Impact of the aggregation state of amphotericin B on its biopharmaceutical properties. Design of micro- and nanocarriers for oral delivery.
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Par

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Impact of the aggregation state of amphotericin B on its biopharmaceutical properties. Design of micro- and nanocarriers for oral delivery.

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There are many people who love to say the expression “everything is relative” and attribute to the famous scientist Albert Einstein. Well, for my knowledge, he did not say that. In fact, he hypothesized that the speed of light is a constant and all the other “parameters” around would vary according to the proximity of the objects to the speed of light. However, during the last couple of years I might have travelled on really different speeds. There were seconds that lasted for days, and days that lasted for seconds. There are things I will miss from this time, that are other things I will regret to had done it, but for sure to many of them I will be thankful. In the following text I will try to thank everyone who somehow was a part of this journey, trying to stabilize my speed, or at least showing at which speed I was travelling, so I would not miss what it was just besides me.

First, my family! I may say, as I often do, we are not exactly close, but we do stick together (“One for all, and all for one”), and definitely we do not need to talk much to know if we are good or not. Without them, I would not be able to even start this journey and to have the tools to adapt myself through all the situations I have been through these last couple of years. To them, my biggest thank you.

There is also the family we choose during life, the ones we simply call friends. Well, I have a family like that, the “Top & Amigos”. Victor (Top), Daniel (Guerra), Danilo (Gira), Carla (Carlinha), Rodrigo (Motoki), Ana Luisa (Lulu), Alan, Leidson (Leixow), Mila (Ôôô... Mila.), Loiziane (Loize). There are also few other friends, such as Thiago Alves, Gabrielle Azevedo, Rodrigo Araújo (Alf), etc… that I can also not forget to say thank you.

Getting back to the ones who provide me the opportunities I have had, I can not describe how important are my advisors and friends Prof Sócrates Egito and Prof Gilles Ponchel who were, are, and will be really important in the next steps that life has to offer me. Also, to Prof Hartmut Derendorf, who accepted me to work in his lab for a short training.

Now a little bit of history, which as usual starts from the beginning. This one starts in Brazil, at the laboratory of dispersed systems, where my teammates and I were often working. In the beginning (even before the PhD) I met Elquio Oliveira, Toshiyuki Nagashima and Acarilia Eduardo, who first directly guided me. However, they were not the only ones, there were Gyselle Holanda and Amanda Andriola, who also were there from the beginning of my
“Scientific carrier” and still are there helping me and being my friends. Alongside, during the PhD new team members were “recruited”, Bartolomeu Santos, Alexandrino Junior, Cybelle Holanda, Marjorie Freire, Thiago Gomes and Ibrahim Carlos to them my thank you. Therefore, the lab is not only our team, there are others and to them I also have to be thankful for donating a diversity of subjects and themes inside of the lab and, of course, to the friendship relations developed there, in special I would like to cite a few: André Leandro, Francine Johansson, Andreza Rochelle, Miguel Adelino and Julieta Genre. Furthermore, I would like to kindly thank Prof Arnóbio Silva and Prof Matheus Pedrosa for all the support provided.

Within a few months I had started the PhD I went to the US, more precisely to Gainesville/Florida to work with Prof Hartmut Derendorf. By then, I have never been in the US before and I had a lot of expectations, which were matched or not once I arrived and lived there. For sure, the nicest part were the people I met there: Prof Guenther Hoccahhaus, Patricia (Pat) Khan, Kim, Sara, Ravi Singh, Nívea Falcão, Alex Voelker, Maria Seabra, Benjamin (Ben) Weber, Matthias Fueth, Mongjen Chen, Karin Haug, Ruan Oliveira and many others. However, I would like to specially thank Sandra Hänner (and the Hänner family), Saskia Furhmann, Steffanie Schmidt, Claudia Al-Kawari and Julia Richards, who later became close friends.

A year later, I went back to Brazil and restarted my research. However, in a few months I received the confirmation that I would come to France, start a new project and defend my thesis (by then I was not sure if it would be in French). I was more than happy, but also cautious about how it would be, once I only knew how to say “bonjour”, and the French do not have the stereotype of having good English skills.

Then, I arrived in France. Here I thought I would have the tough years of my thesis, first because I would try to work hard (which I tried) and also because I always heard that the “French society” is a really close one (which is not a false statement, but it is not that hard either). I remember on my first days, when I could only catch few words of a conversation among francophone people, and I tried to give them a context. At this time I was living in the Brazilian house in the “Cité Universitaire”. I kept studying, but it was only when I moved to the Swedish house, a month later, that I felt I had started to learn some French. And how could I not speak correctly with such picky friends as Alexis Bourdon (and eventually his family 😊), Leila Dehman (“et ça maman”) and Maëlle Robert. Of course, I can not forget...
Astrid Andersson (for the commentaries and lessons), as well as Alizé Bona for the fluency (I still remember how happy I was the first time I could understand everything she said). Well, the French language would not be the only thing I would learn from the people of Swedish house, Ok… I did try to learn some Swedish, but it is hard. Then I just learned few important things, such as a real Swedish usually say things twice (“Hej, hej!”, “Tack, tack!” 😊). The knowledge of the Swedish house people about music, mostly the Brazilian music, always surprised me, and the way they shared this knowledge on Saturday afternoons in the garden listening to a good live concert Jazz/Bossa nova/MPB music, with some beers and jokes (Thank you Fanny, Lucas, Lily, Olof and Albin). No, I did not forget that there were you who introduced me to the Chinese ravioli (and that the waitress would say “see you tomorrow”, and not “bye” once we were living). Finally, the atmosphere full of diversity we always had at the house, helped me a lot. How many times would we open “one” bottle of wine, or “just had a couple of tea” to talk about life and nothing at the same time. To the talks about subjects that we were directly touched or not, the 3rd floor (Louis, Nasim, Mijhaelo, Sarahbit and the aggregates Alexis and Hanna) I am more than grateful to you. Also, there are some really special people that during this memorable sojourn in the Swedish house: Martin Trulsson, Sabrina Valetti, Gabrielle (bébé) Daumen, Evelina Claes, Linnea G. (the president 😊), Tiphaine Bruder, Julia Barceló, Elena Martín, Sofia Lisboa, Kerstin Jonhsson, Simon Mortier, Johanna Hock, and many others. To conclude, I would like to enormously thank Åsa Ekwall, Lisabeth Cotene, Marc, Manuela and Kadja for all the organization to keep the house a really nice place to live.

Well, I did not spend all my days at home, on the contrary, I spent most of the hours of my journey in France at the University (mostly in the lab). Then, how come not say thank you to all the people who made my days better here. From different countries, different regions, with different accents and different habits, here I learned (not by discussions, but “living together”) that diversity brings growth, I also learned I am not a good joker 😷. First, I will be a bit restrict and acknowledge the Team which I am part of, Team 6. To Andre for the friendship and to be as a monitor for me, once we were only two in tour D5, and I did not know anything or anyone here. To Sarah “sareca” Palacio for the dried apricots, and also for the friendship. To Any Tailor for her advices and Spanish lessons. To Tiphany Grisin for being so picking on my French and for the good laughs she provided. To Pierre-Louis Destruel (PiLu) for the honesty and good beer times. To Herman Palacio, as example of not following standards, as for the funny moments, even, when we worked until late, and
for beer time. To my friend, who has 5 papers as first author (probably, by these days she has a few more), Cassiana Mendes for the friendship, good moments with the permeability/mucoadhesion experiments, movie times and for the dried grapes 😊. To Elsa, also known as Gabriela Meirelles, also for the friendship, good moments with the permeability/mucoadhesion experiments and movie times. To Jean-Baptiste Coty (J-B) for the friendship and the help with the experiments. To Fanny Buhler Varenne, to always try to get the two parts of the team together (as by doing a Christmas celebrations). Last but not least, to Christine Vauthier and Kawthar Bouchemal for the good advices provided.

However, the lab is definitely not limited to Team 6. During this year and half I had the opportunity to met people like Mathilde Lorscheider, Sophie Houvenagel (my Taxi driver friend 😊), Marion (Cahier du labo) Quaillet, Claire Albert (Clairealbert!), Tanguy Boissenot, Guilherme Picheth, Elise Guegain, Giovana Giancalone, Gopan Gopalakrishnan, Adam Bohr and many others who cheered one another up by smiling, speaking bad French, or just jokes (good and bad ones). Also, how not to thank Helene Chacun for her help and sympathy every single day. Furthermore, to Prof Elias Fattal and Dr Nicolas Tsapis for the help and advices during the entire sojourn in France. To Dr Audray Solgadi and Stephanie Nicolaï, for the patience, the teaching and the work on the liquid chromatograph/mass spectrum.

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In memoriam: Aurea Fernandes de Oliveira

Maria do Carmo Rodrigues de Paiva

Francisca Lucia Carlos (Biu)
“Life is about choices. Some we regret, some we’re proud of. Some will haunt us forever. The message — we are what we chose to be.”

Graham Brown
RESUMO

Esta tese foi realizada com o objetivo geral de desenvolver e a caracterizar nanocarreadores com potencial para sobrepujar as propriedades biofarmacêuticas não-favoráveis da antocericina B (AmB), uma molécula extremamente eficaz no tratamento de infecções sistemicas fungicas e leishmaniose, mas difícil de formular independentemente da via de administração desejada. Acredita-se que essa molécula hidrofóbica possui limitações devido a pronunciada tendência de agregar sob condições fisiológicas humanas. A primeira parte desta tese foi conduzida pela hipótese de que o estado de agregação da AmB teria um forte impacto sobre as propriedades farmacocinéticas da mesma. Por tal razão, complexos de albumina e amB foram produzidos de forma a controlar o estado de agregação da AmB. A estrutura dos coloides obtidos foi caracterizada através de ensaios de espectroscopia UV-Vis e dicroismo circular. Adicionalmente, o impacto do estado de agregação na permeabilidade intestinal e no possível reconhecimento dos agregados pelo sistema imunológico foram investigados. A segunda parte deste trabalho teve como objetivo o desenvolvimento de micro- e nanocarreadores para sobrepor as barreiras para absorção da AmB após a sua administração pela via oral. Para este fim, AmB foi incorporada em micro- e nanoemulsões para observação da habilidade destes sistemas de incrementar a permeabilidade intestinal de moléculas. Tal habilidade foi avaliada através do método ex vivo de Câmaras de Ussing, onde o tecido intestinal é utilizado como barreira entre duas semi-câmaras. Nenhuma permeação foi detectada nas condições experimentais utilizadas. No entanto, os dados obtidos através da medidas eletrofisiológicas demonstraram que a velocidade da perda da viabilidade do tecido é dependente do estado de agregação da AmB em contato com o tecido. Também foi observado, através dos ensaios de permeabilidade que as rotas de absorção paracelular e transcelular devem ser rotas marginais quando a absorção da AmB é observada in vivo, como descrito na literatura. Como alternativas, as rotas de absorção pela captura de agregados e partículas pelas placas de Peyer e a rota de absorção linfática têm sido discutidas. Finalmente, um outro sistema particulado que objetiva a liberação em nível de colón e baseada na utilização da xilana, um biopolímero natural e enzimaticamente degradado. A xilana é polissacarídeo presente em grãos, cereais e plantas angiospermas que é especificamente degradado na região colônica, especificamente pela microbiota lá presente. A técnica aplicada de forma original consiste na formação de uma emulsão água-água de xilana em...
presença de PEG, seguida por uma etapa de reticulação com o trisódio trimetafosfato. Através da aplicação desta técnica foi possível produzir partículas à base de xilana que podem ter seu tamanho médio, de forma controlada, variado entre 380 nm e 4.5 µm, de acordo com os parâmetros utilizados. Esta técnica também é livre do uso de solventes orgânicos e possui potencial aplicação para a liberação controlada de AmB em nível de colón.

**Palavras-chave:** Anfotericina B, agregação, permeabilidade intestinal, xilana, emulsão água-água, micropartículas.
ABSTRACT

This thesis is part of the development and evaluation of nanomedicines potentially able to overcome unfavorable biopharmaceutical properties of amphotericin B (AmB), a highly effective molecule used for the treatment of systemic fungal infections and leishmaniasis, but difficult to formulate efficiently, whatever the route of delivery. It is believed that this hydrophobic molecule suffers from severe limitations due to its pronounced tendency to aggregate under physiological conditions. The first part of the thesis was driven on the hypothesis that the degree of aggregation of AmB could have a strong impact on some of its pharmacokinetics properties. For this purpose albumin has been used to produce controlled complexes between albumin and AmB in order to control AmB aggregation states. The morphological characteristics of the resulting colloidal objects have been carefully characterized by UV-Vis spectroscopy and circular dichroism. Furthermore, the impact of aggregation state on both the intestinal permeability and a possibly expected recognition of the aggregates by the immunological system were investigated. The second part of this work was focused on the development of micro- and nanocarriers intended to overcome the absorption barrier raised against AmB after oral delivery. For this purpose, AmB was loaded into micro- and nanoemulsions to evaluate a possible permeability enhancement effect through the intestinal membrane, which was evaluated in rats using the Ussing chamber model. No detectable permeation was seen in any of the experimental conditions. However, the electrophysiological data showed tissue viability losses due to the strong toxicity of AmB, that were dependent on the aggregation state of AmB when in contact with the tissue. It was also concluded from detailed permeation experiments in healthy tissues that paracellular and transcellular routes were likely to be only marginal pathways when oral absorption are observed in vivo, as reported in the literature. The likeness of other possible absorption pathways, including Peyer's patches capture and lymphatic pathway implication for aggregated particles has been discussed. Finally, another particulate system intended for colonic delivery and based on xylan, a natural and enzymatically degradable biopolymer, has been investigated. Xylan is a polysaccharide present in grains, cereals and angiosperm plants that is specifically degraded on colon region, by the microbiota. An original process consisting in a water-in-water emulsion of xylan in presence of PEG followed by a crosslinking phase using trisodium trimetaphosphate has been developed, making possible
the production of xylan-based biocompatible micro- and nanospheres ranging from 380 nm to 4.5 µm, depending on the parameters in the process. This eco-friendly process is free of harmful solvents and has potential application for the delivery of AmB at the colonic level.

**Keywords:** Amphotericin B, aggregation, intestinal permeability, xylan, water-in-water emulsions, microparticles.
RESUMÉ

Le développement de nanomédicaments capables de contrecarrer les propriétés biopharmaceutiques défavorables de l'amphotéricine B (AmB) représente un enjeu important. L'AmB est en effet une molécule très efficace pour le traitement des infections fongiques systémiques et aussi pour la leishmaniose, mais difficile à formuler efficacement, quelle que soit la voie d'administration. Cette molécule particulièrement hydrophobe souffre de limitations importantes en raison de sa tendance prononcée à l'agrégation dans les conditions physiologiques. La première partie de cette thèse a consisté à vérifier l'hypothèse selon laquelle le degré d'agrégation de l'AmB pourrait avoir un fort impact sur certaines de ses propriétés biopharmaceutiques et pharmacocinétiques. Dans cet objectif, l'albumine a été utilisée pour produire avec l'AmB des complexes de taille contrôlée. Les caractéristiques morphologiques des objets colloïdaux formés ont été déterminées par spectroscopie UV-Vis et par dichroïsme circulaire. Ainsi, l'impact de l'état de l'agrégation sur la perméabilité intestinale d'une part et la reconnaissance éventuellement des agrégats par le système immunitaire d'autre part a été étudié. La deuxième partie de ce travail a été axée sur le développement des micro et nanotransporteurs destinés à surmonter la barrière d'absorption élevée contre AmB après son administration orale. À cet effet, des micro- et nanoémulsions chargées en AmB ont été préparées afin d'estimer leur capacité à améliorer la perméabilité de l'AmB au travers de l'épithélium digestif de rat. Le modèle de la Chambre d'Ussing a été utilisé à cet effet. Aucun passage de l’AmB n'a pu être détecté dans chacune des conditions expérimentales testées. Toutefois, les données électrophysiologiques ont montré une diminution de la viabilité des tissus, attribuable à la grande toxicité de l'AmB, et dépendante de l'état d'agrégation de l'AmB lorsque ces objets sont au contact avec le tissu. Ces essais de perméation menés sur des tissus sains au niveau jéjunal suggèrent que le transport de l'AmB par les voies paracellulaire et/ou transcellulaire est sans doute marginal. Cependant, la littérature rapporte que qu'une absorption par voie orale de l'AmB, bien que peu importante, peut être observée in vivo. Ceci suggère donc que d'autres voies d'absorption pourraient être mises en œuvre, parmi lesquelles la capture d'agrégats d'AmB au niveau des plaques de Peyer et l'accès à la voie lymphatique pourraient représenter des voies d'absorption alternatives. Enfin, l'emploi d'un autre système de transporteur conçu pour atteindre le colon et assurer la délivrance colonique grâce à l'action enzymatique bactérienne locale a été
envisagé. Dans cet objectif, un biopolymère naturel et dégradable par des enzymes, le xylane, a été sélectionné. Le xylane est un polysaccharide présent dans les grains, de céréales et de plantes angiospermes qui est spécifiquement dégradé dans la région du côlon, grâce à l'action enzymatique du microbiote. Pour cela, un procédé original de préparation de particules a été mis en œuvre consistant tout d'abord à produire une émulsion eau-dans-eau de xylane en présence de PEG, suivie d'une phase de réticulation du xylane au moyen du triméthaphosphate de trisodium. La méthode a permis la production de nano et de microparticules allant de 380 nm à 4,5 µm et les paramètres contrôlant le processus ont été identifiés. Ce processus, respectueux de l'environnement et ne nécessitant pas l'emploi de solvants organiques, pourrait être appliqué à la délivrance colonique de AmB.

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SECTION I

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GENERAL INTRODUCTION
GENERAL INTRODUCTION

Infectious diseases are one the main causes of mortality in the world. In tropical regions specific diseases caused by parasites, viruses or bacteria are major concerns for public health systems. Among parasitic diseases, leishmaniasis receives much attention. Indeed, the visceral form of leishmaniasis has a really bad prognostic, when untreated. Further, acquired resistance to the existing drugs makes mandatory the development of efficient medicines able to match a complex set of characteristics, satisfying both efficacy and tolerance standards, as well as, in agreement to pharmaco-economical constrains. Nowadays, there are efficient treatments against leishmaniasis, even against the antimonial resistant strains, based on the use of amphotericin B (AmB). However, the efficiency of this molecule is counterpartyed by the complex process necessary to its safe delivery [1, 2].

AmB is the archetype of a drug for which an in-depth understanding of its physico-chemistry is crucial for the development of really efficient dosage forms. So far, different types of formulations, including liposomal formulations are available. However, these formulations are very expensive, require intravenous administration and therefore they are not always accessible to patients [3]. Not surprisingly, this pharmaco-economical context continues to stimulate pharmaceutical research in this area since existing formulations of amphotericin B are still not fully satisfactorily. The present work has been conducted partly in the LaSiD (Natal/RN, Brasil) and in the IGPS (Châtenay-Malabry, France), within the framework of a scientific cooperation program (CAPES-Cofecub) aiming to improve the potential of anti-infectious drugs by the mean of nanotechnologies.

As aforementioned, AmB exhibits a remarkable tendency to strongly aggregate in biological media, which has major consequences on its absorption and its biodistribution in the body. In this work we focused on two aspects. First, we aimed to evaluate the impact of the AmB aggregation state on (i) its ability (or inability!) to cross different barriers in the body. This knowledge is of interest for being able to conceive rationale and scientifically sounded formulations. Therefore, we addressed both (i) the intestinal barrier (oral delivery) and (ii) the possible recognition of AmB aggregates by the reticulum-endothelial system once in the body, which was investigated by the quantification of their interactions with proteins of the
immune complement system.

In a second step different types of micro- or nanostructures aimed to counterbalance the unfavorable properties of amphotericin B were prepared, including AmB-loaded micro- and nanoemulsions. Furthermore, the development of micro- and nanoparticulate systems intended for colonic delivery and based on xylan, a natural and enzymatically degradable polysaccharide, was also carried out, using an original process based on a solvent-free technology.

**The potential of nanotechnologies for improving oral drug delivery of poorly absorbed drugs**

The administration of drugs by the oral route is widely used due to the innumerable advantages for patients and clinicians, such as painless and easy self-administration. These two factors alone can increase patient’s compliance leading to an efficient therapeutic treatment [4, 5]. Additionally, dosage forms are usually produced as tablets and capsules, which can be manufactured at low cost by pharmaceutical industries [5]. However, systemic absorption can be limited if not negligible depending on the drug characteristics despite the 300 – 400 m$^2$ of absorptive surface area of the gastrointestinal tract (GIT). Furthermore, intra or inter individual variability can lead to ineffective treatments. The continuously regenerating absorptive surface of the GIT is mostly formed by enterocytes, goblet cells and M cells. The enterocytes are cells that have mainly absorption functions; the goblet cells are mucus-secreting cells and the M cells are either disseminated or on the top of lymphoid regions, known as Peyer’s patches, and are responsible for immunological and absorption activities [4]. The ability of M cells to absorb molecules and small supramolecular structures has received much attention in the last decades as it is sometime envisioned as a possible pathway to increase drug absorption, as it will be described in detail during this document.

Even though the GIT has an enormous surface offering different uptake mechanisms for molecules and particles, the oral absorption of drugs can still be insufficient to reach effective therapeutic concentrations in the body, mainly, due to their physicochemical properties, such as their solubility in the gastrointestinal fluids, and their sensitivity to enterocyte metabolism and/or efflux pumps [6]. In general, small molecules (MW < 500 Da) can easily permeate the GIT barriers and be absorbed. Additionally, different molecular characteristics including the presence of ionic groups, the presence of too numerous chemical groups behaving both as
hydrogen bonds acceptors and donors, an important lipophilicity, can impair the permeation of molecules through the GIT barriers [7, 8]. Due to these close relationships between physicochemical properties and drug absorption the Biopharmaceutical Classification System (BCS) was purposed in 1995 (Figure 1) by Amidon et al. [9]. The BCS has since then been used by regulatory agencies, such as FDA for the faster approval of drugs in case they classified as BCS Class I (for which biowavers can be used to prompt medicines evaluation).

**Figure 1** Biopharmaceutical Classification System purposed by Amidon et al (Amidon, 1995). The green square identifies the Class I drugs that can be waived by the FDA, while the red square identifies Class IV drugs, which are drugs hardly approved by regulatory agencies for their use by oral administration.

However, the occurrence of differences in the bioequivalence tests of similar formulations (e.g. generics) have attracted attention to the fact that excipients, formulation and processes variables could also have an impact on the class II and class III molecules dissolution and permeability properties. In fact, regulatory agencies have high interest on the understanding of the biopharmaceutical effects of excipients over drugs, in order to improve the quality and when possible, to provide biowavers for immediate release dosage forms [10]. The influence of excipients on the biopharmaceutical phase, as well as on the pharmacokinetics of drug does not depend on the complexity of the excipients used, once these effects can be seen when lactose or polymeric nanoparticles are used. Indeed, the use of lactose in different amounts can induce considerable modifications on the dissolution profile of drugs, consequently altering their absorption kinetics [11].

If simple compounds are able to change the bioavailability of a given drug, what are the
modifications on drug absorption that could be observed with the use of supramolecular structures (in the nanometric range) able to interact differently with biological barriers than to the same material on its bulk macroscopic form? The answer to this question has been left “under construction” over the last two decades by pharmaceutical scientists that have been working on the development of nanoengineered materials in order to enhance the absorption of drugs, straightforwardly BCS Class IV drugs (e.g. amphotericin B) [12, 13] and also some BCS Class II and BCS Class III molecules which also have shown some inconsistencies with their dissolution and absorption profiles when administered by oral route [5]. In fact, the engineering of original structures at the nanoscale offers new physico-chemical characteristics, which may be of benefit for improving the delivery of such drugs, e.g. by tremendously increasing the specific surface offered to dissolution processes, and changing the interactions with biological materials.

Based on the raw the materials used for their production, the nanocarriers for the enhancement of drug absorption might be classified in two types: (1) lipidic and (2) polymeric. The lipid-based nanocarriers comprise nanoemulsions and self-nanoemulsifying systems (Figure 2) which are the most described carriers [14, 15]. Polymer-based carriers exhibit different morphologies (e.g. nanocapsules, nanospheres), while the combination of different materials gives rise to hybrid structures including polymer/lipids conjugates, micelles, and/or a large variety of other structures. Additionally, inumerous attempts are being made to control the surface of these systems which can be for exemple hydrophilized (e.g. PEGylated) and/or functionalized by the addition of recognition ligands or enzymes on their surface with the aim of implementing the ability to cleave some polysaccharides chains of the mucus layer, for example [4, 16].

These different nanocarriers that aim to enhance drug absorption through the oral route apparently do not interact with the GIT fluids and barriers so differently, as it can be seen in the Figure 2. In fact, both types of systems can be designed for increasing the apparent solubility of the drug, and/or to protect from enzymatic and chemical degradation, as well as, to interact with cells that belong to the lymphatic system leading to their uptake by non-conventional routes of absorption [17].
Figure 2 Overview of the described mechanisms by which polymeric and lipid-based nanocarriers could enhance drug absorption. (Based on Desai, 2011[17])

Lipid-based nanocarriers

As mentioned before, the most used lipid-based systems that are under development for the enhancement of drug absorption by the oral route are nanoemulsions and/or self-nanoemulsifying systems. However the development of microemulsions and hybrid carriers containing liposomes have also been reported [13, 14, 18-22].

Carriers based on mixtures of lipids, surfactants and water have been described as being able to mix with chylomicrons in order to pave the way of the drugs absorption. Therefore, the choice of the ingredients for their production plays an important role on the behavior of these systems into the human body. For example, the use of medium-chain triglycerides and bile salts (e.g. sodium taurocholate) can lead to the disruption of the cellular membranes or turn them more fluid [23], which may be favorable to absorption, but simultaneously create toxic effects. On the other hand, the use of long chain fatty acids, such as Peceol® and olive oil, have been considered due to their biocompatibility and the successful results that have been reported revealing their ability to enhance drug absorption [24]. Additionally, these systems have been reported as able to inhibit efflux pumps (i.e.: P-gp) and also to improve drug
absorption by the lymphatic route, which make these materials really interesting for the production of drug nanocarriers [25].

Nonetheless, the nanocarriers often show different properties compared to bulk materials, in such a manner that the impact of its utilization should be carefully evaluated. A historical example for these choices are the formulations of AmB approved in the middle of the 90’s, which are mostly based on the use of phospholipids and long chain lipids. These compounds were chosen due to their ability to keep the AmB in its monomeric state and consequently reduce the AmB toxicity when compared to AmB micellar solution [26]. However, the simple use of lipids did not lead to AmB absorption in the GIT, and that is probably one of the reasons why AmB still does not have any oral dosage form for systemic treatments approved by regulatory agencies. Instead, reports in the literature reveal the efforts that have been done to achieve the desired plasma concentrations and absence of toxicity by the administration of AmB by the oral route [24, 27].

**Polymer-based nanocarriers**

Polymeric carriers are usually classified as nanocapsules and nanospheres due to the presence or absence of a inner core into their polymeric structure [28]. However, in the lastest years nanocarriers that are composed by polymer/lipid conjugates have also been described in the literature in order to associate various functionalities to these particles [12].

The surface of polymer-based nanocarriers can be functionalized with PEG chains (or other hydrophilic moieties, e.g. polysaccharides) grafted on their shell (slippery nanoparticles). Additionally, their surface can be decorated with enzymes, which can have different abilities, such as to cleave the mucus and allow the release of the drug closer to the enterocytes layer (mucus penetrating nanoparticles). Likewise, these nanoparticles may have mucoadhesive properties due to the presence of specific chemical groups or specific recognition ligands, which can increase the residence time of the nanoparticles into the GIT due to their strong interaction with mucus [29-32].

Regarding the different properties that polymeric nanoparticles can display, many polymers have to be screened at any time that a new polymeric nanocarrier is going to be developed. On the latest years, natural polymers received much attention of researchers, and among them chitosan has been highlighted [33]. Its mucoadhesive property, which has also been enhanced by the addition of thiol groups in its structure, is one of the major reasons that make this
polymer a promising material for drug delivery applications [29, 34]. Chitosan conjugation with other chemical groups, mainly long chain lipids have already been successfully reported in the literature (including attempts to entrap AmB into intramolecular and/or supramolecular structures). However, no dosage form based on such polymers have reach the market yet [12].

Additionally, another polysaccharide that has been studied for the development of drug carriers to oral delivery is xylan, which is a biopolymer with attractive properties due to its potential specific drug release in the colon region. Xylan is a polysaccharide that can be found in cereals, grains and angiosperm plants [35-39]. Its complex structure formed by a backbone of D-xylopyranose makes this biopolymer insoluble and not degradable in most of the GIT fluids. However, in theory, its degradation would occur due to enzymatic activity in the colon region, because of the presence of the microbiota and mainly Bacterioides, which secrete xylanases into the lumen of the colon [40]. These systems shave potential applications for the treatment of local diseases, such as Crohn’s Disease and Ulcerative Colitis, as well as, a potential application to enhance drug absorption once the residence time of the chyme in that region is long (up to 5 days) [41]. Therefore, they could be envisioned as a possible platform for colonic delivery of AmB too.

**General organization of the manuscript**

The present manuscript is divided in two sections. The first section describes the research undertaken in order to understand the impact of aggregation on the biopharmaceutical properties of AmB, and the second section describes the development and characterization of xylan-based micro- and nanocarriers.

The first section is divided into three chapters. The first chapter consists in a review of the literature concerning the oral absorption of amphotericin B with the help of nanomedicines. The second chapter consists in an experimental part describing the effect of aggregation of AmB, as well as its association to micro- and nanoemulsions, on the intestinal AmB permeability using the experimental model of Ussing’s chambers. The third chapter describes the possible influence of AmB aggregation state on AmB biodistribution by the the activation of the immunologic complement system. Indeed, depending on the delivery patterns used during patients’ infusion in clinics, the possibility of an aggregation of amphotericin B directly in the body may not be excluded. Therefore, the fate of these aggregates (i.e. their
distribution in the different organs) may be modified, prompting us to check the capacity of these aggregates to activate the system of the immune complement system in the body, which is a pre-requisite prior to a possible recognition of these aggregates as a xenobiotic, which in turn would modify AmB distribution into the body.

The second section of this document is devoted to the development of xylan-based systems intended for colon specific delivery. It is divided in two chapters. Chapter IV presents how xylan-based microcapsules can be obtained and their characteristics, while chapter V is dedicated to the development of an original process making possible the production of micro- or nanostructures without the use of harmful solvents.

A general discussion and a conclusion section finish the manuscript. Furthermore, two already published manuscripts are equally attached to the document, which have been prepared in collaboration with other researchers and have constituted a contribution to the development of xylan-based micro- and nanostructures that were specifically developed on during this thesis.

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RÉSUMÉ ÉTENDU
RÉSUMÉ ÉTENDU

Les maladies infectieuses sont une des principales causes de mortalité dans le monde. Dans les régions tropicales les maladies causées par des parasites, des virus ou des bactéries sont des préoccupations majeures pour les systèmes de santé publique. Parmi les maladies parasitaires, la leishmaniose reçoit beaucoup d'attention. En effet, quand elle n'est pas correctement traitée, la forme viscérale de la leishmaniose présente un très mauvais pronostic. En outre, la résistance acquise aux médicaments existants rend éminemment désirable le développement de médicaments efficaces et bien tolérés, tout en satisfaisant aux contraintes pharmaco-économiques. De nos jours, il existe des traitements basés sur l'utilisation de l'amphotéricine B (AmB) qui sont efficaces contre la leishmaniose, y compris contre les souches résistantes aux médicaments antimoniés. Cependant, ces médicaments ne sont pas sans inconvénients parce que leur mise en œuvre est pharmaceutiquement complexe et onéreuse.

L’AmB est l'archétype d'une substance active pour laquelle une compréhension profonde de ses propriétés physico-chimie est indispensable au développement de formes galéniques efficaces. Jusqu'à présent, parmi les différents types de formulations disponibles, les suspensions de liposomes se sont révélées les plus efficaces. Cependant, ces formulations sont très coûteuses, nécessitent une administration par la voie intraveineuse et, par conséquent, ne sont pas toujours accessibles aux patients. Sans surprise, ce contexte pharmaco-économique continue à stimuler la recherche pharmaceutique dans ce domaine, alors même que ces formulations ont été commercialisées dans les années 90. Le présent travail a été réalisé pour partie au LaSiD (Natal / RN, Brésil), un laboratoire fortement impliqué dans la formulation des substances anti-fongiques et à l'Institut Galien Paris-Sud (IGPS, Châtenay-Malabry, France), dans le cadre d'un programme de coopération scientifique bilatéral Brésil-France (CAPES-Cofecub) visant à améliorer le potentiel des substances anti-infectieuses grâce à la mise en œuvre de nanotechnologies.

Au plan physico-chimique, l’AmB présente une remarquable tendance à s'agréger très fortement dans les milieux biologiques, ce qui a des conséquences majeures sur son devenir pharmacocinétique et particulièrement sur son absorption et sa biodistribution dans
l'organisme. Dans ce travail, nous nous sommes concentrés sur ces deux aspects. Tout d'abord, nous avons cherché à évaluer l'impact de l'état d'agrégation de l'AmB sur sa capacité (ou son incapacité !) à traverser les différentes barrières dans l'organisme. Cette connaissance est indispensable pour être en mesure de concevoir et de justifier scientifiquement les formulations proposées. Par conséquent, nous avons successivement cherché à évaluer l'impact de l'agrégation sur: (i) le passage de la barrière intestinale (administration par voie orale) et (ii) sa distribution dans l'organisme, liée à une reconnaissance éventuelle des agrégats d'AmB par le système réticulaire endothélial.

Dans une deuxième étape, nous nous sommes intéressés à la mise au point et la préparation de deux types de micro- ou nano-transporteurs visant à contrebalancer ou masquer transitoirement les propriétés défavorables de l'AmB. Il s'agit d'une part de micro- et nano-émulsions lipidiques chargées en AmB et d'autre part de micro- et nanoparticules constituées de xylane, un polysaccharide naturel et enzymatiquement dégradable. Ces particules, destinées à favoriser l'absorption colonique, ont été préparées grâce à un procédé original mettant en œuvre une technologie sans solvant.

**Impact de l'aggrégation de l'AmB et de ses interactions avec les protéines sur sa capacité à être absorbée au niveau jéjunal.**

Le développement de formes galéniques permettant l'administration orale de l'AmB représente un énorme défi, même si certains résultats encourageants ont été récemment publiés dans ce domaine. Ainsi, le manque de connaissance des voies de passages possibles et ouvertes à l'absorption orale de l'AmB ne permet pas facilement de choisir des stratégies de formulation efficaces. Un certain nombre de tentatives de formulation visent à développer des systèmes particulaires lipidiques capables de rejoindre la voie lymphatique, avec plus ou moins de succès en terme de quantité absorbée. En revanche, il n'existe pratiquement pas de données relatives à l'absorption de l'AmB par les voies trans- ou para-cellulaires. Clairement, l'AmB présente des caractéristiques connues pour être particulièrement défavorables à l'absorption par ces voies, étant ionisée sur la gamme des pH intestinaux, possédant un caractère amphiphile marqué et présentant une très grande tendance à l'agrégation. Cependant, à notre connaissance, il n'existe pas d'étude approfondie à ce sujet. Ainsi, l'objectif de la première partie du travail expérimental a consisté à étudier l'impact de cette agrégation sur les voies d'absorption au niveau jéjunal (chez le rat) en mettant en œuvre la
technique bien contrôlée de la chambre de Ussing.

Pour cela, l'AmB a d'une part été complexée à l'albumine (BSA) de manière à former des agrégats de taille parfaitement connue et d'autre part associée à des microémulsions ou à des nanoémulsions constituées de lipides à chaîne moyenne, ces deux stratégies permettant de "présenter" l'AmB dans différents états physico-chimiques devant la membrane intestinale. Une technique HPLC avec une détection en spectrométrie de masse (LC/MS) a été mise en oeuvre dans ce but (limite de détection 50 ng/mL). Ainsi, aucune apparition de l'AmB dans le compartiment sèreux n'a pu être mise en évidence quelle que soit les conditions expérimentales adoptées (cinétiques conduites durant 3 heures). En revanche, une disparition systématique d'une fraction de l'AmB dans le compartiment muqueux a été mise en évidence, ce qui suggère l'incapacité des trois systèmes utilisés à maintenir l'AmB dans son état monomérique et à des concentrations suffisantes devant la muqueuse pour assurer un passage passif trans ou paracellulaire. En revanche, l'analyse des données électrophysiologiques suggère clairement une diminution de la viabilité tissulaire dans le cas des complexes AmB-BSA tandis que l'encapsulation de l'AmB dans les micro et nanoémulsions ne l'affectent pas. Cet effet protecteur vis à vis de la toxicité était plutôt inattendue dans la mesure où ces structures contenant des lipides à chaînes moyennes et des agents tensioactifs sont précisément conçues pour maximiser la quantité d'AmB libre. En conclusion, l'incompréhension des phénomènes mis en jeu à l'échelle moléculaire, au niveau des quelques centaines de microns constituant la couche de mucus recouvrant la membrane et/ou au contact direct des entérocytes, subsiste. Elle résulte de la très grande difficulté expérimentale d'être capable de mesurer directement, à cette échelle, l'état physico-chimique dans lequel existe l'AmB au plus près de la barrière d'absorption.

**Influence de l'agrégation de l'AmB sur sa biodistribution : Interaction avec l'albumine et interactions des agrégats avec le système du complément**

L'activité pharmacologique de l'AmB ainsi son profil toxicologique dépend directement de ses propriétés physico-chimiques. En particulier, il est connu que l'agrégation de l'AmB dans les milieux aqueux est à l'origine d'effets toxiques. C'est d'ailleurs pour cette raison que différentes formulations, notamment liposomales, ont été développées dans les dernières décades. Toutefois, les observations relevées en clinique montrent une certaine variabilité des effets dont l'origine pharmacocinétique n'est pas élucidée.
Pour cette raison, nous avons formé l'hypothèse que lors de son administration (par voie parentérale notamment), l'AmB pourrait, selon les conditions d'administration (concentration, nature de la formulation, durée du bolus, etc) former des agrégats dans le sang, soit par l'auto-association de la forme monomérique de l'AmB et/ou par la formation d'agrégats d'AmB avec les protéines plasmatiques. Ainsi, la présence d'agrégats circulants pourrait alors être détectée par le système immunitaire, conduisant à leur capture par le système réticulo-endothélial et, par conséquent, à une modification de la distribution tissulaire de l'AmB selon les modalités de l'administration.

Afin d'étudier cette hypothèse nous avons caractérisé finement les interactions se développant entre l'AmB et l'albumine (sélectionnée en raison de son abondance dans le plasma), ainsi que la structure des agrégats formés, de manière à obtenir différents états d'agrégation de l'AmB, dans des conditions les plus proches possibles des conditions physiologiques. Le degré d'agrégation de l'AmB seule ou en présence d'albumine a été mesuré par spectrophotométrie UV-Visible ainsi que par dichroïsme circulaire et diffusion dynamique de la lumière. Dans un second temps, nous avons tenté de déterminer la capacité de ces agrégats à activer le système du complément grâce à une technique d'immunoélectrophorèse dédiée. Il est en effet connu que l'activation du système du complément joue un rôle majeur dans l'épuration de l'organisme des particules étrangères (microorganismes ou particules volontairement administrées). Dans le cas des particules inertes, la reconnaissance ou la non reconnaissance aboutit à leur capture par le système réticuloendothélial et donc à des modifications de leur distribution dans l'organisme. Les essais menés sur les agrégats d'AmB et d'albumine AmB-BSA semblent ainsi mettre en évidence une certaine tendance à l'activation, laissant ouverte l'hypothèse d'une possible implication de l'état d'agrégation dans la distribution tissulaire de l'AmB. Toutefois, l'intensité de cet effet étant dépendant des quantités d'agrégats exposées aux protéines plasmatiques et ces quantités étant petites dans les conditions de mesures adoptées, ces déterminations devraient être menées de manière systématique et couplées à des études de distribution tissulaires pour permettre de conclure.

**Production et caractérisation de microcapsules constituées de xylane**

Le xylane est un polysaccharide extrait du bois et de plantes annuelles. Il est principalement composé de D xylopyranose et dans une moindre proportion d'acide glucuronique, d'arabinose et d'autres sucres. Sa structure complexe le rend insoluble dans l'eau notamment aux pH acides et neutres. Dans cette partie du travail, nous avons montré qu'il était possible…
de produire facilement des microparticules de xylane par réticulation interfaciale au moyen du chlorure de téréphthaloyle. Leurs caractéristiques physiques et thermiques, la nature des liaisons formées ont été déterminées afin d'essayer d'établir une relation entre la toxicité cellulaire des microcapsules obtenues et leurs caractéristiques. Comme attendu, la cytotoxicité est dépendante de la concentration en microsphère. Toutefois, les conditions de la réticulation et la présence résiduelle d'agent réticulant ont été identifiées comme étant des facteurs de la cytotoxicité et pas uniquement la stoechiométrie utilisée entre le polymère et l'agent réticulant. Ces résultats suggèrent donc la nécessité d'utiliser d'autres approches pour la fabrication de systèmes particulaires à base de xylane.

Développement de micro et de nanosphères constituées de xylane

Les micro et nanoparticules constituées de polysaccharides sont universellement utilisées dans de nombreux domaines industriels. Dans le domaine pharmaceutique, ces transporteurs sont particulièrement intéressants, notamment en raison de leur biocompatibility, de la possibilité de les charger efficacement en molécule active et de contrôler leur cinétique de libération une fois administrées, aussi de pouvoir les préparer de manière reproductible par des procédés industrialisables. Cependant, ces procédés nécessitent habituellement d'utiliser des solvants potentiellement toxiques, ce qui nécessite de mettre en place des procédés de purification efficaces et de contrôler les taux de solvants résiduels en fin de production. Il est donc intéressant de rechercher des procédés alternatifs, évitant le recours à de tels solvants. Dans cet objectif, nous avons sélectionné un procédé original de préparation, basé sur un phénomène de séparation de phase se produisant naturellement lorsqu'on met en contact deux solutions aqueuses contenant deux polymères hydrophiles de nature différente. Ce phénomène repose sur le manque d'affinité l'un pour l'autre des deux polymères, ce qui aboutit à leur ségrégation et à la formation de systèmes dispersés parfois décrits comme étant de véritables "émulsions eau dans l'eau".

Ainsi, nous avons utilisé les propriétés de ségrégation du polyéthylene glycol (PEG 20000) et du xylane et recherché les conditions dans lesquelles il est possible de former des micro- ou nano domaines contenant du xylane, par simple mélange des deux solutions aqueuses. La mise en œuvre d'un plan d'expérience factoriel de type $2^2$ a permis de préciser l'influence des deux solutions (concentrations, quantités) sur la taille des gouttelettes ou micro-domaines formés. Dans une deuxième étape, ces particules ont été stabilisées par un procédé de réticulation chimique par l'action d'une solution aqueuse de trimétaphosphate de sodium.
(TSTP). La microscopie optique et/ou la diffusion dynamique de la lumière ont permis d'établir que selon les conditions, il était possible d'obtenir des particules microniques ou au contraire submicroniques. La tension superficielle des phases ainsi que la différence des masses volumiques sont certainement deux déterminants importants de la taille des domaines de xylane obtenus par ségrégation. Toutefois, il n'a pas été possible de relier la taille des domaines de xylane obtenus au moment du mélange des solutions et la taille des particules après leur réticulation par le TSTP. En conclusion, la méthode proposée permet d'obtenir facilement des micro ou nanoparticules de xylane réticulées. Des études complémentaires de cytotoxicité menées sur la lignée Caco2 ayant montré la faible cytotoxicité de ces substances, ces travaux suggèrent que ces particules pourraient être utilisées pour assurer l'administration colonique de substances actives, puisque le xylane est un polysaccharide connu pour subir une dégradation enzymatique préférentielle au niveau du colon.

Organisation générale du manuscrit

Le manuscrit est divisé en deux sections. La première section décrit les recherches entreprises afin de comprendre l'impact de l'agrégation sur les propriétés biopharmaceutiques de l'AmB. La deuxième section décrit le développement et la caractérisation des micro et nanotransporteur constitués de xylane.

La première section est divisée en trois chapitres. Le premier chapitre consiste en une revue de la littérature concernant les essais d'amélioration de l'absorption orale de l'amphotéricine B par la mise en œuvre de nanomédecines. Le deuxième chapitre comprend une partie expérimentale décrivant les modalités d'agrégation de l'AmB, ainsi que son association aux micro- et nanoémulsions, et l'effet de cette agrégation sur la perméabilité intestinale, en utilisant le modèle expérimental de la chambre de Ussing. Le troisième chapitre décrit l'influence possible de l'état d'agrégation AmB sur la biodistribution de l'AmB. En effet, selon les modes d'administration utilisés en clinique, notamment par perfusion de patients, la possibilité d'une agrégation d'amphotéricine B directement dans le corps ne peut pas être exclue. Il pourrait en résulter une modification de leur distribution dans les différents organes selon les conditions d'administration, la nature des formes administrées, etc.

La deuxième partie de ce document est consacrée au développement de systèmes à base de xylane destinés à la livraison spécifique du colon. Il est divisé en deux chapitres. Le quatrième et le cinquième chapitre présentent des techniques permettant d'obtenir d'une part
des microcapsules à base de xylane et d'autre part des micro- ou nanoparticules de xylane grâce à une méthode ne nécessitant pas l'utilisation de solvants nocifs.

Une discussion générale et une section de conclusion et perspective terminent le manuscrit. En outre, deux manuscrits déjà publiés sont également joints au document, correspondant à des travaux menés en collaboration avec d'autres chercheurs et qui ont constitué une contribution au développement des micro et nanoparticules à base xylane spécifiquement mises au point au cours de cette thèse.

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SECTION I

EVALUATING AMPHOTERICIN B INTERACTIONS WITH BIOLOGICAL FLUIDS AND BARRIERS
INTRODUCTION

Amphotericin B is one of the most effective antifungal drugs used to treat life-threatening systemic fungal infections, and is also indicated for the treatment of visceral leishmaniasis. Amphotericin B is also well known for its severe side effects, such as nephrotoxicity intrinsic from the molecule accumulation on the kidneys. However, the use of this drug is also restricted to hospitals due to the long infusions necessary for its administration, which hampers the access of the population with lower resources, mostly in developing countries.

The development of an oral dosage form for amphotericin B could reduce the risks of its administration and also increase the total amount of the population that could be treated due to the possible decrease on the costs of the treatment.

Nowadays, there have been quite successful reported showing the absorption enhancement of hydrophobic drugs by the use of drug delivery systems, most of them using nanotechnology. Even for amphotericin B, there have showed nanocarries able to enhance drug’s absorption. However, none of the reports (for the knowledge of the authors) revealed the mechanism used for these nanocarriers to overcome the biological barriers present in the whole length of the gastrointestinal tract.

Then, one of the goals of this thesis was to try to observe physic-chemical properties that would be involved on amphotericin B absorption, and if there is any influence if the drug is on its monomeric or aggregated form. Additionally, it was tried to see if lipid-based nanocarriers would enhance the transport by paracellular or transcellular route.

Furthermore, it was tried to observe how amphotericin interacts with different blood proteins, such as albumin and protein C3 (immunological complement system).
CHAPTER I

OVERCOMING INTESTINAL BARRIERS TO AMPHOTERICIN B ORAL ABSORPTION WITH NANOMEDICINES: WHERE ARE WE?
BRIEFING

The utilization of amphotericin B (AmB) dates from 1958, when it started to be commercialized for the treatment of fungal infections, despite its important toxicity, mainly nephrotoxicity. For this reason, pharmaceutical scientists have kept the studies with this molecule aiming to better understand its molecular properties and to find better tolerated dosage forms as well as making possible an oral delivery of this therapeutically useful molecule.

In the middle of the 1990’s, new dosage forms for AmB obtained their approval from the FDA. Most of them were lipid-based nanocarriers able to keep AmB in its monomeric state, and AmBisome® have found clinical utility. However, these already approved AmB dosage forms only partially solved the problem of AmB delivery due to their prohibitive costs and the necessity of hospitalization to carry out the treatment.

The drawbacks evolving AmB mentioned above may explain why studies with this drug are still being carried out with the majority of them focusing on the development of oral dosage forms. The aim of this review was to compile some data about the history of AmB utilization on clinics and the approaches based on nanotechnology that are currently being investigated to develop an oral delivery system for this effective antifungal drug.
Overcoming intestinal barriers to amphotericin B oral absorption with nanomedicines: where are we?

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ABSTRACT

The development of a dosage form for oral effective delivery of amphotericin B (AmB) is an attractive goal for pharmaceutical scientists, which is prompted by the necessity to overcome many inconveniences of intravenous administration, and costly commercialized formulations, as well as the aim of reducing the high incidence of adverse and toxic effects. In the 1980’s AmB and other poorly or very poorly water soluble molecules led to the development of nanocarriers, such as AmBisome® and Doxil®, conceived for being intravenously infused. The aim of this review was to focus on the possible pathways for AmB oral absorption and to analyze the trends in the development of nanocarriers for AmB intended to oral administration. Within this purpose, a systematic search on the Web of Science® database was carried out and data analysis was performed in order to clarify the scenario of the development of nanocarriers for the oral route. It was observed that the research in this subject is concentrated in few countries and the increasing number of publications in the latest’s years suggests that the subject is becoming a “hot topic”. Basically, two classes of nanomaterials are being investigated, lipids and polymers. Furthermore, some in vivo data comparing these investigational nanocarriers with the commercialized AmB formulations such as AmBisome®, showed that promising results could be expected. In conclusion, the analysis of the literature corpus suggests that the successful development of an AmB nanomedicine for an efficient delivery of this molecule requires both an in-depth understanding of the unique aggregative characteristics of this molecule, as well as the development of new delivery technologies, which obviously are not yet available.

Keywords: Amphotericin B; oral; nanotechnology.
1. Introduction

Amphotericin B (AmB) is antifungal molecule successfully used for the treatment of life-threatening systemic fungal infections, such as visceral leishmaniasis. Its use on therapeutics dates from 1958, when it was launched on the market under the form of a micellar solution containing sodium deoxycholate, well known as Fungizone®. Very rapidly, adverse effects, such as fever, chilling, vomiting, headache, nauseas and nephrotoxicity, were reported once this drug was used for chronic treatments [1, 2].

For this reason, in the following four decades researchers tried to develop new dosage forms that would reduce its toxicity. Viable alternatives were finally available only recently on the market [1, 3]. This long time for the development of a new dosage form can not only be attributed to the bureaucracy of the regulatory agencies on the approval of new drug dosage forms, but mostly to the complex properties of AmB, stemming from its chemical structure (Figure 1a).

In fact, it is now well known that some uncommon physicochemical properties of AmB are responsible for its pharmacological activity, its toxicological profile and the huge difficulties to find safe and efficient pharmaceutical formulations [4]. AmB structure (Figure 1a) comprises a glycosylated lactone, a polyhydroxylated region, a seven carbon chain comprising intercalated double and single bonds, as well as the simultaneous presence of a carboxylic group and an amino group, which provide to this molecule amphoteric and amphiphilic characteristics, a very low solubility in solvents and a pronounced tendency to self-aggregate. AmB pharmacological activity is based on the ability of this molecule to strongly associate to ergosterol, specifically present in the membrane bilayer of fungi, where it forms pores (Figure 1b). Therefore, any uncontrolled aggregation in the body will result in the loss of its selectivity to ergosterol-based membranes and an increase on toxicity [5-9].
Thus, the general strategy to obtain safe and efficient dosage forms of AmB has consisted for long and still consists on the attempt to control its aggregation tendency and to conceive formulations which would be able to keep AmB in its monomeric form all over the ADME pathway. For obvious reasons, lipids/lipophilic or amphiphilic molecules have constituted up to now the best candidates for attempting to solve these problems.

The approvals for Abelcet®, Amphotec® and AmBisome® were given by the FDA in the middle of the 1990’s considering their efficacy and the reduction of the adverse effects [3]. Nonetheless, these dosage forms still have high costs and need patient’s hospitalization due to their administration by parenteral infusions. Thus, these new dosage forms only partially solved the problem. Further, many countries under development, such as India and Brazil, are not be able to spread this treatment in their entire territories due to their high costs [10, 11]. In this context, the development of oral dosage forms looks as a an interesting alternative in order to increase the feasibility of the treatments by preventing patient’s hospitalization, not only increasing patient’s compliance but also simplifying medicine distribution circuits and logistics concerns [11].

In this context, the objective of this review was to compile the different strategies that have been followed so far to develop AmB oral dosage forms. For this purpose, a systematic methodology of research has been used consisting in browsing systematically the Web of Science® database, chosen because it gathers a very large part of publications in the field of drug delivery. Hence, the keywords “amphotericin B”, “oral” and “nano*” were selected and the analysis was carried out up to 16/January/2015. Additionally, searches combining these three keywords in different ways have been also done, as well as searches using the keywords “nasal” and “intravenous” by the replacement of the word “oral” in the search engine. In total, when the words “amphotericin b”, “oral” and “nano*” were used, the research yielded
70 references, which were reduced to a number of 54 once the refinement “Articles” was used which excluded most of the reviews about amphotericin B and oral drug delivery systems which were present in the raw analysis. From these 54 research publications, 30 were later excluded because they were not related with the use of AmB or its utilization by the oral route. or were still reviews about close related themes, which were not initially filtered, or at the articles that were not accessible from the local network. The refined 24 articles were all identified by a “*” after their title in the References section. Additionally, the U.S. Food and Drug Administration (FDA) database (FDA Orange Book) was used to obtain information about the market dosage forms that contain amphotericin B and the discontinued commercialization and/or authorizations. Furthermore, other references were inserted into the text to support the necessary information about the different types of nanocarriers described in this review.

2. Who is interested in this subject?

The inherent right to life ought to be of interest for the entire world as explicit in the article 6.1 of the International Covenant on Civil and Political Rights – “Every human being has the inherent right to life. This right shall be protected by law. No one shall be arbitrarily deprived of his life.” [12]. However, if we consider that this right to life can be extended to health preservation it also means that prevention and cure of systemic fungal infections along with leishmaniasis are directly concerned. Coincidently, some of the countries which are the more affected by leishmaniasis, such as India, are the ones that are investing more in the development of nanocarriers to improve AmB oral bioavailability, as can be seen in Figure 2. The interest of India in the development of an oral dosage form for AmB is straightforward, once this country is an endemic area for leishmaniasis according to the World Health
Organization (WHO), and due to the probable reduction of the costs of the treatment, which for a country under development is really important in order to provide a good health care to its population. Further, the use of AmB for the treatment of antimony-resistant infections in the Region of Bihar, India has been reported, making interesting attempts to offer improved formulations to these populations [13]. Whatsoever, the interest in the development of an AmB oral dosage forms is not restricted to endemic areas. Other countries, such as England, France and Canada have also been working on the development of nanocarriers for the oral administration of AmB (Figure 2).

Obviously, the interest of pharmaceutical scientists in investigating the delivery of AmB by the oral route has been a major subject of research when compared with other routes of administration. However, when the delivery approach involve the use nanotechnologies (here identified by the concomitant use of the keywords "oral" and “nano*”) a considerable shrinkage in the number of publications was seen (Figure 3). Furthermore, when considering the combination of the keywords "nano*" and "oral" this combination gave more results than the other routes, such as “nasal” and “intravenous” routes, suggesting that the effective enhancement of AmB absorption by the oral route is a common goal of pharmaceutical scientists around the world.

3. **Amphotericin B dosage forms available on the market.**

AmB is a molecule with unusual physicochemical properties, such as very low water solubility, and simultaneous amphoteric and amphiphilic characteristics. These are properties that make the design of AmB dosage forms a huge challenge, whatever the route of administration. Nowadays, four dosage forms of AmB are available on the market, namely Fungizone®, AmBisome®, Abelcet®, and Amphotec®.
Fungizone® was the first dosage form approved and is based on the mixture of AmB with sodium deoxycholate in order to form a micellar solution. However, the prolonged use of this dosage form led to nephrotoxicity and other adverse effects, leading to the development of new drug delivery systems to try to diminish these side effects. Therefore, unsuccessful new AmB dosage forms were progressively marketed from the beginning of the 1980’s until the middle of the following decade, when Abelcet®, Amphotec® and AmBisome® were approved in 1995, 1996 and 1997, respectively. Abelcet® was the second nanotechnological-based dosage form ever approved by the FDA, only three days after the Doxil® (doxorubicin) approval on the 17th November 1995, while Abelcet® had its approval on the 20th November 1995 [3, 14].

All these new AmB dosage forms are based on the use of lipids able to interact with AmB and attempting to keep AmB in the monomeric state (i.e. to avoid AmB aggregation and consequently reduce the toxic effects caused by the presence of aggregates into the human body) [15-19]. As it will be described in the next sections, the use of lipids is also one of the currently adopted approaches to try to enhance AmB absorption through the GIT, since the role of aggregation state of AmB during the absorption process could be major in decreasing its diffusibility through the intestinal epithelium. In this context, it should be remembered that the marketed lipid-based nanomedicines never showed an acceptable level of absorption in order to be used by this route. The FDA Orange book proves this fact, once no mention of oral administration of AmB for these dosage forms is described [3].

4. Nanocarriers proposed for oral delivery of AmB accordingly to their raw materials

Despite the prefix "nano", nanocarriers are very large objects compared to the drug molecules they are carrying, since they are often constituted by auto-assembling of different materials
(lipids, polymers, drug molecules, etc) selected to achieve different functionalities into a single object. In the context of oral delivery, nanocarriers could be of interest because of the existence of two well-documented mechanisms for the absorption in living organisms (Figure 4). First, once they are large objects compared to drug molecules, they can reach the lymphatic route, which implies that they ought to be captured by the gut associated lymphoid tissues (GALT) including Peyer's patches. This phenomenon is especially significant for particles with hydrophobic surfaces, such as the one based on lipids. The second mechanism (which does not excludes the occurrence the first one) is a mucoadhesion phenomenon. In this case, depending on its properties (surface, morphology, etc) the nanocarriers can diffuse into the mucus hydrogel lining to the epithelium and be temporarily immobilized in front of the enterocytes. From this location at the mucosal surface, and depending on the kinetics of release of the drug out of the carrier, considerable increases in the absorption can be observed and sometimes it can result in a spectacular oral bioavailability improvement [20]. Additionally, direct translocation of the nanocarriers through the intestinal membrane can be observed, although it is likely that such a phenomenon is quantitatively not predominant and often unwanted, due to possible toxicity concerns, especially when chronic treatments are envisioned. Finally, as it can be seen in Figure 5 that strategies are almost equally shared between polymeric and lipid-based nanocarriers.

4.1. Lipid-based nanocarriers

As previously mentioned, the new marketed parenteral dosage forms of AmB are all based on the use of lipids in their composition. However, when delivered by the oral route, these lipid-based carriers are not able to increase AmB bioavailability [21]. In fact, it can be hypothesized that the type of the lipids used to produce the supramolecular structures may
play a key role on their ability to generate an adequate release of AmB from the nanocarriers to control the aggregation state of AmB into the physiological fluids, which may not be adapted to enhance AmB oral absorption [22].

Among these structures, nanoemulsions and solid lipid nanoparticles are the most reported ones [23, 24]. Nanoemulsions are colloids produced with liquid lipids such as medium chain triglycerides and essential oils [27, 28]. Solid lipid nanoparticles are colloidal dispersions made from solid lipids at the human body temperature, such as glycerides and fatty acids (or waxes) [25, 26]. However, their stabilization is usually obtained by using biocompatible surfactants such as phospholipids, Tween® and Span® [25-28].

The analyses of the articles selected for this review revealed that in general, the control over the droplet/particle size distribution of these lipid-based nanocarriers does not seem essential for oral delivery. In fact, the average sizes of the described nanocarriers were in the range of 50 – 250 nanometers and the values of polydispersity indexes (PdI) close to 0.6 as it can be seen in Table I. Additionally, nothing was mentioned in the articles about an optimization step of the technique of production to obtain sharper droplet/particle size distributions [29].

Moreover, the zeta potential of these nanocarriers is usually not described, even though this property has been often related with mucoadhesion properties [30].

The reports that tried to reveal or to hypothesize the possible pathways taken by these nanocarriers to enhance the AmB absorption only showed a possible role of the gut-associated lymphoid tissue (GALT) (Peyer’s patch and M cells) (Figure 4) on the uptake of the lipid-based particles/complexes [2, 23]. Nevertheless, the increase in the drug residence time due to the interaction of the lipids with the mucus has also been cited [23]. Additionally, and quite surprisingly, the biodistribution of AmB after oral delivery also seems to be under the influence of the nanocarriers used, once no similar accumulation into the organs has
been described among the different nanocarriers under development. Gupta et al. observed an accumulation of AmB into the spleen and the liver when using copaiba oil-based nanoemulsions, while Ibrahim et al observed a low AmB concentration in the spleen but higher concentration into the liver by the use of lipid-complexes systems [2, 31]. It has also been suggested that the biodistribution of the AmB is linked to the phagocytic activity of macrophages considering that AmB was locally accumulated in the infected tissues [2, 32]. Interestingly, these experimental evidences suggest that differences in AmB distributions in the body could result of the direct absorption of the nanocarriers themselves, which would be differently distributed in the body, in accordance to differences in their composition and their structure.

4.2. Polymer-based nanocarriers

Polymers are very often used as building blocks for the preparation of nanocarriers. These nanocarriers can adopt various structures belonging either to the class of nanospheres or nanocapsules. The FDA has already approved the use of few polymers including PEG and PLGA, which were found in these publications. Also, partially hydrolyzed chitosan, a biopolymer that shows biocompatibility and is often selected for the development of nanocarriers due to considerable mucoadhesion properties [1, 33-36].

Polymer-based nanocarriers have been gathered in Table 2, including their average particle sizes, polydispersity indexes (PdI-values) and zeta potentials. The PdI-values for these nanocarriers were reported as in the range of 0.15 and 0.40, which are large polydispersions and suggest that not much efforts has being made to obtain narrowed size-distributions when these nanocarriers are being developed for oral delivery [30, 33, 35]. Zeta potential measurements are normally used for characterizing the surface properties of particles due to
the insertion of specific polymers in the structure of the nanocarriers. Indeed, the purpose of
this analysis can be easily seen when chitosan is used, due to the correlation between its
positive electrical charge and its mucoadhesion properties. Consequently, if chitosan was
chemically cross-linked [33] or modified through any process that could change its electrical
charges, this modification could imply on a reduction of the mucoadhesion properties of the
new material in comparison with the raw chitosan [34, 35].

The same pathways described for lipid-based nanocarriers can be invoked for explaining why
polymer-based nanocarriers are able to some extent to enhance the absorption of AmB. Indeed, the uptake of these supramolecular structures by the GALT endocytic mechanisms
has been reported [34, 36]. Enhancements in AmB absorption have been observed for these
nanocarriers with varying intensities, ranging from 4 – 8 fold, when compared with the free
drug or the Fungizone®, for which intestinal absorption is almost null [30, 34, 35]. In
addition, when the pharmacological efficacy of AmB was measured using either polymer-
based nanocarriers or AmBisome® it was showed that polymeric micelles administered
during 10 days could be as efficient as an intravenous delivery of AmBisome®, and could
allow a reduction higher than 90% in the parasitic replication. [36].

4.3. Other nanocarriers

Apart of lipidic and polymer-based nanocarriers some applications based on the use of
dendrimers (monodispersed macromolecules with a regular and highly branched three
dimensional structure) [37-39], carbon nanotubes (rolled up graphene sheets in multiple or
single layers in forming concentric cylinders) [40] and silica mesoporous nanoparticles
(nanocarriers based on silica e.g. tetraethyl orthosilicate that have on their structure many
porous cavities able to entrap active molecules, and protect them from degradation) have
been reported [13, 41, 42]. Due to the scarcity of the publications in this area, no particular trends can be given, except by the fact that neither dendrimers nor silica mesoporous nanoparticles had any enhancing effects. Further, the potential of carbon nanotubes intended to increase AmB absorption, has been only evaluated through pharmacological activity determinations [13].

Furthermore, other approaches can be reported, including the association of AmB into cubosomes (liquid crystalline particles exhibiting a cubic crystallographic symmetry and formed by self-assembling of amphiphilic or surfactant-like molecules) [43, 44] and nanocrystals/nanosuspensions (In general, it is considered a nanocrystal a crystal of the drug in the nanometric range under suspension) [10, 45].

### 5. General discussion

AmB is a hard drug molecule to formulate and the development of an oral dosage form for this drug is still a challenge, which keeps pushing pharmaceutical scientists to develop new technologies, as it happened in the past with AmBisome®. From a clinical point of view, there is a growing interest in the design of oral dosage forms suitable for allowing a safe and an efficient delivery of AmB, which is can be observed by a significant raise in the number of publications in the field. Likewise, it is possible to see that this interest is not only from countries which are considered endemic by the WHO, but also from countries that do not have high incidence of these parasitic diseases (Figure 2).

These oral nanocarriers mainly fall into two categories, namely lipid- and polymeric-based nanocarriers. Additionally, when AmB absorption enhancements are observed, the global analysis of these publications suggests that an uptake of the lipid and/or polymeric droplets/particles by the cells related to GALT and the lymphatic route could be the more
likely pathway for explaining AmB absorption. Moreover, some polymeric nanocarriers have been shown to be able to interact with the mucus layer by ionic interactions that increase their residence time and lead to an increase on drug absorption by longer exposure (Figure 3). Furthermore, some of the carriers attempt to take advantage of the two approaches (described above) concomitantly in order to reach the desired enhancement effect [46].

An interesting fact about the nanocarriers here reviewed is the use of a common ingredient, independently of the designed nanocarrier. D-alpha-tocopherol polyethylene glycol succinate is an amphiphilic molecule that has been used as a stabilizer for micro- and nanocarriers. It is often used for improving AmB entrapment efficiency into the nanocarriers, not only because of a high emulsification efficiency, but also because it is known as an inhibitor of P-glycoprotein, which could result in an increased cellular uptake and in a reduction of the oxidative stress caused by scavenging free radicals [1, 47]

6. CONCLUSIONS

Successful approaches for oral delivery of AmB have been reported, which in some cases are comparable with AmBisome® administered by the IV route. However, none of these nanocarriers already reached the market. The detailed analysis of the literature showed that so far lipidic and polymer-based nanocarriers have been prepared and evaluated. It is quite reasonable to hypothesize that AmB absorption from these carriers implies their capture by the GALT. Whatsoever, these absorption mechanisms should be the subject of deepest investigations for consolidating and improving formulation strategies for AmB oral delivery.

ACKNOWLEDGEMENTS

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Figure Captions

**Figure 1:** (A) Chemical structure of amphotericin B. (B) Molecular poring mechanism explaining the pharmacological effect of AmB against fungi and parasites; Step 1: interaction of mycosamine ring with the exposed hydroxyl of ergosterol, Step 2: Insertion of AmB molecule among ergosterol molecules and stabilization of the physical binding through the carbon chains interactions and the hydroxyl at the carbon 35, Step 3: Formation of porous in the membrane and consequently flow of ions.

**Figure 2:** (A) Location of the countries where researches dealing with the development of nanomedicines conceived for an oral delivery of AmB are located. (B) Worldwide prevalence of visceral leishmaniasis, according to WHO (accessed 06/January/2016, http://gamapserver.who.int/mapLibrary/Files/Maps/Leishmaniasis_VL_2013.png?ua=1). Not surprisingly, the comparison of the two sets of data demonstrate that research efforts for improving AmB delivery are concentrated in concerned countries.

**Figure 3:** Ranking of the different routes investigated for improving AmB delivery and the corresponding part of nanomedicines among the different formulation strategies investigated.

**Figure 4:** Different strategies for enhancing oral absorption by associating drugs to nanocarriers. The design of nanocarriers to exploit these different absorption pathways has been investigated so far to improve AmB absorption.
**Figure 5:** Publications describing the use of nanocarriers aiming to enhance AmB oral bioavailability and depending on the materials used for their preparation.

**Figure 6:** Kinetics of publications devoted to the oral delivery of AmB by the mean of nanocarriers accordingly to an analysis of the Web of Science® database, using “amphotericin B”, “oral” and “nano*” as the keywords and filtered by year of publication.
**Figure 1:** (A) Chemical structure of amphotericin B. (B) Molecular poring mechanism explaining the pharmacological effect of AmB against fungi and parasites; Step 1: interaction of mycosamine ring with the exposed hydroxyl of ergosterol, Step 2: Insertion of AmB molecule among ergosterol molecules and stabilization of the physical binding through the carbon chains interactions and the hydroxyl at the carbon 35, Step 3: Formation of porous in the membrane and consequently flow of ions.
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![Frequency of Visceral Leishmaniasis](image)

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<td>2</td>
</tr>
<tr>
<td>Portugal</td>
<td>2</td>
</tr>
<tr>
<td>Thailand</td>
<td>2</td>
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<tr>
<td>UK</td>
<td>8</td>
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</table>

![Status of Endemicity](image)
Figure 3: Ranking of the different routes investigated for improving AmB delivery and the corresponding part of nanomedicines among the different formulation strategies investigated.
Figure 4: Different strategies for enhancing oral absorption by associating drugs to nanocarriers. The design of nanocarriers to exploit these different absorption pathways has been investigated so far to improve AmB absorption.
**Figure 5:** Publications describing the use of nanocarriers aiming to enhance AmB oral bioavailability and depending on the materials used for their preparation.
Figure 6: Kinetics of publications devoted to the oral delivery of AmB by the mean of nanocarriers accordingly to an analysis of the Web of Science® database, using “amphotericin B”, “oral” and “nano*” as the keywords and filtered by year of publication.
Table Captions

**Table 1:** Description of the lipid-based nanocarriers physical properties, as well as, their composition.

**Table 2:** Description of the polymer-based nanocarriers physical properties, as well as, their composition.
Table 1: Lipid-based nanocarriers developed to try to enhance AmB absorption into the gastrointestinal tract.

<table>
<thead>
<tr>
<th>Nanocarrier</th>
<th>Material</th>
<th>Size (nm)</th>
<th>PdI</th>
<th>Zeta Potential (mV)</th>
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<tbody>
<tr>
<td>Nanoemulsion</td>
<td>Propylene glycol monocaprilate</td>
<td>67.32</td>
<td>0.635</td>
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<td>[29]</td>
</tr>
<tr>
<td></td>
<td>Polysorbate 80</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D-alpha-tocopherol polyethylene glycol succinate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Peceol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gelucire 44/14</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Polysorbate 80</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Poly(ethylene glycol) 400</td>
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<tr>
<td></td>
<td>Lecithin</td>
<td>298</td>
<td></td>
<td>-55</td>
<td>[48]</td>
</tr>
<tr>
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<td>Tween 20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chitosan + EDTA microparticles</td>
<td>90</td>
<td>0.316</td>
<td>1.1</td>
<td>[46]</td>
</tr>
<tr>
<td></td>
<td>Captex 355: Labrasol (70:30)</td>
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<tr>
<td>Self-emulsifying</td>
<td>Copaiba oil</td>
<td>127</td>
<td>0.11</td>
<td>-38.5</td>
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<tr>
<td>Nanocarriers</td>
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<td></td>
<td></td>
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<tr>
<td></td>
<td>D-alpha-tocopherol polyethylene glycol succinate</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>lecithin</td>
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<td>0.19</td>
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<td>beewax</td>
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<td></td>
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<tr>
<td></td>
<td>sodium cholate</td>
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<tr>
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<td>lecithin</td>
<td>222</td>
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</tr>
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<td>oleic acid</td>
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<td>theobroma oil</td>
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<td>sodium cholate</td>
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<tr>
<td>Solid lipid</td>
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<td>171</td>
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<tr>
<td></td>
<td>Cholesterol</td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td>Ceramides</td>
<td></td>
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</tr>
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<td>Liposomes</td>
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<td>Nanocochleates</td>
<td>Dioleoylphosphatidylserine cholesterol</td>
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### Table 2: Description of the polymer-based nanocarriers physical properties, as well as, their composition.

<table>
<thead>
<tr>
<th>System</th>
<th>Material</th>
<th>Size (nm)</th>
<th>PdI</th>
<th>Zeta Potential (mV)</th>
<th>Ref</th>
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<tr>
<td>Nanocapsules</td>
<td>Poly(lactic co-glycolic acid) – Poly(ethylene glycol)</td>
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<td>-</td>
<td>-</td>
<td>[1]</td>
</tr>
<tr>
<td></td>
<td>Capric acid /caprilic acid</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Polyvinylpyrrolidone</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Polyoxylethylenepolyoxypropylene block copolymer</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D-alpha-tocopherol polyethylene glycol succinate</td>
<td>-</td>
<td>-</td>
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<td>0.274</td>
<td>15.3</td>
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<td>gelatin</td>
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<tr>
<td></td>
<td>glutaraldehyde</td>
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<td>Nanospheres</td>
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<td>0.37</td>
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<td>Porphyran tripolyphosphate</td>
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<td></td>
<td>Poly(lactic co-glycolic acid)</td>
<td>113</td>
<td></td>
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<td>[34]</td>
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<tr>
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<td></td>
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<tr>
<td></td>
<td>Poly(lactic co-glycolic acid)</td>
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<td>-15.4</td>
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<tr>
<td>Polymeric micelles</td>
<td>N-Palmitoyl-N-monomethyl-N,N-dimethyl-N,N-trimethyl-6-O-glycol chitosan</td>
<td>216 and 35 (bimodal distribution)</td>
<td>-</td>
<td>-</td>
<td>[36]</td>
</tr>
</tbody>
</table>

*Université Paris-Saclay*
Université Paris Sud / Institut Galien Paris Sud (CNRS UMR 8612), 5 rue Jean-Baptiste Clement, 92290, Châtenay-Malabry, France
CHAPTER II

INTESTINAL PERMEABILITY OF AMPHOTERICIN B AND THE ROLE OF NANOMEDICINES ON ITS ENHANCEMENT.
BRIEFING

The use of amphotericin B is always related with intravenous administration due to its poor solubility and low permeability in biological fluids and membranes. Indeed, the four major market dosage forms of AmB have their administration based on infusions procedures. Then, the development of an oral dosage form for this drug is a big challenge for pharmaceutical research groups.

Additionally, the infusions are also related with the formation of AmB aggregates into the body. The AmB aggregates are related with the many of its undesirable properties in the human body, mainly due to the AmB aggregates lose its specificity to ergosterol membranes and bind to cholesterol-based membranes.

On this work we tried to evaluate the permeability of AmB on the intestinal level at different aggregation states. Also there were performed assays with AmB-loaded micro- and nanoemulsions, which have their ability of enhance hydrophobic drug absorption often described in the literature.
Amphotericin B aggregation and intestinal absorption: Effect of AmB association to serum albumin or to micro- and nanoemulsions.

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ABSTRACT

Amphotericin B (AmB) is a polyenic molecule highly effective for the treatment of life threatening systemic fungal infections and leishmaniasis. AmB is mostly delivered by the intravenous administration, which requires patient’s hospitalization and increases the costs of therapeutics. However, the development of oral dosage forms is still a challenge for pharmaceutical scientists, even though, some successful results have recently been published. One of the challenges is the lack of knowledge about the pathways that are really opened for AmB systemic absorption. Various attempts to reach the lymphatic vessels by using lipids and particulate systems have been made with few successful ones. Thus, the aim of this work was to check the influence of the aggregation state of AmB on its jejunal permeability. In this purpose, AmB was complexed to albumin and also associated to micro- (ME) and nanoemulsions (NE) droplets. No AmB permeability was observed in any of the experimental conditions even though losses in the donor compartment were systematically detected. The electrophysiological data revealed a decrease in tissue viability in presence of AmB-BSA complexes, which were designed to control the presence of AmB monomers, while the viability was unaffected by the use of AmB-loaded ME and NE. In conclusion, the the AmB-albumin complexes were unable to control the AmB aggregation level. Also, the non permeability in all conditions suggests that AmB absorption through the classical (passive or active) pathways faced huge obstacles, probably partly due to the difficulty to create high enough concentrations of AmB in the monomeric form. Additionally, the different interactions that happen due to the entrapment of AmB into the micro- and the nanoemulsions had unexpected effects on tissue viability as it could be observed that supramolecular structures containing surfactants and medium chain fatty acids were much less toxic as the AmB-BSA complexes, conceived for maximizing the amount of free monomeric AmB.

Keywords: amphotericin B, Ussing’s Chamber, permeability, electrophysiology.
1. INTRODUCTION

Amphotericin B (AmB) is a polyenic molecule widely used for the treatment of systemic fungal infections and leishmaniasis [1-3]. Due to its very poor aqueous solubility and ability to cross efficiently the epithelial barriers, AmB is usually delivered through intravenous infusions, with essential patient hospitalization due to the long duration of the infusions [4]. In fact, many efforts have been done in order to develop a suitable oral dosage form for AmB. Different formulation approaches have been investigated, many of them successfully reported as absorption enhancers for other molecules characterized by low water solubility and/or a permeability through the gastrointestinal membrane, but unfortunately with limited success for AmB so far. The resolution of this challenge of AmB oral absorption could have a significant impact for the treatment of these pathologies, because of an expected increase in patients compliance, as well as a decrease in the risks of infections and financial costs [5-7].

The use of nanocarriers, as an approach to enhance drug absorption, has received much attention during the last decades [8-11]. Nanocarriers under development are mostly based on lipids and polymers, which are able to interact with fluids and barriers of the gastrointestinal tract. The lipid-based systems, such as micro- and nanoemulsions, usually have their enhancement ability justified by the presence of long chain lipids, which forms complexes with the drugs and allows them to be absorbed by the lymphatic route [12]. The polymeric systems also have their ability to enhance drug absorption explained by their uptake by cells associated with the lymphatic system, and also by the interaction between the nanoparticles and the epithelium, which would provoke the aperture of the tight junctions [13, 14].

Many techniques have been used to identify the pathways used by the nanocarriers to enhance drug absorption. Those techniques could be easily classified according to the level of complexity, as it follows: *in vitro*, by the use of monolayers of Caco-2 cells or co-cultured
with other cell lines, such as HT29-MTX mounted on Transwell® plates or in Ussing’s Chambers (making it possible the measurement of electrophysiological parameters) [15, 16]; 
ex vivo, using mounted fresh tissues, mostly from rats and human biopsies, using Ussing’s Chambers; and in vivo in animals or from clinical trials in humans under controlled conditions. Indeed, quite satisfactorily correlations between the results obtained with these approaches and the in vivo approach in humans have been established, which suggest that the use of those techniques is of interest for helping formulators on evaluating their strategies to enhance drug absorption [17].

The objective of this work was to evaluate the permeability of AmB over the jejunal tissue from rats by focusing on the impact of physicochemical state of AmB in front of the intestinal membrane. Indeed, once AmB has been released from its formulations, it is possible that it can exist under different physicochemical states, ranging from large aggregates to "monomeric" forms (i.e., molecular state). In this context, soluble AmB complexes with albumin, AmB aggregates in the presence of AmB-BSA complexes), and finally AmB-loaded micro- and nanoemulsions were used to create different physicochemical presentations of AmB and evaluate its absorption using the Ussing’s chamber model.

2. METHODOLOGY

2.1. Materials

Amphotericin B was purchased at Spectrum Chemicals (USA), methanol HPLC grade was purchased at Merck (France), acetonitrile HiperSolv® CHROMANOFORM was bought at VWR- PROLABO Laboratories (France). Bovine serum albumin, Tween® 80, Span® 80, calcium chloride, glucose, natamycin, potassium phosphate monobasic and sodium chloride were bought at Sigma-Aldrich (France). Sodium bicarbonate was purchased at Fluka
(France). Magnesium sulphate and formic acid was purchased at Carlos Erba (France). Ultrapure water was obtained by Milli® Q purification system. Lipoid® S100 and Mygliol® 812 N were donated by LIPOID GmbH (Germany) and Sasol (Germany), respectively.

2.2. Preparation of AmB-BSA complexes
Stoichiometry-controlled complexes of AmB and BSA (AmB-BSA) were prepared accordingly to Marcelino et al. [18]. First, AmB was dissolved in methanol and added into 4 %\(_{w/v}\) BSA dispersion on Krebs-Ringer-Bicarbonate Buffer (KRB Buffer). Then, methanol was evaporated under vacuum and the final dispersion was kept under room temperature until its use.

2.3. Preparation of AmB-loaded micro and nanoemulsions
In a first step non-loaded nanoemulsions were prepared accordingly to the methodology first described by Silva et al [19] with few minor modifications. Briefly, all the ingredients of the formulation were weighted according to Table 1 and after being stirred with a sonic probe for 2 minutes, they were placed in an ultrasound bath for 3 minutes. This cycle was repeated 3 times for each batch produced. Microemulsions were produced accordingly to the same procedure, but different compounds, as it can be seen in Table 1. However, stirring cycles were performed till translucent dispersions had been obtained. The loading of AmB in both type of emulsion-based systems was carried by the alkalization (NaOH 1N) of the nanocarriers, followed by the addition of the AmB powder under magnetic stirring. Then, the nanocarriers were let under stirring for 1h and their pH was neutralized (pH =7) with HCl 1N, as described by Silveira et al.[20].
2.4. Spectroscopic studies

UV-Vis spectra measurements were carried out with a Lambda 25 Perkin Elmer® UV-Vis spectrometer (France), using a 1cm length optical pathway quartz cell. Quantification and evaluation of AmB aggregation was evaluated at $\lambda_{\text{max}}= 415$ nm and 334 nm, respectively [21]. Circular dichroism analyses were performed in a Jasco® J-810 dichrograph (France), with 0.5, 1 and 2 cm length quartz cells. Wavescans were recorded from 300 to 450 nm and the identification of AmB aggregates was observed near to $\lambda = 325$ nm [18].

2.5. Droplet size and Zeta potential determinations

The particle size distribution of the micro- and nanoemulsions was determined by dynamic light scattering (incident light $\lambda = 633$ nm) in presence or absence of AmB in a calibrated NanoZS (Malvern®, England) at 25°C.

2.6. Intestinal permeability assays

Ussing’s chambers were used to determine the permeability of AmB through fresh jejunal rat tissues to AmB, depending on AmB state of aggregation (aggregated versus monomeric state), as well as from AmB-loaded micro- and nanoemulsions. Electrophysiological parameters (electrical current and voltage) were recorded along the experiments to determine the tissue viability. The detailed method was carried out as described below:

**Preparation of intestinal tissues.**

Jejunum from fresh small intestine of sacrificed male Wistar rats (200–250 g) (Charles River, Paris) were excised and rinsed with cold Krebs-Ringer-Bicarbonate (KRB) Buffer [22] and
cut into segments of 2–3 cm length. After visual examination of the tissue, sections containing Peyer’s Patches were discarded from the studies [14].

**Permeation experiments.**

Jejunum sections were mounted in Ussing’s chambers (intestinal surface was 1 cm\(^2\) and chamber volume was 3.5 mL) bathed with 4 %\(_{(w/v)}\) BSA dispersion on KRB Buffer at pH 7.3. The system was maintained at 37º C and continuously oxygenated with O\(_2\) /CO\(_2\) 95%/5%. At pre-set time intervals, aliquots of 400 \(\mu\)L were recovered from the acceptor chamber and replaced with the same volume of fresh medium. The assays were carried out for 3 h, and additional samples were taken at times \(t_0\) and \(t_{180}\) from both half-chambers (i.e. apical and basolateral compartments). Samples were analyzed for AmB content by liquid chromatography. Tissue viability (electrophysiological analysis) was also verified with a 4 %\(_{(w/v)}\) BSA dispersion on both sides of the mucosa in order to verify the suitability of the chosen carrier for AmB. The experiments were repeated on different days (\(n=6\), days =3).

**Electrical parameters measurements.**

During the experiments, four electrodes systems were used to perform the electrophysiological measurements. Transmucosal potential difference (PD) was continuously recorded between two KCl saturated agar bridges connected to a voltage clamp (Biomecatronics\(^{®}\), France) via calomel electrodes filled with saturated KCl solution. Electric potential difference was short-circuited throughout the experiment by establishing a short-circuit current (Isc) via agar bridges placed in each half-cell and adapted to platinum electrodes connected to an automatic voltage clamp (Biomecatronics\(^{®}\), France). Delivered Isc (short-circuit current) was corrected for fluid resistance and recorded at pre-set times. The
trans-epithelial electrical resistance (often referred as TEER) was calculated from Ohm’s law: TEER = PD/Isc. For comparing the different experiments runs, TEER values were converted to percentage (100% = 101 ± 46 Ω x cm²).

2.7. AmB quantification by UPLC-mass spectrometry

AmB quantification was carried out based by an UPLC technique previously described by Qin et al [23], except few modifications were done as described below.

Chromatographic conditions.
A Thermo Scientific UPLC system (Thermo Scientific Instuments, France) consisting of DIONEX® U-3600 pump, with a thermal controlled auto sampler and room for 96 samples. was used for AmB determination. The stationary phase was a 5 µ Gemini C18 column (2 x 100 mm, Phenomenex®). A gradient mobile phase comprising a solution A (0.1 %<sub>v/v</sub> formic acid aqueous solution) and solution B (methanol-acetonitrile, 2/3, v/v + 0.1 %<sub>v/v</sub> formic acid) was achieved on a 7 minutes duration at a total flow rate of 400 µL/min accordingly to the following steps, with 1.5 min of 80 % solution A at the beginning and followed by 0.5 min for solution A to decrease to 20 %, 3 minutes of 20 % solution A, then 0.5 to increase to 80% solution and finally 1.5 min of 80 % solution A. The column was kept at ambient temperature and the auto sampler temperature was kept at 4°C. The sample injection was set at 10 µL.

Mass spectrometric conditions.
A Quattro Ultima triple-quadrupole mass spectrometer (Waters®, France) with a spray ion source was used to analyze samples. AmB and natamycin (internal control) were detected by tandem mass spectrometry using MRM with a mass transition of m/z 924.9 → 743.5 and
666.5 → 503.2, respectively. The Mass Spectrum parameters were: Ionization mode, positive; Source gas, azote; Collision gas, argon; Ion Spray Voltage (Capillary), 3.0 kV; Cone, 35 V; Source temperature, 120 ºC; Desolvation temperature, 350ºC; Desolvation gas flow, 500 L/Hr; LM and HM Resolutions, 15.0. All the data were acquired using MassLynx software V4.1 SCN627 (Waters, France).

_Preliminary treatment of the samples._

Once the aliquotes are likely to contain contaminating proteins or glycoproteins, samples from the mounted tissues (300 µL from the 400 µL withdrawn), they were diluted in 900 µL of methanol in order to lead contaminants precipitation. These dilutions were vortexed for 30 s on a TopMix FB15024 (Fisher Scientific, France) and centrifuged (Sigma 201M, Fisher Scientific) for 20 minutes at 11000 rpm. Then, 10 µL of a 50 µg/mL natamycin solution was added to an aliquot of 380 µL the supernatants and transferred to the vials for chromatographic analysis.

_Preparation of standards._

Stock solutions of AmB (0.25 mg/mL) were prepared by dissolving 1 mg of AmB in 4 mL of methanol. These stock solutions were diluted in a 1:3 (v/v) mixture of KRB Buffer:methanol to produce seven standards plus one limit of detection (LOD). Solutions containing AmB at the following concentrations: 2500, 1000, 500, 250, 100, 50, 30 and 10 ng/mL were prepared. A stock methanolic solution (1 mg/mL) of natamycin, which was prepared and further, diluted in methanol at 50 µg/mL. Afterwards, 10 µL of this dilution were added into 380 µL of AmB solutions previously prepared.
2.8. Statistical analysis
The t-Student tests and ANOVA performed during data analysis were carried out with RStudio v0.99.467 loaded with R version 3.2.3 (2015-12-10), psych and lattice libraries.

3. RESULTS AND DISCUSSION

3.1. AmB-BSA complexes
In the situation of passive diffusion, it is widely recognized that the size of the permeating molecule is an important determinant of the permeability coefficient. In order to overcome the strong aggregation leaning of AmB in aqueous fluids, it was attempted to generate monomeric AmB molecules by taking advantage of the dissociation equilibrium of AmB-BSA complexes in water. Thus, AmB-BSA complexes were prepared under two conditions by introducing AmB in presence of bovine serum albumin (BSA) at the concentration of 4% (w/v) either below AmB critical aggregation concentration (CAC_{AmB-BSA}) ([AmB] = 3x10^{-6} M) or under ([AmB] = 1x10^{-5} M), taking in account the Critical Aggregation Concentration of AmB in the conditions used, CAC_{AmB-BSA} = 5x10^{-6} M [18].

3.1.1. Spectroscopic characterization
The UV-Vis spectra of AmB in aqueous media and in the presence of albumin have already been described in the literature [21, 24]. Figure 1 presents AmB spectra for AmB in the presence of BSA, either below or over the critical aggregation concentration of AmB in water. On this figure, it is possible to observe the AmB spectra changing along with an increase on the molar attenuation coefficient at the region of λ = 334 nm at higher AmB concentration. In fact, this phenomenon has been attributed to the formation of AmB aggregates [18]. Additionally, circular dichroism spectroscopy (Figure 2) revealed the
presence of a doublet, with its highest positive value in the region $\lambda \approx 325$ nm, for the highest AmB concentration used. These observations corroborated with the hypothesis that at the concentration of $1 \times 10^{-5}$ M there are AmB aggregates into the dispersion, while at the AmB concentration of $3 \times 10^{-6}$ M there are none or few AmB aggregates.

### 3.2. AmB-loaded emulsion-based systems

The production of AmB loaded micro- and nanoemulsions (ME and NE, respectively) was successfully achieved. At the end of the process, both systems showed proper macroscopic characteristics. The NE had the characteristic milky aspect (blank opaque homogenous dispersions), while the ME had a translucid yellowish aspect.

#### 3.2.1. Droplet size and zeta potential

Size distribution (average size and polydispersity index) as well as zeta potential of the blank and AmB-loaded ME and NE are presented in Figures 3a,b. Nanoemulsions displayed an average size of $198.0 \pm 0.6$ nm with a PdI = 0.2, which was not significantly affected by the addition of AmB into the formulation ($p = 0.583$). The average size of the droplets in the microemulsion was much smaller ($22.0 \pm 0.1$ nm) and was also not significantly changed after the addition of AmB ($p > 0.05$). However, the size distribution was quite heterodisperse (PdI = 0.25), which was clearly evidenced by the presence of two intensity peaks in the raw data graphs (data not shown) and once AmB was added the PdI-value increased to a value of 0.37. Additionally, both formulations showed strongly negative zeta potential values (Figure 3b), which was expected due to of the presence of acidic fatty acids (capric and caprilic acids) [19]. Furthermore, the addition of AmB into both systems reduced the average zeta potential, with the reduction in the ME being statistically significant ($p$-value $= 7.982 \times 10^{-7}$).
3.3. Amphotericin B quantification by UV/Vis spectroscopy and LC/MS

3.3.1. Drug loaded emulsion-based systems and AmB-BSA dispersions.

It was important to determine the amount of AmB present in the different preparations under the conditions encountered in Ussing’s chambers. In this purpose, AmB-BSA dispersions were quantified by the UV-Vis method described by Marcelino et al. [18]. The actual AmB concentrations were $3.2 \times 10^{-6} \pm 4.2 \times 10^{-7}$ M and $9.6 \times 10^{-6} \pm 7.0 \times 10^{-7}$ M for the samples theoretically containing $3 \times 10^{-6}$ M and $1 \times 10^{-5}$ M of AmB, respectively. Differently, the quantification of AmB into the ME and NE was performed by analyzing the first samples ($t_0$) placed in the donor chamber in permeability experiments. For them, only 1.5% and 5% of the total AmB loaded into the ME’s and NE’s were quantified, respectively. However, this low quantification may have different reasons to happen. First, the instability phenomena led by the dilution of ME and NE into the chamber by the buffer utilized to carry out the experiment, this dilution can consequently cause AmB precipitation into the chamber and once an aliquot is taken the AmB added was already precipitated (even though, no precipitation could be seen by naked eye during all the experiments). A second possible explanation, would be the rapid interaction between the AmB and the molecules of the mucus, which would retain them into its polymeric net. Furthermore, this quantification might be misled by the small amount of methanol used to extract the AmB from the aliquots. In general, when the drug entrapment efficiency of these ME and NE are carried out the ratio between the sample and the methanol is usually around 1:1000 v/v (formulation:methanol) and not 1:3 v/v as done in the permeability assays [20]. However, once the technique used for AmB entrapment was the same used by Silveira et al for the ME’s, the results here obtained are theoretically equal to theirs [20].
3.3.2. Extraction of AmB from AmB-BSA complex

Bovine serum albumin has been used to increase the apparent solubility of AmB in the KRB buffer into the receiver half-chamber of Ussing’s chambers, which was necessary to create a concentration gradient across the jejunal membrane. Therefore, the quantification of AmB in the donor compartment needed an extraction step of AmB from the albumin and possibly other materials from the tissue. An extraction technique was developed in this purpose using methanol and derived from the work of Polson et al [25]. Briefly, in order to get a calibration curve, mixtures of the samples and methanol in the ratios 1:1, 1:2, 1:2.5, 1:3, (v/v) were prepared from AmB-BSA dispersions containing AmB at the concentration of 4x10^{-6} M. After centrifugation, the supernatants were measured by UV-Vis spectroscopy. The degrees of albumin precipitation were obtained by the use of the linear equation previously obtained (y = 0.6636x + 0.0011, R^2 = 0.9999), while AmB extraction rates were calculated accordingly to the equation y = 12000x + 0.0023, R^2 = 0.9995, previously obtained. The average BSA precipitation was 99.5 ± 0.1 %, and the AmB extraction rates for 1:2.5 and 1:3 v/v were 86.5 ± 4.2 % and 88.7 ± 5.5 %, respectively. Even though, the ratio 1:2.5 v/v had an efficiency similar to the ratio 1:3, the last one was the ratio used, due to the later realized interference of methanol amount over AmB and natamycin peaks on the LC/MS measurements.

3.3.3. Evaluation of the method used to quantify AmB

The feasibility of the method was evaluated by the performance of calibration curves at the beginning and at the end of each day devoted to analyses. Additionally, due to the fast degradation rate of AmB, new AmB dilutions were prepared everyday. Then, the equation y = 4x10^{-5}x - 0.07, r^2 = 0.99895, where “y” is the “area of the peak” obtained in the LC/MS
analysis and “x” is the AmB concentration into the sample, was obtained and used for all the experiments.

3.4. Interaction between AmB and the intestinal epithelia

3.4.1. AmB permeability through the jejunum

AmB has been described as a very poorly water-soluble molecule and a very low permeable molecule. These characteristics classify AmB as a Class IV molecule, according to the Biopharmaceutical Classification System [26]. Therefore, it is crucial to understand the reasons for such a poor absorption before attempting to design an oral dosage forms suitable for this molecule. In this work it was attempted to evaluate whether AmB intestinal absorption could be related to its aggregation state. Indeed, it is now pretty obvious that AmB can exist in different aggregation states, ranging from "monomeric" state (i.e. molecular) to large "super-aggregates", and with a series of intermediate aggregates, including dimers and trimers, which have a strong impact on both pharmacological activity and toxicity. These different AmB species are believed to co-exist in dynamic equilibrium, that can become very complex when in presence of other binding molecules such as proteins and polysaccharides. In this work, the intensity of AmB fluxes through the intestinal membrane were thus evaluated in different conditions, corresponding to different aggregation states of AmB, using the Ussing’s chambers model. Two strategies were used to produce monomeric AmB molecules in front of the absorptive membrane, including the complexation of AmB to BSA and its entrapment in ME and NE. Rats jejunal fragments without Peyer's patches were selected in experiments in order to minimize any possible capture of AmB aggregates which could reach the lymphatic vessels. Diffusion experiments were performed in both directions, i.e. mucosal → serosal and serosal → mucosal. Strikingly, no AmB presence could
(detection limit by LC/MS = 50 ng/mL) be detected in the receiver chambers, whatever the direction, and whatever the system tested (AmB-BSA complexes, ME and NE), suggesting that AmB fluxes through the membrane were very low or at least under the detection limits. However, for all the experiments carried out, decreases in AmB concentration were observed in the donor side of the chambers, independently of the direction investigated (mucosal → serosal; serosal → mucosal) and on the type of systems investigated. It should be pointed out that duration of experiments in Ussing’s chambers are limited to 3 hours at the best, period during which tissue viability can be maintained.

Many hypotheses can be made to try to explaining these results, which are summarized in Figure 5. First, when exploring the possibility of molecular diffusion through the mucosa (passive or transporter mediated), the possibility that only a small amount of monomeric AmB reaches the enterocytes layer could be one of the reasons for this lack of absorption. Indeed, by comparison, it has been reported that the maximal solubility in the monomeric form was less that 1 µg/mL in serum [27] or even less (0,7 µg/mL in serum [28]), which may be the same rank of order in luminal intestinal fluids. Further, an efflux of AmB from the basolateral to the apical side of the membrane is still not identified, even though its efflux transportation by P-gp has been reported in the blood-brain barrier and one of the reasons for AmB resistance in some parasites [29-31]. Further, no difference was noticed in the disappearance of AmB from the donor compartment, whatever the orientation of the membrane, which would be likely in case of any efflux transport. Another hypothesis is in case of the release of monomers of AmB from the delivery systems that can lead to different phenomena in the vicinity of enterocytes, including direct re-aggregation of free monomeric AmB molecules to form larger aggregates which are unable to efficiently permeate the tissue. Alternatively, AmB binding to mucus glycoproteins and/or intra cellular components are
quite likely to happen. Indeed, AmB binding to proteins has not been reported only for albumin, but for many proteins [27]. Further, it has been reported that in plasma conditions the percentage of binding could even be increased when AmB concentrations are increased, due to the amphoteric nature of the molecule [27].

Finally, in the case of massive aggregation of AmB in front of the absorptive membrane or AmB encapsulated in ME or NE droplets, the Ussing’s chamber model was not adapted to detect such a particulate transport if any. Indeed, it is commonly accepted from the literature that lymphatic drainage becomes a significative absorption pathway for particulate materials, including nanocarriers. From our results it is possible to infer that the major pathways used by the nanocarriers to enhance drug absorption are not based on the paracellular or the transcellular transport, once these are the pathways most detected when Ussing’s Chambers methodology is used [32].

### 3.4.2. Tissue viability through electrophysiological data

The toxicity of AmB is strongly related to its aggregation state. The toxicity of AmB on jejunal tissues was observed by continuous measurement of the electrophysiological parameters of the jejunal fragment under study, including its electrical resistance. Figure 4 shows that jejunal tissue the transepithelial resistance (TEER) progressively decreased almost linearly with time until 180 min. TEER can be regarded as an indicator of tissue viability and thus of the toxicity of the formulations in contact with the mucosa. The exposition patterns had a considerable effect on the transepithelial resistance (TEER). Compared to the control (4 % w/v BSA dispersion), the contact with AmB-BSA dispersions accelerated considerably the loss of viability of the tissues (2-4 folds depending on the side of the tissue exposed to AmB). This reduction could be attributed to the presence of an increased amount of AmB able to
interact directly with the cell membranes of enterocytes and/or other epithelial cells. Indeed, amphotericin B has been shown to perforate enterocytes membranes pores, which are not controlled by the cell machinery \([33, 34]\).

Surprisingly, the tissue viability was less affected when the serosal side of jejunal fragments was exposed to AmB (again compared with 4 \(\%_{(w/v)}\) BSA dispersion), although the cells membranes are quite directly exposed to the drug because of the lack of any protective mucus layer on this side. The reason behind this phenomenon is unknown, but a possible hypothesis could be that under these conditions the AmB affinity for BSA could be higher than for the cell membranes lipids, while on the mucosal side AmB interactions with the abundant MUC2 type polysaccharides could create a local reservoir of AmB, which in turn could lately reach the tissue membranes and exert a toxic effect.

Furthermore, the exposition of the mucosal side of the jejunal membrane to ME and NE formulations had only a mild effect on tissue viability. Indeed, combined toxicity of AmB and the surfactants and phospholipids contained in the formulation \([35]\) were expected. On the contrary, these formulations had a very moderate toxicity (comparable to the control itself), which could be originated from the entrapment of AmB in the oil droplets of the micro- and nanoemulsions, and possibly the stabilization of the droplets, leading to decreases in their availability and toxicity over the membrane bilayers. Actually, most of the reports in the literature show concentration-dependency of the activity of the surfactants over the tissues \([35]\). If beneficial from the point of view of local toxicity, such a sequestration of AmB into the droplets of ME or NE would be detrimental to the release of AmB outside of the droplets, thus to AmB direct absorption under this physicochemical state.
4. CONCLUSIONS

During this work attempts were made to measure the permeation of AmB through the jejunal epithelium when it is presented in front of the mucosa under different physicochemical situations, ranging from monomeric to aggregated states. No absorptive fluxes across the membrane could be detected using the Ussing’s chamber model independently of the conditions used, which suggests that the dramatically low water-solubility of AmB, possibly combined to its amphoteric character in luminal conditions, forbid an efficiently entry of AmB through classical diffusive pathways. The exploration of the particulate absorption pathways either through enterocytic endocytosis and/or lymphatic drainage would deserve attention. Furthermore, the electrophysiological determinations showed indirectly that AmB association to proteins or lipids used in formulations (in ME or NE systems) could strongly modulate the toxic effects, as suggested by the large differences in contact toxicity when AmB was associated to supramolecular structures (micelles in ME or NE) or the AmB-BSA complexes. In summary, the intimate physicochemical mechanisms occurring at the epithelial surface should be investigated for being better understood at the molecular level. However, from the formulator point of view, it is suggested that the targeting the lymphatic system could be an interesting alternative in attempting to enhance AmB oral absorption.

ACKNOWLEDGEMENTS

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Figure Captions

**Figure 1:** UV-Vis spectra ($\lambda = 300-450$ nm) of AmB-BSA complexes at the concentrations of $3 \times 10^{-6}$ M $1 \times 10^{-5}$ M ([BSA] = 4 % (w/v); $\text{CAC}_{\text{AmB-BSA}} = 5\times10^{-6}$ M).

**Figure 2:** Circular Dichroism spectra ($\lambda = 300-450$ nm) of AmB-BSA complexes at the concentrations of $3 \times 10^{-6}$ M $1 \times 10^{-5}$ M ([BSA] = 4 % (w/v); $\text{CAC}_{\text{AmB-BSA}} = 5\times10^{-6}$ M).

**Figure 3:** Mean size (DLS) in microemulsions and nanoemulsions with and without AmB (A) and corresponding zeta potentials (B). ($n = 3$)

**Figure 4:** Transepithelial resistance (TEER) for (a) AmB-BSA dispersions $\text{M} \rightarrow \text{S}$; (b) AmB-BSA dispersions $\text{S} \rightarrow \text{M}$; (c) BSA dispersion; (d) AmB-loaded emulsion based systems. ($n = 6$, days =3)

**Figure 5:** Expected equilibria created by the presence of the AmB containing formulations in front of the intestinal membrane and possible absorption pathways or shortcuts to AmB absorption, depending on possible interactions between AmB and biological environment. In (a) it is represented the possible pathways for the AmB-BSA, while in (b) for the micro- and nanoemulsions. The descriptors 1, 2, and 3 identify the possible behaviors of AmB in the jejunal tissue; (1) Local aggregation/precipitation of AmB in the mucus layer; (2) AmB absorption; (3) unknown pathway.
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![UV-Vis spectra](image-url)
Figure 2: Circular Dichroism spectra ($\lambda = 300$-$450$ nm) of AmB-BSA complexes at the concentrations of $3\times10^{-6}$ M $1\times10^{-5}$ M ($[\text{BSA}] = 4 \%_{(w/v)}$; $\text{CAC}_{\text{AmB-BSA}} = 5\times10^{-6}$ M).
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INTESTINAL PERMEABILITY OF AMPHOTERICIN B AND THE ROLE OF NANOMEDICINES ON ITS ENHANCEMENT

(c)

(d)

NE or ME
M → S

NE
ME
**Figure 5:** Expected equilibria created by the presence of the AmB containing formulations in front of the intestinal membrane and possible absorption pathways or shortcuts to AmB absorption, depending on possible interactions between AmB and biological environment. In (a) it is represented the possible pathways for the AmB-BSA, while in (b) for the micro- and nanoemulsions. The descriptors (green circles) 1, 2, and 3 identify the possible behaviors of AmB in the jejunal tissue; (1) Local aggregation/precipitation of AmB in the mucus layer; (2) AmB absorption; (3) unknown pathway.
SECTION I
Chapter II
INTESTINAL PERMEABILITY OF AMPHOTERICIN B AND
THE ROLE OF NANOMEDICINES ON ITS ENHANCEMENT

[Diagram showing the process of particulate transepithelial capture and the role of nanomedicines on its enhancement.]
Table Captions

**Table 1:** Composition of micro- (ME) and nanoemulsions (NE).
### Table 1: Composition of micro- (ME) and nanoemulsions (NE).

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Nanoemulsion Amount (%) [18]</th>
<th>Microemulsion Amount (%) [19]</th>
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<td>Tween® 80</td>
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<tr>
<td>Lipoid® S100</td>
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<td>Water</td>
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<td>68</td>
</tr>
</tbody>
</table>
CHAPTER III

IMMUNOGENICITY OF AMPHOTERICIN B BOVINE SERUM ALBUMIN AGGREGATES
BRIEFING

The pronounced tendency of amphotericin B (AmB) to self-aggregate and/or to interact with proteins is well documented, although it is still the subject of intensive researches. In the previous chapter it was shown that AmB was almost unable to cross jejunal epithelium, which demonstrated a rarely encountered situation. The use of AmB and albumin complexes was inefficient as well to favor AmB diffusion across this membrane.

This behavior prompted us to investigate another physiological situation in which AmB is associated to proteins, i.e. when AmB is delivered by the intravenous route. Drug complexation with albumin has been broadly reported and the importance of protein binding to pharmacological responses had also been showed. Hydrophobic drugs, such as AmB usually are highly bound to albumin, with rates close to 95% in the case of AmB. More specifically, AmB binding to albumin, as well as the different affinities of AmB for these binding sites have already been reported. Therefore this work attempted to investigate the self-aggregation of AmB, the role the interactions between amphotericin B and albumin as well as its interaction with immunological proteins (protein C3) belonging to the complement system.
Immunogenicity of amphotericin B in the presence of bovine serum albumin

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ABSTRACT

Amphotericin B (AmB) is a polyenic molecule with antifungal and leishmanicidal activities. The AmB pharmacological activity as well as toxicological profile are closely related to its physicochemical properties, and its aggregation in aqueous media has been related to the presence of toxic effects. In the last decades, new drug delivery systems were developed to increase the safety of AmB. However, until now not much is known about the origin of the differences in the pharmacokinetic parameters observed with these new drug delivery systems. The aim of this work was to characterize the interaction between AmB and albumin and to evaluate the immunogenicity of the different aggregation states of AmB under these conditions, which are close to blood physiological conditions. To reach this goal, spectroscopic and immunoelectrophoretic techniques were used in order to determine the presence of AmB aggregates as well as the capacity of AmB to form complexes with albumin binding sites. Afterwards, a correlation between the presence of AmB aggregates and the activation of the immunological system was found. Our data suggest that the activation of the immunological system by the complement route could be a consequence of the presence of aggregates of AmB and/or the formation of AmB interactions with blood proteins, which might alters its pharmacokinetic parameters. To conclude, this work suggests that albumin-binding of AmB could impact both toxicity and biodistribution of AmB, by possibly implying the immunological system in the clearance of the into the body.

Keywords: Amphotericin B; Aggregation; Immune complement system; Spectroscopy; 2-D immunoelectrophoresis.
1. INTRODUCTION

Amphotericin B (AmB) is a polyene molecule broadly used for the treatment of systemic fungal and leishmania infections [1, 2]. Its pharmacological activity is closely related to its complex chemical structure, as well as unique physico-chemical properties associating both amphiphilic and amphoteric characteristics. AmB chemical structure has a macrocyclic compound comprising a conjugated chain with 7 double bonds and a saturated carbon chain bearing hydroxyls and aminohydrophilic groups, thus creating an hydrophobic face and a polar face in the AmB chemical structure are responsible for its pharmacological activity. The binding of AmB to phospholipidic membranes has been described as the main mechanism for pharmacological and toxic effects [3, 4]. The primarily mechanism for it has been recently described, as an interaction between the amine group of the AmB mycosamine ring and the hydroxyl group present in the ergosterol specifically present into membrane of fungi microorganisms [3, 5]. However, the most reported mechanism for AmB toxic effects on mammalian cells is based on Van der Waals interactions between the AmB polyenic chain and the phospholipidic membrane, even though the importance of the hydroxyl group from carbon 35 for AmB insertion into membranes was recently reported [3]. The insertions of AmB into membranes generate pores, which are not controlled by the internal machinery of the cell, and lead to rapid loss of cell ionic balance [3, 6, 7]. Further, from a pharmacokinetics standpoint, AmB is usually associated with other molecules, mainly blood proteins such as albumin and α1-acid glycoprotein, which are able to change drug pharmacokinetics/pharmacodynamics properties [8-10].

Despite the knowledge on AmB pharmacological activity and its mechanisms of action, there is a lack on the determinants driving its biodistribution. Additionally, it has been shown that nanostructures, such as liposomes (AmBisome®) and lipid complexes (ABCL®) are able to
decrease AmB toxicity by controlling the presence of the free-drug into the plasma through a reinforcement of their strong interactions between AmB and phospholipids in the formulation. However, the origin of the differences in the pharmacokinetic parameters (e.g. fecal elimination) of these new drug delivery systems, when compared to the classical dosage forms (e.g. Fungizone®) are not completely understood [10].

This work aimed to investigate the impact of the AmB aggregation in the presence of albumin, which has been selected because it is the most abundant protein in the human plasma, and to evaluate its possible impact on the activation of the immunological complement system due to the presence of AmB aggregates once AmB concentration reach values higher than the CAC_{AmB-BSA4%}, and provide a clue to explain the differences in pharmacokinetic parameters observed for the newly launched AmB delivery systems.
2. METHODOLOGY

2.1. Materials

Amphotericin B was purchased at Spectrum Chemicals (USA), methanol HPLC grade was purchased at Merck (France). Bovine serum albumin, calcium chloride, glucose, potassium phosphate monobasic, tricine, Tris base (Sigma 7-9®) and sodium chloride were bought at Sigma-Aldrich (France). Sodium bicarbonate was purchased at Fluka (France). Magnesium sulphate was purchased at Carlos Erba (France). Ultrapure water was obtained by Milli® Q purification system.

The human serum was prepared from plasma provided by Etablissement Français du Sang (EFS) (France). Polyclonal anti-human C3 antibody raised in goat was purchased from Fitzgerald antibodies (USA). Coomassie brilliant blue R-250, calcium lactate and glacial acetic acid were supplied by Thermo Fisher Scientific (France). Gel-Fix™ for agarose (265x150 mm) was purchased from Serva Electrophoresis (Germany).

2.2. Preparation of AmB dispersions

AmB stock solutions, at the concentration of 0.25 mg/mL, were prepared in methanol. The molecular dispersion was obtained by magnetic stirring and sonication in an ultrasound bath at 25°C. Afterwards, different amounts of the stock solution were added into 4%(w/v) BSA solution in Krebs-Ringer-Bicarbonate Buffer (pH= 7.3) [11]. Then, the solvent was evaporated under vacuum (Büchi R-124, Germany) at 40°C until the volume has reached the initial volume of 4% BSA dispersions. All dilutions were carried out under magnetic stirring.
2.3. UV spectroscopy

UV-Vis spectra were obtained in a Perkin Elmer (France) spectrophotometer with a 1cm length quartz cell. Evaluation and quantification of AmB aggregation was assessed from wavescans from 300 to 450 nm in different media and concentrations. AmB concentrations were obtained at $\lambda_{\text{max}} = 415$ nm were used.

2.4. Circular Dichroism Spectroscopy (CD)

Circular dichroism analyses were performed on a Jasco J-810 dichrograph (France), with 0.5, 1 and 2 cm length quartz cells. Wavescans from 300 to 450 nm were carried out, with the main objective to observe the presence of a doublet with its highest point near to $\lambda = 320$ nm, which identifies the presence of AmB aggregates in the medium.

2.5. Size distribution of amphotericin B - AmB complexes and aggregates

The size distribution of the AmB-BSA complexes and/or aggregates was determined by dynamic laser scattering using a NanoZS (Malvern®, England). This equipment performed the analysis using the principle of quasi-elastic shocks between the light ($\lambda = 633$ nm with the detector at 173º from the light source) emitted and the particles in suspension. Attempts to determine the modifications of the aggregation pattern were made in presence of BSA 4% (w/v) and by varying the concentration of AmB under or above the Critical Aggregation concentration (CACC\textsubscript{AmB-BSA}) into the medium.

2.6. Multi-crossed electrophoresis ($\mu$C-IE) for the determination of protein C3 activation

The method of multi-crossed 2D immunoelectrophoresis described by Coty et al. [12] was
performed for the analysis of complement C3 activation. Briefly, AmB-BSA dispersions (50 µL) were incubated with human serum (25 µL) in Veronal Buffer Saline (VBS2+) (25 µL) prepared as described by [13]. Samples were incubated for 1 h at 37°C. Then, they were immediately cooled to 4°C in an ice bath for immediate analysis by μC-IE.

Agarose gel 1% (w/v) containing anti-C3 antibody was prepared in Tricine buffer (Calcium Lactate 1 mM, TRIS 63 mM, Tricine 27 mM, in milliQ® water, pH 8.6) and then casted on a Gel-Fix™ film. Bands were cutted and then filled with 1% (w/v) agarose gel prepared in Tricine buffer for the 1st dimension. Wells were formed at the same time using a homemade comb. The gel was placed in Multiphor II electrophoresis system (GE Healthcare, Velizy-Villacoublay, France) and wells were filled with 2.5 µL of samples. The samples were then subjected to the first dimension electrophoresis (600 V, 16 mA, 100 W), freely migrating in the agarose gel band according to their molecular weight. Then, the gel was turned by 90° for the second dimension corresponding to the rocket immunoelectrophoresis (500 V, 12 mA, 100 W), for 210 min. Gels were dried and stained as described by J.M. Walker [14].

The gels were scanned and the areas under peaks were integrated using ImageJ software. The peak on the left side was identified as native protein C3, whereas the second peak was attributed to fragments derived from protein C3 cleavage. The complement activation factor (CAF) was expressed as the ratio of peak surface of cleaved protein regarding the total peak surface of C3 native and fragments and also removing the natural activation during the performance of the technique, according to the follow equations (Eq. 1,2,3) [15].

$$ Activation_{sample}^{(\%)} = \left[ \frac{cleavedC3_{sample}}{cleavedC3_{sample} + nativeC3_{sample}} \right] \times 100 $$

(Eq. 1)
\[ Activation_{control}(\%) = \left( \frac{cleavedC3_{control}}{cleavedC3_{control} + nativeC3_{control}} \right) \times 100 \]  
(Eq. 2)

\[ CAF(\%) = \left( \frac{Activation_{sample} - Activation_{control}}{100 - Activation_{control}} \right) \times 100 \]  
(Eq. 3)

2.7. Statistical Analysis
The linear regression models and the graphics were prepared in Microsoft Excel® for Mac (Version 15.17). The t-Student tests used to analyze the complexes average size and PdI were carried out with RStudio v0.99.467 loaded with R version 3.2.3 (2015-12-10), psych and lattice libraries.

3. RESULTS AND DISCUSSION
Independent of the formulation administered, when AmB is delivered by the intravenous route, AmB molecules are likely to interact extensively with serum proteins, as well as to self-aggregate, all phenomena which may considerably affect the biodistribution of the drug in the organs. In this work, an attempt to mimic some of the conditions prevailing in the blood after AmB administration was made by varying both AmB concentrations under and above its critical aggregation concentration and also the BSA concentration. Spectroscopic determinations combined to DLS measurements showed that conditions could be find in which AmB-BSA complexes and/or AmB aggregates could coexist. The impact of those different species on the activation of the immune complement system was further investigated, once it is known that macrophages and the reticuloendothelial system (RES) are able to modulate the clearance of such objects.
3.1. **Aggregation of amphotericin B in presence of BSA**

### 3.1.1. UV-Vis spectroscopy

The AmB aggregation in aqueous media has been broadly reported in the literature, as well as, the identification of the aggregates by spectroscopic methods [16-18]. The increase of the molar absorptivity in the region of from 300 to 350 nm combined to a decrease in the region from 405 to 420 nm are characteristics of the formation of a various aggregation states of AmB aggregation [4]. However, there are no available straightforward techniques to determine and/or to separate the different kinds of AmB aggregates [6].

Once AmB was dispersed into a 4%\(_{w/v}\) BSA dispersion at different concentrations a the spectra showed that AmB existed partially in its monomeric form, even though the concentrations used were clearly above the solubility of AmB in water [17]. This was attributed to the formation of AmB-BSA complexes, which keeps AmB in a molecular state at the BSA binding-sites working as a reservoir of AmB monomers. Such AmB-BSA interactions are also highly observed into the human body, where the percentage of AmB protein binding has been reported to be above 95% [10]. By comparison, as expected, large aggregation of AmB molecules was observed in absence of BSA (Figure 1).

The first AmB concentration where the AmB aggregates could be detected, is here called CAC\(_{\text{AmB-BSA}}\). In order to determine the CAC\(_{\text{AmB-BSA}}\) by UV-Vis spectroscopy the methodology previously described by Aramwit et al was used [19]. After data analysis, a CAC\(_{\text{AmB-BSA}}\) value of 5x10\(^{-6}\) M was obtained. This result is different from the ones previously reported by Aramwit et al and by Egito et al. that obtained both a value of 8x10\(^{-6}\) M, probably due to differences in experimental conditions, such as the buffer solution used [4, 19, 20]. In fact, Aramwit and co-workers, as well as Egito and co-workers, used an isotonic phosphate buffer as a blank (control), while in this work a buffer with higher variety of ions including...
divalent ions was used. It is likely that these different values obtained in vitro are not representative of the one in the plasma, since toxic effects of AmB which are attributed to the presence of aggregates were observed at lower concentrations [10]. A hypothesis could be that under physiological conditions the number of available binding sites on albumin is reduced due to the attachment of other physiological molecules (or even other drugs) present in the human body.

The effect of BSA concentrations has been investigated (2, 4 and 8 % (w/v)). As expected in the number of available binding sites on albumin resulted in a decrease in the concentration in AmB aggregates while monomeric form was favored (Figure 2).

3.1.2. CD spectroscopy

The CD measurements were carried out in order to confirm the results obtained by UV-Vis spectroscopy. Although this technique is mainly used to identify changes in the conformation of proteins and peptides [21], it is often used for the characterization of AmB dispersions since it makes possible the identification of AmB aggregates with high accuracy [17].

As expected, a characteristic doublet for the molar dichroic absorptivity that represents the formation of AmB aggregates (λ = 320 - 345 nm; Figure 3a) was observed in the dispersions containing high amounts of AmB. The highest value of the molar circular dichroism (Δε), which was at λ = 325nm, from the doublet was used as a marker to identify the presence of aggregates into the AmB-BSA dispersions. Thus when Δε_{325nm} = 0 it was considered that it corresponded to the CAC_{AmB-BSA}.

However, differently of what was observed for UV-Vis spectroscopy, an interaction with two different intensities was observed between AmB and BSA. Variations in the spectra close to λ = 325 nm, can be observed in Figure 3b. The differential absorbance started with
negative values of $\Delta \varepsilon$ for $1 \times 10^{-6}$ M and they rise until reaching positive values and passing by the $\Delta \varepsilon = 0$ (CAC$_{AmB-BSA}$). However, the difference in the intensities between $1 \times 10^{-6}$ M and $2 \times 10^{-6}$ M was bigger than among the other concentrations, which might correspond to differences in the protein binding, such differences in the number of BSA available binding sites as previously described by Romanini et al [22].

3.2. Particle size distribution of AmB-BSA complexes and aggregates.

Depending on the respective concentrations of AmB and BSA, AmB-BSA complexes and/or AmB aggregates coexisted in the buffer medium and the DLS was used to attempt a characterization of these different types of objects. As shown in Table 1, BSA and AmB-complexes could be detected by DLS with a diameter close to 8 nm, as generally reported [23]. The hydrodynamic dimensions of the protein were only very slightly affected by the presence of AmB, whatever the concentration (under or above the CAC$_{AmB-BSA}$). This is reasonable since the size increase due to the fixation of two AmB molecules (MW = 924.09 Da) on one BSA molecule (MW = 66.500 Da) is unlikely to be detected by conventional DLS apparatuses. The significant changes in size were only observed when concentrations below the CAC$_{AmB-BSA}$ were compared with concentrations above the CAC$_{AmB-BSA}$. For the BSA alone the size was close to 8 nm, with a peak area $\geq$ 97 % exceptionally [AmB] = $1 \times 10^{-5}$ M, Peak area $\geq$ 93 %, which was used to try to observe some statistical difference. At all the condition used, the increase in size caused by the addition of AmB were only in the order of 0.1 nanometers.

However, during the analysis the presence of BSA aggregates in the range of few hundred nanometers was always detected, which was reflected in the particle size distribution index (PdI) near 0.15. Indeed, PdI-had a tendency to become wider while increasing the
concentration of AmB (Table I). Then, while the concentration of the AmB was below $\text{CAC}_{\text{AmB-BSA}}$ the differences among the PdI-values were not statistically significant (Table 1). Therefore, once AmB concentration was above the $\text{CAC}_{\text{AmB-BSA}}$ the PdI values were significantly different ($p$-value < 0.05) These wider distributions could correspond to the formation of AmB aggregates in parallel to the existence of AmB-BSA complexes in the dispersions containing AmB amounts above the $\text{CAC}_{\text{AmB}}$. This would corroborate with the spectroscopic results that demonstrates the presence of AmB aggregates into these dispersions.

3.3. Activation of complement immunological system

The immune system is able to detect the presence of pathogens or other foreign objects in the blood, thanks to the complement system among other mechanisms. One of the pathways that lead to the detection and the capture of the objects by the macrophages and the cells of the reticulo-endothelial organs requires the activation of C3, a specific protein belonging to the complement system. In this context, AmB-BSA dispersions prepared with different concentrations of AmB were tested in order to investigate their capacity to cleave the C3 protein into two fragments (normally C3a and C3b), which is the result of an activation of the immunological complement system. In fact, our hypothesis was that the activation of the complement system was expected to happen once the concentration of AmB reached higher values than the $\text{CAC}_{\text{AmB}}$, where AmB self-aggregates are present.

The immunoelectrophoretic technique used was able to semi-quantify the activation of the complement by the ratio of the amount of C3 and cleaved C3 proteins [24]. The complement activation fraction (CAF) values were calculated with the integrated values from the areas of the peaks corresponding to the interaction of native C3 and its active proteic fractions with
the Anti-C3 antibody. Figure 4, shows a positive correlation ($r = 0.9861$) between the amount of C3 activated fractions and the AmB concentration, when AmB concentrations were close to or higher than the CAC$_{\text{AmB-BSA}}$.

Additionally, the behavior of $\Delta\varepsilon$ at $\lambda = 325$ nm was similar to the behavior of the activation of complement immunological systems, as it can be seen on Figure 5. The increase on the activation is almost negligible for the AmB concentrations below the CAC$_{\text{AmB-BSA}}$, and a moderate increase could be detected once the CAC$_{\text{AmB-BSA}}$ was reached.

Further, Figure 5 suggests that the complement activation could be directly related to the aggregation status of Am-B in presence of proteins. At low AmB concentrations, where AmB exists mainly in the form of AmB-BSA complexes, the activation was close to the “natural activation”, while it was steeply increasing above the CAC$_{\text{AmB-BSA}}$ in which conditions AmB began to self aggregate.

It would be therefore interesting to investigate other conditions leading to higher aggregations, for example by decreasing the BSA concentration in order to form higher amounts of aggregated AmB (above CAC$_{\text{AmB}}$). Although the findings here seen revealed a possible correlation between AmB aggregates and immune complement activation, which could lead to why the different drug delivery system have different pharmacokinetic parameters, as observed by Bekersky et al. [10]. In fact, the recognition of the AmB aggregates could have different consequences, including: (i) the recruitment of the macrophages active towards fungal infections and which have been hypothesized as the possible explanation for AmB higher accumulation into the sites of infections [25]; (ii) AmB aggregation can induce changes in elimination pathways, possibly inducing unexpected side-effects. Indeed, AmB aggregates might have the ability to activate the immune complement system on a similar way as the non-sthealth polymeric nanoparticles, which are able to
induce C3 cleavage and also have high accumulation on the liver and/or RES organs, when administered intravenously [26, 27].

4. CONCLUSIONS

The pivotal role of BSA on the monomerization of AmB has been confirmed under concentration conditions closed to in vivo conditions. Depending on the saturation of BSA available binding sites, the formation of AmB aggregates occurred with the CAC\textsubscript{AmB-BSA} in BSA 4 %\textsubscript{(w/v)} of 5x10\textsuperscript{-6} M. This limit in the concentration of AmB could have a significant impact on both its activity and toxicity. Further, above CAC\textsubscript{AmB-BSA}, the progressive formation of AmB aggregates was shown to begin to activate the complement system as shown by an increased the cleavage of C3 protein, a marker for the activation of the immunological complement system. This behavior may have an incidence on the pharmacokinetics properties of AmB such as modifications of the biodistribution and the opening of non-conventional elimination pathways (e.g liver biotransformation due to increased capture by the liver). In conclusion, despite experimental difficulties in measurements, it should be recognized that any factors (dosage regimen, dosage form) likely to impact the aggregation status of AmB in the blood should be carefully considered, since the formation of aggregates in the blood might induce changes in the pharmacokinetics, pharmacodynamics and toxicological profiles.

ACKNOWLEDGEMENTS

The authors are grateful to CAPES-Cofecub project 742/11 for the scholarship provided.
REFERENCES


Figure Captions

**Figure 1**: (a) Uv-Vis spectra (λ= 300-450 nm) of AmB-BSA complexes in KRB Buffer pH=7.3 revealing a correlation between AmB concentration and the absorbances in the different region of interest (self-aggregation of AmB was evaluated at λ= 334nm).

**Figure 2**: Evaluation of the influence BSA concentration on AmB aggregation state depending on the concentration in AmB and for different concentrations of BSA: 1x10-6, 5x10-6 and 1x10-5 M. (a) BSA 0 % (w/v), (b) BSA 2 % (w/v), (c) BSA 4 % (w/v) and (d) BSA 8 % (w/v).

**Figure 3**: Circular Dichroïsm spectra (λ= 300-450 nm) of AmB-BSA dispersions with different AmB concentrations.

**Figure 4**: Relative amount of cleaved C3 fractions induced by AmB in presence of BSA when compared to natural activation (addition of VBS+2) in the human serum. The dashed line suggests the existency of a correlation between the presence of C3 derivatives and the AmB concentrations above CAC_{AmB-BSA}.

**Figure 5**: Relationship between the nature of the species formed by Amb in presence of BSA (Differential absorbancy by CD spectroscopy at λ= 325 nm) and activation of the complement immune system quantified by the cleavage of the protein C3.
IMMUNOGENICITY OF AMPHOTERICIN B IN THE PRESENCE OF BOVINE SERUMALBUMIN

Figure 1: (a) Uv-Vis spectra ($\lambda = 300$-450 nm) of AmB-BSA complexes in KRB Buffer pH=7.3 revealing a correlation between AmB concentration and the absorbances in the different region of interest (self-aggregation of AmB was evaluated at $\lambda = 334$nm). The “Agg” and “c” abbreviations stand for AmB aggregates and AmB-BSA complexes, respectively.

(b) The values here used are average values of at least three measurements.
**Figure 2:** Evaluation of the influence BSA concentration on AmB aggregation state depending on the concentration in AmB and for different concentrations of BSA: $1 \times 10^{-6}$, $5 \times 10^{-6}$ and $1 \times 10^{-5}$ M. (a) BSA 0\%\(\text{(w/v)}\), (b) BSA 2\%\(\text{(w/v)}\), (c) BSA 4\%\(\text{(w/v)}\) and (d) BSA 8\%\(\text{(w/v)}\).
**IMMUNOGENICITY OF AMPHOTERICIN B IN THE PRESENCE OF BOVINE SERUMALBUMIN**

*The values here used are average values of at least three measurements.*
Figure 3: Circular Dichroism spectra ($\lambda = 300-450$ nm) of AmB-BSA dispersions with different AmB concentrations.

*The values here used are average values of at least three measurements.
Figure 4: Relative amount of cleaved C3 fractions induced by AmB in presence of BSA when compared to natural activation (addition of VBS$^{+2}$) in the human serum. The dashed line suggests the existence of a correlation between the presence of C3 derivatives and the AmB concentrations above $CAC_{AmB-BSA}$.

![Graph showing correlation between AmB concentration and CAF%](image)

$R^2 = 0.97248$

*The values here used are average values of at least three measurements.*
Figure 5: Relationship between the nature of the species formed by Amb in presence of BSA (Differential absorbance by CD spectroscopy at $\lambda = 325$ nm) and activation of the complement immune system quantified by the cleavage of the protein C3. The “Agg” and “c” abbreviations stand for AmB aggregates and AmB-BSA complexes, respectively.
**Table Captions**

**Table 1**: Particle size and PdI-values of AmB-BSA complexes under AmB critical aggregation concentration. (n = 3)
Table 1: Particle size and Pdl-values of AmB-BSA complexes, under AmB critical aggregation concentration. (mean of n = +/- sd)

<table>
<thead>
<tr>
<th>AmB concentration (M)</th>
<th>0</th>
<th>1x10^{-6}</th>
<th>2x10^{-6}</th>
<th>3x10^{-6}</th>
<th>4x10^{-6}</th>
<th>5x10^{-6}</th>
<th>1x10^{-5}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average (nm)</td>
<td>8.0 ± 0.2</td>
<td>8.0 ± 0.1</td>
<td>8.1 ± 0.0</td>
<td>8.1 ± 0.1</td>
<td>8.1 ± 0.1</td>
<td>8.1 ± 0.1</td>
<td>8.3 ± 0.1</td>
</tr>
<tr>
<td>Pdl-values</td>
<td>0.12 ± 0.01</td>
<td>0.13 ± 0.01</td>
<td>0.17 ± 0.01</td>
<td>0.14 ± 0.01</td>
<td>0.17 ± 0.01</td>
<td>0.16 ± 0.01</td>
<td>0.21 ± 0.01</td>
</tr>
</tbody>
</table>
Graphical Abstract

Highlights

1. The interaction of AmB-albumin increases AmB apparent solubility.
2. AmB aggregation is dependent of the amount of the ions into the medium.
3. AmB aggregates are able to moderately activate the immune complement system.
SECTION II

DEVELOPMENT OF XYLAN-BASED SYSTEMS FOR COLON-TARGETED DRUG DELIVERY
INTRODUCTION

Once the lipid-based system approach to enhance amphotericin B’s intestinal permeability was not successfully observed by the Ussing’s Chamber technique, it was decided to try a new approach to reach the desired goal: to take advantage of the larger spaces among the cells on the colonic region, which are filled by wider tight junctions.

To produce these colon-specific delivery systems, an idea already inserted on our laboratory, however with other purposes, such as the treatment of Inflammatory Bowel Diseases (IBD), was added into this work.

The systems produced in the laboratory were prepared with the use of xylan, a hemicellulose present on the cell-wall of plants, mainly angiosperms, grasses and cereals. The structure of xylan usually has different side chains, even though xylanopyranose groups form the main backbone. For more detailed information, please read the book chapter published by our group (Attachment I).

The production of the xylan-based microcapsules was tried using different techniques, as it can be seen on Attachment II. However, there was no characterization regarding its biological compatibility. Then, on the next chapters there will be demonstrated the biological characterization of the xylan-based microcapsules and the other xylan-based particles develop as a consequence of the results obtained during the first part of the xylan-based systems characterization.
CHAPTER IV

LEADS FROM PHYSICAL, CHEMICAL AND THERMAL CHARACTERIZATION ON CYTOTOXIC EFFECTS OF XYLAN-BASED MICROPARTICLES
BRIEFING

This following manuscript was the result of a detailed characterization of xylan-based microcapsules, which were first described by Nagashima et al., 2008 and were continually studied by Silva et al., 2013 (Attachment II). The properties analyzed were most physical properties, such as size, particle size distribution and the crystalinity of the raw material and the product obtained after the crosslinking reaction. Therefore, some chemical and thermal characterization of the xylan before and after the crosslinking was also done.

Afterwards, the biocompatibility results from the raw material (xylan) and the xylan-based microcapsules were brought together in order to correlate the physical and chemical characterization carried out with the cytotoxicity studies.

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Article

Leads from Physical, Chemical, and Thermal Characterization on Cytotoxic Effects of Xylan-Based Microparticles

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Abstract: Interfacial cross-linking (ICL) has been considered a feasible technique to produce polysaccharide-based microparticles (PbMs), even though only a few studies have been concerned with their biocompatibility. In this work, PbMs were prepared by the ICL method and characterized in regard to their in vitro biocompatibility, chemical linkages, and physical and thermal properties. First, the cell viability assay revealed that PbMs toxicity was concentration-dependent. Then, it was observed that the toxicity may be related to the way in which the binding occurred, and not exclusively to the stoichiometry between the polymer and the cross-linking agent. Moreover, the PbMs biosafety was predicted by the use of physicochemical procedures, which were able to identify unbound cross-linking agent residues and also to reveal the improvement of their thermal stability. Accordingly, this work suggests a step-by-step physicochemical procedure able to predict potential toxicity from micro-structured devices produced by polysaccharides. Likewise, the use of PbMs as a drug carrier should be cautiously considered.

Keywords: biocompatibility; interfacial cross-linking; polysaccharide; terephthaloyl chloride, microparticles

1. Introduction

The interfacial cross-linking method (ICL) has been considered a feasible technique to produce polysaccharide-based microparticles (PbMs) since the late 1970s [1]. This technique is based on the preparation of an emulsion as the first step, followed by a chemical reaction that occurs at the emulsion interface, which works as a microreactor to control the process and avoid the formation of chemically bounded aggregates [2].

Combined with the ICL technique, many cross-linkers have been extensively used to produce PbMs. Among them, epichlorohydrin, phosphoryl chloride, terephthaloyl chloride, and glutaraldehyde have been drawing more attention due to the good stability of the resulting particles. In spite of that, all those molecules have been reported as toxic when in their pure state [2–4].

Xylan is a polysaccharide extracted from hardwoods and annual plants, composed of a D-xylopyranose backbone chain with glucuronic acid, L-arabinose, and other sugars. Due to its
complex structure, this polymer is insoluble in different media at both acid and neutral pH. Indeed, xylan’s application in the pharmaceutical field has been studied mainly for colon-targeted drug delivery. When administered by the oral route, this polysaccharide is only degradable at the ascendant and transversal colon region due to the presence of bacteria that produce and excrete xylanases into the gastrointestinal tract [5,6]. However, its use as a raw material for the production of drug delivery systems has emerged in the recent literature [7,8]. Our group has also evaluated the production of colon-targeted drug carriers as a promising application for xylan [9,10].

The development of colon-targeted drug delivery systems has its focus in the treatment of local pathologies such as inflammatory bowel diseases and cancer. Such a strategy also has been used to enhance the bioavailability of peptides and proteins by the protection of such compounds in the gastrointestinal tract and by permeation through the wider tight junctions of this tissue [11].

The aim of this work was to evaluate the effect of ICL on xylan’s biosafety and establish possible correlations among its physical, chemical, and thermal properties.

2. Experimental Section

2.1. Materials

Terephthaloyl chloride (TC), sorbitan triestearate (Span® 65), polysorbate 20 (Tween® 20), polysorbate 80 (Tween® 80), and a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) kit were purchased from Sigma-Aldrich Chemical (St Louis, MO, USA). Sodium hydroxide, chloroform, cyclohexane, and ethanol came from Vetec Chemical (Rio de Janeiro, Brazil). Dulbecco’s modified eagle medium (DMEM) was obtained from Gibco® (Gaithersburg, MD, USA). Xylan was obtained after extraction from corn cobs as previously described by our group [12].

2.2. Microparticle Production

PbMs were produced by the ICL method as previously reported by Nagashima-Junior and colleagues [9]. Briefly, an alkali xylan solution was added into a chloroform:cyclohexane (1:4, v/v) mixture in order to obtain a W/O emulsion and then cross-linked using TC (Figure 1). According to the chemical composition of xylan from corn cobs described by Melo-Oliveira et al., the ratio of hydroxyl radicals to TC molecules was $4.7 \times 10^{-4}$ (molecules of xylan/molecules of TC) [13]. The PbMs were then washed three times with (1) an ethanolic solution containing 2% (v/v) polysorbate (Tween® 80 and Tween® 20, 1:1); (2) an ethanolic aqueous solution, 95 °GL; and (3) distilled water. After washing, the PbMs
were dispersed into 50 mL of water. Afterwards, five samples containing 10 mL of PbMs were subjected to an ultra-rapid freezing process by immersion in liquid nitrogen (T = −196 °C) for 5 min. Freeze-drying was performed in an Alpha 1-2 freeze-dryer (Christ, Germany) at −63 °C and 0.0018 mbar for 24 h to allow a complete freeze-drying cycle.

![Figure 1. Scheme of cross-linking reaction between xylan and terephthaloyl chloride.](image)

The dashed polygone (---) highlight the linkage between terephthaloyl chloride molecules and xylan chains. The black circle represents the chlore atom that is lost by the terephthaloyl chloride molecule during the cross-linking reaction.

### 2.3. Xylan Dispersions for Biocompatibility Assay

Xylan dispersion at 49.6 mg·mL\(^{-1}\) was prepared, under magnetic stirring, by the addition of xylan powder into a known amount of distilled water. Then, all dilutions utilized were produced according to the values described in Table 1.

**Table 1. Chart of products used in the in vitro biocompatibility assay.**

<table>
<thead>
<tr>
<th>Tested Product</th>
<th>Dilutions (Product:DMEM, v/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylan dispersion in water (49.8 mg·mL(^{-1}) of xylan)</td>
<td>1:1 (corresponding to 24.8 mg·mL(^{-1}))</td>
</tr>
<tr>
<td></td>
<td>1:3 (corresponding to 12.4 mg·mL(^{-1}))</td>
</tr>
<tr>
<td></td>
<td>1:7 (corresponding to 6.2 mg·mL(^{-1}))</td>
</tr>
<tr>
<td></td>
<td>1:15 (corresponding to 3.1 mg·mL(^{-1}))</td>
</tr>
<tr>
<td>PbMs suspension in water (1.488 mg·mL(^{-1}) of xylan)</td>
<td>1:1 (corresponding to 0.744 mg·mL(^{-1}))</td>
</tr>
<tr>
<td></td>
<td>1:3 (corresponding to 0.372 mg·mL(^{-1}))</td>
</tr>
<tr>
<td></td>
<td>1:7 (corresponding to 0.186 mg·mL(^{-1}))</td>
</tr>
<tr>
<td></td>
<td>1:15 (corresponding to 0.093 mg·mL(^{-1}))</td>
</tr>
<tr>
<td></td>
<td>1:31 (corresponding to 0.0465 mg·mL(^{-1}))</td>
</tr>
</tbody>
</table>

### 2.4. Morphology and Particle Size Distribution Analysis
The PbMs morphology was evaluated by scanning electronic microscope (XL 30 ESEM, Phillips, Amsterdam, Netherlands). The particle analysis was carried out by dynamic laser scattering (920L Cilas, Beckman Colter, Villepinte, France). The span index was also evaluated by the following equation:

\[
\text{Span Index} = \frac{(D90 - D10)}{D50}
\]  

Where D90, D10, and D50 are the particle sizes determined to the 90th, 50th, and 10th percentile of undersized particles, respectively.

2.5. Powder X-Ray Diffraction (XRD)

XRD analyses were performed for the xylan powder, TC, and PbMs using an XRD-6000 diffractometer (Shimadzu, Kyoto, Japan) with a 2\(\theta\) range between 20° and 80° using Cu Ka radiation \((\lambda = 1.54056\text{Å})\). The XRD patterns were recorded at room temperature.

2.6. Fourier Transform Infrared (FT-IR) Spectroscopy

The FT-IR analyses were performed in solid state with a Nicolet Nexus 470 FT-IR spectrometer (Thermo Scientific, Waltham, MA, USA). The xylan powder and the PbMs dried samples were crushed with KBr and compressed into pellets. Spectral scanning was run in the range of 4000 to 400 cm\(^{-1}\). Prior to recording, the spectra were transformed against a KBr background.

2.7. Thermal Analysis

Thermogravimetry analysis (TGA) and derivative thermogravimetric curve (DTG) were obtained with an STA 409 PC Luxx device (Netzsch, Selb, Germany), using an aluminum pan with about 5 mg of sample under dynamic nitrogen atmosphere (10 mL·min\(^{-1}\)) at the heating rate of 10 °C·min\(^{-1}\), from 25 to 600 °C. Differential scanning calorimetry (DSC) curves were obtained with a Phoenix 204 calorimeter (Netzsch, Selb, Germany) using an aluminum pan with about 5 mg of sample under dynamic nitrogen atmosphere (10 mL·min\(^{-1}\)) at a heating rate of 10 °C·min\(^{-1}\), from 25 to 600 °C.

2.8. In Vitro Biocompatibility Assay

To evaluate the biocompatibility of the formulation (PbMs) and its raw material (xylan), the metabolic activity was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Firstly, human cervical adenocarcinoma cells \((5 \times 10^4 \text{ HeLa cells/well})\) were plated on 96-well microplates and were treated with 200 µL of the tested dispersions (Table 1) for 24 h at 5% CO\(_2\) and 37 °C.
The control cells were incubated with only DMEM medium under the same conditions. The MTT was dissolved in sterile PBS at 0.5 mg/mL. Then, 100 µL of the MTT solution was added into each well after removing old culture medium and washed twice with PBS at 37 °C, followed by incubation at 5% CO\textsubscript{2} atmosphere and 37 °C for 2 h. After removing the MTT reagent, 100 µL of dimethyl sulfoxide (DMSO) was added to dissolve the purple formazan crystal inside the cells. Finally, the absorbance of the purple formazan crystal in DMSO was measured at 540 nm wavelength using a microplate reader (μQuant™, Bio-Tek instruments, Winooski, VT, USA), and cell viability was expressed as ratios versus untreated cells (OD\textsubscript{Control}/OD\textsubscript{Sample} = 100%/x), where “x” is the percentage value for the cell viability tested with samples. All samples were tested in 15 and 18 replicates for each concentration of PbMs and xylan, respectively.

2.9. Statistical Analysis

To evaluate the data obtained from the in vitro biocompatibility assay, a one-way ANOVA was performed, followed by the Tukey post hoc test. A p-value less than 0.05 was assumed for the statistically significant differences. Moreover, linear regressions were performed to better describe data behavior. For all analyses, RStudio version 0.98.501 was used with the following packages loaded: lattice, ggplot2, and pwr.

3. Results and Discussion

The development of PbMs as potential drug carriers by ICL with TC has been reported over the last two decades [14,15]. However, a small number of papers investigated the biocompatibility of these systems, and only a few of them showed success in its biological application [16–18]. In the following sections, PbMs biocompatibility will be described and possible correlations among physical, chemical, and thermal properties with their biosafety will be discussed.

3.1. Physical and Chemical Characterization

3.1.1. Morphology and Size Distributions

The scanning electron microscopy (SEM) images revealed PbMs defined by a tiny polymeric membrane, a smooth surface, and an oblong shape (Figure 2a,b). No aggregates were observed, although many broken PbMs were found. This probably occurred because the freeze-drying process is a stressing process and involves ice crystal formation and ice sublimation that can disrupt the complex structure of the PbMs as observed in other freeze-dried microparticles [19]. Also, Nagashima et al. observed no broken structures in the PbMs morphology evaluation by optical microscopy.
LEADS FROM PHYSICAL, CHEMICAL AND THERMAL CHARACTERIZATION ON CYTOTOXIC EFFECTS OF XYLAN-BASED MICROPARTICLES

Figure 2. SEM images of polysaccharide-based microparticles after freeze-drying at (a) 200×; and (b) 2000×.

The PbMs presented symmetric distribution as might be observed by the particle size distribution analysis (Figure 3a,b). In fact, 10%, 50% and 90% of the microparticle samples were smaller than 13, 34 and 63 µm, respectively. Also, the mean particle diameter was found to be 37 µm (Figure 3a). The mean particle sizes of 10%, 50%, and 90% of PbMs before freeze-drying were smaller than 14, 41 and 74 µm, respectively, and their mean diameter was 43 µm (Figure 3b). The span index for both formulations was found to be 1.47, indicating good control over the particle size distribution using the ICL method [20].

Figure 3. Cont.
LEADS FROM PHYSICAL, CHEMICAL AND THERMAL CHARACTERIZATION ON CYTOTOXIC EFFECTS OF XYLAN-BASED MICROPARTICLES

3.1.2. FT-IR Spectroscopy

The infrared analyses were performed in order to observe the formation of terephthalic esters bound to the PbMs due to the reaction between the cross-linking agent and the xylan. Initially, the xylan infrared spectrum (Figure 4) was found to be similar to those reported by our group and elsewhere [12,21]. Concerning the FT-IR spectrum of PbMs, peaks ascribed for polysaccharides were also detected. Moreover, an absorption band at 1280 cm\(^{-1}\), which can be attributed to the halogen bond in the carbonyl group of the TC structure, was detected (Figure 4). Also detected was a peak at 1724 cm\(^{-1}\), which is attributed to C=O stretching vibrations related to the aromatic acid esters, such as terephthalic esters [16,22].

The presence of terephthalic esters on the PbMs spectrum is evidence of the cross-linking reaction between xylan and TC, which is the reason for the polymeric membrane stabilization since no other known approach for cross-linking, such as heating, pressure, or UV light exposure, was used to reinforce the polymer chains [23].

Figure 3. (a) Size distribution of polysaccharide-based microparticles before; and (b) after the freeze-drying process. The red line present the cumulative index line.

Figure 4. Cont.
Figure 4. (a) FT-IR spectra of polysaccharide-based microparticles; (b) terephthaloyl chloride; and (c) and xylan.

Additionally, the halogen bond identified at the PbM spectrum corroborates with the hypothesized mechanism of toxicity, as described in the Cell Viability through MTT assay section. The toxicity appears to be related to the cross-linking agent molecules that are bound to xylan, but would remain with some unreacted radicals free to react with the media.

3.1.3. XRD Analysis

XRD has been reported as a helpful tool to strengthen the results from the FT-IR spectroscopy because cross-linking agents usually show crystallinity while polymers are described as amorphous compounds [24,25]. As previously suggested by Oliveira and colleagues, the XRD analysis of xylan presented a profile of amorphous polymers displaying no crystallinity peaks. On the other hand, the cross-linking agent showed several peaks due to its crystallinity [12] (Figure 5a,b). In fact, the analysis of PbMs detected two peaks with different intensities near the angle for TC peaks [25]. The absence of other peaks from TC suggests that the molecules self-arranged while maintaining a minimal crystallinity. However, the crystallinity observed in the PbMs may be due to the organization of the xylan chains after the cross-linking reaction with TC, which might be an additional factor together with the unreacted TC radicals observed by FT-IR, as the reason for PbMs toxicity.
Indeed, the results from the cell viability, FT-IR, and XRD showed that the toxicity of PbMs is related with the cross-linking of xylan, which may not be merely related to the stoichiometry between the cross-linking agent and the polymer, but to the way in which the TC acts on the binding. Moreover, the toxicity could also be predicted by using a step-by-step physicochemical procedure to identify the crystalinity changes that might induce toxicity.

3.1.4. Thermal Analysis

Even though PbMs revealed to be more toxic than xylan itself, and similar citotoxicity has been described in starch microcapsules [14], its use at low concentrations might be considered for drugs with high potency, where the administration of PbMs would be reduced due to the low payload of the drug. For this reason, the analyses of the PbMs thermal characteristics were carried out.

The TGA of xylan powder (Figure 6a) showed two steps of mass loss, −8.9% and −49.8% at 55–150 °C and 180–380 °C, respectively. These events were clearly fingerprinted on the DTG curve, where a small shoulder in the second mass loss event suggests that it is a two-stage process (Figure 6a). The first event, occurring between 100 and 150 °C, may be related
to hydration water loss from the xylan powder. The main event, occurring subsequently at higher temperatures up to about 380 °C, is related to the onset of polymer degradation processes.

These results are in agreement with the complex structure of carbohydrates, which have a degradation process determined by dehydration reactions such as desorption of physically adsorbed water and removal of structural water, respectively. Additionally, depolymerization followed by rupture of C–O and C–C bonds in the ring units, such as of 4-O-methylglucuronic acid and acetyl groups, results in the increase of CO, CO$_2$, and H$_2$O, and the formation of polynuclear aromatic and graphitic structures [26–29]. Thus, the observed events can be related to the release of water and the pyrolysis of xylan. The xylan pyrolysis was driven by the evolution of several volatile gases as shown by Shen et al. [27]. Both mass loss processes were also evidenced in the DSC curves by an endothermic and an exothermic peak, respectively (Figure 6a). A third endothermic event had an onset temperature of 485.5 °C. Even though it has no counterpart in the TGA curve, this event could be related to the CH$_4$ evolving, as observed by Shen et al. [27], at the same temperature.

On the other hand, the TGA of PbMs showed only a one-step mass loss of 83.3% between 230 °C and 450 °C (Figure 6b). Similar to the previous case, the DTG curve showed that three subsequent events were involved in the PbMs decomposition. These events were clearly observed in the DSC curves as an exothermic peak was followed by an endothermic one. The third event, a weak shoulder after the main mass loss process, was not observed in the DSC curve, probably because of the little residual mass resulting from the decomposition. The different thermal behaviors of xylan and PbMs at 100 °C may be a consequence of the freeze-drying process that removes almost all water content from the PbMs dispersion. Additionally, the increase in the temperature of degradation for PbMs may be related to the complexation between the cross-linking agent and the xylan. In fact, it has been demonstrated that TC is able to increase the thermal and mechanical properties of some
materials in aerospace and military areas [30]. Thus, the only identified mass loss event is related to the breaking of the bonds between glycoside compounds and depolymerization [27]. These features are supported by the results of the FT-IR and the XRD analyses, which revealed the correlation between the polymer and the cross-link agent, and the crystallinity of PbMs.

3.2 Cell Viability through MTT Assay

The MTT assay, based on NAD(P)H-dependent cellular oxidoreductase activity, has been widely used for assessing cytotoxicity induced by several compounds in in vitro cell culture models [31]. The data obtained from the MTT assay showed that PbMs are more cytotoxic than xylan, since PbMs caused reduction in cell viability at lower concentrations than the ones used for xylan. To standardize the used xylan concentrations for PbMs samples (Table 1), 1.488 mg·mL⁻¹ was considered as the initial studied concentration in this assay. This value corresponds to the initial amount of xylan in the final volume of the PbMs suspension.

Xylan was shown to be satisfactorily biocompatible in the range of 4.1–12.4 mg·mL⁻¹, as a cell viability degree of about 70% was found (Figure 7a,c). Indeed, these results were expected because this material is considered highly stable, nontoxic, and hydrophilic [10,32], even though anti-proliferative activity of xylan against HeLa cells has been reported as its AC50 = 1 mg/mL, as well as the activity of other polysaccharides [13]. Despite its low toxicity, at 24.8 mg·mL⁻¹ the cell viability decreased (Figure 7a,c), probably as a result of the saturation of the medium with xylan and, consequently, the precipitation of xylan as result of its low solubility in aqueous medium at physiological pH [33]. Moreover, concerning the biosafety use of xylan in normal cells, this result might lead to the conclusion that concentrations smaller than 24.8 mg·mL⁻¹ should be used. In fact, this prediction might be supported, as HeLa cells (used here as a model of study) are much less sensitive than the non-carcinogenic cells.
**Figure 7.** Cell viability of HeLa cells after 24 h incubation with xylan dispersions at different concentrations and polysaccharide-based microparticle (PbMs) dilutions. (a) Scatterplots for xylan dispersions; and (b) PbMs suspensions with trend lines. The lines in the graphics are the trending line (blue) and the direct line that connect all the points (black).

The cell viability in the presence of PbMs was close to 70% at the theoretical xylan concentration of 0.372 mg·mL⁻¹ at the formulation (Figure 7b). The cytotoxicity exhibited by PbMs might be explained by the presence of the free unreacted radicals from TC, displayed in the FT-IR (Figure 4a). The chloride toxicity is induced due to its ability to oxidize the main cell components such as amino groups of amino acids [34].

Afterwards, the performance of the statistics analysis for different groups of xylan dispersion and PbMs suspensions showed p-values higher than 0.05 (Table S1). However, when concentration dependency was analyzed for xylan and PbMs separately, p-values smaller than 0.05 were observed. Therefore, a ratio between xylan concentration (for xylan dispersions) and the theoretical xylan concentration (for PbMs suspensions) was performed. As a result, it was found that in order to present the same toxicity as PbMs, the xylan dispersion would have to be presented at 33.3-fold in the media. This ratio value supports the hypothesis that polysaccharides are safe as broadly reported [35]. Therefore, further physicochemical analyses were mandatory to identify eventual residues of the cross-linking agent, as previously mentioned.

Furthermore, the evaluation of the concentration of xylan dispersions and PbMs suspensions able to reduce the cell viability in 50% was calculated by the equation from the linear regression study (Figure 7, Table 2). The values of 17.93 and 0.59 mg·mL⁻¹ were found for xylan dispersions and PbMs, respectively. The ratio between these concentrations was 30.27 mg·mL⁻¹, which is close to the value obtained in the analysis among the non-statistically different groups.

Overall, the statistical results pointed out the biosafety of the xylan polymer and the toxicity related to the cross-linking agent, TC, in the production of PbMs.

**Table 2.** Linear regression from cell viability vs. xylan concentrations in the xylan dispersions and in PbMs suspensions.

<table>
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<th>Slope (Angular Coefficient)</th>
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**4. Conclusions**

This work suggests a step-by-step procedure to evaluate the biosafety of polysaccharides and
the micro-structured devices produced with them. Moreover, xylan showed to be a biocompatible raw material for the production of microparticles. However, the use of TC as a cross-linking agent for the PbMs production prompts a meaningful reduction, approximately 30-fold, in its biocompatibility due to the residual chloride radicals. The FT-IR showed remains of a halogen bond, while XRD revealed that no crystals from TC remain in the particle. The evaluation of these results together suggests that the halogen-aromatic bond from TC would be the probable source of toxicity of PbMs. Additionally, the xylan cross-linking with TC would be the reason for PbMs crystallinity. Thermal analysis confirmed the formation of a more stable compound, as the temperatures to observe weight loss and the variation on enthalpy were higher for PbMs than for xylan itself, indicating that if biological safety is not a concern, the utilization of PbMs is chemically feasible. Furthermore, the ICL technique showed to be a very controlled process able to produce a short range of particle size distributions, as observed by the microscopy and particle size distribution. Finally, the ICL with TC seemed to be a viable technique for the production of drug carriers, although PbMs showed some toxicity. However, its good chemical, morphological, and thermal characteristics also provide a potential applicability in textile and tissue engineering.

**Supplementary Materials**

Supplementary materials can be accessed at: http://www.mdpi.com/2073-4360/.

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**Author Contributions**

Henrique Rodrigues Marcelino: Design of experiments, performance of experiments, data analysis, and writing of the manuscript; Acarília Eduardo da Silva: Data analysis and writing of the manuscript; Monique Christine Salgado Gomes: Performance of experiments; Elquio Elenam Oliveira: Design of experiments, data analysis, and writing of the manuscript; Toshiyuki Nagashima-Junior: Data analysis and of writing the manuscript; Gardênia Sousa Pinheiro: Performance of experiments and data analysis; Acarízia Eduardo Silva: Performance of experiments and data analysis; Ana Rafaela de Souza Timoteo: Performance of experiments and data analysis; Lucymara Fassarela Agnez Lima: Data analysis and editing manuscript;
Alejandro Pedro Ayala: Data analysis and editing manuscript; Anselmo Gomes Oliveira: Editing manuscript; Eryvaldo Sócrates Tabosa do Egito: Concept of the Project, funding, and editing manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

References


### Table S1. Statistically difference among groups after the performance of the post-hoc Tukey test.

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* p-value > 0.05
CHAPTER V

THE USE OF PEG SEPARATION PHASE TO PRODUCE XYLAN-BASED MICRO- AND NANOSTRUCTURES
BRIEFING

The development of a new drug carrier based on xylan was defined once the drug release experiments performed by Silva et al (Attachment II) were finished, and the results from the biocompatibility were obtained. As aforementioned, it was seen that the methodology applied also showed to have intrinsic toxicity.

The microcapsules were toxic against different cell lines, as showed on the previous chapter of this thesis (Manuscript 4 and Attachment I). Additionally, the inability of the microcapsules to retain 5-aminosalicylic molecules inside of its inner structure (aqueous nucleus) was a major problem to overcome and the coating by other pH-sensitive polymers, such as Eudragit® S100 was also not effective (Attachment II).

Thus, to develop new xylan-based drug delivery systems the choice of the raw materials was done carefully, by choosing polymers and even a crosslinking agent that are not toxic against Caco-2 cells under their physiological conditions. Additionally, there was avoided the use of organic solvents.
Xylan-based nano and micrometric structures using PEG phase separation process

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ABSTRACT

Polysaccharide-based micro- or nanoparticles are widely used and have broad applications in various industrial areas. In the field of drug delivery, they are often considered as promising drug carriers, due to an interesting set of characteristics, including biocompatibility, drug loading and controlled release capacities for various drugs, availability of preparation process, etc. However, the techniques to produce these polysaccharide-based particles usually require the use of potentially harmful organic solvents, with the risk for toxicity due to the presence of residual solvents at the end of purification processes. In this respect, processes based on polymers phase separation have recently attracted attention for producing various micro- or nanostructures not only for avoiding the use of organic solvents but also to access to original structured objects. The objective of this work was to use the segregation capacity of PEG 20000 to form xylan-containing domains in water and to produce xylan-based particles without the use of organic solvents. Initially, the cytotoxicity of xylan and trisodium trimethaphosphate solutions on Caco-2 cells has been determined. Then, a $2^2$ factorial design was performed and a modeling of the results was done in order to describe the influence of each polymer solution on the mean droplet size of the dispersions. Also, some physicochemical characterization was carried out for a better understanding of the interaction between the two polymer solutions. Afterwards, two of the studied mixtures were used to produce xylan-based particles through a chemical crosslinking approach. Spherical particles were produced but there were no correlations between their size distributions (after cross-linking) and the size of the domains. Additionally, the possibility to obtain mixtures of polymers with different droplet sizes in micro- and nanometric range and to obtain information about their structure were related to their surface tension characteristics. In conclusion, it was suggested that TSTP could be used as a safe chemical crosslinker, as well
as the biocompatibility of xylan against caco2 cells. Furthermore, it also showed the potential of this method for the production of both micro- and nanoparticles in the absence of organic solvents.

Keywords: xylan, PEG, water-in-water emulsion, biocompatibility, surface tension, nanoparticles.
1. INTRODUCTION

Polysaccharide-based micro- or nanoparticles are widely used and have broad applications in various industrial areas [1, 2]. In the field of drug delivery, they are often considered as promising drug carriers, due to an interesting set of characteristics, including biocompatibility, drug loading and controlled release capacities for various drugs, availability of preparation process, modulation of their morphologies, etc [3].

Xylan is a polysaccharide extracted from grains, such as corn cobs and beechwoods. This biopolymer often exists as fibers, has a lower, if any, solubility in the gastrointestinal fluids and consequently it is only degradable in the region of the colon by the enzymes produced by the microbiota, which makes this polymer a potential candidate, as an excipient, to produce colon-targeted drug delivery systems. In fact, our research group has previously showed its potential as a drug carrier [4-7].

However, many techniques to produce polysaccharide-based particles require the use of potentially harmful organic solvents, with the risk for toxicity due to the presence of residual solvents at the end of purification processes [8, 9]. In this respect, processes based on polymers phase separation have recently attracted attention for producing various micro- or nanostructures as a mean to avoid the use of organic solvents but also to access to original structured objects [10, 11].

The phase separation of polymer solutions or polymer blends has attracted great attention of scientists in recent years. Due to long relaxation time and large scale of polymer molecules, phase separation can occur, leading to water in water emulsions, which behave very differently compared with that in small molecule systems and are likely to produce original structures at the mesoscopic level [12]. However, there are still lacks of knowledge on the process, which is one of the reasons for its low application for the production of micro- and
nanocarriers. Additionally, the use of high amounts of polymers to produce these emulsions is another limiting factor [11].

In the recent years, the characterization of few pairs of polymers in water solutions has been described, including starch and others. Once phase separation has been obtained, the process comprises a second step consisting in a mechanical reinforcement of the particles, generally obtained by using conventional cross-linking strategies [13, 14].

In the present work the aim was to obtain and to optimize xylan/PEG20000 (PEG) water-in-water emulsions, in order to find the smallest amount of polymers necessary to their formation and to understand the mechanisms controlling droplet sizes and if possible to obtain a prediction model for it. Additionally, it was also aimed to proof the feasibility of this technique for the production of polysaccharide-based micro- and nanoparticles, in absence of organic solvents and other harmful compounds.

2. MATERIALS AND METHODS

2.1. Materials

Xylan from beechwood, trisodium trimetaphosphate (TSTP), Dulbelco’s Eagle Modified Medium (DMEM) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) kit were purchased from Sigma Chemical Co. (USA). Polyethylene glycol (PEG, $M_w = 20000$ Da) was purchased from Merck (Brazil). Sodium hydroxide and toluene were purchased from Vetec Chemical (Brazil). Deionized water was used at lab grade.

2.2. Cell viability assay

Caco-2 cells (ATCC® HTB 37) were disposed in a 48-well plate at $6 \times 10^3$ cells/well plate. The plates were incubated for $24h$ at $37^\circ C$ in humidified atmosphere of $5\%$ CO$_2$ in air. Then,
the media was removed and replaced by 200 µL of dilutions prepared with DMEM containing xylan and TSTP at the final concentrations of 2 mg/mL and 1 mg/mL, respectively. The addition of the MTT reagent was performed 24h later to evaluate cell viability. All the assays were performed in triplicate.

2.3. Xylan-PEG mixtures morphological characterization
Initially, PEG and xylan solutions, in the concentrations described at Table 1 (in accordance with a $2^2$ factorial design), were produced under magnetic stirring for 18 h in water and sodium hydroxide 1N, respectively. Afterwards, the xylan solution was added into the PEG solution at 1:9 (v/v) ratio. All mixtures were homogenized by magnetic stirring for 5 minutes and incubated at 45°C for 6 h, without stirring. The samples were stored into glass vials and their macroscopic aspects, such as color and presence of precipitate were evaluated, as well as microscopic aspects and particle size distribution. All samples were produced and analyzed in triplicate.

2.4. Light scattering methods
Static (SLS) and dynamic light scatterings (DLS) were carried out on a NanoZS (Malvern) instrument. All measurements were made at the temperature of 25°C, $\lambda = 633$nm and the detector at 173°. For SLS measurements the xylan differential index refraction (dn/dC) was calculated from the slope of a linear regression of refractive index versus xylan solutions in different concentrations [15].
2.5. Density of xylan and PEG solutions

The density ($\rho$) of xylan and PEG solutions in water were measured using the pycnometry methodology. A 5 mL pycnometer was used for all measurements, and the $\rho$ were calculated using the densities of water at 25ºC as the reference.

2.6. Surface tension analysis

The surface tension values for the mixture of polymers in water were obtained through the Du Noüy ring method using a Tensiometer K100 (KRUSS, Germany). Measurements were performed in duplicate at 25ºC.

2.7. Production of xylan-based particles

To obtain rigid solid structures, such as micro- and nanospheres, the addition of an amount of TSTP, in order to have the final concentration of 1% (w/v) at pH $\approx$ 12, was done into xylan solution before its mixture with PEG solutions. The mixtures were produced using the same ratio used for the formulations 2 and 4 (Table 1). The crosslinking reaction was carried out for 6 h, then the reaction was stopped by the addition of ethanol. After centrifugation (2000 RPM), the precipitate was later re-suspended and dialyzed against water during 18h in order to remove the excess of PEG from the dispersions. At the end, the dispersions were kept in glass vials with a final volume of 10 mL. Aliquots of these dispersions were freeze-dried for further characterizations.

2.8. Morphology, droplet and particle size distribution analysis

The morphology and the size distribution of both xylan-containing droplets before and particles after cross-linking were determined by optical microscopy (Leica DM500, coupled
with a digital camera (Leica, ICC50). Methylene blue was added previously to these analysis and Ferret’s diameter of the coloured droplets was measured on 1500 droplets/particles per samples (n=3), adn finally expressed as a mean. Additionally, scanning electron microscopy (TM3000, Hitachi) were conducted. Particles in formulation 2, which showed smaller droplet and particle size, were analysed by transmission electron microscopy (MET 1400, JEOL) and DLS.

### 2.9. Statistical analysis

Statistical analysis of the data was obtained by applying the RStudio version 0.98.501 (R version 3.2.0) which was used with the following loaded libraries (lattice version 0.20-31, ggplot2 version 1.0.1 and psych version 1.5.4). Additionally, Microsoft® Excel® for Mac version 15.14. was also used to plot some graphics.

### 3. RESULTS AND DISCUSSION

Polysaccharide-based micro- or nanoparticles are of interest for drug delivery. Among many, xylan-based particles have been described as potential colon-specific drug delivery systems, which may be useful for local treatment of Inflamatory Bowel Diseases (IBD) or site-specific cancers delivery. The aim of our study was to investigate an innovative process for the preparation of xylan particles, avoiding the use of potentially harmful solvents and using biocompatible excipients [16]. PEG and xylan where chosen once they are reported as biosafe in the literature.
3.1. Biological evaluation of the raw materials.

As previously reported by Silva et al. and Melo-Silveira et al. [5, 17] for HeLa and 3T3 fibroblast cell lines, xylan did not present itself cytotoxic for Caco-2 cells. We studied Caco-2 cell viability for xylan solutions at the same concentration used in previous reports (Figure 1). This result corroborates with the statement of xylan’s biocompatibility in this range of concentration (0.03-0.5 mg/mL). Additionally, TSTP demonstrated no adverse effect against Caco-2 cells in the same range of concentration (0.03-0.5 mg/mL) and at neutral pH. This is an interesting result, since cross-linkers are generally suspected for toxicity concerns. Indeed, reactivity of TSTP with hydroxyls groups of polysaccharides has been often used for crosslinking of polysaccharides and to produce microparticles [10, 13, 18, 19]. In fact, the absence of toxicity for Caco-2 cells suggests a lack of reactivity of TSTP with biological materials, which may be related to the fact that reactions with other molecules requires pH=12 conditions, which is very far from the neutral pH used during cytotoxicity tests and in biological environments (pH=7.4) [20].

3.2. Xylan characterization

Xylan is a polysaccharide that has its chemical characterization (by infrared measurements) extensively described in the literature, even though its optimal extraction conditions are still being studied. Therewith, the lack of fully standardized extraction methods can play an important role in the variations of the properties of the raw material and, consequently, on the characteristics of the polymeric systems that will be produced [21]. Among these properties molecular weight has been suggested to be responsible for changes in particle shape [3]. In this respect, the molecular weight of xylan was determined by SLS. Therefore, to perform this analysis, it was first necessary to determine experimentally the refractive index dn/dC of
Xylan solutions in sodium hydroxide 1N. Xylan solutions at different concentrations were prepared and analyzed in a refractometer. Afterwards, a plot to of concentration of xylan solution versus refractive index was made and the angular coefficient was obtained after linearisation of the data that describes the influence of xylan concentration over the refractive index of xylan solutions, which has been reported as the dn/dC value (dn/dC\textsubscript{xylan} = 0.1142) [15]. SLS analysis revealed that the xylan had a molecular weight of 66 ± 18 KDa with second virial coefficient of 2.6 ± 1.7 mL x mol / g\textsuperscript{2}.

DLS analysis showed that xylan had a wide and multimodal chain distribution (Figure 2a). This indication led us to further observe whether the cross-linking reaction could lead to a homogenization of the particle size, or if the particles would show similar polydispersity.

### 3.3. Polymer mixture characterization

#### 3.3.1. Morphology and droplet size distribution

In a first step, four mixtures of xylan and PEG were prepared and their macroscopic properties were examined. The mixtures with low amount of xylan (Formulations 1 and 3, Table 1) had a translucid aspect. However when higher amounts of polymers were used (Formulations 2 and 4) a yellowish color was observed after magnetic stirring. The samples were also evaluated after incubation, and at this point all samples had a slightly orange color with an increasing in intensity from Formulations 1 to 4. In spite of the color changing the hypothesis of xylan thermal degradation might be discarded due to thermal stability of xylan as previously described [5, 16].

Phase separation between xylan and PEG resulted in the formation of discrete domains containing xylan (dyed in blue by methylene blue) and PEG transparent solution, forming an external phase. Different techniques were used to determine the size distributions of these
domains. Subsequently, all xylan-PEG mixtures were analyzed by DLS. Differently from xylan solutions, where 3 different populations were observed (Figure 2a), all mixtures showed only one major population (Figure 2b, Table 1) with a PdI ≤ 0.2 for almost all of them. This effect could be attributed to a strong phase separation effect induced by the presence of PEG 20000 in the solution.

On the contrary, when the size of the domains was in the micrometric range (mixtures 3 and 4) the particle size distribution was evaluated by optical microscopy based on Ferret’s diameter measurement (Figures 3a,b) and PdI was calculated by the following equation:

\[ \text{PdI} = \left( \frac{\sigma}{\mu} \right)^2 \]

Where \( \sigma \) is the standard deviation and \( \mu \) is the average values for droplet size. The fact that homogenous populations were found in both measurements may show that the technique applied for the production of emulsions overcomes the properties of the polymer on the shape of the particles that will be produced, being always spherical, in opposite to what has been reported for the nanoprecipitation technique [3].

### 3.3.2. Mathematical modeling of the effect of polymers on particle size distribution

A modeling of the data obtained from the \( 2^2 \) full-factorial design was performed in order to evaluate which of the polymer solutions would have the degree of influence on the mean droplet size. Even though, as it will be further discussed it is the physicochemical interaction between the two phases that really describes the formation of the distinct phases into the mixture.
After data analysis an equation \( y = 1060.62 - 80.38 \times [\text{xylan}] + 265.33 \times [\text{PEG}] - 170.88 \times [\text{xylan}] \times [\text{PEG}]; \) \( \text{Radj} = 0.6333, \) p-value = 0.001617, “\( y \)” equal to mean droplet size) was obtained. Its capacity to predict the droplet size in the different mixtures was checked and was considered to be satisfactorily with an average-error of 13.1 ± 9.8 %. Additionally, the analysis of the equation and the Pareto’s plot (Figure 4) showed that the concentration of the external phase concentration (PEG solution) played an important role on the determination of the mean droplet size, probably due to an increase in the viscosity of the dispersive phase that could diminish the diffusibility of xylan chains into the external phase, as expected from the Stokes-Einstein equation for diffusibility. Therefore, the interaction [xylan] * [PEG] also seemed to be important for this property, and its influence will be further discussed in the next section.

Additionally, to the authors knowledge this is the first time that a quantitative relationship between the concentration of the two phases and the mean droplet size both in micro and nanometric ranges is reported. Obviously, the mathematical model here obtained must not be applied to predict droplet size out of the range here studied. In fact, when concentrations over 32 % (w/v) of PEG were tested it was not possible to obtain mixtures without a xylan precipitation (data not shown).

### 3.3.3. Physicochemical characterization of the polymers mixtures in solution

A relationship between \( \Gamma \) and \( \Delta \rho \) has been reported which could help to predict not only phase separation between polymers in solution but also the morphology of the domains formed in polymer mixtures [12]. An increase in \( \Delta \rho \) has been shown to lead to a decrease in the mean droplet size, although a low limit must be carefully taken in order to avoid the miscibility between the two phases at low concentrations. The surface tensions (\( \Gamma \)) of all the
mixtures were measured in order to evaluate a possible correlation with the droplet size or the mass balance between the two phases (Table 2). As it can be observed in Figure 5 there was a negative correlation between the superficial tension and the difference of density between the two phases. In fact, this phenomenon might be explained by an increase in the interactions between xylan and PEG, which would leave less free PEG chains onto the interface air/water. This hypothesis is corroborated by the fact that xylan is a hydroxyl-rich polymer, and these groups can strongly interact with other hydrophilic compounds by forming hydrogen bonds. Additionally, this phenomenon has also been indirectly reported by other research groups that revealed an increase in the interfacial tension with the increase of the density difference ($\Delta \rho$) between the two phases [12, 22, 23]. Furthermore, when the results were analyzed from the point of view of the impact of xylan concentration into the medium it was clearly possible to observe (Figure 6) an increase in the surface tension of the mixtures with xylan concentration.

It was also possible to correlate the droplet size, and the increase on droplet size distribution with the $\Delta \rho$ (Figure 7). Briefly, an increase of xylan concentration led to an increase of the interactions between the two phases, as previously hypothesized. Indeed, the increase in the interaction forces can be observed by the plot of the experimental $\Delta \rho$ versus a theoretical $\Gamma$ using the non-linear model ($b = 3, \Gamma = a(\Delta \rho)^b$) previously reported to apply and to explain the interfacial behavior of mixtures of dextran and gelatin [12, 22, 23]. Thus, such a high tension could make unable the free movement of polymer chains from one droplet to another, which may explain the smaller variation in mean droplet size.
3.4. Production of polymeric particles

3.4.1. Morphology and droplet size distribution

TSTP has been used as a cross-linking agent in order to obtain rigid structures (particles) from the domains contained in the mixture of polymers. In practice, TSTP was added into xylan solutions before its mixture with the PEG solution. Afterwards, particles were produced and their size distributions were analyzed by optical and electron microscopy. Through these analysis it was possible to observe spherical particles (Figure 8a,b) and the reproducibility of the method was observed, even though large distributions were observed when higher concentrations of PEG were used.

Mean particle sizes and PdI values are gathered in Table 1. Differently of what was observed for the mixtures, where higher mean values were followed by narrow droplet size distributions, the nanoparticles (formulation 2) showed a much sharper particle size distribution than the microparticles (formulation 4). Similar effects of high concentrations of polymers on size distribution were also found by others research groups that tested different polymers, such as dextran and Pluronic® F68 [11].

Additionally, this result shows that different techniques for particles’ production are under influence of different parameters (e.g. the nanoprecipitation technique is under influence of polymer molecular weight and the presence of side chains, while in this work the presence of polymer chains with different sizes did not cause the production of particles with different populations) [3].

4. CONCLUSIONS

The present study showed that xylan, a biocompatible polysaccharide can be segregated in
presence of PEG 20,000 and further cross-linked by TSTP. Because these particles are intended to be used under physiological conditions, TSTP proved to be of real interest as a cross-linker due to its lack of reactivity in such conditions, while being effective at cross-linking xylan chains at pH=12 during the preparation process.

Interestingly, it was possible to produce polymer mixtures with different droplet size distributions by the mixture of two polymeric solutions (xylan and PEG 20000) in different concentrations. An increase in the amount of PEG in the external phase resulted in larger particles in the micro-range.

This is the first report, for the knowledge of the authors, that describes a method to produce biocompatible xylan-based particles both in the range of micro- and nanometric and with potential applications in food and pharmaceutical fields, with the advantage of no concerns about organic solvents residues.

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REFERENCES


**Figure Captions**

**Figure 1:** Cell viability after 24h of Caco-2 cells against xylan and TSTP at different concentrations through the evaluation of their mitochondrial activity (MTT assay).

**Figure 2:** DLS analysis of (a) xylan solution at 10mg/mL in NaOH 1N and (b) xylan-PEG mixture (Formulation 2).

**Figure 3:** Optical microographies of xylan domaines into PEG 20000 dyed with methylene blue (a) Formulation 3 (xylan 1 %\(\text{w/v}\) : PEG 32 %\(\text{w/v}\)) and (b) Formulation 4 (xylan 4 %\(\text{w/v}\) : PEG 32 %\(\text{w/v}\)).

**Figure 4:** Pareto’s plot for the multivariate analysis to predict mean droplet size of xylan/PEG mixtures.

**Figure 5:** Correlation between surface tension and \(\Delta \rho\). The inner graph shows the theoretical correlation between \(\Delta \rho\) and interfacial tension, when \(\Delta \rho\) is close to the critical point (\(\Delta \rho \to 0\)).

**Figure 6:** Correlation between surface tension and total polymer concentration. Xylan’s interference on the interaction between the two polymer phases.

**Figure 7:** Correlation between mean droplet size and droplet size distribution with \(\Delta \rho\).

**Figure 8:** Microscopy of xylan microparticles produced by the reaction between xylan and TSTP in xylan-PEG mixtures (a) optical microscopy, (b) scanning electron microscopy.
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$R^2 = 0.9517$
Figure 6: Correlation between surface tension and total polymer concentration. Xylan’s interference on the interaction between the two polymer phases.
Figure 7: Correlation between mean droplet size and droplet size distribution with $\Delta \rho$.

$y = -126.2 \ln(x) + 249.52$

$R^2 = 0.87311$
**Figure 8:** Microscopy of xylan particles produced by the reaction between xylan and TSTP in xylan-PEG mixtures (a) optical microscopy and scanning electron microscopy of microparticles, (b) transmission electron microscopy of xylan-based nanoparticles.
Tables Captions

Table 1: Description of the mixture of polymers and the outcomes obtained by the performance of the experiments related to $2^2$ factorial design, as well as, the outcomes of the xylan-based particles.

Table 2: Measurements of polymeric solutions density, surface tension and interfacial tension of the all the mixtures studied.
Table 1: Description of the mixture of polymers and the outcomes obtained by the performance of the experiments related to $2^2$ factorial design, as well as, the outcomes of the xylan-based particles.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>[xylan] (mg/mL)</th>
<th>[PEG] (mg/mL)</th>
<th>Coded values (xylan)</th>
<th>Coded values (PEG)</th>
<th>Mean droplet Size (nm)</th>
<th>PdI</th>
<th>Mean particle Size (nm)</th>
<th>PdI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>100</td>
<td>-1</td>
<td>-1</td>
<td>704 (± 61)$^a$</td>
<td>0.243 (±0.09)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>100</td>
<td>+1</td>
<td>-1</td>
<td>885 (± 119)$^a$</td>
<td>0.237 (±0.04)</td>
<td>378 (± 9)</td>
<td>0.12 (± 0.04)</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>320</td>
<td>-1</td>
<td>+1</td>
<td>1450 (± 620)$^b$</td>
<td>0.213 (±0.06)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>40</td>
<td>320</td>
<td>+1</td>
<td>+1</td>
<td>1080 (± 240)$^b$</td>
<td>0.058 (± 0.01)</td>
<td>4390 (± 530)</td>
<td>0.442 (± 0.03)</td>
</tr>
</tbody>
</table>

(n=3)

$^a$ particle size distribution of sub-micrometric particles measured by DLS measurements.

$^b$ particle size distribution of sub-micrometric and micrometric particles determined by Ferret’s technique.
Table 2: Measurements of polymeric solutions density, surface tension and interfacial tension of all the mixtures studied.

<table>
<thead>
<tr>
<th>Solution / Dispersion</th>
<th>Concentration (mg/mL)</th>
<th>Surface Tension (mN/m)**</th>
<th>Density (g/mL)</th>
<th>Δρ</th>
<th>Calculated interfacial tension (N/m)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>xylan solution</td>
<td>10</td>
<td>-</td>
<td>1.122</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>-</td>
<td>1.133</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PEG solution</td>
<td>100</td>
<td>-</td>
<td>1.092</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>320</td>
<td>-</td>
<td>1.122</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Formulation 1</td>
<td>-</td>
<td>54 (±0.8)</td>
<td>-</td>
<td>0.030</td>
<td>2.6x10^-5</td>
</tr>
<tr>
<td>Formulation 2</td>
<td>-</td>
<td>53 (±0.8)</td>
<td>-</td>
<td>0.041</td>
<td>6.8x10^-5</td>
</tr>
<tr>
<td>Formulation 3</td>
<td>-</td>
<td>57 (±0.1)</td>
<td>-</td>
<td>0.000</td>
<td>1.4x10^-11</td>
</tr>
<tr>
<td>Formulation 4</td>
<td>-</td>
<td>57 (±0.2)</td>
<td>-</td>
<td>0.011</td>
<td>1.5x10^-6</td>
</tr>
</tbody>
</table>

* The interfacial tension was calculated from the expression $\Gamma = a(\Delta \rho)^b$ where $a = 1$ and $b = 3$.

** All measurements were performed 10 times using the same samples.
GENERAL DISCUSSION
The experimental work performed during this thesis addresses two main questions, which will be discussed separately in this chapter.

1. Impact of the aggregative behaviour of amphotericin B in its biopharmaceutical properties

The oral route is the preferred route of administration when the feasibility and the compliance of patients to therapeutic treatments is taken into account. The dosage forms formulated for this route are usually solids, e.g. tablets and capsules, even though liquid dosage forms, such as solutions and suspensions are also used [1-3]. However, the availability of various dosage forms does not mean that they will be suited to a given drug and will maximize its pharmacological activity. In fact, biopharmaceutical parameters must be carefully evaluated, previous to any formulation program, mainly the drug solubility degree in gastrointestinal fluids and the drug permeability through the gastrointestinal membrane in order to guarantee that the extent of absorption will be maximal for the drug and consequently produce the expected pharmacological activity [1, 2, 4, 5]. Once many drugs have no optimal characteristics, there are often limitations to their delivery by the oral route. Among these drugs that are not well absorbed there is amphotericin B (AmB), an efficient polyene antifungal molecule that has its use restricted to hospitals [6-13].

In the last three decades much has been done to try to solve the problem of drug solubility into physiological fluids, including the gastrointestinal fluids, as take advantage of the properties of the obtained materials produced by the use of nanotechnologies [7, 9, 14-18]. Many drug nanocarriers have been developed, most of them being based on the use of lipids or/and polymers, probably due to their versatility on having different properties accordingly to the material and the technique used to produce them [6, 9, 19-22]. Clear examples of such efforts are reported in the literature through the use of glycerol monooleate (type 40) (for lipid carriers) and chitosan derivatives (for polymeric carriers) [23-29]. Both approaches have been attempted in order to achieve an enhancement of AmB oral absorption, and both have showed promising results for AmB [6, 7]. However, there is an uncertainty about the pathways by which these nanocarriers act into the gastrointestinal tract and on how they promote absorption [7, 8, 11]. Therefore, one of the aims of this thesis was to investigate
some possible pathways, using or not lipid-based systems.

During this work, the impact of different parameters on intestinal absorption of AmB were evaluated. Many absorption models can be used for measuring intestinal absorption, ranging from side by side chambers experiments using either cell cultured monolayers or intestinal tissues from sacrificed animals or human biopsies, intestinal loop models, in situ absorption models, to in vivo delivery and deconvolution of the absorption profile from pharmacokinetics data. Here, the model of Ussing’s chambers has been selected, since it is relatively simple to handle compared to others and allow us to parallelize a reasonable number of experiments, while at the same time it makes possible to investigate absorption directly on living tissues, thus presenting all the know (and unknown) transporters, and moreover on tissues which will be used in any preclinical trials in living animals, such as rats or mice. However, obviously important physiological determinants of absorption in vivo are not reproduced in this model, mainly the gastrointestinal transit and nervous interaction of the mucosa.

First, the influence of AmB concentration as been selected as a major parameter since the propensity of AmB to aggregate in different aggregation states (dimers, trimers, larger aggregates, etc) has been better and better understood through the years, and may extensively impact the passage of AmB through biological membranes [30]. Afterwards, AmB was entrapped into micro- and nanoemulsions in order to reproduce and to investigate the mechanisms by which previously reported absorption enhancements could occur, including the different routes of entry which are theoretically available to intestinal passage, i.e.: (i) active or passive transport, paracellular or transcellular, possible direct uptake of AmB aggregates by cells belonging to the gut-associated lymphatic tissue (GALT) (lymphatic route) [24, 31]. However, in contrast to what has been reported in the literature no permeation through the jejunal epithelium was seen in any of the conditions used. Indeed, decreases of the concentrations in the donor side of the chamber were systematically observed, which probably implies to some extent drug interactions with the mucus and/or the tissue, but no AmB was detected in the receiver chamber, whatever the conditions, the formulations, etc. A possible hypothesis for this phenomenon will be given as it follows. Drug aggregates could diffuse into the mucus layer and then remain trapped into these layers, without any possibility for the aggregates to dissociate and to form monomeric species of AmB, which are more likely to be absorbed. Indeed, it has been shown with nanoparticles that their accumulation into the mucus layer could be quantitatively important, which in turns
can be favorable to the absorption of an encapsulated drug, once the entrapped molecules can be easily released or not when drug release is impaired due to inadequate structure of the nanoparticle. Huge enhancements of the permeability have been observed for paclitaxel loaded nanoparticles able to penetrate the mucus layer and to release the drug as close as possible to the epithelium [32-35].

Additionally, it was decided in the experimental protocol to discard any tissue fragment presenting naked eye visible Peyer’s patches (even for the assays performed with nanocarriers). The reason for it was to avoid any possible capture of the particles by the GALT and thus, to be able to attribute any observed passage only to diffusive processes or carrier-mediated transporters on the enterocytes membrane. As previously mentioned, no permeation of AmB by active or passive mechanisms could be detected, leading us to the conclusion that in vivo observed absorption enhancements for AmB might mainly result from an interaction and an uptake of the nanocarriers by GALT as already hypothesized in the literature [7, 24, 36].

Interestingly, the Ussing’s chamber model allow the determination of the electrophysiological conditions of the tissue fragment all along the permeation experiments, which may help to evaluate the toxicity of AmB and the formulations. Indeed, an increase in AmB concentrations, meaning an increase in the aggregation of AmB resulted in a reduction of tissue viability. Indeed, this phenomenon may be directly related to the pharmacological and toxicological mechanisms described in the literature for AmB. Basically, AmB has the property to be inserted into the cells membranes where they form pores, which leads to an impairment of the electrolytic balance inside of the cells causing their death [37, 38]. In our study, a progressive loss of jejunal tissue viability was detected by the reduction of the Trans-epithelial electric resistance when compared to control experiments after 180 min.

The remaining uncertainties related to AmB and its behavior into the human body are not only linked to its oral absorption pattern. Indeed, when commercial preparations are dosed by the intravenous route and depending on the dosing schedule in clinics, as well as the posology also, important differences is the biodistribution of AmB into the human body have been observed and reported. Because inconsistencies or variabilities in biodistribution may impact both the therapeutics as well as the side effects of AmB.

Due to the fact that AmB is known for the formation of aggregates, by itself and also to
adsorb to plasmatic proteins we formulated the hypothesis that AmB biodistribution into the body could be influenced by: (i) the formation of aggregates following its delivery in the body, which in turn can be recognized as "non self" by the immune system, and (ii) by the fact that AmB could act as an hapten able to adsorb on albumin or other plasmatic protein, which in turn can lead AmB to immune cells and also to elicit an immune response. Indeed, in the case of the formation of aggregates there may be a parallelism with the recognition phenomena by which is known (but not yet well understood!) to happen with lipidic or polymeric carriers after intravenous delivery. Their recognition, and thus their biodistribution in the different organs, is known to be dependent of chemical and physical properties, such as size and the surface characteristics [39-41]. In therapeutics, it is expected that AmB accumulation would occur where the fungal infection is developing because of its own physico-chemical characteristics but not because of the one of the dosage form used for its delivery. In fact, there is an hypothesis that the biodistribution of the AmB-loaded nanocarriers are under influence of the macrophages movements towards the sites of infection [7]. In this context, we attempted to check if there would be any interaction between the AmB monomers and/or aggregates with the proteins of the immunological complement system, mostly protein C3, which could result in fast and easy AmB engulfment by immune cells, such as macrophages.

To check this hypothesis we attempted to control the aggregation state of AmB by using albumin, which works in the human body as “solubilizing agent” due to adsorb approximately two AmB molecules and therefore to its ability to keep AmB in monomeric form in its structure. Indeed, this interaction is well-known and it has been studied before in order to determine AmB binding sites and their affinities. However, in this work we did not use the same medium as utilized by Romanini et al and Aramwit et al, when they carried out their studies [42-45]. This is an important parameter since the behavior (or more precisely the AmB aggregation) can change depending on the medium (or the ions nature and concentration) changes [46, 47]. Additionally, we also used a set of spectrometric techniques, including, circular dichroism instead of the traditional fluorescence spectroscopy, which allowed us to clearly identify the beginning of the formation of AmB aggregates, while the fluorescence spectroscopy could probe AmB-albumin interactions by a change of the conformation of albumin that allows the tryptophan to emit a specific fluorescence [42, 48, 49], although it does not allow the determination of the aggregation state of AmB into the medium.
The Critical Aggregation Concentration for AmB into a dispersion of bovine serum albumin 4%\%(w/v) in Krebs-Ringer-Bicarbonate buffer (CAC_{AmB-BSA}) was determined to be 5x10^{-6} M which was obtained from both UV-Vis spectroscopy and circular dichroism spectroscopy. This value was slightly different from the previous values reported for the Critical Aggregation Concentration of AmB into a specific medium, which corroborates with the hypothesis that AmB aggregation could be slightly depending on medium composition [45, 50, 51].

In order to investigate the possibility that AmB aggregates could be recognized as a xenobiotic material by the immune system, a test of activation of the complement system was carried out, which suggested that AmB aggregation state could be an important determinant. Indeed, relevant increases of the cleavage of protein C3 once the AmB concentration reaches values equal or above the CAC_{AmB-BSA} aggregation value were evidenced. In fact, this phenomenon might mean that the AmB aggregates may behave similarly to non-stealth nanoparticles that lead to the cleavage of the protein C3 once it interacts with it [52, 53]. Such non-stealth nanoparticles are known for being rapidly captured and taken by the cells of the reticulum-endothelial system, which are mainly phagocytic cell, such as macrophages and be quickly eliminated from the blood stream, while being concentrated in organs such as the liver or the spleen [52, 54, 55].

Thus, we might have found an evidence that the biodistribution as well as elimination pathways of AmB (liver and passive elimination by kidneys) could depend at least partly from immune recognition phenomena in the body. This is a reasonable hypothesis once the physicochemical characteristics of AmB (aggregation, adsorption to proteins) is likely to be varied depending on the modalities of delivery as well as the nature of the formulations. It would be interesting to investigate these phenomena more in depth. However, this raises considerable experimental problems since the detection of low amounts of AmB aggregates would not only require highly sensitive techniques such has Mass spectroscopy coupled to a chromatograph, but also adequate techniques to detect aggregates in the nanoscale range. Finally, this phenomenon may also happen with other poorly or very poorly water-soluble molecules, such as paclitaxel and griseofulvin.
2. Development of xylan-based micro and nanoparticulate systems for colonic delivery of drugs

The development of drug delivery systems able to ensure drug delivery specifically in the colon is aimed usually for two reasons: (1) for the local treatment of different pathologies at this anatomical level and/or (2) to enhance drug absorption taking advantage of the wider tight junction this region, as well as a prolonged duration of contact in this region [56-58]. Such strategies could be very helpful for (1) improving the treatment of Inflammatory Bowel Diseases (IBD) pathologies or (2) for the enhancement of AmB absorption in order to treat systemic fungal infections. However, other applications can be imagined.

In the recent years, the influence of the mycobiota in the balance of the healthiness of the gastrointestinal tract has started to be reported. In fact, the role of the mycobiota has been described as multifaceted and complex, involving fungal-bacterial, fungal-fungal and fungal-host interactions [59-61]. Additionally, alterations of the microbiota in diseased states occur. For example, differences between the mycobiota of healthy volunteers and patients suffering from IBD, such as Crohn’s Disease, has already been reported [59, 62].

Indeed, the patients affected by the Crohn’s disease might have their gastrointestinal tract heavily colonized by Candida albicans, as well as, they might produce antibodies against oligosaccharides of the C. albicans cell wall [59]. In healthy volunteers the C. albicans population corresponds to approximately 40 % of the total mycobiome, while the production of the antibodies against the components of the epithelium are reduced or absent [59]. Thus, the control over the mycobiome by the use of antifungal molecules could be an alternative for treatment of IBD patients, who show a discrepant mycobiome when compared to healthy humans.

The control over the mycobiome could be reached by the use of antifungal molecules, however the amount of the drug released into the inflamed regions would have to be carefully controlled, so it would not cause a reduction of the mycobiome population to levels below normal. In addition to the challenges of the controlled release of molecules there is also the need for a determination of the “normal” mycobiome, since not all the species hosted are known and the ones known do not have their standard population determined [59, 63, 64].

Despite this lack of knowledge about the composition, the specificity and the total amount of fungi composing the mycobiome, it is known that the population of Candida can be altered
by the diet when it is enriched in carbohydrates, probably due to their ability to degrade in nutrients, e.g. starch [59]. Thus, in this work we aimed to develop a polysaccharide-based nanocarrier using xylan, a polymer which adequate properties for reaching the colon without being degraded in the gastro-intestinal tract, due to its resistance to the conditions found in the stomach and the in small intestine, as well as, the need for specific degrading enzymes which are only produced in the colonic region [65-68].

Xylan-based microcapsules were first developed by Nagashima et al, then lately studied by Silva et al. [69, 70]. However, no characterization of their physical and chemical properties along with their biocompatibility had been done before this project. Then, we confirmed the possibility to prepare microcapsules by the interfacial cross-linking process, using terephthaloyl chloride as a crosslinking agent in order to cause the cross-linking of the polymer and produce a polymeric wall capable of separating the two aqueous phases (inner core and dispersive phase) obtained after the dispersion of the microcapsules in water at the end of the process.

The reaction between xylan and terephthaloyl chloride was followed by different techniques, including Fourier-transformed infrared spectroscopy (FT-IR), X-Ray Diffraction (XRD), thermo-gravimetric analysis (TGA) and differential scanning calorimetry (DSC). The utilization of these techniques to confirm the binding between other polymers and crosslinkers has been described in the literature [71, 72]. The infrared spectra of different xylans have been broadly reported and they do not differ much from one to another since these polymers are very repetitive in their compositions although comprising small amounts of monosaccharides and small acid molecules containing mostly carbon, hydrogen and oxygen in their composition. Additionally, the use of different methods does not lead to a change on the main constituents, only the ratios among them [73, 74]. Thus, once an halogen bound, naturally present in the terephthaloyl chloride was found during the analysis of xylan-based microcapsules it evidenced that a chemical change was obtained. The identification of some crystallinity in the xylan-based microcapsules by the XRD technique corroborated with the results obtained by the FT-IR.

The thermal analyses revealed changes in the pattern of degradation of xylan. The most prominent change was an increase in the beginning temperature of degradation. Similar changes have also been reported for other materials cross-linked with terephthalic acid derivatives [75].
The evaluation of xylan-based microcapsules cytotoxicity against HeLa cells revealed a certain extent of toxicity. This reduction on the biocompatibility of xylan after crosslinking was attributed to the presence of residual terephthaloyl chloride in the microcapsules suspension as previously identified by FT-IR and XRD. For this reason, and also because drug release studies carried out by Silva et al (see attachment II) had revealed that the porosity of the polymeric wall of the microcapsules was too important and prevented an efficiently controlled release of drugs, the development of a new carrier based on xylan was started.

To develop this new xylan-based carrier, a preliminary screening of the cytotoxicity of the raw materials was performed in the Caco-2 cell line. At the end, three materials were selected, including xylan, poly(ethylene glycol) (PEG) and sodium trimetaphosphate (STMP). The use of PEG was based on our decision of develop an eco-friendly technique for the preparation process, which would avoid the use of harmful solvents, such as chloroform and cyclohexane used for the production of xylan-based microcapsules previously described.

The preparation method adopted was based on the formation of “water-in-water” emulsions. The principle of this technique is based on the fact that two aqueous solutions containing different water soluble polymers can be immiscible in some conditions, due to the segregation effects occurring between the polymeric chains of differing nature. Discrete domains of one or the other polymeric solutions can be formed, which in turn require to be cross-linked. So far, it has been mainly applied to the production of microspheres and/or polymeric (semi-solid) dispersions [76-79]. In this work, we revealed the potential use of this technique to produce particles in the range of 700 nm to 5 µm with the control over the polydispersity which varied accordingly to the desired average value.

The use of STMP as a cross-linking agent necessitated to operate the crosslinking between xylan chains reaction at pH ≈12, as stated by Lack et al, 2007 [80]. According to mechanism proposed in the above mentioned study, the crosslinking reaction can be stopped by decreasing the pH below 10, which very interestingly, induce a self hydrolysis of the crosslinking agent itself. Therefore, it is likely that under homeostatic conditions prevailing in the body, any traces of STMP in the preparations would rapidly disappear, which would be in favor of the safety of these drug carriers, as corroborated by Caco-2 viability assays carried out in this work.

In summary, this original approach used for the production of the xylan-based micro or
nanospheres is interesting due to: (1) the specificity of the cross-linking reaction allowing the particles to be biocompatible independently of the ratio of the polymers used and (2) the ability to control the particle size distribution by acting on the variable of the process. However, deeper characterization of the already produced xylan-based carriers must be done in order to evaluate the influence of the drug loading on the characteristics of these systems, the efficiency of drug encapsulation, the specificity of drug release.


[22] A.E. Silva, G. Barratt, M. Cheron, E.S. Egito, Development of oil-in-water


[53] S. Hussain, J.A. Vanoirbeek, P.H. Hoet, Interactions of nanomaterials with the immune


[77] B.-z. Li, L.-j. Wang, D. Li, B. Adhikari, Z.-h. Mao, Preparation and characterization of


CONCLUSIONS AND PERSPECTIVES
CONCLUSIONS AND PERSPECTIVES

During this work we attempted to understand how AmB aggregation could impact on its intestinal absorption, also we tried to enhance amphotericin B absorption by the use of micro- and nanoemulsions. Obviously, the paracellular or the transcellular routes are ineffective for contributing to AmB intestinal absorption, which was confirmed in the well established model of Ussing’s chamber. Alternatively, our results suggest that an enhancement of AmB absorption by the lymphatic route is probably a more realistic outreach, although the doses that can be systemically delivered by this route are probably limited. In this respect, it can be noted on the basis of a review of the literature, that AmB absorption in the gastrointestinal tract can be observed in vivo, mostly when it is associated to particulate drug delivery systems, such as lipid-based micro- and nanoparticles or micro- and nanoemulsions that are able to permeate the intestinal tissue by this specific route. However, we believe that other approaches could be imagined and investigated, such as the use of polymeric particles able to enhance local concentrations near to the epithelial cells by an increase of their residence time in the mucus layer. Alternatively, the colon could be a region of interest for attempting AmB absorption, which requires the use of colon-targeted delivery systems, such as the one developed during this work, but which unfortunately could not reach the biological assays.

Furthermore, an interesting process has been found for the production of xylan-based micro- or nanoparticles, a solvent-free process with the addition of a biocompatible crosslinking agent. Obviously, the particles developed during this work still need to be characterized in order to proof their ability to achieve specific release in the colon region and their resistance to gastrointestinal fluids (gastric and intestinal fluids), as well as their degradability in presence of xylanases produced by bacteroides. Therefore, once these characteristics will have been proven, their capacity to deliver AmB could be envisioned, not only for enhancing colonic absorption of AmB but also for attempting new therapeutic strategies for the treatment of inflammatory intestinal diseases by acting directly on the regulation of the mycobionta in the colon region, as such inflammatory pathologies have been correlated to an expansion of the mycobiote. In this respect, we suggest that a strategy worth to be investigated could consist to ensure a continuous release of AmB directly in the large bowel by the mean of the xylan particles already developed, which may have potential clinical applications to treat inflammatory bowel diseases by controlling the local expansion of the
mycobiota on the colon region.
1. Social

On my first year I had the opportunity to go to the United States of America (US) at University of Florida (UF) located on Gainesville/Florida, US. There, I could improve a lot my English skills (mostly oral and written English), and probably that is the reason why this thesis was all written on that language, even though sometimes I doubt myself if I was not on a German territory inside the US (which I will explain on the next paragraphs).

Gainesville is a small city on north of the Florida state in US that has as it’s heart the University of Florida. It is really possible to see the difference between the classes’ period and the holidays, when I was there I heard many times people saying that the population decrease on half during holydays, which means from 130.000 to 65.000 people, approximately. Also, all the people related with the UF are known as gators, the reason? (Please, see the photo below).

![Photo taken on 25th November 2015 by Oliveira, R. at University of Florida Commuter Lot](image)

So, why did I feel that sometimes I was on German territory? Briefly, at that time my supervisor was the Distinguished Professor Hartmut Derendorf, PhD. and him together with Pr Guenther Hocchaus have a lot of contact from their origin country (Germany). So, every semester that would be around 20 new interns (Masters students) doing internship at UF, and from them around 15 would be from Germany, together with most of the PhD students, which were also German, it was like a small community. Then, ten months later I went back to Brazil to family and friends.

A little less than a year I was back to Brazil my Brazilian advisor tells me that there is an opportunity to go France; and that I could spend 12 months with a probable extension of 6 months if we sign a co-advisorship agreement. Thus, I started to study French and to
prepare all the papers needed to obtain the scholarship and the visa to stay in France as a student.

On 1\textsuperscript{st} September 2014 I was in France, and here I had another cultural shock, the life style is different from Brazil and US. Here I learned many the stereotypes (clichés) are true, but more important, I saw many were also wrong.

The first main goal to be achieved was to be able to communicate with others, because in France you can survive (in Paris) without knowing French (meaning knowing English), but you will only have the “full experience” if you learn enough to communicate with others. Personally, I do think this goal was reached, even though I learn still an “expression per day” or at least every “Friday beer”.

The sojourn in Paris also provided the opportunity to meet the few Swedish people and sometimes be immersed on their culture. Do one \textit{fika} per day is as much as important to them than to have cheese during a meal for a French (“un repas sans frommage n’est pas un repas” Bourdon, G.). Living in the Swedish house allowed me to see the diversity existent in Europe.

In conclusion, I did got know the different cultures and languages from where I have been, and too be hones that was one of the great results I had from this PhD. Also, I hope to be able to bring the open-mildness I developed during these years wherever I go, and apply not only to meet people but to always be open to new ideas and approaches on science.

2. Technical

On the first year of my PhD I had a project well defined with a particulate system that was promising as a possible carrier to colon delivery, although with time we found out the system developed did not work properly, and also had some undesirable properties. Thus it was necessary to start all over again, which from the technical point of view was one of the major topics I could learn about. So, I had to set up a new platform to produce microparticles, which was later extended to the production of nanoparticles. Additionally, I also learned some techniques to produce micro- and nanoemulsions with my colleagues in order to be able to test their ability to enhance drug solubility and absorption.

During the four years I carried out experiments for this project I could learn about different characterization techniques, how to explore the data they would give and possible links among these characterization methods. Additionally, once I arrived in France in order to
evaluate the permeability of amphotericin B I had to set up a method to try to perform the assays with this drug. Furthermore, I listed below some of the techniques I learned or improved my skills during the PhD:

**Table 1: Techniques learned or improved during PhD.**

<table>
<thead>
<tr>
<th>Biological</th>
<th>Physical</th>
<th>Chemical</th>
</tr>
</thead>
<tbody>
<tr>
<td>• MTT</td>
<td>• Powder X-ray Diffraction</td>
<td>• UV-Vis spectroscopy</td>
</tr>
<tr>
<td>• Permeability assays</td>
<td>• Particle Size distribution</td>
<td>• Fourier-transformed infrared spectroscopy</td>
</tr>
<tr>
<td>o Ussing’s Chamber</td>
<td>o Ferret diameter;</td>
<td>• Liquid chromatography</td>
</tr>
<tr>
<td>• Immunological complement system activation</td>
<td>o Laser Diffraction;</td>
<td>o UV-Vis</td>
</tr>
<tr>
<td>(in vitro)</td>
<td>o Dynamic Laser Scattering</td>
<td>o MS	extsuperscript{n}</td>
</tr>
<tr>
<td></td>
<td>• Thermal Characterization</td>
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<td></td>
<td>o Differential Scanning</td>
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<td></td>
<td>o Calorimetry</td>
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<td></td>
<td>o Thermogravimetric Analysis</td>
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<td></td>
<td>• Surface Tension</td>
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<td>o Ring’s method</td>
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<td>• Microscopy</td>
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<td>o Scanning electron microscopy</td>
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</table>

In addition to the experiments and the reports that were needed to write, some additional skills were developed during the PhD, such as management skills. Actually, during these four years I was not only enrolled on research activities, which demanded I had a quite well organized schedule. Also, the processes to obtain the scholarships sometimes had shorts deadlines, then it was necessary to be in advance with other activities, sometimes.
Table 2: Skills learned or improved during PhD.

<table>
<thead>
<tr>
<th>Academic</th>
<th>Management</th>
</tr>
</thead>
<tbody>
<tr>
<td>• English language</td>
<td>• Application for Grants</td>
</tr>
<tr>
<td>• French language</td>
<td>o Brazilian funding agencies</td>
</tr>
<tr>
<td>• Statistics</td>
<td>• Scientific supervision</td>
</tr>
<tr>
<td>o R programming</td>
<td>o Undergraduates</td>
</tr>
<tr>
<td>• Bibliography management</td>
<td>o Master’s students</td>
</tr>
<tr>
<td>o EndNote®</td>
<td>• Collaboration with other research teams</td>
</tr>
<tr>
<td>• Teaching (bachelor’s level)</td>
<td>o Local level (Brazil)</td>
</tr>
<tr>
<td>o Classes (theory and practice)</td>
<td>o Internationally (US and France)</td>
</tr>
<tr>
<td>o Exams (elaboration and correction)</td>
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</tbody>
</table>

3. Academic

The last, but for sure, not less important were the academic results obtained with this PhD project. At the end of this PhD I will have at least one manuscript as first author and few others as part of others PhD student projects. In total there were already 3 manuscripts and 1 book chapter, with 3 other manuscripts on the writing/editing process. Additionally, I could help my university to establish an important collaboration with Pr Hartmut Derendorf, at University of Florida, as well as, to keep the collaboration existing between Brazil and France more precisely with the Institut Galien Paris Sud.
CURRICULUM VITAE
Henrique Rodrigues Marcelino  
Curriculum Vitae

Personal Information

Name: Henrique Rodrigues Marcelino  
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Formal Education

2012 - 2016  
PhD in Biopharmacy and Pharmaceutical Technology (Health Sciences).  
Institut Galien Paris Sud, CNRS 8612, Université Paris-Sud, France.  
(Co-advisorship) Universidade Federal do Rio Grande do Norte, Brazil.  
**Title:** Impact of the aggregation state of amphotericin B on its biopharmaceutical properties. Design of micro- and nanocarriers for oral delivery.  
**Advisors:** Pr Gilles Ponchel and Pr E. Sócrates Tabosa do Egito.  
**Funding:** CAPES-Cofecub (Agreement between Brazil and France)

2011 - 2011  
MSc in Pharmaceutical Sciences.  
Universidade Federal do Rio Grande do Norte, Brazil  
**Title:** Development and application of xylan-based microparticles for controlled release  
**Advisor:** Pr. E. Sócrates Tabosa do Egito  
**Funding:** CNPq (Brazil)

2006 - 2010  
Bachelor in Pharmacy.  
Universidade Federal do Rio Grande do Norte, UFRN, Natal, Brazil  
**Title:** Physical characterization of xylan-based microparticles cross-linked with terephthaloyl chloride  
**Advisor:** Pr E. Sócrates Tabosa do Egito

Courses (formal education related)

2013  
V	extsuperscript{th} training School on Bioencapsulation.  
Bioencapsulation Research Group, BRG, France.

2013  
Nanomaterials: Synthesis and characterization by electronical microscopy.  
Universidade Estadual de Campinas, UNICAMP, Campinas, Brazil

2012  
Winterschool: Nanotechnology for the design of functional materials.  
Technische Universität Dresden, TUD, Dresden, Alemanha

Exchanges and professionally meaningful internships

2012 - 2013.  
University of Florida – UF, Gainesville / Florida, United States  
Research Scholar (Supervisor: Distinguished Pr Hartmut Derendorf)
**Description:** Development of chromatographic methods for drug analysis.

**2010 - 2010.** OPHTHALMOS S.A, São Paulo / São Paulo, Brazil
Intern (Supervisor: Andreia Lourenço)

**Description:** Internship on Quality Control and Quality Assurance of parenteral medicines.

**Periodic Peer-reviewer**


**Research Papers Published in Peer-Reviewed Periodic Journals**


**Book Chapters**


**Published Abstracts in Conference Proceedings.**

- **Full work** 1
- **Extended Abstracts** 8
- **Abstracts** 34

**Personal Interests**

Travelling (10% of the world, Cities I’ve visited – Trip Advisor), Learning languages (Spanish, Swedish), Computer Programming (R!), Guitar (Beginner)
ATTACHMENTS

RESEARCH DONE IN COLLABORATION WITH COLLEAGUES
ATTACHMENT I

XYLAN, A PROMISING HEMMICELLULOSE FOR PHARMACEUTICAL USE
BRIEFING


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Author's major contribution: Section 3. Xylan microparticles
Xylan, a Promising Hemicellulose for Pharmaceutical Use

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¹Universidade Federal do Rio Grande do Norte
²Universidade Estadual da Paraíba
³Universidade Federal de Campina Grande
Brazil

1. Introduction

Polymers are versatile materials with wide use in several industry fields, such as engineering, textile, automobile, packaging and biomedical. In the pharmaceutical industry, both natural and synthetic polymers have been largely used with different applications for the development and production of cosmetics and traditional dosage forms and novel drug delivery systems. For instance, a number of polymers are used as fillers, lubricants, disintegrants, binders, gldiants, solubilizers, and stabilizers in tablets, capsules, creams, suspensions or solutions. Additionally, biodegradable and bioadhesive polymers may play an important role in the development of novel drug delivery systems, especially for controlled drug release.

Polymers microparticles have been studied and developed for several years. Their contribution in the pharmacy field is of utmost importance in order to improve the efficiency of oral delivery of drugs. As drug carriers, polymer-based microparticles may avoid the early degradation of active molecules in undesirable sites of the gastrointestinal tract, mask unpleasant taste of drugs, reduce doses and side effects and improve bioavailability. Also, they allow the production of site-specific drug targeting, which consists of a suitable approach for the delivery of active molecules into desired tissues or cells in order to increase their efficiency.

Lately, the concern with environment and sustainability has been rising progressively and renewable sources of materials have been increasingly explored.

The aim of this chapter is to summarize some of the research findings on xylan, a natural polymer extracted from corn cobs, which presents a promising application in the development of colon-specific drug carriers. Physicochemical characterization of the polymer regarding particle size and morphology, composition, rheology, thermal behavior, and crystallinity will be provided. Additionally, research data on its extraction and the development of microparticles based on xylan and prepared by different methods will also be presented and discussed.
2. Xylan

For thousands of years, nature has provided humankind with a large variety of materials for the most diversified applications for its survival, such as food, energy, medicinal products, protection and defense tools, and others. The pharmaceutical industry has benefitted from such diversity of biomaterials and has exploited the use of natural products as sources of both drugs and excipients. One example of a promising biomaterial for pharmaceutical use is xylan, a hemicellulose largely found in nature, being considered the second most abundant polysaccharide after cellulose.

Xylan has drawn considerable interest due to its potential for packaging films and coating food, as well as for its use in biomedical products (Li et al., 2011). Because it is referred to as a corn fiber gum with a sticky behavior, xylan has been used as an adhesive, thickener, and additive to plastics. It increases their stretch and breaking resistance as well as their susceptibility to biodegradation (Ünlü et al., 2009). Xylan has also been studied because of its significant mitogenic and comitogenic properties, which enable it to be compared to the commercial immunomodulator Zymosan (Ebringerova et al., 1995). Another interesting application for xylan may be found in the food industry as an emulsifier and protein foam stabilizer during heating (Ebringerova et al., 1995). Previous papers have investigated the suitable use of xylan in papermaking (Ebringerova et al., 1994) and textile printing (Hromadkova et al., 1999). In the drug delivery field, xylan extracted from birch wood has been used for the production of nanoparticles after structural modification by the addition of different ester moieties, namely those with furfate and pyrogallate functions (Heinze et al., 2007). On the other hand, the esterification of xylan from beech wood via activation of the carboxylic acid with N,N'-carbonyldiimidazole has been carried out in order to produce prodrugs for ibuprofen release (Daus & Heinze, 2010).

Egito and colleagues have been working for over a decade on the extraction of xylan from corn cobs and its use for the development of microparticles as drug carriers for colon-specific delivery of anti-inflammatory and toxic drugs, such as sodium diclofenac (SD), 5-aminosalicylic acid (5-ASA), and usnic acid (UA). Xylan-coated microparticles have also been developed by Egito and co-workers in order to deliver magnetite particles (Silva et al., 2007). Different microencapsulation techniques have been used for the production of xylan-based microparticles. Coacervation, interfacial cross-linking polymerization, and spray-drying have been shown to be the most successful methodologies for that purpose (Garcia et al., 2001; Nagashima et al., 2008).

Xylan degradation occurs by the action of hydrolytic enzymes named xylanases and β-xylanosidases. Those enzymes are produced by a number of organisms, such as bacteria, algae, fungi, protozoa, gastropods, and arthropods (Kulkarni et al., 1999). The degradation of xylan in ruminants has been well reported, while some human intestinal bacteria have been investigated for their ability to produce xylan-polymer degrading enzymes. Among those intestinal species able to degrade complex carbohydrates, lactobacilli, bacteroides, and non-pathogenic clostridia have demonstrated that ability (Grootaert et al., 2007). Because of the presence of those bacteria in the human colon whether by induction of prebiotics or not, it is believed that xylan is a promising polymer for the composition of biodegradable drug carriers for colonic delivery. They would be able to undergo the upper gastrointestinal tract mostly intact, being degraded by xylanases when reaching the colon.
Xylan, a Promising Hemicellulose for Pharmaceutical Use

Additionally, corn cobs correspond to an abundant and low-cost renewable material in several countries worldwide and their recycling plays a very important role in the reduction of waste products. Consequently, such approach would lead to a relevant increase in the sustainability of agriculture around the world.

2.1 Sources, extraction, and structure

Hemicelluloses are the second most abundant polysaccharides in nature after cellulose. They occur in close association with cellulose and lignin and contribute to the rigidity of plant cell walls in lignified tissues. Hemicelluloses constitute about 20–30% of the total mass of annual and perennial plants and have a heterogeneous composition of various sugar units, depending on the type of plant and extraction process, being classified as xylans (β-1,4-linked D-xylene units), mannans (β-1,4-linked D-mannose units), arabinans (α-1,5-linked L-arabinose units), and galactans (β-1,3-linked D-galactose units) (Figure 1) (Belgacem & Gandini, 2008).

Xylans are the main hemicelluloses in hardwood and they also predominate in annual plants and cereals making up to 30% of the cell wall material and one of the major constituents (25–35%) of lignocellulosic materials. The most potential sources of xylans include many agricultural crops such as straw, sorghum, sugar cane, corn stalks and cobs, and hulls and husks from starch production, as well as forest and pulping waste products from hardwoods and softwoods (Ebringerova & Heinze, 2000; Kayserilioglu et al., 2003).

The structural diversity and complexity of xylans are shown to depend on the botanic source. Various suitable extraction procedures for the isolation of xylans from different plant sources are described and compared in the literature. It is suggested that certain structural types of xylans, such as glucuronoxylan, arabinogluconoxylan, and arabinoxylan, can be prepared from certain plant sources with similar chemical and physical properties. Its general structure has a linear backbone consisting of 1,4-linked D-xylpyranose residues, a reducing sugar with five carbon atoms. These may be substituted with branches containing acetyl, arabinosyl, and glucuronosyl residues, depending on the botanic source and method of extraction (Den Haan & Van Zyl, 2003; Habibi & Vignon, 2005).

![Chemical structure of xylan](image)

Fig. 1. Chemical structure of xylan (Shalom & Shoham, 2003).
A frequently used classification is based on the degree of substitution and types of side groups for characterization (Ebringerová, 2005; Sedlmeyer, 2011):

a. Homoxylans are linear polysaccharides common in some seaweeds.

b. Glucuronoxylans can be partly acetylated and have units substituted with α-(1→2)-4-O-methyl-D-glucopyranosyl uronic acid (MeGlcUA). They are found in hardwood, depending on the treatment.

c. (Arabinoglu)curonoxylans have a substitution with α-(1→3)-L-arabinofuranosyl (ArbF) next to MeGlcUA. They are typical for softwoods.

d. Arabinoxylans with a substitution of the β-(1→4)-D-xylopyranose backbone at position 2 or 3 with ArbF can be esterified partly with phenolic acids. This type is frequently found in the starchy endosperm and the outer layers of cereal grains.

e. (Glucurono)arabinoxylans can be disubstituted with ArbF units, acetylated, and esterified with feric acid. This form is typical of lignified tissues of grasses and cereals.

f. Heteroxylans are heavily substituted with various mono- or oligosaccharides and are present in cereal bran, seed, and gum exudates.

Investigation of the xylan structure by various researchers is necessary. The use of xylan as a raw material is directly related to its structure. There is an interest in the application of the xylan polymer in the paper, pharmaceutical, cosmetic, biofuel and food industries. Several medical applications are cited in the literature. The films based on xylan show low oxygen permeability and thus have a potential application in the food packaging and pharmaceutical areas. Numerous studies use the xylan polymer as a specific substrate for xylanases. Besides that, xylan can be hydrolyzed into xylose and subsequently be converted into ethanol (Ebringerova & Heinze, 2000; Ebringerova & Hromadkova, 1999; Ebringerova et al., 1998; Garcia et al., 2000; Kayserilioglu et al., 2003; Oliveira et al., 2010; Sedlmeyer, 2011; Yang et al., 2005).

Previous studies on the corn cob xylan revealed the existence of at least two structurally different components. One is a low-branched arabinoglucuronoxylans, which is mostly water-insoluble (wis-X), and the second is a highly branched, water-soluble heteroxylan (ws-X), which possesses significant mitogenic and comitogenic activities (Ebringerova et al., 1995). The ws-X could be useful also as a food additive because of its emulsifying activity and ability to stabilize protein foam during heating. The wis-X has the ability to remain intact in the physiological stomach environment and small intestine. This property, together with the presence of xylanases (a group of enzymes which degrade the xylan) in the human colon, makes this polymer a suitable raw material for the medical field, especially as a constituent of colon-specific drug carriers (Oliveira et al., 2010; Rubinstein, 1995; Silva et al., 2007).

The most common method to extract xylan is the alkaline extraction. Several pretreatment methods can be used in association in order to break the covalent bonds that exist between xylan and other carbohydrates during the extraction (Wang & Zhang, 2006). A number of articles studied the use of ultrasound on the xylan extraction. Hromadkova and coworkers reported that 36.1% of xylan was extracted from corn cobs with 5% NaOH solution at 60°C for 10 min of ultrasonication in comparison with 31.5% of xylan in the classical extraction. Both extractive methods yielded xylan with immunogenic properties (Hromadkova et al., 1999).
Wang and Zhang also investigated the effects on the xylan extracted from corn cobs enhanced by ultrasound at various lab-scale conditions. Results showed that the optimization conditions of xylan extraction should be carried out using (i) 1.8 M NaOH, (ii) corn cobs to NaOH solution ratio of 1.25 (w/w), (iii) sonication at 200 W ultrasound power for 30 min at 5 min intervals, and (iv) 60 °C (Wang & Zhang, 2006).

The process of the alkaline extraction of xylan from corn cobs was studied by Egito and colleagues (Unpublished data). The methodology applied in this work consisted of milling the corn cobs and separating the powder into different sizes. After that, the dried corn cobs were dispersed in water under stirring for 24h. The sample was treated with 1.3% (v/v) sodium hypochlorite solution in order to remove impurities. Then, an alkaline extraction was carried out by using NaOH solution. The bulk was neutralized with acetic acid, and xylan was extracted by settling down after methanol addition. Afterwards, several washing steps were performed by using methanol and isopropanol. Finally, the sample was filtered and dried at 50°C.

The efficiency of extraction was observed to be inversely proportional to the corn cob particle size. This was expected because the size reduction corresponds to an increase in total particle surface area. An increase in the time of the alkaline extraction and in the NaOH concentration also improves the efficiency of xylan extraction. This happened because when the NaOH concentration was lower, the xylan present in corn cobs could not be fully dissolved in the solution. Thus, it resulted in lower efficiency of xylan extraction. However, when the NaOH concentration was higher than 2 M, the yields decreased with continuously increasing of the NaOH concentration. This is probably due to the alkaline degradation of xylan chains, proceeding at the higher NaOH concentration, which indicated that the ideal NaOH concentration in the extraction was between 1.5 and 1.8 M (Unpublished data).

2.2 Characterization of corn cob xylan

Comprehensive physicochemical characterization of any raw material is a crucial and multi-phased requirement for the selection and validation of that matter as a constituent of a product or part of the product development process (Morris et al., 1998). Such demand is especially important in the pharmaceutical industry because of the presence of several compounds assembled in a formulation, such as active substances and excipients, which highlights the importance of compatibility among them. Besides, variations in raw materials due to different sources, periods of extraction and various environmental factors may lead to failures in production and/or in the dosage form performance (Morris et al., 1998). Additionally, economic issues are also related to the need for investigating the physicochemical characteristics of raw materials since those features may determine the most adequate and low-cost material for specific procedures and dosage forms.

After the extractive process described by Oliveira and colleagues, corn cob xylan appears to be an off-white fine powder with limited flowability. The xylan powder consists of a mixture of aggregated and non-aggregated particles with irregular morphology, a spherical shape, and a rough surface, as could be observed through the scanning electron microscopy (SEM) (Figure 2) (Oliveira et al., 2010).
Fig. 2. SEM image of xylan powder after extraction from corn cobs (Oliveira et al., 2010).

The xylan particle size distribution was determined by laser diffraction. It was observed that approximately 90%, 50%, and 10% of the dry extract of xylan was smaller than 65.39 ± 1.76, 23.34 ± 1.2, and 7.68 ± 0.54 μm, respectively, while the mean particle size of xylan was found to be 30.53 ± 1.5 μm (Oliveira et al., 2010) (Figure 3).

Fig. 3. Particle size distribution of xylan powder after extraction from corn cobs (Oliveira et al., 2010).

As a consequence of the irregular and rough structure of the xylan particles, entanglements between particles are promoted and this fact may explain the poor flow properties of this polymer (Kumar et al., 2002; Nunthanid et al., 2004). Additionally, rheological parameters of xylan powder have also been studied, such as bulk and tapped densities, Hausner ratio, Carr’s index, and angle of repose values, and they are summarized in Table 1.
Xylan, a Promising Hemicellulose for Pharmaceutical Use

<table>
<thead>
<tr>
<th>Property</th>
<th>Value (± standard deviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk density</td>
<td>0.1336 (± 0.0029) g/ml</td>
</tr>
<tr>
<td>Tap density</td>
<td>0.2256 (± 0.0059) g/ml</td>
</tr>
<tr>
<td>Compressibility index</td>
<td>40.77 (± 0.0035) %</td>
</tr>
<tr>
<td>Hausner ratio</td>
<td>1.68 (± 0.01)</td>
</tr>
<tr>
<td>Compactability</td>
<td>32.6 (± 0.1) mL²</td>
</tr>
<tr>
<td>Angle of repose</td>
<td>40.70 (± 3.2318)°</td>
</tr>
</tbody>
</table>

*extrapolating the values to 100 mL.

Table 1. Rheological properties of xylan powder extracted from corn cobs.

The bulk density of a powder is calculated by dividing its mass by the volume occupied by the powder (Abdullah & Geldart, 1999). Tapped bulk density, or simply tapped density, is the maximum packing density of a powder achieved under the influence of well-defined, externally applied forces (Oliveira et al., 2010). Because the volume includes the spaces between particles as well as the envelope volumes of the particles themselves, the bulk and tapped density of a powder are highly dependent on how the particles are packed. This fact is related to the morphology of its particles and such parameters are able to predict the powder flow properties and its compressibility.

Hausner ratio and the compressibility index measure the interparticle friction and the potential powder arch or bridge strength and stability, respectively (Carr, 1965; Hausner, 1967). They have been widely used to estimate the flow properties of powders. A Hausner ratio value of less than 1.20 is indicative of good flowability of the material, whereas a value of 1.5 or higher suggests a poor flow (Daggupati et al., 2011). The compressibility index is also called the Carr index. According to Carr, a value between 5 and 10, 12 and 16, 18 and 21, and 23 and 28 indicates excellent, good, fair, and poor flow properties of the material, respectively. The Hausner ratio and Carr’s index values obtained for xylan are listed in Table 1 and suggest that xylan presents extremely poor flow properties. Although the Hausner ratio and the Carr index correspond to indirect measurements of flowability of materials during preliminary studies, the values obtained for xylan suggest the characterization of this biopolymer as a cohesive powder.

Another parameter of the flow behavior of a powder is the angle of repose, which evaluates the flowability of powders through an orifice onto a flat surface. It is considered a direct measurement. Angles of repose below 30° indicate good flowability, 30°-45° some cohesiveness, 45°-55° true cohesiveness, and > 55° sluggish or very high cohesiveness and very limited flowability (Geldart et al., 2006). The angle of repose for xylan is 40.70°, which confirms its cohesive nature predicted by the aforementioned indirect measurements. This is due to the irregular shape of the xylan particles. Besides, the fine particles of xylan, having high surface-to-mass ratios, are more cohesive than coarser particles; hence, they are more influenced by gravitational force. In addition, it is generally believed that the flowability of powders decreases as the shapes of particles become more irregular (Oliveira et al., 2010).

Regarding the characterization of corn cob xylan by Fourier-transform infrared (FT-IR) spectroscopy, two main absorption bands at 3405 cm⁻¹ and 1160 cm⁻¹ are revealed. They can
be attributed to the OH stretching characteristic of glycosidic groups and to CC and COC stretching in hemicelluloses, respectively (Figure 4).

![FT-IR spectrum of xylan powder extracted from corn cobs.](image)

Fig. 4. FT-IR spectrum of xylan powder extracted from corn cobs.

Moreover, an absorption band near 1375 cm⁻¹ is detected and it is assigned to the CH bending vibration present in cellulose and hemicellulose chemical structures (Sun et al., 1998). The prominent band at 1044 cm⁻¹ is also associated with hemicelluloses and is attributed to the C-OH bending. Finally, a sharp band at 897 cm⁻¹, which is typical of β-glycosidic linkages between the sugar units in hemicelluloses, was detected in the anomeric region (Sun et al., 2005).

A solid-state $^{13}$C nuclear magnetic resonance (NMR) experiment was carried out in 4 mm double bearing rotor made from ZrO₂ on a Bruker DSX 200 MHz spectrometer with resonance frequency at 75.468 MHz. The pulse length was 3.5 μs and the contact time of 1H-13C CP was 2–5 ms.

The NMR spectrum of the dry sample showed broad unresolved peaks that correspond to a typical mixture of 4-O-methyl-D-glucuronic acid, L-arabinose and D-xylose, and proteins (Oliveira et al., 2010) (Figure 5).

Concerning the analysis of crystallinity of xylan, the X-ray diffraction detects a few and small peaks, which indicate that xylan presents a low crystallinity (Figure 6).

On the other hand, thermal analysis of xylan by thermogravimetry demonstrates a first event of 8.9% weight loss detected in the range of 62 and 107°C due to dehydration. The second and most relevant event of 49.8% weight loss appears in the range of 250 and 300°C due to the polymer decomposition (Figure 7). The differential scanning calorimetry curve reveals an endothermic peak at 293.04°C, which is attributed to the melting point of the polymer (Figure 7).
Fig. 5. Solid-state $^{13}$C nuclear magnetic resonance spectrum of corn cob xylan.

Fig. 6. X-ray diffraction pattern for corn cob xylan (Unpublished data).
Fig. 7. Thermogravimetry and differential scanning calorimetry curves for corn cob xylan (Unpublished data).

3. Xylan microparticles

As previously described, xylan has been considered as a suitable raw material to produce colonic drug delivery systems due to the ability of enzymes produced by the colonic microflora to degrade the β-glycosidic bonds between the sugar units of the polymer backbone (Kacurakova et al., 2000; Oliveira et al., 2010; Saha, 2000). Regarding the colonic environment, it presents a neutral pH range of the colon and a local blood circulation that prevents the rapid distribution of the drug into the body before circulating into the intestinal blood vessels. As a result, the colonic absorption of drugs is an alternative approach to deliver molecules that are degraded in the stomach medium and are toxic in small quantities in the body (Luo et al., 2011).

A large variety of drug delivery systems are described in the literature, such as liposomes (Torchilin, 2006), micro and nanoparticles (Kumar, 2000), polymeric micelles (Torchilin, 2006), nanocrystals (Muller et al., 2011), among others. Microparticles are usually classified as microcapsules or microspheres (Figure 8). Microspheres are matrix spherical microparticles where the drug may be located on the surface or dissolved into the matrix. Microcapsules are characterized as spherical particles more than 1μm containing a core substance (aqueous or lipid), normally lipid, and are used to deliver poor soluble molecules.
in hydrophilic medium (Couvreur et al., 2002; Kumar, 2000; Ribeiro et al., 1999). Furthermore, microcapsules may have one or more cores while the microspheres may show a homogeneous or heterogeneous aspect with the drug distributed equally or aggregated into the particle.

![Diagram of microcapsules and microspheres](image)

Fig. 8. Structural differences between microcapsules and microspheres.

In the past, microparticles were considered as mere carriers, usually micronized dry material without sophisticated attributes (Vehring, 2008). However, nowadays they have found a number of applications in the pharmaceutical field. For instance, microparticles have been used in order to achieve controlled release of drugs, deliver two or more agents in the same system, improve the bioavailability and the biodistribution of molecules, target drugs to specific cells or issues, or mask the unpleasant taste of some active molecules (Simó et al., 2003; Tran et al., 2011; Vehring, 2008). Xylan microparticles have been successfully produced by the following methods: coacervation (Garcia et al., 2001), interfacial cross-linking (Nagashima et al., 2008) and spray-drying (Unpublished data), all of which are described in the following subsection.

3.1 Methods of production
3.1.1 Coacervation

The coacervation technique is defined as a partial desolvation of a homogeneous polymer solution into a polymer-rich phase (coacervate) and the poor polymer phase (coacervation medium). It was the first process to be scaled-up to an industrial process (Jyothi et al., 2010). However, for the optimization of this method, some changes in the methodology were made and the technique was classified into two types: simple and complex. In simple coacervation the desolvation agent is added to form the coacervate, while the complex coacervation process is guided by the presence of two polymers with different charges, and divided into three steps: (i) formation of three immiscible phases, (ii) deposition of the coating, and (iii) strengthening of the coating (Gouin, 2004; Jyothi et al., 2010; Qv et al., 2011).

After the first step, which includes the formation of three immiscible phases (liquid manufacturing vehicle, core material, and coating material), the core material is dispersed in a solution of the coating polymer. The coating material phase, which corresponds to an immiscible polymer in liquid state, is formed by (i) changing the temperature of the polymer solution, (ii) adding a salt, (iii) adding a non-solvent, (iv) adding an incompatible polymer to the polymer solution, and (v) inducing polymer-polymer interaction. The second step includes deposition of the liquid polymer upon the core material. Finally, the prepared
microcapsules are stabilized by cross-linking, desolvation, or thermal treatment (Jyothi et al., 2010; Stuart, 2008).

Xylan-based micro- and nanoparticles have been produced by simple coacervation (Garcia et al., 2001). In the study, sodium hydroxide and chloride acid or acetic acid were used as solvent and non-solvent, respectively. Also, xylan and surfactant concentrations and the molar ratio between sodium hydroxide and chloride acid were observed as parameters for the formation of micro- and nanoparticles by the simple coacervation technique (Garcia et al., 2001). Different xylan concentrations allowed the formation of micro- and nanoparticles. More precisely, microparticles were found for higher concentrations of xylan while nanoparticles were produced for lower concentrations of the polymer solution. When the molar ratio between sodium hydroxide and chloride acid was greater than 1:1, the particles settled more rapidly at pH=7.0. Regarding the surfactant variations, an optimal concentration was found; however, at higher ones a supernatant layer was observed after 30 days (Garcia et al., 2001).

3.1.2 Interfacial cross-linking polymerization

The production of microparticles by this technique involves basically two experimental steps: (i) emulsification and (ii) cross-linking reaction (Figure 9). In fact, the emulsification is the major step of the process to determine the particle size distribution and the aggregation arrangement of the microparticles. Therefore, the chemical reactivity of the cross-linking agent is also important to determine the required time to complete the entire process (Chang, 1964; Jiang et al., 2006; Levy & Andry, 1990; Li et al., 2009).

In the first step of the interfacial cross-linking polymerization, the polymer is dissolved into the solvent, which is the internal phase of the emulsion, and another phase with a non-solvent to the polymer is produced; then the aqueous phase is poured to the organic phase to produce the emulsion. Afterwards, a solution containing the cross-linking agent is added to the emulsion to form a rigid structure of the microparticles (Couvreur et al., 2002; Rao & Geckeler, 2011).

![Scheme for interfacial cross-linking polymerization](https://www.intechopen.com)
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The influence of the lipophilic external phase on the production of xylan-based microparticles by interfacial cross-linking polymerization has been investigated (Nagashima et al., 2008). Three different external phases were investigated: a 1:4 (v/v) chloroform:cyclohexane mixture, soybean oil, and a medium chain triglyceride, with viscosities below 1, 24, and 52 cP, respectively. It was observed that the use of these different lipid phases results in different macroscopic and microscopic aspects of the system (Figure 10).

a) 1:4 (v/v) Chloroform: cyclohexane mixture;
b) Soybean oil;
c) Medium chain triglycerides.

Fig. 10. Optical microscopy images of xylan microcapsules produced by interfacial cross-linking polymerization with different lipophilic external phases (Nagashima et al., 2008).

Because emulsions are susceptible to many destabilizing phenomena occurring since the formation of these systems, such as Ostwald rippling (Anton et al., 2008) and coalescence (Li et al., 2009), the formation of the microcapsules may be influenced by those phenomena, which can form aggregates and agglomerates, respectively. Also, the higher viscosity of the lipid phase may support the shaping of microcapsules with a bigger size than the oil phases with a lower viscosity (Nagashima et al., 2008).

The cross-linking agent is present in the interfacial area, where the polymer should be adsorbed due to the poor solubility of the polymer at the external medium. It is known that the chemical reactivity of the cross-linking agent is a limiting parameter to determine the duration and the yield of the process (Li et al., 2009). Terephthaloyl chloride is a cross-linking agent used to produce microcapsules based on polysaccharides, and it was extensively studied by Levy to produce starch derivate microcapsules for pharmaceutical uses. According to Levy, the pH medium, the concentration of the polymer, the stirring speed, and the concentration of terephthaloyl chloride are significant parameters for the formation of the microparticles and their structure (Andry et al., 1996; Andry & Lévy, 1997; Edwards-Lévy et al., 1994; Levy & Andry, 1990).

Cross-linked xylan-based microparticles are produced by the emulsification of an alkaline solution of xylan with a lipophilic phase formed by a mixture of chloroform and cyclohexane by using 5% (w/v) sorbitan triesterate as the surfactant. Subsequently, the cross-linking reaction is carried out for 30 minutes with 5%(w/v) terephthaloyl chloride in order to yield a hard and rigid polymeric shell (Nagashima et al., 2008).

The interfacial cross-linking polymerization has been demonstrated to be a suitable method for the production of xylan microcapsules with high drug encapsulation efficiency. SD-
loaded cross-linked xylan microcapsules have been produced with three different amounts of the drug (3.1, 6.2, and 60mg). At the end of the process, yellowish suspensions of spherical polymeric microcapsules were produced. The mean particle size was found to be approximately 12.5 μm (Figure 11). Regarding the encapsulation efficiency, high and inversely concentration-dependent rates were achieved. While the SD concentration of 3.1 mg induced a load ability of 99 ± 2%, 6.2 mg of SD promoted 75.8 ± 1 %, and 60mg of SD yielded a 30.4 ± 6 % load efficiency. Accordingly, the results demonstrated the feasibility of producing xylan microcapsules with and without SD, presenting the same aspect and homogeneity, but concentration-dependent encapsulation rates (Unpublished data).

Regarding the stability of those formulations after storage, studies have been performed in order to evaluate the SD release. As a result of storage for 30 days, it was found that approximately 30 ± 5% of SD had been released to the external medium. This fact may be evidence that some adjustments in the methodology need to be made. One approach that has been shown as a promising strategy to avoid the drug release to the external medium is the spray-drying technique, which will produce a dried product instead of an aqueous suspension of microparticles. It may be used as a complement to the interfacial cross-linking polymerization and is described in the following subsection.

Fig. 11. Optical microscopy of SD-loaded cross-linked xylan microcapsules at 40x magnification.

Cross-linked xylan microcapsules have also been successfully developed in order to protect superparamagnetic particles from gastric dissolution (Silva et al., 2007). First, magnetic particles were synthesized by coprecipitation using solutions of ferric chloride and ferrous sulphate as a source of iron. Subsequently, xylan was dissolved in 0.6 M NaOH solution and the magnetic suspension was added to the xylan solution after neutralization and sonication. Finally, the emulsification was carried out in chloroform:cyclohexane containing 5% (w/v) sorbitan tristerate followed by the cross-linking reaction with terephthaloyl chloride. As a result, polymeric microparticles with a mean diameter of 25.26 ± 0.42 μm and roughly spherical in shape were produced. They were suggested to involve more than one magnetic particle entity due to their five-fold
larger size. Additionally, dissolution studies revealed that only 2.3% of the magnetite content was dissolved in 0.1 M HCl solution at 37 ± 0.1 °C after 120 min. This fact corroborates the feasibility of xylan as a material for colon delivery.

3.1.3 Spray-drying

The spray-drying technique is a one-step continuous operation characterized by the atomization of suspensions or solutions into fine droplets followed by a drying process that leads to the formation of solid particles (Tewa-Tagne et al., 2007). When compared to other approaches for producing and drying systems, this technique exhibits the advantages of low price, rapid process, and the possibility of modulating the physicochemical properties of particles, such as particle size, polydispersity, bulk and tapped densities, and cohesion (Raffin et al., 2006; Tewa-Tagne et al., 2006; Vehring, 2008). Briefly, the main steps of the process are (1) atomization of the feed into a spray, (2) spray-air contact, (3) drying of the spray, and (4) separation of the dried product from the drying gas (Tewa-Tagne et al., 2007; Tewa-Tagne et al., 2006). Because of the dry state of the final product obtained by the spray-drying technique, this method is highly appropriate to improve the stability of microparticulate systems due to the reduction of microbiological contamination, polymer hydrolysis, and physicochemical instability because of the elimination of the water content.

The production of xylan-based microparticles by spray drying has provided useful results. Although some limitation may be observed due to the sticky nature of xylan, which may lead to scarce amounts of final dry product, the use of other materials is very helpful. With that purpose, derivatives of methacrylic acid and methyl-methacrylate, also known as Eudragit®, have been used to prepare suitable xylan-based microparticles. In addition, Eudragit® S-100 (ES100) plays an additional role in the pharmacokinetic properties of the polymeric microparticles. ES100 is a synthetic gastroresistant polymer that has been largely used in the pharmaceutical industry due to its safety and degradation behavior. It is a pH-sensitive copolymer and, because of that, it is able to prevent drug release until the formulation passes through the stomach and reaches some distance down the small intestine (Friend, 2005).

Thus, spray-dried xylan/ES100 microparticles were produced at different polymer weight ratios dissolved in alkaline and neutral solutions, separately. More precisely, xylan and ES100 were dissolved in 1:1 and 1:3 weight ratios in 0.6 N NaOH and phosphate buffer (pH 7.4). Then, the suspensions were spray-dried at the feed rate of 1.2 mL/min (inlet temperature of 120°C) using a Büchi Model 191 laboratory spray-dryer with a 0.7 mm nozzle, separately. Cross-linked xylan microcapsules were also coated by ES100 after spray-drying at the same conditions.

It was observed that this technique was able to produce microparticles with a mean diameter of approximately 10.17 ± 3.02 μm in a reasonable to satisfactory yield depending on the formulation. This value was observed to be higher for the polymer weight ratio of 1:3 (87.00 ± 4.25 %), which indicates that ES100 improves the final result of the spray-drying process. According to the SEM analysis, the polymeric microparticles were shown to be quite similar in shape. Regardless of the formulation, they appeared to be mostly concave and asymmetric (Figure 12).
4. Biocompatibility of xylan and its products

Among other natural products, biopolymers have been largely studied, due to their numerous applications in which their contact to cells and tissues via their surface is of utmost importance. For instance, micro- and nanocapsules, film coatings, excipients for traditional dosage forms, and novel drug delivery systems have taken much advantage by using biopolymers, especially due to their biocompatibility and biodegradability properties (Drotleff et al., 2004; Villanova et al., 2010). Biopolymers are subject to degradation in vivo by hydrolysis or enzymatic attack. The use of these polymers may represent a lower cost compared to other conventional biodegradable polymers (Villanova et al., 2010).

During the development of pharmaceutical products, the toxic effect of biomaterials on cells is considered one of the most important issues to be evaluated. For instance, cell death, cell
proliferation, cell morphology, and cell adhesion are features directly correlated with the toxicity in vitro. Therefore, loss of viability could be a consequence of a toxic biomaterial (Marques, 2005). Although biopolymers are considered non-toxic and biocompatible, residues from their extraction methodology may cause toxicity issues.

In order to assess the effect of the corn cob xylan on the cell viability and proliferation rate, xylan solutions at concentrations of 0.1, 0.25, 0.50, 0.75, and 1 mg/ml were placed in contact with human cervical adenocarcinoma cells (HeLa cells) for 24 and 72 h. Finally, the cell viability was determined by the MTT assay. It was observed that regardless of the xylan concentration, the samples tested did not affect the viability of HeLa cells after incubation for 24 h (Figure 13) (Unpublished data).

Besides, the statistical analysis of the results obtained confirmed that the xylan samples did not present a significant effect on the cell viability and cell proliferation rate when in direct contact with HeLa cells at the concentrations used in this study and compared to the control.

Similarly, after a longer time of incubation, no significant changes in the cell proliferation rate was detected, as can be seen in the data for 72 h (Figure 13). In fact, this was expected due to the biocompatible nature of xylan. As a natural polysaccharide, this type of biomaterial is considered to be highly stable, non-toxic and hydrophilic (Liu et al., 2008). Accordingly, the alkaline extraction of xylan from corn has proved to be a safe approach for obtaining the polymer with no relevant toxicity (Unpublished data).

![Graph showing cell proliferation (% of control) vs. xylan concentration (mg/ml)](image)

Fig. 13. Viability of HeLa cells after incubation for 24 and 72h with solutions of xylan at different concentrations.

Xylan-based microparticles were also evaluated regarding their in vitro toxicity. In fact, cross-liked (CLM) and spray-dried microparticles (SDM) based on xylan and E5100 were produced in order to carry UA and avoid its side effects, namely hepatotoxicity and nephrotoxicity. Additionally, CLM and SDM dispersions at concentrations of 50, 125, 250, and 500 µg/ml were placed in contact with human embryonic lung fibroblasts (MRC-5 cells)
for 24 h and the MTT assay was carried out to assess the cell viability. According to the MTT assay results, the cells treated with CLM presented an initial decrease in the cell viability of 56% at the lowest tested concentration (50 µg/mL) while the cell viability rate reached only 12.6% at the highest concentration (500 µg/mL) (Figure 14).

Nevertheless, SDM showed a maximum decrease in the cell survival rate of approximately 12% and 27% at the lowest and highest concentrations of microparticles, respectively (Figure 14). The massive cytotoxicity induced by CLM may be explained by the presence of remaining molecules of terephthaloyl chloride, which plays the role of cross-linking agent during the formation of CLM and is well known as a toxic substance.

In contrast, the MTT assay for SDM did not show high cytotoxicity. This fact confirms the advantage of using spray-drying in order to avoid toxic and hazardous reagents such as terephthaloyl chloride and other cross-linking agents. Additionally, such results indicate a relevant biocompatibility of spray-dried xylan/ES100 microparticles containing UA.

![Figure 14](image.png)

Fig. 14. Viability of MRC-5 cells after incubation for 24h with spray-dried (SDM) and cross-linked xylan microparticles (CLM) containing UA.

5. Conclusions

The need of modern science to achieve a sustainable future development has been shown in many circumstances in society. Finding strategies less harmful to the environment has been a quest for research in several areas, such as pharmaceuticals, biotechnology, and food industries. With that purpose, the increase in research and development of more applications of xylan and its derivatives has shown the versatility of this biopolymer, thus helping the search for sustainable alternatives.

Xylan may be extremely useful in the pharmaceutical field, especially for the production of colon-specific drug carriers, such as micro- and nanoparticles, and film coatings. In addition, because of its abundant sources in nature, its use would bring many benefits, including reducing costs to industry, optimizing the use of natural resources, and reducing environmental damage due to its biodegradability and biocompatibility.
Large amounts of agricultural waste products, such as corn cobs, are continuously provided in several developing countries. Xylan is considered to be a green polymer that may play an essential role in the renewability of waste products due to its biodegradable and biocompatible nature. Furthermore, as shown in this chapter, xylan presents particular properties that allow a wide range of applications.

6. Acknowledgements

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It is interesting to consider that biopolymers are by no means new to this world. It is only because of our fascination with petrochemical products that these wonderful materials have been neglected for so long. Today we face a different challenge. Environmental pressure is pushing away from synthetic or petro-chemically derived products, while economic factors are pulling back from often more expensive "green" options. This book presents two aspects of biopolymers; potential products and some applications of biopolymers covering the current relevance of biopolymers.

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ATTACHMENT II

PRODUCTION OF XYLAN/EUDRAGIT® S100-BASED MICROPARTICLES BY CHEMICAL AND PHYSICAL-MECHANICAL APPROACHES AS CARRIERS FOR 5-AMINOSALICYLIC ACID.
BRIEFING


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**Author’s major contribution:** Production of xylan microparticles by interfacial crosslinking reaction and Spray-Drying technique.
ORIGINAL ARTICLE

Producing xylan/Eudragit® S100-based microparticles by chemical and physico-mechanical approaches as carriers for 5-aminosalicylic acid

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Abstract

Xylan is a biopolymer found in a variety of cell wall plants. Eudragit® S 100 (ES100), a pH-dependent polymer, is used as a coating material in gastrointestinal delivery systems. In this study, microparticles based on both polymers were produced by interfacial cross-linking polymerisation and/or spray-drying technique in order to investigate feasibility and stability of the systems. Size and morphology of the microparticles were characterised by optical and SEM while FT-IR, thermal analysis (TG/DTA), and X-ray diffraction (XRD) evaluated the drug-polymer interactions and the thermal behaviour of the systems. FT-IR confirmed the absence of chemical interaction between the polymers. TG/DTA analysis showed a higher stability for spray-dried microparticles and XRD data proved the amorphous feature of both carriers. The results reveal that xylan/ES100 microparticles can be produced by chemical or physico-mechanical ways, the latter being the best option due to the lack of toxic cross-linking agents and easy scale-up.

Keywords

Cross-linking, infrared spectroscopy, polymer–biopolymer interaction, spray-drying, thermal analysis

Introduction

Polymeric drug carriers have been largely designed to release drugs, cells and proteins for the treatment of several conditions, such as neuregenerative disorders and infections as well as inflammatory bowel diseases (IBD; Leonard et al., 2009; Wilson et al., 2009; Tang et al., 2010; Mistry et al., 2011). As the first-line therapy for patients with IBD, 5-aminosalicylic acid (5-ASA) has been recommended since the 1960s by the rectal administration of gels, foams and enemas with satisfactory efficacy due to its successful action in the induction and maintenance of clinical remission in patients with ulcerative colitis (Sonu et al., 2010). However, those dosage forms offer some issues regarding inconvenience and difficulty of administration, problems with retention and leakage and, consequently, low patient compliance (Sonu et al., 2010). In addition to aminosalicylates, corticosteroids are very effective for the treatment of mild to severe IBD. Nevertheless, systemic side effects after oral and intravenous administration limit their use. Therefore, targeted delivery systems have been suggested as a new way of treatment for IBD (Vardhoszas et al., 2011).

Biopolymers have been extensively studied and used for drug development in the pharmaceutical field due to their biodegradability and biocompatibility properties. As a hemicellulose found in a variety of cell wall plants, xylan is considered one of the most abundant biopolymer in hard wood and grass (Kučuráková et al., 2000). Besides the great advantage of a renewable material, this natural polymer has also been related to several profitable properties in the pharmaceutical field such as anti-phlogistic effects, immune function, inhibitory action on the growth rate of tumours, mutagenicity activity and use in the preparation of pH-responsive hydrogels for the controlled release of oral drugs (Sun et al., 2013). Because it is degraded by enzymes exclusively presented in the colon, xylan seems to be an eligible polymer for colon-specific drug carriers (Oliveira et al., 2010). On the other hand, Eudragit® S 100 (ES100) is a synthetic polymer based on methacrylic acid and methyl methacrylate in the ratio of 1:2 of the free carboxyl groups to the ester groups. Among several applications in the pharmaceutical field such as its use as a coating material and in drug delivery systems, ES100 has been utilised in the development of colonic drug carriers due to its pH-dependent dissolution properties (Yoo et al., 2011). Therefore, the association of two polymers with different responsive activities, such as biodegradability or pH-sensitive degradation, corresponds to a very interesting approach to develop novel drug delivery systems (Tian et al., 2012).

Depending on the formulation and the application of microparticulate drug carriers, several methods have been used for their production, such as coacervation, emulsion solvent extraction, emulsion solvent evaporation, interfacial cross-linking polymerisation and spray-drying (Oliveira et al., 2010). This latter one has become the easiest and widely used method to produce microparticles, which is a general term to address polymeric particles in the range of few μm in size (Keating, 1996). Briefly, the spray-drying method consists of the atomisation of a liquid
feed into a spray under hot air contact followed by the drying stage initiated by heat transfer. After the drying process, the dried particles are collected by Tewa/Tagne et al., 2006). Accordingly, it is considered a one-step, but complex method whose output control depends on a combination of many parameters (Durrigl et al., 2011). On the other hand, the interfacial cross-linking polymerisation is based on a chemical reaction occurring in the interface around droplets between functional groups of polymers and requires the use of organic solvents and cross-linking agents (Li et al., 2009; Yulen & Rochefort, 2010; Salaun et al., 2011). As a result, this method produces microparticles regarded by this paper as microcapsules due to their aqueous inner core enclosed by a polymeric shell.

The interactions among drugs and excipients in pharmaceutical dosage forms and their thermal stability is key points to be investigated during pre-formulation studies. For that purpose, the aim of this work was to produce 5-ASA-loaded microcapsules based on xylan and ES100 and prepared by a chemical and a physico-mechanical approach (cross-linking polymerisation and spray-drying, respectively) in order to evaluate the influence of the spray-drying process on the thermal stability of the microparticles. 5-ASA was chosen as a model molecule because of its traditional use as the first-line therapy in IBD (Markowitz, 2008; Ford et al., 2012).

**Materials and methods**

**Materials**

Terephthaloyl chloride, sorbitan triesterate and 5-aminosalicylic acid (5-ASA) were purchased from Sigma Aldrich, USA. Chloroform, cyclohexane, ethanol, sodium hydroxide (NaOH), Polysorbate® 20 and Polysorbate® 80 were obtained from Vetec chemical, Brazil. Eudragit® S-100 was purchased from Degussa Röhm Pharma Polymers, Germany. All the chemicals were used as received from manufacturers. Xylan samples were obtained after alkaline extraction from corn cobs as reported in the literature (Garcia et al., 2001; Oliveira et al., 2010).

**Methods**

**Preparation of microparticles**

**Interfacial cross-linking polymerisation method.** Xylan microcapsules (F1) were produced by means of interfacial cross-linking polymerisation (Nagashima et al., 2008). This method comprises a w/o emulsification step followed by a polymer cross-linking reaction (Lévy & Andry, 1990). First, 6 mL of an alkaline solution containing xylan, 5-ASA and (NaOH) was prepared and, then, emulsified in 30 mL of 1:4 (v/v) chloroform:cyclohexane containing 5% (w/v) sorbitan triesterate. After 10 min, under stirring, the interfacial cross-linking reaction was triggered by adding 40 mL of a 5% (w/v) terephthaloyl chloride chloroform:cyclohexane 1:4 (v/v) solution. Stirring was maintained for 30 min at room temperature. The reaction was ended by dilution with 30 mL of cyclohexane. Afterwards, the microcapsules were separated by centrifugation at 2300 g and withdrawal of the supernatant after three washing steps: first with a 2% (v/v) Polysorbate® 20 and 80 mixture (1:1; HLB = 18.85) in ethanol, then with ethanol, and finally with water. At the end the final volume of the formulation was set up at 50 mL and the nominal concentration of 5-ASA was 15 mg (30 μg/mL).

**Spray-drying technique.** Spray-drying was used for preparing three formulations: one, in which cross-linked xylan microcapsules (F1) were coated with ES100 at the polymer weight ratio of 1:3, producing formulation F2; and two others in which xylan and ES100 at two weight ratios (1:1 and 1:3), named F3 and F4, respectively, were spray-dried generating microparticles in a one single-step process. F3 and F4 have a 5-ASA loading weight of 15 mg.

In order to prepare ES100-coated xylan microcapsules (F2), ES100 was solubilised in 0.6 N NaOH. Subsequently, F1 was dispersed in this alkaline solution and spray-dried at the feed rate of 1.2 mL/min (inlet temperature: 120°C) using a laboratory spray-dryer (Büchi, Model B-191, Geneva, Switzerland) with a 0.7 mm nozzle.

On the other hand, F3 and F4 were produced by spray-drying dispersions containing 5-ASA and the polymers xylan and ES100 at the weight ratios of 1:1 and 1:3, respectively, in 0.6 N NaOH solution. Table 1 summarises the composition of all prepared formulations.

**Table 1. Composition of the studied formulations.**

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Xylan (mg)</th>
<th>ES100 (mg)</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>124</td>
<td>–</td>
<td>Interfacial cross-linking polymerisation</td>
</tr>
<tr>
<td>F2</td>
<td>74.4</td>
<td>223.2</td>
<td>Interfacial cross-linking polymerisation followed by spray-drying</td>
</tr>
<tr>
<td>F3</td>
<td>150</td>
<td>150</td>
<td>Spray-drying</td>
</tr>
<tr>
<td>F4</td>
<td>150</td>
<td>450</td>
<td>Spray-drying</td>
</tr>
</tbody>
</table>

*For all formulations the solvent was a 0.6 N NaOH solution containing 15 mg of 5-ASA.

**Determination of the entrapment efficiency**

Entrapment efficiency is the percentage of drug encapsulated in the microcapsules compared to the initial (nominal) quantity of the drug loaded in the formulation. A total of 20 mg of microcapsules were weighted and crushed in a glass mortar-pestle. Following, 3 mL of phosphate buffer pH 7.4 were added, one by one mL, to the ground microcapsule powder and the crushing process were continued for 5 min each time to get the maximum extraction of 5-ASA in the solvent. Then, the sample obtained was centrifuged (Excelsa™ II centrifuge, Model 206 BL, FANEM™, São Paulo, Brazil), at 2300 g, for 3 min to obtain a clear solution and assay of the drug content was quantified by spectrophotometry at 328 nm. Entrapment efficiency was determined by using the formula below:

\[ \text{Entrapment efficiency} = \frac{\text{Real drug content}}{\text{Nominal drug loading}} \times 100 \]

For F1, which was not a dried powder, 3 mL of the sample were withdrawn for this study.

**Characterisation of the microparticles**

**Microscopic evaluation.** The shape and the surface of the microparticles were analysed by optical microscopy (Zeiss, Model Axioscope 50, Oberkochen, Germany) and scanning electronic microscopy (SEM; Philips, Model XL30, Eindhoven, the Netherlands), respectively.

**Determination of particle size.** The microparticles were subjected to particle analysis under optical microscopy (Leica, Model 020507.010, Olympus, Center Valley, PA). The samples were placed on glass slides and size measurements of 1500 microparticles of each sample formulation were performed according to Feret’s diameter principle using an optical microscope calibrated with a stage micrometer scale (Al-Thyab et al., 2007).
Fourier transform infrared (FT-IR) spectroscopic analysis. FT-IR spectroscopic analysis of the polymers, their physical mixture, and microparticulate formulations (F2 – F4) were carried out at room temperature, in the range of 400-4000 cm⁻¹ using KBr pellets in a FT-IR spectrometer, 470 FT-IR (Thermo Nicolet Nexus, Model 470 FT-IR, Waltham, MA).

Thermal analysis. Thermogravimetric analysis (TGA) and differential thermal analysis (DTA) were carried out for xylan, ES100 and 5-ASA, separately. Additionally, the thermal behaviour of the microparticulate systems was also evaluated. TGA and DTA curves were obtained from approximately 20 mg of samples with a thermobalance (NETZSCH, Model STA 409 PC/PG, Selb, Germany), using platinum pans under dynamic nitrogen atmosphere (50 mL·min⁻¹) at a heating rate of 10°C·min⁻¹ and temperature range from 25 to 600°C.

X-ray diffraction (XRD). XRD experiments were performed for all the microparticulate systems on a diffractometer (Shimadzu, Model XRD-6000, Kyoto, Japan) with Cu-Kα radiation (30 kV x 30 mA). The XRD pattern was recorded for 2θ min range from 10° to 80°.

In-vitro drug release studies

For the in-vitro drug release profile of the formulations F2, F3 and F4, 40 mg of the microparticulates were weighted and placed in the beaker containing 30 mL of phosphate buffer, pH 7.4. The release study was started by magnetic stirring of the system at 75 RPM and continued for a period of 24 h. Aliquots of 3 mL were withdrawing on time 0.5, 1, 2, 3, 4, 5 and 24 h and analysed by spectrophotometry at 328 nm (the volume of the aliquots were replaced to maintain sink conditions). To quantify the 5-ASA all the aliquots were previously centrifuged at 2300 g, for 3 min to settle down remain traces of microparticulates and only the lipmid supernant was measured. The cumulative drug release was calculated and expressed as the percentage of release. For F1, which was not a dried sample, the release profile was performed with the 50 mL of the formulation and the time of study was set up between 15 and 2400 min. The study was repeated in triplicate.

Results and discussion

Preparation of microparticulates

Interfacial cross-linking polymerisation

According to the particle size analysis, the mean diameter of xylan microcapsules produced by means of interfacial cross-linking polymerisation (F1) was found to be 21.2 ± 8.0 μm. Optical microscopy analysis also confirmed the capular structure of the microparticulates, which were shown to be quite spherical in shape (Figure 1). The entrapment efficiency for this formulation was 24.98 ± 0.12%. The loss of 75% of the 5-ASA on this process can be inferred for the several washing process, which is mandatory to avoid any residual organic solvent and terephthaloyl chloride content.

Coating of cross-linked microcapsules by spray-drying

ES100-coated xylan microcapsules by spray-drying (F2) were successfully obtained and they were shown to be regular in shape. They appeared to be concave and shrivelled in a manner that is typically related to the particles derived from macromolecules, such as starch, after the spray-drying process (Figure 2; Fu et al., 2012).

The mean particle size of F2 was found to be 10.17 ± 3.02 μm, which is half value of F1, showing that the spray-drying process reduces the particle size of xylan microparticulates probably due to the dehydration followed by the coating process with the ES100 polymer. At the end of the spray-drying process, the yield provided for F2 was 50.56 ± 0.15%. The entrapment efficiency of this formulation was 23.61 ± 0.15%, which is quite similar to the F1 formulation. Therefore, as expected, the spray drying process although generating a loss of the totality of microparticulates (represented by the 50% of yield) did not degrade the 5-ASA.

One-step spray-drying technique

Concerning the xylan/ES100 microparticulates produced directly by one step spray-drying process, all the proposed formulations were
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Figure 3. SEM images of spray-dried xylan and ES100 microparticles in the weight polymer ratio of (a) 1:1 and (b) 1:3 in 0.6N NaOH (F3 and F4, respectively) at 938x and 400x, respectively.

satisfactorily produced. They appeared to be an off-white to yellowish fine powder with good apparent flowability. According to the SEM analysis, the spray-dried microparticles were shown to be quite similar in shape—mostly shriveled and asymmetric (Figure 3). Such characteristic profile is quite similar to the ones found by Moretti et al. for microspheres of ketoprofen (Moretti et al., 2001) and Kim et al. for the thermo-sensitive microparticles of PNIPAM-grafted ethylcellulose (Kim et al., 2002), both produced by the spray-drying technique. According to the polymer ratio used for each formulation, the following yields were provided for F3 and F4: 87.00 ± 4.25% and 74.03 ± 8.81%, respectively. The entrainment efficiency for these formulations was 47.30 ± 0.68% and 91.06 ± 0.42% for F3 and F4, respectively. Such variation on the content of 5-ASA on these formulations can be attributed to the final density of both systems. In fact, the formulation F4 has two times more mass than F3 due to the high content on ES-100 (Table 1). Therefore, F4 is much denser and more 5-ASA can be entrapped on the polymer network.

The feasibility of spray-drying as a technique for successful production of microparticulate delivery systems has also been evaluated in several studies. It has been used not only as an effective method for scale-up, but also for improvement of photo-stability of drugs, such as pantoprazole (Raffini et al., 2006b, 2008) or ketoprofen (Moretti et al., 2001). However, such microencapsulation technique has not been reported in the literature as a method to produce microparticles based on xylan.

Polymer interaction

FT-IR spectroscopy

FT-IR analyses were performed in order to investigate the interaction between the polymers. Therefore, the analyses were carried out for xylan, ES100, their physical mixture and the microparticles produced by both interfacial cross-linking polymerisation and spray-drying.

As expected, the spectra of the raw materials were similar to those found in the literature (Ciurzu et al., 2003; Oliveira et al., 2010). Thus, the FT-IR spectrum of xylan (Oliveira et al., 2010) showed a broad absorption band at 3405 cm⁻¹, attributed to the OH stretching associated with polar groups linked through intra- and intermolecular hydrogen bonding (Sun et al., 2005). This band in addition to the one found at 1160 cm⁻¹ is characteristic of glycosidic groups, the latter being assigned to CC and COC stretching vibrations in hemicelluloses (Sun et al., 1998; Xu et al., 2004). Additionally, a band at 2920 cm⁻¹, indicative of CH stretching vibrations due to CH₂ and CH₃ groups, and a band due to CH₃ stretching vibrations, near 1460 cm⁻¹, were also observed. A sharp band around 1635 cm⁻¹ can be ascribed to the HOH stretching due to hydration water present in xylan-type polysaccharides (Kačuráková et al., 1998). Moreover, other bands at 1375 cm⁻¹, 1044 cm⁻¹ and a sharp one at 897 cm⁻¹ were detected and were typically composed of cellulose and hemicellulose chemical structures (Sun et al., 1998, 2005). Concerning ES100, the FT-IR spectrum showed a typical absorption band at 1730 cm⁻¹ that is attributed to C=O vibration from esterified carboxylic groups, besides the bands related to ester at 1150, 1190 and 1275 cm⁻¹. Stretching vibrations attributed to methyl groups were observed between 2900 and 3000 cm⁻¹. Additionally, the presence of bands at 1385, 1450 and 1485 cm⁻¹ corroborates those findings while a broad band near 3500 cm⁻¹ may be attributed to the presence of hydroxyl groups and water (Ciurzu et al., 2003; Oliveira et al., 2010).

The spectrum of the physical mixture (Figure 4a) is dominated by the bands of ES100 (2920, 1730, 1450, 1275, 1190 and 1150 cm⁻¹). However, the xylan content can be fingerprinted by the wide OH-stretching band at 3405 cm⁻¹, the shoulder of the carbonyl stretching around 1630 cm⁻¹ (HOH stretching), and the low wave number structure around 550 cm⁻¹.

The FT-IR spectra of powdered samples of cross-linked xylan microcapsules (F1) showed a prominent absorption band at 1276 cm⁻¹ that can be attributed to the formation of terephthaloyl chloride as the cross-linking agent. This band was observed to be less intense at the FT-IR spectra of cross-linked xylan microcapsules coated with ES100 by spray-drying (F2; Figure 4b).

Moreover, the absorption band at 1276 cm⁻¹ did not appear at the spectra of xylan/ES100 directly spray-dried microparticles (F4) probably because of the absence of terephthaloyl chloride. Also, absorption bands at 1717 and 1276 cm⁻¹, which 1717 reflect the formation of ester bonds from hydroxyl groups of xylan (Devy et al., 2006), could be detected at neither F2 nor F4 (Figure 4c). This fact may be evidence that xylan and ES100 do not present chemical interaction during the spray-drying process. In addition, when ES100 is ionised, the carboxylate band shifts from 1728 to 1560 cm⁻¹, corresponding to the anti-symmetrical vibration of COO⁻ (Raffini et al., 2006a). As this band did not show any shift at F2 or F4 spectra, it is suggested that no new chemical bond was formed after spray-drying and the results would confirm that the polymers are physically aggregated to the microparticles (Figure 4c).

thermal analysis

TGA curves for xylan, ES100 and 5-ASA revealed relevant events of weight loss for each compound in the range of 250-350 °C, 360-430 °C and 280-300 °C, respectively (Figure 5a). In order to evaluate the influence of the spray-drying process on the polymers and the drug during the microencapsulation process,
Figure 4. FT-IR spectrum of (a) the physical mixture of xylan and ES100 in the weight ratio of 1:1, (b) cross-linked xylan microcapsules (F1) and (c) ES100-coated cross-linked xylan microparticles (F2).

Figure 5. TGA (a) and DTA (b) curves for xylan, ES100, 5-ASA and the physical mixture of the polymers and the drug, and (c) TGA curves for F1, F2, F3 and F4 (microparticles produced by single cross-link reaction (F1)), microcapsules produced by cross-link reaction followed by coating with ES100 polymer by spray-drying (F2), microcapsules produced with xylan and ES100 (1:1) by the spray-drying technique (F3) and microparticles produced with xylan and ES100 (1:3) by the spray-drying technique (F4).

TGA and DTA data of these compounds were correlated with their physical mixture (Figure 5a and b).

From the TGA/DTA data for xylan, a first event of 6.62% weight loss was detected in the range of 62 and 107°C due to dehydration, and the second and most relevant event of 45% weight loss in the range of 250 and 300°C due to the polymer decomposition. TGA/DTA data for ES100 revealed a first event between 60°C and 104°C with weight loss of 2.73% due to dehydration and the second endothermic event around 360 and 430°C with weight loss of approximately 90% corresponding to the polymer decomposition. Regarding the thermal behaviour of 5-ASA, one single and well-defined endothermic event was observed between 270°C and 280°C with weight loss of 85% due to decomposition of the drug.

Data obtained from the thermogravimetric experiments were used to determine the reaction order by Arrhenius equation (Equation 1):

$$k = Ae^{-E/RT}$$  \hspace{1cm} (1)

where $E$ is the activation energy, $A$ is the pre-exponential factor, $R$ is the universal gas constant, $T$ is the absolute temperature, and $k$ is the reaction rate. Thus, the decomposition events of xylan, ES100 and 5-ASA were found to present zero-order kinetics and the Arrhenius constant for those materials were $7.428 \times 10^{-2}$, $1.31 \times 10^{-2}$, and $4.426 \times 10^{-2}$, respectively. As expected, xylan presented lower stability when compared to ES100 and 5-ASA most likely due to the fact that it is a natural organic material with variable composition depending of its source and its extraction procedure.

The TGA/DTA curves for the physical mixture of both polymers and the drug at a proportional weight ratio revealed behaviour similar to that demonstrated by TGA/DTA curves for xylan, ES100 and 5-ASA, separately. Endothermic events attributed to weight losses were detected at approximately the same temperature ranges for each compound. An interesting fact is that the decomposition of xylan and 5-ASA occurred at very similar temperature ranges and because of that it may not be possible to clearly identify the decomposition events individually.

Concerning the thermal behaviour of the microparticulate systems, ES100-coated cross-linked xylan microparticles (F2) and directly spray-dried ES100/xylan microcapsules (F3 and F4) were evaluated and thermal data were correlated with cross-linked xylan microcapsules (F1; Figure 5c). Table 2 summarises the thermal analysis data.

By analysing the TGA curve for F1, it was possible to observe two events. The first one corresponded to a weight loss of approximately 80% at the range of 200–300°C while the second event occurred from 300°C to 400°C and corresponded to the
final 20% of the sample weight. Such events may be attributed to both the decomposition of 5-ASA and non-cross-linked xylan likely present in the formulation and the decomposition of cross-linked xylan, respectively. The fact that the decomposition of 5-ASA occurred at the same temperature may be explained by the absence of chemical interaction with the polymer during interfacial cross-linking polymerisation (Agnihotri & Aminabhavi, 2004). When ES100 was added to the formulation in order to coat the cross-linked xylan microcapsules or produce spray-dried xylan microparticles, a higher thermal stability was clearly evidenced by the weight loss of only 60% for F2 and at more than 400°C. This fact may be explained by the high stability of ES100, corroborated by its low Arrhenius constant when compared to xylan and 5-ASA.

F3 and F4 showed a weight loss of approximately 20% and 10% at nearly 200°C and 300°C, respectively, probably due to dehydration of xylan, and their masses remained unchanged until 600°C. A possible reason for the absence of weight loss at the 5-ASA temperature of decomposition is its complete entrapment in the polymer matrix as reported elsewhere (Simonoska Cccarevska et al., 2008). Additionally, it is possible that a significant reduction in drug crystallinity happened after encapsulation as reported by other studies and corroborated by the absence of the crystallinity profile of the drug in the XRD curves (Mladenovska et al., 2007; Simonoska Cccarevska et al., 2008; Stulzer et al., 2009). According to the Arrhenius equation, the decomposition of F3 presents first-order kinetics. Thus, concentration of its formulation has a considerable influence on the decomposition of the system. On the other hand, concerning F4, this phenomenon did not occur because it presents zero-order kinetics decomposition.

When interfacial cross-linking polymerisation (F2) was compared to the spray-drying technique (F4) regarding the thermal stability, TGA curves demonstrated a relatively slight increase in the stability of the spray-dried system (F4). Although some studies have demonstrated the importance of adding a cross-linking agent after the spray-drying process in order to stabilise the drug release from the microcapsules (Desai & Park, 2005b; Mladenovska et al., 2007), depending of the polymer and the drug encapsulated, the strategy of spray-drying previously cross-linked microparticles has proven to be a suitable method to increase the stability of drug release by polymeric carriers (Desai & Park, 2005a; Simonoska Cccarevska et al., 2008; Möbus et al., 2012). No other studies focusing on the concurrent comparison between the thermal stability of cross-linked and spray-dried polymeric particles were found in the literature. Nevertheless, Silva-Junior et al. (2008, 2009) performed a comprehensive thermal analysis of spray-dried particles containing ciprofloxacin hydrochloride and triamcinolone, which gave rise to this work (Silva-Junior et al., 2008, 2009).

X-ray diffraction (XRD)

Physical properties of drugs and pharmaceutical excipients in the solid state are of great interest in that they can affect not only the product development and formulation but also their biological
effect. Crystallinity of a substance may affect its stability in the solid state, its flowability properties and its dissolution rate, which affects the substance bioavailability. Total or partial loss of crystallinity results in a significant increase in solubility and dissolution rate of drugs (Shoukri et al., 2009).

The spray-drying technique generally produces amorphous compounds. This fact is usually attributed to the speed of the drying process, which hinders the organisation of a crystalline structure. It is well known that amorphous solid molecules are organised randomly and, thus, low energy is required to separate them and, consequently, their dissolution is faster (Cheow & Hadinoto, 2012; Van den Moortel, 2012). Based on this fact, the development of formulation containing a drug in its amorphous form is normally advantageous regarding dissolution and bioavailability concerns.

By analysing the XRD curves for both cross-linked (F1 and F2) and spray-dried (F3 and F4) microparticulate systems, one broad peak related to the presence of ES100 at approximately 30° could be detected for F2, F3 and F4 (Figure 6). Besides, the intensity of this peak increased according to the increment in the amount of ES100 in the formulation (Figure 6). Typical peaks of 5-ASA could also be detected for all the samples, although they indicated a less intense drug crystallinity probably due to their partial solubilisation in the amorphous polymer, xylan, as similarly occurs during the hot-melt extrusion (Bruce et al., 2005).

Additionally, in the XRD curves for xylan/ES100 microparticles produced directly by the spray-drying technique, it was possible to observe the profile of a predominantly amorphous compound with a slight crystallinity due to the presence of a large halo at 2θ from 10° to 40°, which is typical of materials subjected to the spray-drying process (Figure 6c and d).

In vitro drug release studies

The release profile of the formulation F1 can be fitted by the Higuchi model within the almost totality of release at around 40h (2400 min; Figure 7). It is interesting to note that although presenting a low entrapment efficiency, F1 releases 5-ASA into the media and this phenomenon happens at the pH 7.4. Our team has previously demonstrated that the xylan polymerisation process by itself is not able to produce microparticles with no pores on their structure, probably due to the intrinsic molecular weight variation of this polymer produced during its extraction process (Nagashima et al., 2008). In fact, this is the reason why on this work it was attempted to produce xylan microparticles coated with Eudragit® S100. The objective was to avoid the drug release through the pores on the polymeric shell by the utilisation of a pH sensitive polymer to fill out the pores and confine the drug inner the microcarrier.

On the other hand, the release profile of F2, F3 and F4 formulations was quite dissatisfying (Figure 8). All the formulations released the 5-ASA as soon as the powder was dispersed into the phosphate buffer media. The reason for such release was that the NaOH used to dissolve the polymers and the drug was still present on the spray dried formulation and the buffer was not strong enough to neutralise it. In fact, the pH measured on the release media was around 9.0, which completely dissolves the microparticles into the media.

Besides the unfortunate data revealed by the release studies, the totality of physico-chemical data presented here demonstrated that the xylan-based microparticles coated with ES100 can be a very promising system to treat IBD in the colon. The NaOH presented on the formulation prepared by the spray drying process.
can be easily removed by the use of a diluent with an acid pH to neutralise it.

Conclusion

Both cross-linking interfacial polymerisation and/or the spray-drying technique successfully produced well-defined micro-particles based on xylan and ES100 containing 5-ASA and presenting suitable physical characteristics and satisfactory yields. However, the spray-drying technique produced more stable micro-particles regarding the thermal behaviour when compared to the cross-linked ones. In addition, it was also possible to coat cross-linked xylan microcapsules by means of the spray-drying technique.

Thermogravimetric analysis provided data to the prediction of the higher stability of spray-dried micro-particles when compared to the cross-linked ones. FT-IR spectroscopy demonstrated the lack of relevant interactions among the compounds of the formulation. Finally, XRD was able to evidence the influence of the microencapsulation methods on the crystallinity of the systems.

The totality of the results presented here reveals that although the biopolymer xylan can be used to produce micro-particles by chemical or physico-mechanical ways, the latter could be the better option because it avoids the use of cross-linking agents, frequently responsible for important side effects in pharmaceutical products. In addition, spray-drying is a technique easily transposable for an industrial scale.

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Declaration of interest

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

References


Title: Impact of the aggregation state of amphotericin B on its biopharmaceutical properties. Design of micro- and nanocarriers for oral delivery.

Mots clés: amphotericine B, agrégation, perméabilité intestinale, xylene, émulsions eau-dans-l'eau, nanoparticules.

Résumé: Le développement de nanomédicaments capables de contrecarrer les propriétés biopharmaceutiques défavorables de l'amphotéricine B (AmB) représente un enjeu important. L'AmB est en effet une molécule très efficace pour le traitement des infections fongiques systématiques et aussi pour la leishmaniose, mais difficile à formuler efficacement, quelle que soit la voie d'administration. Cette molécule particulièrement hydrophobe souffre de limitations importantes en raison de sa tendance prononcée à l'agrégation dans les conditions physiologiques. La première partie de cette thèse a consisté à vérifier l'hypothèse selon laquelle le degré d'agrégation de l'AmB pourrait avoir un fort impact sur certaines de ses propriétés biopharmaceutiques et pharmacocinétiques. La deuxième partie de ce travail a été axée sur le développement des micro- et nanotransporteurs destinés à surmonter la barrière d'absorption élevée contre AmB après son administration orale. Enfin, l'emploi d'un autre système de transporteur conçu pour atteindre le colon et assurer la délivrance colonique grâce à l'action enzymatique bactérienne locale a été envisagé. Dans cet objectif, un biopolymère naturel et dégradable par des enzymes, le xylane, a été sélectionné.

Title: Impact de l'état d'agrégation de l'amphotéricine B sur ses propriétés biopharmaceutiques. Mise en œuvre de micro- et de nano-transporteurs en vue de son administration orale.

Keywords: Amphotericin B, aggregation, intestinal permeability, xylan, water-in-water emulsions, microparticles.

Abstract: This thesis is part of the development and evaluation of nanomedicines potentially able to overcome unfavorable biopharmaceutical properties of amphotericin B (AmB), a highly effective molecule used for the treatment of systemic fungal infections and leishmaniasis, but difficult to formulate efficiently, whatever the route of delivery. It is believed that this hydrophobic molecule suffers from severe limitations due to its pronounced tendency to aggregate under physiological conditions. The first part of the thesis was driven on the hypothesis that the degree of aggregation of AmB could have a strong impact on some of its pharmacokinetics properties. The second part of this work was focused on the development of micro- and nanocarriers intended to overcome the absorption barrier raised against AmB after oral delivery. Finally, another particulate system intended for colonic delivery and based on xylan, a natural and enzymatically degradable biopolymer, has been investigated.