Faculdade de Ciências Farmacêuticas de Araraquara
Universidade Estadual Paulista "Júlio de Mesquita Filho"
Programa de Biociências e Biotecnologia Aplicadas à Farmácia

Desenvolvimento, síntese e caracterização de lipossomas conjugados com análogos de sideróforos e estudo *in vitro* como estratégia terapêutica para tuberculose

Camila Maríngolo Ribeiro

Orientador: Prof. Dr. Fernando Rogério Pavan

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Araraquara, SP
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"Estou entre aqueles que acham que a ciência tem uma grande beleza"
Marie Curie

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RESUMO

A tuberculose (TB) é uma doença infecciosa que afeta milhões de pessoas anualmente, é necessário um longo tratamento para a cura, especialmente para os casos de resistência a antibióticos. Outra particularidade de *Mycobacterium tuberculosis,* principal agente etiológico de TB, é a sua habilidade de sobreviver dentro de macrófagos, células imunológicas que são a primeira defesa no tecido pulmonar. Em uma busca de um tratamento eficaz, seguro, que promova uma entrega do antibiótico no local da infecção e, possivelmente, contribua para a adesão ao tratamento, propusemos uma abordagem nanotecnológica baseada em lipossomas funcionalizados, ou seja, com a superfície modificada com um análogo de sideróforo (Sid) para carrear moxifloxacino (Mox) para tratar TB para uma administração intrapulmonar. Os sideróforos são a principal estratégia das bactérias para captar ferro, um metal essencial para seu metabolismo. Foram desenvolvidos com fosfatidilcolina de soja, colesterol e ácido oleico, quatro lipossomas: lipossoma vazio (LipV); lipossoma contendo Mox (LipMox); lipossoma funcionalizado com Sid e sem fármaco (LipS) e lipossoma funcionalizado com Sid e contendo Mox (LipSMox). A caracterização dos lipossomas foi feita pela técnica de espalhamento de luz dinâmica (DLS), análise de rastreamento de nanopartículas (NTA) e microscopia eletrônica de transmissão (TEM). Por todos os métodos, observamos partículas esféricas e de aproximadamente 200 nm. O potencial zeta foi a principal diferença entre eles, e foi aproximadamente -40mV para LipV e LipMox e +15mV para LipS e LipSMox. A NTA mostrou a concentração de ~10^{11} partículas/mL. Um método de quantificação de Mox em cromatografia líquida de alta eficiência foi desenvolvido e validado para determinar a eficiência de encapsulação (EE) e o perfil de liberação (RP). A EE foi 51,31% para LipMox e 45,76 % para LipSMox e a RP seguiu o modelo proposto por Korsmeyer-Peppas, comum para lipossomas. A concentração inibitória mínima (MIC) frente a *M. tuberculosis* foi determinada em condições padrão (pH 6,6 e [Fe] = 0,04 µg/mL), com acréscimo de ferro (múltiplos de 2x, 5x e 10x em relação ao meio comercial), pH 7,4 e acréscimo de 10% de soro fetal bovino (FBS). A alteração de pH do meio para 7,4 não afetou a MIC. A MIC de LipMox e LipSMox foram na maioria das vezes menor que 0,5%. Já LipS teve MIC próxima a 10% em todas as condições, exceto na presença de FBS (MIC >25%), em que há ferro ofertado, mas de não livre e sim complexado com ferritina. O ensaio de *time-kill* não mostrou diferença significativa entre Mox livre e encapsulada, a ação bactericida foi concentração-dependente. O ensaio de citotoxicidade foi realizado para quatro linhagens celulares: macrófagos (J774A.1), fibroblastos (MRC-5), células hepáticas (HepG-2) e células intestinais (Caco-2) e observou-se uma heterogeneidade nos resultados de índice de citotoxicidade (IC_{50}) com maior toxicidade com lipossomas contendo Sid. A atividade frente a *M. tuberculosis* intramacrofágico apresentou reduções de mais de 90% dos bacilos intracelulares (LipMox 1 e 5%; Mox 1 e 5% e LipSMox 1%). A microscopia eletrônica de varredura e transmissão confirmaram a fagocitose das vesículas lipossomais pelos macrófagos, confirmando a possibilidade de um direcionamento natural dos lipossomas para células fagocíticas. Embora a funcionalização com Sid não trouxe melhora na atividade biológica dos lipossomas, ainda houve um direcionamento natural para os macrófagos, células de extrema importância na patogênese de TB. Diante das vantagens de *drug delivery* dos lipossomas, propõe-se uma administração intrapulmonar *in vivo* deste sistema para o tratamento de TB.

Palavras-chave: *Mycobacterium tuberculosis*, tuberculose, lipossomas, caracterização de nanopartículas, sideróforo.

ABSTRACT

Tuberculosis (TB) is an infectious disease that affects millions of people annually and a long treatment is necessary for a cure, especially for antibiotic resistant cases. Another particularity of *Mycobacterium tuberculosis*, the main etiologic agent of TB, is the ability to survive inside macrophages, immune cells in lungs. In a search for an effective, safe treatment that promotes antibiotic delivery at the site of infection and, possibly, contributes to treatment adherence, we proposed a nanotechnological approach based on functionalized liposomes, that is, with the surface modified with an analogue of siderophore (Sid) to carry moxifloxacin (Mox) to treat TB for an intrapulmonary administration. Siderophores are the main strategy of bacteria to take up iron, an essential metal for their metabolism. Four liposomes were developed with soy phosphatidyl, cholesterol and oleic acid: empty liposome (LipV); liposome containing Mox (LipMox); drug-free and Sid-functionalized liposome (LipS) and Sid-functionalized and Mox-containing liposome (LipSMox). The characterization of these liposomes was performed by dynamic light scattering technique (DLS), nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM). By all methods, we observed spherical particles and approximately 200 nm. The zeta potential was the main difference between them and was approximately -40mV for LipV and LipMox and +15mV for LipS and LipSMox. NTA showed a concentration of ~10^{11} particles/mL. A method of quantification of Mox in high performance liquid chromatography was developed and validated to determine the encapsulation efficiency (EE) and the release profile (RP). The EE was 51.31% for LipMox and 45.76% for LipSMox and the RP followed the model proposed by Korsmeyer-Peppas, common for liposomes. The minimal inhibitory concentration (MIC) against *M. tuberculosis* was determined under standard conditions (pH 6.6 and [Fe] = 0.04 µg/mL), with addition of iron (multiples of 2x, 5x and 10x in relation to the commercial medium), pH 7.4 and with addition of 10% fetal bovine serum (FBS). Changing the pH of the medium to 7.4 did not affect the MIC. The MIC of LipMox and LipSMox were less than 0.5%. LipS had MIC close to 10% in all conditions, except in the presence of FBS (MIC >25%), in which there is iron offered, not free, but complexed with ferritin. The time-kill assay showed no significant difference between free and encapsulated Mox, the bactericidal action was concentration-dependent. The cytotoxicity assay was performed for four cell lines: macrophages (J774A.1), fibroblasts (MRC-5), liver cells (HepG-2) and intestinal cells (Caco-2). Heterogeneity was observed in the cytotoxicity index (IC50) results with greater toxicity for liposomes containing Sid. The activity against intramacrophagic *M. tuberculosis* showed reductions of more than 90% of intracellular bacilli (LipMox 1 and 5%; Mox 1 and 5% and LipSMox 1%). Scanning and transmission electron microscopy confirmed the phagocytosis of liposomal vesicles by macrophages, confirming the possibility of a natural targeting of liposomes to phagocytic cells. Although functionalization with Sid did not improve the biological activity of liposomes, there was still a natural targeting of macrophages, cells of extreme importance in the pathogenesis of TB. Given the advantages of drug delivery of liposomes, an intrapulmonary *in vivo* administration of this system is proposed for the treatment of TB.

Keywords: *Mycobacterium tuberculosis*, tuberculosis, liposomes, nanomedicine, siderophore.

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CAPÍTULO II

LISTA DE ABREVIATURAS E SIGLAS

DLS	*Dynamic light scattering* – Espalhamento de luz dinâmica
FBS	*Fetal bovine sérum* – Soro fetal bovino
HD	*Hydrodynamic diameter* – Diâmetro hidrodinâmico médio
HPLC	*High performance liquid chromatography* – Cromatografia líquida de alta eficiência
IC_{50}	*Cytotoxicity index* - Índice de citotoxicidade
INH	*Isoniazid* - Isoniazida
SI / IS	*Selective index* - Índice de seletividade
LipMox	*Liposome containing moxifloxacin* - Lipossoma contendo moxifloxacino
LipS	*Siderophore-functionalized liposome* - Lipossoma funcionalizado com sideróforo vazio
LipSMox	*Siderophore-functionalized liposome containing moxifloxacin* - Lipossoma funcionalizado com sideróforo e contendo moxifloxacino
LipV	*Empty liposomes* - Lipossoma vazio
MIC	*Minimal inhibitory concentration* – Concentração inibitória mínima
MN	*Micronucleus* - micronúcleo
Mox	*Moxifloxacin"* - Moxifloxacino
NTA	*Nanoparticle tracking analysis* – Análise de rastreamento de nanopartículas
PBS	*Phosphate buffer saline"* – Tampão salina fosfato
PDI	*Poly dispersivity index* – Índice de polidispersividade
OADC	*Culture medium supplement made with oleic acid, albumin, dextrose and catalase* - Suplemento de meio de cultura, a base de ácido oleico, albumina, dextrose e catalase
OLA	*Oleic acid* – ácido oleico
OMS	Organização Mundial da Saúde
REMA	*Resazurin microtiter assay* – Ensaio de microdiluição para determinação da MIC em placas de 96 poços em que a resazurina é o indicador de viabilidade celular
RFP	*Rifampicin* - Rifampicina
SEM	*Scanning electron microscopy* – Microscopia eletrônica de varredura
Sid	*Siderophore analogue* – Análogo de sideróforo
TB	*Tuberculosis* - Tuberculose
TEM	*Transmission electronic microscopy* – Microscopia eletrônica de transmissão
ZP	*Zeta potential* – Potencial Zeta

SUMÁRIO

Capítulo I

1. Introdução

1.1. Tuberculose

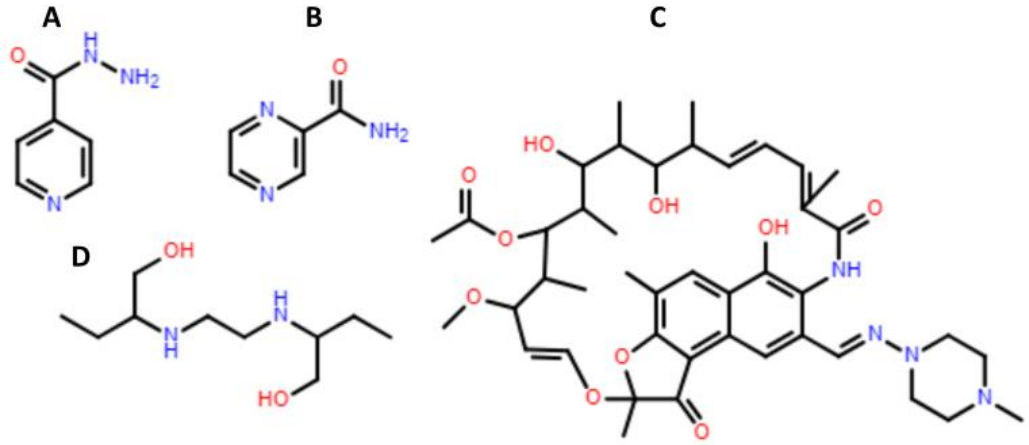
A Organização Mundial da Saúde (OMS) reportou que, em 2019, houve 10 milhões de casos de tuberculose (TB) e 1,4 milhão de mortes em decorrência da doença sendo, então, uma das doenças infecciosas que levou mais pessoas a óbito (WORLD HEALTH ORGANIZATION, 2020).

Os dados nacionais indicam 75.717 casos novos de TB em 2018 e 4.614 mortes em decorrência da doença em 2017, um pouco mais que nos anos anteriores, pois foram diagnosticados 69.569 casos novos em 2017 e 4.426 óbitos por tuberculose em 2016 (MINISTÉRIO DA SAÚDE - SECRETARIA DE VIGILÂNCIA EM SAÚDE, 2018; MINISTÉRIO DA SAÚDE (BRASIL), 2019). Já em 2020, o Brasil registrou 66.819 casos novos de TB e foram notificados cerca de 4,5 mil óbitos pela doença no ano anterior (MINISTÉRIO DA SAÚDE DO BRASIL, 2021).

A pandemia de SARS-COV-2, assumiu o posto de doença infecciosa que mais matou pessoas no mundo e ainda prejudicou o avanço constante que vinha acontecendo em relação a TB no mundo (número de casos e mortalidade). O número de novos casos de TB diagnosticados caiu drasticamente de 7,1 milhões (2019) para 5,8 milhões (2020), a mesma taxa de 2012 e que representa redução de 18% do estimado para 2020. Esta redução é vista como um retrocesso, pois possivelmente não representa a realidade do número de casos, mas sim uma falha nos sistemas de saúde em diagnosticar e reportar adequadamente os casos de TB devido à sobrecarga enfrentada devido pandemia de Covid-19, especialmente em países em desenvolvimento como Índia, Indonésia e Filipinas. O cenário ainda pode piorar para os anos de 2021 e os seguintes (WORLD HEALTH ORGANIZATION, 2021).

Quando o paciente é diagnosticado com infecção por *M. tuberculosis* sensível aos fármacos chamados de 1ªlinha/escolha, o esquema terapêutico básico empregado para o tratamento é composto por uma fase intensiva com 2 meses de rifampicina (RFP), isoniazida (INH), pirazinamida (PZA) e etambutol (ETB) seguido de uma fase de manutenção de 4 meses de RFP e INH totalizando seis meses de terapia. As estruturas moleculares destes fármacos estão na figura 1(WORLD HEALTH ORGANIZATION, 2010, 2016a).

Figura 1. Fármacos de primeira linha utilizados no tratamento de tuberculose: (A) isoniazida, (B) pirazinamida, (C) rifampicina e (D) etambutol.



Fonte: Autora.

Esse tipo de tratamento que combina antibióticos é chamada de poliquimioterapia e foi introduzido há mais de 50 anos com o objetivo de reduzir a emergência de cepas resistentes, por minimizar a possibilidade de seleção de mutantes espontaneamente resistentes a algum fármaco, e consequentemente visando obter maiores taxas de sucesso do tratamento (WORLD HEALTH ORGANIZATION, 2010, 2016a).

No Brasil, o tratamento é oferecido pelo Sistema Único de Saúde (SUS) e totalmente gratuito. Na primeira etapa do tratamento os fármacos são administrados na forma de um único comprimido, no esquema de dose fixa combinada. Adultos com mais 50kg, por exemplo, devem ingerir um comprido ao dia que contém RFP + INH + PZA + ETB (150mg + 75mg + 400mg + 275mg). Na segunda etapa do tratamento os comprimidos também são únicos, e tomados uma vez ao dia, e contém RFP + INH (300mg +150mg) (COMISSÃO NACIONAL DE INCORPORAÇÃO DE TECNOLOGIA AO SUS, 2018; INSTITUTO DE TECNOLOGIA EM FÁRMACO, 2019).

Infelizmente nem todos os pacientes finalizam seus tratamentos da maneira correta, o desaparecimentos dos sintomas da doença, possíveis efeitos adversos e até mesmo a falta de informação da importância da conclusão adequada do tratamento, resulta em uma taxa de abandono de 12% no Brasil (MINISTÉRIO DA SAÚDE DO BRASIL, 2021).

Embora tenha havido um avanço em relação à taxa de cura com o emprego da poliquimioterapia, a falha no tratamento ainda é recorrente. As principais razões para tal falha são: o abandono da terapia e a resistência bacteriana aos antibióticos. Quando um paciente inicia seu tratamento e o abandona ou o faz de forma inadequada, se estabelece uma pressão seletiva, uma vez que os antibióticos não atingiram concentração e tempo suficiente para

eliminar todos os bacilos. Esta pressão seletiva permite que bacilos resistentes sejam selecionados e, estes por sua vez, podem vir a se multiplicar causando um novo quadro de infecção e/ou transmitidos a outros indivíduos. Deste modo, quando a terapia anti-TB não é feita corretamente pode haver prejuízo tanto para o indivíduo quanto para a comunidade (JOHNSON et al., 2009; SZUMOWSKI; ADAMS; EDELSTEIN, 2013; WORLD HEALTH ORGANIZATION, 2016b).

A resistência a antibiótico é uma grande crise na saúde pública e este cenário se repete no combate a TB. As definições de resistência para TB são: TB resistente a RFP (RR-TB [*rifampicin resistant TB*]), que é o principal fármaco no tratamento, TB multifármaco resistente (MDR-TB [*multidrug resistant TB*]), que além de RFP, são resistentes a INH (WORLD HEALTH ORGANIZATION, 2021).

Há ainda a classificação de TB pré-extensivamente resistente a fármacos (pré-XDR-TB [*pre-extensively drug-resistant TB*]), quando além de RFP e INH, verificou-se a resistência a pelo a uma fluoroquinolona (Por exemplo: moxifloxacino (Mox) ou levofloxacino) e, por fim, TB extensivamente resistente a fármacos (XDR-TB [*extensively drug-resistant TB*]) quando resistente a RFP, qualquer fluoroquinolona e à bedaquilina ou linezolida, já que estas últimas são utilizadas como último recurso no tratamento e são os fármacos mais recentes na terapia contra TB (WORLD HEALTH ORGANIZATION, 2021).

A taxa de cura dos novos casos de TB no Brasil em 2020 foi de 70,1%, já a taxa de cura para casos de RR/MDR-TB é de 51,1%. Enquanto a taxa de cura global foi de 85% para novos casos e 59% para RR/MDR-TB (MINISTÉRIO DA SAÚDE DO BRASIL, 2021; WORLD HEALTH ORGANIZATION, 2021).

Com a redução do diagnóstico de TB em decorrência da pandemia de Covid-19, a queda na detecção e tratamento dos casos de TB resistente a antibióticos é uma consequência direta e foi de 15% (~177 mil em 2019 para ~150 mil em 2020). Foram aproximadamente 130 mil casos de MDR/RR-TB e 25 mil casos de pré-XDR/XDR-TB (WORLD HEALTH ORGANIZATION, 2021). No Brasil foram mais de 7 mil casos de TB resistente a pelo menos um fármaco (MINISTÉRIO DA SAÚDE DO BRASIL, 2021).

Diante do apresentado, é notável a necessidade de novos fármacos e de novas estratégias terapêuticas contra TB, especialmente as que visem reduzir o tempo de tratamento e aumentar a adesão do paciente à terapia (FREITAS et al., 2014; DA SILVA et al., 2016a, 2016b; WORLD HEALTH ORGANIZATION, 2016b).

1.2. Nanotecnologia

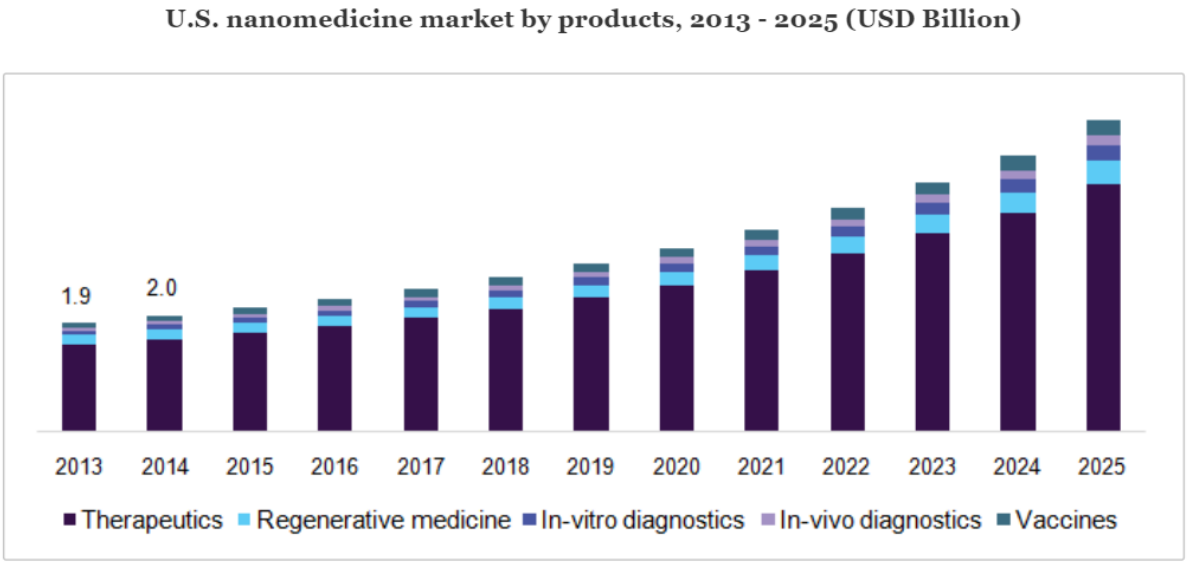
Dentre as diversas estratégias terapêuticas, as abordagens nanotecnológicas recebem certo destaque, pois através de diferentes tipos de formulação, como por exemplo: nanopartículas poliméricas, nanopartículas metálicas, nanotubos de carbono, nanopartículas lipídicas sólidas, sistemas líquido-cristalinos, lipossomas e microemulsões, é possível conferir adesão, proteção frente ao sistema digestivo, redução da ligação a proteínas plasmáticas, proteção da metabolização hepática, entre outras características desejáveis no desenvolvimento de um novo tratamento anti-TB (DA SILVA et al., 2016b).

A nanotecnologia é uma ferramenta que vem sendo bastante explorada no cenário biomédico, tanto com aplicações terapêuticas, mas também aplicações em medicina regenerativa, vacinas e diagnóstico. Em 2016, o mercado global de produtos nanotecnológicos para aplicações médicas foi de 138,8 bilhões de dólares, valor que vem evoluindo nos últimos anos e que tem expetativa de continuar em evolução para os próximos, fato este que corrobora com a avaliação de novas estratégias terapêuticas pautadas em nanotecnologia. A figura 2 representa a evolução e a projeção dos próximos anos do mercado norte-americano para produtos nanotecnológicos com fins medicinais, o que evidencia a atualidade e relevância da proposta aqui relatada (GRAND VIEW RESEARCH, 2017).

Ainda nas abordagens nanotecnológicas para uma possível terapia anti-TB podemos destacar os lipossomas. Eles surgiram no início da década de 1960 e foram utilizados em estudos de modelo de membrana plasmática, pois são compostos por bicamadas lipídicas separadas por um conteúdo aquoso. Para a obtenção de lipossomas com aplicação biomédica são empregados componentes biocompatíveis como esteróis e fosfolipídeos. Estas nanoestruturas podem carrear substâncias hidrofílicas ou lipofílicas, fato que as torna ainda mais versáteis (BANGHAM; STANDISH; WATKINS, 1965; GREGORIADIS; RYMAN, 1971; KIMELBERG H.K., TRACY T.F., BIDDLECOME S.M., 1976; DA SILVA et al., 2016b).

Diversos estudos já empregaram lipossomas para encapsular compostos com potencial anti-*M. tuberculosis*, mas também há relatos na literatura do uso de lipossomas em aplicações diagnósticas e até preventivas da doença, como potentes adjuvantes vacinais (SOUZA et al., 2010; PINHEIRO et al., 2011; CUI et al., 2013; TIWARI et al., 2017; TIAN et al., 2018).

Figura 2. Evolução e projeção do mercado de produtos nanotecnológicos com aplicações médicas nos Estados Unidos.

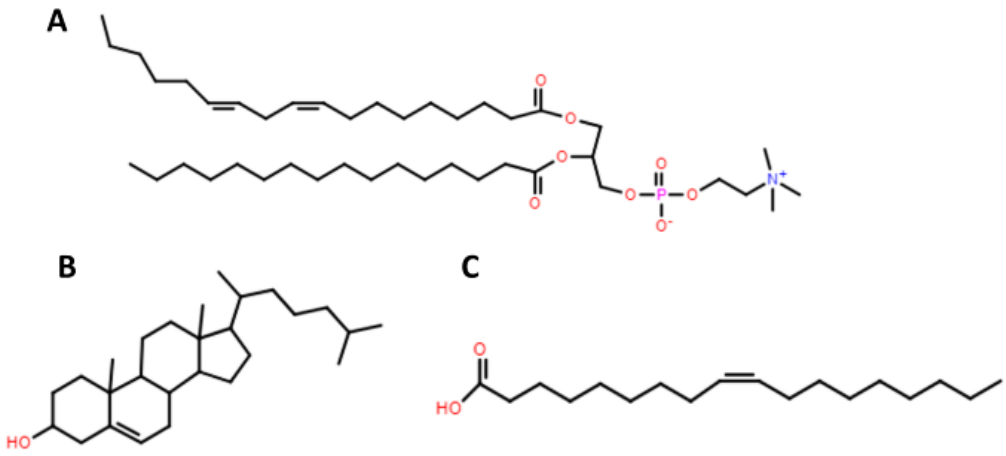


Fonte: https://www.grandviewresearch.com/industry-analysis/nanomedicine-market.

A Agência Europeia de Medicamentos (EMA) aprovou em 2020 a comercialização de uma formulação de amicacina lipossomal em suspensão para inalação (ALIS) para o tratamento infecções pulmonares por bactérias do complexo *Mycobacterium avium*, também chamado de MAC. O medicamento em questão é comercializado com o nome ARIKAYCE e é produzido pela Insmed Corporation. Sabe-se que a amicacina é um fármaco que apresenta muitos efeitos adversos e farmacocinéticos, exigindo uma administração intravenosa, a administração na forma de lipossomas ainda não é capaz de mitigar completamente os eventos adversos, mas consegue promover uma administração indolor e que não requer profissionais e estrutura de saúde, possibilitando uma melhor adesão ao tratamento e, possivelmente, uma taxa de cura maior (ROSE et al., 2014; ZHANG et al., 2018; B.V., 2020; CROMMELIN; VAN HOOGEVEST; STORM, 2020; INSMED INCORPORATED, 2020; CHALMERS et al., 2021; EUROPEAN MEDICINES AGENCY (EMA), 2021; HOY, 2021).

A aprovação de um medicamento na forma lipossomal, nos encorajou nesta proposta ainda mais pela administração intrapulmonar que é visada. Os lipossomas que serão empregados neste trabalho são constituídos de fosfatidilcolina de soja, colesterol e ácido oleico (OLA) e a figura 3 apresenta as estruturas moleculares destes componentes lipídicos.

Figura 3. Componentes lipídicos do lipossoma: (A) fosfatidilcolina de soja, (B) colesterol e (C) ácido oleico.



Fonte: autora.

A fosfatidilcolina de soja foi o fosfolipídeo escolhido devido a sua biocompatibilidade e estabilidade em relação a alterações de pH e concentração de sais (BANGHAM; STANDISH; WATKINS, 1965; DA SILVA et al., 2016b). Outro componente escolhido foi o colesterol que confere maior estabilidade as vesículas, mesma função que exerce nas membranas das células (GREGORIADIS; RYMAN, 1971; KIMELBERG H.K., TRACY T.F., BIDDLECOME S.M., 1976).

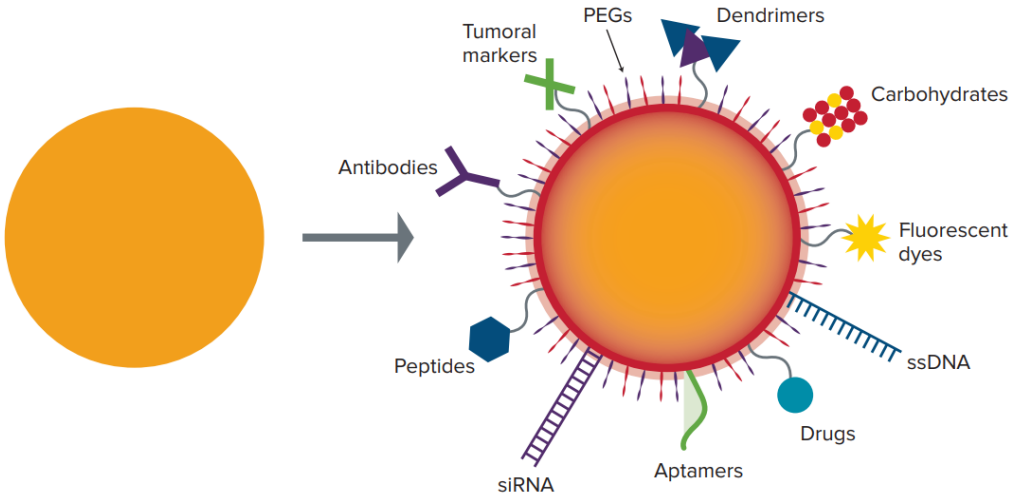
O último componente lipídico é o OLA, este por sua vez é um ácido graxo saturado, e sua ação em biológica é um pouco divergente entre os autores, mas já foi observado redução de citocinas pró-inflamatórias e produção de oxigênio reativo pelas mitocôndrias, ações estas que podem ser interessantes para o tratamento de TB (SOUZA et al., 2018).

A funcionalização de nanocarreadores, ou seja, a modificação da superfície de nanocarreadores com o objetivo de torná-los específicos a um alvo também é uma estratégia disponível para *"drug delivery"*(SUNDAR; PRAJAPATI, 2012). Esta é uma estratégia já bastante explorada na pesquisa de antitumorais, visando uma diferenciação entre células tumorais e sadias e com isso a redução de efeitos adversos (SUNDAR; PRAJAPATI, 2012; SALOUTI; AHANGARI, 2014; POETA et al., 2016).

Algumas moléculas utilizadas para funcionalizar nanocarreadores, ou seja, torná-los dirigidos a um alvo são, por exemplo: anticorpos monoclonais, folato, aptâmeros, peptídeos, transferrina e outras, como exemplificado na figura 4. Estas nanoestruturas com a superfície modificada podem ter diversas aplicações biomédicas, tais como: ação antitumoral, diagnóstico, ação teranóstica, tratamento de doenças infecciosas, doenças autoimunes, entre

outras (KIM E. SAPSFORD, W. RUSS ALGAR, 2013; RAMESHKUMAR; RAMARAJ, 2013; HURST et al., 2016).

Figura 4. Estratégias de funcionalização e direcionamento de nanopartículas.



Fonte: Hurst *et al*, 2016.

Para avaliar ainda se a utilização de uma modificação na superfície destes lipossomas pode trazer benefícios à sua atividade anti-*M. tuberculosis*, utilizou-se a estratégia da funcionalização com uma molécula semelhante a um sideróforo.

1.3. Sideróforos

Sideróforos são moléculas secretadas pelos microrganismos para promover o *uptake* de ferro para o citoplasma, isto porque uma molécula de sideróforos, no ambiente externo, se complexa com um átomo de ferro, através de interações eletrostáticas decorrentes da presença de átomos de oxigênio e nitrogênio com pares de elétrons livres e a nuvem eletrônica do átomo de ferro e, então, este complexo é internalizado pela bactéria, sendo que o sideróforos é reciclado e o ferro cumpre diversas funções intracelulares. Os sideróforos são específicos para cada gênero e até para espécies em alguns casos. Em *M. tuberculosis* os sideróforos encontrados são: carbomicobactina e micobactina (RATLEDGE, 2004; BARRY; BOSHOFF, 2005; LUO; FADEEV; GROVES, 2005; AHMED; HOLMSTRÖM, 2014).

Na literatura podemos encontrar diversos trabalhos que exploram a conjugação de sideróforos ou moléculas análogas a sideróforos com fármacos como estratégia de direcionamento, uma analogia usada para esta estratégia é conhecida como “cavalo de troia” (WENCEWICZ et al., 2009; JI; JUÁREZ-HERNÁNDEZ; MILLER, 2012; JUÁREZ-

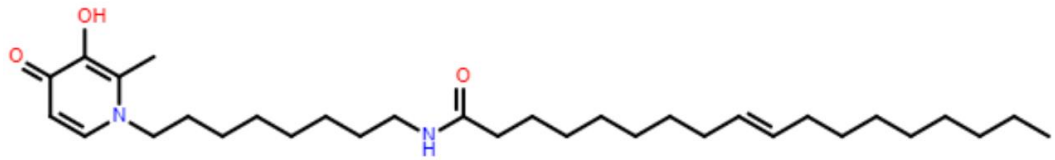
HERNÁNDEZ; FRANZBLAU; MILLER, 2012; JUÁREZ-HERNÁNDEZ; MILLER; MILLER, 2012; MCMAHON; ZIJL; GILAD, 2015); no entanto, o ineditismo de nossa proposta é a utilização do sideróforo como um direcionador de um lipossoma que carrega um potente antibiótico.

Outra possível vantagem de utilizar um nanocarreador é a fagocitose deste pelos macrófagos, fato já relatado na literatura, visto que este tipo celular é naturalmente responsável pela fagocitose de corpos estranhos e está intimamente relacionado com a patogênese de TB (GREF et al., 1994; PROSPERI et al., 2017).

Esta estratégia é chamada de ***Natural targeting***, ou seja, os macrófagos são as células naturalmente responsáveis pela fagocitose de corpos estranhos, e por este motivo, as nanopartículas são fagocitadas por estas células, no entanto, como o *M. tuberculosis* é um microrganismo capaz de sobreviver dentro dos macrófagos, é possível se aproveitar de um mecanismo imunológico de defesa para direcionar um tratamento para os bacilos intracelulares (GREF et al., 1994; PROSPERI et al., 2017).

A figura 5 ilustra a molécula usada para a síntese dos lipossomas funcionalizados análoga aos sideróforos (Sid).

Figura 5. Ácido oleico conjugado a uma molécula capaz de complexar com o átomo de ferro mimetizando a função do sideróforo.

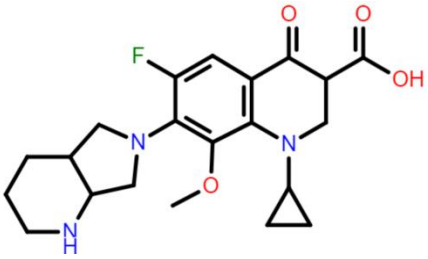


Fonte: autora.

1.4. Moxifloxacino

Sabendo a nanoestrutura que será empregada no desenvolvimento deste trabalho, que são os lipossomas, é o momento de dar destaque ao fármaco que será encapsulado: a Mox, cuja estrutura molecular está apresentada a figura 6, uma 8-metoxi quinolona.

Figura 6. Estrutura molecular de moxifloxacino da classe das fluoroquinolonas.



Fonte: autora.

A Mox é um antibiótico da classe das fluoroquinolonas, que vem ganhando destaque nos últimos anos no tratamento anti-TB, embora sua ação não seja seletiva contra as bactérias do gênero *Mycobacterium sp.,* Mox sempre está presente em ensaios clínicos tanto em protocolos para tratamento de MDR-TB quanto em propostas de regimes mais breves de tratamento de cepas sensíveis e os resultados vem demonstrando seu grande potencial para o tratamento de TB (NATIONAL INSTITUTE OF HEALTH, 2014, 2015a, 2015b). Possivelmente, daqui a alguns anos, tornando-se um fármaco de primeira escolha para o tratamento de TB.

Além disso, a resistência a Mox faz parte das classificações de pré-XDR e XDR-TB, dada sua importância no tratamento (WORLD HEALTH ORGANIZATION, 2021)

O mecanismo de ação da Mox é a inibição da enzima DNA gyrase (codificada pelos genes *gyrA* e *gyrB*) e envolvida na replicação do DNA, consequentemente, inibindo a divisão celular (BRYSKIER, 1993; COLE et al., 1998; DRLICA; MALIK, 2003; MALIK et al., 2012).

Sabe-se que a Mox tem bons parâmetros farmacocinéticos, ou seja, baixa metabolização, alta biodisponibilidade, e conhecer a sua segurança é uma boa vantagem em estudar uma nova estratégia de *"drug delivery"* (STASS; KUBITZA, 1999; DESHPANDE et al., 2010; GILLESPIE, 2016).

Diante do apresentado, espera-se avaliar se a estratégia de usar um nanocarreador (lipossoma) contendo Mox traz alguma vantagem terapêutica com potencial para utilização contra TB e ainda avaliar se a funcionalização destes lipossomas com análogos de sideróforos são relevantes para a atividade biológica.

2. Objetivos

2.1. Objetivo geral

Desenvolver lipossomas funcionalizados com sideróforos e contendo moxifloxacino, caracterizá-los e avaliar o potencial *in vitro* contra *M. tuberculosis*.

2.2. Objetivos específicos

- Desenvolver os lipossomas: LipV (lipossoma vazio), LipMox (lipossoma contendo Mox), LipS (lipossoma vazio e funcionalizado com Sid) e LipSMox (lipossoma funcionalizado com Sid e contendo Mox);
- Determinar o diâmetro hidrodinâmico médio (HD), o índice de polidipersividade (PDI) e Potencial Zeta (ZP) dos lipossomas pela técnica de espalhamento de luz dinâmica (DLS);
- Avaliar a estabilidade dos lipossomas armazenado em geladeira por 85 dias via DLS;
- Avaliar a estabilidade dos lipossomas na presença dos meios de cultura utilizados nos ensaios microbiológicos e de citotoxicidade via DLS;
- Avaliar a estabilidade dos lipossomas em pH ácido (pH = 1,5) e pH neutro (pH = 7,4) mimetizando condições do trato gastrointestinal via DLS;
- Avaliar a estabilidade dos lipossomas após exposição de 15min a luz ultravioleta (UV) via DLS;
- Determinar o número de nanopartículas por mililitro e a distribuição de tamanho das nanopartículas pela análise de rastreamento de nanopartículas (NTA);
- Desenvolver e validar um método de cromatografia líquida de alta eficiência (HPLC) para quantificar Mox;
- Determinar a eficiência de encapsulação;
- Determinar o perfil de liberação;
- Determinar a concentração inibitória mínima (MIC) dos lipossomas frente a *M. tuberculosis* sob diferentes condições (maiores concentrações de ferro (2x, 5x e 10x em relação a concentração tradicional), pH 7,4 e com a adição de 10% de soro fetal bovino (FBS);
- Determinar a curva de morte bacteriana (*time-kill*) frente ao antibiótico Mox, LipV, LipMox, LipS, LipSMox e Sid;

- Determinar a citotoxicidade dos lipossomas frente a macrófagos murinos (linhagem J774A.1), fibroblastos pulmonares (linhagem MRC-5) hepatócitos (linhagem HepG-2) e células intestinais (Caco-2);
- Determinar o índice de seletividade (IS) dos lipossomas frente as quatro linhagens celulares;
- Avaliar a genotoxicidade em células hepáticas (HepG-2) pela técnica de detecção de micronúcleos;
- Determinar a atividade anti-*M. tuberculosis* intramacrofágico dos lipossomas;
- Avaliar a interação de macrófagos (J774A.1) com os lipossomas por microscopia eletrônica de transmissão (TEM) e varredura (SEM).

3. Conclusões

A presença do Sid nos lipossomas funcionalizados (LipS e LipSMox) resultou em um perfil físico-químico e biológico diferente dos lipossomas não funcionalizados (LipV e LipMox), como por exemplo: a carga superficial (ZP), positivo para LipS e LipSMox e negativo para LipV e LipMox, e a citotoxicidade (IC_{50}), em que se notou maior citotoxicidade para aqueles com o Sid na superfície dos lipossomas.

Entretanto a funcionalização com o Sid não mostrou aumento na atividade biológica contra TB *in vitro,* como observado nos ensaios de determinação de MIC em condições padrão, *time-kill* e atividade intramacrofágica. Entretanto, vale ressaltar que tanto a Mox livre quanto a Mox encapsulada (LipMox e LipSMox) apresentaram excelente atividade contra os bacilos de *M. tuberculosis* e até mesmo contra os bacilos intracelulares.

Sendo assim, a possibilidade de um direcionamento ativo (*active targeting*) dos lipossomas pela ação do Sid, nestas condições de composição, estrutura e características físico-químicas, parece não ser essencial para o bom desempenho dos lipossomas desenvolvidos, já que tanto LipMox quanto LipSMox demonstraram potencial excelente e muito semelhante contra *M. tuberculosis*.

Por outro lado, quando analisamos a possibilidade de um direcionamento natural (*natural targeting*) através da ação fagocítica dos macrófagos, células de extrema importância na patogênese de TB, fica evidente pelas imagens de microscopia eletrônica a fagocitose das partículas lipossomais e, com isso, a relevância de uma abordagem nanotecnólogica contra *M. tuberculosis,* uma vez que o bacilo é capaz de sobreviver dentro dos macrófagos.

Propomos uma aplicação intrapulmonar destas nanopartículas para o tratamento de TB *in vivo*, pois supomos que uma administração intrapulmonar combinada a uma liberação controlada de Mox e capacidade de atingir aqueles bacilos intracelulares (através da fagocitose) é uma estratégia passível de bons resultados.

O fato de já existir no mercado um tratamento baseado no mesmo sistema nanotecnológico (lipossomas) e para um tratamento similar (infecção pulmonar por micobactérias), nos encoraja ainda mais a propor uma administração intrapulmonar e exemplifica que há espaço no mercado para produtos como este.

Capítulo II

Liposomes containing moxifloxacin and functionalized with a siderophore analog for tuberculosis treatment

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1. Introduction

The etiology of tuberculosis (TB) caused mostly by *Mycobacterium tuberculosis* was described by Robert Koch more than a century ago, but TB is still one of the most killer infectious diseases in the world. In 2019, 10 million fell ill and 1.4 million died of TB (KOCH, 1882; WORLD HEALTH ORGANIZATION, 2020). In 2020, there was a reduction in the number of newly diagnosed TB cases from 7.1 million to 5.8 million as a result of the overload of health systems due to the SARS-COV-2 pandemic (WORLD HEALTH ORGANIZATION, 2021).

The pathogenesis of TB has some particularities, the main one being the ability of the bacillus to survive inside of macrophages, the main defense cells of the immune system, reducing their metabolism to a latent state (PARBHOO; SAMPSON; MOUTON, 2020).

The particular pathogenesis added to the resistance to the available antibiotics that has been advancing beyond the discovery of new ones, even with more financial resources, makes the need for new therapeutic strategies urgent, especially aiming at a shorter treatment regimen and with more patient adherence (WORLD HEALTH ORGANIZATION, 2021).

Nanotechnology is a set of strategies capable of employing different types of materials that can be applied in the encapsulation of drugs and it can promote pharmacokinetic changes such as protection against chemical and enzymatic degradation, increased circulation time,

reduced binding to blood proteins, drug release targeted, sustained release and more (DANAEI et al., 2018).

Liposomes are one of these nanocarriers and were first described in 1965 by Bangham, Standish and Watkins during research on the plasma membrane and liposomes are nanovesicles widely explored in the biomedical field. They are spherical vesicles formed by at least a double layer of a phospholipid, a hydrophobic compartment and an aqueous core, a hydrophilic compartment (BANGHAM; STANDISH; WATKINS, 1965; BOZZUTO; MOLINARI, 2015).

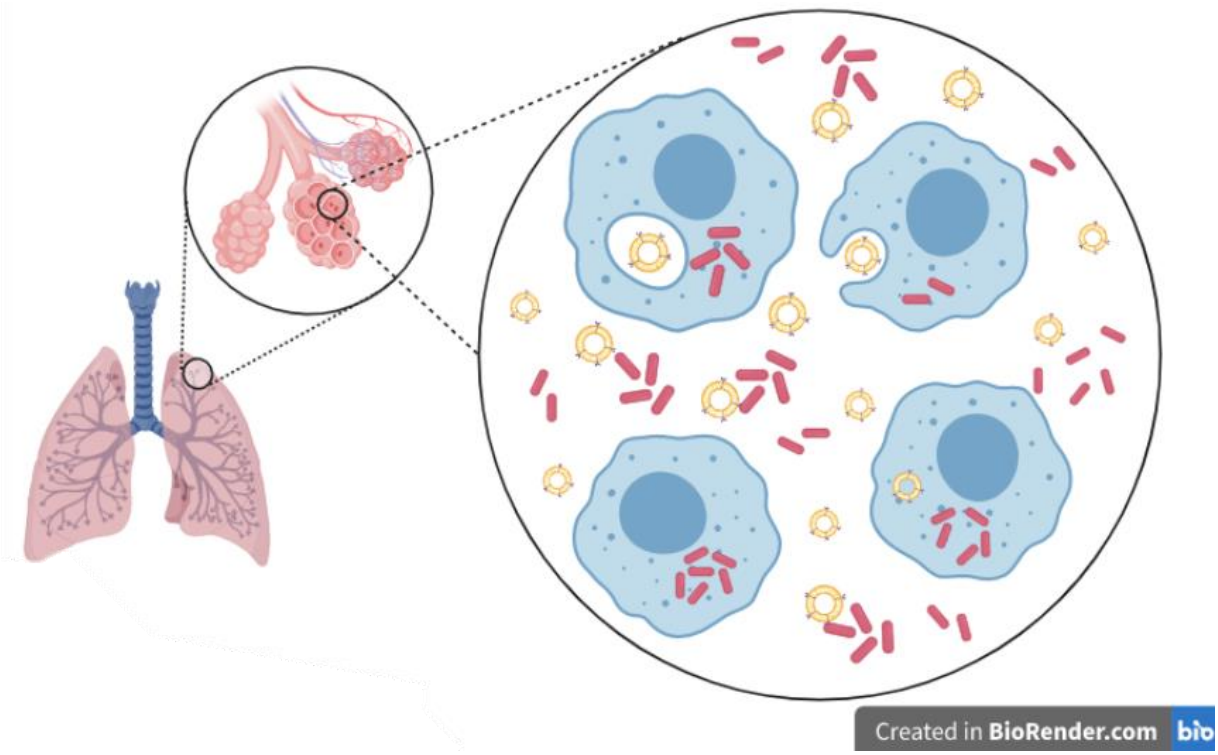
Some molecules can be attached to the surface of nanoparticles in search of greater specificity in drug delivery and this is called functionalization. These kinds of strategies are extensively applied in anti-cancer research (KIM E. SAPSFORD, W. RUSS ALGAR, 2013; RAMESHKUMAR; RAMARAJ, 2013; HURST et al., 2016).

Bacteria need iron for their metabolism, and they secrete siderophores for extracellular complexation with iron and have superficial receptors to uptake them into the cell. Thus, a siderophore attached to the nanoparticle surface maybe can promote an active targeting to *M. tuberculosis.* The conjugation of siderophores and siderophore analogs (Sid) with drugs is already described in the literature as a drug delivery strategy against *M. tuberculosis* and other bacteria which is called the "*Trojan horse*" approach, but there are no reports of a Sid functionalizing nanoparticle for a specific drug delivery (RATLEDGE, 2004; BARRY; BOSHOFF, 2005; LUO; FADEEV; GROVES, 2005; JI; JUÁREZ-HERNÁNDEZ; MILLER, 2012; JUÁREZ-HERNÁNDEZ; FRANZBLAU; MILLER, 2012).

Moxifloxacin (Mox) was the antibiotic chosen for nanoencapsulation, because it has excellent pharmacokinetic parameters and is used in the treatment of resistant strains, and may be a possible first-line treatment in the future, as it is present in several clinical trials (PAI et al., 2016).

A siderophore analog (Sid) was obtained and conjugated with an oleic acid (OLA) molecule and this conjugate Sid+OLA was used in the development of liposomes containing (functionalized) or not Sid and encapsulating Mox, the physicochemical characterizationof the liposomes were performed and an evaluation of their potential against *M. tuberculosis* as well their *in vitro* safety were evaluated to analyse whether the functionalization of liposomes under these conditions is relevant to their biological activity and whether the use of these liposomes can bring benefits to anti-TB therapy. The strategy behind a possible innovative drug delivery system to treat TB is illustrated in Figure 1.

Figure 1. Drug delivery strategy based on a siderophore analog functionalized liposome to treat tuberculosis infection.



Liposome size = 0.1-0.3µm; *Mycobacterium tuberculosis* bacillus = 2-4 x 0.2-0.5 µm and macrophages = 21-22 µm.

2. Material and methods

2.1.Liposomes's synthesis

The film-hydration technique was employed and the composition of liposomes was soybean phosphatidylcholine (180 mg – 10.45 M), cholesterol (45 mg- 5.27 M) e oleic acid (OLA) (25 mg – 4M) to the empty liposome (LipV) and the liposome containing Mox (LipMox). For the functionalized liposomes instead of OLA, was used a siderophore analog (Sid) conjugated with OLA at the same proportion to make the liposomes functionalized with Sid (LipS) and the liposomes functionalized with Sid and containing Mox (LipSMox). The Sid+OLA was solubilized in methanol (10 mg/mL).

The lipidic components were solubilized in 13mL of ethylic ether and rotary evaporated (10 0rpm at 65 ºC for 60 min under vacuum pressure of 200 mmHg - IKA RV 10 Control) and the hydration phase was with 22mL of phosphate buffer saline (PBS) or Mox 0.1 mg/mL by a sonicator step for 10 min, a vortex agitation step for 1 min and a magnetic stirrer step for 60 min (SBS Stirrer – 300rpm).

An phase high-pressure homogenizer was applied to size decreased at 1500 bar for 10 minutes in EmulsiFlex-C3, Avestin Inc. as adapted from Von Zuben et al, 2021 (DE SOUZA VON ZUBEN et al., 2021). Liposomes were kept in a refrigerator (2-8°C) for up to 30 days.

2.2.Liposomes's characterization

2.2.1. Dynamic Light Scattering (DLS) and Zeta Potential (ZP)

The liposomes were evaluated using the Nano ZS equipment (Zetasizer®)and the hydrodynamic diameter (HD) and the polydispersity index (PDI) that represents how homogeneous the size of the particles in the sample are determined by analyzing the DLS and the zeta potential (ZP) that represents the interaction electrostatics of the nanoparticle surface with the medium by the microelectrophoresis technique using the Zetasizer Nano ZS equipment (WU et al., 2004).

The stability of the liposomes was analyzed after exposure to the culture media used in biological assays, in the face of pH variations (1.5 for 1h and pH = 7.4 for 24h), stability after 15 min of ultraviolet exposure (decontamination method) and the stability for 85 days under refrigeration.

For these analyzes, aliquots of the liposomal suspensions (100 µL) were diluted in Milli-Q water until the volume was 10 mL (dilution 1:100).

2.2.2. Nanoparticles tracking analysis

The nanoparticle tracking analysis (NTA) is based on the theory of Brownian motion and it analyses the individual movement of particles according to the intensity of the scattered light (FILIPE; HAWE; JISKOOT, 2010; RIBEIRO et al., 2018). The liposomes were diluted 1:1000 before injection into the NanoSight NS300 (Malvern Pananalytical).

2.2.3. Mox quantification by high-performance liquid chromatography (HPLC)

Mox quantification method was adapted from Ashour and Kattan, 2016 and partial validation was performed (ANVISA, 2017). Conditions: C18 column, mobile phase: acid water (acetic acid [99.8%] until pH 3) and methanol (55:45). The rate was 0.8 mL/min, the injection volume was 20 µL. Device: Infinity 1290 I Agilent Technologies with detection by ultraviolet (UV) light *Diode Array Detector* (DAD) (ASHOUR; KATTAN, 2016).

2.2.4. Encapsulation efficiency (EE)

Ultracentrifugation of 1 mL of was performed liposomes using Amicon®-100 kDa ultrafiltration filter at 1,000 rpm for 30 min. The filtrate containing the free and non-encapsulated Mox was diluted and quantified by the HPLC method described. The EE was calculated by the formula EE (%) = [(Total amount of Mox - Free amount of Mox) / Total amount of Mox] x 100 (DE SOUZA VON ZUBEN et al., 2021).

2.2.5. Release profile (RP)

Before performing the assay, the sink concentration of Mox in PBS (pH = 7.4) was determined. The supernatant was collected from a saturated solution of Mox, which was filtered and quantified on HPLC (SAARINEN-SAVOLAINEN et al., 1997).

Liposomes (1mL) were packaged in dialysis bags (molecular weight cut off 10 kDa,- Thermo Fisher Scientific) which were submerged in 25 mL of PBS. The flasks were kept at 37° with slow agitation. At time intervals, 100 µL of PBS was collected and the same volume was replaced. Aliquots were quantified on HPLC as described in 2.2.3 (HUA, 2014).

The mathematical models of release kinetic: Baker and Lonsdale (Zero order); Korsmeyer-Peppas; Hixon and Crowell; Higuchi and First-order were analyzed to find the best match with Mox, LipMox and LipSMox results (KALAM et al., 2007; LARACUENTE; YU; MCHUGH, 2020).

2.2.6. Transmission electronic microscopy (TEM)

The size and morphology of nanoparticles were characterized using a transmission electron microscope - TEM (JEOL 1011, Tokyo, Japan) operated at an accelerating voltage of 100 kV. Samples were prepared by placing a drop of the sample (5 µL) using a 400-mesh copper grid coated with formvar film, and then it was air-dried, following contrast enhancement with osmium tetroxide vapor. Grids were kept at room temperature until analysis.

2.3. *In vitro* anti-*M. tuberculosis* activity and safety

2.3.1. Minimal inhibitory concentrations against *Mycobacterium tuberculosis*

The laboratorial and antibiotic susceptible strain of *Mycobacterium tuberculosis* H_{37}Rv (ATCC 27294) was cultivated in Middlebrook® 7H9 medium with 10% OACD homemade (oleic acid, albumin, catalase and dextrose). Activity against *M. tuberculosis* was determined under standard conditions and in the presence of modified media. The regular conditions of

7H9 medium are 0.04 mg/mL of ferric ammonium citrate (1x [Fe]) and pH 6.6. The modifications were increasing the iron concentration to 2x, 5x and 10x (added as ferric ammonium citrate, same salt present in the commercial medium), pH 7.4 (adjusted with sodium hydroxide 0.1 M) and addition 10% SFB (presence of iron complexed with ferritin (KAKUTA et al., 1997)).

Briefly, the samples were diluted 0.10 – 25 % or µg/mL (100 µL) in a 96-wells plate and then the mycobacterial inoculum (~10^5 Colony-forming unit [CFU]/mL) was added (100 µL). The plate was incubated for seven days (37ºC and 5% CO_2). After that, the resazurin solution was added (30 µL – 0.01%) and after one more day, the fluorescence was read (530/590 nm). Based on an untreated control it was possible to determine the concentration with 90% of growth inhibition, the minimal inhibitory concentration (MIC) (PALOMINO et al., 2002; CAMPOS et al., 2019).

2.3.2. Time-kill

The *M. tuberculosis* inoculum (50 mL - 10^5 CFU/mL) in 7H9 medium supplemented with 10% OADC was placed in a 125mL Erlenmeyer flask. One of these flasks received no treatment, being the positive control of mycobacterial growth, while the others received Mox, LipV, LipMox, LipS, LipSMox, or Sid. On days 1, 3, 6, 9 and 12, aliquots (100µL) were taken, serially diluted and plated on 7H11 solid media plates with 10% OADC. The plates were incubated at 37ºC and 5% CO_2 for colony growth. After 4 to 6 weeks, the colonies were counted and the results were plotted in a graph of Log_{10} CFU/mL according to time. The bactericidal effect is considered a 3 Log_{10} CFU/mL reduction (99.9%) otherwise it is considered a bacteriostatic effect (STEENWINKEL et al., 2010).

2.3.3. Cytotoxicity and selectivity

The cells used were macrophages (J774A.1), pulmonary fibroblasts (MRC-5), hepatocytes (HepG-2) and intestinal cells (Caco-2) according to the method previously described (SILVA et al., 2017) to determine the cytotoxicity index (IC_{50}), the concentration with 50% of cell viability. Selective index (SI) was calculated by the ratio of MIC and IC_{50} and their values higher than 10 indicate potential as a possible treatment (EKINS et al., 2013).

2.3.4. Genotoxicity by micronucleus (MN) analysis

In 6-wells plates, HepG-2 cells were seeded (2.5x10^5 cells/mL). After 24h hours, the treatments were added in non-toxic concentration (previous determinate): LipV, LipS,

LipMox and LipSMox at 10% (v/v), Mox at 100 µg/mL, H_2O_2 1mM as a positive control (for four minutes) and an untreated control. After 24h, the treatments were washout and cytochalasin B (5mg/mL) was added for 48h to prevent cytokinesis (cell separation after mitosis).

The cells were labeled with the fluorophore Hoechst 33342 and the images were taken by Axiocan 503 color microscope and Zen image D2 software.

Based on the images, it was calculated the nuclear division index (NDI) in 500 viable calls to determine the frequency of cells with 1, 2, 3 or 4 nuclei and calculate the NDI using the following formula: NDI = (M1 + 2M2 + 3M3 + 4M4) / N, where M1 – M4 represents the number of cells with 1-4 nuclei and N is the total number of viable cells scored, excluding necrotic and apoptotic cells. Acceptable NDI should be between 1.3 and 2.2, the percentage of the binuclear cells should be between 30 and 60% and MNs were counted in 1000 binuclear cells (FENECH, 2007; SILVA et al., 2017). The experiments were done three independent times.

2.3.5. Intramacrophagic activity

The macrophages were cultivated and then seeded ($5x10^5$ cell/mL) in a 24-well plate, after 24 hours (37°C and 5% CO_2) the mycobacterial suspension ($1x10^6$ CFU/mL) was added for phagocytosis for 2 hours. The extracellular bacilli were gently removed by three washes with PBS. The treatments were added at non-toxic concentrations (previously determined) and stayed in contact with the infected cell for 72h as adapted from Solcia et al, 2021 (SOLCIA et al., 2021).

The cells were lysates with triton 0.1%, diluted and seeded on agar-plates (7H11 plus 10% OADC). After approximately four weeks, the CFU were counted and the Log_{10} CFU/mL graph was elaborated.

2.3.6. TEM of macrophages and liposomes

The macrophages were seeded ($5x10^5$ cell/mL) in coverslips sterile and previously treated with poly-L-lysine in a 12-wells plate. After 24h, the liposomes (25% [v/v]) were placed in the wells for 2h. Then, cells were fixed with Karnovsky fixative and post-fixed with 1% osmium tetroxide containing potassium ferricyanide for 1 h at room temperature. Samples were dehydrated in grading acetone (30-100%) and embedded in Epon resin. Ultrathin sections were stained with 5% uranyl acetate. Micrographs were performed using a TEM (JEOL 1011, Tokyo, Japan) at an accelerating voltage of 80 kV.

2.3.7. Scanning electronic microscopy (SEM) of macrophages and liposomes

Cells already treated with 25% (v/v) of liposomes (2h) were seeded in coverslips and were fixed using Karnovsky fixative overnight. Cells were washed with cacodylate buffer (0,1M), post-fixed with osmium tetroxide and dehydrated using graded acetone (30-100%). Then, they were critical-point-dried (Balzers, CPD 030, Germany) from liquid CO_2 and gold-sputtered. The cell morphology was observed using SEM (Jeol, JSM-7001F).

2.4.Statistical analysis

The results presented were the mean and standard deviation of two or three independent experiments and these results were calculated by the Prism5 GraphPad software, which was also used to prepare the graphs presented in this report and for the statistical analyses.

The statistical analysis used on the stability results of the liposomes in the refrigerator for 85 days was One-way ANOVA with Tukey's post-test ($p<0.05$) always considering day 1 of the respective sample.

For the results of intramacrophage activity, the MN and NDI of the genotoxicity analysis and the comparison of the MIC in the different media, the One-way ANOVA analysis with Newman-Keuls post-test was used ($p<0.05$).

3. Results and discussion

3.1.Liposome's development and characterization

DLS analysis determines hydrodynamic diameter (HD) which represents the average particle size; the polydispersity index (PDI) which represents the particle size homogeneity and the zeta potential (ZP) which is the electrostatic interaction of the liposome surface with the liquid it is in, in this case it is deionized water, and therefore we generalize as surface charge.

To assess the stability of the liposomes in the presence of the culture media, a 1: 2 dilution of the liposomes was made in the respective culture media (500µL of liposome + 500µ of culture medium) and this mixture was stored in an oven at 37ºC for 7 days for the medium used in the microbiological tests (Medium 7H9 + 10% OADC [oleic acid, albumin, catalase and dextrose]) and for 24h for the medium used in the cytotoxicity test (Medium RPMI + 10% fetal bovine serum (FBS), DMEM medium + 10% FBS and DMEM high glucose medium (DMEM HG) + 20% FBS), mimicking the period of biological experiments. After the incubation period, 200µL of this mixture was diluted in Milli-Q water until the

volume was 10mL and the analysis was performed to determine DHM, PDI and PZ and the results are in Table 1.

Likewise, it was done to assess the stability of liposomes at acid pH (0.1M hydrochloric acid solution - pH = 1.5 for 1h) and neutral pH (PBS - pH = 7.4 for 24h), which mimic the pH and the time that the substances spend, after oral intake, in the stomach and intestinal environment, respectively (KELLUM, 2000; HOLZER, 2007) and table 2 present the results.

Ultraviolet light, especially type C, between 200 and 280 nm has a germicidal action (DAI et al., 2012), and, for this reason, it was used to decontaminate liposomal formulations for use in biological tests. As the culture media used in these tests are very rich in nutrients, some decontamination method needed to be used to avoid contamination of the tests with environmental microorganisms, since the liposomes are prepared aseptically, but they are not steril. As table 3 showed the HD, PDI and ZP were not altered after exposure to ultraviolet light.

It is known that liposomes are phagocyted from the circulatory system by cells and tissues and that plasma proteins play an important role in this process. Albumin is one of the proteins most associated with interaction with liposomes in the circulatory system, as described by Semple et al 1998(SEMPLE; CHONN; CULLIS, 1998). Albumin is present in the mycobacterial culture medium 7H9 + 10% OADC and in the SFB of cell culture media and this interaction can explain the changes in HD, PDI and ZP observed.

Changes in ZP have already been reported as a result of the modification of the pH divided by the protonation of the phospholipids used in the synthesis of liposomes, and therefore the interaction between the particles can also be altered resulting in modifications of HD and PDI as well(SUŁKOWSKI et al., 2005; ROY et al., 2016).

The stability of LipMox and LipSMox for 85 days analyzed by DLS is presented in figure 2. The LipV and LipS followed the same pattern (data not shown) of LipMox and LipSMox, respectively.

Table 1. Analysis by the dynamic light scattering technique of liposomes after contact with the culture media of biological assays.

		Normal condition	7H9 + 10%OADC	RPMI + 10%SFB	DMEM +10% SFB	DMEM HG + 20% SFB
LipV	**HD**	97.9±0.7	256.8±7.9	275.5±4.3	292.3±16.9	317.1±15.5
	PDI	0.166±0.005	0.494±0.004	0.463±0.018	0.453±0.025	0.575±0.086
	%	98.63%	96.9%	79.03%	88.97%	71.67%
	ZP	-42.3±0.7	-36.8±2.1	-67.3±0.4	-57.0±1.6	-57.3±0.9
LipMox	**HD**	151.1±6.2	190.3±1.2	408.7±6.4	460.9±8.1	406.0±7.4
	PDI	0.196±0.022	0.303±0.026	0.481±0.022	0.528±0.015	0.487±0.024
	%	98.03%	94.4%	72.47%	77.50%	85.00%
	ZP	-41.6±0.4	-40.5±4.2	-64.9±1.0	-60.9±0.7	-59.5±1.1
LipS	**HD**	246.8±4.9	200.2±0.3	130.7±1.5	167.7±3.4	141.7±6.7
	PDI	0.333±0.022	0.349±0.005	0.375±0.013	0.632±0.026	0.537±0.028
	%	97.40%	91.8%	75.3%	69.97%	71.4%
	ZP	15.3±0.6	-24.8±1.1	-30.1±1.37	-30.7±1.4	-27.6±0.4
LipSMox	**HD**	126.1±0.2	131.2±1.3	134.6±4.0	134.8±4.4	138.7±6.7
	PDI	0.162±0.006	0.277±0.009	0.502±0.018	0.498±0.013	0.559±0.046
	%	99.07%	93.7%	70.4%	78.73%	67.4%
	ZP	13.7±0.8	-20.4±1.1	-23.9±0.7	-31.1±1.1	-26.6±0.3

HD = hydrodynamic diameter (nm); PDI = polydispersity index; ZP = zeta potential (mV); % = how much of the sample is in this HD and PDI range; 7H9 = culture medium for *Mycobacterium tuberculosis*; OADC = supplement for the culture medium of mycobacteria based on oleic acid, albumin, dextrose, and catalase; RPMI = “Roswell Park Memorial Institute” medium for cell culture; DMEM = “Dulbecco's Modified Eagle Medium” for cell culture; FBS = fetal bovine serum.

Table 2. Analysis by the dynamic light scattering technique of liposomes after pH variation.

		Normal condition	After 1h at pH 1.5	After 24h at pH 7,4
LipV	**HD**	97.9±0.7	326.4±9.0	209.5±5.6
	PDI	0.166±0.005	0.416±0.064	0.232±0.004
	%	98.63%	95.13%	96.63¨%
	ZP	-42.3±0.7	-26.4±2.1	-50.5±0.1
LipMox	**HD**	151.1±6.2	293.3±57.1	335.7±14.6
	PDI	0.196±0.022	0.635±0.070	0.392±0.028
	%	98.03%	100%	84.23%
	ZP	-41.6±0.4	-29.4±0,3	-50.5±1.1
LipS	**HD**	246.8±4.9	163.2±14.3	120.8±4.0
	PDI	0.333±0.022	0.537±0.006	0.347±0.043
	%	97.40%	71.23%	85.63%
	ZP	15.3±0.6	-8.6±1.7	-12.5±0.5
LipSMox	**HD**	126,1±0,2	138,2±7,8	114,9±3,8
	PDI	0,162±0,006	0,510±0,036	0,372±0,038
	%	99,07%	69,70%	83,73%
	ZP	13,7±0,8	-6,9±0,4	-9,0±0,4

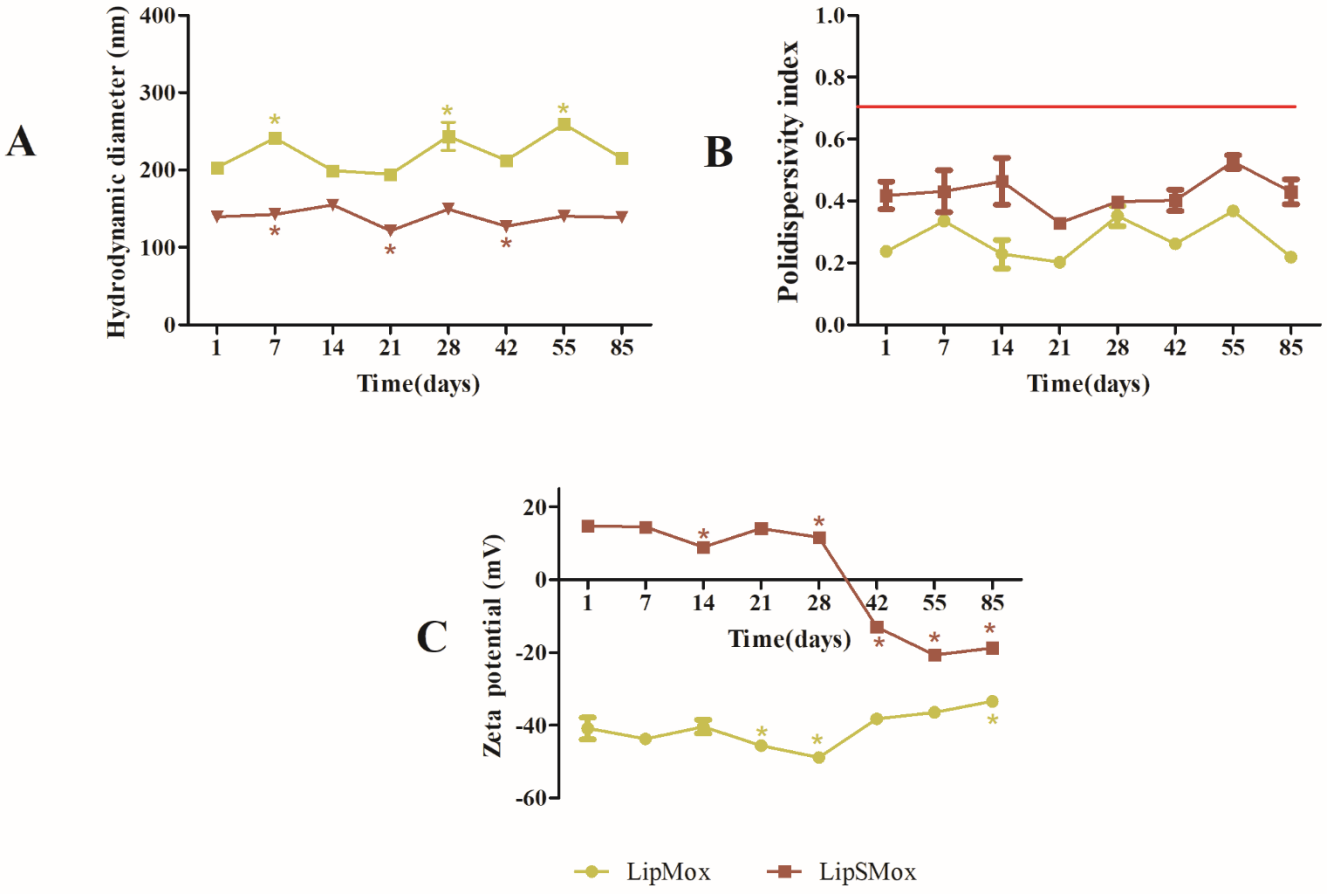
HD = hydrodynamic diameter (nm); PDI = polydispersity index; ZP = zeta potential (mV); % = how much of the sample is in this HD and PDI range.

Table 3. Analysis by the dynamic light scattering technique of liposomes after 15 minutes of exposure to ultraviolet light. Ultraviolet light was used for decontamination of liposomes before biological tests.

	LipV	LipMox	LipS	LipSMox
HD (nm)	94.26±1.0	236.4±10.8	124.6±4.4	133.1±3.1
PDI	0.195±0.01	0.329±0.04	0.329±0.03	0.390±0.04
ZP (mV)	-43.3±0.75	-46.1±1.19	16.3±0.50	18.0±0.35
%	97.2%	95.1%	85.13%	77.2%

HD = hydrodynamic diameter (nm); PDI = polydispersity index; ZP = zeta potential (mV); % = how much of the sample is in this HD and PDI range

Figure 2. Evaluation of the stability of the hydrodynamic diameter, polydispersity index and zeta potential of LipMox and LipSMox for 85 days under refrigeration.



A. Hydrodynamic diameter (nm) is determined by the dynamic light scattering (DLS) technique and represents the average particle size. **B**. Polydispersity index represents the homogeneity of the samples detemined by DLS analisys and must be below 0.7 (red line) but preferentially below 0.5. **C**. Zeta potential (mV) refers to the interaction between the surface

of the liposomal particles and the solution. LipMox = liposome containing moxifloxacin; LipSMox = liposome functionalized with a siderophore analog and containing moxifloxacin. * = significantly different compared to day 1 (p<0.05), according to the analysis: One-way ANOVA with Tukey's post-test by the Prism5 software.

Statistical analyzes of the stability based on the DLS results were performed concerning day 1 of the respective sample, except for the PDI analysis, since the recommended in the literature is PDI less than 0.700, as represented in the graph by a red line (DANAEI et al., 2018).

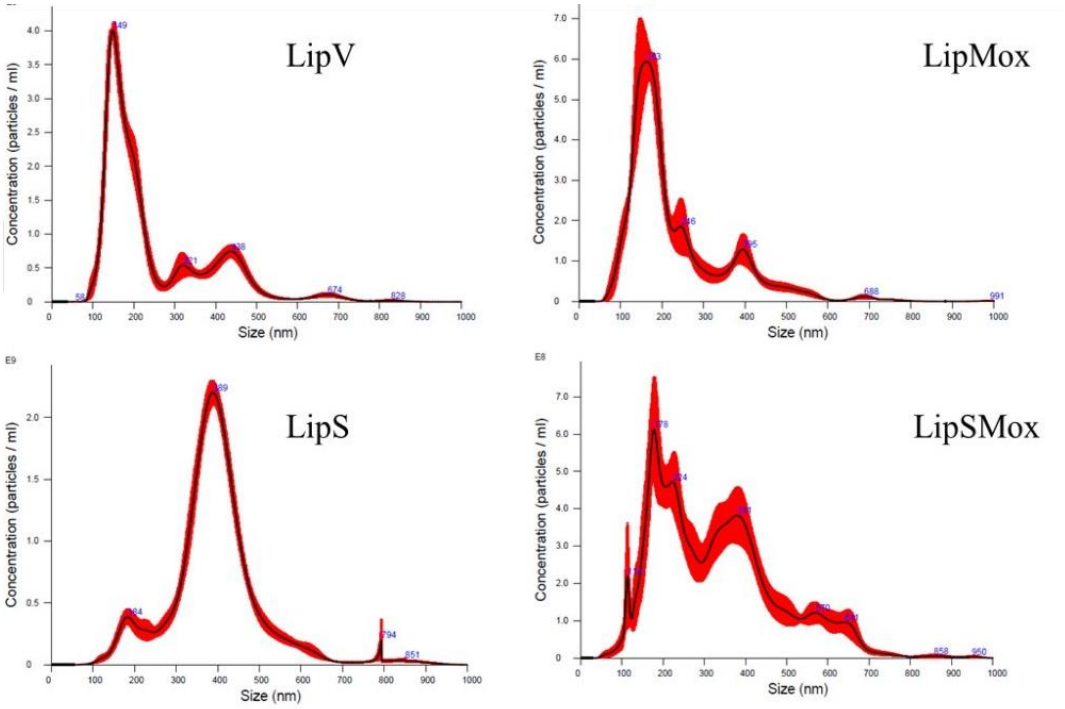
The HD, PDI and ZP of LipV, LipMox, LipS and LipSMox after exposure to the culture media used in biological assays, after 15 min of exposure to ultraviolet light and after the challenge of pH variations are in the supplemental material.

The NTA analysis showed a concentration of ~10^{11} particles/mL for the four liposomes analyzed. NTA also shows the average size and the most frequent of size, as it is called mode. The results are shown in table 4 and Figure 3.

Table 4. Particle concentration per milliliter (mean ± standard deviation), average size and mode (the most frequent value) determined by the nanoparticle tracking analysis technique.

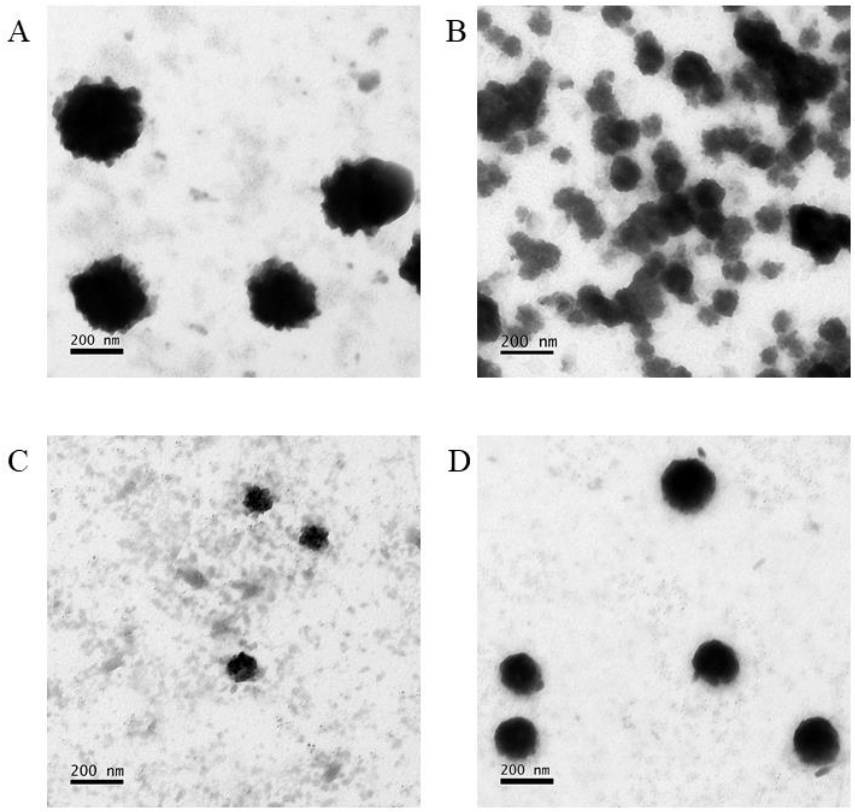
Sample	**Concentration (particles/mL)**	**Size (nm)**	**Mode (nm)**
LipV	$4.44x10^{11} \pm 9.58x10^{9}$	247.5±4.7	148.9±3.2
LipMox	$8.08x10^{11} \pm 2.12x10^{10}$	231.9±3.8	164.5±7.7
LipS	$3.55x10^{11} \pm 7.55x10^{9}$	393.1±1.9	390.4±3.8
LipSMox	$1.45x10^{11} \pm 2.09x10^{9}$	329.6±5.9	211.9±43.9

Figure 3. Concentration of LipV, LipMox, LipS and LipSMox particles according with the size (nm).



The TEM images of the liposomes were evaluated to confirming the size of the particles and their morphology. The results are shown in figure 4 and confirmed the size around 200nm and a spherical morphology, expected to liposomes.

Figure 4. Images of the liposomes: LipS (A); LipSMox (B); LipV (C) and LipMox (D) obtained by transmission electronic microscopy (10,000 times magnification).



The size of liposomes determined by NTA (LipSMox = ~ 330nm average and LipMox = ~230nm average) was a little higher than the values found by DLS analysis (LipSMox = ~ 200nm average and LipMox = ~130nm average). Some difference was also observed by the TEM images (Size ~200nm) which is perfectly acceptable since we are talking about different and complementary methods.

About the liposomes characterization, the ZP values close to -30 and 30 mV are ideal, as they promote greater stability of the nanoparticles, preventing them from aggregating and, consequently, modifying the HD and PDI of the entire formulation (JOSEPH; SINGHVI, 2019; SAMIMI et al., 2019). The ZP results showed LipV and LipMox (approximately -40mV) (~50 days) more stable than LipS and LipSMox (approximately +15mV) (~30 days).

The mycobacteria have a negative surface charge, the ZP values found were: -29.7mV for *M. smegmatis*; -22.6mV for *M. tuberculosis* and -28.1mV for *M. bovis* due to the presence of mycolic acids on the cell wall surface (NIKAIDO, 2001; AYALA-TORRES et al., 2014), besides that was not relevant in biological activity of LipV and LipMox or LipS and LipSMox.

Farzaneh *et al* evaluated the influence of cholesterol (38.3mM) combined with four different types of phosphatidylcholines (PC) (distearoyl PC, dipalmitoyl PC, dimyristoyl PC and egg FP) and the DLS analysis of the synthesized liposomes revealed that addition of cholesterol to the liposomal formulation increased the liposomes's particle size stability (FARZANEH et al., 2018). All liposomes formulation of this project has more than 30 days of stability in DLS parameters and the cholesterol could have some responsibility for it even in a smaller concentration.

For LipMox and LipSMox, whose COL concentration is 5.27, the DHM was between 194-259 and 121-154 nm, respectively, and, despite some instability, even after 85 days, the DHM was not statistically different from the first day.

The presence of cholesterol also favored the release of the drug studied, doxorubicin whose LogP is 1.18, as cholesterol reduced the hydrophobicity of the lipid bilayer and facilitates the transit of hydrophilic molecules, such as doxorubicin (FARZANEH et al., 2018). The MOX antibiotic is also a hydrophilic drug, LogP 1.48 and may benefit from this same mechanism (UNITED STATES - ENVIRONMENTAL PROTECTION AGENCY, 2020).

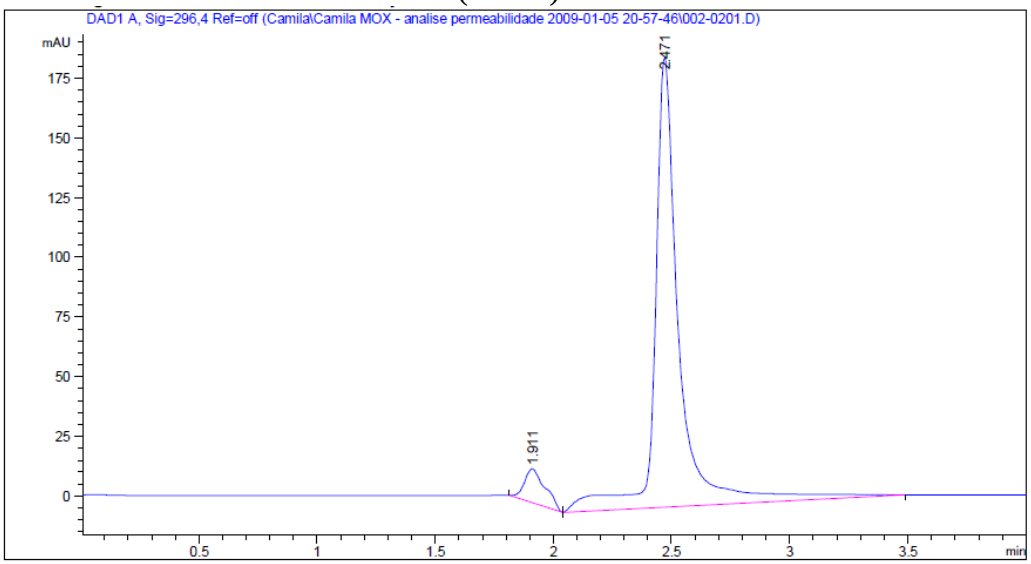
About the NTA, it is possible to note that LipMox and LipSMox presented a wider base in size distribution curve and more peaks, which is due to the drug nanoencapsulation process (supplementary material). It is known that Mox is a hydrophilic drug (QIAO et al., 2019) and, therefore, its accumulation occurs preferentially in the internal compartment of the liposomes, thus a greater heterogeneity of particle sizes is expected when the drug is present as well we can see in PDI values of the DLS analysis.

To determine EE and RP, it was necessary a quantification method of Mox, so it was developed and validated a quantification method in HPLC.

The UV detection wavelength (λ) was 296 nm in the HPLC method. The HPLC method was selective for Mox. The precision and accuracy found were: 2.5% and 101.10 and the detection and quantification limits were calculated and the values were 0.45 µg/mL and 1.35 µg/mL respectively.

A Mox detection chromatogram (10 µg/mL) is illustrated in Figure 5 and the retention time was 2.471 minutes.

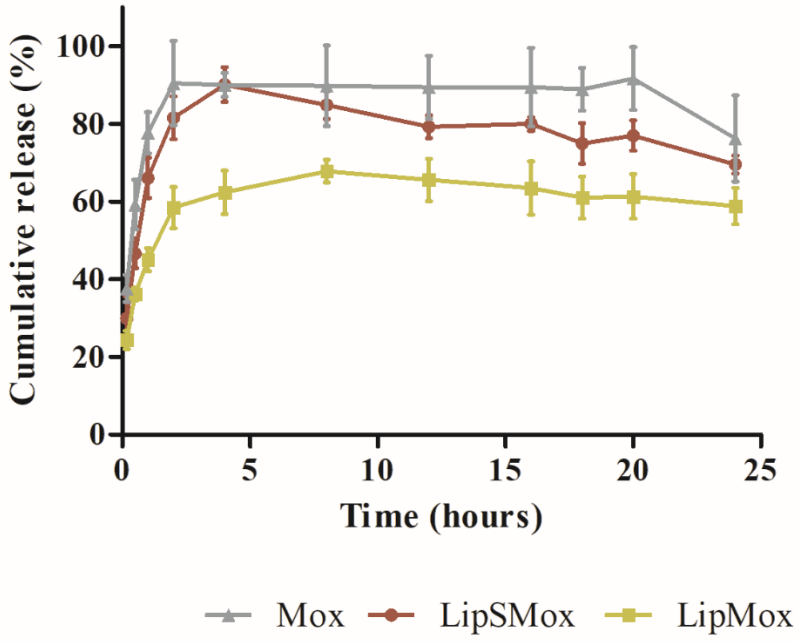
Figure 5. Moxifloxacin detection chromatogram by high performance liquid chromatography (HPLC).



The Mox EE was 51.31 ± 13.89% to LipMox and 45.76 ± 9.67% to LipSMox.

The sink concentration of Mox in PBS was 4,183.75 µg/mL and that was expected because of its hydrophilicity. The RP is shown in figure 6.

Figure 6. Cumulative release of the moxifloxacin (Mox – 100 µg/mL) free and encapsulated by a liposome (LipMox- 100 µg/mL) and a siderophore-functionalized liposome (LipSMox - 100 µg/mL) in 24 hours.



The RP pattern was analyzed comparatively with the Baker and Lonsdale (Zero order), Peppas, Hixon and Crowell, Higuchi and First Order's models and the result are presented at Table 5. The highest R^2 indicates the best match and it was the Korsmeyer-Peppas model for both liposomes: LipMox and LipSMox.

Table 5. Parameters k, R^2 and n were analyzed to determine the best release patterns model to LipMox and LipSMox.

	LipMox			LipSMox		
	k	**R^2**	**n**	**k**	**R^2**	**n**
Baker and Lonsdale (Zero order)	0,008	0		0,028	0	
Korsmeyer-Peppas	44,67	**0,720**	0,129	60,23	**0,493**	0,104
Hixon and Crowell	0,021	0		0,060	0	
Higuchi	16,86	0		21,23	0	
First Order	0,09	0		1,13	0	

The release profile followed the mathematical model proposed by Korsmeyer-Peppas, which is very common to liposomes (SIEPMANN; SIEPMANN, 2008; WU et al., 2019; SINGH; PILANI, 2021).

The liposome's characterization results showed no restriction to a biological application. The first analysis was the anti-*M. tuberculosis* activity by MIC determination and time-kill followed by the analysis of the interaction between the liposomes and mammalian cells.

3.2.Biological activity

The anti-*M. tuberculosis* activity of Mox, LipV, LipMox, LipS, LipSMox and Sid are presented in table 6 (MIC).

Regarding the MIC values under different [Fe], the excessive increase of [Fe] led to a mycobacterial resistance to liposomes and even Mox, for example, LipMox at 5x[Fe] showed a MIC >25%, LipSMox maintained its activity up to 10x[Fe] (MIC 5%~). These results may indicate that Fe is indeed complexed by Sid from the LipSMox surface and is less available in the medium, not interfering as prominently with MIC.

During the execution of these assays, it was observed that with the increase of [Fe] in the culture medium, the liposomes functionalized with Sid showed a color change, becoming

more reddish and this also corroborates the hypothesis that the Sid on the surface of the liposomes is complexing with the Fe from the medium.

Table 6. Minimal inhibitory concentration *Mycobacterium tuberculosis* $H_{37}Rv$ of the liposomes (%), siderophore (Sid - µg/mL) and moxifloxacin (Mox - µg/mL) in a regular and modified culture media.

	1x [Fe] pH 6,6	2x [Fe]	5x [Fe]	10x [Fe]	pH 7,4	10% de SFB
LipV	>25$^{\#}$	>25$^{\#}$	>25$^{\#}$	>25$^{\#}$	>25$^{\#}$	>25
LipMox	0.69±0.18	0.36±0.01	>25*$^{,\#}$	>25*$^{,\#}$	0.34±0.15	0.57±0.14
LipS	11.79±0.16	7.69±1.76*	9.13±3.17	13.48±4.80	10.63±0.31*	>25*
LipSMox	0.32±0.15	0.27±0.06	0.43±0.09*	5.63±0.20*	0.29±0.12	0.34±0.01
Sid	>25	>25	>25	>25	>25	>25
Mox	0.21±0.01	0.22±0.03	0.22±0.02	>1	0.16±0.04	0.33±0.10

FBS = fetal bovine serum; LipMox = Liposome containing Mox; LipV = empty liposome; LipS = empty liposome functionalized with a siderophore analogue (Sid); LipSMox = liposome Sid-functionalized and containing Mox. Results are the average ± standard deviation. * = significantly different from the commercial medium (1x [Fe] - 0.04 mg/mL of ferric ammonium citrate - and pH 6.6) and # = significantly different from respective functionalized liposome (LipV compared to LipS and LipMox compared to LipSMox) under the same condition, according to the One-way ANOVA analysis with Newman-Keuls post-test using Prism5 software (p<0.05).

Fe is essential for *M. tuberculosis* to survive inside macrophages because the [Fe] internally is low (1-10 ng/mL) and this metal is also related to the virulence of the bacillus (RATLEDGE, 2004).

LipS presented a MIC between 7 and 13%, except for the condition with the presence of FBS (MIC>25%), while LipV has a MIC>25% for all conditions and the only difference between them is the presence of the Sid on the surface of the LipS. It is known that FBS is a source of Fe, but complexed with the ferritin protein (KAKUTA et al., 1997). It can be assumed that the lack of free Fe would be the reason for this LipS's lack of antimycobacterial activity.

The Mox antibiotic acts by DNA gyrase enzyme inhibition that is involved in the process of DNA replication (BRYSKIER, 1993; COLE et al., 1998; DRLICA; MALIK, 2003; MALIK et al., 2012) and probably in face of a stressful condition (like 10x[Fe]) is a less available target.

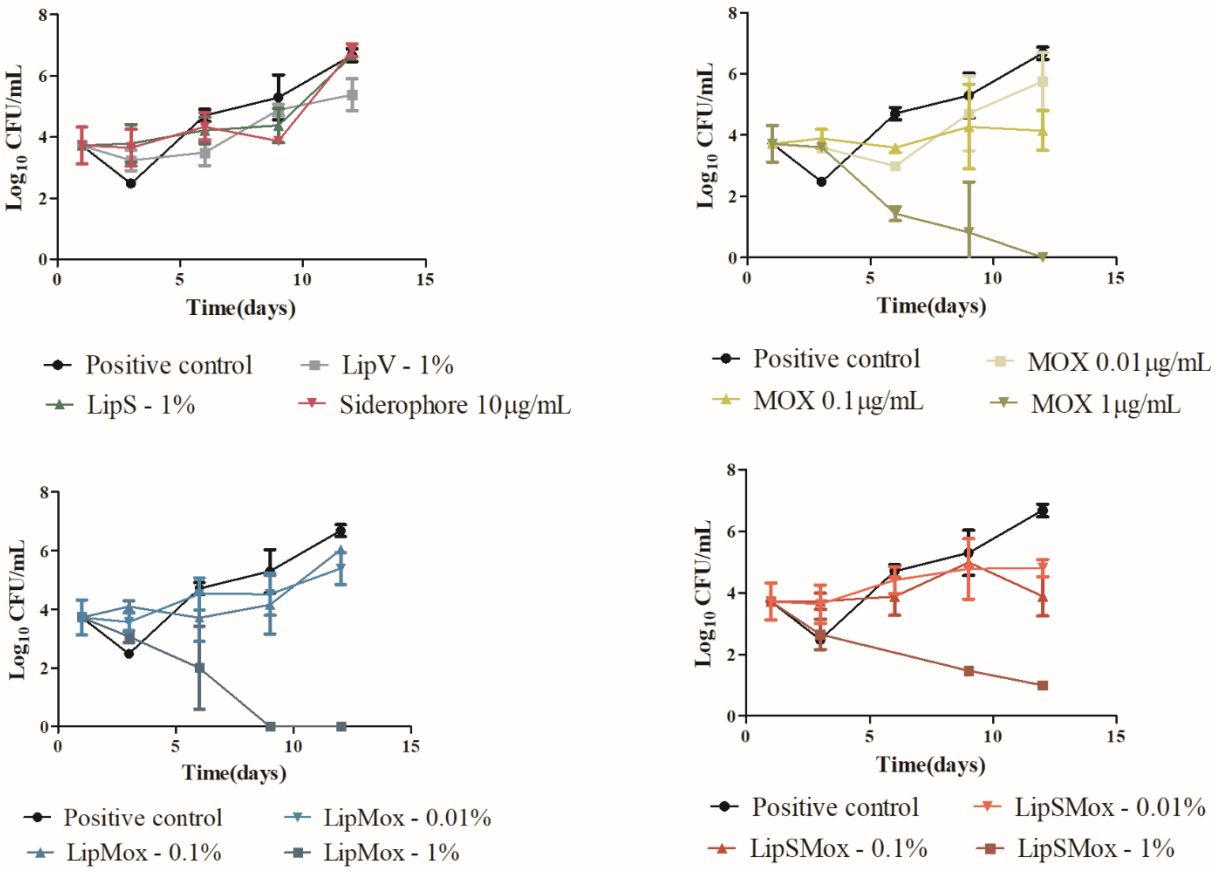
Other nanoparticles containing Mox have already been reported in the literature for several applications and in general, the results are promising (GADAD et al., 2012;

KASKOOS, 2014; MAHOR et al., 2016; YURTDAS-KIRIMIOGLU et al., 2018; QIAO et al., 2019)

Kisich, et al 2007 synthesized and characterized polymeric nanoparticles (poly [butyl cyanoacrylate] - PBCA) containing Mox and evaluated their potential against *M. tuberculosis*. The results showed that free Mox inhibited *M. tuberculosis* a little more efficiently than when encapsulated, since there is one more barrier to be overcome by the drug molecules (KISICH et al., 2007). This corroborates with the findings in this work, as the MIC of free Mox (MIC = 0.21 µg/mL) is lower when nanoencapsulated by LipMox (MIC = 0.69 µg/mL) or LipSMox (MIC = 0.32 µg/mL). Note that LipMox and LipSMox encapsulated 100 µg/mL of Mox, so the concentration was presented in % (v/v) is equivalent to free Mox.

The time-kill of *M. tuberculosis* in face of Sid, Mox, LipV, LipMox, LipS or LipSMox are presented in figure 7.

Figure 7. Time-kill of *Mycobacterium tuberculosis* ($H_{37}Rv$) in presence of Mox, LipV, LipMox, LipS, LipSMox, or Sid for 12 days.



Mox = moxifloxacin; Sid = siderophore analogue; LipMox = Liposome containing Mox; LipV = empty liposome; LipS = empty Sid-functionalized liposome; LipSMox = Sid-functionalized liposome and containing Mox. Results are the average ± standard deviation of three independent assays.

According to Steenwinkel et al., 2010, a bactericidal effect is the reduction of 3 Log_{10} CFU/mL (99,9%) of the initial inoculum and this result was observed only with LipMox 1% (Day 9) and Mox 1µg/mL (Day 12). LipV, LipS and Sid under these conditions were not active against *M. tuberculosis* which is corroborating with the MIC results once LipV and Sid presented MIC > 25 (% and µg/mL, respectively) and LipS presented MIC of 11,79% (STEENWINKEL et al., 2010).

The time-kill results showed no difference in the profile of Mox, LipMox and LipSMox regarding bactericidal or bacteriostatic effect and what mattered was the concentration of free or encapsulated Mox.

The next step evaluated was the *in vitro* safety of Mox, Sid and the liposomes. The calculation of the IS (IC_{50}/MIC) aims to determine if a sample can eliminate *M. tuberculosis* without causing cell damage and that means the higher IS is better and the results are presented in table 7 (EKINS et al., 2013).

Table 7. Minimal inhibitory concentration (MIC) *Mycobacterium tuberculosis* $H_{37}Rv$ (Mtb), the cytotoxicity index (IC_{50}) against four cell lines and the selective index (SI=IC_{50}/MIC) of the liposomes (%), siderophore (Sid - µg/mL) and moxifloxacin (Mox -µg/mL).

	Mtb	Macrophages (J774A.1)		Fibroblasts (MRC-5)		Hepatic cells (HepG2)		Intestinal cells (Caco-2)	
	MIC	IC_{50}	SI	IC_{50}	SI	IC_{50}	SI	IC_{50}	SI
LipV	>25	21.05	>0.84	19.31	>0.77	>25	>1	24.95	>1
LipS	11.79	4.2	0.36	>25	>2.12	>25	>2.12	2.33	0.20
LipMox	0.69	23.12	33.46	18.35	26.56	12.67	18.33	15.34	22.20
LipSMox	0.32	4.29	13.31	3.54	10.99	>25	77.49	1.55	4.81
Mox	0.21	>200	938.27	>200	938.27	>200	938.27	109.42	513.31
Sid	>25	46.13	>1.84	>50	>2	>50	>2	>50	>2

Mox = moxifloxacin; Sid = siderophore analogue; LipMox = Liposome containing Mox; LipV = empty liposome; LipS = empty Sid-functionalized liposome; LipSMox = Sid-functionalized liposome and containing Mox. Results are the average ± standard deviation of three independent assays. The concentration of Mox free or encapsulated are equivalent since the entrapped concentration was 100 µg/mL.

Mox and Sid did not show cytotoxicity against the cell lines evaluated. Mox's SI was undoubtedly high due to the low MIC against *M. tuberculosis* and on the other hand, Sid's SI was not so good as the MIC was not meaningful.

It is necessary to highlight the heterogeneity of the results in the different cell lines and evidence how important was this careful selection.

LipS (IC_{50} = 2.33 % to Caco-2) and LipSMox (1.55% to Caco-2) presented an IC_{50} lower than LipV (19.31% to MRC-5) and LipMox (12.67% to HepG-2), which could be explained by the Fe complexation and lack of enough Fe for the cell metabolism.

Souza et al 2010 evaluated the cytotoxicity of liposomes composed of synthesized with soybean phosphatidylcholine and cholesterol (HD and PDI approximately 130 nm and 0.300 respectively) against macrophages (J774A.1 cell line) and also didn´t observe toxicity in the analyzed conditions, corroborating with our findings (SOUZA et al., 2010).

Naturally, cytotoxicity and genotoxicity are warning signs, but in a scenario of severe infection and if anti-M. *tuberculosis in vivo* is relevant, these results are still relevant. Toxicity can be assessed in several ways; cytotoxicity is just one of them and basically determines cell viability. Another thing to be evaluated is the genotoxicity and the method chosen was the MN detection and the results are presented in table 8.

Table 8. Genotoxicity of Mox, LipV, LipMox, LipS and LipSMox by the micronucleus analysis in hepatic cells (HepG-2 cell line): nuclear division index (NDI) and micronucleus counting in 1000 binuclear cells.

Treatment	NDI	Micronucleus
Damage control - H_2O_2 (1 mM)	1.39 ± 0.06*	17.31 ± 7.74*
Untreated control	1.74 ± 0.02	1.60 ± 1.07
Mox – 100 µg/mL	1.49 ± 0.03*	5.32 ± 4.40
LipV – 10%	1.45 ± 0.07*	5.65 ± 3.77
LipMox – 10%	1.51 ± 0.08*	1.88 ± 0.49
LipS – 10%	1.42 ± 0.04*	28.26 ± 7.87*
LipSMox – 10%	1.43 ± 0.01*	23.74 ± 7.17*

Mox = Moxifloxacin; LipMox = Liposome containing Mox; LipV = empty liposome; LipS = empty functionalized with a siderophore analogue (Sid) liposome; LipSMox = Sid-functionalized liposome and containing Mox. Results are the average ± standard deviation of three independent assays. * Significantly different from untreated control according to One-way ANOVA test and Tukey's post-test (p<0,05).

As mentioned, the NDI must be between 1.3 and 2.2 and all samples got this requirement. The lowest MN counting is the best result, as the untreated control (FENECH, 2007). The LipS and LipSMox showed more MN presence than H_2O_2 (1mM), but H_2O_2 stayed in touch with the cell for just four minutes, but the liposomes stayed for 24 hours.

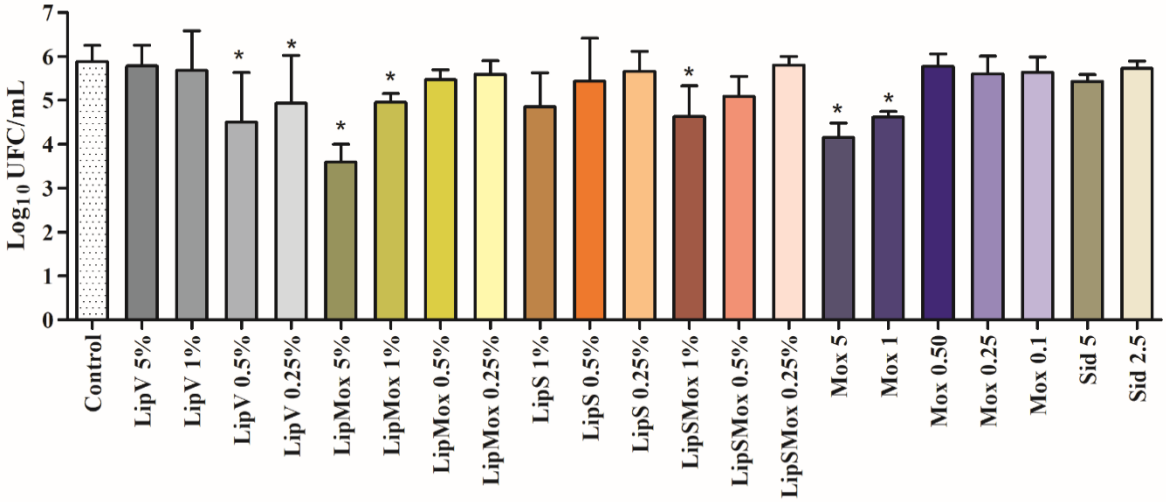
The MN originate from entire chromosomes or chromosomal fragments that fall behind in anaphase during nuclear division. They are smaller and present a diameter up to 1/3 of the main nucleus. The MN assay has international validation and applies to any nucleated cell type, being one of the best-established cytogenetic tests to assess genotoxicity (FENECH, 2007; GRANDIS, 2016; SILVA et al., 2017; GUO et al., 2020).

We observed the LipSMox and LipS more genotoxic than LipMox and LipV, which could be considered a disadvantage of functionalization with Sid. SHAH et al., 2013, showed that there is a strong correlation between MN formation and the surface charge of nanoparticles, measure by ZP, and they concluded that nanocarriers with positive surface charges induced MN formation and could also have toxic effects (SHAH et al., 2013).

This suggestion supports our findings because LipS and LipSMox had positive ZP values, the IC_{50} were lower and more MN were detected. LipV and LipMox besides that presented a negative superficial charge, compatible with their composition, and the IC_{50} and MN results were better compared with LipS and LipSMox in general.

The next assessment was the intracellular action of the liposomes. The intracellular bacilli count after 72h of treatment with Mox, Sid, LipV, LipMox, LipS and LipSMox is presented in figure 8. The concentrations tested respect a non-toxic concentration to macrophages at the same time of this experiment (data not showed).

Figure 8. Anti-*M. tuberculosis* intracellular (macrophages – J774A.1 cell line) activity of liposomes (LipV, LipMox, LipS and LipSMox), moxifloxacin (Mox) and the siderophore analogue (Sid) (µg/mL).



LipMox = Liposome containing Mox; LipV = empty liposome; LipS = empty functionalized with Sid liposome; LipSMox = Sid-functionalized liposome and containing Mox. * Statistically different than untreated control according to one-way ANOVA with Dunnett's post-test (p<0.05).

The drop of 1 Log_{10}UFC/mL means a 90% reduction of the mycobacterial inoculum compared to the untreated control, 2 Log_{10}UFC/mL reduction means 99%, and so on.

It is worth mentioning that LipSMox and LipSMox could not be evaluated at higher concentrations due to their cytotoxicity, which was previously evaluated.

Pienaar et al 2017, investigating which drug of the fluoroquinolone class (Mox, levofloxacin or gatifloxacin) would have the best effect against *M. tuberculosis*, observed, under the conditions analyzed, that Mox sterilizes intramacrophagic bacilli faster than levofloxacin or gatifloxacin, which corroborates the findings once Mox at 5µg/mL eliminated 98.26% of intramacrophage *M. tuberculosis* (PIENAAR et al., 2017).

Kisich et al 2007, shows that when macrophages infected with *M. tuberculosis* were challenged with free or nanoencapsulated Mox (polymeric nanoparticles made of poly [butyl cyanoacrylate] - PBCA), the concentration required to reduce 99.9% of intracellular mycobacteria was significantly lower for nanoencapsulated Mox (KISICH et al., 2007).

Clemens et al 2012, using mesoporous silica nanoparticles (MSNP) for drug delivery of RFP and an MSNP functionalized with a pH-responsive cyclodextrin system and carrying INH, against macrophages (THP-1 strain) infected with *M. tuberculosis*, observed that the nanoparticles promoted a greater reduction of intracellular *M. tuberculosis* (CLEMENS et al., 2012).

Differently from our results that show an equivalent activity between free Mox, LipMox and LipSMox when in equivalent conditions.

The evaluation by transmission and scanning electron microscopy clearly showed the interaction between macrophages and liposomes, including the phagocytosis of the liposome particles at different stages, and these results support our natural targeting hypothesis.

Kisich et al 2007 and Clemens et al 2012 in agreement observed an accumulation of their nanoparticles within macrophages by confocal fluorescence microscopy (KISICH et al., 2007; CLEMENS et al., 2012)

In figures 9-18 it is possible to observe the liposomal vesicles on the macrophages and the interaction between them. This interaction can withstand processing that includes several washing steps. The indication of liposomal vesicles with arrows in the figures was based on their morphology and size.

Figure 9. Scanning electronic microscopy with the interaction of macrophages (J774A.1 cell line) and empty liposomes (LipV) in the orange arrows (12.000x) according to size and morphology of them.

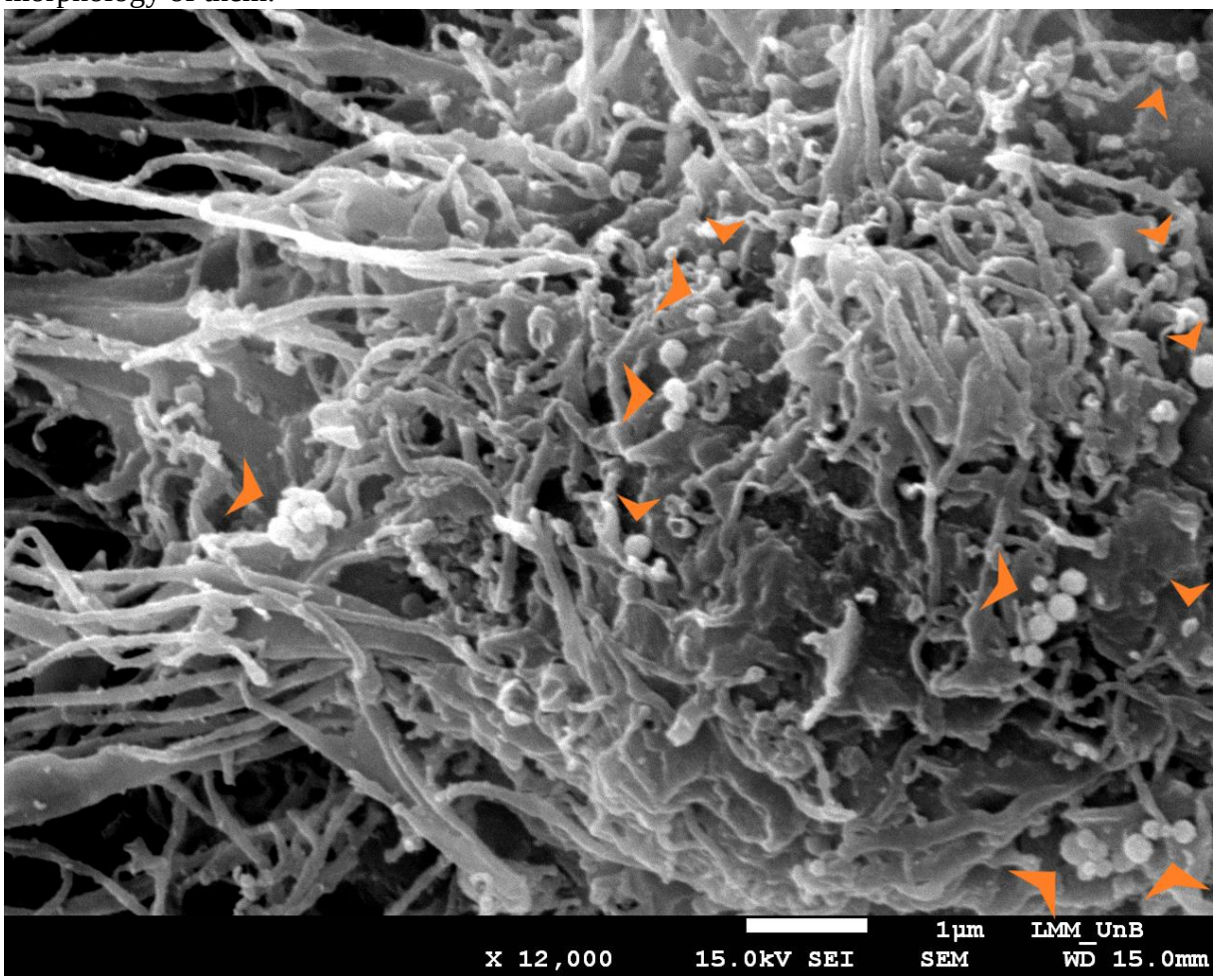


Figure 10. Scanning electronic microscopy with the interaction of macrophages (J774A.1 cell line) and liposomes containing moxifloxacin (LipMox) in the white arrows (12.000x) according to size and morphology of them.

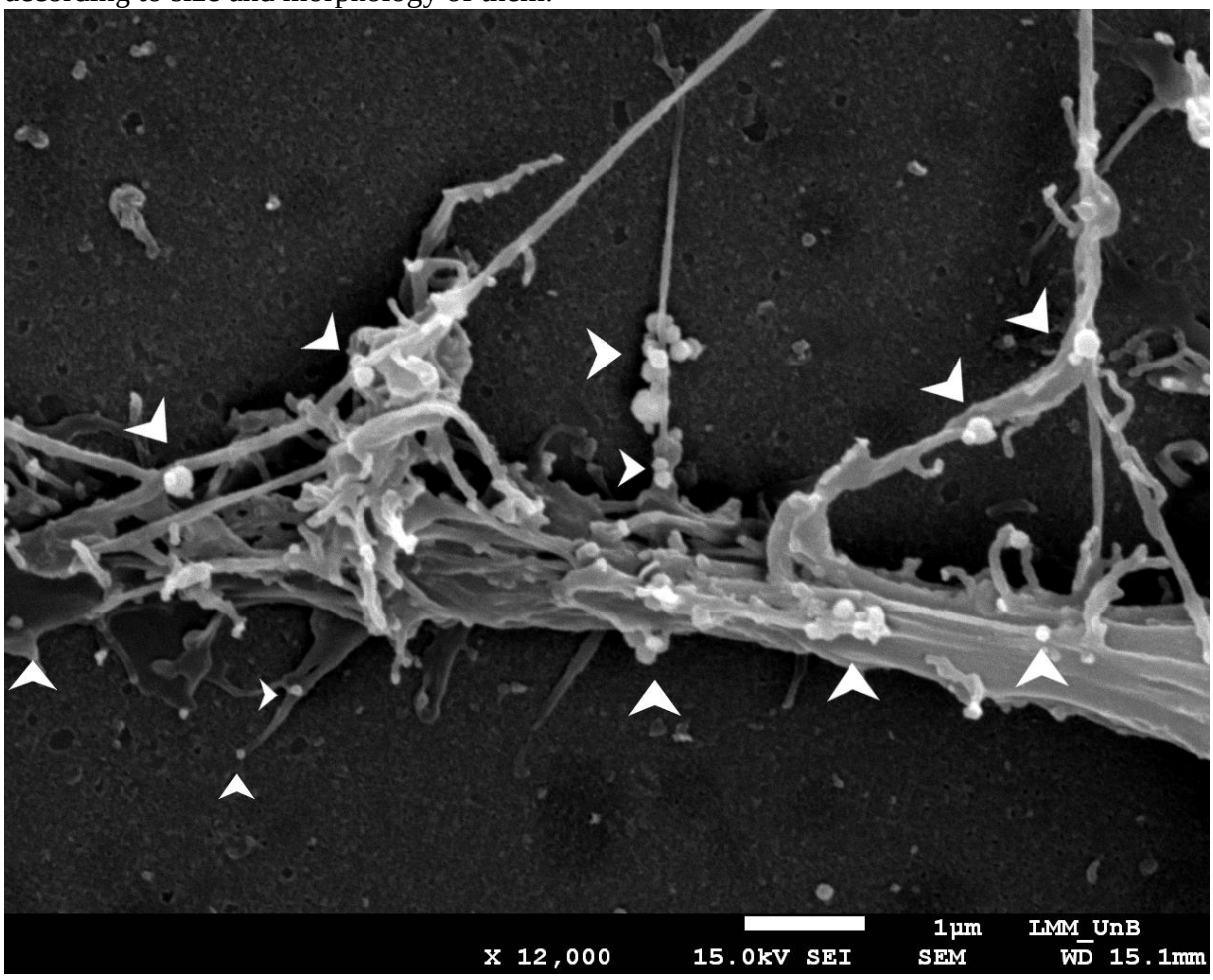


Figure 11. Scanning electronic microscopy with the interaction of macrophages (J774A.1 cell line) and liposomes functionalized with a siderophore analog (LipS) in the blue arrows (12.000x) according to size and morphology of them.

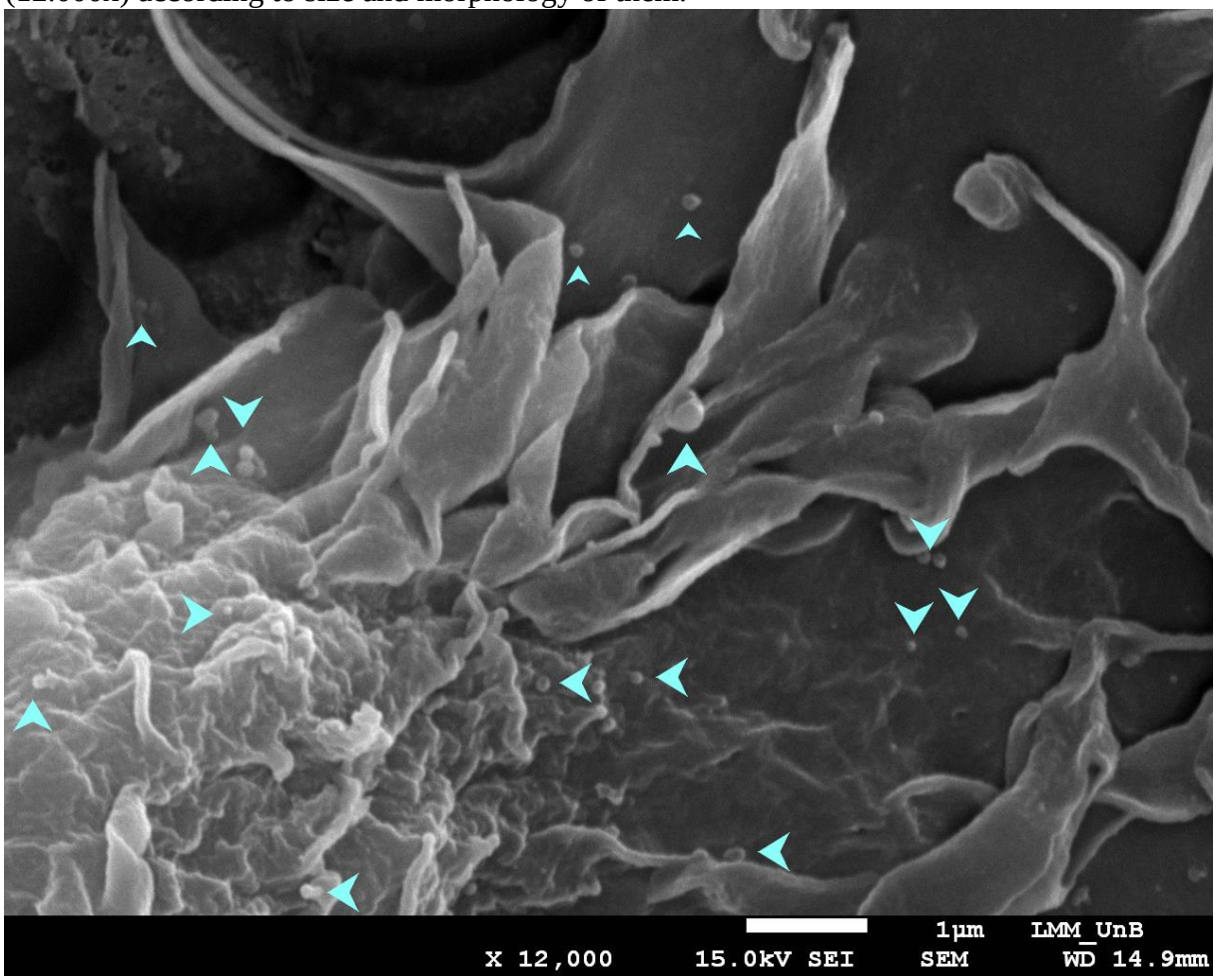
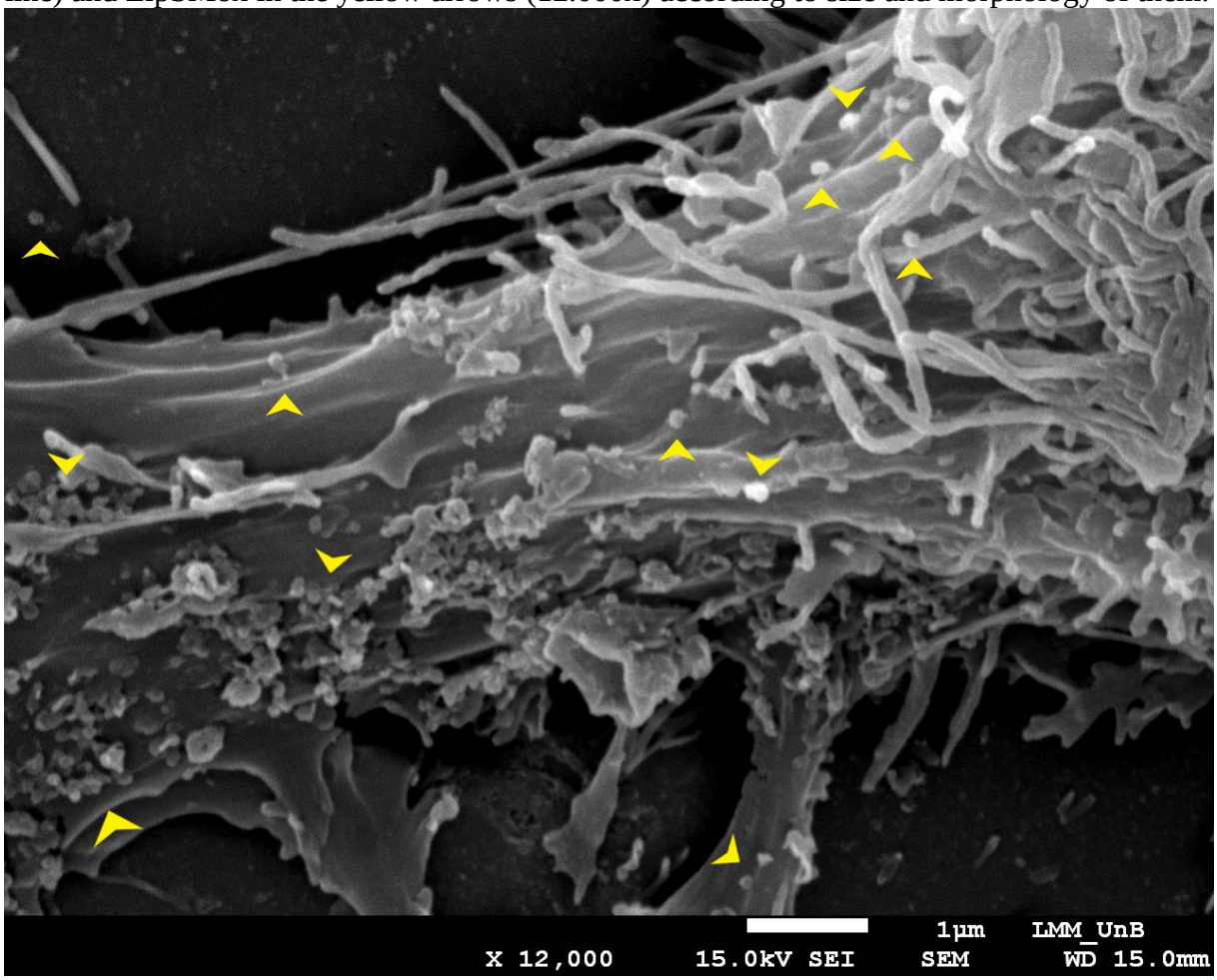
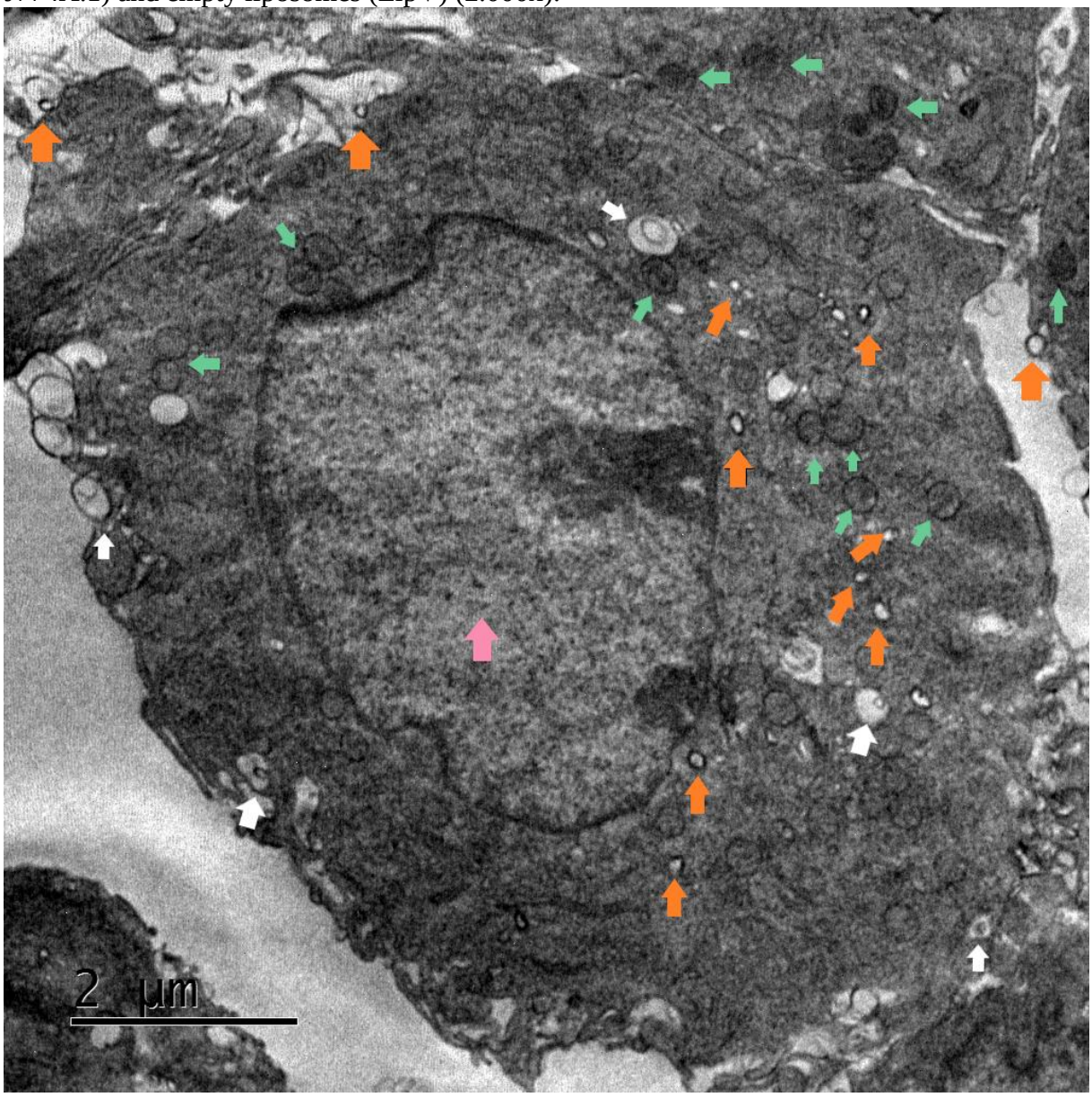


Figure 12. Scanning electronic microscopy with the interaction of macrophages (J774A.1 cell line) and LipSMox in the yellow arrows (12.000x) according to size and morphology of them.



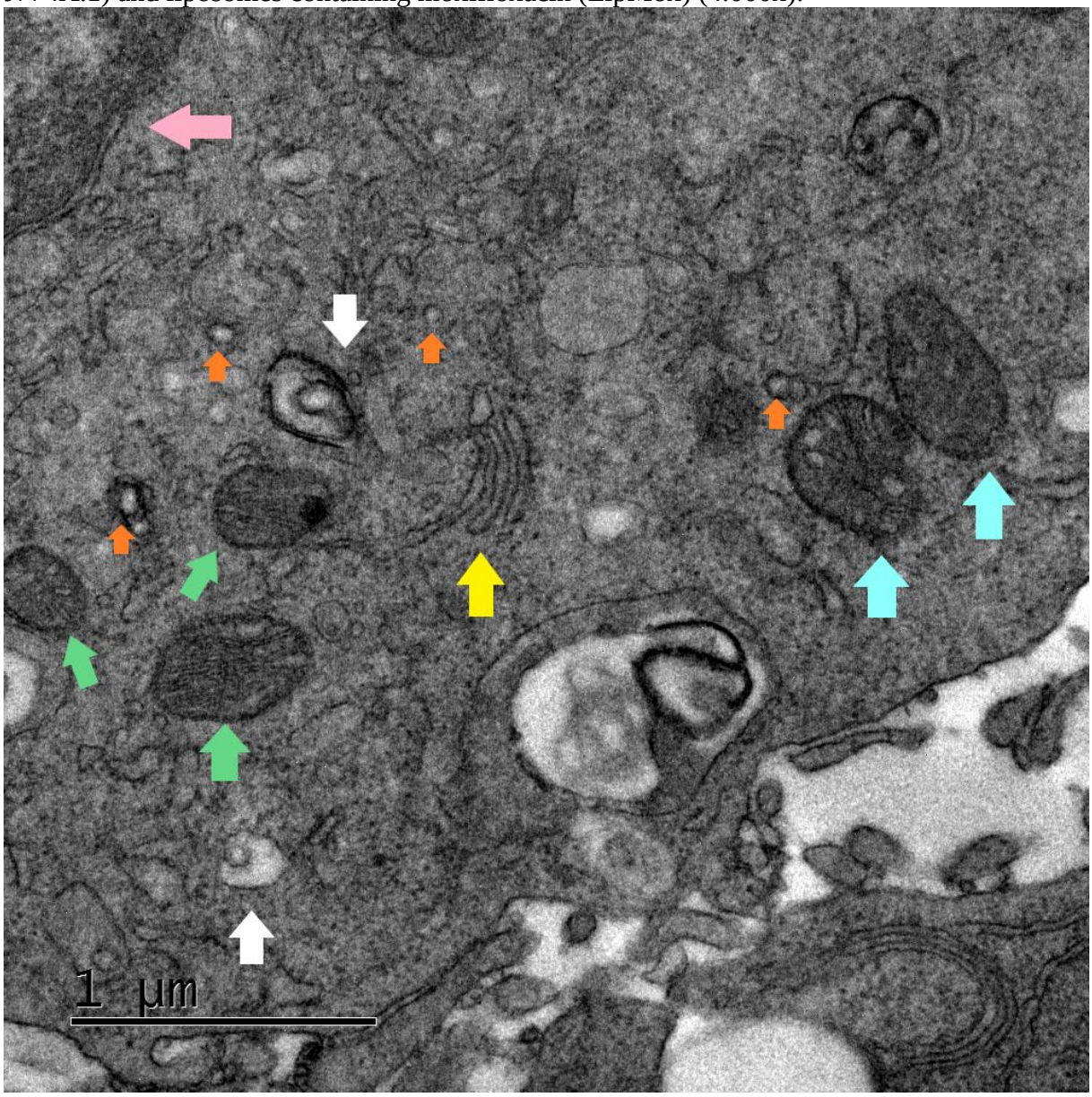
LipSMox = liposomes functionalized with a siderophore analogue and containing moxifloxacino.

Figure 13. Transmission electron microscopy of the interaction of macrophages (cell line J774A.1) and empty liposomes (LipV) (2.000x).



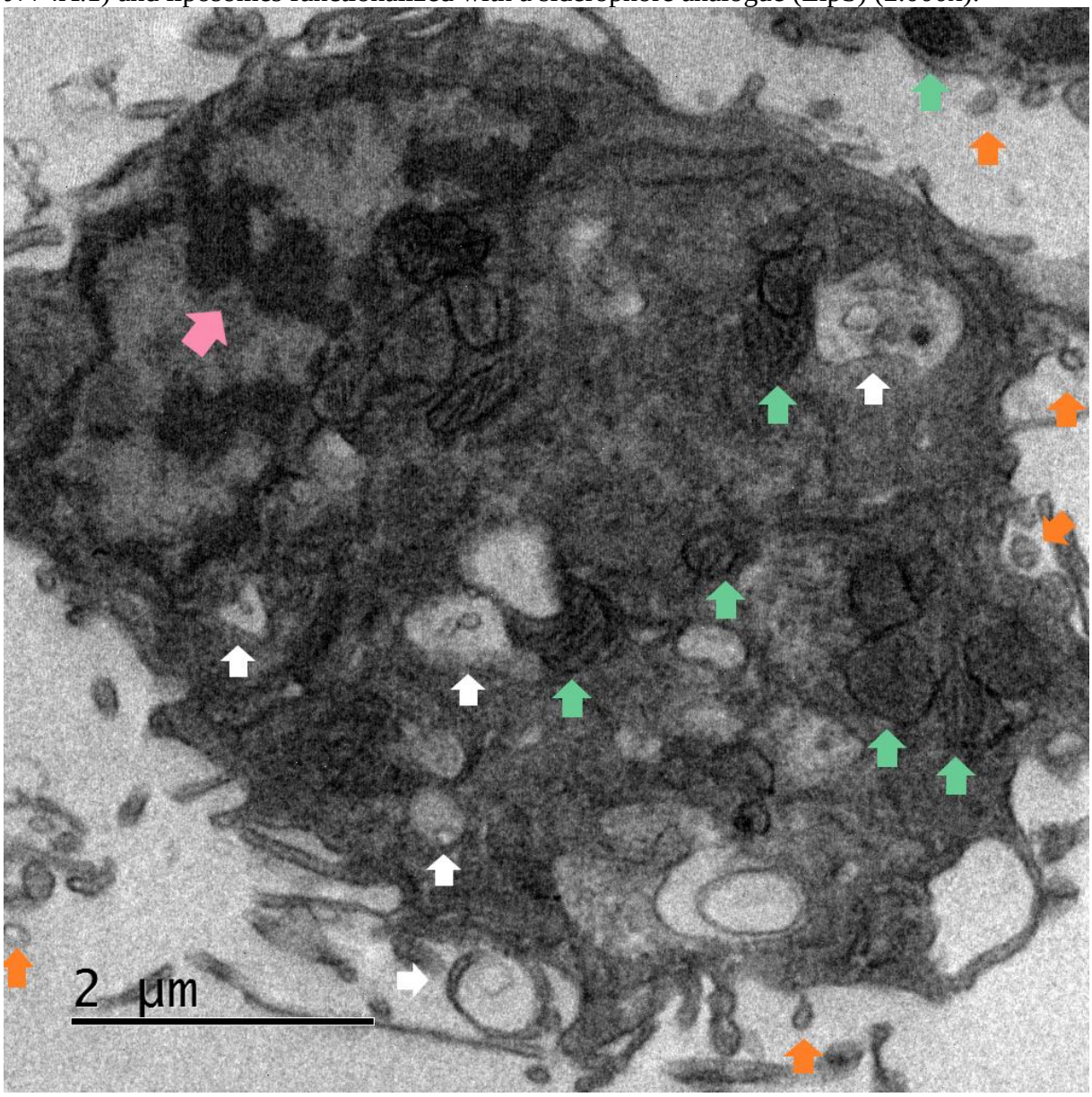
Pink arrow = nucleus. Green arrow = lysosomes. White arrow = phagosome containing liposomes. Orange arrow = liposomes.

Figure 14. Transmission electron microscopy of the interaction of macrophages (cell line J774A.1) and liposomes containing moxifloxacin (LipMox) (4.000x).



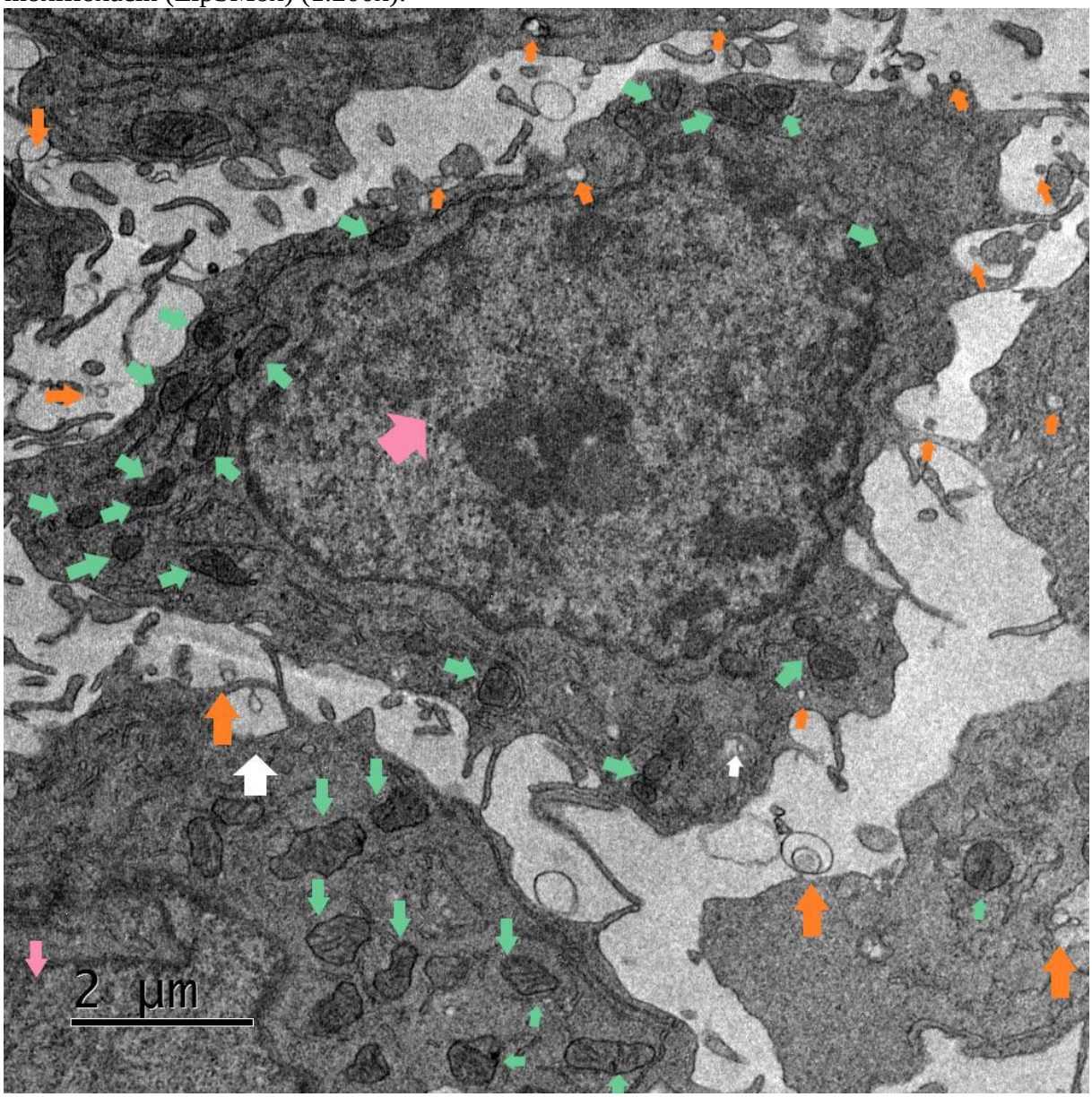
Pink arrow = nucleus. Green arrow = lysosomes. White arrow = phagosome containing liposomes. Orange arrow = liposomes. Blue arrow = phagolysosome. Yellow arrow = Golgi apparatus.

Figure 15. Transmission electron microscopy of the interaction of macrophages (cell line J774A.1) and liposomes functionalized with a siderophore analogue (LipS) (2.000x).



Pink arrow = nucleus. Green arrow = lysosomes. White arrow = phagosome containing liposomes. Orange arrow = liposomes.

Figure 16. Transmission electron microscopy of the interaction of macrophages (cell line J774A.1) and liposomes functionalized with a siderophore analogue and containing moxifloxacin (LipSMox) (1.200x).



Pink arrow = nucleus. Green arrow = lysosomes. White arrow = phagosome containing liposomes. Orange arrow = liposomes.

In TEM images, it is possible to observe different stages of phagocytosis such as pseudopod emission, engulfing the liposomal particle, the phagosome containing the liposomal vesicle inside the cell, the phagosome and the lysosome approaching for fusion and even the phagolysosomes after the fusion (URIBE-QUEROL; ROSALES, 2020).

The propose is an intrapulmonary administration of these nanoparticles for the treatment of TB *in vivo*. A direct administration at the site of infection, combined with a

controlled release of Mox and capable of reaching those intracellular bacilli (through phagocytosis) is a strategy likely to have good results.

In an active infection process, there are bacilli outside and inside of macrophages and other immune cells with the ability of phagocytosis, like neutrophile for example. That way, the liposomes like LipMox and LipSMox that present approximately 50% of EE of Mox and it means that there are Mox molecules outside and inside of liposomes vesicle have a huge advantage since part of this Mox molecule are already available for antibacterial action against the extracellular bacilli and the Mox molecules in liposomes could be phagocytosed and promote their antibacterial action intracellularly.

The pioneer drugs on the market in the liposomal form were Doxil (doxorubicin) and AmBisome (amphotericin B). They were launched in the 90's, they strengthened the nanomedicine market and paved the way for others to do the same (BELTRÁN-GRACIA et al., 2019; ALLAHOU; MADANI; SEIFALIAN, 2021).

The European Medicines Agency (EMA) recently approved in 2020 a formulation of liposomal amikacin in suspension for inhalation (ALIS) for the treatment of lung infections by bacilli of the *Mycobacterium avium* complex, also called MAC or nontuberculous mycobacteria (NTM). The drug in question is marketed under the name ARIKAYCE and is produced by Insmed Corporation®. It is known that amikacin is a drug with many adverse effects and its administration in the form of liposomes is still not able to completely mitigate them (ROSE et al., 2014; ZHANG et al., 2018; B.V., 2020; CROMMELIN; VAN HOOGEVEST; STORM, 2020; INSMED INCORPORATED, 2020; CHALMERS et al., 2021; EUROPEAN MEDICINES AGENCY (EMA), 2021; HOY, 2021).

The growth of the nanomedicine market and the approval of a medicine similar of ours (liposomes for the treatment of pulmonary infection by mycobacteria) only encourages us to seek more information about the activity of these liposomes *in vivo* by an intrapulmonary administration.

McCallum et al, 2020 analyzed plasma and alveolar cells from humans undergoing TB treatment to quantify the first-line drugs of treatment (rifampicin, isoniazid, ethambutol and pyrazinamide) and found that the concentration of rifampicin in alveolar cells is lower than expected (MCCALLUM et al., 2021). Reinforcing that an intrapulmonary administration could overcome some pharmacokinetic difficulties that a long-term multidrug therapy such as anti-TB therapy may face.

In conclusion, we observed that the functionalization had no positive relevance in the activity of the liposomes LipS and LipSMox against *M. tuberculosis* compared with LipV and

LipMox. On the other hand, LipMox results were comparable to free Mox, showing that nanoencapsulation preserves drug activity and safety, in this case.

In addition, it brings great benefits to the treatment of TB such as the possibility of drug delivery targeted to intracellular bacilli by phagocytosis of liposome particles by cells such as macrophages and the possibility of adequate drug concentration at the site of infection by an intrapulmonary route of administration.

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