta potential was negative (Table 1), ranging from -18 to -31 mV, indicating electrostatic repulsion among the particles and suggesting the stability of liposomes containing lysozyme. These results show that the incorporation of negatively charged polysaccharides seem to have formed an outer coating, providing increased electrostatic repulsion between the nanoparticles, since PC-pectin and PC-polygalacturonic acid showed more negative zeta potential values, in comparison with PC liposomes.

Liposomes developed showed high entrapment efficiency for lysozyme, as shown in table 1. The incorporation of pectin or polygalacturonic acid to the formulations caused a decrease in encapsulation efficiency, which was more accentuated in the case of PC-pectin liposomes (table 1). The decrease in EE values is possibly related to a rearrangement of the liposomes after incorporation of polysaccharides (Silva et al., 2014), thus releasing some lysozyme. Previous EE values obtained for liposomes encapsulating lysozyme were 75.36%, found by Wu et al. (2016). The percentage of EE of lysozyme into chitosan nanoparticles was

examined by Wu et al. (2017). The study verified that the percentage of EE increase as the concentration of lysozyme increased from 0.25 to 1.25 mg/mL, suggesting that more than 80% of lysozyme was efficiently loaded in nanoparticles. Thus, the concentration of lysozyme used in this study (2 mg/mL) may have contributed to the high encapsulation efficiency in liposomes.

Images by transmission electron microscopy of liposomes encapsulating lysozyme are presented in Fig. 1. The liposomes showed presence of predominantly spherical structures, which have been typically observed by Lopes, Pinilla and Brandelli (2017) when nisin was encapsulated in liposomes containing pectin or polygalacturonic acid, and similar to that observed by Amara et al. (2016) which used pectin particles to encapsulated lysozyme.

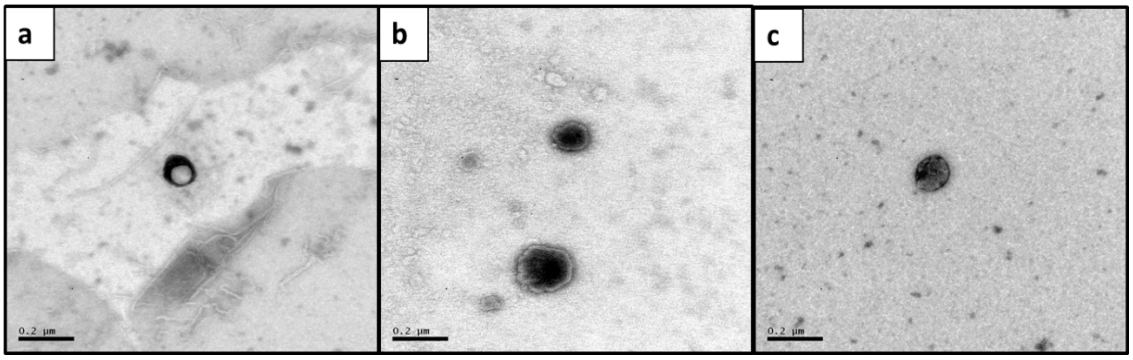

Fig. 1. Transmission electron microscopy of (a) PC liposomes, (b) PC-pectin and (c) PC-polygalacturonic acid encapsulating lysozyme.

The antimicrobial activity of liposomes encapsulating lysozyme were evaluated using TTC assay, during storage under refrigeration for 14 days, against Gram-positive bacteria *L. monocytogenes* ATCC 7644 and Gram-negative bacteria *S.* Enteritidis ATCC 13076, with the purpose to check the formulations stability. Free lysozyme 2 mg/mL tested, showed 100% and 78,1% of activity against *L. monocytogenes*, and *S.* Enteritidis respectively. Fresh samples of PC liposomes, PC-pectin and PC-polygalacturonic liposomes contend lysozyme showed 100% antimicrobial activity against *L. monocytogenes*, however, no activity was observed against *S.* Enteritidis. After 14 days, the antimicrobial activity of liposomes remained the same behavior

in both bacteria. Therefore, the addition of polymers coatings to the liposomes with lysozyme, not affect its antimicrobial activity against *L. monocytogenes*.

The chemical structure of lysozyme is characterized by single polypeptide chain. Its antimicrobial activity is related to the ability to hydrolyze the beta 1–4 glycosidic bonds present in peptidoglycans, which comprise 90% of the cell wall of Gram-positive bacteria, making them very susceptible to lysozyme antimicrobial activity. In addition, the bactericidal activity of lysozyme is independent of its enzymatic activity, that can be altered for factors as interaction with other cell wall components and with the compounds resulting by the disruption of the cell wall (Bugatti, Sorrentino, & Gorrasi, 2017; Chung & Hancock, 2000). Although lysozyme is more active against Gram-positive bacteria, in this study, free lysozyme showed activity against Gram-positive bacteria and Gram-negative bacteria. However, lysozyme liposomes were not effective against *S.* Enteritidis, possibly due to the less quantity of enzyme available in the medium by the controlled release caused for the liposome encapsulation and also a reduction of the activity can be attributed for the partial denaturation of the enzyme in the process of sonication during liposomes production, resulting in lower solubility and loss of lysozyme activity (Were et al., 2003). In this way, other alternatives have been studied to increase the spectrum of action of the liposomes.

Lysozyme was integrated into chitosan nanoparticles (CS-NPs) to improve the antibacterial activity by Wu et al. (2017). In this study, the antibacterial activities of CS-NPs and chitosan-lysozyme nanoparticles (CS-Lys-NPs) against *E. coli* and *B. subtilis* were investigated, where the results showed that CS-Lys-NPs had higher antimicrobial activities against both *E. coli* and *B. subtilis* than CS-NPs based on inhibition zone diameters, indicating that the lysozyme can increase the activity against Gram negative bacteria in combination with others antimicrobials. The inhibition with lysozyme liposomes against five *L. monocytogenes* strains was investigated by Were et al. (2003). Growth inhibition was strain dependent, with

greater inhibition observed for strains 310 and Scott A with PC-cholesterol and PC-PG-cholesterol liposomes. Our results showed that the lysozyme liposomes were less effective than free lysozyme against *S.* Enterica growth, however, indicated the potential of liposomes contend protein based antimicrobials to serve as delivery vehicles for antimicrobials in foods.

3.2. Encapsulation of lysozyme-nisin

Combinations of nisin and lysozyme have been tried in food systems, reducing and controlling Gram-positive bacteria, besides inhibiting *Salmonella* Typhimurium and *E. coli* O157:H7 using different methodologies (Gill & Holley, 2000; Gill & Holley, 2000b). Synergy between nisin and lysozyme was also seen against *L. monocytogenes* and food spoilage lactobacilli (Chung & Hancock, 2000). Lysozyme may facilitate the access of nisin to the cell membrane, leading to a larger reduction of viable cells, but the actual mechanism of synergy between nisin and lysozyme is not clearly understood.

For determination of the optimal concentration of antimicrobials which would be encapsulated, the antimicrobial effect of four different concentrations of nisin with lysozyme (1:1 ratio) was tested against *L. monocytogenes* and *S.* Enteritidis as described by Pinilla and Brandelli (2016) (data not shown) and the concentration of 16 μg/mL nisin to 2 mg/mL lysozyme was chosen for liposome production due to this is the minimum concentration of nisin that maintain optimal antimicrobial activity. The results of the characterization of the liposomes containing lysozyme and nisin are presented in Table 2. In this case, liposomes containing polysaccharides had less particle size when compared to the PC liposome. The mean diameter of liposomes co-encapsulating nisin and lysozyme was from 77 to 86 nm. These parameters mainly depended on the polymer nature and characteristics of the nisin and lysozyme, as well as the probable interactions among them. The polydispersity index indicated a narrow size distribution to all samples (around 0.2), with the exception of PC-polygalacturonic acid liposomes, which showed lower polydispersity index (0.176±0.01).

Values of zeta potential below -30 and above +30 mV are conventionally considered high zeta potentials, and can be expected to be more stable because the repulsive interactions increase, reducing the frequency of liposome collisions (Pinilla & Brandelli, 2016). The liposomes produced presented are cationic (table 2), showing values below -30 in the case of PC-pectin and PC-polygalacturonic acid liposomes, suggesting stable properties. Lysozyme and nisin were encapsulated into PC liposomes and the samples showed high encapsulation efficiency (table 2). The percentage of EE of lysozyme was not affected by the addition of pectin or polygalacturonic acid, and the load of nisin showed if a little higher in formulations containing polysaccharides, however, was found not statistical difference.

Table 2. Characterization of PC liposomes containing lysozyme-nisin.

	PC	PC-pectin	PC-polygalacturonic acid
Particle size (nm)	85.6±5.7	77.3±2.5	79.9±4.9
Polydispersity	0.208±0.004	0.227±0.01	0.176±0.01
Zeta potential (mV)	-24.89±5.00	-35.66±8.34	-38.47±6.90
EE lysozyme (%)	86.91±3.61	86.85±1.59	86.86±1.12
EE nisin (%)	79.35±2.93	83.86±2.21	81.23±3.89

The values of liposomes co-encapsulating nisin were similar to those our previous work (Lopes, Pinilla, & Brandelli, 2017), for the developed nanoliposomes containing pectin or polygalacturonic acid for nisin encapsulation. For lysozyme encapsulation was obtain superior values as compared with the work of Matouskova et al. (2016). In these works, were prepared liposomes by different methods (sonication, thin film evaporation and ethanol injection) and the EE was not superior to 60% for lysozyme and less than 30% for nisin, both alone. To determinate its morphology, liposomes encapsulating lysozyme and nisin were observed by transmission electron microscopy (figure 2), showing spherical structures as often observed for nanoliposomes incorporating nisin (Lopes, Pinilla, & Brandelli, 2017; Malheiros et al., 2010;

Silva et al., 2014) and similar to nisin-loaded pectin particles (Khaksar et al., 2014; Krivorotova et al., 2016).

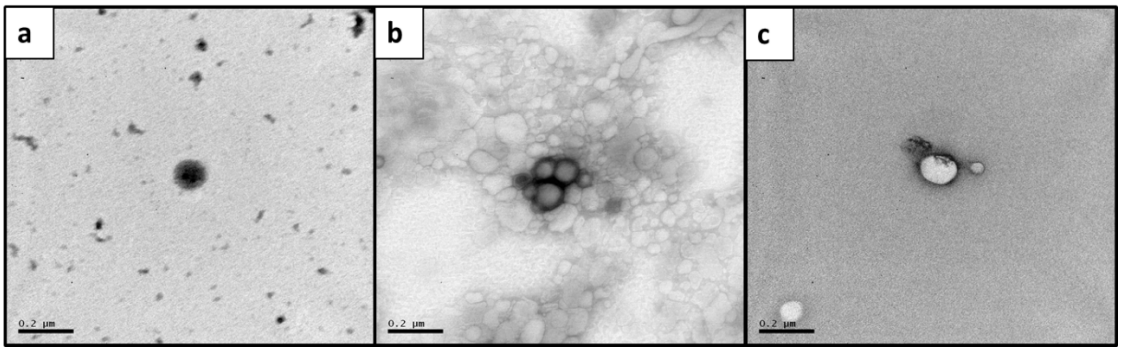

Fig. 2. Transmission electron microscopy of (a) PC liposomes, (b) PC-pectin and (c) PC-polygalacturonic acid encapsulating lysozyme-nisin.

The stability of liposomes encapsulating lysozyme and nisin was evaluated for 14 days by the determination of its antimicrobial activity against *L. monocytogenes* ATCC 7644 and *S.* Enteritidis ATCC 13076, using TTC method. The activity against *L. monocytogenes* was 100% during the 14 days tested and the coating with polysaccharides of the liposomes had not influence in its the antilisterial activity. This result was expected, since we have also observed good activity against the food pathogen *L. monocytogenes* using encapsulated lysozyme.

On the other hand, at the first day, the mixture nisin/lysozyme encapsulated, showed an activity against *S.* Enteritidis of 62.8% for PC liposomes, and 62.9% for PC-pectin and PC-polygalacturonic acid. After 7 days, was observed that the liposomes were losing their antimicrobial activity, but this decrease was less for the formulation nisin/lysozyme in PC-pectin. PC liposomes showed 52.9% of activity, while the liposomes containing PC-pectin and PC-polygalacturonic acid showed 54.3 and 38.6%, respectively. Thus, the incorporation of pectin improved stability of liposomes, since they maintained a higher activity against *S.* Enteritidis, when compared with the other formulations tested. Furthermore, the results

demonstrate the benefits of using mixtures of nisin and lysozyme against undesirable food bacteria, than the use of the individual agents.

Nisin acts on the cytoplasmic membrane of Gram-positive bacteria to cause lesions. On the other hand, lysozyme hydrolyzes the bond between N-acetylglucosamine and N-acetylmuramic acid of the peptidoglycan layer of the wall of susceptible bacteria, increasing their permeability and causing the rupture of cells. Thus, nisin could be inhibiting energy dependent processes that repair lysozyme damage (Chung & Hancock, 2000; Were et al., 2003). The mechanism of synergy is not known, however, liposomes produced with the combination of nisin/lysozyme increased cell lysis in Gram-negative bacteria and has potential to prevent spoilage in food systems.

3.3. Fourier transform infrared spectroscopy (FTIR)

The FTIR analysis was performed to confirm the presence of lysozyme and nisin and to investigate possible interactions with the membrane compounds. In our previous study, the infrared characteristic absorption peak of liposomes containing pectin and polygalacturonic acid for nisin encapsulation was analyzed in detail (Lopes, Pinilla, & Brandelli, 2017). Briefly, the region 1000-1250 cm^{-1} and 1600-1750 cm^{-1} may indicate the presence of pectin and polygalacturonic acid on the surface of liposomes prepared with polysaccharides, and the peaks around 1520-1400 cm^{-1} was attributed to amide groups of nisin.

Lysozyme has three characteristic infrared regions, including amide I (1700–1600 cm^{-1}), amide II (1600–1500 cm^{-1}) and amide III (1320–1230 $cm^{-}1$) (Wu et al., 2017; Wu et al., 2018). The broad peak at 3290 cm^{-1} arises from N-H stretching of the free amino groups, and the absorption at 2943 cm^{-1} is characteristic of C-H stretching. As can be seen in the Figure 3b, lysozyme and nisin peaks appear in similar regions. Thus, the peak appearing 1657 cm^{-1} was probably due to the absorption of lysozyme and nisin, suggesting the existence of lysozyme and

nisin in liposomes. The more intensity observed at about 3,437 cm−1 resulting from N–H and O–H stretching vibrations for the nisin and the lysozyme in the PC-PA liposomes than that of the others formulations is due to the presence and incorporation of the antimicrobials outside of membrane vesicle via physicochemical interactions with the polygalacturonic acid and this interaction may change the dynamic of its release.

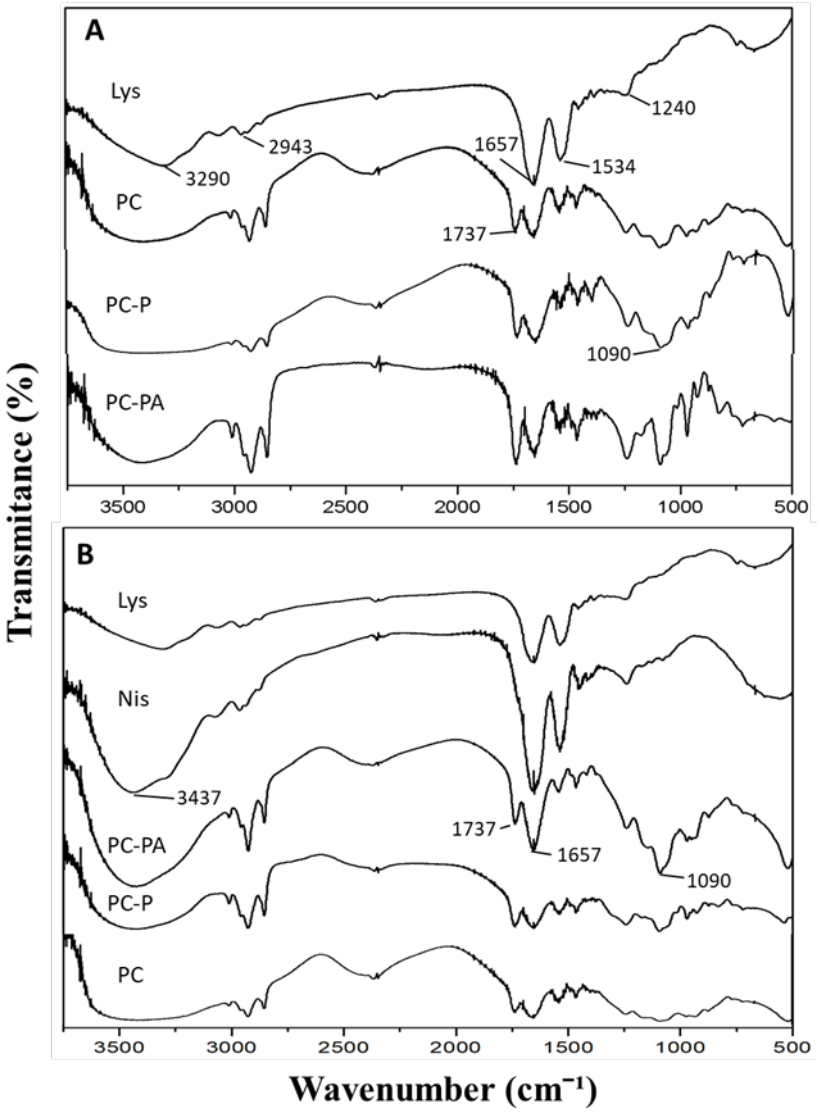

Fig. 3. FTIR spectra of A) liposomes encapsulating lysozyme, and B) liposomes encapsulating lysozyme-nisin. PC-P= PC-pectin and PC-PA= PC-polygalacturonic acid.

3.4. Inhibitory effect of free and encapsulated lysozyme/nisin in milk

The antimicrobials lysozyme, nisin, and its mixture, were studied to ascertain their abilities to control the growth of the *L. monocytogenes* ATCC 7644 and *S.* Enteritidis ATCC 13076, with the goal to optimize an antimicrobial for potential use in preservation of foodstuffs using whole and skim milk as model. The antimicrobial activities of free lysozyme and nisin and its encapsulated combination were evaluated for 10 h at 37 °C (Figure 4). Besides that, the addition of pectin and polygalacturonic acid in liposomes was evaluated, aiming a controlled release of antimicrobials.

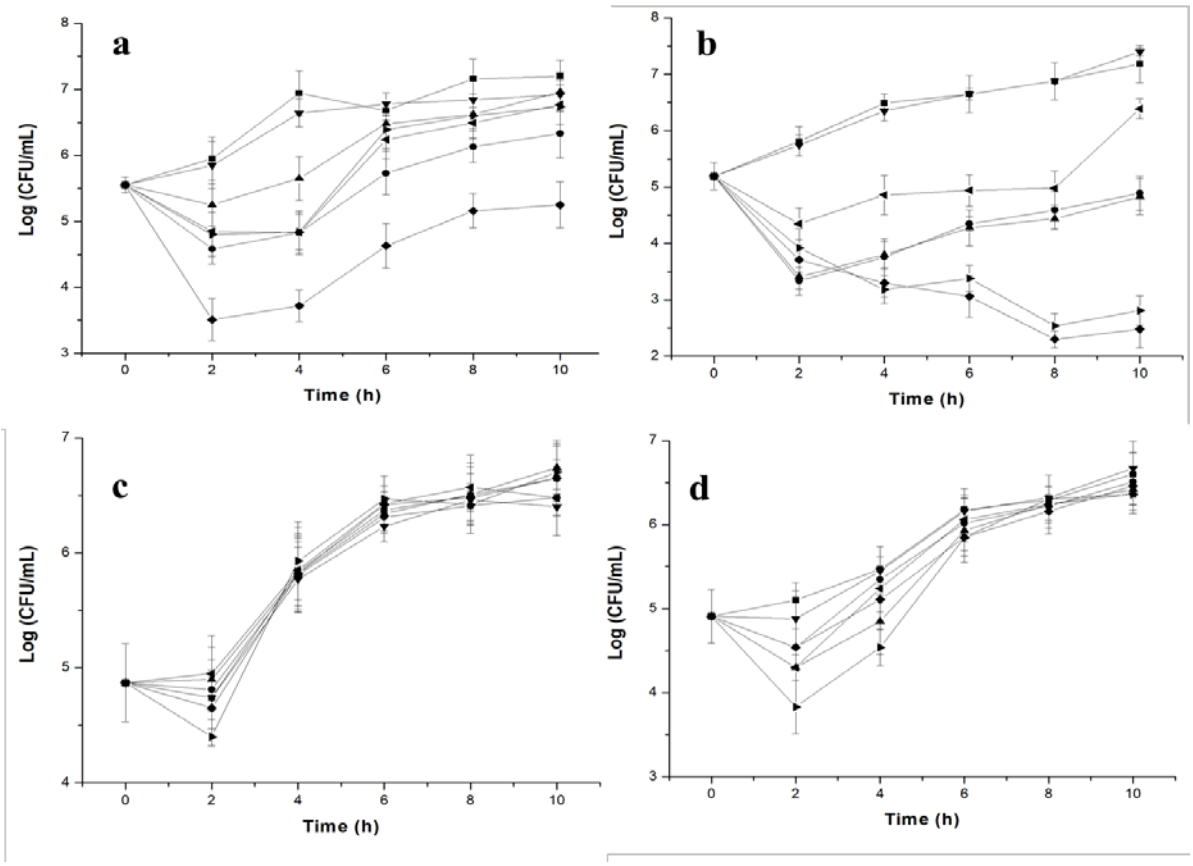

Fig. 4. Growth of *L. monocytogenes* ATCC 7644 in whole milk (a) and skim milk (b), *S.* Enteritidis ATCC 13076 in whole milk (c) and skim milk (d) at 37 °C containing free or encapsulated lysozyme-nisin. Viable counts were monitored of PC liposomes (●), PC-pectin (♦), PC-polygalacturonic acid (◄), free lysozyme-nisin (►), free lysozyme (▼), free nisin (▲), and control (■). Each point represents the mean ±SEM of three independent experiments.

From the results presented in Figure 4, it can be observed that in whole milk the combination of lysozyme with nisin had synergistic antimicrobial effect against *L. monocytogenes* and the greater inhibition was achieved in the presence of PC-pectin liposomes. At 4 h, these liposomes reduced the population to 3 log CFU/mL, and after 10 h the decrease was 2 log CFU/mL compared to control, suggesting that the controlled release of nisin and lysozyme is taking place during incubation, preventing *Listeria* growth. In skim milk, similar viable counts were observed for free and PC-encapsulated lysozyme-nisin, but the PC-pectin liposomes showed greater inhibition and decreased the bacterial counts for up to approximately 5 log CFU/mL for *L. monocytogenes* at 10 h incubation. Silva et al. (2014) observed similar viable counts for free and PC-encapsulated nisin at 24 h, but the inhibition of *L. monocytogenes* was greater in the presence of PC-chitosan nanovesicles. The treatments not effective to inhibit the growth of *S.* Enteritidis in whole milk. The skimmed milk presented a small decrease (about 1 log CFU/mL) at 2 and 4 h for free lysozyme-nisin, however, no significant difference between the treatments was observed after 10 h.

The effect of lysozyme-nisin liposomes during storage of whole and skim milk at 7 °C was investigated against *L. monocytogenes* and against the mixture of strains of *Listeria* sp. str1, *L. innocua* 6a, *L. monocytogenes* 4b and *Listeria* sp. str2, to simulate a food model. In the case of whole milk (figure 5a and 5c), similar viable counts were observed for free and PC-encapsulated lysozyme-nisin for 5 days, but the addition of pectin in liposomes increased retention of the encapsulated material, showing greater inhibition. PC-pectin was effective in reducing the initial population of *L. monocytogenes* to about 3 log CFU/mL for 5 days, and to final counts was about 1.5 log CFU/mL after 25 days. The growth of mixture of strains of *Listeria* showed a small decrease (about 1.5 log CFU/mL) after 5 days, however, after 15 days the growth was not inhibited.

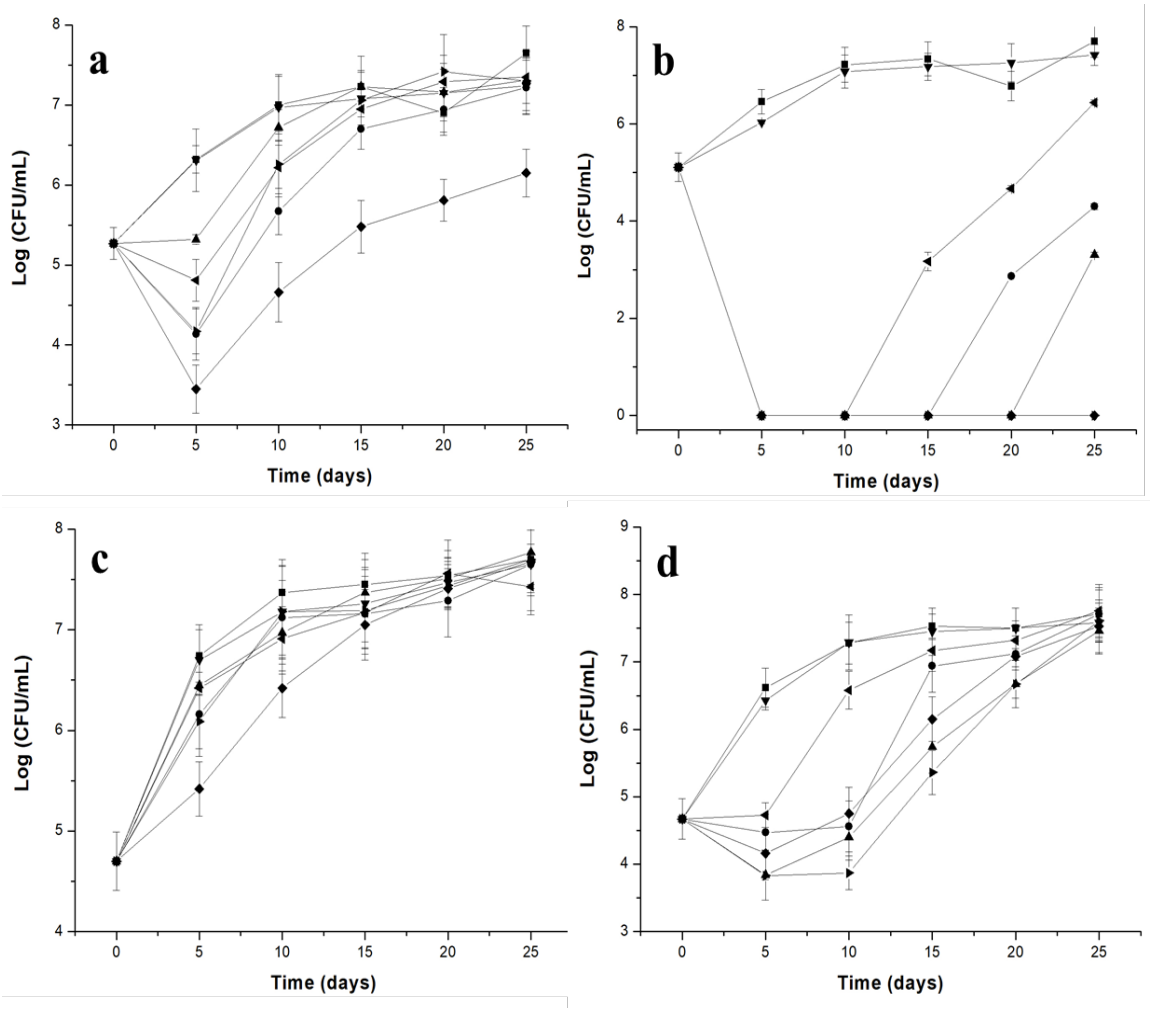

Fig. 5. Growth of *L. monocytogenes* ATCC 7644 in whole milk (a) and skim milk (b), mixture of strains of Listeria sp in whole milk (c) and skim milk (d) at 7 °C containing free or encapsulated lysozyme-nisin. Viable counts were monitored of PC liposomes (●), PC-pectin (♦), PC-polygalacturonic acid (◄), free lysozyme-nisin (►), free lysozyme (▼), free nisin (▲), and control (■). Each point represents the mean±SEM of three independent experiments.

In the treatment with skim milk was observed that only free lysozyme did not inhibit *L. monocytogenes* up to 5 days (figure 5b). After 10 days, the antimicrobial effect of free nisin was surpassed and *L. monocytogenes* grows back, followed by PC-polygalacturonic acid and PC-liposome at 15 and 20 days, respectively. In addition, was observed that the free lysozyme-nisin and PC-pectin did not present a significant difference and reduced the population of *L.*

monocytogenes to almost zero for up to 25 days. The growth of mixture of strains of *Listeria* showed decrease about 3.5 log CFU/mL after 10 days for free lysozyme-nisin, but no significant difference between the treatments was observed after 20 days (figure 5d).

The negative effect of fat on nisin antimicrobial activity is extensively reported (Chollet et al., 2008; Sobrino-López & Martín-Belloso, 2008; Pinilla & Brandelli, 2016). Thus, the most efficient action of nisin in skim milk was somewhat expected, due to the adsorption of nisin onto fat globules. In this work, the synergy between lysozyme and nisin ensured a more efficient inhibition of *Listeria* spp. Matouskova et al. (2016) related that liposomes containing lysozyme or nisin release a significant mass fraction of the antimicrobials in fatty medium, but also showed that this process can be affected by the temperature. At 28 °C significant mass fraction of lysozyme and nisin were released, while at 5 °C minimum mass fraction of lysozyme was found in the medium and at the same temperature a higher concentration of nisin was determinate. This fact showed that the presence of fat and the temperature may modify the dynamic of release of each component, affecting its synergetic effect against of *L. monocytogenes* and *S.* Enteritidis in whole milk. However, the encapsulation into liposomes did not restrain the antilisterial activity and the addition of pectin increased retention of the lysozyme-nisin in whole and skim milk stored in temperature of refrigeration and at 37 °C, evidencing potential for food application. In addition, factors as the highly negative zeta potential of nisin-lysozyme loaded liposomes that may influence its interaction with bacteria, due to the electrostatic repulsion between negatively charged liposomes and the cell surface (negative charge) may reduce the direct contact between liposomes and bacteria, and the release of antimicrobials (Were et al., 2004).

Thus, the use of nanoliposomes coated with polysaccharides as carriers of natural antimicrobials could be beneficial to samples of raw, pasteurized, or even extended shelf life milk that would be used for further processing, such as cheese manufacturing, considering that

Listeria spp could be introduced in milk or dairy products by post-pasteurization contamination (Pinilla & Brandelli, 2016)

4. Conclusion

The liposomes developed in this work showed low polydispersity index, and high entrapment efficiency for lysozyme and nisin. In addition, the synergy between lysozyme and nisin ensured a more efficient inhibition of *Listeria* spp, and the addition of pectin in liposomes increased retention of the lysozyme-nisin in milk stored at 7 and 37 °C. The results indicate that liposomes containing polysaccharides can be a promising technology for the controlled release of lysozyme and nisin, allowing maintenance of antimicrobial activity.

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4 DISCUSSÃO GERAL

Lipossomas revestidos com pectina ou ácido poligalacturônico foram preparados para a encapsulação de nisina. Para o desenvolvimento dos lipossomas, três concentrações diferentes dos polissacarídeos foram inicialmente testadas (0,5, 1,0 e 1,5 mg/mL). Os lipossomas preparados apresentaram resultados semelhantes com relação ao diâmetro médio e potencial zeta nas três concentrações testadas. No entanto, o PDI das amostras nas concentrações de 0,5 e 1,5 mg/mL foi acima de 0,4, indicando alto índice de polidispersão nessas amostras. Na concentração de 1,0 mg/mL os lipossomas revestidos com polissacraídeos mostraram valores de PDI em torno de 0,2. Segundo Malheiros et al. (2011) em sistemas preparados a partir de materiais biológicos, como os lipossomas, os valores de PDI em torno de 0,2 são considerados adequados, indicando estreita distribuição de tamanho das partículas. Assim, a concentração de 1,0 mg/mL de polissacarídeo foi selecionada como a melhor condição para o desenvolvimento de lipossomas com nisina revestidos com pectina ou ácido poligalacturônico.

Os lipossomas preparados tiveram alta eficiência de encapsulação em todas as formulações testadas, mostrando valores acima de 80%. Nos lipossomas revestidos, a eficiência de encapsulação depende do grau de esterificação da pectina, mostrando-se maior para pectina de alta metoxilação e ácido péctico a pH 7,0, sugerindo que não só as interações eletrostáticas, mas também interações hidrofóbicas contribuem para a formação do complexo em valores de pH neutro (Krivorotova et al., 2016).

As características físico-químicas dos lipossomas contendo pectina ou ácido poligalacturônico foram avaliadas por 28 dias, onde os lipossomas foram mantidos em refrigeração ou temperatura ambiente. Ao longo do tempo, as formulações avaliadas apresentaram diferentes tamanhos de partículas, mas um diâmetro médio dentro da escala nanométrica, variando de 90 a 160 nm. Os lipossomas PC-ácido poligalacturônico diminuíram o diâmetro médio nos primeiros 7 dias, permanecendo estável ao longo de 28 dias. Em geral, os valores para o PDI foram em torno de 0,2 e 0,3 indicando que os lipossomas foram fisicamente estáveis durante o armazenamento. A incorporação de polissacarídeos parece ter formado um revestimento externo nos lipossomas, mostrando carga líquida negativa, mantendo-se ao longo do tempo, tanto em 7 °C quanto 25 °C. Esses resultados indicam que os polissacarídeos de carga negativa podem ser revestimentos interessantes para lipossomas encapsulados com nisina, aumentando sua estabilidade durante o armazenamento à temperatura ambiente, provavelmente devido a maior repulsão eletrostática entre as nanopartículas.

Além disso, foi realizada a avaliação da atividade antimicrobiana nos lipossomas armazenados a 7 e 25 °C por até 14 dias. Foi observado que os lipossomas perderam a atividade antimicrobiana após 14 dias, porém, essa redução foi menor para aqueles armazenados a 7 °C. Os lipossomas revestidos com ácido poligalacturônico mantiveram maior atividade antimicrobiana quando comparados com os lipossomas revestidos com pectina. A perda de atividade antimicrobiana pode ser associada ao estabelecimento de fortes interações entre a nisina (catiônica) e os polissacarídeos carregados negativamente, durante o armazenamento (Krivorotova et al., 2016). Assim, maiores concentrações de nisina poderiam ser encapsuladas para garantir que a atividade antimicrobiana seja prolongada.

O ensaio com membranas de diálise mostrou que os lipossomas revestidos com polissacarídeos apresentaram menor taxa de liberação da nisina, mantendo a liberação controlada por até 30 h, em comparação com os lipossoas sem revestimento. A maior taxa de liberação da nisina nos lipossomas sem revestimento pode ser atribuído à nisina localizada na borda externa das bicamadas de fosfolipídios (Silva et al. 2014). Esses resultados revelam uma diferença importante no padrão de liberação de nisina para as formulações contendo polissacarídeos, uma vez que a interação da nisina com os polissacarídeos com carga negativa provavelmente garante uma taxa de liberação mais lenta.

Na sequência, a caracterização estrutural dos lipossomas foi realizada, mostrando que a relação (1:2) entre os picos de Bragg no SAXS confirmou a presença de estruturas lamelares em todas as amostras de lipossomas analisados. Além disso, a presença de fases cúbicas também foi observada nos lipossomas contendo nisina, produzidos pelos métodos de filme lipídico e evaporação em fase reversa. Diferentes fatores podem ter contribuído para a formação das fases cúbicas (Koynova e Tenchov, 2013), entre eles, a utilização de água em excesso durante a produção dos lipossomas, combinada com o uso de fosfolipídios negativamente carregados com polissacarídeos, através do aumento da repulsão eletrostática entre as bicamadas lipídicas e redução da energia necessária para dissipação da fase lamelar antes da sua conversão em fase cúbica. Embora a fosfatidilcolina não influencie a formação de fases cúbicas invertidas (Koynova e Tenchov, 2013), a nisina parece induzir a formação (Jastimi e La£eur, 1999; Rzeszutek e Willumeit, 2010), através da perturbação da estrutura das membranas formadas por fosfatidilcolina, enquanto que as amostras sem adição de nisina não apresentaram a formação de fases cúbicas.

Desta forma, provavelmente entre os empilhados de bicamadas a presença de nisina pode ter levado a estruturas contendo fases cúbicas dentro delas, ou seja, parte da estrutura multilamelar tornou-se cúbica. A formação de fase cúbica tem atraído muita atenção, devido

suas estruturas internas e propriedades físico químicas altamente ordenadas, que podem garantir a liberação lenta de compostos bioativos (Chen, Ma e Gui, 2014; Guo et al., 2010). Assim, a descoberta de estruturas cúbicas corrobora os resultados obtidos na primeira parte do trabalho, onde os lipossomas produzidos promoveram a liberação controlada de nisina.

Na última parte do trabalho, lisozima e nisina foram encapsuladas em lipossomas contendo polissacarídeos. Inicialmente, a lisozima livre foi avaliada, apresentando atividade contra *L. monocytogenes* e *S.* Enteritidis. Com isso, foram preparados lipossomas encapsulados com lisozima e revestidos com pectina ou ácido poligalacturônico, visando proteger o composto e prolongar a atividade antimicrobiana frente a bactérias Gram-positivas e Gram-negativas. Os lipossomas produzidos apresentaram boas características físico químicas, porém, não mostrou atividade contra bactérias Gram negativas, ou seja, a lisozima quando encapsulada perdeu sua atividade contra a *S.* Enteritidis.

Combinações de nisina e lisozima têm sido experimentadas, reduzindo e controlando bactérias Gram-positivas e Gram-negativas (Gill & Holley, 2000; Gill & Holley, 2000b). A lisozima pode facilitar o acesso da nisina à membrana celular, diminuindo o número de células viáveis, mas o mecanismo real de sinergia entre esses compostos não é claramente entendido. Assim, visando ampliar o espectro de ação, a mistura de nisina e lisozima foi testada, e os lipossomas produzidos com essa mistura foram avaliados quanto a caracterização físico química e microbiológica.

Os lipossomas contendo a mistura de nisina-lisozima, revestidos com polissacarídeos, apresentaram menor tamanho de partícula quando comparados ao lipossoma sem revestimento. Esse parâmetro depende da natureza dos polímeros e das características da nisina e da lisozima, bem como das prováveis interações entre eles. O índice de polidispersividade indicou uma distribuição de tamanho estreito para todas as amostras. Os valores de potencial zeta (em torno de -30 mV) se mostraram elevados, indicando aumento das interações repulsivas e redução da frequência de colisões nos lipossomas. Com relação a atividade antimicrobiana, os resultados demonstraram os benefícios de utilizar misturas de nisina e lisozima contra bactérias indesejáveis, quando comparado com o uso dos agentes individuais. A mistura encapsulada mostrou atividade contra *L. monocytogenes* e *S.* Enteritidis, além disso, a incorporação da pectina melhorou a estabilidade dos lipossomas, uma vez que mantiveram uma maior atividade contra *S.* Enteritidis, quando comparada com as demais formulações testadas. O mecanismo de sinergismo entre nisina e lisozima não está totalmente esclarecido, no entanto, os lipossomas produzidos com a combinação de nisina-lisozima aumentaram a lise celular nas bactérias Gram-negativas, podendo prevenir a contaminação nos alimentos.

Quando avaliados em um sistema complexo, como o leite integral e desnatado, armazenado a 7 °C e 37 °C, a formulação contendo pectina como revestimento foi mais eficiente na inibição de *Listeria* spp. No leite desnatado o efeito antimicrobiano foi maior, quando comparado ao leite integral. O efeito negativo da gordura sobre a atividade antimicrobiana da nisina é amplamente relatado (Chollet et al., 2008; Sobrino-López e Martín-Belloso, 2008; Pinilla & Brandelli, 2016). Assim, a ação mais eficiente da nisina no leite desnatado era de certa forma esperada, devido à adsorção de nisina nos glóbulos de gordura. Os resultados mostraram que a presença de gordura e a temperatura podem modificar a dinâmica de liberação de cada componente, afetando o efeito sinérgico contra *L. monocytogenes* e *S.* Enteritidis no leite integral. No entanto, a encapsulação da mistura não restringiu a atividade antilisterial e a adição de pectina aumentou a retenção da lisozima-nisina no leite integral e desnatado, armazenado em temperatura de refrigeração e a 37 ° C, evidenciando seu potencial para aplicação em alimentos.

De uma forma geral, os lipossomas produzidos nesse estudo apresentaram boas características físico químicas e, quando adicionados de pectina ou ácido poligalacturônico, promoveram a liberação controlada de agentes antimicrobianos, mostrando-se estáveis quando armazado em diferentes temperaturas. Desta forma, a adição de polímeros em lipossomas pode ser uma alternativa promissora, com potencial aplicação em alimentos.

5 CONCLUSÃO GERAL

Com a realização deste trabalho, foi possível concluir que lipossomas revestidos com pectina ou ácido poligalacturônico podem ser uma alternativa interessante para a liberação controlada de agentes antimicrobianos, tais como nisina e lisozima.

Os lipossomas contendo nisina apresentaram alta eficiência de encapsulação, baixo índice de polidispersão e foram estáveis durante 21 dias, na temperatura de 7 e 25 °C, evidenciando sua aplicabilidade tecnológica.

A caracterização aprofundada das estruturas lipídicas dos lipossomas mostrou que, de uma forma geral, a estrutura lamelar se encontra em maior quantidade, porém, também é encontrada a presença de estrutura cúbica nos lipossomas produzidos. É provável que entre os empilhados de bicamadas lipídicas, a presença de nisina tenha levado a formação de estruturas cúbicas, com isso, parte da estrutura lamelar tornou-se cúbica. Esses resultados corroboram com os anteriores, pois a fase cúbica apresenta estruturas internas definidas, que favorecem a liberação controlada de compostos bioativos.

A mistura de nisina e lisozima demonstrou benefícios, quando comparado com o uso dos agentes individuais. Os lipossomas desenvolvidos apresentaram baixo índice de polidispersão e alta eficiência de encapsulação. O sinergismo entre lisozima e nisina garantiu a inibição mais eficiente de *Listeria* spp, e a adição de pectina nos lipossomas aumentou a retenção da mistura, quando adicionados em leite armazenado em diferentes temperaturas. Assim, os resultados demonstram que os lipossomas contendo polissacarídeos podem ser uma tecnologia promissora para a liberação controlada de lisozima e nisina em alimentos.

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7 OUTRAS PUBLICAÇÕES

- **Artigo de revisão publicado:**

LOPES, N. A.; BRANDELLI, A. Nanostructures for delivery of natural antimicrobials in food. **Critical Reviews in Food Science and Nutrition**. 10, 1–11, 2017. doi: 10.1080/10408398.2017.1308915. 2017

Abstract

Natural antimicrobial compounds are a topic of utmost interest in food science due to the increased demand for safe and high-quality foods with minimal processing. The use of nanostructures is an interesting alternative to protect and delivery antimicrobials in food, also providing controlled release of natural compounds such as bacteriocins and antimicrobial proteins, and also for delivery of plant derived antimicrobials. A diversity of nanostructures are capable of trapping natural antimicrobials maintaining the stability of substances that are frequently sensitive to food processing and storage conditions. This article provides an overview on natural antimicrobials incorporated in nanostructures, showing an effective antimicrobial activity on a diversity of food spoilage and pathogenic microorganisms.

Keywords: Bacteriocin; nanobiotechnology; nanoparticles; nisin; phytochemicals

- **Capítulos de livros publicados:**

BRANDELLI, A.; LOPES, N.A.; BOELTER, J.F. Food applications of nanostructured antimicrobials. **In: Nanotechnology in the Agri-Food Industry**, Vol. 6, Food Preservation, A.M. Grumezescu (Ed.). Elsevier: Londres, 35–74, 2017.

Abstract

The demand by consumers for more natural foods with an extended shelf life and without the addition of chemical preservatives is increasing, challenging industry and research institutions to develop new conservation technologies. To meet this demand, bioconservation emerges as an interesting alternative, in which natural antimicrobial compounds are used in food in order to control the growth of pathogenic and spoilage microorganisms. The application of these

antimicrobial compounds in food can be improved using nanotechnology, which becomes increasingly important for the food industry. Nanostructured antimicrobials can have diverse applications in food formulations and packaging, and promising results are found with the use of the nanostructures that are addressed and discussed throughout the chapter. Nanovesicles, nanospheres, metallic nanoparticles, nanofibers, and nanotubes are capable of trapping bioactive substances, increasing and maintaining the stability of compounds that are often sensitive under typical food processing and storage conditions. The effective utilization of nanostructured antimicrobials in real food systems and toxicological studies are topics that still need additional investigation. Strategies for studies on the potential toxicity of antimicrobial nanostructures are discussed.

Keywords: antimicrobial; controlled release; drug delivery; liposome; nanoparticle; nanotoxicology

BRANDELLI, A.; Pinilla, C. M. B.; LOPES, N. A. Nanoliposomes as a platform for delivery of antimicrobials. **In: Mahendra Rai**; Carolina Alves dos Santos. (Org.). Nanotechnologies Applied to Pharmaceutical Technology. 1ed.Cham: Springer International Publishing, 2017, v., p. 55-90.

Abstract

The recent expansion of pharmaceutical nanotechnology (nanomedicines) and targeting strategies for antimicrobial delivery are highlighted and utilized in the applied fields of biomedicine, cosmetology, pharmaceutical, and food technologies. Nanoliposomes are artificially prepared membranous vesicles often composed of natural phospholipids and cholesterol. Their structure is similar to the cell membrane, and they can trap and release compounds with different properties, including both hydrophobic and hydrophilic drugs. Nanoliposomes can be used to achieve a slow release of drugs; the reduced particle size enhances the surface area, improving bioavailability and solubility, and thereby reducing its toxicity. As pharmaceutical nanocarriers, liposomes have been extensively studied for topical/dermal use against fungal diseases and as alternative therapy for diseases such as tuberculosis, due to the increase of antimicrobial efficacy, improving the interactions between pathogen and encapsulated drugs. Conventional liposomes have some limitations, such as low stability for long periods of storage and rapid uptake. Actually, a new generation of liposomes is developed with modifications in their surface, optimized size, high loading efficiency, ease

of interaction with the cell membrane, and increased target specificity, gaining novel applications and increasing attention in the medicine area.

Keywords: liposome; antibiotic; drug delivery; controlled release

- **Capítulo de livro submetido para publicação:**

BRANDELLI, A.; Pinilla, C. M. B.; LOPES, N. A. Nanotechnology in food preservation. **In: Advances in Processing Technologies for Bio-Based Nanosystems in Food.** CRC Press, 2018.

- **Participação em trabalho de conclusão de curso com artigo submetido para publicação:**

BERTOLINI, D.; CORRÊA, A. P. F.; VERAS, F. F.; LOPES, N. A.; GREGORY, G.; BRANDELLI, A. **Characterization of nanovesicles containing bioactive peptides obtained from sheep whey.** Trabalho de conclusão do curso de Biomedicina - Universidade Federal do Rio Grande do Sul.

Abstract

Whey is a by-product formed in the milk casein production. It corresponds to the remaining aqueous fraction of milk, containing about half of its soluble components. Despite having excellent nutritional qualities, its production may have a big environmental impact. The production of bioactive peptides from proteins is a viable alternative to solve this problem, turning the residue into an applicable food bioconservative. This application, however, is only possible with microencapsulation methods which protect the peptides from degradation. In this work, bioactive peptides from sheep whey were encapsulated in liposomes, which had their stability characterized and their ability to maintain the biological activities evaluated. Though slightly unstable, liposomes have shown constant parameters over a month, in addition to preventing accentuated loss of biological activities of the peptides.

Keywords: bioactive peptides; microencapsulation; liposomes; whey

- **Trabalhos apresentados em congressos:**

LOPES, N. A.; PINILLA, C. M. B.; BRANDELLI, A. **Encapsulação de lisozima em nanolipossomas contendo polímeros: caracterização e avaliação antimicrobiana.** In: II CINA – II Congresso Internacional de Nanotecnologia e V Simpósio de Nanobiotecnologia e suas Aplicações, 2017.

ISAIA, H. A.; PINILLA, C. M. B.; LOPES, N. A.; BRANDELLI, A. **Desenvolvimento e caracterização de nanocápsulas contendo o peptídeo antimicrobiano P34.** In: II CINA – II Congresso Internacional de Nanotecnologia e V Simpósio de Nanobiotecnologia e suas Aplicações, 2017.

BERTOLINI, D.; CORRÊA, A. P. F.; VERAS, F. F.; LOPES, N. A.; GREGORY, G.; BRANDELLI, A. **Caracterização de nanovesículas contendo peptídeos bioativos obtidos do soro de leite ovino.** In: II CINA – II Congresso Internacional de Nanotecnologia e V Simpósio de Nanobiotecnologia e suas Aplicações, 2017.

BARRETO, C.; LOPES, N.; BRANDELLI1, A. **Melhoramento da estabilidade térmica de nanolipossomas contendo a mistura de nisina e extrato de alho.** In: IX Congresso Brasileiro de Termodinâmica Aplicada e V Escola de Termodinâmica, 2017.

LOPES, N. A.; PINILLA, C. M. B.; BRANDELLI, A. **Caracterización de nanoliposomas con incorporación de ácido galacturónico para liberación controlada de péptidos antimicrobianos.** In: XXIII Congreso Latinoamericano de Microbiologia, 2016, Rosario. Libro de Resumenes del XXIII CLAM, 2016. v. 1.