



Study of amphotericin B molecular aggregation into different carrier system

André Silva

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André Silva. Study of amphotericin B molecular aggregation into different carrier system. Pharmaceutical sciences. Université Paris Saclay (COMUE); Universidade federal do Rio Grande do Norte (Natal, Brésil), 2017. English. NNT : 2017SACLS326 . tel-02284561

HAL Id: tel-02284561

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Étude de l'état d'agrégation de l'amphotéricine B dans différents systèmes d'administration

Thèse de doctorat de l'Université Paris-Saclay
préparée à l'Université Paris-Sud

École doctorale n°569 Innovation thérapeutique : du fondamental à
l'appliqué (ITFA)
Spécialité de doctorat: Pharmacotechnie e Biopharmacie

Thèse présentée et soutenue à Châtenay-Malabry, le 19 octobre 2017, par

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MINISTÉRIO DA EDUCAÇÃO
UNIVERSIDADE FEDERAL DO RIO GRANDE DO NORTE
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA (RENORBIO)

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**Study of amphotericin B molecular aggregation into different
carrier systems**

ORIENTADOR: Prof. Dr. ERYVALDO SÓCRATES TABOSA DO EGITO
CO-ORIENTADOR: Prof. Dr. GILLES PONCHEL

CHÂTENAY-MALABRY, FRANCE

2017



UNIVERSITÉ PARIS-SACLAY
ÉCOLE DOCTORALE 569 – INNOVATION THÉRAPEUTIQUE: DU
FONDAMENTAL À L'APPLIQUÉ
UMR CNRS 8612 – INSTITUT GALIEN PARIS SUD (ÉQUIPE 6)

UNIVERSIDADE FEDERAL DO RIO GRANDE DO NORTE
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA (RENORBIO)
LABORATÓRIO DE SISTEMAS DISPERSOS – LASID

ANDRÉ LEANDRO SILVA

STUDY OF AMPHOTERICIN B MOLECULAR AGGREGATION INTO
DIFFERENT CARRIER SYSTEMS

Tese apresentada ao Programa de Pós-Graduação em Biotecnologia e à École Doctorale 569 como requisito para a obtenção do grau de Doutor em Biotecnologia e em Innovation Thérapeutique, em cotutela celebrada entre a Universidade Federal do Rio Grande do Norte e a Université Paris-Saclay.

ORIENTADOR: Prof. Dr. ERYVALDO SÓCRATES TABOSA DO EGITO
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CHÂTENAY-MALABRY

2017

ACKNOWLEDGEMENTS

The conclusion of this PhD thesis closes a very important cycle in my life, the one of scholar formation. Looking for knowledge, I have quite my cozy home too early, when I was only 14 years old. When I was 17, I was living thousand kilometers away of my family because I would like to become a pharmacist, and then to get my master and PhD degrees. All along this journey I had the luck and pleasure of meeting people to whom I would like to address my thanks and recognition.

The first person I would like to say a big “*obrigado*” (thank you in Portuguese) is my mom, Mrs. Teresinha Lins. Back in 2003, it was her the one who encouraged me to seek for a better education. I can’t guess how much questions passed through out her mind and how much distress her heart felt seeing his son, that was only 14 years old, going to live alone, in other federation state. Then, during the college, in spite of financial difficulties, she encouraged me and payed for my interchange in Santiago do Chile. She never let anything lack to me, in spite of the distance we live since 15 years ago, the thing we have the much is love. - Mom, your efforts were not in vain! Your son is becoming doctor, and he owes a lot to you! “*Muito obrigado!!!*”

I would like to thank to my family: my siglings (Adriana, Adriale, Júlio and Maria), my cousins, uncles and aunts for all the support and love you’ve dedicated to me, each one in its own way.

Thank you, my friends! You are an important part of this history. Thank you for the good moments we have spent together. I’d like to mention Angélica and Renato, Iury, Claudinha, Karol, Simone, Henedy, Vanessa, Hendo, Duda, Diego, Ed, George and Andrew. Sharing my live with you guys made this process much more “soft”.

This thesis was produced in a cooperation accord celebrated between the Federal University of Rio Grande do Norte (Brazil) and the University of Paris-Sud (France). I would like to thank to Capes and CNRS for the financial support and to the Capes-COFECUB (Project 742/11) for the fellowship provided.

Many were the people contributing on my formation and construction of this thesis. In Natal, the first person I would like to thank is my advisor, Professor Sócrates Egito. Ten years ago, I was a little boy entering (maidenly) in your lab, the Laboratory of Dispersed Systems (LaSiD). In this journey, I am very proud of the history we built together, I am talking about science, respect, partnership and friendship. Thank you so

much for believing in me in so many opportunities. I hope the future reserves us much more partnership, from now as colleagues. *“Muito obrigado, meu amigo!”*

I would like to thank to my friends from the LaSiD. Thank you so much for your kindness and friendship, Julieta Genre, Francine Johansson, Franceline Reynaud, Lourena Veríssimo, Henrique Marcelino, Belle Holanda, Bartô Santos, Katia Lira, Alexandrino Junior, Andreza Morais, Junior Xavier, Éverton Nascimento, Sarah Rafaelly, Renato Ribeiro, Marjorie Freire. I really appreciate all the scientific discussion we have had and all the million liters of coffee we have shared along these 10 years.

A very important part of this work was prepared at the *“Institut Galien Paris-Sud”*, in Châtenay-Malabry (France). Then, I would like to thank to Professor Elias Fattal and all the UMR 8612 staff (Mme. Zemmour, Mme. Livet, Mme. Martin) for welcoming me in your prestigious research unity.

I would also thank to Mme Lucie Landry, the secretary of the ED 569 for all the attention and comprehension during this period.

I may address a special thanks to my French advisor, Professor Gilles Ponchel, for welcoming me as part of his team and for being open to study one molecule that wasn't part of his arsenal, at that time. Thank you very much for all you have taught me and for all the scientific discussion we have had. I cannot forget to thank you for the rides up to the *“Cité U”* and for helping me to improve my spoken French (I hope someday I'll be able to pronounce *“Je, J'ai, J'ait”* properly). *“Merci beaucoup, mon cher!”*

As a member of *“Équipe 6”*, I had the opportunity of sharing the bench and the office with friends that I hope to bring with me for good, coming from Italy, Mexico, France, USA, Colombia and Brazil. *“Muchas gracias”* Any Taylor and Herman Palacio, *“Obrigado”* Sarah Palácio, Henrique Marcelino, Mariane Lira, Élquio Eleamen and Junior-Xavier, *“Grazie”* Martina Bombardi and Valeria Candioli, *“Merci”* Pierre-Louis Destrudel, Tiphany Grisin, Aurélia Nemo, Fanny and Jean Baptiste, and finally, thank you Nick Frazier. Still in the team 6, I wish to thank to Dr. Christine Vauthier and Dr. Kawthar Bouchemal, for the scientific discussions.

Thank you, the colleagues from other teams, partners of *“cafeteria”* and *“on va manger !?”*. Especially the ones which became great friends, Thais Leite and Eloisa Berbel.

To finalize the acknowledgments at the University of Paris-Sud, I would thank to Magali Noiray for the training on ITC, to Hélène Chacun, for the training on

radiolabeling, to Gillian Barrat, for the training on DLS facility and especially to Monique Chéron, for performing the circular dichroism assays.

The life in Paris was much more pleasurable because of the friends I have met on my way: Alex de Carvalho and Thiago Queiroz, at *Maison du Brésil* Kellen Tjioe, Barbara Fonseca and Georges, Cauê Souza, Monize Moura, Juliane Tamara. *Muito obrigado, meus amigos!*

The *Maison du Liban* welcomed me during the 18 months I lived in Paris. Thanks to Mme Atié and all the personal of MDL. Living at this place I've learned a little more about the Lebanese culture, had the pleasure of tasting their food and dancing their dances. I want to mention the ones which really made the difference during my stay in the house: Rim Lebar, Ramy Bechara, Christelle El Haddad, Zahi Daoui, Ziad Salem, Marwan Hu, Saly Barakat, Naila Bou Haidar, Rami El Zein and Mhamad El Houssaini.

شكراً جزيلاً

Last, but not least, I would like to thank to the jury for your time and considerations, Prof. Dr. Phillippe Legrand, Prof. Dr. Frédéric Frezard, Prof. Dr. Franceline Reynaud, Dr. Christine Vauthier. Other professors that already evaluated this work in many other opportunities in Brazil, Prof. Dr. Maria Aparecida Maciel, Prof. Dr. Arnóbio Silva-Junior, Prof. Dr. Hugo Rocha, Prof. Dr. Bolívar Damasceno and Prof. Dr. Matheus Pedrosa.

« Muito obrigado a todos! *Merci à tous !* »

« Celui que ne connaît pas les tourments de l'inconnu doit ignorer les joies de la découverte qui sont certainement les plus vives que l'esprit de l'homme puisse jamais ressentir »

“Aquele que não conhece as tormentas do desconhecido deve ignorar as alegrias da descoberta, que são certamente as mais vivas que a mente do homem pode experimentar”

Claude Bernard

ABSTRACT

The amphotericin B (AmB) is a drug of peculiar physicochemical features: being amphiphilic and amphoteric. These characteristics turn difficult the drug load into therapeutic systems. AmB is currently available in the market as micelles, liposomes and lipid complex for injection. The literature show that there is an intimate correlation between the AmB bound to the carrier and its biological response. However, there is a deficiency concerning the physicochemical characterization of the available AmB-containing products. Therefore, the aim of this work was to characterize AmB-containing carriers seeking a prediction to its biological response. The AmB-containing micellar system was the first product available for clinical use. The patent of this product has already expired some years ago. In this work we have characterized the original system and two other similar micellar products. In addition, we studied the stability increase of heated systems, by the formation of AmB “super-aggregates”. AmBisome[®], an AmB-containing liposomal system, was also characterized and, for the first time, tested for the possibility of super-aggregates formation. The AmB incorporation into nano and microemulsion systems was presented and the physicochemical characteristics evaluated, focusing mainly on applications for the treatment of fungal ocular diseases and also for visceral leishmaniasis. The main techniques used for characterization were electronic spectroscopy, circular dichroism and dynamic light scattering. The isothermal titration calorimetry (ITC) was used as an attempt to measuring the super-aggregates energy formation. Besides, an AmB soluble derivative was developed and characterized by atomic mass spectroscopy, infra-red, UV-Vis and circular dichroism. Then, this AmB-derivative was loaded into a microemulsion as a vehiculation strategy. The overall results show that the AmB-containing systems presented different molecular aggregation states that depends on the carrier, the way the drug is incorporated and also on the diluent. According to the literature, the aggregation state is associated with both, drug efficiency and toxicity. In nanoemulsion systems, the drug is found aggregated and multi-aggregated. In microemulsions, AmB is loaded as monomers. The heated micellar systems form AmB super-aggregates while the liposomal system is unable to form such molecular structure. Moreover, the AmB soluble derivative presented distinct features when compared to the original molecule. However, once incorporated into the microemulsion, the aggregation state is similar to that of the original AmB molecule, as

supported by UV-Vis and circular dichroism results. It can be concluded that the AmB aggregation state varies according to the kind of carrier, the drug concentration and also the way of drug incorporation, even into one same carrier. Finally, the soluble derivative opens the possibility for drug carrying into aqueous vehicles for the treatment of many diseases by different administration routes.

RESUMO

A anfotericina B (AmB) é um fármaco de características físico-químicas bastante peculiares: de caráter anfifílico e anfotérico. Essas características tornam difícil sua veiculação em sistemas terapêuticos. Atualmente, a AmB é veiculada por via intravenosa nas formas de micelas, lipossomas e complexos lipídicos. A literatura demonstra que existe uma íntima relação entre a forma como a AmB está complexada ao sistema carreador e sua resposta biológica. Entretanto, há uma deficiência nos dados de caracterização físico-química dos produtos disponíveis contendo AmB. Portanto, o objetivo deste trabalho foi realizar a caracterização físico-química de sistemas carreadores de AmB visando uma predição de sua resposta biológica. Os sistemas micelares de AmB foram os primeiros produtos disponíveis à prática clínica, de forma que sua patente expirou há alguns anos. Neste trabalho, o sistema original e dois de seus similares foram caracterizados e o aumento da estabilidade destes sistemas após aquecimento, pela formação dos super-agregados de AmB foi estudado. O sistema liposomal AmBisome[®] também foi caracterizado e, pela primeira vez, foi estudada a possibilidade de super-agregação, a exemplo dos sistemas micelares, a partir de lipossomas. A incorporação de AmB em sistemas nano e microemulsionados foi apresentada e as características físico-químicas destes sistemas foram estudados, com demonstração de suas aplicações no tratamento ocular de infecções fúngicas e também para o tratamento de leishmaniose visceral. As principais técnicas de caracterização aplicadas foram espectroscopia UV-Vis, dicroísmo circular e espalhamento dinâmico de luz. A técnica de calorimetria de titulação isotérmica (ITC) foi utilizada numa tentativa de medir a energia de formação dos superagregados. Adicionalmente, um derivado solúvel de AmB foi desenvolvido e caracterizado por espectroscopia de massa atômica, infra-vermelho, UV-Vis e dicroísmo circular, bem como incorporado em sistema microemulsionado como estratégia de veiculação deste derivado solúvel. Os resultados revelam que os sistemas contendo AmB apresentam diferentes formas de agregação molecular dependendo do carreador, da forma de incorporação do fármaco e do diluente empregado para redispersar o sistema. Segundo a literatura, o estado de agregação está intimamente ligado à eficácia e à toxicidade da molécula. Nos sistemas nanoemulsionados a AmB apresenta-se na forma agregada e multi-agregada. Na microemulsão, está incorporada na forma monomérica. Os sistemas micelares aquecidos

dão lugar à formação de super-agregados de AmB enquanto os sistemas lipossomais são incapazes de se modificar em super-agregados. O derivado solúvel de AmB apresentou características que diferem da AmB original. Contudo, quando incorporado na microemulsão, o estado de agregação é similar ao da molécula original tanto nas análises de UV-Vis quanto de dicroísmo circular. Pode-se concluir que a forma de agregação de AmB varia não somente de acordo com o tipo de carreador, mas também com sua concentração no meio e com o tipo de incorporação, ainda que num mesmo tipo de carreador. Finalmente, o derivado solúvel abre a possibilidade de veiculação do fármaco em carreadores de caráter aquoso para o tratamento de diversas enfermidades e por várias vias de administração.

RÉSUMÉ

L'amphotéricine B (AmB) est une molécule utilisée en thérapeutique pour ses propriétés antifongiques remarquables. Cependant, ses caractéristiques physico-chimiques très particulières, puisqu'elle est à la fois amphiphile et amphotère, rendent difficile la conception et la fabrication de systèmes thérapeutiques chargés en AmB qui soient simultanément efficaces et peu toxiques. Au plan physico-chimique, les médicaments contenant de l'AmB et disponibles sur le marché, sont constitués de micelles, de liposomes ou de complexes lipidiques auxquels l'AmB est physiquement associée. Ces médicaments sont tous administrés par la voie parentérale. La littérature montre qu'il existe une relation intime entre la façon dont l'AmB est associée au système transporteur et les effets pharmacologiques et toxicologiques qui sont observés. Malgré de très nombreuses études, l'état d'association des molécules d'AmB dans les différentes formulations commercialisées contenant de l'AmB n'est toujours pas connu avec suffisamment de précision. Pour cette raison, le but de ce travail expérimental est de caractériser différents systèmes contenant de l'AmB, dans l'objectif de prédire les effets biologiques induits par l'état d'association de cette molécule à ces systèmes supramoléculaires. Les systèmes micellaires contenant de l'AmB ont été les premiers produits mis sur le marché dans les années 1960 et les brevets protégeant ces produits ont expiré depuis quelques années. Dans ce travail, nous avons caractérisé un système micellaire original ainsi que deux autres produits similaires tout en les comparant. De plus, nous avons étudié les mécanismes par lesquels se forment des super-agrégats d'AmB par l'augmentation de la stabilité des systèmes chauffés. Dans un second temps et pour la première fois, la capacité de l'AmBisome[®], un système liposomique commercialement disponible d'AmB, à former des super-aggrégats a également été caractérisée et testée. Enfin, l'incorporation de l'AmB dans des systèmes de type nano- et micro-émulsion a été étudiée, ainsi que ses caractéristiques physico-chimiques lorsqu'elle est associée à ces systèmes, avant d'être appliquée au traitement des maladies oculaires fongiques et de la leishmaniose viscérale. Les principales techniques utilisées pour la caractérisation physico-chimiques de l'état d'agrégation ont été : (i) la spectroscopie électronique (UV-Vis), (ii) le dichroïsme circulaire (DC) et (iii) la diffusion dynamique de la lumière (DLS). La calorimétrie à titrage isotherme (ITC) a été utilisée afin de tenter de mesurer l'énergie de formation des super-agrégats. De plus, un dérivé soluble de

l'AmB a été développé et caractérisé par spectroscopie de masse atomique, infrarouge, UV-Vis et dichroïsme circulaire (DC). Afin de disposer d'un système d'administration adéquat, ce dérivé soluble a été ensuite incorporé dans une micro-émulsion. Au total, l'ensemble des travaux expérimentaux conduits, montrent que l'état d'agrégation moléculaire de l'AmB dépend très largement du système d'administration utilisé, ainsi que des procédés par lesquels l'AmB est associée à ces systèmes. Ces résultats ont une réelle importance pratique puisque la littérature montre sans ambiguïté que l'efficacité du médicament ainsi qu'à sa toxicité dépendent étroitement de l'état d'agrégation de l'AmB. Ainsi, dans la nanoémulsion, l'AmB se trouve dans des états agrégés et multi-agrégés. Au contraire, dans la micro-émulsion, l'AmB se présente plutôt sous forme « monomère ». Une fois chauffés, les systèmes micellaires forment des super-agrégats d'AmB, tandis que les liposomes étudiés sont incapables de donner naissance à cette structure supramoléculaire. Enfin, le dérivé soluble d'AmB que nous avons préparé présente des caractéristiques distinctes par rapport à la molécule d'origine. Cependant, une fois associé à une microémulsion, son état d'agrégation est modifié et redevient similaire à celui de l'AmB originale, comme l'indique les études en UV-Vis et en dichroïsme circulaire. On peut donc conclure de ce travail que l'état d'agrégation d'AmB varie considérablement en fonction du type de système d'administration utilisé, de la concentration de l'AmB ainsi que du mode d'incorporation de la molécule, y compris pour un même système. Enfin, ce travail a permis la mise au point d'un dérivé soluble original de l'AmB qui offre la possibilité d'utiliser des formulations aqueuses adaptées à différentes voies d'administration et pourrait renouveler l'intérêt de cette molécule ancienne dans le traitement de différentes pathologies fongiques pour lesquelles il n'existe pas de formulations réellement adaptées.

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General Introduction

GENERAL INTRODUCTION

The emergence of the antibiotics has revolutionized the practice of medicine by enabling breakthroughs across the spectrum of clinical medicine (1). In this context, the nature has been a source of medicinal products for millennia, with many useful active substances obtained from plant sources and then, in the 20th century, the discovery of penicillin was the start pointing for drug discovery from microbial sources.

The majority of drugs have been developed from lead structures on the basis of natural products synthesized by bacteria. It is estimated that during the golden era (1940 – 1970) of microbial natural product screening, tens of millions of soil microorganisms have been screened, given birth to substances widely prescribed as antibacterial therapeutics nowadays (Figure 1) (2).

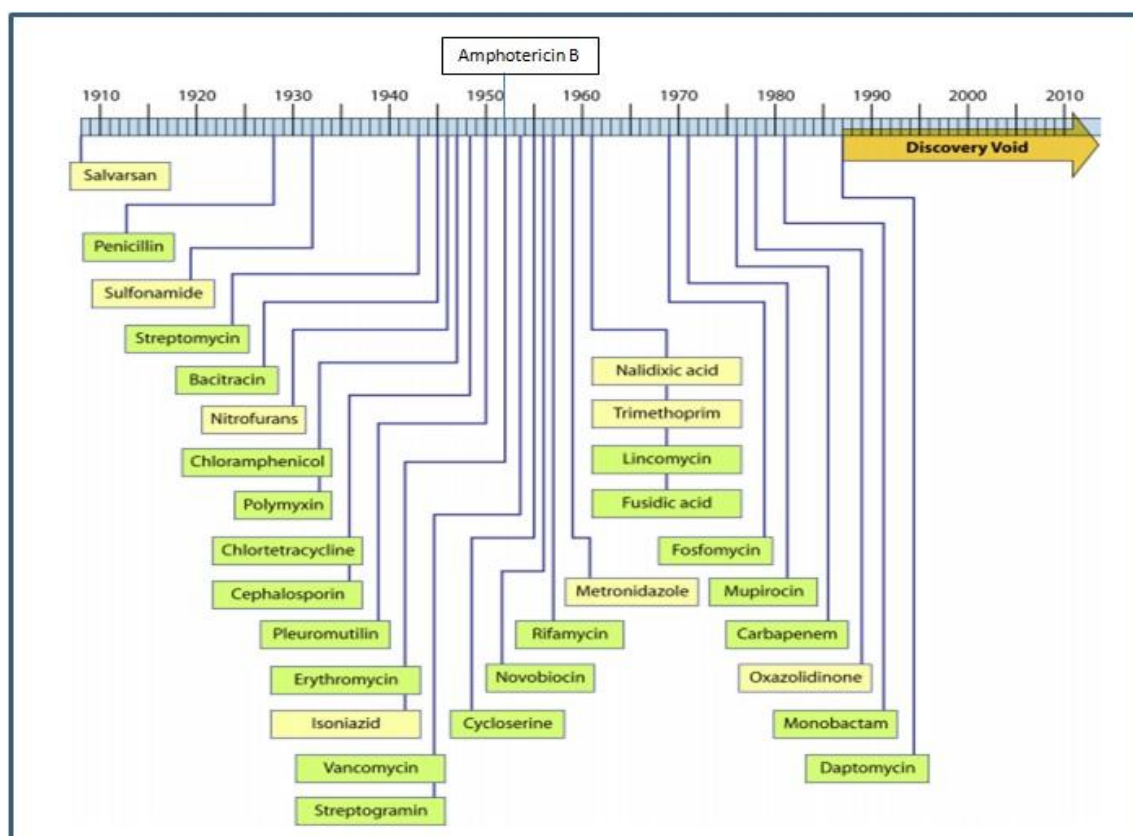


Figure 1. Timeline of antibiotic discovery, modified from Silver (2011)(3).

Among the antibiotics discovered during the golden era figures the amphotericin B (AmB), which is a polyene macrolide derived from the actinomycete *Streptomyces nodosus*. AmB was discovered in 1953, three years after the discovery of nystatin, from

an isolate culture of soil obtained in the Orinoco basin, in Venezuela. Although the antifungal spectrum and general chemical properties were similar for both drugs, AmB presented several pronounced differences when compared to nystatin (4).

After complete purification, two new antifungal substances had been revealed: amphotericin A and amphotericin B. The A component possessed a tetraene chromophore in the ultraviolet (UV), whereas the B component was a heptaene with considerably greater inhibitory activity than either nystatin or amphotericin A (4).

Considering the exposed, one might wonder “why to study such an old molecule?” For which we can answer with the slow speed of drug discovery, the development of drug resistance and the lack of information needed about AmB behavior in different situations.

As observed in Figure 1, an antibiotic discovery void period occurred. In fact, after the golden era few new antibiotics have been registered by the FDA. For example, from the end of 2012 up today, only 5 antimicrobials have been approved by the agency (5), highlighting the need of a better understanding and exploitation of available antimicrobial agents.

The slow rate of antibiotic discovery is due to the difficulty in discovering novel chemicals that have selectivity and the increased regulatory scrutiny for safety and efficacy. Furthermore, the demonstration of superiority of the new compound is essential in order to rationalize pricing and to assure a return on the financial investment made (6).

Besides, the antimicrobial resistance is an important point that has to be taken in consideration. The antibiotic resistance emergence varies in time according to the drug class and spread use. For example, if in one hand vancomycin did not present drug resistance for more than three decades’ period of use, in other hand, daptomycin experienced it in a period of less than five years after its discovery. A relation of time between drug discovery and the report of clinical resistance is presented in the Figure 2.

It can be observed that the more recently discovered antibiotics experienced microorganism resistance in a short period of time (less than five years). This fact raises a concern about the arsenal of drugs we will have to fight against infections in the future. In this context, it is interesting the fact that only few cases of AmB resistance have been reported in humans up to now. In general, resistance to AmB is related to modification

on the parasite membrane sterol (7), an essential component for the drug mechanism of action, the pore formation that allows ions leakage and cell death.

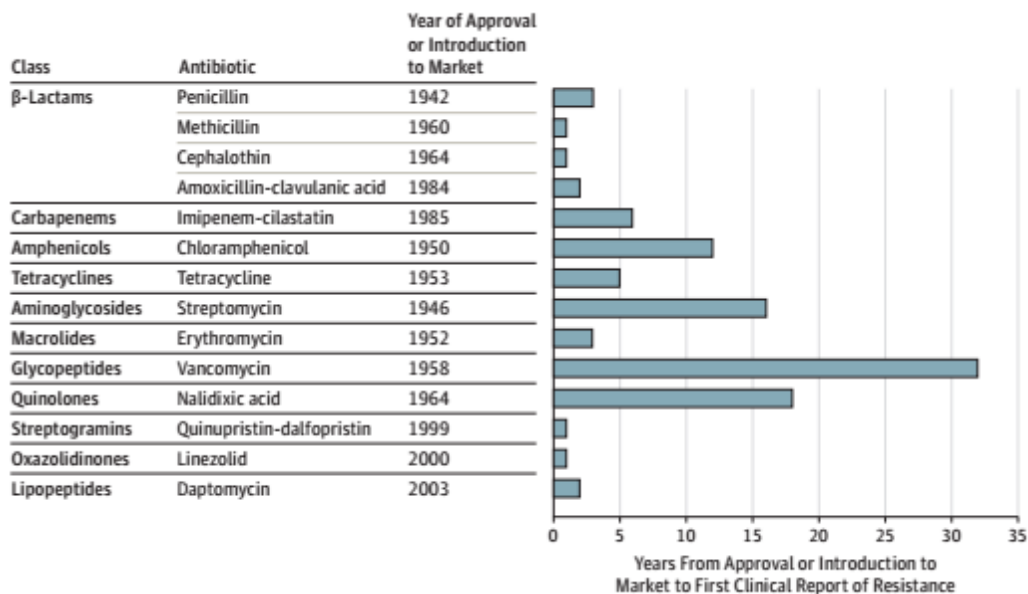


Figure 2. Time from antibiotic approval or introduction to detection of resistance in clinical samples, extracted from Marston et al. (2016) (1).

The AmB mechanism of action has been recently reinforced by Grudzinski et al. (2016) (8), showing that the presence of sterols in the membrane lipid phase promoted formation of supramolecular structures of AmB and their penetration into the membrane hydrophobic core (Figure 3).

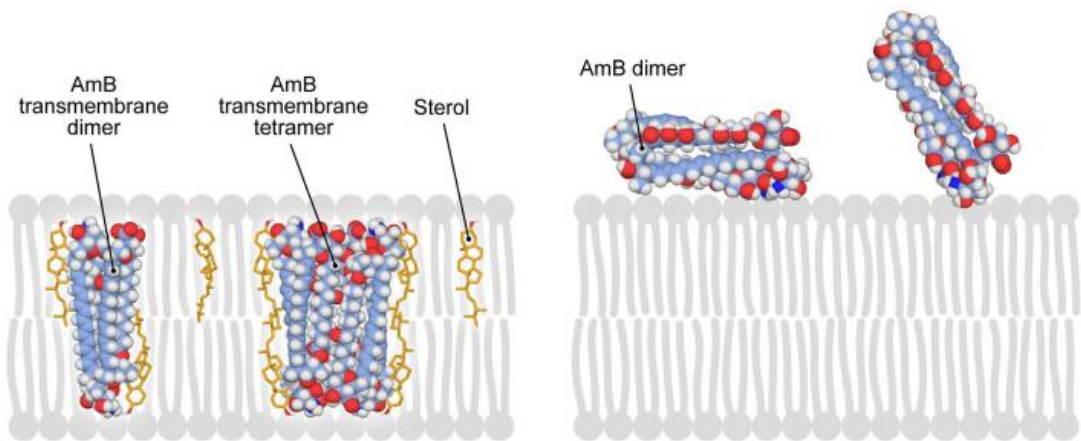


Figure 3. Model presenting localization, molecular organization and orientation of AmB with respect to a lipid membrane with and without molecular sterols. Extracted from Grudzinski et al. (2016)(8).

The effect of AmB on lipid membrane has showed to be multi-modal, and among various molecular mechanisms associated with interaction of AmB with lipid membranes we can mention the destabilization of molecular order of lipids and self-assembly of the drug molecules within the lipid phase (8).

AmB tends to self-associate in aqueous media due to its physicochemical features. This characteristic is intimately linked to the drug activity, but also with toxic effects (9). Thus, the importance of knowing the AmB aggregation state: drug monomers are supposed to bind preferable to ergosterol while aggregates tends to lose specificity, binding to both cholesterol and ergosterol (10, 11). Super-aggregates, in turn, are supposed to act as a monomer reservoir, delivering monomers to the medium (12).

A scheme showing the importance of the aggregation state on the pore formation in cholesterol- and ergosterol-containing membranes is displayed in the Figure 4.

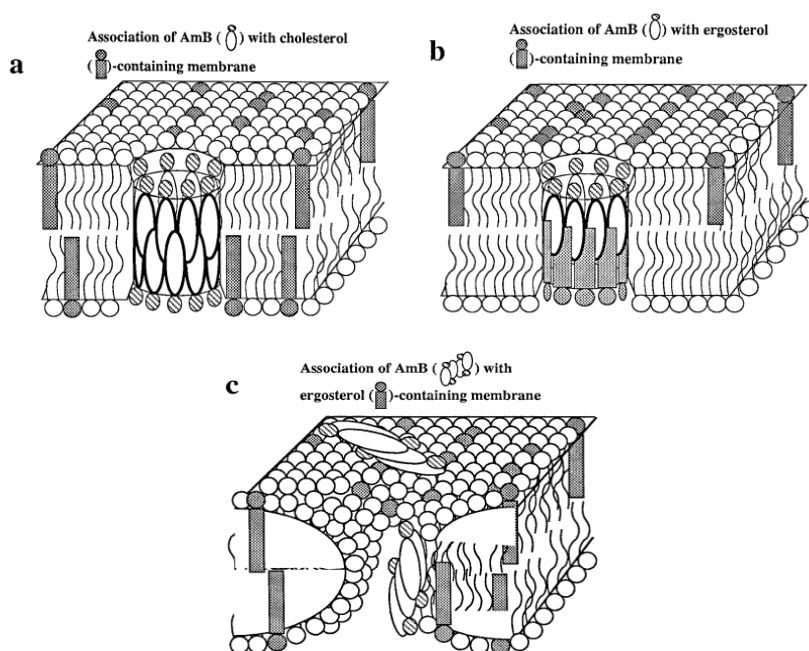


Figure 4. Schemes of pore formation by AmB monomers in cholesterol-containing membranes a) and ergosterol-containing membrane b). AmB aggregates forming pores in ergosterol- or cholesterol-containing membranes. Extracted from Bratjburg and Bolard (1996) (13).

In the proposed model, more AmB would be recruited in order to form pores in cholesterol-containing membranes, while in the ergosterol-containing membrane the

sterol makes a part given support to the pore formation. When self-associated or aggregated, AmB lose selectivity making pores in both sterol types.

Concerning the sterol types, ergosterol is found in parasitic membranes such as the ones of fungi and some protozoa species, as *Leishmania sp.*. That is why AmB is already used as the golden standard for the treatment of deep fungal infections and leishmaniasis in many countries. But, on the other hand, when self-associated, the drug can bind to cholesterol, the sterol present in the membrane of mammalian cells, being responsible for the drug toxic effect.

The AmB molecular association can be studied from spectroscopy since the heptaene chain composing the AmB molecule gives a good fingerprint to it on UV-Visible (UV-Vis) and circular dichroism (CD) (14), being sensible tools for follow up of molecular changes. Then, when the aggregation phenomena occurs, it readily affects spectra record (Figure 5).

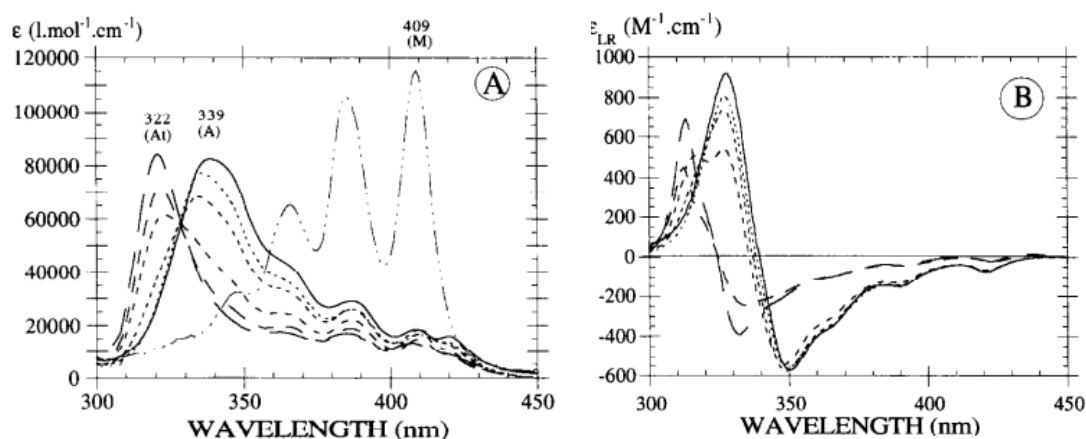


Figure 5. A) UV-Vis spectra for monomers (M), aggregates (A) and super-aggregates (At); B) CD spectra for monomers, aggregates and super-aggregates, extracted from Gaboriau et al. (1997) (15).

The monomeric form is presented in the graphic as “M”. Three maxima are found when the drug is in the form of monomers and no signal is observed by CD. When the drug is aggregated, one new spectra appears, blue shifted, in the expenses of monomers when observed by UV-Vis. “A” represents self-associated AmB and “At” the mild heated solution of AmB, forming drug super-aggregates. In CD analysis, AmB aggregation causes a bisignated spectra that is also blue shifted when super-aggregates are analyzed.

Due to the AmB low water solubility, the drug has been loaded in specially designed pharmaceutical preparations. The first AmB-based product was a micellar system, launched in the 1960's. Almost thirty years later, in the middle of the 1990's, other products such as lipid complexes, colloidal dispersion and liposomes were approved and put on the market for clinical use (11). However, the interaction among the drug and its carrier influenced on the drug aggregation state, and consequently, on its biological activity (13). Then, this work was dedicated to the study of AmB aggregation state into different carrier systems, in which marketed and new systems were analyzed.

This thesis memoire is divided in four sections. In the first section we have one review paper about pharmaceutical strategies for AmB delivery: this article presents the already marketed products as well as new approaches aiming the improvement of AmB safety. The second section is devoted to the micellar systems of AmB: they were the first product launched and still useful in therapeutics. We have analyzed similar products from three different countries and also the possibility of forming super-aggregates, comparing micellar and liposomal systems. In the third section we report the AmB incorporation into emulsion systems, reinforcing the potential of such preparations for ocular application and antileishmanial treatment. The fourth and last section, presents a patent of the production process of an AmB-soluble derivative and a report of the derivative incorporation into a microemulsion system.

Concluding remarks closes this thesis, dedicated to the AmB. In addition, as an appendix, there are an article and one patent dedicated to emulsion systems intended to gene delivery, a contextualization is given as appendix introduction.

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SECTION I

PHARMACEUTICAL STRATEGIES FOR AMPHOTERICIN B DELIVERY

BRIEFING

Amphotericin B (AmB) is a quite old molecule, discovered in the 1950's. However, the studies dealing with this molecule are still in evidence. Much has been done in the last 60 years: the antibiotic structure was fully described and the drug features clarified. The AmB mechanism of action is still in discussion, despite the evidences of mechanistic pore formation and membrane oxidation.

The drug is available in the market in a few pharmaceutical presentations and this fact is mainly due to the drug physicochemical features. Then, the first section of this thesis is composed of one chapter bringing a review manuscript discussing important aspects of the AmB molecule, such as its physicochemical characteristics, aggregation patterns, systems marketed worldwide and mainly the new pharmaceutical strategies for AmB delivery.

CHAPTER I

Pharmaceutical strategies for amphotericin B delivery

Pharmaceutical strategies for amphotericin B delivery

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Abstract

The amphotericin B (AmB) is an antibiotic used for the treatment of systemic fungal infections and leishmaniasis. The AmB molecule has amphiphilic and amphipathic properties, which make difficult its load into pharmaceutical carriers. Since its discovery in the 1950's, just a few kind of pharmaceutical products were developed and are available in the market worldwide. However, these products show problems regarding toxicity (micelles) and high cost, besides the fact of been available only by intravenous administration. Then, the development of new pharmaceutical systems is mandatory. In this context we reviewed the strategies used for the development of new nanotechnological devices that could improve AmB solubility and safety. In conclusion, AmB is an old molecule that has not yet achieved its maximum on pharmaceutical use, due to the lack of specific drug carriers, even though much has been done in the scenery of lipid-based AmB-loaded systems, evidencing challenges and opportunities.

Key-words: amphotericin b; micelles; colloidal systems; liposomes; microemulsion; nanoemulsions; cochleates; nanoparticles;

I. INTRODUCTION

The amphotericin B (AmB) is an antibiotic widely used for the treatment of life-threatening systemic fungal infections and more recently used against the protozoa *Leishmania* (1, 2). The drug presents peculiar physicochemical characteristics that make difficult its solubilization in aqueous solvents (3).

Nowadays, there are in the market micelles, lipid-based and liposomal systems containing AmB, which are administered only intravenously. Researchers are still looking for systems that would be safer and/or of lower cost than the available products (4) and also that could be administered for other routes.

Many approaches have been applied to the development of new AmB products. However, when the molecule is loaded into a carrier, new chemical interactions are built and may change the AmB aggregation state, consequently modifying the activity and toxicity profile of the final product (5, 6).

In this review, pharmaceutical strategies for AmB delivery are revisited beginning with the first micellar formulation up to recent AmB-delivery approaches.

II. AMPHOTERICIN MOLECULAR CHARACTERISTICS

The amphotericin molecule is an antibiotic extracted from the *Streptomyces nodosus* fungi, which was first isolated from a ground sample obtained on the region of the Orinoco River, in South America (3, 7). The amphotericin was patented in 1959 as the general name given for two molecules: amphotericin A (AmA) (8) and amphotericin B (AmB) (7). The structure of both molecules of amphotericin were elucidated and they are extremely similar, the only difference is a single bond between carbons 28 and 29 in AmA, instead of a double bond in the AmB molecule (9-11) (Figure 1). As demonstrated on the amphotericin's patents (7, 8), the molecules are extracted together as a pool and

then purified. The amphotericin purification is an important step, once the AmA molecule has low antifungal activity when compared to AmB. Its concentration into the commercial preparation is frequently below 5% (12).

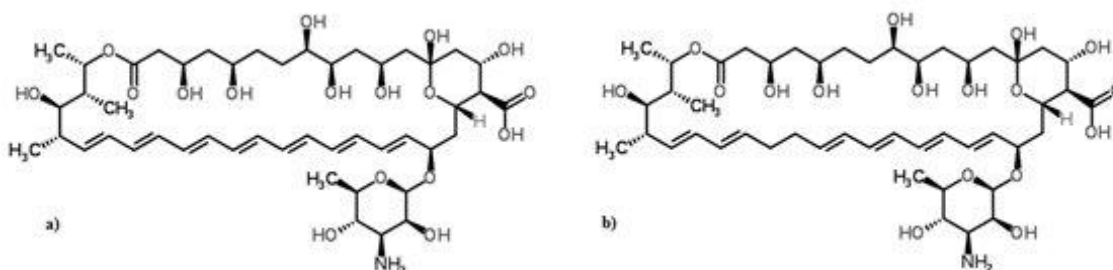


Figure 1. The molecular structure of amphotericin: a) amphotericin B; b) amphotericin A.

The AmB molecule (C₄₇H₇₃NO₁₇) has hydrophilic (due to the presence of seven hydroxyl groups and an ester carbonyl group) and hydrophobic portions (due to a polyene group, a rigid heptaene chain) (13) (Figure 1a). Besides its amphiphilic properties, this molecule also shows amphoteric characteristics, due to the presence of an amine group (pK_a 8.12) and one carboxyl group (pK_a 3.72) (14). These characteristics have an important role on the high insolubility of the molecule in aqueous medium (6). Hence, its load within pharmaceutical preparations is hard to be achieved.

Due to the amphiphilic properties of the molecule, the AmB tends to self-aggregate in aqueous medium (15). Then, the molecule can be found as monomers, soluble aggregates, insoluble aggregates and also as superaggregates (Figure 2), depending on the medium and drug concentration. The drug aggregation state plays an important role on drug toxicity (16).

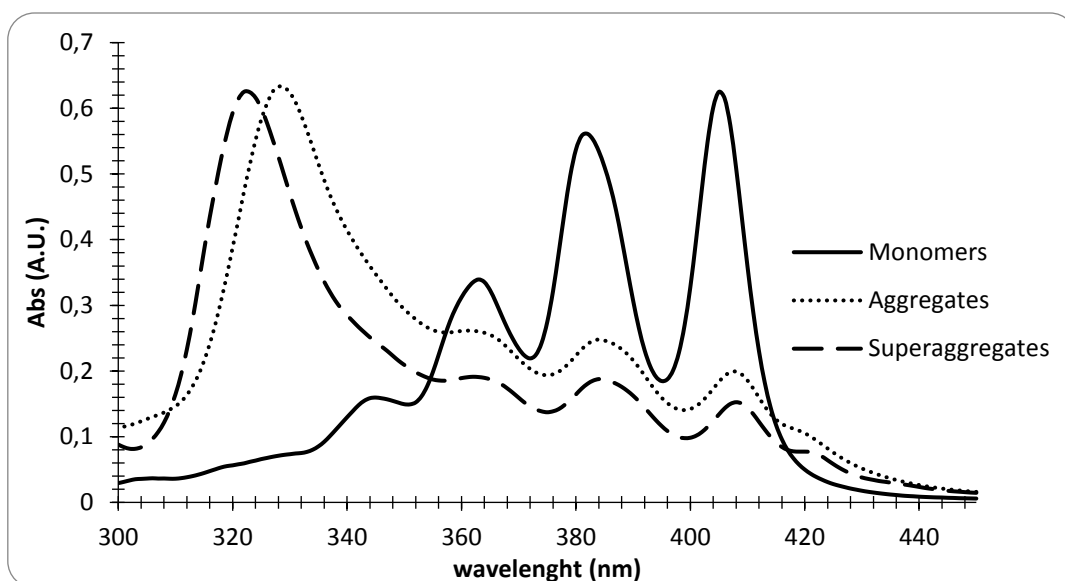


Figure 2. AmB electronic configuration spectra: monomers, aggregates and superaggregates.

The AmB mechanism of action is controversial. It is generally believed that it binds to sterol of biological membranes forming trans-membrane pores that cause the leakage of ions and consequent disruption of the cell (3, 17). However, the drug is not specific for the parasite sterol (ergosterol) and can also bind to the mammalian cholesterol, since they are structurally quite similar (2). As aforementioned, AmB aggregation state seems to play an important role on the drug toxicity, it was reported that when it is delivered as monomers, AmB preferably binds to ergosterol, being less toxic for the mammalian cells (5, 18).

III. MARKETED SYSTEMS

After AmB discovery in the 1950's, the first AmB-based product was approved by the Food and Drug Administration (FDA) in 1966, under the form of a micellar solution called Fungizone®. This presentation was for decades the only AmB product available worldwide. After that, in 1990's, other products were approved by FDA and

launched into the market such as the Fungizone[®] generic, AmB-lipid complexes, and lately, a liposomal-based product. Once the patents of such products have expired, similar products have appeared worldwide (Table 1).

Table 1. List of some available AmB-based products

Product	Supplier / Country	FDA approval (year)
<i>Micellar system</i>		
Fungizone[®]	Bristol-Meyers Squibb/ US	1966
Amphotericin B	X-Gen Pharms/US	1992
Anforicin[®]	Cristália/BR	
Funtex B[®]	Cibla/IN	
Uniant[®]	União Química /BR	
Amphotret[®]	Bharat Serum/IN	
Photericin B[®]	Cipla/IN	
Amphotin-Vit[®]	Health Biotech LTDA /IN	
Fungitericin[®]	Lifecare Innovations/IN	
Amphotin[®]	United biotech/IN	
Anfocam[®]	Dabur India ltd/IN	
Hospicin B[®]	Alkem Laboratories ltd/IN	
Amtericin[®]	VHB lifesciences inc/IN	
Ambilip[®]	United biotech/IN	
Amphonex[®]	Bharat serum/IN	
Amfitas[®]	Intas/IN	
<i>AmB Lipid complex</i>		
Abelcet[®]	Sigma Tau Pharm / US	1995

Anolip[®]	Samarth/IN	
Amphotin-Lip[®]	United Biotech/IN	
Ampholip[®]	Bharat serum/IN	
<i>AmB Colloidal dispersion</i>		
Amphotec[®]	Three Rivers Pharm. /US	1996
Amphocil[®]	Sequus Pharmaceuticals	
<i>Liposomal system</i>		
AmBisome[®]	Gilead/UK	1997
Fungisome[®]	Lifecare Innovations/IN	
Phosome 10[®]	Cipla/IN	
Anfogen[®]	Richmond Laboratorios/AR	
Lambin[®]	Sun Pharma Laboratories/IN	
Amphotin-LS[®]	United Biotech Pvt Ltd/IN	
Ambihope[®]	Abbott/IN	
Amphogard[®]	Zydus cadila/IN	
<i>AmB naniosuspension for injection</i>		
Amfy[®]	Intas Pharmaceuticals ltd/IN	
<i>Emulsion for injection</i>		
Amphomul[®]	Bharat Serum and Vaccines /IN	
<i>Lozanges for oral administration</i>		
Fungilin[®]	Bristol-Meyers Squibb/AU	

US = United States of America; BR = Brazil; IN = India; AR = Argentina; AU = Australia;

The FDA has approved some dosage forms for AmB (3% ointment; 3% cream; 100mg/mL oral suspension; 3% topical lotion) but just the Fungizone[®] for injection is

currently available in the USA. In 1992, X-Gen Pharm approved the first generic for Fungizone[®], marketed as “Amphotericin B[®] for injection”. After that, in 1995, the AmB lipid complex Abelcet[®] was approved, being followed for Amphotec[®] colloidal dispersion (1996) and the liposomal dosage form AmBisome[®] (1997) (19). The approval of these three dosage forms, together with Doxil[®] (an anticancer liposome), were the first FDA approvals to nanoengineered medicines (20). The approval of new biotechnological drugs has received much more attention due to their increased specificity and lower toxic effects. The structural schemes of FDA approved products are depicted on Figure 3.

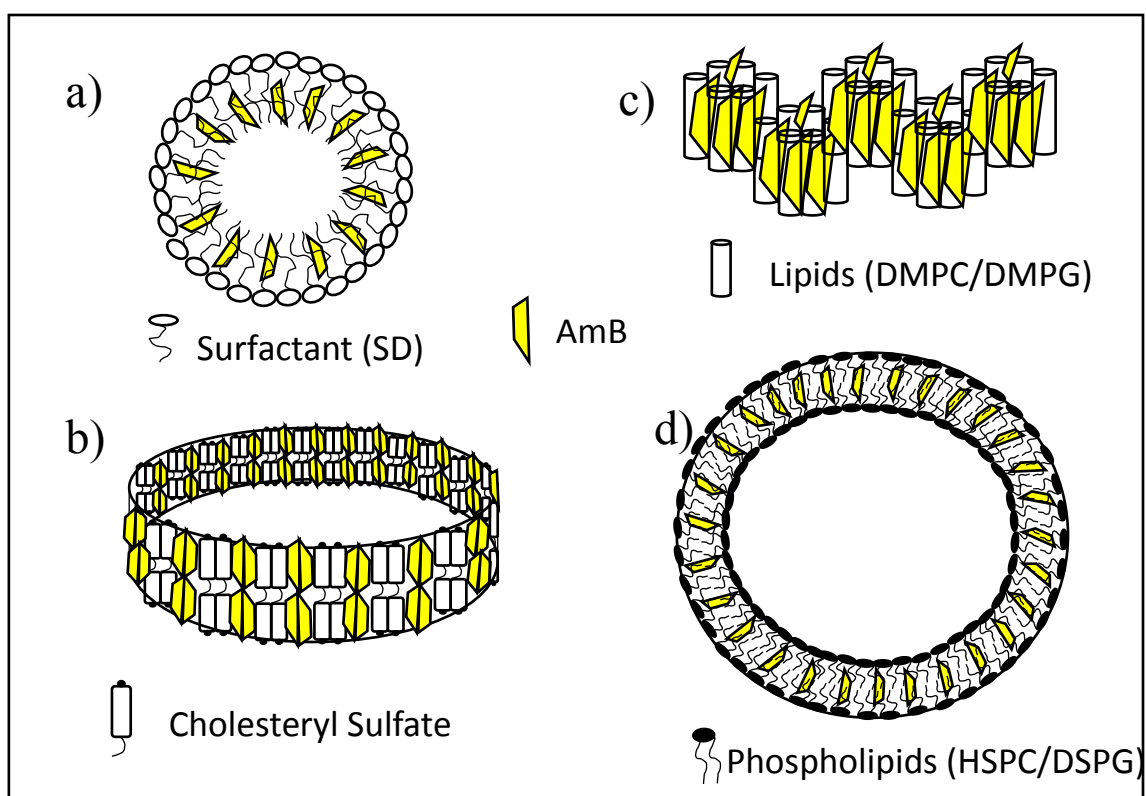


Figure 3. Structural schemes of AmB-based formulations already in the market: a) micellar systems (Fungizone[®] and others); b) disk-like lipid complex (Amphotec[®]); c) ribbon-like colloidal dispersion (Abelcet[®]); d) liposomal systems (AmBisome[®] and others). Based on (5, 6).

Most of AmB-based products listed on the Table 1 are micellar systems, probably due to the easier production when compared to the other known systems. Another interesting data is that most of the products listed (~70%) are produced in India. Indeed,

this fact is understandable once this country presents endemic leishmania infection areas where patients are refractory to the treatment with antimonials (first line treatment) (21). Some efforts have been made in India since 2005 in order to fight against leishmaniasis and a great number of clinical trials using AmB probably gave birth to new AmB-containing products (22).

All the systems listed in the Table 1 are intended for intravenous administration, except Fungilin[®] lozanges, In fact the advances in the engineer of drug carriers allowed the development of new AmB-based products, however their ability to cross the intestinal barrier has not yet been achieved. Fungilin[®] lozanges are indicated for oral delivery but with a topic pharmacological effect on the treatment of oral and intestinal candidiasis.

In the following lines it will be presented the AmB-based products already approved by the FDA that are/were available in the market.

Fungizone[®]

The Fungizone[®] was launched in the 1960's consisting of a mix of 50 mg of AmB and 41 mg of sodium deoxycholate (SD) (4, 5, 17) constituting for a long period of time the only AmB-based product available in the market. Side effects such as headache, fever, chills, nausea and mainly nephrotoxicity have been related to its infusion (2, 17, 23-26). However, due to its low cost and high antifungal efficacy, it is broadly used nowadays.

To avoid the Fungizone's drawbacks and to increase its therapeutic index some strategies have been applied. The AmB aggregation state has an important role on the host toxicity (18), when the AmB-SD is dissolved in water, the drug molecules are mainly in the aggregated form (16, 27, 28) that is supposed to bind the cholesterol of host cells, causing toxic effects. One strategy for increasing AmB-SD therapeutic index could be the delivery of AmB monomers. In fact, it was demonstrated that AmB-SD has its deleterious effects balanced by the presence of serum albumin, which influences the AmB

aggregation state towards monomers (29). In this way, other strategies have been published such as the use of other micellar (30) or lipid systems (4, 31, 32) to dilute Fungizone[®] as well as the mild heating of the product, in order to form AmB superaggregates (25, 26, 28, 33).

Abelcet[®]

The product is an AmB lipid complex (AmBLC) consisting of a 1:1 molar ratio of drug to lipids. The lipids herein used are dimyristoyl phosphatidylcholine (DMPC) and dimyristoyl phosphatidylglycerol (DMPG) (7:3 molar ratio) (5, 6, 34-38).

The formation of the system is possible dependent of an AmB concentration higher than 3 mol% (34, 38). Into this system, the drug is complexed with a ribbon-like non-liposomal structure forming lipid-stabilized AmB aggregates. It is suggested that the new structure is responsible for the drug toxicity attenuation (36). In fact, a low concentration of monomers on the medium is enough to kill the fungal cell but insufficient to cause toxicity to mammalian (34). Indeed, phospholipases are required to hydrolyzate the lipids in order to allow the drug release (35).

Due to Abelcet[®] low toxicity, it is possible to increase the quantity of drug administered to the patient. In this way, better fungicidal level is achieved. Besides, even in higher concentration, this formulation is better tolerated than AmB-SD (39). Moreover, it is effective against fungal infections on bone marrow transplanted patients who had failed to respond to previous antifungal therapy (even treated with AmB) (40).

Amphotec[®]

The Amphotec[®], an AmB colloidal dispersion (ABCD) was the third FDA approved AmB based product (34), it is presented as uniform disk shaped particles of

around 115 nm where the drug is complexed with cholesteryl sulphate in a 1:1 proportion (41-43). ABCDs were marketed as Amphotec[®] (USA) and Amphocil[®] (Europe) (6, 17).

The disc-resemble particles of this system have similar antifungal activity and decreased toxicity when compared to Fungizone[®]. Such an improvement in safety is due to the strong interaction of the drug with cholesteryl sulfate, reducing the amount of free AmB available in the blood (6, 42). However, ABCD showed up regulation of inflammatory genes and consequently, similar or even more infusion-related reaction compared to AmB-SD. It is probably why its production was discontinued in 2011 (17).

AmBisome[®]

The AmBisome[®] is a single bilayer liposome that was introduced in the European market in 1989 and approved by the FDA for the treatment of leishmaniasis in 1997 (6). The product consists of AmB, hydrogenated soya phosphatidylcholine (HSPC), cholesterol, distearoyl phosphatidylglycerol (DSPG) and sucrose, where the AmB molecule is intercalated within the liposomal bilayer (5, 17).

The low toxicity of liposomal systems is due to their small size which makes possible their prolonged circulation in the blood, as well as due to their composition: once cholesterol is a component of the system, AmB binds to it and remains attached until it binds to an ergosterol-containing membrane. There is also a negative charged component in the system (DSPG) that complex the positive AmB amine group (6, 44, 45). Consequently, less free AmB is found in the medium interacting with mammalian cells and causing side effects (17, 46).

The AmB encapsulation into liposomes could protect the drug against degradation processes such as chemical inactivation, enzymatic degradation and immunological neutralization (47). Indeed, it was demonstrated that liposomal-based eye drops were stable for up to six months after reconstitution (48).

AmBisome[®] has its high cost (when compared to other marketed systems) justified by the advantages in terms of high bioavailability and few side-effects (6). There are already some AmBisome[®] similars on the market. However, studies showed that some of these products do not have equivalence with AmBisome[®] regarding safety or efficacy (44, 49). Indeed, even products having the same composition presented different results concerning antifungal activity and toxicity both *in vitro* and *in vivo*. It is suggested that the manufacture process could play an important role on the differences between products (47, 50).

So far, many studies have been performed comparing the effectiveness of AmB-loaded marketed products. As a common consensus, AmB-based micelle is the most toxic system despite its high efficiency. The new lipid-based formulations are safer and well tolerated even for immunocompromised and HIV co-infected patients, but they are cost-limiting mainly for people in developing countries (17, 51, 52). That is the reason why pharmaceutical strategies for AmB delivery are still under development.

IV. NEW STRATEGIES FOR AMB DELIVERY (NOT MARKETED)

The strategies described above are not completely new. In fact, the majority of researchers deal with the nanotechnology in order to produce nanodevices well known such as micelles, nanoparticles, microemulsions, nanosuspension among other systems. However, the use of some components or even the methodology applied in the production process may turn the drug more soluble and the product more effective or less toxic.

IV.A. Micellar Systems

The most used strategy is the one applied to the first AmB-based product, micelization. It is well established that micelles are able to improve the solubility of many drugs and that the location of the drug depends on its hydrophobicity (53). Besides, the

micelle dynamics (surfactant unimers exchanges) could be responsible for the release of the AmB from the system (54).

The problem behind the use of AmB-loaded micellar systems is the aggregation state of the drug. The AmB de-aggregation in Fungizone[®] was achieved by using a new micellar system composed of poly (ethylene glycol) distearoyl phosphatidylethanolamine (PEG-DSPE), a mixture that makes possible the delivery of the drug as monomers. The antifungal efficacy of this new system did not change and the hemolytic activity was lower than that of the original product (30). In the same way, but using pure AmB instead of AmB-SD, self-assembled PEG-block-poly(ϵ -caprolactone-*co*-trimethylenecarbonate) (PEG-p(CL-*co*-TMC)) system was able to solubilize AmB and to de-aggregate it. The AmB solubility in a 20% PEG-p(CL-*co*-TMC) micellar solution was increased more than 100 times when compared to the drug solubility in water. AmB was found as monomers in the micelles and this fact was reflected on the reduction of the hemolytic drug induced effect. However, the antifungal effectiveness of the system was reduced (55). A similar behavior was observed when a gemini surfactant derived from cysteine was used for AmB solubilization (56).

Another strategy developed to increase the therapeutic index of AmB-SD was introduced with the concept of the AmB super-aggregates. These super-aggregates consist of the same AmB-SD mixture of components, therefore this dispersion passes through a heat treatment (70 °C for 20 minutes) that changes the supramolecular organization of the micellar system (27). The new structure has been reported as less toxic maintaining the drug activity, when compared to the un-heated AmB-SD (25, 26, 28, 33, 57). Recently, our group have demonstrated how micelles can be re-arranged to obtain the super-aggregated structure after a heat process (26). Additionally, this re-built

system can be freeze-dried and maintain its physicochemical and biological properties (33).

The surfactant used in the Fungizone[®] preparation, SD, is a bile-acid detergent. Other micellar systems were already prepared with SD-like components sodium cholate, sodium deoxycholate sulfate (SDS) and potassium deoxycholate. These micelles were tested for membrane permeation and they were able to increase this parameter two to five folds when compared to pure AmB. Among these systems, the AmB-SDS was the less toxic and the one who showed best permeability (58). The use of AmB-SDS showed to be an interesting approach for the AmB pulmonary delivery, as an aerosol dosage form, effective against *C. albicans* and *C. neoformans* (24).

The use of polymers to built micelles has been well studied for AmB delivery. Modified alginate micelles were able to improve AmB water solubility up to 160 times, and to control the drug release (90% AmB release within 14 hours) (59). Using poly-(dimethylmalic acid), AmB water solubility was increased 1000 times (60).

For copolymers-based systems, the composition (ratio) of the blocks influences both the self-assemble and the drug incorporation. Due to it, for a system based on poly(isoprene-b-ethyleneoxide) copolymers, the final product did not present good polydispersity compromising AmB loading efficiency, droplet size and drug release (61). This highlights the importance of controlling the molecular weight of copolymers. For PDMAEMA-b-PCL-bPDMAEMA triblock copolymers, the micelles built from low molecular weight polymers encapsulated more AmB than those produced with polymers of high molecular weight. The consequence of high drug load was molecular aggregation. Nanocontainers prepared with high molecular weight copolymers incorporated less drug, presented as monomers into the system that had small droplet size and low hemotoxicity, without loss of effectiveness (54). A study conducted with δ -decalacton homopolymers

and copolymers revealed slow release and long degradation time of the drug when loaded in its micelles (62). It gives rise to the possibility of the use of polymeric micelles as a kind of depot AmB-based system.

Cyclodextrin (CD) could be used to improve AmB solubilization and further micelle preparation. The use of CD for the solubilization of AmB in poloxamer micelles was described (63) as well as the use of hydroxypropyl- γ -CD for the treatment of pulmonary aspergilosis (64). Indeed, when the drug is included in a CD, only the polyene macrolide ring fits the CD cavity (65). Then, the arrangement of the cyclic polysaccharides as monomer, dimer or hybrid cyclodextrins, influences the AmB binding power (66).

As it could be seen, the use of micelles are very often intended for AmB solubilization. New approaches such as the use of polymers on micelles formation have raised the possibilities of the use of this kind of systems not only for AmB solubilization but also for drug controlled release, consequently decreasing its toxicity.

IV.B. Emulsion systems

Emulsion systems are composed by two immiscible liquids dispersed within each other and stabilized for a surfactant film (67). These kind of systems are of easy preparation and can be classified as thermodynamically stable (microemulsions) or unstable (true emulsions and nanoemulsions) systems (68). Both of them were already used as AmB carriers, and the drug is supposed to be located on the droplet surface or liquid interfaces (14).

Some techniques such as spontaneous emulsification, microfluidization, high pressure homogenization and sonication were already employed for the development of AmB-containing emulsion systems (69-72). Even the use of the already prepared

emulsion Lipofundin® have been related in the literature as an strategy for AmB therapeutic index improvement (4, 31, 32).

Some studies have showed high AmB loading efficiency (> 90%) into emulsion systems (73-75). Indeed, AmB had its solubility improved 1000 times in a microemulsion system (71) and different load charges have been related, depending on the components used. When Captex® 200 was used as oil core 6.8mg/mL (76) and 5.0 mg/mL (23) AmB were loaded. For Miglyol® core, 3.0 mg/mL (72) and for cholesterol core, 2.5 mg/mL (77). However, there is evidence that the AmB solubilization is due to the formulation microstructure and not to the components themselves (78).

When loaded into the system, the AmB molecules are mainly in the monomeric form (23, 70-73) specially in microemulsion formulations. It was also found in the literature a case in which the drug was complexed within the emulsion droplet, but released as monomers (79) in a safe and efficient manner (80).

In fact, the aggregation state of the drug was closely related to the formulation safety profile. Emulsions in which the drug was mainly in the monomeric state presented less side effects *in vivo* (73) and was 10-fold less toxic than AmB-SD (23). The presence of self-aggregates in the formulation could be responsible for its time-dependent cytotoxicity (71).

These systems were already intended for different administration routes such as topical (75, 81), ocular (72), oral (71, 73, 74) and parenteral (4, 31, 32). For the treatment of different diseases, mainly fungal-caused diseases (4, 31, 32, 72, 81), but also cutaneous (75) and visceral leishmaniasis (73, 74).

Special attention should be paid for formulations intended for oral and topical delivery. In the first case, the system must be stable in gastric and intestinal simulated media, promote the absorption of the drug and be well distributed to the target organ (74).

In the second case, it could be interesting the use of a permeability enhancer such as the transcutol-P (81). A formulation lacking such component failed to bypass the skin (75).

IV.C. Liposomal systems

Liposomes are vesicles made out of the same material as a cell membrane. It could be multilamellar or unilamellar and the drug may be entrapped into the aqueous and/or in the lipid phase (82). The launch of AmBisome[®], in 90's, improved the safety use of the AmB. Researchers are now using liposomes seeking other administration routes such as topical (83, 84), vaginal (85), oral (86) and pulmonary (87).

Comparing liposome components, the most common ingredient is cholesterol (83, 85, 86). Other components, like phospholipids, change in type and concentration among formulations. Indeed, the use of cholesterol is a strategy to maintain the drug loaded into the liposome.

Even the use of Fungizone[®] for liposome preparation is related in the literature (83). In this case, liposomes were prepared with HSPD/cholesterol/stearylamine (7:2:1 molar ratio) intended for the topical application of AmB. The liposomes were of high stability and deep penetration into the skin. The use of charged liposomes was also a strategy for the production of a vaginal intended delivery product (85). For this purpose, 1,2 dioleoyl-sn-glycero-3-phosphoethanolamine, 1,2-dioleoyl-3 trimethylammonium-propane, and cholesterol (4:5:1 molar ratio) were used for liposome prepare and then incorporated in a thermosensitive gel of Poloxamer[®] 407 and Poloxamer[®] 188, gelling at 37 °C.

The use of a plant sterol (stigmasterol) was the strategy of Iman et al. (2011) (88) to produce liposomes, mixing it with the new lipid 1,2-Distigmasterylhemisuccinoyl-sn-glycero-3-phosphocholine. The system was characterized and compared with AmBisome[®], showing similar activity against fungal and leishmania infections. The use

of vegetal ceramides was described by Skiba-Lahiani et al. (2015) (86), who developed liposomes intended for AmB oral delivery. Ceramides were important to give liposome membrane stability in the digestive medium. However, cholesterol and phospholipids were required to prevent drug leakage and to provide liposomal lamellar structure.

A modification of the liposome preparation method was already suggested. In this case, proliposomes were employed (87, 89). The concentrated proliposomes were used to prepare unilamellar liposomes *in situ*, resulting in high entrapment efficiency (> 95%) and AmBisome® comparable release and efficacy (89). Also a chitosan-coated formulation intended for nebulisation was prepared by this method, showing to be active against *C. albicans* and *C. tropicalis*, comparable to Fungizone® (87).

The most different liposome produced used ethanol in its composition (30-45%) and was called ethosome (84). It was intended for the treatment of cutaneous fungal infection, once the etholic nature of this carrier is supposed to enhance skin permeation. Besides, the AmB-loaded ethosome was mixed with Carbopol® gel, in order to prepare a final nanoethosome gel that could be retained for more time in the skin. The size of the carrier was in the range of 180 – 300 nm, showing up to 89% AmB loading efficiency. The drug was controlled released from the system (87% in 24 hours), although 95% of the free drug was released to the medium within 2 hours. When compared with marketed AmB by topical delivery, the new system was able to enhance drug permeation as well as drug deposition and improve activity against *C. albicans*.

IV.D. Nanoparticles

Nanoparticles are very versatile systems due to the possibility of easy modulation of important characteristics such as size, shape and surface charge, and are of particular interest for the encapsulation of insoluble drugs such as AmB. It is suggested that this

sort of carrier could improve drug solubility and increase its distribution in biological tissues (90).

Many polymers and copolymers such as N-Palmitoyl-N-monomethyl-N,N-dimethyl-N,N,N-trimethyl-6-O-glycol chitosan (GCPQ)(91), poly(ϵ -caprolactone)/poly(N,Ndimethylamino-2-ethyl methacrylate)(92), poly(lactide)-poly(ethylene glycol) (PLA-PEG) (93), poly(D,L-lactide-co-glycolide) (PLGA) - D- α -tocopheryl polyethylene glycol 1000 succinate(94), PLGA-PEG (90, 95), PLGA-b-poly(L-histidine)-b-PEG (96), bovine serum albumin (BSA) (97) and hydroxypropylmethylcellulose (98) have already been used for the production of AmB-containing nanoparticles. Besides, AmB-containing magnetic nanoparticles were also developed for the treatment of pulmonary infections (99, 100).

In general, the drug is loaded into the nanoparticle matrix (92) and, as aforementioned for polymeric micelles (54), according to the polymer molecular weight more or less AmB can be loaded into the nanoparticle matrix. As a consequence of the high AmB incorporation amount, the drug is self-aggregated into the system (92, 93). However, the drug could also be loaded on the nanoparticle surface (99, 100) and be found as monomers (97, 101) depending on the nanoparticle type and polymer used. The nanoparticle drug load varies, the herein reviewed articles loaded > 68% AmB (90, 92-98). However, strategies using cluster dextrin (101) and solid lipid nanoparticles built with theobroma oil/bees wax (102) did not present high drug load.

The nanoparticle structure permits the decoration of its surface. Tang et al. (2015) (96) did it with anti-*Candida albicans* antibody. Besides the particle decoration, the polymer used by them was pH responsive, which is a very interesting approach when it is considered that during a fungal infection the local pH is diminished, lowering the AmB activity. A modification of a chitosan-based nanoparticle with a ligand 4-sulfated N-

acetyl galactosamine (4-SO₄GalNAc) is also related in the literature for the treatment of leishmania infected macrophages (103). These strategies lie on the drug targeting. If achieved, the dose may be diminished causing side effects decrease while activity is maintained.

In fact, most of the nanoparticle systems were able to increase efficacy and decrease the toxicity when compared to free-AmB (92, 94, 96, 99) and AmB-SD (93), or, at least, maintain the drug efficacy while decreasing drug toxicity (96, 97, 101). Such results could be explained by the sustained release of the drug from the system (93, 96, 98) that could be intended for alternative administration routes such as pulmonary (98-100) and oral delivery (91, 95, 102).

IV.E. Other colloidal systems

Other colloidal systems have been studied in order to improve AmB solubilization and target delivery. Nanodisks produced from phospholipids and amphipathic apolipoproteins (104) and calcium sulfate and hydroxyapatite (105) have been described, opening the possibility for the treatment of diseases such as fungal osteomyelitis (105), that is not covered by using the marketed systems.

Carbon nanotubes could also be used for AmB delivery (106). Pruthi et al. (2012) (107) loaded carbon nanotubes with AmB (which was physically adsorbed on the external sidewalls of the nanostructure) called AmBitubes. This system was able to load up to 75% AmB and sustain the drug delivery in a pH dependent manner. The system was 3 times less hemotoxic than the free AmB solution and had improved drug uptake by macrophages, resulting in an interesting strategy for AmB delivery in leishmaniasis.

Some natural products such as bixin have demonstrated ability to disperse AmB in aqueous medium (108). This compound is antibacterial itself, and more effective than AmB-SD against *C. albicans* (109).

The use of new colloidal systems as nanogels (110) and cochleates (111) opens the possibility of AmB delivery by two important routes: topical and oral, respectively. The nanogel developed by Qasim et al. (2014) (110) was based on poly(*N*-isopropylacrylamide), achieving globule sizes of around 430 nm from where the drug was well solubilized, controlled released and efficient against *C. albicans*.

The cochleates are nano or sub-micron sized multi-lamellar cylindrical structures spontaneously generated by the fusion of negatively charged liposomes with metal cations (6, 111-115) that were already used for the oral treatment of systemic candidiasis (111). Results showed the system as efficient as AmB-SD by intraperitoneal injection. When AmB-containing cochleates were tested for the treatment of leishmania, they were also effective as AmB-SD with the advantage of being non-toxic for macrophages (114).

V. CONCLUDING REMARKS

Despite all drawbacks concerning AmB physicochemical properties and adverse effects, scientists have been successful in creating dosage forms for this drug. Nowadays, there are available in the market systems based on micelles, lipids and liposomes. The studies comparing these products usually show the micellar system as the most effective one. However, using the other systems it is possible to safely increase the drug posology (116).

The main concern for the AmB lipid-based systems is the cost. Then, the majority of the researchers try to develop systems with properties that overcome AmB-SD concerning safety.

From the last 5 years, researches have been focused mainly on the development of polymeric micelles and nanoparticles. In the first case, researchers try to have the drug loaded as monomers once this presentation is supposed to be less toxic than AmB

aggregates, binding preferably to ergosterol-containing membranes. In the second case, as the drug is mainly entrapped into the particle matrix, the drug could be aggregated and the strategy lies on the AmB controlled release. Again, the aim is to release AmB monomers, that are less toxic and more effective against parasites and fungi.

Besides the improvement on the AmB therapeutic index, led by the development of new AmB delivery systems, many attempts are still being made on the development of carriers to turn possible the administration of AmB by other routes, such as oral and topical. So far, good results have been demonstrated by the use of cochleates and emulsion systems.

The research for the “ideal carrier” continues. The society still waits for the AmB-containing system able to treat infections without damaging kidneys, that would treat diseases not only by intravenous, but by other routes and mainly, that would be affordable by the most needed patients.

Acknowledgments

Authors are grateful to Dr. H. R. Marcelino and Dr. J. Genre for the paper critical read before its publication and to CAPES-COFECUB (Project 742/11) for the scholarship provided for A. L. Silva.

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SECTION II

AmB-CONTAINING MICELLAR SYSTEMS: CHARACTERIZATION
OF SIMILARS AND SUPER-AGGREGATES FORMATION

BRIEFING

Micelles are known for their ability of solubilizing many water insoluble molecules. Not surprisingly, this was the first successful strategy for amphotericin B (AmB) solubilization.

In the 1960's a mix of sodium deoxycholate and AmB was launched in the market and after 50 years, in spite of the side-effects of this medicine, it is still used in the therapeutics.

The second section of this thesis is dedicated to the AmB micellar systems, bringing 2 chapters in which are presented and discussed issues such as the characteristics of similar products and the formation of AmB super-aggregates.

CHAPTER II

Amphotericin B-containing sodium deoxycholate micellar systems: a comparison of physicochemical characteristics of marketed products

Amphotericin B-containing sodium deoxycholate micellar systems: a comparison of physicochemical characteristics of marketed products

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Abstract

The amphotericin B (AmB) is an antibiotic used to treat deep life-threatening fungal infections and also some protozoa-caused diseases such as leishmaniasis. The AmB physico-chemical characteristics turn difficult the drug loading into pharmaceutical carriers. Despite this fact, since the 1960's, the drug has been commercialized as a mix of AmB and Sodium Deoxycholate (SD). Many side-effects are attributed to this system. However, after Fungizone's patent expiration, similar products emerged on the market worldwide. The aim of this work was to compare the characteristics of three AmB-containing SD micellar systems. Drug label, carrier size and AmB aggregation state were evaluated. The products presented size of around 30 nm. However, the drug label differed between them and according to it, systems have different aggregation state. Besides of evidencing the impossibility of product interchange, the findings of drug aggregation pattern may suggest product efficacy and safety.

Key-words: Amphotericin B; UV-Vis; Circular Dichroism; Aggregation State;

Introduction

The amphotericin B (AmB) is a polyene antibiotic extensively used to treat life-threatening systemic fungal diseases (1, 2). The mechanism of action of the drug is not completely clear, it is believed that the clinic effect results from the interaction of the drug with the sterols of the cellular membrane, mainly the ergosterol of fungal cells. The drug forms pores in the cell membrane increasing its permeability and causing cell death (3, 4).

The molecule was discovered in the 1950's (5) and had its structure elucidated in the 1970's (6). Its structure is composed by a rectangular ring containing a transconjugated heptane chromophore justaposed by an acyl chain linked with many hydroxyls (Figure 1) (3, 7).

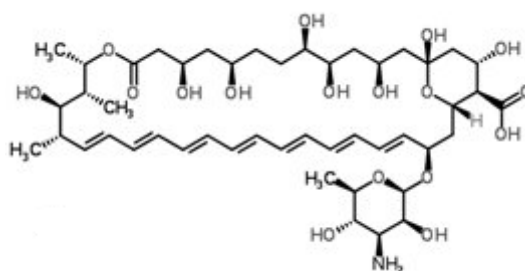


Figure 1. Amphotericin B molecular structure.

Besides of being amphiphilic, AmB is also amphoteric, once it is a zwitterionic molecule in solution at neutral pH: the carboxylic acid group is deprotonated while the amino acid group is protonated (4). Because of that, the molecule is soluble just in few organic solvents and insoluble in water (3). This fact turns difficult the drug loading into pharmaceutical carriers.

In early 1960's, it was developed a micellar system in which sodium deoxycholate (SD) solution was able to solubilize the drug. This system was patented as Fungizone[®] and is broadly used as “gold standard” to treat life-threatening fungal infections up to now (8-10).

Fungizone[®] was the only AmB-based product approved by the Food and Drug Administration Agency (FDA) up to 1990's. In the last 20 years it appeared in the market lipid-based products such as Abelcet[®], Amphotec[®] and AmBisome[®] that improved AmB therapeutic index, since many reactions such as vomiting, headache, chills, fever and hard nephrotoxicity, linked to Fungizone's infusion (8, 10, 11) were overcome by these products. However, those systems have much more technology involved in its preparation and are cost-limiting mainly for developing countries.

After Fungizone's patent expiration some similar products were launched in the market worldwide (Table 1). Mainly in India, a developing country of pungent pharmaceutical industry, where the use of AmB has augmented with the increase of the leishmaniasis refractory treatment with antimonials (12, 13).

Table 1. List of some AmB-based micellar systems available in the market.

Product	Supplier	Country
Fungizone[®]	Bristol-Meyers Squibb	USA
Amphotericin B[®]	X-Gen Pharms	USA
Unianf[®]	União Química	Brazil
Anforicin[®]	Cristália	Brazil
Funtex B[®]	Cibla	India
Amphotret[®]	Bharat Serum	India
Photericin B[®]	Cipla	India
Amphotin-Vit[®]	Health Biotech	India
Fungitericin[®]	Lifecare Innovations	India
Amphotin[®]	United Biotech	India
Anfocam[®]	Dabur India	India

Hospicin B[®]	Alkem Laboratories	India
Amtericin[®]	VHB Lifesciences	India
Ambilip[®]	United Biotech	India
Amphonex[®]	Bharat serum	India
Amfitas[®]	Intas	India

The emergence of such a number of similar products could be relate to the ease of prepare of the micellar system. However it raises the doubt about the safe of the use and interchangeability between these systems. It is important to highlight that they are not generic, but similars.

The aim of this work was to compares the physicochemical characteristics of AmB-based micellar systems. For its purpose, three products were analyzed for AmB content, system size and degree of AmB aggregation, by UV-Vis and circular dichroism (CD).

Methodology

Materials

Amphotericin B (99% purity) was purchased from Indofine Chemical Company (USA). The micellar systems were: Fungizone[®] from Bristol-Myers Squibb (France), Amphotret[®] from Bharat Serums (India) and Anforicin[®] B, from Cristália (Brazil). Methanol was HPLC grade from Merck[®] (France) and the ultrapure water was obtained by Milli[®] Q purification system.

Calibration curve

An AmB stock solution (1×10^{-4} M) was prepared by diluting 1.71mg AmB in 17.1 mL methanol. In order to completely dissolve the drug, the container was put in a ultra-

sound bath during 5 minutes. Then, the stock solution was diluted in methanol for nine points of AmB concentration (1.6×10^{-5} ; 8.0×10^{-6} ; 6.0×10^{-6} ; 5.0×10^{-6} ; 4.0×10^{-6} ; 2.5×10^{-6} ; 2.0×10^{-6} ; 1.0×10^{-6} and 5.0×10^{-7} M) that were read in a Perkin Elmer (France) spectrophotometer with a 1cm length quartz cell. A screening was performed from 300 to 450 nm wavelength and $\lambda_{\max} = 405$ nm were used for AmB quantification.

AmB-SD solution preparation

All the products: Fungizone[®], Amphotret[®] and Anforicin[®] B are presented as lyophilized yellow powder containing 50 mg AmB and 41 mg SD. They were diluted in 10 mL water for injection, as recommended for the suppliers and reserved for further use. The resulting solution was 5×10^{-3} M AmB concentrated. The formulations were identified in this study as A; B and C, randomly, to avoid commercial appeal.

AmB quantification

The calibration curve was used to quantify the AmB-content into the products. After water suspension (5×10^{-3} M), the products were diluted 1000-fold in methanol, in order to result a 5×10^{-6} M AmB concentration, well fitting the calibration curve and assuring accurate drug quantification at $\lambda_{\max} = 405$ nm.

Evaluation of AmB aggregation state

The systems were diluted in ultra-pure water in order to have its aggregation state evaluated. The drug conformation was analyzed for both electronic and circular dichroic (CD) spectroscopy.

UV-Vis spectra were recorded in a PerkinElmer (France) spectrophotometer after 1000x dilution of the re-suspended product, using a 1cm length quartz cell. The wavescan was from $\lambda = 300$ to 450 nm (14).

For circular dichroism (CD), transitions of AmB were recorded from dilutions of 5×10^{-8} up to 5×10^{-5} M on a Jasco J-810 dichrograph (France), by using 10, 5, 1 and 0.1

cm path length quartz cells respectively. Wavescans from $\lambda = 300$ to 450 nm were carried out with the main objective of observing the presence of a doublet that identifies the presence of AmB aggregates in the medium.

Size measurement

The globular size was measured using a Malvern Zetasizer Nano-ZS (France) by dynamic light scattering (DLS) at 173° angle, 25 °C. The powder was reconstituted in water and the system had its size measured. Results were reported as size distribution by intensity and number.

Results

Calibration curve

The AmB molecule in a monomeric conformation absorbs at 363, 382 and 405 nm in methanol (Figure 2).

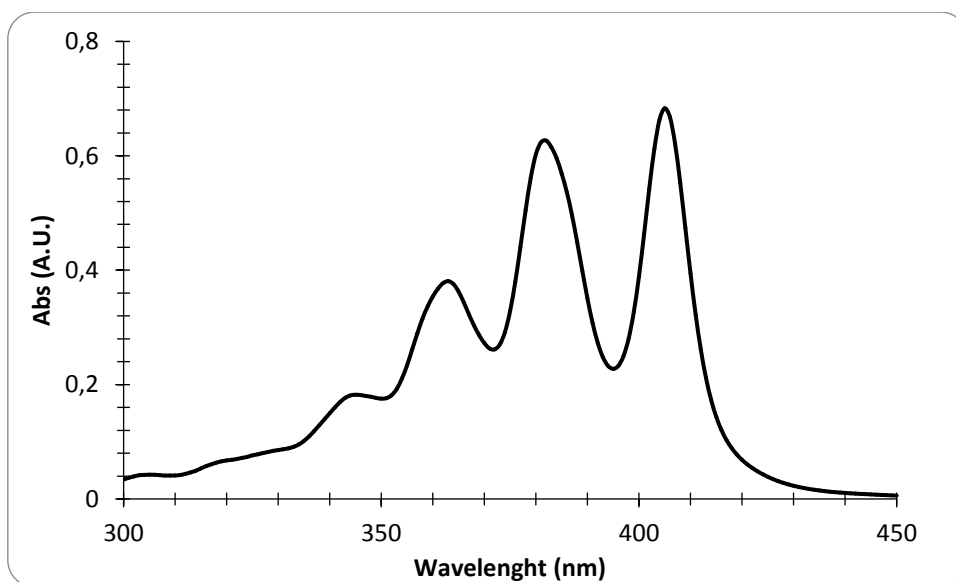


Figure 2. UV-Vis electronic spectrum of pure AmB in methanol.

A calibration curve was built using an UV-Vis spectrophotometer and showed to be linear ($R^2 = 0,9996$) (Figure 3).

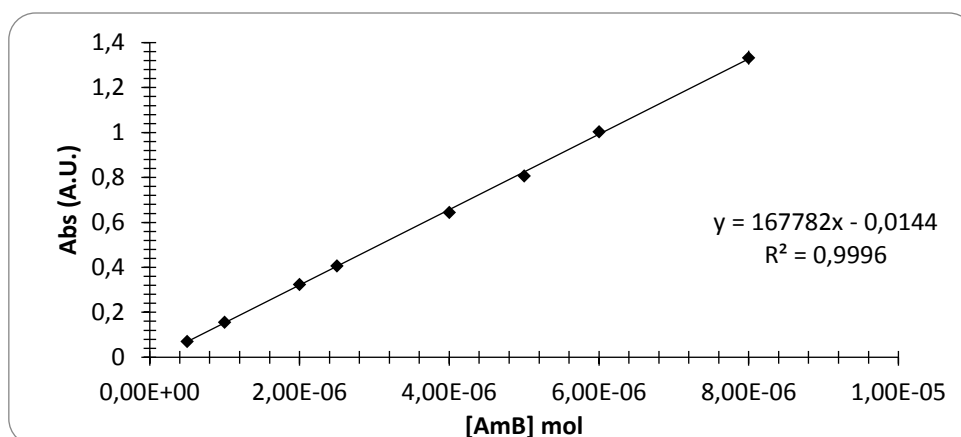


Figure 3. AmB calibration curve in methanol.

Eight from nine concentration points were used in the curve once the concentration 1.6×10^{-5} M gave 2.16 A.U. absorbance, very out of the Beer-Lambert theoretical principles. Indeed, considering the absorbance range 0.2 to 0.8, only the concentrations from 1.0×10^{-6} to 5.0×10^{-6} M AmB would be considered and the correlation coefficient R^2 would be 0.9999 (Figure 4).

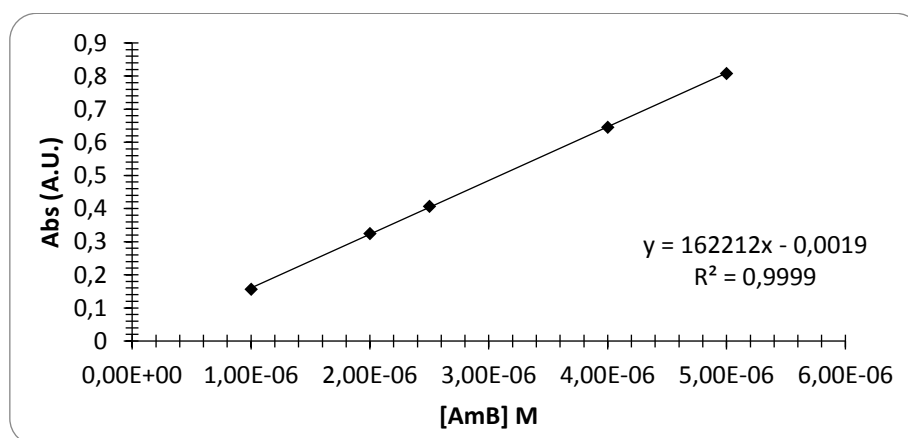


Figure 4. AmB calibration curve in the range of Beer-Lambert law.

The dilution for 5.0×10^{-6} M AmB was that chosen for further sample quantification.

AmB quantification

The AmB quantification was performed applying the equation from the calibration curve aforementioned. The micellar products were suspended in water and then, 1000-times diluted in methanol. From the wavescan 300 – 450 nm, the value of the main peak $\lambda_{\max} = 405$ nm was considered for AmB quantification (Table 2).

Table 2. Quantification of the AmB content in AmB-based micellar systems.

Product	Abs	[AmB] M
A	1.1017	6.65 ± 0.09
B	1.039	6.28 ± 0.18
C	0.8197	4.97 ± 0.11

AmB aggregation state

The AmB absorption spectra were recorded by UV-Vis for all the samples after 1000x ultrapure water dilution from the 5mg/mL reconstituted product and are depicted on the Figure 5.

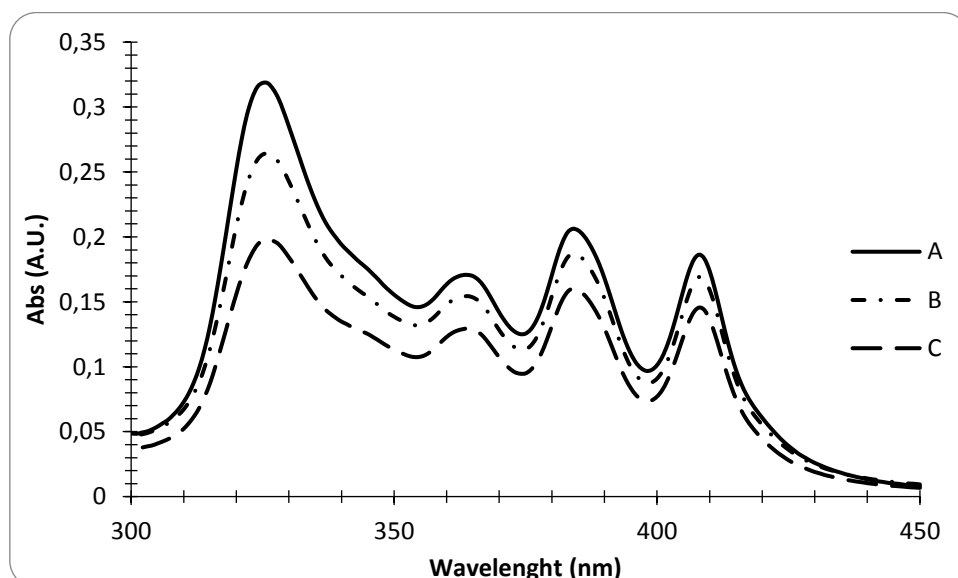


Figure 5. Electronic absorption spectra of samples A, B and C diluted in water. Labeled concentration 5×10^{-6} M AmB.

The spectra were characterized for the presence of both monomers and aggregates. The aggregation level in the samples was then calculated as the ratio between the absorption of aggregates ($\lambda = 325\text{nm}$) by the absorption value of the main peak of monomers ($\lambda = 408\text{nm}$) (Figure 6).

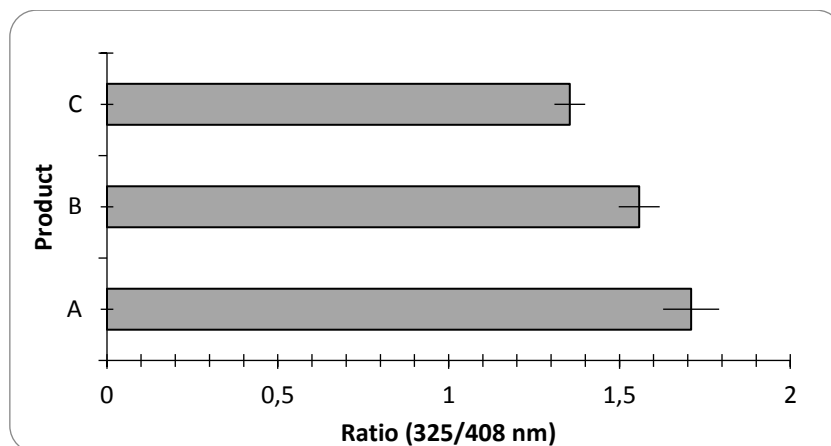


Figure 6. Relation between AmB species: aggregates and monomers, calculated from UV-Vis absorbances at 325 and 408 nm respectively.

The aggregation state of the samples was also evaluated by CD. All of them presented an exciton couplet characteristic of aggregated AmB forms (Figure 7).

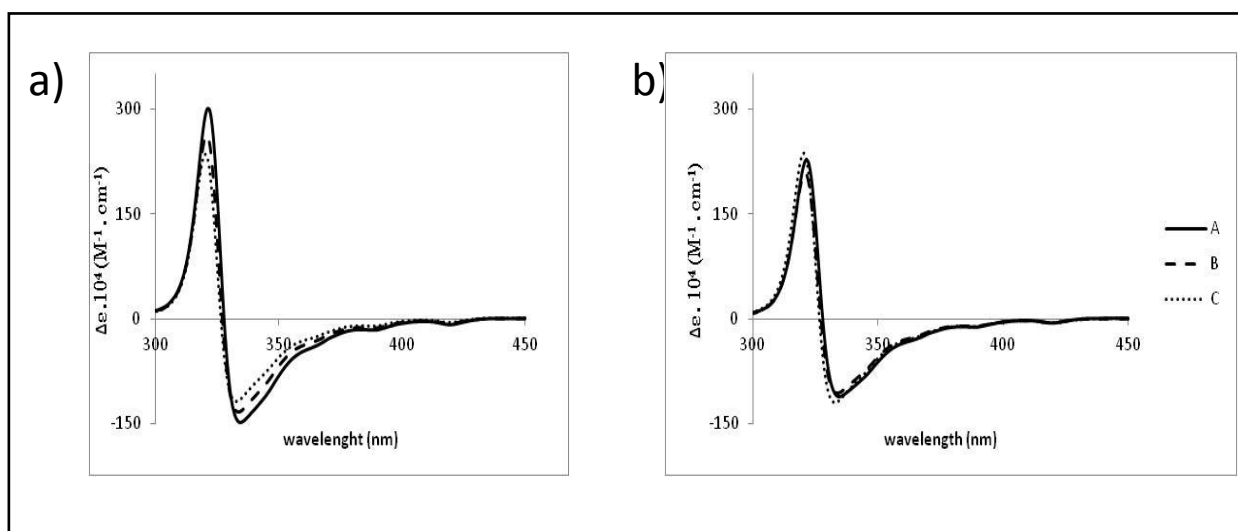


Figure 7. CD spectra of AmB-containing micellar systems at $5 \cdot 10^{-5}$ M AmB in water. Values were corrected in b) according to the quantified AmB.

The CD spectra were also recorded for different AmB concentrations. All the samples presented same aggregation behavior while being diluted (result not shown). The dilution of the product A is showed on the Figure 8.

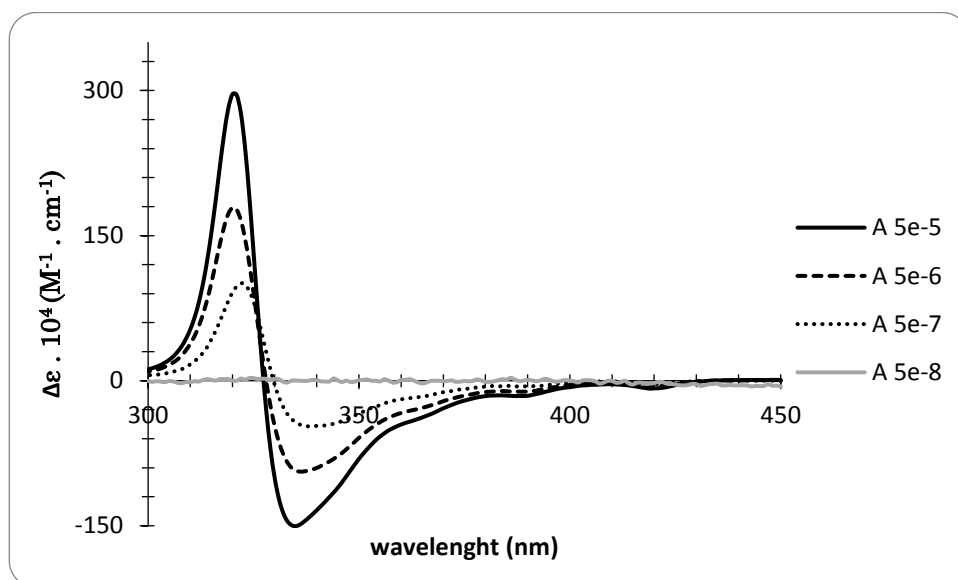


Figure 8. CD spectra of product A in different concentrations: from 5.10^{-5} to 5.10^{-8} M AmB.

The ratio among the positive and negative couplet peaks were 2.0, 1.9 and 1.9 for the dilutions from 5.0×10^{-5} to 5.0×10^{-7} M AmB, respectively. The spectra recorded at 5.0×10^{-8} M AmB did not present excitonic couplet.

Size distribution

The globular size of the products were measured by DLS and reported as size distribution by number and intensity (Figure 9).

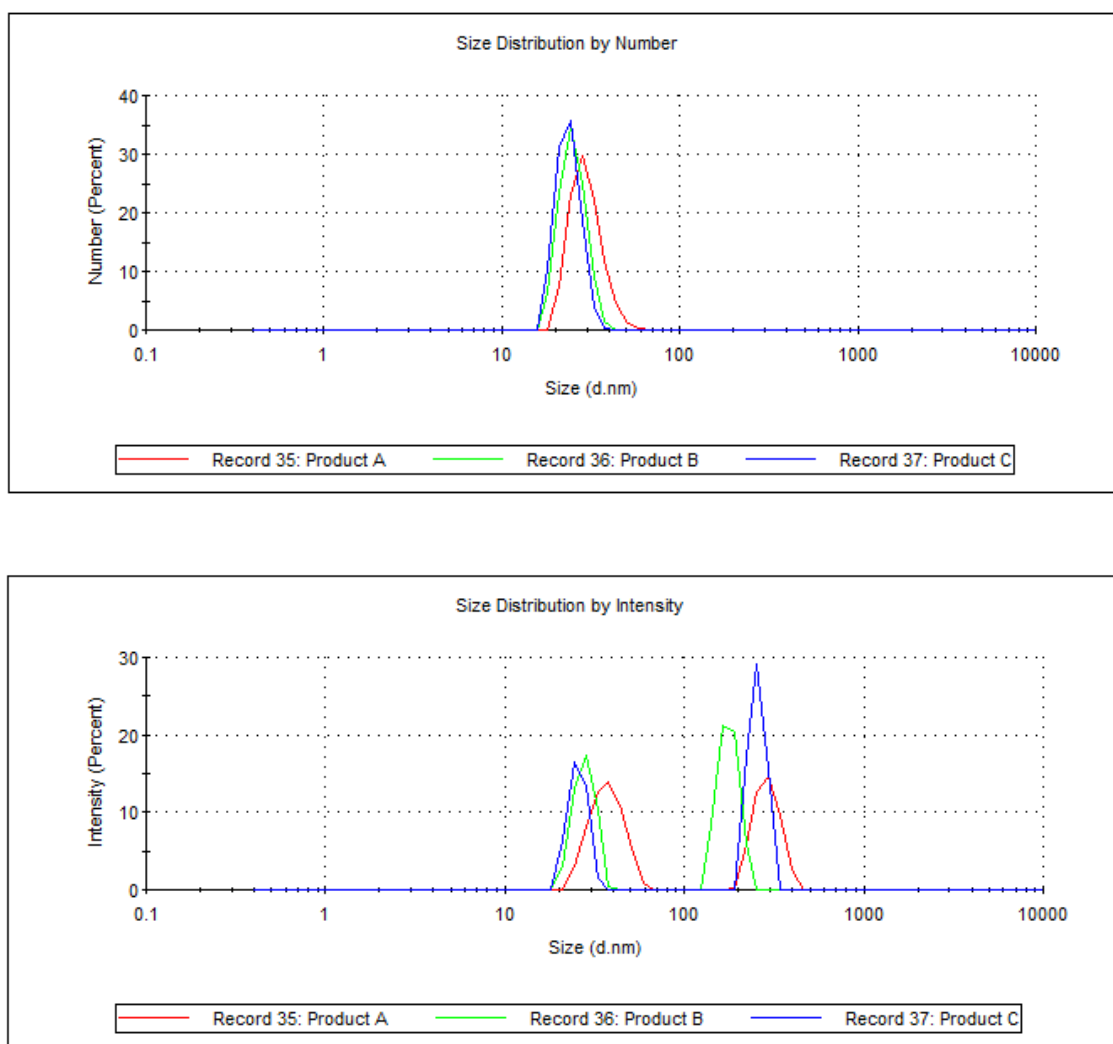


Figure 9. Size distribution for products A, B and C: a) size distribution for number; b) size distribution for intensity.

In the first distribution (Figure 9a) all the products presented the same range of size by number, the micelles were under 100 nm size (~30 nm). By intensity (Figure 9b), the distribution showed to be bimodal with peaks from 20 – 40 nm and from 150 – 300 nm.

Discussion

The optical properties of AmB, due to the presence of a system of seven conjugated double bonds (Figure 1), make the spectroscopic methods particularly

appealing for the drug study (15, 16). Indeed, polyene antibiotics absorb light between 300 and 450 nm (7, 17).

In this work we have prepared stock solutions of AmB in methanol, for drug quantification. Solvents classified as stronger or weaker proton acceptor are able to display AmB as monomers (7). In fact, when AmB is dissolved in organic solvents such methanol or in dimethyl sulfoxide at concentrations lower than 10^{-4} M, the drug is totally presented in the monomeric form (14, 18) and behaves as a normal solute in its adherence to Beer's law (7). Results are in agreement with the literature, once the AmB spectra in methanol presented characteristic bands (362, 382 and 405 nm) related for the monomeric form of the drug (Figure 2) and the absorption on the main band ($\lambda_{\text{max}} = 405$ nm) permitted the construction of a standard curve (Figure 3) which presented good linearity ($R^2 = 0,9996$), permitting drug quantification in methanol in the range of 10^{-6} M AmB, using a 1 cm path length cuvette. In the case of need of quantifying AmB below 10^{-6} M AmB a stationary cuvette may be used (19). The use of electronic absorption for the AmB quantification was already described in the literature as a simple, reliable and cheap analytical method (20-23).

The AmB-SD products are sold as a lyophilized mixture of 50 mg AmB and 41 mg SD (10, 24), to be dissolved firstly in 10 mL injection water prior to patient administration. Concerning drug label, results show that just one of the three products evaluated was in the concentration announced. Products A and B were more than 25% AmB concentrated than the labeled amount. Such results were reflected on the AmB aggregation state, once UV-Vis spectra of polyenes are very sensitive to conformation changes induced by molecular interactions (8).

It is known that AmB concentration is directly linked to the molecular self-association (16, 25-27). The drug aggregates in aqueous media due to its chemical

characteristics. Aggregated and monomers species exhibit significantly different absorption spectra (7, 28) as it can be observed in the Figure 5. Besides of the bands characteristics of monomeric AmB, an intense band appeared at $\lambda = 325$ nm, counting for the molecular aggregated form (14, 18). So, in AmB-SD products the UV-Vis spectra is that of monomeric and aggregated AmB (27, 29).

In water, AmB dissociates from SD forming monomers and oligomers of various aggregation states (10). Indeed, in aqueous suspension, three forms of AmB coexist: monomers, water-soluble oligomers and non-water soluble aggregates (14, 25). In general, the degree of AmB aggregation is calculated by the ratio between the absorption of the aggregated band for the monomeric band, in this case $Abs_{325\text{ nm}} / Abs_{408\text{ nm}}$ (8, 17, 18, 27). Figure 6 shows the results for such a calculation. In agreement with the literature, the degree of AmB aggregation increased according to the increase in drug concentration.

However, electronic spectroscopy is not the best way to differ aggregated species (18). Spectra of different organization forms can be recorded by many other spectroscopy techniques such as circular dichroism (CD), fluorescence, Fourier transform infra red spectroscopy and Raman scattering (30). The CD of AmB-SD systems is expected to be a better tool for the evaluation of AmB aggregation than electronic absorption due to it is elevated sensitivity to environmental modifications (9, 15). Indeed, AmB contains many centers of dissymmetry. Then, the use of chiroptical spectroscopy techniques can provide additional insight in the nature of aggregation tendencies (7). By definition, CD is a measure of the differential absorbance between left- and right-circularly polarized light (31). Therefore, any modification on AmB conformation could be identified by CD (9), showing changes that would be undetectable by UV-Vis (18).

The spectrum for AmB-SD alone presents a strong dichroic doublet typical of AmB dispersed as oligomers (3, 9, 25, 26). The bisignated shape of the spectrum is an

indication of a multimolecular structure in which heptane chromophores are in excitonic interactions (3, 7, 15, 18). The specific nature of the CD signal results from the fact that neighboring molecule have preferred orientations (9). Moreover, the couplet shape is unique for both, specific structural aggregate or a mixture of several aggregates. Then, any changes in the shape could be taken as evidence for a change in aggregate type or assembly of aggregates (18).

In agreement with the literature, results showed in the Figure 7a indicated that all AmB-SD products presented AmB in its molecular aggregated form. The signals were equal in shape but slightly different in intensity. It can be explained by the difference in AmB concentration between products (18). In fact, intensities decreased with the decrease on AmB concentration. However, when the intensities values were adjusted for the same concentration, by calculation, both intensity and shape were similar for all the products analyzed (Figure 7b). An indication that the products present same oligomeric species into the suspension (18). The spectra obtained by electronic absorption and CD looks like contradictory, since the UV-Vis showed difference in the aggregation degree and for CD sign just a slight difference in intensities was found. In fact, these spectroscopy methods do not monitors the same species (15). Bolard et al. (1980) (16) explained that at intermediate AmB concentration the peaks wavelength of these methods no longer coincide. Then, electronic absorption monitors the exact amounts of the different amphotericin B forms, while CD allows the detection of associated species in minor amounts.

The increase in product dilution evidenced the AmB dissociation towards monomers (Figure 8). In fact, the affinity between AmB and SD is so week that under dilution they are completely dissociated (10). Reduction in CD amplitude is related to the formation of aggregates with lesser number of the molecule in the solvent (3). In fact,

there is an AmB concentration threshold around 10^{-7} M from where CD records performed under and above showed the drug as monomers and aggregates, respectively (27). This kind of experiment, using products dilution were conducted by CD because the disappearance of the dichroic doublet is much more easily monitored by this mean than by its corresponding excitonic absorption band (17). The disappearance of CD signal was demonstrated in our findings at 5×10^{-8} M AmB concentration (Figure 8), indicating that only monomers of AmB were found (9, 18, 25, 32).

The nature of aggregates seems to be the same, once the ration between the positive and negative peaks of AmB CD couplet (~ 2) did not vary under dilution (18). According to the CD couplet signature, AmB molecules are aggregated probably as antiparallel dimmers (26).

The aggregation state of the drug seems to have influence on the size of the systems. It can be observed in the Figure 9 that the evaluated systems are quite similar in size (~ 30 nm) concerning number of droplets. However, when volume is taken in account, the signal became bimodal with less intense peaks around 30 nm and more intense peaks in the range of 150 – 300 nm. Hartsel and Bolard (1996) (11) and Espada et al. (2008)(14) indicate the size of Fungizone® micelles as < 400 nm. However, this kind of measurement could be not exactly reflex of the structure size (14) since the dilution of the system have direct influence on the disruption of the micelle structure and AmB aggregation.

The most important point concerning AmB aggregation state in AmB-SD systems is about the drug mechanism of action. Both, the pharmacological activity and the toxic side effects are strongly dependent on the AmB molecular organization (8, 33). It is well discussed in the literature that AmB binds to sterol membranes, forming pores in it and making possible the leakage of important ions or, more than that, in dependence of AmB

concentration, causing the disruption of the cell, with consequently cell death (15, 16). It has been proved that AmB-SD of high aggregation state is more toxic than the same mixture at lower aggregation state (4, 8, 14, 25).

The drug bind to ergosterol and/or cholesterol is suggested to be the key for understanding the appearance of AmB side effects (4, 26). It is admitted that the interactions between AmB and sterols occur through hydrophobic interactions, where the seven conjugated AmB double bonds interacts with ergosterol or cholesterol (4). AmB is not specific for the binding to ergosterol, the sterol of fungi and protozoa cells. However, it has been demonstrated that when the drug is in its monomeric form, it is not able to cause toxicity for cholesterol-containing cells (4, 14). Then, the difference in AmB aggregation in products supposed to be equal could result in differences in the therapeutic response, incurring in patient's hard side effects and consequently augmentation in the time of hospital housing.

Conclusion

To our knowledge, this is the first time that a comparison between AmB-SD micellar systems is made. The overall results showed that the marketed products analyzed have similar mean diameter size, despite the unmatched labeled content. Only the product C was in the designated concentration of 5 mg/mL. Products A and B were more than 25% up concentrated. All of the products were presented as a mix of AmB monomers and oligomers as expected, and the level of AmB aggregation was in accordance to the drug concentration. CD studies revealed that the same species of oligomers are presented into the three formulations. This study suggests, in the light of AmB aggregation that the AmB-SD systems in here evaluated cannot be interchangeable.

Acknowledgments

Authors are grateful to CAPES-COFECUB (project 742/11) for the scholarship provided to A. L. Silva.

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CHAPTER III

*Amphotericin B super-aggregates: formation and influence on the drug
stability*

Amphotericin B Super-aggregates: formation and influence on the drug stability

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Abstract

The amphotericin B (AmB) is a polyene antibiotic used to treat life-threatening systemic fungal infections. The drug is currently available as AmB-micelles and -lipid-based products. The micelles are a mix of AmB and sodium deoxycholate (SD), a biliar salt that acts as a surfactant. This is a quite simple delivery system, and its injection is responsible for important side-effects of the drug. The mild heating has been proved to improve the safety of AmB-SD micelles by the formation of super-aggregates. In this work we tested the possibility of super-aggregates formation from a liposomal system and tried to measure the energy involved in the super-aggregation process. In addition, the heated system was evaluated along the time, to evaluate the influence of the process on the drug stability. It was demonstrated that only AmB-SD micelles can form super-aggregates that improved the drug stability along the time. The process was accompanied of spectral changes by UV-Vis and circular dichroism (CD). These changes, caused by the mild heating seem to be irreversible. Isothermal titration calorimetry (ITC) was not able to clarify about the energy spent/generated on super-aggregation process, but suggested that some energy is generated during the re-organization of oligomers towards monomers of AmB.

Key-words: amphotericin B; micellar system; liposomal system; super-aggregates; circular dichroism; isothermal titration calorimetry;

Introduction

The amphotericin B (AmB) molecule is an antibiotic discovered in the 1950's and broadly used in the treatment of life-threatening systemic fungal infections up to now (1). More recently, the drug has been used to fight against the leishmanial protozoa, in refractory treatment with antimonials (2).

Drug physico-chemical characteristics are marked by hydrophobicity, due to a polyene chain and hydrophilicity, due to a polyhydroxylated region. Besides, AmB has amphoteric character. All together are responsible for the poor solubility of the drug (3). Indeed, it is the main drawback to the development of AmB-containing pharmaceutical formulations (4).

AmB was firstly marketed in the form of AmB-containing micelles, a mix of the drug with sodium deoxycholate (SD). In spite of the problems such as headache, nausea, vomiting, phlebitis and nephrotoxicity related to the AmB-SD micelles infusion, the product is commonly used, mainly in developing countries (5, 6). In the last decades, AmB-containing lipid-based products have been introduced in the market (7-9), increasing AmB safety, mainly by nanomodification (10-13). Indeed, the lipid systems developed are less toxic and have the possibility of improvement of doses, that was not possible for AmB-SD (8, 14). However, due to their cost, this sort of systems are not affordable for most of poor countries (4, 9).

Strategies those are still in the field of research deal with the use of micelles themselves being diluted for other systems like nanoemulsions and microemulsions (6, 15), as an attempt of de-aggregate the drug, delivering monomers of AmB instead of oligomers, proved to be more toxic AmB species (16).

The mild heat of AmB-SD has been related to the formation of a different supramolecular organization of the drug. Through this process a new spectroscopic AmB

species with same efficacy and improved safety is born. The structural re-organization is accompanied of increase in aggregates size and then, has been called as “super-aggregates” (3, 17-20).

The super-aggregates were proved to be formed from AmB dissolved in water (17) and most commonly, from AmB-SD (21), which is more convenient since it is the widely available form of AmB (9). From our knowledge, there is no reference about the super-aggregation from different AmB-containing systems. In this work we have applied the heat process to an AmB-SD as well as to an AmB-liposomal system. It was verified the capacity to form super-aggregates from them and the stability of the generated products along the time. In addition, we tried to measure the drug super-aggregation and de-aggregation energy by Isothermal Titration Calorimetry (ITC).

Methodology

Materials

The AmB-SD micellar system used was Anforicin[®] B, a gift from Cristália (Brazil). AmBisome[®] was acquired from Gilead Sciences (USA). Methanol was HPLC grade from Merck[®] (France) and the ultrapure water was obtained by Milli[®] Q purification system.

Products suspension

Both Anforicin[®] B and AmBisome[®] were prepared according to supplier's recommendation. Briefly the yellow lyophilized powder was re-suspended in water for injection. Each vial was re-suspended in 10 mL water, reaching 5×10^{-3} M AmB concentration and reserved for further studies.

Preparation of AmB super-aggregates

The super-aggregates were prepared as described for Gaboriau et al (1997) (17) . After Anforicin[®] B and AmBisome[®] re-suspension, samples were placed in a water bath at 70 °C (\pm 2) for 20 min and then reserved for further characterization.

Super-aggregates characterization

Spectroscopic studies

The AmB aggregation state was analyzed in the samples before and after heat process by electronic spectroscopy (UV-Vis) and circular dichroism (CD) (22, 23).

UV-Vis analyzes were carried out in a PerkinElmer (France) spectrophotometer after 1000x dilution of the re-suspended product, using a 1cm length quartz cell in different days (D): D0; D3; D7; D14 and D28.

CD transitions of AmB were recorded using a Jasco J-810 dichrograph (France), by using 0.1, 1, and 5 cm path length quartz cell, according to the sample dilution, in the first day of analysis.

The wavescans for both, UV-Vis and CD were from $\lambda = 300$ to 450 nm.

Drug degradation

The AmB degradation was evaluated by the decrease in drug absorption, calculated in this work as percentage (%) of remained AmB. Aliquots of the samples were diluted 1000x in methanol and read at $\lambda_{\text{max}} = 405$ nm. The absolute absorbance value was taken and expressed as percentage. The first day absorbance was considered as 100% AmB and the next days' percentage were calculated in function of the first day.

Isothermal Titration Calorimetry studies

The ITC experiments were performed in a MicroCal[®] VP-ITC (Malvern) microcalorimeter. Briefly, 1200 μL of the AmB-SD system (5×10^{-3} M) was placed into the cell and left for 20 min, solution injections of 5 μL were made for a continuously

rotating (264 rpm) syringe at time 0 and 20 min, in order to specify the beginning and end of the experiment. The titrations were performed at different temperatures: 40, 50, 60 and 70 °C.

ITC is supposed to measure directly the energetics (via heat effects) associated with reactions or processes occurring at constant temperature. The heat released or absorbed as a result of the reaction is monitored by this means (24).

Afterwards, the liquid was recovered from the cell and analyzed by dynamic light scattering (DLS) at 173 ° angle, 25 °C in a Malvern Zetasizer Nano-ZS (France) for size measurement and UV-Vis, for molecular aggregation evaluation.

Additionally, a dilution assay was performed at 20 °C. The AmB-SD samples were diluted from 5×10^{-5} to 5×10^{-8} M AmB by 148 injections of 2 µL water.

Results

Spectroscopic studies

The spectrum of heated and unheated AmB-SD and AmB-liposome were recorded by both, UV-Vis and CD spectroscopy.

Figure 1 shows the electronic spectra of the systems in water. AmB-SD (Anforicin[®] B) presented bands at 326, 364, 385 and 408 nm. After heat treatment the first band was blue-shifted to 323 nm while the other bands remained unchanged.

The liposomal system presented a main band at 322 nm and smooth peaks at 365, 388 and 418 nm. The heat treatment did not shift any peak, in this case.

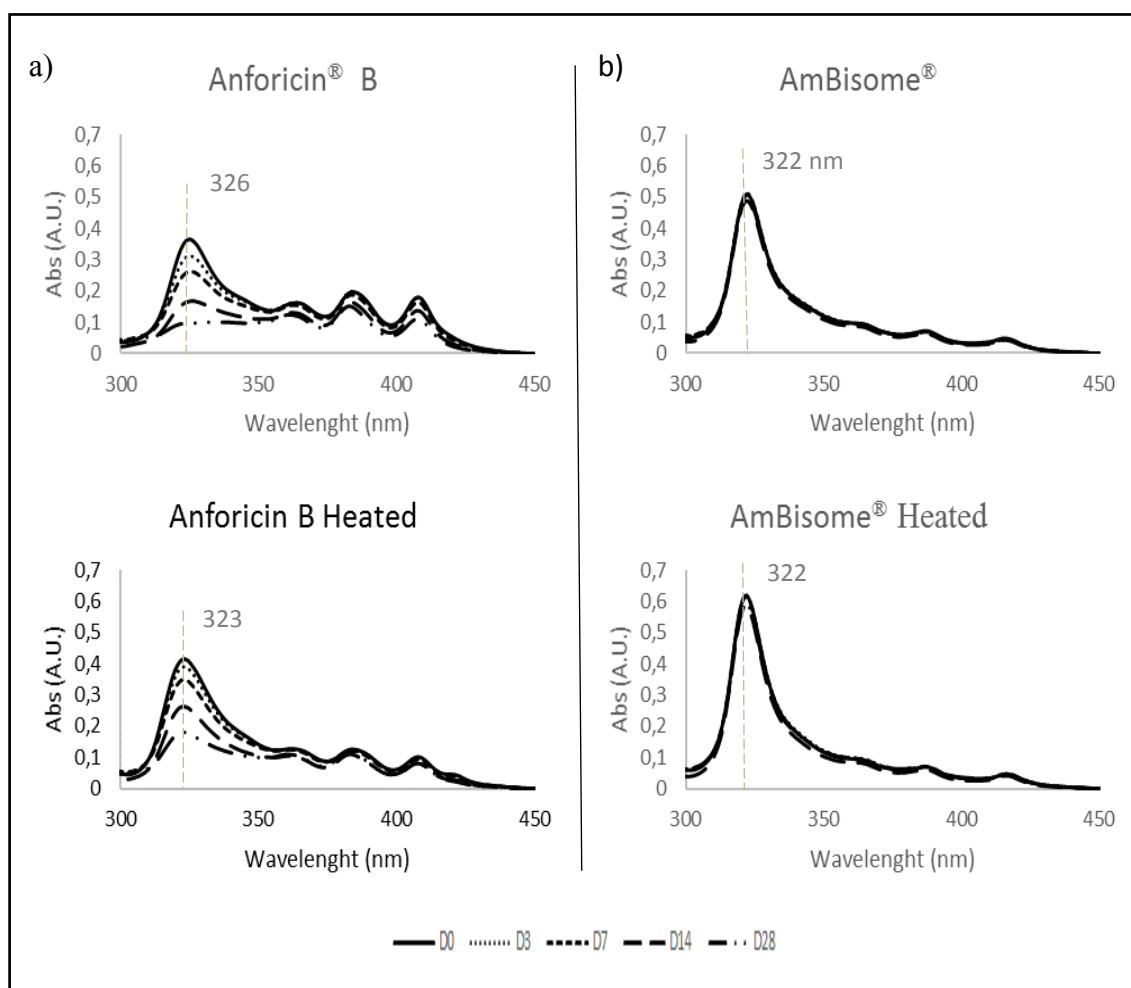


Figure 1. UV-Vis spectra in water for a) Anforicin® B and b) AmBisome® before (upper panel) and after (bottom panel) heating process, recorded along 28 days from the initial concentration of 5×10^{-6} M AmB.

The spectra shape of AmB-SD was modified along the time for the unheated system. The peak at 326 nm was progressively decreasing in intensity while increasing at 408 nm. For the heat treated system, it was observed a decrease in intensity for all bands along the time, spectra shape remaining unchanged. The liposomal system did not present any change. Besides, it was observed that both of systems presented slightly more intense peaks after heat treatment (Figure 1).

The Figure 2 presents the drug CD spectra in water at three different concentrations, before and after heating treatment.

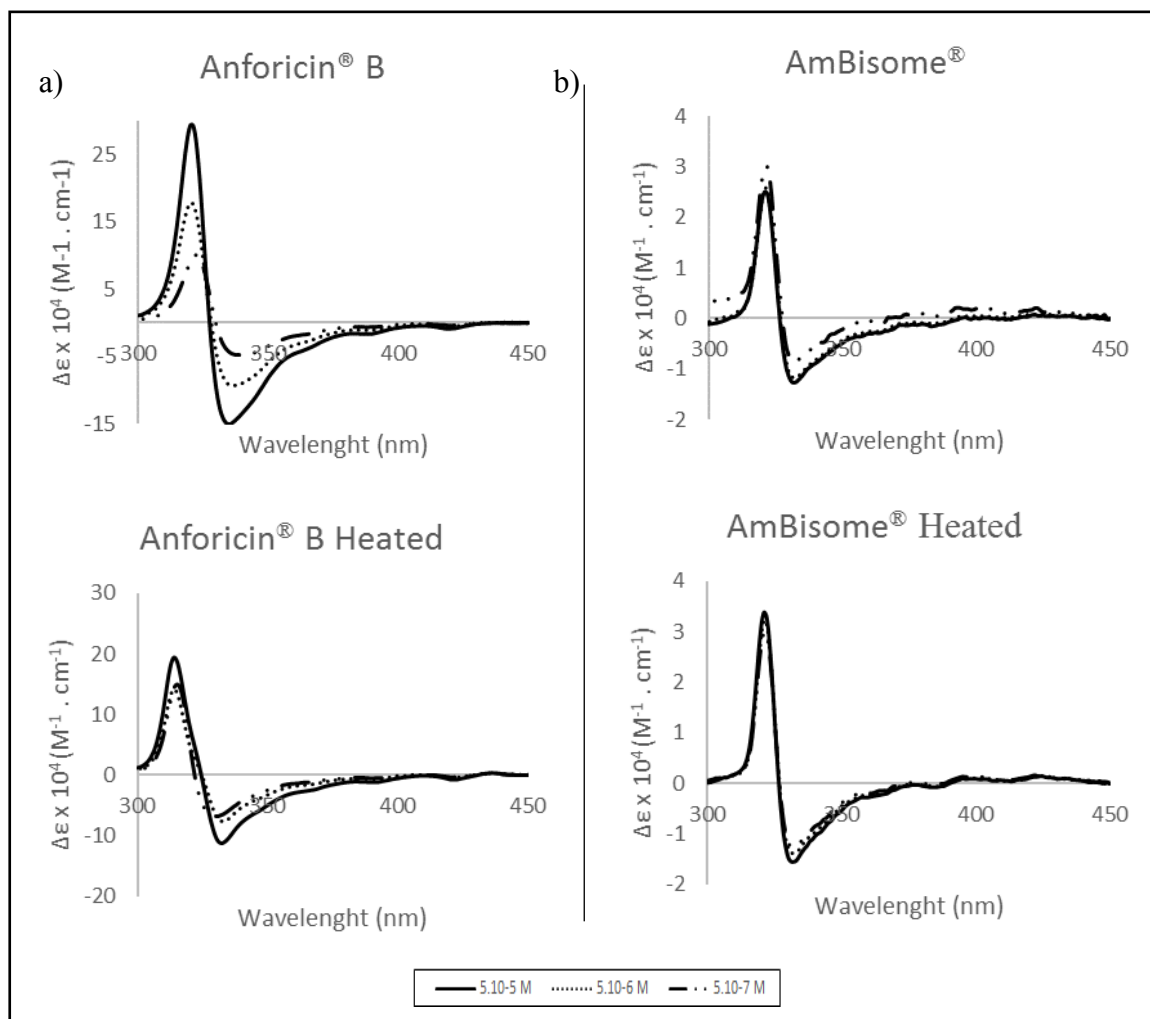


Figure 2. AmB CD spectra in water for a) micellar and b) liposomal preparation. Before (upper panel) and after (bottom panel) heating process, in three concentrations.

The unheated micellar system presented an intense dichroic doublet centered at 330 nm, with a positive band at 321 nm and a negative band at 335 nm, where the doublet intensity progressively decreased upon system dilution. After heat process, the dichroic doublet center was blue-shifted to 321 nm and positive and negative peaks shifted to 315 nm and 333, respectively. Two interesting results were observed for the micellar system after heat treatment: dichroic intensity decrease and almost unchanged intensity upon dilution.

The liposomal system did not change characteristics after heat treatment and dilution. The intensity was ~10 times lower than Anforicin[®] B and the dichroic doublet was centered at 328 nm, with positive and negative bands at 322 and 333 nm respectively.

Drug degradation

The AmB degradation was followed by UV-Vis during 28 days. Drug concentration is given in percentage of absorption with basis in the first day's absorption absolute value in methanol.

As it could be observed in the Figure 1, the intensity of the AmB-SD systems in water decreased along the time while the liposomal system remained unchanged. Such behavior was a reflex of drug degradation, as demonstrated in Figure 3.

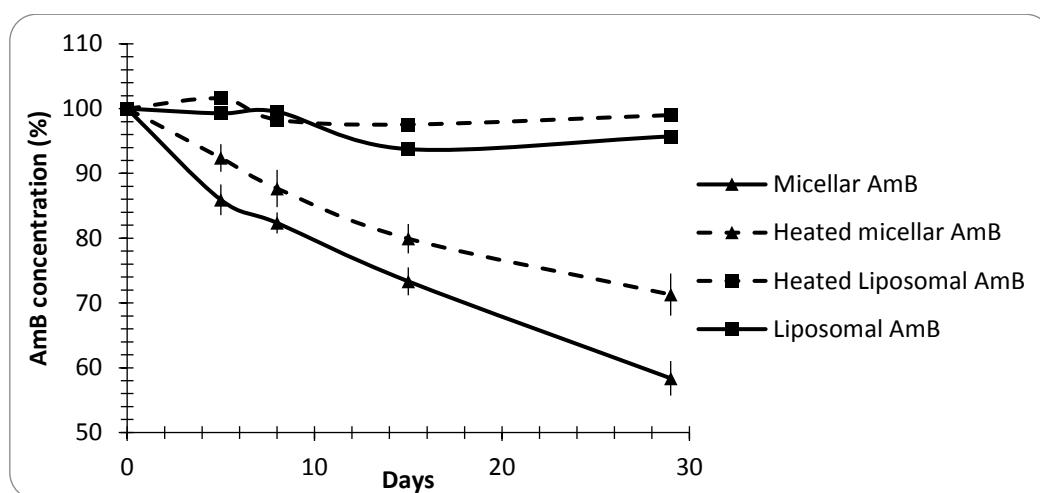


Figure 3. UV-Vis quantification (%) of AmB-containing micellar and liposomal products. Heated (discontinuous lines) and unheated products were analyzed.

After 5 days in water solution, AmB-SD 14% degraded, while heated AmB-SD was 7% degraded. In the end of 29 days, the heated system presented 13% more drug than the unheated one.

The liposomal system was always upper than 94% drug content during the whole study, for heated and unheated systems.

As the super-aggregates were formed using AmB-SD, we tried to measure the energy of molecular re-organization of this system using four different temperatures, from 40 to 70°C. Figure 4 shows the thermograms for each assayed temperature.

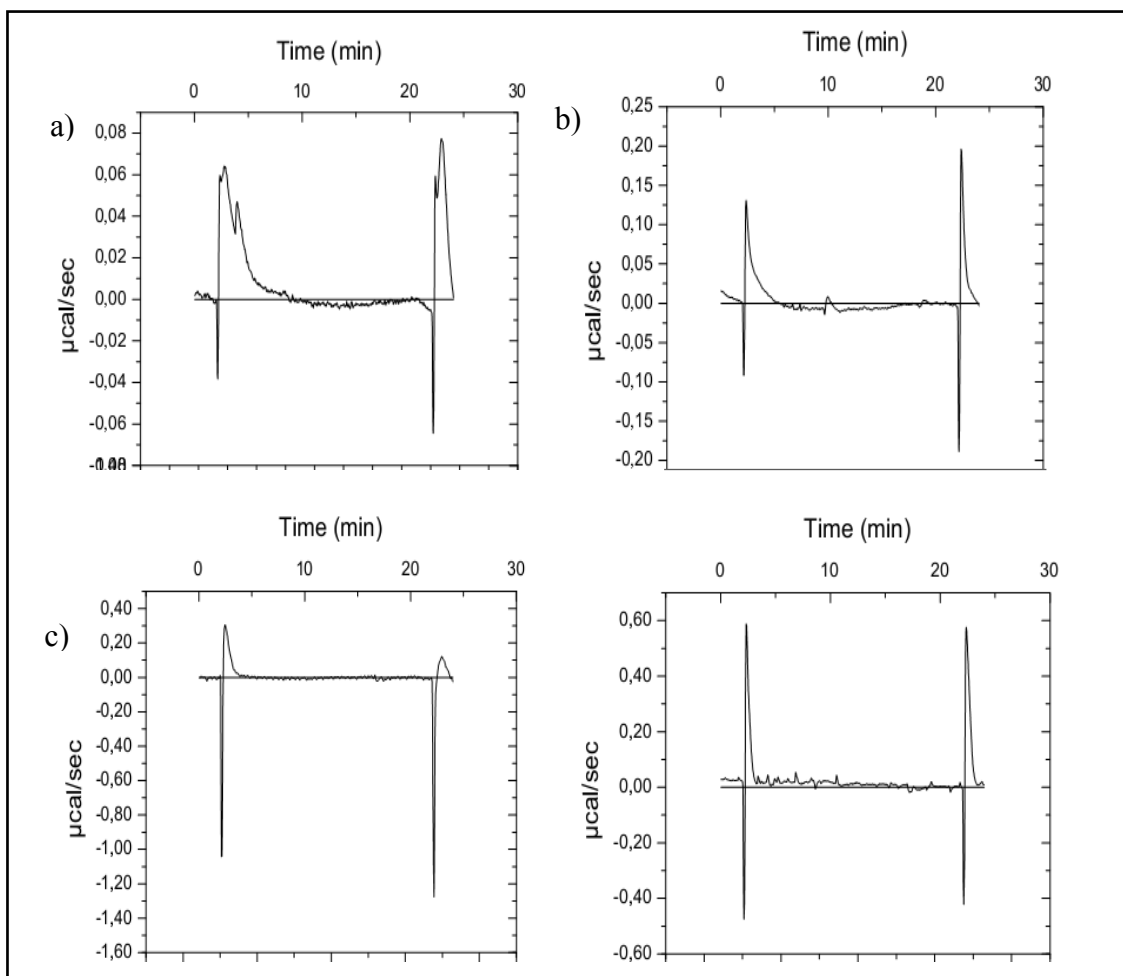


Figure 4. Thermograms of the Anforicin® B ($5e^{-3}$ M) record in different temperatures: a) 40 °C; b) 50 °C; c) 60 °C and d) 70 °C. The injections define the beginning and the end of the counted time (20 minutes).

The signal for all of experiments were too low, making impossible the measurement of the heat signal for any temperature tested. The two more intense signals refers to the injection moments used to indicate beginning and ending time points.

However, the collected samples changed in turbidity that reflected the increase in size, confirmed by DLS measurement (Figure 5).

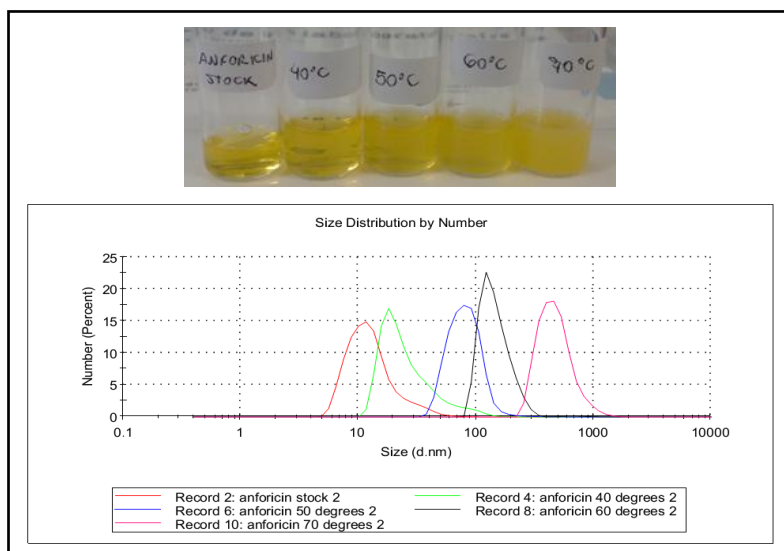


Figure 5. Comparison of the size of an AmB micellar solution after heating process at four different temperatures by naked eye (upper panel) and DLS (bottom panel).

As the temperature increased, the size increased: stock solution (20 °C) 12 nm; 40 °C – 18 nm; 50 °C – 79 nm; 60 °C – 122 nm and 70 °C – 459 nm (size/number).

Besides, the drug aggregation peak was blue-shifted in different levels according to the temperature applied (Figure 6).

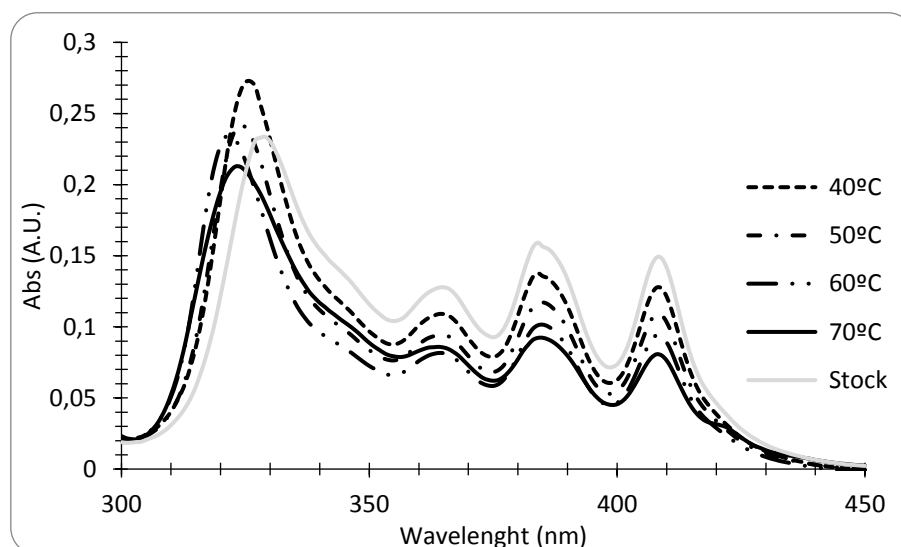


Figure 6. Spectra of Anforicin® B solution (5×10^{-3} M AmB) heated at different temperatures in an ITC equipment.

Then, a dilution from aggregated to monomeric form was performed by water titration and the thermogram is depicted in the Figure 7.

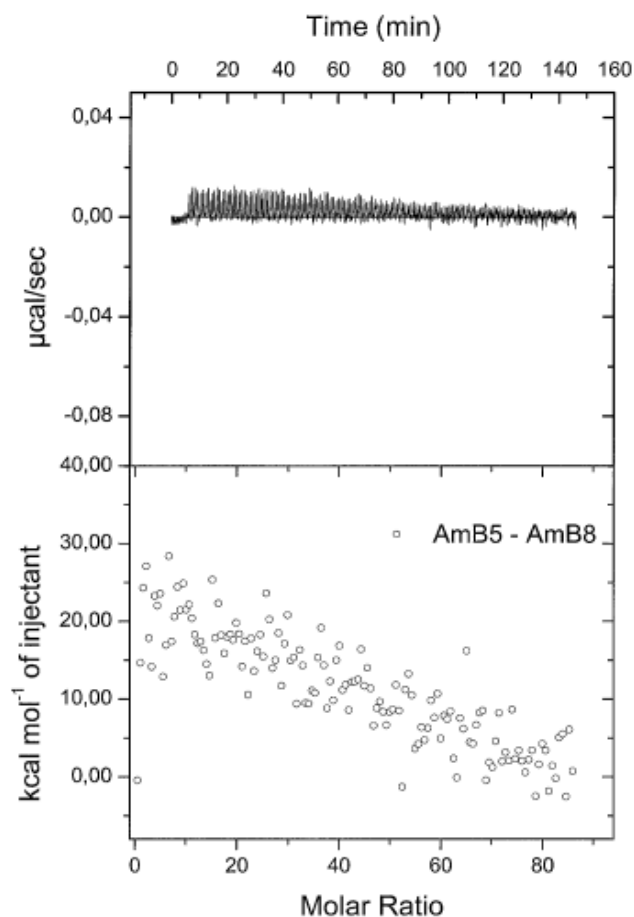


Figure 7. Thermogram for the dilution of an AmB-SD solution, from 5×10^{-5} to 5×10^{-8} M AmB.

Although the heat signal was too low, it can be observed that the heat was more pronounced in the beginning of the reaction.

CD spectra were recorded for the samples before and after dilution, evidencing the differences in the dichroic doublet in unheated systems, from AmB aggregated to monomeric forms (Figure 8).

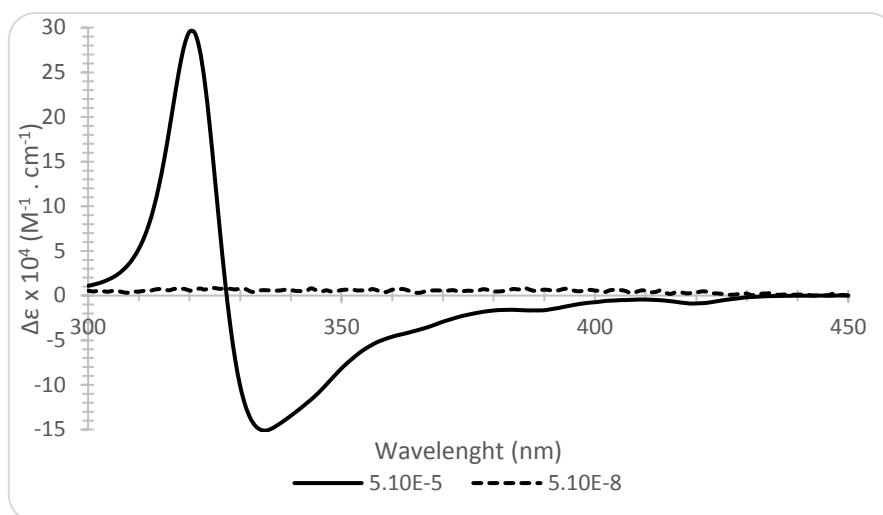


Figure 8. CD spectra of AmB-SD solution as oligomer aggregates (5×10^{-5} M) and AmB monomers (5×10^{-8} M).

The concentrated drug presented a pronounced excitonic doublet while the diluted drug did not present any signal.

Discussion

Super-aggregates formation

The AmB is a polyene macrolide antibiotic in which a hydrophilic and hydrophobic portion coexists (3). In the hydrophobic portion, there are seven double bonds that give a good spectroscopic fingerprint for this drug: a chromophore region absorbing light between 300 and 450 nm (25, 26).

In the aqueous media, AmB can be found in equilibrium as monomers and aggregates, this last species could be soluble or insoluble in the medium (17, 18, 23). Due to the chromophore organization, each aggregation state exhibits an unique absorption spectrum (27). Sánchez-Brunete et al. (2004) (28) studied the different AmB aggregation states in aqueous medium. They have showed the spectra for monomers (λ_{max} 363, 383 and 406 nm), soluble aggregates (oligomers) (λ_{max} 328 nm) and a new spectrum for a species that they have called as multi-aggregates (λ_{max} 358, 370, 392 and 420 nm). However, shifts in the peaks could appear under specific conditions such as the presence

of polar solvents, the method of preparing solutions, temperature changes (20, 23) and association with other lipids (9).

In the Figure 1 it is showed the electronic spectra of AmB-SD and AmB-liposomes before and after heat treatment. It can be observed that AmB-SD presents peaks for aggregates as well as for monomer species (28), while the liposomal preparation presents a main peak, blue-shifted, probably due to the interaction of the chromophores groups with the liposome-composing lipids and low signal for the red-shifted monomer peaks.

Besides of electronic spectroscopy, circular dichroism (CD) is a very sensitive tool to identify the presence of aggregated AmB in the medium (20). A characteristic excitonic couplet in the CD signal is noted when the drug is aggregated (29-32) due to the many AmB centers of dissimetry (26). Then, the couplet is assumed to reflect excitonic interactions between AmB molecules within the aggregates (17). Therefore, any modification on AmB conformation can be identified by CD (30). In fact, Figure 2 displayed a 10 times low signal for liposomal system, when compared to AmB-SD, reflecting the difference in aggregation between those systems.

The AmB super-aggregates are formed under mild heating in the expenses of the drug smaller oligomers (mainly dimmers) and monomers (18, 19, 27) (Figure 9). The two most probable chemical modification caused by heating on AmB would be oxidation of the polyene backbone or lactone ring cleavage (9), both of them sensitive to spectroscopic methods. As they not occur, it is suggested that the changes are merely in the supramolecular structure (9, 20), which is reflected in a blue-shift on the drug spectrum by CD and UV-Vis (Figures 1 and 2) (17, 27).

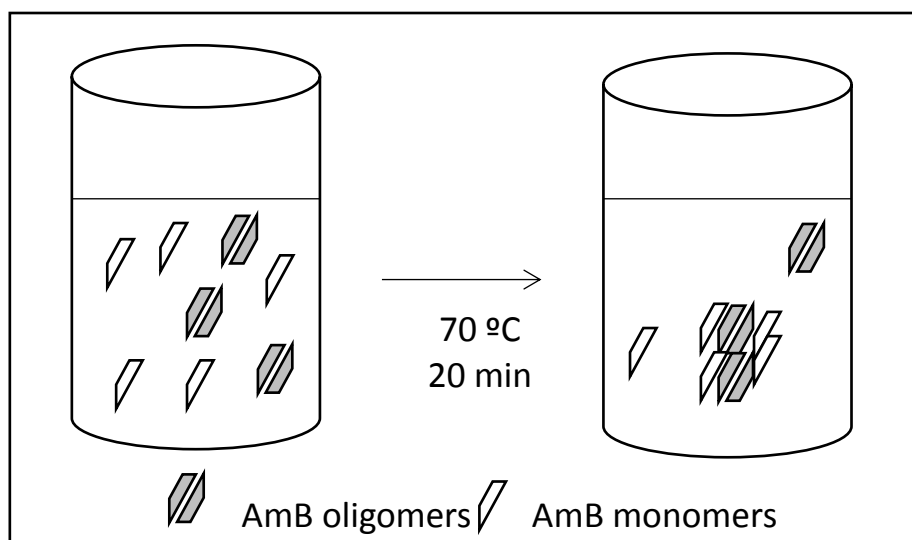


Figure 9. Scheme of super-aggregate formation: AmB coexists as monomers and oligomers in solution; after mild heating super-aggregates are formed in the expenses of the previous AmB forms (Based on Gaboriau et al. (1997) (18)).

The formation of super aggregates has been reported from AmB aqueous solution and mainly from micellar solutions (drug mixed with sodium deoxycholate) (3, 17-19, 21, 22, 33). There is no report of AmB super-aggregates formation from liposomal systems. Maybe, because the super-aggregation is a strategy to lower the toxicity of the micellar systems and that lipid-based systems already present improved safety. But, on the other hand, it have to be taken in mind that in the liposomal system, AmB is strongly associated with cholesterol (14, 23, 34), making difficult AmB release upon dilution. This is probably why the CD signal is ~10 times lower than the AmB-SD signal (Figure 2): as the drug remains bound to the lipid, less oligomers are free in the medium to form an intense CD doublet signal. Consequently, the essential species above mentioned (AmB aggregates and monomers) for the super-aggregate formation are not available in the required concentrations.

Moreover, there is no shift in both UV-Vis (Figure 1) and in the couplet former peaks (Figure 2), reinforcing the assumption of non-formation of AmB super-aggregates from the liposomal system.

After a mild heating, super-aggregates were formed from AmB-SD systems as evidenced by the blue-shift on the electronic band from 326 nm to 323 nm (Figure 1) and also in the excitonic doublet observed by CD (Figure 2) (9, 17, 23). Indeed, increases in temperature enhance the hydrophobic strength while decrease ionic and polar stabilization. Then, the blue shift in the maximum band indicates a new aggregation form in which the chromophore groups are highly organized in a nonpolar environment, where the frequency of hydrophobic interactions is assumed to increase (17, 20, 27).

Although the Bee-Lambert law does not hold in the case of CD for aggregated AmB (20), it is important to mention that the CD signal decreased after heating process. Such a behavior was also found by super-aggregates characterized by other researchers (17, 23). A change in CD bands amplitude is likely to reflect a change in the number of molecules in an aggregated form, and variations of the other spectral characteristics are related to conformational changes of aggregates (20). Decrease in CD doublet has also been explained by the increased size of super-aggregates, that makes a blue-shift on the electronic transition and could also be accompanied of weak CD doublets at lower wavelength (17).

Stability after heating process

The super-aggregates appear to be more stable than the previous system. In our study this assumption is supported for both, the maintenance of the electronic spectrum shape (Figure 1) and the low change in intensity of CD signal upon dilution (Figure 2) that happened only for heated AmB-SD and that is going to be discussed in the following lines.

In the first case, it is seemed that the heat was able to change the AmB conformation in a stable way. Along the study, for the un-heated micellar system, the peak of aggregates decreased while the peak of monomers increased (Figure 2),

suggesting a modification on the aggregation state. The ratio between the peak of aggregates and that of monomers is an interesting tool to evaluate the degree of drug aggregation into the system (35, 36). In fact, when the concentration of the drug decreases, the balance between monomers and aggregates moves towards monomers (19, 32). However, for the heated system, both peaks decreased in intensity and the spectra remained similar in shape during the complete study.

Diluting an aliquot of the sample in methanol and analyzing it by UV-Vis, it was confirmed that the heated system had its stability increased when compared with the unheated counterpart (Figure 3). The literature had already showed that heating AmB solutions increase its chemical stability for at least 2h (17). However, it is the first time that it is presented a study for such a long period as one month.

Such a behavior can be explained by the mechanism of drug degradation summed with the dissociation of AmB species from the super-aggregates. The decrease in AmB concentration is a process dependent of oxygen, corresponding to the drug auto-oxidation, mainly related to the various oligomers types present in AmB-SD (9). Then, the increase in stability might be explained by the drug re-structuration itself. The super-aggregates would be a more cohesive particle, hiding the chromophore group and consequently lowering its exposure to the aqueous solvent and also acting as a reservoir, being slowly dissociated as monomers, those are not involved in auto-oxidation (17).

Besides of electronic spectroscopy, it was observed that after heat treatment CD signal was quite constant even upon dilution (Figure 2), indicating that the drug remained associated in its new structure (37). This behavior is explained by Silva-Filho et al. (2012) (19) for the stronger bound of AmB to the surfactant when compared to un-heated system, while Brajtburg and Bolard (1996) indicate that the signal is constant due to

uniformization of the type of oligomers in solution (11). All together suggests super-aggregates as more stable organization than the previous micellar system.

The super-aggregates showed to be less degraded than the un-heated system. However, as the drug is in solution, drug degradation does not stop occurring. Recent studies have shown the possibility of freeze-drying AmB super-aggregates, maintaining its physical and biological features (3).

The liposomal system was stable along the study, once no differences in concentration were found among days and the heating process did not affect the system aggregation behavior.

ITC experiments

Super-aggregates formation

Once observed that the super-aggregates only are formed from micellar systems of AmB, it was conducted a study seeking to measure the energy dispensed or generated during the AmB molecular re-organization.

ITC technique is highly used for evaluation of molecular interactions or conformational changes based in the knowledge that any interaction can release or absorb heat (24). The titrations performed in this work were set at 40, 50, 60 and 70 °C, considering that super-aggregation can also occurs at low temperature, spontaneously (17).

Despite the sensibility of ITC equipment, the amount of heat generated/dispensed in the process was not enough for track any molecular re-organization. In the titrations obtained at different temperatures (Figure 4), it was possible to measure the two water injections performed in the beginning and end of the titration. However, the tracked heat during the 20 min does not show any significant heat peak.

The ITC recovered liquid evidenced physical changes in size at naked eye that was further confirmed by DLS size measurement. Siqueira et al. (2014) (3) have also reported a prominent increase in turbidity upon increased temperature, suggesting molecular structural re-organization.

The size increased as the temperature increases: by naked eye, the cloudier the sample, the larger it was found to be. The mean size of the stock solution was around 12 nm, and increased sequentially up to around 450 nm (volume/number), when 70 °C was used in the heating process (Figure 5). Such an increase in size justify the name give for the new structure: super-aggregates (19).

It is assumed that the size of AmB-SD increases along time, probably due to AmB release from the micelle and consequent aggregation. The heat process may accelerates the dissociation process from the SD micelles, being followed by AmB re-association (9). Such a greater size is responsible for an increase on the uptake of the drug by the cells (22).

Previous studies have showed that thermal treatment does not induces molecular dispersion, but seems to increase the size of aggregates until flocculation occurs (17). Increasing the temperature upper than 75 °C have been reported in the literature with loss of drug solubility (27), probably due to precipitation caused by increased particle size. Because of that, and also because that at 70 °C practically all the self-associated form is converted in the super-aggregated form (17), this temperature was set as the best one to form super-aggregates.

The same samples, recovered from the ITC experiments conducted at different temperatures were then analyzed by UV-Vis. It reinforced the role of the temperature in the formation of the super-aggregates.

Different level of blue-shift and drug aggregation were found in accordance to the temperature employed (Figure 6) (27). Gaboriau et al. (1997) (17) showed that solution absorption spectrum stop to change for temperature higher than 70 °C, reinforcing the choose of such a temperature to super-aggregates formation.

De-aggregation

A final attempt to measure the energy of the aggregation process was tried by the inverse of the process: diluting an aggregated AmB-SD solution (5×10^{-5} M AmB) to the monomeric form of the drug, at 5×10^{-8} M AmB (Figure 7). Since the biding isotherms are defined in terms of reaction heats, allowing a direct estimation of enthalpy changes related to association/dissociation constant (24).

Unfortunately, the heat measurement done by ITC was quite low and did not permit many inferences: the signal was more intense in the beginning of the dilution, suggesting that the molecular re-organization generates heat. Then, in the end of the dilution the signal was even weaker, probably due to the reduction in shocks of different organized oligomeric structures.

Luengo-Alonso et al. (2015) (4) have performed studies in which AmB (1.1×10^{-5} M) was de-aggregated with PEG_{5kDa}-cholane (0.6×10^{-3} M). The isothermal results highlighted the complexity of AmB interactions of AmB, which takes place with different AmB soluble species.

Despite the failure of measuring the energy involved in the de-aggregation process, CD spectra demonstrated that, in fact, the drug changed its conformation upon dilution, from aggregated to monomeric form (Figure 8). The monomeric units do not presents specific optical activity (17) (parallel transition moments do not lead to excitonic CD), while the intense CD couplet is explained by interactions between the small units (20).

Conclusion

The overall results showed that the formation of super-aggregates is possible by heating AmB-micellar, but not liposomal systems. The process is responsible for the increase in size of the nanostructures. Such an increase was dependent on the temperature applied in the process. The super-aggregates seem to play an interesting role improving the stability of the drug along the time, since the new structure was maintained for at least 28 days, although drug degradation occurred also for super-aggregates. Unfortunately, ITC experiments were not able to accurately measure the heat involved in the process of super-aggregates formation. However, it seems that the de-aggregation generates heat, that becomes weaker when the drug is presented as monomers. Finally, it is assumed that super-aggregates are less toxic than AmB aggregates, once its reorganization do not like to have the propensity of reconvert to the toxic soluble oligomer species. The effect of drug reservoir reported to super-aggregates means the release of a limited amount of monomeric AmB to the medium, that would increase both drug safety and efficacy, reinforcing the study of super-aggregates as an alternative to toxic AmB-SD products.

Acknowledgment

Authors would like to thanks to Mme. Magali Noiray for the training on the ITC facility and to CAPES-COFECUB (Project 742/11) for the fellowship provided to the first author, A. L. Silva.

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SECTION III

INCORPORATION OF AMPHOTERICIN B INTO EMULSION SYSTEMS

BRIEFING

Emulsion systems are able to load hydrophilic and hydrophobic molecules, once they are systems composed of oil and water. Micro-emulsion is one type of emulsion characterized for presenting high amounts of surfactants and this fact helps this system to better penetrate into different barriers. Nano-emulsion, in turn, are mainly aqueous system composed of low amounts of surfactants.

These systems are eligible for the encapsulation of the amphotericin B (AmB), due to its physicochemical characteristics, the drug is supposed to be placed in the interface oil/water.

This section is dedicated to the AmB incorporation into emulsion systems. The first chapter is a scientific report about the details of the drug loading into nano- and micro-emulsion. The drug aggregation state into these systems is presented in the first chapter for the nano-emulsion and in the second chapter for the micro-emulsion. The second chapter was conducted in collaboration with other Ph.D student from the LaSiD (Mr. Walteça Silveira) and present applications for AmB-loading micro-emulsions treating fungal infections.

CHAPTER IV

AmB loading into micro- and nano-emulsion systems

AmB loading into micro- and nano-emulsion systems

(Scientific Report)

Introduction

The “*Laboratório de Sistemas Dispersos*” (LaSiD) is dedicated to the development of emulsion systems, aiming to carry many types of molecules, mainly those fragile (1-3) and insoluble (4-7).

Emulsions are biphasic systems, composed of oil and water, stabilized by a film of surfactant molecules. These systems can be thermodynamically stable (e.g. micro-emulsions) or unstable (e.g. nano-emulsions, real emulsions). Nano-emulsion systems, in spite of being thermodynamically unstable, are considered to be kinetically stable, due to their small droplet size. The main instability phenomena playing in this case is Ostwald ripening (8).

Once emulsion systems present both aqueous and lipid phases, the incorporation of different kind of substances of hydrophilic and hydrophobic character is believed to be possible (9). In this scenery, emulsions are suggested to be promising carriers for amphotericin B (AmB), a molecule of 924 Da, counting with a hydrophobic chain and polyhydroxylated region (10).

Indeed some studies have been dealing with emulsions as carriers for AmB delivery by different administration routes (4-6, 11-13)

In this work, the incorporation of AmB into nano- and micro-emulsion is reported, evidencing the limit of drug loading according to the system and the correspondent drug aggregation state.

Methodology

Micro-emulsion preparation

The micro-emulsion system was prepared based on the work of Silveira et al. (2016) (4) (complete work in the next chapter), with some modifications. Briefly, Lipoid® S100 and water were weighed in a flask and left under magnetic stirring up to the formation of a milky suspension. Then, Tween® 80 was added to the suspension and left

under stirring. Finally, Miglyol® 812N was added to the mixture that was homogenized by cycles of probe sonication (1.5 min) and ultra-sound bath (3 min). At least three cycles were needed.

Table 1. List of ingredients for micro-emulsion prepare.

Ingredient	(%)
Miglyol® 812N	11
Tween® 80	14.7
Lipoid® S100	6.3
Water	68

The full characterization of this micro-emulsion system is presented in the next chapter.

Nano-emulsion preparation

The nano-emulsion was prepared based on the work of Silva et al. (2016) (2) (Complete work in appendix). Briefly, the water phase composed of Tween® 80 and water was weighed and homogenized, under magnetic stirring. The oil phase, comprising Span® 80 and Miglyol® 812N was weighed and homogenized into another flask. The aqueous phase was poured into the oil phase and sonicated for 4 min in a cold water-bath and reserved for further use.

Table 2. List of ingredients for nano-emulsion prepare.

Ingredient	(%)
Miglyol® 812N	5
Tween® 80	1.2
Span® 80	0.8
Water	93

Two incorporation ways were tested for nano-emulsion. In the first method, the system was produced and then the drug was incorporated, this method will be represented in the results as “Nano + AmB”. In the second method, AmB was solubilized in water before nano-emulsion preparation, this formulation will be represented as NanAmB.

Solubility test

AmB solubility was tested in water, micro- and nano-emulsion, adapted from Mazerski et al. (1990) (14). 5mg AmB was added for each mL of liquid and homogenized under magnetic stirring. Determined volumes of NaOH 1N was added to the mixture and then centrifuged, 10 000 rpm, 5 min. After each centrifugation, an aliquot was taken from the supernatant and diluted in water for drug aggregation assessment and in methanol, for drug quantification. In case of pellet formation, more NaOH was added, the pellet resuspended restarting the process up to no pellet was formed or maintenance of supernatant drug concentration.

AmB aggregation state and concentration

These studies were fully described in the Chapter II. Briefly, the drug concentration was evaluated by UV-Vis, using a calibration curve constructed in methanol, at 10^{-6} M AmB range. The aggregation state was evaluated for both, UV-Vis and circular dichroism (CD).

Droplet size measurement

Measurements were made by dynamic light scattering. Samples were diluted 200-times in pure water before analysis.

Results and Discussion

Drug solubility

It is well described in the literature that AmB is water-soluble in alkaline pH, mainly above pH 10 (14). We were wondering if importance would be credited for the pH value or for the alkaline agent concentration. Then, in the drug solubility study the pH was evaluated after each NaOH (1N) addition and the results are displayed in the Table 3.

Table 3. Relation between alkaline agent volume and solution pH.

NaOH (μ L)*	pH**	SD (\pm)
0	4,5	0,021
5	11,2	0,008
10	11,6	0,005
15	11,8	0,031
20	12,0	0,027

25	12,2	0,008
30	12,3	0,006
40	12,5	0,006

* 1N solution; ** average;

If just the pH value would be considered for drug solubilization, we could assume, from the Table 3, that the addition of 5 μ L NaOH 1N would be enough to solubilize the drug in the medium. However, we have found that adding this NaOH quantity was not enough for full drug solubilization. Besides, the amount of alkali agent interfered on the AmB aggregation state (Figure 1).

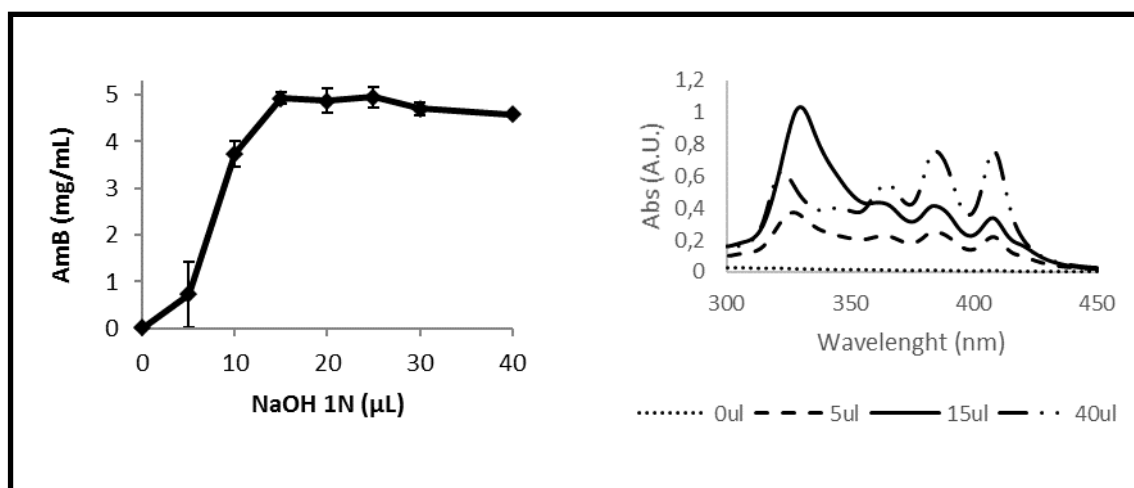


Figure 1. AmB solubilization in function of NaOH 1N addition and its influence on the drug aggregation state.

The solubilization increases with the increment of NaOH concentration in the medium, reaching a plateau after 15 μ L of alkali agent addition, in which 100% drug was in solution. It was observed that after total drug solubilization (achieved after 15 μ L NaOH addition) the increment in alkali agent influenced on the aggregation state. In the beginning, drug was solubilized and presented mainly as AmB aggregates. In the end, when an excess of NaOH 1N was added, the drug was mainly as monomers and had its aggregation peak blue-shifted, indicating a different form of drug association.

Then, it was evidenced that not only the pH, but the concentration of alkali agent, plays an important role on AmB solubilization in aqueous media, evidencing the importance of the net charge inducing the solubility of the polyene antibiotic (14).

Results for the solubilization of the drug into the emulsion systems are displayed in the Figure 2. Nano-emulsion presented results quite similar to the water solubility, being able to solubilize up to 5mg AmB. In spite of solubilizing less amount of drug (up to 3mg/mL), AmB presented similar aggregation patterns into the micro-emulsion system, with bands of aggregates and monomers (Figure 2).

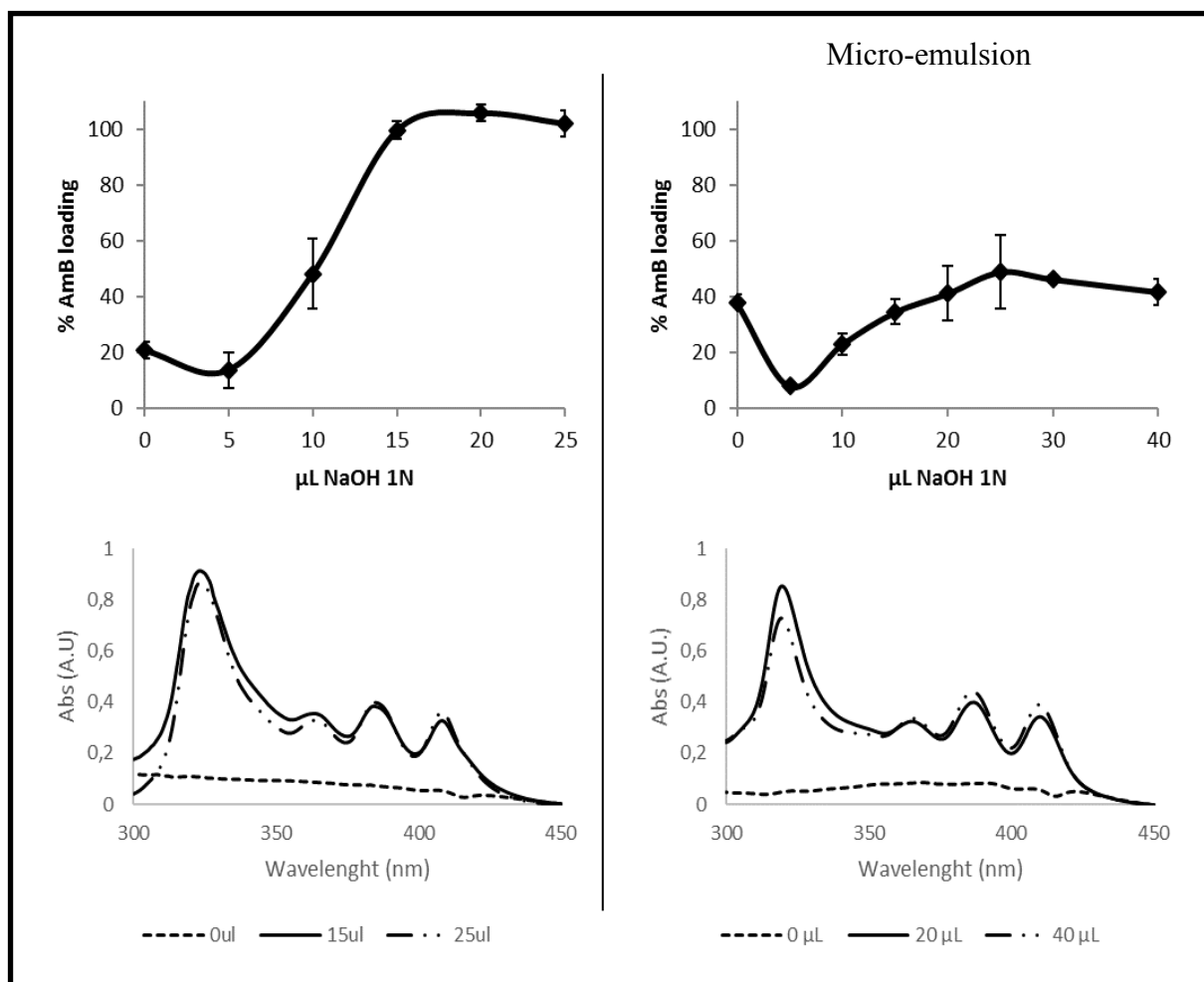


Figure 2. AmB solubility profile in nano- and micro-emulsion. The percentile of drug solubility (5mg/mL max) is showed in the upper panel and the aggregation pattern in function of the added NaOH 1N, by UV-Vis, is presented in the bottom panel.

The fact that same concentration of alkali agent was needed to solubilize the drug in water and in nano-emulsion could be due to the high water content (93%) in this system. However, the excess of NaOH did not cause changes in the aggregation pattern of the drug. Probably because AmB was associated with the lipids into the nano-emulsion system.

The maximum drug solubilization in micro-emulsion was around 60%. However, the drug aggregation pattern was similar to that of nano-emulsion. The lower amount of water and greater amount of other components that are placed in the interface oil/water help to explain the solubilization rate into this system. A fully study concerning AmB loading into micro-emulsion is presented in the Chapter VI of this thesis.

AmB-loaded nano-emulsions

As mentioned in the methodology, the loading into nano-emulsions was done in two ways. In the first loading method, the drug was incorporated after the prepare of the system (Nano + AmB). We have tested if the amount of drug loaded has influence on the size of the system (Table 4) and also in the AmB aggregation pattern (Figure 3).

Table 4. Droplet size and polydispersity index (Pdl) of nano-emulsion loaded with different amounts of AmB.

Sample	Z-Ave d.nm	SD	Pdl	SD
Nano + 0,5 mg AmB	163,0	0,44	0,131	0,016
Nano + 1,0 mg AmB	163,7	2,79	0,132	0,011
Nano + 3,0 mg AmB	163,0	3,29	0,122	0,014
Nano + 5,0 mg AmB	162,8	1,96	0,147	0,015

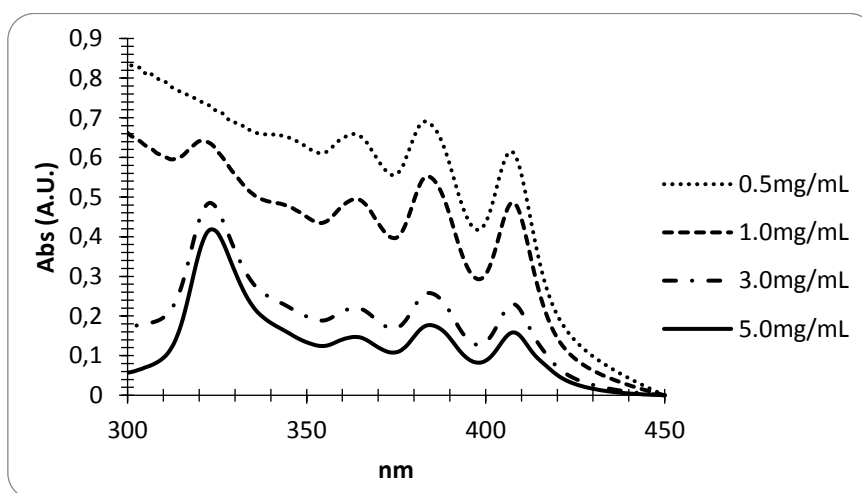


Figure 3. AmB aggregation pattern according to the drug concentration loaded into the nano-emulsion.

The amount of drug loaded into the nano-emulsion did not influence the droplet size. However, as the amount increased, peaks of drug association emerged (15).

After drug loading, at high pH, the system was neutralized. During this process the AmB aggregation pattern changed (Figure 4).

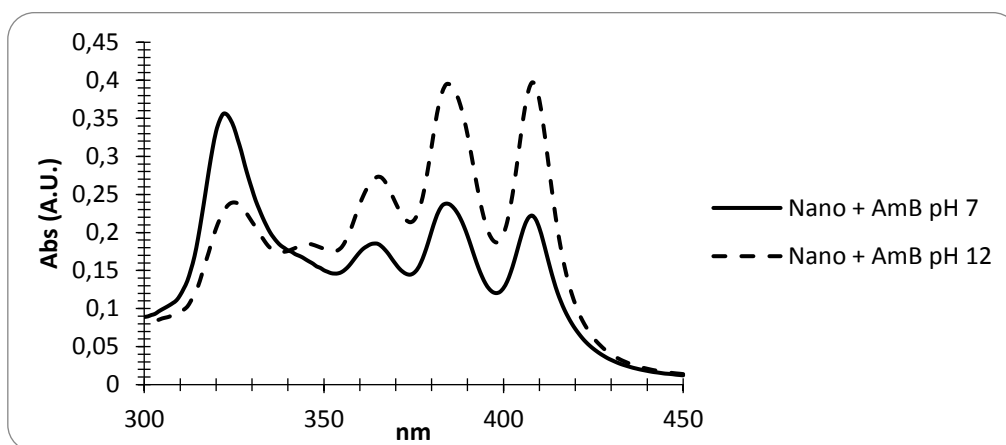


Figure 4. Change of drug aggregation pattern into nano-emulsion according to the pH.

When the drug is loaded, at high pH, it is mainly as monomers. Then, at neutral pH, the drug is associated to the lipids or self-associated, being blue-shifted in the UV-Vis spectra.

Compared to the blank system, the drug incorporation caused an increase in size of about 10 nm and drastically decreased de zeta potential of the system (Figure 5).

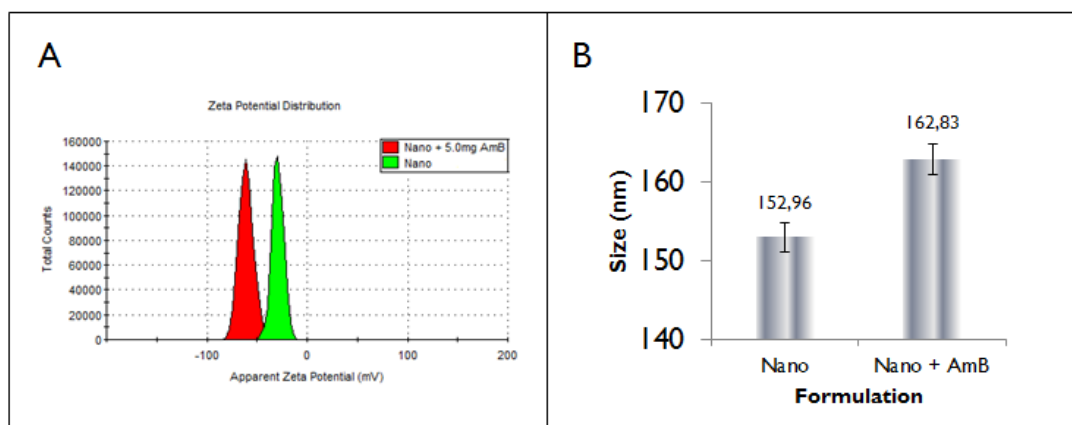


Figure 5. Characterization of nano-emulsion before and after AmB loading. A) zeta potential; B) droplet size.

These behaviors might be explained by the AmB placement mainly at the droplet oil/water interface. Increasing the thickness of the surfactant layer and as the drug is ionized, it contributes to the negative zeta potential.

Another way of loading AmB into the nano-emulsion was preparing the system with an aqueous solution of the drug. Such approach produced a system in which the drug was loaded in a different aggregation pattern (Figure 6).

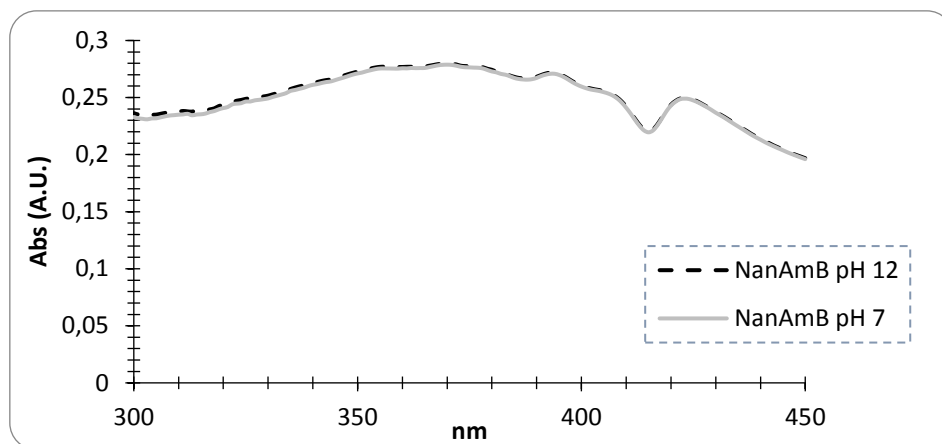


Figure 6. AmB aggregation pattern into NanAmB according to the pH.

This new aggregation state has been reported in the literature as poly-aggregates of AmB (16). Interesting, the pattern did no change according to the pH.

This form of AmB aggregation has been reported as less toxic than the other associated forms *in vivo*, permitting an alternative dosing regimen (17) .

An experiment using circular dichroism (CD) evidenced the differences on the structural arrangement of the drug into both types of nano-emulsions (Figure 7).

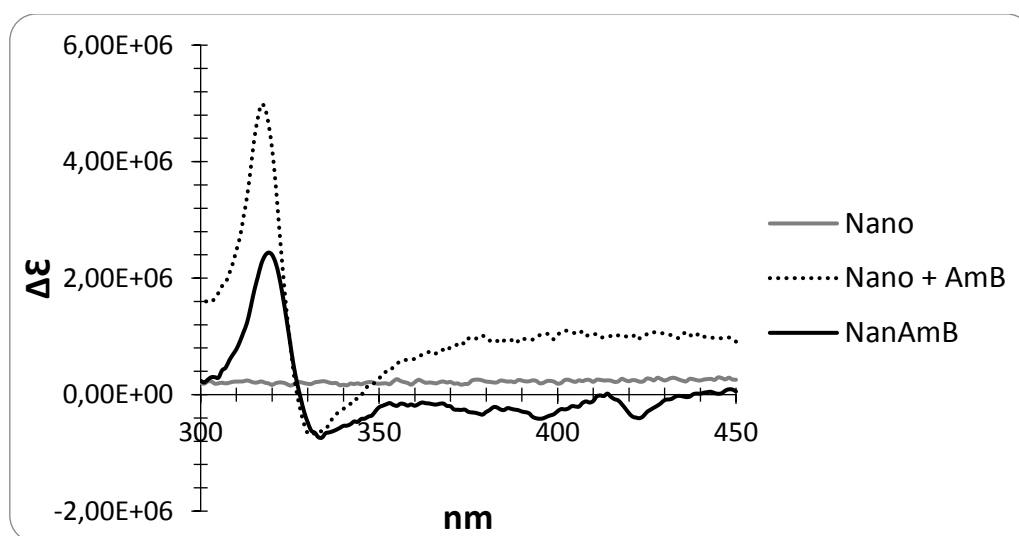


Figure 7. CD spectra of blank nano-emulsion (Nano) and after AmB loading by two incorporation methods.

The nano-emulsion free of AmB did not present signal by CD analysis. When loaded into the systems, the drug gives a bisignated spectra and the mismatch of intensity and shape evidences the difference on the arrangement of the drug into the systems.

The multi-aggregation pattern of the drug into NanAmB was reflected on the emulsion droplet size (Figure 8).

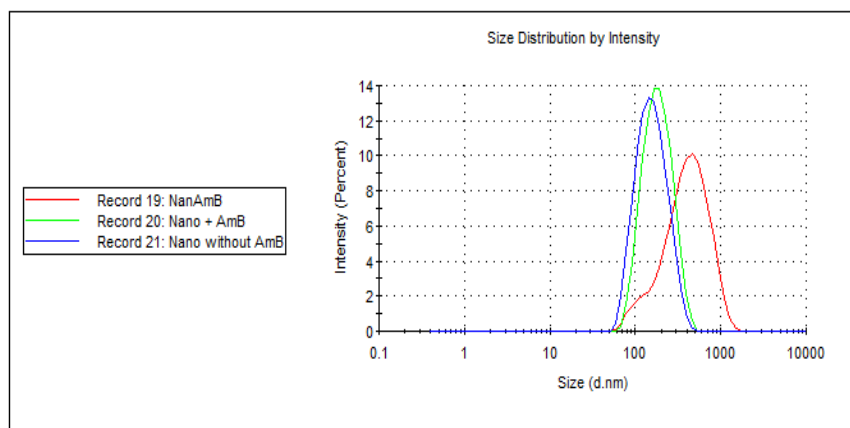


Figure 8. DLS analysis of blank nano-emulsion and AmB-loaded systems during (NanAmB) and after (Nano + AmB) nano-emulsion preparation.

This result is in agreement with Espada et al. (16), who has studied the physicochemical and toxic characteristics of poly-aggregated AmB.

Concluding remarks

The AmB loading into emulsion systems is promising, once they are of easy prepare, of high water content and built of low toxicity ingredients. Some experiences using nano-emulsion for carry AmB has been successful in the treatment of leishmaniasis (13) and fungal infections (18). Besides, emulsion systems open the possibility for AmB delivery by different routes such as topical (11) or even oral (6), an alternative for the therapy of nowadays, which is only possible by intravenous route.

The systems developed in our laboratory were able to load up to 5mg/mL, same concentration found in the products marketed as micelles and liposome worldwide. The method used for AmB incorporation into nano-emulsions had influence on the drug arrangement, being possible to form poly-aggregates of AmB that was already ascribed in the literature as less toxic then the conventional dosage form of this drug.

The overall results reported in here show the possibilities provided by emulsion systems on the search for an ideal carrier for the old molecule amphotericin B.

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CHAPTER V

*Development and characterization of a microemulsion system containing
amphotericin B with potential ocular applications*

Development and Characterization of a Microemulsion System Containing Amphotericin B with Potential Ocular Applications

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Abstract: Background: Amphotericin B eye drops are widely used in the treatment of ocular infections. However, amphotericin's toxicity leads to low patient compliance and aggravation of symptoms. This work describes the development of a microemulsion system containing amphotericin B, aiming for its use in ocular applications.

Methods: The microemulsion was developed by the titration technique. The physicochemical characteristics were determined with both loaded and unloaded amphotericin B-microemulsion. The nanostructures were analyzed by polarized light microscopy. The microdilution method was used to establish the minimum inhibitory concentration against fungal strains, and, therefore, evaluate the microemulsion activity. Additionally, in order to evaluate the microemulsion toxicity an *in vitro* toxicity assay against red blood cells was performed.

Results: The performed studies showed that the presence of amphotericin B loaded into the system did not induce serious changes in the physicochemical properties of the microemulsion when compared to the unloaded system. The spectrophotometric studies depicted amphotericin B-self-associated species, which allow predicting its behavior *in vitro*. The high pressure liquid chromatography results revealed high drug content entrapment in the microemulsion droplet. Finally, the amphotericin B- microemulsion *in vitro* susceptibility test showed high activity against *Candida* strains and a low toxicity profile against red blood cells when compared to Fungizone®.

Conclusion: The physicochemical characterization of the microemulsion demonstrated that its characteristics are compatible with the topical ocular route, making it eligible for consideration as a new and interesting amphotericin B-delivery-dosage form to be used as eye drop formulation.

Keywords: Amphotericin B, Drug carriers, Eye drops, Microemulsions, Pharmaceutical nanotechnology, Red Blood Cells.

Received: April 28, 2015

Revised: August 18, 2015

Accepted: November 04, 2015

1. INTRODUCTION

“Fungal keratitis is a common cause of corneal infection and blindness in the developing world” [1]. Prompt treatment for fungal keratitis depends on a number of factors related to antifungal agents, “including the molecular mass, the concentration of the drug and the route by which it was

administered, the duration of contact with the target ocular tissue, and the ability of the compound to penetrate the eye” [2]. Indeed, the effects of the static barriers (cornea, sclera, and retina) and the dynamic barriers (tear dilution) lead to a poor bioavailability of these drugs [3].

“Amphotericin B (AmB) is a water-insoluble compound widely used to effectively treat systemic fungal infections” [4]. For therapy of ocular infections, “AmB has been administered by intravenous, topical, intravitreal, and intracameral routes” [5] with many drawbacks [6]. The current formulation of AmB (AmB-M) eye drops (micelle system,

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Fungizone[®], Bristol-Myers Squibb, USA) contains deoxycholate, “which renders their instillation painful and leads to poor compliance as well as aggravation of symptoms” [7]. Moreover, the corneal penetration of AmB, in the presence of an intact corneal epithelium, is also reduced and one persistent concern is the possibility of corneal toxicity [8].

Conventional ocular drug delivery systems, as eye drops, in general, present poor bioavailability not only because of the inherent ocular anatomical and physiological barriers, but also because of the low retention time of these formulations on the cornea. Such drawback leads to the need for a stable and effective new ocular drug delivery system that would be able to control the drug release and to enhance the residence time in the ocular cavity, thereby, attaining better ocular delivery [9].

The development of stable and safe new pharmaceutical formulations for the topical ocular administration of AmB would, therefore, be desirable. “Microemulsions (MEs) appear to be an interesting alternative to topical ocular delivery of AmB because of their intrinsic properties and specific structures” [10]. They can be easily prepared, have higher ability to dissolve drugs, and are easily sterilized by filtration. They can also have their viscosity adjusted for the regular use in ocular administration [10].

Recently, our research team has demonstrated the ability of MEs to change the physicochemical and biological behavior of AmB. However, it would be necessary to move forward to a highly biocompatible and safe formulation for future human use. Therefore, the aim of this work was the development and characterization of a ME system intended for ocular administration of AmB by eye drops. The physicochemical characteristics were evaluated for attempting the particularities of the topical ocular route.

2. MATERIALS AND METHODS

2.1. Materials

Standard AmB and Tween[®] 80 were purchased from Sigma-Aldrich (Saint Louis, MO, USA). The micelle dosage form, AmB-M, was a gift sample from UNICAT (Natal-RN-Brazil). Miglyol[®] 812N was a gift sample from Sasol, Witten, Germany. Soy phosphatidylcholine, Lipoid[®] S100, was a gift sample from Lipoid (Ludwigshafen, Germany). Mono-basic and dibasic sodium phosphate, sodium hydroxide, and hydrochloric acid were purchased from Vetec Fine Chemicals (São Paulo, SP, Brazil). Methanol was purchased from Tedia Company (Fairfield, OH, USA). All other chemicals were of analytical grade and used without further purification.

2.2. Methods

2.2.1. Pseudo-ternary Phase Diagram (PTPD) and Preparation of the Blank Microemulsion

For the pseudo-ternary phase diagram (PTPD) construction, an adequate surfactant system [(Lipoid[®] S100)/Tween[®] 80] (3:7), in which the weight/weight ratio was previously determined to produce a large ME region, and oil (Miglyol[®] 812N) weight ratios were used in the range from 1:9 to 9:1,

respectively, to obtain the phase diagram. For the mixture, the aqueous phase (isotonic phosphate buffer pH 7.4) was titrated with an automatic pipette, stirred with a sonic probe for 1.5 min, and placed in an ultrasound bath for 3 min in order to remove the air bubbles generated during the sonic probe stirring process. The transition from the separated phase to the optically clear microemulsion systems was observed by naked eye after 24 hours in order to reach the equilibrium [11].

For preparation of the blank ME, one point from the PTPD was chosen taking into account the homogeneity, the transparency, and the optical isotropy of the system. To prepare the blank ME bulk (30mL), both aqueous (68%_(w/w)) and oil (11%_(w/w)) phases were magnetically stirred with the Tween[®] 80 (14.7%_(w/w)) and the Lipoid[®] S100 (6.3%_(w/w)) for 20 min and 3 min, respectively, until complete dispersion. The ME was achieved by addition of the aqueous phase in the oil phase followed by sonic stirring (1.5 min) and ultrasound bath (3.0 min) aiming to reduce the time used to achieve the formulation. After the production, the ME system was stored at room temperature for 24 hours to reach the equilibrium before further characterization assays.

2.2.2. Amphotericin B Loading Process

AmB was incorporated at 3.0 mg/mL concentration into the ME (AmB-ME). Briefly, an appropriate amount of AmB was weighed and added directly into the ME previously prepared, under continuous magnetic stirring. After 1 min, the AmB was dissolved by addition of sodium hydroxide (NaOH) solution 1 N ($\approx 150 \mu\text{L}$). After solubilization ($\text{pH} \geq 10.0$), the pH was neutralized using hydrochloric acid (HCl) solution (1 N). Finally, for the assays of quantitative analysis and sterility, the AmB-loaded-ME (AmB-ME) was filtered through 0.45 μm membrane filters (surfactant-free cellulose acetate) in order to remove eventual AmB suspended or not properly incorporated in the disperse system and, then, sterilized through a sterile filter of porous size of 0.22 μm (Sartorius Minisart[®] filters, Grottingen, Germany).

2.2.3. Physicochemical Characteristics of ME and AmB-ME [12]

Both the ME and AmB-ME were characterized in terms of macroscopic aspect, pH, rheological behavior and viscosity, refractive index, conductivity, particle size, surface tension, and zeta potential. The nanostructures were analyzed by polarized light microscopy (PLM). All parameters were determined at $25^\circ \pm 1^\circ\text{C}$ and the analysis was carried out in triplicate.

2.2.3.1. Macroscopic Aspects

The macroscopic parameters observed by naked eye were color, consistency, and homogeneity.

2.2.3.2. Polarized Light Microscopy (PLM) Evaluation

Isotropic and anisotropic materials can be distinguished by PLM. Therefore, structural changes in the systems were evaluated by PLM [13]. The ME was placed on a glass slide covered with cover slip and analyzed using a microscope (Jenamed 2, Carl Zeiss, Jena).

2.2.3.3. pH Evaluation

The pH values were performed by digital pH meter PG2000 (Gehaka, São Paulo, SP, Brazil) equipped with a glass electrode, which was previously calibrated with 4.0 and 7.0 pH standard solution. The electrode was placed directly in the ME and AmB-ME samples, which were previously stored in a scintillation flask (15 mL).

2.2.3.4. Osmolarity Evaluation

Similarly, the osmolarity of the formulation was measured by direct sampling of ME and AmB-ME using a discarded syringe of 25 µL of capacity. The syringe was injected on the Micro-Osmolimeter (Model 3300, Advanced Inst., Norwood, MA, USA) and after sample freezing the reading was performed at 25°C. The assay was performed in triplicate.

2.2.3.5. Rheological Evaluation

The rheological analysis (RA) of the ME (10mL) and AmB-ME (10mL) was performed using a controlled stress rheometer fitted with concentric cylinder geometry (DC-41), connected to a Haake K20-DC50 thermostatic bath (Thermo Electron, Newington, NH, USA). The experiments were recorded with a shear rate in the range from 1 to 100 (1/s) and performed for both up and down curves.

2.2.3.6. Refractive Index Evaluation

Using an Abbé refractometer (Analytik, Jena, AG, Germany), the refractive index (RI) was determined. The equipment was calibrated with distilled water, and 30 µL of the samples was placed on the refractometer prism for RI evaluation.

2.2.3.7. Conductivity Evaluation

The conductivity values were measured by a conductimeter MC 226 (Toledo/Mettler, São Paulo, SP, Brazil), equipped with an Inlab 730 electrode (TTLER/Toledo, São Paulo, SP, Brazil), which was previously calibrated with standard solution. The electrode was placed directly in the ME and AmB-ME previously stored in a scintillation flask (15 mL).

2.2.3.8. Particle Size Analysis Evaluation

Particle size analysis of samples was carried out by photon correlation spectroscopy using a Particle Sizing System by *Dynamic Light Scattering* (DLS)– Brookhaven Instruments Corporation, EMI 9863 model (Holtville, NY, USA) –with laser source He-Ne 10 mW, 532 nm-HUGHES, and self-correlator with 64 channels. Before the analysis, the samples were diluted in phosphate buffer (pH 7.4) to yield a 1:32 dilution [12a, 12c, 14], as this instrument requires sufficiently diluted samples to minimize multiple scattering of the laser light by a more concentrated system and to avoid experimental errors [15]. It has been shown that this dilution process is not able to produce changes in ME systems [12a]. The size measurements were carried out at a fixed angle of 90° and the correlator was operated in parallel mode. The cumulate method of analysis was used to calculate the droplet size according to the intensity of scattered light. To per-

form these analyses, the RI was in accordance with the value requested for each analyzed sample.

2.2.3.9. Morphology Evaluation by Transmission Electronic Microscopy (TEM)

The ME samples were also evaluated by Transmission Electronic Microscopy (TEM) using an electron microscopy JEOL 1400 (SamX-Plus, France) equipped with a high resolution CCD Gatan digital camera (SC1000 Orius, France) and operated at 60 kV as the acceleration voltage to observe the morphology of the droplets. They were placed on a carbon-coated copper grid and then a drop of 1% phosphotungstic acid covered the systems. The superfluous phosphotungstic acid on the sample was wiped off by filter paper.

2.2.3.10. Surface Tension Measurements

The surface tension of the samples was measured using a SensaDyne QC-6000 tensiometer (Chem-Dyne Research Corp, Mesa, AZ, USA) under azote atmosphere. The electrode was placed into the samples where bubbles of nitrogen were released measuring the reached tension [16].

2.2.3.11. Zeta Potential Evaluation

The electrophoretic mobility was evaluated by measuring the mobility of particles by nano-zetasizer equipment (Microtrac Zetatrak, York, PA, USA). The samples were placed directly into the cell in which a potential difference of 150 V, at a modulation frequency of 1000 Hz, was applied. Therefore, the movement of the charged particles to the electrode of opposite polarity and the speed of the charged particle were observed.

2.2.4. Quantitative Analysis of AmB

The AmB content of the ME was quantified by using HPLC analysis [17]. A hypersil BDS C18 column (250 x 4.6 mm) was used in a Chromatograph Finnigan Surveyor Plus HPLC system (USA) equipped with a UV-Vis detector at a wavelength of 408 nm and a loop of 10 µL. The flow rate was 1.0 mL/min and the mobile phase consisted of a mixture of analytical grade methanol and EDTA solution (0.005 M) 90:10 v/v. An analytical curve was built in a range concentration from 0.1 to 8.0 µg/mL.

2.2.5. Characterization of the Interaction between the AmB and the ME

To evaluate the interaction between the AmB and the ME, AmB-ME samples were diluted in phosphate buffer pH 7.4 and in the blank ME to yield a concentration of 3×10^{-6} mg/mL (3×10^{-9} M). The absorption UV-Vis spectrum of these “solutions” was recorded at the wavelength from 300 to 450 nm and compared to the ones obtained for the AmB-M in phosphate buffer (pH 7.4) and standard AmB dissolved in methanol at the same concentration [3×10^{-6} mg/mL]. “The optical path of the quartz cuvette used was 1 cm and this path was chosen to obtain spectra with absorbance values less than 0.8. The molar extinction coefficients (ϵ) were calculated using the Beer-Lambert equation (Eq. 1)”, in which Abs was the absorbance; c , the absorber’s concentration; and dx , the cuvette path length [4].

$$\text{Abs} = \epsilon c dx \quad \text{Eq. (1)}$$

2.2.6. In vitro Antifungal Susceptibility

The minimum inhibitory concentration (MIC) was determined by the microdilution method proposed by the Clinical Laboratory Standards Institute (CLSI) against clinical isolates of *Candida* (M27-A2 protocol) and *Fusarium* (M38-A protocol) strains with adaptations [18]. These tests were performed using the AmB-ME, AmB-M as positive control, and blank ME as negative control. 96-well plates previously seeded with *Candida albicans*, *Candida parapsilosis*, and *Candida krusei* incubated at 35°C for 24 h. *Fusarium* strains, *Fusarium oxysporum*, *Fusarium solani*, and *Fusarium napiforme* were incubated at 35°C for 48 h. The MICs were defined as the lowest AmB concentration at which there was a complete absence of growth.

2.2.7. In vitro Toxicity Evaluation

The safety of the formulation was assessed by an *in vitro* toxicity assay in which red blood cells (RBC), from human healthy donors were used (Ethical Research Committee protocol number 002/2009). A dose response pattern study was designed, and potassium (K^+) and hemoglobin (Hb) leakage from these cells were monitored, respectively, “as a measure of acute and chronic toxicity” [4]. The profile of toxicity of AmB-ME was compared to that produced by AmB-M – positive control. ME alone was used as negative control. The results were expressed as the mean \pm the standard deviation. “The data were grouped according to each formulation and analyzed through a two-way ANOVA, followed by the Duncan Test” [4].

3. RESULTS

The methodology used for classifying the ME systems and building the phase diagrams turned out to be quite simple and reproducible [11]. After the titrations, it was possible to define different regions in the phase diagram [11]. The area of existence of oil-in-water ME systems as well as the point chosen to produce the blank ME is denoted in Fig. (1).

The resulting system was, therefore, easily prepared and presented a yellow color, a homogeneous aspect, a transparent appearance, and the absence of precipitates. Such characteristics are typical for a Winsor IV ME system [19]. Because the isotropic system does not change the polarized light angle, a dark field in the PLM was exhibited demonstrating the clear characteristic of the dispersed nanodroplets.

The overall physicochemical characteristics of the ME and the AmB-ME (Table 1) revealed a great compatibility with the characteristics ascribed for ME systems used for the topical ocular route [10, 20]. Indeed, the systems, loaded and unloaded with AmB, were clear and showed acceptable parameters for topical ocular use. All systems can be diluted with water without producing instability [12a] (data not shown). The pH values lie within physiological range. As expected, the osmolality of the formulation was 228 ± 0.5 mOsm/kg and 246 ± 0.0 mOsm/kg for unloaded (ME) and loaded ME (AmB-ME), respectively. It can also be speculated that no impairment of visibility will be generated because of the proximity of the RI of the ME to that of the water (Table 1) [10, 20]. The unloaded (ME) and loaded ME

(AmB-ME) exhibited a nonlinear relationship between shear rate and shear stress. The non-linear reduction of the viscosity ascribed to the rising of the shear rate (data not shown) was observed for all samples, which is characteristic of a shear thinning flow behavior. The flow characteristics for the samples, obtained according to the power law model [$\tau = K(\dot{\gamma})^n$], in which τ is the shear stress, $\dot{\gamma}$ is shear rate, K is consistency index, and n is the flow index, confirmed that the systems presented a non-Newtonian flow, pseudoplastic type (flow index $n < 1.0$) with presence of thixotropic behavior Fig. (2). The drug loading was not able to induce relevant changes in the rheological behavior of ME, which was attributed to the similar particle size of AmB-ME compared with ME (Table 1). In addition, the viscosity value (Table 1) allowed the sterile filtration and dispensing as eye drops. A water continuous phase was observed at high values of conductivity (Table 1) and an oil-in-water ME, which is preferred for use in the eye, was produced [10, 20].

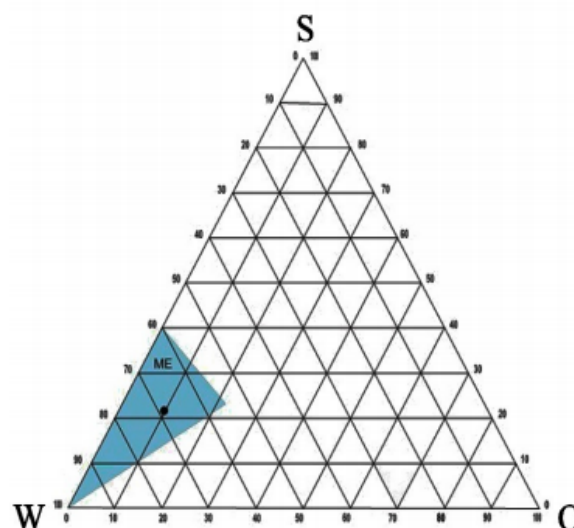


Fig. (1). Pseudo-ternary phase diagrams of the system containing Miglyol® 812N (oil phase- O), isotonic phosphate buffer pH 7.4 (aqueous phase -W), Lipoid® S100, and Tween® 80 (surfactants - S), showing the area of existence of microemulsions (MEs) and the point chosen to produce the blank ME formulation for future studies.

The sterility was achieved by filtration through sterile 0.22 μm pore size filters, and microbiological assays were performed with the aim to prove the absence of microorganisms. No growth was observed for both bacteria and fungi, showing that this method was effective in sterilizing these systems (data not shown).

The AmB was completely incorporated in the ME after the addition of NaOH solution (1 N) into the media. Moreover, the system kept its homogeneity and transparency (transmittance value of $96.5 \pm 1.92\%$) as the blank ME. The values of transmittance for MEs vary not only by the droplet size of internal phase, but also by some factors that affect the color of the system, such as the refractive index of the internal and the continuous phase, the volume of the internal phase, and the presence of any color agent [21]. Once the drug was dissolved in the ME, the measured transmittance value decreased to $58.0 \pm 0.93\%$ due to the absorbance of AmB at the

selected wavelength. However, drug dissolution was not able to induce further changes in the transparency of the ME.

Table 1. Physicochemical parameters of blank microemulsion (ME) and amphotericin B-loaded-microemulsion (AmB-ME) (Mean \pm SD).

Parameter	ME	AmB-ME
pH	7.40 \pm 0.04	7.16 \pm 0.31
Viscosity (mPa.s)	48.28 \pm 0.36	47.20 \pm 0.31
Refractive index	1.373 \pm 0.00	1.374 \pm 0.00
Conductivity (μ S)	733.00 \pm 3.46	1811.00 \pm 7.64
Particle size (nm)		
X ₁₀	7.22 \pm 0.41	7.39 \pm 0.52
X ₅₀	9.79 \pm 0.42	9.87 \pm 0.64
X ₉₀	13.96 \pm 0.49	14.15 \pm 0.59
PDI	0.218 \pm 0.014	0.361 \pm 0.020
Surface tension (mN/m)	71.40 \pm 0.57	71.10 \pm 1.06
Zeta potential (mV)	-16.33 \pm 3.11	-9.98 \pm 1.20

PDI = Polydispersity Index.

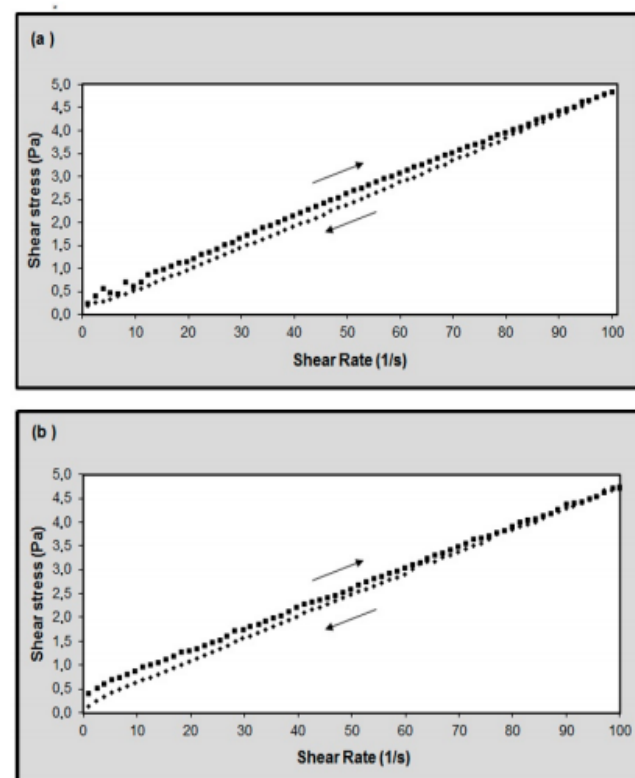


Fig. (2). Rheological behavior of the blank microemulsion (ME-2a) and Amphotericin B-loaded-microemulsion (AmB-ME -2b).

The TEM results revealed, however, a spherical shape and a uniform droplet size of the system Fig. (3) and corroborated those found by the DLS. Indeed, the droplet size of

the ME and the AmB-ME from DLS, reported as X₁₀, X₅₀, and X₉₀, respectively for the 10th, 50th, and 90th cumulative volume percentiles [22], revealed an average of around 14 nm (Table 1). Additionally, the polydispersity index of the ME and the AmB-ME was 0.218 \pm 0.014 and 0.361 \pm 0.020, respectively.

In order to evaluate the entrapment efficiency of the ME, an HPLC procedure for AmB was validated based on the method used by Moreno *et al.* (1998) [17]. The validation protocol was performed in terms of specificity, precision (by repeatability and intermediate precision), linearity, and ruggedness. A good linear behavior was observed and could be expressed as $y = 483347x + 9805$, $R^2 = 0.9998$. In agreement with the method proposed by Moreno *et al.* (1998) [17], all the evaluated parameters presented specificity, precision, and ruggedness. The AmB-ME system had an AmB content of 98.31 \pm 1.93%, proving that the alkaline environment was useful for incorporating the drug in the ME. It is important to note that the filtration procedures at 0.45 μ m and 0.22 μ m did not remove any AmB content from the formulation, because all the AmB molecules were completely entrapped within the ME droplets.

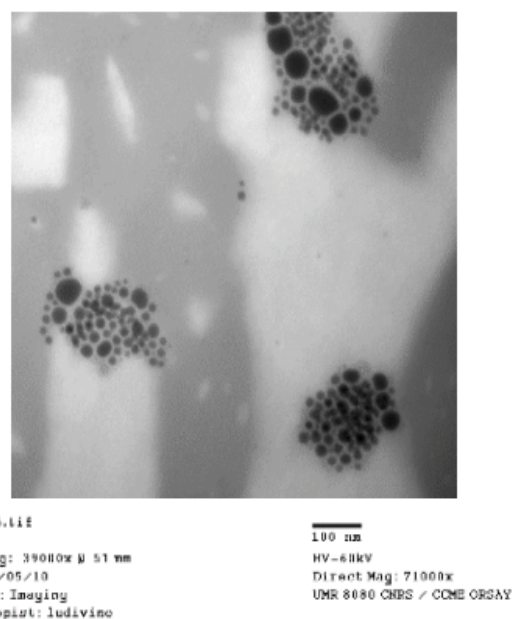


Fig. (3). TEM microphotography of the microemulsion.

The spectrophotometric results revealed that, in phosphate buffer, the AmB-M presented four characteristic absorption bands at 329, 364, 385, and 408 nm Fig. (4b). In methanol, four bands with decreasing intensities at 406, 383, 363, and 345 nm could be seen Fig. (4a). These spectral bands indicate the existence of aggregate states and monomers, located at 329 and 409 nm, respectively [23]. As it can be clearly seen in the spectra of AmB-ME diluted in phosphate buffer, the presence of one intense band at 323 nm indicates a clear interaction between the AmB and the ME due to the blue shifting of the band. Similar results were found by Bolard and co-workers for other lipidic matrix [24]. Indeed, the shift of such band from 329 to 323 indicates that the aggregation complex generated with the AmB and the ME

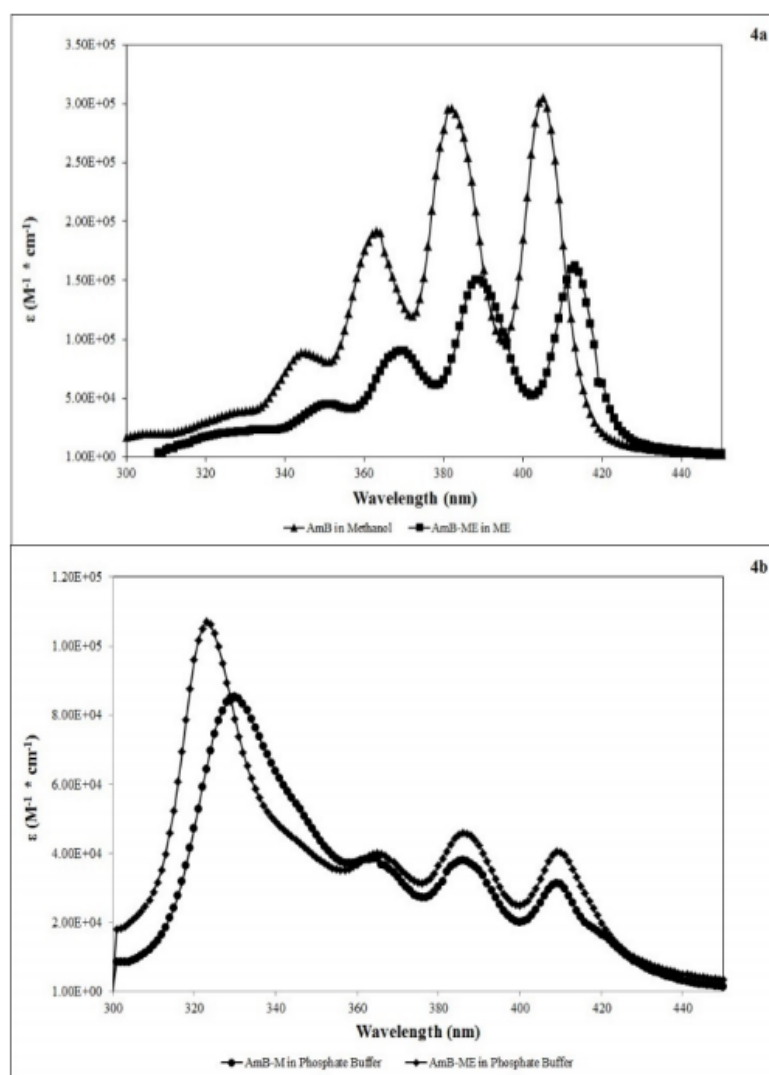


Fig. (4). UV-Vis absorption spectra of the standard Amphotericin B (AmB) in methanol and the Amphotericin B-loaded-microemulsion (AmB-ME) in microemulsion (ME) (4a). UV-Vis absorption spectra of the micelle dosage form of amphotericin B (AmB-M) and the Amphotericin B-loaded-microemulsion (AmB-ME) in phosphate buffer (pH 7.4) (4b).

was different from that presented for the AmB-M Fig. (4). Also, the intensity of the AmB-ME band was much higher ($\epsilon = 1.1 \times 10^5$) than that for AmB-M ($\epsilon = 8.5 \times 10^4$). These results predict that AmB-ME will have a different mechanism of action when in contact with mammalian and fungal cells, as was well demonstrated by Bolard and co-workers with other AmB formulations [24].

To verify the efficacy of the AmB-ME formulation, antifungal susceptibility tests against *Candida* and *Fusarium* strains were performed. Both strains were considered sensitive to AmB, in which the MIC values were lower than 1 $\mu g/mL$, except for *Fusarium napiforme*, which was marginally inhibited (Table 2). These strains showed susceptibility, demonstrating that this system can be useful in treating fungal infections in the eye. Blank ME, on the other hand, was not able to induce antifungal susceptibility.

Concerning the toxicity evaluation assay, at the concentrations of 50 mg/mL and 5 mg/mL, AmB-ME presented an Hb release of 87.49 ± 0.02 and 1.41 ± 0.05 , respectively (Fig. 5a). For AmB-M, these values changed to 100% and 5.88%, respectively ($p < 0.05$). Therefore, it can be verified

that the lethal effect or acute toxicity demonstrated by the cellular lysis was shown to be larger for AmB-M than for the AmB-ME.

4. DISCUSSION

“Ocular drug delivery through aqueous eye drops is the most common approach for administration of ocular drugs, in spite of the low residence time of a few minutes, which limits the bioavailability to less than 5%” [25]. Developing new topical ocular delivery systems that improve the ocular retention and increase the corneal drug absorption create a great challenge [26].

ME of AmB has been studied over the last 15 years. Brime *et al.* (2002) have developed an AmB-loaded microemulsion intended for parenteral route [27]. The authors were able to incorporate 4mg of AmB in the ME. However, it is important to mention that, different from our findings, the size distribution of this ME was bimodal, with a large majority of droplets in the range of 10 nm. This probably happened because of the AmB loading quantity, which was 25%

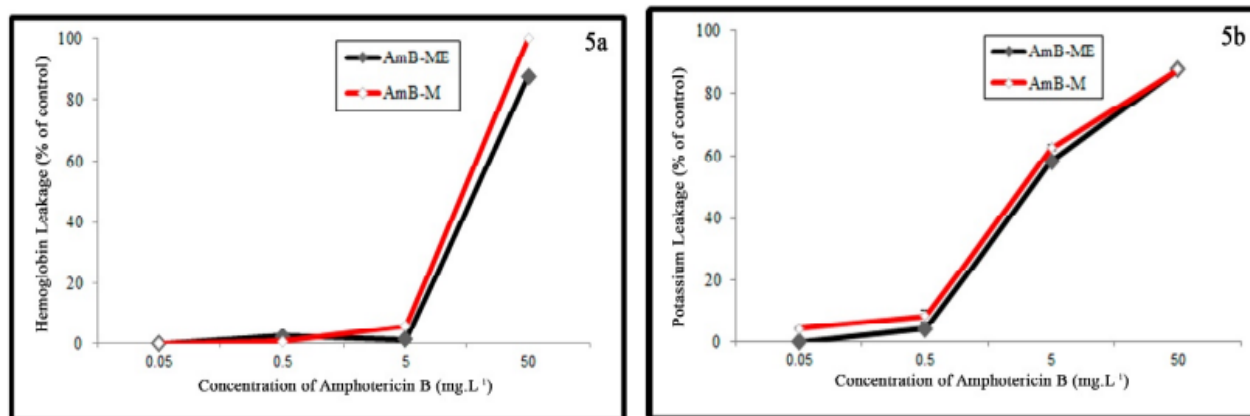


Fig. (5). Profile of toxicity of AmB-M and AmB-ME on RBC. (5a) Hb leakage, and (5b) K⁺ leakage.

more than in our study. Our group recently developed an ME intended for the oral delivery of AmB [28]. This system was able to improve 1000 times the solubility of the drug when its water solubility was compared. Ustuendag-Okur *et al.* (2014), on the other hand, developed an ofloxacin-loaded ME for ocular use [29]. They also added chitosan to improve its adhesivity to the cornea. However, the formulation could not be sterilized by filtration due to its high viscosity.

Table 2. Antifungal susceptibility of amphotericin B-loaded-microemulsion (AmB-ME) against *Candida* and *Fusarium* strains.

Fungal Strain	MIC (μg/mL)*	
	AmB-M	AmB-ME
<i>Candida ssp</i>		
<i>Candida albicans</i>	0.0312	0.0039
<i>Candida parapsilosis</i>	0.0156	0.0078
<i>Candida krusei</i>	0.1250	0.0078
<i>Fusarium ssp</i>		
<i>Fusarium oxysporum</i>	0.250	0.125
<i>Fusarium solani</i>	0.250	0.250
<i>Fusarium napiforme</i>	0.250	8.000

*MIC = Minimal Inhibitory Concentration.

One of the best characteristics of an ME system is its ability to entrap drugs into the oil phase, thereby enhancing their solubility [30]. Because of its high solubilization capacity, this system can be used as a carrier for drugs that have poor water solubility. "MEs can be defined as thermodynamically stable, isotropically clear dispersions of two immiscible liquids, consisting of microdomains of one or both liquids stabilized by an interfacial film of surface active molecules" [31]. These immiscible liquids, usually water and oil, coexist in thermodynamic equilibrium due to the presence of a surfactant film at the oil-water interface [15]. MEs are superior to simple micellar systems not only in terms of

their solubilization potential, but also because of their thermodynamic stability, "which offers advantages over unstable dispersions, such as emulsions and suspensions, since they are manufactured with little energy input (heat, mixing) and have a long shelf life" [32].

Besides the high stability of ME, this system has shown a great capacity for transcutaneous delivery due to its high solubilizing capacity, the adjustable steady-state flux provided by the use of different internal phases and the eventual addition of permeation enhancers [33]. Indeed, ME as a transcutaneous delivery carrier has been widely studied [12a, 34]. Paolino *et al.* [35] has shown that a ketoprofen-loaded ME was able to not only improve the ketoprofen permeability through human skin, but also improve the skin tolerability. The improvement of permeability was credited to the ability of lecithin, which was in the formulation, to influence lipid-fluidization, which leads to the decreased barrier function in a reversible way. More recently, Fouad *et al.* [12c] showed that diclofenac epolamine (DE) loaded ME (DE-ME) presented a higher DE cumulative level on the skin compared to the topical gel and patch in the market (Flector®). Therefore, Fouad *et al.* [12c] demonstrated that ME can permeate the skin and act as an in-skin drug depot, achieving sustained release and in-skin depot function.

4.1. Pseudo-ternary Phase Diagram (PTPD) and Preparation of the Blank ME

In general, the phenomenon of "micro emulsification is mainly governed by factors such as nature and concentration of the oil, surfactants, aqueous phase, oil/surfactant and surfactant/surfactant ratio, temperature and pH of the environment, and physicochemical properties of the drug" [36]. Formulation concerns regarding the ME components are also important [37]. Very good reviews about this subject were produced by Date (2008)[37] and Vandamme (2002)[38], who reviewed the excipients available for formulation of parenteral and ocular MEs, respectively, reporting the investigations for the various classes of therapeutic agents.

Graphically, ME systems are represented as stability areas in the triangular phase diagram in which each triangular corner designates a certain component [30a]. These diagrams, namely PTPD, describe the experimental condition in which the components must be combined to form clear

formulations. PTPD has also been widely used due to its relevance in the characterization and further classification of ME systems. In fact, PTPD is a tool used to describe the wide range of possibilities to obtain areas of a clear ME in which oil-in-water systems would prevail (Fig. 1) [39].

In this work the ME components chosen from the PTPD were based on their characteristics for ocular drug delivery applications (Table 1) [20, 38]. The surfactant concentrations were usually high to decrease the interfacial tension and provide the thermodynamic stability of these systems [22, 40]. The phase behavior of ternary systems containing oil, water, and surfactants can be studied with the aid of a PTPD “in which each corner of the diagram represents 100% of that particular component” [40]. This method plays a crucial role in development of lipid-based colloidal or isotropic (emulsified) systems, which is an important characteristic for their application in the eye [31].

Soy phosphatidylcholine and Tween® 80 (amphoteric and non-ionic surfactants, respectively) were chosen for this formulation because they exhibit excellent biocompatibility [38]. The oil and the aqueous phase proportion should be chosen in a way that not only produces a clear ME, but also allows a high loading of lipophilic drugs. On the other hand, the external lipophilic property of the ME system is mandatory in order to have it well tolerated by the eye. Thus, according to the literature, the aqueous, the oil and the surfactant substances composing this formulation would be very safe for ocular application [38].

To prepare the blank ME, not only the proportion and addition order of the components, but also the mechanical stirring and the heating process promoted by the sonication method were of utmost importance. In fact, the control of these parameters speeds up the process of microemulsification [38]. The sonication method is often used, particularly in systems which contain non-ionic surfactants, and was very important for the process, allowing the formation of a transparent system and assisting in reducing the size of dispersed droplets [41].

The transparency of MEs arises from their small droplet diameter, which is usually within the range of 20–200 nm [42]. ME is the simplest representation of a structure in which oil droplets are surrounded by an interfacial film consisting of surfactant molecules [42]. A widely accepted definition to classify MEs by their equilibrium state was suggested by Winsor in 1948 [19]. In a Winsor IV system, the oil phase and the water are mutually solubilized and, thus, there are no phase-separated components [19]. Because of such characteristic, MEs have been widely used in the pharmaceutical field as drug delivery systems for several routes of administration, comprising oral, parenteral, topical, and ocular routes [39].

For topical ocular drug delivery, recent research efforts have focused on the development of MEs [10, 20, 25, 38, 43]. They have been proposed to achieve sustained release of a drug applied to the cornea and higher penetration into the deep layers of the ocular structure and aqueous humor. This is possible due to the low surface tension, which guarantees a good spreading effect on the cornea and mixing with the film constituents, thus, possibly improving the contact between

the drug and the cornea epithelium [20]. These properties, combined with the ability to deliver insoluble water compounds by carrying the drugs in their oil core or on water-oil interface, allow the use of MEs as biotechnological carriers for large water insoluble molecules such as AmB for ocular use [26]. However, although some scientific approach has been demonstrated in the literature [44], no ME carrying AmB can be found as a pharmaceutical product on the market.

4.2. Amphotericin B Loading Process

Some works have described the use of alkaline pH to solubilize AmB in the internal phase of the ME [45]. “Indeed, this procedure is of great help in the entrapment process of this drug into lipidic structures, such as MEs” [15]. If this process is done quickly, no instability is observed for the AmB molecules. Concerning an eventual instability of the ME system, the process of AmB loading into the ME was not able to dramatically modify the droplet-size diameter profile of the internal oil phase, even though it caused a slight increase in the oil droplet volume (Table 1).

The adjustment of the initial pH at 7.0–8.0 is very important for blank MEs and must be performed not only to minimize the discomfort upon instillation, but also to maximize ME stability [43]. This is important in order to minimize the hydrolysis of the presented phospholipids into the formulation, which can decrease the pH of the ME, and to guarantee the solubility, stability, and corneal permeability of the drugs, which are also quite dependent on this parameter [38]. Moreover, the pH of the ocular formulations, as the presented ME and AmB-ME (Table 2), should be very similar to that of the human tear film to minimize changes in tear pH after eye drop instillation [43]. This would ensure the maximum comfort and absence of eye irritation when this preparation is instilled in the eye [10]. Additionally, the osmolarity of the formulation was in the range of tolerability for topical ocular administration, which is predicted to be between 171 mOsm/kg to about 1711 mOsm/kg [46].

4.3. Physicochemical Characteristics of ME and AmB-ME

Originally, several research groups suggested that a negative free energy was necessary for the ME formation [31]. This could be achieved when large reductions in surface tension are followed by significant favorable entropic changes [31]. However, the micro emulsification process, which would be spontaneous if a high concentration of surfactants was added, could lead to ocular toxicity [38]. On the other hand, the ME process described here could not happen spontaneously not only because of the high concentration of the aqueous phase, which allows the formation of oil-in-water dispersion, but also because of the other components of the formulation and the nature of the surfactants (amphoteric for Lipoid® S100 and non-ionic for Tween® 80). Therefore, a preparation method using a sonication apparatus was necessary [41]. Thus, the results (Table 1) indicated high surface tension for both the blank ME and the AmB-ME, suggesting that no free surfactant molecules in the system were located in the interfacial area. This result allows us to speculate that the AmB-ME will present no toxicity caused by the existence of free surfactant molecules.

The AmB addition to the obtained blank ME system did not have an effect on its rheology. However, a slight decrease in the viscosity value of the AmB-ME system was observed (Table 1). Both systems presented a viscosity that allows sterile filtration and these values can still increase the ocular residence time of AmB without the occurrence of “discomfort, blurred vision, or foreign body sensation with the cornea surface” [10, 20]. The higher viscosity value of the ME formulation favors, therefore, a better penetration of this drug into the eye when compared to the viscosity value of the conventional ocular solutions (≈ 20 mPa.s) [10]. In addition, the identified pseudoplastic flow behavior contributes to the suitable drug distribution on the surface of the cornea, since during the shear the ME formulations showed a sharp decrease in viscosity, which permitted us to postulate that blinking would have a positive effect, decreasing the viscosity during the application procedure (Fig. 2) [20]. The thixotropic behavior observed for both formulations occurred possibly due to the less ordered arrangement of droplets after the shearing process, which affects drug release rate. The occurrence of pseudoplastic and thixotropic behavior depends on the composition of the ME. Both the formulations, AmB-ME and ME, have presented a considerable intern phase volume ($10\%_{(w/w)}$) and surfactant ratio (about $22\%_{(w/w)}$). Streck *et al.* [47] found soybean oil in water emulsion systems with pseudoplastic flow ($n \approx 0.5$) by using 10% w/w of intern phase stabilized with 5% $_{(w/w)}$ of surfactant mixture (composed by polysorbate 80 and monooleate of sorbitan). Similar behavior was also demonstrated for MEs containing 10% $_{(w/w)}$ of CaptexTM 200 as oil phase stabilized in phosphate buffer as aqueous phase by using $20\%_{(w/w)}$ of surfactant mixture composed of soy phosphatidylcholine and Tween[®] 20 ($1:1_{(w/w)}$) [15, 48].

The decrease in the transparency of the formulation, traced by a variation on the transmittance of the formulation, could be due to the organization of the AmB molecules into the ME droplet. AmB is a strong yellow powder (due to its seven double bonds in its structure, the chromophore group). Therefore, this change in the ME transparency can be credited to the presence of AmB, which instantaneously increases the structural organization of the system [13], reducing the transmittance of the formulation. Additionally, the results of the interaction between the AmB and the ME revealed that the AmB is presented in a monomeric state (Fig. 4 - presence of a strong band at 409nm on the UV-Vis spectrum). Therefore, it can be speculated that no crystals would be found in the formulation. Indeed, some centrifugation (15,000 g for 15 minutes) studies on the AmB-ME formulations were performed and no precipitates were found in the formulation (results not shown). However, because the AmB-ME RI value (Table 1) was quite close to that of the water (1.33) and the corneal and lachrymal fluids (1.34-1.36) [10, 20], no impairment of vision or discomfort to the patient after instillation was expected.

The high value of conductivity found for the system allows us to classify it as an oil-in-water type (Table 1). This characteristic was maintained after the incorporation of AmB, showing that this drug was not able to cause changes in the intrinsic properties of the ME. This type of ME is pre-

ferred for use in the eye because the droplet structure can often be retained during the dilution process by the lachrymal fluid [20]. Indeed, the administration of oil-in-water systems could be advantageous because they increase the membrane permeability and facilitate the corneal drug delivery, achieving prolonged release and “higher penetration into the deeper layers of the ocular structure and aqueous humor than the native drug” [25]. Moreover, the increase in the value of conductivity after the incorporation of the AmB can be explained by the addition of ionic solutions (NaOH and HCl), which were mandatory to incorporate this drug into the system.

The ME and AmB-ME zeta potential values, presented in Table 1, revealed that this parameter was dependent on the combination of both the surfactants and the drug at the interface [49]. These values are important in that the charge of the internal phase provides information on the efficacy of the induced electrical barrier [49]. Indeed, the presence of phosphatidylcholine, which is a zwitterionic compound, in the ME composition (Lipoid[®] S100) has a negative charge that turns the zeta potential into a negative value [49]. Moreover, this negative charge could influence their absorption in the ocular route because the corneal area is also negatively charged [38]. Such surface charge could be an advantage or a drawback, depending on whether the molecules carried in the system have to be absorbed or not.

The AmB was totally entrapped (100%) in the ME droplets favored by the alkaline pH values. The AmB solubilization process into the ME was observed not only by the clarity of the solution, but also, spectrophotometrically, by the presence of the AmB typical monomeric band at 405 nm (Fig. 4a). Moreover, the final AmB content determined by HPLC [17] was found to be $98.31 \pm 1.93\%$ when compared to the nominal AmB content (3 mg/mL).

The entrapment efficiency of AmB in the ME formulation demonstrates that the drug was partitioned into the oil/water interface, confirming that the alkaline environment promotes the better performance in terms of content in these structures [15]. Moreover, these results reveal the great potential of the ME as a carrier for amphiphilic molecules such as AmB.

The changes in the droplet size of AmB-ME compared to the blank ME can be explained by both the amphoteric and the amphiphilic properties of AmB. In fact, the AmB molecule has an amphoteric behavior due to the presence of ionizable carboxyl and amine groups. Besides, since the pKa value of the amino group of AmB was taken as 5.7 for $-\text{COOH}$ and 10.0 for $-\text{NH}_2$, under the experimental conditions (pH 7.0-7.4), the AmB is amphoteric and very slightly water-soluble. Therefore, it can be favorably partitioned into the oil/water interface, increasing the droplet volume [15, 50]. This condition is able to modify the droplet-size diameter profile of the internal oil phase of the ME, causing a slight increase in the oil droplet [15, 48]. In spite of these changes in the droplet size, the systems showed a good droplet size distribution and homogeneity, which can be observed by the morphology depicted in the TEM microphotography (Fig. 3).

4.4. Characterization of the Interaction between the AmB and the ME

As described earlier, AmB is an amphiphilic molecule [50]. Therefore, it is able to “self-associate in aqueous media and to form supramolecular aggregates, coexisting in monomers, and soluble and insoluble aggregates” [4, 51]. Clinically, the aggregation state of AmB is directly related to its mechanism of action and toxicity [51, 52]. The water-soluble monomer, which can be detected by UV-Vis spectrophotometry by a band at 409nm, is usually associated with a low toxic form of AmB, while water-insoluble oligomers, detected by a band at 329nm, have been defined as the most toxic [4]. In this study, the evaluation of the interaction between the AmB with the ME and the different AmB aggregation state behavior of the formulations was inferred by the variation in the absorption spectra calculated by the molar extinction coefficients (ϵ) [23]. Our overall results are in complete agreement with those of Bolard and co-workers with other lipidic matrices [24].

The most interesting result from the study of the interaction between the AmB and the ME is the one described in (Fig. 4a). As is widely known, all the pharmaceutical formulations containing AmB, although lipid based, possess an external hydrophilic character. This is mandatory for biocompatibility reasons and is true for AmB liposomes [53], AmB lipidic complex [54], and other AmB unusual lipid structures [55]. However, all of them present a self-aggregated band when diluted with the aqueous solvent as a phosphate buffer. In the AmB-ME case, it was demonstrated that when diluted with ME, which possess an aqueous character, a completely different spectrum appeared (Fig. 4a). This spectrum is quite similar to those found for AmB in organic solvents such as methanol or DMSO [12b], in which the AmB molecules lie in a monomeric state with a maximum band, detected by UV-Vis spectrophotometry, at 409nm [12b]. To the best of our knowledge, this was the first time that an AmB formulation presented such spectrum in water media. Also, this predicts that only monomers are present in the formulation, and, therefore, it will present a low toxicity against mammalian cells, as predicted by Bollard and co-workers and other research groups [24a][4]. Indeed, depending on its aggregation state, AmB may present several patterns of activity against fungal cells compared with the toxicity of mammalian cells, showing that a lipid carrier such as ME can “modify the equilibrium between monomeric and oligomeric forms of this drug in the aqueous media and change its overall activity and toxicity” [51].

4.5. In vitro Antifungal Susceptibility

Candida species are the most common pathogens found in fungal keratitis in temperate areas [23]. Early diagnosis and rational therapy against these infections reduce the dreaded complications of ulcer [56]. AmB remains widely used in the treatment of *Candida* keratitis [57]. Table 2 summarizes the MIC values of the AmB-ME against *Candida* and *Fusarium* strains. All these strains were considered sensitive to AmB and the MIC values were lower than 1 $\mu\text{g/mL}$ [18]. For all *Candida* strains, the AmB-ME was revealed to be much more active than the AmB-M. The best results were found for *C. albicans* and *C. krusei*, in which AmB-ME

showed to be 8 and 16 times more active than AmB-M, respectively. Blank ME was not able to induce antifungal susceptibility.

Additionally, the results showed that the ME systems presented an important action against the *Fusarium oxysporum* and *Fusarium solani* strains and a discrete action against the *Fusarium napiforme* strain. The ME was able to release the AmB, making it bioavailable to act against these fungal cells (Table 2). These results suggest that AmB-ME may be helpful for treatment of *Fusarium* keratitis even though the AmB has not been considered the drug of choice for this disease [58].

Therefore, regarding antifungal drug therapy for ocular fungal infection, in which “antifungal drug susceptibility testing has become especially important because of the increasing infection cases and the problem of drug resistance” [1], the ME was revealed to be an effective carrier for AmB. The improvement of AmB efficacy in such carrier could be due to the existence of many more monomeric forms in the ME system and to the protective effect that this system can produce for the AmB molecule in this aqueous media [59].

4.6. In vitro Toxicity Evaluation

RBCs have been used as a great cell model to evaluate efficacy of pharmaceutical products, mostly containing AmB. Indeed, they are sensitive to the tonicity and the pH from the media, and they possess a high content of cholesterol in their membranes [23]. Such features allow them to interact with AmB and create pores or to interact with the ions from the aqueous media disrupting the ionic balance [24a]. Therefore, they are used to evaluate the ME toxicity. In fact, both ME and AmB-ME revealed to be very safe. Blank ME did not induce any change in RBCs, which predicts its safe use by ocular route. On the other hand, AmB-ME showed a slight toxicity only at high concentration, translated by the leakage of K^+ , which is a reversible toxicity in human cells. This behavior could be credited to the new complex of self-associated AmB structures formed with the ME, which was able to change the AmB toxicity profile when compared to AmB-M [4]. For K^+ leakage (Fig. 5b), AmB-M and AmB-ME presented a similar profile. However in RBC, the K^+ ions inside the cells can be released by any disturbance in the cell membrane due to the pore formation. Indeed, the generated pores enable the output of K^+ , but not the release of Hb, “which occurs only after the total destruction of the membrane barrier” [4]. Such profile of safety was already detected for other lipid-based AmB carriers [12b, 14, 24b, 60].

CONCLUSION

AmB is a quite old molecule that has been used as a micelle system since its discovery. Due to the high nephrotoxicity induced by this conventional formulation of AmB, several ways involving nanostructured systems were pursued aiming to reduce its toxicity. In this work we have focused on the development of an ME-based system for AmB towards a future ocular application of the drug in ocular delivery. The challenge was not only to develop an AmB-nanostructured carrier but also to assemble in such system all the physicochemical and physiologic properties required for

an ocular delivery. A step-by-step procedure to achieve an AmB-ME was fully developed. Then, a primary characterization of the system was performed by looking at the physicochemical needs properties to provide a good bioavailability and the physiological needs of the ocular local tissue for the ocular delivery. Additionally, a deep rheological evaluation was carried out in order to keep the drug in the inner part of the eye cavity, avoiding the drug drainage through the lacrimal duct. A microbiological assay was also developed to demonstrate the robustness of the AmB-ME formulation against fungal strains. The results revealed success in such task. AmB-ME is a formulation that keeps all of these properties, and can be produced by a single step in a very easy way. Also, the manufacturing process used in this work is of easy scale-up. The entrapment process using a pH gradient was successful for AmB loading into the lipid nanodroplets, yielding a high incorporation level. The aggregation state of the formulation and its correlation to the improvement in the AmB activity make ME systems appropriate for ocular applications of AmB.

CONFLICT OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

ACKNOWLEDGEMENTS

The authors wish to thank BNB and CNPq (Brasília, Brazil) for financial support in the development of this work, LAPET-UFRN for rheological analyses, the Opthalmos for the osmolarity measurements, and the PROCAD NF 2008 for *in vitro* antifungal susceptibility assay. The authors are also grateful to Glenn Hawes, M.Ed. English from the University of Georgia, for editing this manuscript.

PATIENT CONSENT

Declared none.

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SECTION IV

VEHICULATION OF AN AmB-SOLUBLE DERIVATIVE INTO A MICRO-EMULSION SYSTEM

BRIEFING

The amphotericin B (AmB) physicochemical features cause the drug insolubility in various media, mainly in water. Such an insolubility influences on the development of new pharmaceutical products containing AmB, once the drug is soluble in a few types of solvents that are, in most cases, incompatible with the human use.

We have observed the aggregation behavior of alkaline solubilized AmB under the conditions of “super-aggregates” formation and developed a process in which the insoluble molecule passes through out, becoming soluble in water at neutral or slightly acid pH.

This section presents, in the chapter VI, a scientific report about the incorporation of an AmB-derivative, developed by our group (under protection) into a micro-emulsion system.

CHAPTER VI

Microemulsion encapsulation of an AmB soluble derivative

Microemulsion encapsulation of an AmB soluble derivative

(Scientific Report)

Introduction

The amphotericin B (AmB) is a polyene macrolide antibiotic which presents amphoteric and amphipathic character. Such features make difficult the drug load into pharmaceutical systems. Then, the development of drug derivatives has been tried as an attempt of improving drug solubility in the last decade (1, 2).

Our research group developed a water-soluble AmB derivative by following simple physical and chemical steps of drug modification (in process of patenting). When compared by infra-red spectroscopy, the interaction C=O is lost during the derivation process (Figure 1).

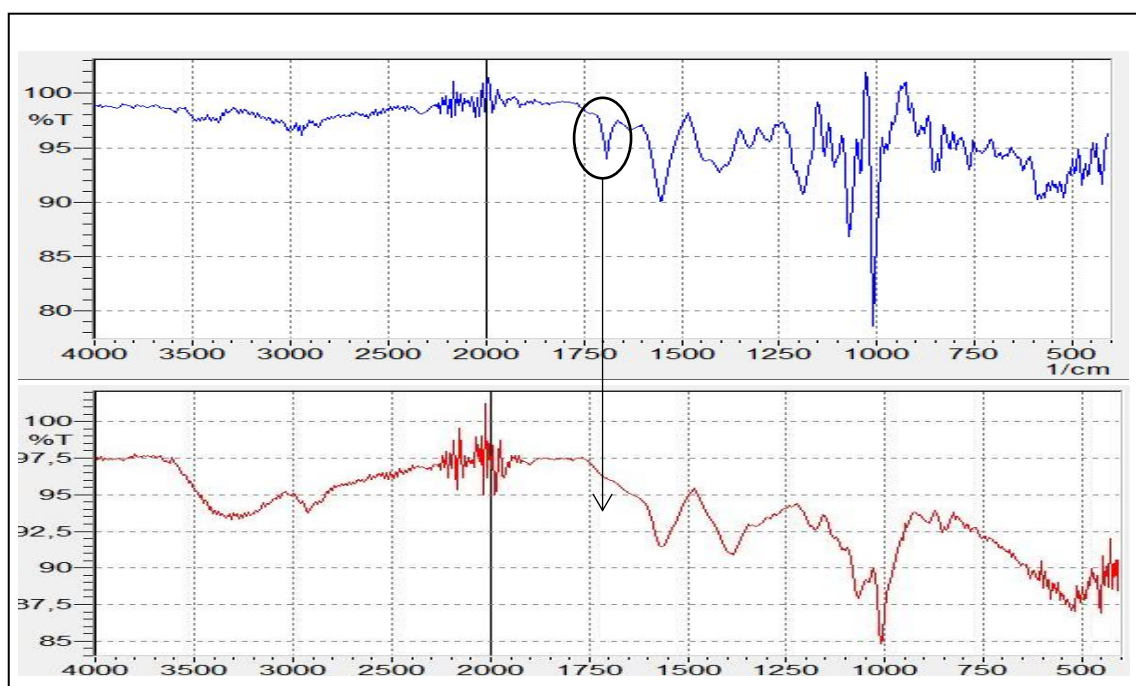


Figure 1. Infra-Red spectra of AmB (blue line) and its soluble derivative (red line). The lost of a signal band is evidenced.

This derivative product presented the same spectrum of the parent molecule by UV-Vis, evidencing no changes in the polyene chain when dissolved in organic solvents (data in Chapter VIII).

Emulsion systems are composed mainly of water and oil, and among the possible emulsion systems, microemulsion has been already used for loading AmB (3-7). Indeed,

the molecule hydrophobic part might interact with the lipids of the system, being supposed placed at the oil/water interface.

We hypothesised, in view of the polyene chain maintenance, the derivative could also be placed in the interface oil/water of emulsion systems. Therefore, the aim of this work was to encapsulate the AmB-derivative into a microemulsion without using organic solvents neither pH changes and evaluate the derivative aggregation state into the system.

Methodology

Materials

Amphotericin B (99% purity) used for the derivative preparation was from Indofine Chemical Company (USA). Tween[®] 80 was from Sigma-Aldrich (USA), Lipoid[®] S100 was from Lipoid (Germany). Miglyol[®] 812N was a gift from Sasol (Germany). The ultra-pure water was obtained by Milli[®] Q purification system.

Microemulsion production

The microemulsion was prepared as described elsewhere (3), with some modifications. Briefly, Lipoid[®] S100 (6.3%_(w/w)) was dissolved in water (68%_(w/w)) under magnetic stirring up to the formation of a milk liquid. Then, Tween[®] 80 (14.7%_(w/w)) and Miglyol[®] 812N were added to the mixture that was sonicated during 1.5 min using a probe sonicator Vibra-Cell 75041 (Bioblock Scientific, France), followed by 3.0 min of ultrasound bath USC-1800A (Unique, Brazil). Three sonication cycles were enough for microemulsion preparation.

Drug encapsulation

The soluble derivative was weighed to the corresponding weight of 1mg AmB and directly added to the microemulsion. Mechanical stirring was applied by using magnetic bars, and the sample was left to homogenize for 1.0 min. Centrifugation at 10.000 rpm, 5 min in a Gusto mini centrifuge (Heathrow Scientific, USA) was performed and three aliquots of supernatant were taken and dissolved in methanol to drug quantification by UV-Vis Lambda 25 (PerkinElmer, USA), using the equation: $y=167782x - 0.0144$ ($R^2 = 0.9996$).

Spectroscopic characterization

The loaded system was characterized in terms of drug aggregation by UV-Vis, using a Lambda 25 (PerkinElmer, USA) spectrophotometer and by Circular Dichroism (CD), using a J-810 (Jasco, USA) dichrograph.

Results and Discussion

The AmB derivative was easily dispersed into the microemulsion system. Any precipitation was found after centrifugation and 100% drug was recovered, as measured by UV-Vis.

The AmB-derivative molecular spectra after microemulsion encapsulation showed peaks related to drug monomers as well as a shoulder in the region of aggregates (Figure 2).

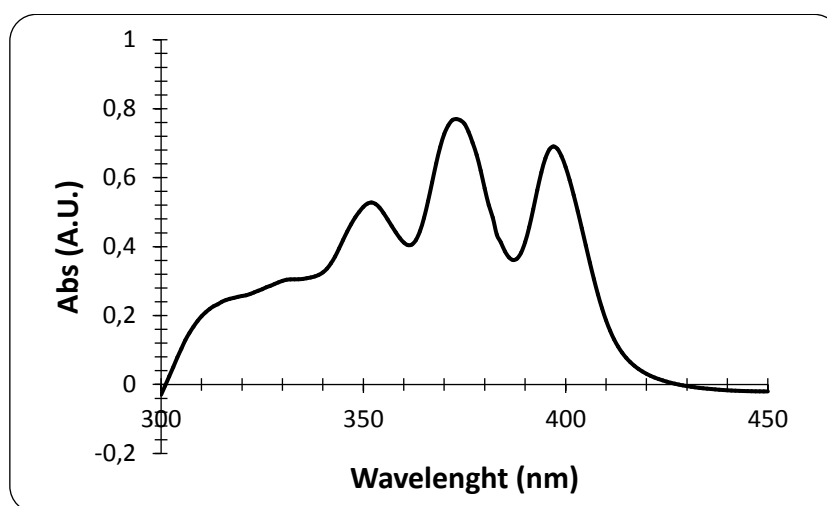


Figure 2. UV-Vis spectrum of AmB-derivative-containing microemulsion diluted in water.

This behavior of aggregation was quite different from the one of the derivative in water, in which the molecule was mainly aggregated, in spite of being soluble (data on Chapter VIII). In fact, although both media were aqueous, the derivative aggregation state changed from the dilution in water to the dilution in microemulsion. A scheme is showed in the Figure 3.

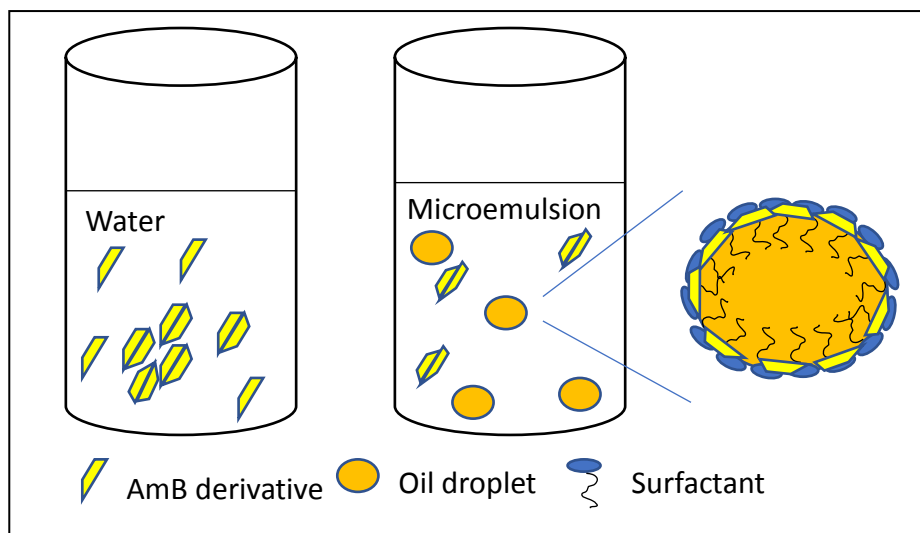


Figure 3. Scheme of AmB derivative aggregation in water and in microemulsion medium.

As the polyene chain was not damaged, the derivative may do hydrophobic interactions, self-aggregating, as occurs with the parent molecule, AmB. It is supposed that the derivative hydrophobic part interacts with the microemulsion lipid core, being placed at the interface oil/water, mainly as monomers.

Once water counts for the main component of the microemulsion system, some derivative may be self-aggregated and dissolved into the system aqueous phase, responding for the shoulder found by UV-Vis in the region of AmB aggregates.

The aggregation state can be also observed by CD studies due to AmB many centers of dissymmetry. Then, chiroptical spectroscopy techniques can provide additional insight in the nature of aggregation tendencies (8).

The encapsulation also changed the derivative CD spectra in water. Before encapsulation, one positive peak was found in addition to the characteristic excitonic doublet (data on Chapter VIII). This behavior changed and the signal of the microemulsion-encapsulated derivative was quite similar to the one of the parent molecule, which was re-analyzed after one week (Figure 4).

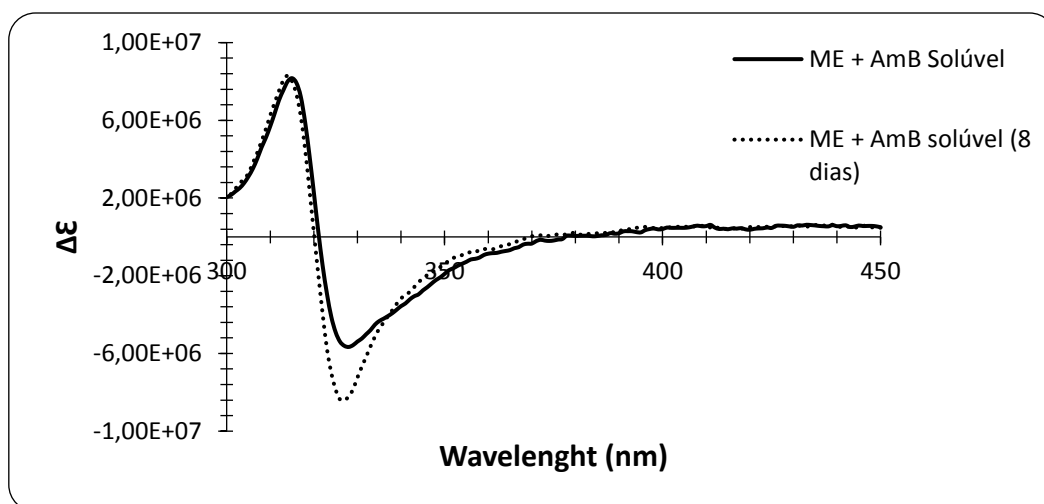


Figure 4. CD spectra of AmB-derivative encapsulated in microemulsion. Full line represents Day 0 and dotted line, Day 7.

The excitonic doublet formed could be related to self-aggregation as well as complexation of the drug with the lipids composing the emulsion system. Our finds suggest that the encapsulation was maintained at least for one week, since the doublet did not change in shape and/or intensity along time.

Concluding Remarks

It can be concluded that the AmB-derivative may be encapsulated into the microemulsion by simple magnetic stirring, without the need of organic solvents neither alkali agents, common strategies applied to the parent molecule, AmB.

The encapsulation was responsible for a change on the aggregation behavior towards derivative monomerization, in spite of observation of some signal of derivative aggregates in the aqueous medium.

Finally, it was demonstrated that the encapsulation maintained the derivative stability into the system for at least one week.

The overall results motivate us to evaluate the efficacy and toxicity of this AmB-derivative microemulsion-encapsulated, as a possible agent for the treatment of fungal and protozoa infections.

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General Discussion

Amphotericin B (AmB) has been used since the discovery in the 60's of its efficacy in the treatment of fungal infections. However, because of unusual and specific physicochemical features, not many products have reached the market, due to formulation issues. Indeed, for at least 25 years the micellar system launched in 1966 (Fungizone®) has long remained as the only clinical option before the arrival of new formulations in the 90's. After Fungizone's patent expiration, at least 15 generic products appeared in the market. The emergence of these products raised the doubt about the use and interchangeability between them. In spite of these issues, different micellar systems are still present in the market, being an inexpensive alternative to the treatment of fungal infections and leishmaniasis, mainly in the developing countries.

Interestingly, only few cases of resistance have been reported for this drug along the time. This reason, besides of the high drug efficacy, have boosted researchers to develop new strategies for AmB formulation, aiming to control its release in physiological environments, aiming mainly to reduce drug toxicity and medicine costs besides of providing safer products that would be administered by other routes than parenteral delivery. This research effort has led in the 1990's to the launching of new AmB-based products, including: Abelcet® (1995), Amphotec® (1996) and AmBisome® (1997). Such products had mainly improved drug safety but were also very costly compared to Fungizone®. These advances in drug delivery of AmB have been made possible both by a better understanding of AmB behavior at molecular level and the rationale development of formulations based on this knowledge.

The drug mechanisms of action is depicted in the Figure 1, an updated scheme in which AmB molecules are able to interact in different ways with sterols, and particularly with ergosterol, which is specific from parasitic membranes, leading not only to the creation of ionic channels within the membrane, but also to direct interactions with ergosterol out of the membrane, leading to membrane depletion. Even, the formation of "sponges" made of ergosterol amphotericin B, outside of the parasitic membrane have been recently described by Anderson et al. (2014) (1).

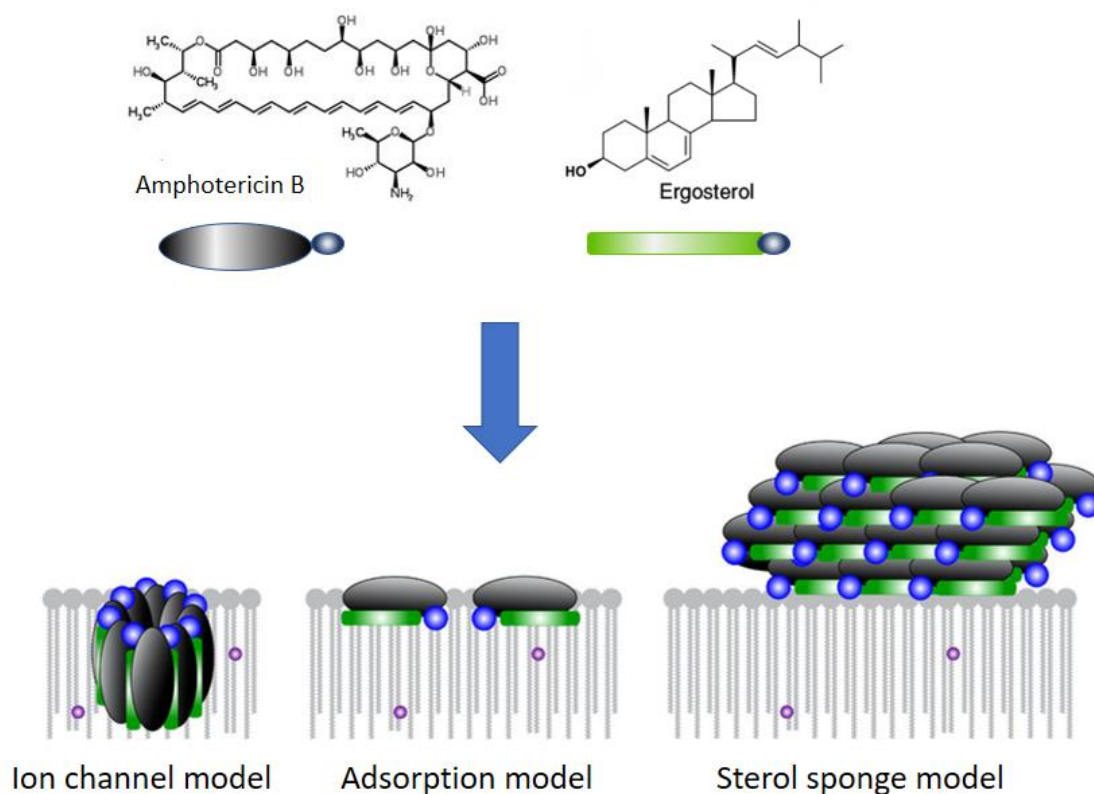


Figure 1. Molecular structure of amphotericin B and ergosterol and proposed unifying model for amphotericin B antifungal activity involving two complementary AmB mechanisms of antifungal activity including membrane permeabilization (1), AmB ergosterol binding (2), and sterol sponge formation (3).

In this revised model, these authors wrote that: “In the widely accepted ion channel model for its mechanism of cytotoxic action, amphotericin forms aggregates inside lipid bilayers that permeabilize and kill cells. In contrast, (the authors) report that amphotericin exists primarily in the form of large, extra membranous aggregates that kill yeast by extracting ergosterol from lipid bilayers.”(1)

It is important to note that membrane permeabilization leads to different types of ion channels formation in ergosterol containing membranes (2), as depicted in Figure 2.

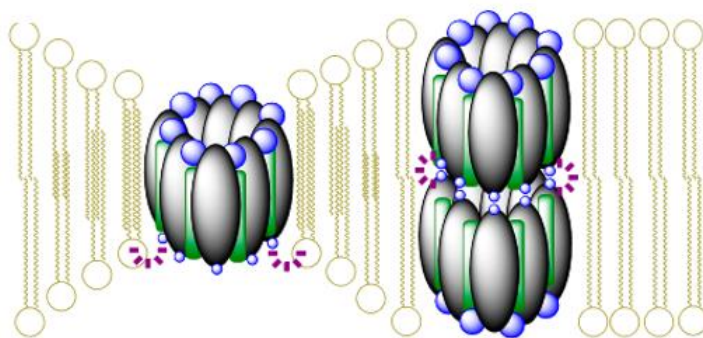


Figure 2. Scheme of ion channel formed by AmB membrane permeabilization.

In view of these activity pathways, it is therefore not surprising that any modification in the parasitic cells membrane environment is likely to affect both AmB killing activity and toxicity. Therefore, coming to AmB formulation issues, it is not surprising that most of researchers in the field cared about the aggregation state of the drug into the carrier and about the optimal mean to control the aggregation state of AmB in physiological media, since the activity and the toxicity are linked to the drug molecular arrangement in the medium. In fact, considerable knowledge has now been gained concerning the biophysics of AmB aggregation, which should have to be taken in account during the development of original AmB formulations.

Figure 3 schematically depicts how an AmB-containing formulation, when placed in a physiological medium after delivery to patient, will give rise to a complex equilibrium between monomeric AmB (i.e. single molecule in water), dimers and different types of aggregates. In turn, these different AmB species constituting this equilibrium will interact with fungi membrane ergosterol by different mechanisms as discussed above.

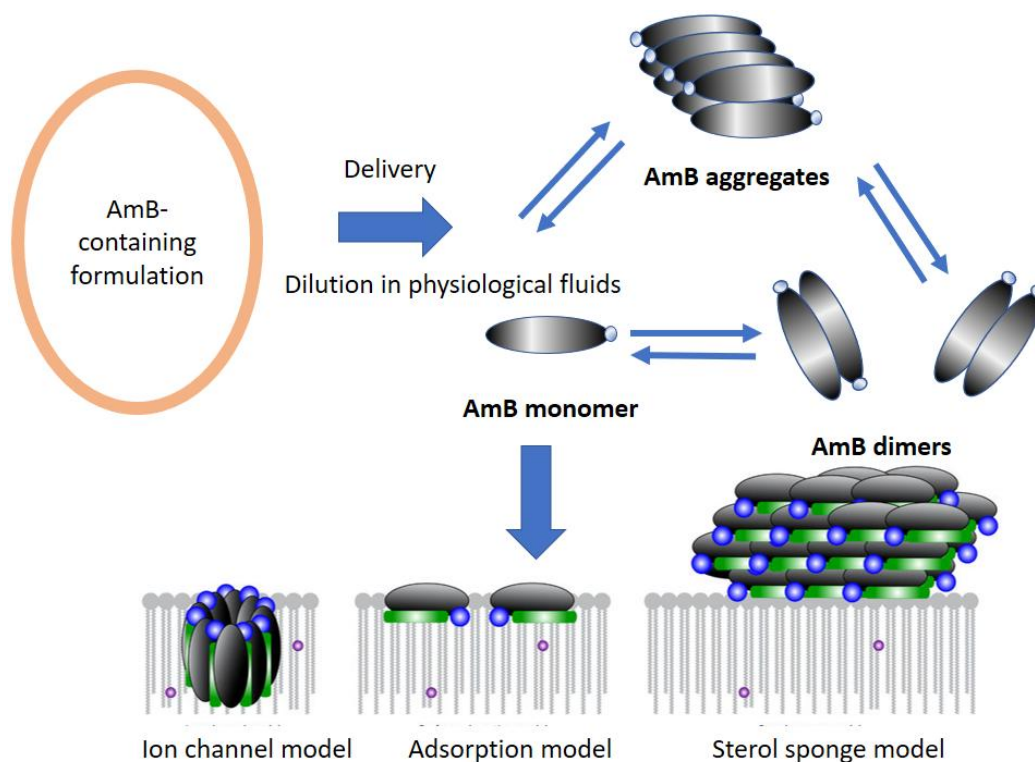


Figure 3. Schematically, after AmB-containing formulation delivery, complex equilibria are created between monomeric, dimeric and aggregates states. In turn, these species interact with fungi membrane ergosterol by different mechanisms, including ion-pore formations within the membrane, ergosterol-AmB complexes adsorption at membrane surface or creation of large ergosterol-AmB “sponges” outside of the fungi, all mechanisms leading to kill the parasite.

1. Attempts to control AmB aggregation state and release from pharmaceutical formulations

Not surprisingly, since the molecular state of AmB in formulation could be a critical parameter for releasing AmB in a molecular form favorable to cytotoxic activity, many formulation strategies have been proposed, which have been reported in the first chapter of this thesis (*Pharmaceutical strategies for amphotericin B delivery*), including: (i) micellar systems comprising polymers and co-polymers, biodegradable surfactants and even cyclodextrins; (ii) emulsified systems such as micro- and nano-emulsion, prepared from different oil cores and surfactants; (iii) liposomal systems using different types of phospholipids and preparation methods; (iv) nanoparticles made of different polymers and co-polymers, including different shapes and surface charges; and finally (v) various

other colloidal systems including nanodisks, carbon nanotubes, nanogels and cochleates (3).

In view of this variety of carrier systems, this thesis was dedicated to the study of AmB aggregation state into both marketed systems and newly conceived delivery systems.

2. Variability of AmB aggregation state in marketed micellar pharmaceutical formulations

In the second chapter (*Amphotericin B-containing sodium deoxycholate micellar systems: a comparison of physicochemical characteristics of marketed products*) we have compared three micellar systems, having same composition and produced in three different countries: Brazil, India and France (4).

The labeled concentration of all products was 50 mg AmB per flask, that has to be diluted in 10 mL water (theoretical final concentration: 5mg AmB/mL). Quite curiously, two out of the three formulations were over-dosed, leading to over-concentrated solutions (more than 25%), which may have consequences on the aggregation state after delivery.

Indeed, the increase in concentration was correlated to an increase on the drug aggregation level, which was observed in UV-Vis spectra, highlighting the close relationship between concentration and drug aggregation. Meanwhile, the other physico-chemical parameters analyzed such as aggregates size distribution and circular dichroism spectra showed no differences among products.

The coexistence in the molecule of an hydrophilic and an hydrophobic moiety of the molecule makes that AmB molecules are prone to extensive self-aggregation. Consequently, in aqueous media, AmB can be found simultaneously in the form of monomers, dimers (head-head or head-tail) and aggregates, related by different equilibria. Talking about micellar formulations, when diluted (e.g. in physiological media), the drug is released in the medium and is likely to self-aggregate, accordingly to the concentration.

Circular dichroism (CD) helped to clarify two important points. At the first, the reversibility of aggregation was demonstrated by diluting the different micellar systems, leading to AmB dis-aggregation, proving the aforementioned link between concentration and aggregation. The second point concerns the type and stoichiometry of oligomers formed. Our results showed that they were of the same type in all the samples tested, despite the differences in drug concentrations.

Once CD and UV-Vis spectra were recorded for these samples, it is important to clarify that these methods no longer coincide. Electronic absorption measured by UV-Vis monitors the exact amounts of the different AmB forms, while CD allows the detection of associated species in minor amounts.

The nature of aggregates seemed to be the same, once the ratio between the positive and negative peaks of AmB couplet did not vary under dilution and according to the CD couplet signature, AmB molecules are aggregated probably as antiparallel dimers.

The size of the structures in solution is quite controversy since different values were obtained when DLS technique was employed, depending if the data were expressed in number or in intensity. It seems that the aggregation state played a role on the droplet size. However, as mentioned, this kind of measurement could be cautiously examined, as different bias could affect the estimation of the aggregates actual size.

3. Formation of AmB super-aggregated from marketed micellar pharmaceutical formulations

AmB super aggregates are known from the 90s, which result from the extended aggregation of the different species of the “classical” equilibrium between monomeric, dimeric and aggregated species. Super aggregates have been initially produced by heating different existing formulations.

It is well known that mild heating of AmB micellar solutions promotes AmB super-aggregation. Such super-aggregates are less toxic than the original preparation while keeping drug efficiency. Since, this behavior has been observed in other systems, including nanodisks (5), and more recently, attempts to engineer AmB-based

nanoparticles has been described (6). The interest of these strategy is to create larger objects, which are known to demonstrate less toxicity against mamallian cells, while keeping antiparasitic activity.

During the comparative study of different commercial formulations undertaken during this work, significant differences in the labelled drug concentration were observed, which were likely to directly impact AmB aggregation state. In the present work, we have studied the super-aggregation process of AmB-sodium deoxycholeate (SD) micelles and also evaluated the possibility of super-aggregation of the drug contained in a liposomal suspension (7).

In the case of the micellar AmB-SD solution, we have confirmed by UV-Vis spectrophotometry that the formation of super-aggregates was accompanied of an electronic blue-shift. The main peak centered at 326 nm moved towards 323 nm for the sodium-deoxycholate micellar system, confirming super-aggregation. In fact, the super-aggregates formation could also be observed by naked eye, since the solution become blurred. Such an increase in turbidity was confirmed by DLS measurements.

From clinical experience, AmB liposomal suspension is a safe product and maybe, because of that, there are no reports in the literature describing super-aggregation formation with this kind of lipidic colloidal systems. Not very surprisingly, we observed no changes in spectral UV-Vis and CD signatures, suggesting that this system do not form super-aggregates on heating. We can hypothesize that super-aggregates heating of liposomal systems cannot lead to super-aggregation because the presence of oligomers and monomers in solution seems to be mandatory for the new entity formation. In the liposomes, AmB is strongly associated to the phospholipids and not released by dilution, as demonstrated by UV-Vis and CD, even along the time. Interestingly, this case study demonstrates that the physico-chemical state of aggregation within the formulation is able to control the nature of AmB equilibrium outside of the formulation. In the present case, if the reagents are not available, the new product cannot be produced.

An interesting point is that following the spectral changes of heated and unheated systems along the time, it was observed that the unheated systems balanced the ratio among dimers and monomers as a function of drug concentration. Then, the spectral shape

changed along the time. Such behavior was not observed when the system micellar system was heated. In this case there was a maintenance of the spectral shape along the time. A decrease in intensity was described for all the species, super-aggregates and monomers. This experience allowed us to suggest that the super-aggregates had a more cohesive structure, which resulted in an improvement of drug protection along the time. Heated systems after 28 days of shelf-storage, contained at least 10% AmB more than the unheated counterparts.

4. Consequences of super-aggregates delivery in the body by parenteral routes.

When AmB formulations are to be delivered by intravenous route of delivery, the behavior of super aggregates in the blood stream and possibly their recognition by the immune system may be an issue. Indeed, the possible recognition and capture of polymeric nanoparticles is a well know phenomenon. As described by Coty et al. (2017) (8), the adsorption of specific plasmatic proteins at the surface of the particles may lead, or not, to their rapid recognition by specialized phagocytic cells in different organs, not only leading to fast clearance of the particles out of the blood, but also to unadequate organs/cells distribution within the body.

For this reason, we attempted first to get a better understanding of the architecture of AmB super aggregates, by attempting to measure the energy necessary to self-aggregation by using an isothermal titration calorimetry equipment (ITC). However, the signals were quite low, just suggesting qualitatively that molecular re-organization generated heat but making impossible to gain any insight on the aggregation process itself.

However, we used a test known as the complement activation test, to evaluate the possible impact of AmB aggregation on the immune response. This part of the work was conducted mainly by Dr. Marcelino, in which we collaborated on the aggregates characterization. Briefly, preliminary results were obtained, which showed a correlation between the presence of AmB aggregates in (human) plasma and the activation of the immunological system (9). Independent of the formulation administered, when AmB is delivered by the intravenous route, AmB molecules are likely to bind to serum proteins, as well as self-aggregate, all phenomena which may considerably affect its uptake by

macrophage cell-like. From a practical point of view, immune system activation would be detrimental to safety of self-aggregated formulations and we suggest that these aspects should be explored in more detail for a better understanding.

5. AmB incorporation into micro- and nano- emulsions and aggregation state control

Emulsions are systems able to carry lipophilic and hydrophilic molecules once they are composed mainly of oil and water. According to the composition, way of production and final characteristics, emulsions can be classified as micro-, nano- or regular-emulsion. Having in mind that drug aggregation can occur in very different carrier systems, we focused on AmB incorporation into micro- and nano-emulsion systems and followed the drug aggregation patterns into these systems (10).

We reported AmB incorporation into micro- and nano-emulsion systems, trying at first to figure out the solubility of the drug in the media by using pH alkalization. In order to avoid the use of organic solvents, researchers take advantage of the alkaline pKa of the drug. Once the pH is higher than 10, AmB is mainly ionized and becomes soluble in the medium. However, up to the moment, there was not a study concerning the best alkali concentration in order to have AmB totally soluble or in the monomeric state.

In a first step, $1,48 \cdot 10^{-2}$ N NaOH alkalization to $\text{pH} > 11$ helped to solubilize AmB, which existed mainly as dimers as demonstrated by UV-Vis spectrophotometry. A further increase in alkali (to $3,85 \cdot 10^{-2}$ N) was able to displace the UV-Vis spectra towards monomeric bands, indicating that not only the pH but the alkali agent concentration played a role on the drug solubility and aggregation.

In a second step, AmB solubility in nano- and micro-emulsion media was also evaluated. These emulsions consisted of medium chain triglycerides associated to different surfactants and water and had a constant composition in the whole study. It was found that the nano-emulsion was able to solubilize a higher amount of AmB (5 mg AmB), when compared to the micro-emulsion (3 mg AmB).

Drug aggregation of AmB into the nano-emulsion increased as the drug concentration was increased similarly to the behavior of already reported for micellar

systems. Interestingly, the increase in droplet size when comparing loaded and drug-free droplets did not change in line with drug concentration.

When AmB was incorporated as a powder in the preparation, AmB was present into the nano-emulsion both as monomers and aggregates. Changing the incorporation process, when the drug was first solubilized into an alkaline aqueous phase, the drug was loaded as poly-aggregates, an AmB species already described in the literature and which have been described as being less toxic than the other AmB species.

When AmB was loaded into the micro-emulsion, AmB existed mainly in the monomeric form. The studies conducted in collaboration with Dr. Silveira (11) and Dr. Morais (12) showed how promising the AmB-containing micro-emulsion are. In Dr. Silveira's study the development of the micro-emulsion and all the characterization for its use in ocular infections are showed. The system was less toxic than the micellar formulation, having efficacy saved and even improved for the treatment of many fungal species.

Dr. Morais and co-workers have tested, *in vitro* and *in vivo*, formulations which would be efficient and inexpensive to treat leishmaniasis. They have tested heated and unheated micellar systems as well as the AmB-loaded micro-emulsion, in comparison to the liposomal system and no differences among the inexpensive systems (micro-emulsion and heated micellar system) and the liposomal products were found in toxicity and efficacy against *L. donovani*.

Up to this moment we have been dealing with AmB into different carrier systems: micelles, liposomes, micro- and nano-emulsion. Caring mainly about characterizing the aggregation state of the drug into the system and on how the aggregation pattern could influence the drug toxicity and we have reported at least two successful uses of the micro-emulsion system as an inexpensive alternative to the toxic micellar system. Then, we have loaded the AmB derivative into a micro-emulsion system(13). The new AmB species was easily dispersed in the micro-emulsion media, presenting UV-Vis spectra of monomers and a slight shoulder corresponding to AmB aggregates.

7. Conclusions

Issues concerning AmB loading into pharmaceutical systems stems from the unique, and unfavorable physicochemical features of this molecule. We hope that the present work will contribute to bringing new strategies for its delivery and opening possibilities for other administration routes, based on a better understanding of the relationships between AmB aggregation and formulation characteristics.

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Conclusions

CONCLUSIONS

The amphotericin B (AmB) molecule has been studied since its discovery, in the 1950's. Since that time many strategies have been considered aiming to improve drug availability for the clinics. The first developed product containing this drug was a micellar system produced as a mix of the biliary salt sodium deoxycholate and AmB. This system, available up to now, is of easy prepare and was largely produced for many pharmaceutical companies around the world, after Fungizone's patent expiration.

In this work we made a comparison among Fungizone's similar products, concluding that they are not equivalents physic-chemically. The ratio among monomers and aggregates is not reproducible on the different brands, even the labeled content was not respected in some cases, evidencing no interchangeability of tested similar products. Such result might be due to the difference on the production process or event to the raw material employed for the different pharmaceutical companies.

The AmB molecular re-organization by mild heating is responsible for the formation of the called "super-aggregates". This procedure is already known for the scientific community and the decrease on the drug toxicity is attributed to the "super-aggregated" conformation. Many researchers have demonstrated the possibility of re-arranging the drug from micellar systems but no reports are found about forming "super-aggregates" from AmB-containing liposomes. We have showed that the "super-aggregates" are AmB species more stable than the ones of the original product and, for the first time, it was showed that is not possible to form "super-aggregates" from the liposomal system AmBisome[®].

Lipid systems loaded with AmB are known for being safer than micellar systems. In fact, lipid complex and liposomes are available in the market and can be administered in higher doses without inducement of nephrotoxicity or other side-effects related to the infusion of AmB-micelles. Nevertheless, those are quite expensive products that impairs their broadly use, mainly in developing countries.

We have showed that AmB can be loaded into emulsion system of easy production and low cost, such are micro- and nano-emulsion. We have deepen the discussion about drug incorporation dependence on pH medium, highlighting the importance of the alkali

agent concentration. Besides, applicability of microemulsions as AmB-carrier was demonstrated as an alternative treatment for ocular infections and visceral leishmaniasis.

Finally, we understand that the main problems concerning AmB loading are linked to its low solubility. Then, we reported the production process of an AmB-soluble derivative that can be loaded into a micro-emulsion system, opening the possibility of administering the drug for the treatment not only for various fungal and protozoa diseases, but also for new administration routes.

The overall results presented in this Ph.D thesis are very important for the built of the scientific knowledge about antibiotics, mainly the amphotericin B, in which was evidenced that if one hand the drug aggregation state changes according to the system, on the other hand one can control these changes towards the aggregation states supposed to be less toxic and more efficient for the patients.

Synthèse de la Thèse

RESUME ETENDU

Depuis la découverte de son activité antifongique dans les années 60, l'amphotéricine B (AmB) est devenue une molécule majeure de l'arsenal thérapeutique en parasitologie. Cependant, en raison de ses caractéristiques physico-chimiques tout à fait particulières et qui la distingue de nombreuses autres molécules actives, la formulation de cette molécule est extrêmement délicate et il n'existe qu'un nombre limité de spécialités commercialisées. Ainsi, depuis son lancement en 1966, la suspension micellaire Fungizone[®], est restée pendant 25 ans la seule option médicamenteuse disponible. Il a fallu attendre les années 90 pour voir arriver sur le marché d'une quinzaine de médicaments contenant de l'AmB et destinées à l'administration intraveineuse, après l'expiration des brevets couvrant la Fungizone[®]. Cependant, la question de leur interchangeabilité s'est rapidement posée en raison des réelles difficultés de formulation de cette molécule. Malgré ces difficultés, ces différentes formulations sont toujours disponibles sur le marché car dans de nombreux pays en développement elles représentent une alternative peu coûteuse en vue du traitement de diverses infections fongiques et principalement des différentes formes de la leishmaniose.

Au plan clinique, l'amphotéricine B est une molécule particulièrement intéressante car elle ne semble pas favoriser l'émergence de résistances majeures. A ce jour, seuls quelques cas ont été rapportés. Dans ce contexte, et en raison de sa grande efficacité fungicide, de nombreux groupes de recherche dans le monde ont intensifié leurs recherches en vue de proposer de nouvelles stratégies de formulation, avec simultanément pour objectifs : (i) de contrôler l'état moléculaire dans lequel l'AmB est libérée de ces formulations, (ii) de réduire la toxicité (importante) des formulations existantes, (iii) de diminuer le coût des formulations et enfin (iv) d'envisager l'usage de voies d'administration alternatives aux voies parentérales. Ces efforts ont conduit dans les années 90 à la mise sur le marché de différentes formulations lipidiques avec notamment l'Abelcet[®] (1995), l'Amphotec[®] (1996) et l'AmBisome[®] (1997). Ces formulations originales ont principalement permis d'améliorer la sécurité d'emploi en comparaison de la Fungizone[®]. Il est important de relever combien la compréhension des mécanismes du comportement de l'AmB au niveau moléculaire, grâce aux progrès de la physico-chimie ont permis ces avancées thérapeutiques.

Afin d'illustrer cela, la Figure 1 décrit schématiquement les mécanismes actuellement connus et par lesquels les molécules d'AmB interagissent avec les stérols et tout particulièrement l'ergostérol, qui est un stérol spécifiquement constitutif des membranes des cellules des parasites. Pour expliquer l'action antifongique de l'AmB, les progrès récents en physico-chimie font appel, non seulement à la formation de pores ioniques transmembranaires, mais aussi à d'autres mécanismes impliquant une interaction avec l'ergostérol d'origine parasitaire et sa déplétion progressive de la membrane cellulaire, suivie de la mort du parasite.

Dans ce dernier modèle, la production de véritables éponges constituées par l'agrégation de molécules d'ergostérol et d'AmB a récemment été décrite par Thomas M Anderson et al. 2014 (1).

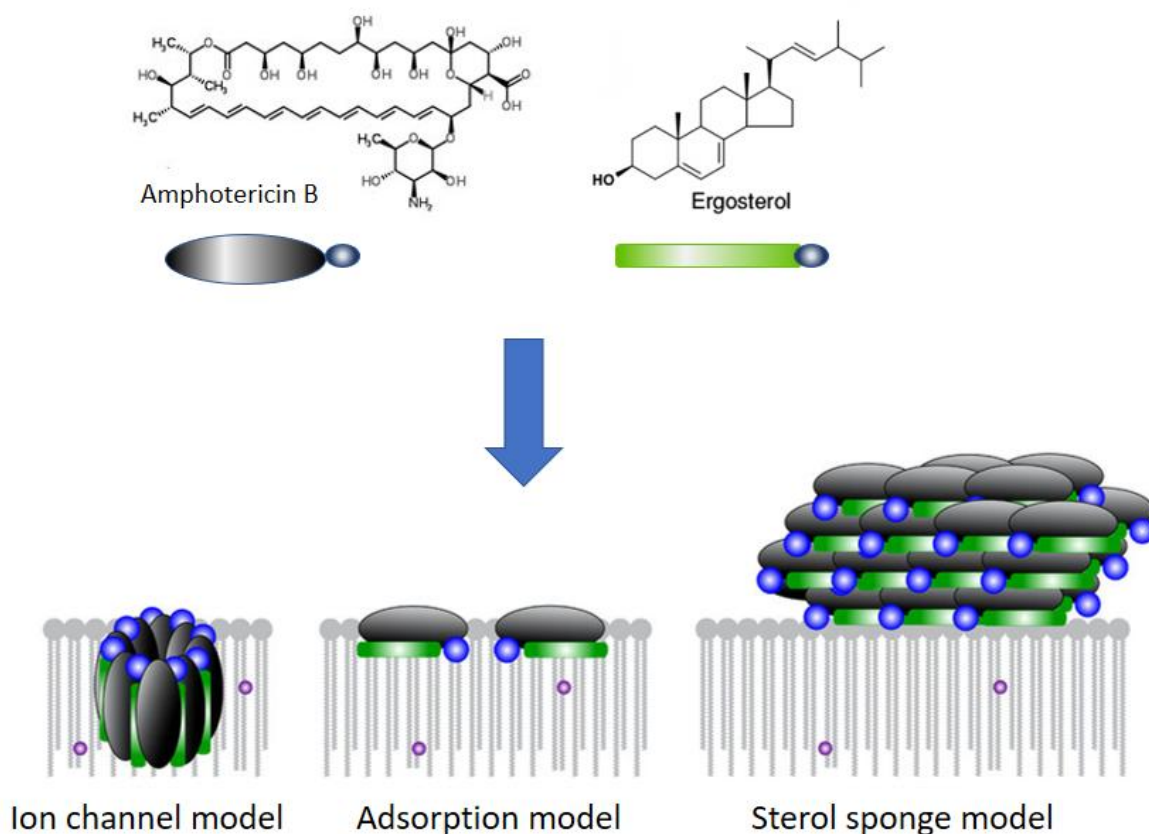


Figure 1 : Structures moléculaires de l'amphotéricine B et de l'ergostérol et modèle unifié décrivant les mécanismes de l'action antifongique par trois mécanismes complémentaires, avec : (i) la perméabilisation membranaire par l'insertion de canaux ioniques néoformés, (ii) la liaison d'une molécule d'AmB à une molécule d'ergostérol

et (iii), la formation extracellulaire d'éponges constituées d'ergostérol extrait de la membrane parasitaire et d'AmB.

Dans leur vision de ces interactions, ces auteurs ont ainsi écrit : “In the widely accepted ion channel model for its mechanism of cytotoxic action, amphotericin forms aggregates inside lipid bilayers that permeabilize and kill cells. In contrast, (the authors) report that amphotericin exists primarily in the form of large, extra membranous aggregates that kill yeast by extracting ergosterol from lipid bilayers” (1).

On notera que deux types d'organisation (au moins) des canaux ioniques ont été décrites conduisant à la très grande toxicité de ces molécules pour les parasites (Figure 2).

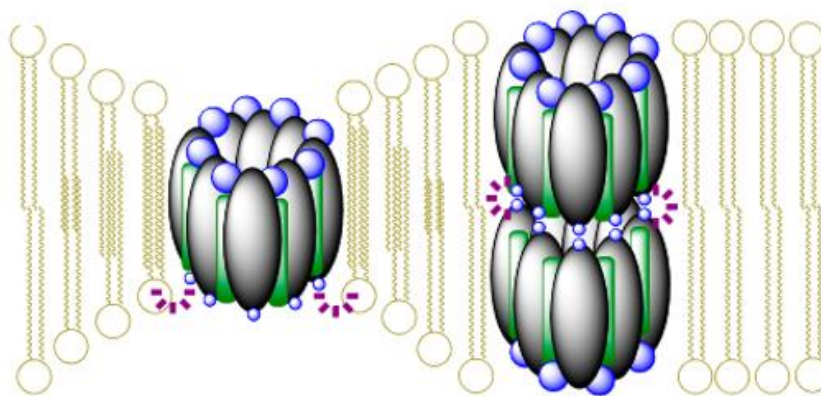


Figure 2 : Schéma d'organisation moléculaire pour la formation des canaux ioniques d'AmB et ergostérol. D'après Gray et al. (2012) (2).

Ainsi, l'état d'agrégation et la nature des interactions moléculaires avec les stérols gouvernent simultanément l'activité antiparasitaire, mais aussi la toxicité de cette molécule, de manière extrêmement fine. Il n'est donc pas surprenant de constater que les formulateurs se sont attachés à concevoir des systèmes d'administration pour lesquels il soit possible de contrôler non seulement l'état d'agrégation dans la forme galénique, mais aussi dans les liquides physiologiques et au moins ceux du site d'administration, le sang quand il s'agit de la voie intra-veineuse. De ce point de vue, il est permis d'espérer que les progrès considérables de ces dernières années dans la compréhension du comportement biophysique de l'AmB pourront être pris en considération dans le but de formuler des médicaments à base d'AmB, plus efficaces et plus sûrs. La Figure 3

représente schématiquement comment une formulation d'AmB, placée dans un milieu physiologique donné, va donner naissance à un équilibre complexe entre différents arrangements des molécules d'AmB, avec non seulement des molécules existant à l'état « monomérique », mais aussi des dimères et différents types d'agréats. Toutefois, ces connaissances mériteraient largement d'être complétées. Ainsi, force est de reconnaître que les interactions non spécifiques avec les molécules présentes dans le plasma sanguin sont fort peu étudiées. Dans tous les cas, il faut considérer que ce sont ces différentes espèces d'AmB en équilibre entre elles qui vont ensuite interagir avec l'ergostérol originaire des membranes parasitaires, par les différents mécanismes décrits ci-dessus.

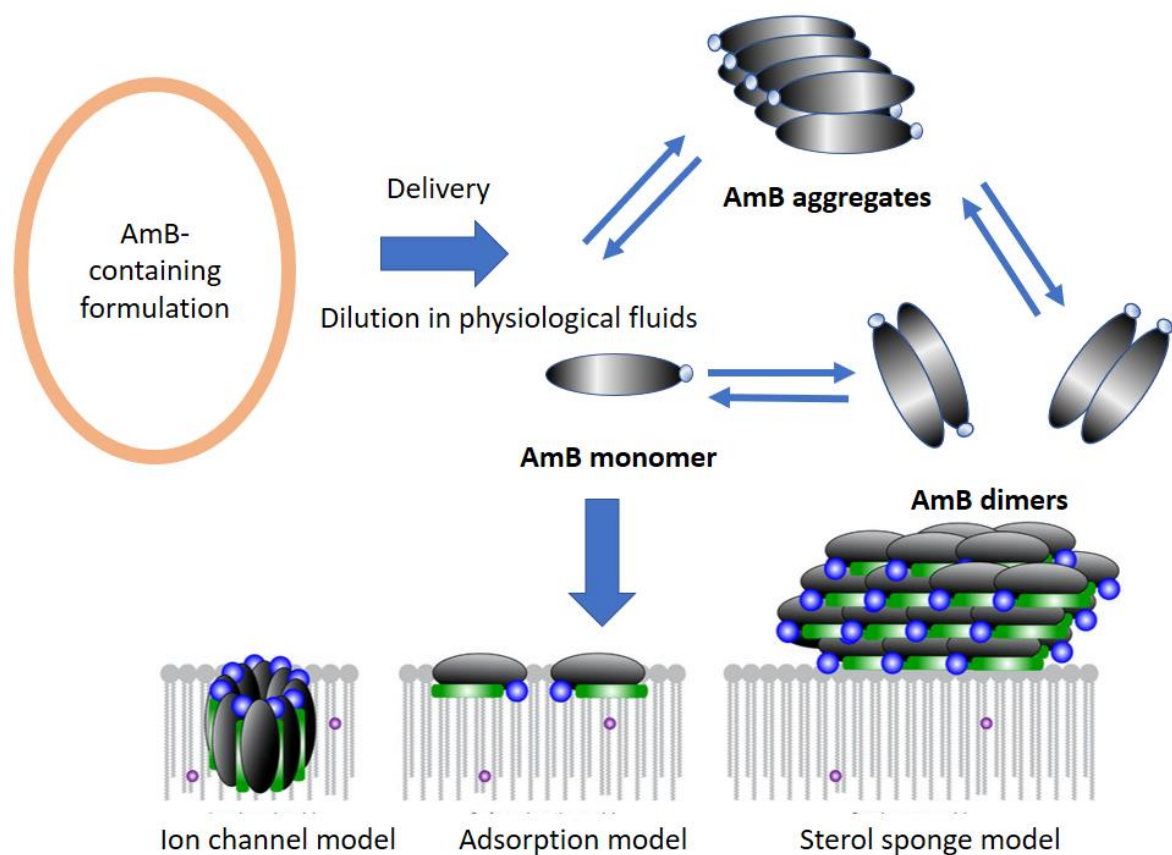


Figure 3 : Description schématique des événements survenant après l'administration d'une formulation contenant de l'AmB. Dans un premier temps, la dilution de la préparation aboutit à la formation d'un équilibre complexe entre les formes monomériques, dimériques (parallèles et anti-parallèles) et les états agrégés. Ces différentes espèces interagissent ensuite avec l'ergostérol spécifiquement contenu dans la membrane des parasites, ce qui aboutit par différents mécanismes à la fragilisation de la membrane du parasite et à sa mort.

1. Contrôle de l'état d'agrégation de l'AmB dans les formulations pharmaceutiques et des modalités de sa libération.

Dans ce contexte, il n'est pas surprenant de constater que de nombreuses stratégies de formulation de l'AmB ont été proposées. Pour cette raison, le premier chapitre de ce travail a consisté à passer en revue ces différentes stratégies (*Pharmaceutical strategies for amphotericin B delivery*), et plus particulièrement : (i) les systèmes micellaires mettant en œuvre des polymères et des copolymères, des agents tensioactifs biodégradables et même des cyclodextrines, (ii) des systèmes émulsifiés du type micro- et nano-emulsions, préparées à partir de différentes huiles et différents agents tensioactifs; (iii) des liposomes préparés à partir de différents phospholipides et par différentes méthodes de préparation; (iv) des nanoparticules polymères à morphologie contrôlée (géométrie, taille, charge de surface) et enfin (v) divers systèmes colloïdaux tels que des nanodisques, des cochleates, des nanotubes de carbone, des nanogels (3).

La grande variété de ces systèmes conduit à s'intéresser à l'état d'agrégation permis dans chacun d'eux. Toutefois, pour une question d'efficacité, nous nous sommes focalisés dans un premier temps sur la caractérisation de cet état dans les différents médicaments actuellement sur le marché et dans un second temps au développement de nouveaux systèmes.

2. Variabilité de l'état d'agrégation de l'AmB dans les formulations micellaires commercialement disponibles.

Le second chapitre de ce travail (*Amphotericin B-containing sodium deoxycholate micellar systems: a comparison of physicochemical characteristics of marketed products*) a consisté à comparer trois systèmes micellaires possédant la même composition et produits au Brésil, en Inde et en France (4). La dose nominale d'AmB affichée par flacon était de 50 mg, ce qui correspond à une concentration finale de 5mg AmB/mL après dilution dans 10 mL d'eau.

Assez curieusement, deux de ces préparations se sont révélées sur-dosées, conduisant à des solutions sur-concentrées d'environ 25%, ce qui pourrait avoir des conséquences en terme d'agrégation après leur administration. Expérimentalement, l'augmentation de concentration constatée a conduit à une augmentation du niveau d'agrégation de l'AmB,

tandis que d'autres paramètres physico-chimiques tels que la taille et la distribution des agrégats, les spectres en dichroïsme circulaire (DC), ne présentaient pas de différences.

Toutefois, les études de dichroïsme circulaire (DC) ont permis de préciser les points suivants. En premier lieu, la réversibilité de l'agrégation a été mise en évidence lors d'essais de dilution des différents systèmes micellaires, conduisant à la dissociation des agrégats d'AmB, confirmant l'existence d'une relation entre concentration et agrégation. Dans un second temps, les états d'agrégation obtenu à partir des trois systèmes ont été comparés mais n'ont pas montré de différences appréciables en dépit des différences de concentration. Cependant, la comparaison des spectres UV-Vis a permis de mettre en évidence des modifications que le DC n'était pas capable de révéler. La combinaison de ces études suggère néanmoins que les agrégats formés paraissent de même nature. Par ailleurs, le rapport des pics positifs et négatifs du couplet observé en DC correspondant à l'AmB ne variait pas au cours de la dilution, suggérant que les molécules d'AmB étaient probablement associées sous forme de dimères antiparallèles.

La taille des différentes structures formées en solution est assez mal connue et les données sont difficilement comparables aux données de la littérature dans la mesure où les mesures de diffusion dynamique de la lumière (DLS) sont parfois exprimées en nombre et parfois en intensité.

3. Formation de super agrégats d'AmB à partir des formes pharmaceutiques commercialement disponibles

L'existence de super agrégats d'AmB a été mise en évidence dans le courant des années 90. Ces structures paraissent résulter de l'agrégation d'agrégats préexistants et des autres espèces d'AmB présentent dans l'équilibre « classique » entre les formes monomériques, dimériques et agrégées. Les super agrégats ont d'abord été produits par simple chauffage modéré des formulations micellaires existantes, notamment la Fungizone. La préparation obtenue est alors moins toxique alors qu'elle garde son efficacité thérapeutique. Par la suite, ce comportement a également été rapporté pour d'autres systèmes, tels que les nanodisques (5), et plus récemment, différents travaux ont été rapportés, consistant à préparer des « nanoparticules » d'AmB (6), ouvrant la voie à

diverses stratégies dans ce domaine, avec l'objectif de diminuer la toxicité de ces médicaments vis-à-vis des cellules des mammifères, tout en conservant l'activité antifongique.

Ainsi, durant ce travail, nous avons étudié la capacité de différentes formulations commerciales constituées de micelles d'AmB et de sodium deoxycholate (SD) et aussi de formulations liposomales à former des super-agrégats en les soumettant à un chauffage modéré (7).

Dans le cas des solutions micellaires d'AmB et de déoxycholate de sodium, l'étude des spectres UV-vis a permis de mettre en évidence une dérive du pic centré à 326 nm vers 323. Cette dérive (dite « dérive vers le bleu ») a ainsi confirmé l'obtention d'un état de super agrégation, d'ailleurs confirmé par une étude en DLS.

L'expérience acquise en clinique a montré que l'AmB liposomale est un produit sûr. C'est peut-être la raison pour laquelle jusqu'à présent leur capacité à former des super agrégats n'avait pas été évaluée. Quoiqu'il en soit, le chauffage modéré de la suspension liposomale n'a pas abouti à la formation de super agrégats détectables par spectrophotométrie UV-Vis et/ou DC. Ainsi, la présence de monomères et de dimères paraît indispensable à la formation des super agrégats, alors que dans les formulations liposomales, les molécules d'AmB sont fortement associées aux phospholipides constitutifs du liposome, comme le montrent d'ailleurs des essais de libération par dilution des suspensions et au cours desquels la spectrophotométrie UV-Vis et le DC ne montrent jamais de libération d'AmB dans ces conditions. De façon intéressante, cette étude du cas des formulations liposomales suggère donc que l'état de l'agrégation au sein de la formulation est parfaitement capable de contrôler la nature des espèces d'AmB disponibles à l'extérieur des objets, ainsi que leurs équilibres.

En suivant les changements spectraux des systèmes chauffés et non chauffés au cours du temps, il a été observé dans le cas des systèmes non chauffés que le rapport des dimères et des monomères s'équilibrait en fonction de la concentration en AmB. Alors que des modifications de la forme des spectres ont été observées en fonction du temps, dans le cas des liposomes un tel comportement n'a pas été observé. Dans le cas des systèmes micellaires, une diminution des quantités de toutes les espèces décrites a été

observée. Ce résultat suggère que les super agrégats pourraient avoir une structure plus cohésives que les autres espèces connues. D'un point de vue pratique, le chauffage présentait également un avantage en terme de conservation de l'AmB puisque, après 28 jours de stockage, les préparations contenant environ 10% d'AmB en plus, comparativement aux contrôles non chauffés.

4. Conséquences de la présence de super agrégats lors de l'administration parentérale des préparations.

Lors de l'administration intraveineuse des formulations d'AmB contenant des agrégats ou des super-agrégats, la possible reconnaissance par le système immunitaire de ces particules étrangères à l'organisme doit être prise en considération. Ce phénomène connu dans le cas des microorganismes bien sûr, mais plus largement des nanoparticules administrées par voie IV résulte de l'adsorption de certaines catégories de protéines plasmatiques à la surface de ces objets qui sont alors rapidement phagocytées par diverses cellules spécialisées présentes dans différents organes. Bien évidemment, il en résulte non seulement une diminution des quantités de molécules actives réellement disponibles aux sites d'action, mais aussi des modifications importantes de la distribution dans les organes ou les cellules concernées par l'action (ou la toxicité d'ailleurs).

Afin d'étudier la possibilité de ce phénomène, nous avons tenté dans un premier temps de mieux connaître la structure des agrégats de molécules d'AmB en utilisant la technique calorimétrique de titration isotherme (ITC). Cependant, les signaux étaient trop faibles pour être quantitativement exploitables.

En revanche, la mise en œuvre d'un test d'activation du complément classiquement utilisé dans le cas des nanoparticules polymères, a été menée avec les différentes formulations étudiées afin d'évaluer l'impact de l'agrégation sur en terme de réponse immunitaire (8). Cette partie du travail a été menée en collaboration avec le Dr Marcelino et ma contribution à cette partie du travail a consisté à caractériser les états d'agrégation dans les préparations testées. Bien que préliminaires, ces premiers essais suggèrent que lorsqu'on place les différentes préparations contenant des agrégats au contact de plasma, on observe une activation du système du complément. Cette partie du travail mériterait d'être développée dans la mesure où le déclenchement d'une réponse

immunitaire du fait de l'agrégation constituerait un effet négatif, en dépit d'autres avantages observés par ailleurs.

5. Incorporation de l'AmB dans des micro- et des nano- émulsions et contrôle de l'état d'agrégation obtenu

En fonction de leur composition, les émulsions constituées d'eau, d'huile et d'agents tensioactifs, peuvent donner naissance, non seulement aux émulsions conventionnelles, mais aussi aux micro-émulsions et aux nano-émulsions, selon leur nature et leur mode de fabrication. Dans la mesure où l'agrégation de l'AmB dépend fortement de la nature des systèmes auxquels elle est associée, nous nous sommes ainsi focalisés sur le cas des micro- et des nano-émulsions puisque ces deux systèmes constituent des systèmes différents de ceux étudiés précédemment, et avec pour objectif d'étudier l'état d'agrégation de l'AmB qu'ils permettent d'obtenir (9).

Afin d'associer l'AmB à ces systèmes et alors qu'elle est naturellement insoluble dans les excipients qui les composent, nous avons étudié dans un premier temps la possibilité d'obtenir des solutions aqueuses alcalines d'AmB, facilement neutralisables, et de préférence à l'emploi de solvants organiques difficilement éliminables. Ainsi, nous avons montré qu'à $\text{pH} = 11$, et concentration $1,48 \cdot 10^{-2} \text{ N}$, l'AmB existait majoritairement à l'état de dimères. Une alcalinisation supplémentaire (jusqu'à $3,85 \cdot 10^{-2} \text{ N}$) permet d'observer un déplacement supplémentaire du spectre UV-Vis indiquant l'apparition de monomères. Il s'est ainsi révélé possible de trouver une concentration en base permettant d'obtenir la dissolution totale de l'AmB dans un état monomérique.

Dans une deuxième étape, l'incorporation de l'AmB dans des micro- et des nano-émulsions de composition équivalente et classiquement préparées à partir de triglycérides à chaîne moyenne (Mygliol 812) et de différents tensioactifs a été réalisée d'une part en incorporant l'AmB directement sous forme de poudre dans les préparations et d'autre part, par ajout de solutions alcalines d'AmB.

Ainsi, quand l'AmB était ajoutée sous forme de poudre dans les nano-émulsions, l'AmB existait dans ces préparations simultanément à l'état de monomères et d'agrégats. En revanche, lorsque l'AmB était ajoutée sous forme de solution alcaline lors de la

préparation de la nano-émulsion, l'AmB apparaissait principalement sous forme de poly-agrégats. Comme cela a été discuté plus haut, l'existence de l'AmB sous forme d'agréats est de nature à diminuer la toxicité des préparations. Toutefois, le maintien de l'efficacité de ces préparations reste à démontrer.

L'association de l'AmB aux micro-émulsions, permet d'observer qu'elle existait principalement sous forme monomère. Plus prometteuses, l'efficacité de ces micro-émulsions a été évaluée en collaboration avec les Dr. Silveira (10) et Dr. Morais (11). Ainsi, l'étude du Dr. Silveira qui décrit le développement pharmaceutique et la caractérisation de ces systèmes, a montré le grand intérêt de cette formulation dans le traitement d'infections fongiques à localisation oculaire. Ainsi, cette formulation s'est montrée moins toxique que les formulations micellaires commerciales, tout en ayant une efficacité maintenue, voire accrue selon la nature des espèces fongiques étudiées. Par ailleurs, le Dr. Morais et ses collaborateurs ont testé ces formulations, *in vitro* and *in vivo*, dans le traitement de la leishmaniose en leur comparant les systèmes micellaires non chauffés et chauffés, en utilisant les suspensions liposomales comme contrôle. Dans ces conditions, les microémulsions et les systèmes micellaire chauffés avaient un profil d'efficacité et de toxicité vis-à-vis de *L. donovani* comparable à celui des préparations liposomales. Ainsi, ces micro-émulsions d'un coût très inférieur à celui des préparations liposomales, pourraient représenter une alternative très prometteuse aux formulations micellaires commerciales grâce à un profil de toxicité plus favorable (12).

7. Conclusions

Les caractéristiques physico-chimiques de l'AmB sont à la fois à l'origine de ses propriétés fongicides remarquables, mais simultanément sont la source de sérieux problèmes en terme de formulation pharmaceutique. Au cours de ce travail, en nous basant sur une meilleure compréhension des phénomènes d'agrégation de l'AmB en lien avec les caractéristiques des formulations étudiées, nous espérons avoir contribué à clarifier les différentes stratégies d'administration actuellement développées, notamment la technique du chauffage modéré. Ce travail pourrait également ouvrir la voie au développement de stratégies d'administration par des voies d'administration alternatives aux voies parentérales

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Enclosures

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Stearylamine-containing cationic nanoemulsions as a promising carrier for gene delivery

In 2013, when I have started my Ph.D studies, my mentor and I presented to the RENORBIO a project in biotechnology entitled “Cationic nanoemulsions for gene delivery: intracellular process and *in vitro/in vivo* evaluation of gene expression”, a natural continuation of the work developed for my master degree, in which we have developed a cationic system able to pack a plasmid DNA, clarifying the physical factors influencing the genetic material compaction into a pharmaceutical system.

Still in the first months of Ph.D, due to collaboration with the Université Paris-Sud, by means of a Capes/COFECUB cooperation project, we have modified my thesis project resulting in the works presented in the previous nine chapters.

Although in a short period of time (around four months), summed to some results from the master period, the previous Ph.D project resulted in paper entitled “Stearylamine-Containing Cationic Nanoemulsion as a Promising Carrier to Gene Delivery”, published in the Journal of Nanoscience and Nanotechnology.

Stearylamine-Containing Cationic Nanoemulsion as a Promising Carrier for Gene Delivery

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The drawbacks related to the use of viral vectors in gene therapy have been stimulated the research in non-viral strategies such as cationic nanoemulsions. The aim of this work was to develop a stearylamine-containing nanoemulsion for gene therapy purpose. The formulation was chosen from a Pseudo-Ternary Phase Diagram and had its long-term stability assessed by Dynamic Light Scattering and Phase Analysis Light Scattering during 180 days at 4 °C, 25 °C and 40 °C. Besides, studies of sterilization and scale up of the product were conducted. It was demonstrated that the proposed system was stable up to 180 days when stored at 4 °C and could be sterilized by a 0.22 μ m filter pore without changes on its characteristics. The scale up was possible by adjusting the volume to the sonication time. Because the nanoemulsion presented a droplet size smaller than 200 nm and a zeta potential higher than 30 mV, this system was able to correctly complex the plasmid model PIRE2-EGFP, as confirmed by the agarosis gel electrophoresis assay. The nanoemulsion toxicity evaluated over lung fetus human cells (MRC-5) was dose-dependent. However, it does not appear to be a limiting factor for further experiments aiming gene transfection. As a conclusion, stearylamine-containing cationic nanoemulsions can be used for gene therapy, since it presents suitable characteristics, stability and possibility of sterilization.

Keywords: Cationic Nanoemulsion, Stearylamine, Non-Viral Carriers, Gene Delivery.

1. INTRODUCTION

Gene therapy has been seen as a promising approach to prevention and treatment of acquired and genetic diseases.^{1–4} The suggested therapy is based on the transfer of an exogenous genetic material into the cell⁵ by replacing defective genes⁶ inhibiting pathogenic gene expression⁷ or even, given new functions to the cell.⁸ However, to be successful, the genetic material must be efficiently delivered and targeted to the nucleus.⁹

Regarding the targeting purpose, two different carriers have been suggested: viral and non-viral. Viral carriers were firstly developed for gene delivery due to their natural ability to infect cells. However, there are many disadvantages related to their use, mainly concerning safety.¹⁰ On the other hand, non-viral carriers as liposomes,⁵

nanoparticles,¹¹ nanoemulsions,^{3,12,13} dendrimers,¹⁴ etc. have been extensively investigated in the latest decades.

Concerning non-viral carriers, cationic nanoemulsions appear as a promising approach to gene delivery. They consist of two immiscible liquids containing an oil core stabilized by surfactants (including a cationic one, which is responsible for the positive charge on the droplet surface).^{3,9,12,13} They are able to complex the genetic material, which is negatively charged, via electrostatic interactions^{11–13,15,16} forming lipoplexes, useful in gene targeting purpose.¹⁷

Nanoemulsions might be produced by high or low energy methods.^{18–20} The use of sonication (a high energy method) was chosen in this work because its ability to easily and fast produce emulsified systems without use of organic solvents. Additionally, the use of non-ionic surfactants can not only predict that the formulation will have a low toxicity profile, as they are very safety surfactants for

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The full CV can be assessed at: <http://lattes.cnpq.br/07124887678709>

Titre : Etude de l'état d'agrégation de l'amphotéricine B dans différents systèmes d'administration

Mots clés : amphotéricine B, états d'agrégation, super agrégats, micelles, liposomes, émulsions

Bien qu'ancienne, l'amphotéricine B (AmB) reste une molécule de référence pour le traitement des infections fongiques profondes et particulièrement la leishmaniose. Bien qu'étant commercialisée depuis les années 1960, les états d'agrégation de cette molécule dans les formulations pharmaceutiques restent mal connus. Pourtant, on sait que l'auto-association de la molécule joue un rôle important dans la toxicité des médicaments qui la contiennent, ce qui justifie pleinement les efforts pour comprendre et maîtriser ses états d'agrégation.

Cette thèse est divisée en quatre sections. La première section consiste en une revue de la littérature destinée à faire le point sur l'état des connaissances des caractéristiques physico-chimiques de l'AmB, ainsi que les stratégies pharmaceutiques mise en œuvre pour la délivrance de l'AmB.

La deuxième section est consacrée à la comparaison des systèmes micellaires utilisés pour l'administration de l'AmB et disponibles sur le marché, ainsi qu'à la formation des «super-agrégats». Notre travail nous a permis d'expliquer pourquoi il n'était pas possible de produire des "super-agrégats" à partir de liposomes.

La troisième partie de ce travail décrit la préparation d'émulsions lipidiques d'AmB, notamment de type micro- et nano-émulsion. A quantité d'AmB incorporée, son état d'agrégation et ses possibilités d'applications au traitement des infections fongiques et leishmaniques sont discutés dans cette section.

Enfin, la dernière partie de cette thèse décrit la mise au point d'un procédé de production d'un dérivé soluble de l'AmB, protégé par un brevet. L'encapsulation de ce dérivé dans une microémulsion est également décrite.

Title : Study of amphotericin B molecular aggregation into different carrier system

Keywords : amphotericin B ; aggregation state ; super-aggregates; micelles ; liposomes ; emulsion ;

Abstract : The amphotericin B (AmB) is an old molecule used as golden standard for the treatment of diseases such as deep fungal infection and leishmaniasis. In spite of being in the market since the 1960's, there is a lack of studies about the aggregation state of this drug into the carrier systems. Such an information gains importance when one knows that the self-association of the molecule plays an important role on the drug toxicity.

This thesis was divided in four sections. In the first one, a revision on the literature was made in order to highlight the physicochemical characteristics of the drug and mainly the pharmaceutical strategies for AmB delivery. Among the strategies there is the solubilization of the drug into micelles. This strategy was studied in the second section, dedicated to the

comparison of micellar systems available in the market and to the "super-aggregates" formation. Here we explained why it was not possible to produce "super-aggregates" from liposomes.

The third section dealt with lipid emulsions: micro- and nano-emulsion; drug loading, aggregation state and applications on the treatment of fungal and leishmania infections were discussed in this section.

The last section of this thesis was about the production of an AmB-soluble derivative. A patent for the production process was presented and finally, the derivative encapsulation into a microemulsion was conducted.