UNIVERSIDADE FEDERAL DE PERNAMBUCO
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LABORATÓRIO DE IMUNOLOGIA KEIZO-ASAMI
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS

DESENVOLVIMENTO E ATIVIDADE BIOLÓGICA DE LIPOSSOMAS SÍTIO-ESPECÍFICOS CONTENDO ÁCIDO ÚSNICO PARA O TRATAMENTO DA TUBERCULOSE

Rafaela de Siqueira Ferraz Carvalho

Março, 2015
Recife-PE

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Doutoranda: RAFAELA DE SIQUEIRA FERRAZ CARVALHO
Orientadora: Profa. Dra. NEREIDE STELA SANTOS MAGALHÃES
Co-orientadora: Profa. Dra. MARIANE CAJUBÁ DE BRITTO LIRA

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COMISSÃO EXAMINADORA

Profa. Dra. Nereide Stela Santos Magalhães (Orientador/UFPE)

Profa. Dra. Mariane Cajubá De Britto Lira Nogueira (Co-orientadora/UFPE)

Profª Drª Maria Tereza S. Correia (1º Examinador/UFPE)

Profª Drª Luana Cassandra Breitenbach Barroso Coelho (2º Examinador/UFPE)

Profª Drª Maria da Paz C. Silva (3º Examinador/UFPE)

Drª Milena Sales Ferraz (4º Examinador/UFPE)

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RESUMO

A utilização de sistemas de liberação controlada de fármacos de base nanotecnológica, a exemplo dos lipossomas, representa atualmente um grande avanço no tratamento e diagnóstico de várias doenças. Modificações na superfície desses nanossistemas vêm sendo foco nas pesquisas, com objetivo de maximizar a eficácia e segurança dos mesmos, a partir do desenvolvimento de sistemas de longa circulação e/ou sítio-específicos. Neste trabalho, a fucana, um polissacarídeo sulfatado, foi utilizada como molécula de revestimento da superfície de lipossomas, uma vez que apresenta duas propriedades importantes: a capacidade de não ativar o sistema complemento, e a de ser reconhecida e poder se ligar à receptores encontrados na superfície de macrófagos. Essas duas propriedades nos encorajaram a utilizar a fucana com o propósito de desenvolver um novo sistema lipossomal de longa circulação e direcionado para macrófagos. Assim, a primeira etapa deste estudo envolveu uma síntese química, na qual a fucana foi modificada quimicamente por meio de sua conjugação com o colesterol (fucana hidrofobizada), através de um novo procedimento de irradiação por micro-ondas. Posteriormente, lipossomas revestidos com fucana contendo ácido úsnico foram preparados pelo método de hidratação do filme lipídico, variando as quantidades de fucana hidrofobizada. Os lipossomas foram caracterizados por determinação do tamanho da partícula, índice de polidispersão (PDI), carga de superfície (ζ) e eficiência de encapsulação do fármaco. Além disso, os lipossomas foram avaliados em estudos *in vitro* de citotoxicidade, captura celular e pelo método de competição de inibidores por receptores específicos frente a macrófagos RAW 264.7. Por fim, a atividade antimicobacteriana e interações entre fármacos foram avaliada pelo método *chekerboard* contra isolados de *Mycobacterium tuberculosis* multirresistentes. Os resultados obtidos evidenciaram que a fucana modificada quimicamente foi capaz de ser utilizada no desenvolvimento de lipossomas revestidos. Observou-se que a variação da quantidade de fucana de 5 para 20 mg promoveu um aumento no tamanho e no PDI das vesículas de 168 ± 2,82 nm, 0,36 para 1,18 ± 0,01µm, 0,46, respectivamente. Por outro lado, com o aumento da concentração de fucana, o ζ diminuiu (1,35 ± 0,185 mV para -5,41 ± 0,234 mV), visto que a mesma é um polissacarídeo negativo. Em relação à taxa de encapsulação, todas as formulações apresentaram valor superior a 98% indicando que o revestimento com fucana não alterou a encapsulação do ácido úsnico. Os estudos de citotoxicidade revelaram que as formulações lipossomais revestidos com fucana, exibiram valores de IC_{50} inferiores (12.70 ± 1,56µM) em comparação aos lipossomas sem revestimento IC_{50}= 18,37 ± 3,24 µM). Em relação à captura celular, os resultados mostraram que os lipossomas revestidos foram capturados de forma mais rápida (15 min) que os lipossomas sem revestimentos (60 min) podendo sugerir que foram mais rapidamente reconhecidos pelos macrófagos. Adicionalmente, os resultados obtidos no experimento de inibidores competitivos sugerem que os lipossomas revestidos pela fucana ($Lipo_{fuc5}$) foram internalizados através do "scavenger receptors" (SR) presente na superfície dos macrófagos. Nos estudos utilizando isolados de *M. tuberculosis* multirresistentes, a encapsulação do ácido úsnico em lipossomas potencializou a atividade antimicobacteriana deste composto liquênico, além de apresentar um efeito sinérgico com a ripamficina. Esses sistemas apresentam, portanto, um potencial como candidatos à melhoria da atividade antimicobacteriana da rifampicina. Diante do exposto, os lipossomas revestidos com fucana contendo ácido úsnico apresentam-se como uma alternativa para o direcionamento eficiente de fármacos aos macrófagos.

Palavras-chaves: Fucana, ácido úsnico, lipossomas, macrófagos e tuberculose.

ABSTRACT

The use of controlled drug delivery systems, such as liposomes, currently represents a major advance in the treatment and diagnosis of various diseases. Changes in the surface of these nanosystems have been focusing on research, aiming to maximize their effectiveness through the development of stealth or site-specific systems. In this work, the fucoidan, a sulfated polysaccharide, was used for coating liposomes, since it presents two important properties: the ability to not activate the complement system and of being recognized and bound by receptors found on macrophages. These two activities have encouraged us to use the fucoidan for the purpose of developing a new long-circulating liposomal system and directed to macrophages. Thus, the first stage of the study involved a synthesis, wherein the fucoidan was chemically modified by conjugating with cholesterol through a new microwave irradiation procedure. Subsequently, fucoidan-coated liposomes containing usnic acid were prepared by lipid film hydration method and varying the amounts of hydrophobized fucan. Liposomes were characterized by determining particle size (Ø), polydispersity index (PDI), surface charge (ζ) and drug encapsulation efficiency. Finally, liposomes have been evaluated *in vitro* study of cytotoxicity, cellular uptake and competition assay using RAW 264.7. In addition the antimycobacterial activity and interactions between the drugs were evaluated through chekerboard method against multidrug resistant strains of *M. tuberculosis*. The results showed that (hydrophobized fucoidan) were able to be used in the development of coated liposomes. It was observed that by varying the amount of fucoidan from 5 to 20 mg, an increase in size and PDI of the vesicles from 168 ± 2.82nm, 0.36 and 1.18 ± 0.01µm, 0.46, was found respectively. Moreover, with the increase in fucoidan concentration the ζ lower (1.35 ± 0.185 ± 0.234 -5.41 mV mV), due to its negative charges exposed to the surface of liposomes. Regarding drug encapsulation efficiency, all formulations showed greater than 98% value, indicating that the coating did not affect the encapsulation of usnic acid. In relation to cellular uptake, the results showed that the fucan coated liposomes were captured faster (15 min) as compared with uncoated liposomes (60 min), suggesting that were quickly recognized by macrophages. In addition, the results obtained in the experiments of competitive inhibitors suggested that the coated liposomes ($Lipo_{fuc5}$) were internalized through C-type carbohydrate recognition domain present in the scavenger receptors (SR). In studies using multidrug resistant *M. tuberculosis* isolates, the encapsulation of usnic acid into liposomes enhanced the antimycobacterial active both in the free and encapsulated forms. In this way, usnic acid may be candidates for improving the antimycobacterial activity of rifampicin. Given the above, fucoidan-coated liposomes containing usnic acid are an alternative approach for targeting efficiently drugs to macrophages.

Keywords: Fucoidan, usnic acid, liposomes, macrophages, tuberculosis.

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LISTA DE ABREVIATURAS E SIGLAS

AU - Ácido úsnico
BCG - Bacilo Calmette-Guerin
BK- Bacilo de Koch
CCS - Cicloserina
CF - Clofazima
CIM - Concentração Inibitória Mínima
EMB - Etambutol
EST - Estreptomicina
ETN - Etionamida
FDA - Administração de Alimentos e Medicamentos (Food and Drug Administration)
IFN-γ - Interferon Gama
INH – Isoniazida
$Lipo_{42}$ – Lipossoma com concentração lipídica de 42 mM
$Lipo_{60}$ – Lipossoma com concentração lipídica de 60 mM
$Lipo_{80}$ - Lipossoma com concentração lipídica de 80 mM
LUV- Vesículas Unilamelares Grandes
MLV- Vesículas multilamelares
MSR – Macrophages Scavenger Receptors
Mtb – *Mycobacterium tuberculosis*
OMS- Organização Mundial de Saúde
O-SAP - O-esteroil Amilopectina
PAS - Ácido Paraminossalicílico
PDI – Indíce de polidispersão
PRZ - Pirazinamida
RF - Rifabutina
RIF - Rifampicina
SLC - Sistema de Liberação Controlada
SUV - Vesículas Unilamelares Pequenas
TB - Tuberculose
TB-MDR - Tuberculose Multirresistente
TB-XDR- Tuberculose Extensivamente Resistente
TNF - Fator de necrose tumoral
TOD - Tratamento Diretamente Observado
UA-$Lipo_{80}$ - Lipossoma com concentração lipídica de 80 mM contendo ácido úsnico
UA - $Lipo_{fuc5}$ – Lipossoma revestido com fucana (5 mg) contendo ácido úsnico
UA - $Lipo_{fuc10}$ – Lipossoma revestido com fucana (10 mg) contendo ácido úsnico
UA - $Lipo_{fuc20}$ – Lipossoma revestido com fucana (20 mg) contendo ácido úsnico
UFC- Unidade Formadora de Colônia

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1. INTRODUÇÃO

A tuberculose (TB) é uma doença causada pelo *Mycobacterium tuberculosis*, patógeno intracelular que se localiza nos macrófagos, e há 20 anos é considerada um problema de saúde global. Em 2012, 8,6 milhões de pessoas foram contaminadas e 1,3 milhões foram levadas a óbito (WHO, 2013). A despeito dos esforços desempenhados pelos governos mundiais na erradicação da TB e milhões de dólares serem gastos anualmente, a doença é considerada como a segunda causa de morte por doenças infecciosa em todo mundo, ficando atrás apenas da Síndrome da Imunodeficiência Adquirida (SIDA) (WHO, 2013; KAUR; SINGH, 2014).

Apesar de ser uma patologia curável, a TB continua sendo uma das principais causas de morte evitável no mundo. A ineficiência no tratamento envolve vários fatores, como a dificuldade dos antibióticos em ultrapassar as membranas celulares (com exceção da rifampicina a maior parte dos medicamentos usados na clínica é hidrofílico), administração diária de múltiplos medicamentos por período prolongado e graves efeitos colaterais. Esses fatores tem sido responsáveis pela baixa adesão por parte dos pacientes aos esquemas terapêuticos, contribuindo assim com surgimento de cepas resistentes (DUBE et al., 2012; KAUR; SINGH, 2014). Desta forma, o principal desafio para a quimioterapia intracelular é projetar e desenvolver um carreador para antibióticos que possa ser eficientemente capturado pelos macrófagos e, uma vez no interior dessas células prolonguem a liberação dos antibióticos de modo que o número de doses e toxicidade dos fármacos associados possa ser reduzido.

Os problemas associados à liberação do fármaco no espaço intracelular levou a investigação de novos carreadores para o tratamento de infecções intracelulares. Dos vários nanocarreadores existentes, os lipossomas apresentam como importante vantagem a sua capacidade de encapsular uma gama de compostos, além de apresentar uma flexibilidade e versatilidade estrutural (TYAGI et al., 2012). Ao longo dos anos, modificações estruturais, em especial relacionadas à superfície dos lipossomas vêm sendo realizada com o intuito de potencializar a atividade terapêutica do fármaco encapsulado. Uma dessas modificações foi a adição de moléculas como PEG, anticorpos, proteínas, peptídeo, polissacarídeos dentre outras à superfície. Como consequência, os sistemas são capazes de permanecer por mais tempo na circulação sistêmica ou ainda de reconhecer e se ligar a tecidos e órgãos alvos aumentando assim a atividade terapêutica.

Atualmente grande atenção tem sido dada a adição de polissacarídeos à superfície de nanosistemas com o intuito de torná-los furtivos e ou sítio-específicos. Nesse contexto, acredita-se que a fucana, um polissacarídeo sulfatado extraído principalmente de algas marrons, pode ser utilizado no desenvolvimento de nanocarreadores, devido a sua capacidade de não ativar o sistema complemento, conforme descrito por Clement e colaboradores (2010), além de se ligar aos receptores de membrana do tipo AI e AII encontrados nos macrófagos chamados "*macrophages scavenger receptors"* (SR) (KIM; ORDIJA; FREEMAN, 2003). Essas duas propriedades são de grande interesse para obtenção de nanosistemas de longa circulação e capazes de carrear fármacos diretamente aos macrófagos infectados.

O aumento no número de casos de resistência associado ao fato de que nenhum fármaco contra o *M. tuberculosis* foi introduzido nos últimos 40 anos, tem impulsionado os pesquisadores a buscar novas substâncias para o tratamento da tuberculose (LUO et al., 2013). Dentre estas, o ácido úsnico um dos mais bem conhecidos e estudados derivados liquênicos, destaca-se por apresentar várias atividades biológicas, incluindo a atividade antimicobacteriana podendo ser uma alternativa promissora para quimioterapia da TB (MÜLLER, 2001; HONDA et al., 2010). No entanto, o ácido úsnico apresenta algumas características desfavoráveis, tais como baixa solubilidade em água e uma elevada hepatotoxicidade (INGÓLFSDÓTTIR, 2002; PRAMYOTHIN et al., 2004). Dessa maneira, a comunidade científica tem se empenhado no sentido de minimizar este problema através da utilização da nanotecnologia.

Diante do exposto, o principal desafio deste trabalho consistiu em desenvolver lipossomas, furtivos e sítios-específicos, através do revestimento das vesículas com a fucana, visando aumentar o direcionamento para os macrófagos e em contrapartida viabilizar a utilização do ácido úsnico na terapêutica da tuberculose. Desta forma, os resultados oriundos desta tese serão apresentados na forma de artigos científicos. O primeiro artigo versará sobre a síntese química da fucana com o colesteroil e da sua utilização nos estudos de uptake em macrófagos RAW 264.7. O segundo artigo trará os resultados obtidos no desenvolvimento e caracterização dos lipossomas revestidos com a fucana contendo ácido úsnico, assim como a avaliação da citotoxicidade em macrófagos RAW 264.7 e o terceiro artigo trará os resultados obtidos com os testes de atividade antimicobacteriana e de interações entre os fármacos padrões (isoniazida e rifampicina) com o ácido úsnico livre e ecapsulado em lipossomas frente a isolados clínicos multirresistentes de *M. tuberculosis.*

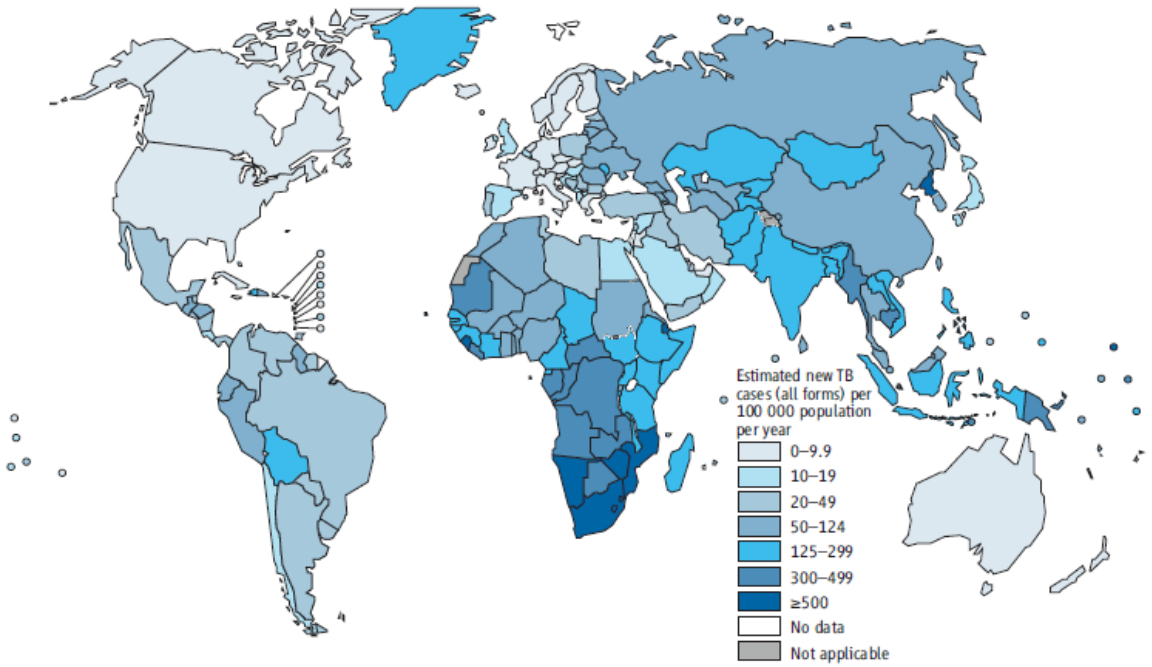
2. REVISÃO BIBLIOGRÁFICA

2.1. Tuberculose

2.1.1. Epidemiologia

A tuberculose (TB), uma doença infecciosa e mortal do sistema respiratório, é considerada um dos principais desafios da saúde pública há 20 anos. Em 2012, registraram-se mundialmente o surgimento de 8,6 milhões de casos da TB, o que corresponde aproximadamente a 126 casos por 100.000 habitantes. A maioria destes casos ocorreu na Ásia (58%) e na África (27%), e em menores proporções na região do Mediterrâneo Oriental (8%), na Europa (4%) e nas Américas (3%) (Figura 1) (WHO, 2013).

Figura 1. Taxa de incidência de tuberculose em 2012.



Fonte: WHO, 2013.

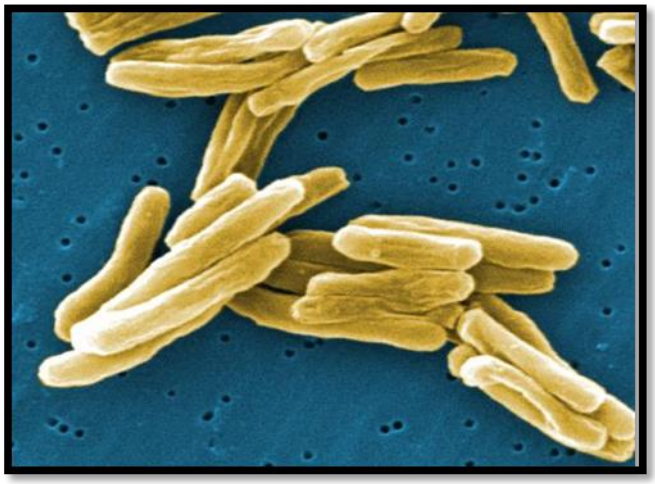
No Brasil, a tuberculose é um problema de saúde emergencial, pois juntamente com outros 21 países em desenvolvimento, representam 81% dos casos mundiais da doença. Em 2011, foram notificados 69.245 novos casos de TB (equivalente a 36 casos por 100.000 habitantes) e, segundo o Ministério da Saúde, pela primeira vez esta estimativa foi inferior a 70.000. Com relação aos óbitos, o país registrou 2,4 mortes para cada grupo de 100.000 habitantes. No país, a

tuberculose representa a quarta causa de óbitos por doenças infecciosas e a primeira entre pacientes com a Síndrome da Imunodeficiência Adquirida (MINISTÉRIO DA SAÚDE, 2011).

2.1.2. Etiologia e patogênese

A tuberculose vem atingindo a humanidade ao longo dos anos e está diretamente associada à pobreza, desnutrição, superpopulação e imunosupressão. Em 1882, Robert Koch descobriu o agente etiológico da doença, o *Mycobacterium tuberculosis* (Mtb), conhecido como Bacilo de Koch (BK) (Figura 2).

Figura 2. Micrografia eletrônica do *M. tuberculosis*



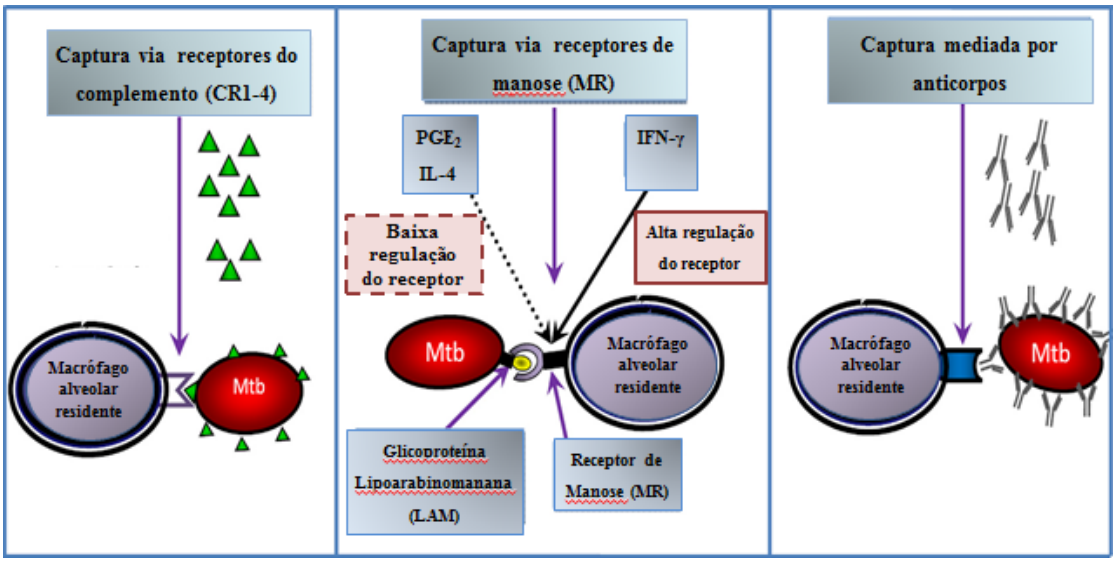
Fonte: www.textbookofbacteriology.net

O *M. tuberculosis* é um parasita intracelular facultativo, não-móvel, aeróbio, com tamanho entre 0,8-4 mícrons, sensível a luz solar e a radiação ultravioleta, que uma vez fagocitado pelos macrófagos são capazes de sobreviver e se multiplicar dentro da célula hospedeira (GASPAR et al., 2008; ORCAU et al., 2011).

A tuberculose, geralmente é iniciada pela entrada do Mycobacterium no sistema respiratório, através de gotículas contendo um pequeno número do bacilo. Esse estágio inicial de infecção do pulmão não está totalmente descrito na literatura, acredita-se que a captura do Mtb pelos mácrofagos pode ocorrer através de receptores do complemento, receptores de manose ou mediada por anticorpos, como descrito na Figura 3 (GARG et al., 2014). Uma vez no pulmão, as bactérias são fagocitadas pelos macrófagos alveolares que processam os antígenos bacterianos e os apresentam aos linfócitos T. Após a fagocitose dos bacilos, os macrófagos alveolares tornam-

se ativos e devido ao vasto arsenal microbicida que possuem, tentam destruí-los através da digestão lisossomal, entretanto alguns bacilos são capazes de escapar e sobreviver nos macrófagos (LIU; MODLIN, 2008; KAUFMANN, 2001). Em seguida, o número de agentes patogênicos aumenta exponencialmente, ocasinando a morte das células hospedeiras e dissimenando a infecção para os nódulos linfáticos da região pulmonar através da circulação linfática (3 a 8 semanas após a infecção). Mais tarde, ocorre a disseminação do bacilo dos pulmões infectados para outros órgãos, como sistema nervoso central, fígado, rins e órgãos genitais, configurando a tuberculose extrapulmonar (SMITH, 2003).

Figura 3. Interação do *Mycobacterium tuberculosis* aos macrófagos, através de um ou mais mecanismos: (1) Captura via receptores do complemento; (2) receptores de manose e (3) anticorpos.

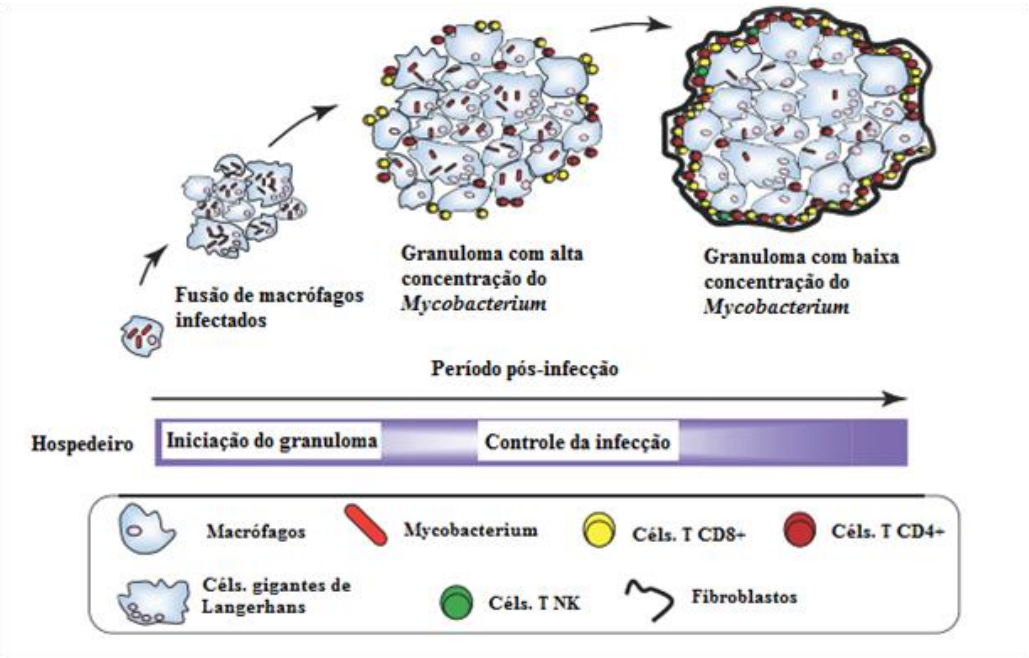


Fonte: Adaptado de Garg et al., 2014.

A interação entre os componentes das micobactérias e os receptores de macrófagos, resulta na produção de quimiocinas e citocinas, que sinalizam a infeção ocasinando a migração de macrófagos e células dendríticas para o pulmão. Essa migração celular culmina na formação do granuloma, uma característica da TB (Figura 4). O granuloma é formado por células T, macrófagos, células dendríticas e células epiteliais, o que gera um microambiente imunológico que intensifica a interação entre citocinas, produzidas pelos macrófagos e células T, o que é imprescindível para defesa antimicrobiana efetiva. Por outro lado, no centro do granuloma surge

uma porção caseosa (área de necrose sólida e hipóxica), uma condição em que a cultura do Mtb não se replica. A maioria dos granulomas persisti durante toda a vida do indivíduo sem causar a tuberculose ativa (JORDAO; VIEIRA, 2011; WELSH et al., 2013; GARG et al., 2014).

Figura 4. Formação do granuloma na tuberculose humana



Fonte: Adaptado de DUBE; AGRAWAL; VYAS, 2012.

2.1.3. Tratamento da tuberculose

Apesar de ser uma patologia curável e com farmacoterapia disponível há mais de 50 anos, a tuberculose continua sendo uma das principais causas de morte em todo mundo. Até o momento, a única medida profilática disponível contra Mtb é a vacina Bacilo Calmette-Guerin (BCG), que é obtida a partir de um cepa de *Mycobacterium bovis* (M. bovis) atenuada. Essa vacina confere proteção contra várias formas de tuberculose na infância, mas não consegue evitar a tuberculose pulmonar (PTB) em adultos, sendo esta a forma mais contagiosa da TB (MEERAK et al., 2013).

O tratamento preconizado pela OMS envolve a administração diária de quatro antibíoticos orais por um período de seis meses ou mais. Na fase inicial (intensiva – 2 meses) as drogas utilizadas são: isoniazida (INH), rifampicina (RIF), pirazinamida (PRZ) administradas em conjunto com o etambutol (ETB). A apresentação farmacológica desse esquema passa a ser em comprimidos de doses fixas combinadas dos quatro medicamentos, nas seguintes dosagens: RIF

150mg, INH 75mg, PRZ 400mg e ETB 275mg. A segunda fase (quatro meses) compreende exclusivamente RIF e INH. Estas quatro drogas em conjunto com estreptomicina (EST) constituem a chamada terapia de primeira linha (Tabela 1) (SOSNIK et al., 2010; SHEGOKAR et al., 2011; MINISTÉRIO DA SAÚDE, 2011).

Tabela 1. Fármacos utilizados na terapia antimicobacteriana.

	Fármacos	Mecanismo de Ação	Dose (mg)
Fármacos Primeira linha	Rifampicina (RIF)	Inibe a síntese do RNA bacteriano por formar um complexo com a RNA-polimerase DNA-dependente, impedindo a ação desta enzima.	300
	Isoniazida (INH)	Pró-fármaco ativado pela enzima KatG, uma catalase-peroxidase micobacteriana, o qual exerce efeito letal através da interrupção da biossíntese dos ácidos micólicos, principais constituintes da parede celular micobacteriana.	125
	Pirazinamida (PRZ)	O ácido pirazinóico inibe o crescimento do bacilo por diminuir o pH intracelular. PRZ inibe a trans-tradução, a chave do processo celular que gere os danos a proteínas e recruta ribossomos não funcionantes da micobactéria. Age como um antimetabólito da nicotinamida que interfere na síntese do NAD, inibindo a síntese dos precursores de ácidos graxos.	500
	Etambutol (ETB)	Inibe a enzima arabinosil transferase involvida na polimerização da D-arabinofuranose para arabinogalactanas, um componente essencial da parede celular.	400
	Estreptomicina (EST)	Inibe irreversivelmente a síntese protéica ao ligar-se à subunidade 30S do ribossomo bacteriano.	500
Fármaco Segunda linha	Ácido paraminossalicílico (PAS)	Antimetabólito que interfere na incorporação do ácido para-aminobenzóico no ácido fólico- é um antagonista da síntese do folato.	500
	Cicloserina (CCS)	Análago estrutural da D-alanina, inibe a incorporação da D-alanina no peptideoglicano pela inibição da alanina racemase.	500
	Etionamida (ETN)	Assim como a isoniazida, inibe a sítese do ácido micólico.	500
	Rifabutina (RF)	Inibe a síntese do RNA bacteriano por formar um complexo com a enzima RNA-polimerase DNA-dependente. Similar a rifampicina.	150
	Clofazima (CF)	Mecanismo desconhecido. Possui propriedade antimicobacteriana e imunossupressora.	100

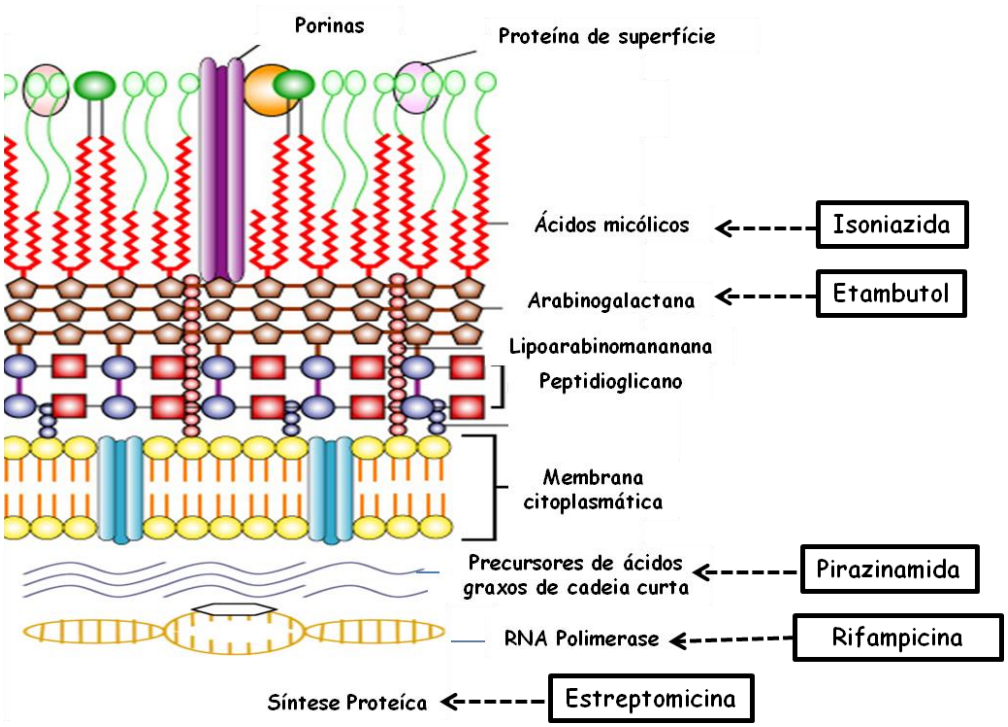
Fonte: SOSNIK et al., 2010 DUBE; AGRAWAL; VYAS, 2012.

O tratamento da tuberculose multi-resistente (MDR-TB) é constituída pela administração de PRZ simultaneamente com medicamentos de segunda linha (Tabela 1). Estes medicamentos de segunda linha são mais tóxicos, mais caro e menos ativos do que os agentes de primeira linha. Além disso, tratamentos prolongados (9-12 meses) são necessários a fim de garantir a eficácia terapêutica (SOSNIK et al., 2010).

Nos casos de falência, ou seja, quando ocorre persistência da positividade do escarro no final do tratamento, o paciente é medicado com um novo esquema terapêutico por mais um ano. Nos primeiros três meses, associa-se EST, PZA, ETB e ETN e, nos nove meses restantes, permanece o uso apenas da ETN (MINISTÉRIO DA SAÚDE, 2011).

Vários fármacos são usados no tratamento da TB e a terapia adequada deve ter capacidade bactericida precoce, evitar a seleção de cepas mutantes e ter atividade esterilizante. Na Figura 5 são mostrados os principais alvos de ação dos fármacos de primeira linha na estrutura celular do *M. tuberculosis.*

Figura 5. Sítio de ação dos fármacos de primeira linha usados na tuberculose.



O tratamento da tuberculose através da quimioterapia antimicobacteriana é uma tarefa árdua. Além de ser uma terapia de longa duração, necessita da administração diária de múltiplas doses, uma vez que os fármacos de referência apresentam baixa permeabilidade, além de uma

toxicidade elevada (Tabela 2). Esses fatores associados aos diversos efeitos colaterais justificam a baixa adesão por parte dos pacientes aos esquemas terapêuticos utilizados, contribuindo assim para o desenvolvimento de cepas multirresistentes (KAUR et al., 2014).

Tabela 2. Fármacos de primeira linha utilizados na tuberculose, características físico-químicas e efeitos colaterais.

Fármacos	**Coeficiente de partição (LogP)**	**Solubilidade em água (mg/mL)**	**Efeitos colaterais**
Isoniazida (INH)	- 0,639	140	Hepatotoxicidade/Neuropatias
Rifampicin (RIF)	3.719	1.4	Hepatotoxicidade
Etambutol (EMB)	- 0,14	10	Hepatoxicidade/Toxicidade ocular
Estreptomicina (EST)	- 6,400	-	Ototoxicidade/nefrotoxicidade
Pirazinamida (PRZ)	- 1,884	15	Hepatotoxicidade

Fonte: Adaptado de KAUR et al., 2014.

Dependendo da resistência, as cepas de micobactérias podem ser classificadas em multirresistente (MDR) quando apresenta resistência pelo menos a isoniazida e a rifampicina (fármacos de primeira linha) e extensivamente resistente (TB-XDR) as que apresentam uma resistência adicional a fluoroquinolonas e a pelo menos um fármaco de segunda linha (Tabela 1) (DUBE; AGRAWAL; VYAS, 2012; KIDENYA et al., 2014).

Na ocorrência da tuberculose multirresistente (MDR) e extensivamente resistente (TB-XDR), a terapêutica adequada deve ser avaliada por profissionais experientes no manejo dos fármacos de segunda linha. Em geral, o esquema para multirresistência deve ser composto por, pelo menos, quatro fármacos com atividades efetivas que, preferencialmente, não tenham sido utilizados anteriormente. A esses princípios soma-se o Tratamento Diretamente Observado (TDO), no qual um profissional treinado passa a observar a tomada da medicação do paciente desde o início do tratamento até a sua cura, sendo uma estratégia fundamental para o sucesso da terapêutica (MINISTÉRIO DA SAÚDE, 2011).

Apesar da disponibilidade de tratamentos eficazes para TB, as taxas de cura permanecem baixas. Nesse sentindo, pesquisadores tem convergido esforços na tentativa de desenvolver novos medicamentos, melhorar a eficácia terapêutica e diminuir o tempo do tratamento desta doença.

Uma estratégia bastante inovadora e promissora é o desenvolvimento de nanocarreadores de fármacos associados a novos agentes antituberculostáticos, que podem ser direcionados às células fagocíticas infectadas por micobactérias, os quais podem exercer um papel fundamental no aumento da adesão do paciente a terapia com consequente aumento da incidência de cura (GARG et al., 2014; VYAS et al., 2004).

2.2. Nanotecnologia farmacêutica e sua utilização na terapia da tuberculose

Nos últimos anos, a nanotecnologia tem emergido como uma tecnologia altamente sofisticada e avançada. Com o tamanho em nanoescala os átomos, moléculas e macromoléculas apresentam propriedades físico-químicas exclusivas e significativamente melhores (com respeito à droga livre), o que torna possível tratar de forma mais eficaz doenças como tuberculose e AIDS (HAUCK et al.,2010).

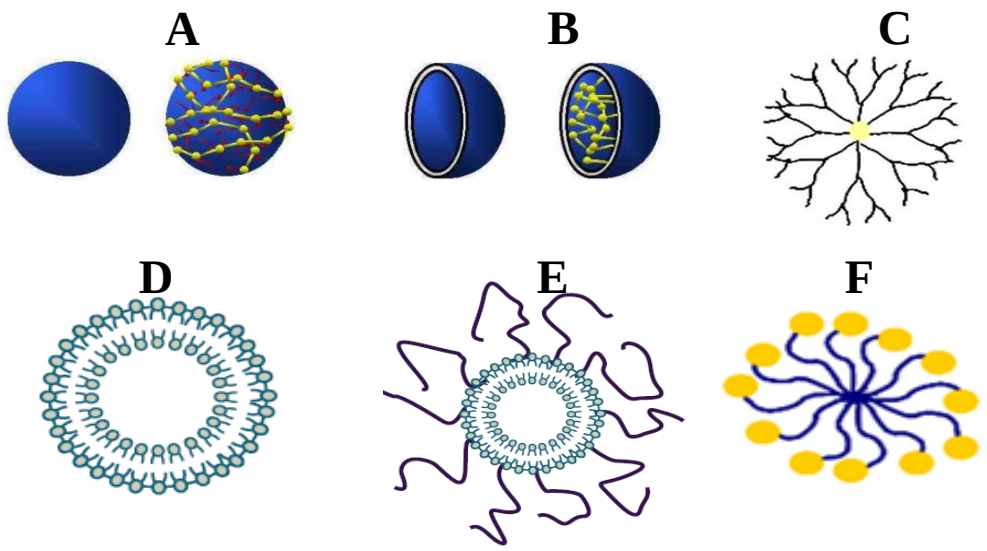
As falhas terapêuticas frequentes e o surgimento de cepas multirresistente, levaram os pesquisadores a desenvolverem novas formas de superar a resistência do mycobacterium aos medicamentos, restringir a duração do tratamento e diminuir os efeitos colaterais e interações medicamentosas existentes na terapêutica atual. Esses fatores, associado ao fato que nas últimas quatro décadas nenhum medicamento contra a TB foi lançado, sugerem a importância do desenvolvimento de sistemas nanoestruturados capazes de potencializar a ação dos fármacos antituberculostáticos (GARG et al., 2014; KAUR et al., 2014; HAUCK et al., 2010).

A farmacocinética e a farmacodinâmica de um fármaco são dependentes de suas características físicas e químicas e são influenciadas também pela forma farmacêutica utilizada. Ao utilizar fármacos em carreadores na escala nanométrica é possível melhorar e modular o seu desempenho quando comparados a sua utilização em formas farmacêuticas convencionais. Nesta faixa de tamanho, os pesquisadores podem manipular as propriedades ópticas, magnéticas e elétricas de nanoestruturas, alterando seu tamanho, forma ou composição atômica. Estudo de tais sistemas tem sido realizado ativamente com o propósito de direcionar e controlar a liberação de fármacos (SAKATA et al., 2007; ANDRADE et al., 2013).

A tecnologia associada à modificação da liberação de fármacos, ou de outras substâncias bioativas, a partir de preparações farmacêuticas sofreu um avanço notório nas últimas décadas na tentativa de maximizar as vantagens inerentes às formas farmacêuticas de liberação controlada (DAS; DAS, 2003). A figura 6 apresenta nanodispositivos com potenciais atividades biomédicas.

Dentre os principais dispositivos utilizados como sistemas de liberação controlada, destacam-se os sistemas poliméricos como as nanopartículas e as nanocápsulas. As nanocápsulas são constituídas por um invólucro polimérico disposto ao redor de um núcleo oleoso, podendo o fármaco estar dissolvido neste núcleo e/ou adsorvido à parede polimérica. Por outro lado, as nanoesferas, que não apresentam óleo em sua composição, são formadas por uma matriz polimérica, onde o fármaco pode ficar retido ou adsorvido. Os dendrímeros representam séries repetidas de compostos macromoleculares que formam uma matriz em seu interior. As micelas que são partículas coloidais formadas por copolímeros anfifílicos e os lipossomas são definidos como vesículas com núcleo aquoso envolvido por uma bicamada lipídica. Os lipossomas furtivos são revestidos com PEG que é um polímero hidrofílico que minimiza as interações das nanopartículas com proteínas do plasma (Loira-Pastoriza et al.,2014).

Figura 6. Representação esquemática de sistemas de liberação controlada: (A) nanopartícula, (B) nanocápsula, (C) dendrímeros, (D) lipossomas, (E) lipossomas peguilhado e (F) micelas

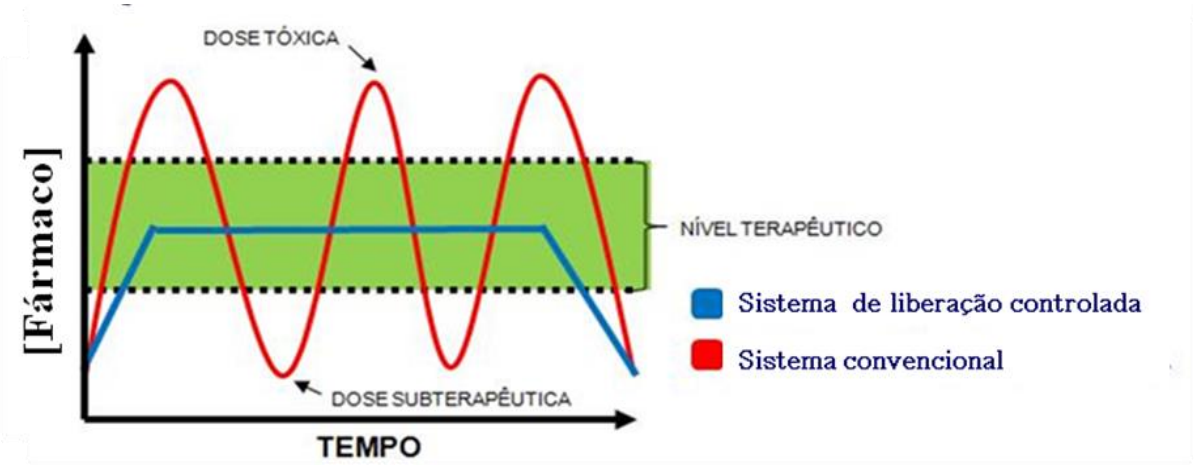


Fonte: Adaptado de Loira-Pastoriza et al., 2014.

Os sistemas de liberação controlada de fármacos são desenvolvidos para se obter uma concentração plasmática ou níveis de concentração tecidual de fármacos dentro da faixa terapêutica com velocidade de liberação controlada, atingindo o efeito terapêutico desejado e evitando possíveis reações tóxicas inerentes ao composto. Estes sistemas podem retardar a liberação do princípio ativo, sustentar a sua liberação e/ou direcioná-lo a sítios específicos de

ação (células, órgãos, micro-organismos). Na terapia medicamentosa utilizando formas farmacêuticas convencionais como comprimidos não-revestidos, soluções, suspensões, emulsões, cápsulas, etc.; torna-se difícil manter as concentrações plasmáticas de muitos fármacos em nível terapêutico por longo período de tempo, isto porque, essas apresentações liberam todo seu conteúdo de imediato gerando inicialmente um pico máximo de concentração plasmática que pode atingir níveis tóxicos, e logo após, uma concentração insuficiente de ação, ou seja, uma dose subterapêutica, o que proporciona flutuações aleatórias da biodisponibilidade do princípio ativo (Figura 7) (BOISSEAU; LOUBATON, 2011).

Figura 7. Farmacocinética de sistemas farmacêuticos convencionais e sistemas de liberação controlada de fármacos.



Fonte: Adaptado de GOONOO et al., 2014.

As principais vantagens dos sistemas de liberação controlada de fármacos são (TORCHILIN, 2005; ANDRADE et al., 2013):

- Proteger o conteúdo encapsulado;
- Possibilitar a incorporação tanto de substâncias hidrofílicas quanto lipofílicas;
- Promover a vetorização do fármaco;
- Promover liberação controlada do princípio ativo ao longo do tempo;
- Manter os níveis plasmáticos do fármaco em concentração constante dentro da faixa terapêutica;
- Reduzir o número de administrações do fármaco;
- Reduzir a toxicidade devido à menor liberação do princípio ativo em tecidos saudáveis;

- Melhorar a adesão do paciente à terapêutica.

Um sistema perfeito de liberação controlada de fármacos deve ser capaz de direcionar fármacos para o sítio alvo desejado, com a mínima exposição dos demais tecidos não desejados. Esse sistema de liberação de fármacos, por si só, deve ser farmacologicamente inativo, ter toxicidade mínima, ser prontamente metabolizado e depurado da circulação após ter exercido sua função. Além de ser confortável para o paciente, simples de se administrar e remover, fácil de fabricar e esterilizar (ZHOU et al., 2002).

2.2.2. Lipossomas

A história do sucesso dos lipossomas foi iniciada por Bangham e seus colegas em 1960, que observaram por microscopia que o esfregaço de lecitina de ovo em água formava estruturas muito complexas. Nos anos seguintes, os lipossomas foram utilizados principalmente como modelos de membranas artificiais que imitavam os sistemas celulares simples para a investigação das funções e mecanismos de transporte e propriedades de permeação (BANGHAM 1972).

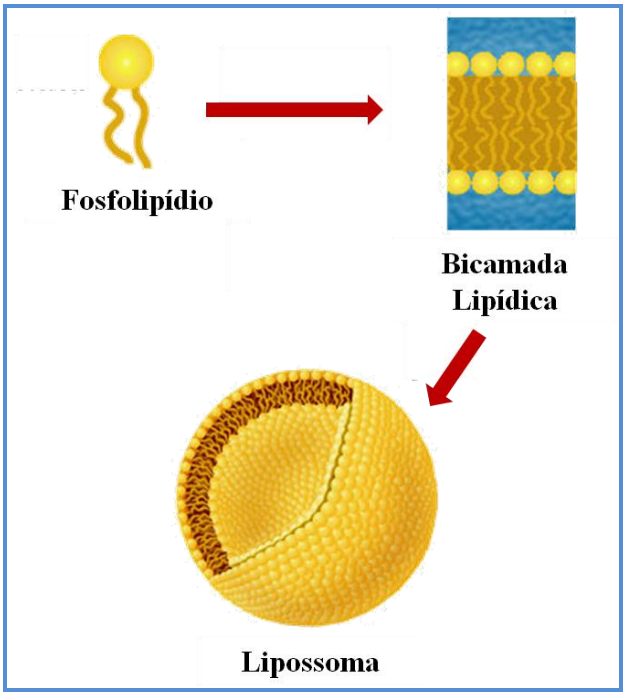
A partir dos estudos pioneiros de Bangham, os lipossomas passaram de simples objetos de pesquisa na área biofísica a carreadores terapêuticos para numerosas aplicações clínicas (TORCHILIN, 2005; FRÉZARD et al., 2005). Em 1971, Gregory Gregoriadis propôs pela primeira vez a utilização dos lipossomas como sistema transportador de fármacos, mantendo desde então um papel preponderante no desenvolvimento desta área (SANTOS; CASTANHO, 2002).

Lipossomas são vesículas esféricas formadas por uma ou mais bicamadas lipídicas que se organizam espontaneamente em meio aquoso (Figura 8) (BRANDL; GREGORIADIS, 1994). Eles despertam grande interesse devido a sua flexibilidade estrutural seja no tamanho, composição e fluidez da bicamada lipídica, seja na sua capacidade de incorporar uma variedade de compostos tanto hidrofílicos como hidrofóbico (VOINEA; SIMIONESCU, 2002; TYAGI et al 2012). Além disso, apresentam atrativas propriedades biológicas tais como: são biodegradáveis, biocompatíveis e não imunogênicos o que os tornam altamente versáteis para pesquisa, terapêutica e aplicações analíticas (BRANDL; GREGORIADIS, 1994; TORCHILIN, 2005; EDWARDS; BAEUMNER, 2006; DRULIS-KAWA et al., 2010).

A composição da bicamada lipídica é um fator preponderante na preparação dos lipossomas e na obtenção de formulações estáveis. Os lipossomas podem ser obtidos a partir de substância anfifílica formadora de fase lamelar (FRÉZARD et al., 2005), basicamente

constituídos por fosfolipídios e esteróis (VEMURI; RHODES, 1995). Os fosfolipídeos (Figura 9) são substâncias graxas de origem natural ou sintética, sendo os principais componentes das membranas celulares apresentando a capacidade de auto-organização quando em soluções. Portanto, os lipossomas são compostos por uma porção hidrofílica (cavidade interna aquosa) e outra hidrofóbica (bicamada lipídica) (TORCHILIN, 2006). Os fosfolipídeos mais utilizados nas formulações de lipossomas são os que apresentam forma cilíndrica como a fosfatidilcolina, fosfatidilserina, fosfatidilglicerol e esfingomielina, que tendem a formar uma bicamada estável em solução aquosa. As fosfatidilcolinas são as mais empregadas nas preparações lipossomais, pois apresentam grande estabilidade frente a variações de pH ou da concentração de sal no meio (BATISTA et al., 2007).

Figura 8. Esquema de formação dos lipossomas: estrutura do fosfolipídio, da bicamada lipídica e do lipossoma.



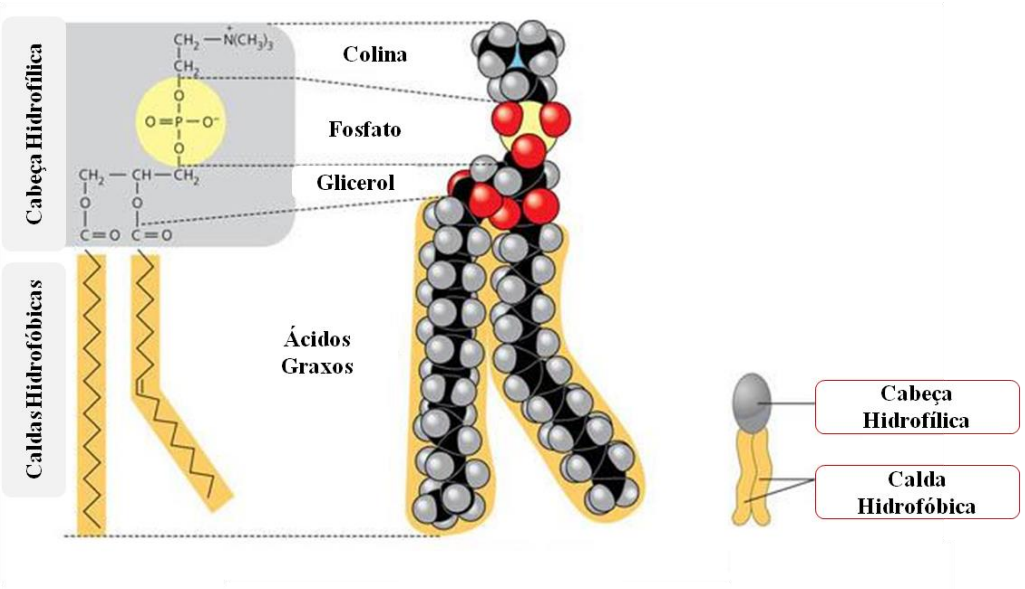
Fonte: Adaptado de GOMBOTZ 2013.

O colesterol é um componente lipídico importante uma vez que atua aumentando a rigidez das membranas no estado “cristal-líquido” e reduz a rigidez e os defeitos estruturais das membranas no estado “gel” (FRÉZARD et al., 2005). Mesmo incapaz de se organizar em bicamadas, esse lipídio pode ser incorporado em membranas de fosfolipídios para modular a fluidez da membrana fosfolipídica, reduzindo a permeabilidade da bicamada e melhorando a

estabilidade da membrana em fluidos biológicos. Além disso, O colesterol também melhora a estabilidade da vesícula na presença de fluidos biológicos como o sangue, através da redução da interação das proteínas plasmáticas com as membranas dos lipossomas, diminuindo assim a desestrututuração dos mesmos (VEMURI; RHODES, 1995; KIM, 2007).

Algumas preparações também possuem lipídios apresentando carga efetiva negativa, por exemplo, fosfatidilserina e fosfatidilglicerol, ou positiva, como a estearilamina. A presença de cargas nos lipossomas pode influenciar a taxa de incorporação de substâncias, impedir a agregação/fusão das vesículas lipídicas por repulsão eletrostática e modular o seu destino no organismo, por exemplo, lipossomas carregados negativamente são mais capturados pelo baço do que os carregados positivamente (BANERJEE, 2001; FRÉZARD et al., 2005) .

Figura 9. Estrutura do fosfolipídio.



Fonte: Adaptado de GOMBOTZ 2013.

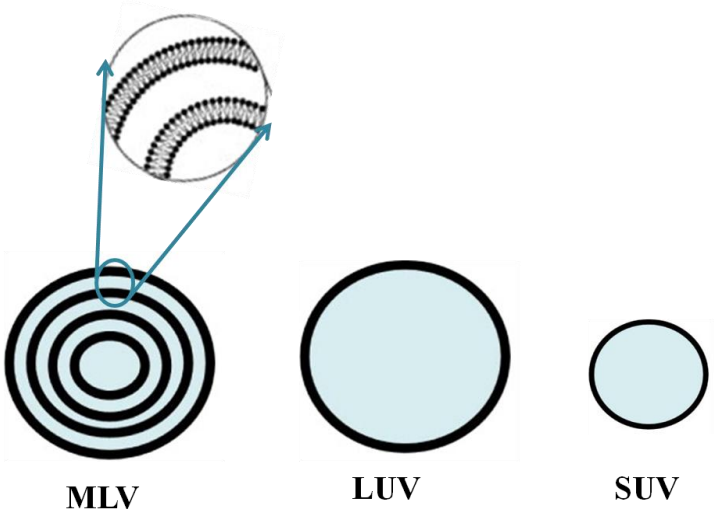
2.2.2. Classificação dos Lipossomas

Os lipossomas são sistemas altamente versáteis, cujo tamanho, número de lamelas e composição química dos lipossomas podem ser modificado em função dos requisitos farmacêuticos e farmacológicos (FRÉZARD et al, 2005).

Os lipossomas podem conter uma única bicamada lipídica ou bicamadas múltiplas em torno do compartimento aquoso interno e, portanto, são classificados em unilamelares e

multilamelares, respectivamente. As vesículas multilamelares (*MLV- multilamellar vesicles*) possuem um tamanho que varia de 500 a 5000 nm. Já as vesículas unilamelares podem ser pequenas ou grandes. Lipossomas unilamelares pequenos (*SUV- small unilamellar vesicles*) tem o tamanho em torno de 100 nm e os lipossomas unilamelares grandes (*LUV- large unilamellar vesicle*) podem ter o diâmetro variando entre 200 e 800 nm (Figura 10) (ŠEGOTA; TEŽAK, 2006; BATISTA; CARVALHO; SANTOS-MAGALHÃES, 2007).

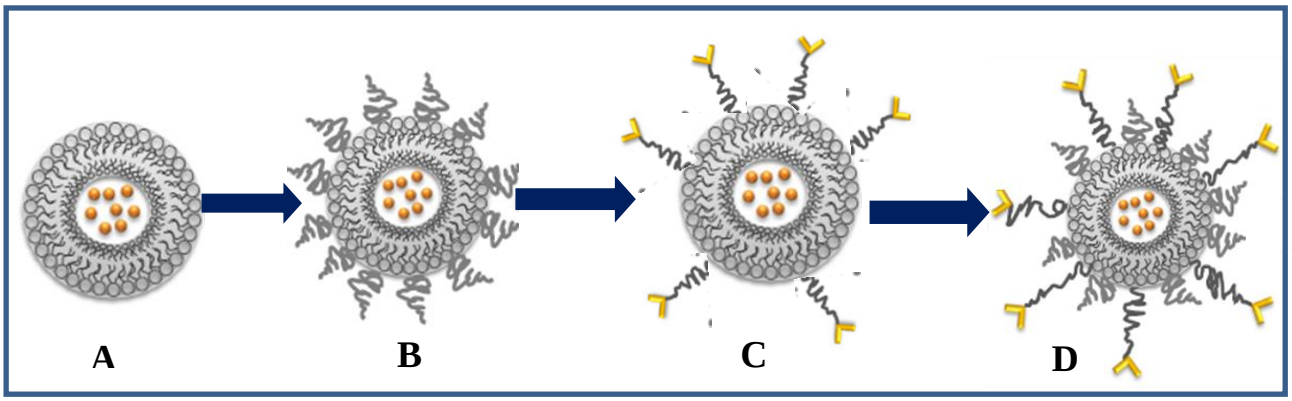
Figura 10. Classificação dos lipossomas quanto ao tamanho e número de lamelas.



Fonte: Adaptado de KHOSRAVI-DARANI et al., 2013. Vesículas multilamelares (MLV); vesículas unilamelares grande (LUV) e vesículas unilamelares pequena (SUV).

Na evolução de seu emprego como carreadores de fármacos, algumas alterações foram realizadas na estrutura básica dos lipossomas possibilitando uma maior aplicação terapêutica. De acordo com a composição química, os lipossomas podem ser classificados em convencionais, furtivos e sítio-específicos (TORCHILIN et al., 2005) (Figura 11).

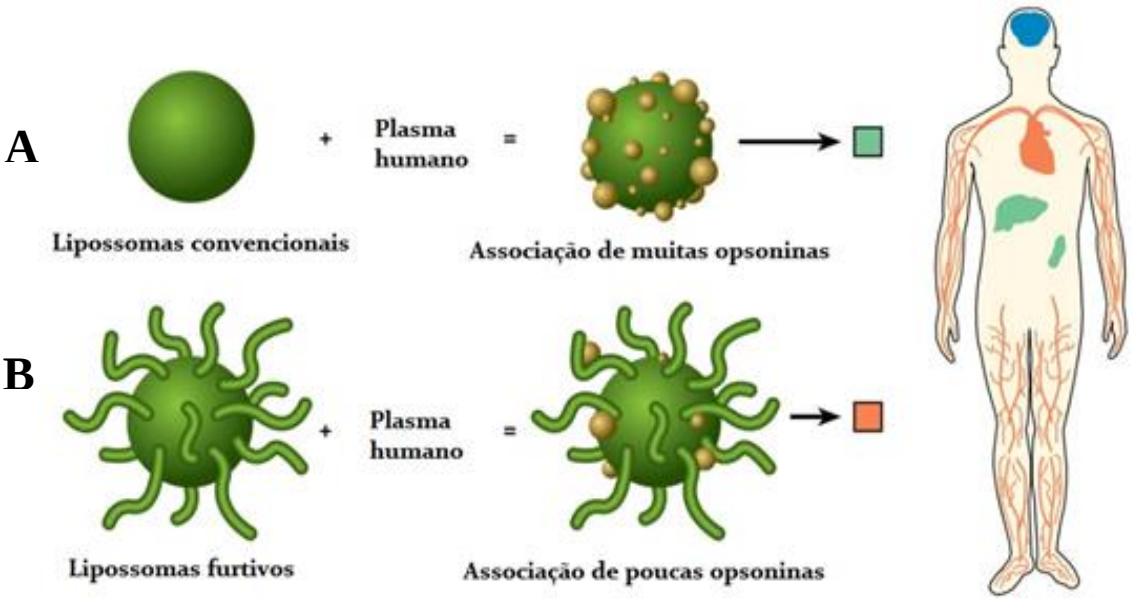
Figura 11. Classificação dos lipossomas quanto à constituição: **A-** Lipossomas convencionais. **B-** Lipossomas furtivos. **C-** Lipossomas sítio-específicos. **D-** Lipossomas direcionados de longa circulação.



Fonte: Adaptado de KOSHKARYEV et al., 2013.

Lipossomas convencionais são compostos de fosfolipídeos e colesterol, além de um lipídeo com carga negativa ou positiva para evitar a agregação das vesículas, aumentando a estabilidade em suspensão. *In vivo* os lipossomas convencionais são rapidamente removidos da circulação devido à adsorção das opsoninas na superfície desses nanocarreadores, desencadeando o seu reconhecimento e captura pelo sistema fagocitário mononuclear. Essa captura está diretamente relacionada ao tamanho das vesículas e sua superfície hidrofóbica, o que resulta em uma curta permanência desses lipossomas na corrente sanguínea após o contato com componentes do sangue (Figura 12 A) (VEMURI; RHODES, 1995; PINTO-ALPHANDARY et al., 2000; BATISTA et al., 2007). Lipossomas do tipo LUV e MLV são rapidamente reconhecidos e eliminados da circulação, por outro lado, os SUV podem permanecer por mais tempo na circulação. Portanto, lipossomas com diâmetro maior que 100 nm necessitam de estratégias adicionais para prevenir a opsonização (KOO; RUBINSTEIN; ONYUKSEL, 2005; IMMORDINO; DOSIO; CATTEL, 2006). Esse problema levou ao desenvolvimento de lipossomas de longa duração na circulação sanguínea, conhecidos como furtivos ou *stealth liposomes* (TORCHILIN, 2005).

Figura 12. Processo de opsonização de lipossomas convencionais (A) e furtivos (B).



Fonte: modificado de AGGARWAL et al., 2009.

Os lipossomas de longa circulação são obtidos pelo revestimento da superfície da bicamada lipídica com componentes hidrofílicos naturais, como o monossialogangliosídeo GM1, fosfatidilinositol e polissacarídeos, ou com polímeros hidrofílicos sintéticos inertes e biocompatíveis, especificamente os polietilenoglicóis. A característica mais importante dos lipossomas furtivos é a prevenção da opsonização e consequente diminuição da captura pelas células do sistema fagocitário mononuclear, o que aumenta a sua biodisponibilidade na corrente sanguínea (Figura 12 B). Além disso, os lipossomas de longa duração também tem a propriedade de serem direcionados passivamente para alguns tecidos e órgãos patológicos, ou seja, para os que possuem maior vascularização (como tumores e inflamações), acumulando-se nestes locais (TORCHILIN, 2009; IMMORDINO; DOSIO; CATTEL, 2006).

O mecanismo de prevenção da opsonização dos lipossomas furtivos é denominado estabilização estérica. Ele é resultante da blindagem da superfície lipossomal pelo polímero hidrofílico, que ocasiona uma maior repulsividade entre a superfície da partícula e os componentes do sangue. O polímero de revestimento ideal tem que apresentar uma excelente solubilidade em soluções aquosas, uma alta flexibilidade na sua cadeia principal e ser biocompatível e biodegradável (TORCHILIN, 2009). Outro avanço relacionado às modificações estruturais dos lipossomas trata-se de uma combinação entre longa circulação e a capacidade de

direcionamento a local específico, esses lipossomas são conhecidos como sistemas direcionados de longa circulação. Eles são obtidos através do acoplamento de ligantes específicos a superfície das vesículas conferindo seletividade para distribuir o fármaco encapsulado no sítio de ação desejado (tecidos e órgãos alvos), mesmo quando há uma diminuição do fluxo sanguíneo no órgão ou mesmo quando a expressão do sítio de reconhecimento da molécula sinalizadora é baixa (TORCHILIN, 2005, 2006). Alguns exemplos de ligantes de reconhecimento são anticorpos, proteínas, peptídeo, polissacarídeos e outras moléculas, tais como, folato e transferrina (SAPRA; ALLEN, 2003; TORCHILIN, 2009). Essas moléculas ficam acopladas na superfície dos lipossomas, sem comprometer a integridade da membrana e as propriedades do ligante, através de ligação covalente ou pela inserção na membrana lipídica após serem modificadas com resíduos hidrofóbicos.

2.2.1. Lipossomas como sistemas de liberação para fármacos tuberculostáticos

O aumento da frequência de cepas MDR como resultado de um regime posológico irregular, associado a uma deficiência na obtenção de um diagnóstico rápido e eficiente para a TB, revela a necessidade imediata pela busca de novas estratégias que melhorem a eficácia terapêutica desta patologia. Além disso, apesar do surgimento de novos antibióticos, o tratamento de infecções intracelulares ainda continua difícil, uma vez que os patógenos estão localizados no interior das células fagocíticas e boa parte dos antibióticos, embora altamente ativos, não conseguem atravessar as membranas celulares e, portanto não atingem a faixa terapêutica desejada no interior das células infectadas (BRIONES et al., 2008; DUBE et al., 2012; KAUR et al., 2014).

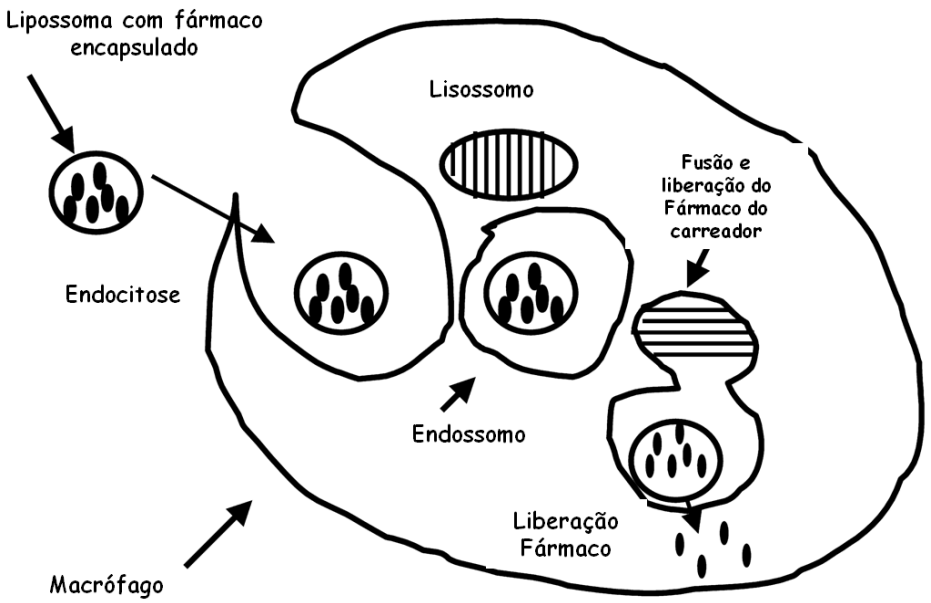
O principal desafio para a quimioterapia intracelular consiste em desenvolver um carreador para antibióticos que possa ser eficientemente fagocitado e uma vez dentro das células prolongue a liberação do fármaco, de modo a que o número de doses e a toxicidade seja reduzida (BRIONES et al., 2008). Neste contexto, a nanotecnologia farmacêutica, em especial os lipossomas, surge como uma ferramenta destinada a melhorar a eficiência e reduzir a toxicidade associada à farmacoterapia da tuberculose.

A utilização de lipossomas como sistemas de liberação controlada no tratamento de várias patologias tem sido explorado extensivamente por mais de 20 anos (AGRAWAL; GUPTA, 2000). Considerando os mecanismos de resistência bacteriana, o desenvolvimento de sistemas coloidais de liberação controlada de fármacos, como os lipossomas, para o carreamento de

antibióticos, poderia aumentar sua atuação em cepas não resistentes e superar essa resistência, no caso de cepas resistentes. Lipossomas e bactérias podem interagir diretamente por processos de fusão, levando à liberação do antibiótico encapsulado diretamente ao alvo bacteriano (SACHETELLI et al., 2000).

Os lipossomas são considerados um dos sistemas mais antigos e mais simples de carreamento de antibióticos para o tratamento de infecções intracelulares como a tuberculose. A interação dos lipossomas com os macrófagos ocorre em quatro etapas: 1) as partículas se ligam à membrana dos macrófagos pelo reconhecimento das moléculas presente na superfície das partículas, ou através de forças eletrostáticas; (2) transferência de sinal para ativação dos receptores; (3) englobamento das partículas pela membrana, formando um endossomo; (4) degradação lisossomal do endossomo com liberação do fármaco (Figura 13) (AHSAN et al., 2002; GEISER, 2010). Entretanto, a ligação dos lipossomas e sua subsequente ingestão pelos macrófagos dependem de um número de fatores relacionados aos lipossomas, como composição, tamanho e carga de superfície. Em geral, lipossomas pequenos (0,05 – 0,1µm) e carregados positivo ou negativamente são capturados de forma mais eficiente em relação as sua contrapartida (SCHWENDENER et al., 1984; WANG et al., 2014).

Figura 13. Fagocitose dos lipossomas pelo macrófago.



Fonte: Adaptado de ASHAN et al., 2002.

A utilização de lipossomas na infecção por *M. tuberculosis* tem sido descrita há décadas e suas modificações estruturais vêm potencializando a atividade dos fármacos tuberculostático. Além disso, novos agentes de origem natural ou sintética vêm sendo testado frente ao *M. tuberculosis*, a fim de se descobrir novas classes de compostos estruturais capazes de substituir ou complementar os medicamentos estabelecidos na terapia da TB (DUBE et al., 2012).

Vladimirsky e Ladigina (1982) realizaram um trabalho pioneiro, onde eles compararam uma injeção intravenosa de estreptomicina livre e encapsulada em lipossomas convencionais em camundongos infectados com o Mtb. Nesse experimento foi possível observar uma maior sobrevida dos animais tratados com a dispersão lipossomal e uma diminuição da toxicidade aguda. Klemens e colaboradores (1990) mostraram que a gentamicina encapsulada em vesículas lipossomais foi capaz de reduzir significantemente o Mtb no fígado e no baço dos camundongos, relatando, então, uma diminuição dose-dependente da carga bacteriana. Resultados semelhantes a estes foram obtidos em outras pesquisas com lipossomas e fármacos antituberculostáticos de segunda linha (DUZGUNES et al., 1996; LEITZKE et al., 1998).

Labana e colaboradores (2002) administraram intravenosamente lipossomas contendo INH e RIF semanalmente por seis semanas e conseguiram reduzir significativamente a carga micobacteriana nos pulmões, fígado e baço de ratos infectados em comparação com animais tratados com a droga livre. Em pesquisas mais recentes, lipossomas contendo a pirizamida e a rifabutina foram produzidos e também mostraram uma alta eficácia terapêutica quando comparados ao fármaco livre, com redução significativa da carga bacteriana no fígado, baço e pulmão (EL-RIDY et al., 2007; GASPAR et al., 2008).

As referências descritas anteriormente tiveram em comum o desenvolvimento de lipossomas convencionais. Sabe-se, porém, que este tipo de vesícula é rapidamente reconhecido pelo sistema fagocitário mononuclear e, principalmente, captado por macrófagos e encaminhado ao baço e fígado, o que justifica apenas a redução da carga micobacteriana ao invés de uma total eliminação do patógeno.

Neste contexto, modificações na superfície dos lipossomas, que proporcionam o aumento do tempo de circulação na corrente sanguínea e os direcionam especificamente para os macrófagos pulmonares vêm sendo desenvolvidas. Estes novos sistemas ainda permitem ser administrados pela via intravenosa e pela via respiratória (VYAS et al., 2000; PANDEY; KHULLER, 2006; CHONO et al., 2007).

Deol e colaboradores (1997) produziram lipossomas furtivos e sítio-específicos, revestidos com PEG e tendo como molécula sinalizadora a O-esteroil amilopectina (O-SAP), para o carreamento de izoniazida e rifampicina. Eles observaram que, dentro de 30 min, o acúmulo dos nanocarreadores nos pulmões foi de 31% dos lipossomas modificados e apenas 5,1% dos convencionais. Adicionalmente, uma significante diminuição dos efeitos tóxicos dos antibióticos foi observada devido a sua encapsulação. Além disso, a administração desses lipossomas direcionados e de longa duração, mesmo em dose sub-terapêutica, levou a uma alta diminuição de UFC, quando comparado à ação dos fármacos livres e em doses terapêuticas.

Em estudos clínicos de fase II realizados por Whitehead e colaboradores (1998) foi possível observar que MiKasome® (Lipossomas contendo amicacina) foi bem mais tolerado que o fármaco livre, uma vez que houve uma acumulação 30 vezes maior do MiKasome® no escarro em comparação com o fármaco livre. Além disso, alterações auditivas ou renais significativas não foram observadas. No entanto, apesar da melhoria na farmacocinética do antibiótico na forma lipossomal,o *M. tuberculosis* não foi erradicado totalmente e nenhuma sensibilidade à droga foi detectada, em contraste com o fármaco livre que teve o MIC aumentado de 2 para 8 mg/L.

Por sua vez, Vyas e colaboradores (2004) desenvolveram sistemas micrométricos lipossomais contendo rifampicina para administração por nebulização (aerossol). Moléculas de O-SPA (O-esteroil amilopectina) e albumina metilada do soro bovino (MBSA) foram ancoradas na superfície das vesículas com a intenção de aumentar a seletividade para os pulmões. As partículas administradas apresentaram tamanho de partícula de 3.85±0.59 µM e 3.64±0.65 µM e potencial zeta de -7 e -5 mV, respectivamente. A O-SPA é reconhecida pelos receptores do tipo *scavenger* dos macrófagos e a MBSA apresenta afinidade pelos macrófagos alveolares. Os sistemas lipossomais sítio-específicos mostraram uma maior diminuição da viabilidade do patógeno (7-11%) quando comparados com o fármaco livre (45,7%) e lipossomas convencionais (31,6%). Houve preferencialmente um acúmulo dos lipossomas direcionados nos macrófagos alveolares, porém os sistemas revestidos com O-SPA, por terem sido mais captados pelos fagócitos, foram mais eficazes do que o com MBSA.

Lipossomas revestidos com manose também apresentaram *in vitro* uma pronunciada captação pelos macrófagos quando comparados com lipossomas sem revestimento. Além disso, após administração pulmonar desses dois sistemas, constatou-se um maior acúmulo dos lipossomas modificados nos pulmões dos ratos (CHONO et al., 2007). Nesta mesma abordagem, Wijagkanalan e colaboradores (2008) estudaram *in vitro* e *in vivo* a influência da concentração de

manose na superfície lipossomal em relação à captura feita pelos macrófagos. Constatou-se que quanto maior a concentração de manose maior seria o acúmulo nas células fagocíticas. Além disso, observou-se que a captação dos lipossomas manosilados foi inibida quando se acrescentou excesso de manana solubilizada no meio, mostrando que a interação carreador-receptor é específica. E, adicionalmente, após administração intratraqueal, esses sistemas demostraram maior afinidade pelos macrófagos alveolares do que pelos epiteliais.

Outra classe de polissacarídeo que vem recebendo destaque no desenvolvimento de sistemas de liberação controlada de fármacos são os sulfatos (4-sulfato N-acetil galactosamina e a fucana). Sua utilização tem sido descrita em sistemas de liberação, como fucoesferas (SEZER et al., 2008); nanopartículas (LIRA et al., 2011; LEE et al., 2013); nanocápsulas (PINHEIRO et al., 2015) e lipossomas (SINGODIA et al., 2012).

Em 2011, Lira et al. desenvolveram nanopartículas de poli-butilcianoacrilato revestidas com fucana e avaliaram a citotoxicidade *in vitro* e a captura celular desses nanossistemas. Eles observaram que estas partículas apresentaram baixa toxicidade celular e foram englobadas preferencialmente por macrófagos J774 em relação às células fibroblásticas. Sugerindo que a fucana incorporada na superfície das nanopartículas pode promover uma interação da nanopartícula com os macrófagos através do reconhecimento específico deste polissacarídeo aos receptores de manose expresso nos macrófagos.

Por sua vez, Singodia e colaboradores (2012) desenvolveram dois ligantes diferentes, o palmitoil manose (Man-Lip) e o 4-sulfato N-acetil galactosamina (Sulf-lip) para direcionar lipossomas contendo anfotericina B a macrófagos residentes. Os estudos *in vitro* e *in vivo* forneceram evidências de que o Suf-Lip apresentou um maior direcionamento aos macrófagos residentes em comparação ao Man-Lip, sendo uma alternativa em potencial no tratamento de patologias intracelulares como tuberculose e leshimaniose.

Portanto, o direcionamento aos receptores de macrófagos através da utilização de polissacarídeos sulfatados tem sido explorado por vários grupos de pesquisa como descrito acima. Diante disso, o desenvolvimento de lipossomas sítio-específicos revestidos pela fucana podem fornecer uma nova estratégia no direcionamento aos macrófagos infectados com o *M. tuberculosis* potencializando a terapia da tuberculose.

2.3. Fucana

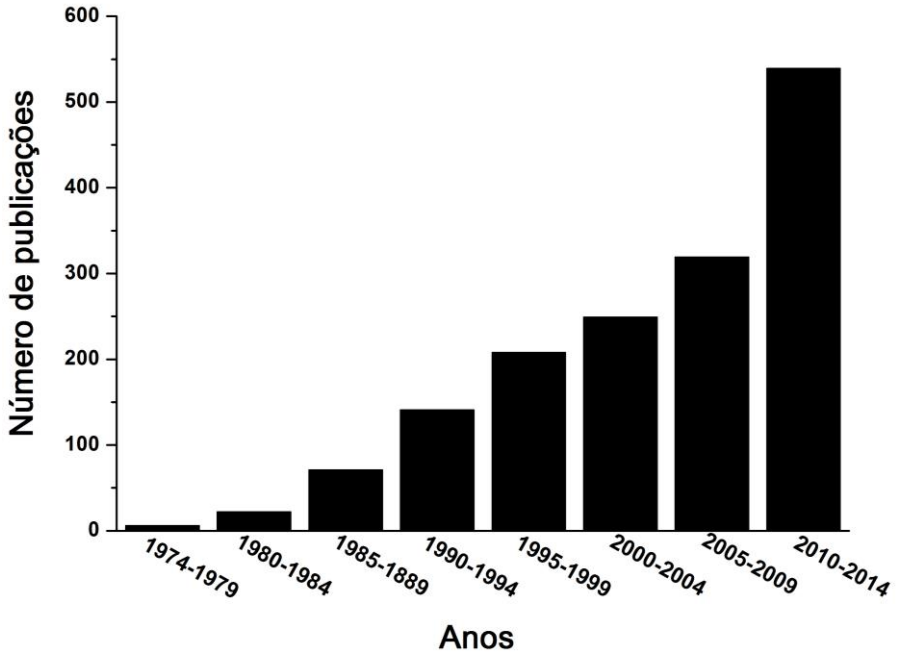
Os oceanos são particularmente ricos em biodiversidade, cobrindo mais de 70% da superfície da Terra. O ambiente marinho contém uma variedade de plantas, animais e microrganismos, que oferecem uma grande diversidade de produtos naturais (COSTANTINO et al., 2004). Diante da incapacidade para curar muitas doenças, as fontes naturais tem sido usada como base para o desenvolvimento de novos medicamentos. Ao longo dos anos, polissacarídeos sulfatados com potencial farmacológico e nutracêutica tem sido isolados a partir de algas marrons (Phaeophyceae) e utilizados no desenvolvimento de novas formas farmacêuticas (HOLDT et al., 2011; WIJESINGHE; JEON, 2012).

A fucana é um tipo complexo de polissacarídeo sulfatado (JIAO et al., 2011) encontrado principalmente em várias espécies de algas marrons como, por exemplo, *Fucus vesiculosus* (VEENA et al., 2007), *Fucus serratus* (BILAN et al., 2006), *Laminaria japonica* (WANG et al., 2008), *Sargassum cymosum* (PARDO; SOLÉ, 2007) e *Stoechospermum marginatum* (LI et al., 2008) e alguns invertebrados marinhos, como o ouriço-do-mar e o pepino-do-mar (HOLTKAMP et al., 2009).

A fucana foi isolada pela primeira vez em 1913 por Kylin, sendo denominada de Fucoidina. Atualmente, de acordo com a IUPAC, ela é nomeada de fucoidana, contudo também é conhecida como fucana, fucosana e fucana sulfatada (BERTEAU, 2003; LI et al., 2008). Nos últimos anos, a fucana tem despertado grande interesse científico, fato este comprovado pelo crescimento progressivo no quantitativo de publicações, totalizando um número aproximado de 539 entre os anos de 2010 e 2014 (Figura 14).

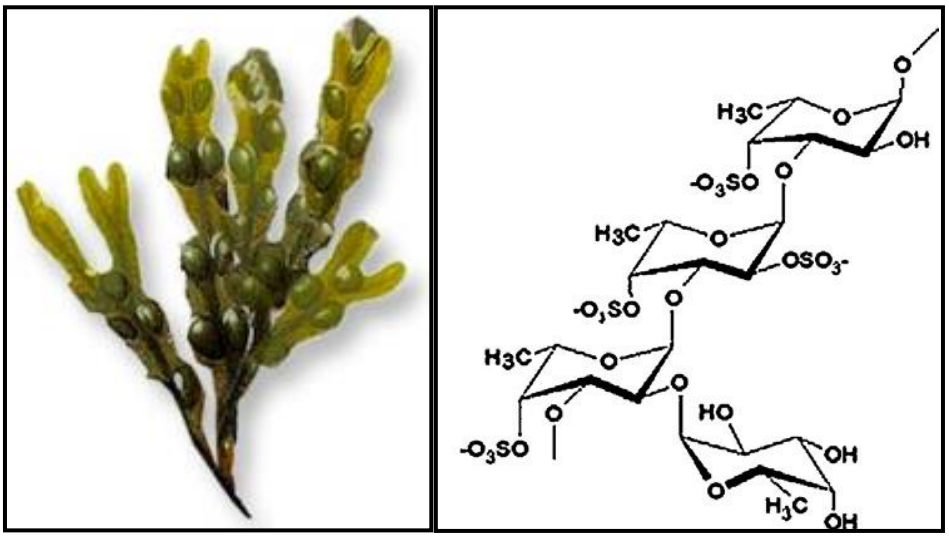
A estrutura química da fucana de muitas espécies de algas marrons, por exemplo, *Fucus vesiculosus*, em geral é bem simples (Figura 15), sendo composta principalmente de L-fucose (12,6 a 36,0%) e grupos sulfatos (8 a 25%), que conferem carga negativa a essa molécula. Outros monossacarídeos (manose, galactose, glicose, xilose) e ácidos glucurônicos também podem fazer parte em menor quantidade. Além disso, a estrutura, a massa molecular e a quantidade de grupos sulfatos desta molécula variam de espécie para espécie e sofrem influência dos métodos de extração e purificação utilizados (WIJESINGHE; JEON, 2012; SENTHILKUMAR et al., 2013).

Figura 14. Número de citações científicas sobre a fucana no decorrer dos anos, baseando-se na busca no banco de pesquisa científica internacional, Scopus (www.scopus.com) e tendo como palavra-chave: *Fucoidan*.



Dentre todas as espécies, a fucana extraída da *F. vesiculosus* é a que tem sido mais investigada, cuja estrutura está elucidada. Sabe-se que ela é composta basicamente por fucose, grupos sulfatos e traços de aminoglicose (LI et al., 2008). Em 1993, Pantankar e colaboradores descreveram sua forma estrutural. Eles sugeriram que, na cadeia principal, as fucoses são associadas por ligações glicosídicas do tipo α- 1,3 e a sulfatação pode ocorrer no C-2 ou C-3 e C-4 da L-fucopiranose. Além disso, às vezes, ramificações na cadeia linear acontecem a cada dois ou três resíduos de fucose através de ligações α- 1,2 e α- 1,4 (LI et al., 2008).

Figura 15. *Fucus vesiculosus* ***(A)*** e estrutura química do polissacarídeo sulfatado (B).



Fonte: Adaptado de SENTHILKUMAR et al., 2013.

A literatura relata várias atividades biológicas para a fucana: anticoagulante (WIJESINGHE et al., 2011), antitrombótica (CUMASHI et al., 2007), antioxidante (SOUZA, et al., 2007; WANG et al., 2008), antiviral (MANDAL, et al., 2007; HAYASHI et al., 2008), antiinflamatória (CUMASHI et al., 2007; KANG et al., 2011), antiangiogênica (CUMASHI et al., 2007), antitumoral (ALEKSEYENKO et al., 2007; TERUYA et al., 2007), antiproliferativa (MARUYAMA et al., 2003, 2006), imunomoduladora (KIM; JOO, 2008; TERUYA et al., 2009), cardioprotetora (THOMES et al., 2010), gastroprotetora (RAGHAVENDRAN.; SRINIWASAN; REKHA., 2011), hipolipidêmica (LI et al., 2001), renoprotetora (VEENA et al., 2006, 2007, 2008) e hepatoprotetora (HAYASHI et al., 2008). Além disso, possui a propriedade de inibir a atividade do sistema complemento (ZVYAGINTSEVA et al., 2000; TISSOT; DANIEL, 2003), a capacidade de interagir com receptores de macrófagos do tipo s*canvenger receptors* (SR) (KIM; ORDIJA; FREEMAN, 2003), a habilidade de seqüestrar metais pesados tóxicos como Cd^{2+}, Cu^{2+}, Zn^{2+}, Pb^{2+}, Cr^{3+} e Hg^{2+} (DAVIS; VOLESKYA; MUCCI, 2003) e pode ser utilizada como suporte para imobilização de antibióticos (ARAÚJO et al., 2004).

Duas propriedades biológicas da fucana são bastante interessantes e apropriadas para o desenvolvimento de nanocarreadores e, por isso, devem ser detalhadas. A primeira corresponde ao fato da fucana não ativar o sistema complemento, que é um importante componente do sistema imunológico e está envolvido tanto na resposta inata quanto na humoral. O sistema complemento

é o principal mediador do processo inflamatório junto aos anticorpos, sendo constituído por proteínas solúveis no plasma ou encontradas em membranas celulares. Ele está essencialmente envolvido na opsonização, no reconhecimento e na eliminação de corpos estranhos ao organismo, incluindo os nanodispositivos (MOGHIMI et al., 2011). Assim, o desenvolvimento de novos sistemas de liberação controlada tem se focalizado na busca por moléculas que sejam capazes de revestir os nanodispositivos, aumentando, então, o seu tempo de circulação na corrente sanguínea. Portanto, polímeros naturais, como a fucana, que sejam biocompatíveis, biodegradáveis, hidrofílicos, com flexibilidade na cadeia principal e não ativadores do sistema complemento são ideais para este fim.

Há quase duas décadas, a fucana vem sendo estudada como inibidor do sistema complemento e as observações iniciais mostraram que as frações da fucana de *Ascophylum nodosum* inibiram fortemente as vias clássica e alternativa de ativação do complemento (BLONDIN et al., 1996). Em seus estudos Tissot e colaboradores estudaram extensivamente essa atividade e determinaram que as fracções de baixo peso molecular das fucanas se ligam à subunidade C1q do complexo C1, que desencadeia o complemento através do reconhecimento e ligação de complexos imunológicos (TISSOT et al., 2003; CLEMENT et al., 2010). Essa ligação da fucana parece interferir com a capacidade de C1q para desencadear a ativação total de C1. Polissacarídeos sulfatados também podem se ligar a subunidade C4, evitando assim a sua degradação e a geração do seu produto de clivagem (C4b), necessário para a propagação da cascata de reações de ativação do complemento (TISSOT et al., 2003b; TISSOT et al.,2005).

Outra propriedade de grande interesse é a capacidade da fucana se ligar aos *scavenger receptors* (SR), de classe A tipos I e II, encontrados na superfície de macrófagos (KIM; ORDIJA; FREEMAN, 2003). Esses receptores são glicoproteínas triméricas e integrais de membrana, que promovem a internalização do ligante ao reconhecê-lo.

2.4. Ácido úsnico

Nos últimos anos, a comunidade científica tem despertado o interesse pela descoberta de novas compostos com aplicações farmacológicas, seja oriundas de fontes naturais ou provenientes de modificações químicas a partir de moléculas preexistentes. Tendo em vista a vasta biodiversidade, essas moléculas bioativas podem ser extraídas de animais, vegetais, algas, bactérias, fungos, dentre outros. Dentre esses organismos, os líquens chamam atenção devido a sua ampla distribuição geográfica, podendo ser encontrado sobre rochas, solos e troncos de

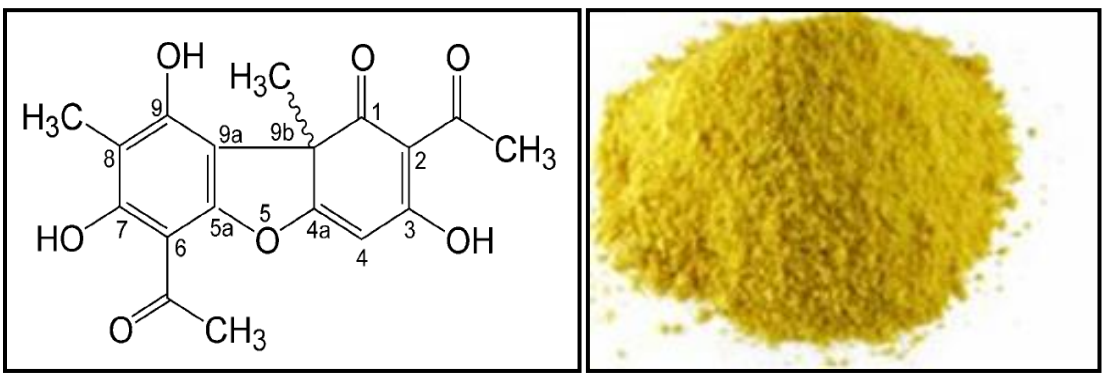
árvores. A atenção dada a estes organismos é devido a sua habilidade de produzir substâncias atrativas, tais como os metabólitos secundários sintetizados pelas hifas do fungo, os quais são utilizados como mecanismo de defesa e proteção contra intempéries (MÜLLER, 2001).

Dos diversos metabólitos secundários de líquens, o ácido úsnico (AU), um dibenzofurano de baixo peso molecular (2,6-diacetil-7,9-dihidroxi-8-9b-dimetil-1,3(2H,9bH)-dibenzofurandiona; $C_{18}H_{16}O_{7;}$ PM = 344,3 g/mol) e pigmentação amarelada, é sem dúvida o mais extensivamente estudado (Figura 15). O AU ocorre na natureza em duas formas enatioméricas (-) e (+) decorrente da projeção angular do grupamento metil localizada na posição 9b (COCCHIETTO et al., 2002; INGÓLFSDÓTTIR, 2002). Os enantiômeros do AU podem apresentar atividades biológicas e mecanismos de ação distintos (COCCHIETTO et al, 2002; INGÓLFSDÓTTIR, 2002).

O ácido úsnico apresenta caráter hidrofóbico, sendo praticamente insolúvel em água (10 mg/100 mL a 25 ^{o}C); solúvel em solventes orgânicos, como acetona, benzeno, metanol, clorofórmio, acetato de etila e diclorometano (INDEX MERCK, 1995); e possui um ponto de fusão é em torno de 204 ºC (KRISTMUNDSDÓTTIR et al., 2002). A lipofilia do AU pode ser explicada pela presença dos três grupos cetônicos, bem como ao anel furano que une os anéis aromáticos (ASAHINA & SHIBATA, 1954), além de pontes de hidrogênio intramoleculares (MÜLLER, 2001). Sua acidez é justificada pela presença do anel fenólico, cuja estrutura é instável (SHIBATA, 2000).

Este composto é produzido por várias espécies liquênicas, tais como *Cladonia* (Cladoniaceae), *Usnea* (Usneaceae), *Lecanora* (Lecanoraceae), *Ramalina* (Ramalinaceae), *Parmelia* (Parmeliaceae) e *Alectoria* (Alectoriaceae) (INGÓLFSDÓTTIR, 2002).

Figura 16. Ácido úsnico: (A) Estrutura química; (B) aspecto macroscópico em forma de pó com coloração amarelada.



Segundo Ingólfsdóttir e colaboradores (1985), o interesse terapêutico do ácido úsnico foi despertado após a utilização dos liquens na medicina popular, desde a antiguidade, no combate da tuberculose pulmonar e algumas doenças de pele. Posteriormente, outras atividades biológicas lhe foram atribuídas, tais como: antimicrobiana (LAUTERWEIN et al., 1995), antiinflamatória, analgésica, antipirética (YAMAMOTO et al., 1995), antifúngica (BROSKA et al., 1996), antiparasitária (FOURNET et al., 1997), antitumoral (KUMAR & MÜLLER, 1999), antiviral (CAMPANELLA et al., 2002), de inibição enzimática (HUNECK, 1999) e gastroprotetora (ODABASOGLU et al., 2006).

Dentre as atividades farmacológicas do AU, a atividade antimicrobiana merece destaque. As investigações sobre a atividade de extratos e de compostos puros isolados a partir de líquens tem sido realizadas há muitos anos, em especial, contra microrganismos, incluindo o gênero Mycobacterium (VARTIA 1973). Em 1998, Ingólfsdóttir e colaboradores avaliaram, *in vitro*, a atividade antimicobacteriana de cinco compostos liquênicos contra o *Mycobacterium aurum*, um organismo não patogênico e de crescimento rápido, mas com o perfil de sensibilidade semelhante ao *M. tuberculosis.* Eles observaram que, dos cinco compostos avaliados, o ácido úsnico apresentou a melhor ação, tendo a concentração inibitória mínima (CIM) de 32 µg/mL.

Em outro estudo mais recente, a atividade antimicrobiana do ácido úsnico foi avaliada contra cepas susceptíveis e resistentes a isoniazida, estreptomicina e rifampicina, fármacos usados na terapia atual, demonstrando que não ocorreu resistência cruzada, sugerindo que o mecanismo de ação do acido úsnico seja diferente dos fármacos atualmente usadas na terapia da tuberculose (RAMOS e DA SILVA, 2010), fator que viabiliza sua utilização contra o *Mycobacterium tuberculosis*.

Adicionalmente, o ácido úsnico vem sendo utilizado como alternativa contra cepas resistentes aos antibióticos usados na clínica, como por exemplo, *Enterococos* resistentes à vancomicina e *Staphylococcus aureus* resistente à meticilina (SARM) (ELO et al, 2007). Ainda sobre estas cepas, Pompilio e colaboradoes em 2013, além de comprovarem a atividade antimicrobiana do ácido úsnico, identificaram a sua ação antibiofilme.

Um grande número de pesquisadores tem dedicado esforços para descobrir novas classes de compostos, a fim de desenvolver fármacos para substituir ou complementar os medicamentos estabelecidos. Os efeitos sinérgicos são produzidos, quando os constituintes de um extrato ou de uma combinação de drogas afetam diferentes alvos ou interagem um com o outro, a fim de melhorar a solubilidade e, deste modo aumentar a biodisponibilidade de uma ou mais substâncias

na mistura (HONDA et al., 2010). Segatore e colaboradores (2012), a fim de superar a resistência da SARM, avaliaram ação do ácido úsnico combinado a outros antibióticos. Neste estudo foi averiguada uma ação sinérgica do ácido úsnico com a gentamicina, e antagônica com a levofloxacina.

Uma vez que o seu mecanismo de ação não está completamente elucidado, a explicação para sua toxicidade também não é totalmente compreendida. Pramyothin e colaboradores (2004) constataram que o (+) ácido úsnico aja alterando a integridade da membrana celular permitindo a liberação de enzimas hepatoespecíficas (aspartato aminotransferase e alanina aminotransferase), além de causar destruição da função mitocondrial. Diante disto, os dados expostos sugerem que o dano oxidativo esteja relacionado à toxicidade deste composto liquênico, principalmente em células hepáticas.

Além dos danos hepáticos induzidos pelo ácido úsnico, ensaios *in vitro* evidenciaram a sua neurotoxicidade. Em tal estudo o tratamento com o ácido (20 µg/mL) favoreceu o aumento de radicais livres intracelulares, levando a modificações neuronais e consequente morte celular (RABELO et al., 2012).

Apesar de apresentar atividades biológicas promissoras, o uso do ácido úsnico na terapêutica é bastante limitado devido a sua, hepatotoxicidade, baixa hidrossolubilidade e consequentemente eficácia reduzida. Deste modo, uma opção bastante eficaz para diminuir os efeitos tóxicos, otimizar a ação antimicobacteriana e possibilitar o uso do ácido úsnico na terapêutica é a encapsulação deste composto em sistemas de liberação controlada de fármacos.

A literatura relata que o desenvolvimento de nanocápsulas e microesferas, contendo o ácido úsnico, potencializou a ação antitumoral e também diminuiu de forma significativa a hepatotoxicidade deste bioativo (RIBEIRO-COSTA et al., 2004; SANTOS et al., 2006). Por sua vez, Lira e colaboradores (2009) encapsularam AU em lipossomas. Os resultados indicaram uma forte interação entre lipossomas e os macrófagos J774, facilitando a penetração do ácido úsnico nessas células, como também, uma potencialização de sua atividade contra o *Mycobacterium tuberculosis* após a nanoencapsulação. A formulação lipossomal assegurou que o AU permanecesse por mais tempo no interior dos macrófagos (92% por 30h), quando comparado com o AU livre (46% por 8h), o que proporcionou um aumento da atividade antibiótica desta molécula. O desenvolvimento de sistemas lipossomais de longa circulação sistêmica e sítio-específicos, revestidos com a fucana contendo o AU pode ser uma alternativa em potencial para o tratamento da tuberculose, além de ser uma promissora inovação técnico-científica.

3. REFERÊNCIAS BIBLIOGRÁFICAS

AGGARWAL, P.; HALL, J. B.; MCLELAND, C. B.; DOBROVOLSKAIA, M.A.; MCNEIL, S.E. Nanoparticle interaction with plasma proteins as it relates to particle biodistribution, biocompatibility and therapeutic efficacy. **Advanced Drug Delivery Reviews**, v. 61, p. 428-437, 2009.

AGRAWAL, A. K.; GUPTA, C. M. Tuftsin-bearing liposomes in treatment of macrophage-based Infections, **Advanced Drug Delivery Reviews**, v. 41, p. 135-146, 2000.

AHSAN, F.; RIVAS, I. P.; KHAN, M. A.; SUÁREZ, A. I. T. Targeting to macrophages: role of physicochemical properties of particulate carriers—liposomes and microspheres—on the phagocytosis by macrophages, **Journal of Controlled Release**, v. 79, p. 29-40, 2002.

ALEKSEYENKO, T. V.; ZHANAYEVA, S.Y.; VENEDIKTOVA, A.A.; ZVYAGINTSEVA, T.N.; KUZNETSOVA, T.A.; BESEDNOVA, N.N.; KOROLENKO, T.A. Antitumor and antimetastatic activity of fucoidan, a sulfated polysaccharide isolated from the Okhotsk sea Fucus evanescens brown alga. **Bulletin of Experimental Biology and Medicine**, v. 143, p.730–732, 2007.

ANDRADE, F.; RAFAEL, D.; VIDEIRA, M.; FERREIRA, D.; SOSNIK, A.; SARMENTO, B. Nanotechnology and pulmonary delivery to overcome resistance in infectious diseases, **Advanced Drug Delivery Reviews,** v. 65, p. 1816-1827, 2013.

ARAÚJO, P. M.; OLIVEIRA, G. B.; CÓRDULA, C. R.; LEITE, E. L.; CARVALHO JR, L. B.; SILVA, M.P. Sulfated fucan as support for antibiotic immobilization. **Brazilian Journal of Medicine and Biology Research**, v. 37, p. 301-305, 2004.

BANERJEE, R. Liposomes: aplications in medicine. **Journal of Applied Biomaterials,** v. 16, p. 3-21.

BANGHAM, A. D. Model membranes. **Chemistry and Physics of Lipids**, v. 8, p. 386-392, 1972.

BATISTA, C. M.; CARVALHO, C. M. B.; SANTOS-MAGALHÃES, N. S. Lipossomas e suas aplicações terapêuticas: Estado da arte. **Revista Brasileira de Ciências Farmacêuticas,** v. 43, p 167-179, 2007.

BERTEAU, O.; MULLOY, B. Sulfated fucans, fresh perspectives: structures, functions, and biological properties of sulfated fucans and an overview of enzymes active toward this class of polysaccharide. **Glycobiology,** v. 13, p. 29R-40R, 2003.

BILAN, M. I.; GRACHEV, A. A.; SHASHKOV, A. S.; NIFANTIEV, N. E.; USOV, A. I. Structure of a fucoidan from the brown seaweed Fucus serratus L. **Carbohydrate Research**, v. 341, p.238-245, 2006.

BLONDIN, C. et al. Relationship between chemical characters and anticomplementary activity of fucans. **Biomaterials**, v. l7, p. 597-603, 1996.

BOISSEAU, P.; LOUBATON, B. Nanomedicine, nanotechnology in medicine. **Comptes Rendus Physique,** v. 12, p. 620-636, 2011.

BRANDL, M.; GREGORIADIS, G. Entrapment of hemoglobin into liposomes by the dehydratation-rehydratation method: vesicles characterization and in vivo behavior**. Biochimica et Biophysica Acta – Biomembranes,** v.1196, p. 65-75, 1994.

BRIONES, E.; COLINO, C. I.; LANAO, J. M. Delivery systems to increase the selectivity of antibiotics in phagocytic cells. **Journal of Controlled Release**. v. 125, p. 210-227, 2008.

CHONO, S.; TATINO, T.; SEKI, T.; MORIMOTO, K. Efficient drug targeting to rat alveolar macrophages by pulmonary administration of ciprofloxacin incorporated into mannosylated liposomes for treatment of respiratory intracellular parasitic infections. **Journal of Controlled Release.** v. 127, p. 50–58, 2008.

CHONO, S.; TATINO, T.; SEKI, T.; MORIMOTO, K. Uptake characteristics of liposomes by rat alveolar macrophages: influence of particle size and surface mannose modification. **Journal of Pharmacy and Pharmacology**. v.59, p.75–80, 2007.

CLEMENT, M.J.; TISSOT, B.; CHEVOLOT, L.; ADJADJ, E.; DU, Y.; CURMI, P.A.; DANIEL, R. NMR characterization and molecular modeling of fucoidan showing the importance of oligosaccharide branching in its anticomplementary activity. **Glycobiology**. v.20, p.883–894, 2010.

COCCHIETTO, M.; SKERT, N.; NIMIS, P. L.; SAVA, G. A review on usnic acid, an interesting natural compound. **Naturwissenschaften.** v. 89, p. 137-146, 2002.

CUMASHI, A.; USHAKOVA, N. A.; PREOBRAZHENSKAYA, M. E.; D'INCECCO, A.; PICCOLI, A.; TOTANI, L.; TINARI, N.; MOROZEVICH, G. E.; BERMAN, A. E.; BILAN, M. I.; USOV, A. I.; USTYUZHANINA, N. E.; GRACHEV, A. A.; SANDERSON, C. J.; KELLY, M.; RABINOVICH, G. A.; IACOBELLI, S.; NIFANTIEV, N. E. A comparative study of the anti-inflammatory, anticoagulant, antiangiogenic and antiadhesive activities of nine different fucoidans from brown seaweeds. **Glycobiology**. v. 17, p. 541-552, 2007.

DAVIS, T.A.; VOLESKYA, B.; MUCCI, A. A review of the biochemistry of heavy metal biosorption by brown algae. **Water Research**. v. 37, p. 4311–4330, 2003.

DEOL, P.; KHULLER, G. K.; JOSHI, K. Therapeutic efficacies of isoniazid and rifampin encapsulated in lung-specific stealth liposomes against Mycobacterium tuberculosis infection induced in mice. **Antimicrobial Agents and Chemotherapy**. v. 41, p. 1211–1214, 1997.

DRULIS-KAWA, Z.; DOROTKIEWICZ-JACH, A. Liposomes as delivery systems for antibiotics. **International Journal of Pharmaceutics**. v. 387, p. 187-198, 2010.

DUBE, D.; AGRAWAL, G. P.; VYAS, S. P. Tuberculosis: from molecular pathogenesis to effective drug carrier design. **Drug Discovery Today.** v. 0, n.0, 2012.

DUZGUNES, N.; FLASHER, D.; REDDY, M. V.; LUNA-HERRERA, J.; GANGADHARAM, P. R. Treatment of intracellular Mycobacterium avium complex infection by free and liposome encapsulated sparfloxacin. **Antimicrobial Agents and Chemotherapy**, v. 40, p. 2618–2621, 1996.

EDWARDS, K. A.; BAEUMNER, A. J. Liposomes in analyses. **Talanta**. v. 68, p. 1421-1431, 2006.

ELO, H.; MATIKAINEN, J.; PELTTARI, E. Potent activity of the lichen antibiotic (+)-usnic acid against clinical isolates of vancomycin-resistant enterococci and methicillin-resistant Staphylococcus aureus. **Naturwissenschaften.** v. 94, p. 465–468, 2007.

EL-RIDY, M. S.; MOSTAFA, D. M.; SHEHAB, A.; NASR, E. A.; EL-ALIM, S. A. Biological evaluation of pyrazinamide liposomes for treatment of Mycobacterium tuberculosis. **International Journal of Pharmaceutics**. v. 330, p. 82-88, 2007.

FRÉZARD, F.; SCHETTINI, D. A.; ROCHA, O. G. F.; DEMICHELI, C. Lipossomas: propriedades físico-químicas e farmacológicas, aplicações na quimioterapia à base de antimônio. **Química Nova**. v. 28, p. 511-518, 2005.

GARG, N. K.; DWIVEDI, P.; JAIN, A.; TYAGI, S.; SAHU, T.; TYAGI, R. K. Development of novel carrier(s) mediated tuberculosis vaccine: More than a tour de force. **European Journal of Pharmaceutical Sciences**. v. 62, p. 227-242, 2014.

GASPAR, M. M.; CRUZ, A.; PENHA, A. F.; REYMÃO, J.; SOUSA, A. C.; ELEUTÉRIO, C. V.; DOMINGUES, S. A.; FRAGA, A. G.; FILHO, A. L.; CRUZ, M. E.; PEDROSA, J. Rifabutin encapsulated in liposomes exhibits increased therapeutic activity in a model of disseminated tuberculosis. **International Journal of Antimicrobial Agents**, v. 31, p. 37–45, 2008.

GEISER, M. Update on Macrophage Clearance of Inhaled Micro- and Nanoparticles. **Journal of aerosol medicine and pulmonary drug delivery.** v. 23, p. 207-217, 2010.

GOONOO, N.; BHAW-LUXIMON, A.; UJOODHA, R.; JHUGROO, A.; HULSE, G. K.; JHURRY, D. Naltrexone: a review of existing sustained drug delivery systems and emerging nano-based systems. **Journal of Controlled Release**. v. 183, p. 154 – 166, 2014.

HAUCK, T. S.; GIRI, S.; GAO, Y.; CHAN, W. C. W. Nanotechnology diagnostics for infectious diseases prevalent in developing countries. **Advanced Drug Delivery Review**. v. 62, p. 438-448, 2010.

HAYASHI, K.; NAKANO, T.; HASHIMOTO, M.; KANEKIYO, K.; HAYASHI, T. Defensive effects of a fucoidan from brown alga Undaria pinnatifida against herpes simplex virus infection. **International Immunopharmacology.** v. 8, p. 109–116, 2008.

HOLDT, S. L.; KRAAN, S. Bioactive compounds in seaweed: functional food applications and legislation. **Journal of Applied Phycology**, v. 23, p. 543-597, 2011.

HOLTKAMP, A. D.; KELLY, S.; ULBER, R.; LANG, S. Fucoidans and fucoidanases — focus on techniques for molecular structure elucidation and modification of marine polysaccharides. **Applied Microbiology and Biotechnology**, v. 82, p.1 – 11, 2009.

HONDA, N. K.; PAVAN, F. R.; COELHO, R. G.; DE ANDRADE LEITE, S. R.; MICHELETTI, A. C.; LOPES, T. I. B.; MISUTSU, M. Y.; BEATRIZ, A.; BRUM, R. L.; LEITE, C. Q. F. Antimycobacterial activity of lichen substances. **Phytomedicine**. v. 17, p. 328 – 332, 2010.

IMMORDINO, M.L.; DOSIO, F.; CATTEL, L. Stealth liposomes: review of the basic science, rationale, and clinical applications, existing and potential. **International Journal of Nanomedicine**, v.1, p. 297–315, 2006.

INGÓLFSDÓTTIR, K. Molecules of Interest Usnic Acid. **Phytochemistry,** v. 61, p. 729-736, 2002.

INGÓLFSDÓTTIR. K.; CHUNG, G. A. C.; SKÚLASON, V.G.; GISSURARSON, S. R.; VILHELMSDÓTTIR, M. Antimycobacterial activity of lichen metabolites in vitro. **European Journal of Pharmaceutical Sciences.** v. 6, p.141-144, 1998.

JIAO, G.; YU, G.; ZHANG, J.; EWART, H. S. Chemical Structures and Bioactivities of Sulfated Polysaccharides from Marine Algae. **Marine Drugs**. v. 9, p. 196-223, 2011.

JORDÃO, L.; VIEIRA, O. V. Tuberculosis: New Aspects of an Old Disease. **International Journal of Cell Biology,** p. 1-13, 2011.

KANG, S. M.; KIMB, K.N.; LEEA, S.H.; AHNA, G.; CHAC, S.H.; KIMA, A.D.; YANGA, X.D.; KANGA, M.C.; JEON, Y.J. Anti-inflammatory activity of polysaccharide purified from AMG-assistant extract of Ecklonia cava in LPS-stimulated RAW264.7 macrophages. **Carbohydrate Polymers**, v. 85, p. 80-85, 2011.

KAUFMANN, S.H. How can immunology contribute to the control of tuberculosis? **Nature Reviews Immunology**, v.1, p. 20–30, 2001.

KAUR, I. P.; SINGH, H. Nanostructured drug delivery for better management of tuberculosis, **Journal of Controlled Release**, v. 184, p. 36-50, 2014.

KIDENYA, B. R.; WEBSTER, L. E.; BEHAN, S.; KABANGILA, R.; PECK, R. N.; MSHANA, S. E.; OCHERETINA, O.; FITZGERALD, D. W. Epidemiology and genetic diversity of multidrug-resistant tuberculosis in East Africa, **Tuberculosis**, v. 94, p. 1-7, 2014.

KIM, K.Y. Nanotechnology platforms and physiological challenges for cancer therapeutics. **Nanomedicine: Nanotechnology, Biology and Medicine,** v. 3, p. 103–110, 2007.

KIM, M. H.; JOO, H. G. Immunomodulatory effects of fucoidan on bone marrow-derived dendritic cells. **Immunology Letters**, v. 115, p. 138–143, 2008.

KIM, W. S.; ORDIJA, C. M.; FREEMAN, M. W. Activation of signaling pathways by putative scavenger receptor class A (SR-A) ligands requires CD14 but not SR-A. **Biochemical and Biophysical Research Communications,** v. 310, p.542–549, 2003.

KOO, O.M.; RUBINSTEIN, I.; ONYUKSEL, H. Role of nanotechnology in targeted drug delivery and imaging:a concise review. **Nanomedicine: Nanotechnology, Biology and Medicine**. v. 1, p. 193– 212, 2005.

KOSHKARYEV, A.; SAWANT, R.; DESHPANDE, M.; TORCHILIN, P. Immunoconjugates and long circulating systems: Origins, current state of the art and future directions. **Advanced Drug Delivery Reviews**. v. 65, p. 24-35, 2013.

KRISTMUNDSDÓTTIR, T.; ARADÓTTIR, H. A.; INGÓLFSDÓTTIR, K.; OGMUNDSDÓTTIR, H. M. Solubilization of the lichen metabolite (+)-usnic acid for testing in tissue culture. **Journal of Pharmacy and Pharmacology**, v.54, p.11, 2002.

KUMAR, M.; KUMARI, P.; TRIVEDI, N.; SHUKLA, M. K.; GUPTA, V.; REDDY, C. R. K.; JHA, B. MINERALS, PUFAs and antioxidant properties of some tropical seaweeds from Saurashtra coast of India. **Journal of Applied Phycology.** v. 23, p. 797-810, 2011.

KUMAR, S.; MÜLLER, K. Lichen metabolites 2: antiproliferative and cytotoxic activity of gyrophoric, usnic and diffractaic acid on human keratinocyte growth. **Journal of Natural Products.** v.62, p.821-823, 1999.

LABANA, S. et al. Chemotherapeutic activity against murine tuberculosis of once weekly administeres drugs (isoniazid and rifampicin) encapsulated in liposomes. **International Journal of Antimicrobial Agents,** v. 20, p. 301-304, 2002.

LAUTERWEIN, M.; OETHINGER, M.; BELSNER, K.; PETER, T.; MARRE, R. In vivo activities of the lichen secondary metabolites vulpinic acid, (+) usnic acid, and (-) usnic acid

against aerobic and anaerobic microorganisms. **Antimicrobial agents and chemotherapy**. v. 39, p. 2541-2543, 1995.

LEITZKE, S.; BUCKE, W.; BORNER, K.; MÜLLER, R.; HAHN, H.; EHLERS, S. Rationale for and efficacy of prolonged-interval treatment using liposome-encapsulated amikacin in experimental Mycobacterium avium infection. **Journal of Pharmacy and Pharmacology**. v. 42, p. 459–461, 1998.

LI, B.; LU, F.; WEI, X.; ZHAO, R. Fucoidan: Structure and Bioactivity. **Molecules**. v. 13, p. 1671-1695, 2008.

LI, D.Y. et al. Effect of fucoidan of L. japonica on rats with hyperlipidaemia. **Food Science**, v. 22, p.92-95, 2001.

LIRA, M. C. B.; SIQUEIRA-MOURA, M. P.; ROLIM-SANTOS, H. M. L.; GALETTI, F. C. S.; SIMIONI, A. R.; SANTOS, N. P.; EGITO, E. S. T.; SILVA, C. L.; TEDESCO, A. C., SANTOS-MAGALHÃES, N. S. In vitro uptake and antimycobacterial activity of liposomal usnic acid formulation. **Journal of Liposome Research**. v. 19, p. 49-58, 2009.

LIRA, M.C.B.; SANTOS-MAGALHÃES, N. S.; NICOLAS, V.; MARSAUD, V.; SILVA, M. P.; PONCHEL, G.; VAUTHIER, C. Cytotoxicity and cellular uptake of newly synthesized fucoidan-coated nanoparticles. **European Journal of Pharmaceutics and Biopharmaceutics**. v. 79, p. 162 - 170, 2011.

LIU, P. T.; MODLIN, R. L. Human macrophage host defense against Mycobacterium tuberculosis. **Current Opinion in Immunology.** v.20, p.371-376, 2008.

LOIRA-PASTORIZA, C.; TODOROFF, J.; VANBEVER, R. Delivery strategies for sustained drug release in the lungs. **Advanced Drug Delivery Reviews.** v. 75, p. 81 - 91, 2014.

LUO, X.; PIRES, D.; AÍNSA, J.A.; GRACIA, B.; DUARTE, N.; MULHOVO, S.; ANES, E.; FERREIRA, M.J.U. Zanthoxylum capense constituents with antimycobacterial activity against

Mycobacterium tuberculosis in vitro and ex vivo within human macrophages. **Journal of Ethnopharmacology.** v.146, p.417–422, 2013.

MANDAL, P.; MATEU, C. G.; CHATTOPADHYAY, K.; PUJOL, C. A.; DAMONTE, E. B.; RAY, B. Structural features and antiviral activity of sulphated fucans from the brown seaweed Cystoseira indica. **Antiviral Chemistry and Chemotherapy**. v. 18, p. 153–162, 2007.

MARUYAMA, H.; TAMAUCHI, H.; HASHIMOTO, M.; NAKANO, T. Antitumor activityand immune response of mekabu fucoidan extracted from sporophyll of Undaria pinnatifida. **In Vivo.** v. 17, p. 245–249, 2003.

MARUYAMA, H.; TAMAUCHI, H.; IIZUKA, M.; NAKANO, T. The role of NK cells in antitumor activity of dietary fucoidan from Undaria pinnatifida sporophylls (mekabu). **Planta Médica**. v. 72, p. 1415–1417, 2006.

MEERAK, J.; WANICHWECHARUNGRUANG, S. P.; PALAGA, T. Enhancement of immune response to a DNA vaccine against Mycobacterium tuberculosis Ag85B by incorporation of an autophagy inducing system, **Vaccine**, v. 31, p. 784-790, 2013.

MINISTÉRIO DA SAÚDE. **Manual de recomendações para o controle da tuberculose no Brasil**, 2011. Disponível on-line em: portal.saude.gov.br/portal/arquivos/pdf/manual_de_recomendacoes_tb.pdf. Acessado em: 02/05/2014.

MOGHIMI, S. M.; ANDERSEN, A. J.; AHMADVAND, D.; WIBROE, P. P.; ANDRESEN, T. L.; HUNTER, A. C. Material properties in complement activation. **Advanced Drug Delivery Reviews**. v. 63, p. 1000 - 1007, 2011.

MÜLLER, K. Pharmaceutically relevant metabolites from lichens. **Applied Microbiology and Biotechnology**. v. 56, p. 9 - 16, 2001.

ORCAU, À.; CAYLÀ, J. A.; MARTÍNEZ, J. A. Present epidemiology of tuberculosis. Prevention and control programs. **Enfermedades Infecciosas Microbiologia Clínica.** v. 29, p. 2-7, 2011.

PANDEY, R.; KHULLER, G. K. Nanotechnology based drug delivery system(s) for the management of tuberculosis. **Indian Journal of Experimental Biology**. v. 44, p. 357-366, 2006.

PARDO, P.; SOLÉ, M.A. Marine flora of the Peninsula of Macanao, Margarita Island, Venezuela. I. Chlorophyta and Phaeophyceae. **Acta Botanica Venezuelica**. v. 30, p. 291-325, 2007.

PATANKAR, M. S.; OEHNIGER, S.; BARNETT, T.; WILLIAMS, R.L.; CLARK, G. F. A Revised Structure for Fucoidan May Explain Some of Its Biological Activities. **The Journal of Biological Chemistry**. v. 268, p. 21770 – 21776, 1993.

PINTO-ALPHANDARY, H.; ANDREMONT, A.; COUVREUR, P. Targeted delivery of antibiotics using liposomes and nanoparticles: research and applications, **International Journal of Antimicrobial Agents**. v. 13, p. 155-168, 2000.

POMPILIO, A.; POMPONIO, S.; VINCENZO, V.D.; CROCETTA, V.; NICOLETTI, M.; PIOVANO, M.; GARBARINO, J.A.; BONAVENTURA G.D. Antimicrobial and antibiofilm activity of secondary metabolites of lichens against methicillinresistant Staphylococcus aureus strains from cystic fibrosis patients. **Future Microbiology**. v. 8, 281-292, 2013.

PRAMYOTHIN, P.; JANTHASOOT, W.; PONGNIMITPRASERT, N.; PHRUKUDOMC, S.; RUANGRUNGSI, N. Hepatotoxic effect of (+)usnic acid from Usnea siamensis Wainio in rats, isolated rat hepatocytes and isolated rat liver mitochondria. **Journal of Ethnopharmacology**. v. 90, p.381-387, 2004.

RABELO, T.K.; ZEIDÁN-CHULIÁ, F.; VASQUES, L.M.; DOS SANTOS, J.P.A.; DA ROCHA, R.F.; PASQUALI, M.A.B.; RYBARCZYK-FILHO, J.L.; ARAÚJO, A.A.S.;

MOREIRA, J.C.F.; GELAIN, D.P. Redox characterization of usnic acid and its cytotoxic effect on human neuron-like cells (SH-SY5Y). **Toxicology in Vitro**. v. 26, p. 304-314, 2012.

RAGHAVENDRAN, H. R.; SRINIWASAN, P.; REKHA, S. Immunomodulatory activity of fucoidan against aspirin-induced gastric mucosal damage in rats. **International Immunopharmacology**. v. 11, p.157–163, 2011.

RAMOS, D.F.; DA SILVA, P.E.A. Antimycobacterial activity of usnic acid against resistant and susceptible strains of Mycobacterium tuberculosis and non-tuberculous mycobacteria. **Pharmaceutical Biology**. v. 48 p. 260-263, 2010.

RANJITA, S.; LOAYE, A. S.; KHALIL, M. Present Status of Nanoparticle Research for Treatment of Tuberculosis. **Journal of Pharmaceutical Sciences**. v. 14, p. 100-116, 2011.

RIBEIRO-COSTA, R. M.; ALVES, A. J.; SANTOS, N. P.; NASCIMENTO, S. C.; GONÇALVES, E. C. P.; SILVA, N. H.; HONDA, N. K.; SANTOS-MAGALHÃES, N. S. In vitro and in vivo properties of usnic acid encapsulated into PLGA-microspheres. **Journal of Microencapsulation.** v. 21, p. 371-384, 2004.

SACHETELLI, S.; KHALIL, H.; CHEN, T.; BEAULAC, C.; SÉNÉCHAL, S.; LAGACÉ, J. Demonstration of a fusion mechanism between a fluid bactericidal liposomal formulation and bacterial cells. **Biochimica et Biophysica Acta**. v. 1463, p. 254-266, 2000.

SANTOS, N. C.; CASTANHO, M. A. R. B. Lipossomas: a bala mágica acertou? **Química Nova**. v. 25, p. 1181-1185, 2002.

SANTOS, N. P. S.; NASCIMENTO, S. C.; WANDERLEY, M. S. O.; PONTES-FILHO, N.T.; SILVA, J. F.; CASTRO, C.M.M.B.; PEREIRA, E. C.; SILVA, N. H.; HONDA, N. K.; SANTOS-MAGALHÃES, N. S. Nanoencapsulation of usnic acid: An attempt to improve antitumour activity andreduce hepatotoxicity. **European Journal of Pharmaceutics and Biopharmaceutics**. v. 64, p. 154-160, 2006.

SAPRA, P.; ALLEN, T.M. Ligant-target lipossomal anticancer drugs. **Progress in lipid Research.** v. 42, p. 439–462, 2003.

SCHWENDENERER, R. A.; LAGOCKI, P. A.; RAHMAN, Y. E. The effects of charge and size on the interaction of unilamellar liposomes with macrophages. **Biochimica et Biophysics Acta**. v. 772, p. 93-101, 1984.

SEGATORE, B.; BELLIO, P.; SETACCI, D.; BRISDELLI, F.; PIOVANO, M.; GARBARINO, J.A.; NICOLETTI, M.; AMICOSANTE, G.; PERILLI, M.; CELENZA, G. In vitro interaction of usnic acid in combination with antimicrobial agents against methicillin-resistant Staphylococcus aureus clinical isolates determined by FICI and ΔE model methods. **Phytomedicine.** v. 19 p. 341-347, 2012.

SAKATA, S. et al. Programming control of intelligent drug releases in response to single and binary environmental stimulation signals using sensor and electroresponsive hydrogel. **Radiation Physics and Chemistry,** v.76, p.733–737, 2007.

ŠEGOTA, S.; TEŽAK, D. Spontaneous formation of vesicles. **Advances in Colloid and Interface Science.** v. 121, p. 51-75, 2006.

SENTHILKUMAR, K.; MANIVASAGAN, P.; VENKATESAN, J. Brown seaweed fucoidan: Biological activity and apoptosis, growth signaling mechanism in cancer. **International Journal of Biological Macromolecules**. v. 60, p. 366-374, 2013.

SHEGOKAR, R.; AL, S.; MITRI, K. Present Status of Nanoparticle Research for Treatment of Tuberculosis. **J Pharm Pharmaceut Sci**. v. 14, p. 100 - 116, 2011.

SOSNIK, A.; CARCABOSO, A. M.; GLISONI, R. J.; MORETTON, M. A.; CHIAPPETTA, D. A. New old challenges in tuberculosis: Potentially effective nanotechnologies in drug delivery. **Advanced Drug Delivery Reviews.** v.62, p. 547-559, 2010.

SOUZA, M.C.R.; MARQUES, C. T.; DORE, C. M. G.; SILVA, F. R. F.; ROCHA, H. A. O.; LEITE, E. L. Antioxidant activities of sulfated polysaccharides from brown and red seaweeds. **Journal of Applied Phycology**, v. 19, p. 153–160, 2007.

TERUYA, T.; TATEMOTO, H.; KONISHI, T.; TAKO, M. Structural characteristics and in vitro macrophage activation of acetyl fucoidan from Cladosiphon okamuranus. **Glycoconjugate Journal**, v.26, p. 1019-28, 2009.

TERUYA, T.; KONISHI, T.; UECHI, S.; TAMAKI, H.; TAKO, M. Anti-proliferative activity of oversulfated fucoidan from commercially cultured Cladosiphon okamuranus TOKIDA in U937 cells. **International Journal of Biological Macromolecules**, v. 41, p. 221–226, 2007.

THOMES, P.; RAJENDRAN, M.; PASANBAN, B.; RENGASAMY, R. Cardioprotective activity of Cladosiphon okamuranus against isoproterenol induced myocardial infraction in rats. **Phytomedicine**. v.18, p.52–57, 2010.

TISSOT, B.; DANIEL, R. Biological properties of sulfated fucans: The potent inhibiting activity of algal fucoidan against the human complement system. **Glycobiology**. v.13, p.29–31, 2003.

TISSOT, B.; GONNET, F.; IBORRA, A.; BERTHOU, C.; THIELENS, N.; ARLAUD, G.J.; DANIEL, R. Mass spectrometry analysis of the oligomeric C1q protein reveals the B chain as the target of trypsin cleavage and interaction with fucoidan. **Biochemistry.** v.44, p.2602–2609, 2005.

TISSOT, B.; MONTDARGENT, B.; CHEVOLOT, L.; VARENNE, A.; DESCROIX, S.; GAREIL, P.; DANIEL, R. Interaction of fucoidan with the proteins of the complement classical pathway. **Biochimica et Biophysica Acta.** v.1651, p.5–16, 2003.

TORCHILIN, V. Multifunctional and stimuli-sensitive pharmaceutical nanocarriers, **European Journal of Pharmaceutics and Biopharmaceutics**, v. 71, p. 431-444, 2009.

TORCHILIN, V. Multifunctional nanocarriers. **Advanced Drug Delivery Reviews**. v. 58, p. 1532– 1555, 2006.

TORCHILIN, V. Recent advances with liposomes as pharmaceutical carrier. **Nature Reviews Drug Discovery**. v. 4, p. 145-160, 2005.

VARTIA, K.O. Antibiotics in lichens. In: Ahmadjian, V., Hale, M.E. (Eds.), **The Lichens. Academic Press**. p. 547–561, 1973.

VEENA, C. K.; JOSEPHINE, A.; PREETHA, S. P.; VARALAKSHMI, P. Beneficial role of sulfated polysaccharides from edible seaweed Fucus vesiculosus in experimental hyperoxaluria. **Food Chemistry**. v. 100, p. 1552–1559, 2007.

VEMURI, S.; RHODES, C. T. Preparation and characterization of liposomes as therapeutic delivery systems: a review. **Pharmaceutica Acta Helvetica**. v. 70, p. 95-111, 1995.

VOINEA, M.; SIMIONESCU, M. Designing of 'intelligent' liposomes for efficient delivery of drugs. **Journal of Cellular and Molecular Medicine.** v. 6, p. 465-474, 2002.

VYAS, S. P.; KATARE, Y. K.; MISHRA, V.; SIHORKAR, V. Ligant directed macrophage targeting of amphotericin B loaded liposomes. **International Journal of Pharmaceutics**. v. 210, p. 1-14, 2000.

VYAS, S.P.; KANNAN, M.E.; JAIN, S.; MISHRA, V.; SINGH, P. Design of liposomal aerosols for improved delivery of rifampicin to alveolar macrophages. **International Journal of Pharmaceutics.** v. 269, p. 37–49, 2004.

WANG, Y.; WATTS, A. B.; PETERS, J. I.; WILLIAMS III, R. O. The impact of pulmonary diseases on the fate of inhaled medicines—A review. **International Journal of Pharmaceutics.** v. 461, p. 112-128, 2014.

WANG, J. et al. Antioxidant activity of sulfated polysaccharide fractions extracted from *Laminaria japonica*. **International Journal of Biological Macromolecules,** v. 42, p. 127–132, 2008.

WELSH, K. J.; HUNTER, R.; ACTOR, J. K. Trehalose 6,6'-dimycolate – A coat to regulate tuberculosis immunopathogenesis. **Tuberculosis**. v. 51, p. 53-59, 2013.

WHO (World Health Organization). **Global tuberculosis report 2013,** 2013. Disponível on-line em: http://apps.who.int/iris/bitstream/10665/91355/1/9789241564656_eng.pdf. Acessado em: 20/03/2014.

WIJESINGHE, W. A. J. P.; JEON, Y. Biological activities and potential industrial applications of fucose rich sulfated polysaccharides and fucoidans isolated from brown seaweeds: A review. **Carbohydrate Polymers.** v. 88, p. 13-20, 2012.

ZHOU, S.; LIAO, X.; LI, X.; DENG, X.; LI, H. Poly-DL-lactide-co-poly (ethylene glycol) microspheres as potential vaccine delivery systems. Jornal of Controlled Release, v. 8, n. 2-3, p. 195-205, 2002.

4. OBJETIVOS

4.1. Objetivo geral

Desenvolver, caracterizar e avaliar a atividade biológica de lipossomas revestidos com fucana contendo ácido úsnico.

4.2. Objetivos específicos

- Extrair, purificar e hidrolizar a fucana;
- Sintetizar e caracterizar quimicamente o derivado colesteroil-fucana;
- Desenvolver lipossomas convencionais e sítio-específicos revestidos com o derivado colesteroil-fucana contendo o ácido úsnico;
- Avaliar as características físico-químicas das formulações desenvolvidas;
- Determinar a citotoxicidade, frente à macrofagos Raw 264,7, do ácido úsnico livre e nanoencapsulado em lipossomas convencionais e sítio-específicos;
- Avaliar o mecanismo de captura celular por microscopia de fluorescência;
- Avaliar mecanismo de ação dos lipossomas sítio-específicos desenvolvidos através da citometria de fluxo.
- Determinar a Concentração Inibitória Mínima (CIM) do ácido úsnico livre e encapsulado em lipossomas frente ao *Mycobacterium tuberculosis* H37Rv e a isolados clínicos multirresistentes;
- Mensurar a interação *in vitro* entre os fármacos de referência (isoniazida e rifampicina) e o ácido úsnico livre e encapsulado em lipossomas convencionais frente a isolados clínicos multirresistentes.

5. CAPÍTULO 1

A ser submetido ao Bioorganic & Medicinal Chemistry Letters

Cholesteryl-fucoidan: A versatile hydrophobized polysaccharide synthetized by microwave-assisted reaction for the development of site-specific liposomes

Taciana L. Salviano [a,b], Rafaela S. F. Carvalho [a], Vanessa X. Barbosa [c], Maria da Paz C. Silva [a], Mariane C. B. L. Nogueira [b], Nereide S. Santos-Magalhães [a], Alexandre J. S. Góes [c,*]

[a]Laboratório de ImunopatologiaKeizo-Asami, Universidade Federal de Pernambuco, Recife, PE, Brazil
[b]Centro Acadêmico de Vitoria, Universidade Federal de Pernambuco, Vitória de Santo Antão, PE, Brazil
[c]Departamento de Antibióticos, Universidade Federal de Pernambuco, Recife, PE, Brazil

[*]Corresponding author:
Dr. Alexandre José S. Góes
Universidade Federal de Pernambuco (UFPE)
Departamento de Antibióticos
Av. Prof. Moraes do Rego, 1235, Cidade Universitária,
50670-901, Recife, PE, Brazil
Phone: + 55-81-21268587; fax: + 55-81-21268485
E-mail: ajsg@ufpe.br

Abstract

A method involving microwave-assisted synthesis of cholesteryl-fucoidan has been developed with several advantages including a high yielding of 75 %, a short reaction time of 45 minutes, operational simplicity and possible applicability to synthesize others hydrophobized polysaccharides. In addition, the cholesteryl-fucoidan was used to prepare targeted fucoidan-coated liposomes, which were taken up by Raw 264.7 macrophages faster than uncoated-liposomes (15 min and 60min, respectively). Fucoidan-coated liposomes present as potent drugs carrier for infectious diseases

Keywords: hydrophobized polysaccharides, cholesteryl-fucoidan, microwave, fucoidan-coated liposomes.

Hydrophobized polysaccharides have been investigated intensively during recent decades, due to their ability to improve the physical and biochemical stability of drug delivery systems and directed them to specific organs and cells.[1,2] These molecules are amphiphilic polymers used for coating liposomes with the aim of providing physical and biochemical stability, stealth characteristics and the ability to target the vesicle system to specific organs and cells.[3] The approach of the sugar-recognition mechanism to liposomal targeting has been shown to significantly improve their therapeutic benefits.[4,5,6]

Among the reported methods for chemically modified sugars, the synthesis consists basically of reacting covalently polysaccharides with cholesterol moieties as hydrophobic side group.[4,7,8,9] These partially hydrophobized polysaccharides could be anchored into the outer phospholipid bilayer of liposomes by hydrophobic interaction. Recently, some derivatives of carbohydrates, such as pullulan,[4] chitosan,[8] alginate,[10] chitin,[11] have already been synthetized using different methods. However, the most of those techniques require long reaction times. Moreover, despite the innumerous advantages of covering liposomes with hydrophobized polysaccharides, until now few sugar molecules were explored for this purpose.

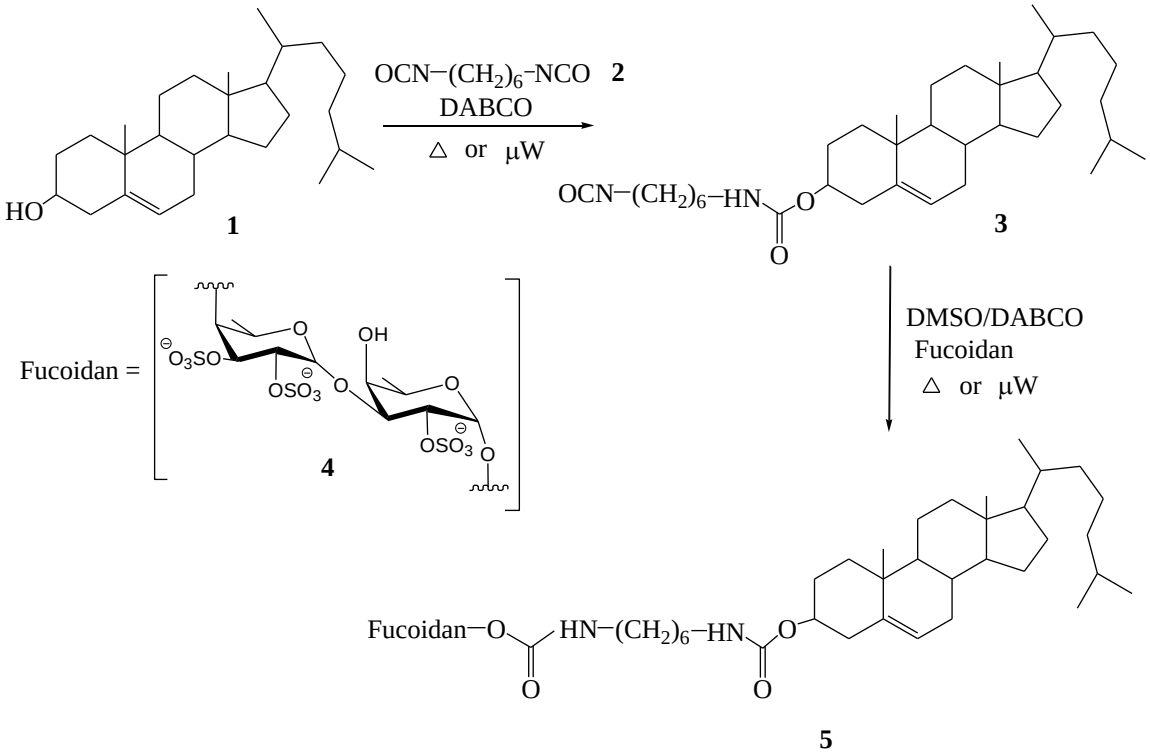
In the past years, fucoidan, a water-soluble sulphated complex polysaccharide, has attracted increasing attention due to its functional properties and characteristics, such as stability, safety, non-toxicicity, biocompatibility and biodegradability.[12] This molecule is extracted mainly from the cell wall of brown seaweed as *Fucus vesiculosus* and its structure predominantly consists of sulphated L-fucose units linked via α-1,2, α-1,3 or α-1,4 glycosidic bounds.[13] In respect of biological functions of fucoidan, this polysaccharide presents several activities including anticomplementary activity [14] and the ability to recognize and bind itself to macrophage scavenger class A type I and II receptors (MSR-I and MSR-II), which are expressed abundantly in alveolar macrophages.[2] Therefore, these two biological findings open new fields of fucoidan uses mainly in the pharmaceutical nanotechnology aiming to develop new long-circulating and targeted liposomal systems toward infected macrophages, as potential carriers of drugs to improve the treatment of lung diseases such as tuberculosis.

As part of our search for the development of a novel and versatile nanocarrier systems, we focused on the possibility of synthesize a hydrophobized fucoidan derivative, for the development of newly fucoidan-coated liposomes as a site-specific system, following two different methods: a conventional–method[4] and an optimized procedure by microwave-assisted reaction.

Previously, we extracted[15] and hydrolyzed[16] the fucoidan from *Fucus vesiculosus* and obtained a yield of 1.32% of dry weight. Our results were similar to those obtained by Rioux et al., 2007, with a yield of 1.4% for *F. vesiculosus*.[13] The average molecular weight of 30 KDa was determined by HPSEC-RI[17]. Furthermore, the amorphous aspect of fucoidan was observed by scanning electron microscopy (SEM) (Figure 1A) and confirmed by X-ray powder diffraction pattern (XRPD) (Figure 2).

In order to synthesize the hydrophobized fucoidan, two step sequences were carried out (Scheme 1, Table 1).[18] Using the conventional procedure, the reaction between cholesterol (**1**, 1 equiv) and hexamethylene diisocyanato (**2**, 2 equiv) was performed in the presence of 1,4-diazobicyclo(2,2,2)octane (DABCO) (0.06 equiv) as catalyst, in dry toluene under reflux, to give cholesteryl N-(6-isocyanatohexyl)carbamate (**3**) in 64% of yield (entry 1).[19] Then, the cholesteryl-fucoidan (**5**) was prepared by refluxing compound **3** (8.43equiv) in dry DMSO with catalytic DABCO (0.15 equiv) and fucoidan (**4,** 1 equiv) obtaining 77% yield (entry 4). These two conventional reactions got completed in 8 h and 6 h, consecutively. The morphological aspect of **5** was analyzed by electron microscopy (Figure 1B) and XRPD (Chol-Fuc Δ) (Figure 2). It showed to be more amorphous than compound **4**.

By continuing our work to design a cholesteryl compound, we aimed to optimize this procedure using a microwave-assisted reaction. Microwave irradiation has become an important source of energy in organic synthesis because it improves the selectivity avoiding side reactions and it accelerates the rate of procedure.[20] In that way, we wish to report an important improvement of this synthetic route.



Scheme 1 Synthesis of cholesteryl-fucoidan (**5**).

Table 1

Comparison of stepwise reactions using regular thermal and microwave heating.

Entry	Reagents	Conditions	Solvent (dry)	Additive DABCO	Product yield (%)
1	**1** (1equiv), **2** (2 equiv)	Δ, reflux, 8h	toluene	0.06 equiv	**3** (64)
2	**1** (1 equiv), **2** (2 equiv)	μW, 100^{o}, 20min	toluene	0.06 equiv	**3** (66)
3	**1** (1 equiv), **2** (2 equiv)	μW, 100^{o}, 25min	-	0.06 equiv	**3** (50)
4	**3** (8.43 equiv), **4** (1 equiv)	Δ, reflux, 6h	DMSO	0.15 equiv	**5** (77)
5	**3** (8.43 equiv), **4** (1 equiv)	μW, 100^{o}, 40min	DMSO	0.15 equiv	**5** (75)

Keeping this view, in the first step, the optimal conditions for the microwave-assisted synthesis were determined to be a mixture of compounds **l** (1 equiv) and **2** (2 equiv) in dry toluene with DABCO (0.06 equiv) at 100ºC (120 W) for 20 min (scheme 1, entry 2). Cholesteryl N-(6-isocynatohexyl) carbamate (**3**) was found in 66% overall yield. In addition, a reaction to obtain compound **3** without solvent was also carried out, but the product was achieved in only 50% yield (entry 3). Differently, in the synthesis of cholesteryl-fucoidan (**5**), the best condition was achieved mixing the limiting reactant **3** (8.43 equiv), compound **4** (1 equiv) and DABCO (0.15 equiv) with dry DMSO under microwave irradiation (120 W) at 100°C. The reaction got completed in 40 min affording a 75% yield of final product (**5**). Figure 1C shows the SEM of hydrophobized fucoidan obtained by the microwave procedure. The morphological aspect of compound **5** obtained by microwave reaction was similar to the conventional synthesis. This characteristic was also confirmed by XRPD (Chol-Fuc μw) (Figure 2) Thus, as expected, reaction time of both steps was drastically decreased (around 90%) under microwave irradiation, but the overall yield of **3** and **5** was maintained. Clearly, the cholesteryl derivatives were synthetized in a fast and efficient manner comparing to the conventional method overcoming its time-consuming disadvantage.

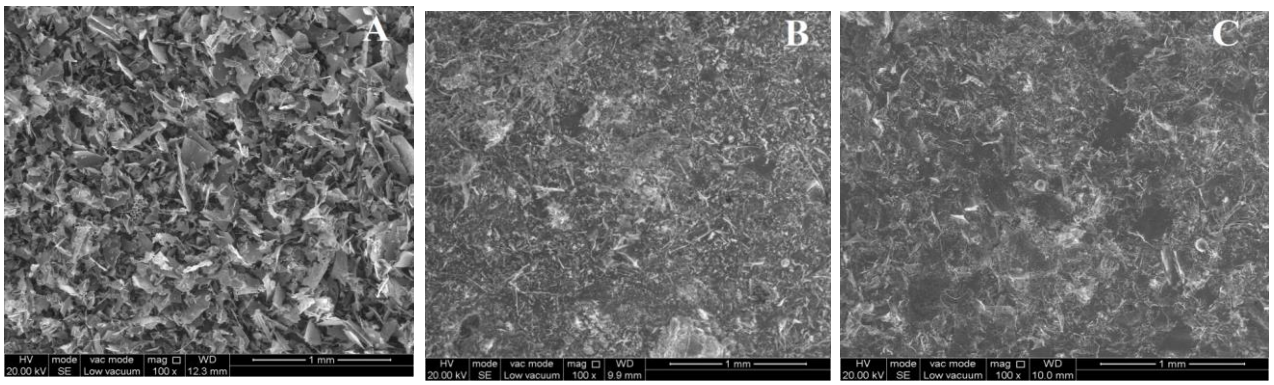


Figure 1. SEM images of Fucoidan (A) and Cholesteryl-Fucoidan obtained by conventional method (B) and microwave-assisted reaction (C).

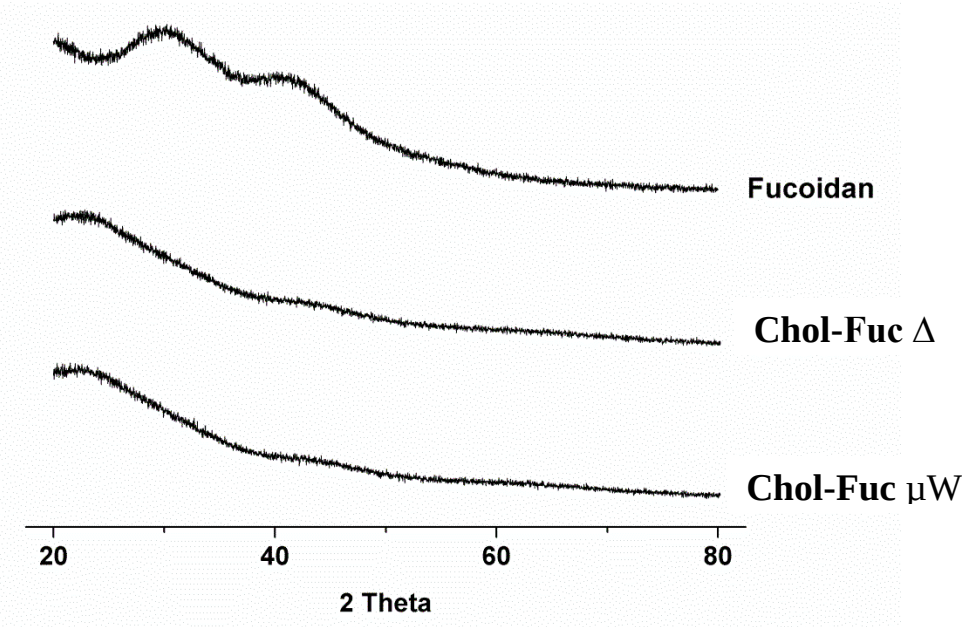


Figure 2. XRDP of Fucoidan and Cholesteryl-Fucoidan obtained by conventional method (Chol-Fuc Δ) and microwave-assisted reaction (Chol-Fuc μW).

The Cholesteryl-Fucoidan was used to prepare site-specific liposomes obtained by the lipid thin film.[21] Spherical vesicles were formed and observed by transmission electronic microscopy (TEM) (Figure 3).[22] Those liposomes presented a less electron dense corona, characterizing the presence of fucoidan on the vesicle surface. The mean diameter size of fucoidan-coated liposomes was 228 ± 3.74 nm, PDI of 0.360 and zeta potential value of 2.07 ± 0.18 mV. Comparing with uncoated-liposomes, particle size 117 ± 0.14 nm, PDI of 0.296 and zeta potential of +21.3 ± 0.51 mV was found. The results of the surface charge suggest, in addition to TEM, that the external bilayer of liposomes was recovered by the polysaccharide.

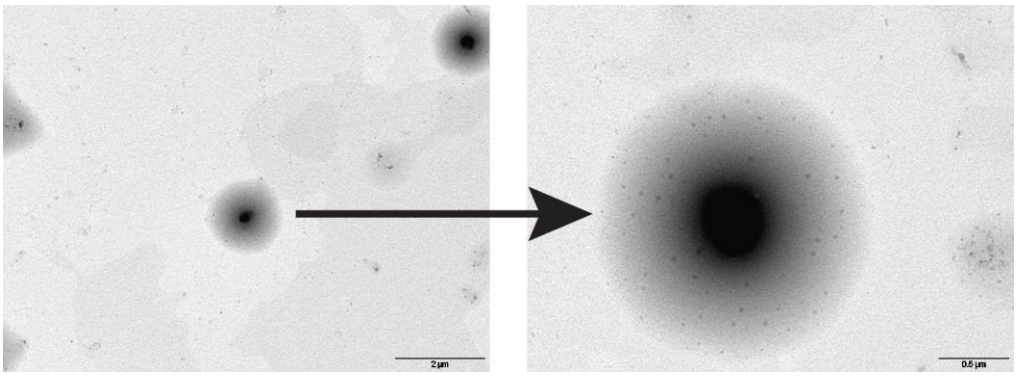


Figure 3. TEM images of cholesteryl-fucoidan liposomes obtained by the lipid thin film method.

To further evaluate whether the presence of fucoidan at the surface of liposomes can target the nanosystems through macrophages, an uptake study was performed aiming to investigate the cellular internalization of fluorescent-labeled uncoated-liposomes and also fucoidan-coated liposomes, at different incubation time (15, 30 and 60 minutes).[23] The cellular up take of fucoidan-coated liposomes and conventional liposomes occurred at 60 minutes. On the other hand, different observations were highlighted at 15 and 30 minutes (Figure 4). While no significant fluorescence was observed after incubation with conventional liposomes, a clearly increase on fluorescence intensity was detected after incubation with fucoidan-coated nanoparticles. According to Lira et al., (2011)[23] the presence of fucoidan on the surface of poly (isobutylcyanoacrylate) nanoparticles could influence the way nanoparticles are interacting with macrophages (J774) and the fate of the nanoparticles within the cells. In our work the results suggest that the uptake of fucoidan-coated liposomes by cells was faster than conventional liposomes, indicating a targeting to macrophages.

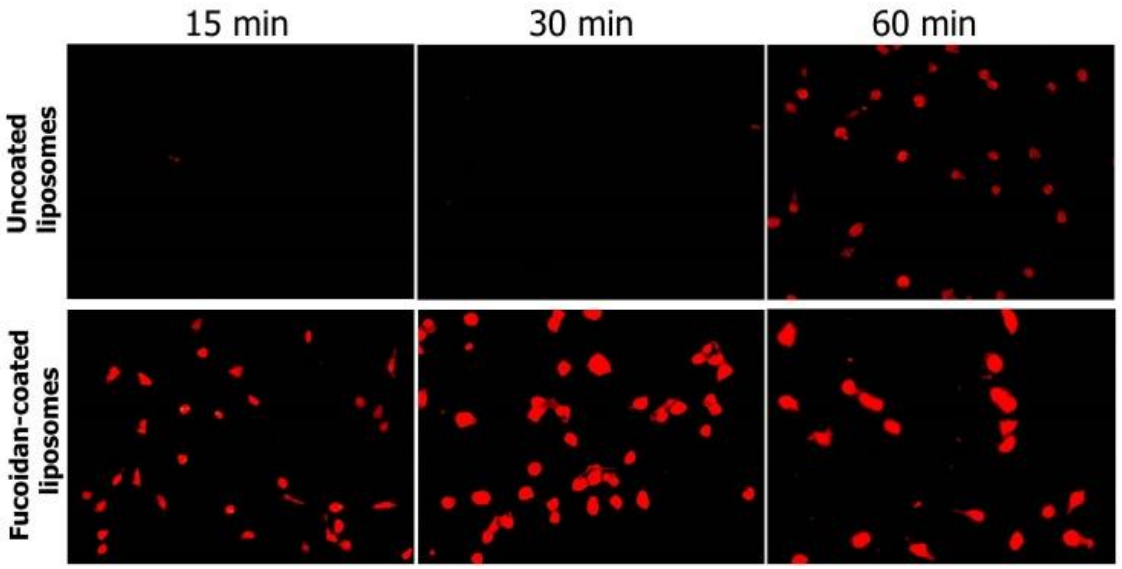


Figure 4. Fluorescence microscopy of Raw 264.7 macrophages incubated with rhodamine-labelled fucoidan-coated and uncoated liposomes at different incubation time.

In summary, an efficient and fast procedure involving microwave-assisted synthesis of cholesteryl-fucoidan has been developed. This protocol has several advantages including a good yield of the final product, short reaction time, simple workup and possible applicability to synthesize others hydrophobized polysaccharides. Further assays will be performed to optimize

the formulation aiming to obtain a novel system designed as a potential drug carrier for infectious diseases.

Acknowledgment

The first author thanks the Brazilian Education Ministry (MEC/CAPES) for a PhD schoolarship. The authors are grateful to the Foundation for Science and Technology Development of the Pernambuco State of Brazil – FACEPE for a financial support (grant #APQ0676-4.03/10). NSSM thanks the Brazilian National Council for Research – CNPq for financial support (grant# APQ484574/2011-6). Also much appreciated was the collaboration with the Institute of Technologies of the Northeast Brazil – CETENE, Department of Fundamental Chemistry of the Federal University of Pernambuco (DQF-UFPE) and Laboratory of Immunophatology Keizo-Asami (LIKA).

References and Notes

1. (a) Sihorkar, V.; Vyas, S.P. *J. Pharmaceut. Sci.* **2001**, 4, 138. (b) Jain, K.; Kesharwani, P.; Gupta, U.; Jain, N.K. *Biomaterials* **2012**, 33, 4166.
2. Liu, Z.; Jiao, Y.; Wang, Y.; Zhou, C.; Zhang, Z. *Adv. Drug Del. Rev.* **2008**, 60, 1650.
3. (a) Sivakumar, P.A.; Rao, K.P. *Carbohydr. Polym.* **2003**, 51, 327. (b) Vyas, S. P.; Kannan, M.E.; Jain, S.; Mishra, V.; Singh, P. *Int. J. Pharm.* **2004**, 269, 37. (c) Chono, S.; Tanino, T.; Seki, T.; Morimoto, K. *J. Control. Release* **2008**, 127, 50.
4. (a) Akiyoshi, K.; Deguchi, S.; Moriguchi, N.; Yamaguchi, S.; Sunamoto, J. *Macromolecules* **1993**, 26, 3062. (b) Wang, Y.; Tu, S.; Li, R.; Yang, X. Y.; Liu, L.; Zhang, Q. *Nanomedicine: Nanotech. Biol. Med.* **2010**, 6, 471. (c) Boridy, S.; Takahashi, H.; Akiyoshi, K.; Maysinger, D. *Biomaterials* **2009**, 30, 5583.
5. Yang, L.; Zhang, B.; Wen, L.; Liang, Q.; Zhang, L.M. *Carbohydr. Polym.* **2007**, 68, 218.
6. Tong, Y.; Guan, H.; Wang, S.; Xu, J.; He, C. *Carbohydr. Res.* **2011**, 346, 495.
7. Li, B.; Lu, F.; Wei, X.; Zhao, R. *Molecules* **2008**, 13, 1671.
8. (a) Holtkamp, A. D.; Kelly, S.; Ulber, R.; Lang, S. *Appl. Microbiol. Biotechnol.* **2009**, 82, 1. (b) Wijesinghe, W.A.J.P.; Jeon, Y. J. *Carbohydr. Polymers* **2012**, 88,
9. Zvyagintseva, T.; Shevshenko, N.; Nazarova, I.; Scobun, A.; Lukyanov, P.; Elyakova, L. *Comp. Bioch. Phys. Part C* **2000**,126, 209.
10. Kim, W. S.; Ordija, C. M.; Freeman, M. W. *Biochem. Biophys. Res. Commun.* **2003**, 310, 542.
11. Patankar, M. S.; Oehniger, S.; Barnett, T.; Williams, R.L.; Clark, G. F. *J. Biol. Chem.***1993**, 29, 21770.
12. Rupérez, P.; Ahrazem, O.; Leal, J. A. *J. Agric. Food Chem.* **2002**, 50, 840.
13. Rioux, S.E.; Turgeon, S.L.; Beaulieu, M. *Carbohydr. Polym.* **2007**, 69, 530.
14. M.J. Clement; B. Tissot; L. Chevolot; E. Adjadj; Y. Du; P.A. Curmi; P.A. R. Daniel, Glycob., 2010, 20, 894.
15. **Extraction procedure:** Fucoidan was extracted from the powered algae *Fucus vesiculosus* (100 g) using selective solvents under constant stirring at 150 rpm and controlled temperature. First, ethanol 85% (v/v) at 25°C (2 x 12h) and 70°C (2 x 5h) was used to extract pigments and proteins. The solvent was separated from residual seaweeds by filtration. Residual seaweeds were treated with $CaCl_2$ 2% (w/v) at 70°C (3 x 3h) in order to precipitate alginates as well to extract a mix of laminaran and fucoidan and then, separated. Fucoidan

was extracted from residual algae with 0.01 M, pH 2, at 70°C (3 x 3h), centrifuged and dialyzed (cut off 1000 Da) during 48h. Then, Fucoidan was freeze-dried. ^{1}H NMR (400 MHz, D_2O): δ= 5.5-4.7 ppm (*m*, α-glycosidic configuration), δ= 4.4-1.8 ppm (*m*, anomeric-H),*δ*=1.5 ppm (*s*, 6-deoxi-sugar-H). IR (KBr, cm^{-1}): 3446 (OH), 1261 (S=O), 1029-1168 (hemiacetal stretching), 849 (SO_3 at the axial C-4 position of fucose). Elemental analysis: C, 26%; H, 5.5%; S, 7.2%.

16. **Hydrolysis of Fucoidan**: Fucoidan solution at a concentration of 20 mg/mL was prepared and acidified with 0.1M HCl solution to achieve pH 2.5. Then, the solution was heated in a water bath at 60°C for 40 min under stirring. After, the sample was neutralized with 0.1M NaOH (pH=7) and freeze-dried.
17. **Molecular weight determination:** The molecular weight of fucoidan was determined by HPSEC-RI. Solutions (1 mg/mL) of dextran standards with different molecular weights (5, 25, 50, 150 and 410 KDa) were used to construct an analytical curve. A column Ultrahydrogel Linear (300 × 7.8 mm d.i., Waters) was used to analyze the standards and samples. The mobile phase consisted of 0.1 M $NaNO_3$ solution. The flow rate was 0.5 mL/min and analyses were performed at room temperature and detected by refractive index detector. The regression equation was Log MM = Retention time – 28.38663/-1.34508 (r^2= 0.9853, n= 3).
18. **Synthesis of cholesteryl-fucoidan:** Compound **2** (50 mg, 90.1 µmol, 8.43 equiv), fucoidan (**3**, 321 mg, 10.7 µmol, 1 equiv) and DABCO (1.5 mg, 13.1 µmol, 0.15 equiv) were added in 5 mL of dry DMSO in a 25 mL round bottom flask and heated at 100°C with continuous stirring. After complete consumption of **3** (checked by TLC), 60 mL of ethanol were added to the reaction mixture and stored overnight at 4°C. The precipitate obtained was separated, purified by dialysis against distillated water and lyophilized.^{1}H NMR (300 MHz, D_2O): δ= 4.8-4.7 ppm (*m*, fucose-H), δ= 3.1-4.0 ppm (*m*,fucose-H), *δ*=1.0-2.0 ppm (*m*, cholesterol-H). IR (KBr, cm^{-1}): 3448 (OH), 2937 (CH_2), 1624 (C=O), 1259 (S=O), 1032 (COC).
19. **Synthesis of cholesteryl N-(6-isocyanatohexyl)carbamate:** Cholesterol (**1,** 3.9 g, 10.08 mmol, 1 equiv), hexamethylenediisocyanate (3.38 g, 20.17 mmol, 2 equiv), DABCO (0.07 g, 0.61 mmol, 0.06 equiv) and dry toluene (50 mL) were placed in a 125 mL round bottom flask and heated at 100°C under continuous stirring until completed reaction. The proceeding of reaction was monitored by TLC (appearance of a single spot of cholesterylN-(6-

isocyanatohexyl)carbamate (**2**) at R_f 0.58 developed by chloroform). Then, the mixture was concentrated in vacuum. The residue was poured in to 400 mL of petroleum ether and stored overnight at -10°C. The precipitate obtained was separated by centrifugation, washed with petroleum ether and dried in a vacuum desiccator. ^{1}H NMR (300 MHz, $CDCl_3$): δ= 5.31 ppm (1H, m, cholesterol 14-H_1), δ= 3.22 ppm (2H, *t*, J= 12 Hz, CH_2CNO), δ=3.1 ppm (2H, *m*, CH_2N), δ=0.78-2.3 ppm (28H, CH_2, CH_1), δ= 1.25-1.56 ppm (*m*, C_4H_8) ,δ= 0.6 ppm (3H, *s*, cholesterol 18-H_3). IR (KBr, cm^{-1}) : 3445 (NH), 2936 (CH_2), 2258 (NCO), 1690 (C=O), 1137 (OCO).

20. Shukla, P.; Choudhary, M.K.; Nayak, S.K. Synlett 2011, 11, 1585.

21. Lira, M. C. B.; Siqueira-Moura, M. P.; Rolim-Santos, H. M. L.; Galetti, F. C. S.; Simioni, A. R.; Santos, N. P.; Tabosa do Egito, E. S.; Silva, C. L.; Tedesco, A. C.; Santo-Magalhães, N. S. *J Liposome Res.* **2009**, 19, 49. Briefly, liposomes were prepared using the dried thin lipid hydration method (Lira et al., 2009) followed by sonication. Briefly, lipids (soya phosphatidylcholine, cholesterol, and stearylamine) were dissolved in a mixture of chloroform and methanol (3:1 v/v) under magnetic stirring. The solvents were completely removed and the thin film formed was hydrated with 10mL of phosphate buffer solution (7.4 pH). In order to obtain fucoidan-coated liposomes, the lipid film was hydrated with a phosphate buffer solution containing the ligand corresponding to hydrophobized fucoidan (57:1 lipid:ligand). Fluorescent-labelled liposomes were prepared following the method described, using 150µL of a rhodamine solution (1mg/mL) in methanol added in organic phase.

22. **Liposomes characterization:** Liposome formulations were characterized by mean hydrodynamic diameter, polydisperse index (PDI), surface charge (zeta potential) of vesicles and morphology by TEM. The morphology of the liposomes was observed by transmission electron microscopy (TEM) (FEI, Tecnai20) at the Centro de Tecnologias Estratégicas do Nordeste (CETENE), Recife, PE, Brazil. The liposomes suspensions were spread 200 mesh copper grid coated with carbon and stained with a drop of 1% phosphotungstic acid. The excess fluid was removed and the grid was dried for 2-5 minutes in desiccator and after observed by TEM. The zeta potential (ζ mV) was measured at 25 °C using the electrophoresis technique (Malvern Zetasizer Nano ZS90, Malvern Instruments Ltd., Worcestershire, UK). The polydisperse index and diameter of the liposomes were analyzed using photon correlation spectroscopy (Delsa™ Nano S Particle analyzer, Beckman Coulter, Brea, CA, USA), at 25 °C

at a fixed angle of 90°. For the analysis, the samples were adequately diluted in purified water. The results represent the average of three determinations.

23. Lira, M. C. B.; Santo-Magalhães, N. S.; Nicolas, V.; Marsaud, V.; Silva, M. P. C.; Ponchel, G.; Vauthier, C. *Eur. J. Pharm. Biopharm.* **2011**, 79, 162. The uptake of liposome was investigated on Raw 264.7 macrophages (10^5 cells/well) using fluorescence microscopy (Carl Zeiss Microscopy GmbH 37081, Gottingen, Germany). Cells were grown on a glass cover-slip placed in 12-well culture plate, and incubated with fluorescent-labeled liposomes coated and uncoated with fucoidan for 15, 30 and 60 minutes at 37^oC. Subsequently, cells were fixed with 4% paraformaldehyde (800 µL) for 20 min. Next 1 mL of 50 mM ammonium chloride (NH_4Cl) was added and incubated for 10 min. Finally, the cover-slips were sealed on microscope glass slide and analyzed.

6. CAPÍTULO 2

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Fucoidan-coated liposomes as a system for targeting usnic acid to macrophages

Rafaela S. Ferraz[1], Mariane C. B. Lira-Nogueira[1-2], Marcela A. Pereira[1], Terezinha G. Silva[3], Jaciana G. Aguiar[3], Taciana L. Salviano[1-2], Fabrício O. Souto[1], Nereide S. Santos-Magalhães[1*]

[1]Laboratório de Imunopatologia Keizo-Asami, Universidade Federal de Pernambuco, Recife, PE, Brazil

[2]Centro Acadêmico de Vitória, Universidade Federal de Pernambuco, Vitória de Santo Antão, PE, Brazil

[3]Departamento de Antibióticos, Universidade Federal de Pernambuco, Recife, PE, Brazil

* Corresponding author:
Nereide Stela Santos-Magalhães
Universidade Federal de Pernambuco (UFPE)
Laboratório de Imunopatologia Keizo-Asami (LIKA)
Av. Prof. Moraes do Rego, 1235, Cidade Universitária,
50670-901, Recife, PE, Brazil
Tel: + 55-81-21268587; fax: + 55-81-21268485
E-mail addresses: nssm@ufpe.br

ABSTRACT

The present study investigated the potential of chemically modified cholesteryl-fucoidan in the development of fucoidan-coated liposomes and their ability in guiding liposomal delivery system to macrophages. Fucoidan was coated liposomes surface during preparation and usnic acid (UA), a secondary lichen metabolite, was encapsulated. The fucoidan coated and uncoated liposomes were evaluated for particle size (Ø), polydispersity index (PDI), surface charge (ζ), UA encapsulation efficiency, *in vitro* cytotoxicity and cellular uptake on RAW 264.7 macrophage. It was observed that when the amount of fucoidan in liposomes formulation was increased from 5 to 20 mg, vesicles size significantly increased from 168 ± 2.82nm to 1.18 ± 0.01µm. PDI values varied from 0.36 to 0.46, and, as expected, ζ decreased (from 1.35 ± 0.185 mV to -5.41 ± 0.234 mV) once fucoidan is a negatively charged polysaccharide. The change in zeta potential from + 20.0 ± 0.41 mV for uncoated liposomes (UA-$Lipo_{80}$) to – 5.41 ± 0.23 mV for fucoidan coated liposomes (UA-$Lipo_{fuc20}$) suggest that the liposome were coated by the fucoidan. Cytotoxicity study revealed that all formulations of fucoidan-coated liposomes exhibited low IC_{50} values (8.26 ± 1.11 µM) as compared with uncoated liposomes (18.37 ± 3.34 µM), both containing usnic acid. These results indicate that fucoidan coated liposomes were uptake more rapidly and consequently the concentration of usnic acid within the cell increased, potentializing its action. In addition, the IC_{50} values found for $Lipo_{fuc20}$, which presents the highest amount of fucoidan, was similar to $Lipo_{80}$, both without UA, indicating that fucoidan was not cytotoxic. The results from cellular uptake experiments suggested that the fucoidan-coated liposomes were internalized through the scavenger receptors (SR) found on the surface of macrophages. Targeting ability of fucoidan-coated liposomes suggest that this polysaccharide could be used to promote specific interactions of a drug delivery system with macrophages, can be used in the therapy of intracellular diseases, such as tuberculosis.

Keywords: fucoidan, scavenger receptors, usnic acid, liposomes, macrophages, tuberculosis.

1. Introduction

Treatment of intracellular diseases, in particular tuberculosis, is intimately related to targeting the drugs until macrophages. However, most of antibiotics used in clinical, although highly active in vitro, do not actively pass through cellular membranes, because of their hydrophilic characteristics. Thus, the main challenge for intracellular chemotherapy is to develop an alternative to carrier antibiotics, which could be able to target the drug into phagocytic cells and, once inside the cells prolong release of the drug [1,2]. In this context nanosystems, such as liposomes, are well suited as vehicles for the delivery of antimicrobial agents to macrophages [3].

Conventional liposomes are not capable to deliver the drug to target cells, being necessary to evaluate changes in its surface by the addition of ligants. These ligants allows them to recognize and bind target tissues and organs, promoting an increase in therapeutic properties while decrease drugs untoward aspects. [4]. currently, several compounds have been used as ligands to modify the surface of liposomes. Among the ligands, polysaccharides attract the attention once macrophages express pattern recognition receptors carbohydrates such as mannose receptor (MR), Toll-Like receptors and macrophages scavenger receptors (SR) [5]. In this way, it is reported the use of carbohydrate conjugated to the liposomes surface, such as pullulan [6], dextran [7], 4-sulfated N-acetyl galactosamine [2] and arabinogalactan [8] to actively drive systems to carbohydrate receptors expressed on the surface of macrophages.

The present study explored the use of fucoidan, a sulfated polysaccharide isolated from brown algae, as a binder for targeting liposomes to macrophages. Fucoidan was chosen mainly because of two major properties: the first one is related to its ability to bind membrane receptors of type AI and AII called macrophages scavenger receptors (SR-A) [9], which contains a C-type carbohydrate recognition domain at the carboxy terminus-hence named SR with C type lectin I (SRCL-I) [10]; and the second major property is his ability of not activate complement system [11,12]. These two properties are of great interest to obtain site-specific nanosystems capable of carrying drugs directly to infected macrophages, being an alternative in the therapy of intracellular diseases such as tuberculosis and leshimaniose. In parallel to the development of a site-specific system, we propose to encapsulate usnic acid, once this secondary lichen metabolite presents antimycobacterial activity [13,14].

Within this framework, the greatest interest of our work was to develop and characterize fucoidan-coated liposomes containing usnic acid, as a site-specific and long-circulating

nanosystem. Furthermore, the assessment of cytotoxicity and uptake cellular of these systems was evaluated on RAW 264.7 macrophages.

2. Materials and Methods

2.1. Materials

Cholesterol (Chol), stearylamine (SA), (+)-Usnic acid, Albumin from bovine serum (BSA), Mannan from S. cerevisiae and galactose were purchased from Sigma-Aldrich (St. Louis, USA). Soybean phosphatidylcholine (PC) (94% Epikuron 200®) was obtained from Lipoid GMBH (Ludwigshafen, Germany). The brown algae *Fucus vesiculosis*, which fucoidan was extracted and purified, was provided by M. J. Flag (Recife, Brazil). All solvents and other chemicals were supplied by Merck (Darmstadt,Germany).

2.2. Synthesis of hydrophobized fucoidan

The cholesteryl-fucoidan (Chol-fuc) was synthesized as follows, Cholesteryl N-(6-isocynatohexyl) carbamate, fucoidan and DABCO were added to of dry dimethyl sulphoxide (DMSO) under microwave irradiation (120 W and 40 min) at 100°C with continuous stirring. Subsequently, 60 mL of ethanol were added to the reaction mixture and stored overnight at 4°C. The precipitate obtained (cholesteryl-fucoidan) was filtrated and dialyzed against distillated water and lyophilized. The material was stored in desiccator until use.

2.3. Preparation of liposomes containing usnic acid

Liposomes containing usnic acid where prepared using the dried thin lipid hydration method followed by sonication. Initially, formulations without surface changes, i.e. without the polysaccharide on the surface, were prepared aiming to choose best constituent concentrations due to physicochemical characteristics. Briefly, lipids (soya phosphatidylcholine, cholesterol, and stearylamine (8:1:1) ranging from 42 to 80 mM and usnic acid (2.90 to 5.80 mM) were dissolved in a mixture of chloroform and methanol (3:1 v/v) under magnetic stirring (Table 1).

The solvents were completely removed under pressure (37 °C, 80 rpm) and the thin film formed was hydrated with 10 mL of phosphate buffer solution (7.4 pH). The liposomal suspension was then sonicated (Vibra Cell, BRANSON, USA) at 200W and 40 Hz for 300 s aiming to obtain small unilamellar vesicles in a nanoscale.

2.3.1. Preparation of fucoidan-coated liposomes containg usnic acid

Finally, in order to prepare fucoidan-coated liposomes, in addition to PC/CH/SA (80 mM) in a molar ratio of 8:1:1 the film formed was hydrated using different amounts (5, 10 and 20 mg) of cholesteryl-fucoidan solution (in phosphate buffer pH 7.4). Table 1 shows the different formulations developed in relation to the concentrations of drug and fucoidan for coating liposomes.

For in vitro assays, fluorescent-labelled $Lipo_{Fuc}$ were prepared using 1 mL of a rhodamine solution (1.5 mg/mL) in methanol added to organic phase.

Table 1.

Uncoated and Fucoidan-coated liposomes containing usnic acid

Liposome Formulations	Code Formulations	Lipids (mM)	PC (mM)	Chol (mM)	SA (mM)	UA (mM)	Lipid:ligant (w/w)
UA-loaded Liposomes	UA-$Lipo_{42}$	42	35.73	4.42	4.19	2.90	-
	UA-$Lipo_{50}$	50	42.54	5.30	4.97	5.80	-
	UA-$Lipo_{60}$	60	51.06	6.38	5.97	5.80	-
	UA-$Lipo_{80}$	80	68.08	8.50	7.97	5.80	-
UA loaded Fucoidan-coated Liposomes	UA-$Lipo_{fuc5}$	80	68.08	8.50	7.97	5.80	114:1
	UA-$Lipo_{fuc10}$	80	68.08	8.50	7.97	5.80	57:1
	UA-$Lipo_{fuc20}$	80	68.08	8.50	7.97	5.80	28:1

PC= Phosphatidylcholine; Chol= Cholesterol; SA= Stearylamine; UA= Usnic acid; Chol-fuc= Cholesteryl-fucoidan; Lipo= Liposomes

2.4. Physicochemical characterization of liposomes

Developed liposomal formulations were characterized after surface ligand anchoring. First, liposomes were characterized by vesicle size and size distribution studies (PDI) which were carried out using a photon correlation spectroscopy (Beckman Coulter Delsa™ Nano S Particle analyzer). Measurements were made at 25 °C with a fixed angle of 90° and size values are the mean of the liposomal hydrodynamic diameter (nm). The polydispersity index was measured by the same method and equipment. Zeta potential (ζ mV) was measured at 25 °C using the

electrophoresis technique (Malvern Zetasizer Nano ZS90, Malvern Instruments Ltd., Worcestershire, UK). In this experiment liposome dispersions (20 µL) were diluted in 1 mL of purified water. The results represent the average of three determinations.

2.4. Usnic acid content and drug entrapment efficiency

Usnic acid content into liposomes was determined using the UV spectrophotometric method previously validated by Lira et al., (2009b). An aliquot of liposomal sample (50 µL) was diluted in methanol (10 mL) and analyzed using a spectrophotometer (Ultrospec® 300, Armshan Pharmaceutical) at 280 nm. Samples were analyzed for determining the UA content which was then calculated using the equation: % drug content = (Measured UA amount/ Theoretical UA amount) X 100.

Drug encapsulation efficiency was determined by the ultrafiltration/ultracentrifugation technique, using Ultrafree® units (Millipore, USA, MW cut-off = 10.000 Da). Liposomes (400 µL) were inserted in filtration unit and submitted to ultracentrifugation at 8792 g for 1 h. The sample passed through the filter (50 µL), corresponding to unloaded UA amount, was diluted in 2 mL of methanol and UA was quantified as described above. Data of drug entrapment efficiency are calculated using the equation: $\%EE = (UA_{content} - UA_{content\ filtrate}/\ UA_{content}) \times 100$.

2.5. Cytotoxicity studies

The cytotoxicity of uncoated and fucoidan-coated liposomes containing usnic acid was evaluated on RAW 264.7 macrophages line, purchased from the cells bank of Rio de Janeiro (Brazil). Cells were maintained in culture bottles of 25 cm^2, in an atmosphere of 5% CO_2 and 37°C throughout the experimental period. The number of viable cells was determined by the estimation of their mitochondrial reductase activity (MTT). Briefly, cells (1 x 10^5 cells/well) were seeded into 96-well microplates and grown in a humidified 5% CO_2 incubator at 37°C, for 24 h. After incubation, samples of uncoated liposomes ($Lipo_{80}$ and UA-$Lipo_{80}$) and fucoidan-coated liposomes with and whithout usnic acid, at concentrations ranging from 0.7 to 40 µM, and 0.81 to 29.03 µM, respectively, were added to the plates for a 24 h incubation period. Then, MTT (5.0 mg/mL) was added and incubated for more two hours. Lately, the formazan product was dissolved in DMSO (100 µL) and absorbance was measured using a multi-plate reader at 560 nm. Fucoidan-coated liposomes without usnic acid ($Lipo_{fuc20}$) were used to demonstrate that the

fucoidan is non-cytotoxic. The cytotoxicity was expressed as the concentration required inhibiting 50% of the cell proliferation (IC_{50}). All experiments were carried out in triplicate, and the statistical significance was determined by using the Student's *t*-test.

2.6. Competition assay

RAW 264.7 macrophages were cultured in full phenol red-free DMEM in 12-well plates for 24 h at 10^5 cells/mL (500 µL/well). The competition assay was carried out as described by Ruan et al. [15]. D-galactose, used as negative control, was prepared at 20 mM, mannan and fucoidan used as inhibitors, was prepared at 1 mg/mL. Competitive inhibitors (mannan and fucoidan) and D-galactose were preincubated with the cells for 30 min. Then, fluorescent-labelled $Lipo_{Fuc5}$ (3.53 µg/mL) was added to the plates and was incubated for 2 h.

After, the cells were washed three times with PBS, detached by scraping and centrifuged (1000 g for 10 min). Then, the pellet was resuspended in 2% BSA and analyzed by flow cytometry (FACS BD Accuri C6, BD Biosciences) measuring seven thousand events in each sample. The experiments were performed in duplicate. The toxicity of the competitive inhibitors was evaluated using MTT assay.

3. Results and discussion

3.1. Physicochemical characterization of liposomes

The literature vastly describes the surface modification of nanocarriers, after the addition of ligands, including polysaccharides, aiming to develop site-specific systems [16-19]. Beyond polysaccharide, a multitude of ligands are currently being assessed including peptides, antibodies, proteins, glycolipids, glycoproteins, and lectins which make use of mononuclear phagocytes characteristic receptor expression and phagocytic innate processes [20]. In that way, we harnessed that knowledge to hypothesize that adding fucoidan on the surface of liposomes, it would be possible to target them through a specific cells type, such as macrophages.

Initially, different batches of usnic acid-loaded liposomes were prepared using different lipid concentrations (from 42 to 80 mM) and varying drug concentration (2.90 to 5.80 mM), in order to achieve a stable formulation containing the highest amount of drug (Table 2). Lately, UA-loaded liposomes were evaluated according to particle size, PDI, ζ potential and drug efficiency (Table 3). All formulations were stable in dispersion form for 30 days, except liposomes prepared at 50 and 60 mM with usnic acid which presented drug precipitation after 24

h. UA-loaded liposomes presented particle sizes ranging from 117 ± 0.14 to 172 ± 0.70 nm and polydispersity index was lower than 0.330, suggested that this system are homogeneus [21], presenting a positive surface charge due to the use of cationic lipid (SA) and the drug efficiency ratio was near 100%. It is known that lipophilic drugs tend to present high encapsulation efficiency in liposomes when compared to hydrophilic compounds [22]. The high ratio of usnic acid encapsulation was reported previously by Lira et al. [13]. They prepared liposomes consisting of PC:CH:SA in the ratio 7:2:1 containing UA (1 mg/mL) and showed an encapsulation rate of approximately 96%. These results indicate that the change in lipid ratio from 7:2:1 to 8:1:1 (PC:CH:SA) did not influence on the drug encapsulation ratio efficiency.

Table 2. Physicochemical characterization of usnic acid loaded-liposomes.

Liposomes formulations	UA (mM)	**Size (nm)**	**PDI**	**ζ (mV)**	**EE (%)**
UA-Lipo$_{42}$	2.90	117 ± 0.14	0.29	+12 ± 0.50	99.07 ± 0.10
*UA-Lipo$_{50}$	5.80	172 ± 0.70	0.32	+13 ± 2.33	98.93 ± 0.02
*UA-Lipo$_{60}$	5.80	139 ± 0.79	0.33	+15 ± 1.50	98.61 ± 0.05
UA-Lipo$_{80}$	5.80	133 ± 0.90	0.33	+21 ± 0.51	99.16 ± 0.19

UA: Usnic acid; UA-Lipo$_{42,50,60,80}$: AU-liposomes loaded with different concentrations of total lipids; * Usnic acid precipted after 24h

3.2. Physicochemical characterization of fucoidan-coated liposomes

After physicochemical characterization of UA-loaded liposomes, the chosen formulation to prepare fucoidan-coated liposomes containing UA was that composed of 80 mM of lipids and 5.80 mM of usnic acid. From this formulation, the total lipid:ligand ratio was varied, ranging from 114:1 to 28:1 (w/w), in order to achieve maximum coating. Table 3 describes the physicochemical characteristics of fucoidan-coated liposomes. The size and polydispersity index of the fucoidan-coated liposomes increased from 168 ± 2.82 nm and 0.36 to 1.18 ± 0.01µm and 0.46 when the amount of fucoidan was increased from 5 to 20 mg, respectively. In addition, all formulations exhibited higher encapsulation efficiency of usnic acid (98%) after the anchoring of cholesteryl fucoidan, indicating that the coating has not affected drug encapsulation. The increase in particle size after coating polysaccharide was also observed in liposomes developed by

Venkatesan; Vyas [23]. The authors described that uncoated liposomes presented 2.5 µm, O-palmitoylpullulan-coated liposomes exhibited a mean size of 4.5 µm.

It can be noted that, while the proportion of anionic polysaccharide increase, a decrease in the zeta potential value from 1.35 ± 0.185 to -5.41 ± 0,234 mV was found. This change in the surface charge of +20 mV (UA $Lipo_{80}$) to -5mV (UA $Lipo_{Fuc20}$) is due to the negative charge of the fucoidan [24], that coat the liposomes. The increase in particle size, in addition to a decrease in the zeta potential measurements could suggest that fucoidan is located on the surface of liposomes. Alterations in the zeta potential values due to changes on the amount of ligand have been reported by Vyas et al. [25]. The authors developed liposomes at a ratio of 7:2:1 (PC:CH:SA) coated with mannan-cholesteryl (CHM) in different proportions of lipid:ligant (10:0 to 1:1). The initial positive value of zeta potential (+40 mV) decreased on increasing the ratio of anionic polysaccharides (CHM) and approached towards a minimum of +5 mV. Singodia et al. [2] evaluated the potential of 4-SO_4GalNAc (Sulf-Lip) to target mannose receptors on macrophages after surface decoration of Amphotericin B loaded liposomes. About Sulf-Lip, the 4-SO4GalNAc was adsorbed through electrostatic interaction on cationic liposomes, which was confirmed by change in zeta potential from +48.2 ± 3.7 mV for Lip to +12.2 ± 1.3 mV for Sulf-Lip.

Table 3. Physicochemical characteristics of fucoidan-coated liposomes containing usnic acid.

Code Formulation	**Lipid:Ligand (w/w)**	**Vesicles sizes**	**PDI**	**ζ (mV)**	**UA EE (%)**
UA-$Lipo_{80}$	-	133 ± 0.90 nm	0.33	20 ± 0.41	99.16 ± 0.19
UA-$Lipo_{Fuc5}$	114:1	168 ± 2.82 nm	0.36	1.35 ± 0.18	99.31± 0.01
UA-$Lipo_{Fuc10}$	57:1	252 ± 3.53 nm	0.38	- 2.15 ± 0.05	99.21 ± 0.03
UA-$Lipo_{Fuc20}$	28:1	1.18 ± 0.01 µm	0.46	-5.41 ± 0.23	98.67 ± 0.03

UA: Usnic acid (5.80 mM); UA-$Lipo_{80}$: UA-loaded liposomes; UA-$Lipo_{Fuc}$: UA loaded fucoidan coated liposomes; Fuc: Fucoidan (5, 10 and 20 mg)

3.3. *In vitro* cytotoxicity of UA-loaded fucoidan coated liposomes

The idea of developing fucoidan-coated liposomes ($Lipo_{Fuc}$) was to improve the affinity for macrophages. Thus, to investigate whether the presence of fucoidan at the surface of

liposomes actually target the liposomes through macrophages in comparison with uncoated liposomes the in vitro cytotoxicity was studied. This study was based on the cytotoxicity evaluation of different fucoidan coating liposomes on Raw 264.7 macrophages and was used as a parameter for choosing the best formulation.

Figure 1 shows the IC_{50} of fucoidan-coated liposomes containing UA after incubation with RAW 264.7 macrophage cell line. UA-$Lipo_{fuc20}$ had IC_{50} values seven-fold lower than the pure usnic acids, as well as 2.22-fold lower than UA-Lipo80. These results suggest that the encapsulation of the UA into fucoidan coated liposomes increased the liposomes capture by the macrophages, leading to an increase of UA cytotoxicity. Lira et al., 2009 also observed a decrease in IC_{50} values of UA after encapsulation into liposomes (IC_{50_UA}= 63.84 µM and $IC_{50\_UA\text{-}Lipo}$= 36.29 µM).

The cytotoxicity results suggest that the fucoidan is a promoter of recognition by macrophages. This recognition leads to an increase in the internalization of the fucoidan coated liposomes in these cells. In addition, fucoidan was capable to increase the amount of usnic acid within cells justifying a decrease of the value of IC_{50} in comparison with uncoated liposomes containing usnic acid. In parallel, the $Lipo_{fuc20}$ was evaluated on RAW macrophages and no statistically difference was observed in IC_{50} values compared with $Lipo_{80}$. This was in accordance to results expected, and support our hypothesis that fucoidan anchored at the outer layer of liposomes could promote interactions of liposomes with macrophages thanks to a specific recognition of this polysaccharide by macrophage scavenger receptors (SR) exposed at the surface of the macrophages [26,27].

Despite UA-$Lipo_{fuc20}$ (8.26 ± 1.11 µM) presented similar IC_{50} value than UA-$Lipo_{fuc10}$ (10.47 ± 0.60 µM), it was excluded due to the micrometer particle size range once. According Epstein-barash et al. [28], large liposomes (190 ± 24 nm) activate cytokines and tend to deposit in the lungs. $Lipo_{fuc5}$ was chosen to continue the experiments once it presented a particle size lower than 200 nm and no statistica difference in the IC_{50} value in relation to $Lipo_{fuc10}$.

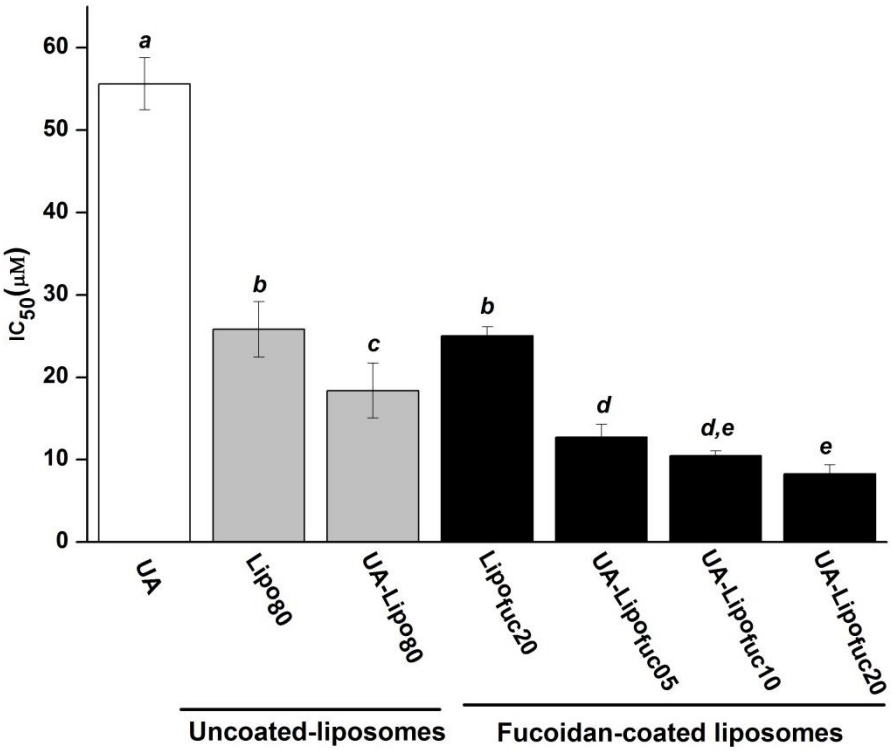

Figure 1. Cytotoxicity of fucoidan-coated liposomes at different fucoidan concentrations (5, 10 and 20 mg Fuc) on 264.7 RAW macrophages for 24 h (n=3). Different letters differ statistically by Student's *t*-test ($p < 0.05$). UA: Usnic acid; $Lipo_{80}$: Liposome without UA; UA-$Lipo_{80}$: UA-loaded liposomes; $Lipo_{Fuc20}$: fucoidan coated liposomes without UA; UA-$Lipo_{Fuc}$: fucoidan coated liposomes containing UA; Fuc = Fucoidan (5, 10 and 20 mg).

3.4. Competition assay

To assess that fucoidan coated liposomes phagocytosis occurs through the C-type carbohydrate recognition domain present in the SR-A, mannan, fucoidan and galactose were used as different competition inhibitors. In the competition assay on Raw 264.7 cells, mannan and fucoidan at 1 mg/mL inhibited in 71.43 % and 51.02 % uptake of control respectively, whereas galactose did not inhibit the uptake (Fig. 2).

Ruan et al. [15] investigated the specific mannose receptor internalization of spermine-mannan systems by a competition assay. They used different concentrations of carbohydrates (mannan and galactose) as competition inhibitors. They found that mannan, at 1 mg/mL, was capable to inhibit the uptake of their systems, related to control. Galactose, at different concentrations, presented no inhibition effect in cellular uptake. These results corroborate with

our study, indicating that fucoidan coated liposomes was internalized through a specific SR-A mediated pathway. Free fucoidan (1 mg/mL) was also tested as a competitive inhibitor and was able to inhibit the cellular uptake of fucoidan coated liposomes, confirming this hypothesis.

We also investigated the cytotoxic effect of all competitive inhibitors at the same conditions of the competition assay. They presented no cytotoxicity and functioned at biocompatible concentrations in RAW cells. These results corroborate with the IC_{50} values found in the cytotoxicity assay mentioned above. The coated liposomes exhibited lower IC_{50} values compared to uncoated systems. This is due to the binding of fucoidan with SR, increasing the internalization of these systems. The increase in the internalization of the coated systems could result in a higher amount of usnic acid within the infected macrophages enhances the activity of this drug against *M. tuberculosis*. Thus, fucoidan coated systems appear as a promising approach for drug target to macrophages the treatment of tuberculosis.

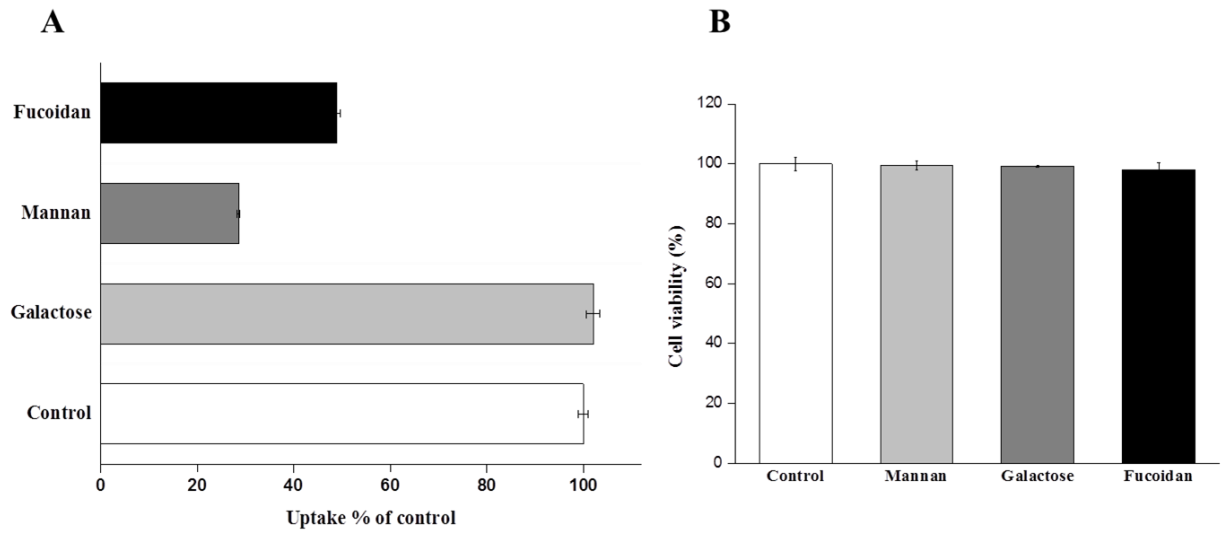

Figure 2. Competition assay of fucoidan (1 mg/mL) with fluorescent-labelled $Lipo_{Fuc5}$ using mannan (1 mg/mL) and galactose (20 mM) as positive and negative controls, respectively (A). Citotoxicity evaluation of fucoidan on Raw 264.7 cells (B).

4. Conclusion

Different batches of fucoidan-coated liposomes were developed and characterized as a system for targeting usnic acid to macrophages. All coated liposomes exhibited lower IC50 values than the uncoated liposomes, suggesting that fucoidan promoted an increase in liposomes uptake. In addition, the results obtained from the competition assay on RAW 264.7 suggested that

fucoidan coated liposomes was internalized through a specific SR-A mediated pathway, justifying in that way, the cytotoxicity data. To our knowledge, this is the first study which demonstrate an interactions of fucoidan coated liposomes with macrophage through of the SR-A. In summary, in a specific manner, the fucoidan coated liposomes developed could be an interesting strategy for delivery drugs to macrophages infected by *M. tuberculosis.*

Acknowledgments

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REFERENCES

[1] D. Dube, G. P. Agrawal, S. P. Vyas, Tuberculosis: from molecular pathogenesis to effective drug carrier design, Drug Discovery Today, 17 (2012), 13-14.

[2] D. Singodia, A. Verma, R.K. Verma, P.R. Mishra, Investigations into an alternate approach to target mannose receptors on macrophages using 4-sulfated N-acetyl galactosamine more efficiently in comparison with mannose-decorated liposomes: an application in drug delivery, Nanomedicine: NBM, 8 (2012), 468-477.

[3] Z. Drulis-kawa, A. Dorotkiewicz-jach, Liposomes as delivery systems for antibiotics, Int. J. Pharm., 387 (2010), 187-198.

[4] V. P. Torchilin, Multifunctional nanocarriers, Adv. Drug Delivery Rev., 64 (2012), 302-315.

[5] P.R. Taylor, L. Martinez-pomares, M. STACEY, Macrophage receptors and immune recognition, Annu. Rev. Immunol., 23 (2005), 901-944.

[6] P. A. Sivakumar, K. P. Rao, The use of cholesteryl pullulan for the preparation of stable vincristine liposomes**,** Carbohydr. Polym., 51 (2003), 327-332.

[7] S. Ning, Q. Huang, J. Li, Y. Zhang, Y. LIU, Functionalized dextran-coated liposomes for doxorubicin loading, J. Controlled Release., 152 (2011), 1-11.

[8] S. M. Shah, P. N. Goel, A. S. Jain, P. O. Pathak, S. G. Padhye, S. Govindarajan, S. S. Ghosh, P. R. Chaudhari, R. P. Gude, V. Gopal, M. S. Nagarsenker, Liposomes for targeting hepatocellular carcinoma: Use of conjugated arabinogalactan as targeting ligand, Int. J. Pharm., 477 (2014), 128-139.

[9] W. S. Kim, C. M. Ordija, M. W. Freeman, Activation of signaling pathways by putative scavenger receptor class A (SR-A) ligands requires CD14 but not SR-A. Biochem. Biophys. Res. Commun., 310 (2003), 542-549.

[10] S. Mukhopadhyay, S. Gordon, The role of scavenger receptors in pathogen recognition and innate immunity, Immunobiology, 209 (2004), 39-49.

[11] B. Tissot, B. Montdargent, L. Chevolot, A. Varenne, S. Descroix, P. Gareil, R. Daniel, Interaction of fucoidan with the proteins of the complement classical pathway, Biochim. Biophys. Acta., 1652 (2003), 5-16.

[12] M.J. Clement, B. Tissot, L. Chevolot, E. Adjadj, Y. Du, P.A. Curmi, P.A. R. Daniel, NMR characterization and molecular modeling of fucoidan showing the importance of oligosaccharide branching in its anticomplementary activity. Glycob., 20 (2010), 883-894.

[13] M. C. B. Lira, M. P. Siqueira-moura, H. M. L. Rolim-santos, F. C. S. Galetti, A. R. Simioni, N. P. Santos, E. S. T. Egito, C. L. Silva, A. C. Tedesco, N. S. Santos-magalhães, In vitro uptake and antimycobacterial activity of liposomal usnic acid formulation, J. Liposome Res., 19 (2009), 49-58.

[14] N. K. Honda, F. R. Pavan, R. G. Coelho, S. R. DE Andrade Leite, A. C. Micheletti, T. I. B. Lopes, M. Y. Misutsu, A. Beatriz, R. L. Brum, C. Q. F. Leite, Antimycobacterial activity of lichen substances, Phytomedicine, 17 (2010), 328-332.

[15] G. X. Ruan, Y. Z. Chen, X. L. Yao, A. Du, G. P. Tang, Y. Q. Shen, Y. Tabata, J. Q. Gao, Macrophage mannose receptor-specific gene delivery vehicle for macrophage engineering, Acta Biomaterialia, 10 (2014), 1847-1855.

[16] A. C. Pinheiro, A. I. Bourbon, M. A. Cerqueira1, É. Maricato, C. Nunes, M. A. Coimbra, A. A. Vicente, Chitosan/Fucoidan multilayer nanocapsules as a vehicle for controlled release of bioactive compounds, Carbohydr. Polym., (2014), 1-31.

[17] A. Bhardwaj, L. Kumar, R. K. Narang, R. S. R. Murthy, Development and characterization of ligand-appended liposomes for multiple drug therapy for pulmonary tuberculosis, Artif Cells Nanomed Biotechnol., 41 (2013), 52-59.

[18] S. Arpicco, C. Lerda, E. D. Pozza, C. Costanzo, N. Tsapis, B. Stella, M. Donadelli, I. Dando, E. Fattal, L. Cattel, M. Palmieri, Hyaluronic acid-coated liposomes for active targeting of gemcitabine, Eur. J. Pharm. Biopharm., 85 (2013), 373-380.

[19] A. Štimac, S. Šegota, M. D. Sikirić, R. Ribić, L. Frkanec, V. Svetličić, S. Tomić, B. Vranešić, R. Frkanec, Surface modified liposomes by mannosylated conjugates anchored via the adamantly moiety in the lipid bilayer, Biochim. Biophys. Acta., 1818 (2012), 2252 – 2259.

[20] C. Kelly, C. Jefferies, S-A. Cryan, Targeted liposomal drug delivery to monocytes and macrophages. J. Drug Deliv., 2011 (2011), 2-11.

[21] W.L.J. Hinrichs, F.A. Mancenido, N.N. Sanders, S.C. Smedt, J. Demeester, H.W. Frijlink, The choice of a suitable oligosaccharide to prevent aggregation of PEGylated nanoparticles during freeze thawing and freeze drying, Int. J. Pharm., 311 (2006), 237-244.

[22] M. Gulati, M. Grover, S. Singh, M. Singh, Lipophilic drug derivatives in liposomes. Int. J. Pharm., 165 (1998), 129-168.

[23] N. Venkatesan, S.P. Vyas, Polysaccharide coated liposomes for oral immunization — development and characterization, Int. J. Pharm., 203 (2000), 169–177.

[24] W. A. J. P. WIJESINGHE, Y. JEON, Biological activities and potential industrial applications of fucose rich sulfated polysaccharides and fucoidans isolated from brown seaweeds: A review. Carbohydr. Polym., 88 (2012), 13-20.

[25] S.P. Vyas, V. Sihorkar, S. Jain, Mannosylated liposomes for bio-film targeting, Int. J. Pharm., 330 (2007), 6-13.

[26] H.Y. Hsu, S.L. Chiu, M.H. Wen, K.Y. Chen, K.F. Hua, Ligands of macrophage scavenger receptor induce cytokine expression via differential modulation of protein kinase signaling Pathways, J. Biol. Chem., 276 (2001), 28719-28730.

[27] T. Nakamura, H. Suzuki, Y. Wada, T. Kodama, T. Doi, Fucoidan induces nitric oxide production via p38 mitogen-activated protein kinase and NF-K_B- dependent signaling pathways through macrophage scavenger receptors, Biochem. Biophys. Res. Commun., 343 (2006), 286–294.

[28] H. Epstein-barash, D. Gutman, E. Markovsky, G. Mishan-eisenberg, N. Koroukhov, J. Szebeni, G. Golomb, Physicochemical parameters affecting liposomal bisphosphonates bioactivity for restenosis therapy: Internalization, cell inhibition, activation of cytokines and complement, and mechanism of cell death. J. Control. Release, 146 (2010), 182-195.

7. CAPÍTULO 3

A ser submetido à Tuberculosis

Effects of the encapsulation of usnic acid into liposomes and interactions with antituberculous agents against MDR-TB clinical isolates

Rafaela S. Ferraz[1], Marcela A. Pereira[1], Lilian M. L. Montenegro[2], Leonardo A. Linhares[2], Mariane C. B. Lira-Nogueira[3], Isabella M. F. Cavalcanti[3], Nereide S. Santos-Magalhães[1*]

[1]Laboratório de Imunopatologia Keizo-Asami, Universidade Federal de Pernambuco, Recife, PE, Brazil

[2]Department of Immunology, Aggeu Magalhães Research Center – Fiocruz, Recife, Brazil

[3]Centro Acadêmico de Vitoria, Universidade Federal de Pernambuco, Vitória de Santo Antão, PE, Brazil

* Corresponding author:
Nereide Stela Santos-Magalhães
Universidade Federal de Pernambuco (UFPE)
Laboratório de Imunopatologia Keizo-Asami (LIKA)
Av. Prof. Moraes do Rego, 1235, Cidade Universitária,
50670-901, Recife, PE, Brazil
Tel: + 55-81-21268587; fax: + 55-81-21268485
E-mail addresses: nssm@ufpe.br

Abstract

The prevalence of multidrug-resistant *Mycobacterium tuberculosis* (MDR-TB) strains has increased globally. Consequently, the development of novel dosage forms of drug administration or the association with antituberculous agents is necessary to achieve effective TB control. Thus, the aim of this study was to evaluate the effect of the encapsulation of usnic acid into liposomes as well as its combinations with antituberculous agents, such as rifampicin (RIF) and isoniazid (INH), against MDR-TB clinical isolates. The UA-loaded liposomes (UA-Lipo) were prepared using the dried thin lipid hydration method. The *in vitro* antimycobacterial activity against MDR-TB was evaluated by microdilution method according to the *Clinical and Laboratory Standards Institute* (CLSI). The *in vitro* interaction of UA was evaluated using the checkerboard method. MIC values were 31.25 and 0.98 µg/mL for UA and UA-Lipo, respectively. Based on the FICI values (fractional inhibitory concentration index), the results suggested a synergistic interaction between RIF and UA (FICI = 0.31) or UA-Lipo (FICI = 0.28). Regarding INH, the combinations tested showing indifferent effect, with FICI values ranged from 1.30 to 2.75. In this way, it is concluded that the encapsulation of UA into liposomes increased significantly its antimicrobial activity. UA-lipo may be candidate to improve the antimycobacterial activity of RIF, a first-line drug for the treatment of infections caused by *Mycobaterium tuberculosis*.

Keywords: MDR-TB; Antimycobacterial activity; Synergism; Usnic acid; Liposomes.

1. Introduction

Tuberculosis (TB) is a chronic bacterial infection caused by an airborne bacterium known as *Mycobacterium tuberculosis* (Mtb). Treatment for susceptible Mtb isolates is based on isoniazid (INH), rifampicin (RIF), ethambutol (EMB) and pyrazinamide (PZA) [2]. Patients with TB infection requires long duration therapy and the non-compliance with the full therapeutic regimen could lead to patient relapse and to the emergence of multi- and extensively-drug resistant *M. tuberculosis* (MDR-TB and XDR-TB) strains [1]. Globally in 2010, only 48% of MDR-TB patients were successfully treated with the currently used anti-TB drugs [2]. The therapeutic options for the treatment of MDR-TB are PZA concurrently with second-line drugs such as ethionamide, prothionamide, clycloserine, capreomycin, p-aminosalicylic acid or fluoroquinolones (Mukherjee et al., 2004). Once the second-line drugs exhibit more toxicity, are more expensive and are less potent than the first-line agents [5], there is an urgent need to find new effective drugs, new drug administration or the association of antituberculous agents for TB treatment.

In the last years, new agents, synthetic and natural, have been tested against Mtb in order to find new classes of structural compounds capable of substitute or complement the established medicine used in TB therapeutic [1]. The usnic acid (UA), a lichen dibenzofuran derived secondary metabolite, has been shown to display an interesting antimycobacterial activity. However its weak potency (MIC > 32 μg/mL), compared to reference antimycobacterial drugs, does not allow its use as an antituberculous drug [3,4].

Accordingly, the nanotechnology rises as an efficient tool that is able to enhance the drug efficacy and overcome the resistance that *Mycobacterium* presents against known antibiotics used in clinic [5].These advantages, associated to the fact that the last four decades only one anti-TB drug has been approved by the FDA (TMC207), suggest the importance of developing nanosystems, such as liposomes [1]. Liposome encapsulation has been shown to improve the therapeutic efficacy of pharmaceutical drugs [6-8]. More recently, proliposome containing levofloxacin were used to the *in vivo* treatment of *Mycobacterium* with promised results [8]. With regard the association of drugs, pulmonary tuberculosis and drug resistant cases requires a combination of multi-drug regimens over long periods [9]. Therefore, the combination of first-line drugs, such as rifampicin and isoniazid, with natural product or drug delivery systems, appears as an alternative in tuberculosis therapy [1, 10].

Although the antimicrobial activity of pure UA and UA encapsulated into liposomes (UA-Lipo) against Mtb is known [11-13], their activity against MDR-TB as well as the interaction between UA or UA-Lipo with other anti-TB drugs has not yet been investigated. Thus, the aim of our study was to evaluate the effect of usnic acid encapsulation into liposomes (UA-Lipo) and its combinations with antituberculous agents, such as rifampicin (RIF) and isoniazid (INH), against MDR-TB clinical isolates, since multidrug resistance became a problem in the management of TB.

2. Materials and Methods

2.1 Materials

Isoniazid (INH), rifampicin (RIF), (+)-Usnic acid (UA), Cholesterol (Chol), stearylamine (SA) and Middlebrook 7H9 medium were obtained from Sigma-Aldrich (St Louis, MO). Middlebrook OADC Enrichment was purchased from Becton Dickinson (New Jersey, USA). Soybean phosphatidylcholine (PC) (94% Epikuron 200®) was furnished by Lipoid GMBH (Ludwigshafen, Germany). All solvents and other chemicals were supplied by Merck (Darmstadt,Germany).

2.2 Methods

2.2.1 Preparation and characterization of liposomes containing usnic acid

Liposomes containing usnic acid (UA-Lipo) were prepared using the dried thin lipid hydration method previously reported by Lira et al. [11]. Briefly, lipids (soya phosphatidylcholine, cholesterol and stearylamine; 8:1:1; 80 mM) and usnic acid (2 µg/mL) were dissolved in a mixture of chloroform and methanol (3:1 v/v) under magnetic stirring. The solvents were removed under pressure (37 °C, 80 rpm) and the thin film formed was hydrated with 10 mL of phosphate buffer solution (7.4 pH). The liposomal dispersion was then sonicated (Vibra Cell, BRANSON, USA) at 200W and 40 Hz for 300 s aiming to obtain small unilamellar vesicles.

The mean particle size and polydispersity index of liposomes were measured by photon autocorrelation spectroscopy (PCS) using a laser particle analyzer DelsaTMNano-S (Beckman Coulter, UK). Zeta potential of the liposomes was determined by electrophoretic mobility using a ZetasizerNano-ZS90 (Malvern, Worcestershire, UK). Analyses were performed using samples diluted with deionized water (2:1) at 25 °C. Moreover, the encapsulation efficiency of UA into

liposomes was determined using ultrafiltration/centrifugation by a previously method validated [11].

2.2.2 Antimycobacterial activity

2.2.2.1 *Mycobacterium* strains and growth conditions

Mycobacterium tuberculosis H37Rv ATCC27294 strain and six multi-drug resistant (MDR-TB) clinical isolates were studied (MDR-TB 1412, 1619, 0729, 1411, 1409 and 1484). MDR-TB isolates were obtained from the Central Laboratory of Public Health, Pernambuco, Brazil (LACEN/PE). Drug susceptibility of MDR-TB isolates to first line drugs (ethambutol, streptomycin, isoniazid and rifampicin) was verified out by LACEN using proportional agar methods. *Mycobacterium tuberculosis* strains were cultured in Middlebrook 7H9 broth supplemented with 10% (v/v) OADC, 0.05% (v/v) Tween 80 (Sigma-Aldrich) and 0.2% (v/v) glycerol (Sigma-Aldrich) and incubated at 37°C for approximately 10 days.

2.2.2.2 Determination of the Minimum inhibitory concentration (MIC)

The antimycobacterial activity of the tested compounds (UA and UA-Lipo) and the standard drugs, isoniazid (INH) and rifampicin (RIF), was determined in triplicate through the Microplate Alamar Blue Assay (MABA) [14]. Initially, the 96-well microplates were filled with 100 µL of Middlebrook 7H9 broth into each well. Serial two-fold dilutions of drugs were made by transferring 100 µL from the first to the last well. INH was prepared in sterile distilled water to a concentration from 16 to 0.25 µg/mL. RIF and UA were dissolved in methanol (8 – 0.125 µg/mL and 125 – 1.95 µg/mL, respectively) and UA-Lipo were dissolved in Middlebrook 7H9 broth (500 – 0.122 µg/mL). Finally, H37Rv ATCC27294 and MDR-TB clinical isolates concentrations were adjusted to a density corresponding to 1.0 McFarland turbidity standards, following by dilution (1:20 v/v) in the culture medium 7H9. Each well was inoculated with 100 µL of this bacterial suspension. Plates were incubated at 37 ± 1 °C for approximately 10 days. Next, 30µL of freshly resazurin was added to each well and incubated by 24h. Growth of the organisms was determined after re-incubation at 37 ± 1 °C for 24 h by visual determination of a color change from blue to pink. The MIC is defined as the lowest concentration that prevents the color change. The positive control well consisted of Middlebrook 7H9 broth with bacterial suspension and the negative control well comprised Middlebrook 7H9 broth with the tested

drugs. Methanol, DMSO and unloaded liposomes were used negative as controls to evaluate any possible effects of vesicles on bacterial growth. All the experiments were performed in triplicate.

2.2.2.3 Checkerboard method

The *in vitro* interactions between RIF or INH with UA or UA-Lipo were evaluated by checkerboard bi-dimensional method [15]. The checkerboard was carried out to verify the possible effects that the combination of drugs may cause in the antimicrobial activity of the compounds against Mtb. Initially, the 96-well microplates were seeded by dispersing 100 µL of Middlebrook 7H9 broth into each well. After, the drugs and liposome formulations were appropriately diluted and added into 96-well microplates to obtain a final concentration equal to the MIC or dilutions lower than the MIC of the respective samples. Finally, the bacterial concentration was adjusted to a density corresponding to 1.0 McFarland turbidity standards, followed by dilution (1:20 v/v) in the culture medium 7H9. Each well was inoculated with 100 µL of this bacterial suspension. Then, the plates were incubated at 37 ± 1 °C for approximately 10 days. The analysis of result was performed and interpreted as described above. All the experiments were performed in triplicate.

The data were interpreted after calculating the fractional inhibitory concentration index (FICI) as follows:

$$\sum \mathrm{FICI} = \mathrm{FIC_A} + \mathrm{FIC_B} = \mathrm{MIC_{AB}}/\mathrm{MIC_A} + \mathrm{MIC_{BA}}/\mathrm{MIC_B}$$

where, MIC_{AB} equals the MIC of drug A in combination with drug B, MIC_A is the MIC of drug A alone, MIC_{BA} equals the MIC of drug B in combination with drug A and MIC_B is the MIC of drug B alone. The interaction is considered synergistic for FICI ≤ 0.5; additive (0.5 ≤ FICI ≤ 1), indifferent (1 < FICI ≤ 4) and antagonistic (FICI > 4) [15].

3. Results and Discussion

3.1 Physicochemical characterization of liposomes

In this paper, conventional liposomes were chosen as a vehicle for UA because they are excellent carriers for antibiotics used in the treatment of intracellular pathogens, such as MB [17].

Liposomes exhibited mean particle size of 146.46 ± 1.91 nm. The PDI of this formulation was 0.32 ± 0.01 showing the homogeneity of this system, since formulations with this index ranges from 0 for monodispersed samples up to 1 for polydispersed ones [18]. Surface charge

was + 21.30 ± 0.51 mV due to the presence of a cationic lipid (stearylamine) [19] and the drug efficiency ratio was practically 100% (99.56 ± 0.74%). The high ratio of usnic acid encapsulation was also previously reported by Lira et al. [11], in their pioneer study about liposomes containing usnic acid. They prepared liposomes consisted of PC:CH:SA in the ratio 7:2:1 containing UA (1 µg/mL) and showed an drug encapsulation ratio of approximately 96%. These results indicate that the change in lipid ratio from 7:2:1 to 8:1:1 (PC:CH:SA) did not influence on the drug encapsulation efficiency.

3.2 Antimycobacterial activity

3.2.1 Minimum inhibitory concentration (MIC)

The antimycobacterial activity of UA and UA-Lipo is shown in Table 1. MIC values obtained for the pure usnic acid (31.25 µg/mL) are in agreement with the literature. Honda et al. [12] evaluated the anti-tubercular activity of lichen derivatives against *M. tuberculosis* (H37Rv), including usnic acid, with a MIC value of 62.5 µg/mL. In the same year, Ramos and Silva [13] determined the antimycobacterial activity of UA against resistant and susceptible *M. tuberculosis* clinical isolates. The MIC value of 12.25 µg/mL was found for sensitive strains (H37Rv) and 1.56 to 12.5 µg/mL for *Mycobacterium tuberculosis* clinical isolates with mono-resistance only to one standard antibiotic, such as INH, streptomycin (SMR) or RIF.

Yempala et al. [4] carried out chemical modifications of dibenzofuran derivatives through molecular hybridization and evaluated antimycobacterial activity against Mtb H37Rv. Among the derivatives analyzed the lowest MIC value was found as 3.12 µg/mL. In comparison with our results, we can say that the encapsulation of UA into liposomes was 3-fold more effective than the new usnic acid derivatives.

Regarding the UA-Lipo, all isolates exhibited MIC values (0.98 µg/mL) more than 30-fold lower compared to pure usnic acid. One possible explanation for the improvement of antimicrobial activity promoted by encapsulation of usnic acid into liposomes can be related to a supposed electrostatic interaction between negatively charged carboxyl groups of mycolic acids which are the main components of the MTb cell wall [20] and positively charged liposomal vesicles. In addition, liposomes and bacteria can interact directly by fusion processes, leading to the release of the encapsulated antibiotic into bacterial [21].

The MIC values of the standard drugs, INH and RIF, against MDR-TB clinical isolates are shown in Table 1. These clinical isolates exhibit MIC values of 4 to ≥ 16 and 4 to ≥ 8 µg/mL

for INH and RIF, respectively. The resistance of all of clinical isolates used in this study was confirmed, since Ganihigama et al. [1] referred multidrug-resistant *M. tuberculosis* isolates based on the resistance of these isolates to isoniazid and rifampicin (MIC values of 2 to ≥ 8 µg/mL).

Other authors also demonstrated that the drug encapsulation into liposomes enhanced the antimycobacterial activity compared to pure drugs. In 2009, Changsan et al. [22] encapsulated rifampicin into liposomes using the thin film method. The MIC value of RIF encapsulated into liposomes against *M. bovis* was 0.2 µM whereas the MIC of pure RIF was higher (0.8 µM), suggested that liposomes were more efficient. Recently, Rojanarat et al. [8] prepared proliposomes containing levofloxacin (LEV-proliposome) and its activity against *M. bovis* were assessed, as well as the pure drug activity. The antimycobacterial activity of LEV-proliposomes against *M. bovis* was higher than pure LEV (MIC = 0.5 and 1 µg/mL, respectively).

Table 1

Antimycobacterial activity of compounds tested against *Mycobacterium tuberculosis* clinical isolates.

	MIC (µg/mL)			
***Mycobacterium tuberculosis* strains**	**Isoniazid**	**Rifampicin**	**Usnic acid**	**UA-Lipo**
H37Rv	< 0.25	< 0.12	31.25	0.98
1412	≥ 16	4	31.25	0.98
1619	16	8	31.25	0.98
0729	≥ 16	≥ 8	31.25	0.98
1411	16	8	31.25	0.98
1409	4	≥ 8	31.25	0.98
1484	≥ 16	≥ 8	31.25	0.98

H37Rv: Virulent strains; MIC: Minimum Inhibitory Concentration; UA-Lipo: usnic acid encapsulated into liposomes.

As expected, DMSO, methanol and empty liposomes, used as negative controls, did not exhibit antimycobacterial activities.

3.3.2 Checkerboard method

The drugs combination regimen could lead to creating successful therapeutic schemes for TB treatment, since MDR-TB isolates may rapidly resist the new drugs, especially those redesigned from the existing scaffolds of the currently used anti-TB drugs [2].

The *in vitro* interactions was evaluated in two MDR-TB isolates (1619 and 1411), because both isolates presented the MIC values set for the reference and tested drugs. The interaction results of tested compounds (UA and UA-Lipo) with INH and RIF, against MDR-TB isolates are exhibited in Table 2. The FICI of the combinations UA/INH and Lipo-UA/INH ranged from 1.30 to 2.75, showing indifferent effect. However, the combinations of UA/RIF and UA-Lipo/RIF exhibited synergistic effect showing a value below the threshold used to determine synergism ($FICI \leq 0.5$).

In the synergism, the drugs involved can affect different targets in the microorganisms or interact with each other in order to improve their solubility and bioavailability [15,23]. Thus, the result obtained with the combinations of UA and UA-Lipo with RIF demonstrated an improvement in their antimycobacterial activity, when compared with the effect of both drugs separately. Additionally, it important mentions that the *in vitro* synergism between RIF and UA or UA-Lipo can permit the use of this first line-drugs in therapy with combination of drugs against MDR-TB.

Bapela et al. [10] were the pioneer in the experiments combining natural products with antituberculous agents, such as INH and RIF. The combinations of isoniazid or rifampicin with 7-methyljuglone, a natural product isolated from *Euclea natalensis*, reduced MIC values found four-fold and eight-fold, respectively. Results showed synergistic effect against *M. tuberculosis* isolates for both combinations.

In 2013, Rey-Jurado et al. [16] evaluated the *in vitro* effect of combinations between antituberculous agents such as INH, RIF and ethambutol (EMB) against drugs-susceptible clinical isolates. The FICI values for all isolates were 1.5, showing indifferent activity. Thus, we can say that our results of combinations between UA or UA-Lipo with RIF were more effective than the first-line drug combinations proposed by Rey-Jurado et al. [16]

Table 2

Effect of UA or UA-Lipo in combination with INH or RIF against MDR-TB clinical isolates.

Combination	MDR-TB clinical isolates	FICI	Interaction
UA/INH	1619	1.30	Indifferent
	1411	2.50	Indifferent
UA-Lipo/INH	1619	2.00	Indifferent
	1411	2.75	Indifferent
UA/RIF	1619	0.31	Synergistic
	1411	0.38	Synergistic
UA-Lipo/RIF	1619	0.25	Synergistic
	1411	0.28	Synergistic

FICI: Fractional Inhibitory Concentration Index; INH: Isoniazid; RIF: Rifampicin; UA: Usnic acid; UA-Lipo: usnic acid encapsulated into liposomes.

4. Conclusion

UA exhibit antimicrobial activity against all MDR-TB clinical isolates tested and its encapsulation into liposomes enhanced significantly the antimycobacterial activity. Once the UA and UA-lipo had synergistic interaction with RIF, these systems can be candidates to improve the antimycobacterial activity of this first-line drug for the treatment of infections caused by *Mycobaterium tuberculosis*.

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Competing interests: None declared.

Ethical approval: Not required

References

[1] Dakshina G, Sanya S, Sasithorn S, Poonpilas H, Thammarat A, Chulabhorn M, Somsak R, Prasat K. Antimycobacterial activity of natural products and synthetic agents: Pyrrolodiquinolines and vermelhotin as anti-tubercular leads against clinical multidrug resistant isolates of *Mycobacterium tuberculosis*. Eur J Med Chem 2015; 89:1-12.

[2] World Health Organization (WHO) global tuberculosis report. 2013. www. who.int/iris/bitstream/10665/91355/1/9789241564656_eng.pdf.

[3] Kristín I, Gavin C, Vilhjálmur S, Stefán G, Margrét V. Antimycobacterial activity of lichen metabolites *in vitro*. Eur J Pharm Sci 1998; 6: 141-144.

[4] Thirumal Y, Jonnalagadda S, Perumal Y, Darmarajan S, Srinivas K. Design, synthesis and antitubercular evaluation of novel 2-substituted-3H-benzofuro benzofurans via palladium–copper catalysed Sonagashira coupling reaction. Bioorg Med Chem 2013; 23: 5393–5396.

[5] Indu K, Harinder S. Nanostructured drug delivery for better management of tuberculosis. J Control Release 2014; 184: 36-50.

[6] El-Ridy M, Mostafa D, Shehab A, Nasr A, Abd El-Alim, S. Biological evaluation of pyrazinamide liposomes for treatment of *Mycobacterium tuberculosis*. Int J Pharm 2007; 330: 82-88.

[7] Gaspar M, Cruz A, Penha F, Reymão J, Sousa C, Eleutério V, Domingues A, Fraga G, Filho L, Cruz E, Pedrosa J. Rifabutin encapsulated in liposomes exhibits increased therapeutic activity in a model of disseminated tuberculosis. Int J Antimicrob Agents 2008; 31: 37–45.

[8] Wipaporn R, Titpawan N, Ekawat T, Niracha Y, Teerapol S. Levofloxacin-Proliposomes: Opportunities for Use in Lung Tuberculosis. Pharmaceutics 2012; 4; 385-412.

[9] Dinh-Duy P, Elias F, Nicolas T. Pulmonary drug delivery systems for tuberculosis treatment. Int J Pharm 2015; 478: 517–529.

[10] Bapela B, Lall N, Fourie B, FranzblauG, van Rensburg, E. Activity of 7-methyl-juglone in combination with antituberculous drugs against *Mycobacterium tuberculosis*. Phytomed 2006; 13: 630–635.

[11] Lira M C B, Siqueira-Moura M P, Rolim-Santos H M L, Galetti F C S, Simioni A R, Santos N P, Egito E S T, Silva C L, Tedesco A C, Santos-Magalhães N S. *In vitro* uptake and antimycobacterial activity of liposomal usnic acid formulation. J Liposome Res 2009; 19: 49-58.
[12] Honda N K, Pavan F R, Coelho R G, De Andrade Leite S R, Micheletti A C, Lopes T I B, Misutsu M Y, Beatriz A, Brum R L, Leite C Q F. Antimycobacterial activity of lichen substances. Phytomed 2010; 17: 328 – 332.
[13] Ramos D F, Da Silva P E A. Antimycobacterial activity of usnic acid against resistant and susceptible strains of *Mycobacterium tuberculosis* and non-tuberculous mycobacteria. Pharm Bio 2010; 48: 260-263.
[14] Clinical and Laboratory Standards Institute. 2011. In: NCCLS/CLSI document M24-A2. Wayne, PA: NCCLS; 2011.
[15] Agertt V A, Marques L, Bonez P C, Dalmolin P C, Manzoni de Oliveira G N, Anraku de Campos M. Evaluation of antimycobacterial activity of a sulphonamide derivative. Tuberculosis 2013; 93: 318-321.
[16] Rey-Jurado E, Tudó G, Bellacasa J P, Espasa M, González-Martín J. *In vitro* effect of three-drug combinations of antituberculous agents against multidrug-resistant *Mycobacterium tuberculosis* isolates. Int J Antimicrob Agents 2013; 41: 278-280.
[17] Drulis-Kawa Z, Dorotkiewicz-Jach A. Liposomes as delivery systems for antibiotics. Int J Pharm 2010; 387:187-198.
[18] Hinrichs W L J, Mancenido F A, Sanders N N, De Smedt S C, Demeester J, Frijlink H W. The choice of a suitable oligosaccharide to prevent aggregation of PEGylated nanoparticles during freeze thawing and freeze drying. Int J Pharm 2006; 311: 237–244.
[19] Villasmil-Sánchez S, Drhimeur W, Ospino S C S, Alvarez A M R. Positively and negatively charged liposomes as carriers for transdermal delivery of sumatriptan: *in vitro* characterization. Drug Develop Industrial Pharm 2010; 36: 666-675.
[20] Pinheiro M, Giner-Casares J, Lúcio M, Caio J M , Moiteiro C, Lima J L F C, Reis S, Camacho L. Interplay of mycolic acids, antimycobacterial compounds and pulmonary surfactant membrane: A biophysical approach to disease. Biochim Biophys Acta 2013; 1828: 896-905.
[21] Sachetelli S, Khalil H, Chen T, Beaulac C, Sénéchal S, Lagacé J. Demonstration of a fusion mechanism between a fluid bactericidal liposomal formulation and bacterial cells. Biochim Biophys Acta 2000; 1463: 254-266.

[22] Changsan N, Nilkaeo A, Pungrassami P, Srichama T. Monitoring liposomes containing rifampicin on respiratory cell lines and *in vitro* against *Mycobacterium bovis* in alveolar macrophages. J Drug Target 2009; 17: 751-762.
[23] Rastogi N, Goh K S, Horgen L, Barrow W W. Synergistic activities of antituberculous drugs with cerulenin and transcinnamic acid against *Mycobacterium tuberculosis*. FEMS Immunol Med Microbiol 1998; 21:149-157.

8. CONCLUSÕES

- O derivado hidrofóbico da fucana, colesteril-fucana, foi eficientemente sintetizado por irradiação de micro-ondas, obtendo-se altos rendimentos que variaram de 75-77%.
- Lipossomas revestidos com fucana, em escala nanométrica, homogêneos e com elevada eficiência de encapsulação do ácido úsnico foram obtidos.
- Os estudos de citotoxicidade frente a células RAW 264.7 revelaram que todas as formulações revestidas com fucana exibiram baixos valores de IC_{50}, em comparação aos lipossomas convencionais.
- Os lipossomas revestidos com fucana foram capturados mais rapidamente (15min) que os lipossomas sem revestimentos (60 min) sugerindo que foram mais rapidamente reconhecidos pelos macrófagos.
- Os resultados obtidos no experimento de inibidores competitivos sugerem que os lipossomas revestidos pela fucana ($Lipo_{fuc5}$) foram internalizados através do domínio de reconhecimento de carboidrato do tipo C presentes nos *scavengers receptors* (SR).
- A encapsulação do ácido úsnico em lipossomas potencializou a atividade antimicobacteriana deste composto liquênico frente a isolados clínicos de *M. tuberculosis* multirresistentes.
- O ácido úsnico livre e encapsulado apresentou efeito sinérgico com rifampicina contra isolados de *M. tuberculosis* multirresistentes, podendo ser candidato em associação à melhoria da atividade antimicobacteriana da rifampicina.

Anexo

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The pharmaceutical biotechnology section provides a forum for the publication of papers on scientific, developmental, regulatory and manufacturing issues concerning the development of biopharmaceuticals, uniquely combining expert contributions and opinions of authors from the fields of pharmaceutics and biotechnology. This scope encompasses modern biopharmaceuticals (e.g., proteins and nucleic acids), biosimilars/biogenerics as well as 'traditional' products such as antibiotics.

"THE RULES OF 3"

The Editors and Editorial board have developed the "Rules of 3". Authors must consider the following three criteria before they submit a manuscript and set the whole process of editing and reviewing at work. The rules are also used as guidelines for the peer review of manuscripts. CLICK HERE to view the "Rules of 3".

Authors are encouraged to submit video material or animation sequences to support and enhance your scientific research. For more information please see the paragraph on video data below.

Types of paper

The following types of papers are considered by the Editors: Research Papers, Review Articles, Short Reviews, and Notes.

Preparation

NEW SUBMISSIONS

Submission to this journal proceeds totally online and you will be guided stepwise through the creation and uploading of your files. The system automatically converts your files to a single PDF file, which is used in the peer-review process.

As part of the Your Paper Your Way service, you may choose to submit your manuscript as a single file to be used in the refereeing process. This can be a PDF file or a Word document, in any format or lay-out that can be used by referees to evaluate your manuscript. It should contain high enough quality figures for refereeing. If you prefer to do so, you may still provide all or some of the source files at the initial submission. Please note that individual figure files larger than 10 MB must be uploaded separately.

References

There are no strict requirements on reference formatting at submission. References can be in any style or format as long as the style is consistent. Where applicable, author(s) name(s), journal title/book title, chapter title/article title, year of publication, volume number/book chapter and the pagination must be present. Use of DOI is highly encouraged. The reference style used by the journal will be applied to the accepted article by Elsevier at the proof stage. Note that missing data will be highlighted at proof stage for the author to correct.

Formatting requirements

There are no strict formatting requirements but all manuscripts must contain the essential elements needed to convey your manuscript, for example Abstract, Keywords, Introduction, Materials and Methods, Results, Conclusions, Artwork and Tables with Captions.

If your article includes any Videos and/or other Supplementary material, this should be included in your initial submission for peer review purposes. Divide the article into clearly defined sections.

Figures and tables embedded in text

Please ensure the figures and the tables included in the single file are placed next to the relevant text in the manuscript, rather than at the bottom or the top of the file.

REVISED SUBMISSIONS

Use of word processing software

Regardless of the file format of the original submission, at revision you must provide us with an editable file of the entire article. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with Elsevier: http://www.elsevier.com/guidepublication). See also the section on Electronic artwork.

To avoid unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your word processor.

LaTeX

You are recommended to use the Elsevier article class *elsarticle.cls* (http://www.ctan.org/tex-archive/macros/latex/contrib/elsarticle) to prepare your manuscript and BibTeX (http://www.bibtex.org) to generate your bibliography. For detailed submission instructions, templates and other information on LaTeX, see http://www.elsevier.com/latex.

RESEARCH PAPERS

Should contain the following sections: Introduction, Theoretical Development (if applicable), Materials and Methods, Results, Discussion (or Results and Discussion) and References. For purely theoretical papers, appropriate sections may be selected.

It should define the purpose of the research and reveal its connections with the principal work of other authors in the field.

Materials and methods

It should include materials, standard techniques and procedures relevant to the study. Published procedures and techniques should be cited unless significant modifications are involved. Exact specification of relevant materials and equipment must be given. Chemical terms must conform with IUPAC rules. Trademarks of commercial products must be labelled using a superscripted '(r)'. Names of products and equipment mentioned in the Materials and Methods section must be accompanied by the name of the manufacturer or distributor, location and state or country. This information must be stated in parentheses in the text, and not as a footnote. Any potential hazards connected with materials and procedures must be mentioned. A precise and detailed description should be given of those steps which are of vital importance in carrying out any repetition of the work. The Declarations of Helsinki and Tokyo for humans, and the European Community guidelines as accepted principles for the use of experimental animals, must be adhered to. Therefore, *EJPB* will only consider manuscripts that describe experiments that have been carried out under approval of an institutional or local ethics committee. **Authors must state in the manuscript that the protocol complies with the particular recommendation and that approval of their protocols was obtained.**

Equations must be part of the text and consecutively numbered on the right hand side using numbers in parentheses. References to equations in the text are also to be made with parentheses, e.g. using Eq. (3), etc.

Organic formulas, both in figures and in the text, should be numbered in boldface arabic numerals.

SI units must be used throughout.

Theory/calculation

A Theory section should extend, not repeat, the background to the article already dealt with in the Introduction and lay the foundation for further work. In contrast, a Calculation section represents a practical development from a theoretical basis.

Results

Results may be presented in tables, figures or schemes which must be referred to in the accompanying text, using appropriate numbering, e.g. Fig. 1, Table 2.

Discussion

It should focus on the interpretation of the results. It might be appropriate to combine RESULTS AND DISCUSSION in one section. If necessary at all, use CONCLUSIONS only to illustrate the general implication of the results and do not summarize the previous text.

Conclusions

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

Appendices

If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

Essential title page information

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

• **Author names and affiliations.** Where the family name may be ambiguous (e.g., a double name), please indicate this clearly. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.

• **Corresponding author.** Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. **Ensure that phone numbers (with country and area code) are provided in addition to the e-mail address and the complete postal address. Contact details must be kept up to date by the corresponding author.**

• **Present/permanent address.** If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

It should contain a brief and clear description of the aim of the paper, its principal results and major conclusions (100--200 words). The abstract should include all keywords pertinent to the subject.

Graphical abstract

A Graphical abstract is mandatory for this journal. It should summarize the contents of the article in a concise, pictorial form designed to capture the attention of a wide readership online. Authors must provide images that clearly represent the work described in the article. Graphical abstracts should be submitted as a separate file in the online submission system. Image size: please provide an image with a minimum of 531 × 1328 pixels (h × w) or proportionally more. The image should be readable at a size of 5 × 13 cm using a regular screen resolution of 96 dpi.

Preferred file types: TIFF, EPS, PDF or MS Office files. See http://www.elsevier.com/graphicalabstracts for examples. Authors can make use of Elsevier's Illustration and Enhancement service to ensure the best presentation of their images also in accordance with all technical requirements: Illustration Service.

Keywords

Indicate 5 to 10 English keywords. They should be carefully selected in order to improve accessibility of scientific information in the manuscript. Keywords commonly used in international abstracting services will be preferred.

Chemical compounds

You can enrich your article by providing a list of chemical compounds studied in the article. The list of compounds will be used to extract relevant information from the NCBI PubChem Compound database and display it next to the online version of the article on ScienceDirect. You can include up to 10 names of chemical compounds in the article. For each compound, please provide the PubChem CID of the most relevant record as in the following example: Glutamic acid (PubChem CID:611). The PubChem CIDs can be found viahttp://www.ncbi.nlm.nih.gov/pccompound. Please position the list of compounds immediately below the 'Keywords' section. It is strongly recommended to follow the exact text formatting as in the example below: Chemical compounds studied in this article
Ethylene glycol (PubChem CID: 174); Plitidepsin (PubChem CID: 44152164); Benzalkonium chloride (PubChem CID: 15865) More information is available at: http://www.elsevier.com/PubChem.

Abbreviations

Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

Acknowledgements

Acknowledgements of financial support, gifts, technical help or other assistance may be given in an unnumbered paragraph under this heading preceding the references.

Database linking

Elsevier encourages authors to connect articles with external databases, giving their readers one-click access to relevant databases that help to build a better understanding of the described research. Please refer to relevant database identifiers using the following format in your article: Database: xxxx (e.g., TAIR: AT1G01020; CCDC: 734053; PDB: 1XFN). See http://www.elsevier.com/databaselinking for more information and a full list of supported databases.

Math formulae

Present simple formulae in the line of normal text where possible and use the solidus (/) instead of a horizontal line for small fractional terms, e.g., X/Y. In principle, variables are to be presented in italics. Powers of e are often more conveniently denoted by exp. Number consecutively any equations that have to be displayed separately from the text (if referred to explicitly in the text).

Footnotes

Footnotes should be used sparingly. Number them consecutively throughout the article. Many wordprocessors build footnotes into the text, and this feature may be used. Should this not be the case, indicate the position of footnotes in the text and present the footnotes themselves separately at the end of the article. Do not include footnotes in the Reference list.

Figure captions

Ensure that each illustration has a caption. A caption should comprise a brief title (**not** on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

Tables

Tables must be presented on separate sheets in consecutive order using Arabic numerals. The table headings must include a descriptive title and additional information to make the table self-explanatory. Some information may be given by using lower-case letter designations referring to footnotes at the bottom of the table. Indicate SI units of measure in parentheses.

References

References must be arranged as follows:

[1] A.– L. Cornaz, P. Buri, Nasal mucosa as an absorption barrier, Eur. J. Pharm. Biopharm. 40 (1994) 261– 270.

[5] C. Lanczos, Applied Analysis, Prentice-Hall, Englewood Cliffs, NJ, 1967, pp. 272– 280.

[10] D.M. Barends, Stability of active ingredients, in: H. Mü ller, W.H. Oeser (Eds.), Drug Master Files, Wissenschaftliche Verlagsgesellschaft mbH, Stuttgart, Germany, 1992, pp. 121– 128.

[14] E.A. Balazs, Ultrapure hyaluronic acid and the use thereof, U.S. Patent 4,141,973 (1979).

The system used by Chemical Abstracts (Chemical Abstracts Service Source Index) must be followed for the abbreviations of journals. Reference (4) is for patents, including the status, international country code, number of patent and year.

TUBERCULOSIS

DESCRIPTION

Tuberculosis is a speciality journal focusing on basic experimental research on **tuberculosis**, notably on **bacteriological**, immunological and **pathogenesis** aspects of the disease. The journal publishes original research and reviews on the **host response** and **immunology** of tuberculosis and the molecular biology, genetics and physiology of the organism.

Areas covered include: Immunology **Immunogenetics Pathogenetics Microbiology Microbial physiology** Pathogenesis **Pathology Molecular epidemiology Diagnostics Vaccine development Drug resistance** The resurgence of interest in tuberculosis has accelerated the pace of relevant research and *Tuberculosis* has grown with it, as the only journal dedicated to experimental biomedical research in tuberculosis.

Article types

The following types of manuscripts are routinely accepted for consideration by Editors (please note that word count is from abstract to references but excluding references):

Original Articles: The form of these articles is discussed fully below under manuscript preparation; an abstract is required.

Letters: Headings should not be used in a letter; no abstract or keywords are required. The text should be no more than 800 words; there should be a maximum of 5 references and one table or figure may be included.

Reviews: An abstract and keywords are required. The text should be divided into sections by suitable headings. Tables and figures may be used as appropriate for the text.

Comments: Editorial comments are generally invited by the Tuberculosis Editorial team. They are 1,500 words in length with no abstract or keywords.

Short Communications: These should be no more than 2,500 words, with up to 15 references and a maximum of 3 figures or tables.

PREPARATION

Papers should be submitted in journal style and must be written in good English. Failure to do so may lead to significant delays in publication. Spelling may be British or American, but this must be consistent throughout the paper. It is highly recommended that authors use the "spell-checking" facility on their word processing software.

Format the entire manuscript using 1.5 double spacing and 2 cm margins. Your paper should include page numbers and consecutive line numbering. Ensure that each new paragraph is clearly indicated. Present tables and figure legends on separate pages at the end of the manuscript. If possible, consult a recent issue of the journal to become familiar with layout and conventions. Number all pages consecutively.

Authors should note that upon submission of their manuscripts to the Editor, they must ensure that they have adhered to the following checklist:

One author is designated as the corresponding author and provides the following information:

• E-mail address

• Full postal address

• Telephone and fax numbers

Papers should be set out as follows, with each section beginning on a separate sheet: title page, summary, keywords, text, acknowledgements, references, tables, captions to illustrations.

• Title page: The title page should give the following information (in the order stated):

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

• Author names and affiliations. Where the family name may be ambiguous (e.g., a double name), please indicate this clearly. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name, and the e-mail address of each author.

• Corresponding author. Clearly indicate who is willing to handle correspondence at all stages of refereeing and publication, also post-publication. Ensure that telephone and fax numbers (with country and area code) are provided in addition to the e-mail address and the complete postal address.

• Present/permanent address. If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Abstract: Original articles should include an abstract of no more than 200 words. Abstracts for review articles may be up to 300 words, and for short communications 150 words. A recent copy of the journal should be consulted as a guide. An abstract is often presented separate from the article, so it must be able to stand alone.

Keywords: Immediately after the abstract, provide a minimum of 3maximum of 6 keywords, avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.