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MARIANO MICHELON

PRODUCTION OF LIPOSOMAL SYSTEMS BY MICROFLUIDIC DEVICES AIMING FOOD APPLICATIONS

PRODUÇÃO DE SISTEMAS LIPOSSOMAIS EM DISPOSITIVOS MICROFLUÍDICOS VISANDO APLICAÇÕES ALIMENTÍCIAS

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RESUMO

Este projeto teve como objetivo o desenvolvimento tecnológico de processos microfluídicos para a obtenção de estruturas lipossomais visando à sua utilização como sistemas de encapsulação para as indústrias de alimentos. Inicialmente foi avaliada a viabilidade técnica de obtenção de nanovesículas incorporadas com β-caroteno utilizando diferentes lecitinas comerciais de soja pelo método de injeção de etanol. O tipo de lecitina utilizada para a produção de nanovesículas não influenciou na capacidade de carregamento do β-caroteno, porém influenciou nas propriedades estruturais dos lipossomas formados. Assim, foi possível utilizar uma lecitina comercial de soja de baixo custo contendo triacilgliceróis para a produção de lipossomas visando à veiculação do β-caroteno. Na segunda etapa, lipossomas foram produzidos continuamente utilizando dispositivos microfluídicos de focalização hidrodinâmica. Este estudo objetivou um aumento da taxa de produção de lipossomas para uma redução no número de canais visando a um sistema microfluídico paralelizado mais eficiente. Diferentes estratégias para o aumento da produtividade foram avaliadas em função dos impactos causados nas distribuições de tamanho dos lipossomas formados. Os resultados indicam que lipossomas com baixa polidispersidade podem ser obtidos continuamente com uma alta taxa de síntese, utilizando dispositivos microfluídicos com canais de seção retangular de elevada relação largura/altura em altas vazões volumétricas e alta concentração de lecitina. Na terceira etapa, lipossomas gigantes baseados em emulsões duplas A/O/A foram produzidos em um dispositivo microcapilar de vidro, empregando uma mistura de acetato de etila e pentano em substituição às misturas contendo clorofórmio e/ou hexano normalmente utilizadas para solubilizar os fosfolipídios. O tamanho das gotas das emulsões duplas foi precisamente ajustado com uma variação da vazão da fase contínua. Assim, de forma inédita, foi possível desenvolver um processo microfluídico para produção de lipossomas gigantes utilizando ingredientes e materiais com possibilidades reais de aplicação em sistemas alimentícios. Todos os resultados experimentais obtidos sugerem que lecitinas comerciais de soja amplamente disponíveis como emulsificantes para indústria de alimentos podem ser utilizadas como uma alternativa de baixo custo para obtenção de sistemas lipossomais incorporados com β-caroteno. Além disso, podemos concluir que diferentes processos microfluídicos podem ser empregados para obtenção de diferentes estruturas lipossomais.

Palavras-chave: Lipossomas; Microfluídica; Lecitinas

ABSTRACT

The project evaluated the technological development of microfluidic processes for production of liposomal structures aiming their use as delivery systems for food industries. In the first step, it was evaluated the technical feasibility of obtaining β-carotene-incorporated nanovesicles using different commercial soybean lecithin by the ethanol injection method. The lecithin-type used did not influence on loading capacity of β-carotene, but it influenced on the structural properties of the liposomes generated. Thus, it was possible to use commercial low-cost soybean lecithin containing triacylglycerol for the production of liposomes for β-carotene delivery. In the second step, liposomes were produced in a continuous mode using hydrodynamic flow-focusing microfluidic devices. This step aimed at increasing the production rate of liposome for a reduction in the number of channels to obtain a more efficient parallelized microfluidic system. Different strategies to increase liposome throughput were evaluated as a function of the impacts caused on the size distributions of the liposomes generated. The results indicate that liposomes with low polydispersity can be obtained in continuous mode at a high synthesis rate. The best results were observed using microfluidic devices with cross-section area of high aspect-ratio channels, at high volumetric flow rates and lecithin concentration. In the third step, giant liposomes based on W/O/W double emulsions were produced using a glass capillary device through an ethyl acetate/pentane mixture in replacement of a mixture containing chloroform and hexane normally used for phospholipid dissolution. The droplet size of the double emulsions was tuned by a flow variation of continuous phase. Thus, in an unprecedented way, it was possible to develop a microfluidic process for the production of giant liposomes using ingredients and materials with true possibilities of application in food systems. All experimental results obtained suggest that commercial soybean lecithins widely available as emulsifiers for the food industry can be used as a low-cost alternative to obtain β-carotene-incorporated liposomal systems. Apart that, we can conclude that different microfluidic processes can be used to produce different liposomal structures.

Keywords: Liposomes; Microfluidics; Lecithin

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- INTRODUÇÃO GERAL
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1.1. Introdução geral

Lipossomas são sistemas auto-organizados que constituem vesículas esféricas de uma ou mais bicamadas concêntricas de lípidios que isolam um ou vários compartimentos internos do meio aquoso externo. Dependendo do tamanho e de suas propriedades, os lipossomas podem ser utilizados para veiculação de compostos hidrofílicos, hidrofóbicos ou anfifílicos como ingredientes bioativos de interesse industrial. A veiculação em lipossomas pode aumentar a estabilidade dos compostos ativos e promover a manutenção da atividade em condições que normalmente levariam a uma degradação rápida. Além disso, em comparação com alguns sistemas carreadores, os lipossomas apresentam elevada biocompatibilidade, são biodegradáveis e atóxicos. Por estes motivos, o uso dos lipossomas como sistemas de encapsulação despertam o interesse das indústrias de alimentos, farmacêutica e agrícola (Frézard et al., 2005; Laye et al., 2008; Mozafari, et al., 2008a; Mozafari, et al., 2008b).

Os lipossomas são sistemas metaestáveis, portanto a maior limitação para sua utilização é a tendência de agregação em função do tempo, além da possibilidade de ruptura da bicamada fosfolipídica e a liberação prematura dos compostos bioativos encapsulados. Esta estabilidade limitada se deve em parte à natureza da carga superficial da maior parte dos lipossomas, pois fosfolipídios zwiteriônicos são majoritariamente utilizados nas formulações, como fosfatidilcolinas purificadas. Uma alternativa para elevar a estabilidade coloidal é promover a repulsão eletrostática entre as vesículas com emprego de fosfolipídios aniônicos, como os presentes minoritariamente em lecitina natural de soja, como o ácido fosfatídico e o fosfatidilglicerol. Além disso, lecitinas naturais de soja, que são utilizadas largamente como emulsificantes na indústria de alimentos, custam aproximadamente 20% do valor de fosfolipídios purificados, que são normalmente empregados em aplicações farmacêuticas (Yokota et al., 2012).

Além das limitações devido à estabilidade, outro desafio que deve ser considerado são as técnicas de produção de lipossomas. Nas últimas décadas uma variedade de métodos de formação de lipossomas foi desenvolvida. Entre estes métodos, estão incluídos dentre outros, técnicas clássicas como a hidratação do filme lipídico (Bangham et al., 1965), a evaporação em fase reversa e a injeção de etanol (Batzri & Korn, 1973). No entanto, uma maior versatilidade nas aplicações dos lipossomas exige o desenvolvimento de processos mais robustos, já que os métodos clássicos proporcionam um controle limitado do processo e uma baixa reprodutibilidade. Além disso, nessas técnicas clássicas é difícil controlar o tamanho

das vesículas formadas, sendo necessárias etapas adicionais de processamento, tais como cromatografia de exclusão de tamanho, forças mecânicas de cisalhamento e extrusão com membranas, para atingir uma distribuição monodispersa de tamanho. Os processos microfluídicos, que foram recentemente desenvolvidos, seriam uma alternativa para superar estas dificuldades, seja como uma adaptação aos processos convencionais ou como processos totalmente inovadores (Mijajlovic et al., 2013; Swaay & deMello, 2013).

Como tecnologia inovadora, a microfluídica se refere ao processamento de fluidos realizados com pequenos volumes escoando em dutos de dimensões submilimétricas e, consequentemente, números de Reynolds muito baixos. A grande vantagem da operação dos processos microfluídicos para formação de lipossomas é o regime estritamente laminar. Através do escoamento paralelo de dois fluidos miscíveis sob condições laminares, a região de difusão entre as fases é considerada uma interface dinâmica, cuja transferência de massa pode ser facilmente controlada, obtendo estruturas lipossomais uniformes com dimensões coloidais. Além disso, as técnicas microfluídicas podem ser empregadas para o escoamento de duas fases imiscíveis para geração de intermediários micrométricos, como gotas de emulsões duplas, originando estruturas lipossomais diferenciadas. A microfluídica se destaca também pela perspectiva de intensificação dos processos, buscando reduzir o tamanho da planta e o consumo de energia em várias ordens de grandeza. A técnica de produção de lipossomas em dispositivos de microfluídica de focalização hidrodinâmica foi desenvolvida e aplicada com sucesso (Jahn et al., 2004; Atencia & Beebe, 2005; Jahn et al., 2008; Jahn et al., 2010; Swaay & deMello, 2013).

A produção microfluídica de lipossomas ainda está fortemente baseada na utilização de dispositivos planares de focalização hidrodinâmica, sendo conduzida através da introdução de uma dispersão de fosfolipídios em solvente orgânico no canal central do dispositivo, e posteriormente pela constrição dessa corrente central por uma ou mais correntes aquosas perpendiculares. A focalização da corrente central resulta na depleção controlada da concentração do solvente orgânico através da sua difusão para a fase aquosa, desencadeando assim a auto-organização dos fosfolipídios em bicamadas e posteriormente em lipossomas. Foi demonstrado que, através da microfluídica de focalização hidrodinâmica, são formados lipossomas unilamelares com uma distribuição de tamanho que pode ser facilmente controlada por meio da razão entre as vazões volumétricas das correntes aquosa e orgânica. Desta forma, a microfluídica se caracteriza como um método simples e de alta reprodutibilidade para produção de lipossomas unilamelares com distribuição de tamanho

controlado (Jahn et al., 2008; Jahn et al., 2010; Mijajlovic et al., 2013; Swaay & deMello, 2013).

Neste contexto, o desenvolvimento de processos microfluídicos para produção de lipossomas com elevada reprodutibilidade que permitam a formação de partículas uniformes resultará na obtenção de sistemas de encapsulação estáveis e de interesse para as indústrias de alimentos. O desenvolvimento de dispositivos microfluídicos para formação de vesículas lipossomais com distribuição de tamanho com baixa polidispersidade continua sendo um grande desafio científico. Além disso, processos microfluídicos de geração de gotas utilizando dispositivos capilares para obtenção de estruturas lipossomais diferenciadas ainda são pouco explorados na literatura científica. Portanto, este trabalho teve como objetivo o desenvolvimento tecnológico de processos microfluídicos para a obtenção de diferentes tipos de estruturas lipossomais. Além disso, objetivou-se a sua utilização na veiculação do β-caroteno como um composto ativo hidrofóbico modelo de interesse para indústria de alimentos e o entendimento das propriedades estruturais, tecnológicas e funcionais dos sistemas lipossomais obtidos.

1.2. Objetivos

1.2.1. Geral

Este projeto de pesquisa teve como objetivo geral o desenvolvimento tecnológico de processos microfluídicos para a obtenção de estruturas lipossomais visando a sua utilização como sistemas de encapsulação para as indústrias de alimentos.

1.2.2. Específicos

(i) Produzir e caracterizar lipossomas utilizando lecitinas comerciais de soja pela técnica convencional de injeção de etanol.

(ii) Desenvolver e avaliar uma plataforma microfluídica planar de focalização hidrodinâmica para produção contínua de lipossomas.

(iii) Desenvolver e avaliar um microdispositivo capilar de vidro para obtenção de estruturas lipossomais baseadas em emulsões duplas.

(iv) Avaliar a viabilidade técnica da incorporação do β-caroteno nos lipossomas como um composto ativo hidrofóbico modelo.

1.3. Estruturação da tese

As etapas do desenvolvimento do projeto de pesquisa estão apresentadas na forma de capítulos. Neste Capítulo I são abordados o tema de estudo e os objetivos pretendidos. No Capítulo II é apresentada uma breve revisão bibliográfica sobre temas gerais relacionados aos lipossomas e aos processos microfluídicos. O Capítulo III contempla um artigo que avaliou a utilização de lecitinas comerciais de soja para obtenção de lipossomas incorporados com β-caroteno utilizando a técnica convencional de injeção de etanol. O Capítulo IV apresenta os resultados sobre a produção de lipossomas utilizando dispositivos microfluídicos planares de focalização hidrodinâmica. No Capítulo V são apresentados dados experimentais sobre a produção de lipossomas gigantes, baseados em emulsões duplas, utilizando dispositivos microcapilares de vidro. O Capítulo VI traz uma discussão integrada dos capítulos experimentais anteriores, bem como os resultados mais relevantes, melhorando assim o entendimento geral da tese. No Capítulo VII são apresentadas, de forma sucinta, as conclusões gerais obtidas durante o desenvolvimento do projeto de pesquisa. Por fim, o Capítulo VIII traz algumas sugestões para o desenvolvimento de pesquisas futuras e a memória do período de doutorado.

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CAPÍTULO II

- REVISÃO BIBLIOGRÁFICA

2.1. Revisão bibliográfica

2.1.1. Lipossomas

Os lipossomas são vesículas aproximadamente esféricas com dimensões variando de alguns nanômetros a alguns micrômetros, constituídas de uma ou mais bicamadas concêntricas de lipídios, que podem isolar um ou vários compartimentos aquosos internos do meio externo, conforme ilustra a Figura 2.1. Os lipossomas são formados por lipídios anfifílicos, caracterizados pela presença de grupos hidrofílicos e hidrofóbicos na mesma molécula. De acordo com a teoria de formação de lipossomas por técnicas de injeção de solventes, quando os fosfolipídios dispersos numa fase orgânica são injetados em uma solução aquosa, os mesmos têm a capacidade de auto-organização em intermediários planares denominados de fragmentos de bicamadas fosfolipídicas, expondo suas cabeças hidrofílicas e escondendo as caudas hidrofóbicas do meio aquoso. A difusão contínua do solvente orgânico na água provoca uma redução na solubilidade dos fosfolipídios e uma instabilidade termodinâmica nas bordas dos fragmentos, induzindo sua curvatura e seu fechamento em vesículas. Portanto, a auto-organização dos fosfolipídios em bicamadas é a configuração de menor energia livre do sistema. No entanto, os lipossomas formados não estão em equilíbrio termodinâmico, devido à curvatura inerente da forma esférica. Este fato confere aos lipossomas uma estabilidade relativa, que irá depender da composição, temperatura e pressão do meio onde se encontram dispersos (Lasic, 1989; Mertins et al., 2005; Mertins et al., 2006; Akbarzadeh et al., 2013; Frézard et al., 2005; Keller, 2001; Mozafari, et al., 2008a; Ullrich et al., 2013).

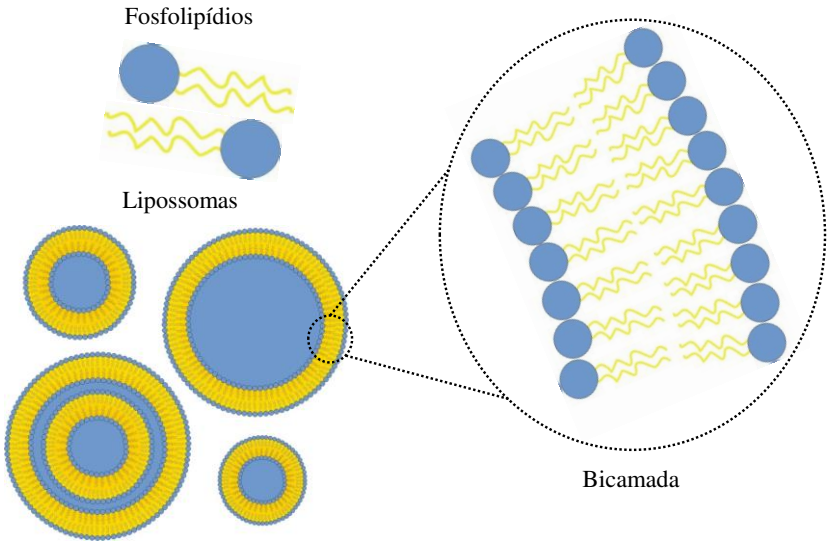

Figura 2.1. Diagrama esquemático das características estruturais dos fosfolipídios e do mecanismo de formação dos lipossomas.

Um aspecto importante para a caracterização estrutural dos lipossomas é a sua lamelaridade, que é definida pelo número de bicamadas. De acordo com a Figura 2.2, um lipossoma constituído por uma série de bicamadas concêntricas é classificado como uma vesícula lipossomal multilamelar (MLV), enquanto que lipossomas compostos de muitas vesículas pequenas, não concêntricas, encapsuladas dentro de uma única bicamada são denominados sistemas multivesiculares (MVV). Outros tipos de lipossomas são as vesículas unilamelares, constituídas por uma única bicamada lipídica, podendo ser pequenas (SUV, <100 nm) ou grandes (LUV, >100 nm). Alguns autores definem os LUVs como lipossomas compreendidos entre 100 nm e 1 µm, e classificam os lipossomas unilamelares superiores a 1 µm como lipossomas gigantes (GUV) (Batista et al., 2007; Mozafari, et al., 2008b; Swaay & deMello, 2013).

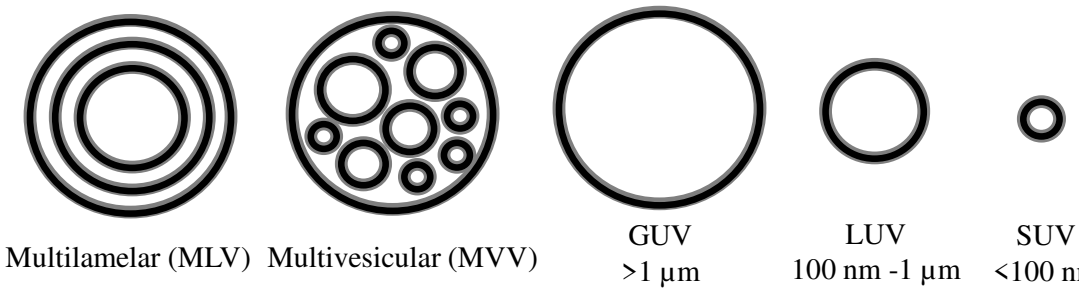

Figura 2.2. Classificação dos lipossomas em relação ao tamanho e a lamelaridade; MLV (*multilamellar vesicles*), MVV (*multivesicular vesicles*), GUV (*giant unilamellar vesicles*), LUV (*large unilamellar vesicles*) e SUV (*small unilamellar vesicles*). Adaptado de Swaay & deMello (2013).

As moléculas anfifílicas mais comuns que apresentam a capacidade de auto-organização em bicamadas são os fosfolipídios de duplas cadeias de ácidos graxos. Fosfolipídios comuns, também conhecidos como ésteres de ácido fosfatídico ou lecitinas, como por exemplo, as fosfatidilcolinas, apresentam duas moléculas de ácidos graxos esterificadas no primeiro e segundo grupo hidroxila do glicerol. Já o terceiro grupo hidroxila forma uma ligação éster com o ácido fosfórico. As fosfatidilcolinas ainda apresentam um grupo amina esterificado ao ácido fosfórico, formando assim a cabeça polar da molécula. A Figura 2.3 (a) apresenta a estrutura do 1,2-dipalmitoil-*sn*-glicero-3-fosfatidilcolina (DPPC), um exemplo de fosfatidilcolina saturada, em que a região polar é formada pelo grupo colina e a região apolar por duas cadeias de ácido palmítico.

A classificação dos fosfolipídios está relacionada a aspectos estruturais dos grupos polares e das cadeias de ácidos graxos. Os grupos polares podem ser zwiteriônicos, catiônicos ou aniônicos. A fosfatidilcolina é um exemplo clássico de fosfolipídio zwiteriônico, pois em pH igual a 7 o grupo fosfato tem uma carga negativa e o grupo colina é carregado positivamente, conforme se verifica na Figura 2.3 (a). Já o fosfatidilglicerol é um exemplo de fosfolipídio aniônico, cuja região polar apresenta uma carga negativa única, como é exemplificado pelo 1,2-dioleoil-*sn*-glicero-3-[fosfo-rac-(1-glicerol)] (DOPG) na Figura 2.3 (b). Na região apolar as cadeias de ácidos graxos podem variar em função do número de carbonos e de insaturações. Portanto, a variação destas características moleculares possibilita a formação de lipossomas com bicamadas de características específicas para uma determinada função (Laye et al., 2008).

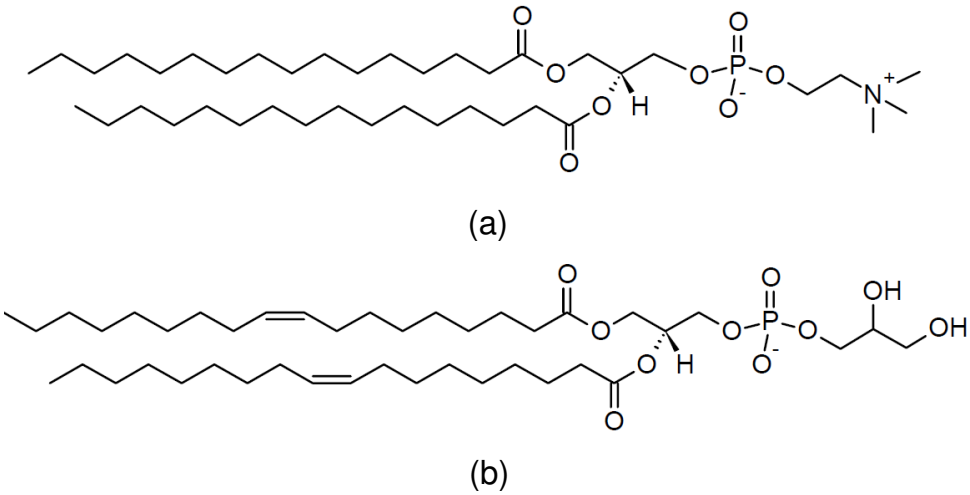

Figura 2.3. Estruturas moleculares de uma fosfatidilcolina **(a)** 1,2-dipalmitoil-*sn*-glicero-3-fosfatidilcolina (DPPC) e de um fosfatidilglicerol **(b)** 1,2-dioleoil-*sn*-glicero-3-[fosfo-rac-(1-glicerol)] (DOPG).

A composição dos lipossomas desempenha um papel crítico na distribuição de tamanho e na carga elétrica superficial, podendo ter um impacto direto na funcionalidade dos compostos carreados. Lipossomas formados por fosfatidilcolinas, contendo uma pequena quantidade de fosfatidilglicerol apresentaram uma estabilidade térmica superior, em relação aos lipossomas obtidos somente com fosfatidilcolinas (Taylor et al., 2007). A estabilidade coloidal dos lipossomas pode ser alcançada utilizando fosfolipídios carregados negativamente, como o fosfatidilglicerol, o fosfatidilinositol e o ácido fosfatídico presentes em pequenas quantidades em lecitinas naturais não purificadas extraídas da gordura do leite (Thompson & Singh, 2006; Thompson et al., 2007). Além disso, estudos mostram que

lipossomas estáveis podem ser obtidos utilizando lecitinas comerciais de soja não purificadas (Yokota et al., 2012). A lecitina comercial de soja corresponde a um conjunto formado majoritariamente de fosfolipídios, os quais são obtidos através da degomagem do óleo bruto de soja com água quente, sendo seus parâmetros de qualidade normatizados pela AOCS (*American Oil Chemistry Society*) para ser utilizada como um aditivo alimentar emulsificante (Marconcin, 2008).

Os fosfolipídios são caracterizados por uma temperatura de transição de fases, na qual a bicamada passa de um estado ordenado, para o estado de cristal líquido, no qual as moléculas apresentam liberdade de movimentação e os radicais hidrofílicos estão completamente hidratados. O comprimento e o número de insaturações da cadeia lipídica influenciam na temperatura de transição de fases dos fosfolipídios (Frézard et al., 2005). Portanto, lipossomas compostos por fosfolipídios distintos podem exibir diferentes níveis de permeabilidade em uma determinada temperatura. A permeabilidade dos lipossomas é relativamente baixa quando a temperatura é menor que a temperatura de transição de fases dos fosfolipídios. A adição de colesterol aos fosfolipídios faz com que a transição de fases ocorra de forma mais suave, podendo até ser eliminada em concentrações mais elevadas (Lasic, 1989; Mozafari, et al., 2008a).

A estabilidade dos lipossomas pode ser afetada por fenômenos químicos, físicos e biológicos. A estabilidade química depende da composição dos lipossomas, estando associada à prevenção da hidrólise do éster e da oxidação das insaturações localizadas na cadeia lipídica. Neste caso, a associação de compostos antioxidantes com os fosfolipídios pode minimizar estes efeitos (Woodall et al., 1997). O principal processo físico que causa a instabilidade dos lipossomas é a agregação e/ou coalescência das vesículas. A inclusão de pequenas quantidades de fosfolipídios carregados pode promover uma repulsão eletrostática, garantindo a estabilidade coloidal do sistema. Além disso, uma estabilidade estérica pode ser alcançada através da deposição/complexação de um polímero na superfície do lipossoma, tais como a quitosana, o ácido hialurônico e o polietilenoglicol. Por outro lado, a estabilidade biológica depende de fatores mais complexos que estão relacionados à presença de agentes que interagem com os lipossomas em seu meio de aplicação (Batista et al., 2007).

Com o desenvolvimento de novos materiais e a evolução do emprego dos lipossomas como carreadores de compostos ativos, algumas modificações na composição básica foram realizadas para satisfazer condições específicas de utilização, sendo estas alterações classificadas quanto às características da interação com os sistemas biológicos (Torchilin,

2005; Batista et al., 2007). Dentre as diferentes classes de lipossomas podemos destacar: (i) convencionais: são lipossomas formados por bicamadas fosfolipídicas, bastante estudados para aplicações na indústria de alimentos como carreadores de antimicrobianos, antioxidantes e vitaminas por apresentarem uma grande variabilidade em suas propriedades físico-químicas e pelo curto tempo de circulação na corrente sanguínea (Batista et al., 2007; Isailović et al., 2013; Moraes et al., 2013; Taylor et al., 2007; Malheiros et al., 2010); (ii) longa-circulação: são lipossomas formados por bicamadas de fosfolipídios e glicolipídios conjugados com polietilenoglicol, sendo geralmente empregados na indústria farmacêutica como carreadores de anticancerígenos e anti-inflamatórios (He et al., 2010; Akbarzadeh et al., 2013); (iii) fusogênicos: são lipossomas formados por bicamadas de fosfolipídios catiônicos ou sensíveis ao pH do meio, muito utilizados como sistemas carreadores de material genético para terapia e vacinação gênica devido à capacidade de interação com DNA, aumentando assim a transfecção de genes (Torchilin, 2005; Balbino et al., 2012; Balbino et al., 2013a; Balbino et al., 2013b); (iv) mucoadesivos: são lipossomas formados por bicamadas de fosfolipídios complexadas eletrostaticamente com quitosana, utilizados em condições nas quais propriedades adesivas são necessárias para aumentar o tempo de residência do composto ativo nas mucosas do trato gastrointestinal (Henriksen et al., 1994; Filipovic-Grcic et al., 2001; Takeuchi et al., 2003).

Diversas técnicas de produção de lipossomas foram desenvolvidas nas últimas décadas. Cabe ressaltar que a técnica empregada é um fator determinante nas características estruturais e na estabilidade dos lipossomas obtidos. A hidratação do filme lipídico (Bangham et al., 1965) é a técnica clássica mais difundida e utilizada para produção de lipossomas em escala laboratorial. Nesta técnica, os fosfolipídios são solubilizados e posteriormente submetidos a um processo de secagem para obtenção de um filme lipídico. Posteriormente, este filme é hidratado com uma solução aquosa para o desencadeamento da auto-organização em lipossomas (Malheiros et al., 2010). Outros processos clássicos são as técnicas de injeção de etanol (Batzri & Korn, 1973) e de evaporação em fase reversa (Akbarzadeh et al., 2013), sendo que ambas estão baseadas na obtenção de uma solução fosfolipídica utilizando um solvente orgânico antes da sua dispersão em uma fase aquosa. No entanto, os lipossomas formados através destas técnicas apresentam distribuição de tamanho com elevada polidispersidade, fator que limita suas aplicações. Além disso, todos esses métodos necessitam de uma operação unitária posterior de redução e/ou uniformização de tamanho, para atender aplicações específicas (Swaay & deMello, 2013).

Algumas tecnologias emergentes foram recentemente desenvolvidas para a produção de lipossomas e abordam princípios que utilizam altas temperaturas, liofilização de soluções monofásicas, fluidos supercríticos e microfluídica. Os processos que utilizam gases densos e fluidos supercríticos utilizam uma pequena quantidade de solvente orgânico, além de possibilitarem a esterilização *in situ*. Todas estas tecnologias emergentes estão inseridas nas perspectivas de intensificação de processos, visando atender às especificações do programa de Boas Práticas de Fabricação, a eliminação da utilização de solventes orgânicos, a diminuição da complexidade do processo, a redução do tempo de produção e a eficiência energética. No entanto, apesar de algumas técnicas emergentes ou clássicas de produção de lipossomas serem passíveis de escalonamento para a indústria de alimentos, com exceção da microfluídica, atualmente nenhum desses processos permite a operação em regime contínuo (Bridson et al., 2006; Meure et al., 2008; Swaay & deMello, 2013; Ushikubo et al., 2015).

2.1.2. Aplicações alimentícias e veiculação do β-caroteno em lipossomas

Lipossomas apresentam diversas aplicações no setor alimentício (Mozafari et al., 2008a; Mozafari et al., 2008b) principalmente na veiculação de ingredientes funcionais com estabilidade físico-química limitada. Trabalhos reportam a utilização dos lipossomas na veiculação de ingredientes, tais como: curcumina (Karewicz et al., 2013; Liu et al., 2015); polifenóis como o resveratrol (Isailović et al., 2013), extrato de hibiscos (Gibis et al., 2014b) e extratos de sementes de uva (Gibis et al., 2012; Gibis et al., 2013; Gibis et al., 2014a); carotenoides como a luteína (Tan et al., 2013; Tan et al., 2014ab), licopeno (Tan et al., 2014ab) e o β-caroteno (Carvalho et al., 2015; Moraes et al., 2013; Zômpero et al., 2015; Tan et al., 2014ab). Aplicações de lipossomas em matrizes alimentícias complexas também vêm sendo realizadas com o intuito de aproximar as pesquisas científicas da realidade tecnológica industrial, como a aplicação de lipossomas contendo ácido ascórbico em sucos de laranja (Marsanasco et al., 2011); lipossomas contendo sulfato ferroso em leite (Xia & Xu, 2004) e lipossomas contendo β-caroteno em iogurtes (Toniazzo et al., 2014).

O β-caroteno é o carotenoide que apresenta maior atividade provitamínica A, podendo ser encontrado em óleos, frutas e vegetais. O β-caroteno é considerado um carotenoide modelo, sendo o mais efetivamente convertido em retinol no organismo, apresentando propriedades que resultam em possíveis funções benéficas à saúde, tais como o fortalecimento do sistema imunológico e a diminuição do risco de doenças degenerativas. Além disso, o β-

caroteno apresenta atividade antioxidante, neutralizando os radicais livres, atuando como doador de elétrons e evitando os danos causados pelos radicais livres às células vivas (Burton & Ingold, 1984). A Figura 2.4 apresenta a estrutura molecular do β-caroteno com quarenta átomos de carbono, contendo dois anéis β-ionona nas extremidades e uma cadeia poliênica de nove ligações duplas conjugadas ligando ambos os anéis.

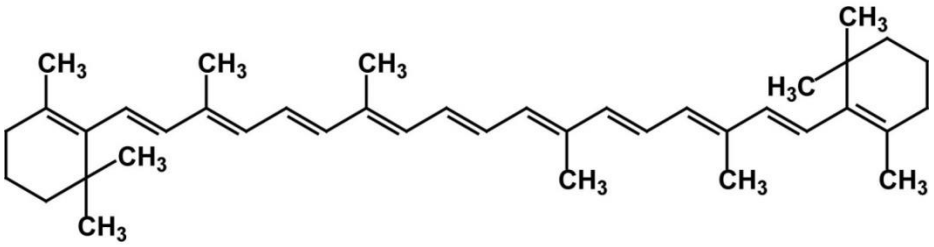

Figura 2.4. Estrutura molecular do β-caroteno constituída pela cadeia poliênica e os anéis β-ionona.

Diferentemente dos carotenoides polares, que possuem terminais oxigenados e apresentam modelos de orientação estabelecidos no interior das bicamadas fosfolipídicas, o β-caroteno não apresenta grupos polares na sua estrutura molecular, o que o torna altamente hidrofóbico. Dessa forma, sua orientação na bicamada será restringida somente ao ambiente da membrana lipídica e regulada por interações de van der Waals com as cadeias de ácidos graxos (Gruszecki et al., 2005). Estudos mostraram que a orientação do β-caroteno no interior da membrana dos lipossomas provavelmente é dependente da composição fosfolipídica, sendo paralelamente orientada ao plano da bicamada no caso de lipossomas compostos por fosfatidilcolina de dupla cadeia de ácido oleico (DOPC) e perpendicular ao plano da bicamada quando compostos de lecitinas naturais de soja (van de Ven et al., 1984).

A orientação vertical somente será possível se a espessura lamelar for grande o suficiente para comportar a molécula de β-caroteno nessa posição. Nesta configuração, a membrana lipossomal apresenta menor permeabilidade devido a um maior grau de compactação resultando numa proteção adicional para a oxidação dos fosfolipídios por radicais livres (Gonnet et al., 2010). Sabe-se que o β-caroteno se localiza na porção hidrofóbica das bicamadas fosfolipídicas de maneira aleatória, aumentando a liberdade de movimentação dos grupos polares dos fosfolipídios e reduzindo a organização da membrana no estado cristalino (Socaciu et al., 2002). No entanto, é geralmente aceito que a orientação da molécula de β-caroteno, devido à elevada flexibilidade conferida pelos anéis β-ionona nas extremidades da molécula, é mais próxima do plano horizontal do que do plano vertical

(Gonnet et al., 2010; Tan et al., 2014a). A Figura 2.5 apresenta a provável orientação do β-caroteno e de outros carotenoides no interior da bicamada fosfolipídica.

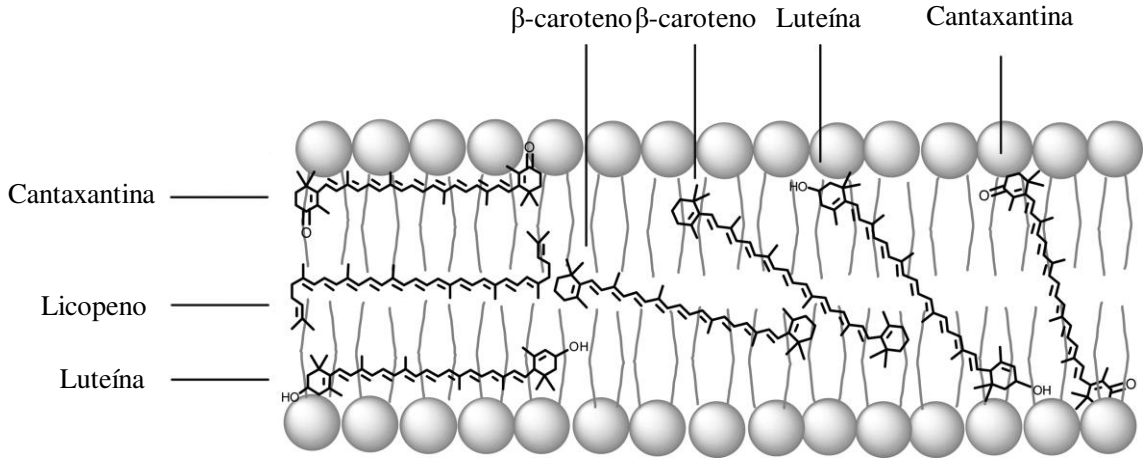

Figura 2.5. Diagrama esquemático da provável localização e orientação de alguns carotenoides no interior da bicamada fosfolipídica. Adaptado de Tan et al. (2014a).

2.1.3. Microfluídica

A microfluídica pode ser definida como o estudo do comportamento dos fluidos na escala microscópica. Desde 1990, com a tecnologia emergente de miniaturização dos dispositivos, a microfluídica foi diversificada e propagada para diversos campos de aplicação, sendo difundida nas áreas de química, biologia e física. A microfluídica pode ser definida também como a ciência de projetar, fabricar e operar dispositivos e processos com pequenas quantidades de fluidos em regime laminar. Os dispositivos microfluídicos são portáteis e possuem canais com dimensões que variam de poucos milímetros até micrômetros e são caracterizados por apresentarem canais com uma dimensão menor que 1 mm (Skurtys & Aguilera, 2008; Fang & Cathala, 2011; Vladisavljević et al., 2012). A utilização de dispositivos constituídos de materiais inertes como o vidro e polímeros é amplamente utilizada, pois estes materiais apresentam baixo custo, além de permitirem a fácil estruturação dos sistemas que constituem o dispositivo, como canais, orifícios, colunas, bombas e válvulas. Atualmente, diversas tecnologias para a fabricação de dispositivos microfluídicos podem ser empregadas, das quais, entre outras, destacam-se: ablação a laser, litografia macia e estereolitografia (Lippert & Dickinson, 2003; Moreira et al., 2009; Kanai & Tsuchiya, 2016; Ushikubo et al., 2015; McDonald et al., 2000).

A microfluídica tem se destacado também por estar inserida na perspectiva de intensificação de processos, em que as inovações buscam reduzir o tamanho da planta e

consumo de energia em várias ordens de grandeza para determinadas finalidades. A intensificação de processos visa desenvolver tecnologias que também reduzam substancialmente a produção de resíduos, a razão tamanho de equipamento/capacidade de produção, e que sejam mais eficientes energeticamente, resultando em tecnologias sustentáveis com menor demanda financeira. Outra vantagem desses sistemas é com relação à ampliação de escala de produção, a qual ocorre de maneira diferente dos processos convencionais, uma vez que estes requerem aumento do tamanho dos equipamentos. O aumento de produção dos processos microfluídicos ocorre em termos de amplificação de processos, onde os dispositivos não necessitam de uma alteração em suas dimensões, e sim de montagem em paralelo de forma a elevar a produção por réplica, reduzindo custos e tempo de estudo para a transposição do processo laboratorial para o industrial (van Gerven & Stankiewicz, 2009; Jahn et al., 2010).

A obtenção de nanopartículas por síntese, formação ou auto-organização utilizando técnicas microfluídicas somente é possível devido à flexibilidade de operação dos dispositivos com uma ampla variedade de materiais. Os processos microfluídicos para obtenção de nanopartículas são capazes de superar os obstáculos existentes nos métodos convencionais, como o controle da polidispersidade e do tamanho de partícula, e os problemas relacionados com reprodutibilidade. A obtenção de nanopartículas pelo método microfluídico muitas vezes dispensa uma etapa posterior de processamento para a redução e homogeneização de tamanho (Lo et al., 2010; Debus et al., 2012). Além disso, dentre outras vantagens dos sistemas microfluídicos com relação aos sistemas convencionais destacam-se o tamanho mínimo de dispositivos, o controle preciso de fenômenos de transferência de energia e massa, o escoamento estritamente laminar, o baixo consumo e dissipação de energia e, por fim, o baixo custo relativo de produção por dispositivo. Em aplicações na área de biotecnologia, o uso de dispositivos microfluídicos permite a manipulação de diferentes biomateriais, com investigações em diversas áreas, desde o desenvolvimento de biorreatores enzimáticos e microbianos, processos de recuperação e purificação, quantificação da expressão gênica até a obtenção de nanopartículas (Bettinger & Borenstein, 2010).

Processos de auto-organização para obtenção de estruturas de escala nanométrica requerem ambientes que sejam controláveis sobre a dimensão da própria estrutura. Sistemas microfluídicos têm várias características que permitem o controle do processo neste nível. Nestes sistemas, as forças interfaciais são predominantes e as forças inerciais são muitas vezes desprezíveis, resultando em melhores propriedades de transferência difusional de calor

e massa que são naturalmente observadas em nível celular. Além disso, as condições de escoamento laminar nos canais microfluídicos podem ser utilizadas para criar uma região de interface bem definida e previsível entre os dois fluidos. Estas características permitem controlar processos em um regime que antes era de difícil implementação experimental (Jahn et al., 2004; Jahn et al., 2008).

2.1.4. Produção de lipossomas em dispositivos microfluídicos

Diferentes abordagens baseadas em princípios microfluídicos podem ser utilizadas na produção de lipossomas, sejam elas adaptações e/ou melhoramento de técnicas já existentes na macroescala ou técnicas totalmente inovadoras. Basicamente, os processos microfluídicos de produção de lipossomas podem ser divididos em duas grandes categorias: (i) sistemas de mistura através do escoamento paralelo contínuo de duas fases miscíveis, os quais utilizam geralmente dispositivos planares de focalização hidrodinâmica para obtenção de lipossomas (SUVs e LUVs), sendo esta caracterizada como um melhoramento da técnica de injeção de solventes; (ii) sistemas baseados na geração de gotas utilizando fases imiscíveis em dispositivos microfluídicos para posterior obtenção de lipossomas (GUVs). Além dessas abordagens, outras técnicas baseadas em fundamentos microfluídicos foram desenvolvidas objetivando a produção de lipossomas, tais como: eletroformação/hidratação (LUVs, GUVs) (LeBerre et al., 2008); jato pulsado (GUVs) (Funakoshi et al., 2007; Stachowiak et al., 2009) e transferência de gotas (GUVs) (Hu et al., 2011; Nishimura et al., 2012; Matosevic & Paegel, 2011).

2.1.4.1. Sistemas microfluídicos de focalização hidrodinâmica

Recentemente, a abordagem baseada na focalização hidrodinâmica em dispositivos microfluídicos foi desenvolvida e aplicada com sucesso na formação de lipossomas (SUVs e LUVs). A Figura 2.6 apresenta um esquema hipotético de formação de lipossomas em dispositivos microfluídicos planares através do mecanismo de focalização hidrodinâmica. A formação de lipossomas é realizada pela introdução de uma corrente orgânica contendo fosfolipídios dispersos em um álcool, geralmente etanol ou isopropanol, através de um canal central e a posterior constrição dessa corrente por uma ou duas correntes aquosas perpendiculares ou oblíquas. A focalização hidrodinâmica da corrente central provoca difusão

controlada do álcool para a fase aquosa e vice-versa. Como consequência, as moléculas de água irão substituir as moléculas de álcool em torno dos fosfolipídios, provocando uma mudança na solubilidade do sistema. Este fato desencadeia a auto-organização dos fosfolipídios em fragmentos de bicamadas e posteriormente em pequenas vesículas esféricas unilamelares (Jahn et al., 2004; Jahn et al., 2007; Jahn et al., 2008; Jahn et al., 2010; Huang et al., 2010; Balbino et al., 2013a; Mijajlovic et al., 2013; Hood & DeVoe, 2015).

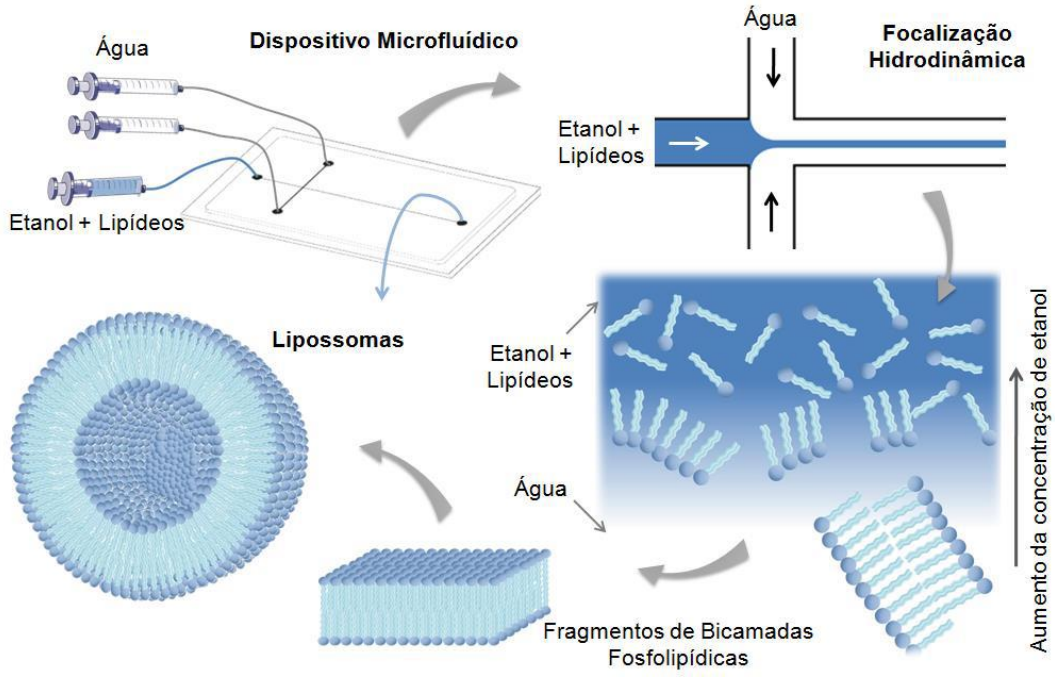

Figura 2.6. Esquema hipotético da formação de lipossomas em dispositivos microfluídicos planares através da técnica de focalização hidrodinâmica. Adaptado de Balbino et al. (2013a).

O tamanho dos lipossomas obtidos através da focalização hidrodinâmica é aproximadamente constante quando a vazão total de escoamento no interior do dispositivo varia entre 30 e 200 µL/min, desde que a razão entre as vazões volumétricas das fases aquosa e orgânica seja mantida constante. No entanto, o diâmetro dos lipossomas formados pode ser altamente controlável quando a razão entre as vazões volumétricas é alterada. Uma redução nos valores dessa razão provocará um aumento na largura da corrente orgânica, proporcionando uma zona central com elevada concentração de solvente orgânico. Assumindo que os lipossomas são formados através de uma estrutura intermediária, uma maior concentração de solvente pode estabilizar as bicamadas e permitir uma maior agregação dos fosfolipídios, resultando em lipossomas grandes com ampla distribuição de tamanho. Por outro lado, com um aumento da razão entre as fases a corrente orgânica será focalizada em

um jato mais fino em que o solvente orgânico estará mais diluído. Essa concentração reduzida de solvente orgânico limita a formação dos compostos intermediários, resultando em lipossomas menores e com uma distribuição de tamanho mais estreita (Jahn et al., 2008; Jahn et al., 2010; Balbino et al., 2013a).

Pioneiramente, Jahn et al. (2004) descreveram a formação de lipossomas em dispositivos microfluídicos através do mecanismo de focalização hidrodinâmica. Os lipossomas foram formados em um processo controlado pela difusividade, em que uma corrente contendo fosfolipídios dispersos em isopropanol mais um corante fluorescente ($DiIC_{18}$) foi focalizada hidrodinamicamente por duas correntes aquosas perpendiculares. O $DiIC_{18}$ é um corante que exibe maior eficiência quântica quando aprisionado na membrana lipídica em comparação a sua forma livre. A Figura 2.7 apresenta o perfil de concentração no interior do dispositivo, onde o valor máximo de intensidade fluorescente significa uma maior concentração de lipossomas formados. Assim, lipossomas foram formados em pontos do sistema onde a concentração da mistura alcançou uma condição crítica de solubilidade para os fosfolipídios. A máxima intensidade fluorescente foi observada após a ocorrência da largura mínima da corrente de isopropanol. Os lipossomas formados neste processo apresentaram tamanhos entre 50 e 150 nm e uma distribuição de tamanho de partícula monodispersa.

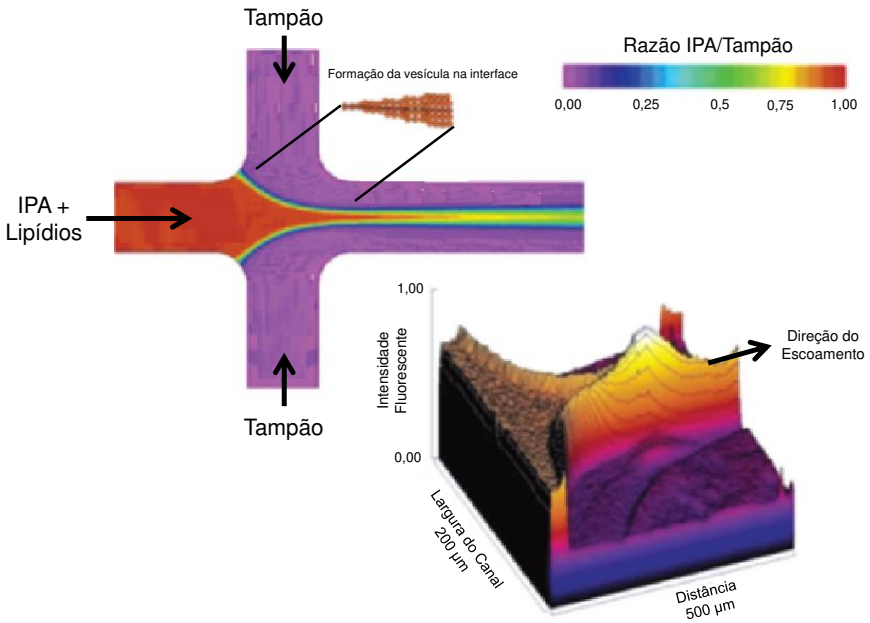

Figura 2.7. Diagrama esquemático do processo de formação de lipossomas em canal microfluídico, onde a relação de cores representa as razões de concentração isopropanol (IPA)/tampão aquoso e mapa tridimensional de intensidade fluorescente do $DiIC_{18}$ durante a formação de lipossomas. Adaptado de Jahn et al. (2004).

Posteriormente, Jahn et al. (2010) estudaram a influência das dimensões de dispositivos planares de focalização hidrodinâmica, utilizando microcanais com 10 μm e 65 μm de largura. Os resultados mostraram que os lipossomas de menor diâmetro (~80 nm) foram obtidos nos dispositivos de menor largura. Balbino et al. (2013a) estudaram a produção contínua de lipossomas com altas concentrações de fosfolipídios empregando dispositivos microfluídicos com uma ou duas focalizações hidrodinâmicas paralelas, com intuito de aumentar a taxa de produção. Em condições ótimas, os lipossomas produzidos nos dispositivos de focalização dupla apresentaram diâmetro médio de 100 nm, distribuições de tamanho monodispersas e produtividade superior em comparação com os dispositivos de focalização simples. Nesta mesma linha, Hood & DeVoe (2015) avaliaram a influência de canais com diferentes áreas de seção retangular, observando que canais maiores promovem a formação de lipossomas com distribuições de tamanho de menor polidispersidade. Além destes, na última década diversos trabalhos foram publicados, em sua maioria com o objetivo de avaliar a utilização de dispositivos de focalização hidrodinâmica e a influência de variáveis operacionais na formação de lipossomas (Jahn et al., 2007; Huang et al., 2010; Balbino et al., 2013b; Mijajlovic et al., 2013; Hood & DeVoe, 2015; Hood et al., 2013; Wi et al., 2012; Zook & Vreeland, 2010; Hong et al., 2010).

O processo de formação de lipossomas por microfluídica através do mecanismo de focalização hidrodinâmica não pode ser visualizado diretamente devido às altas velocidades de escoamento, com valores de até 1 m/s, associados a números Reynolds inferiores a 100. Além disso, os tamanhos dos lipossomas formados (<1 μm) através dessa técnica estão abaixo do limite da resolução óptica. Com isso, até o presente momento, é experimentalmente impossível observar a formação desses intermediários fosfolipídicos. No entanto, a fundamentação teórica clássica proposta na literatura para formação dos lipossomas por métodos baseados na injeção de solventes corrobora os resultados experimentais de produção por focalização hidrodinâmica em dispositivos de microfluídica obtidos até o presente momento.

2.1.4.2. Sistemas microfluídicos de gotas

Os sistemas microfluídicos para produção de lipossomas baseados na geração de gotas manipulam fases imiscíveis para formar emulsões, geralmente duplas do tipo água-óleo-água (A/O/A) com dimensões micrométricas ou submicrométricas, dentro de dispositivos

microfluídicos. Neste processo, misturas de solventes orgânicos são utilizadas para solubilizar os fosfolipídios e formar a fase intermediária das emulsões duplas. Além disso, essa mistura de solventes orgânicos deve ser parcialmente miscível na água para acelerar o processo de evaporação do solvente orgânico, conforme a Figura 2.8. Após a evaporação parcial da fase intermediária, devido à elevada pressão de vapor dos solventes orgânicos utilizados, o invólucro formado pela fase intermediária da emulsão dupla A/O/A em torno do núcleo aquoso será inferior a 1 µm de espessura, permitindo a formação de lipossomas gigantes (Shum et al., 2008; Arriaga et al., 2014; Akamatsu et al., 2015). A produção de lipossomas através dessa abordagem está fortemente baseada na utilização de dispositivos capilares de vidro com suas superfícies funcionalizadas e também na utilização de solventes orgânicos com elevado potencial tóxico como o tolueno, clorofórmio, octanol e hexano (Arriaga et al., 2014; Kim et al., 2011; Shum et al., 2008; Kim et al., 2013; Deshpande et al., 2016; Kong et al., 2014).

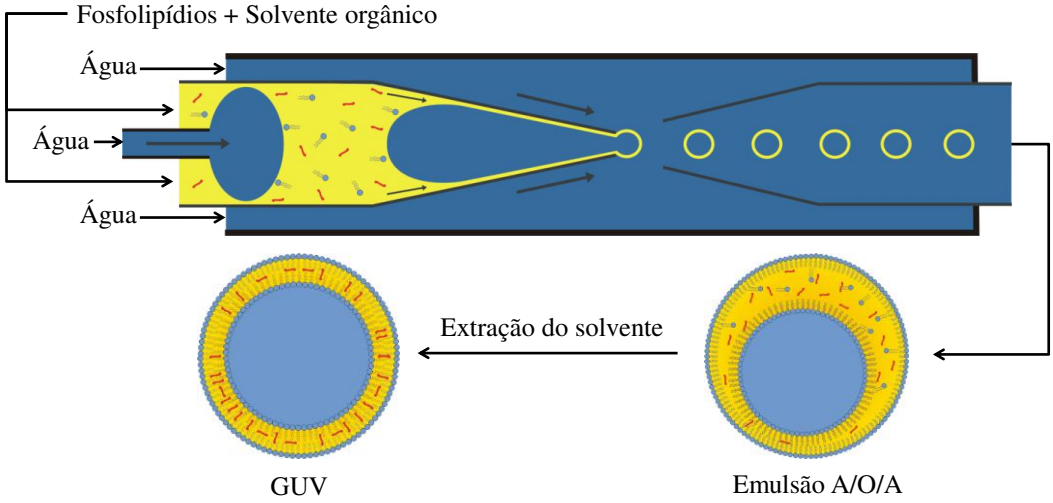

Figura 2.8. Microdispositivo capilar de vidro para formação de emulsões A/O/A e mecanismo de formação de lipossomas gigantes. Adaptado de Shum et al. (2008).

De maneira similar, a produção microfluídica de lipossomas utilizando sistemas de geração de gotas também pode ser alcançada através do uso de dispositivos poliméricos planares, geralmente polidimetilsiloxano, utilizando uma junção com cinco canais de entrada e um canal de saída (Deshpande et al., 2016; Arriaga et al., 2015), conforme apresenta a Figura 2.9. Neste processo, a fase aquosa interna e a fase lipídica intermediária são focalizadas através da sua constrição por duas correntes aquosas perpendiculares de fase contínua externa. Assim, é possível produzir gotas de emulsões duplas A/O/A estáveis e monodispersas em uma etapa única (Deshpande et al., 2016). No entanto, o uso de

dispositivos microfluídicos de polidimetilsiloxano para formação de emulsões A/O/A requer que as superfícies dos canais de entrada das fases aquosas sejam funcionalizadas para torná-las hidrofílicas, o que se caracteriza como um desafio a ser superado. Além disso, produção microfluídica de lipossomas em sistemas de geração de gotas ainda é pouco explorada na literatura científica e a utilização de materiais complexos de grau alimentício ainda não foram reportadas. A grande maioria dos trabalhos para produção de lipossomas gigantes utiliza fosfolipídios com elevado grau de pureza. Assim, a utilização de fosfolipídios de baixo custo como lecitinas de soja não purificadas e a utilização de solventes orgânicos com baixo potencial tóxico para a saúde humana se caracterizam como um grande desafio científico a ser superado.

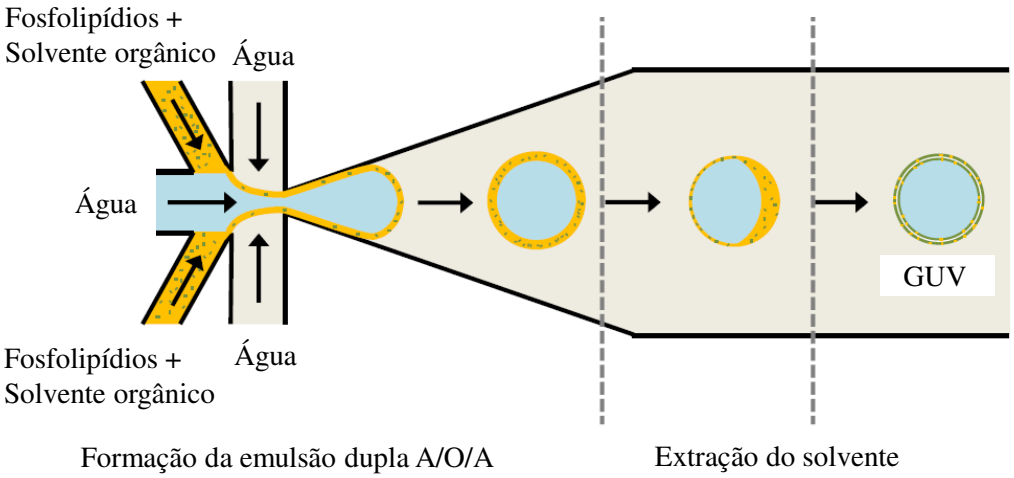

Figura 2.9. Diagrama esquemático da formação de lipossomas utilizando emulsões duplas A/O/A em dispositivos microfluídicos planares com focalização hidrodinâmica e o mecanismo de extração do solvente orgânico. Adaptado de Deshpande et al. (2016).

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CAPÍTULO III

Structural characterization of β-carotene-incorporated nanovesicles produced with non-purified phospholipids

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Structural characterization of β-carotene-incorporated nanovesicles produced with non-purified phospholipids

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Highlights

- Low-cost non-purified lecithin can be employed for production of liposomal systems.

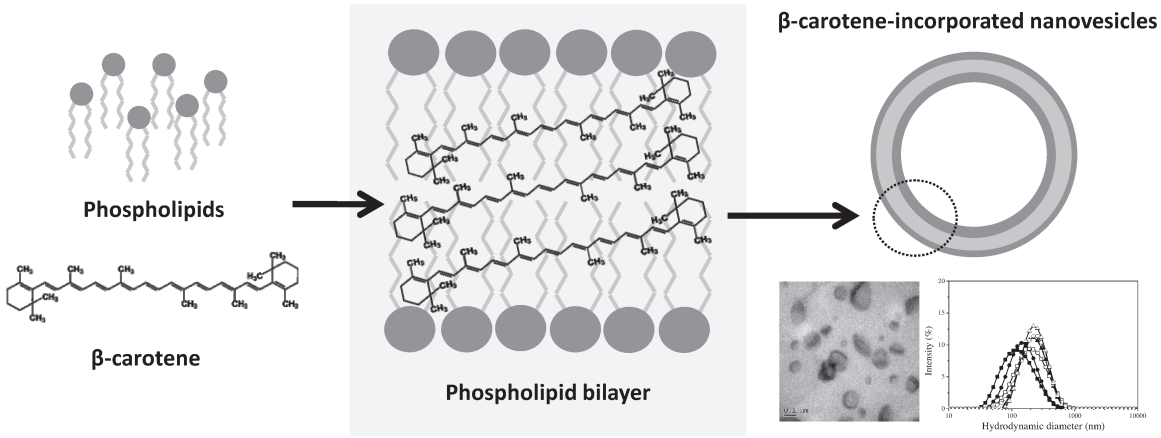
- Lecithin-type did not influence on the β-carotene loading capacity.

- The results expand opportunities of using liposomal systems in the food industry.

Abstract

The technical feasibility of obtaining β-carotene-incorporated phospholipid nanovesicles using non-purified soybean lecithins was studied. For this purpose, three lecithin-types were evaluated. Nanovesicles were characterized by average hydrodynamic diameter, particle size distribution, polydispersity index, ζ-potential, transmission electron microscopy, membrane microviscosity, small angle X-ray scattering and capacity of lipid peroxidation inhibition. In general, the β-carotene incorporation did not promote a significant increase on average hydrodynamic diameter, but vesicles produced from lecithins containing triglycerides showed lower polydispersity. The lecithin-type used to produce nanovesicles did not influence the β-carotene loading capacity, but significantly influenced the microviscosity of liposomal membrane and lipid peroxidation inhibition capacity. Non-enzymatically modified lecithin (containing or not triglycerides) showed similar efficiency and peroxidation inhibition capacity considering β-carotene incorporation. Therefore, low-cost non-purified lecithin can be employed for production of liposomal systems as an encapsulating and/or delivery system to be used in food products.

Graphical abstract



Keywords: β-Carotene; Peroxidation; Liposome; Phospholipid bilayer

3.1. Introduction

β-Carotene is an important member of the carotenoid family found in many fruits and vegetables, which has a molecular structure containing forty carbon atoms and two β-ionone rings at the end of its conjugated double bond chain (Silva et al., 2011). β-Carotene has important biological activity due to provitamin A activity which results in potential health benefits such as strengthening of immune system and decreasing the risks of degenerative diseases (Michelon et al., 2012). However, its poor physicochemical stability and low bioavailability related to its high hydrophobicity makes difficult the effective utilization of β-carotene as a nutraceutical ingredient (Tan et al., 2014a). Several delivery systems for β-carotene have been evaluated to improve its application in aqueous systems and to achieve targeted and/or sustained release, such as microemulsions (Roohinejad et al., 2015), O/W emulsions (Lim, Griffin, & Roos, 2014), nanoemulsions (Silva et al., 2011), nanostrucutured lipid carriers (Helganson et al., 2009 and Hejri et al., 2013) and liposomes (Moraes et al., 2013 and Zômpero et al., 2015).

Liposomes are colloidal vesicles formed by the self-assembling of amphiphilic lipids that in excess of water aggregate forming lipid bilayers and enclosing aqueous compartments (Tan et al., 2014a and Balbino et al., 2012). The most common lipids used for industrial applications of liposomes are phospholipids. Among delivery systems, liposomes have great

potential for food applications because of their biocompatibility, non-toxicity, sustained release potential, ability to act as vehicle for both hydrophobic and hydrophilic compounds and utilization of natural food-grade ingredients as raw material (Moraes et al., 2013 and Tan et al., 2013). Besides, the phospholipid bilayer can provide a physicochemical barrier against pro-oxidant elements through the incorporation of β-carotene making it an attractive ingredient to be used in aqueous food formulations (Tan et al., 2014b and Tan et al., 2014c).

The major challenges for application of phospholipid nanovesicles as a delivery system in food industries are the process scale-up and the high cost of the phospholipids (Yokota, Moraes, & Pinho, 2012). Moreover, most of the conventional methods for liposome production require dispersion of phospholipids in organic solvents which could be a drawback for nutraceutical or food use (Gonnet, Lethuaut, & Boury, 2010). Phospholipid nanovesicles with sizes of approximately 250 nm and low polydispersity index without need of a subsequent processing step can be obtained using the ethanol injection technique (Zômpero et al., 2015), which is considered a simple method to scale up the process. Besides, the use of ethanol as a pharmacological and food processing organic solvent is well accepted (Tan et al., 2013).

Therefore, the high cost of raw materials is the largest limitation of liposome applications, even for high aggregate-value in food products. Most studies about liposomes aimed at food applications employ materials suitable for the pharmaceutical field, such as synthetic phospholipids or purified natural phospholipids, which are costly for large-scale production. The average cost of purified natural phospholipids is around 980 euros per kg, while non-purified soy lecithin costs approximately 20% of this value (Yokota et al., 2012). To overcome these drawbacks, the utilization of commercial lecithins arises as an alternative. Lecithin consists of a mixture of phospholipids in oil obtained by water degumming of oil seeds generally extracted from soybean. Natural lecithin presents acyl chains with high unsaturation content which has been shown to have a number of health benefits, including liver protection and cholesterol absorption (Thompson & Singh, 2006).

Therefore, the objective of this work was to evaluate the technical feasibility of obtaining phospholipid nanovesicles by ethanol injection technique using non-purified soy lecithins with low cost and widely available for the food industry. The phospholipid nanovesicles were used as vehicle for β-carotene that is recognized as a model molecule for evaluation of food delivery systems and a highly hydrophobic bioactive compound of great

interest to the food industry. The nanovesicles were characterized by hydrodynamic diameter, size distribution, polydispersity index, ζ-potential, transmission electron micrography, membrane microviscosity and small angle X-ray scattering. Finally, β-carotene-incorporated nanovesicles were subjected to a highly oxidative environment and capacity of lipid peroxidation inhibition was estimated.

3.2. Materials and methods

3.2.1. Materials

The nanovesicles were obtained using three types of food-grade natural soybean lecithin: standardized fluid lecithin (SFL) Solec™ SGTN containing 23% *w/w* phosphatidylcholine, 20% *w/w* phosphatidylethanolamine and 37% *w/w* triglycerides; enzyme-modified fluid lecithin (EFL) Solec™ AEIP containing 16% *w/w* phosphatidylcholine, 15% *w/w* lysophosphatidylcholine, 14% *w/w* phosphatidylethanolamine and 37% *w/w* triglycerides, both obtained from The Solae Company™ (St. Louis, USA), and fat-free powder lecithin (DPL) Lipoid S45 containing >45% *w/w* phosphatidylcholine, 10-18% *w/w* phosphatidylethanolamine, <4% *w/w* lysophosphatidylcholine and <3% *w/w* triglycerides acquired from Lipoid GmbH (Ludwigshafen, Germany). Absolute ethanol chromatographic-grade (99.9% *v/v*) was purchased from Panreac® (Barcelona, Spain). DPH fluorescence probe (98% *w/w*, 1,6-diphenyl-1,3,5-hexatriene) and synthetic β-carotene powder (>93% *w/w*) were purchased from Sigma-Aldrich (St. Louis, USA). TBA (>99% *w/w*, 2-thiobarbituric acid) was purchased from Merck KGaA (Darmstadt, Germany). Ascorbic acid (99% *w/w*), hexane (98.5% *w/w*), hydrochloric acid (35-37% *v/v*), trichloroacetic acid (analytical-grade) and iron(III) chloride (>97% *w/w*) were purchased from Synth® (Diadema, Brazil).

3.2.2. Methods

3.2.2.1. Nanovesicle preparation

The nanovesicle dispersions with final concentration of lecithin of 1 mM to 10 mM, considering an average molar weight of 780 g mol^{-1}, were prepared by the ethanol injection method according to the protocol proposed by Zômpero et al. (2015) with some modifications. The system used for ethanol injection was basically composed of a glass

jacketed reactor, useful volume of 150 ml, equipped with a four baffle system and a straight blade impeller, both manufactured in stainless steel. Firstly, lecithin stock solutions in different concentrations were prepared from lecithin dispersion in ethanol at 40 °C using an ultrasonic bath at 20 kHz (USC450, Unique, Brazil). Subsequently, this organic phase (10 ml) was fed at a constant flow rate of 10 ml min^{-1} using a peristaltic pump (MasterFlex®, Cole-Parmer Inc., USA) into the reactor which was filled with 90 ml of ultrapure water (Direct-Q3, Millipore, USA). During this process the temperature of the system was kept at 40 °C using a thermostatic bath and the stirring was kept at 500 rpm using a mechanical stirrer (RW, Ika Works Inc., Germany). A similar procedure was used to incorporate β-carotene into nanovesicles. In this case, after the complete dispersion of lecithin in ethanol, β-carotene was added to the organic phase. The concentration of β-carotene in the organic phase (IC) was expressed relative to amount of lecithin (% *w/w*). Incorporation of β-carotene was performed only at the highest lecithin concentration (10 mM) using different IC values (0.125 to 2.5% *w/w*). Afterwards, the dispersion containing the nanovesicles in 10% (*v/v*) ethanol/water were cooled at 8 °C for 12 h and centrifuged (Allegra 25R, Beckman Coulter, USA) at 2450×*g* for 10 min to separate the β-carotene-unincorporated. Finally, the supernatant fractions containing nanovesicles with β-carotene incorporated were physico-chemically characterized.

3.2.2.2. Nanovesicle characterization

Hydrodynamic diameter and polydispersity index

The average hydrodynamic diameter of the nanovesicles dispersed in 10% (*v/v*) ethanol/water was determined at 25 °C by Dynamic Light Scattering (DLS) using the equipment Zetasizer Nano-ZS (Malvern Instruments, UK). This technique is based on the dependence of intensity fluctuations of light scattering as a function of time due to Brownian motion of the particles in suspension. Hydrodynamic diameter and polydispersity index of the liposomes was evaluated in terms of scattered light intensity which is proportional to the sixth power of the particle diameter. The polydispersity index was calculated from cumulant analysis of the measured DLS intensity autocorrelation function. The polydispersity index varied from 0 to 1, where higher values indicate a less homogeneous nanoparticle size distribution.

ζ-potential

The determination of surface electric charge of the nanovesicles dispersed in 10% (*v/v*) ethanol/water was performed at 25 °C using the equipment Zetasizer Nano-ZS (Malvern Instruments, UK). The electrophoretic mobility was obtained by Laser Doppler Anemometry technique, which makes use of the relationship between the electrophoretic mobility and the light scattering produced by the particles dispersed in the medium. The mathematical model proposed by Smoluchowski was used to convert electrophoretic mobility measurements in ζ-potential values.

Transmission electron microscopy (TEM)

The transmission electron micrographs were obtained by negative staining method according to the protocol proposed by Théry et al. (2006) with some modifications. The supernatant obtained after separation of β-carotene-unincorporated from the vesicles was ultracentrifuged (L8-80 M, Beckman Coulter Inc., USA) at 100,000×*g* for 3 h. The pellet obtained was resuspended in 100 µL of buffered formaldehyde solution (2% *v/v*) and diluted 2-fold. Nickel grids coated with the Formvar® resin (vinyl polyacetate) were placed on 5 µL of the resuspended pellet. Afterwards, the grids were washed with phosphate buffer solution (PBS 10 mM, 150 mM NaCl, pH 7.4) for approximately 7-fold and kept at room temperature for 20 min. For the negative staining, grids were deposited on drops of saturated uranyl oxalate solution (pH 7.0) and kept for 5 min under room conditions. Grids were then transferred to drops of a mixture (1:9 *v/v*) of uranyl oxalate solution and methyl cellulose (2% *w/v*) under room temperature for 10 min. Finally, the grids were transferred to drops of methyl cellulose (2% *w/v*) on ice for 10 min. Grids were retrieved with a stainless steel loop of diameter slightly larger than the grid. Excess of methyl cellulose and uranyl oxalate solutions were removed with filter paper (Whatman 50) and air-dried. Samples were examined under the electron microscope (1200 EX II, JEOL Inc., Japan). Transmission electron micrographs were obtained by using a digital camera (Multiscan 791, GATAN, USA).

Small angle X-ray scattering (SAXS)

Qualitative information about assembly of the nanovesicles was obtained by means of SAXS measurements performed at room temperature using the SAXS1 beamline of the

National Synchrotron Light Laboratory (CNPEM, Campinas, Brazil). The beamline is equipped with an asymmetrically cut and bent silicon (111) monochromator that yields a monochromatic (λ=1.54 Å) and horizontally focused beam. A position-sensitive X-ray detector and a multichannel analyzer were used to record the SAXS intensity, as a function of modulus of scattering vector (q), where $q = (4\pi/\lambda)\text{sen}(\theta/2)$, with θ being the scattering angle. The SAXS measurements were performed with β-carotene-free nanovesicles obtained from different lecithin-types at 10 mM and each pattern corresponding to a data collection time of 90 s.

Loading content and process efficiency

Firstly, β-carotene was extracted from the aqueous liposomal fractions by liquid–liquid extraction using absolute ethanol-hexane mixture. In flasks containing 0.5 ml of the dispersion of nanovesicles was added 2 ml of absolute ethanol and 3 ml of hexane. The systems were shaken every 10 min during a period of 30 min and the extraction was repeated twice. Subsequently, the phases containing hexane were removed from the extraction system, combined and diluted with hexane in volumetric flasks (Hung et al., 2011 and Toniazzo et al., 2014). β-Carotene content was measured by reading the absorbance of hexane phase at 450 nm in spectrophotometer (SP-220, Biospectro, Brazil) and relating to the β-carotene molar absorption coefficient in hexane, which is 2592 M^{-1} cm^{-1} (Rodriguez-Amaya, 2001). The process efficiency of β-carotene incorporation into nanovesicles (%) was estimated according to ratio between the amount of β-carotene-incorporated (present in the supernatant) and the total amount of β-carotene of the samples before centrifugation. The effective loading capacity in nanovesicles (% *w/w*) was also estimated by the ratio between the amount of β-carotene-incorporated and lecithin content used to form nanovesicles (Tan et al., 2013, Tan et al., 2014b and Tan et al., 2014c).

Microviscosity

The liposomal membrane microviscosity was measured by fluorescence anisotropy (Pandey & Mishra, 1999 and Tan et al., 2013). Firstly, a stock solution of DPH probe (10 mM) was prepared in tetrahydrofuran. Subsequently, aliquots of the stock solution were diluted 1000-fold in PBS (10 mM, 150 mM NaCl, pH 7.4) to obtain a DPH-PBS solution 0.01 mM. For labeling, 1 ml of nanovesicle suspension was diluted 10-fold in PBS, mixed with 1

ml of DPH-PBS probe solution, and the mixture was incubated for 3 h at 25 °C. The fluorescence anisotropy of DPH in labeled nanovesicles was measured at 25 °C (λ_{em}=357 nm and λ_{ex}=425 nm) by spectrofluorimeter (PC-1 model, ISS Inc., USA). The apparent microviscosity (η) of liposomal membrane was calculated by the Perrin equation (Shinitzky & Barenholz, 1978) modified by Pandey & Mishra (1999) that considered the DPH probe properties.

3.2.2.3. Kinetics of lipid peroxidation

The kinetics of induced lipid peroxidation and inhibition capacity of the β-carotene-incorporated in nanovesicles were determined by measurement of 2-thiobarbituric acid-reactive substances (TBARS). For induced peroxidation assays, samples of nanovesicles (1 ml) were firstly mixed with 1 ml of iron(III) chloride solution (400 μM) and 1 ml of ascorbic acid solution (400 μM) and incubated for 3 h at 37 °C. Afterwards, an aliquot (1 ml) of each sample was removed at predetermined intervals and combined with 5 ml of a reaction solution composed by TBA (2-thiobarbituric acid, 0.37% *w/v*), trichloroacetic acid (15% *w/v*), and hydrochloric acid (1.8% *v/v*). The reaction mixture was kept in a boiling bath for 30 min to promote formation of the malondialdehyde (MDA) - a final product of fatty acid peroxidation, which reacted with TBA to form a colorful complex (Tan et al., 2013 and Tan et al., 2014c). Afterwards, the mixture was cooled rapidly in an ice bath, centrifuged at 613×*g* for 5 min and absorbance of the supernatant was measured at 532 nm using a spectrophotometer (SP-220, Biospectro, Brazil). TBARS values were expressed as nmol MDA equivalent/g lecithin using a molar absorption coefficient equal 1.56×10^5 M^{-1} cm^{-1} (Pandey & Mishra, 1999, Balachandran & Rao, 2003 and Karaca et al., 2013). The lipid peroxidation inhibition capacity (LPIC) after 3 h of iron-induced assays was determined by percentage (%) of TBARS values of β-carotene-incorporated in nanovesicles relative to TBARS values of β-carotene-free nanovesicles (Tan et al., 2014c).

3.2.2.4. Statistical analysis

The assays for nanovesicles preparation using different lecithin-types and incorporation of different amount of β-carotene were performed in triplicate (*n*=3) and analytical determinations in duplicate. The results obtained were submitted to variance

analysis (ANOVA) and Tukey's test was applied to evaluate significant differences between the mean values (p<0.05).

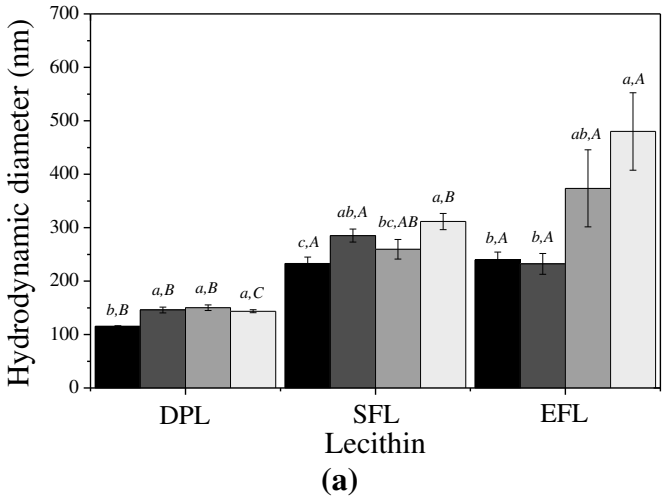
3.3. Results and discussion

3.3.1. β-carotene-free nanovesicles

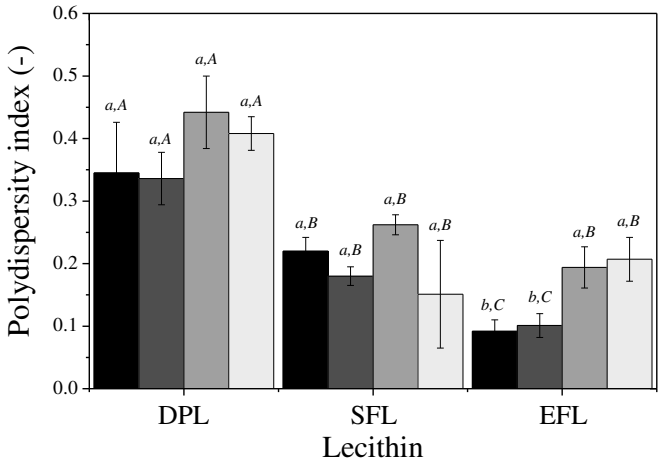
3.3.1.1. Structural characterization

The effect of the lecithin-type and concentration on the average hydrodynamic diameter and polydispersity index is shown in the Figure 3.1 (a,b). SFL and EFL nanovesicles showed higher hydrodynamic diameters than DPL nanovesicles, according to Figure 3.1 (a). These higher diameter values may be associated with the presence of triglycerides (37% *w/w*) that could be trapped inside bilayers. The results are in agreement with another study which aimed to incorporate long-chain triglycerides (e.g. soybean and corn oils) in saturated liposomes and a significant increase in hydrodynamic diameter was observed (Hong, Kim, & Lim, 2015). In contrast, the nanovesicles obtained with these lecithins showed lower polydispersity index. In this case, the presence of triglycerides incorporated inside the bilayer probably favored the obtaining of systems with lower polydispersity than the nanovesicles obtained from fat-free lecithin.

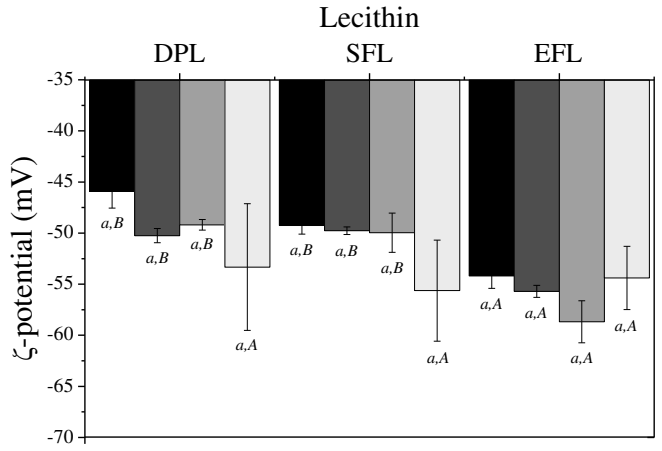
In general, the increase of lecithin concentration caused a significant increase (p<0.05) in the average hydrodynamic diameter of the vesicles for the three lecithin-types. However, DPL nanovesicles were less dependent on the lecithin concentration (Figure 3.1 a). This tendency could be explained by the liposome formation process involving diffusion of a lipid organic dispersion into an aqueous medium. Planar intermediate structures, denominated lipid bilayer fragments, are formed during the process, characterizing a resistance to mass transfer for organic solvent diffusion. Thus, at high phospholipid concentration the organic solvent diffusion will occur slowly and free lipid molecules and lipid bilayer fragments will aggregate in an uncontrolled way leading to the formation of larger vesicles with higher polydispersity (Balbino et al., 2013 and Mijajlovic et al., 2013). In addition, the triglycerides incorporated into liposomes may have promoted an additional resistance to mass transfer, resulting in larger vesicles. However, a significant increase (p<0.05) of the polydispersity index was observed only for EFL nanovesicles, according to Figure 3.1 (b).



(a)



(b)



(c)

Figure 3.1. Influence of lecithin concentration on the **(a)** hydrodynamic diameter; **(b)** polydispersity index and **(c)** ζ-potential of the empty nanovesicles obtained from different lecithin-types. Data represent means ± standard deviations (n=3), different lowercase letters (a-c) represent significant differences (p<0.05) between the lecithin concentrations used and different uppercase letters (A-C) represent significant differences (p<0.05) between lecithin-type used, where: ( ) 1 mM; ( ) 2.5 mM; ( ) 5 mM and ( ) 10 mM.

Enzymatic modification of lecithins is carried out to improve their emulsifying properties and thermal stability, generally using lipases *sn*-2 or *sn*-1,3-specific, leading to partially hydrolyzed phospholipids containing only one fatty acid chain (Guo, Vikbjerg, & Xu, 2005). For this reason, in an aqueous solution phosphatidylcholine organizes phospholipid bilayers while lysophospholipids form micelles (Nakagaki, Komatsu, & Handa, 1986). Lysophospholipids, in particular lysophosphatidylcholine is a potent fusogenic agent capable of promoting cell fusion that can cause changes in membrane permeability even in small amounts. It was suggested that lysophosphatidylcholine can induce the leakage of multilamellar liposome contents (Ralston et al., 1980). Thus, the amount of lysophospholipid on the enzyme-modified lecithin probably may have caused a disorder in the bilayer leading to the nanovesicle coalescence increasing significantly the hydrodynamic diameter and polydispersity with increasing lecithin concentration, according to Figure 3.1 (a,b).

According to Figure 3.1 (c), the nanovesicles obtained from different unsaturated soybean lecithin-types showed negative ζ-potential values varying between -45 mV and -60 mV. These results suggest the existence of a strong electrostatic repulsion between the nanovesicles, indicating a good stability of the colloidal system. Since phosphatidylcholine is a zwitterionic phospholipid, the negative ζ-potential values could be related to the phosphatidic acid and phosphatidylinositol present in natural soybean lecithins (Thompson & Singh, 2006 and Nongonierma et al., 2009). The colloidal stability of the nanovesicles depends on the balance between repulsive electrostatic forces and attractive van der Waals interactions, the latter being responsible for coalescence and/or aggregation phenomenon. Therefore the use of charged phospholipids as non-purified soybean lecithin could be an alternative to improve colloidal stability of phospholipid nanovesicles.

Figure 3.2 (a-c) shows the nanovesicle size distribution obtained from different lecithin-types at different concentrations and unimodal distribution was observed for all conditions. Besides, in Figure 3.2 (a-c) the electron micrographs show spherical or rod-like shaped nanovesicle and in some cases a well-defined region surrounding the particle, suggesting the phospholipid bilayer formation. The DPL nanovesicles showed a higher sphericity, typical of liposomes, than other vesicles, probably due to the higher phosphatidylcholine content and absence of triglycerides and lysophosphatidylcholine. The non-occurrence of nanovesicle coalescence and/or aggregation on the micrographs confirmed the ζ-potential results (Figure 3.1 c).

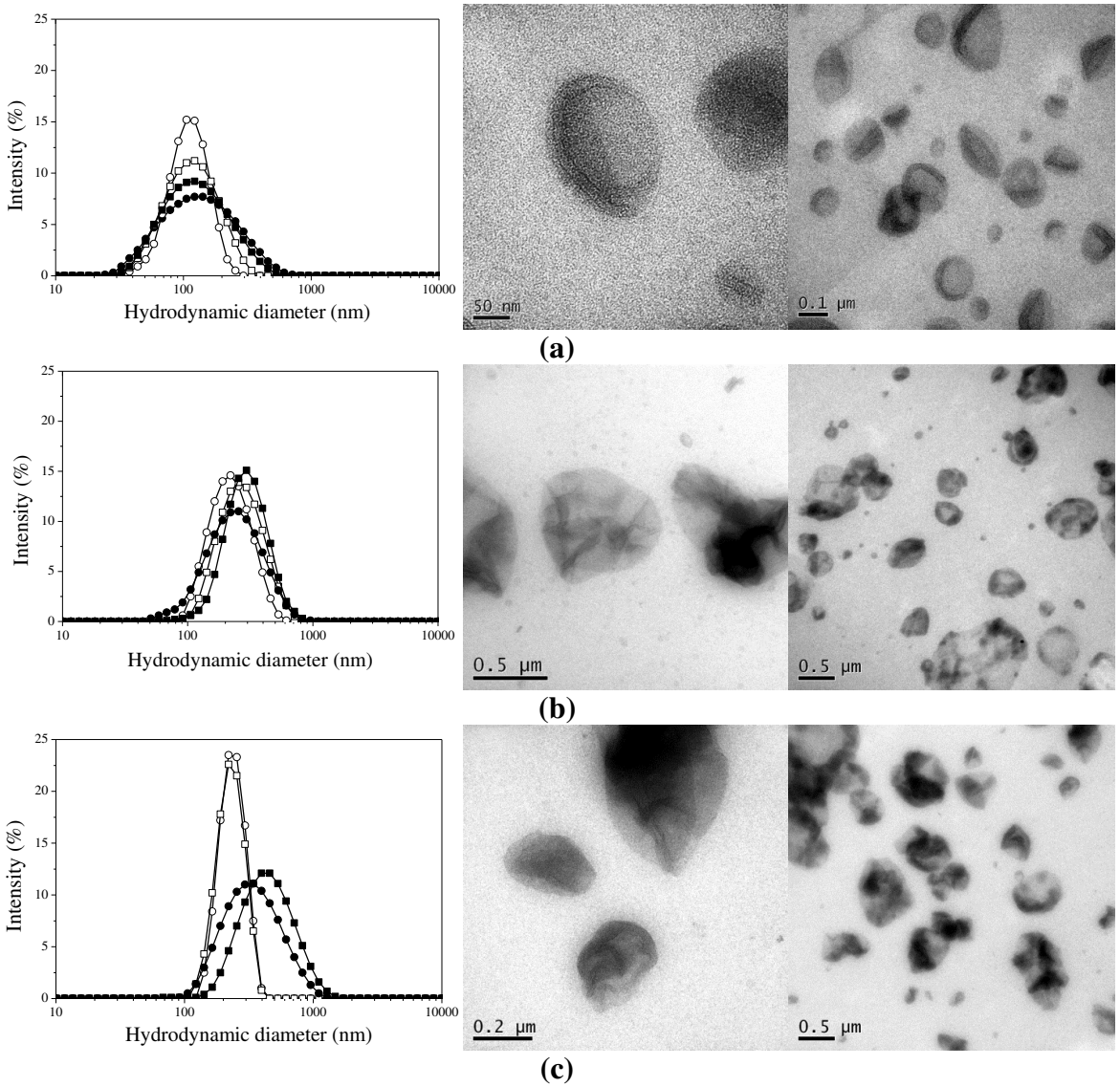


Figure 3.2. Particle size distribution by dynamic light scattering and electron micrographs of empty nanovesicles obtained at 10 mM for **(a)** fat-free powder lecithin (DPL); **(b)** standard fluid lecithin (SFL) and **(c)** enzyme-modified fluid lecithin (EFL) at different lecithin concentrations, where: (○) 1 mM; (□) 2.5 mM; (●) 5 mM and (■) 10 mM. Scale bar (a) 50 nm and 0.1 µm, respectively; (b) both with 0.5 µm and (c) 0.2 µm and 0.5 µm, respectively.

Nanovesicles showed differences in the SAXS profile as a function of lecithin-type, according to Figure 3.3. SAXS is a fundamental technique for the study of assembling vesicular systems at the molecular level. High definition of Bragg peaks is related to a more ordered bilayer structure at molecular level, while extended curves correspond to an inconsistency of molecular assembling (Bowstra et al., 1993). An increase of the scattering peak definition was observed with higher phosphatidylcholine content of the lecithin. SAXS profiles with intense and well defined peaks, as observed for the DPL nanovesicles, probably correspond to a high content of multilamellar vesicles in the systems. On the other hand,

extended curves with low intensity peaks, as observed for the other nanovesicles, correspond to unilamellar vesicles and/or multivesicular systems (Bowstra et al., 1993 and Mertins et al., 2005). Therefore, these results suggest that higher content of phosphatidylcholine in lecithins exert a positive influence on assembling at the molecular level. Indications of a more intense assembling at the molecular level for DPL nanovesicles could also be observed in the electron micrographs (Figure 3.2 a). SAXS results reported previously also indicated a small increase of vesicle lamellarity and stiffness in DPL nanovesicles (Manca et al., 2015).

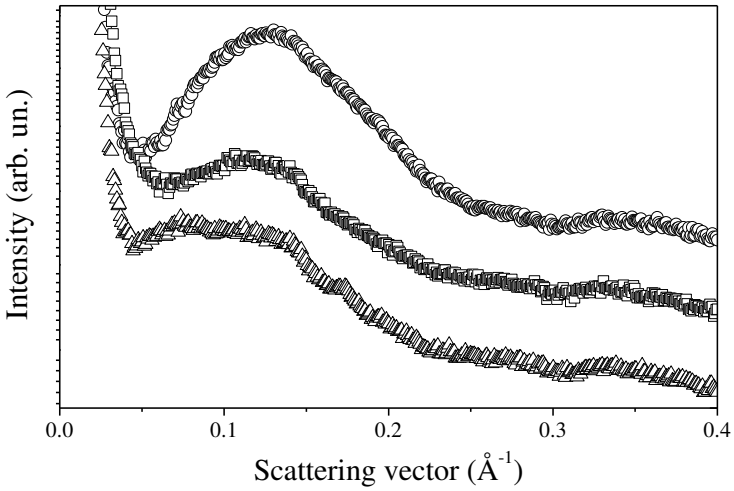


Figure 3.3. SAXS profiles of nanovesicles obtained at 10 mM, where: (○) fat-free powder lecithin (DPL); (Δ) enzyme-modified fluid lecithin (EFL) and (□) standard fluid lecithin (SFL).

3.3.2. β-carotene-incorporated nanovesicles

3.3.2.1. Physicochemical characterization

The influence of initial β-carotene concentration (IC) in the average hydrodynamic diameter of the nanovesicles produced from different lecithin-types can be observed in Figure 3.4 (a). The hydrodynamic diameter showed a significant increase (p<0.05) depending on the initial β-carotene amount only for the DPL nanovesicles. The effect of size expansion in these vesicles could be associated with the orientation of the β-carotene molecule inside the liposomal membrane (Tan et al., 2014b). On the other hand, SFL and EFL nanovesicle size did not change and these results can be related to the presence of the triglycerides (37% *w/w*). The inclusion of triglycerides increases the fluidity providing spaces in the vesicle structure, which results in an easy-fit of β-carotene inside bilayers (Hong et al., 2015).

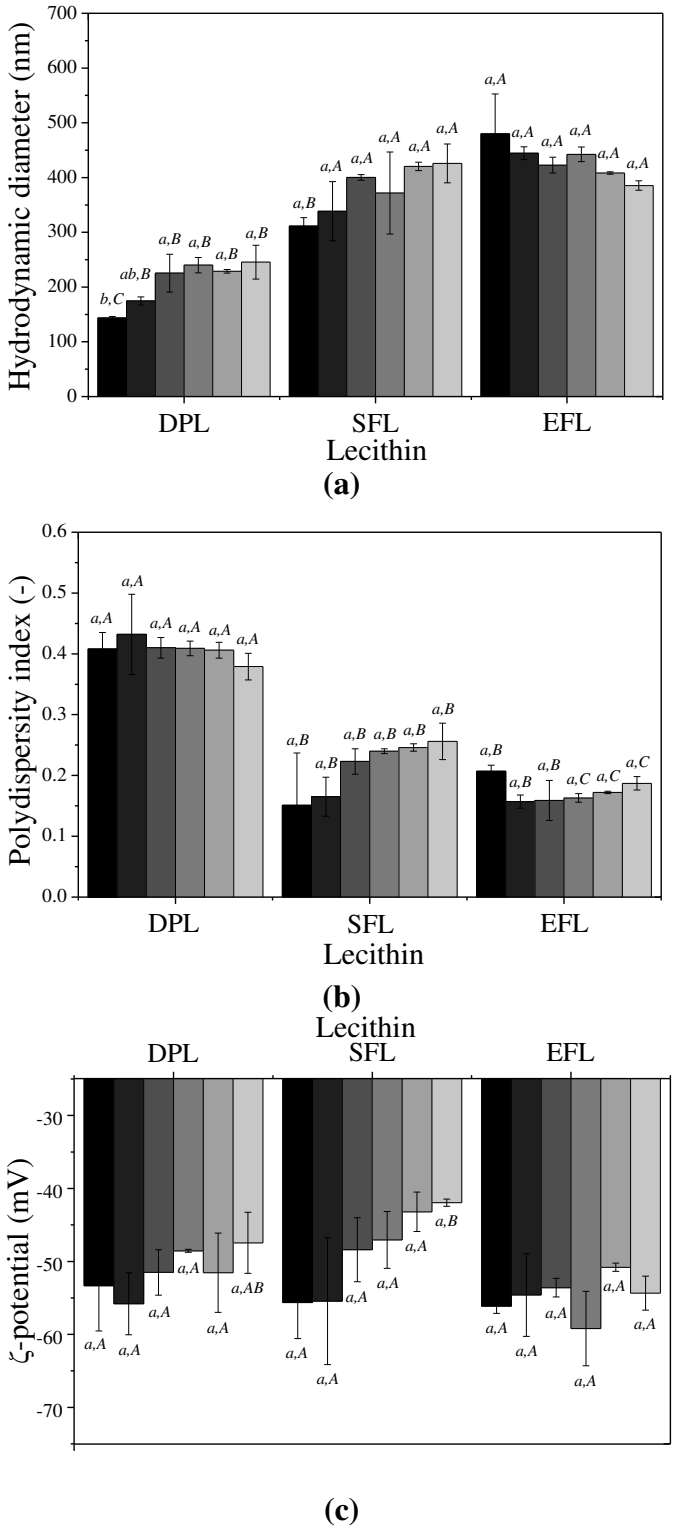


Figure 3.4. Influence of IC (% *w/w*) values on the **(a)** hydrodynamic diameter; **(b)** polydispersity index and **(c)** ζ-potential of the nanovesicles obtained from different lecithin-types at 10 mM. Data represent means ± standard deviations (n=3), different lowercase letters (a-b) represent significant differences (p<0.05) between the initial amount of β-carotene used and different uppercase letters (A-C) represent significant differences (p<0.05) between lecithin-type used, where: (■) 0% (*w/w*); (■) 0.125% (*w/w*); (■) 0.25% (*w/w*); (■) 0.5% (*w/w*); (■) 1% (*w/w*) and (■) 2.5% (*w/w*).

The increase of initial β-carotene concentration did not exert significant influence (p<0.05) on polydispersity index and ζ-potential values of all the nanovesicle system, according to Figure 3.4 (b,c). Besides, according to Figure 3.5 (a-c), for all experimental conditions it was possible to obtain nanovesicle systems with unimodal population. Moreover, the presence of β-carotene did not influence the nanovesicle morphology, since the micrographs of empty nanovesicles were similar to those obtained for β-carotene-incorporated nanovesicles, according to Figures 3.2 (a-c) and 3.5 (a-c).

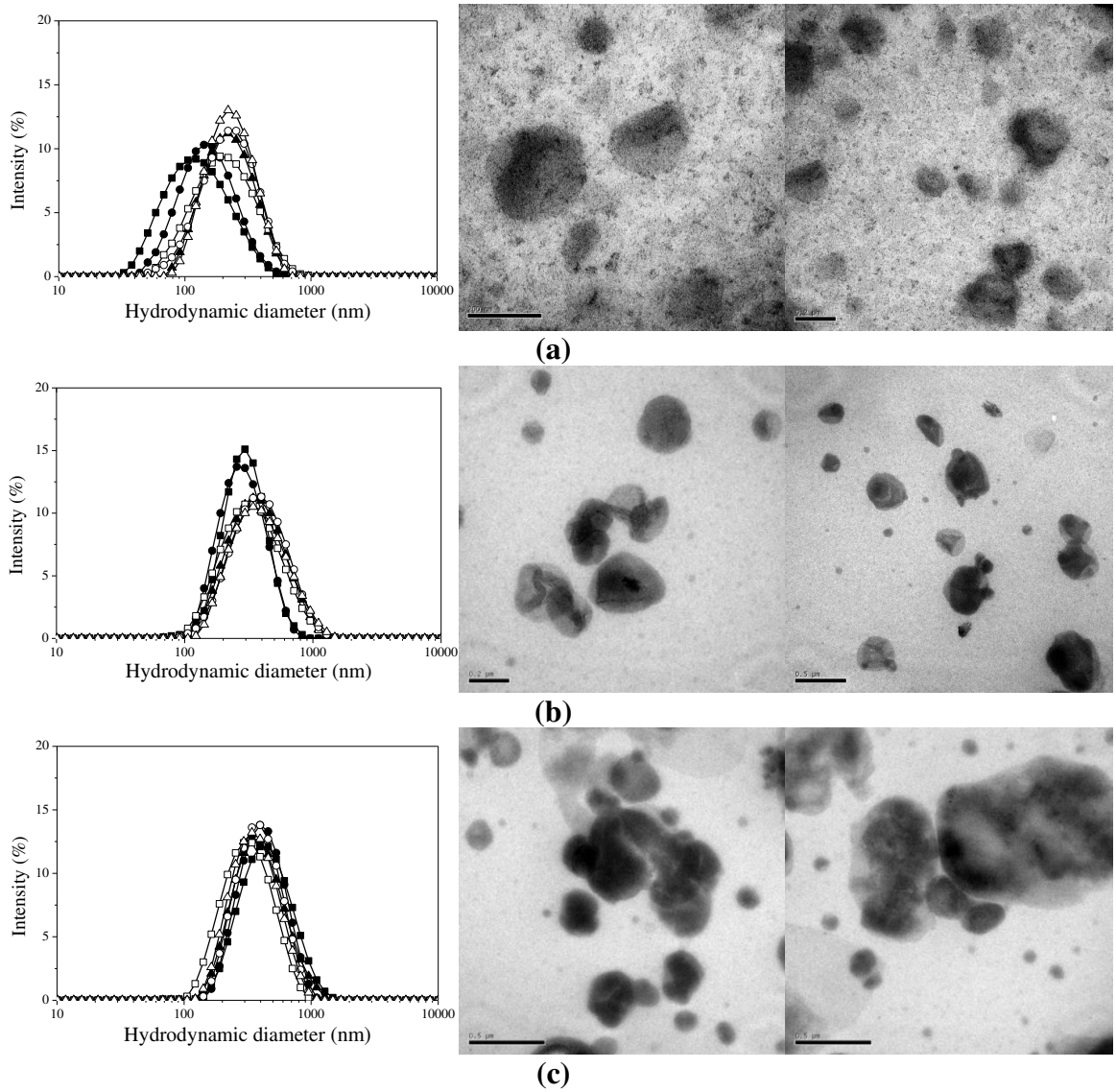


Figure 3.5. Particle size distribution by dynamic light scattering and electron micrographs of β-carotene-incorporated nanovesicles at IC value equal 2.5% (*w/w*) for **(a)** fat-free powder lecithin (DPL); **(b)** standard fluid lecithin (SFL) and **(c)** enzyme-modified fluid lecithin (EFL) at different IC values (% *w/w*), where: (■) 0% (*w/w*); (●) 0.125% (*w/w*); (▲) 0.25% (*w/w*); (□) 0.5% (*w/w*); (○) 1% (*w/w*) and (Δ) 2,5% (*w/w*). Scale bar (a) both with 0.2 µm; (b) 0.2 µm and 0.5 µm, respectively and (c) both with 0.5 µm.

3.3.2.2. Loading capacity and process efficiency

The process efficiency and the β-carotene loading content were measured after separation of β-carotene that was not incorporated into nanovesicles (centrifugation step) and these results are shown in Figure 3.6 (a,b). For the DPL nanovesicles at lower initial concentration (0.125 and 0.25% *w/w*) the β-carotene amounts incorporated inside the bilayer were closer to the initial loading tested, i.e., the best process efficiency. On the other hand, the higher initial concentration of β-carotene (2.5% *w/w*) resulted in a maximum loading capacity of 0.55% (*w/w*). However this result means a high loss of material non-incorporated and may reflect a tendency to maximum incorporation of β-carotene into liposomes rich in phosphatidylcholine. The same behavior was observed for the EFL and SFL nanovesicles and no significant differences were detected ($p<0.05$). Our results suggest that lecithin-type did not interfere in the β-carotene loading capacity. However, in some cases the loading capacity may be increased due to the presence of triglycerides, which creates spaces in the liposomal membrane and may increase the insertion of hydrophobic molecules (Hong et al., 2015).

In general, nanovesicles showed the higher process efficiency values at low initial concentrations of β-carotene and tended to reach constant values when initial concentrations used were equal to or higher than 0.5% *w/w*. This result can indicate the maximum capacity of β-carotene loading in nanovesicles. However, EFL nanovesicles at higher β-carotene concentration tested (2.5% *w/w*) showed a decrease in efficiency, which can be associated with the presence of lysophosphatidylcholine that is responsible for liposomal membrane alterations and limits the β-carotene insertion. Probably, the low process efficiency values is due to the lack of polar groups in the molecule of β-carotene, so that its location is only in the hydrophobic core of the lipid bilayer (Palozza et al., 2006, Tan et al., 2013 and Tan et al., 2014c). The technical feasibility of β-carotene incorporation into liposomes obtained from commercial lecithin with low degree of purity is evidenced, without losses in the loading capacity and process efficiency.

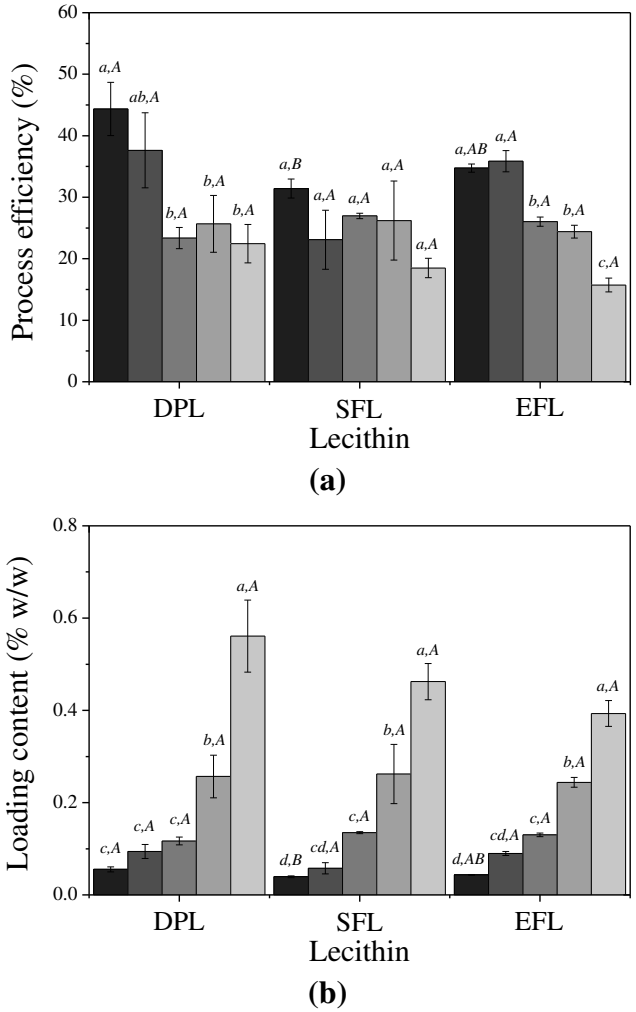


Figure 3.6. (a) Process efficiency of β-carotene incorporation and **(b)** loading content of β-carotene in nanovesicles obtained from different lecithin-types at different IC values (% *w/w*). Data represent means ± standard deviations (*n*=3), different lowercase letters (a-d) represent significant differences ($p<0.05$) between the initial amount of β-carotene used and different uppercase letters (A-B) represent significant differences ($p<0.05$) between lecithin-type used, where: (■) 0.125% (*w/w*); (■) 0.25% (*w/w*); (■) 0.5% (*w/w*); (■) 1% (*w/w*) and (■) 2.5% (*w/w*).

The β-carotene vehiculation efficiency process is directly associated with its localization and orientation inside the lipid bilayer. Because of the absence of polar groups in its structure, the localization of β-carotene is restricted only to the environment of the lipid membrane being regulated by van der Waals interactions with the fatty acid chains (Gruszecki & Strzayk, 2005). With respect to the orientation, studies indicate its dependency on the phospholipid composition. By resonance-Raman spectroscopy studies, it was observed that β-carotene molecules were oriented parallel to the bilayer surface with a high degree of alignment in liposomes obtained from 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC). In contrast, the β-carotene molecules were oriented preferentially forming certain angles to the

surface, i.e., along the lipid chains in bilayers from soybean phosphatidylcholine (van de Ven et al., 1984 and Gonnet et al., 2010).

Despite several indications that orientation of the β-carotene molecule in the liposomal membrane of natural lecithin is closer to the vertical plane than horizontal plane, this conformation is only possible if the lamellar thickness is large enough to keep the β-carotene in this position (Gonnet et al., 2010). A vertical orientation could contribute to a stronger capacity to β-carotene incorporation because more molecules could be inserted in the bilayer besides their presence in the hydrophobic core (Tan et al., 2014b). However, in general it is possible to conclude that β-carotene molecule is located in the hydrophobic portion of phospholipid bilayers with random orientation, increasing the freedom of movement of phospholipid polar groups and reducing the organization of the membrane in the crystalline state (Socaciu et al., 2002).

3.3.2.3. Microviscosity

The average packing order of lipid bilayer is directly proportional to the microviscosity of the lipid bilayer. Therefore, high microviscosity values represent high degree of molecule organization and as a consequence, low permeability of the bilayer (Tan et al., 2013). According to Figure 3.7, the nanovesicles obtained from the three lecithin-types showed an increase of microviscosity with increasing β-carotene content. It was also observed that the presence of triglycerides in the lecithins resulted in a permeability increase of the liposomal membrane. This increase was more pronounced for the EFL nanovesicles (lecithin containing lysophosphatidylcholine), which may explain the low assembling level observed in Figure 3.3 and electron micrographs in Figure 3.5 (c).

In general, the increase of microviscosity was more pronounced up to initial β-carotene amount equal to 0.5% (*w/w*) and 1% (*w/w*). The nanovesicles obtained from fat-free lecithin (DPL) with greater phosphatidylcholine content showed higher microviscosity values (~700 cP) when compared to the others and consequently membranes with less fluidity, indicating that β-carotene molecule was incorporated inside bilayers. The fluorescence anisotropy assays led to the hypothesis that β-carotene incorporation into lipid membranes was governed not only by polarity but by its ability to change membrane anisotropy. Membranes tolerate only a very limited change of rigidity and beyond that limit they do not

incorporate more carotenoid molecules (Socaciu, Lausch & Diehl, 1999). These results confirm the saturation point (0.5% w/w) of the lipid bilayer as shown in Figure 3.6 (a,b).

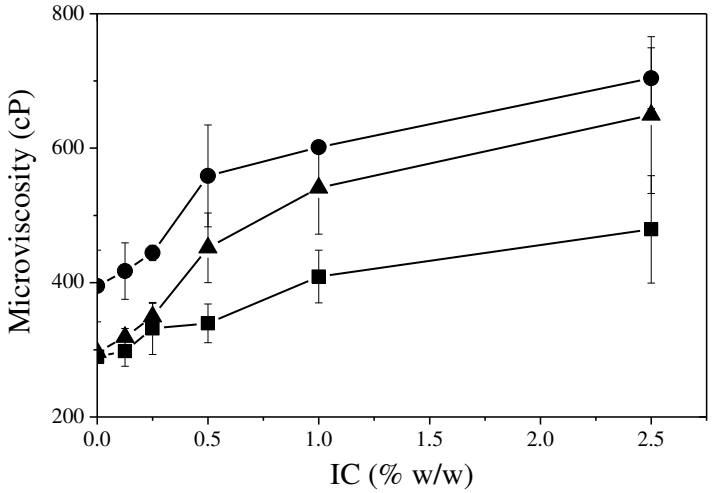


Figure 3.7. Microviscosity changes of the liposomal membrane with increasing the IC values (% w/w). Data represent means ± standard deviations (*n*=3), where: (●) fat-free powder lecithin (DPL); (■) enzyme-modified fluid lecithin (EFL) and (▲) standard fluid lecithin (SFL).

3.3.3 Lipid peroxidation

Natural lecithins may undergo peroxidation due to the presence of polyunsaturated acyl chains in its phospholipid molecules, leading to increased bilayer permeability of the nanovesicles. The iron-induced peroxidation in the presence of ascorbate leads to the formation of water-soluble free radicals that attack the vesicles from the external medium (Schnitzer, Pinchuk, & Lichtenberg, 2007). The kinetics of iron-induced peroxidation and residual β-carotene during assays as a function of lecithin-type and initial amount of β-carotene incorporated in nanovesicles are shown in Figure 3.8 (a-f).

Different behavior of peroxidation kinetics was observed depending on the lecithin-type. EFL nanovesicles showed a linear increase of the TBARS formation over time and an induction period (0 to 0.5 h) where no lipid peroxidation occurred depending on β-carotene concentration (Figure 3.8 b). On the other hand, for DPL and SFL nanovesicles the formation of oxidation products tended to reach equilibrium at the final stage of the period. In general, the TBARS values observed for the DPL nanovesicles were higher than values observed for the EFL and SFL nanovesicles prepared with the commercial lecithins dispersed in oil. This behavior could be related to the presence of α-tocopherol and other antioxidants in the soybean oil, which were removed from the deoiled lecithin.

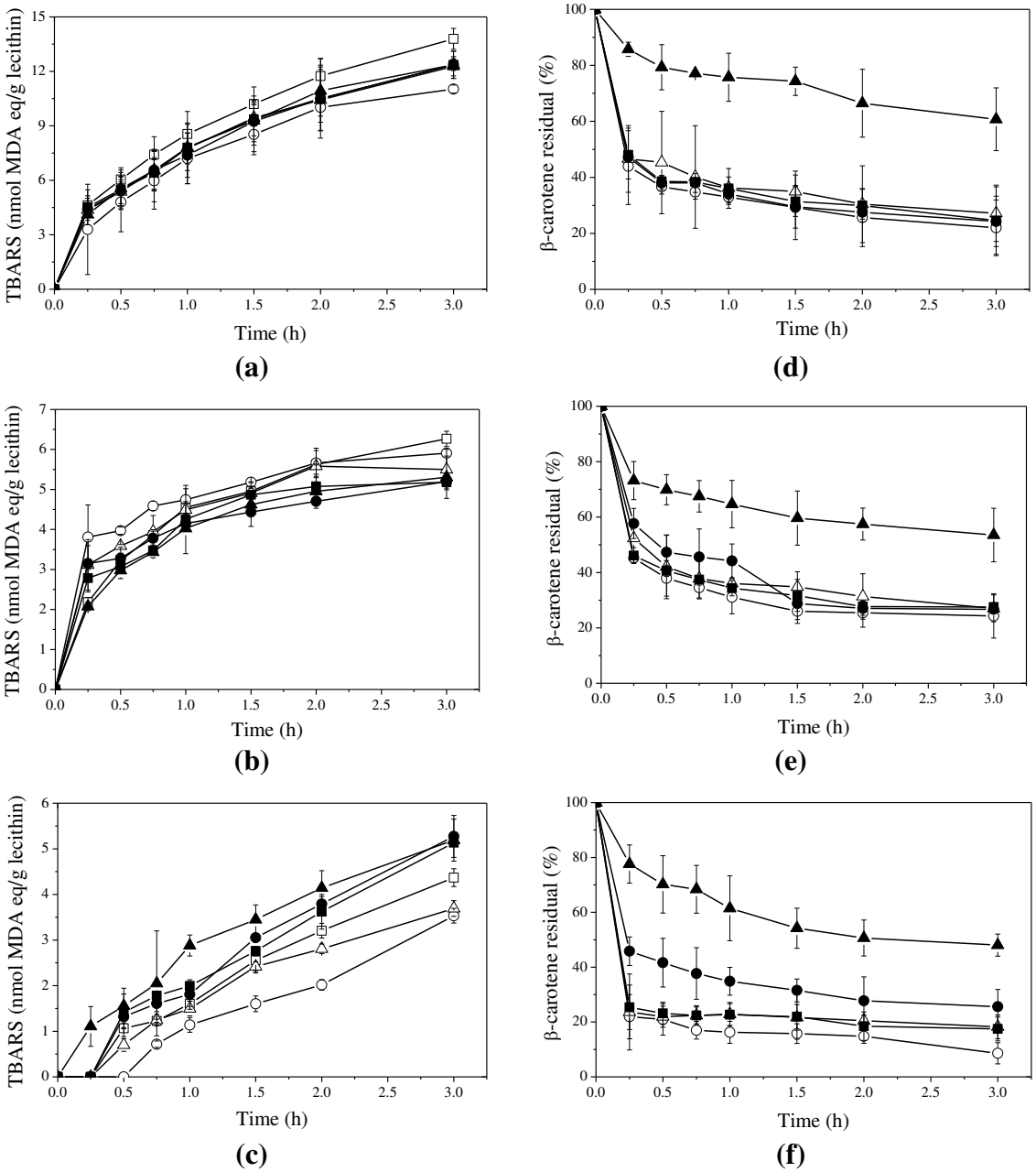


Figure 3.8. Kinetics of TBARS formation and β-carotene oxidation during iron-induced lipid peroxidation at 37° C for the nanovesicles obtained from **(a,d)** fat-free powder lecithin (DPL); **(b,e)** standard fluid lecithin (SFL) and **(c,f)** enzyme-modified fluid lecithin (EFL), respectively. Data represent means ± standard deviations (*n*=3), where: (□) 0% (w/w); (○) 0.125% (*w/w*); (Δ) 0.25% (*w/w*); (■) 0.5% (*w/w*); (●) 1% (*w/w*) and (▲) 2.5% (*w/w*).

According to Figure 3.9 (a), the lipid peroxidation was inhibited after 3 h with incorporation of all IC values used in vesicles obtained from deoiled and commercial standard lecithins. The LPIC varied between 5 and 20% and these results seem not to be related with the initial amount of β-carotene incorporated in the nanovesicles. On the other hand, the inhibition was observed only at low amounts of β-carotene (0.125 to 0.25% *w/w*) for the EFL

nanovesicles, while high initial amounts of β-carotene (0.5 to 2.5% *w/w*) showed significant pro-oxidant properties (p<0.05). According to Figure 3.9 (d-f) a significative amount of β-carotene was degraded during iron-induced peroxidation. The lipid peroxidation inhibition capacity of the β-carotene incorporated into vesicles is related to its direct reaction with pro-oxidant elements instead of lipids or the modulating effect of carotenoids on the physicochemical properties of lipid bilayers (Tan et al., 2014c).

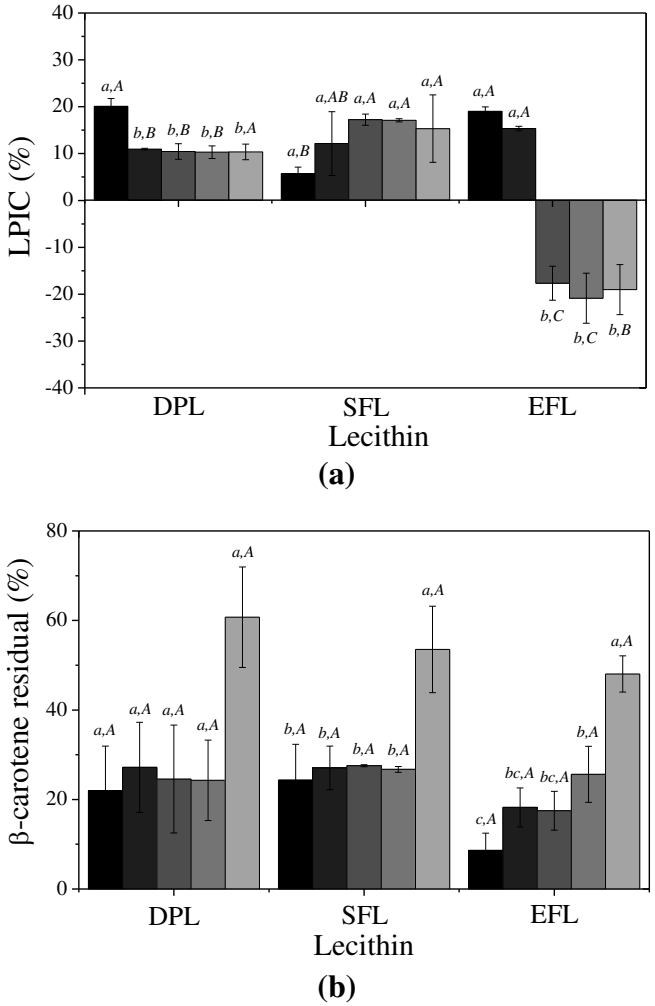


Figure 3.9. (a) Lipid peroxidation inhibition capacity (LPIC) and **(b)** residual amounts of β-carotene after 3 h of iron-induced peroxidation at 37° C. Data represent means ± standard deviations (n=3), different lowercase letters (a-c) represent significant differences (p<0.05) between the initial amount of β-carotene used and different uppercase letters (A-C) represent significant differences (p<0.05) between lecithin-type, where: (■) 0.125% (*w/w*); (■) 0.25% (*w/w*); (■) 0.5% (*w/w*); (■) 1% (*w/w*) and (■) 2.5% (*w/w*).

On the other hand, the action of β-carotene as a pro-oxidant agent in the nanovesicles (Figure 3.9 a) is probably related to three distinct mechanisms: (i) the direct reaction of β-carotene with reactive oxygen and nitrogen species resulting in its cleavage generating

potentially pro-oxidant elements (Woodall et al., 1997 and Tan et al., 2014c); (ii) higher oxygen tension (pO2) that possibly promoted the formation of carotenoid peroxyl radicals and hence pro-oxidant effects (Young & Lowe, 2001 and Woodall et al., 1997); and (iii) the incorporation of β-carotene inside the bilayer packing altering the dynamics of the fatty acid chains and consequently increasing the fluidity of the membrane facilitating the permeation of reactive oxygen species (McNulty et al., 2007 and Tan et al., 2014c).

The antioxidant behavior of β-carotene is partially dependent on the oxygen tension in the medium of application (Woodall et al., 1997 and Young & Lowe, 2001). Under physiological conditions, i.e., at low pO2 values as in the lung alveoli (100 mm Hg), venous blood (40 mmHg) and human tissues (5-15 mm Hg) the β-carotene acts as a chain breaking antioxidant consuming peroxy radical. In contrast, our results were obtained at very high pO2 (760 mm Hg) favoring the loss of antioxidant ability due to its auto-oxidation. Despite β-carotene's being characterized as a pro-oxidant element in the EFL nanovesicles, this phenomenon probably would not occur in biological systems.

According to Figure 3.9 (b), the residual amount of β-carotene did not present significant differences (p<0.05) between EFL and the others nanovesicles considering conditions in which the pro-oxidation phenomenon was observed (0.5 to 2.5% w/w). These results indicate that lipid peroxidation inhibition capacity can be more strongly associated with the modulating effects of β-carotene on the physicochemical properties of lipid bilayers than other mechanisms. Besides, in other studies a strong correlation between the peroxidation and the disorder of lipid bilayers was observed (McNulty et al., 2007). Bilayer organization in the EFL nanovesicles was disturbed due to the increased amount of β-carotene leading to the formation of more permeable membranes and an increased lipid peroxidation, which is in agreement with the microviscosity results shown in Figure 3.7.

The β-carotene probably promoted a disturbance on the lipid acyl chains and increased the motional freedom of lipids in the headgroup region, although the apolar carotenoid remained in the hydrophobic core. Such a disturbing effect could decrease the penetration barrier for molecular oxygen and other small molecules to the liposomal membrane. As a consequence, β-carotene was rapidly degraded by iron or oxygen, and the lipid was not effectively protected from peroxidation. Thus, the contrasting effects of β-carotene on lipid peroxidation can be attributed to their orientation and location inside the membrane (Woodall

et al., 1997, Young & Lowe, 2001, McNulty et al., 2007, Tan et al., 2013 and Tan et al., 2014c).

3.4 Conclusion

In general, the β-carotene incorporation did not promote a significant increase in average hydrodynamic diameter of the phospholipid nanovesicles. However, lower polydispersity indexes of the nanovesicles obtained from oiled lecithins were observed. Although the lecithin-type did not influence the β-carotene loading capacity, the incorporated β-carotene presented contrasting behavior after iron-induced peroxidation process depending on the lecithin-type. In the conditions of higher loading capacity, a residual amount of β-carotene higher than 60% was observed after 3 h exposure in a strongly oxidative environment. Besides, nanovesicles obtained from lecithin with triglycerides showed lower amounts of oxidation compounds than nanovesicles obtained from deoiled lecithin. Finally, our results suggest that low-cost non-purified lecithin containing triglycerides could be employed for nanovesicle production aimed at β-carotene incorporation with the same efficiency of nanovesicles produced from purified phospholipids.

Acknowledgments

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CAPÍTULO IV

Continuous formation of phospholipid vesicles using hydrodynamic flow-focusing microfluidic devices: a focus in higher-throughput for food applications

Submitted to Colloids and Surfaces B: Biointerfaces

Continuous formation of phospholipid vesicles using hydrodynamic flow-focusing microfluidic devices: a focus in higher-throughput for food applications

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Highlights

- β-carotene was successfully incorporated into liposomes.

- The higher aspect-ratio enabled the most rapid liposome formation.

- The potential technical feasibility of microfluidic processes was demonstrated.

- The results expand opportunities of using liposomal systems in the food industry.

Abstract

The microfluidic hydrodynamic flow-focusing is a simple technique for nanoscale liposome formation that provides several advantages compared to the traditional manufacturing techniques. This work aimed to perform a systematic study of the liposome formation using planar microfluidic devices with different channel aspect-ratios, as an alternative to enhance the throughput of liposome synthesis. In general, liposomes with a low polydispersity and a precise control of the size were successfully produced from alteration of the flow rate ratio and channel aspect-ratio. The higher aspect-ratio enabled the most rapid generation of liposomes with similar diameter and significant lower polydispersity index than the obtained by other batch technique. Besides, β-carotene was successfully incorporated into liposomes with efficiency of approximately 60% and the incorporation ability was not specific to a choice of microfluidic device aspect-ratio. The results suggest that the use of microfluidic devices could be employed for liposome production with a possible advantage to minimize

the degree of parallelization of processes. These results demonstrate the potential technical feasibility applications of microfluidic processes for food systems.

Keywords: β-Carotene; Liposome; Microfluidics; Lecithin

4.1. Introduction

Liposomes, or phospholipid vesicles, are spherical supramolecular aggregates composed mainly by natural or synthetic phospholipids, showing shells with one or more bilayers, or lamellae, and an aqueous inner core (Balbino et al., 2013; Carugo et al., 2016). Vesicle systems could be prepared using biocompatible, biodegradable, and non-toxic ingredients enabling faster and easier implementation in food systems, and overcoming regulatory barriers that might hamper their application (Isailović et al., 2013). These structures present the ability to incorporate water-soluble, lipid-soluble and amphiphilic bioactive compounds, which makes them attractive to lipid-based carrier systems for food industry (Đorđević et al., 2015). The phospholipid vesicle systems have been used for delivery and/or protection of compounds such as curcumin (Liu et al., 2015), resveratrol (Isailović et al., 2013), lutein (Tan et al., 2013; Tan et al., 2014a), lycopene (Tan et al., 2014a) and β-carotene (Carvalho et al., 2015; Moraes et al., 2013; Zômpero et al., 2015). Liposome application in complex food matrices has also been performed aiming to bring bench assays closer to technological scenario of food industry, such as the use of ascorbic acid-loaded liposomes in orange juice (Marsanasco et al., 2011); iron(III) sulfate-loaded liposomes in milk (Xia & Xu, 2004) and β-carotene-incorporated liposome in yogurts (Toniazzo et al., 2014).

On the other hand, the production of phospholipid vesicles aiming food applications has several drawbacks. Among other critical factors, conventional batch techniques of liposome formation, such as film hydration, reverse-phase evaporation or alcohol injection present limitations in terms of suitability for scaling up from the bench-scale to the industrial production, since size distribution of generated liposomes show a low reproducibility from batch-to-batch (Justo & Moraes, 2011). Apart that, these processes are discontinuous and typically should be combined with post-processing steps to reduce the diameter and polydispersity of the final liposomes. Thus, the great challenge is the continuous production of monodisperse liposomes, with tunable sizes below the limits achieved current batch production techniques. In this context, the microfluidic techniques may be used to overcome the challenges imposed for large-scale food industrial production.

Microfluidics is defined as the science of designing, manufacturing and operating processes and devices with small amounts of fluids in laminar regime. Microfluidic devices have dimensions ranging from a few millimeters to micrometers, which are characterized by exhibiting at least one channel with dimension smaller than 1 mm (Ushikubo et al., 2015; Whitesides, 2006). The most common microfluidic approach for liposomes production is the hydrodynamic flow-focusing technique using planar microfluidic devices with cross-shaped geometry, described firstly by Jahn et al. (2004). The microfluidic hydrodynamic flow-focusing is a simple technique for nanoscale liposome formation that provides several advantages compared with the conventional manufacturing methods (Hood & DeVoe, 2015). In this approach, the liposome formation occurs by introducing a stream containing phospholipids dispersed in alcohol through a central channel and subsequent constriction of phospholipid dispersion by two perpendicular or oblique aqueous streams (Jahn et al., 2004; Jahn et al., 2010; Balbino et al., 2013; Mijajlovic, et al., 2013). The controlled co-diffusion of phospholipids, alcohol and aqueous streams results in the self-assembly of unilamellar lipid nanovesicles, with diameters that may be adjusted by changing the ratio of water to alcohol volumetric flow rates (Hood & DeVoe, 2015).

Microfluidics technology offers additional advantages over batch techniques, including the possibility of liposome continuous production and scaling up by microchannels parallelization (Carugo et al., 2016). Despite of these advantages, the hydrodynamic flow-focusing technique using planar microfluidic devices for liposome formation is characterized as a low-throughput process due to the limited volumetric flow rates imposed by the small channel dimensions generally used. Scarce alternatives have been proposed to improve liposomes throughput by hydrodynamic flow-focusing, such as the increase of phospholipids concentration or total volumetric flow rate (Mijajlovic et al., 2013; Jahn et al., 2010; Kastner et al., 2015), the use of microfluidic devices with a double hydrodynamic flow-focusing region (Balbino et al., 2013) or with vertical flow-focusing device (Hood & DeVoe, 2015). The maximum flow rate and phospholipids concentration are generally restricted by limitations associated to fluid rheological behavior, which can increase internal pressure leading to clogging issues. Alternatively, the use of multiple microfluidic flow-focusing elements operating in parallel can be explored for liposomes formation. However, it is desirable to minimize the degree of parallelization, because a large number of parallel microfluidic elements can have a negative impact on the overall reliability of the continuous-flow system (Hood & DeVoe, 2015).

Thus, as an alternative to enhance the throughput of liposome synthesis by hydrodynamic flow-focusing, this work aimed to perform a systematic study of the liposome formation using planar devices with higher aspect-ratio (width/height) of channels. Thus, the high aspect-ratio may support industrial production of liposomal food systems with a minimal parallelization degree using a smaller number of devices. Finally, β-carotene shows important biological activity due to its provitamin A activity, but its effective utilization as nutraceutical ingredient in aqueous formulations is hampered. For this reason, β-carotene, a hydrophobic model molecule, was incorporated in the liposomal systems, aiming the evaluation of technical feasibility of the microfluidic hydrodynamic flow-focusing as a potential technique for industrial production of food systems.

4.2. Materials and methods

4.2.1. Materials

The liposomal systems were obtained using a food-grade and deoiled powder soybean lecithin commercially named Lipoid S45 (>45% *w/w* phosphatidylcholine, 10-18% *w/w* phosphatidylethanolamine, <4% *w/w* lysophosphatidylcholine and <3% *w/w* triglycerides) acquired from Lipoid GmbH (Ludwigshafen, Germany). Absolute ethanol chromatographic-grade (99.9% *v/v*) and synthetic β-carotene powder (>93% *w/w*) were purchased from Panreac® (Barcelona, Spain) and Sigma-Aldrich (St. Louis, USA), respectively. The rigid replication masters were obtained using SU-8 25 UV photoresist and the SU-8 developer solution (MicroChem Co., Newton, MA, USA). The microfluidic devices were prepared using the commercial mixture Sylgard 184 Silicone Elastomer kit (The Dow Corning Co., Midland, MI, USA), containing the polydimethylsiloxane pre-polymer and curing agent. Polycarbonate microfluidic connectors 1.6 mm, 1/16" (Cole-Parmer Instrument Co., Chicago, IL, USA), rigid polyethylene tubes with outer diameter 2 mm (Mark Med, Bragança Paulista, SP, Brazil), silicone adhesive glue (TekBond®, Embu das Artes, SP, Brazil) and syringe needles 0.8×32 mm (Nipro Medical Co., Bridgewater, NJ, USA) were used to assemble the microfluidics devices.

4.2.2. Methods

4.2.2.1. Device fabrication

Microfluidic devices were obtained using the conventional soft lithography technique, according to the protocol described by McDonald et al. (2000) with some modifications proposed by Moreira et al. (2008). Firstly, four different mask layouts with flow-focusing mechanism geometry were designed using the software AutoCAD 2012 (Autodesk Inc., San Rafael, CA, USA). These flow-focusing microfluidic devices (cross-shaped junction) were designed with different channels aspect-ratio (width/height): 1:1 (50×50 μm), 2:1 (100×50 μm), 4:1 (200×50 μm) and 6:1 (300×50 μm), according to Figure 4.1 (a). Next, designs were plotted in photo-masks (Figure 4.1 b) with 8000 dpi resolution by DGM Design (Curitiba, Brazil). For the production of rigid replication masters, silicon wafers were spin-coated with negative SU-8 25 photoresist at 1000 rpm during 30 s. The layouts were transferred from the photo-masks to the rigid replication masters by UV exposure during 70 s using a contact mask aligner (model MJB-3 UV300, KarlSuss, Garching, Germany). Afterwards, the rigid replication masters were revealed through a washing with developer solution for SU-8 25 photoresist. The microfluidic devices were casted using a polydimethylsiloxane pre-polymer and curing agent mixture (10:1 *w/w*). The mixture was degassed at 6 Pa during 1 h to eliminate air bubbles, and spilled over the replication masters using an appropriate experimental holder. The polymer curing process was performed during 1 h on a hot plate at 95 ºC. The devices were firstly washed with ultrapure water, then with isopropyl alcohol and finally dried with nitrogen gas. The devices were irreversibly sealed to a glass slide through oxygen plasma treatment at 16 Pa during 20 s (model PLAB SE80, Plasma Technology Ltd., Wrington, England). Finally, syringe needles were inserted and sealed with silicone adhesive glue for injection of the phases and for collection of generated liposomes. Figure 4.1 (c) shows a microfluidic device after the fabrication process.

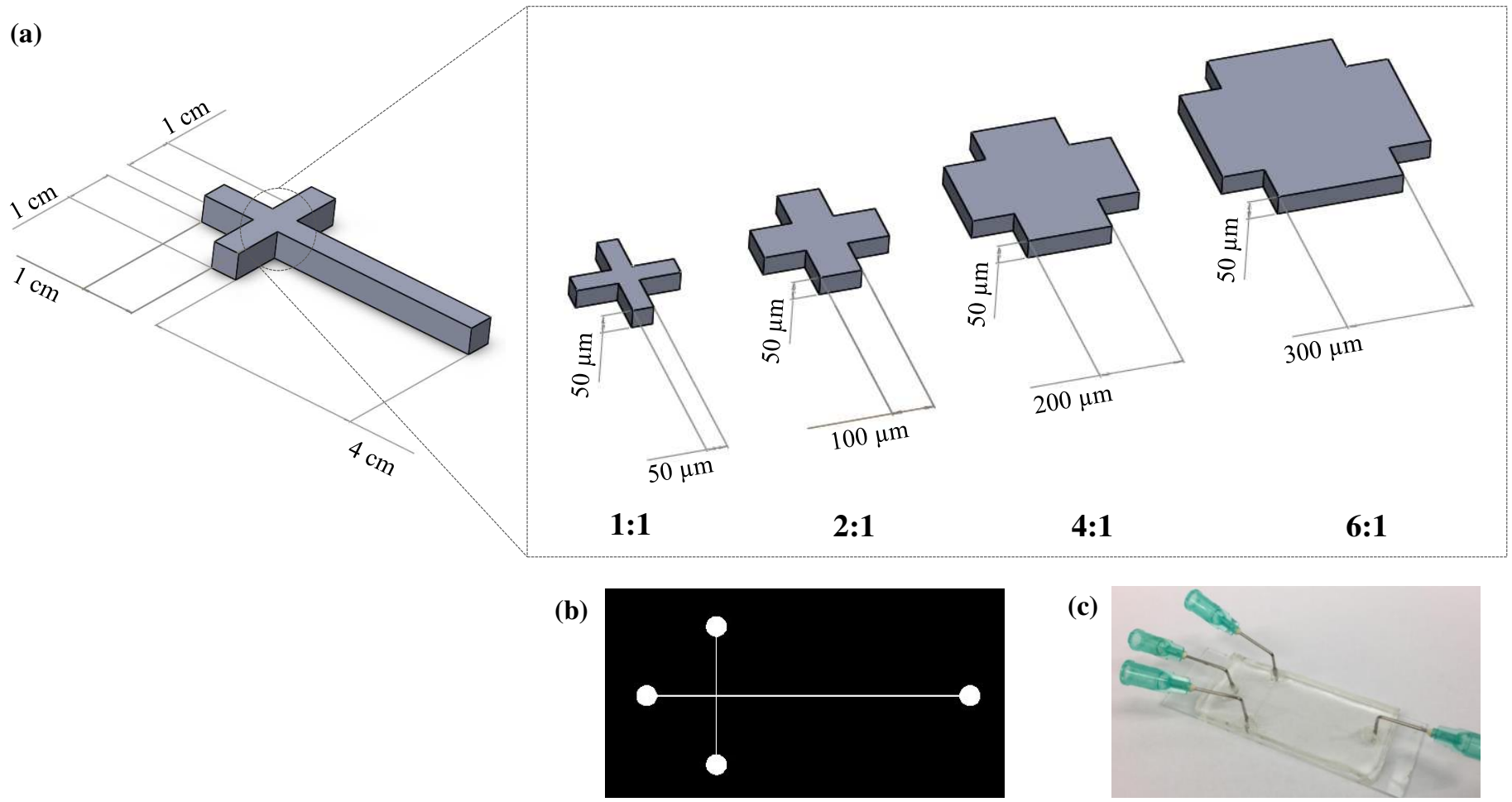


Figure 4.1. **(a)** Schematic diagram of microfluidic device aspect-ratios, **(b)** an example of photo-mask used and **(c)** image of a fabricated microfluidic device.

4.2.2.2. Liposome production

The microfluidic production of liposomal systems was performed by hydrodynamic flow-focusing method. Basically, an alcoholic phase containing soybean lecithin dispersed in ethanol was injected between two phases containing ultrapure water, resistivity 18.2 MΩ/cm (Direct-Q3 System, Millipore Co., Billerica, MA, USA). Phases flowed through microfluidic devices with the volumetric flow rate controlled by syringe-type pumps (model PHD 2000, Harvard Apparatus, Inc.; South Natick, MA, USA). Polyethylene tubing and connectors for microfluidic were used to link the microfluidic device to the syringe-type pumps. All assays were performed at room temperature (~22 ºC) and hydrodynamic flow-focusing was observed through an optical microscope (model Multizoom AZ100, Nikon Co., Tokyo, Japan). Bright field micrographs were obtained and used to measure the width of the focused stream using the open-source software Image J (version Java 1.6.0_24, National Institutes of Health, Bethesda, MD, USA). The liposomal systems were collected in the channel output using a glass-flask. The flow rate ratio (FRR) was defined as the ratio of volumetric flow rate of aqueous stream (Q_a) to the volumetric flow rate of alcoholic stream (Q_l) according to Equation 4.1, where Q_t is the total volumetric flow rate calculated by Equation 4.2.

$$FRR = \frac{2Q_a}{Q_l} \quad \text{(Equation 4.1)}$$

$$Q_t = (2Q_a) + Q_l \quad \text{(Equation 4.2)}$$

Firstly, we used a microfluidic device with aspect-ratio of 2:1 (100×50 µm) operated at a constant flow rate ratio of 10, to evaluate the influence of soybean lecithin concentration (20, 40, 60, 80 and 100 mM) in the center stream and average flow velocity (0.075, 0.1, 0.125, 0.15, 0.2, 0.3, 0.4 and 0.5 m/s, corresponding to volumetric flow rates of 22.5, 30, 37.5, 45, 60, 90, 120 and 150 µl/min, respectively) on the liposome particle size distribution. On the other hand, the influence of microfluidic device aspect-ratio on the liposome size was performed at different flow rate ratios (5, 7.5, 10, 12.5, 15, 20, 30, 40 and 50) with constant average flow velocity at 0.5 m/s, corresponding to volumetric flow rates of 75, 150, 300 and 450 µl/min for microfluidic aspect-ratios of 1:1 (50×50 µm), 2:1 (100×50 µm), 4:1 (200×50 µm) and 6:1 (300×50 µm), respectively. In parallel, liposomal systems were also produced by ethanol injection technique for comparison with microfluidics. The system used for ethanol injection was basically composed of a glass jacketed reactor, useful volume of 150 ml, equipped with a four baffles system and a straight blade impeller (Michelon et al., 2016). The

soybean lecithin dispersion (100 mM) was fed (1:10, *v/v*) at a constant flow rate of 10 ml/min using a peristaltic pump (model MasterFlex® L/S, Cole-Parmer Instrument Co., Chicago, IL, USA) into the reactor which was filled with ultrapure water. During this process, the temperature of the system was kept at ~25° C using a thermostatic bath and at 500 rpm using a mechanical stirrer (model RW 20, Ika Works Inc., Germany). All liposomal dispersions were cooled to 8 °C for 12 h before their physicochemical characterization.

4.2.2.3. β-carotene-incorporated liposome production and stability

The same processes described previously were used to produce β-carotene-incorporated liposome. In this case, β-carotene was mixed with soybean lecithin dispersed in ethanol (100 mM), in order to reach a β-carotene concentration relative to amount of soybean lecithin of 2.5% (*w/w*). The influence of microfluidic device aspect-ratio was evaluated at a constant flow rate ratio of 10 and average flow velocity of 0.5 m/s. The liposomal dispersions obtained were cooled to 8° C for 12 h before their physicochemical characterization. In addition these dispersions were centrifuged (model Allegra 25R, Beckman Coulter, Miami, FL, USA) at 2,450×*g* for 10 min to separate the unincorporated β-carotene amount. The stability of liposomal systems was evaluated during storage at room temperature under uncontrolled light conditions during 28 days. For β-carotene quantification, firstly β-carotene was extracted from the aqueous liposomal fractions by a liquid-liquid extraction using absolute ethanol-hexane mixture (Toniazzo et al., 2014; Hung et al., 2011). Subsequently, β-carotene content was measured by reading the absorbance of hexane phase at 450 nm in UV-vis spectrophotometer (model SP-220, Biospectro, Brazil) which was related to the β-carotene molar absorption coefficient in hexane (2,592 M^{-1} cm^{-1}), according to proposed by Rodriguez-Amaya (2001). The relative β-carotene-incorporated amount (%) into liposomes was estimated according to the ratio between the amount of β-carotene-incorporated that is present in the supernatant after centrifugation and the total initial amount of β-carotene.

4.2.3. Liposome characterization

4.2.3.1 Particle size distribution and ζ-potential

The particle size distribution, hydrodynamic diameter and polydispersity index of the liposomal systems were determined at 25° C by Dynamic Light Scattering using the

equipment Zetasizer (model Nano-ZS, Malvern Instruments, UK). This technique is based on the dependence of intensity fluctuations of light scattering as a function of time due to Brownian motion of the particles in suspension. Hydrodynamic diameter of the liposomes was evaluated in terms of scattered light intensity which is proportional to the sixth power of the particle diameter. The mode of the intensity peak for the particle size distribution was considered as the average hydrodynamic diameter. The polydispersity index was calculated from cumulant analysis of the measured light scattering intensity autocorrelation function. The polydispersity index varied from 0 to 1, where higher values indicate a less homogeneous size distribution. Besides, the same equipment was used to measure the surface electric charge of the liposomes. In this case, the electrophoretic mobility was obtained by Laser Doppler Anemometry technique, which makes use of the relationship between the electrophoretic mobility and the light scattering produced by the particles dispersed in the medium. The mathematical model proposed by Smoluchowski was used to convert electrophoretic mobility measurements in ζ-potential values.

4.2.3.2 Transmission electron microscopy

The transmission electron micrographs were obtained by negative staining method according to protocol proposed by Théry et al. (2006) with some modifications proposed by Michelon et al. (2016). The liposomal fractions were ultra-centrifuged (model L8-80M, Beckman Coulter Inc, USA) at 100,000×*g* for 3 h. The pellet obtained was suspended in 100 µl of buffered formaldehyde solution (2% *v/v*) and diluted 2-fold. Nickel grids coated with the polyvinyl formal resin (Formvar) were placed on 5 µl of the suspended pellet. For the negative staining, grids were deposited on drops of saturated uranyl oxalate solution (pH 7.0) and kept for 5 min under room conditions. Afterwards, the grids were transferred to droplets of methyl cellulose (2% *w/v*) on ice for 10 min. Excess of methyl cellulose and uranyl oxalate solutions were removed with filter paper and air-dried. Samples were examined under the electron microscope (model 1200 EX II, JEOL Inc., Japan) at 80 kV and the micrographs were obtained by using a digital camera (model Orius 832J06W1, GATAN, USA).

4.2.4. Statistical analysis

All assays for liposome production using microfluidic device and ethanol injection techniques were performed in triplicate (n=3), and the analyses for liposome characterization

were also performed in triplicate. The results obtained were evaluated using the open-source software Sisvar 4.6 (University of Lavras, Lavras, MG, Brazil) by variance analysis and Tukey's test was applied to evaluate significant differences between the average values (p<0.05).

4.3. Results and discussion

4.3.1. Soybean lecithin concentration influencing liposome characteristics

The phospholipid concentration is an important parameter contributing to the increase in the throughput of liposomes in hydrodynamic flow-focusing microfluidic devices, since an increase in concentration could result in an increase of liposome productivity. Thus, the influence of soybean lecithin concentration on hydrodynamic diameter, polydispersity index and particle size distribution was investigated. Liposome size did not show significant differences (p<0.05) when concentration was increased from 20 to 80 mM (Figure 4.2 a) but an increase of soybean lecithin concentration from 80 to 100 mM led to higher hydrodynamic diameter of liposomes. On the other hand, according Figure 4.2 (b,c) the polydispersity did not show any significant variation (p<0.05). This behavior was in agreement to that obtained for other authors using different phospholipid mixtures and microfluidic systems by hydrodynamic flow-focusing technique (Balbino et al., 2013; Mijajlovic et al., 2013; Hood & DeVoe, 2015).

The increase of hydrodynamic diameter as a function of lecithin concentration could be related to the theory of liposome formation by methods involving the diffusive mixture of an alcohol and an aqueous phase. In these processes, initially phospholipids dispersed in alcohol are transformed into planar intermediate structures in contact with water, such as disk-like fragments or oblate micelles (Jahn et al., 2010; Carugo et al., 2016). Then, the diffusive mixture of water and alcohol during liposome formation will cause thermodynamic instabilities at edges of planar intermediates, inducing their bending and closing. The planar intermediates formed simultaneously to the diffusion process characterize an additional resistance to mass transfer of the alcohol stream (Huang et al., 2010; Balbino et al., 2013). Thus, probably the alcohol diffusion will occur more slowly at higher soybean lecithin concentrations, which will allow intermediates and free phospholipid molecules to link between themselves more intensively, thus resulting in larger liposomes.

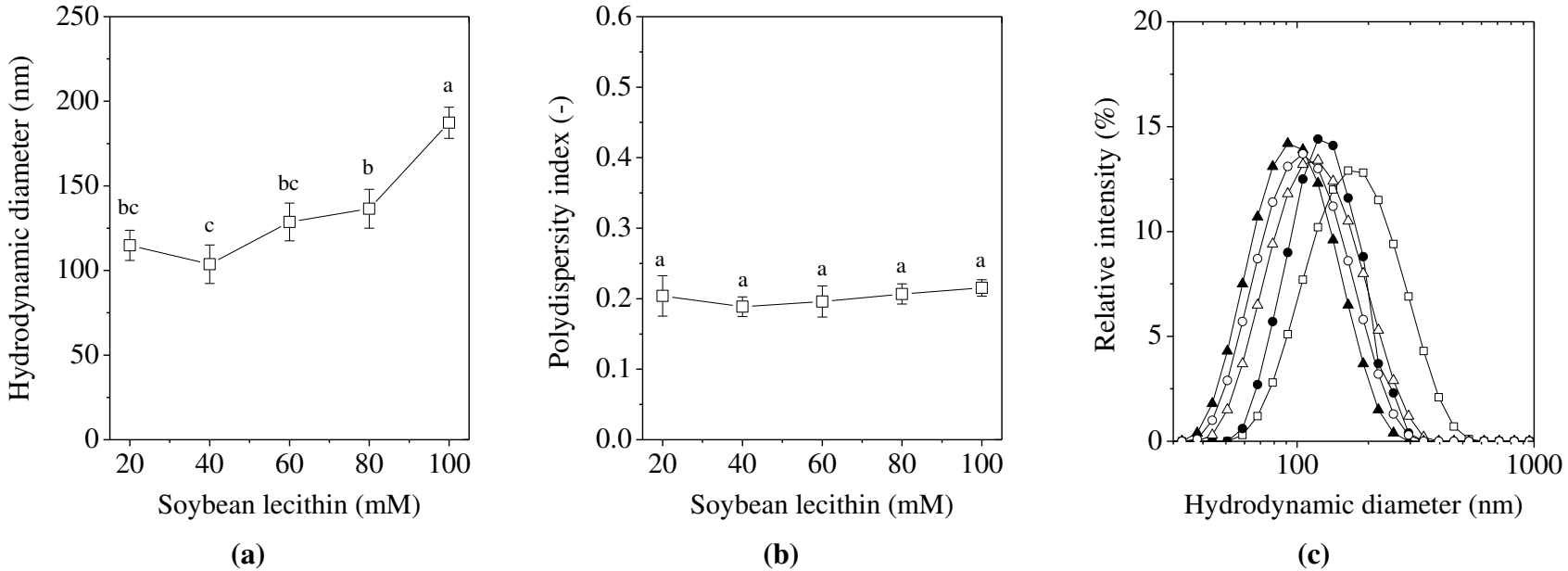


Figure 4.2. **(a)** Hydrodynamic diameter, **(b)** polydispersity index and **(c)** particle size distribution of liposomes produced using a microfluidic device aspect-ratio of 2:1 (100×50 μm), flow rate ratio of 10 and average flow velocity of 0.125 m/s, varying soybean lecithin concentration. Data represent average ± standard deviations (n=3). Different letters represent significant differences (p<0.05) between the soybean lecithin concentrations used: (○) 20 mM, (▲) 40 mM, (Δ) 60 mM, (●) 80 mM and (□) 100 mM.

Most published works based on hydrodynamic flow-focusing using microfluidic devices for liposome formation employed lower values of phospholipid concentration than that used in the present study, for instance 5 mM (Jahn et al., 2007; Jahn et al, 2010; Zook & Vreeland, 2010; Wi et al., 2012), 6.25 mM (Hong et al., 2010), 2-20 mM (Mijajlovic et al., 2013) and 20 mM (Hood & DeVoe, 2015). Low concentrations (~5 mM) are generally used since this is the approximate solubility of phospholipids in alcohols. This ensures that all phospholipids remain below their solubility limits and do not form aggregates prior to liposome formation (Hood & DeVoe, 2015; Balbino et al., 2013). However, due to dilution inherent of the hydrodynamic flow-focusing technique, the final liposomal formulations obtained using these concentration ranges will be highly diluted, and thus their industrial application will be limited. Thus, a lecithin concentration of 100 mM was chosen for use in all experiments, because a diameter of 175 nm was observed, resulting in higher-throughput of liposomes than other tested concentrations.

4.3.2. Influence of total volumetric flow rate, or average flow velocity on liposome properties

Apart that lecithin concentration, the average flow velocity, or total volumetric flow rate is a process condition that impacts directly on throughput of liposomes. Thus, the hydrodynamic diameter, polydispersity index and particle size distribution were evaluated at 100 mM soybean lecithin as a function of total volumetric flow rate, and consequently as a function of average flow velocity, according to Figure 4.3. Results showed that diameter, polydispersity and particle size distribution did not present significant differences (p<0.05) varying flow velocity (0.075 to 0.5 m/s) at a constant flow rate ratio of 10. These results are in agreement with some reports that obtained similar liposome size distribution by an increasing of flow velocity at a constant flow rate ratio, in hydrodynamic flow-focusing microfluidic devices (Jahn et al., 2007; Lo et al., 2010). However, liposome formation could depend on average flow velocity within certain focusing regimes at a specific magnitude of flow rate ratio (Jahn et al., 2010), which did not occur at this focusing condition tested, i.e., constant flow rate ratio of 10 and high soybean lecithin concentration of 100 mM.

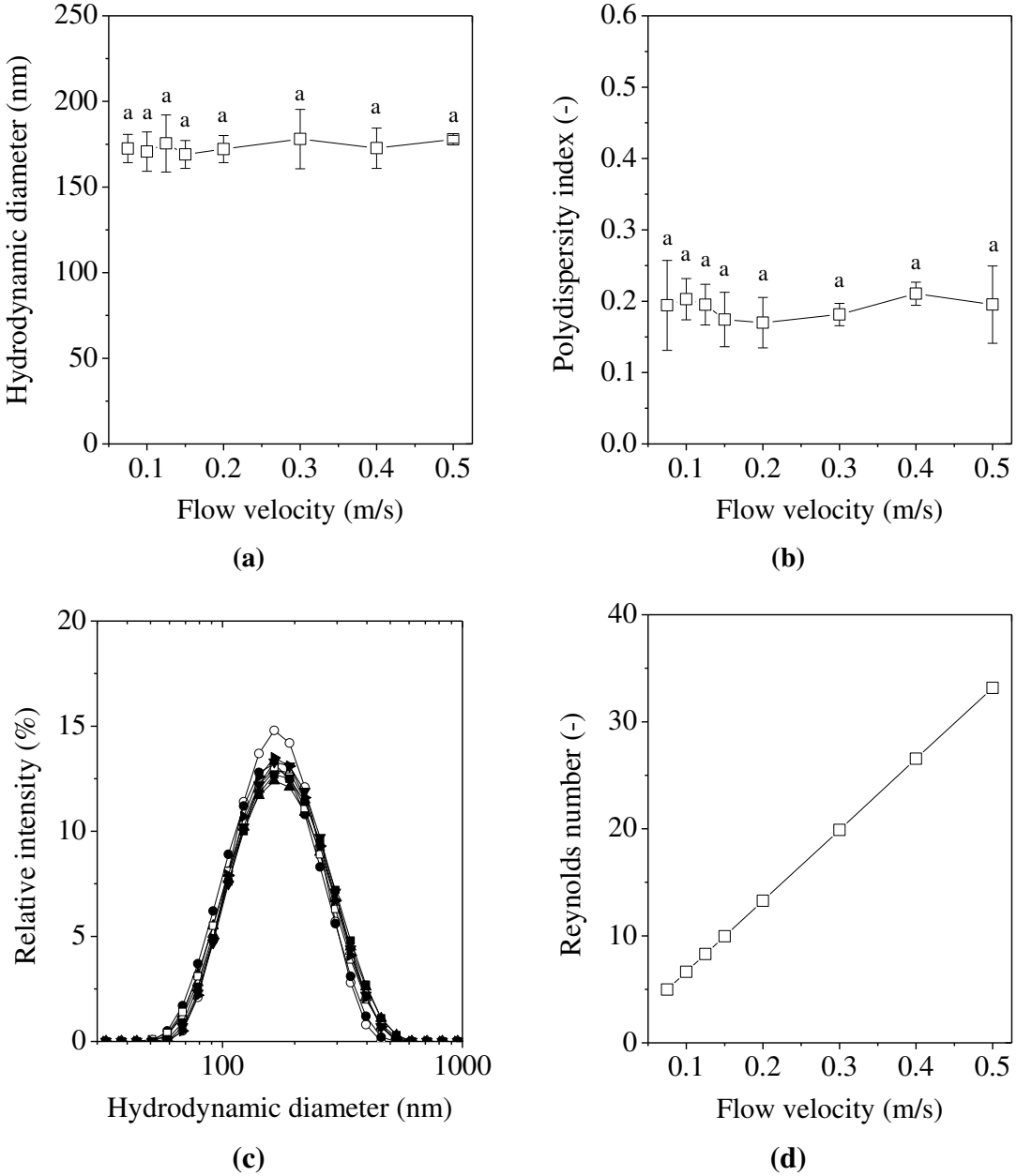


Figure 4.3. (a) Hydrodynamic diameter, **(b)** polydispersity index, **(c)** particle size distribution and **(d)** Reynolds number for liposomes produced using microfluidic device aspect-ratio of 2:1 (100×50 µm), flow-rate ratio of 10 and soybean lecithin concentration of 100 mM, varying average flow velocity. Data represent average ± standard deviations (n=3). Same letters represent non-significant differences (p<0.05) between the average flow velocity used: (○) 0.075 m/s; (▲) 0.1 m/s, (Δ) 0.125 m/s, (●) 0.15 m/s, (□) 0.2 m/s, (▼) 0.3 m/s, (►) 0.4 m/s and (■) 0.5 m/s.

The influence of flow velocity was evaluated at the same flow rate ratio value keeping constant the focused width stream. Thus, the dilution rate was also held constant, since the liquids are incompressible, despite of the shear forces on the pseudo-interface of the two streams were significantly increased with the increase of average flow velocity (Jahn et al.,

2007; Jahn et al., 2010). Therefore, our results indicate that the absolute magnitude of the shear forces between the parallel layered streams has no significant impact on diameter and polydispersity of produced liposome. According to previously described in literature for microfluidic droplet generation using devices of polydimethylsiloxane, the high pressure due to increasing average flow velocity can cause a substantial deformation at the channel walls. Deformation of channel walls could lead to perturbations in focusing, promoting the formation of liposome with a wider particle size distribution or higher polydispersity (Lo et al., 2010). However, our results did not show influence on particle size distributions by increasing flow velocity from 0.075 to 0.5 m/s (Figures 4.3 b,c), demonstrating that possible effects of polydimethylsiloxane deformation on liposome size can be neglected.

Reynolds number (Re) was calculated according to Equations 4.3 and 4.4, where ρ is the density, v is the average flow velocity and μ is the apparent viscosity. Hydraulic diameter (d_h) is obtained from the relation between channel cross-sectional area (A) and wet perimeter (P). Reynolds number expresses the magnitude of inertial to viscous forces ratio. According to Figure 4.3 (d), the non-significant influence of average flow velocity on the particle size distribution can be related to the maximum Reynolds number observed (33.17) at the highest velocity tested (0.5 m/s). It means a laminar flow regime, which is typical in microfluidic devices (Re<<1,800-2,000, Sharp & Adrian, 2004), considering that their characteristic dimension is very small becoming the inertial effects irrelevant. Thus, higher flow velocities could be used maintaining the laminar flow regime, but, in practice, the maximum flow velocity generally is restricted by limitations imposed by fluid rheological behavior and device characteristics.

$$\mathrm{Re} = \frac{\rho\, d_h\, v}{\mu} \quad \text{(Equation 4.3)}$$

$$d_h = \frac{4A}{P} \quad \text{(Equation 4.4)}$$

The average flow velocity is inversely proportional to the residence time inside of microfluidic device. Thus, residence time ranged from 80 ms (0.5 m/s) to 530 ms (0.075 m/s) considering the length of mixing channel of 4 cm (Figure 4.1 a). It was considered that the flow profile was not significantly altered, since the fluid behavior (Newtonian) and viscosity are similar for phospholipid dissolved in ethanol (~1.2 cP) and aqueous streams (1 cP). Besides, we assumed that diffusive mixing occurred mainly after the three fluid streams have

converged in the downstream mixing channel. Thus, the mixing time (τ) for liposome self-assembly in planar flow-focusing microfluidic devices can be estimated using a simple two-dimensional model proposed by Karnik et al. (2008) according to Equation 4.5, where W_F is the width of the focused stream, W is the channel width, FRR is the flow rate ratio and *D* is the diffusion coefficient of the alcohol.

$$\tau \sim \frac{W_F^2}{4D} \approx \frac{W^2}{9D}\frac{1}{(1+FRR)^2} \quad \text{(Equation 4.5)}$$

The time needed to completely mix the two phases, obtained according to Equation 4.5 is 7.17 ms. The mixing time can be interpreted as the minimum residence time required to complete the liposome formation (Balbino et al., 2013). The residence time was sufficient for completing diffusive mixture and liposome formation justifying the small influence of average flow velocities on particle size distribution. These results indicate that the hydrodynamic flow-focusing devices can operate at higher volumetric flow rates, which will certainly increase the liposome throughput, leading to liposomes with the same size characteristics.

4.3.3. Influence of aspect-ratio and hydrodynamic focusing conditions

The hydrodynamic diameter and polydispersity index of liposomes produced using a variety of flow rate ratio values in microfluidic devices with different aspect-ratios, at a fixed average flow velocity of 0.5 m/s, are shown in Figure 4.4. Hydrodynamic diameter of the liposomes generated was inversely proportional to the used flow rate ratio, becoming less sensitive to changes at higher flow rate ratios, regardless the aspect-ratio of the microfluidic devices. On the other hand, the liposome diameter is directly proportional to microfluidic device aspect-ratio at the same flow rate ratio condition. Unimodal particle size distributions were observed for all liposomal systems obtained through microfluidic devices with aspect ratio of 1:1 (Figure 4.4 c), 2:1, 4:1 (data not shown) and 6:1 (Figure 4.4 d). In general, low polydispersity indexes were observed, ranging between 0.2 and 0.4 for all microfluidic devices (Figure 4.4 b). For lower aspect-ratio, the polydispersity index showed a slight increase at flow rate ratio higher than 20. A minimum diameter was observed ranging between 80 and 120 nm at the highest flow rate ratio (50). These conditions in which diameter shows a slight variation as a function of flow rate ratio and aspect-ratio suggest that is the

minimal liposome diameter that can be produced using soybean lecithin at the operational tested conditions.

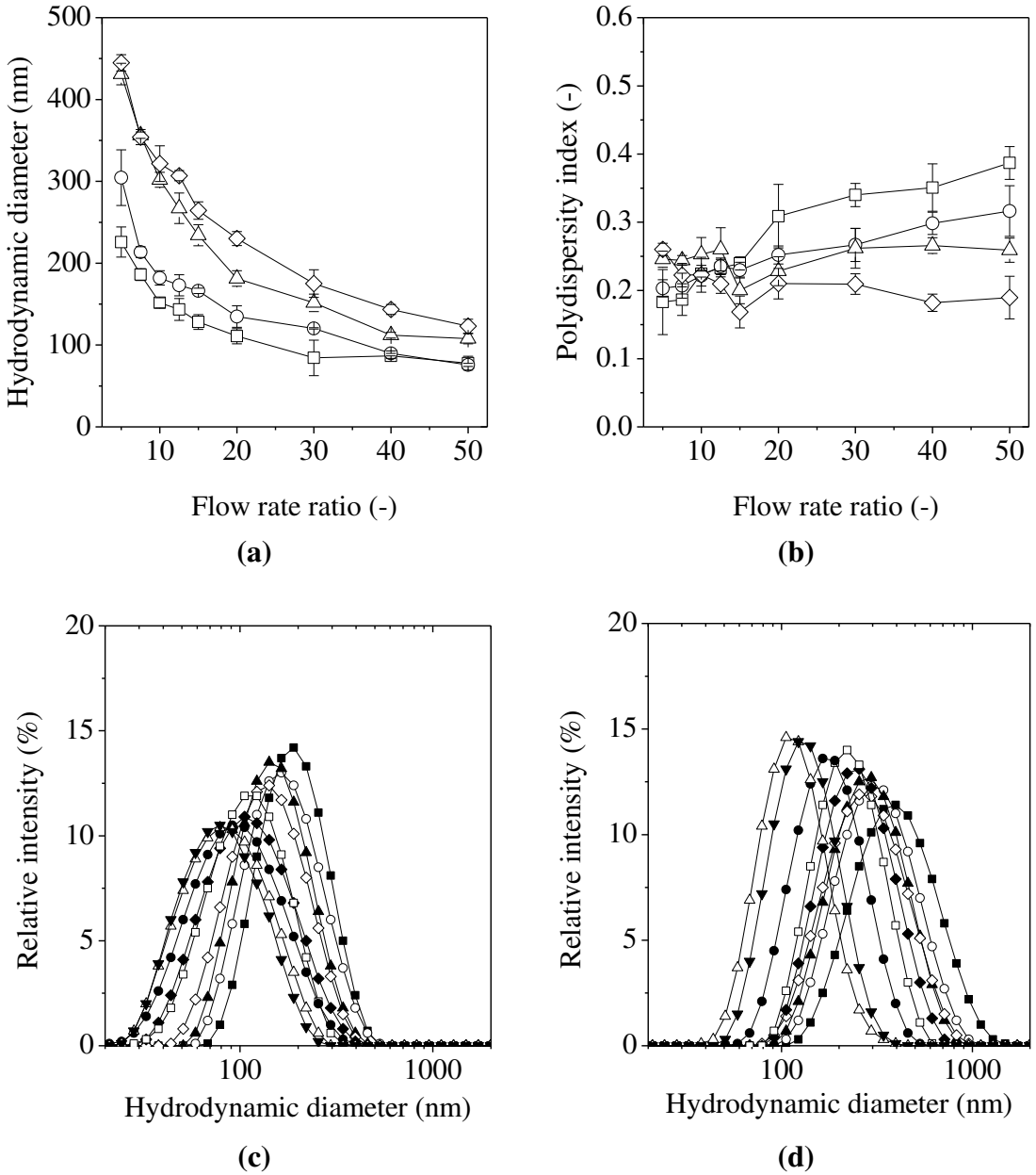


Figure 4.4 **(a)** Hydrodynamic diameter and **(b)** polydispersity index as a function of microfluidic device aspect-ratio and flow rate ratio for liposomes produced at the same flow velocity of 0.5 m/s and soybean lecithin concentration of 100 mM. Data represent average ± standard deviations (*n*=3), where: (□) 1:1 (50×50 µm), (○) 2:1 (100×50 µm), (Δ) 4:1 (200×50 µm) and (◊) 6:1 (300×50 µm). Particle size distributions for liposomes produced using microfluidic device aspect-ratio of **(c)** 1:1 (50×50 µm) and **(d)** 6:1 (300×50 µm) as a function of flow-rate ratio condition, where: (■) 5, (○) 7.5, (▲) 10, (◊) 12.5; (♦) 15, (□) 20, (●) 30, (Δ) 40 and (▼) 50.

Mixture of the miscible liquids flowing in parallel streams occurs predominantly by molecular diffusion during the microfluidic formation of liposomes, but it can be influenced also by convection in the focusing region. At cross-junction, i.e., in focusing region and in the downstream mixing channel, ethanol diffuses into the water and vice-versa up to the point that the solubility of phospholipid decreases exceeding a critical point and causing their self-assembling into planar intermediate structures, according to previously explained. The critical mixing time to occur this self-assembly process is dependent on the extent of focusing of the phospholipid stream (Hong et al., 2010; Balbino et al., 2013), and according to Equation 4.5, the time necessary to completely mix the two phases is inversely proportional to flow rate ratio. On the other hand, the mixing time is directly proportional to channel width, and consequently to microfluidic device aspect-ratio. Thus, according to Figure 4.5, the time required to completely mix the ethanol and water in general is lower than the residence time of 80 ms, calculated considering a mean velocity 0.5 m/s. However, for the highest microfluidic device aspect-ratios at low focusing conditions or low flow rate ratio values, the residence time is not sufficient for total mixing. In these conditions, a partial liposome formation mostly occurred inside microfluidic device and, the total formation could have occurred only in the collection flask, which can be associated to the higher diameter (Figure 4.4 a). Thus, for these process conditions, a higher length of the downstream mixture channel would be necessary for the complete mixture of the two phases.

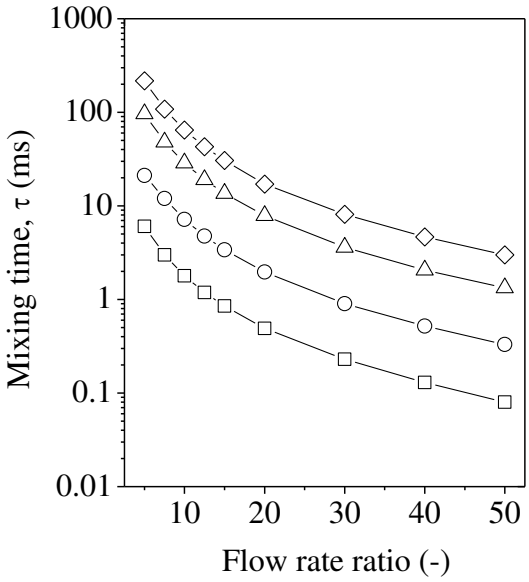


Figure 4.5 Estimated time to complete mixing ethanol and water streams as a function of flow rate ratio for liposomes produced at the same average flow velocity of 0.5 m/s and soybean lecithin concentration of 100 mM, varying microfluidic device aspect-ratio: (□) 1:1 (50×50 μm), (○) 2:1 (100×50 μm), (Δ) 4:1 (200×50 μm) and (◊) 6:1 (300×50 μm).

A low flow rate ratio shows a relatively low constriction, resulting in a low surface/volume ratio of phospholipids. Thus, the ethanol concentration remains high in the interior of the focused stream after the focusing region in the mixing channel. As a result, a large fraction of phospholipid remains dispersed and self-assembles into larger liposomes in the diffusive mixing channel, while the fraction of liposomes that forms in the focusing region is low. On the other hand, a high flow rate ratio results in a relatively narrow center stream with the mixing dominated by two-dimensional convective and diffusive mass transport in the focusing region. Convection abruptly reduces the width of the focused stream in the focusing region, decreasing diffusion length and improving diffusive mixing. This condition results in a relatively high surface/volume ratio and the rapid depletion of the focused center phospholipid stream by convective and diffusive mixing, causing preferentially the phospholipid molecules self-assembling into smaller liposomes (Jahn et al., 2010; Hong et al., 2010; Huang et al., 2010; Hood & DeVoe, 2015; Hood et al., 2013; Zook & Vreeland, 2010). However, focusing conditions higher than 50 were not investigated since the soybean lecithin stream became unstable during the liposome collection time.

According to Figure 4.6, the aspect-ratio of microfluidic device is another parameter that can directly affect the liposomes diameter. At the same flow rate ratio and average flow velocity, a wider channel will also lead to a wider focused stream (Lo et al., 2010; Jahn et al., 2010). This fact resulted in an increase of the mixing time, allowing the formation of bigger liposomes as previously explained. The hydrodynamic diameter increases linearly with the focused stream width and can be up to 4-fold wider in the aspect-ratio of 6:1 than in an aspect-ratio of 1:1. Thus, the linear correlation between liposome size and focused width with r-square values higher than 0.99 for all aspect-ratio evaluated, could be used as an indirect measurement to predict and tune the hydrodynamic diameter of liposomes.

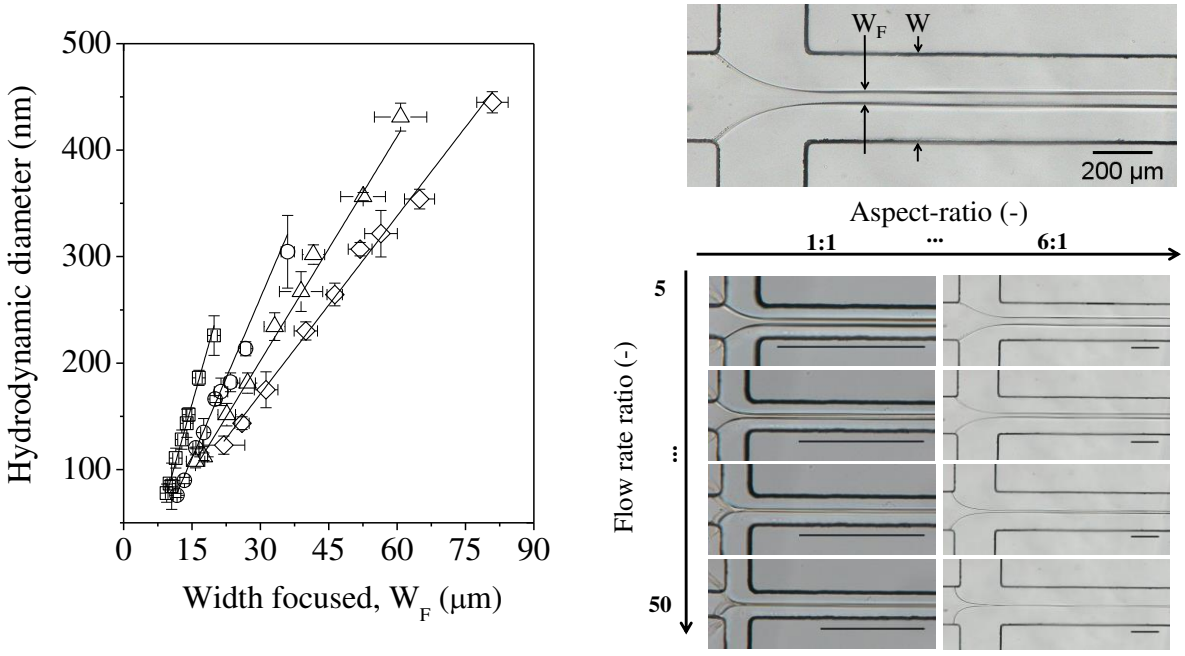
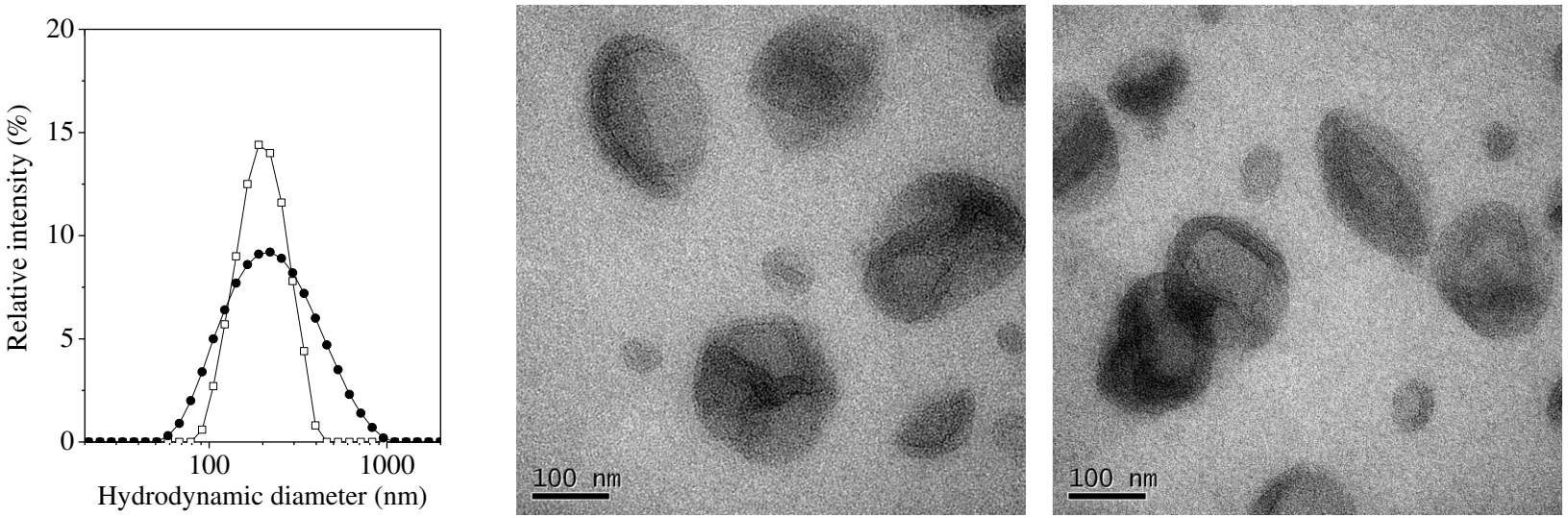


Figure 4.6 (a) Hydrodynamic diameter as a function of width focused (W_F) and **(b)** micrographs of some focusing conditions tested during microfluidic production of liposome as a function of flow rate ratio at the same average flow velocity of 0.5 m/s and soybean lecithin concentration of 100 mM, varying microfluidic device aspect-ratio. Data represent average ± standard deviations (*n*=3), where: (□) 1:1 (50×50 μm), (○) 2:1 (100×50 μm), (Δ) 4:1 (200×50 μm) and (◊) 6:1 (300×50 μm). Scale bar represent 200 μm in all cases.

In an opposite way, the lower polydispersity obtained from higher aspect-ratios can be also explained based on flow velocity profile. Most likely as the microfluidic device aspect-ratio increases, the impact of the non-slip boundary condition which imposes zero velocity at the channel walls diminishes, and consequently the flow velocity distribution becomes more homogenous throughout the mixing region (Hood & DeVoe, 2015). Thus, our results confirm the hypothesis that higher aspect-ratios result in more uniform fluid velocity across the width of the focusing region and in the diffusive mixing channel, thereby enabling the production of liposomes with lower polydispersity. We can conclude that a production of liposomes with narrow particle distribution can be achieved with adequate combination of flow rate ratio and channel aspect-ratio. Besides, a comparison between the particle size distributions of liposomes obtained by hydrodynamic flow-focusing with liposome obtained by batch technique of ethanol injection could be done, according to Figure 4.7 (a). These results highlighted the lower polydispersity of liposome obtained by microfluidic in comparison to conventional batch method of alcohol injection. Besides, the micrographs in Figure 4.7 (b,c) can be used to confirm that similar systems were obtained using both techniques.



(a) **(b)** **(c)**

Figure 4.7 **(a)** Comparison between particle size distributions for liposomes obtained using different production techniques: (□) microfluidic device with aspect-ratio of 2:1, flow rate ratio of 10 at 100 mM, and (●) conventional batch technique of alcohol injection. **(b)** TEM-micrographs of liposomes obtained using microfluidic device and **(c)** obtained from alcohol injection technique.

Considering our results, a number of strategies could be adopted to enhance liposome production rate. First, the initial concentration of soybean lecithin and total volumetric flow rate should be increased aiming a higher throughput and scalable synthesis of liposomes for industrial applications in food formulations. Second, microfluidic device aspect-ratio and flow rate ratio should be adjusted. According to Figure 4.8, the production rate at the same focusing conditions increase with increasing aspect-ratio of microfluidic device. Besides, it was observed that liposome production rate increase ~6 times, while the hydrodynamic diameter showed a slight variation between 80 and 120 nm. These results show that device aspect-ratio could have important technological consequences when aiming industrial application. In addition, larger microfluidic devices are easier to fabricate and operate than smaller aspect-ratios due to increased pressure and clogging issues (Jahn et al., 2010). In summary, for liposome formation with small and similar diameters, the higher aspect-ratio enables the most rapid generation of liposomes with a maximum production rate near 40 mg lecithin per hour. This production rate is relative to a single channel. In parallelized microfluidic systems the overall throughput should be higher.

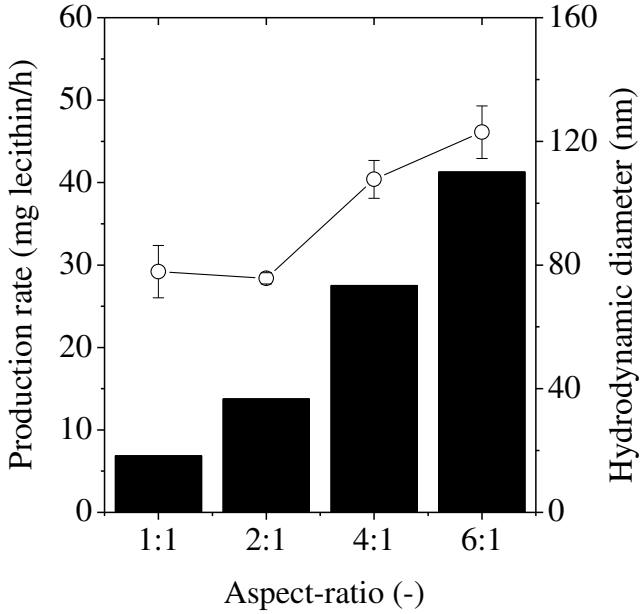


Figure 4.8 Liposome production rate (black columns) and respective hydrodynamic diameter (empty circles) observed at a same flow velocity of 0.5 m/s, soybean lecithin concentration 100 mM and flow rate ratio of 50 as a function of microfluidic device aspect-ratio.

4.3.4. Microfluidic formation of β-carotene-incorporated liposome

β-Carotene is a food bioactive molecule model that shows a poor physicochemical stability and a low bioavailability related to its high hydrophobicity (Tan et al., 2014b). For

this reason, β-carotene molecule was chosen as a bioactive compound to evaluate technical feasibility of microfluidic aiming possible food industrial applications. The influence of β-carotene on hydrodynamic diameter and polydispersity index of the liposomal systems produced using different microfluidic device aspect-ratio can be observed in Figure 4.9 (a,b). The hydrodynamic diameter and polydispersity index shown a non-significant influence (p<0.05) of β-carotene presence for all microfluidic devices tested at the same flow rate ratio. Besides, the incorporation of β-carotene did not exert significant influence (p<0.05) on polydispersity index. Unimodal size distribution was observed (data not show) for all situations, with a polydispersity index relatively low of approximately 0.25.

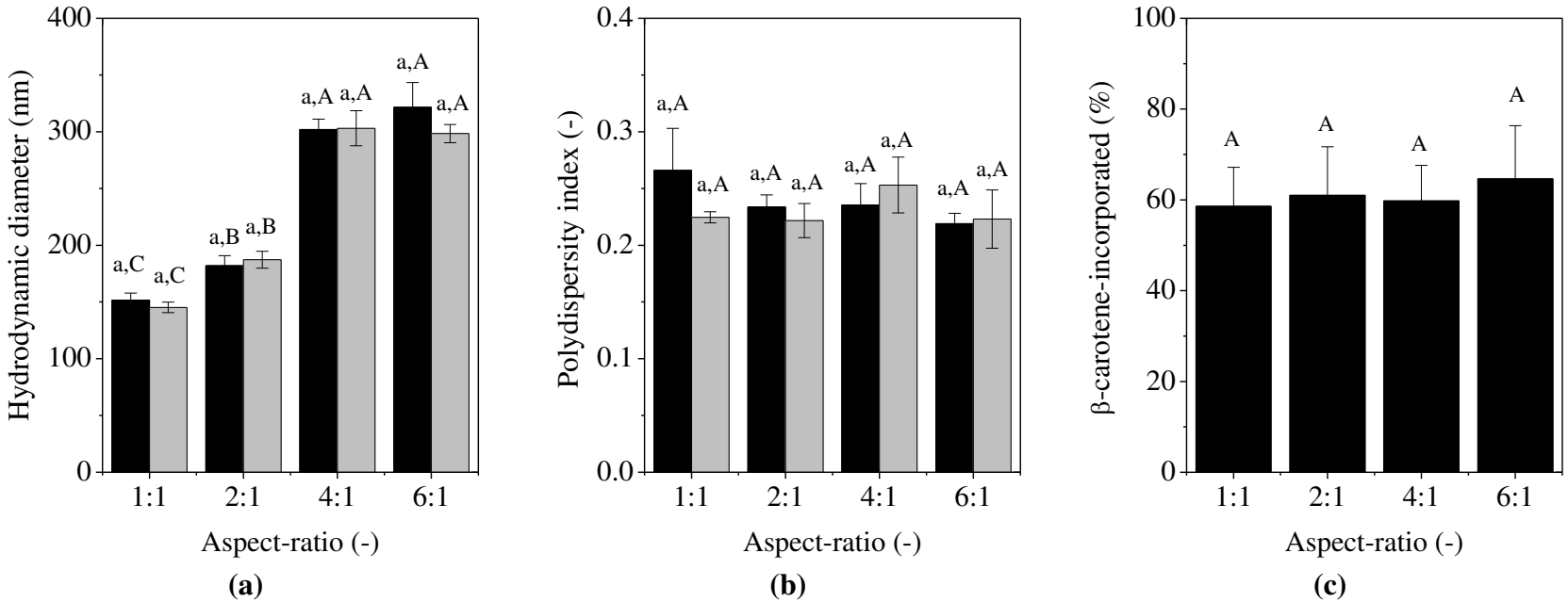


Figure 4.9 (a) Hydrodynamic diameter, **(b)** polydispersity index, and **(c)** relative β-carotene-incorporated amount as a function of microfluidic device aspect-ratio, for liposome obtained at the same average flow velocity of 0.5 m/s, soybean lecithin concentration of 100 mM, and flow rate ratio equal 10. Data represent average ± standard deviations (n=3), different lowercase letters represent significant differences (p<0.05) between the presence and absence of β-carotene in the center stream and different uppercase letters represent significant differences (p<0.05) between microfluidic device aspect-ratio used, where: (■) represent β-carotene-incorporated liposome, and (■) represent free-β-carotene liposome.

The β-carotene-incorporated amount into liposomal system was measured as function of microfluidic device aspect-ratio (Figure 4.9 c). In general, only 60% of β-carotene amount initially used was incorporated into liposomes regardless microfluidic device aspect-ratio. This amount is similar to that observed previously in other study, which aimed β-carotene-loaded liposome formation by alcohol injection technique (Michelon et al., 2016). This result is mostly related to lack of polar groups in the β-carotene molecule restricting its location in the hydrophobic domain of the lipid bilayer (Palozza et al., 2006; Tan et al., 2013). We used 1.95 mg/ml β-carotene in the lipid stream, in order to reach a relative concentration to amount of soybean lecithin of 2.5% (*w/w*). This concentration is higher than the solubility of β-carotene in ethanol at 25 °C of ~0.4 mg/ml (Tres et al., 2007). Thus, besides of saturation of β-carotene on phospholipid bilayers, a significant amount of insoluble β-carotene crystals were adsorbed in the channel walls, due to the hydrophobicity of both β-carotene and polydimethylsiloxane, which help to explain results shown in Figure 4.9 (c). Finally, it can be concluded that β-carotene incorporation ability is not specific to a choice of microfluidic device aspect-ratio. On the other hand, this ability can be directly related to the adequate concentration of β-carotene relative to amount of soybean lecithin reducing loses of β-carotene and increasing process efficiency.

The kinetic stability of liposomal systems obtained using microfluidic device aspect-ratio of 2:1 at flow rate ratio of 10 was evaluated during 28 days at room temperature and under uncontrolled light conditions, as shown in Figure 4.10. The stability of these liposomes was compared with liposomes obtained by alcohol injection technique with presence or absence of β-carotene. In general, both techniques promote the formation of kinetically stable liposomal systems during 28 days, regardless of the β-carotene presence, since no significant differences were observed ($p<0.05$) on hydrodynamic diameter and polydispersity index as function of storage time. Besides, β-carotene presence and production technique did not exert significant influence ($p<0.05$) during storage on ζ-potential and pH values of the liposomal systems (Figure 4.10 c,d). The ζ-potential was approximately -50 mV at natural pH of ~5.5 for all systems, suggesting the existence of a strong electrostatic repulsion between the liposomes that promote a good kinetic stability of the systems. According to Figure 4.10 (b), the polydispersity results can be used to highlight the great advantage of microfluidic devices for liposomes formation in comparison to conventional method of alcohol injection. Although a similar hydrodynamic diameter had been observed for both techniques of liposome

production, in general the systems obtained using microfluidic devices show a polydispersity index approximately 40% lower than liposomes obtained in the ethanol injection method.

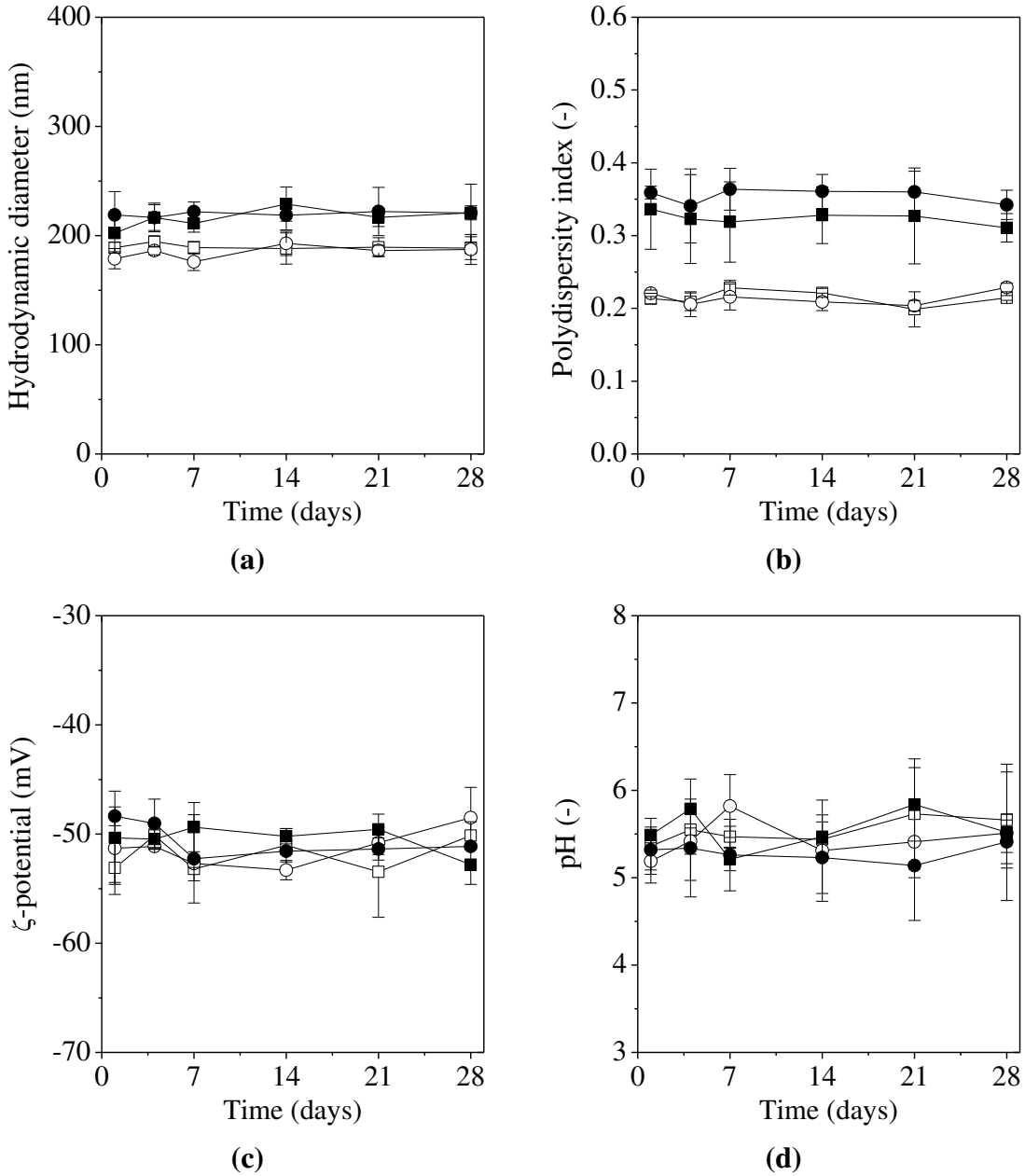


Figure 4.10 Stability of liposomal systems in function of time at room temperature under uncontrolled light conditions during 28 days in terms of: **(a)** hydrodynamic diameter, **(b)** polydispersity index, **(c)** ζ-potential and **(d)** pH. Data represent average ± standard deviations (*n*=3), where: (○) and (□) represent β-carotene-incorporated and β-carotene-free liposomes, respectively, obtained using microfluidic device aspect-ratio of 2:1 at flow rate ratio of 10; (●) and (■) represent β-carotene-incorporated and β-carotene-free liposomes, respectively, obtained by alcohol injection technique.

The β-carotene degradation kinetics was evaluated as a function of techniques used for liposome formation, as shown in Figure 4.11. It was verified that β-carotene was degraded

similarly when incorporated into liposomal systems obtained by both techniques. In general, approximately 40% of β-carotene was degraded in both liposomal systems during 28 days (Figure 4.11 a). It was also verified that β-carotene degradation followed a first-order kinetic model, as shown by the good fitting and correlation higher than 0.98 (Figure 4.11 b). The half-life value, defined as the storage time at which the β-carotene content is reduced by half with respect to the zero time, and degradation kinetic constant (k) can be used to better explain these results. The half-life of β-carotene incorporated into liposomes produced from both formation techniques at room temperature and under uncontrolled light conditions is ~34 days (Figure 4.11 b). The same degradation kinetic constant and half-life values observed for both liposomal systems can be used to confirm that the stability of liposomes obtained from microfluidic devices are very similar to stability of liposomes obtained through conventional techniques.

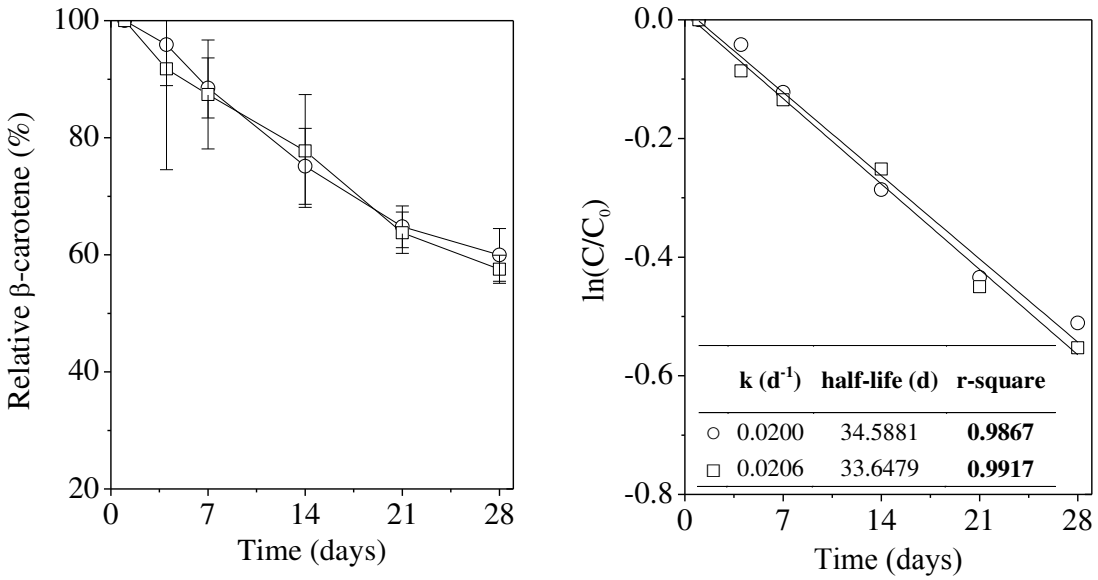


Figure 4.11 (a) Kinetics of β-carotene degradation, **(b)** linear fitting of first-order kinetic model, half-life and degradation kinetic constant of liposomal systems. Data represent average ± standard deviations (*n*=3), where: (□) represent liposome obtained using a microfluidic device aspect-ratio of 2:1 at flow rate ratio of 10 and (○) obtained by injection alcohol technique.

4.4. Conclusion

Liposomes with a narrow particle size distribution and a fine tuning of the average diameter were successfully produced from alteration of the flow rate ratio and channel aspect-ratio. The higher aspect-ratio enabled the most rapid generation of liposomes with similar diameter and significant lower polydispersity index than the obtained by other batch

technique. Besides, the β-carotene was successfully incorporated into liposomes with efficiency of approximately 60% and the incorporation ability was not specific to a choice of microfluidic device aspect-ratio. The liposomal systems present a good kinetic stability with similar results to liposome produced by injection ethanol technique. Finally, our results suggest that the use of microfluidic devices with higher aspect-ratio could be employed for phospholipid vesicles production aiming industrial applications, with a possible advantage to minimize the degree of parallelization of microfluidic processes. These results demonstrate the potential technical feasibility of microfluidic processes to applications for food systems.

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CAPÍTULO V

Single-step microfluidic production of W/O/W double emulsions as templates for β-carotene-loaded giant liposomes formation

Short communication to be submitted to Chemical Engineering Journal

Single-step microfluidic production of W/O/W double emulsions as templates for β-carotene-loaded giant liposomes formation

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Highlights

- W/O/W emulsion templates were successfully produced using soybean lecithin.

- Ethyl acetate/pentane mixture can be used aiming at the W/O/W emulsions formation.

- β-Carotene was incorporated inside phospholipid shell forming giant liposome.

- β-Carotene-incorporated giant liposomes can to applied in aqueous food formulations.

Abstract

We demonstrated the microfluidic production of W/O/W double emulsion droplets aiming at the formation of β-carotene-incorporated giant liposomes for food and/or pharmaceutical applications. For this purpose, glass-capillary microfluidic devices were fabricated to create a truly three-dimensional flow aiming production of giant unilamellar liposomes by solvent evaporation process after W/O/W double emulsion droplet templates formation. The great challenge of microfluidic production of monodisperse and stable W/O/W double emulsion templates for this purpose is the replacement of potentially toxic organic solvents used for phospholipids dissolution. The high cost of several semi-synthetic phospholipids commonly used for giant liposome formation remains as the major technological challenge to be overcome. Thus, β-carotene-incorporated giant liposomes were generated using biocompatible solvents with low toxic potential, such as ethyl acetate and pentane, and non-purified soybean lecithin, a food-grade phospholipid mixture with low cost, by dewetting and

evaporation of the solvents forming the oily intermediate phase of W/O/W double emulsion droplet templates. Our results showed monodisperse β-carotene-loaded giant liposomes with diameter ranging between 100 and 180 μm. Besides, it was observed a time of 10 days to reach complete giant liposomes disruption. In this way, a single-step microfluidic process with highly accurate control of size distribution was developed. This microfluidic process proposed is potentially useful for a broad range of applications in protection and delivery of food and pharmaceutical active compounds.

Keywords: Microfluidic; Glass-capillary; Soybean lecithin

5.1. Introduction

Double water-in-oil-in-water (W/O/W) emulsions are three-phase dispersions composed of inner aqueous droplets dispersed in larger oil droplets, which are themselves dispersed in another aqueous phase [1,2]. A variety of applications has been demonstrated due to their unique and highly hierarchized structure, such as encapsulation and controlled release of active compounds in pharmaceuticals, food, cosmetics, and materials science applications [3]. Besides, W/O/W double emulsions show great potential as templates for preparing functional particles, such as solid lipid capsules [4], polymersomes [5], and giant liposomes [6-9]. The dewetting of the phospholipid middle phase of double emulsion templates creates giant unilamellar liposomes (GUVs, >1 μm) with molecular bilayers [6-9]. However, the preparation of W/O/W double emulsion templates by conventional methods is not trivial and, in general, two emulsification steps are needed. Firstly, W/O single emulsion is produced from high shear mixing of immiscible liquids while in the second step, a W/O/W double emulsion is formed using milder shear conditions to avoid the disruption of the inner water droplet [10,11]. Generally, this two-step process shows poor reproducibility with a broad size distribution of W/O/W droplets being formed, which are not suitable for generation of templates aiming the formation of core-shell structures, such as GUVs. To overcome these drawbacks, microfluidic emulsification devices consisting of networks of flow microchannels have received increased attention as a versatile and powerful tool for preparing in a single-step highly monodisperse W/O/W double emulsions [3].

Several types of microfluidic devices have been developed for W/O/W double emulsion formation, mainly polydimethylsiloxane and glass-capillary microfluidic devices [12,13]. Polydimethylsiloxane devices can be prepared by designing mask patterns using soft

lithography technique, but the fabrication of a device with flow channels in three dimensions is hampered [3]. On the other hand, in microfluidic glass-capillary devices it is possible to create a truly three-dimensional flow and droplets can be produced in two main regimes, dripping and jetting, depending on the balance between interfacial, viscous, inertial and gravity forces [2]. In this short communication, we demonstrated a microfluidic production of W/O/W double emulsion templated β-carotene-loaded giant liposomes using a three-dimensional glass-capillary device by combining co-flow and flow-focusing in coaxial glass-capillaries. The great challenge of W/O/W double emulsion templates by microfluidic approaches is the need to use organic solvents to solubilize the lipids of the oil phase. Generally, organic solvent mixtures with toxic potential to human health are used, such as hexane, chloroform and toluene [6,7]. We proposed the use of biocompatible solvents generally recognized as green, such as ethyl acetate and pentane in replacement to solvents commonly used, aiming food and pharmaceutical applications. Furthermore, our templates were obtained using soybean lecithin, which is a food-grade mixture of phospholipids with low cost. Finally, the β-carotene, a hydrophobic model molecule, was incorporated in the middle oil phase of W/O/W double emulsion templates. The β-carotene shows important biological activity due to provitamin A activity, but it shows poor physicochemical stability and low bioavailability related to its high hydrophobicity, which makes difficult its effective use as nutraceutical ingredient in aqueous formulations [14]. Thus, β-carotene-incorporated giant liposomes can make β-carotene an attractive ingredient to be used in food and/or pharmaceutical formulations.

5.2. Materials and methods

5.2.1. Materials

The W/O/W double emulsion templates were obtained using a food-grade soybean lecithin powder (>45% *w/w* phosphatidylcholine, 10-18% *w/w* phosphatidylethanolamine, <4% *w/w* lysophosphatidylcholine and <3% *w/w* triglycerides), commercially named Lipoid S45 (Lipoid GmbH, Ludwigshafen, Germany); synthetic β-carotene powder (>93% *w/w*), pentane (99.8% *v/v*) and poly(vinyl alcohol) (PVA, molecular weight 13-23 kDa, 87-89% hydrolyzed) supplied from Sigma-Aldrich (St. Louis, MO, USA); sucrose (analytical-grade) and hexane (>98.5% *v/v*) both purchased from BDH Chemicals Ltd. (Poole, Dorset, UK); dextran (molecular weight 70 kDa, TCI Chemical Industry Co., Tokyo, Japan); chloroform

(99.8% *v/v*, Alfa-Aesar, Ward Hill, MA, USA) and ethyl acetate (99.9% *v/v*, Honeywell, Muskegon, MI, USA). The microfluidic devices were obtained using cylindrical (inner and outer diameters 0.58 mm and 1 mm, respectively) and square (inner dimension 1.05 mm) glass capillaries acquired from World Precision Instruments, Inc. (Sarasota, FL, USA) and Atlantic International Technology Inc. (Rockaway, NJ, USA), respectively. Besides, polyethylene tubing of inner diameter 0.86 mm (Scientific Commodities, Inc.; Lake Havasu City, AZ, USA), syringe needles Type 304 (McMaster-Carr, Atlanta, GA, USA) and 5-minute Epoxy® (Devcon Corp., Danvers, MA, USA) were also used. Glass capillaries were treated using 2-[methoxy(polyethyleneoxy)6-9 propyl]trimethoxysilane (Gelest, Inc.; Morrisville, PA, USA) and trimethoxy(octadecyl)silane (90% *w/v*, Sigma-Aldrich, St. Louis, MO, USA).

5.2.2. Methods

5.2.2.1. Fabrication of the glass capillary device

The capillary devices were built on a glass slide, and consisted of two glass cylindrical capillaries inserted into the opposite ends of a square capillary, according to reported by Utada et al. [1]. Briefly, the cylindrical glass capillaries were tapered to an inner diameter of approximately 20 µm with a micropipette puller (model P-97, Sutter Instrument, Co.; San Francisco, CA, USA), and then the tips were carefully sanded to final inner diameters approximately of 60 and 150 µm. The cylindrical tube with smaller inner diameter was treated with trimethoxy(octadecyl)silane for 1 h to render its surface hydrophobic, and the larger diameter tube with 2-[methoxy(polyethyleneoxy)6-9 propyl]trimethoxysilane for ~15-20 min to render its surface hydrophilic. The hydrophobic tube was used as the injection capillary, and the hydrophilic tube was used as the collection capillary. The device was assembled onto a glass microscope slide. For this, the square capillary was fixed to the slide with 5-minute Epoxy®. After, the cylindrical tubes were inserted into the square tubing at both ends, which enabled the alignment of the axes of the injection and collection capillaries, maintaining a separation distance between them of approximately 60 µm, according to shown in Figure 1 (a). For injection of aqueous innermost phase, a third cylindrical capillary was stretched with a burner to an outer diameter of approximately 200 µm, and inserted into the injection capillary. Finally, dispensing needles were placed at the joints between capillaries or their ends, and fix them to the slide with 5-minute Epoxy®.

5.2.2.2. Generation of W/O/W double emulsion templates

The W/O/W double emulsion templates were obtained using an innermost aqueous phase containing 1% (*w/v*) PVA and 9% (*w/v*) dextran. The phospholipid middle phase consisted of a mixture of 0.5% (*w/v*) soybean lecithin and 0.125% (*w/v*) β-carotene dissolved in the following organic solvent mixtures (1:1.8 *v/v*): chloroform/hexane; ethyl acetate/hexane or ethyl acetate/pentane. The continuous phase used in this study was an aqueous solution 10% (*w/v*) PVA. The innermost, middle lipid, and continuous phases flowed into the microfluidic device through connection of glass micro-syringe needles to the dispensing needles of the device with polyethylene tubing using three syringe pumps (model PHD 2000, Harvard Apparatus, Inc.; South Natick, MA, USA). The innermost (q_1) and middle oil (q_2) phases were injected in stretched tube and cylindrical tube with smaller inner diameter, respectively, at a flow rate of 1000 μl/h, according to Figure 1. On the other hand, the continuous phase (q_3) flowed through the interstices between the cylindrical tapered capillary and the square capillary, at a flow rate ranging between 3000 and 12000 μl/h. The droplets were collected in a 50 mM sucrose solution, in order to adjust the osmolarity between the innermost phase, continuous phase and collection solution to 50 mOsm/l, evaluated by a micro-osmometer (model 3300, Advanced Instruments, Inc.; Norwood, MA, USA). All experiments were performed at room temperature and the process was operated in a discontinuous dripping regime, in which the formation of W/O/W double and O/W single emulsions were monitored within the microfluidic device using an 5× objective on an inverted microscope (model DM IRB, Leica Microsystem; Mannheim, Germany) equipped with a high speed camera (model Phantom v9.0, Vision Research; Wayne, NJ, USA).

5.2.2.3. Characterization of giant liposomes

Bright field and fluorescence images were obtained with a 10× objective on an inverted fluorescence confocal microscope (model DM IRBE, Leica Microsystem; Mannheim, Germany) at room temperature. For this, Argon (458 nm) laser was used as excitation source and the fluorescence emission was collected by the PMT detectors, through band pass filters between 488 and 543 nm for β-carotene. The contrast provided by the presence of dextran and PVA in the inner core of the GUVs allowed to visualize them in the bright field. Approximately 20 bright field micrographs were used to determine the particle size distribution, based on diameter measurements of 200 droplets using the open-source

software Image J (version Java 1.6.0_24, National Institutes of Health, Bethesda, MD, USA). The particle size was expressed in terms of mean diameter, while the polydispersity of the system was expressed in terms of coefficient of variation (CV), which relates standard deviation (sd) to mean diameter. Besides, the bright field images were also used to estimate relative kinetic stability by count of GUVs number as a function of time.

5.3. Results and discussion

5.3.1. Formation of W/O/W emulsion templates

The W/O/W emulsion templates were successfully prepared using the glass-capillary device and soybean lecithin by single-step process. This process configuration forces the water droplets to become re-emulsified, leading to formation of monodisperse W/O/W emulsion droplets with an ultra-thin middle oil phase at the orifice of the capillary collection tube, as shown in Figure 5.1 (a,b) and Video 1 of the Supplementary Material. The process was operated in the discontinuous dripping regime, producing intermittently O/W single and W/O/W double emulsion. The W/O/W double and O/W single droplets were separated by density difference between them. The O/W single droplets coalesced and floated on the top of collection flask, which facilitated the oil separation. On the other hand, the W/O/W double emulsion droplets rapidly sank because they are heavier than the collection solution. The micrograph in the Figure 5.1 (d) obtained from inverted microscope shows the GUVs formed in the bottom of glass flask collection. In dripping regime, the breakup of droplets is governed by the balance between the interfacial tension that constrains the droplet to the tip of the tapered tube and the drag force exerted by the continuous phase that pulls the droplet downstream. Therefore, droplets detachment is proportional to the viscosity of the continuous phase (drag forces), but mainly to the velocity difference between the continuous and oil phases. Thus, an accurate control of W/O/W droplet diameter generation was observed by finely tuning the flow rate of continuous phase, as shown in Figure 5.1 (b) and Figure 5.2 (a-c). The diameter of W/O/W emulsion droplets decreased with increasing flow rate of continuous phase, which ranged between approximately 100 and 180 μm for all solvent mixtures. The W/O/W emulsion exhibited high uniformity with coefficients of variation in the range of ~3.0-6.0%.

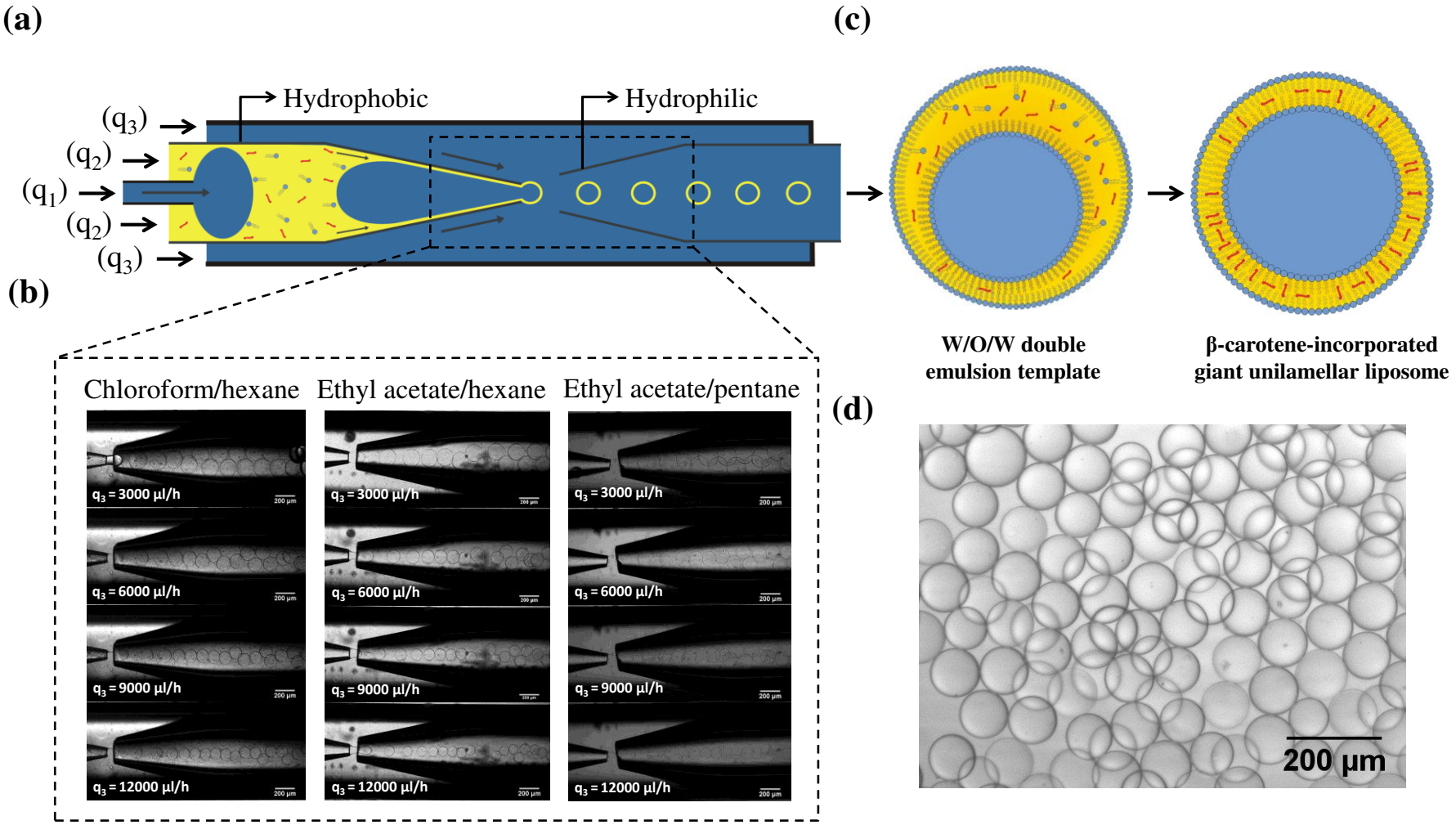

Figure 5.1. **(a)** Microfluidic production of W/O/W double emulsion droplet templates with ultra-thin shells containing β-carotene; **(b)** Optical microscope images of microfluidic process using different solvent mixtures at continuous flow rates (q_3) ranged from 3000 µl/h to 12000 µl/h at flow rate of innermost (q_1) and middle lipid (q_2) phases equal 1000 µl/h; **(c)** Diagram of organic solvent extraction process for GUVs formation; **(d)** Inverted optical microscope images of monodisperse W/O/W double emulsion droplet templates in the bottom of collection solution.

The GUVs formation was only possible using W/O/W double emulsion templates from utilization of a highly volatile organic mixture of good solvents for soybean lecithin, but one of them more soluble in water. Probably, the evaporation of the later, due to its higher vapor pressure, forced the other to dewet from the double emulsion droplets leading to stable giant liposomes formation. Chloroform and ethyl acetate are good solvents for phospholipids, enabling them to remain fully dissolved during double emulsion formation. However, chloroform and ethyl acetate are more soluble in water, dewetting from soybean lecithin more easily, providing formation of a hexane or pentane-rich shell that is poorly soluble for the phospholipid. The reduction of the lipid solubility induces an attractive interaction between the two layers covered by soybean lecithin at the interface of the ultra-thin shell, according to shown Figure 5.1 (c). Besides, the phospholipids also adsorb to the interfaces of the inner core, ultra-thin shell, and the outer aqueous phase, thereby reducing the overall interfacial energy [7]. The thickness of ultra-thin shell shows generally a few micrometers, as shown in Figure 5.1 (d) and Figure 5.3 (a-c), much smaller than in typical double emulsion obtained by conventional methods; this enables the fabrication of giant liposomes containing minimal residual solvent within their structure.

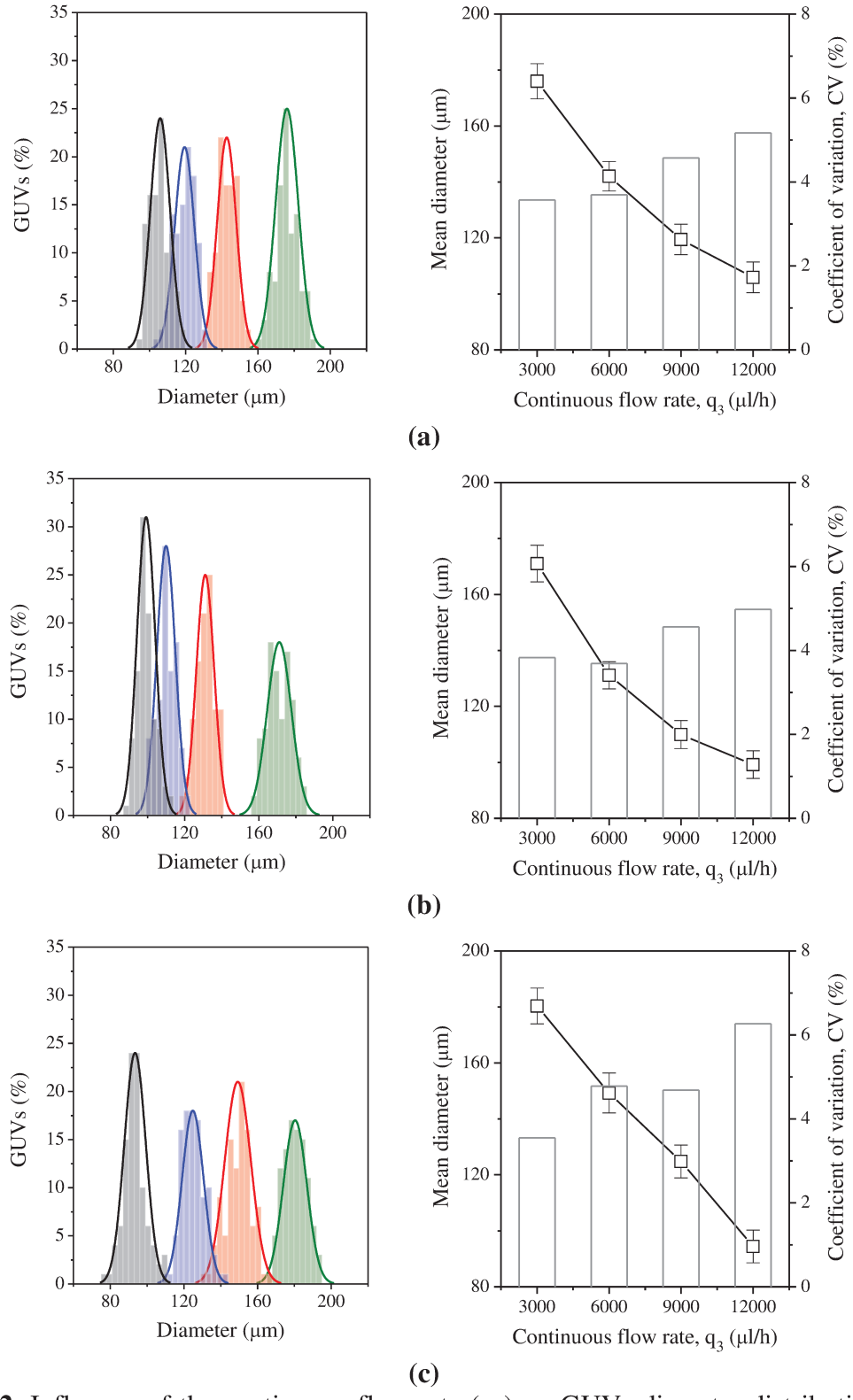

Figure 5.2. Influence of the continuous flow rate (q_3) on GUVs diameter distribution in left column (where green, red, blue and black bars represent 3000 µl/h, 6000 µl/h, 9000 µl/h and 12000 µl/h, respectively, at flow rate of innermost (q_1) and middle lipid (q_2) phases equal 1000 µl/h) and on the mean diameter and coefficient of variation in right column (where empty symbol and empty bars represent mean diameter and coefficient of variation, respectively) using different organic solvent mixtures (1:1.8 *v/v*): **(a)** chloroform/hexane; **(b)** ethyl acetate/hexane and **(c)** ethyl acetate/pentane.

Production of W/O/W double emulsion templates with an ultra-thin middle oil phase is directly associated to the design of glass-capillary devices and principally to chemical functionalization of the glass-capillary surfaces. Such ability is not specific to a particular choice of fluid phases or flow rates used, provided that the inner and outer phases viscosity are in the right ratio and the device is operated in the discontinuous dripping regime [7]. Figure 5.2 (a-c) shows the particles size distribution of GUVs produced with different solvents mixture. According to Figure 5.2 (c) the giant liposome size obtained using ethyl acetate and pentane mixture showed a linear behavior as function of continuous flow rate, and thus a better capacity to fine-tuning of liposome diameter at higher continuous flow rates (9000 and 12000 μl/h). This result can be related with its lower viscosity in comparison to other mixtures, which can facilitate the droplets detachment and improve size control. Thus, it was possible to confirm that single-step microfluidic production of W/O/W emulsion droplet templates aiming giant liposomes formation was efficient using solvents with less toxicity potential to human health, such as ethyl acetate and pentane, replacing solvents with higher toxicity potential, such as chloroform and hexane, commonly used in these processes. The choice of solvent for the industrial processing of products suitable for human consumption must take into account international regulations regarding the safety of consumers as well as the minimization of production costs. The organic solvents ethyl acetate and pentane are classified as Generally Recognized as Safe (GRAS) according to the US Food and Drug Administration (toxicological class III) and could be used in food applications [15-17]. Apart that, the technical feasibility of using low-cost and non-purified food-grade phospholipids was successfully demonstrated for GUVs production.

5.3.2. β-carotene-incorporated giant liposome formation

Figure 5.3 shows that β-carotene was successfully incorporated inside phospholipid ultra-thin shell due to its intrinsic fluorescence for all solvent mixtures. Because of the absence of polar groups in β-carotene structure, its localization of β-carotene was restricted only to the lipid membrane that was regulated by van der Waals interactions with the fatty acid chains [14]. The confocal micrographs and particle size distributions indicated that giant liposomes were highly monodisperse. It is possible to observe that the presence of β-carotene inside oil shell did not affect significantly the mean diameter and coefficients of variation (Figure 5.3 a-c) when compared with liposomes without β-carotene (Figure 5.2 a-c). Within

approximately 10 days, all giant liposomes were disrupted as shown in the Figure 5.4 (a). Kinetic profile of giant liposome rupture obtained in the presence and absence of β-carotene using ethyl acetate and pentane mixture showed similar behavior, as shown in Figure 5.4 (b). Relative stability can be observed in micrographs for the incorporated β-carotene-loaded GUVs (Figure 5.4 c). Our results indicate that solvent-type and β-carotene presence did not exert significant influence on the stability of giant liposomes obtained from soybean lecithin. The stability was probably achieved due to same osmolarity of the inner, continuous and collection aqueous solutions. Such good stability can be also related to the significantly increased shear stress on the innermost droplet because of the lubrication effect associated to a very thin width of the middle phase [9]. The lubricant effect between aqueous phases, continuous and innermost is only possible due to the ultra-thin oily intermediate phase of the W/O/W double emulsion, since the drag force between aqueous phases is minimized which reduces the coalescence phenomenon. Thus, the ultra-thin middle layer provides stability to the W/O/W double emulsion droplets, preventing coalescence between them. The same stability is otherwise difficult to achieve using W/O/W double emulsion templates with oil phase of wider thickness.

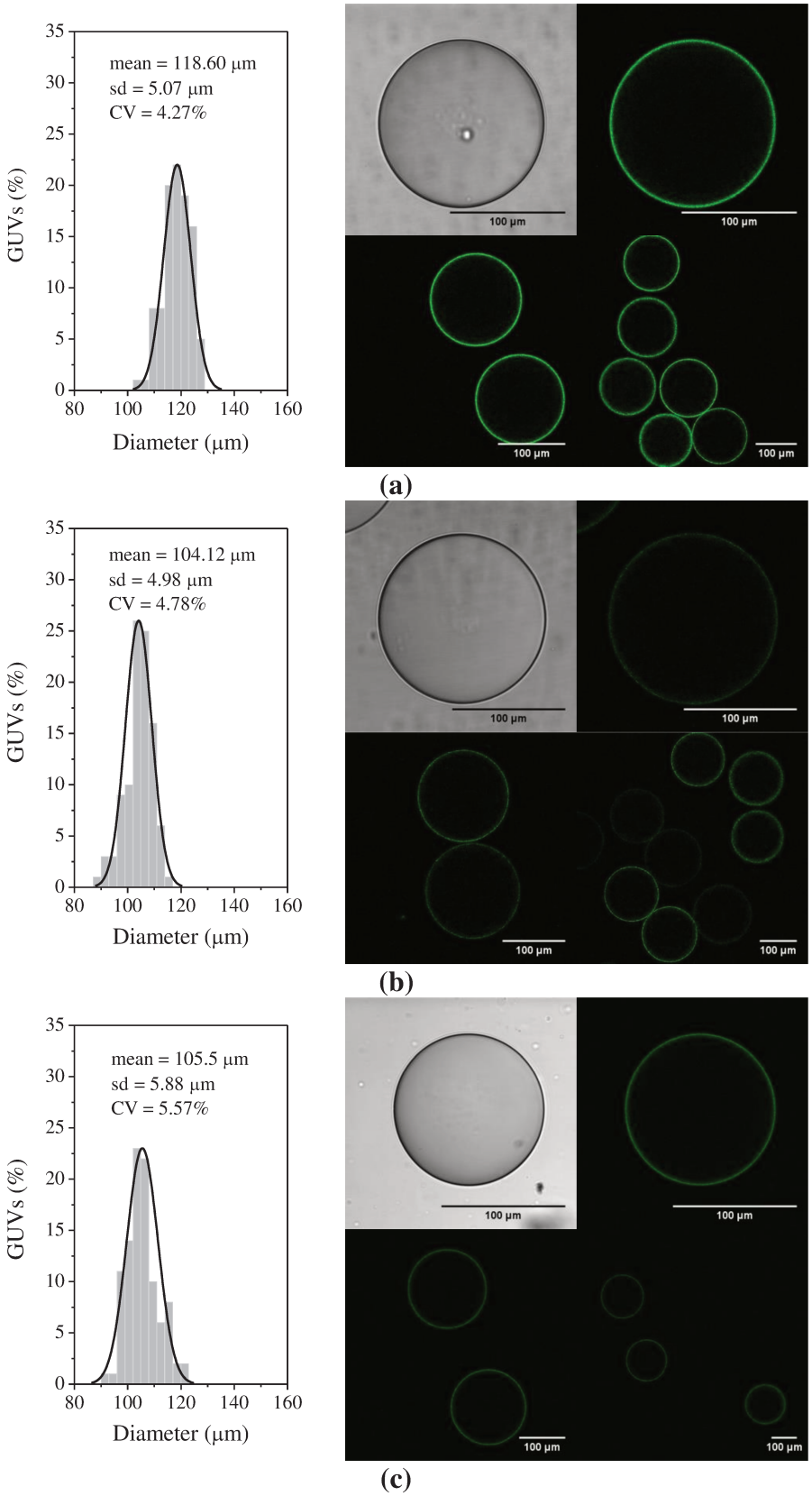

Figure 5.3. Particle size distribution and confocal microscope images of β-carotene-loaded giant unilamellar liposome at flow rate of innermost (q_1), middle lipid (q_2) and continuous (q_3) phases equal 1000 µl/h, 1000 µl/h and 12000 µl/h, respectively, using different organic solvent mixtures (1:1.8 *v/v*): **(a)** chloroform/hexane; **(b)** ethyl acetate/hexane and **(c)** ethyl acetate/pentane.

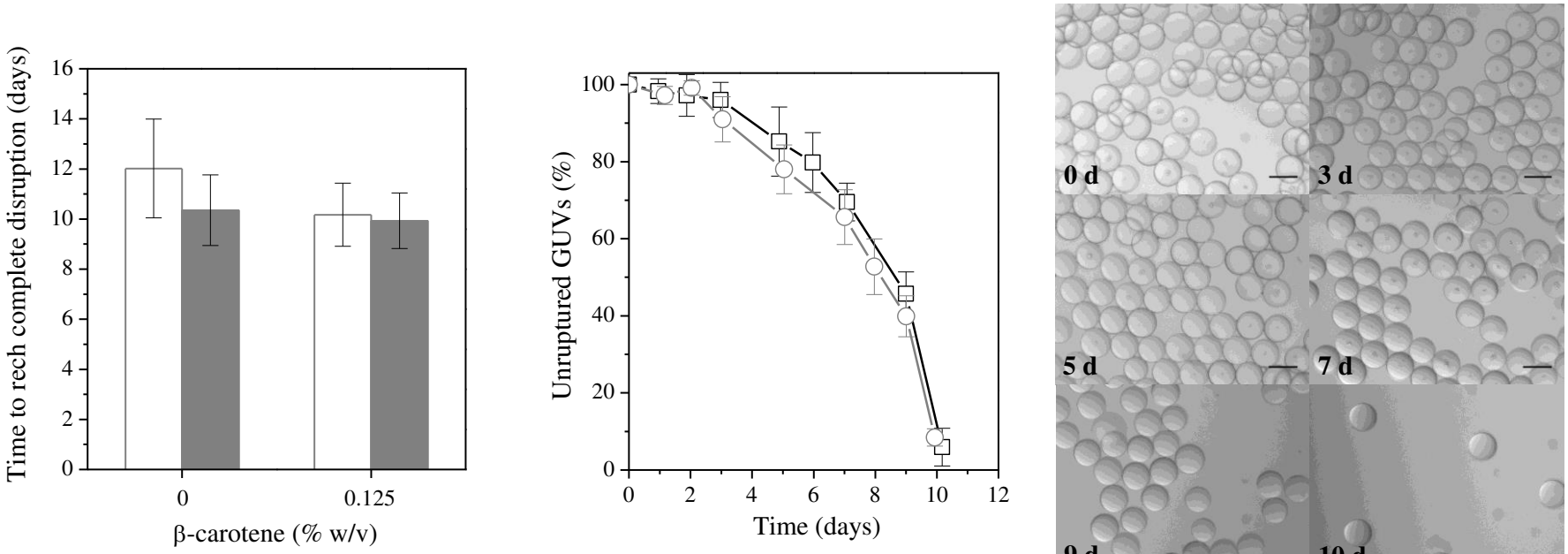

Figure 5.4. Stability of GUVs obtained at flow rate of innermost (q_1), middle lipid (q_2) and continuous (q_3) phases equal 1000 µl/h, 1000 µl/h and 12000 µl/h, respectively. **(a)** Stability time of GUVs obtained using chloroform/hexane mixture (empty bars) and ethyl acetate/pentane mixture (filled bars); **(b)** Fraction of unruptured GUVs as a function of time using ethyl acetate/pentane in presence of β-carotene (circle) and absence of β-carotene (square); **(c)** Optical microscope as a function of time of GUVs obtained using ethyl acetate/pentane in presence of β-carotene, where the scale bars represent 100 µm.

5.4. Conclusion

We demonstrated that W/O/W double emulsion templates aiming formation of phospholipid giant liposomes for food applications can be produced using glass-capillary microfluidic devices. It was possible to successfully confirm the technical feasibility of using non-purified soybean lecithin in the W/O/W double emulsion templates formation. These are much cheaper lipids when compared to other phospholipids commonly used in food and pharmaceutical fields aiming liposomes formation. Besides, the solvent mixtures replacement containing chloroform and/or hexane to green solvents with low toxic potential, such as ethyl acetate and pentane, allowed successfully the formation of stable W/O/W double emulsion templates. Moreover, we did not observe a significant influence of solvent replacement on the particle size distribution, GUVs stability and tunable diameter by varying the continuous flow rate. It was possible to demonstrate β-carotene incorporation inside lipid shell, confirming that GUVs, which are generally used to encapsulate hydrophilic compounds, can be used to load β-carotene and might be extended for other hydrophobic molecules. In general, β-carotene-loaded giant liposomes showed good stability, and its application in aqueous food formulations can be achieved. Thus, this microfluidic process proposed is potentially useful for a broad range of applications in protection and delivery of food and pharmaceutical active compounds.

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CAPÍTULO VI

- DISCUSSÕES GERAIS

6.1. Discussões gerais

Inicialmente a utilização de diferentes tipos de lecitinas comerciais de soja foi avaliada em função das propriedades estruturais dos lipossomas produzidos pela técnica de injeção de etanol, conforme apresentado no Capítulo III. No geral, a utilização de lecitinas contendo triacilgliceróis resultou em sistemas lipossomais com maior diâmetro em comparação à utilização de uma lecitina desengordurada. Este maior diâmetro observado pode estar relacionado com a presença dos triacilgliceróis, os quais provavelmente ficaram aprisionados no interior da bicamada fosfolipídica. Contudo, os lipossomas apresentaram distribuições de tamanho de partículas com menor polidispersidade em relação à lecitina desengordurada. Em geral, o aumento da concentração de lecitina promoveu um aumento do diâmetro, independentemente do tipo de lecitina. Em contraste, um aumento no índice de polidispersidade foi observado somente para os lipossomas obtidos com a lecitina enzimaticamente modificada. Neste caso, acredita-se que os lisofosfolipídios presentes na lecitina modificada levaram à formação de uma bicamada fosfolipídica com menor grau de organização, aumentando assim o diâmetro e a polidispersidade com o aumento da concentração. De acordo com as micrografias eletrônicas de transmissão, todos os lipossomas apresentaram um formato parcialmente esférico, típico para este tipo de sistema. Os lipossomas obtidos apresentam também um potencial-ζ negativo, variando entre -45 e -60 mV, sugerindo a existência de uma forte repulsão eletrostática entre as partículas, garantindo assim a estabilidade do sistema. Por fim, os perfis de espalhamento de raio-X a baixo ângulo sugerem que uma maior quantidade de fosfatidilcolinas nas lecitinas exerce uma influência positiva na auto-organização a nível molecular dos sistemas.

No Capítulo III, também foram avaliados os sistemas lipossomais obtidos com as diferentes lecitinas como sistema de encapsulação de interesse para indústria de alimentos. Para isso, o β-caroteno foi incorporado nos lipossomas em diferentes concentrações iniciais. Observou-se que o diâmetro médio apresentou uma dependência da concentração inicial de β-caroteno somente para os lipossomas obtidos com a lecitina desengordurada. Este efeito pode estar associado com a expansão da espessura da bicamada, dependendo da orientação da molécula de β-caroteno no seu interior. Por outro lado, acredita-se que as moléculas de triacilgliceróis presentes nas demais lecitinas aumentam a fluidez dos lipossomas gerando espaços na bicamada, os quais resultam no melhor ajuste da molécula de β-caroteno no seu interior, justificando assim a invariabilidade no diâmetro. Além disso, o aumento da

concentração de β-caroteno não exerceu influência na polidispersidade e no potencial-ζ dos lipossomas. Em geral, os lipossomas apresentam uma maior eficiência de incorporação nas menores concentrações iniciais de β-caroteno testadas. Provavelmente os baixos valores de eficiência de incorporação observados neste trabalho estão associados à falta de grupos polares na molécula do β-caroteno, de modo que a sua localização seja restrita somente ao interior da bicamada. Os lipossomas obtidos a partir das diferentes lecitinas apresentaram um aumento da microviscosidade da membrana fosfolipídica com o aumento da concentração de β-caroteno. Por fim, foi possível confirmar a hipótese de que a presença de triacilgliceróis nas lecitinas resulta no aumento da permeabilidade da bicamada fosfolipídica.

As lecitinas comerciais utilizadas neste trabalho podem sofrer oxidação devido à presença de cadeias de ácidos graxos poli-insaturados nas suas moléculas. Este fato pode promover um aumento na permeabilidade da membrana lipossomal. Os lipossomas obtidos com a lecitina modificada enzimaticamente apresentam um aumento linear na formação de produtos de oxidação em função do tempo e também um período de indução. Por outro lado, os lipossomas obtidos utilizando as demais lecitinas tenderam a atingir um equilíbrio no estágio final do processo de peroxidação. No geral, os valores absolutos de concentração dos produtos de oxidação foram superiores para os lipossomas obtidos a partir da lecitina desengordurada. Além disso, a peroxidação lipídica foi inibida após 3 h para todos os sistemas lipossomais obtidos com as lecitinas não submetidas à modificação enzimática. Esses efeitos contrastantes na peroxidação possivelmente estão associados à orientação e à localização da molécula de β-caroteno no interior da bicamada. Os resultados apresentados nesse capítulo sugerem que as lecitinas de soja contendo triacilgliceróis podem ser utilizadas para a produção de lipossomas visando à incorporação de β-caroteno, com a mesma eficiência de uma lecitina desengordurada. Além disso, essas constatações ampliam as oportunidades de uso de sistemas lipossomais na indústria de alimentos devido ao custo reduzido das matérias-primas e da sua eficiência como um sistema de encapsulação.

O desafio tecnológico para o desenvolvimento de um processo reprodutível com a possibilidade de operação em modo contínuo, que possa resultar em lipossomas monodispersos, impulsionou os estudos apresentados no Capítulo IV. A ideia foi a exploração das possibilidades e limitações tecnológicas de um dispositivo planar de focalização hidrodinâmica. Este estudo objetivou um aumento da taxa de produção de lipossomas para uma redução no número de canais visando a um sistema microfluídico paralelizado mais

eficiente. Inicialmente foi investigada a influência da concentração de lecitina nas propriedades de tamanho dos lipossomas. Neste caso, observou-se um aumento do diâmetro com o aumento da concentração, enquanto que o índice de polidispersidade permaneceu constante. Neste caso, a maior concentração de lecitina de soja testada foi escolhida, pois resultou em um diâmetro aceitável para aplicações em sistemas alimentícios, resultando assim em uma maior taxa de produção. Além da concentração de lecitina, a velocidade média de escoamento, ou a vazão volumétrica total é um parâmetro que está diretamente associado com a produtividade. Os resultados mostraram que o aumento da velocidade média de escoamento a uma razão constante entre as vazões volumétricas das fases não afetou significativamente as distribuições de tamanho de partículas. Estes resultados podem estar associados ao fato de o tempo de residência no canal ser superior ao tempo de mistura necessário para a difusão completa do etanol na água. Lipossomas com baixa polidispersidade podem ser obtidos utilizando dispositivos microfluídicos de focalização hidrodinâmica operados continuamente em maiores vazões volumétricas e concentrações de alimentação em comparação às condições microfluídicas comumente empregadas.

Após o estudo dos impactos da concentração de lecitina e da velocidade média de escoamento nas distribuições de tamanho de partículas, foram avaliadas a influência da área da seção retangular de canais planares e a influência da razão entre as vazões volumétricas de água e da dispersão fosfolipídica. Observou-se que o diâmetro foi inversamente proporcional à razão entre vazões para todas as áreas de seção retangular testada. Por outro lado, o diâmetro foi diretamente proporcional à área de seção a uma mesma razão entre as vazões. Para todas as condições estudadas, a distribuição de tamanho de partículas foi monomodal, apresentando índices de polidispersidade variáveis entre 0,2 e 0,4. O índice de polidispersidade diminuiu com aumento da área da seção retangular do canal para valores de razão entre as vazões volumétricas maiores que 20. O nossos resultados confirmaram a hipótese de que canais com maiores área de seção resultam em um perfil de velocidade mais uniforme ao longo da região de focalização, permitindo assim a produção de lipossomas com menor polidispersidade e maior produtividade. Os lipossomas incorporados com β-caroteno obtidos pela técnica microfluídica apresentaram uma estabilidade cinética semelhante aos obtidos por injeção de etanol, no entanto o índice de polidispersidade observado foi inferior. Por fim, a relação largura/altura da seção retangular do canal microfluídico não influenciou na capacidade de incorporação do β-caroteno.

No Capítulo V, um processo microfluídico para produção de outro sistema lipossomal de grande interesse industrial foi estudado. Lipossomas gigantes baseados em emulsões duplas A/O/A foram produzidos, empregando uma mistura de acetato de etila/pentano em substituição às misturas contendo clorofórmio e/ou hexano normalmente utilizadas para solubilizar os fosfolipídios. Emulsões duplas A/O/A com uma fase intermediária ultrafina foram produzidas com sucesso, empregando um dispositivo microcapilar de vidro com as paredes funcionalizadas. O tamanho das gotas das emulsões duplas A/O/A foi finamente ajustado entre 100 e 180 μm com a vazão da fase contínua variando entre 3000 e 12000 μL/h. Após a evaporação parcial da mistura de solvente orgânico presente na fase intermediária, as emulsões duplas deram origem a lipossomas gigantes com uma fase intermediária semelhante a uma bicamada fosfolipídica com uma espessura menor que 1 μm. O β-caroteno foi incorporado com sucesso no interior da bicamada, conforme mostraram as micrografias confocais de varredura a laser. No geral, os lipossomas gigantes permaneceram estáveis durante 10 dias. Assim, de forma inédita, através desses resultados foi possível desenvolver um processo microfluídico para produção de lipossomas gigantes, utilizando ingredientes e materiais com possibilidade reais de aplicação em sistemas alimentícios.

CAPÍTULO VII

- CONCLUSÕES GERAIS

7.1. Conclusões gerais

Lecitinas comerciais de soja contendo triacilgliceróis promoveram a formação de lipossomas com maior diâmetro e menor polidispersidade em relação à utilização de uma lecitina desengordurada. Porém, os lipossomas obtidos utilizando lecitina desengordurada apresentaram maior organização em nível molecular. No geral, o aumento da concentração de lecitina promoveu um aumento no diâmetro dos lipossomas, mantendo a polidispersidade constante. A incorporação de β-caroteno não promoveu mudanças significativas na forma esférica, na polidispersidade e no potencial-ζ dos lipossomas, porém o diâmetro aumentou nos lipossomas obtidos utilizando lecitina desengordurada. O tipo de lecitina utilizada não influenciou significativamente na eficiência do processo e na capacidade de carregamento do β-caroteno, que foi incorporado no interior da membrana fosfolipídica. No geral, uma maior eficiência de incorporação de β-caroteno foi observada nas menores concentrações de β-caroteno utilizadas. Os lipossomas incorporados com β-caroteno apresentaram a capacidade de inibir a peroxidação fosfolipídica, e por fim, concluiu-se que as lecitinas comerciais de soja podem ser empregadas na produção de lipossomas, para veiculação do β-caroteno através do método de injeção de etanol.

A produção contínua de lipossomas utilizando dispositivos microfluídicos de focalização hidrodinâmica também foi avaliada. Um aumento da concentração de lecitina de soja na fase orgânica promoveu a formação de lipossomas maiores, sem provocar modificações no índice de polidispersidade. O aumento da velocidade média de escoamento a uma mesma razão entre as vazões não exerceu influência significativa no perfil de distribuição do tamanho das partículas. Por outro lado, o aumento da relação largura/altura da seção retangular dos canais levou à formação de lipossomas maiores com distribuições de tamanhos de menor polidispersidade. Uma máxima taxa de produção de lipossomas foi observada utilizando o canal retangular de maior relação largura/altura (300×50 μm). A capacidade de incorporação de β-caroteno não foi influenciada com o aumento da relação largura/altura do canal, assim como os lipossomas não apresentaram variações nas distribuições de tamanho de partículas e no potencial-ζ durante 28 dias de armazenamento. O aumento da área de seção retangular dos canais permitiu a obtenção de lipossomas incorporados com β-caroteno com propriedades semelhantes aos obtidos pela técnica convencional de injeção de etanol. Por fim, os dispositivos microfluídicos podem ser

utilizados como uma alternativa para produção contínua de lipossomas com um nível mínimo de paralelização de canais.

Os resultados referentes à produção de lipossomas gigantes utilizando dispositivos microfluídicos capilares de vidro permitiram a conclusão de informações relevantes sobre a utilização de solventes orgânicos com baixo potencial tóxico para saúde humana. No geral, concluiu-se que emulsões duplas A/O/A contendo uma fase intermediária ultrafina composta por lecitina de soja, acetato de etila e pentano podem ser produzidas com sucesso. Neste mesmo processo, emulsões duplas A/O/A e emulsões simples O/A foram produzidas de forma descontínua, utilizando um dispositivo capilar de vidro com as paredes funcionalizadas. O tamanho das gotas das emulsões A/O/A formadas foi precisamente ajustado com uma variação na vazão da fase contínua. A incorporação do β-caroteno na fase intermediária das gotas não exerceu influência significativa no controle do tamanho das emulsões duplas A/O/A formadas. Após a evaporação do solvente residual da fase intermediária, as emulsões duplas A/O/A originaram lipossomas gigantes, os quais foram estáveis por até 10 dias quando armazenados à temperatura ambiente com a osmolaridade das fases aquosas ajustadas. Por fim, demonstrou-se de forma inédita que lipossomas gigantes podem ser obtidos utilizando dispositivos microfluídicos, com materiais passíveis de aplicação na indústria de alimentos.

Todos os resultados experimentais obtidos nesta tese sugerem que lecitinas comerciais de soja amplamente disponíveis como emulsificantes para indústria de alimentos podem ser utilizadas como alternativas de baixo custo para obtenção de sistemas lipossomais. Além disso, podemos concluir que diferentes processos microfluídicos podem ser empregados para obtenção de diferentes estruturas lipossomais. Assim, os resultados obtidos para veiculação do β-caroteno podem ser expandidos para a encapsulação de outros compostos ativos hidrofílicos, hidrofóbicos ou anfifílicos de interesse industrial. Por fim, esses resultados são relevantes para superação parcial dos desafios existentes para uma produção industrial de lipossomas como um sistema de encapsulação utilizando dispositivos microfluídicos.

CAPÍTULO VIII

- SUGESTÕES PARA TRABALHOS FUTUROS

- MEMÓRIA DO PERÍODO DE DOUTORADO

8.1. Sugestões para trabalhos futuros

(i) Avaliar a viabilidade técnica de produção de complexos eletrostáticos entre os lipossomas obtidos com as lecitinas naturais e biopolímeros catiônicos;

(ii) Avaliar a produção de lipossomas gigantes baseados em emulsões duplas A/O/A utilizando proteínas e polissacarídeos para o ajuste da osmolaridade entre as fases aquosas;

(iii) Avaliar a influência do impacto das condições do sistema gastrointestinal na estabilidade dos lipossomas e na bioacessibilidade dos compostos ativos veiculados.

8.2. Memória do período de doutorado

O aluno Mariano Michelon ingressou no programa de Doutorado em Engenharia de Alimentos da Faculdade de Engenharia de Alimentos da Universidade Estadual de Campinas em 03/2013. Durante o período, o bolsista usufrui de uma bolsa de doutorado do CNPq (Processo N° 140283/2013-7) com vigência de 03/2013 até 02/2017. Durante o período de doutorado o bolsista cursou três disciplinas: TP199 Seminários; TP143 Reologia; IQ064 Encapsulação de Agentes Bioativos. Para atingir o número de créditos exigidos pelo programa, outras duas disciplinas excedentes cursadas no mestrado foram convalidadas: TP319 Engenharia Bioquímica; TP333 Planejamento Experimental e Otimização de Processos. Além das disciplinas cursadas, outros créditos foram cumpridos através da participação no Programa Estágio Docente do grupo C (PED C) com apoio parcial à docência atuando como voluntário nas disciplinas TA736 Engenharia de Bioprocessos (1s/2013) e TA431 Mecânica dos Materiais (2s/2013) e como bolsista na disciplina TA431 Mecânica dos Materiais (2s/2014).

O bolsista coorientou durante 24 meses o trabalho de iniciação científica da aluna Maria Fernanda Boldim Vaggione intitulado “Produção de quitossomas para encapsulação de resveratrol” com bolsa financiada pela FAPESP (Processo Nº 2014/08442-5) de 08/2014 até 07/2016. Além disso, o doutorando realizou um estágio de doutorado sanduíche no grupo de pesquisa “Experimental Soft Condensed Matter" da John A. Paulson School of Engineering and Applied Sciences da Harvard University, com supervisão do Prof. David A. Weitz. Durante o período de doutorado o candidato publicou artigos em períodos internacionais e trabalhos em anais de eventos nacionais e internacionais. Os resultados referentes à tese e/ou obtidos paralelamente em pesquisas de colaboração com outros membros do grupo de

pesquisa durante este período resultaram até o presente momento em cinco artigos científicos publicados em periódicos e dez trabalhos publicados em anais de eventos, conforme listado a seguir.

8.2.1. Artigos publicados em periódicos

- Ushikubo, F. Y.; Oliveira, D. R. B.; **Michelon, M.**; Cunha, R. L. Designing food structure using microfluidics. Food Engineering Reviews, v. 7, p. 393-416, 2015.

- Michelon, M.; Mantovani, R.A.; Sinigaglia-Coimbra, R.; de la Torre, L.G.; Cunha, R.L. Structural characterization of β-carotene-incorporated nanovesicles produced with non-purified phospholipids. Food Research International, v. 79, p. 95-105, 2016.

- Oliveira, D. R. B.; **Michelon, M.**; Furtado, G. F.; Sinigaglia-Coimbra, R.; Cunha, R.L. β-Carotene-loaded nanostructured lipid carriers produced by solvent displacement method. Food Research International, v. 90, p. 139-146, 2016. (em colaboração)

- Furtado, G. F.; **Michelon, M.**; Oliveira, D. R. B.; Cunha, R.L. Heteroaggregation of lipid droplets coated with sodium caseinate and lactoferrin. Food Research International, v. 89, p. 309-319, 2016. (em colaboração)

- Mantovani, R. A.; Fattori, J.; **Michelon, M.**; Cunha, R.L. Formation and pH-stability of whey protein fibrils in the presence of lecithin. Food Hydrocolloids, v. 60, p. 288-298, 2016. (em colaboração)

8.2.2. Trabalhos publicados em anais de eventos

8.2.2.1 Resumos

- Michelon, M.; de la Torre, L.G.; Cunha, R.L. Production of liposomes using different lecithins aiming food applications. 1st Congress on Food Structure Design, Porto, 2014.

- Sato, A.C.K.; Perrechil, F.A.; **Michelon, M.**; Cunha, R.L. Biopolymeric microbeads for incorporation of lipophilic and hydrophilic compounds. 1st Congress on Food Structure Design, Porto, 2014. (em colaboração)

- Bueno, A.C.; Picone, C.S.F.; **Michelon, M**.; Cunha, R.L. Microcapsules formed by emulsion gelation: design and application as vehicle for *Lactobacillus rhamnosus*. 1st Congress on Food Structure Design, Porto, 2014. (em colaboração)

- Michelon, M.; Okuro, P.K. ; Furtado, G.F.; Cunha, R.L. Caracterização estrutural e reológica de emulsões óleo/água estabilizadas por diferentes lecitinas. 3º Congresso Brasileiro de Reologia, Campinas, 2015.

- Michelon, M.; Furtado, G.F.; Oliveira, D.R.B.; de la Torre, L.G.; Cunha, R.L. Production of monodisperse liposomes by microfluidic devices. 2nd Congress on Food Structure Design, Antalya, 2016.

- Furtado, G.F.; **Michelon, M.**; Oliveira, D.R.B.; Cunha, R.L. Production of heteroaggregated droplets coated with sodium caseinate and lactoferrin. 2nd Congress on Food Structure Design, Antalya, 2016. (em colaboração)

8.2.2.2 Resumos expandidos

- Michelon, M.; Oliveira, D.R.B.; de la Torre, L.G.; Cunha, R.L. Obtenção de nanovesículas lipídicas utilizando dispositivos microfluídicos visando aplicações alimentícias. V Workshop em Microfluídica, Campinas, 2015.

- Vaggione, M.F.B.; **Michelon, M.**; Cunha, R.L. Production and characterization of chitosomes for application as delivery system of bioactive compounds. XXIII Congresso de Iniciação Científica da UNICAMP, Campinas, 2015.

- Michelon, M.; Oliveira, D.R.B.; Furtado, G.F.; de la Torre, L.G.; Cunha, R.L. Development of microfluidic processes aiming β-carotene-incorporated liposomes production. VI Workshop em Microfluídica, Campinas, 2016.

8.2.2.3 Trabalhos completos

- Maximo, G.J.; **Michelon, M.**; Perrechil, F.A.; Cunha, R.L.; Sato, A.C.K. Veiculação de *Lactobacillus rhamnosus* por microcápsulas gelificadas de caseinato de sódio e κ-carragena. XXV Congresso Brasileiro de Ciência e Tecnologia de Alimentos**,** Gramado, 2016. (em colaboração)

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