RAFAEL HENRIQUE DE FREITAS ZOMPERO

Desenvolvimento e otimização do método de injeção de etanol para produção de lipossomas contendo β-caroteno visando sua aplicação na indústria de alimentos

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Desenvolvimento e otimização do método de injeção de etanol para produção de lipossomas contendo β-caroteno visando sua aplicação na indústria de alimentos

Orientadora: Profª Drª Lucimara Gaziola de la Torre

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Este exemplar correponde à versão final da dissertação de mestrado defendida pelo aluno Rafael Henrique de Freitas Zompero e orientado pela Profa. Dra. Lucimara Gaziola de la Torre.

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RESUMO

Este trabalho teve como objetivo principal o desenvolvimento e otimização de um processo escalonável de produção de lipossomas contendo β-caroteno, visando sua posterior aplicação em produtos de interesse da indústria alimentícia. Para tanto, os efeitos das variáveis que influenciam o processo foram analisados, proporcionando um maior conhecimento a respeito da fenomenologia envolvida na produção dos nanoagregados e maior controle sobre as respostas produzidas pelo sistema. O β-caroteno é um antioxidante natural, pró-vitamínico e que pode ser empregado como corante natural em formulações alimentícias. Porém sua elevada hidrofobicidade dificulta a aplicação em alimentos de base aquosa. Dentre as metodologias disponíveis para produção de lipossomas, o método de injeção de etanol apresenta-se como o mais facilmente adaptável as necessidades da indústria, possuindo baixo custo de implantação e operação. Na primeira etapa deste trabalho o método de injeção de etanol foi investigado visando a otimização dos parâmetros operacionais para a produção de nanoagregados tendo como objetivo obter propriedades físico químicas tais como diâmetro médio e polidispersidade de forma. Análises estatísticas dos resultados foram realizadas para determinação dos efeitos de cada variável e modelos foram desenvolvidos para predição do comportamento do sistema em diferentes condições de processo. Em seguida, a incorporação de β-caroteno nos lipossomas obtidos na melhor condição foi avaliada em razões β-caroteno/lipídio pré-definidas. Ensaios de incorporação de β-caroteno aos lipossomas revelaram que proporções molares β-caroteno/lipídio de até 0,5% mostram-se estáveis e solúveis em meio aquoso, resultado confirmado pelos ensaios de monocamadas de Langmuir realizados. A formulação otimizada foi subemtida a testes de estabilidade em condições controladas de stress e, em um segundo momento, incorporada a nanofibras de álcool polivinílico e óxido de polietileno através de processo de electrospinning, conferindo proteção extra ao β-caroteno internalizado. Estas nanofibras produzidas foram caracterizadas quanto a morfologia, diâmetro, presença de fosfolipídios, homogeneidade da distribuição de β-caroteno e estabilidade a exposição a luz ultravioleta. Testes de rehidratação destas nanofibras foram conduzidos, verificando através de microscopia eletrônica de transmissão a liberação de lipossomas na fase aquosa. Dessa forma, a partir dos resultados obtidos conclui-se que o método de injeção de etanol foi otimizado, sendo que os efeitos de cada uma das variáveis de processo foram elucidados, contribuindo para o desenvolvimento tecnológico da técnica. Os ensaios de aplicação de condições de stress mostram que existe uma barreira a ser vencida no que diz respeito a estabilidade de lipossomas em formulações alimentícias complexas, principalmente para situações de elevada concentração de sacarose e altos teores de NaCl. Por fim, os testes de incorporação em nanofibras mostraram-se bastante promissores, mostrando a viabilidade e benefícios que podem ser agregados ao sistema através da utilização de técnica de eletrofiação, contribuindo no desenvolvimento de novos materiais e produtos a serem utilizados pelo setor industrial alimentício.

Palavras-chave: β-caroteno, lipossomas, electrospinning, nanotecnologia, escalonamento de processo.

ABSTRACT

The main goal of the present work was the development and optimization of a scalable process for production of β-carotene loaded liposomes, aiming its application in the food industry. In that way, the effects of the variables that have influence on the process were analysed, providing greater information about the phenomenology evolving the production of these nano aggregates and improved control over the system responses. β-carotene is a natural antioxidant, pro-vitaminic, and can be employed as a natural colorant in food formulations. However, its high hydrophobicity makes it difficult to be applied in water based foods. The development of feasible processes that can be implemented in the food industry for β-carotene encapsulation is a actual research challenge. On the first step of this work the ethanol injection method was investigated aiming process parameters optimization for liposomes production with controlled size and polidispersity. Statistical analyses of the results were performed for variables effects determination and mathematical models development for system behaviour prediction. Then, β-carotene incorporation on liposomes produced at the optimized condition was evaluated studying different β-carotene/lipid ratios. β-carotene incorporation experiments revealed that 0.5% β-carotene/lipid ratios show to be stable and soluble in aqueous media, result confirmed by Langmuir monolayer experiments. The optimized formulation was submitted to stability tests under controlled stress conditions and, in a second stage, incorporated to polyvinyl alcohol and polyethylene oxide nanofibers using electrospinning technique for extra β-carotene protection. The produced nanofibers were characterized regarding morphology, diameter, phospholipids presence, β-carotene homogeneity and stability to UV light exposure. Nanofibers rehydration tests were conducted, verifying using transmission electron microscopy that liposomes were released in the aqueous media. In that way, from the obtained results we can conclude that the ethanol injection method was successfully optimized and the evaluation of the effects of each variable was elucidated, contributing to the technological development of the technique. Experiments regarding application of different stress conditions to liposomes formulations show that there is a barrier that must be overcome related to stability of liposomes to complex food formulations. Finally, incorporation tests on nanofibers showed to be promising, demonstrating the feasibility and benefits that can be aggregated to the system by using electrospinning, contributing to the development of new materials and products to be used by the food processing industries.

Keywords: β-carotene, liposomes, electrospinning, nanotechnology, process scale-up.

Sumário

LISTA DE FIGURAS

Capítulo 2

Capítulo 3

Capítulo 4

LISTA DE TABELAS

Capítulo 3

Capítulo 4

SIGLAS E ABREVIATURAS

α –	Nível de significância
ANOVA –	Analysis of variance
C –	Concentração lipídica
CCRD -	Central Composite Rotatable Design
D –	Diâmetro do impelidor
DCCR –	Delineamento composto central rotacional
DLS –	Dynamic Light Scattering
FTIR –	Fourier Transform Infrared Spectroscopy
GRAS –	Generally Recognized as Safe
k –	Número de fatores estudados no planejamento experimental
TEM –	Transmission Electron Microscopy
R –	Velocidade de rotação do impelidor em rpm
t –	Tempo de processo
T -	Temperatura
Tm –	Temperatura de transição de fase do lipídio
S –	Velocidade de rotação do impelidor em rpm

Capítulo 1 – Introdução Geral

1. Introdução

A vitamina A é um composto essencial a diversas funções fisiológicas dos seres humanos, atuando na expressão gênica, função imune, manutenção da visão, dentre outras inúmeras funções. Esta vitamina pode ser obtida, particularmente, de fontes animais. Porém, outros compostos, denominados carotenoides, possuem atividade pró-vitamínica A e podem ser encontrados em óleos, frutas e verduras. Dentre os diversos tipos de carotenoides existentes, o que é mais eficientemente convertido em vitamina A no organismo é o β-caroteno, molécula altamente hidrofóbica com propriedades corantes e antioxidantes. β-caroteno tem sido reportado na literatura como um composto relacionado à prevenção de doenças como câncer e doenças cardiovasculares, o que tem feito com que o interesse em estudos a respeito deste composto aumentasse significativamente nos últimos anos. Devido a sua elevada hidrofobicidade, sua aplicação em formulações aquosas é dificultada, sendo necessária etapa prévia estabilização. Técnicas de encapsulação de componentes, advindas da micro/nanotecnologia, podem ser utilizadas na tentativa de estabilização prévia e solubilização de β-caroteno em água, facilitando sua aplicação. Além da melhora na solubilidade do composto, técnicas de encapsulação podem conferir proteção da molécula contra a presença de oxigênio, exposição à luz, temperaturas elevadas, além de mascarar possíveis odores ou sabores. Dentre os diversos tipos de estruturas utilizadas para encapsulação de moléculas, os lipossomas, vesículas autoagregadas em bicamadas lipídicas, possuem papel de destaque. Estes são versáteis, além de serem formados por fosfolipídios, compostos geralmente reconhecidos como seguros para aplicação em alimentos (GRAS).

Diversos são os métodos para produção de lipossomas. Um método que pode ser considerado superior aos demais por ser facilmente escalonável e de simples implementação industrial é o método clássico de injeção de etanol. Este método satisfaz muito bem as necessidades do setor industrial alimentício, proporcionando

elevadas taxas de produção com baixo custo de operação, não encarecendo consideravelmente o produto final. Muitos estudos vêm sendo realizados nos últimos anos sobre a fenomenologia e variáveis envolvidas no processo de produção de lipossomas por esta técnica. A partir dos estudos já realizados, pode-se perceber que, além de certas conclusões controversas dos autores, a falta de um estudo completo que relacione todas as variáveis é evidente. A utilização de planejamentos experimentais nesta situação podem ser de grande interesse, listando, além dos efeitos das variáveis isoladas, efeitos sinérgicos entre essas, podendo conduzir a respostas ainda mais otimizadas.

Outra técnica de encapsulação que voltou a estar em voga nos últimos anos são os processos de eletrofiação para produção de nanofibras em escala submicrométrica. Tais processos têm sido muito utilizados no setor alimentício para o desenvolvimento de materiais com novas funções, principalmente no que diz respeito ao desenvolvimento de embalagens funcionais. Materiais com funções inéditas têm sido descobertos graças à exploração da interdisciplinaridade referente a técnicas de nanoprodução e nanoencapsulação. Neste caso, a incorporação de lipossomas contendo β-caroteno em nanofibras pode contribuir para o desenvolvimento de novos materiais a serem utilizados pelo setor industrial, em especial o setor alimentício.

Tendo em vista o exposto acima, o presente trabalho contribui na determinação dos efeitos das variáveis de processo na produção de lipossomas contendo β-caroteno pelo método de injeção de etanol, auxiliando na estabilização e solubilização do carotenoide em soluções aquosas. Este trabalho contribui também na elucidação da fenomenologia envolvida na formação destes nanoagregados, além de proporcionar um maior conhecimento sobre a manipulação das variáveis de forma a se obter respostas desejadas. Por fim, as soluções de lipossomas contendo β-caroteno foram avaliadas quanto sua estabilidade sob diferentes condições de stress e em processos de eletrofiação, auxiliando no desenvolvimento tecnológico de novos materiais e produtos.

2. Objetivos

Sob o ponto de vista científico, tecnológico e de inovação, este projeto de pesquisa contribui no campo da nanotecnologia, mais especificamente para o desenvolvimento de novos produtos e processos na indústria alimentícia. Para isso, o objetivo principal deste trabalho foi o desenvolvimento e otimização do método de injeção de etanol para produção de lipossomas contendo β-caroteno, visando sua estabilização e dispersão em soluções aquosas, facilitando sua aplicação em produtos de interesse pela indústria de alimentos. O presente trabalho foi dividido em três partes, cada uma possuindo objetivos específicos para melhor estruturação da pesquisa, sendo eles:

Estudo das variáveis do processo de injeção de etanol

Objetivo específico I - Obtenção dos efeitos das variáveis do processo de injeção de etanol – (i) tipo de impelidor utilizado no sistema de agitação (Ultra-Turrax e Cowles), (ii) velocidade de rotação do impelidor, (iii) tempo de agitação da dispersão, (iv) concentração lipídica na fase alcoólica e (v) temperatura do reator – sobre as características de diâmetro médio, polidispersidade e potencial zeta da dispersão de lipossomas obtida, visando o estabelecimento da melhor condição de processo para incorporação posterior de β-caroteno.

Estudos de incorporação de β-caroteno aos lipossomas

Objetivo específico II - Obtenção da razão molar ideal β-caroteno/lipídio a ser utilizada nos ensaios de incorporação, nas condições otimizadas determinadas anteriormente, de forma que o β-caroteno seja disperso de maneira satisfatória em solução aquosa. Avaliação da estabilidade dos lipossomas quando aplicado em diversas condições de stress (temperatura, sacarose e NaCl) como estudo preliminar para futuras aplicações na indústria alimentícia.

Aplicação do β-caroteno encapsulado à matriz alimentar padrão e nanofibras de zeína

Objetivo específico III – Avaliação da estabilidade das soluções de β-caroteno encapsulado em condições controladas de stress e em processos de eletrofiação visando, neste último caso, o desenvolvimento de novos materiais para aplicação na indústria alimentícia.

3. Organização da Dissertação em Capítulos

A apresentação desta dissertação está organizada em capítulos conforme mostrado abaixo. Os resultados obtidos nesta pesquisa estão organizados em dois artigos contidos nos capítulos 3 e 4 desta dissertação, os quais serão submetidos a periódicos internacionais de afinidade com o tema abordado. Os itens de introdução, materiais, metodologia, resultados, discussões e conclusões para cada etapa da pesquisa encontram-se atrelados a seus respectivos artigos.

Capítulo 1 – Introdução Geral

Capítulo 2 – Revisão Bibliográfica

Contém o embasamento teórico e o estado da arte referente aos assuntos abordados posteriormente em cada um dos artigos.

Capítulo 3 – Investigation and optimization of ethanol injection method using β-carotene as hydrophobic model molecule

Contém os estudos referentes à avaliação de efeitos das variáveis do processo de injeção de etanol frente às características dos lipossomas produzidos pelo sistema (diâmetro hidrodinâmico, polidispersidade e potencial zeta). Neste estudo, técnicas estatísticas avançadas foram utilizadas visando à identificação de potenciais efeitos sinérgicos entre as variáveis de processo estudadas. Dentre todas as variáveis estudadas, a concentração lipídica foi estabelecida como a mais influente, afetando

significativamente as características da dispersão produzida. Neste capítulo também estão contidos estudos referentes à incorporação de β-caroteno nos lipossomas produzidos na condição estabelecida como ótima. Ensaios de monocamadas de Langmuir demonstraram que a razão molar limite de β-caroteno que pode ser incorporada nos lipossomas produzidos é de 0,5% β-caroteno/lipídio. Por fim, testes de estabilidade das dispersões de lipossomas frente à aplicação de condições controladas de stress demonstram que existe uma barreira a ser vencida no que se refere à aplicação destas nanopartículas em matrizes alimentares complexas.

Capítulo 4 – Incorporation of β-carotene loaded liposomes on Polyvinyl Alcohol and Polyethylene Oxide nanofibers produced by electrospinning

Contém estudos de caracterização das soluções poliméricas (álcool polivinílico e óxido de polietileno) contendo diferentes razões mássicas de lipossomas, relacionando como a presença destes nanoagregados influenciam as características reológicas e de tensão superficial das soluções, afetando diretamente o desempenho do processo de eletrofiação. Neste estudo foram realizados ensaios de caracterização das nanofibras quanto à morfologia, diâmetro, presença de fosfolipídios incorporados na matriz polimérica, homogeneidade da distribuição de β-caroteno e estabilidade do β-caroteno duplamente encapsulado (lipossoma + nanofibra) frente à exposição prolongada à luz ultravioleta. Por fim foram realizados ensaios de reidratação dos filmes poliméricos contendo fosfolipídios e verificou-se, através de microscopia eletrônica de transmissão de amostra da dispersão obtida, que lipossomas podem ser obtidos a partir desta prática.

Capítulo 5 – Conclusões finais

Sintetiza as conclusões obtidas a partir dos estudos realizados e resultados reportados nos diferentes artigos.

Capítulo 6 – Sugestões para trabalhos futuros

Capítulo 2 - Revisão Bibliográfica

2.1. Vitamina A e carotenoides

Vitamina A, também conhecida como retinol, é um composto essencial para os seres humanos. Esta vitamina é importante para manutenção de uma visão adequada, expressão gênica, reprodução, desenvolvimento embrionário, crescimento e função imune (INSTITUTE OF MEDICINE, 2001). Segundo Saari (1994), retinal, uma forma derivada da vitamina A, é requerida no globo ocular para a transdução da luz em sinais neurais necessários à visão. Ela é importante também na prevenção de doenças como xeroftalmia (SOMMER & WEST, 1996), na integridade das células epiteliais (GUDAS et. al., 1994), na regulação da produção de diversos genes que codificam proteínas, na formação de regiões cerebrais, membros, coração, olhos e ouvidos em fetos (DICKMAN & SMITH, 1996; McCAFFERY & DRAGGER, 1995), além de muitas outras funções já conhecidas presentes na literatura.

Vitamina A pode ser obtida, particularmente, em alimentos de origem animal. Porém, outras estruturas, denominadas carotenoides, possuem atividade pró-vitamínica A e podem ser encontrados em óleos, frutas e vegetais. Dentre os mais de 600 tipos de carotenoides existentes, o β-caroteno é o mais abundante e o que apresenta maior atividade pró-vitamínica, sendo o mais efetivamente convertido em vitamina A (retinol) no organismo (INSTITUTE OF MEDICINE, 2001). A representação esquemática da molécula de β-caroteno se encontra na Figura 2.1, mostrando seus dois anéis β-ionona nas extremidades e sua cadeia poliênica contendo nove ligações duplas conjugadas ligando ambos os anéis.

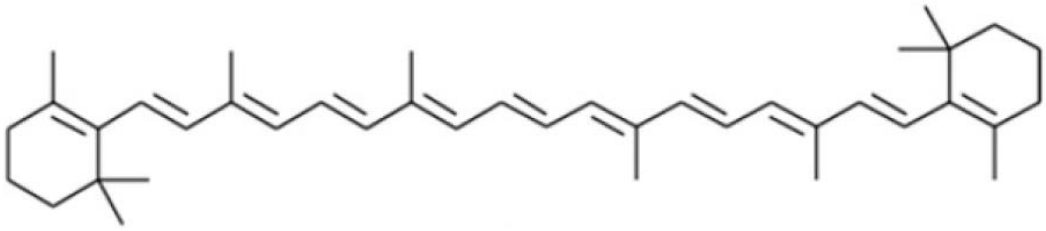

Figura 2. 1 - Representação esquemática da molécula de β-caroteno (MORABITO et. al., 2011).

O β-caroteno é uma molécula da classe dos terpenos, altamente hidrofóbica, com propriedades corantes e antioxidantes comprovadas (FERNANDEZ et al., 2009). Normalmente a coloração amarelada/alaranjada observada em frutas e vegetais é devida a presença deste e outros carotenoides. Desta forma o β-caroteno, além de conferir um aumento do valor nutricional ao alimento quando adicionado, confere adicionalmente coloração amarelada/alaranjada. Esta característica de coloração pode ser desejável ou não, dependendo da aplicação pretendida. Muitos estudos relacionados a coloração de alimentos tem sido realizados nos últimos anos. As fortes restrições impostas ao uso de corantes sintéticos têm motivado o setor industrial a optar pela substituição destes por corantes naturais. Os carotenoides, por exemplo, constituem uma das principais classes de corantes naturais, sendo o β-caroteno o mais estudado e o mais comumente encontrado em frutas e vegetais (PAIVA & RUSSELL, 1999).

A atividade antioxidante associada ao β-caroteno está relacionada a sua capacidade de estabilizar radicais livres e formas reativas de oxigênio chamadas oxigênio singlet (TEFLER et al., 1994). Sua cadeia poliênica está relacionada com a absorção da luz visível e elevada reatividade da molécula (NUNES & MERCADANTE, 2007). Devido a estas propriedades antioxidantes, este carotenoide tem sido bastante referenciado na literatura como possível atuante na prevenção de determinados tipos de câncer e doenças cardiovasculares (DULINSKA et al., 2005; VAINIO, 2000; D'ODORICO, 2000).

Apesar de todas estas características positivas a respeito do β-caroteno, sua elevada hidrofobicidade e sensibilidade à luz, altas temperaturas e oxigênio inviabilizam sua aplicação direta na forma de cristais, principalmente em alimentos de base aquosa. Quando oxidado, o β-caroteno pode gerar diversos subprodutos (BENEVIDES et al., 2011) e perder suas propriedades funcionais e corantes, sendo que estudos cinéticos de descoloração deste em vários sistemas já foram realizados (SAGUY et al., 1985; GOLDMAN et al., 1983; HARALAMPU & KAREL, 1983). O mecanismo de degradação do β-caroteno se mostra bastante complexo, sendo este revisado e descrito por Mordi

(1993). Conforme descrito pelo autor, os principais produtos da degradação desta molécula são diversos tipos de apocarotenos e epóxidos. Outro fato importante está relacionado à baixa biodisponibilidade do β-caroteno quando ingerido. Estudos mostram que a ingestão de compostos lipídicos junto a doses de β-caroteno aumenta a concentração plasmática de β-caroteno, aumentando consideravelmente sua biodisponibilidade (DIMITROV et al., 1988; ROCK et al., 1998; ZANUTTO et al., 2002). Desta forma, com o objetivo de proteger a molécula dos efeitos deletérios da presença de oxigênio, luz e altas temperaturas, além de permitir uma solubilização adequada desta em sistemas aquosos e aumentar a biodisponibilidade do ativo incorporado, técnicas de micro/nano encapsulação em diferentes sistemas são utilizadas.

2.2. Técnicas utilizadas para encapsulação de β-caroteno

A encapsulação é uma estratégia advinda da micro/nanotecnologia e pode ser definida como um processo de aprisionamento de uma substância em matrizes extremamente pequenas, onde o diâmetro pode variar desde alguns nanômetros a milímetros (ZUIDAM & NEDOVIC, 2010). O principal objetivo de processos de encapsulação na área alimentícia é a proteção de componentes contra umidade, oxidação, calor, luz ou condições extremas durante o processamento (SANTOS & MEIRELES, 2010). Sob o ponto de vista nutracêutico, estudos de encapsulação também focam a proteção do componente durante etapas do processo digestivo, permitindo a liberação no local apropriado de absorção (MATALANIS et al., 2011). A encapsulação é uma tecnologia que já vem sendo utilizada com êxito em diversos segmentos industriais como o de cosméticos, farmacêutico e o alimentício (FAVARO-TRINDADE et al., 2008; NEDOVIC et al., 2011; COLE et al., 2007; PARDEIKE et al., 2008). Como exemplos de aplicações no setor alimentício, se destacam a encapsulação de aromas (GIVEN Jr., 2008), óleos (KLAYPRADIT & HUANG, 2007) e corantes (POLYAKOV et al., 2004).

Diversas técnicas para a encapsulação de β-caroteno vêm sendo estudadas e utilizadas nos últimos anos. A escolha do método de encapsulação depende de uma

série de fatores como tamanho de partícula requerido, propriedades físicas e químicas da matriz que encapsulará o β-caroteno, aplicação do produto final, mecanismos desejados de liberação, escala de produção e custos (AZEREDO, 2005). Dentre as diversas técnicas utilizadas destaca-se a secagem por nebulização (*spray-drying*), a coacervação, a encapsulação em lipossomas e, mais recentemente, a estabilização em nano/microfibras através de técnicas de eletrofiação para desenvolvimento de materiais com novas propriedades (FERNANDEZ et al., 2009).

O *spray-drying* é a técnica que possui maior versatilidade operacional, permitindo seu uso tanto em escala laboratorial como em escala industrial. É um processo que se disseminou pela indústria em geral, sendo aplicado especialmente à indústria alimentícia e farmacêutica (ROSA et al., 2003). Loksuwan (2007) testou a utilização de amido de mandioca e maltodextrina como material de parede para a encapsulação de β-caroteno em spray-dryer. Este autor também cita diversos outros materiais que podem ser utilizados, como goma arábica, amidos hidrolisados, proteínas e gelatinas. Apesar da grande aplicação destes equipamentos, algumas desvantagens são inerentes ao processo como a dificuldade no controle de diâmetro das partículas produzidas, gerando um sistema muito polidisperso, e a degradação de ingredientes termosensíveis devido à alta temperatura do ar de secagem (SANTOS & MEIRELES, 2010).

Outro processo de encapsulação bastante utilizado é a coacervação, podendo esta ser simples ou complexa, dependendo do número de materiais de recobrimento empregados. Segundo Santos e colaboradores (2010), este foi o primeiro método de encapsulação estudado, sendo empregado por Green e Sceicher em 1955 para produção de papel de cópia sem carbono. Sua grande vantagem, frente ao processo de spray-drying, é a possibilidade de encapsulação de componentes termosensíveis. Apesar de muito eficiente, a coacervação é um processo caro e complexo, sendo que muitas vezes os coacervados são instáveis ou apresentam traços residuais de solventes (SANTOS & MEIRELES, 2010).

Matrizes como β-ciclodextrinas (TIANTIAN et al., 1998), manitol (SUTTER et al., 2007), poli(hidroxibutirato-co-hidroxivalerato) (PRIAMO et al., 2010), quitosana (DIVYA

et al., 2011) dentre outras, vem sendo utilizadas na tentativa de estabilização do β-caroteno. Tendo em vista as vantagens e desvantagens dos métodos mencionados, a inclusão de componentes em lipossomas mostra-se uma alternativa bastante promissora, sendo estes bastante versáteis quanto ao tamanho, fluidez, carga e número de lamelas, além de serem formados por fosfolipídios, materiais geralmente reconhecidos como seguros para aplicação em alimentos (GRAS), poderem incorporar tanto componentes hidrofílicos como hidrofóbicos e possuírem a capacidade de serem direcionados a locais específicos no organismo (MOZAFARI, 2005).

2.3. Lipossomas e suas aplicações no setor alimentício

Lipossomas são vesículas compostas por uma ou mais bicamadas lipídicas concêntricas. São constituídos predominantemente por fosfolipídios anfifílicos, ou seja, moléculas que são constituídas por um grupo hidrofílico (solúvel em água) e um grupo hidrofóbico (insolúvel em água) (Figura 2.2). Devido a essas propriedades e sua conformação estrutural específica, estes compostos anfifílicos se auto-agregam quando colocados em solução aquosa, expondo a parte hidrofílica e escondendo a parte hidrofóbica. Esta agregação em vesículas pode conter um ou vários núcleos aquosos, com lamelas concêntricas ou apresentar característica unilamelar, dependendo do método de produção (LASIC, 1993). Dependendo da composição e estrutura química dos anfifílicos, estas estruturas formadas podem alternar entre diversas formas de agregação, desde a formação de micelas, estruturas bicontínuas e lipossomas (MYERS, 1999). Fosfolipídios como a fosfatidilcolina são bem conhecidos como formadores de lipossomas devido a sua estrutura característica. A grande vantagem em se trabalhar com esse tipo de molécula em formulações alimentícias está associada ao seu valor nutricional adicional. A fosfatidilcolina, por exemplo, é um dos fosfolipídios mais utilizados para produção de lipossomas, apresentando diversas funções reguladoras no organismo, auxiliando no transporte de impulsos nervosos e no metabolismo de colesterol, além de conferir maior fluidez às membranas celulares (INSTITUTE OF MEDICINE, 1998). Uma das limitações está no fato de fosfolipídios

com cadeias carbônicas insaturadas estarem sujeitos à peroxidação, podendo afetar a permeabilidade da bicamada e a estabilidade dos lipossomas formados. Muitos autores utilizam formas hidrogenadas de fosfatidilcolina justamente para reduzir esta possibilidade de peroxidação (HUANG & CHUNG, 1998).

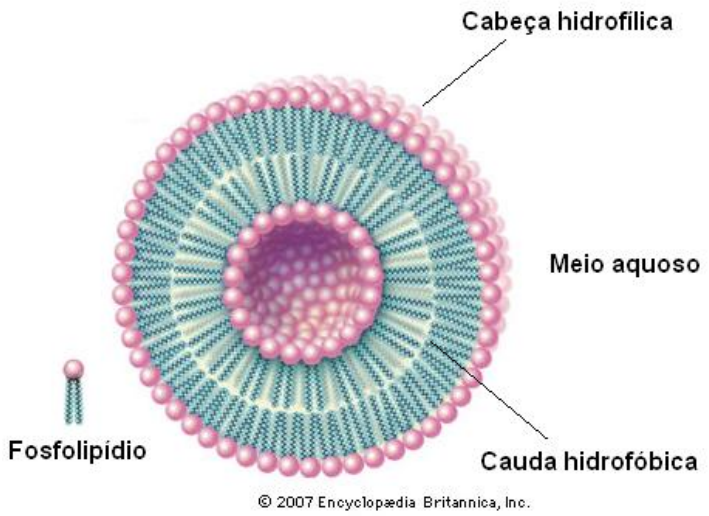

Figura 2. 2 - Esquema de um lipossoma e sua bicamada composta por moléculas anfifílicas (Adaptado de Encyclopedia Britannica, 2007)

Desta forma, os lipossomas são versáteis, pois podem acomodar em sua estrutura coloidal moléculas hidrofóbicas, hidrofílicas e anfifílicas. Quando encapsulados em lipossomas, compostos hidrofílicos se mantêm no cerne aquoso enquanto que compostos hidrofóbicos se acomodam na bicamada lipídica, como no caso do β-caroteno.

Lipossomas possuem diversas aplicações no setor alimentício (MOZAFARI et al., 2008). Aplicações de lipossomas em matrizes alimentares mais complexas já vêm sendo realizadas, como é o caso, por exemplo, do estudo conduzido por Maranasco et al. (2011) que visou aplicação dos lipossomas contendo vitamina C e E em suco de laranja, do estudo conduzido por Xia & Xu (2005) que incorporou lipossomas contendo sulfato ferroso em leite e de muitos outros estudos (FATHI et al., 2012; MOZAFARI et al., 2008; MOZAFARI et al., 2006).

2.4. Produção de lipossomas pelo método de injeção de etanol

Diversos são os métodos de obtenção de lipossomas. O método clássico e pioneiro, descrito por Bangham em 1965, consiste da hidratação de um filme seco de lipídios em um balão de fundo arredondado, sob agitação. Muitos outros métodos foram desenvolvidos a partir deste, porém poucos se apresentam factíveis de serem utilizados em escala industrial, sendo possíveis somente em escala laboratorial. As principais razões são a impossibilidade de aumento de escala, presença de resíduos de solventes orgânicos, esterilidade e reprodutibilidade (RIAZ, 1996). Um método que pode ser destacado dos demais por ser facilmente escalonável e de simples implementação industrial é o método clássico de injeção de etanol. Neste método, o lipídio deve estar solubilizado em etanol (fase orgânica), para em seguida ser injetado em um reator contendo água sob condições controladas de temperatura e agitação. O etanol pode ser substituído por outro solvente orgânico, desde que este seja solúvel em água. Durante a injeção da fase orgânica na aquosa, há a difusão do solvente orgânico na água, deixando o lipídio exposto ao meio aquoso, induzindo sua auto-organização. A auto-agregação em lipossomas ocorrerá quando a concentração lipídica estiver acima da concentração crítica para a formação dos lipossomas (LASIC, 1993). A principal desvantagem deste método é que o lipídio deve estar dissolvido em solvente orgânico, problema que muitas vezes é minimizado quando se utiliza etanol nas formulações, solvente de caráter não tóxico. Como os lipídios normalmente possuem baixa solubilidade em etanol (fosfatidilcolina natural de ovo possui solubilidade de 4 mM em etanol a temperatura ambiente, LASIC, 1993), a dispersão coloidal final contendo os lipossomas possuirá concentração muito baixa, inviabilizando muitas das aplicações industriais pretendidas. Na tentativa de contornar esta limitação imposta pela concentração, maiores proporções de lipídio na fase orgânica foram testadas, sendo esta agora denominada dispersão, pois o lipídio não se encontra mais solúvel como no caso do método clássico de injeção. Este novo método é conhecido na literatura como método modificado de injeção de etanol e vem sendo utilizado com sucesso na produção de lipossomas (JUSTO & MORAES, 2005; MAITANI et al., 2001).

A primeira menção na literatura relacionada ao método de injeção de etanol foi feita por Batzri & Korn (1973). Desde então diversos autores vem trabalhando no estudo e desenvolvimento da técnica, obtendo um conhecimento maior sobre os fenômenos envolvidos na produção dos lipossomas e como cada fator influencia nas características da dispersão final. Entretanto, o que se observa é que poucos dos trabalhos realizados neste campo de investigação, até o presente momento, aplicam técnicas estatísticas mais avançadas para determinação dos efeitos das variáveis sobre as respostas apresentadas pelo sistema. Nota-se também que as variáveis citadas em cada artigo como a mais influente no processo nem sempre é a mesma, mostrando que existe certa controvérsia sobre o assunto ou que, dependendo das condições escolhidas para estudo, o efeito de uma variável pode sobrepor o da outra, gerando influencias mais significativas.

Maitani et al. (2001) estudou a influência da quantidade de etanol sobre o diâmetro dos lipossomas obtidos. Seus resultados mostraram que o aumento da quantidade de etanol no meio aumenta consideravelmente o diâmetro dos lipossomas obtidos. Estes resultados estão de acordo com os obtidos anteriormente por Kremer et al. (1977). No entanto, Wiggenhorn (2007), estudando o mesmo efeito da quantidade de etanol na produção de lipossomas, concluiu que o tamanho das vesículas preparadas pelo método de injeção de etanol diminuiu com o aumento da quantidade de etanol na fase aquosa.

Justo & Moraes (2005) concluíram que o parâmetro de maior influência no diâmetro das vesículas formadas foi a velocidade de rotação do impelidor, sendo que um aumento desta resultou em um decréscimo expressivo do diâmetro observado. As demais variáveis estudadas (concentração lipídica e vazão de alimentação) se mostraram menos significativas, sendo que um aumento destas provocou um aumento no diâmetro observado das vesículas.

Szoka (1996), em sua patente publicada, revela também alguns efeitos da velocidade de rotação em seus resultados. De acordo com o publicado, a velocidade de rotação exerceu um grande efeito sobre a variável resposta, porém somente até o nível de 250 rpm, sendo que após este valor não foram constatadas variações de

diâmetro das vesículas. Outra informação obtida foi que a vazão de alimentação, nos níveis em que foi estudada (0,4 a 6 mL/minuto) não alterou a resposta final.

Kremer et al. (1977) avaliou a influência da concentração final de lipídios na formulação e a velocidade de injeção da fase orgânica. Seus resultados mostraram que a velocidade de adição não causou nenhuma alteração do diâmetro das vesículas formadas, porém o efeito da concentração foi bastante significativo. Os autores concluíram que menores razões lipídio/etanol proporcionaram a formação de vesículas de menor diâmetro.

Justo & Moraes (2011), notando a falta de dados na literatura sobre a influência da temperatura sobre a formação dos lipossomas pelo método de injeção, realizaram estudos em diferentes níveis desta variável, concluindo que maiores temperaturas produziram lipossomas com distribuição de tamanhos mais estreita, principalmente quando temperaturas acima da temperatura de transição de fase foram utilizadas. Desta forma, segundo os autores, é possível controlar a distribuição de tamanhos e polidispersidade dos lipossomas variando este parâmetro. Os mesmos autores, em trabalho publicado um ano antes (JUSTO & MORAES, 2009) avaliaram a viabilidade econômica de uma planta de produção de lipossomas pelo método de injeção de etanol, operando 300 dias por ano e produzindo duas bateladas por dia com 90% de eficiência, resultando diariamente em 288 L de suspensão de lipossomas. Todas as análises e estimativas de investimento de capital, custos de operação e lucratividade foram realizadas, mostrando a viabilidade de implantação do processo, com um tempo total de retorno do investimento estimado em 1,17 anos.

A partir dos estudos já realizados, pode-se perceber que, além de certas conclusões controversas, a falta de um estudo completo que relacione todas as variáveis é evidente. A utilização de planejamentos experimentais nesta situação podem ser de grande interesse, listando, além dos efeitos das variáveis isoladas, efeitos sinérgicos entre essas, podendo conduzir a respostas ainda mais otimizadas.

2.5. Incorporação de β-caroteno em nanofibras produzidas por eletrofiação

Recentemente, diferentes técnicas de estabilização de β-caroteno estão sendo utilizadas com novos objetivos e possíveis aplicações. Um sistema que desde a década de 90 vem sendo investigado para a encapsulação e liberação sustentada de moléculas funcionais são as nano/microfibras. A utilização de nanofibras na indústria alimentícia é uma área ainda pouco explorada para estabilizar e preservar a qualidade dos alimentos e constitui uma nova área para o desenvolvimento de novos produtos (FERNANDEZ et al., 2009).

Diversos métodos podem ser empregados para a produção de nanofibras, desde métodos de alta produtividade a métodos de alta precisão. Dentre eles, a eletrofiação, do inglês, *electronspinning*, tem a vantagem de ser um método relativamente barato e de elevada taxa de produção (RAMAKRISHNA et al., 2006).

No processo de eletrofiação, uma solução polimérica, sujeita a um campo elétrico, é mantida na extremidade de um tubo capilar. À medida que a intensidade do campo elétrico aumenta, o líquido presente na extremidade do tubo se alonga, adquirindo um formato cônico conhecido como cone de Taylor. Quando o campo elétrico atinge um valor que supera a tensão superficial da solução, um jato eletricamente carregado é expelido da extremidade do cone. No trajeto pelo ar, o solvente é evaporado, sendo o polímero seco aleatoriamente recolhido em uma placa de metal (Figura 2.3) (DOSHI & RENEKER, 1995; TAYLOR, 1969).

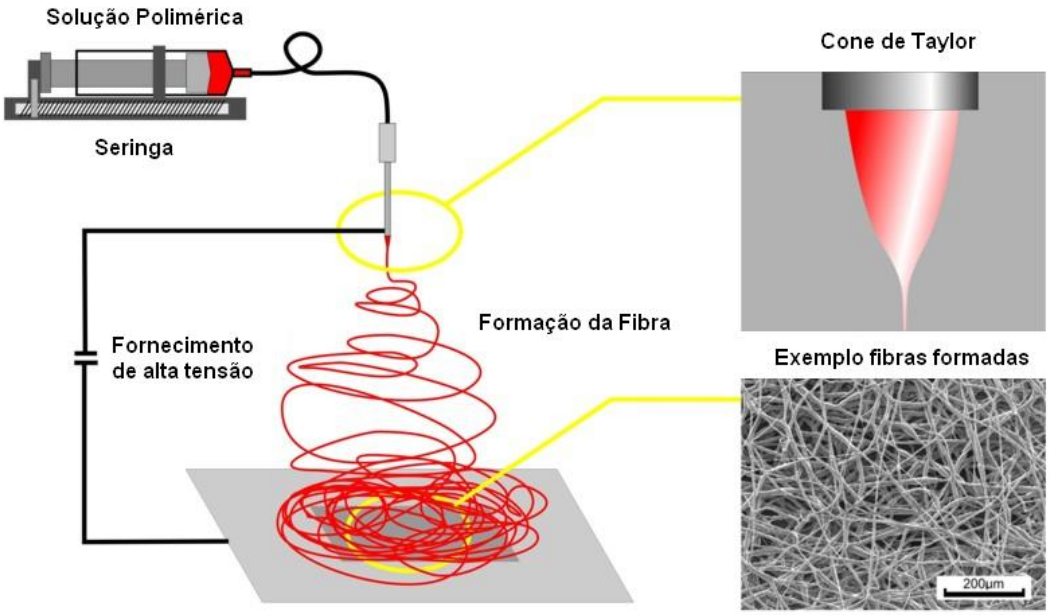

Figura 2. 3 - Esquema do aparato experimental utilizado para produção de nanofibras por eletrofiação. (Adaptado de http://www.centropede.com /UKSB2006/ ePoster/ background.html)

Nas últimas décadas, processos de eletrofiação têm voltado a estar em voga, não somente devido a sua versatilidade e possibilidade de utilização de diversos polímeros, mas também devido a sua habilidade em produzir fibras em escala sub-micrométrica (BHARDWAJ & KUNDU, 2010). Esta habilidade tem incentivado grupos de pesquisa a utilizarem a técnica no desenvolvimento de materiais com novas propriedades. No âmbito da indústria de alimentos, processos de eletrofiação têm contribuído no desenvolvimento de materiais voltados a aplicação em embalagens funcionais, principalmente em embalagens com propriedades de isolamento a componentes gasosos, embalagens comestíveis, embalagens com propriedades antimicrobianas e embalagens com propriedades antioxidantes (TORRES-GINER, et. al., 2008; TORRES-GINER & LAGARON, 2009; LÓPEZ-RUBIO, et. al., 2009; FERNANDEZ et. al., 2009).

A encapsulação de moléculas de β-caroteno em nanofibras de zeína, com o objetivo de produzir embalagens com propriedades antioxidantes, foi estudada por Fernandez et al. (2009). Os resultados apresentados, para produção de fibras na

ausência de β-caroteno, demonstram a formação de fibras com diâmetro médio de 870 nm, sendo que o diâmetro máximo e mínimo observados foram 2910 nm e 310 nm, respectivamente. Já no caso de fibras incorporando β-caroteno, um aumento do diâmetro foi observado, sendo que o diâmetro médio obtido foi 1140 nm e o diâmetro máximo e mínimo observados foram 3580 nm e 540 nm, respectivamente. Microscopias das fibras produzidas mostraram que o carotenoide foi efetivamente incorporado às nanofibras, porém não de forma homogênea ao longo de toda a fibra, formando *clusters* mais concentrados e regiões onde não havia a presença do carotenoide. Tendo em vista o ocorrido, espera-se que processos de estabilização prévia da molécula de β-caroteno possibilitem a dispersão desta de forma mais homogênea ao longo das fibras produzidas. Desta forma, o emprego de lipossomas ou da dispersão do carotenóide em lipídios em etapa anterior ao processo de eletrofiação pode ser uma alternativa promissora no desenvolvimento destas micro/nanoestruturas, apresentando uma solução razoável para o problema em questão (MICKOVA et al., 2012; YU et al., 2011).

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Capítulo 3 - Investigation and optimization of ethanol injection method using β-carotene as hydrophobic model molecule

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Abstract

The development of feasible processes that can be implemented in the food industry for β-carotene encapsulation is an actual research challenge. This work focus on the investigation and optimization of ethanol injection process for production of β-carotene loaded liposomes, since it can be easily adapt to the food industry. We investigated the influence of temperature, stirring speed, ethanol and HSPC concentrations. We produced liposomes with 70 nm, polidispersity of 0.18, -54 mV and ethanol at 10%v/v. Langmuir monolayer studies indicated 0.5% (molar ratio β-carotene/lecithin) as the maximum β-carotene incorporation. In this condition, β-carotene loaded liposomes presented similar physico-chemical properties as “empty” liposomes and we also submitted this formulation to different stress conditions, simulating the behavior in a complex food matrix. In that way, the present work contributes to a better understanding of process variables effects, establishes encapsulation limits and nanoaggregates stability facing different medium compositions.

Keywords: Liposomes, β-carotene, hydrogenated soy lecithin, nanotechnology, ethanol injection

1. Introduction

Over the past few years, the search for reliable production processes of nanoaggregates, with reproducible size and polidispersity, increased rapidly fueled by the rising number of potential applications of these structures in industries, such as pharmaceutical, food and cosmetic (Favaro-Trindade et al., 2008; Nedovic et al., 2011; Cole et al., 2007; Pardeike et al., 2008). In these industries, encapsulation of compounds is the main purpose for the utilization of these produced nanoaggregates. Encapsulation is a technique arising from nano/micro technology and can be defined as the entrapment of substances in tiny matrices, with diameters varying from millimeters to nanometers (Zuidam & Shimoni, 2010). Focusing on the food industry, one of the main purpose of encapsulation are solubilization of hydrophobic compounds, taste and odor control, protection against moisture, oxidation, heat, light exposure and other processing extreme conditions (Santos & Meireles, 2010). On the nutraceutical point of view, encapsulation techniques are also applied to protect compounds against digestive process, promoting controlled release of nutritive compounds on the absorption point (Matalanis et al., 2011). Among all types of possible food additives and nutraceutical compounds, carotenoids are one of the most widely studied molecules due to its natural coloring, antioxidant and nutritive properties, being β-carotene the most common carotenoid found in fruits and vegetables (Paiva & Russel, 1999). β-carotene is a terpene with oxygen singlet and free radicals stabilization properties (Tefler et al., 1994; Fernandez et al., 2009). Due to its antioxidant and nutraceutical properties, β-carotene is being widely referenced on the literature as a possible compound related to cancer and cardiovascular diseases prevention (Dulinska et al., 2005; Vainio, 2000; D'odorico, 2000). Despite all the positive characteristics about β-carotene, its hydrophobicity and high sensitivity to heat, light and oxygen presence make it difficult to be applied readily to food formulations. In that way, micro/nano encapsulation can be performed to protect allow its solubilization in water.

Among several encapsulation methodologies, liposomes are one of the most widely used and studied due to its biocompatibility, nutraceutical properties, ability on encapsulating both hydrophilic and hydrophobic compounds and wide range of possible

diameters and surface charges (Lasic, 1993). Liposomes were first reported on the literature in 1964 (Bangham & Horne, 1964), and since then, many different applications for these vesicles and production methods have been published (Deamer, 2010). However, one of the greatest limitations for the industrial application of liposomes, which is particularly critical for food industry, is difficulty to scale-up the techniques. Most of designed methodologies apply at laboratory scale, but are highly complex for industrial purposes and often lack economical feasibility which is the case of film rehydration technique, microfluidics, super critical processes, and others (Jaafar-Maalej et al., 2011; Meure et al., 2008). Taking into account the necessary characteristics of processes for production of liposomal formulations in the industrial scale, such as sterility, scalability, stability and simplicity, the ethanol injection method is found to be a ready-to-go process, feasible, easy to scale-up, fitting all necessary listed requirements (Wagner et al., 2002; Justo & Moraes, 2010; Pons et al., 1992).

The ethanol injection method consists on the injection of an ethanolic solution containing phospholipids into water, spontaneously forming small sized vesicles (Batzri & Korn, 1973). Since this technological breakthrough, many researchers around the world started studying, modifying and optimizing the methodology in order to understand the impact of system variables on liposomes characteristics and the phenomenology involved (Maitani et al.,2001; Kremer et al., 1977; Wiggenhorn, 2007; Justo & Moraes, 2005; Justo & Moraes, 2011; Szoka, 1996). Justo & Moraes (2005) evaluated the influence of stirring rate, lipid solution flow rate and lipid concentration on the liposomes mean diameter and encapsulation efficiency of kanamycin, an aminoglycosidic antibiotic. They found that the stirring rate was the factor that most affected liposomes size (Justo & Moraes, 2005). Kremer et al. (1977) evaluated the influence of final lipid concentration and stirring rate on liposomes characteristics, concluding that the factor that most influences final nanoaggregate characteristics was the lipid concentration. Justo & Moraes (2011) noticed that there was a lack of studies evaluating the influence of temperature on liposomes final characteristics. In that way, the authors stated that it is possible to control size distribution and polidispersity by varying this parameter. We observed that the cited variable as most influent on certain study is not in accordance with a similar study that states that another variable has

higher influence, what causes controversy. In that way, the present work will contribute to a better understanding on how each process variable affects the system responses as liposomes hydrodynamic diameter, polidispersity index by using advanced statistical tools as CCRD (Central Composite Rotatable Design) statistical design, contributing on the elucidation of involved process phenomenology. This work will also contribute to a better understanding about β-carotene incorporation on liposomes produced at optimum condition, establishing encapsulation limits and stability facing different medium compositions.

2. Experimental Part

2.1 Materials

For liposomes production hydrogenated soybean phosphatidylcholine (HSPC) LIPOID PHOSPHOLIPON 80H® (Ludwigshafen, Germany) (80% purity) was used without further purification. β-carotene was purchaseded from Fluka Analytical (Seelze, Germany) with purity of 97%. Anhydrous ethanol used for lipid dispersion preparations was obtained from Ecibra (Santo Amaro, Brasil). n-hexane from Fluka Analytical (Seelze, Germany) with purity of 98% was used to perform the carotenoid solvent extraction.

2.2. Liposomes production by the ethanol injection method

The HSPC phospholipid was dispersed in ethanol at 60^{o}C (above phospholipid transition temperature), to obtain the organic phase. Different lipid concentrations in ethanol were prepared according to the experimental design (Table 1 and Table 2). The organic phase was then pumped to a jacketed tank under controlled stirring speed and temperature conditions. In all experiments the amount of water inside de reactor was fixed at 100 grams and the organic phase inlet flow rate was 30 mL/min. The influence of impeller type was also studied. For liposomes production two impellers were studied:

IKA® Ultra-Turrax T25 digital equipment with S25N-10G propeller and Cowles propeller type (Figure 1).

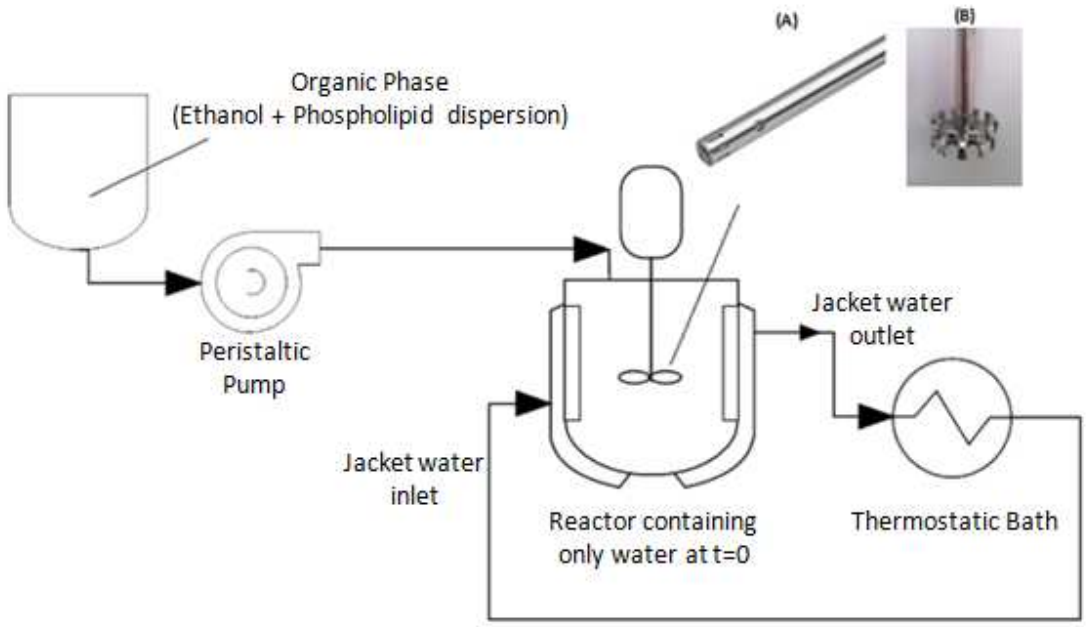


Figure 1 - Schematic process diagram for liposomes production by ethanol injection. In detail the studied impellers (A)IKA® Ultra-Turrax S25N-10G and (B) Cowles impeller type.

2.3. Physicochemical and structural characterization

2.3.1. Hydrodynamic diameter and polydispersity índex

Liposomes hydrodynamic diameter and polidispersity index were determined using Malvern® ZetaSizer Nano ZS equipment. The measurement technique is based on Brownian motion that is related to particle's diffusion coefficient and it is related to particle's hydrodynamic diameter by Stokes-Einstein equation. In all measurements the light incidence angle was fixed at 173° (backscatter) and lipid dispersion concentration was 0.2 mM.

2.3.2. Zeta potential measurement

The zeta potential is a measurement that depends on eletrophoretic mobility of particles, revealing its superficial charge density. The zeta potential of liposomes dispersions was also determined by Malvern® ZetaSizer Nano ZS equipment. For all measurements obtained liposome solutions were diluted in MilliQ water to 0.2 mM and kept at 25 °C.

2.3.3. Colloidal stability

The best liposomal formulation selected after statistical analysis was evaluated weekly regarding their polidispersity index, particle size and zeta potential variation along time and storage at 8 °C.

2.3.4. Morphology

Liposomes had their morphology investigated by Transmission Electron Microscopy (TEM). In that way, liposomes formulations were diluted to 0.5 mM lipid concentration and applied on 400 mesh cooper grids, resting on it for 5 minutes before being dryed carefully by filter paper. For sample coloring it was used uranyl acetate (1% v/v). The prepared samples were visualized using ZEISS CLM 902 transmission electron microscope with image acquisition system CCD Proscan.

2.4. Evaluation of ethanol influence on liposomes hydrodynamic diameter

To evaluate the influence of ethanol in the diameter of the vesicles, liposomes were produced by the ethanol injection method and subsequently subjected to ultra filtration for separation of the liquid phase using Biomax® 10 kDa Millipore® ultrafiltration membrane. After this procedure, the membrane used was sonicated in water and the

retained liposomes were resuspended. This liposome suspension (ethanol free) was divided into eppendorfs with different ethanol/water volumetric ratios. Then the liposomes contained in each ethanol/water concentration were evaluated by DLS measurements and had their hydrodynamic diameter and polidispersity index determined.

2.5. Determination of stirring time influence on liposomes characteristics

To determine the influence of the stirring time on liposomes produced by ethanol injection method, samples were collected at different process times. Experiments were performed to determine the influence of time for both types of impellers studied. The process conditions used in these experiments were defined by the central points of the experimental design for each type of impeller.

2.6. Experimental design and statistical analysis

For evaluation of every variable effect on system response and the synergistic effect among them, a Central Composite Rotatable Design (CCRD) was proposed. All runs were performed in random order defined by sortition. The defined levels for stirring speed variable were defined according to operational limits of the equipment. The results on experimental runs were adjusted to second order mathematical models. The model parameters that were defined as significant were the ones with p-value lower than 0.1. The statistical analysis, mathematical model development, analysis of variance (ANOVA) and the response surfaces were obtained using StatSoft® STATISTICA 8 software.

2.7. Langmuir monolayer experiments

Langmuir monolayer technique was used to determine the ideal β-carotene/phospholipid molar ratio. In that way, mixed β-carotene and phospholipid in chloroform at defined molar ratios were prepared for monolayer studies. The monolayers were prepared by spreading 30 µL of their chloroform solutions over a pure water phase contained in a Langmuir trough of 216 cm^2 total area (Insight, Brasil), resulting on the initial surface pressure. The initial monolayer was left resting for 10 minutes until all chloroform was evaporated. After complete chloroform evaporation, the mobile barrier was activated, slowly going through the surface compressing elements arranged in the interface. In all experiments barrier speed was set to 1.1 mm/s and temperature 25±0.5 °C. The isotherms were recorded in triplicate.

2.8. Influence of stress conditions on the stability of β-carotene loaded liposomes

Liposomes prepared at the best established conditions defined by statistical analysis from experimental design data obtained were evaluated for their particle size distribution stability under different applied stress conditions, simulating the addition to a food matrix. The investigated conditions were 1) pH, 2) sucrose concentration, 3) sodium chloride concentration and 4) storage temperature.

To adjust the pH of the solutions, HCl and NaOH solutions were added to 10 ml of liposome aliquots. Different amounts of sucrose and NaCl were also added to 10 ml of liposomal formulation. Finally another 10 ml aliquots of liposomes dispersion were subjected to temperatures of 35 °C and 70 °C for 5, 15 and 30 minutes. Formulations that do not present phase separation were evaluated in relation to β-carotene content weekly over 1 month. For β-carotene quantification 1 mL of prepared liposomes were added to 9 mL ethanol and homogenized for liposomes rupture and β-carotene release. In addition, the resultant solution was added to 6 mL n-hexane for β-carotene extraction, forming a two phase system. The supernatant phase containing n-hexane with β-carotene was separated and evaluated by spectrophotometry at 450 nm wavelength light. Absorbance results were compared to a previous prepared β-carotene/n-hexane calibration curve (Moraes et al., 2013).

3. Results and discussion

Among all techniques for liposomes production, ethanol injection method presents simplicity and scalability easiness, being a ready-to-go process to be implemented at industrial scale. We divided the ethanol injection method investigation in two sequential parts for a better understanding and logical process approach. In the first section we focused on the investigation of process parameters for liposomes production seeking the optimum process conditions. Unsaturated phospholipids are often subjected to peroxidation process that may influence liposomes stability and bilayer permeability (HUANG & CHUNG, 1998). In that way, even with higher production cost for heating due to higher phase transition temperature (50.4 °C), hydrogenated phospholipids are preferred for stable liposomes production. The priorization of ethanol usage as organic solvent for phospholipid dispersion is due to its easiness for a food grade obtainment. In this context, we focused our efforts on a complete study for variables influence determination using advanced statistical tools and on β-carotene incorporation studies on produced liposomes at the optimized process conditions, contributing to a deeper understanding of process phenomenology.

3.1. Process parameters investigation for hydrogenated soy lecithin liposomes production using ethanol injection method

3.1.1 Ethanol influence on liposomes hydrodynamic diameter

One of the main disadvantages related to ethanol injection method is the residue of this organic solvent at the final liposome dispersion. Traditional protocols include an additional step after liposome preparation for ethanol removal, using for example evaporator or molecular sieve systems (Maitani et al., 2001; Nordlund et al., 1981). Considering this process limitations, we investigated the influence of ethanol on the final

liposome average diameter in order to establish the minimum acceptable amount that did not disturb this physico-chemical parameter. The liposomes hydrodynamic diameter results under different ethanol/water concentrations as function of the volumetric ratio are shown in Figure 2.

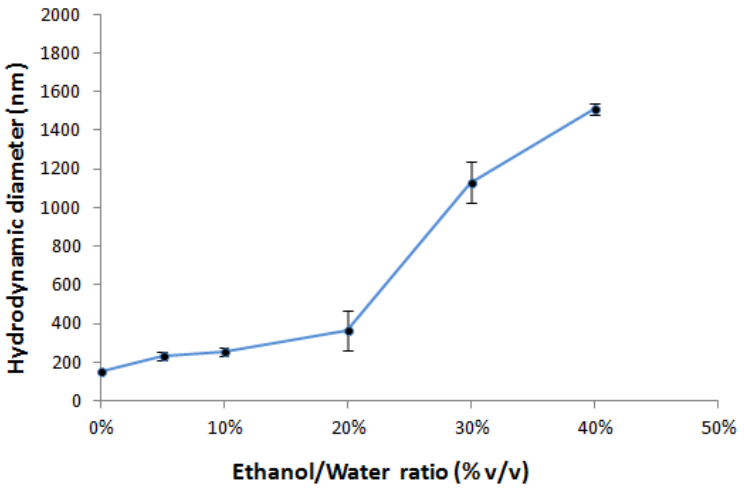


Figure 2 - Influence of different ethanol/water volumetric ratios on liposomes hydrodynamic diameter. The error bars correspond to the standard deviation of independent triplicates. The line is just to guide the eye.

We can observe that as ethanol is added to the final formulation, the average diameter of the nanoparticles tends to increase due to the interaction of the alcohol molecule with the polar heads of phospholipids. We can note that up to a volume ratio of about 10%, liposomes exhibited little change in their diameters (for 20% ethanol the average diameter doubled). Ethanol/Water ratios of 30% or 40% have a very significant change in the diameter of the liposomes, probably causing excessive swelling due to the interaction of ethanol with the polar head groups of the liposome bilayer, the insertion of the alcohol molecules in the membrane, or even forming clumps of vesicles resulting on an increase of average hydrodynamic diameter. It is known that ethanol has influence on liposomes characteristics, especially in their hydrodynamic diameter. The presence of ethanol causes a number of structural changes in the liposomes and

increases their size, interacting with the polar head groups of the lipid bilayers (Vierl et al., 1994; Nagel et al., 1992; Marquês et al., 2011; Löbbecke & Cevc, 1995). Higher ethanol concentrations increased the area per molecule of lipid, increasing its diameter and decreasing vesicle bilayer thickness (Vierl et al. 1994).

We established 10% as the ethanol/water ratio and for all further experiments this limit was respected to prevent results variability due to different ethanol amounts.

3.1.2. Stirring time influence on liposomes characteristics

One of the variables of the ethanol injection process is the stirring time imposed to liposomes. We hypothesised that after organic phase addition on the aqueous phase, the mixing time under shear stress applied by the impeller can influence the characteristics of the final colloidal dispersion. Thus, in order to optimize the production of the liposomes, experiments were conducted to determine what would be the minimum time that the sample should remain in the reactor until the desired specifications of diameter and polidispersity are reached. Figure 3 shows the hydrodynamic diameter and polydispersity index of the liposomes as function of the process time under stirring using Ultra-Turrax and Cowles impeller.

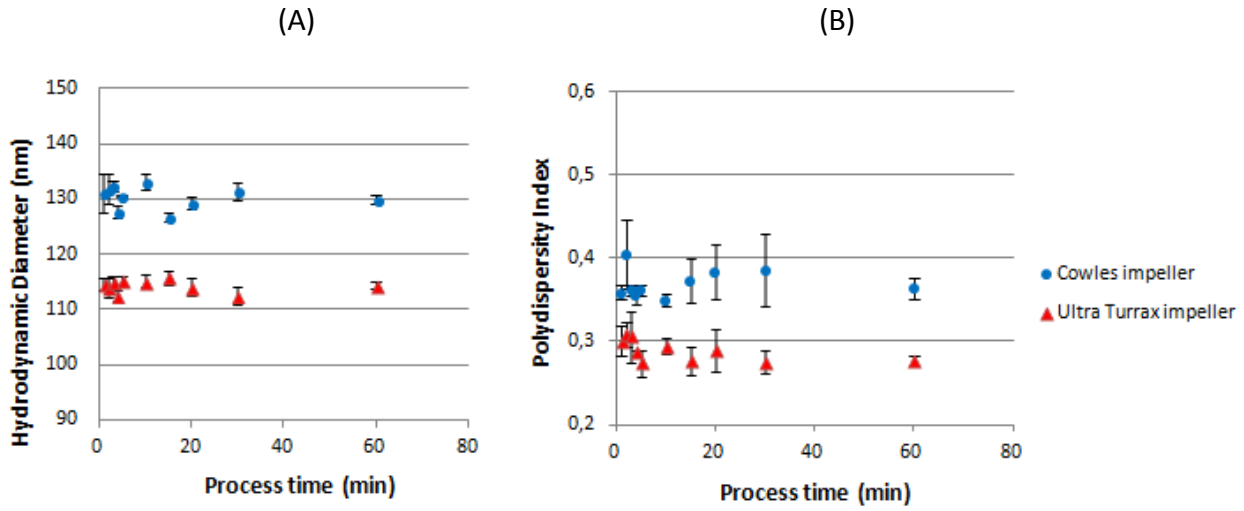


Figure 3 - Hydrodynamic diameter (A) and Polydispersity index (B) variation along process time using Ultra-Turrax impeller and Cowles impeller. The error bars represent the standard deviation of independent triplicates.

We can observe that in all studied cases the time variable is negligible, having small influence on hydrodynamic diameter and polydispersity. In all cases the sample collected after 1 minute process time has essentially the same characteristics as the sample collected, for example, after 60 minutes of process. These results suggest that liposome aggregation time is faster than the evaluated mixing time and the shear rate imposed by both impeller types are not sufficient to cause substantial variation on liposomes characteristics. We conclude that the most important instant in the ethanol injection process, that determines the final characteristics of the colloidal dispersion, is the time at which the lipid organic solution reaches the aqueous phase inside the reactor, currently set as zero time in studies. In this particular moment the liposomes characteristics are determined. To maintain a comparison degree among all performed batches, the stirring time set for all experiments was 5 minutes. We observed controversies about the time needed for liposomes production in scientific literature. Some studies let the liposome formulation under stirring for about 15 minutes (Jaafar-Maalej et al., 2010), some for 30 minutes (Fan et al., 2008) and others do not mention the time used for stirring step (Pons et al., 1992; Maitani et al., 2001). We observed that the amount of time that the liposome formulation is let under stirring do not influence the

final liposomes characteristics like hydrodynamic diameter or polydispersity, but the impeller type does. Comparing Ultra-Turrax impeller type with Cowles type, the first one produced liposomes of smaller hydrodynamic diameter and polydispersity (around 15 nm smaller and 0.1 less polydispersity index). In that way, regarding an industrial liposomes production, it is necessary to evaluate the feasibility of every stirring system related to the final application proposed, depending on the size precision and liposome diameter required.

3.1.3. Temperature, stirring speed and lipid concentration influence on liposomes characteristics

In order to determine the optimal process conditions, which lead to liposomes of smaller diameters and low polidispersity index, two central composite rotatable designs were applied (Table 1 and Table 2).

Table 1 - CCRD matrix with the real and coded values (in parenthesis) for the factors (Temperature (T), Stirring speed (S) and Final lipid concentration (C)) tested and the responses (hydrodynamic diameter and polidispersity index) obtained for the system using Ultra Turrax impeller type.

	Factors			*Responses*	
RUN	***T (°C)***	***S (rpm)***	***C (mM)***	***Hydrodynamic Diameter (nm)***	***Polidispersity Index***
1	60 (-1)	10000 (-1)	6 (-1)	87,2 ± 0,7	0,231 ± 0,006
2	70 (1)	10000 (-1)	6 (-1)	89,0 ± 0,4	0,226 ± 0,003
3	60 (-1)	18000 (1)	6 (-1)	89,3 ± 0,4	0,239 ± 0,008
4	70 (1)	18000 (1)	6 (-1)	88,1 ± 0,7	0,236 ± 0,005
5	60 (-1)	10000 (-1)	10 (1)	157,0 ± 1,5	0,367 ± 0,016
6	70 (1)	10000 (-1)	10 (1)	186,5 ± 4,9	0,432 ± 0,034
7	60 (-1)	18000 (1)	10 (1)	138,4 ± 2,0	0,369 ± 0,007
8	70 (1)	18000 (1)	10 (1)	140,7 ± 0,9	0,377 ± 0,014
9	53,2 (-1,68)	14000 (0)	8 (0)	112,9 ± 0,3	0,329 ± 0,008
10	76,8 (1,68)	14000 (0)	8 (0)	120,0 ± 2,0	0,342 ± 0,020
11	65 (0)	7280 (-1,68)	8 (0)	119,1 ± 1,7	0,329 ± 0,009
12	65 (0)	20720 (1,68)	8 (0)	111,7 ± 1,2	0,258 ± 0,004
13	65 (0)	14000 (0)	4,64 (-1,68)	75,5 ± 2,9	0,241 ± 0,008
14	65 (0)	14000 (0)	11,36 (1,68)	170,3 ± 2,1	0,401 ± 0,016
15	65 (0)	14000 (0)	8 (0)	126,2 ± 0,8	0,263 ± 0,023
16	65 (0)	14000 (0)	8 (0)	111,4 ± 1,0	0,283 ± 0,018
17	65 (0)	14000 (0)	8 (0)	111,1 ± 1,2	0,291 ± 0,033

Table 2 - CCRD matrix with the real and coded values (in parenthesis) for the factors (Temperature (T), Stirring speed (S) and Final lipid concentration (C)) tested and the responses (hydrodynamic diameter and polidispersity index) obtained for the system using Cowles impeller type.

	Factors			*Responses*	
RUN	***T (°C)***	***S (rpm)***	***C (mM)***	***Hydrodynamic Diameter (nm)***	***Polidispersity Index***
1	60 (-1)	800 (-1)	6 (-1)	92,7 ± 0,8	0,226 ± 0,010
2	70 (1)	800 (-1)	6 (-1)	86,4 ± 0,3	0,206 ± 0,009
3	60 (-1)	1200 (1)	6 (-1)	92,4 ± 0,2	0,244 ± 0,013
4	70 (1)	1200 (1)	6 (-1)	84,9 ± 1,4	0,209 ± 0,009
5	60 (-1)	800 (-1)	10 (1)	179,2 ± 0,3	0,439 ± 0,022
6	70 (1)	800 (-1)	10 (1)	217,5 ± 8,5	0,524 ± 0,019
7	60 (-1)	1200 (1)	10 (1)	171,7 ± 2,1	0,417 ± 0,028
8	70 (1)	1200 (1)	10 (1)	182,0 ± 2,3	0,478 ± 0,014
9	53,2 (-1,68)	1000 (0)	8 (0)	126,5 ± 3,9	0,434 ± 0,019
10	76,8 (1,68)	1000 (0)	8 (0)	160,9 ± 2,6	0,444 ± 0,012
11	65 (0)	664 (-1,68)	8 (0)	126,2 ± 0,7	0,273 ± 0,010
12	65 (0)	1336 (1,68)	8 (0)	114,8 ± 0,9	0,339 ± 0,009
13	65 (0)	1000 (0)	4,64 (-1,68)	72,6 ± 1,2	0,191 ± 0,003
14	65 (0)	1000 (0)	11,36 (1,68)	245,8 ± 7,3	0,524 ± 0,018
15	65 (0)	1000 (0)	8 (0)	119,6 ± 0,5	0,322 ± 0,019
16	65 (0)	1000 (0)	8 (0)	123,9 ± 1,4	0,342 ± 0,005
17	65 (0)	1000 (0)	8 (0)	115,6 ± 1,2	0,325 ± 0,015

We can observe that in both designs the condition that provided the smallest liposome hydrodynamic diameter and polydispersity index was the one corresponding to the lowest final lipid concentration (4.64 mM) (run 13, in both Tables 1 and 2). By direct comparison between the results of runs 13 and 14, where all other conditions are kept constant, varying only the concentration from 4.64 mM condition to 11.36 mM, we can observe how strong is the effect of lipid concentration on the final characteristics of colloidal dispersion obtained.

For both impeller systems the responses of zeta potential did not show significant variation with changes in the variable test conditions. None of the three variables significantly affect the zeta potential of the produced liposomes (data not shown).

Despite the lowest temperature (run 9, 53.2 °C) presented acceptable average diameter and PdI, visually the dispersion did not show adequate. There was formation

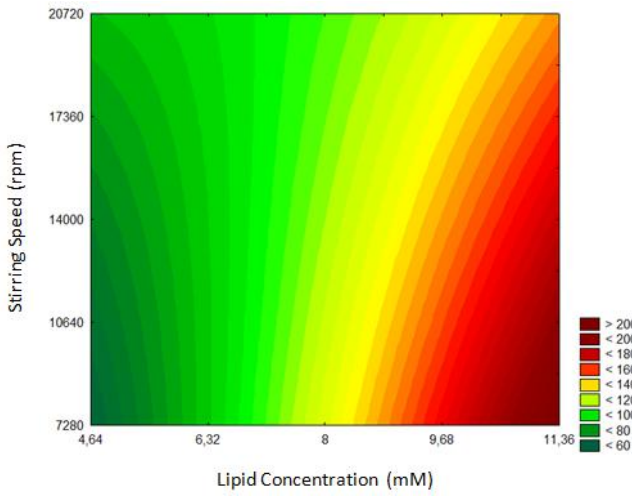
of visible lipid lumps, probably due to possible heat loss through the mixing tank, since this temperature is the closest to the lipid main transition temperature (50.4 °C, Lichtenberg et al., 1988). This occurrence directly affected the choice of optimum temperature condition. We can also observe differences when comparing both impellers. It can be noted that the variation in diameter and polydispersity for Cowles impeller (Table 2) is larger than the one obtained by Ultra-Turrax (Table 1).

Since the analysis of variance generated appropriate regression values for average diameter and polydispersity, it was possible to adjust models to the experimental data (see Supplement Material) and plot contour curves that better express the variables influence on the studied responses (Figure 4).

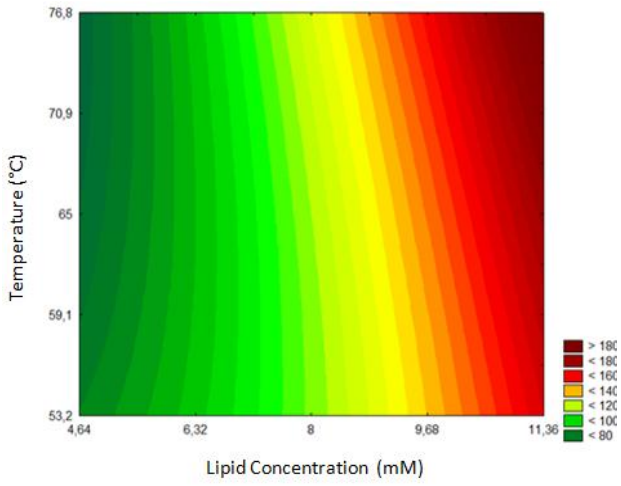
Ultra-Turrax

Response: Hydrodynamic diameter

A

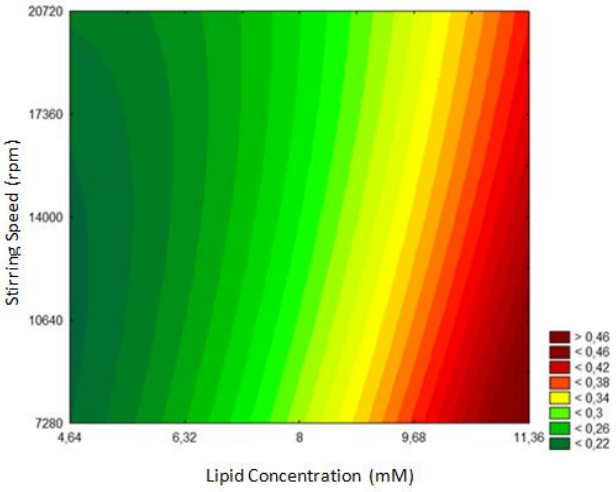


B

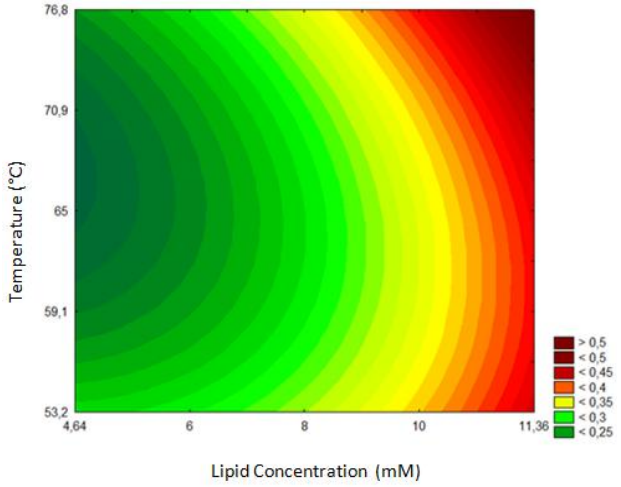


Response: Polydispersity index

C



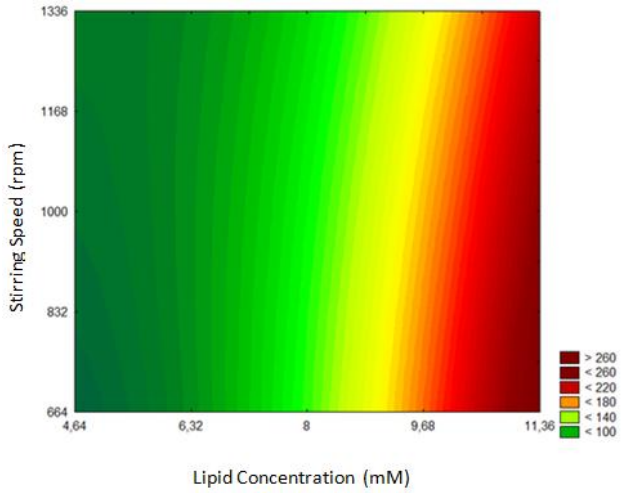
D



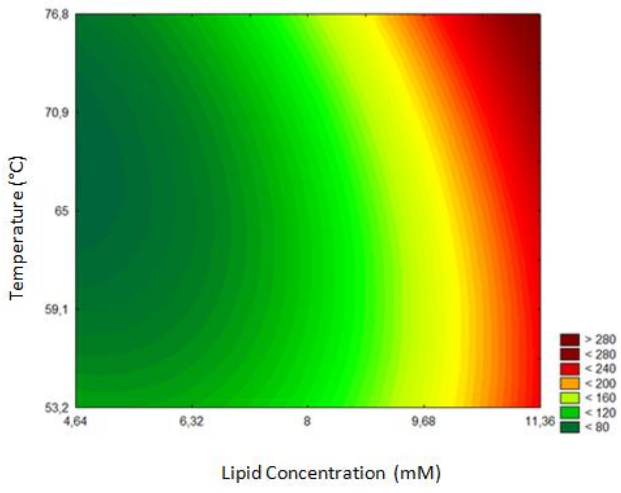
COWLES

Response: Hydrodynamic diameter

E

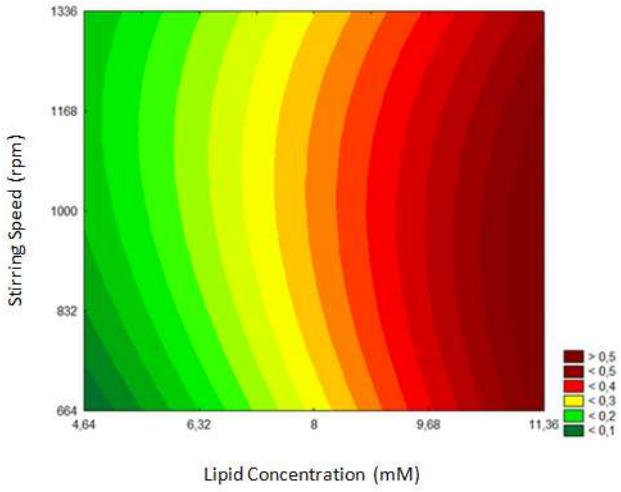


F



Response: Polydispersity index

G



H

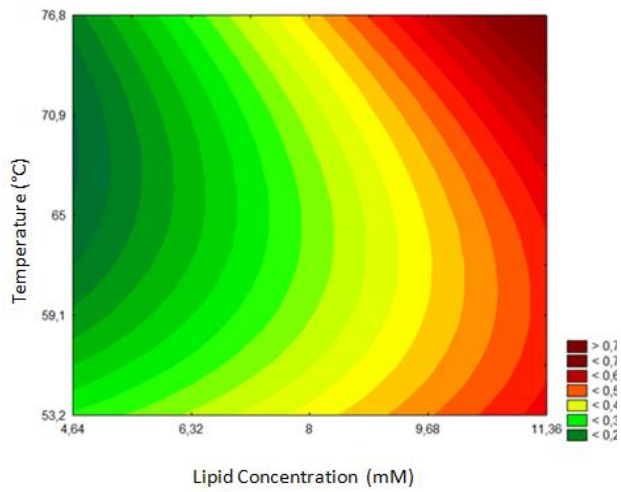


Figure 4 - Contour curves relating the system response with the studied variables. Ultra-turrax: Curves (A) and (B) are related to hydrodynamic diameter response ; Curves (C) and (D) are related to polidispersity index response. Cowles: Curves (E) and (F) are related to hydrodynamic diameter response; Curves (G) and (H) are related to polidispersity index response.

We can observe the magnitude of the influence of concentration parameter on liposomes hydrodynamic diameter response on Figure 4. Varying only this parameter from 4.64 mM to 11.36 mM may cause a difference of more than 100 nm on liposomes diameter using the Ultra-Turrax impeller type. For the case of Cowles impeller, this variation may be more than 200 nm, showing greater variability inherent in the process when this type of impeller is used.

We can also note that the temperature is a most influential factor than the impeller stirring speed on the polydispersity of the liposomes due to the greater curvature of the contour curve. The effect of temperature was also studied by Justo & Moraes (2011) using soy phosphatidycholine for liposomes production by ethanol injection method and, according to their study, higher temperatures lead to the formation of less polydisperse liposomes with narrower size distribution. In the case of our study, it can be noted that there is a temperature threshold that after aprox. 65 °C this begins to present opposing influences on the liposomes characteristics, increasing both the diameter and the polydispersity. In that way we can observe that when the process temperature is below Tm, higher temperatures conduce to the formation of small sized liposomes. On the other hand, when working at temperatures higher than Tm, there is a temperature limit that no longer contributes to liposome size reduction. One may also note the robustness of the temperature variable on the contour curves.

Besides Ultra-Turrax lead to the formation of liposomes with lower average hydrodymanic diameter and polydispersity than cowles impeller, this last one also produced liposomes with proper physico-chemical properties for food application (Taylor & Davidson, 2005). Talking about scale-up possibilities and also application in food, Cowles impeller has many advantages compared to Ultra Turrax system, especially regarding the simplicity of operation, ease of procurement, design, cost and maintenance. In that way, considering all the cited aspects, the cowles impeller choice seems to be the most appropriate for an industrial process scale.

Considering all above statements the optimum condition chosen that produces smaller liposomes with low polidispersity (with higher stability) is: 1) Lipid Concentration = 4,64 mM; 2) Stirring Speed = 1336 rpm; 3) Temperature = 60 °C; 4) Impeller type = Cowles. Experiments were conducted in triplicate on that condition

to verify and validate if it is, in fact, the optimum condition. The average hydrodynamic diameter for the three performed runs was 70.6 ± 0.2 nm with polidispersity index 0.176 ± 0.010. Figure 5 shows the particle size distribution for the liposomes produced under the optimized process conditions.

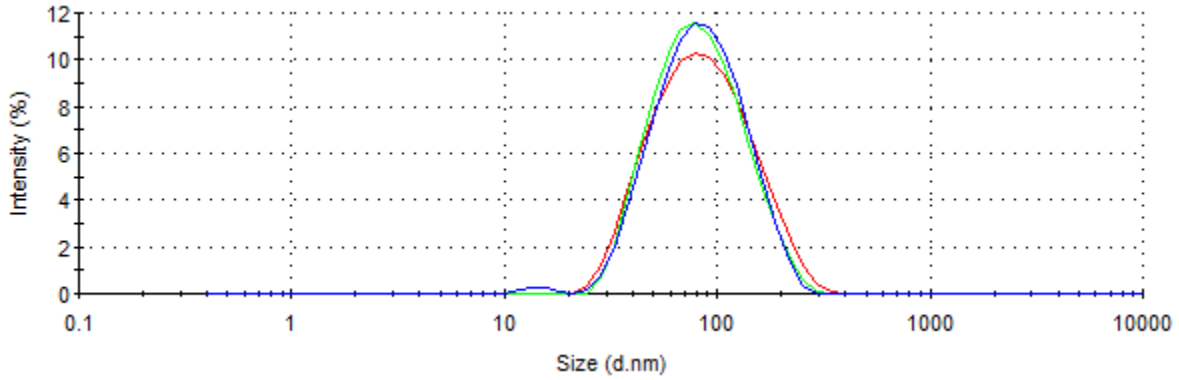


Figure 5 - Size distribution for liposomes obtained at optimized process conditions (T= 60 °C, S= 1336 rpm, C= 4.64 mM) using Cowles impeller. The different curves correspond to independent triplicate.

The results for liposome diameter and polydispersity at the optimized condition, when compared to performed studies contained on the literature (Jaafar-Maalej et al., 2010; Justo & Moraes, 2004; Barnadas-Rodruíguez, 2000; Wagner et al., 2002), show that the liposomes at the present study have lower hydrodynamic diameter and lower polydispersity index, having only one population in terms of size distribution.

The morphology of produced liposomes under the optimized condition was also investigated by Transmission Electron Microscopy (Figure 6).

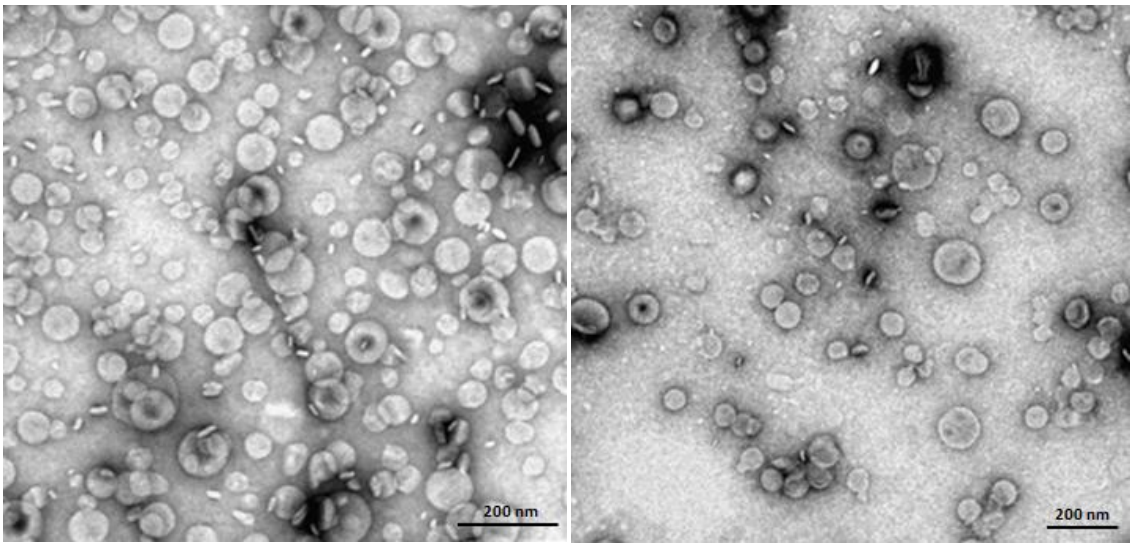


Figure 6 - Transmission Electron Microscopies showing obtained liposomes morphology for best determined process conditions (Temperature = 60 °C; Stirring Speed = 1336 rpm; Final lipid concentration = 4,64 mM; Cowles impeller). Scale bars represent 200 nm.

We can see on Figure 6 that the average hydrodynamic diameter is in accordance with the average population size (Figure 5). Most of nano aggregates obtained are spherical shaped, confirming liposomes formation. It can also be seen that there is another structure, smaller than the rounded liposomes, present among the nanoparticles formed. This rod-like shaped structure could be fragments of liposomes formed due to shear stress imposed by Cowles impeller type. This structure was not visualized in similar studies using ethanol injection method (Maitani et al., 2001; Domazou & Luisi, 2002; Jaafar-Maalej et al., 2010).

Liposomes produced at the best condition of experimental design developed (Run 13) were submitted to stability tests (storage at 8 ^{o}C, under refrigeration). In that way, characteristics as hydrodynamic diameter, polydispersity and zeta potential were evaluated once a week during 5 weeks. In all cases, during the 5 weeks of studies and characteristics evaluation, none of them presented significant variation. Liposomes hydrodynamic diameter showed a variation of less than 3% during the whole period of analysis, demonstrating that the produced liposomes by ethanol injection method are stable even after long periods of storage (data not shown).

3.2. β-carotene incorporation on hydrogenated-soy lecithin liposomes

3.2.1. Langmuir monolayer experiments

The molecular interaction between HSPC and β-carotene was investigated using Langmuir monolayer technique. Different β-carotene to phosphatidylcholine ratios was investigated. The obtained isotherms relating applied pressure on surface molecules and occupied area are presented on Figure 8A. For a better visualization and interpretation of obtained data the graph on Figure 8B was built relating the percentage of applied β-carotene on the surface to the area per molecule, at constant pressure.

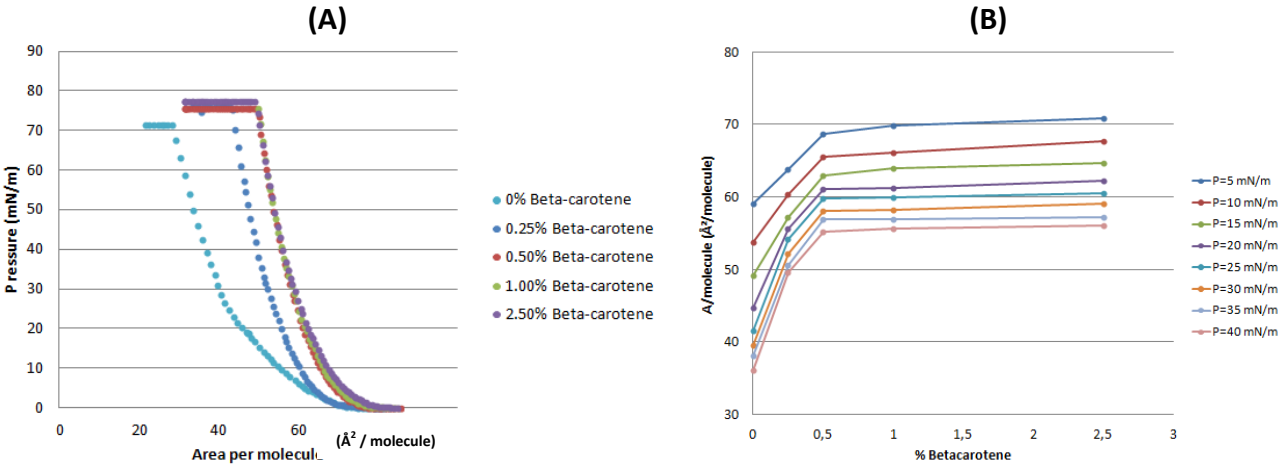


Figure 7 – (A) Surface pressure (π) isotherms for β-carotene/Phosphatidylcholine monolayer on water-air interface. (B) Area per molecule variation due to β-carotene addition to the lipid monolayer on water-air interface, at constant pressure. The lines are just to guide the eye. Barrier speed was set to 1.1 mm/s. All experiments were performed in triplicate under 25 °C.

HSPC used in the experiments is composed of a mixture of different phospholipids with saturated acyl chains. These acyl chains, due to its hydrophobicity, when spreaded on the Langmuir trough, self-organize in a way that they keep at the air phase, away from the aqueous phase. The phospholipid headgroup stays in contact with the water inside the trough, forming a unique monolayer configuration. β-carotene,

due to its high hydrophobicity, when added to the trough, tends to interact with the acyl groups from the lipids, disturbing the monolayer. From Figure 7B we can observe that β-carotene addition changed the HSPC monolayer, increasing the area per molecule as more β-carotene was added until the 0.5% molar ratio. After this molar ratio there was no further area per molecule change, suggesting that the monolayer can not incorporate more β-carotene molecules. The excess of added β-carotene probably formed insoluble aggregates that did not take part on the monolayer. This behavior was observed for all pressures and since it is known that lipids monolayer properties can be related to bilayers properties (liposomes) when the surface pressures are in the range of 30 $mN.m^{-1}$ (Marsh, 1996), we can conclude that β-carotene that can be incorporated in HSPC lipossomes at 0.5% β-carotene/phospholipid molar ratio.

The low β-carotene loading percentage on liposomes can be explained due to its location and position inside liposomes. β-carotene is a non polar hydrophobic carotenoid, being located at the hydrophobic phospholipid bilayer when incorporated into liposomes (Wisniewska et al., 2006). Different carotenoids have different molecular orientations inside the hydrophobic bilayer depending mainly on carotenoid molecule polarity (Gabrielska et al., 1996). Zeaxanthin, a dipolar carotenoid, has its molecular orientation established at 42° in relation to membrane axis due to its 2 polar groups (one connected to each polar head group). On the other hand β-carotene does not have an established position inside phospholipid membrane due to the lack of molecular polar groups, presenting motional freedom inside bilayer (Gruszecki et al., 2004; Gabrielska et al., 1996). Andreeva et al. (2010) reported that β-carotene when incorporated to EPC liposomes promoted an increase on acyl chains and polar head groups mobility. This carotenoids configuration and properties have intimate relation with membrane rigidity and loading capacity (Gabrielska et al., 1996). The influence of β-carotene molecules incorporation on liposomes size distribution was evaluated by DLS measurements and is shown on Figure 8.

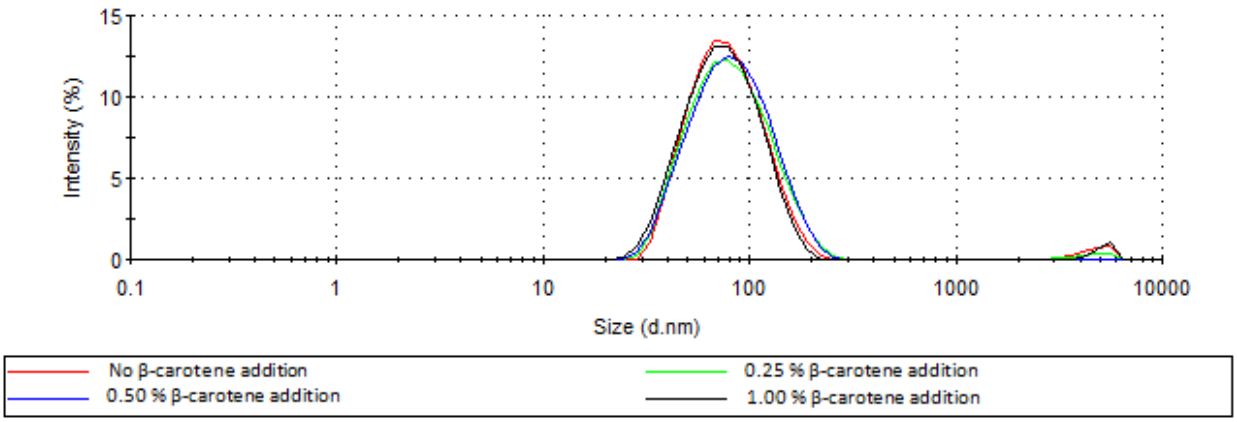


Figure 8 - DLS measurements of liposomes containing different β-carotene/HSPC molar ratios. Process conditions were the ones established as the optimized by the CCRD developed (T= 60 °C, S= 1336 rpm, C= 4.64 mM).

We can observe on Figure 8 that β-carotene addition to liposomes does not interfer on liposomes hydrodynamic diameter distribution. The data shown suggests that the structural changes on liposomes caused by β-carotene addition cannot be detected by DLS measurements. Maybe this kind of technique does not have the required sensitivity to detect variations on liposomes diameter with different β-carotene loadings.

3.2.2. Application of different stress conditions on β-carotene loaded liposomes formulations

To verify β-carotene loaded liposomes stability, different controlled stress conditions were applied to the produced formulations simulating some usual conditions often found on different food systems. Table 3 lists all applied stress conditions and the presence of phase separation or not, revealing stability of liposomes present on the prepared formulations. The results for β-carotene relative quantification during the weeks under each different stress condition are shown on Figure 9.

Table 3 - Different stress conditions applied to β-carotene loaded liposomes produced by ethanol injection method under optimized conditions (T= 60 °C, S= 1336 rpm, C= 4.64 mM). β-carotene/HSPC molar ratio was set to 0.5%.

Applied Stress	Phase Separation
pH	
pH 4,5	No
pH 6,8	No
Saccharose	
1,5%	No
7,5%	Yes
10%	Yes
20%	Yes
Temperature	
35 ºC for 5 minutes	No
35 ºC for 15 minutes	No
35 ºC for 30 minutes	No
70 ºC for 5 minutes	No
70 ºC for 15 minutes	No
70 ºC for 30 minutes	No
NaCl	
0,025 M	Yes
0,1 M	Yes
0,25 M	Yes
0,5 M	Yes
1 M	Yes

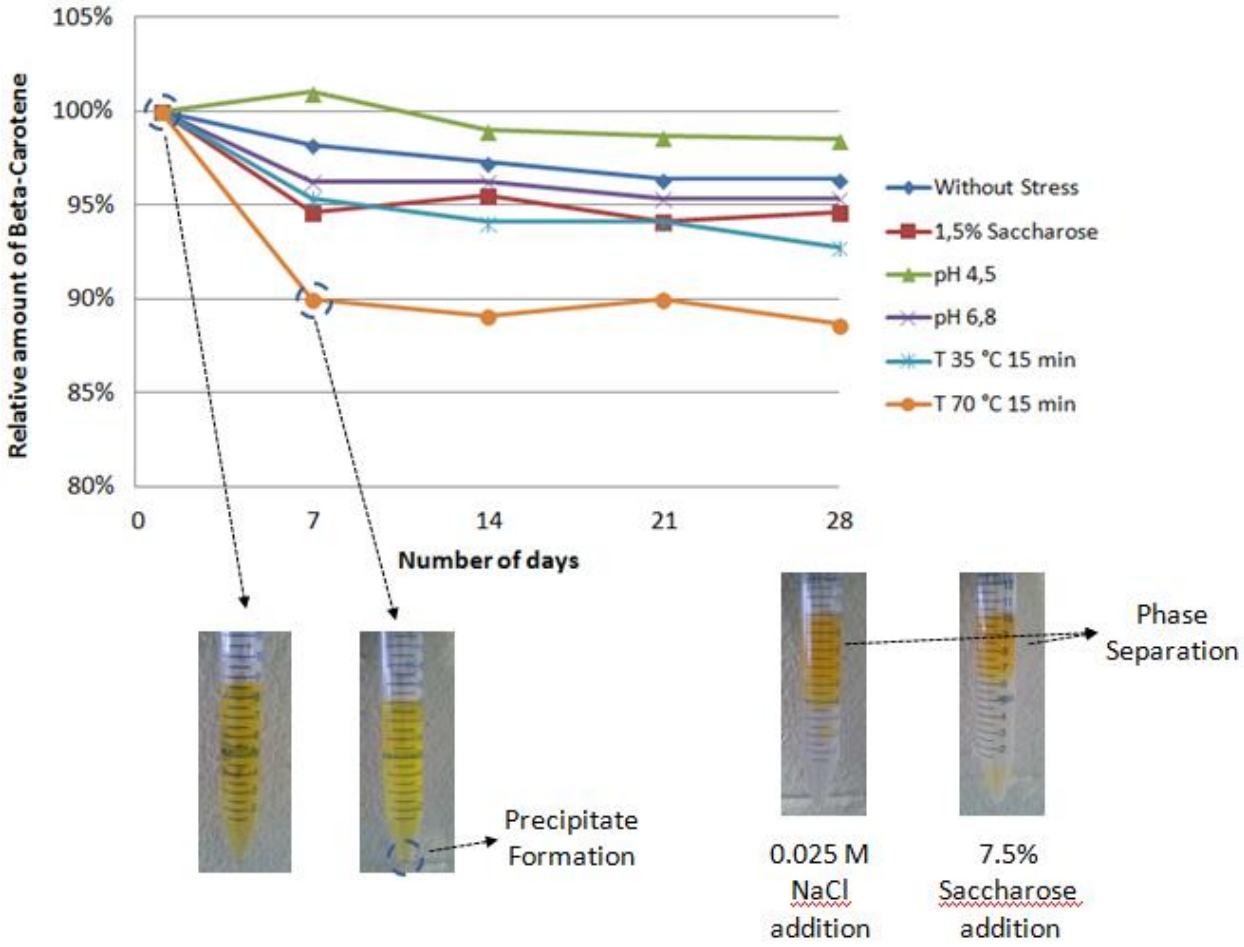


Figure 9 - β-carotene relative amount quantified during the weeks for the formulations that did not present phase separation after applied stress. The samples were stored away from light at room temperature.

We can note by data shown on Table 3 that the stress condition that most affected liposomes stability was the addition of NaCl. Even lower amounts of added NaCl to liposomes formulations caused complete phase separation after few minutes. Sodium chloride is known as a bilayer disruption compound, mainly due to the fact that sodium and chlorine ions assemble to the liposomes surface decreasing its zeta potential and electrostatic repulsion among the nanoaggregates, causing aggregation (McClements, 2004). That can be faced as a great challenge for liposomes application to foods containing large amounts of different salts and ionic compounds. The exposal to different temperatures for different times did not cause any phase separation, even

the formulations that were exposed to 70 °C for about 15 minutes. On that specific condition we could note some β-carotene precipitate formation which interfered on degradation evaluation (Figure 9). In all evaluated conditions the decrease of β-carotene amount on the first week was higher than on further weeks probably due to precipitation of some non-encapsulated β-carotene molecules in suspension. The possible hypothesis to precipitatation at 70 °C stress could be associated to temperature higher than HSPC Tm (70 °C > Tm) possibly causing some release of β-carotene aggregates from the phospholipid bilayer. Different pHs (4.5 and 6.8) did not cause phase separation or increase on degradation rate, showing that liposomes can be applied to this pH range without losing their structural characteristics. Finally, regarding different sucrose concentrations we could note that small amounts of this disaccharide (1.5% m/m) did not cause phase separation, maintaining liposomes physico-chemical characteristics. When we applied 7.5% m/m sucrose we observed phase separation, causing destabilization of liposomes structure and β-carotene release. We could note that when sucrose solution was added to liposome formulation the medium viscosity increased considerably, suggesting a strong chemical interaction between both compounds. Sucrose is known as a cryoprotectant for many kinds of nanoparticles (including liposomes) during freezing and freeze-drying processes, suggesting again that sucrose has a specific interaction with liposomes (Ausborn et al., 1994; Leeuw et al., 1993). The chemical interaction between sucrose and liposomes is not deeply studied on the literature. Regarding all studied conditions that did not present phase separation during stress application we can observe that all of them presented low decay during the weeks, being the highest decay (11.4%) related to liposomes heating to 70 °C during 15 minutes. It is important to note that some of β-carotene decay is due to non encapsulated β-carotene molecules that precipitate and not specifically to the ones that suffered oxidation process.

4. Concluding Remarks

Determination of process parameters effects on liposomes final characteristics, β-carotene incorporation limits and β-carotene loaded liposomes stability during several different stress applications were successfully carried out. Morphology studies confirmed that the liposomes produced at optimized condition are smaller than 100 nm and present low polydispersity. Phospholipid concentration was confirmed as the most influent parameter affecting liposomes final physico-chemical characteristics. Langmuir monolayer studies showed that 0.5% β-carotene/HSPC molar ratio may be the incorporation limit for the carotenoid molecules on that specific phospholipid. β-carotene loaded liposomes showed remarkable stability under several medium compositions and applied stress conditions, showing that this kind of encapsulated compound has great application possibilities on food industries.

5. Acnowledgements

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7. Supplement Material

From the results for each experimental condition, mathematical models have been proposed in an attempt to predict the behavior of the system under different conditions. The central composite rotatable design used allows the obtainment of second order mathematical models, as shown in Table 4. From the statistical analysis performed using the software STATISTICA® 8, regression coefficients were established for each model. The mathematical models obtained are displayed in coded form. The significance level (α), considered for evaluation of the coefficients was 10%, that is, coefficients with a p-value lower than 0.1 are considered statistically significant. This 10% estimation is widely used and accepted in the literature, and this may vary according to the complexity and variability of the process under analysis (Phadke, 2009; Rodrigues & Iemma, 2009). Mathematical models containing the significant regression coefficients and the analysis of variance of each one are shown below.

Table 4 - Mathematical models for Hydrodynamic diameter (D), Polydispersity index (PdI) and Zeta potential (Zeta) prediction.

Ultra-Turrax impeller mathematical models	*Cowles impeller mathematical models*
$D\ (nm) = 119.6-5.5*S+31.3*C-8.20*S*C$	$D\ (nm) = 119.1+6.7*T+7.7*T^2-4.6*S+50.2*C+13.2*C^2+7.8*T*C-5.1*S*C$
$PdI = 0.295+0.014*T^2+0.065*C$	$PdI = 0.321+0.035*T^2+0.112*C+0.025*T*C$
$Zeta\ (mV) = -64.25-1.09*C+2.81*C^2$	$Zeta\ (mV) = -52,53-2,24*C$

Table 5 - Analysis of variance of the mathematical models for different responses prediction.

ANOVA – Hydrodynamic Diameter						
Source of Variation	*Sum of Squares*		*Degrees of Freedom*		*Mean Square*	
	Ultra-Turrax	Cowles	Ultra-Turrax	Cowles	Ultra-Turrax	Cowles
Regression	14398.9	38939.7	3	7	4799.6	5562.8
Error	976.6	438.3	13	9	75.1	48.7
Total	15375.5	39378.0	16	16		
Ultra-Turrax: R^2= 0.936		Cowles: R^2= 0.989				

ANOVA – Polydispersity Index						
Source of Variation	*Sum of Squares*		*Degrees of Freedom*		*Mean Square*	
	Ultra-Turrax	Cowles	Ultra-Turrax	Cowles	Ultra-Turrax	Cowles
Regression	0,0596	0,1932	2	3	0,0298	0,0644
Error	0,0109	0,0121	14	13	0,0008	0,0009
Total	0,0705	0,2053	16	16		
Ultra-Turrax: R^2= 0.846		Cowles: R^2= 0.941				

ANOVA – Zeta Potential						
Source of variation	*Sum of squares*		*Degrees of Freedom*		*Mean Square*	
	Ultra-Turrax	Cowles	Ultra-Turrax	Cowles	Ultra-Turrax	Cowles
Regression	119,1	68,5	2	1	59,5	68,5
Error	62,8	221,1	14	15	4,5	14,7
Total	181,8	289,6	16	16		
Ultra-Turrax: R^2= 0.655		Cowles: R^2= 0.237				

We can note by the above analyzes that models obtained for the dependent variable zeta potential did not show a satisfactory adjustment by analyzing the R^2 value obtained for the models. This lack of fit was expected because of the small variation of the values obtained in the tests, showing that the variables at the levels studied did not present significant effect on the zeta potential of the liposomes. On the other hand, the models obtained for the other responses (hydrodynamic diameter and polidispersity index) were considered very good, properly adjusting the experimental data. The best model is the prediction related to the hydrodynamic diameter of the liposomes using the stirring system with Cowles impeller, with R^2 equal to 0.989. The obtained mathematical models were plotted, generating Due to the lack of fit of mathematical model related to zeta potential prediction, the contour curve was not plotted, avoiding in that way mistaken analysis.

Capítulo 4 - Incorporation of β-carotene loaded liposomes on Polyvinyl Alcohol and Polyethylene Oxide nanofibers produced by electrospinning

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Abstract

Hybrid encapsulation structures based on β-carotene loaded nanoliposomes incorporated within polymeric nanofibers produced by electrospinning were developed for improving the photostability of the antioxidant. These novel materials were also intended for β-carotene incorporation on water-based food formulations, overcoming the current existing limitation associated to its hydrophobic character. Initially, empty and antioxidant-loaded nanoliposomes were developed and incorporated into two different polymeric solutions of Polyvinyl Alcohol (PVOH) and Polyethylene Oxide (PEO). The change in solution properties was evaluated in order to ascertain how this incorporation would affect the electrospinning performance. The mixed polymer solutions were subsequently processed through electrospinning for hybrid nanoliposome-loaded ultrathin fibers production. FTIR analysis confirmed the presence of phospholipid molecules inside the electrospun fibers. These ultrathin fibers were evaluated regarding their morphology, diameter, β-carotene internal distribution and stability against UV irradiation. Liposomes release studies from the electrospun fibers were also carried out, confirming the integrity of the liposomal structures after nanofibers dissolution in water.

Keywords: Liposomes, β-carotene, hydrogenated soy lecithin, nanotechnology, electrospinning.

1. Introduction

Carotenoids are often used as food additive compounds due to its pro-vitaminic, colorant and antioxidant properties. Among naturally occurring carotenoids, β-carotene is the most widely studied and commonly found in fruits and vegetables (Paiva & Russell, 1999). This carotenoid has been reported to potentially have anti-cancer and anti-cardiovascular disease properties (Dulinska et al., 2005; Vainio, 2000; D'odorico, 2000). However, carotenoids (including β-carotene) are sensitive molecules susceptible to oxidation by light, heat and oxygen exposure, besides being hydrophobic compounds, hindering its direct application on aqueous-based food formulations. Micro- and nanoencapsulation techniques are plausible options to overcome the mentioned drawbacks related to carotenoid addition to foods.

Microencapsulation has previously been reported as a technology to protect sensitive substances against the influences of adverse environments. The term "microencapsulation" designates a defined technology of wrapping solids, liquids, or gases in small capsules, which can release their contents under specific circumstances (Champagne & Fustier, 2007). . It is a technique that emerged from the micro/nanotechnology field and has been succesfully used in many industries, conferring remarkable properties to products as pharmaceuticals, foods, cosmetics, textiles and many other products used on daily life (Risch, 1995). Regarding food applications, encapsulation techniques are capable of solubilizing hydrophobic compounds in water based foods, stabilizing reactive compounds and protecting sensitive molecules from oxidation, moisture, light exposure, temperature and other process extreme conditions, thus enabling the development of novel functional food products (Gibbs et al., 1999). Another important application of encapsulation techniques on food technological development is to mask undesirable odors and flavors of some specific compounds, improving the product acceptance by the customer. There are several types of nanoparticles being studied for these applications, being liposomes one of the promising structures due to its wide range of possible diameters and surface charges, nutraceutical properties and ability of encapsulating both hydrophilic and hydrophobic compounds (Lasic, 1993). A more recently explored one for the

encapsulation of compounds for food uses is electrospinning (Bhardwaj & Kundu, 2010; Fernandez et al., 2009). Electrospinning is a simple and highly versatile method to produce fibers in the sub-micron range, presenting a large surface to volume ratio, through the action of an external electric field applied between two electrodes and imposed on a polymer solution or melt. The use of this kind of system for food applications is still on its early stage of development, with many possibilities for design of innovative products with specific new properties. Another advantage of the technique is its low cost and high productivity rate, thus being ideal for industrial purposes (Ramakrishna et al., 2005). This technique, as recently demonstrated, has a tremendous potential in the food science area for development of novel functional ingredients and improved food packages (Fabra et al., 2013; López-Rubio et al., 2009; López-Rubio et al., 2012; Torres-Giner et al., 2008; Torres-Giner & Lagaron, 2009;). The use of electrospinning allied with other technologies arising from the micro/nano encapsulation field can enlarge even more the possibilities for new product development with improved functionalities. One interesting and promising combination of technologies is the entrapment of liposome nanostructures within electrospun fibers for compounds stabilization (Mickova et al., 2012). In this sense, the aim of the present work was to develop novel hybrid materials comprising the two promising technologies for the encapsulation and protection of the antioxidant molecule β-carotene. Thus, β-carotene-loaded nanoliposomes were incorporated inside polyvinyl alcohol (PVOH) and polyethylene oxide (PEO) electrospun fibers, being both water soluble polymers of great interest in food and pharmaceutical industries.

2. Experimental Part

2.1. Materials

PVOH was kindly supplied by Plásticos Hidrosolubles S.L. (Valencia, Spain) in thin film form and PEO, 200 kDa (powder), was supplied by Sigma-Aldrich (Madrid, Spain). Hydrogenated soybean phosphatidylcholine (HSPC) PHOSPHOLIPON 80H

from Lipoid (Ludwisghafen, Germany) and ethanol 96% (v/v) supplied by Panreac (Barcelona, Spain) were used for liposomes production. Synthetic β-carotene (minimum purity 97%) was purchased from Fluka Analytical (Madrid, Spain). For β-carotene quantification n-hexane (98% purity) from Fluka Analytical (Madrid, Spain) was used. The β-carotene was kept under refrigeration and in darkness until use. All samples obtained from the performed experiments were protected from light until analytical characterization.

2.2. Liposomes production

Liposomes were prepared by the ethanol injection method previously described elsewhere (Zompero et al., to be submitted). Briefly, the lipids (or lipids and β-carotene) were dispersed in ethanol (above the main phase transition temperature) and injected at a constant flow rate of 30 mL/min in a jacketed reactor containing water (100 mL) under controlled temperature (60°C) and stirring speed (1336 rpm using a Cowles type impeller), during 5 minutes. This heating process is necessary due to high lipid transition temperature (around 51°C). The final liposome dispersion contains 10% (v/v) ethanol/water proportion and 20 mM lipid concentration. For liposomes production containing encapsulated β-carotene, the alcoholic phase was prepared differently, adding to the formulation β-carotene in a molar ratio β-carotene/Phospholipid of 0.5% (Zompero et al., to be submitted). The average hydrodynamic diameter, polidyspersity and zeta potential of liposomes and β-carotene loaded liposomes were measured using Malvern Zeta-Sizer Nano-ZS equipment.

2.3. Liposome/Polymer Formulations

The solutions used for electrospinning process were composed by polymer (PVOH or PEO) and liposomes previously produced by ethanol injection method. The polymer/water mass ratio was 20%, and this value was kept constant in all formulations.

Three different liposome:polymer mass ratios were studied: 2.5%, 5% and 7,5%, both for empty liposomes and β-carotene loaded liposomes. Polymeric solutions without addition of liposomes were also electrospun as control materials in order to evaluate the effect of empty liposomes or β-carotene loaded liposomes on the properties of polymeric solutions and, thus, in the produced electrospun fibers.

2.4. Electrospinning process

The electrospinning apparatus, a FluidNatek® instrument, trademark of BioInicia S.L. (Valencia, Spain), equipped with a variable high voltage 0–30 kV power supply was used. The anode was attached to a stainless steel needle with internal diameter 0.9 mm that was connected through a PTFE wire to the polymer/nanoliposome solutions kept in a 5 ml plastic syringe. The syringe was disposed horizontally lying on a digitally controlled syringe pump while the needle was horizontally directed towards the collector. The electrospun structures were collected on a stainless steel plate attached to a copper grid used as collector. All of the electrospinning experiments were carried out at room temperature in air. The electrospinning environmental conditions were maintained stable at 24°C and 60% RH by having the equipment enclosed in a specific chamber with temperature and humidity control. The target was placed 10 cm from the capillary tip. The syringe pump delivered the PVOH and PEO polymer solutions at a controlled feed rate of 0.1 ml/h, and the voltage was maintained at 10 kV. The conditions were the same for the electrospinning process of all formulations. The collected materials were stored in a desiccator at 0% RH and protected from light exposure.

2.5. Superficial tension and rheological properties determination

The rheological properties of the various polymeric solutions were evaluated using Thermo Haake RS1 controlled stress rheometer using the cone-plate

configuration, both made of titanium. The angle between the surface of the cone and the plate was of 1 degree, and a distance of 1 mm from the plate was kept during the measurements. The temperature during the measurements was kept constant at 20 ± 1°C using a Phoenix P1 Circulator device (Thermo Haake). Different frequencies and shear rates were applied to the samples and the behaviour of the samples to the determined conditions was evaluated using Rheowin Pro Software v.3.61 Haake. Before the application of stress the samples remained in contact for 10 minutes with the cone-plate system in order to achieve thermal equilibrium. The surface tension of the polymer solutions containing different quantities of liposomes was measured using the Wilhemy plate method in an EasyDyne K20 tensiometer (Krüss GmbH, Hamburg, Germany). All the measurements were made in duplicate.

2.6. Fourier transform infrared spectroscopy

ATR-FTIR spectra of the different electrospun materials obtained (pure polymeric fibers and fibers containing empty or β-carotene-loaded nanoliposomes) were collected in a controlled chamber at 24°C and 60% RH coupling the ATR accessory Golden Gate of Specac Ltd. (Orpington, UK) to a Bruker (Rheinstetten, Germany) FTIR Tensor 37 equipment. All the spectra were collected by averaging 20 scans at 4 cm^{-1} resolution. Comparison of the spectral data was performed using Opus Viewer software (Rheinstetten, Germany).

2.7. Scanning Electron Microscopy

In order to visualize and characterize the morphology and measure the diameter of the produced electrospun fibers, an Hitachi S-4100 Scanning Electron Microscope was used. The different samples were sputtered with a gold–palladium mixture under vacuum. All SEM experiments were carried out at an accelerating voltage of 30 kV.

Fiber diameters of the electrospun fibers were measured by means of the ImageJ software (Maryland, USA) from the SEM micrographs in their original magnification. For higher precision on the mean diameter results a minimum of 100 randomly chosen electrospun fibers were measured.

2.8. Raman spectroscopy

Raman images were taken with a Jasco NRS-3100 Confocal Micro-Raman spectrophotometer (Jasco Inc., Easton, MD) using a 20x objective to evaluate β-carotene distribution within the developed hybrid electrospun fibers. Raman chemical images were carried out in the point by point mode by plotting the area of the β-carotene band at 1500 cm^{-1}, and were constructed by taking 15x15 spectra equally spaced along the selected sample area. The β-carotene is a very large Raman scatterer and, thus, very short acquisition times were needed to record intense spectra, which only showed the signal of the β-carotene in the hydrocolloid films. This signal intensity was therefore used to construct the Raman images.

2.9. Liposomes release from polymeric films

PEO and PVOH nanofibers containing 7.5% (m/m) liposomes and β-carotene loaded liposomes (0.01 g of nanofibers mat) were hydrated in water (5 mL) under gentle stirring at 25^{o}C. After complete dissolution, the aqueous phase was observed by using Transmission Electron Microscopy. The obtained solution was carefully applied on 400 mesh cooper grid, resting on it for 5 minutes before being dried using filter paper. Uranyl acetate (1% v/v) was then added to sample on the cooper grid for coloring, resting on it for 1 minute before being dried using clean filter paper. The prepared samples were put inside the transmission electron microscope sample port for image acquisition, using ZEISS CLM 902 and CCD Proscan systems.

2.10. Stability tests by UV-vis irradiation

PEO and PVOH electrospun fiber mats containing the maximum quantity of added liposomes (7.5% m/m in relation to polymer) and, consequently, the highest quantity of β-carotene incorporated to the matrix were evaluated in relation to their protection level offered to the photosensitive molecule. To evaluate the protection ability of the hybrid systems, the antioxidant degradation rate was compared with that of β-carotene encapsulated in liposomes (aqueous solution) and free β-carotene crystals dissolved in hexane. Samples of each system were placed on glass flasks and exposed to an Osram Ultra-Vitalux 300W UV lamp during 6 hours. The sample-lamp distance was kept constant at 10 cm. Samples were collected every 1 hour and the intact β-carotene concentration was determined using Agilent 8453 spectrophotometer (Santa Clara, USA) at 450 nm wavelength. The β-carotene concentration of the control samples containing β-carotene crystals dissolved in hexane was directly determined by spectrophotometer analysis. For the aqueous solutions of β-carotene loaded liposomes, ethanol was first added for liposomes disruption (releasing entrapped β-carotene) and, then, hexane was added for β-carotene extraction, giving rise to a 2 phase system. The organic upper phase was separated and β-carotene concentration was then determined by spectrophotometry. In the case of the PEO and PVOH electrospun fibers containing the antioxidant-loaded liposomes, the fibers were initially placed in water for polymer complete dissolution before addition of ethanol and hexane for liposomes disruption and β-carotene extraction, respectively.

3. Results and discussion

Electrospinning technique has returned to be in vogue in the last few years. Many authors are studying different polymers and their feasibility regarding nanofibers production (Uyar & Besenbacher, 2009; Arecchi et al., 2010). Among all polymers being studied, polyvinyl alcohol (PVOH) and polyethylene oxide (PEO) have demonstrated to be efficient nanofibers forming compounds, being polymers of great interest for food

and pharmaceutical industries (Peresin et al., 2009; Supaphol & Chuangchote, 2007; Baba et al., 2010; Deitzel et al., 2001). Another reason for their utilization is related to their water solubility, making it easier to introduce liposomal formulations together with both polymers. We divided the investigation of present work in three sequential parts for a logical development approach. In the first part the produced aqueous solutions containing different mass ratios of liposomes and polymers were evaluated in relation to surface tension and their rheological properties, characteristics that affect directly electrospinning performance. On the second part nanofibers were produced from previous studied solutions, linking the different solutions properties with nanofibers morphology and diameters. In the last part studies regarding β-carotene distribution and stability inside nanofibers were performed. β-carotene/phospholipid molar ratio and β-carotene loaded liposomes diameter and polydispersity index were determined according to a previous developed work (Zompero et al., to be submitted). Finally it was verified the possibility to acquire liposomal structures after polymeric rehydration on aqueous phase.

3.1. Superficial tension and rheological properties of polymeric solutions containing "empty" liposomes

It is known that the solution surface tension is an important parameter that directly influences the electrospinning process (Ramakrishna et al., 2005), since the intensity of the electrical field has to overcome the surface tension of the solution, expelling a electrified jet from the Taylor's cone formed on the needle tip (Doshi & Reneker, 1995; Taylor, 1969). Therefore, the surface tension of PEO and PVOH solutions containing different amounts of "empty" liposomes was evaluated and subsequently related to the fibers' morphology (Figure 1).

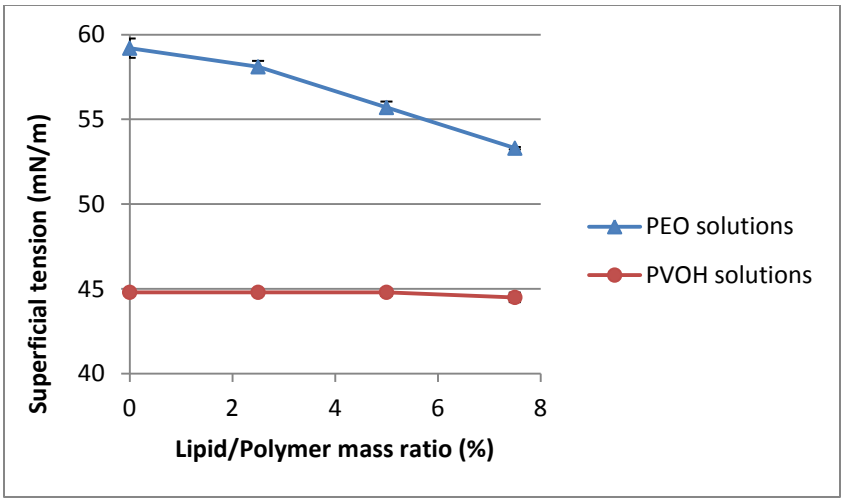


Figure 1 - Superficial tension of PEO and PVOH solutions containing different mass ratios of "empty" liposomes. The mass percentage of liposomes is related to total polymer mass. The error bars correspond to standard deviation of independent duplicates. Measurements performed at 25 °C. The lines are just to guide the eye.

As inferred from Figure 1, the effect of adding empty liposomes on surface tension was different for the PEO and PVOH solutions. While a gradual decrease in surface tension was observed with increasing liposomal content in the PEO solution (from 59.5 mN/m to 53 mN/m), the surface tension of the different PVOH solutions remained constant. PVOH solutions already had a rather low surface tension and, thus, addition of the liposomes had no impact on it.Polymeric solutions containing β-carotene loaded liposomes (0.5% β-carotene/phospholipid molar ratio)(Zompero et al., to be submitted) at the same lipid/polymer mass ratios were also subjected to surface tension measurements, not being observed any difference from the Figure 1 presented results.

Surface tension analysis is an important parameter for electrospinning, but it cannot be taken independently to explain the suitability of a solution for the development of electrospun structures. Addition of liposomes is also expected to affect other important parameters involved in electrospinning such as viscosity and viscoelasticity. These parameters, together with the electrical conductivity of the solutions, are the main factors that determine the suitability of polymeric solutions for electrospinning and the morphology of the structures obtained thereof. Depending on the combination of both

the solution properties and several process parameters (such as applied voltage or distance to collector), three typical types of structures can be formed, mainly beads, beaded fibers and bead-free fibers (Ramakrishna et al., 2005). In order to better understand the morphological features of the hybrid structures obtained, the rheological behavior of the PVOH and PEO-based solutions was analysed. Figures 2A and 2B show the relationship between shear rate and applied stress for PVOH and PEO solutions, respectively, containing different lipid/polymer mass ratios. In addition, Figures 2C and 2D show the correlation between shear rate and apparent solution viscosity.

From Figures 2A and 2C, it can be stated that the pure PVOH solution (without phospholipid addition) presents Newtonian characteristics, since the viscosity was independent from the applied shear rate. Increasing the phospholipid content led to an increase in the solution viscosity, and to a change in the rheological behavior towards a pseudoplastic character, reducing its viscosity with increasing shear rate. The increase in viscosity with increasing lipid/polymer mass ratio was probably consequence of the interactions between hydroxyl groups present along the polymer chain with the phospholipids head groups (Antunes et al., 2009; Boggs et al., 1986).

In the case of PEO, it was observed that addition of phospholipids did not significantly modified the rheological properties of the solution. Figures 2B and 2D show that PEO solutions, even without phospholipids, had a pseudoplastic behavior, which was kept upon nanoliposome addition. This pseudoplastic behavior of PEO solutions indicates that when high shear rates were applied, the molecular structure of the entangled polymer chains acquired a preferential orientation in the flow direction, decreasing the viscosity of the solution (shear thinning flow behavior). In the case of pure PVOH (Newtonian), this phenomenon was not observed and the viscosity is thus referred to as absolute.

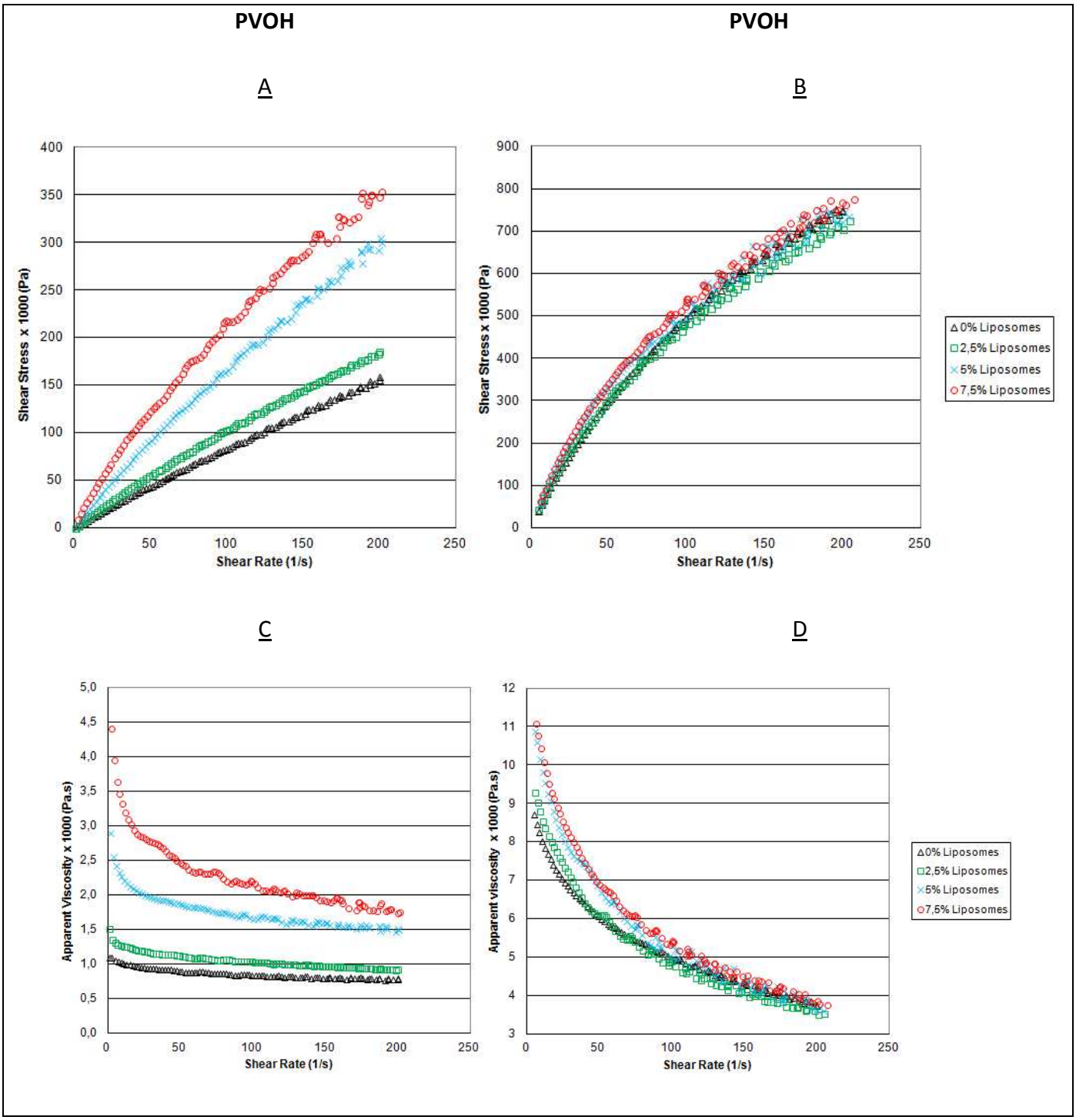
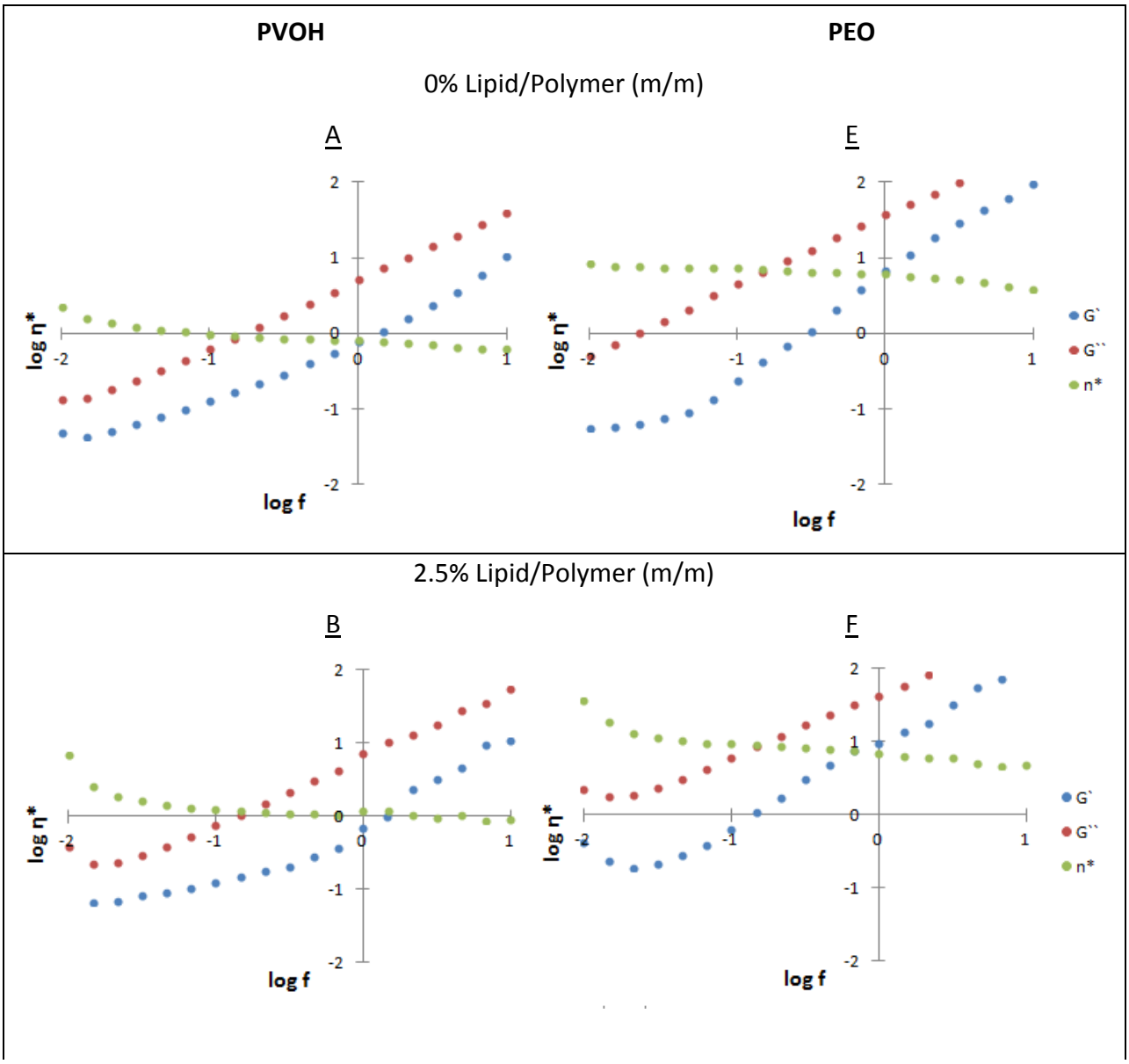


Figure 2 - Rheological behaviour of PVOH and PEO solutions containing different phospholipid mass ratios relating applied stress with shear rate (A,B) and apparent viscosity with shear rate (C,D). All measurements were performed at 20 °C in independent duplicates.

A viscoelastic material is a type of material that, when subjected to shear forces, suffers simultaneously viscous and elastic deformations (Chronakis & Kasapis, 1995). Viscoelasticity, as mentioned earlier, plays an important role in solution electrospinning and, thus, in addition to characterizing the viscosity of the various sample solutions, the viscoelastic behavior in terms of elastic modulus (G') and viscous modulus (G'') was also analysed.


PVOH
PEO
0% Lipid/Polymer (m/m)
A
E
log n*
log f
G`
G``
n*
2.5% Lipid/Polymer (m/m)
B
F

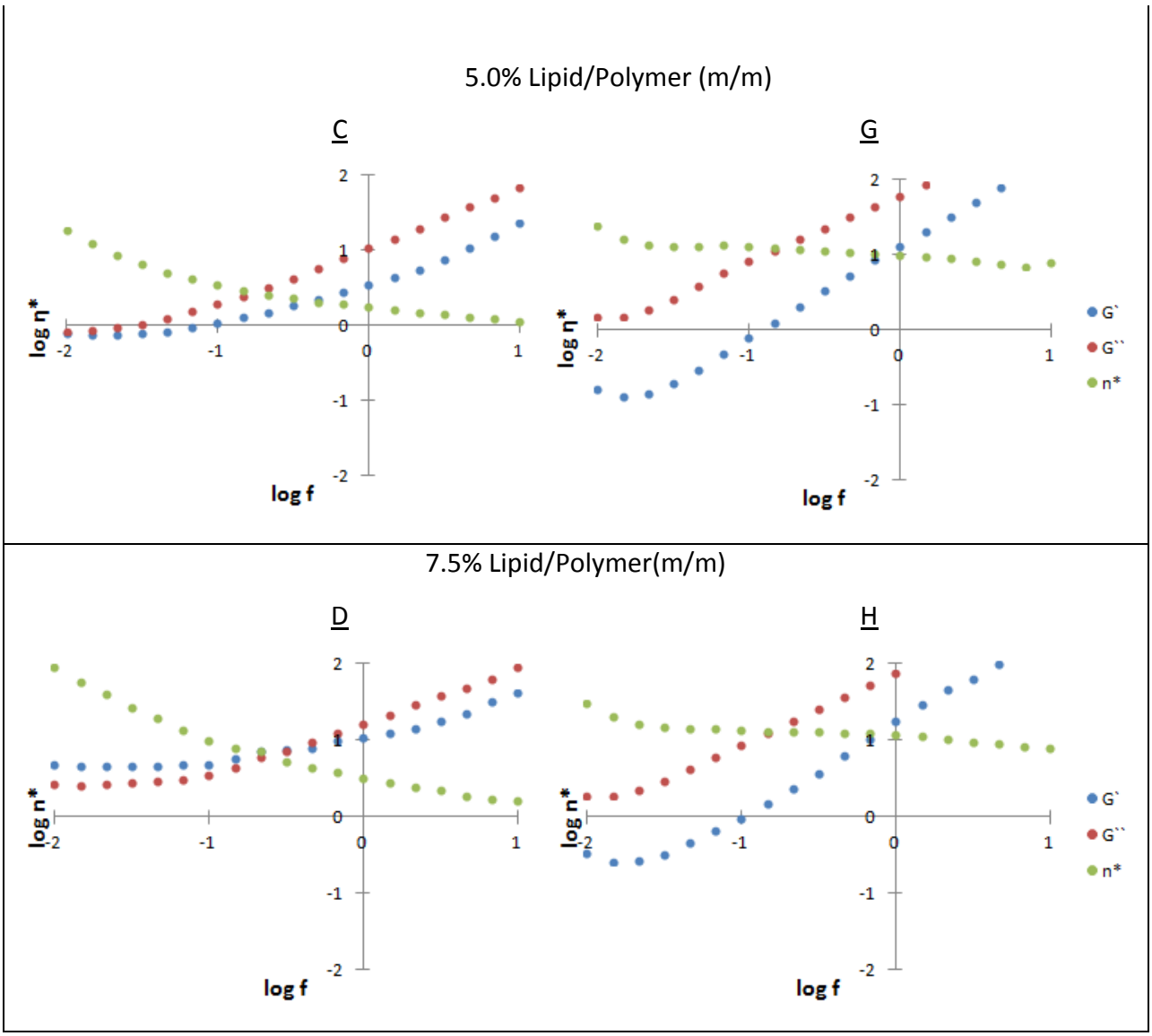


Figure 3 - Elastic modulus (G'), viscous modulus (G'') and aparent viscosity (η*) measured along different applied frequencies (f) to PVOH and PEO samples containing different Liposome/Polymer mass ratios. (A,E) 0%; (B,F) 2.5%; (C,G) 5%; (D,H) 7.5%.

For samples in gel state the storage modulus (G') prevails over the loss modulus (G'') and both of them have no variation with applied frequency. From the obtained data it can be seen that for none of studied solutions such gel state behavior was observed (Chronakis & Kasapis, 1995). Concentrated but not gelled solutions, display slightly higher G'' than G'. In the case of the PEO polymer solution, addition of liposomes did

not significantly changed its viscoelastic behavior and the G' and G'' curves remained virtually the same. On the other hand, for the PVOH polymer, addition of liposomes significantly affected G' and G'' moludus, leading to an increase in storage modulus in comparison with the loss modulus. In other words, the addition of liposomes to PVOH formulations increased the storage and restitution capacities, increasing the rigidity of polymeric networks and the interlacement of polymer chains (Chronakis & Kasapis, 1995; Ambrosio et al., 1999).

3.2. Nanofibers characterizations

3.2.1. Nanofibers morphology studies and diameter determination

The morphology of the different structures obtained with and without nanoliposomes was visualized by Scanning Electron Microscopy (SEM). Both samples containing empty liposomes and liposomes loaded with β-carotene were visualized. Figure 4 shows the obtained images from electrospun fibers.

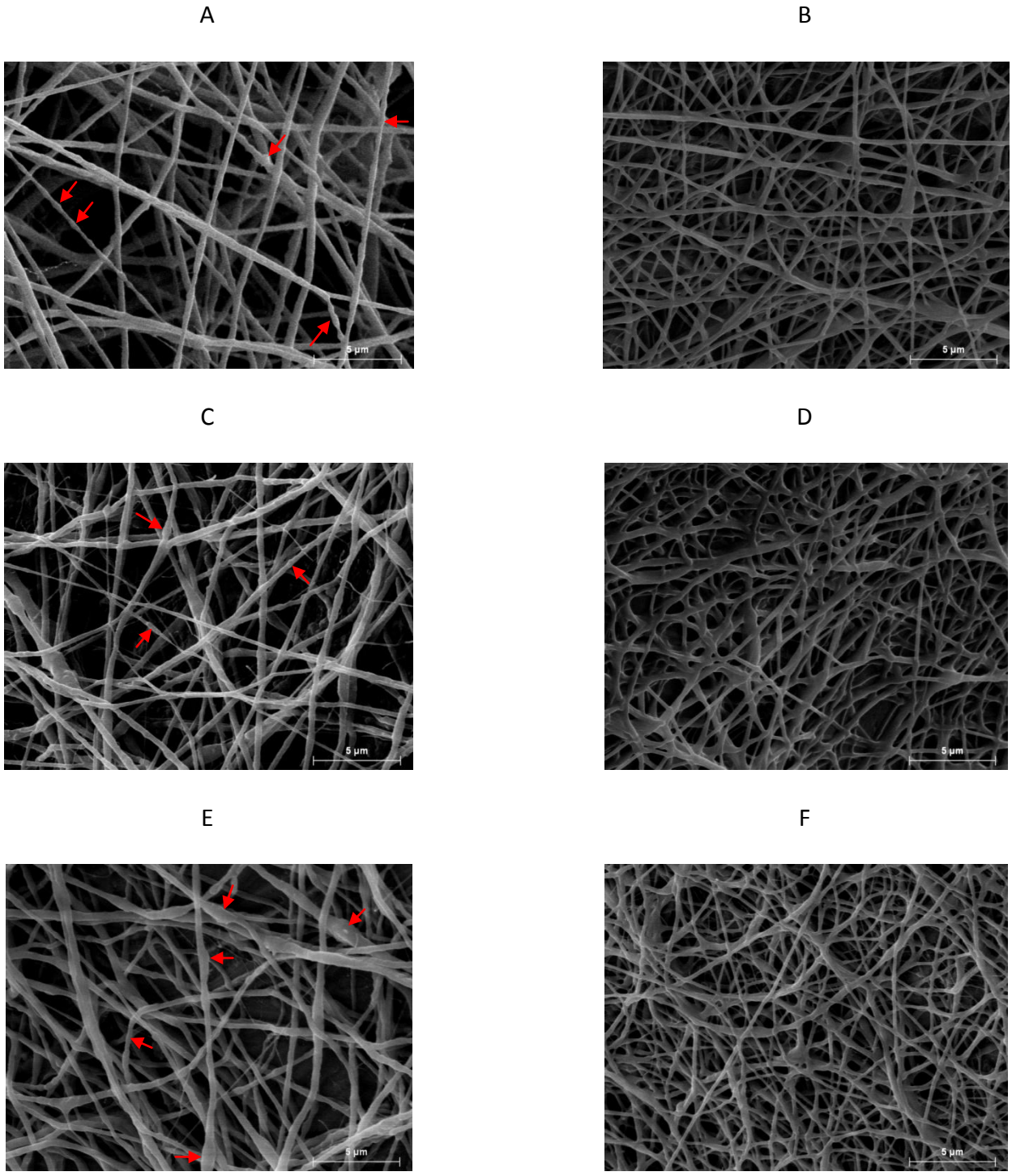


Figure 4 - Scanning Electron Microscopy showing produced nanofibers by electrospinning process. (A) PEO nanofibers, without addition of liposomes; (B) PVOH nanofibers, without addition of liposomes; (C) PEO nanofibers containing 7.5% (m/m) empty liposomes; (D) PVOH nanofibers containing 7.5% (m/m) empty liposomes; (E) PEO nanofibers containing 7.5%

(m/m) liposomes loaded with 0.5% mol β-carotene; PVOH nanofibers containing 7.5% (m/m) liposomes loaded with 0.5% mol β-carotene. Scale bars represent the size of 5 µm. Red arrows represent beads on nanofibers.

Figure 4 shows that ultrathin electrospun fibers were obtained for all the compositions. The average diameter of the fibers was calculated through the analysis of 100 random electrospun structures . The greatest and lowest diameter was also determined. Table 2 contains the summarized diameter results for each lipid/polymer mass ratio studied for PVOH and PEO nanofibers.

Table 1 - Mean, maximun and minimun diameters obtained from the measurement of 100 PVOH and PEO nanofibers containing different Lipid/Polymer mass ratios.

		Lipid/Polymer mass ratio			
		0%	**2,5%**	**5%**	**7,5%**
PVOH	Mean diameter (nm)	194.7 ± 65.6	266.2 ± 99.5	305.9 ± 112.3	407.9 ± 138.6
	Minimun diameter (nm)	85.2	117.0	105.3	148.9
	Maximun diameter (nm)	402.3	613.2	802.2	939.7
PEO	Mean diameter (nm)	287.6 ± 75.5	355,7 ± 84.7	370,1 ± 89.6	379,7 ± 118.8
	Minimun diameter (nm)	64.1	190.5	150.0	150.2
	Maximun diameter (nm)	493.6	717.1	715.2	799.7

Measurements of nanofibers containing β-carotene loaded liposomes resulted on essentially the same results range. Increasing the liposome content in the fibers resulted in an increase on electrospun fibers mean, minimun and maximun diameters. When comparing nanofibers containing empty liposomes and nanofibers containing liposomes loaded with β-carotene the obtained mean diameter was the same. In other words, β-carotene incorporation into liposomes did not have any influence on electrospun fibers diameter. The incorporation of 7.5% (m/m) liposomes on PEO formulations increased around 32% of mean diameter. On the other hand the same liposome addition to PVOH formulations represented an increase of 109% fibers mean diameter. The increase in diameter could be mainly explained by the rheological properties of the electrospun formulations, which directly interfered on electrospinning process dynamics and, consequently, on fibers diameter. The addition of liposomes to

PVOH formulations caused an increase of larger magnitude when compared to PEO formulations. Observing the rheological behavior for both polymer types (Figures 2 and 3) it was noted that the addition of liposomes caused greater variation in viscosity and viscoelasticity characteristics of PVOH than PEO solutions. These observations correlate the rheological characteristics of the solutions to be electrospun with nanofibers diameter, showing that higher solution viscosity leads to greater fibers diameter (Uyar & Besenbacher, 2009).

The morphology of PVOH and PEO electrospun fibers was also different. In the case of fibers produced with PVOH, they are shown as smooth, without the presence of capsules (beads) or occasional agglomerations, characterized as "bead-free nanofibers." In the case of PEO fibers, we note the presence of numerous capsules (beads) along the fibers, characterized as "beaded nanofibers." Initially it was thought that such clusters could be related to the presence of lipid accumulation or even the presence of liposomal structures, but this hypothesis could be readily eliminated when it was observed that the fibers without the addition of liposomes also have the presence of capsules (beads). Thus, the presence of these nanoscale structures (beads) are not related to the presence of liposomes, but with the actual characteristics of the polymer solution (surface tension, viscosity and viscoelasticity) and possible existing molecular interactions. Another factor that greatly influences the morphology of the formed nanofibers is the molar mass of the polymer used. Uyar (2009) and his research group worked with the incorporation of cyclodextrins in PEO nanofibers. From electrospinning of solution containing 3 to 3.5% polymer/water mass ratio they obtained beaded nanofibers morphology whereas for 4% bead-free nanofibers where obtained. Comparing the characteristics of 3.5% and 4% mass ratios, the main difference among them was the viscosity of the solution, varying from 995 cP to 1928 cP. In other words, the increase on solution viscosity decreases the frequency of appearance of capsules along the nanofibers. In this study, the polymer molecular weight used was 900 kDa, allowing the use of low polymer/water mass ratios. In the present study the polymer/water mass ratio studied was 20%, but with a polymer molecular weight of 200 kDa, what causes great structural differences on how the polymeric chains get organized.

3.2.2. FTIR analysis of the encapsulation structures

Fourier transform infrared spectroscopy was used to corroborate that lipidic molecules were effectively incorporated into the electrospun fibers. Characteristic spectra of the lipids from the nanoliposomes and of the various PEO and PVOH fiber compositions were obtained by Fourier Transform Infrared Spectroscopy. Figure 5 shows the spectra for pure PVOH fibers (solid line) and for the hybrid ones containing 7.5% liposomes (dashed line). The dotted line corresponds to the spectrum of the pure lipid and it is used as a reference for evaluating the presence of nanoliposomes in the hybrid fibers. Similarly, Figure 6 shows the spectra corresponding to the PEO materials. A zoom in the spectral range of the CH stretching region is also included in both figures, were the bands associated to the nanoliposomes can be clearly discerned (cf. to arrows).

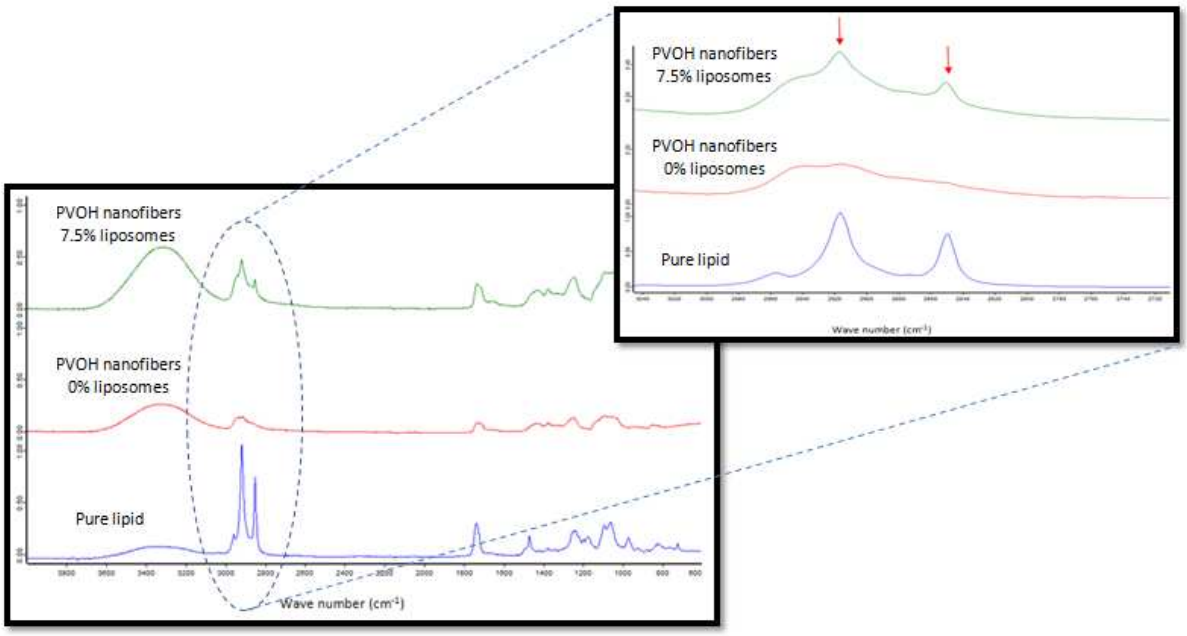


Figure 5 - FTIR spectra for HSPC lipid (blue line), PVOH polymeric film without liposome addition (red line) and PVOH polymeric film with 7.5% (m/m) added liposome (green line). The red arrow stands for the major difference between red and green spectra.

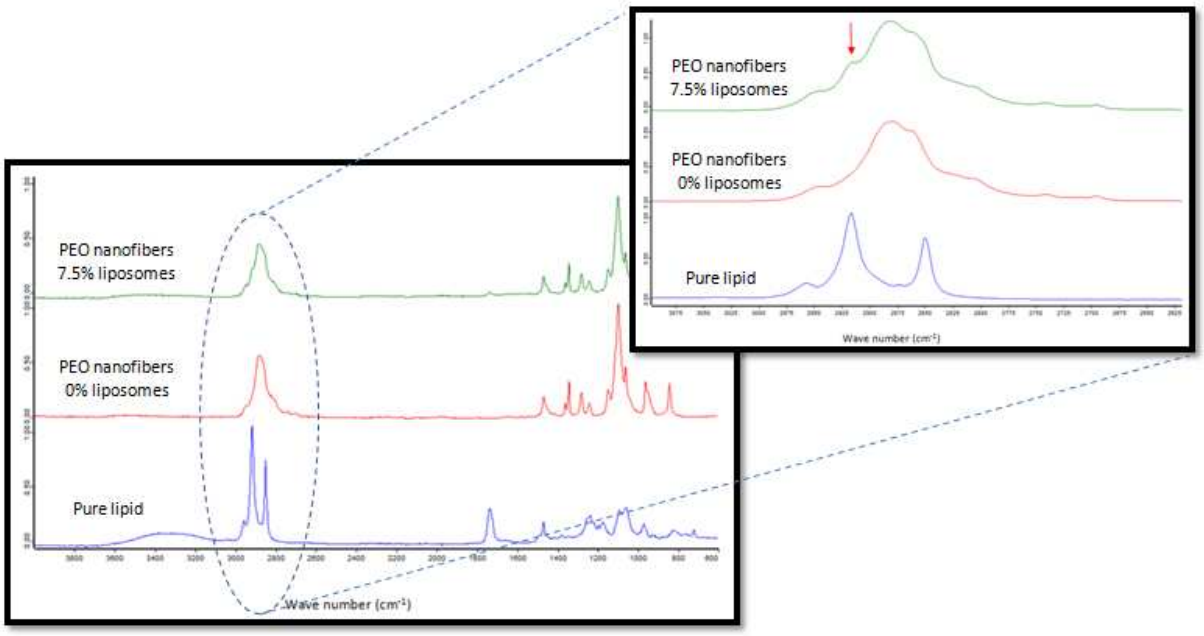


Figure 6 - Expanded obtained FTIR spectra for pure lipid (blue line), PEO polymeric film without liposome addition (red line) and PEO polymeric film with 7.5% (m/m) added liposome (green line). The red arrow stands for the major difference between red and green spectra.

Hybrid fibers containing liposomes loaded with β-carotene were also analysed by Fourier transform infrared spectroscopy, but the obtained spectra was identical to the one containing only empty liposomes. In that way it is possible to state that the amount of β-carotene was too small to be detected by this kind of technique. Addition of the nanoliposomes was mainly characterized by the presence of the CH stretching bands at 2920 cm^{-1} characteristic of the pure lipid. In the case of PEO, only the band at 2920 cm^{-1} was apparent as the other was overlapped with vibrations from the pure polymer.

3.3. Characterization of polymeric nanofibers containing β-carotene loaded liposomes

3.3.1. β-carotene homogeneity determination

The analysis of β-carotene distribution within the electrospun fibers was carried out by means of Raman spectroscopy. This technique has been found to be very useful,

as β-carotene is a strong Raman scatterer. Figure 7 shows the typical β-carotene Raman spectra. The three main vibrational bands obtained are in accordance with previous studies (Fernandez et al., 2009) and they were used to evaluate distribution and stability of the carotenoid in selected pieces of the electrospun fiber mats. Both PVOH and PEO polymers did not present any signal over the tracked Raman shift, thus not causing any interference. To obtain the Raman images, the area of the band at 1500 cm^{-1} was integrated in the 15x15 lm sample area studied. For each of the Raman images, the individual spectrum in different areas with various signal intensities were compared, to estimate whether the antioxidant was degraded in certain areas of the encapsulation structures. Figures 8 and 9 show, as an example, the Raman images of the PVOH and PEO fiber mats, respectively, with the corresponding individual spectra.

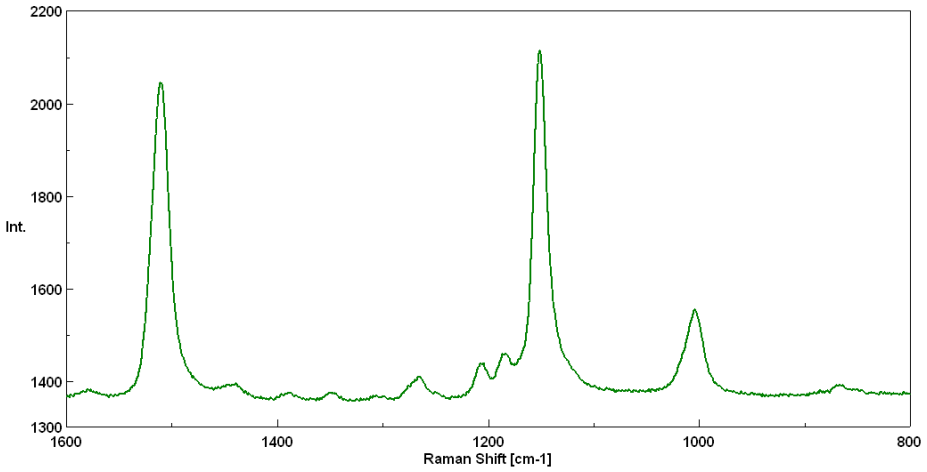


Figure 7 - β-carotene Raman characteristic spectra for β-carotene molecules incorporated on PVOH and PEO nanofibers matrix.

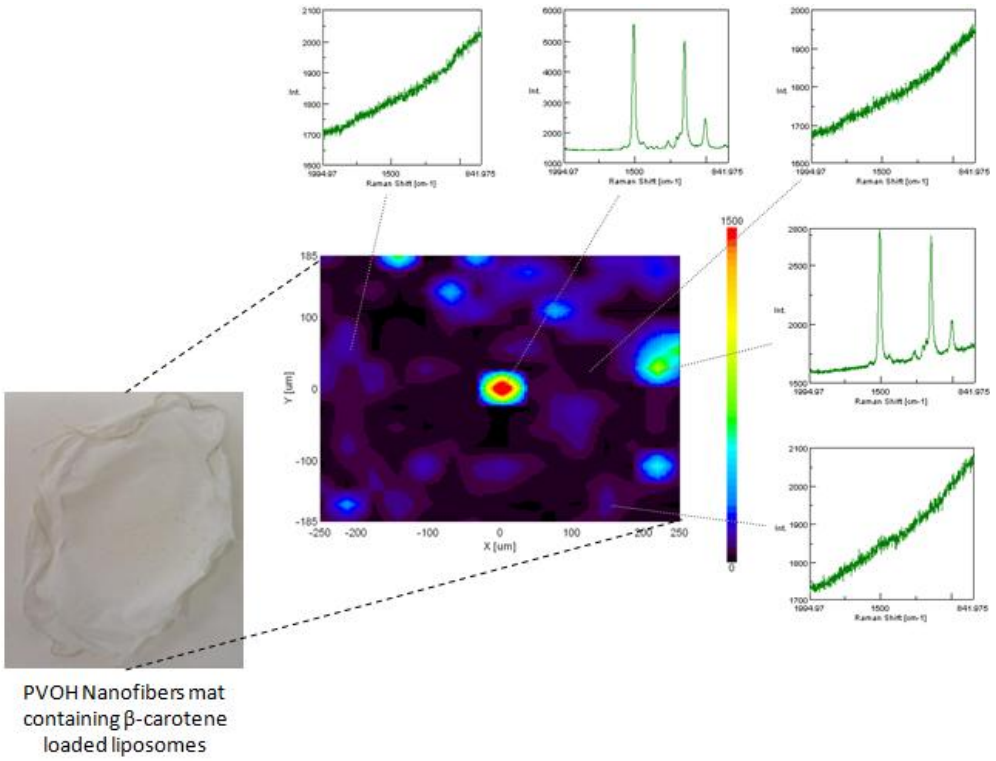


Figure 8 - Image for 1500 cm-1 β-carotene spectra peak tracking inside 185 mm2 PVOH nanofibers mats. The red areas show a great β-carotene intensity signal. Darker regions show lower or none β-carotene intensity signal. Main obtained spectras for different nanofibers mat regions are shown around the central image. Raman image obtained is not related to the whole fiber mat shown on the photo.

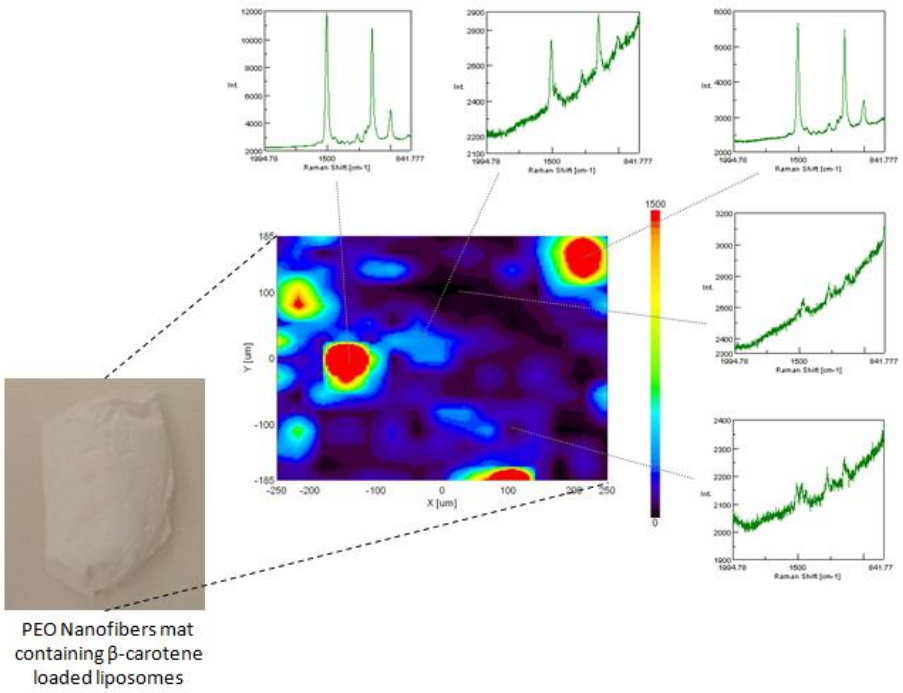


Figure 9 - Image for 1500 cm-1 β-carotene spectra peak tracking inside 185 mm2 PEO nanofibers mats. The red areas show a great β-carotene intensity signal. Darker regions show lower or none β-carotene intensity signal. Main obtained spectras for different nanofibers mat regions are shown around the central image. Raman image obtained is not related to the whole fiber mat shown on the photo.

Fernandez (2009) studied the modification of the β-carotene Raman spectra upon UV irradiation, observing significant differences with the non-oxidised carotenoid spectrum. Comparing the spectra obtained on Figure 7 with the ones obtained by Fernandez and contributors, it can be concluded that the β-carotene molecules inside the obtained electrospun fibers were not degraded, as no peaks related to oxidation were observed. Therefore, it can be stated that the electrospinning technique did not affect the stability of the bioactive during encapsulation. From Figures 8 and 9 the distribution of β-carotene inside the nanofibers mat for both encapsulating polymers can be compared. It is interesting to see that the obtained materials containing encapsulated β-carotene molecules presented white color (not the characteristic orange color from the β-carotene compound), which can be interesting for certain applications. Figures 8 and 9 also show that the distribution of β-carotene inside the electrospun fibers was not

homogeneous, presenting some β-carotene clusters scattered all over the field. β-carotene seemed to be better distributed within the PEO materials as deduced from Figure 9. Even in the regions with lowest Raman signal the presence of small amounts of β-carotene was detected. The difference on β-carotene distributions by changing electrospun polymer is probably due to the different existing molecular interactions. It can be noted on Figure 2 (C,D) (from rheological studies) that PVOH has a greater interaction intensity with the inserted phospholipids, causing a greater variation on solution apparent viscosity. This high intensity interaction occurs probably between phospholipids polar head groups with hydroxyl groups present along PVOH molecules (Boggs et al., 1986). One possible reason for inferior β-carotene homogeneity along PVOH films may be due to this higher intermolecular intensity, causing liposome rupture and β-carotene release and aggregation.

3.3.2. Stability tests by UV-Vis irradiation

The ability of the two polymeric matrices for the protection of β-carotene loaded liposomes was evaluated upon exposure to ultraviolet light. The double encapsulation layer (liposome + nanofiber) is expected to improve the stability of β-carotene when compared to the free carotenoid molecules or those encapsulated using just one of the techniques. For comparison purposes, the degradation of β-carotene-loaded liposomes in aqueous phase and free β-carotene in hexane was also evaluated upon ultraviolet light exposure. β-carotene decay profiles for each system are shown on Figure 10.

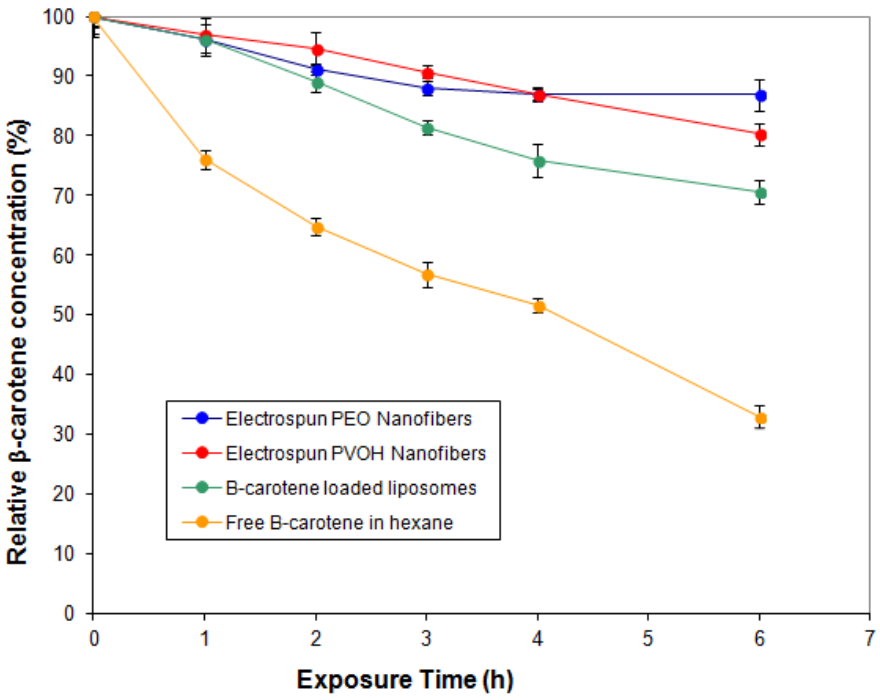


Figure 10 - β-carotene relative concentration level during UV light exposure being protected by different encapsulation systems. For each case 7.5% phospholipid/polymer ratio was used. Samples were kept at 10 cm distance from UV lamp. Experiments were performed in independent duplicates. The lines are just to guide the eye.

From Figure 10 it can be stated that the combination of the nanoliposomes with electrospun fibers significantly decreased β-carotene photooxidation, improving its stability over time. After 6 hours of light exposure 86.8% of initial β-carotene was still intact inside PEO nanofibers and 80.3% inside PVOH nanofibers. These results are considerably better than the ones observed for β-carotene loaded in liposomes (70.7%) and β-carotene in hexane solution (32.9%). The fact that the obtained electrospun fiber mats were white colored (cf. Figures 8 and 9) point out that β-carotene molecules were properly encapsulated within the fibrous polymeric matrices, thus, not being exposed on the surface. Moreover, it is important to note that the solvent extraction procedure from the hybrid structures was not expected to be 100% efficient, and thus, the stability is probably even greater than that reported on Figure 10. Fernandez et al. (2009) studied the degradation of β-carotene encapsulated within zein nanofibers, showing that after 1

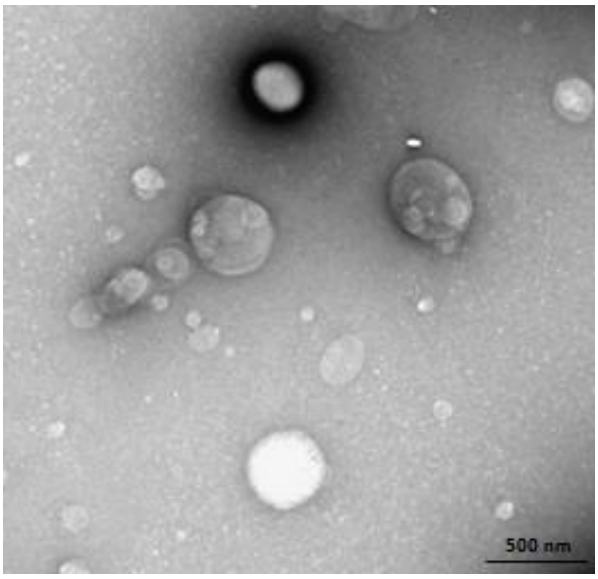
hour UV light exposure there was no substantial variation on β-carotene concentration, which demonstrated that zein has a great protection ability for carotenoid molecules (Rhim et al., 1999). Comparing the protection performance of zein with both PVOH and PEO polymers, it appears that the former was more effective for β-carotene protection over time, but its poor water solubility makes it difficult to be used together with liposomal formulations on electrospun systems.

3.3.3. Liposomes release studies from polymeric nanofibers

In order to verify that the liposomes remained intact within the polymeric fibers produced by electrospinning, small samples of PVOH and PEO fiber mats containing 7.5% phospholipid/polymer mass ratio were immersed in milli-Q water under gentle stirring at room temperature. PVOH and PEO polymers solubilized in the aqueous phase, releasing the entrapped compounds. Samples from the obtained solutions after complete polymer solubilization were evaluated by Transmission Electron Microscopy (TEM) to confirm nanoliposome integrity. The obtained TEM images are shown in Figure 11.

PVOH + 7.5% phospholipids (w/w)

A



PEO + 7.5% phospholipids (w/w)

B

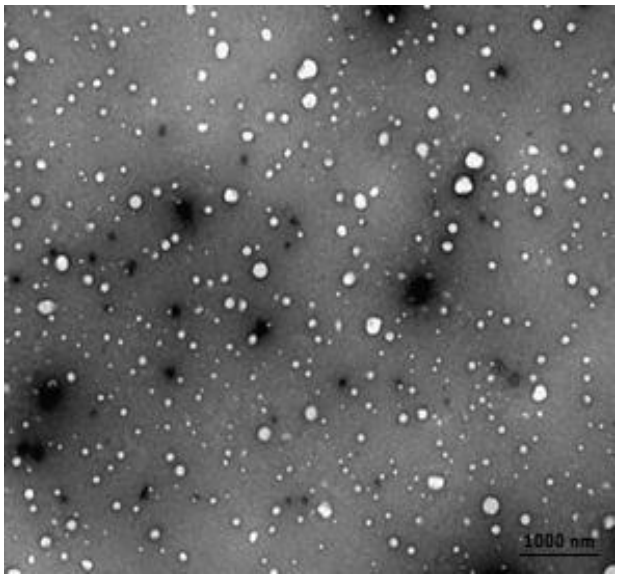


Figure 11 - Transmission Electron Microscopy showing formed nanoparticles after (A) PVOH; (B) PEO nanofibers dissolution on aqueous phase. Dissolution was held under gentle stirring at room temperature. Bottom right scale bar stands for (A) 500 nm; (B) 1000 nm.

Only few studies have developed similar hybrid systems of nanoliposomes within electrospun polymeric fibers (Mickova et al., 2012; Yu et al., 2011) but as far as we know, none of the studies reported about the release of the nanoliposomes from the hybrid structures. From the images in Figure 11 it appears that the integrity of the nanoliposomes (with sizes around 250 nm) is effectively maintained when incorporated within the electrospun fibers. These results demonstrate the potential of combining both encapsulation technologies for the development of novel systems and devices with unique characteristics for a wide range of applications.

4. Concluding Remarks

Incorporation of β-carotene loaded nanoliposomes within PVOH and PEO electrospun fibers was successfully carried out. The effects of nanoliposome addition on the properties of the polymeric solutions were evaluated showing that the lipidic nanoparticles had a greater interaction with PVOH, substantially modifying rheological solution properties and, consequently, electrospinning performance. Therefore, nanoliposome addition to PVOH solutions had a greater effect on electrospun fibers average diameter than when incorporated into PEO solutions. The Raman spectra of the hybrid systems showed that β-carotene molecules were not degraded during the electrospinning process. Moreover, the fact of combining the β-carotene loaded nanoliposomes with electrospun fibers conferred additional UV photostability to the carotenoid molecules as inferred from the spectrophotometry studies. Finally, the integrity of the nanoliposome structures within the electrospun fibers was confirmed after solubilization of the polymeric matrices in aqueous media, highlighting the potential of these hybrid structures for various applications in different industrial fields.

5. Acnowledgements

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Capítulo 5 – Conclusões Finais

A partir do desenvolvimento do presente trabalho pode-se concluir que:

- A quantidade limite de etanol que não afetou o diâmetro final dos lipossomas formados foi estimada em 10% v/v, valor utilizado como base na formulação dos demais ensaios.
- O tempo de agitação não afetou significativamente o diâmetro médio e a polidispersidade dos lipossomas produzidos, tanto para os sistemas de agitação do tipo Ultra-Turrax como para o sistema utilizando o impelidor tipo Cowles.
- Os efeitos das variáveis estudadas nos parâmetros de diâmetro, polidispersidade e potencial zeta dos lipossomas foram estimados com sucesso. Pode-se concluir que o efeito da concentração é o mais estatisticamente significativo, sendo que temperatura, tempo de agitação e velocidade de rotação do impelidor exercem influências menores. As condições determinadas como ótimas para o processo de injeção foram: Temperatura = 60 °C; Velocidade de rotação Cowles = 1336 rpm; Concentração lipídica = 4,64 mM.
- Não foram observadas diferenças expressivas entre os resultados produzidos utilizando-se sistema de agitação Ultra-Turrax e impelidor Cowles. O sistema de agitação Ultra-Turrax produziu diferenças de amplitude pouco menores nos resultados de um ensaio para o outro, fator que não contribuiu suficientemente para a exclusão do impelidor Cowles como sistema a ser adotado, escolha baseada principalmente em sua simplicidade de operação, facilidade de obtenção, projeto, custo e manutenção.
- Os modelos matemáticos obtidos, tanto os completos como os reparametrizados, apresentaram excelentes ajustes aos dados experimentais, predizendo fielmente o comportamento do sistema, a

exceção dos modelos obtidos para a variável resposta potencial zeta, sendo que o valor para esta variável manteve-se praticamente constante ao longo de todos os ensaios realizados.

- Os lipossomas produzidos na melhor condição obtida do planejamento experimental apresentaram-se estáveis em relação ao diâmetro, polidispersidade e potencial zeta durante o período em que foram analisados (4 semanas).
- A morfologia dos lipossomas obtida por microscopia eletrônica de transmissão foi praticamente esférica, de tamanho correspondente às medidas de DLS realizadas. Observou-se também uma população de discos em meio aos lipossomas mostrando que, provavelmente, os lipossomas não sejam a única estrutura estável que pode ser formada a partir dos lipídios utilizados.
- A avaliação visual das soluções de β-caroteno produzidas pelo método de injeção de etanol foi satisfatória, mostrando que parte do β-caroteno efetivamente solubilizou-se em água. Observou-se também que a coloração da solução sobrenadante, para razões molares β-caroteno/lipídio acima de 0,5%, manteve-se constante, indicando um provável limite de incorporação.
- Os ensaios de DLS dos lipossomas contendo β-caroteno não conseguiram captar alterações no diâmetro, polidispersidade ou potencial zeta destes, tanto nas amostras antes e após centrifugação.
- Os estudos das monocamadas de Langmuir mostraram que a maior razão molar β-caroteno/lipídio que pode ser incorporada é de 0,5%, resultado semelhante ao apresentado na avaliação visual das soluções de β-caroteno produzidas pelo método de injeção de etanol.
- Ensaios de adição de lipossomas as formulações poliméricas a serem eletrofiadas mostraram que a interação intermolecular PVOH/Lipossomas é mais intensa que a PEO/Lipossomas. A adição de lipossomas as soluções de PVOH causaram diferenças reológicas significativas, elevando a viscosidade da solução e o módulo elástico G', sugerindo maior entrelaçamento entre as cadeias poliméricas.

- Ensaios de eletrofiação foram conduzidos com sucesso, mostrando a viabilidade da formação de nanofibras a partir das soluções poliméricas contendo diferentes quantidades de lipossomas adicionados.
- Ensaios de espectroscopia de infrevermelho por transformada de Fourier mostraram que moléculas fosfolipídicas foram adicionadas as nanofibras com sucesso, tanto as de PVOH como as de PEO.
- Microscopias eletrônicas de varredura das nanofibras produzidas com ambos polímeros mostraram que, de fato, as nanofibras apresentam-se em escala nanométrica, sendo que a adição de lipossomas causou uma maior variação na amplitude de diâmetros das fibras de PVOH quando comparado as fibras de PEO. Este fato deve-se provavelmente a maior viscosidade da solução de PVOH quando da presença de lipossomas adicionados, causando um maior entrelaçamento das cadeias devido a interação PVOH/Lipossomas.
- Análises de espectroscopia Raman mostraram que as moléculas de β-caroteno internalizadas nas nanofibras de ambos os polímeros mostram-se sem sinais de oxidação, fato que demonstra que a submissão deste carotenoide a elevados valores de campo elétrico durante o processo de eletrofiação não causam efeitos adversos como a oxidação ou desnaturação do composto estudado.
- A distribuição de β-caroteno dentro dos filmes de nanofibras de PVOH mostrou-se menos homogênea quando comparado as nanofibras de PEO, provavelmente devido a maior interação PVOH/Lipossomas relatada anteriormente.
- Ensaios de exposição dos filmes poliméricos contendo β-caroteno em lipossomas mostraram que a incorporação em nanofibras, tanto de PVOH como de PEO, confere uma maior proteção à molécula de β-caroteno quando exposta a radiação UV. O fato de os filmes poliméricos produzidos apresentarem-se na coloração branca é um indício que as moléculas de β-caroteno apresentam-se internalizadas nas nanofibras.

- A viabilidade da formação de lipossomas a partir da reidratação dos filmes poliméricos de PVOH e PEO foi demonstrada a partir de análises de microscopia eletrônica de transmissão realizadas, sendo que nestas observa-se a formação de agregados esféricos em escala nanométrica.

Capítulo 6 – Sugestões para trabalhos futuros

Para a continuação dos estudos e investigações referentes ao desenvolvimento de técnicas de encapsulação de β-caroteno em diferentes sistemas e matrizes, as seguintes sugestões são citadas:

- Estudos realizados neste trabalho demonstraram que o efeito do tempo de agitação durante o processo de formação dos lipossomas é desprezível, sugerindo que os lipossomas formam-se praticamente instantaneamente assim que a solução orgânica entra em contato com a fase aquosa. Desta forma, a utilização de sistemas que possuem tempo de residência menor para produção de lipossomas em processo contínuo pode ser uma alternativa promissora.
- Estudos com diferentes fosfolipídios ou misturas de fosfolipídios podem ser realizados para verificar possíveis diferenças nos diâmetros hidrodinâmicos, polidispersidade e capacidade de incorporação de β-caroteno comparativamente com o presente trabalho desenvolvido.
- Estudos com diferentes carotenoides ou outros compostos hidrofóbicos podem ser realizados para verificar a variação causada nos resultados quando o composto hidrofóbico modelo é alterado.
- Estudos de monocamadas de Langmuir podem ser realizados para maior elucidação da interação intermolecular PVOH/Lipossomas e PEO/Lipossomas. Podem ser utilizados PVOH e PEO de diferentes massas moleculares e lipossomas produzidos a partir de diferentes misturas de fosfolipídios.