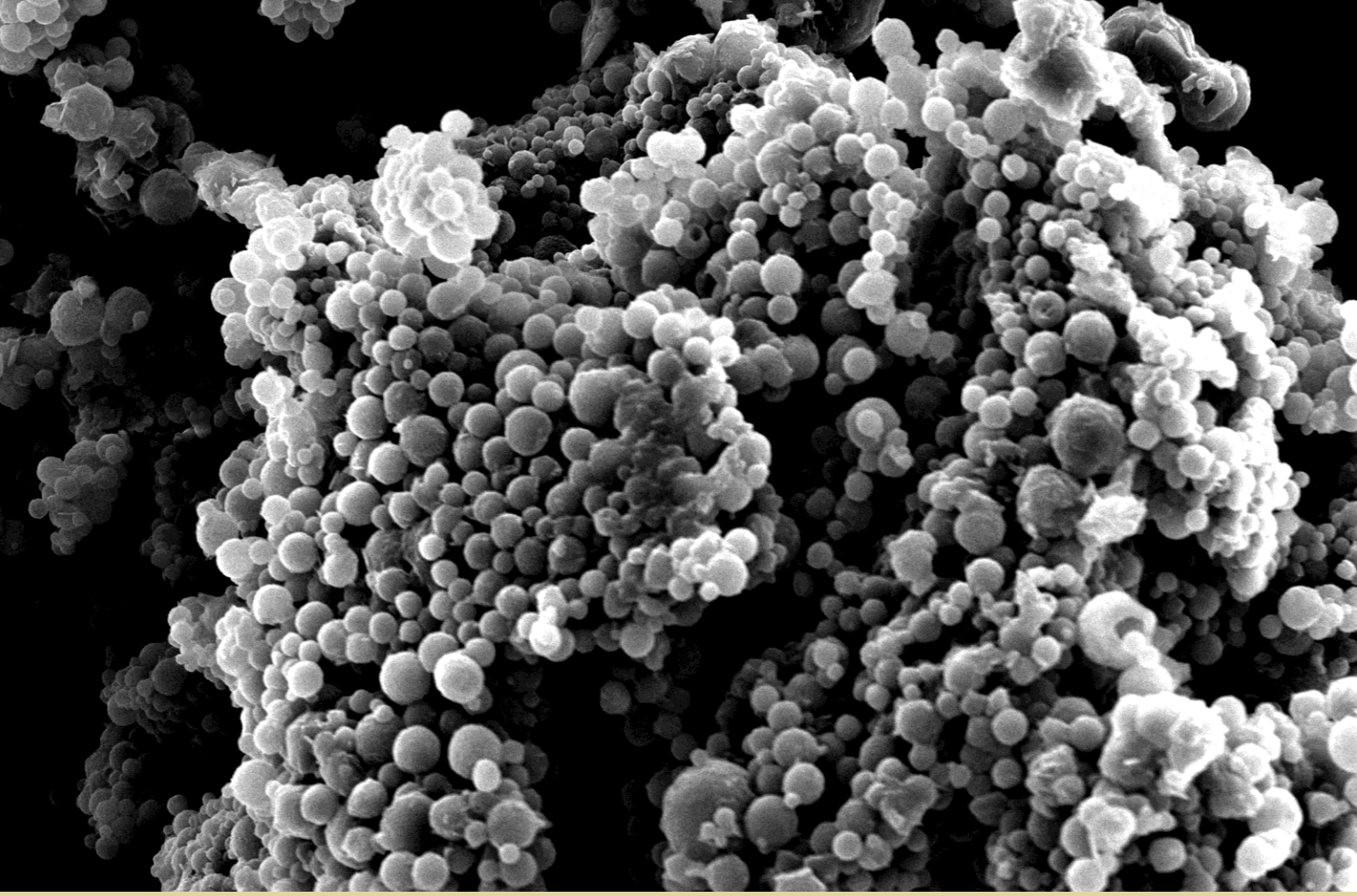

Raquel Maria Pires da Ana

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Dissertação de Mestrado em Tecnologias do Medicamento orientada pela Professora Doutora Carla Sofia Pinheiro Vitorino e apresentada à Faculdade de Farmácia da Universidade de Coimbra

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mas naquilo que ele nos transforma.

John Ruskin

Para os meus pais

Index

List of Abbreviations

AED	Antiepileptic drugs
BBB	Brain-blood barrier
CBZ	Carbamazepine
CHIT	Chitosan
DL	Drug loading
DLS	Dynamic light scattering
DSC	Differential scanning calorimetry
EE	Entrapment efficiency
FDA	Food and Drugs Administration
FT-IR	Infrared spectroscopy
GRAS	Generally regarded as safe
HBL	Hydrophilc-Liphofilic Balance
HPLC	High performance liquid chromatography
HPH	High pressure homogenization
LD	Laser diffraction
LN	Lipid nanoparticles
NLC	Nanostructured lipid carriers
PDI	Polydispersity índex
PS	Particle size
RES	Reticuloendothelial system
SEM	Scanning electron microscopy
SLN	Solid lipid nanoparticles
TEER	Transepithelial/transendothelial electrical resistance
TEM	Transmission electronic microscopy
TRIP	Tripalmitin
ZP	Zeta potential

Resumo

A epilepsia apresenta-se como um distúrbio do sistema nervoso central que afeta milhões de pessoas em todo o mundo. A base do tratamento desta doença são os fármacos antiepiléticos, sendo a carbamazepina um fármaco de primeira linha para o tratamento da maior parte das crises convulsivas. Apesar de este ser um fármaco comercializado há várias décadas já, a sua forma de libertação é lenta e errática, levando a um problema de resistência ao fármaco, tornando a terapêutica ineficaz e comprometendo assim a qualidade de vida dos doentes. As nanopartículas lipídicas são sistemas coloidais compostos por lípidos biocompatíveis e biodegradáveis, podendo estes ser sólidos – nanopartículas lipídicas sólidas – ou, em certos casos, misturados com lípidos líquidos – transportadores lipídicos nanoestruturados – adequadamente estabilizados por soluções de tensioativo(s). As características relacionadas com a sua natureza e com o seu tamanho permitem aumentar a estabilidade e a eficácia do fármaco, bem como controlar a libertação e vetorizar o fármaco para um alvo de interesse, e ainda permitir uma elevada eficiência de encapsulação. Estes sistemas possibilitam a melhoria de propriedades, como a solubilidade e a biodisponibilidade de fármacos hidrofóbicos, tal como a carbamazepina. No entanto, apresenta como desvantagens a agregação em meio ácido.

As nanopartículas híbridas polímero-lípido surgem como um sistema alternativo, tirando vantagem de ambos os materiais. Estes nanossistemas permitem aumentar a estabilidade em meio gástrico, bem como o transporte intracelular de fármacos. Neste sentido, o quitosano é um biopolímero com uma vasta utilização no revestimento de nanopartículas lipídicas, possuindo diversas propriedades interessantes, tais como características mucoadesivas e promotoras da absorção.

Desta forma, o objetivo do presente trabalho consistiu no desenvolvimento de nanopartículas nanopartículas híbridas polímero-lípido, baseadas no revestimento com quitosano, para administração oral de carbamazepina.

As formulações foram produzidas através da técnica de homogeneização a alta pressão a elevada temperatura, e as suas propriedades físico-químicas foram avaliadas, bem como o perfil de libertação *in vitro,* a permeabilidade intestinal e o seu comportamento *in vivo*.

O revestimento das nanopartículas lipídicas com quitosano permitiu uma reversão da carga superficial para valores positivos, tendo sido também observado um menor tamanho de partícula.

A análise do perfil de libertação *in vitro* revelou uma libertação controlada, confirmada pelos ensaios *in vivo*, cujos resultados sugerem também uma vectorização do fármaco para o cérebro.

As partículas foram posteriormente atomizadas, de forma a aumentar a estabilidade das mesmas e permitir a conversão numa forma farmacêutica sólida – comprimidos. Estes foram avaliados em relação ao seu perfil de dissolução, que mostrou ainda controlo sobre a libertação de carbamazepina.

O trabalho desenvolvido pode ser considerado uma base promissora para futuros estudos pré-clínicos no tratamento da epilepsia.

Abstract

Epilepsy is a brain disorder that affects millions of people worldwide. Antiepileptic drugs are the basis for the treatment of this disease, in which carbamazepine (CBZ) is one of the first line drugs that is used in most of the seizure type. Although CBZ has been commercialized for many years, it is released in an erratic and slow way, leading to resistance drug problems, which turns the therapeutic ineffective and, consequently, compromises the patients' quality of life. Lipid nanoparticles (LN) are colloidal carriers composed by biocompatible and biodegradable solid (solid lipid nanoparticles, SLN) and, in some cases, blended with liquid lipids (nanostructured lipid carriers, NLC), that are stabilized in an aqueous emulsifier(s) solution. Their nature and size-dependent properties allow to improve the drug stability and efficacy, control and target drug release, and encapsulate a high drug content. LN also show the ability to improve the solubility and bioavailability of poorly-water soluble drugs, like CBZ. However, under acidic gastric conditions, LN typically tend to aggregate.

Polymer-lipid hybrid nanoparticles arise as alternative nanocarriers that include polymers and lipids in their composition, taking advantage of both materials. These nanosystems allow to increase formulation stability under gastric conditions, also improving drug uptake and intracellular drug transport. Chitosan is a biopolymer with a large use in LN surface coating, with several interesting properties, such as the mucoadhesive and absorption enhancer characteristics.

In this way, the aim of the work was the development of polymer-lipid hybrid nanoparticles, using chitosan as the polymer coating, for oral administration of CBZ.

Nanoparticle formulations were produced by the hot high pressure homogenization technique and were evaluated and characterized in terms of physicochemical properties, *in vitro* release, intestinal permeability, and *in vivo* performance.

Chitosan coating allowed to change physicochemical characteristics of LN once that comparing to non-coated LN, polymer-lipid hybrid nanoparticles presented positive charge and lower particle.

In vitro release profiles suggested a drug controlled release, which is supported by *in vivo* and permeability studies. Due to the higher concentrations of CBZ found

in brain tissue, *in vivo* studies also suggest a drug brain targeting with hybrid lipid nanoparticles comparing with results obtained for CBZ suspension.

Particles were further spray-dried, in order to increase the stability and achieve a solid drug dosage form through the incorporation of the obtained power in a tableting mixture. The nanoparticle based tablets were evaluated and characterized in terms of *in vitro* release, whose results showed that they still keep control over release.

The present work may provide a promising basis for further preclinical studies in the epilepsy treatment.

1. Introduction

Epilepsy is a disease that affects over 60 million people worldwide, which is a brain disorder characterized mainly by recurrent and unpredictable interruptions in normal brain function, termed as epileptic seizures. This non-communicable disorder of the central nervous system (CNS) exhibits a huge increase of electric impulses in one focal locus of the brain and/or in the entire brain, which leads to partial or generalized seizures (Jabir *et al.*, 2015). The drastic and abnormal neuronal excitation has as consequences physical and mental disorders and co-morbidities. Seizures can also affect consciousness, emotional state, memory, cognition, or behaviour. The occurrence of seizures depends on some factors, such as the location of onset in the brain, the propagation forms, the maturity of brain and sleep-wake cycle. In what regards to epilepsy definition, it is important to consider that it encompasses a diverse group of disorders and not only one condition, that have in common the abnormal increase of seizures predisposition (Fisher *et al.*, 2005).

1.1 Epilepsy treatment

The main goals of the epilepsy treatment are the achievement of no seizures, no side effects and optimal quality of life, which is associated to a seizure-free status. Seizure treatment is achieved with anticonvulsant medication. The drug choice depends on the diagnosis of the epileptic syndrome, and on the response variations of specific anticonvulsants to the different syndromes, since different responses reflects distinct physiopathological mechanisms involved in the various types of seizures and the specific epileptic syndromes. Anticonvulsant drugs can present multiple mechanisms of action (as is the case of lamotrigine, topiramate, valproic acid, zonisamide) or, on the other hand, only one known mechanism of action (e.g. phenytoin, carbamazepine, ethosuximide). Based on the mechanism, they can be divided into some large groups (Ko, 2016):

- Blockers of repetitive activation of sodium channels (phenytoin, carbamazepine, oxcarbazepine, eslicarbazepine, lamotrigine, topiramate);
- Enhancers of slow inactivation of sodium channels (lacosamide, rufinamide);
- Gamma-aminobutyric acid (GABA)–A receptor enhancers (phenobarbital, benzodiazepines, clobazam);

- *N* -methyl-D-aspartic acid (NMDA) receptor blockers (felbamate);
- Alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor blockers (perampanel, topiramate);
- T-calcium channel blockers (ethosuximide, valproate);
- N- and L-calcium channel blockers (lamotrigine, topiramate, zonisamide, valproate);
- H-current modulators (gabapentin, lamotrigine);
- Blockers of unique binding sites (gabapentin, levetiracetam, perampanel);
- Carbonic anhydrase inhibitors (topiramate, zonisamide);
- Neuronal potassium channel (KCNQ [Kv7]) opener (Ezogabine).

The treatment strategy involving antiepileptic drugs (AED) should be also individualised, taking into consideration the co-medication and co-morbidities, the patients' lifestyle and the preferences of the people involved (patient and carers). (NICE, 2012). Carbamazepine (CBZ) and derivatives are the most used anti-epileptic drugs, once the majority of adults show partial-onset seizures. Indeed, CBZ is claimed as a first line drug for new-onset partial or generalized seizures, associated to fewer side effects. CBZ mechanism of action is based on the stabilization of excited nervous membranes by inactivation of sodium channels, making brain cells less excitable and also showing capacity to potentiate GABA receptors. It is commercially available as oral suspension, immediate-release, controlled-release (Tegretol XR) and sustained-release tablets (Carbatrol) (DiPiro *et al.*, 2011).

1.2 Epilepsy treatment gap

The advances in the epilepsy treatment are not enough to offer a good quality of life to patients, once just less than 50% of patients become seizure-free. Epilepsy treatments are currently restricted to control brain seizures by exposing brain tissues to sufficient dosages. However, this approach unnecessarily increases the threshold of total body drug, tending to increase resistance to AEDs in epileptic patients. The extension of the therapy for a long period of time can also induce chronic side effects. Drug resistance can lead to epilepsy recurrence after reduction of medication, resulting then in uncontrolled seizures, higher risk of brain damage and higher mortality rates (Jabir *et al.*, 2015).

Carbamazepine released from immediate-release tablets is almost completely absorbed, but in a slow and erratic way, since this drug presents a low water solubility.

The same poor bioavailability is observed for most of the AED, getting ineffective during the treatment. A large variability in the peak-to-trough concentrations of up to 40% is also reported for carbamazepine. This variation in absorption results in peaks of time of 2 to 24 hours (DiPiro *et al.*, 2011).

1.3 Current technological solutions

Anti-epileptic drugs do not just get ineffective by drug resistance, but also because they suffer from low bioavailability. The treatment of this disorder becomes more difficult by the inability of available drugs to cross the adjunctive blood brain barrier, which could be overcome by suitable drug delivery systems. Ideally, the system should provide localized and controlled drug release to target locations in the brain, once it helps to reduce drug associated toxicity and improves the drug effectiveness (Jabir *et al.*, 2015).

Alternatives to immediate-release tablets available in the market are the suspension dosage form, which is absorbed faster than the tablets, controlled-release tablets, and sustained-release tablets. The latter two dosage forms are bioequivalent in twice-daily dosing to a four-time daily dosing regimen with immediate-release carbamazepine. When compared with immediate-release carbamazepine, the formulations show lower peaks and higher troughs, what decrease side effects and improve seizure control (DiPiro *et al.*, 2011).

1.4 Brain drug targeting approaches under investigation

Scientific literature has reported several strategies to effective delivery of AED. Concerning the drug delivery systems for brain targeting, it is possible to find two main approaches – molecular and nanocarrier based. For the molecular approach, the drug targeting is reached by the lipophilicity of the drug, size, receptor mediation, or site-specific enzymes that converts inactive drugs in active forms. However, this is a limited field, since ongoing research is still in development and more studies are necessary. A promising and innovative approach is based on nanotechnology-based systems. This field is gaining interest, because of their ability to cross the BBB, the improved selectivity and the potential to create sustained drug delivery forms to the brain. In order to overcome the elimination of AED at the BBB and increase its persistence, nanostructured drug delivery carriers have been developed for an effective brain delivery. The most important factors to consider for effectiveness of nanocarriers are

the size, molecular weight, co-polymer ratio, mechanism of erosion and surface charge (Jabir *et al.*, 2015).

1.5 Conventional colloidal systems and Lipid Nanoparticles

The main AED nanoformulations reported rely on liposomes, polymeric nanoparticles, nanoemulsions, solid lipid nanoparticles and magnetic nanoparticles.

Liposomes, which consist of unilamellar or multilamellar phospholipid vesicles enclosing an aqueous core, show some interest, because of their biocompatibility, biodegradability, and ability for encapsulation of drugs with different lipophilicities and molecular weights. They are easy to modify, mainly in what concerns the size, membrane fluidity and surface characteristics. It is reported a bioavailability enhancement of drugs across cellular membranes and the decrease of enzymatic degradation by using liposomal carriers. However, liposomes show some limitations, such as a rapid immune-mediated clearance from the blood by reticuloendothelial system (RES), low stability in prolonged storage, fast metabolic degradation of phospholipids, and failure on the maintenance of the continuous release, when compared with other nanocarriers (Jabir *et al.*, 2015; Moghimi e Szebeni, 2003; Suntres, 2011).

Polymeric nanoparticles use several polymers to drug loading. These polymers are usually biodegradable, biocompatible and safe, properly approved by Food and Drug Administration (FDA). By the combination of different mechanisms of diffusion and polymeric degradation, it is possible to release the drug loaded at target places. As advantages, polymeric nanoparticles are easy to prepare, show a great *in vivo* and *in vitro* stability, as well as during storage. Polymeric nanoparticles are also capable of ensuring the continuous and controlled release of drugs for a long time of period. They also present some drawbacks, such as the rapid clearance from plasma due to the activation of immunologic system. Furthermore, the incorporation of some polymers, like PEG, polyvinyl alcohol, poly-acrylamide and polysaccharides, to increase the circulation time, also turns them unrecognized by RES, what leads to the increase of the circulation time from several minutes to hours (Jabir *et al.*, 2015; Lu *et al.*, 2000).

Nanoemulsions, another type of nanoformulation for drug delivery, can be easily prepared by spontaneous emulsification, and allow the improvement of the stability and solubility of the loaded drug. This is a heterogeneous system, composed by oil, water and surfactants that presents a droplet size usually in the range of 10 to 200 nm.

It is reported its efectiveness, by the increase of the absorption rate, rapid drug penetration, the reduction of toxicity, protection of the oily phase from hydrolysis and oxidation phenomena, and it shows potential to be administered by several routes. However, the main advantage of the nanoemulsions loaded with AED is their ability to pass through BBB, even when drugs show low bioavailability (Jabir *et al.*, 2015; Jain *et al.*, 2011; Prajapati *et al.*, 2015).

Magnetic nanoparticles, composed by ferromagnetic elements like cobalt (Co), nickel (Ni) and iron (Fe) for magnetically guidance drug delivery are gaining more attention, because of their effective targeted delivery, and due to the fact of the modulator-type control by external magnetic field characteristic allows to obtain a controlled and sustained release and transportation across the chosen tissues, thus minimizing toxicity in other tissues. The ferromagnetic elements used for their composition and the preparation process lead to their application, mainly as gene therapy or cancer chemotherapy. By the particles surface coating with biodegradable or non-biodegradable polymers, biocompatibility can be improved, which depends on the particle surface characteristics. It also helps to prevent the oxidation of the particles, increases drug loading capacity, and provides good dispersion properties. Despite the mentioned potential as nanocarriers, there are no formulations approved yet for clinical practice (Chomoucka *et al.*, 2010; Jabir *et al.*, 2015; Prijic e Sersa, 2011).

Lipid nanoparticles (LN) are colloidal drug carriers with a submicron size range composed by physiological lipids that are attracting increased attention (Müller, Mäder e Gohla, 2000). Due to their lipid nature and size-dependent properties they present several advantages, like the excellent tolerability, conferred by the physiological, biodegradable, biocompatible, non-toxic and non-allergenic lipids used (with generally recognized as safe, GRAS, status). These lipid-based nanocarriers also provide the improvement of drug stability and efficacy, controlling and targeting drug release, encapsulation of high drug content, and the possibility to carry both lipophilic and hydrophilic drugs. Moreover, they are easy to scale up and sterilize and the associated cost is lower when compared with other carriers. Lipid nanoformulations yield adequate dispersions of poorly water-soluble drugs, leading to the reduction of inherent limitations of slow and incomplete dissolution of this type of drugs. Contrary to other lipid drug delivery systems, lipid particulate systems depend on the size and structure of the particles. During the last 20 years, some lipid based drug delivery systems, such as solid lipid nanoparticles (SLN), nanostructured lipid carriers (NLC)

and lipid drug conjugates, were introduced. When compared to other lipid based carriers, such as liposomes and emulsions, SLN shows clear advantages in the encapsulated drug protection against chemical degradation and it also provides a better modulation of drug release (Attama, Builders e Momoh, 2012). These nanocarriers, besides the physical stability, easy preparation, low toxicity, small size, large surface area, high drug loading and interface interaction, have also shown a great potential as drug delivery system for brain targeting with prolonged drug release profiles, resulting in an improvement of the therapeutic effect in the seizures treatment (Jabir *et al.*, 2015). SLN disadvantages are particle growth, random gelation tendency, polymorphic transitions, and the decrease of incorporation rate due to the crystalline structure of the solid lipid. NLC are characterized by a solid lipid core that consists in a mixture of solid and liquid lipids, with a mean particle size in the nanometer range. Besides the lipid matrix of particles presents a decrease in the melting point when compared with original SLN, this matrix is still solid at body temperature. Their nanostructure improves drug loading and stuck the drug in the lipid core during storage, which means that through these nanocarriers is possible to overcome some problems related to SLN as the low loading and the drug expulsion during storage because of lipid crystallization process. The production method and the composition of the lipid phase allows to obtain different types of NLC with different nanostructures of lipid matrix, considering the main goals of this system, as described above. Also, the mixture of lipids used in the lipid core composition should be carried out considering their ability to trigger and control the drug release (Attama, Builders e Momoh, 2012).

Oral administration is still the preferential route for drug administration. For instance, oral delivery allows to prevent problems related to intravenous administration, such as extravasation of drug or blood, catheter infections, and thrombosis. Nevertheless, oral administration is also limited by the problems related to physicochemical characteristics of the drug, like the poor solubility, low permeability, instability, and rapid metabolism, leading to the decrease of oral bioavailability. Lipid nanoparticles play here an important role, because of their ability to improve the solubility and bioavailability of poorly-water soluble drugs. Absorption in gastrointestinal tract is increased by the acceleration of the dissolution process, facilitating the solubilized phases formation through the particle size reduction to molecular level, generating a solid-state solution within the carriers, modifying drug uptake, efflux and disposition by alteration of enterocyte-based transport and enhance

the drug transport to the systemic circulation through lymphatic system (Figure 1). Lymphatic drug transport shows some advantages, such as the avoidance of first-pass metabolism. Lipids can affect the drug absorption, bioavailability and disposition when orally administrated by mechanisms that include the facilitation of transcellular absorption, due to membrane fluidity increasing, opening tight junctions in order to provide paracellular transport, inhibiting P-gp and/or CYP450 by increasing intracellular concentration of suitable surfactants and lipid stimulation of lipoprotein/chylomicron production (Kalepu, Manthina e Padavala, 2013).

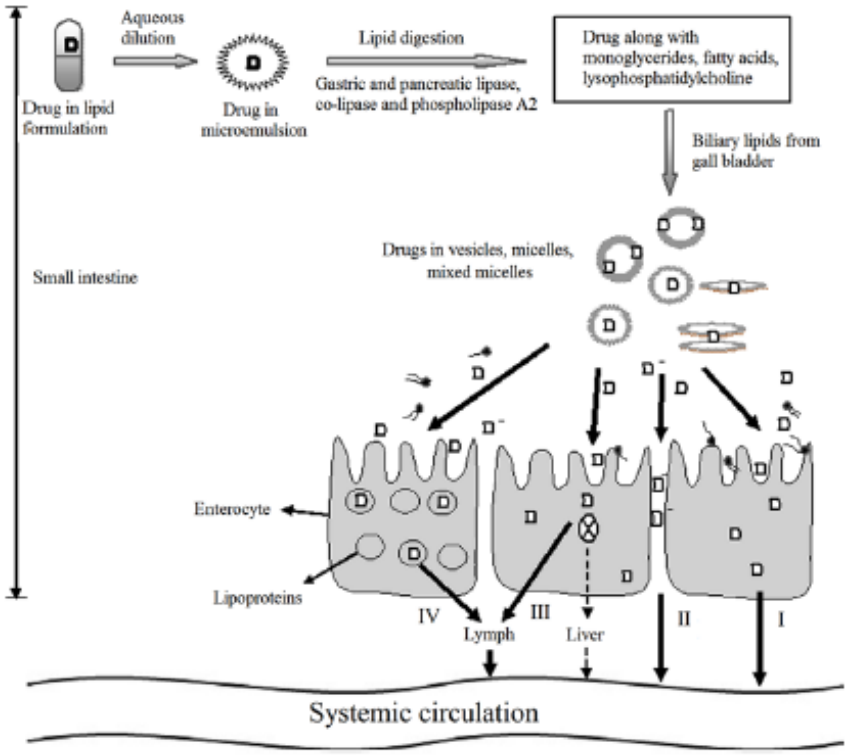

Figure 1: Mechanisms of intestinal drug transport related to lipid nanoparticles. (Kalepu, Manthina e Padavala, 2013)

1.6 Polymer-Lipid Hybrid Nanoparticles

Polymer-lipid hybrid nanoparticles (PLN) are a new variation of nanoparticle drug delivery systems, which take the advantage of polymers and lipids by the inclusion of both in their composition. PLN formulations allows to increase drug loading efficiency, control the drug release, improve drug uptake and intracellular drug transport and circumvent membrane efflux transporter mediated multidrug resistance (MDR). These nanocarriers exhibit some unique advantages when compared with other ones, which

include the wide selection of biocompatible polymers and lipids, as well as a high combination of polymer-lipid, the easy fabrication using a single-step method and the capability to co-encapsulate therapeutic and imaging agents with different properties.

The surface modification of lipid nanoparticles with mucoadhesive natural polymers is being introduced, due to the capacity to improve absorption and oral bioavailability, by improving controlled drug release behaviour. Biocompatible and mucoadhesive polymers have been successfully applied in lipid nanoparticles modification, where chitosan is included (Figure 2). (Amiji, 2010; Ramalingam, Yoo e Ko, 2016; Wu, 2016).

Chitosan is one of the biopolymers most used with lipid nanoparticles as polymer coating, because of its interesting properties. Produced from chitin, it has a rigid crystalline structure. It is positively charged and forms inter and intra molecular hydrogen bonds in acid medium (Hejazi e Amiji, 2003). This makes it chemically interesting, and it is considered as the most versatile polymer for formulation development in the pharmaceutical area. It is also a biopolymer, non-toxic, biocompatible and biodegradable, low immunogenic, and shows mucoadhesive properties. These characteristics make this polymer a good candidate to formulate conventional and new gastrointestinal drug and gene delivery systems and controlled drug release systems (Hejazi e Amiji, 2003). Its pharmaceutical applications have been increasing, and some characteristics, such as the capacity of enhancer the absorption, bioadhesive properties and capability of control the release of drugs, turned chitosan into a biopolymer of choice in formulation development. Chitosan could be also used as a coating agent, wetting agent, bioactive agent, permeation enhancer, among others (Thakur e Thakur, 2015).

Besides, chitosan demonstrated advantages when included in SLN formulation, due to the stability that it can provide to these nanoparticles, especially in acidic environment, like gastric pH. Under these conditions, SLN suffer a large aggregation and this limits their application as oral delivery systems. Chitosan coating SLN can increase the stability under acid conditions by forming a layer around the lipid core. Its inclusion in SLN also allows the improvement of control drug release properties (Luo *et al.*, 2015).

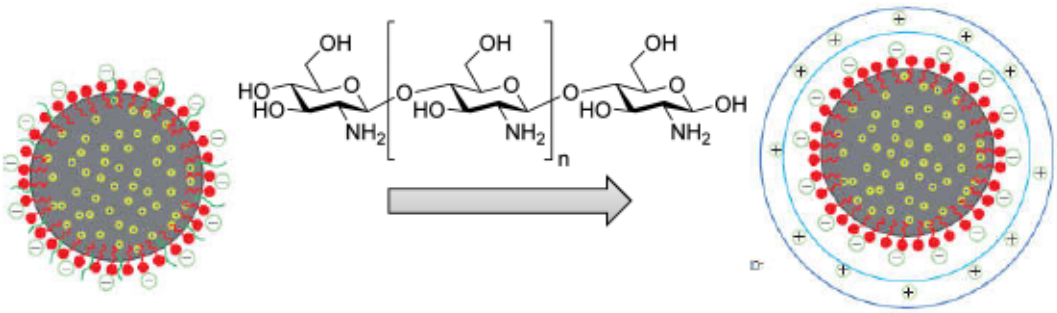

Figure 2: Chitosan coating of lipid nanoparticles. Adapted from (Ramalingam, Yoo e Ko, 2016).

1.7 Lipid Nanoparticles production methods

The main production methods of lipid nanoparticles are high pressure homogenization, microemulsion technique, solvent emulsification-evaporation and, more recently, solvent displacement and emulsification-diffusion technique have also been applied (Souto e Müller, 2007).

1.7.1 High Pressure Homogenization

The two techniques for LN production by high pressure homogenization (HPH) are the hot homogenization and the cold homogenization. In both techniques the drug is dissolved or dispersed in the lipid melted, at a range of 5 - 10°C above its melting point. According to the hot homogenization, the drug-containing lipid melt is dispersed by high-speed stirring or by ultrasound in a hot aqueous surfactant solution at similar temperature. It is produced a pre-emulsion that is then passed into a high pressure homogenizer and the obtained nanoemulsion is cooled down to the room temperature. The lipid phase solidifies and forms an aqueous suspension of lipid nanoparticles. This technique is suitable for drugs that show none or only some temperature sensitivity, because the exposure time to increased temperatures is short. For high temperature sensitive compounds, the cold homogenization should be applied, as well as for hydrophilic drugs, because during the hot homogenization process the drug would partition to the water phase, resulting in a low encapsulation rate. In cold homogenization technique, the drug-containing lipid melt is cooled, the solid lipid precipitates into lipid microparticles and these are dispersed in a cold

surfactant solution by stirring, turning into a macro-suspension. This pre-suspension is then passed through a high pressure homogenizer at the room temperature or lower, and cavitation and shear forces break the microparticles and turn it into solid lipid nanoparticles. This technique avoids or minimizes the melting of the lipid and the loss of hydrophilic drugs to the water phase. For hydrophilic compounds, water can be substituted by liquids that promote low solubility of the drug, like oils and polyethylene glycols, in order to minimize the loss of the hydrophilic compound (Souto e Müller, 2007).

1.7.2 Microemulsion technique

Microemulsions have some particular characteristics. They are clear or lightly bluish solutions composed by a lipophilic phase, a surfactant and, in some cases, a co-surfactant and water. Accordingly, the process for obtaining LN prepared through this technique starts with melting of the lipid material and preparation of an o/w surfactant or co-surfactant containing aqueous phase at the same temperature (60-70°C), and both lipid and aqueous phases are mixed. The hot microemulsion is then diluted into excess of cold water and, consequently, it is converted into an ultrafine nanoemulsion, which recrystallizes the internal lipid phase, forming the particles (Souto e Müller, 2007).

1.7.3 Solvent emulsification-evaporation

Solvent emulsification-evaporation is a production method for preparing LN dispersions by solvent evaporation from o/w emulsions. The lipid is dissolved in an organic solvent water-immiscible, where the drug is dispersed or dissolved. It is produced an organic phase that is emulsified in an o/w surfactant containing aqueous phase by mechanical stirring. The organic solvent is evaporated and nanoparticle dispersion is formed by precipitation of the lipid in the aqueous phase. The step of solvent evaporation must be quick, in order to avoid particle aggregation. Solvent emulsification-evaporation technique has an advantage compared to cold HPH that is the avoidance of thermal stress. In contrast, its biggest disadvantage is the use of organic solvents (Souto e Müller, 2007).

1.7.4 Solvent displacement

According to the solvent displacement method, the lipid material is dissolved into a semi-polar water-miscible solvent. Therefore, the drug is dissolved or dispersed in this phase and at the same time, an o/w surfactant containing aqueous phase is prepared. The organic phase is injected into the aqueous phase under magnetic stirring. The miscibility of both phases causes a huge spreading and droplets of solvent at nanometer scale are formed from o/w interface. They are quickly stabilized by surfactant molecules of the aqueous phase until complete diffusion of the solvent and lipid precipitates. The lipid nanoparticles are formed after total evaporation of the organic solvent (Souto e Müller, 2007).

1.7.5 Emulsification-diffusion

This process starts with the use of a partially water-soluble solvent previously saturated with water to ensure the initial thermodynamic equilibrium between water and solvent. The lipid is dissolved in the saturated solvent and it is produced an organic phase where drug is added. It is emulsified under agitation in an aqueous solution containing a stabilizer agent and an o/w emulsion is obtained. The addition of water to the system under mechanical stirring causes solvent diffusion into the external phase and lipid precipitates. The solvent can be eliminated by ultrafiltration, or by distillation, depending on its boiling point. When the organic solvent is totally eliminated, an aqueous dispersion of lipid nanoparticles it is formed (Souto e Müller, 2007).

1.7.6 Secondary production procedures

1.7.6.1 Sterilization

Preparations for specific uses, such as parenteral administration requires sterile formulations. It is achieved through aseptic production or processes, like filtration, ɣ irradiation and heating that should not change the properties of the formulation respecting to physical and chemical stability, and drug release kinetics. The technique to use should be chosen considering the type and characteristics of the formulations. Filtration sterilization shows some limitations when applied to dispersed systems, since it requires high pressure and is not applicable for particles bigger than 0.2 μm. The major disadvantage of sterilization by radiation is the formation of free radicals that occurs in all samples, because of the high energy of the radiation applied, and lead to

chemical modifications. Some factors, such as the chemical reactivity of the compounds, high molecular mobility – semisolid or liquid state – and the presence of oxygen contributes for a higher degree of degradation. In what regards to the sterilization by heat, this is a reliable procedure, also applied for liposomes. However, the alterations in physical stability induced by temperature is a concern point (Mäder e Mehnert, 2001).

1.7.6.2 Lyophilization

Chemical and physical LN stability for extended periods of time can be increased by lyophilization process, with the respective transformation into a solid form. In this way, it is possible to avoid hydrolysis reactions and avoid crystal grow. It is expected that the lyophilizate in the solid state presents better chemical and physical stability than the aqueous lipid dispersion. However, because of the necessary additional transformations between the formulations, some additional stability problems could be found. The transformation of aqueous dispersion to powder corresponds to the first step, and involves the freezing of the sample and water evaporation under vacuum. During this process, problems due to the freezing out effect can result in osmolarity changes, as well as pH modifications. The second step is characterized by a resolubilization process, which involves in its initial stage, conditions that promote the particle aggregation – low water and high particle content and osmotic pressure. Lyophilization also compromises the protective effect of the surfactant, and have some limitations in its application, being reported that the lipid content of LN dispersion should be lower than 5%, in order to prevent the increase of particle size. To decrease the particle aggregation is necessary the use of cryoprotectors – sorbitol, mannose, trehalose, glucose and polyvinylpyrrolidone – that decrease the osmotic activity of water and promote the glassy state of the frozen sample. Cooling process is another critic process that affects the crystal structure and the properties of the lyophilizate. It is an important factor for sublimation and reconstitution processes.

As such, due to the transformation of liquid forms in solid forms, lyophilization also allows the incorporation of LN into pellets, tablets or capsules (Mäder e Mehnert, 2001).

1.7.6.3 Spray-drying

Spray-drying is a better and cheaper alternative procedure than lyophilization to transform aqueous LN dispersions into a dry product. It is widely used in pharmaceutical technology with the purpose of drying heat-sensitive materials, improving flow properties, by preparing free-flowing granules for production of tablets, increasing the solubility of substances with low water-solubility and produce fine and dust-free powders, as well as agglomerated ones to particular specifications (Mäder e Mehnert, 2001; Ré, 2006). Due to high temperatures needed, shear forces and partial melting of particles, particle aggregation can be observed. To prevent it, it is recommended the use of lipids with melting point higher than 70°C and its application in formulations with low lipid content. The lipid melting can also be minimized with the use of a ethanol-water mixture (Mäder e Mehnert, 2001).

The conversion of a liquid dispersion into a dry product is very useful for the delivery of nanoparticles through some routes, mainly oral administration. Applying this technology, it is possible to obtain LN granulates or powders, which can be used to compress into tablets or incorporate into pellets or capsules. The application of this technique increases the storage stability, due to the reduction of water content, which can be particularly interesting for encapsulation of drugs with susceptibility to suffer hydrolysis, or that are exposed to high temperatures and light. Generally, the process consists in four steps: firstly, the atomization of the feed into a spray, then there is spray-air contact, the spray is dried and occur separation of the dried product from the drying gas. The temperature during the process is usually not very high, depending on the solvent, and the time of residence of the particles is short, which allows the maintenance of the nanoparticle properties after spraying. Because of this, spray-drying come across as an advantageous system for production of the micrometer-sized with low melting point materials, such as lipid material (Freitas e Muller, 1998).

1.8 Lipid Nanoparticles characterization

The variations of formulation composition and the preparation procedure may have effects on the physicochemical properties of LN, such as the particle size, shape and morphology, composition, stability of colloidal structures, and crystalline state of the nanoparticles. Their performance also depends on these characteristics.

1.8.1 Optimization

1.8.1.1 Particle size and size distribution

The most used techniques to measure particle size and its distribution in aqueous suspensions are Laser Diffraction (LD) and Dynamic Light Scattering (DLS). Regarding to LD technique, it is based on the transmission of a laser beam through the sample with dispersed particles. The larger particles scatter the light at small angles and the smaller ones scatter the light at large angles. Sphere diameter of particles is then calculated by application of the Mie scattering solution to the collected data. This method allows to measure particles ranging from dozens of nanometers to thousands of micrometers, depending on the laser source. However, a lack of accuracy of the method is reported for particles in colloidal suspensions that presents a diameter much smaller than the wavelength of the laser. DLS, the technique also known as Photon Correlation Spectroscopy (PCS), is based on time-dependent fluctuations in the scattering intensity produced by small particles in suspension when the laser beam is applied through the sample. The fluctuation, a result of the Brownian motion of the dispersed particles, reflects in a higher velocity of Brownian motion to smaller particles, and lower for the larger ones. By the application of Stokes-Einstein equation to the collected data, it is possible to calculate the particle diameter, considering a sphere shape for particles. This technique is very sensitive, allowing to detect particles lower than 1 nm. Its limitations are related to the micron range, since particles with sizes above 10 μm are not suitable for this analysis.

Concerning the size distribution, given by the PDI – polydispersity Index - parameter, it can be calculated using the DLS method described above and its values ranges from zero to one. Samples monodispersed have values of zero and very polydisperse ones shows values close to one. The same size distribution parameter calculated using LD method is given by Span Value.

Considering the both approaches to particle size measurement, it is suggested that LD and DLS should be used complementary to each other. It should be noted that both techniques allow to measure the particle size in an indirectly way, assuming that particles have a spherical shape. So, it is important to confirm the obtained information with another suitable method, such as microscopic techniques in order to complete the characterization of LN dispersions (Svilenov e Tzachev, 2009).

1.8.1.2 Entrapment efficiency and drug loading

Encapsulation efficiency evidences the success of the drug encapsulation. Factors related to the preparation technique and starting material for preparing nanoparticles can affect it (Douroumis e Fahr, 2013). The amount of drug incorporated in the particles is consequence of its dissolution in the lipid phase. Entrapment efficiency (E.E.), which concerns to the amount of drug incorporated in the particles divided by the total amount present in the formulations, is expressed in % (Svilenov e Tzachev, 2009). It can be indirectly calculated by the following equation (Doktorovova e Souto, 2009):

$$E.E.(\%) = \frac{Total\ drug\ content - free\ drug\ content}{Total\ drug\ content} \times 100$$

This parameter is also influenced by the characteristics of the lipid and the drug, since lipophilic drugs tend to distribute in the lipid phase, leading to a higher E.E., while hydrophilic drugs tend to disperse in the aqueous media, thus decreasing E.E. (Svilenov e Tzachev, 2009).

Drug loading (D.L.) is a parameter that expresses the amount of drug present in the particles divided by the total lipid amount of the system. It is also expressed in percentage (%) and is calculated by the equation(Svilenov e Tzachev, 2009):

$$D.L.(\%) = \frac{Total\ drug\ content - free\ drug\ content}{Total\ lipid\ content} \times 100$$

Drug loading values are low for solid lipid nanoparticles, because of the crystalline nature of the lipid core, what limits the space to drug incorporation. Besides the structure of the lipid matrix and the state of polymorphism of the lipid material, other factors that influence the drug loading are the solubility of the drug in the melted lipid, the miscibility of drug and lipid melting (Douroumis e Fahr, 2013).

The main challenges related to the determination of both parameters are about the drug analysis. Some suitable separation and analytical methods should be combined, in order to provide an effective analysis. The most used techniques are dialysis, ultracentrifugation, gel filtration or membrane filtrations. Drug concentration is measured in the separated aqueous media or in the particles, by spectroscopy or chromatography methods (Svilenov e Tzachev, 2009).

1.8.1.3 Crystallinity

Lipid crystallization is one of the most important points for the evaluation of the particle performance, once there is a relationship between lipid modification and degree of lipid crystallinity, and drug incorporation capability. The methods used for the characterization of lipid status are mainly based on x-ray diffraction (XRD) and differential scanning calorimetry (DSC).

1.8.1.3.1 Differential scanning calorimetry

Differential scanning calorimetry is a technique widely used to study the lipid modifications. It measures differences in the amount of heat required to increase the temperature of the sample, comparing with the reference. The differences detected in heat flow, that can be positive or negative, are presented as function of temperature. The differences between the sample and the reference are detected mainly at phase transition. Analysing the different melting points and melting enthalpies of different lipid modifications, it is possible to obtain information about the sample structure and interactions between the compounds. The confirmation of the results with another technique is recommended, especially in case of drugs with high melting point that dissolve in the melting lipid blend but the crystallization can occur in the solid lipids (Svilenov e Tzachev, 2009).

1.8.1.3.2 X-Ray Diffraction

X-ray diffraction (XRD) is a technique widely used to the analysis of crystallinity structure. This technique is based on the fundamental equation Braggs law which associates the lattice spacing with the angles observed of constructive interference when x-rays scatter from the crystalline material. The measurements for nanomaterials are performed on powders containing a large number of randomly oriented particles, leading to the necessity of income beam by diffraction at various angles. Through the analysis of the position and the intensity of the peaks in power diffraction patterns it is possible to identify and quantify the phase composition of the sample (Linsinger, Roebben e Gilliland, 2012).

1.8.1.4 Structure characterization

1.8.1.4.1 FTIR

Infrared and Raman spectroscopy are useful techniques to investigate structural lipid properties. Comparing the infrared spectra of the sample with the reference, it is possible to conclude the lipid alterations from the original lipid employed. The lipid alteration during the polymorphic transition lead to modifications in the core structure of the lipid assemblies and it can negatively influence the drug-loading capacity and the drug release. It has been observed drug expulsion from different particles compositions when the transition for more stable forms occurs (Douroumis e Fahr, 2013).

1.8.1.4.2 Morphology

Particle morphology can be analysed by microscopical techniques. The most used techniques for LN analysis are Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM). For the visualization of nanoparticles are applied electrons instead of light. The main difference between both techniques is the detention mode. Once that SEM detects the scattered electrons from the particle surface, TEM detects the transmitted electrons that cross the sample. The disadvantages related with processing conditions and sample preparation are common to the both techniques. The sample preparation, that can involve processes as dehydration, staining, conductive coating, and others, and the analysis procedure can cause alterations in the nanoparticles (Svilenov e Tzachev, 2009).

1.8.2 Stability

1.8.2.1 Zeta Potential

Zeta potential is a parameter that measures the magnitude of electrostatic repulsion or attraction between particles in the LN aqueous suspension, and it is useful to predict long term stability of the formulations. High zeta potential values, means that electric repulsion of the particles is enough to stabilize the colloidal system, usually resulting in smaller contact between the particles and, consequently, less aggregation. Particle charge is also important, because it can predict the interaction with tissues and cells. The zeta potential values generally acceptable to consider a dispersion stable are

over |30| mV. However, when steric stabilizers are present in the system this rule is not strictly applied, since adsorption of the steric stabilizers leads to the decreasing of zeta potential. The use of ionic surfactants and the inducing of electrostatic stabilization can turn the values as negative or positive.

The principal method to determine zeta potential is Electrophoretic light scattering, for the measurement of particle velocity in electric field (Müller, Mäder e Gohla, 2000; Svilenov e Tzachev, 2009).

1.8.2.2 Analytical Centrifugation

Stability is a required parameter to characterize any type of products, including nanoparticles dispersions. Alterations in the particle characteristics are often classified and quantified directly by observation or by special fixed or moving sensor. Indirect methods, such as particle size or electrophoretic mobility, are also applied to determine physicochemical parameters. However, the obtained results do not allow the direct calculation of stability parameters, such as sedimentation or flotation velocity. As such, a quick and reliable classification of the stability and shelf live prediction, especially for very stable formulations, that only shows alterations under gravity over a long period of time is useful, allowing to reduce the development cycles and improving the possibilities of quality control. To accelerate the stability measurements, methods are introduced to stress and change the state of the dispersion. They consist of mechanical, thermal, or physicochemical techniques. Phenomena including changes in concentrations or phase separation can be accelerated using physical forces as centrifugal, electric or magnetic. Analytical centrifugation by test the accelerated quality is a promising procedure already used by many research groups. The changes of particle concentration in the sample volume are recorded as a function of centrifugation time and provide information about particle migration. It results in a variation of the local particle concentration, leading to local and temporal variations of transmission. Through the transmission profiles, it is possible to characterize dispersed particles and to quantify the degree of polydispersity (Lerche, 2002; Lerche e Sobisch, 2011).

1.8.3 Performance evaluation

1.8.3.1 Dissolution studies – Dialysis

Several *in vitro* methods have been developed in order to simulate physiological gastric conditions, such as temperature, pH, agitation, enzymes, and chemical composition, and the processes occurring during the digestion in gastrointestinal tract. The simplest techniques applied are static methods that include two or three digestion steps – oral, gastric and intestinal steps. These methods have some limitations, due to the simulation of few parameters of physiological digestion, and the difficulty in mimicking physical processes, such as shearing, mixing, hydration, peristalsis and changes over time. The methods to evaluate the *in vitro* drug release of nanoparticles include side-by-side diffusion cells with artificial or biological membranes, the dialysis-bag diffusion technique, the reverse dialysis sac technique, ultrafiltration, ultracentrifugation, and the centrifugal ultrafiltration techniques.(Douroumis e Fahr, 2013; Verhoeckx *et al.*, 2015) The conventional dialysis bags technique, used to determine drug release from colloidal carriers, is a reliable method when using permeable dialysis membranes and magnetic stirring to provide the separation of free drug and sink conditions during the release. This method is one of preferential for the study of drug release from lipid nanoparticles. However, the type of *in vitro* dissolution technique should be carefully evaluated, since it can influence the drug release kinetics, wherefore components of dissolution media and conditions should be optimal for specific formulation. It is important to refer that bag dialysis have also some limitations, including the retarding of the diffusion, because of the bag and the interaction with drug molecules. Dissolution studies with LN may not predict correctly the *in vivo* release behaviour, due to the enzyme degradation and interaction with cells and lipid membranes (Saarinen-Savolainen *et al.*, 1997; Svilenov e Tzachev, 2009).

1.8.3.2 Permeability

The bioavailability and solubility of a drug molecule determine its effectiveness in the therapeutic. For any drug be absorbed, it is required that it has to be solubilized at the absorption site, and the solubility of the active compounds is determining for drug absorption in a function of permeability and solubility. The intestinal permeability and oral bioavailability can be evaluated by the permeability through a cell monolayer. To

predict passive and transcellular intestinal absorption, the Parallel Artificial Membrane Permeability Assay (PAMPA) can be applied, providing a high output. In this method procedure, it is immobilized an artificial membrane on a filter, that is placed between a donor and an acceptor compartment. In the donor compartment is introduced the drug to study, and during the permeation period, samples of the donor and acceptor medium are collected and quantified. Since gastrointestinal tract has a pH range between 1 and 8, and pH of plasma is 7.4, which should be mimicked in the *in vitro* assays, is possible that this pH gradient affects the transport of the drug. In this way, in the acceptor compartment should be used a pH 7.4 and in the donor compartment a suitable pH, with values of 5, 6.2 and 7.4. PAMPA is an assay well-established and predictive to study the drug absorption, but, once it is an artificial system, it can provide imprecise and ambiguous results. The industry standard for *in vitro* prediction of intestinal drug absorption is the colon carcinoma (Caco-2) cell permeability assay. However, it presents several limitations related to the long-time of the assay and the compound consumption. BBB PAMPA is another variant of this assay which uses brain specific membrane components in artificial monolayers (Douroumis e Fahr, 2013; Sittampalam *et al.*, 2016).

Single cell-culture models are very limited comparing with intestinal epithelium, that consists in absorptive enterocytes and cell as goblet cells, endocrine cells and M cells, Peyer's patches, dendritic cells and macrophages. Ussing chamber is an *ex vivo* technique that uses intestinal tissue segments, allowing the preservation of the morphological and physiological features of the intestine, providing a better representation of the *in vivo* morphology, including multicellular conglomeration and the presence of the mucus layer, and the processes involved. *Ex vivo* techniques also allow the study of regional absorption and immune responses employing intestinal tissues segments from different regions. It has as main limitation the low viability of the used tissues. The Ussing system usually comprises a chamber and a perfusion system, where the epithelia membrane or cell monolayer is supported, and one of its side is isolated, separating the chamber in half. The chamber is filled with a suitable physiological solution.

As mentioned above, the monitoring of cell viability is indispensable in order to ensure the feasibility of the assay. This monitoring is performed by the potential difference, short-circuit current or transepithelial electrical resistance (TEER) in a continuous way, which low resistance indicates tissue damage. The measurements

should be performed for each experiment. However, with a typical transport study, other markers can be employed, including the presence of metabolites, or the paracellular transport, studied by the measurement of the permeability with fluorescein isothiocyanate dextran in the basolateral side (Verhoeckx *et al.*, 2015).

1.8.3.3 Cytotoxicity

Cytotoxicity test is essential in the development and certification not only for medical devices and compounds, but also for all consumer products. Cytotoxicity effects can be the cause of cells death in culture, as well as necrosis and apoptosis. This process is defined as the specifically inducing death of cells, resulting in metabolic failure or cell lysis, which affects the cell viability. It is calculated by the ratio between the total cell number and viable cell number multiplied by 100. Cell death can be monitored by some methods, that measure loss of membrane integrity, loss of metabolic activity, loss of monolayer adherence and cell cycle analysis. The obtained results can be used for the identification of susceptible cell lines to nanoparticle toxicity.

- Cellular damage measured by membrane integrity shows to be important by the evidence of toxicity associated to some cationic nanoparticles that exhibit this effect by disrupting of cell membrane. Some assays that measure membrane integrity are, for example, trypan blue exclusion assay and lactate dehydrogenase leakage assay, that measures the presence of this compound in the release media after cell lysis.
- It is possible that toxic substances interfere or block metabolic pathways or transporters presents in organelles, cytoplasm, and membranes. By chromogenic redox substrates mitochondrial redox cascades are monitored. Some of these assays include the tetrazolium based MTT (dimethyl thiazolyl diphenyl tetrazolium) or XTT (2,3 - bis - (2 - methoxy – 4 - nitro - 5 - sulfophenyl) - 2H - tetrazolium - 5 - carboxanilide) or other derivatives, which quantify the redox potential in cells. MTT is one of the most reduction assays used for the measurement of metabolic activity, being radioactivity free, sensitive and reliable. MTT, a yellow water-soluble tetrazolium, is metabolized by cells in to purple formazan crystals, water insoluble. The latter is dissolved in an appropriated medium, and measured by absorbance. The relative

cytotoxicity estimated is obtained by comparison of the spectra of nanoparticles samples treated with the nanoparticles samples untreated.

- Staining of total protein followed by fixation of adherent cells is a technique to measure monolayer adherence. It is used as a cytotoxicity marker, and, beyond the simplicity of the assay, it is also very sensitive to indicate the loss of cell viability.
- To cell cycle analysis it is used propidium iodide staining of DNA and flow cytometry. The latter can be applied as a screening test for chemical toxicity. The method allows to study the interference of nanoparticle effect on cell cycle progression and it death.

(Amiji, 2010; Oliver e Warzecha, 2012)

1.8.3.4 *In Vivo* Studies

By definition, biological assay is a set of methods that produces a detectable signal to quantify a biological process. The robustness and reproducibility of the signal defines the quality of the assay, when test compound or inactive compounds are not present. Data from *in vivo* studies constitute the reference methods, while bioavailability studies that are based on *in vitro* methods are the background to predictive purposes. *In vivo* methods face many disadvantages that limit their use, such as the limitations in experimental design, difficulties of data interpretation, expensive equipment and labour, ethical issues, inter-individual variations, and lack of certified references to data comparing. Regarding the *in vivo* assay, an experimental design should be applied because of the importance to guide the assay considering the interest of the research, which includes certain aspects, such as the measurements to make, time points, dosing frequency and route, species to use, and the identification of statistical analysis, sample sizes, and randomization scheme (Sittampalam *et al.*, 2016; Verhoeckx *et al.*, 2015). *In vivo* assays in Europe are regulated by Directive 2010/63/EU that regulates the animal use, conferring protection to them for scientific purpose. About animal care for the assay, it is important to select a method to provide the most satisfactory results, but it should also cause the minimum of pain, suffering and distress. The species and the number of animals should be also considered, in order to use the minimum of animals to obtain reliable results and species with lowest capacity to experience pain and suffer. The selected method should avoid death as end-point as far as possible, because of the severe suffer of the process.

The main purposes for carrying out the procedures are basic research, translational or applied research, research in preservation of the species, higher education or skills acquisition and forensic inquiries. (*Directive 2010/63/EU on protection of animals used for scientific purposes, caring for animals aiming for better science*, 2010).

1.9 Aims of the dissertation

Carbamazepine is a well-known and widely used drug for epilepsy treatment. However, its limitations are clear, due to the physicochemical characteristics of this drug, mainly the lipophilic nature, what turns the drug delivery a challenge, especially when oral administration is considered. Issues associated to commercial formulations that lead to several problems in the patients therapeutic have been reported, such as the drug resistance mentioned above, that compromise the therapeutic effectiveness. It is seen that the slow and erratic absorption of carbamazepine can be minimized with some technological improvements, in particular the use of modified release strategies of drug. Although some modified drug release forms are available, it is possible to improve them, in terms of heterogeneity and steady state time. Nanotechnology based strategies show promise to solve the technological issues of commercial carbamazepine formulations.

Polymer lipid hybrid nanoparticles, in particular chitosan coated LN, gathering the unique characteristics of lipid nanoparticles with the several advantages of chitosan as polymer, are the technological solution proposed to overcome the mentioned problems in therapeutic.

In this way, the objectives of the present dissertation are:

- To develop and optimize a chitosan coated lipid nanoparticle formulation by screening different solid and liquid lipids, and surfactants;
- To assess physicochemical characteristics of the developed formulations, including size, morphology and crystalline state, stability and loading properties;
- To evaluate the performance of the developed formulations based on *in vitro* release, permeability, and cytotoxicity studies;
- To evaluate *in vivo* CBZ pharmacokinetics after oral administration to mice of the optimized formulation;
- To develop of a solid dosage form based on hybrid lipid nanoparticles formulation;
- To assess the *in vitro* CBZ release from the nanoparticle based tablet dosage form.

2. Pharmaceutical development of polymer-lipid hybrid nanoparticles for oral delivery of antiepileptics

Drug delivery is an issue of critical importance in the quest for more effective formulations. Oral drug delivery based on nanotechnology is being a challenge, because of the unique characteristics of gastrointestinal system that highly interfere in drug performance. Chitosan has been proposed as a novel excipient for transepithelial drug-delivery systems, providing an improvement in drug delivery.

The present work describes the studies of pre-formulation, *in vitro* release, permeability and *in vivo* pharmacokinetic analysis that were performed, in order to obtain an optimal formulation. The techniques and methods applied are described below.

2.1 Materials

Poly vinyl alcohol (PVA typical MW 13.000-23.000), Glyceryl tripalmitate (tripalmitin, T8127, melting point 66°C), polysorbate 80 (Tween® 80), Carbamazepine, Fluorescein isothiocyanate (FITC, 3',6'-dihydroxy-6-isothiocyanatospiro [2 benzofuran-3,9'-xanthene]-1-one), Vitamin E-Tocopheryl polyethylene glycol 1000 succinate (Vit. E-TPGS) and polyvynilpyrrolidone K30 (PVP k30) were provided by Sigma. Oleic acid, lactose and microcrystalline cellulose were acquired from Fluka. Labrasol® (Caprylocaproyl Polyoxyl-8 glycerides), Compritol® 888 ATO (glyceryl dibehenate), Capryol®90 (propylene glycol monocaprylate) and Transcutol® HP (Diethylene Glycol Monoethyl Ether) was a kind offer from Gattefossé (Gennevilliers, France).Water was purified (Millipore®) and filtered through a 0.22 μ m nylon filter before use. Chitosan was a gift from Lusifar Químico Comercial (Lisboa, Portugal). Acetic acid was provided by Carlo Erba (France). All other reagents and solvents were from analytical or high performance liquid chromatography (HPLC) grade.

2.2 Methods

2.2.1 Lipid nanoparticle dispersions Preparation

The lipid nanoparticles were produced by the hot high pressure homogenization technique previously optimized and described (Vitorino *et al.*, 2013). It was carried out at a temperature 10°C higher than the melting point of the solid lipid. After melting of the lipid phase (composed by different liquid and/or solid lipids – **Table 1 and 2**), it was emulsified in an aqueous solution of Tween® 80 (3% w/V), at the same temperature, during 1 min with an Ultra-Turrax X1020 (Ystral GmbH, Dottingen, Germany) at 25,000 rpm. It was obtained a pre-emulsion that was transferred to a pre-heated Emulsiflex® -C3 (Avestin Inc, Ottawa, Canada) and processed at 1000 bar for 2.5 min. The formulation obtained was stored at 4°C during 24h, to promote the matrix recrystallization and the establishment of nanoparticles, and then it was kept at room temperature. For chitosan coated LN, termed hybrid NLC, the external phase was composed by an aqueous solution of 1% V/V of acetic acid with Tween®80 and chitosan (see Table 2). In the case of loaded formulations, the amount of CBZ was added to the lipid phase.

Table 1:Different compounds considered for formulation development.

Solid lipids	**Liquid lipids**	**Surfactants**
Tripalmitin	Oleic Acid	Tween® 80
Compritol® 888 ATO	Capryol®	Span® 85
	Transcutol®	PVA
		Labrasol®
		Vit. E – TPGS

Table 2: Composition of lipid nanoparticle (SLN and NLC) formulations.

F	Lipid(s)		Surfactant(s)		Chitosan	CBZ	Acetic acid	Water
#	Compounds	% (w/w)	Compounds	%(w/w)	% (w/w)	% (w/w)	% (w/w)	% (w/w)
Unloaded lipid nanoparticles								
1	Compritol	5	Tween 80	3	-	-	-	92
2	Compritol	5	PVA	1.5	-	-	-	93.5
3	Compritol	5	Labrasol	3	-	-	-	92
4	Compritol : Oleic Acid	2.5:2.5	Tween 80	3	-	-	-	92
5	Compritol : Oleic Acid	2.5:2.5	Span 85	3	-	-	-	92
6	Compritol : Oleic Acid	2.5:2.5	Span 85 : Tween 80	1.55:1.45	-	-	-	92
7	Compritol : Oleic Acid	2.5:2.5	Tween 80	5	-	-	-	90
8	Compritol : Oleic Acid	2.5:2.5	Span 85	5	-	-	-	90
9	Compritol : Oleic Acid	2.5:2.5	Span 85 : Tween 80	2.6:2.4	-	-	-	90
10	Tripalmitin : Capryol	2.5:2.5	Tween 80	3	-	-	-	92
11	Tripalmitin : Transcutol	2.5:2.5	Tween 80	3	-	-	-	92
12	Compritol : Capryol	2.5:2.5	Tween 80	3	-	-	-	92
13	Compritol : Transcutol	2.5:2.5	Tween 80	3	-	-	-	92
14	Tripalmitin : Capryol	2.5:2.5	Tween 80	3	0.5	-	0.3	91.2
15	Tripalmitin : Transcutol	2.5:2.5	Tween 80	3	0.5	-	0.3	91.2
16	Tripalmitin : Capryol	2.5:2.5	Tween 80	3	1	-	0.3	90.7
17	Tripalmitin : Transcutol	2.5:2.5	Tween 80	3	1	-	0.3	90.7
18	Tripalmitin : Transcutol	3.75:3.75	Tween 80	3	1	-	0.3	88.2
19	Tripalmitin : Transcutol	5:5	Tween 80	3	1	-	0.3	85.7
Lipid nanoparticles loaded with Carbamazepine								
20	Tripalmitin : Transcutol	2.5:2.5	Tween 80	3	0.5	0.5	0.3	94.2
21	Tripalmitin : Transcutol	2.5:2.5	Tween 80	3	1	0.5	0.3	90.2
22	Tripalmitin : Transcutol	2.5:2.5	Tween 80	3	-	0.5	-	91.5
23	Tripalmitin : Transcutol	3.75:3.75	Tween 80	3	1	0.67	0.3	87.53

24	Tripalmitin : Transcutol	2.5:2.5	Tween 80 : TPGS	3:1	-	0.5	-	90.5
25	Tripalmitin : Transcutol	2.5:2.5	TPGS	1	-	0.5	-	93.5
26	Tripalmitin : Transcutol	5:5	Tween 80	1	1	0.9	0.3	86.8
27	Tripalmitin : Transcutol	2.5:2.5	TPGS	3	-	0.5	-	91.5

2.2.1.1 Characterization

2.2.1.1.1 Particle size

The average particle diameter and polydispersity index (PDI) were determined by dynamic light scattering (DLS). These parameters were determined with a Zetasizer Nano ZS (Malvern, Worcestershire, UK), set at 173° detection angle and at a temperature of 25°C. After a dilution of 100 times with ultrapurified water, the samples were analysed three times. The results were presented as mean±standard deviation, extracted from the cumulants algorithm.

2.2.1.1.2 Morphology

Particles morphology and topography was analysed by scanning electron microscopy (SEM). Samples were previously spread in a double-side carbon tape mounted on an aluminium stud, and further coated with gold at a flow rate of 0.6 nm/s for 15 s in order to turn them conducting. The obtained images were recorded on a JSM 6010LV/6010LA, Jeol (Tokyo, Japan) scanning electron microscope, at acceleration voltages of 20 kV.

2.2.1.1.3 Entrapment efficiency and drug loading

The drug loading (D.L.) and entrapment efficiency (E.E.) were determined by an indirect method, through the measurement of the free drug present in the aqueous phase of the dispersion. Drug Loading, the percentage of entrapped drug divided by total matrix lipid mass, is given by

$$D.L.(\%) = (\,Wtotal\ drug - Wfree\ drug)/\,W\ lipid\ \times 100$$

On the other hand, entrapment efficiency, that corresponds to the amount of drug that is possible to incorporate into the lipid matrix, is given by

$$E.E.(\%) = (Wtotal\ drug - Wfree\ drug)/W\ total\ drug \times 100$$

wherein $W_{total\ drug}$ is the total amount of drug determined in the nanosystem, $W_{free\ drug}$ corresponds to the free drug amount determined in the aqueous phase and W_{lipid} corresponds to the lipid phase amount. Free drug amount was determined by ultrafiltration-centrifugation, using centrifugal filter units (Amicon® Ultra 4, Millipore, Germany) with a 100 kDa molecular weight cut-off. A specific volume of the dispersion was diluted with mobile phase (acetonitrile and water mixture (50:50)) and centrifuged at 3000 x g for 45 min at 4°C. After that, free drug in the aqueous phase was collected of the outer chamber of the centrifuge filter unit, filtered by a 0.22 mm membrane, and CBZ was determined by HPLC. The total drug amount was determined using a specific volume of LN dispersion adequately diluted in mobile phase and heated at 60°C for 15 min. After that, the dispersion was centrifuged for 10 min at 12,000 x g in a Minispin® (Eppendorf Ibérica S.L., Madrid, Spain), the supernatant was collected, filtered by a 0.22 mm membrane and CBZ was determined again by HPLC.

2.2.1.1.4 Crystallinity and structural details

2.2.1.1.4.1 Differential scanning calorimetry

Differential scanning calorimetry (DSC) analysis was performed using a DSC-60 differential scanning calorimeter (Shimadzu, Japan). Pure compounds and spray-dried nanoparticles (1–2 mg) were placed in aluminium pans hermetically sealed, and empty pans were used as reference. The samples were submitted to a heating cycle from 25 to 210°C, at a rate of 10°C/min, with a nitrogen purge of 30 ml/min. Through TA Software (Shimadzu, Japan), parameters, such as onset temperature (Ton), melting point (Tpeak), and enthalpy (ΔH) were determined.

2.2.1.1.4.2 X-Ray diffraction

Spray-dried formulations and pure compounds were analysed by X-ray diffraction using a MiniFlex X-ray diffractometer (Rigaku, Tokio – Japan), with CuKα radiation at 40 kV and 15 mA. The 2θ scan range was 3–50° with a step size of 0.02° and a scan speed of 1 s.

2.2.1.1.4.3 Attenuated total reflectance infrared

Attenuated total reflectance infrared (ATR-FTIR) spectra of particles were recorded using a FT-IR/FT-NIR spectrometer (Spectrum 400, Perkin-Elmer, MA, USA) with an ATR accessory fitted with a Zn-Se crystal plate. The pure compounds and spray-dried formulations were placed in the ATR device and measured using 16 scans for each spectrum, with a resolution of 2 cm^{-1} and a scan speed of 0.5 cm/s. The spectra were collected between 4000 and 650 cm^{-1}.

2.2.1.2 Drug release studies

Dialysis bag method was employed in order to evaluate the CBZ release behaviour of LN formulations (Figure 3). For that, the dialysis membranes were kept overnight in ultrapurified water at 4°C to ensure the wetting of the membrane. A volume of 2 mL of formulation was inserted into the dialysis bag, and subsequently, they were placed into 100 mL of dissolution medium at pH of 1.2 (simulated gastric fluid without enzymes – USP XXV). After 2 hours, the bags were transferred to the dissolution medium at pH of 6.8 (simulated intestinal fluid without enzymes – USP XXV). Both media were kept under magnetic stirring during all the assay period and the temperature was of 37°C. A sample volume of 900 µL of dissolution medium was collected at 0.5h, 1h, 2h, 3h, 4h, 5h, 6h, 7h and 8h, and replaced by an equal volume of fresh medium. The samples were suitably diluted (1:1) with mobile phase and analysed by HPLC. The dissolution profiles were obtained by plotting the cumulative percentage of drug released against time, estimated according to

$$Release\ (\%) = \left(\frac{drug\ amount\ released}{total\ amount\ of\ drug\ in\ formulation}\right) \times 100$$

In order to clarify the possible mechanisms involved in drug release process, the release data were fitted with different mathematical models: zero order, first order, Higuchi, Weibull and Korsemeyer-Peppas equations.

Figure 3: Release study by dialysis bag method.

2.2.1.2.1 HPLC determination of CBZ

The quantification of CBZ was performed through a HPLC method using a Shimadzu LC-2010HT apparatus equipped with a quaternary pump (LC-20AD), an auto-sampler unit (SIL-20AHT), a CTO-10AS oven and a SPD-M2OA detector. A Kinetex Phenyl-Hexyl (5 µm particle size, 3 mm internal diameter and 150 mm length) Phenomenex (USA) analytical column was used. The mobile phase consisted in a mixture of acetonitrile and water in the proportion of 50:50 (V/V) and the chromatography analysis was conducted in isocratic mode with a flow rate of 0.8 mL/min, an injection volume of 10 µL and a run time of 7 min. The detection was carried out at 220 nm. Under these conditions, CBZ was eluted at approximately 1.8 min. The obtained data was processed with a Shimadzu LC-solution version 1.12 software.

2.2.1.3 Stability studies

2.2.1.3.1 Electrophoretic light scattering

Zeta potential was determined by electrophoretic light scattering, in a Zetasizer Nano ZS (Malvern, Worcestershire, UK) apparatus at a temperature of 25°C. Before

the measurements, samples were suitably diluted (100 times) with ultrapurified water. For the ZP calculations, the Helmholtz–Smoluchowsky equation was used.

2.2.1.3.2 Analytical centrifugation

The formulation stability was also assessed by the LUMiFuge (L.U.M. GmbH, Germany) stability analyser, an analytical centrifugation system that measures the intensity of transmitted near infrared (NIR) light during the centrifugation of the sample (Vitorino *et al.*, 2011). The analysis of the formulations was performed for 3 h 30 min of centrifugation, at an acceleration of 2300 x g and 25°C.

2.2.1.4 *Ex vivo* Permeability Studies in Ussing chambers

2.2.1.4.1 Animals and tissue preparation

The animal experimentation was carried out in accordance with European Directive (2010/63/EU) for the accommodation and care of laboratory animals, and the experimental procedures were approved by the Portuguese Veterinary General Division. For the experimentation, healthy adult male CD-1 mice, obtained from Charles River Laboratories (L'Arbresle, France), weighing 25–35 g were used. Mice were sacrificed by cervical dislocation and the entire small intestine was rapidly removed and inserted in cold KBR solution of pH 7.4, for approximately 10 min, in order to minimize the tissue damage during the preparation (Figure 4). Afterwards, jejunum segments with approximately 2 cm and free of visible Peyer's patches were cut along the mesenteric border and carefully placed in vertical Ussing chambers (Harvard Apparatus Inc., Holliston, MA, U.S.A.) (Figure 5) with 0.28 cm^2 of exposed area. Each side of the tissue was bathed with 1 ml of KBR solution with a pH of 7.4 and 6.8 for the acceptor and donor compartments, respectively. Thereafter, chambers were screwed tight and kept at 37°C. In order to evaluate the tissue viability, tissue transepithelial electrical resistance (TEER) was measured with an EVOMX® meter (World Precision Instruments Inc., WPI, Hertfordshire, United Kingdom), and jejunum segments that showed a value of TEER lower than 40 $\Omega.cm^2$ were excluded, since were considered as poorly viable (Stephens *et al.*, 2002).

Figure 4: Mice's small intestine for preparation of *ex vivo* permeability studies.

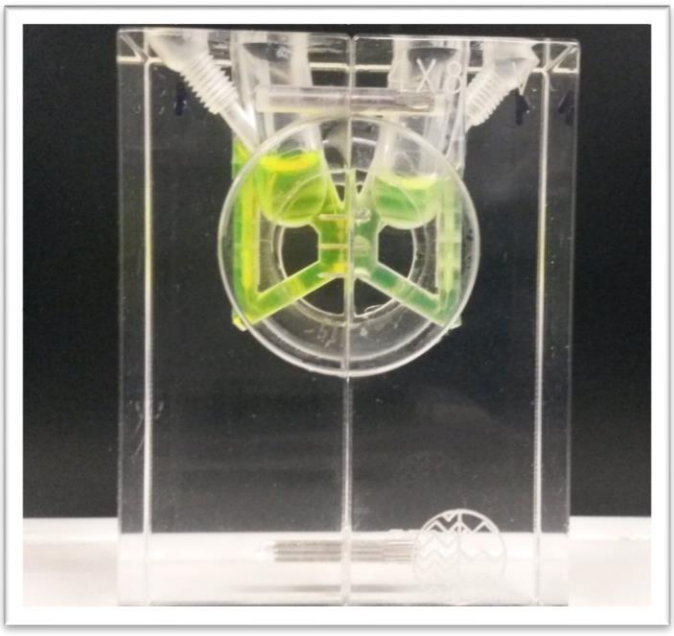

Figure 5: Vertical Ussing chamber used for *ex vivo* permeability studies.

2.2.1.4.2 Apparent permeability absorptive permeation assay

The experiment started after the equilibrium period of 20 min. Donor compartment was the mucosal membrane, whereas acceptor compartment was the serosal membrane (Clarke, 2009). LN formulations were added to the donor compartment and samples of 100 µL were collected from the acceptor compartment at 0.25h, 0.50, 1h, 2h and 3h, in order to measure the amount of drug that permeates

through the membrane. After each sample collection, the same volume of heated KBR solution pH 7.4 was added to acceptor chamber. The experimentation lasted 3 hours and occurred at 37°C. The integrity of the membrane was evaluated by the measurement of TEER and of the apparent permeability (Papp) of fluorescein, a well-known paracellular marker. A stock solution of fluorescein in DMSO was previously prepared and it was added a specific volume of that, in order to obtain a final concentration in the chamber of 0.05%w/V. The collected samples were analysed by fluorimetry (λ excitation: 485 nm; λ emission: 515 nm) in a Synergy HT Multi-Mode Microplate Reader (BioTeK, UK). Standard calibration curves were constructed for fluorescein within appropriate concentration and so it was possible to quantify it. The samples were further suitably treated to HPLC analysis, as described below (2.2.1.5.3).

The P_{app} (apparent permeability coefficient) of CBZ and fluorescein were determined applying the following equation:

$$Papp = Q / (C \times A \times t),$$

where Q (μmol) is the total amount of drug that permeated to the acceptor compartment during the experiment, C (μmol/mL) is the initial concentration of drug in the donor compartment, A (cm^2) is the diffusion area of the ussing chamber, and t (s) is the period of the experiment.

2.2.1.4.3 Mucoadhesiveness study

Particle size and zeta potential were measured in order to study the interference of mucin in these parameters, and mucoadhesive behaviour. A mucin solution of 0.1% (w/v) was prepared and the measurement was performed as described in 2.1.2.1., after a sample dilution of 1:50 with the mucin solution referred, at the temperature of 37°C.

2.2.1.4.4 Cell viability studies

In order to evaluate the cellular viability of the formulations, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed. A stock solution of MTT at a concentration of 5 mg/mL was previously prepared. At the end of permeability assay, a volume of 500 μL was added to mice intestinal membranes. Mice intestinal membranes in contact just with KBR solution, during the same period of time of the permeability experimentation (3h) were used as control. The assay was carried out at 37°C, for 3 hours under stirring. Mice intestinal membranes in contact just with KBR solution, during the same period of time of the permeability experimentation (3h)

were used as control. After the incubation period, MTT was removed and the tissue was rinsed in acidified isopropanol, in order to solubilize formazan crystals. The formazan absorbance was measured at 570 nm and 620 nm in a Synergy HT Multi-Mode Microplate Reader (BioTeK, UK). Cell viability (%) was calculated and compared with the untreated control.

2.2.1.5 *In* vivo studies

2.2.1.5.1 Animals

The animal experimentation was performed with adult male CD-1 mice, purchased from Charles River Laboratories (L'Arbresle, France), that weighted approximately 25-35 g at the time of the study. The animal experimental protocols were carried out in accordance with the European Directive (2010/63/EU) for the accommodation and care of laboratory animals and was approved by the Portuguese Veterinary General Division (Ref. 0421/000/000/2016). Animals were suitably accommodated in local facilities under climate and light-controlled environmental conditions (22 ± 1◦C, 50 ± 5% humidity, 12 h light/darkcycle) until performing the experiments and feed with a regular standard chow diet (4RF21, Mucedola, Italy) and provided with tap water *ad libitum* until drug administration (Fortuna *et al.*, 2013).

2.2.1.5.2 Formulation administration

LN formulation was administered to mice by oral gavage (25 mL/kg) in a single dose (127.5 mg/Kg). Thereafter, mice were sacrificed by decapitation at 0.25h, 0.5h, 1h, 2h, 3h, 6h, 12h and 24h post-dosing. Blood samples were collected to heparinised tubes and then centrifugated at 4000 rpm for 10 min at 4°C. Plasma supernatant was collected and frozen at -30°C until analysis. After exsanguination, the brain was carefully excised. It was weighted and homogenized in a 0.1 M sodium phosphate buffer with pH 5 (4 mL/g of tissue), using a THOMAS® teflon pestle tissue homogenizer and the same homogenates were centrifuged at 4800 rpm and 4°C during 15 min. The supernatants obtained were collected and stored at -30°C until analysis.

For intravenous administration, mice were anaesthetized with an intraperitoneal injection of pentobarbital at a concentration of 5 mg/mL, in a dose of 10 µL/kg, and in order to maintain the body temperature, they were kept in a warm environment. The

LN formulation was intravenously administered by injection via the lateral tail vein (60µL/30g of body weight) with an appropriate syringe. Mice were then sacrificed by decapitation at 0.25h, 0.5h and 1h post-dosing. Blood samples was collected and treated as described above, as well as brain samples.

2.2.1.5.3 Samples pre-treatment and CBZ quantification

Mice plasma and brain samples were processed by solid-phase extraction, followed by HPLC analysis. In this way, 200 µL of plasma was added to 800 µL of 0.1 M sodium phosphate buffer (pH 5) and spiked with an internal standard (IS) working solution. For brain samples, 500 µL of the respective homogenates were added to 500 µL of buffer solution, also spiked with IS. Samples were vortexed and loaded in Oasis® HLB cartridges (30 mg, 1 mL, Waters, Milford, MA, USA). Cartridges were conditioned with 1 mL of methanol, 1 mL of acetonitrile and 1 mL of the solution water-acetonitrile (95:5,v/v). The samples were eluted, and the loaded cartridges were washed under a pressure of -30 kPa four times with 1 mL of water and four times with 1 mL of water-methanol (90:10, v/v). The sorbent was dried during 5 min under airflow and analytes were eluted with 1 mL of ethyl acetate. They were evaporated to dryness under a nitrogen stream at 45◦C and the residues were reconstituted in 100 µL of HPLC mobile phase and injected in HPLC.

The quantification of CBZ in mice samples was carried out on a Shimadzu liquid chromatographic system equipped with a GDU-20A5 degasser, a SIL-20AHT autosampler, a CTO-10ASVP column oven and a SPD-M20A diode array detector, all from Shimadzu Corporation (Kyoto, Japan). Data acquisition and instrumentation control were achieved by means of LC Solution software (Shimadzu Corporation, Kyoto, Japan). The analysis was performed according to a previous validated method (Serralheiro *et al.*, 2014), at 40°C, with a mobile phase of water-methanol-acetonitrile (64:30:6, v/v/v), and a flow rate of 1 mL/min, using an injection volume of 20 µL. CBZ was detected at a wavelength of 235 nm and the running time was 15 min. A C18-reversed-phase column LiChroCART®Purospher®Star (C18, 3 µm, 55 mm x 4 mm; Merck KGaA, Darmstadt,Germany) was used. A work solution of BIA2-024 as internal standard, a compound provided by BIAL, was employed. The CBZ calibration range for plasma was 0.1-30 µg/mL and in brain was 0.1-0.15 µg/mL. The standard solutions for calibration curve were processed as described for plasma and brain samples.

2.2.2 Lipid nanoparticle tablets

2.2.2.1 Preparation

LN based tablets were processed as described elsewhere (Mendes *et al.*, 2016). First, the drying of the nanoparticles dispersion was performed, in which spray-drying was the chosen technique. It was carried out in a Mini Spray-dryer Büchi 190 (Büchi, Göppingen, Germany), with the inlet temperature of 110°C, spray-flow of 4 L/min, aspirator speed of 100% and pump speed of 30%. Tablets were composed of 71.5% (w/w) LN spray-dried powder, 20 % (w/w) microcrystalline cellulose, 2.5% (w/w) crospovidone, 5% (w/w) talc, 0.5 % (w/w) aerosil and 0.5% (w/w) magnesium stearate. Powders were previously sieved (180 µm), and appropriately blended. Tablets were obtained by direct compression in a hydraulic press (Speca Press., UK) at 3 t for 5 s. The final weight of tablets was of 250 mg. In parallel, CBZ tablets were prepared under the same conditions, for comparison purposes, taking into consideration the following composition: 1% (w/w) CBZ, 76% lactose, 14 % (w/w) microcrystalline cellulose, 2.5% (w/w) crospovidone, 5% (w/w) talc, 0.5 % (w/w) aerosil and 0.5% (w/w) magnesium stearate.

2.2.2.2 Particle size analysis

Particle size after spray drying process was analysed by laser diffraction. Results are expressed as volume and number distributions, and diameter values corresponds to percentiles of 10%, 50% and 90%. Through these values, it is obtained the span value, a statistical parameter useful to characterize the particle size distribution calculated by the follow equation:

$$Span\ value = \frac{(d90\% - d10\%)}{d50\%}$$

Higher span value means wider size distribution (Mendes *et al.*, 2016). The diffractometer used was a Beckman Coulter1 LS 13 320 (Miami, Florida) and the assay was performed after re-suspension of atomized powder in ultrapurified water and further adequate dilution. The real refractive index and the imaginary refractive index were set to 1.54 and 0.1, respectively, and a density of 0.997048 g/mL for water at 25 °C was considered.

2.2.2.3 Morphology

The analysis of morphology and topography of spray-dried particles was performed by SEM, with conditions described in 2.1.2.2., after re-suspension of the atomized powder in ultrapurified water.

2.2.2.3.1 Mucoadhesiveness assessment

Mucoadhesive tests were performed by a texture analyzer, providing results of maximum detachment force, and the area under the measured load-extension curve that represents the work of adhesion. Using this technique, mucoadhesion is evaluated by the measurement of maximum force required to separate the probe from the dosage form after contact for a specified time and force, calculating the adhesion (Singh e Rana, 2012). It was used a Texture Analyzer TA.XT Plus (Stable Micro Systems Ltd., Surrey, UK), using an analytical probe (P/10, 10 mm Delrin) that depressed the sample at a defined rate (2 mm/s) to a desired depth (2 mm). Samples were place under an agarose layer, and a standard volume of mucin solution 0.1% (w/v) was added to the same standard volume of the respective formulations. The temperature applied was 37°C in order to mimic the body temperature. Data collection and calculation were performed using the Texture Exponent 3.0.5.0 software package of the instrument, and textural characteristics as hardness and adhesiveness was provided.

2.2.2.4 Dissolution studies

The dissolution tests of tablets were performed using an USP Apparatus I – Basket apparatus (VK 7000 Dissolution Testing Station, VanKel - USA) (Figure 6), initially in pH 1.2 (simulated gastric fluid without enzymes, USP XXV) for 2 hours and, subsequently, in pH 6.8 (simulated intestinal fluid without enzymes, USP XXV) for 6 hours, at 37°C. A volume of 250 mL for each vessel was used, and the stirring speed was 50 rpm. Samples (volume=900 µL) were collected at 0.25h, 0.5h, 1h, 2h, 3h, 4h, 6h and 8h. They were suitably pre-treated and then analysed by HPLC as described in 2.2.1.5.3. Again, dissolution profiles were obtained by plotting the cumulative percentage of drug released against time, calculated according to

$$Release\ (\%) = \left(\frac{drug\ amount\ released}{total\ amount\ of\ drug\ present\ in\ tablets}\right) \times 100$$

Figure 6: Dissolution studies of tablets in Apparatus I (USP)

2.3 Statistical Analysis

Statistical analysis was performed with Microsoft Excel (Microsoft Corp., Redmond, WA). Statistical significance was evaluated using Student t-test and a value of $p < 0.05$ was considered significant.

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3. Results and discussion

The development of a dosage form for oral delivery include several steps, considering the challenges of this administration route and the drug performed desirable. Polymer-lipid hybrid nanoparticles were developed regarding on pre-formulation studies, by the application of suitable tests of optimization of formulation, its characterization, and the particle performance *in vitro* and *in vivo* evaluation. Therefore, quality, safety and effectiveness of the drug product should be assured.

3.1 Formulation optimization

3.1.1 SLN *versus* NLC

Compritol 888 ATO (glyceryl behenate), a mixture of mono- (12-18% w/w), di- (45-54% w/w) and tri- (28-32% w/w) behenate of glycerol was the first solid lipid chosen to be tested. This choice relied not only on the solubility of carbamazepine (CBZ) in this lipid, but above all, on its high melting point (69-74°C). Note that solubility of the drug in the melted lipid is known to be an important precondition to obtain sufficient entrapment efficiency (E.E.) (Vitorino *et al.*, 2013), while a high melting point is critical to support the further atomization process. Moreover, the integrity of the excipients, and consequently the formulation stability, is affected by the processing variables (e.g. high temperature, pressure) they encounter during formulation production (Aburahma e Badr-Eldin, 2014) Compritol 888 ATO was tested either to prepare SLN or NLC formulations (Figures 7-9). SLN based on Compritol 888 ATO presented particles with a large size (higher than 600 nm) and polydispersity indices out of the acceptable values (higher than 0.3), in the following order Labrasol>PVA>Tween®80. Zeta potential was not a discriminatory variable at this preliminary development phase (Figure 7). These results reflect the impact of the emulsifier on the nanoparticle formation. Emulsifiers have a very important role in the lipid nanoparticles dispersion establishment by preventing agglomeration phenomena. Properties like charge, molecular weight, chemical structure and hydrophilic-lipophilic balance (HLB) should be considered to find the ideal surfactant for the stabilization of a lipid matrix (Severino *et al.*, 2012). In this case, non-ionic surfactants were selected, since they exhibit lower toxicity and irritancy, which make them suitable for oral administration. Another advantage that could be taken from this type of surfactants is their effectiveness to inhibit the degradation of lipid matrix (Amiji, 2010). Besides, the HLB values of the surfactants

chosen are in agreement with the typical range (8-18) used for the preparation of oil-in-water dispersions (Tween 80 [Polyoxyethylene sorbitan monooleate], HLB 15 (Vasconcelos, Marques e Sarmento, 2017), Labrasol [Caprylocaproyl macrogol-8 glycerides], HLB 12), PVA [polyvinyl alcohol], HLB 18 (Tuba *et al.*, 2014)). Note that when preparing SLN, the stresses that affect lipid stability are the initial melting process of the lipid to produce the hot pre-emulsion and the high temperature and high pressure during the hot homogenization process. The added surfactants during lipid nanoparticles preparation contributes to the lowering of the melting enthalpy of Compritol 888 ATO by distributing the melted lipid phase and distorting its crystallization. It is claimed that the surfactant may immobilize lipid molecules by interfacial contact and, consequently, avoid reorientation of less-ordered configurations into more organized structural lattice (Aburahma e Badr-Eldin, 2014).

In an attempt to reduce the particle size and the respective distribution, Compritol 888 ATO:Oleic acid NLC formulations were prepared, now taking Tween® 80 and Span 85 (Sorbitan trioleate, HLB 1.8) as surfactants. The use of oleic acid (C8/C10 short-chain fatty acids, HLB 17), as liquid lipid was based on previous work (Mendes *et al.*, 2016). In this case, the method to determine the required hydrophilic lipophilic balance (rHLB) of the lipid phase was applied (Keck *et al.*, 2014). By this method, each lipid phase possesses an individual, i.e., required HLB. By matching the rHLB, it is possible to obtain a more stable and small sized emulsion, than it would have been the case with emulsifiers of any other HLB value (Keck *et al.*, 2014).

Besides the type(s) of surfactant(s), the concentration is also an important parameter to have into consideration. It is described that high concentrations of the emulsifier lead to a decrease in the particle size, since the emulsifier reduces the surface tension and promotes the particle partition during the homogenization process (Mendes *et al.*, 2016). The effect of this variable (concentrations of 3% or 5% w/w of emulsifier(s)) was also inspected. As shown in Figure 8, the change to NLC nanosystem led to the decrease in the particle size, despite the results were not yet satisfactory, even with the rHLB approach. The introduction of a liquid lipid in the lipid core, led to the decrease in particle size and the respective homogeneity, due to the reduction of the lipid core viscosity. Actually, there is a relationship between the lipid melting point and the mean particle size, since a higher melting point leads to high viscosity of the lipid core, and in that way, the differences in the lipid arrangement yield an increase in the mean particle size. (Vitorino *et al.*, 2013) Besides, the inclusion of a liquid lipid

together with a solid lipid favours the formation of a less structured lipid matrix, which avoids the partial formation of lower-energy lipid modifications and reduces the crystallinity arising from the transformation of Compritol 888 ATO into lipid nanoparticles (Doktorovova e Souto, 2009). All these aspects contributed to the reduction in the particle size and, at the same time, to the increase in the physical stability.

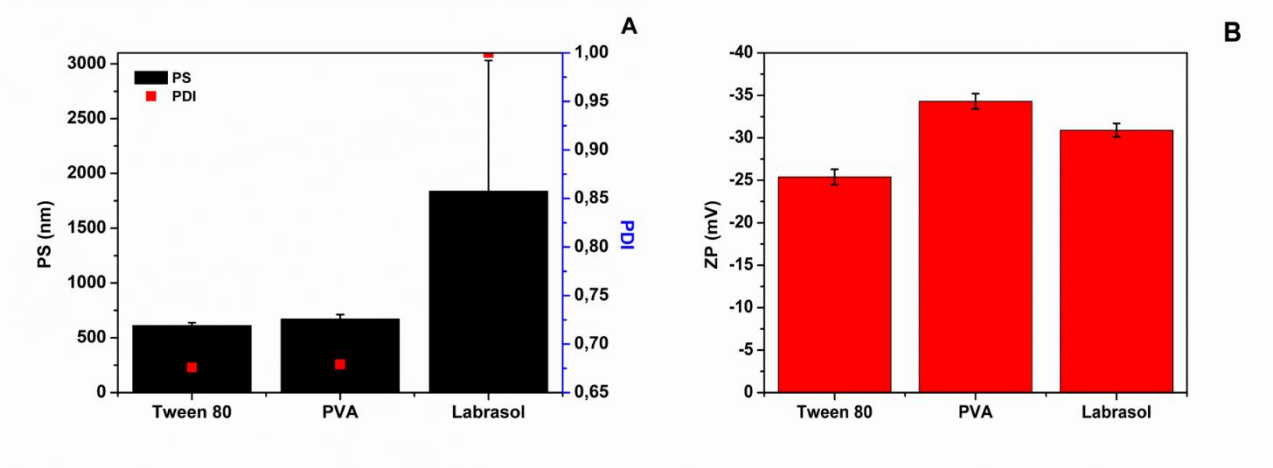

Figure 7: Particle size (PS), and respective distribution (PDI) (A) and zeta potential (ZP) (B) of Compritol SLN formulations prepared with different surfactants - Tween® 80, PVA and Labrasol.

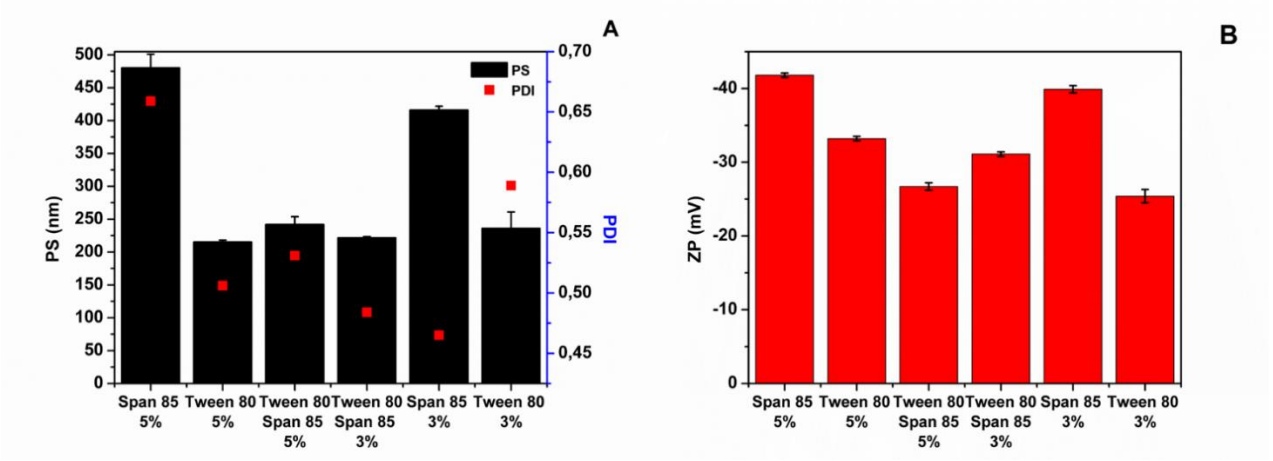

Figure 8: Particle size (PS) and respective distribution, (PDI) (A) and zeta potential (ZP) (B) of Compritol:Oleic Acid NLC formulations prepared with diferent surfactants: Tween® 80 and Span® 85, either alone or combined based on rHLB method– at 3%w/V and 5%w/V concentrations.

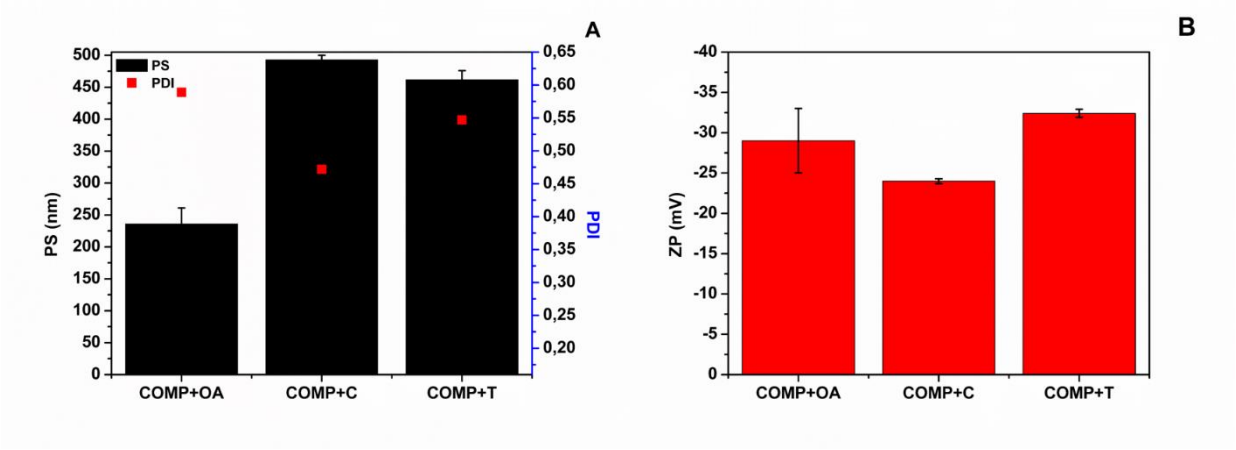

Figure 9: Particle size (PS) and respective distribution, (PDI) (A) and zeta potential (ZP) (B) of NLC formulations of Compritol associated to different liquid lipids - Oleic Acid, Capryol and Transcutol. COMP+OA: Compritol and Oleic Acid; COMP+C: Compritol and Capryol; COMP+T: Compritol and Transcutol.

3.1.2 Lipid selection

The screening of different liquid lipids, including oleic acid (unsaturated long chain fatty acid), Capryol 90 (propylene glycol monocaprylate) and Transcutol (Diethylene monoethyl ether), to obtain Tween®80 stabilized NLC was further carried out. This choice relied on the carbamazepine solubility in each lipid. Accordingly, it is possible to organize the lipids in the following order: Oleic Acid (32,08 mg/mL) (Acharya *et al.*, 2013), Capryol 90 (87,44 mg/mL), and Transcutol (157,23 mg/mL) (Patel *et al.*, 2013). From the combined analysis of solubility, PS, PDI and ZP, as critical quality attributes, Capryol and Transcutol were the liquid lipids chosen to proceed with the study, now changing the Compritol by the tripalmitin, a triglyceride, as solid lipid. Note that this lipid also fits the encapsulation purpose of CBZ, presenting a satisfactory solubility and high melting point (64°C-66°C). The corresponding tripalmitin formulations revealed lower particle sizes, with Transcutol yielding the better performance (PS lower than 250 nm, and acceptable homogeneity with PDI values lower than 0.3). Such a behaviour could be attributed to the lower melting point of tripalmitin in comparison to Compritol 888 ATO. Moreover, Transcutol is also described as an intestinal absorption enhancer, through the inhibition of multidrug resistance protein 2 (MRP2) known as ATP-binding cassette sub-family C member 2, an efflux transporter protein mainly expressed in the liver, kidney and intestine (Table 3) (Vasconcelos, Marques e Sarmento, 2017).

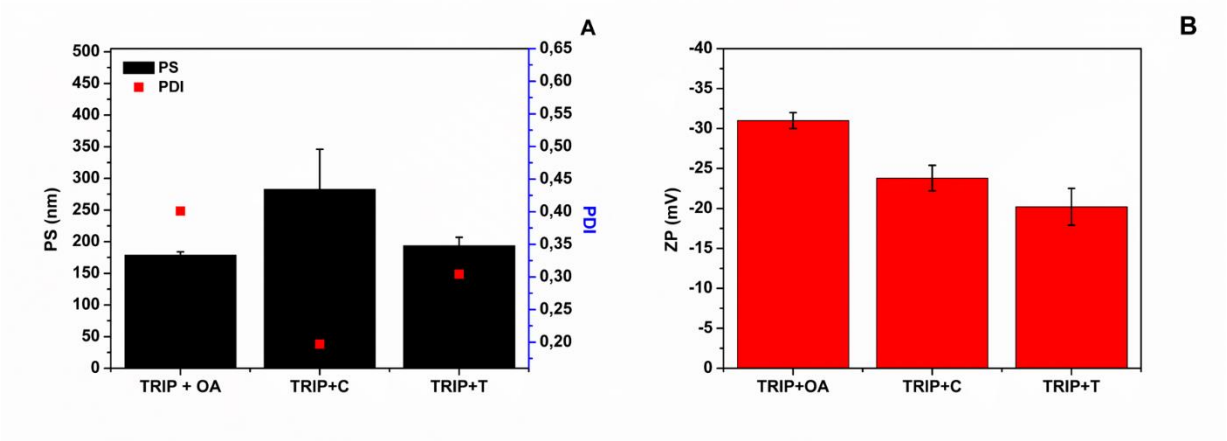

Figure 10: Particle size (PS) and respective distribution, (PDI) (A) and zeta potential (ZP) (B) of NLC formulations of tripalmitin associated to different liquid lipids - Oleic Acid, Capryol and Transcutol; TRIP+OA: Tripalmitin and Oleic Acid (Mendes *et al.*, 2016); TRIP+C: Tripalmitin and Capryol; TRIP+T: Tripalmitin and Transcutol.

Table 3 - Excipients effect on intestinal transporters.

EXCIPIENT	P-GP	MRP2	BCRP
TWEEN 80	±	+	+
VITAMIN E TPGS	+	+	-
TRANSCUTOL		+	

Table 4 - Excipients effect in cytochrome P450.

EXCIPIENT	CYP3A	CYP3A4	CYP3A5	CYP2C9	GLUCURONIDATION
TWEEN 80	+	+	+	+	+
VIT. E TPGS	-	+			

3.1.3 Hybrid nanoparticles

The selection of tripalmitin and transcutol as solid and liquid lipids, respectively, was strongly confirmed when chitosan was introduced in the formulation, irrespective of the concentration employed (0.5% or 1%w/w). Hybrid nanoparticles (chitosan coated

LN) were then obtained with suitable critical quality attributes, with sizes within an acceptable nanoscale range (ca. 200 nm), and ZP values higher than 30 mV. Note that a charge reversion to positive values, compared with conventional lipid nanoparticles, was observed, which is a consequence of their coating with chitosan, a positively charged polymer.

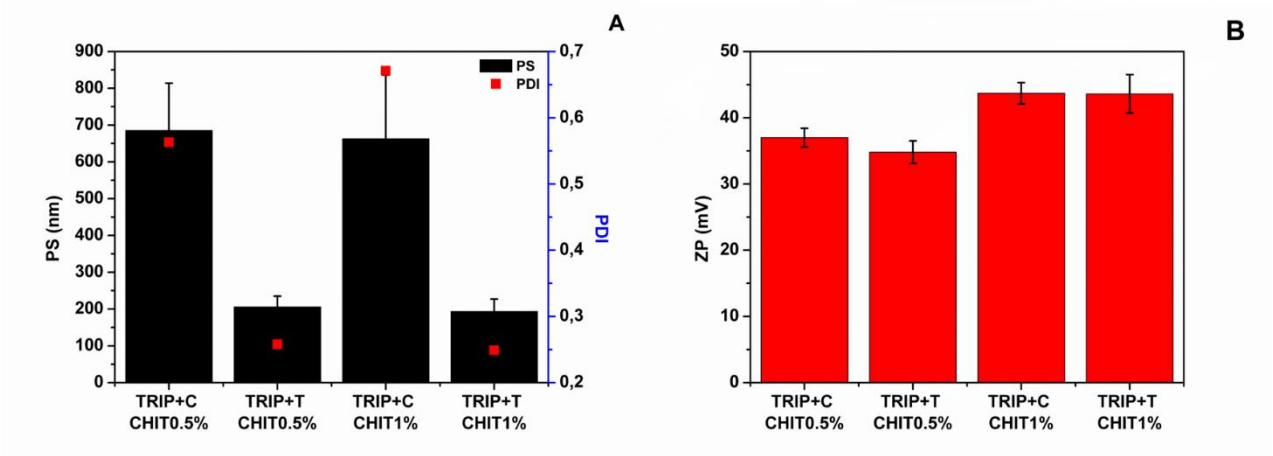

Figure 11: Influence of different chitosan coating concentrations of tripalmitin based NLC with different liquid lipids - Capryol and Transcutol – on particle size (PS) and respective distribution, (PDI) (A) and zeta potential (ZP) (B); TRIP+C: Tripalmitin and Capryol; TRIP+T: Tripalmitin and Transcutol; Chit: Chitosan.

3.2 Carbamazepine encapsulation

3.2.1 Emulsifier effect

The use of excipients as oral bioavailability enhancers through inhibition of efflux mechanisms and/or intestinal metabolization enhancers is currently one of the hot topics in the pharmaceutical field (Vasconcelos, Marques e Sarmento, 2017)

In order to evaluate the effect of Tween® 80 and/or Vitamin E polyethylene glycol succinate (Vit E TPGS, HLB 13) on the system stability and loading properties, four different tripalmitin:transcutol NLC formulations were prepared (Table 3). It should be highlighted that these two surfactants have an advantage, because of their biopharmaceutical behaviour.

Both are intestinal absorption enhancers, since Tween® 80 can inhibit the CYP450 present in the small intestine, while Vit E TPGS interacts with intestinal metabolism by

the specific inhibition of CYP3A4 (Table 4). They have also the ability to affect the efflux transporters: Tween® 80 through the inhibition of MRP2 and BCRP transporters, and Vit E TPGS through the inhibition of P-gp and MRP2 transporters. Vitamin E TPGS is reported to promote an increase in the membrane rigidity, what results in a decrease in ATPase activity, and consequently in the P-pg inibhition. On the other hand, Tween®80 is responsible for its fluidization, leading to the same response. (Vasconcelos, Marques e Sarmento, 2017). It is described that carbamazepine is a strong inducer of CYP3A4 (Giessmann *et al.*, 2004) and acts as an intestinal and renal inducer of MRP2 expression by the nuclear pregnane X receptor (PXR) mechanism of regulation. Note that carbamazepine-10,11-epoxide, the main product of carbamazepine metabolism, is formed by CYP3A4 activity. In what concerns the interaction with P-glicoprotein (P-gp), the developed work mentioned elsewhere (Fortuna *et al.*, 2012), suggest that carbamazepine-10,11-epoxide may interact with P-gp, what is not observed with carbamazepine. In this way, the inclusion of excipients that interact with the same efflux transporters and metabolization enhancers of carbamazepine and carbamazepine-10,11-epoxide, is seen as a technological advantage to modulate the pharmacokinetic/pharmacodynamics (PK/PD) profile.

In what regards the colloidal properties, formulations composed by Vit E TPGS presented a higher particle size and heterogeneity (PDI higher than 0.4). Tween® 80 rendered a better entrapment efficiency and drug loading than formulations with Vit E TPGS, having been taken as a selection criteria. Regarding release studies, the profiles obtained were very similar, irrespective of the surfactant, presenting an initial burst release during the first two hours, in acidic medium, followed by a sustained release up to 8h (Figure 12).

For the reasons presented above, Tween® 80 was chosen as surfactant to proceed to the *in vitro-in vivo* studies, since it provided the best physicochemical and biopharmaceutical characteristics. This reference formulation will be termed as F0.

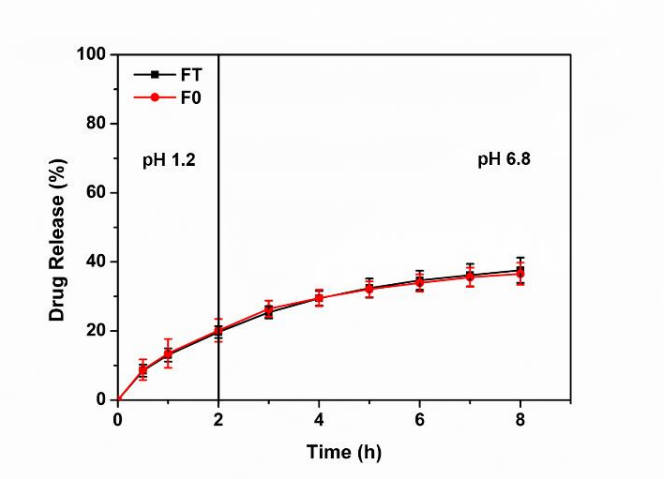

Figure 12: Release profiles of F0 and FT

Table 5: Characterization of NLC with Tween® 80 and Vit E TPGS both alone and combined in different concentrations.

F#	SURFACTANT	PS (nm)	PDI	ZP (mV)	E.E. (%)	D.L. (%)
21	Tween 80 3%	142 ±2	0.255	-21.3 ±0.5	85.2 ±1.1	9.3 ± 0.1
24	Tween 80 3% + TPGS 1%	321 ±78	0.670	-13.9 ±0.6	61 ± 4	2.0 ± 0.8
25	TPGS 1%	182 ±3	0.441	-21.8 ±0	64 ± 5	2.0 ± 0.7
27	TPGS 3%	265 ±5	0.586	-10.0 ±0.1	78 ±2	4.4 ±0.3

3.2.2 Chitosan coating

Conventional NLC are usually negatively charged and it plays an important role on the prevention of the destabilization of these carriers. Nevertheless, it has also some disadvantages, such as the adsorption ions that are present in gastric fluids and the electrostatic repulsion with biological membranes that can occur, since they are also negatively charged (Oyarzun-Ampuero *et al.*, 2010). As such, the incorporation of a polymeric and cationic compound arises as an interesting strategy, in order to provide more stability to the nanoparticles. Besides, lipid nanoparticles exhibit poor stability under acidic conditions, which results in a large aggregation in gastric medium (Luo *et al.*, 2015).

Chitosan was the biopolymer selected for NLC coating, since this surface modification would increase the CBZ absorption rate and improve the delivery

efficacy, both by promoting a closer contact with the negatively charged intestinal epithelium, along with the enhancement over mucoadhesiveness and sustained release NLC properties (Luo *et al.*, 2015).

Note that chitosan coating has here a dual biorelevant and technological function, since it not only increases stability by circumventing the harsh gastric conditions (pH 1.2), but also provides enough stiffness in order to support the further spray-drying procedure.

The incorporation of carbamazepine, irrespective of the type of formulation, did not promote significant variations in the colloidal properties.

In particular, CBZNLC coated with chitosan, either at a concentration of 0.5%w/w (F1CBZ) or 1%w/w (F2CBZ), exhibited generally similar particle sizes and kept acceptable PDI and ZP values – closer to or higher than 30 mV, when comparing with the corresponding unloaded formulations (Table 6). However, NLC with 1%w/w of chitosan coating (F2CBZ) showed more interest, because of the previously mentioned characteristics (higher positive charge).

In an attempt to improve the loading properties (E.E. and D.L.), and considering F2 as the optimal formulation (1% of chitosan), increased lipid phase concentrations up to 7.5% (w/w) (F3CBZ) and 10% (w/w) (F4CBZ) were tested. However, quality attributes such as PS, PDI and ZP did not evidence better characteristics by this improvement, as well as the E.E. and D.L.. The latter parameter was the discriminatory factor, since it decreased significantly when compared with formulations with lower amount of lipid phase (5%), as described in table 6.

Table 6: Characterization of unloaded and carbamazepine (CBZ) loaded NLC and hybrid NLC, with different concentrations of lipid phase and chitosan.

F#	NOTATION	PS	PDI	ZP	E.E.	D.L.
13	F0	194 ±13	0.304	-20.2 ±2.3	-	-
15	F1	206 ±29	0.258	34.8 ±1.7	-	-
17	F2	194 ±33	0.249	43.6 ±2.9	-	-
22	F0CBZ	142 ±2	0.255	-21.3 ±0.5	85.2 ±1.1	9.31 ± 0.11
20	F1CBZ	209.7 ±1.1	0.199	28.4 ±0.6	86 ±2	9.2 ±0.2
21	F2CBZ	178 ±32	0.241	38 ±11	84.0 ±0.4	9.05 ± 0.04
23	F3CBZ	222 ±4	0.463	25.6 ±0.8	76 ± 3	5.8 ± 0.6
26	F4CBZ	175.3 ±1.5	0.252	40.27 ±0.15	84.2 ± 1.4	4.9 ± 0.2

3.3 Physicochemical characterization of formulations

3.3.1 *In Vitro* Release Studies

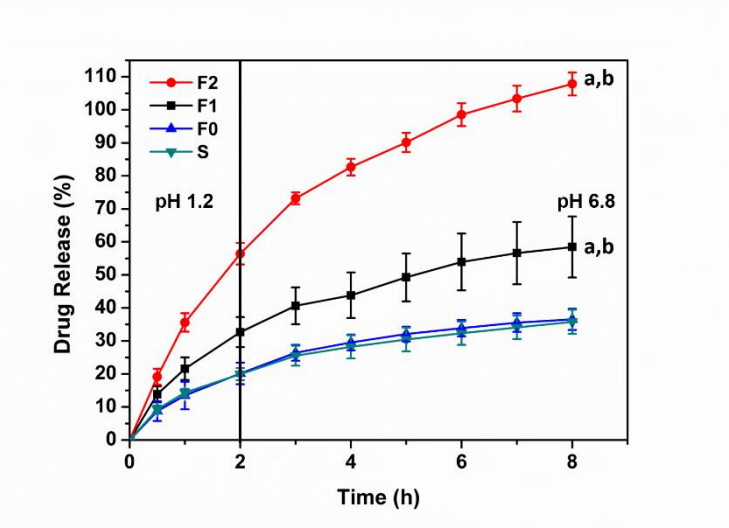

Figure 13: Release profiles of CBZ nanoparticles uncoated (F0), 0.5%w/w chitosan coated (F1), 1% chitosan coated (F2), and commercial oral solution (S). [a] F1 and F2 drug released after 8h is statistically higher than F0 for a $p < 0.05$. [b]F1 and F2 drug released after 8h is statistically higher than S for a $p < 0.05$.

Release studies give preliminary information about the behaviour of the drug formulation in the physiological conditions of the gastrointestinal tract. *In vitro* dissolution tests must be capable of discriminating products in terms of release controlling mechanism. These are studies that take into consideration parameters that should simulate *in vivo* conditions, such as volume, composition, pH, viscosity of the gastrointestinal content, presence of enzymes and motility, although some of these characteristics are difficult to reproduce because of their great variation (Mendes *et al.*, 2016).

Chitosan coating concentration and drug encapsulation led to differences in the release profiles. As shown in Figure 13, release profiles of F0 (uncoated CBZ nanoparticles) and commercial oral solution profile, with non-encapsulated CBZ, are very similar. The faster drug release exhibited for both formulations during the first 2 hours (up to 20%) corresponds to the gastric medium. After 3 hours, a plateau at 35%-36% of CBZ released is reached, which is maintained until the 8 hours. Such a sustained profile can be ascribed to the NLC aggregation in gastric environment, deeming from their poor stability under acid conditions (Luo *et al.*, 2015). This behaviour is confirmed by the PS and ZP values, which evidence an increase in the

particle size for F0 after 2 hours, as well as a decrease in the ZP value (Table 7). In turn, the release pattern of the commercial oral solution is a consequence of the poor solubility of carbamazepine (Lindenberg, Kopp e Dressman, 2004).

The release profile of F1 (CBZ NLC with 0.5% chitosan coating) shows a burst release period more extensive than the last ones (3-4 hours) and a higher amount of CBZ released (around 45%). In the Figure 13, it is also seen a positive relationship between the chitosan coating concentration and the drug released, with F2 formulation exhibiting the largest amount of CBZ released, though still ensuring the desired control over release. This trend could be attributed to the improved stability in acid medium. Table 7 shows that under acidic environment, the particle size of coated lipid nanoparticles, does not suffer a large variation. Chitosan coating also allows to obtain lipid nanoparticles with enhanced mucoadhesive properties, thus enabling an increased absorption rate and improved efficacy of LN delivery, besides the sustained release profile mentioned (Luo *et al.*, 2015). However, it should be noted that, since chitosan is soluble in acidic medium (SGF) and insoluble in basic conditions (SIF), lipid

nanoparticles could loss, part of the coating, which is reflected in a charge neutralization.

Conversely, LN without chitosan coating show a better stability under basic medium, presenting higher negative values of ZP in SIF than SGF, according to Table 7. This behaviour could explain the release of the formulations after 2 hours (Figure 11). The differences found in the release profiles of F0, F1 and F2, highlights the influence of chitosan in different concentrations, pointing F2 as the most interesting formulation.

Table 7: PS, PDI and ZP of formulations F0 and F2 in two different dissolution media – SGF and SIF – at initial time (t0h), after 2 hours (t2h) and at the end time (t8h).

	SGF			SIF		
	PS (nm)	**PDI**	**ZP (mV)**	**PS (nm)**	**PDI**	**ZP (mV)**
F0 t0h	219 ± 2	0.420	0.4 ± 0.9	147 ± 9	0.225	-7.4 ± 0.3
F2 t0h	139 ± 1	0.176	11 ± 1	146 ± 1	0.168	-1.8 ± 0.2
F0 t2h	248 ± 11	0.377	0.01 ± 0.05	-	-	-
F2 t2h	135 ± 2	0.164	1.13 ± 0.09	-	-	-
F0 t8h	-	-	-	143 ± 4	0.237	-8.1 ± 0.6
F2 t8h	-	-	-	147 ± 3	0.179	-0.6 ± 0.5

Table 8: Regression parameters resulting from the application of the different mathematical functions.

Function	**Formulations**	c_1	c_2	c_3	R^2
Zero order $c_1 t$	F0	5.66239 ±0.483724	-	-	0.85384
	F1	8.93502 ±0.75782	-	-	0.89304
	F2	16.3344 ±1.26616	-	-	0.90405
	S	5.48300 ±0.502012	-	-	0.87402
First Order $c_1(1-\exp(-c_2 t))$	F0	38.8595 ±2.56708	0.354771 ±0.0605866	-	0.98140
	F1	59.2007 ±2.15929	0.395807 ±0.0401808	-	0.99189
	F2	112.961 ±2.19942	0.344635 ±0.0170122	-	0.99830
	S	35.2308 ±1.19665	0.445381 ±0.0455632	-	0.99088
Higuchi $c_1 t^{0,5}$	F0	13.6857 ± 0.580225	-	-	0.94755
	F1	21.7274	-	-	0.99281

		±0.295916			
	F2	36.8365 ±2.92921	-	-	0.96776
	S	13.3872 ±0.232153	-	-	0.98942
Weibull $c_1(1-\exp(-c_2t^{c3}))$	F0	1.20347 ±0.220254	36.2337 ±2.42596	0.410480 ±0.0638440	0.98344
	F1	0.716518 ±0.0495658	75.4136 ±6.93680	0.221569 ±0.0520883	0.99882
	F2	0.881078 ±0.0351051	122.322 ±4.26157	0.289624 ±0.0231878	0.99944
	S	0.696791 ±0.0383278	43.9892 ±2.78723	0.256970 ±0.0438890	0.99924
Kormeyer-Peppas c_1t^{c2}	F0	12.7959 ±1.98393	0.541490 ±0.090265	-	0.94624
	F1	22.7604 ±0.961457	0.471161 ±0.024972	-	0.99401
	F2	34.0839 ±1.34993	0.738447 ±0.065602	-	0.99762
	S	14.7600 ±0.594353	0.439220 ±0.023986	-	0.99376

The release profiles obtained were submitted to a fitting with different mathematical models, in order to better understand the drug release mechanism (Dash *et al.*, 2010). The values obtained after the application of the mathematical models are presented in table 8. Through the value of coefficient of determination, R^2, it is possible to assess the goodness of fitting, since it is as better as closer to 1. In this way, it is possible to affirm that Weilbull model provides the best regression function. First order model and Korsmeyer-Peppas also provide a very good regression. Weilbull model (Figure 14) allows to characterize the release profiles by the curve shape through c_3 values. It can be classified as exponential (c_3=1), sigmoidal (c_3>1), which means s-shaped with upward curvature followed by a turning point, and parabolic (c_3<1), with a higher initial slope and consistent with exponential after this. All formulations present a value of c_3<1, what leads to the conclusion that the profiles have a parabolic shape. First order

model allows to predict the asymptotic value through c_1 parameter. It is higher for F2 when compared with all the other formulations, which is in accordance with the results presented in figure 15. Korsmeyer-Peppas model (Figure 15) allows to characterize the drug transport mechanism by c_2 parameter. For c_2 values close to 0.5, it corresponds to a Fickian diffusion process and for values between 0.5 and 1, it corresponds to an anomalous (non-Fickian) transport. When c_2=1, it is applied a zero-order model (Peppas, 2014). Considering the obtained results, it is possible to conclude that F0, F1 and S are characterized by a Fickian diffusion process, since the values of c_2 are close to 0.5. F2 is characterized by a non-Fickian diffusion process, following an anomalous drug transport. This behaviour can be related to polymer relaxation, since the slow displacement of macromolecular polymer chains creates a system that have no mechanical equilibrium (Peppas, 2014). Note that despite F1 and F2 are LN with chitosan coating, this behaviour is just found for F2, where the polymer is present in higher amount. However, this semi-empirical method has a disadvantage, since it just considers the first 60% of the release.

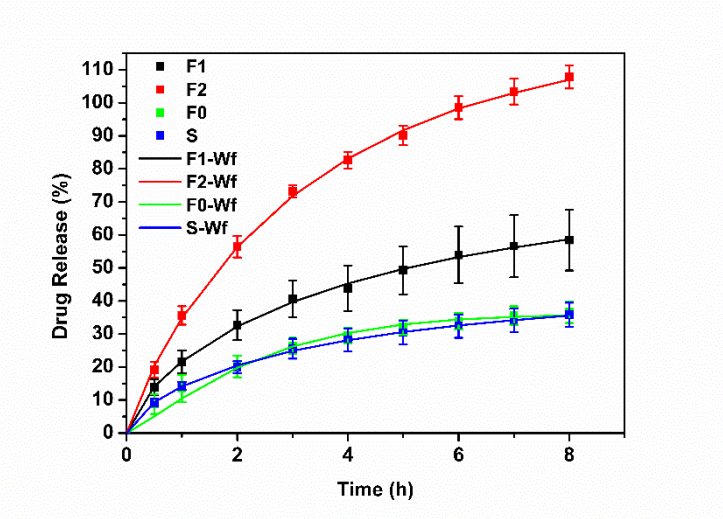

Figure 14: Release profiles of CBZ nanoparticles uncoated (F0), 0,5% chitosan coating (F1), 1% chitosan coating (F2), and commercial oral solution (S) before and after Weibull fitting.

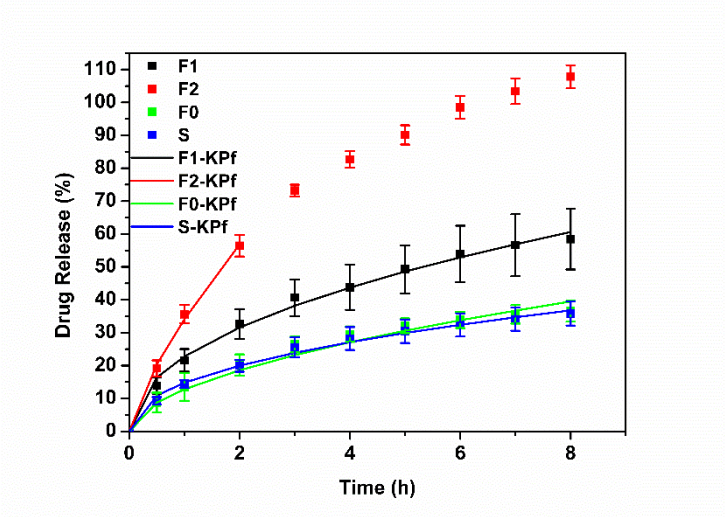

Figure 15: Release profiles of CBZ nanoparticles uncoated (F0), 0,5% chitosan coating (F1), 1% chitosan coating (F2), and commercial oral solution (S) before and after Korsmeyer-Peppas fitting.

3.3.2 Stability Studies

Formulation stability is one of the essential properties of quality that should be monitored. It means that the state of a dispersion must be kept constant throughout the entirety of the product. It emphasizes the need of control any phase separation like sedimentation, flotation or creaming, since these phenomena alters the state of a dispersion (Lerche e Sobisch, 2011).

It is possible to evaluate the stability of a dispersion by particle charge (zeta potential) or by analytical centrifugation. Additionally, size control and nanoparticle growth are important parameters for preparing dispersions and their evolution should also be monitored.

Considering the results obtained by analytical centrifugation, through the instability index (Figure 16) it is possible to conclude that F1 is the formulation with higher instability, which compromises the long-term storage. On the other hand, F0 presents the lower values of instability index, while F2 presents an intermediate relative positioning. It is also seen that the introduction of CBZ do not impact the stability of formulations. These trends are evidenced in transmission profiles (Figure 17), which generally indicate an initial flocculation and creaming, further resulting in sedimentation. This behaviour is more pronounced for chitosan coated formulations. In the case of LN, creaming effects are the main destabilization phenomena.

Moreover, the transmission patterns displayed in Figure 15 C-F, corresponding to chitosan coated formulations, are characteristic from samples with unimodal distributions, which indicate formulation homogeneity. This trend is even more marked with CBZ containing formulations (Figure 15 D and F), being in good agreement with PDI values previously referred (Table 6). Despite the stability studies exhibit higher stability for F0, this result is opposite to zeta potential results, which evidenced a higher stability for F2 formulation. It means that F0 seems to be more stable at long-term, under stress conditions, however F2 is more stable at short/medium time.

The stability evaluated through ZP (Figure 18) after 8 months for F0 and F2 confirm the above mentioned tendency of lower stability for F2, since the reduction observed in ZP is higher for F2 than for F0. However, particle size does not suffer significant alterations, and for F2 it can be even observed a decrease in the PDI. These results are opposite to the ones found by analytical centrifugation, but, considering ZP values, it is possible to affirm that F2 LN could suffer aggregation throughout time, such as predicted before.

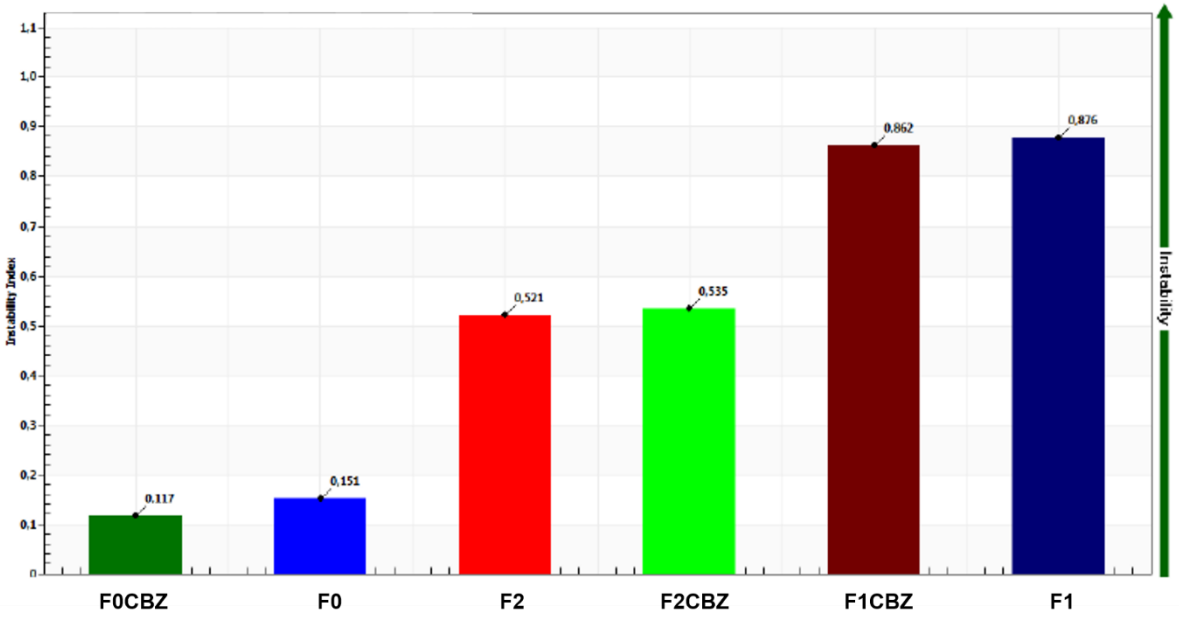

Figure 16: Instability Index values of formulations of LN non-coated and LN chitosan coating, and their similars with CBZ loaded.

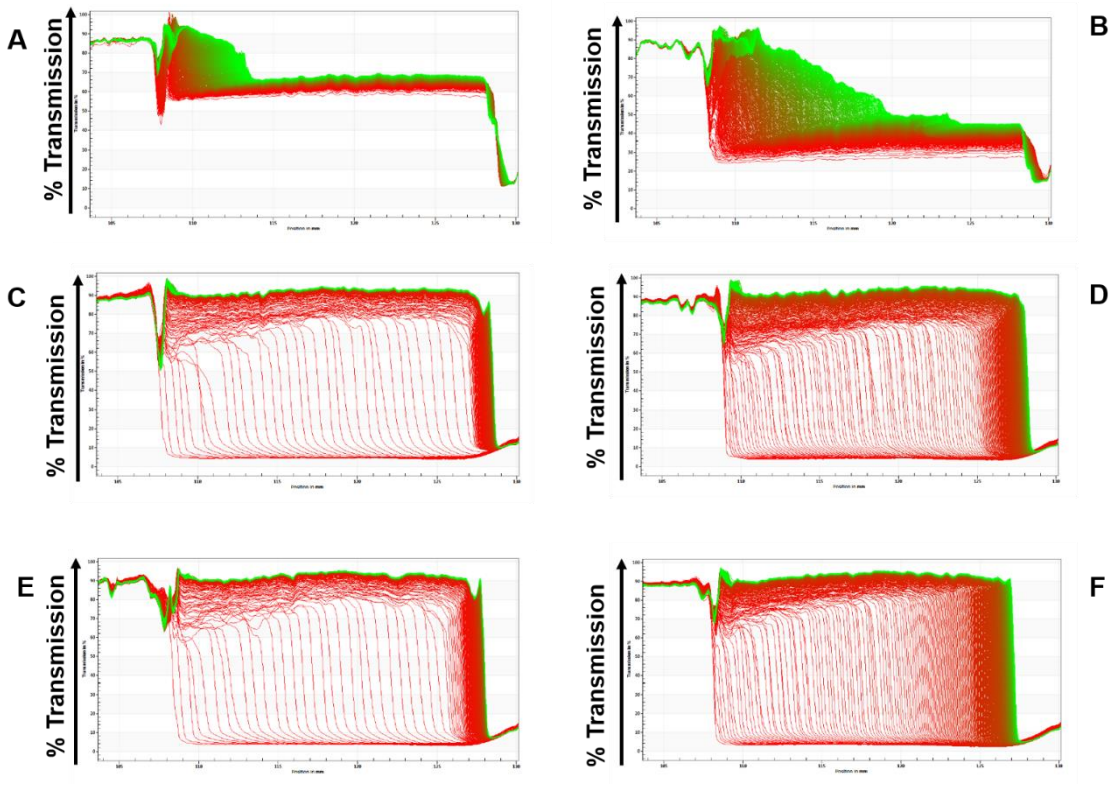

Figure 17: Transmission profiles of F0 (A), F0CBZ (B), F1 (C), F1CBZ (D), F2 (E), F2BCZ (F). The corresponding sequence of profiles should be followed from red for the first profiles to green for the last ones.

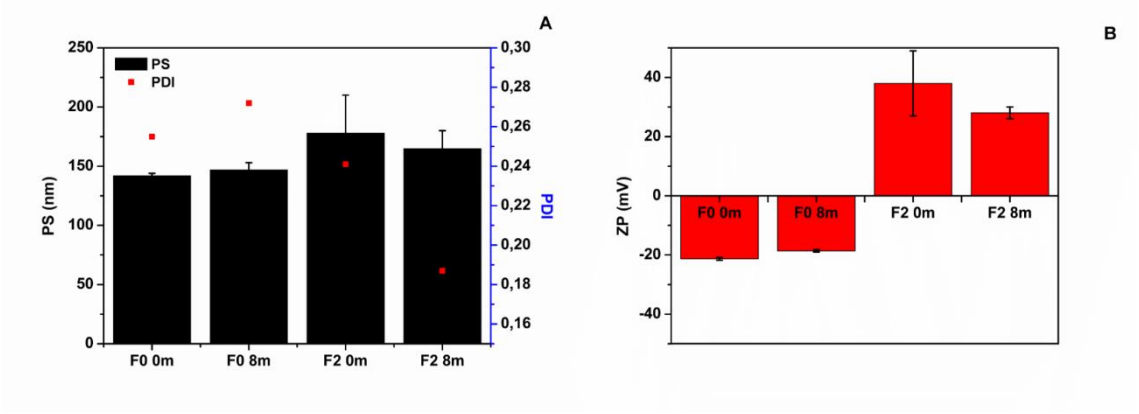

Figure 18: PS, PDI (A) and ZP (B) of F0 and F2 at initial time and 8 months later.

3.3.3 DSC

DSC thermal analysis allows to characterize the status of the lipid matrix in nanoparticles, retrieving information about the respective crystallization behaviour, the

occurrence of different polymorphic forms, the melting point and related enthalpy (Mäder e Mehnert, 2001) The results obtained that can lead to such a characterization are displayed in table 9, and the respective thermograms in figure 19. CBZ typically presents a first endothermic peak, which corresponds to the melting of its form III at 175.64 °C. Thereafter and immediately, CBZ is crystallized in Form I, which can be detected through the exothermic peak detected at 177.19ºC. The second endothermic peak seen in CBZ thermogram (T=192.45°C) corresponds to the melting of form I (Aparecida *et al.*, 2014).

Hybrid-NLC thermograms exhibit one peak corresponding to tripalmitin melting point. This confirms the solid state of the nanoparticles, indicating that they keep their integrity after spray-drying procedure. However, the respective shape in each formulation shows some variations. It can be well-resolved or broader, what could be related to different transition energies and crystallinity degrees/amorphous state. The shape of the peak and the associated enthalpy seems to be associated to the presence or absence of chitosan, with formulations without chitosan coating presenting higher enthalpy values and sharper peaks. Additionally, it is evidenced a slight shift to lower melting temperature (T_{on}) for all the formulations, in comparison to the pure compound. This indicates a high degree of disorder and lower crystallinity, since lower energy enthalpies are involved, probably ascribed to the presence of Transcutol (liquid lipid) in the lipid matrix. (Vitorino *et al.*, 2014). DSC thermograms of the CBZ containing formulations did not exhibit any melting transition peak of the drug, indicating that it is dissolved within the lipid matrix. The absence of the broad endothermic peak at 150.19ºC associated to the weight loss of chitosan, also points to a homogeneous coating of the nanoparticles.

Table 9: DSC melting characteristics of pure compounds and formulations.

	T_{on} (°C)	T_{peak} (°C)	Enthalpy of fusion (J/g)
Tripalmitin	64.36	66.56	-198.76
Carbamazepine	174.2	175.64	-10.07
	176.78	177.19	0.53
	191.09	192.45	-87.16
Chitosan	150.19	151.19	-150.42
F0	62.66	64.82	-69.32
F1	62.12	65.17	-64.49
F2	62.38	65.38	-63.95
F0CBZ	63.27	65.32	-77.2
F1CBZ	62.7	66.61	-69.41
F2CBZ	61.94	65.4	-54.78

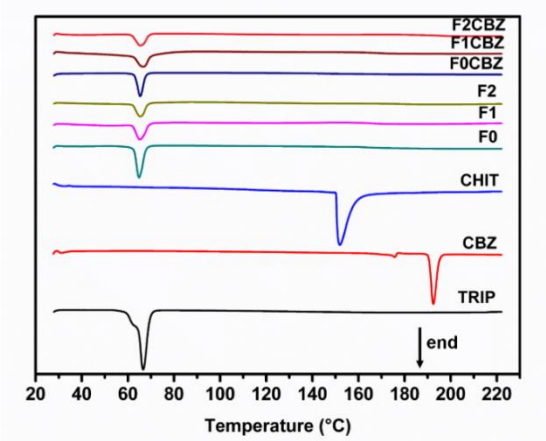

Figure 19: DSC thermograms of pure compounds (Tripalmitin, Carbamazepine and Chitosan), non-loaded formulations (F0, F1, F2) and loaded formulations (F0CBZ, F1CBZ, F2CBZ). Key: CHIT: chitosan, CBZ: carbamazepine, TRIP: tripalmitin.

3.3.4 FTIR

FTIR is an important tool to understand the molecular arrangement of LN when combined with other techniques, such as DSC and X-Ray diffraction. Tripalmitin spectra is characterized by mainly 3 important peaks, visible at 2849 cm^{-1} - 2913 cm^{-1} for the C–H stretching; at 1728 cm^{-1} for the C=O stretching, and at 1472 cm^{-1}

corresponding to the stretching of C–O (Vitorino *et al.*, 2011). Carbamazepine is present in polymorphic form III, which is characterised by the following main bands: at 3465 cm^{-1}(corresponding to the valence vibration of group -NH), at 1675 cm^{-1} for the -CO-R vibration, at 1605 cm^{-1} and 1594 cm^{-1} (for -C=C- and -C=O vibration and -NH deformation), 1383cm^{-1} and a weak band at 762 cm^{-1}. (Rustichelli *et al.*, 2000). The -NH band, located at a lower wave number relate the presence of intermolecular hydrogen bonds (MATSUDA *et al.*, 1994)

Chitosan spectra seems to be like a typical one, composed by three characteristic peaks, at 2913cm^{-1} for the O-H stretching, 1735cm^{-1} for the C=O stretching (amide I) and 1728cm^{-1} to the N–H bending and C–N stretching (amide II). It also shows peaks at 2896cm^{-1} for C-H stretching, and at 1419 cm^{-1} for N-C stretching. (Luo *et al.*, 2015). About NLC formulations spectra, all of them show a similar structure to tripalmitin, the solid lipid and the compound in higher amount. The major differences between pure compounds and NLC are the peak at 1472 cm^{-1} that corresponds to C-O stretching, present in all six formulations, and two sharp peaks at 2914-2915 cm^{-1} and 2849 cm^{-1} both for C-H stretching, which should correspond to the solid lipid (Tripalmitin). A broad, though negligible, band, between 3473 cm-^{1} and 3359 cm^{-1} is also present in chitosan coated LN. The latter also exhibit two peaks between 1596 cm^{-1} and 1559 cm^{-1}, corresponding to C=O stretching, as is present in tripalmitin. There are no peaks related to CBZ, which is in agreement with E.E. and DSC results, thus confirming that drug is mainly encapsulated.

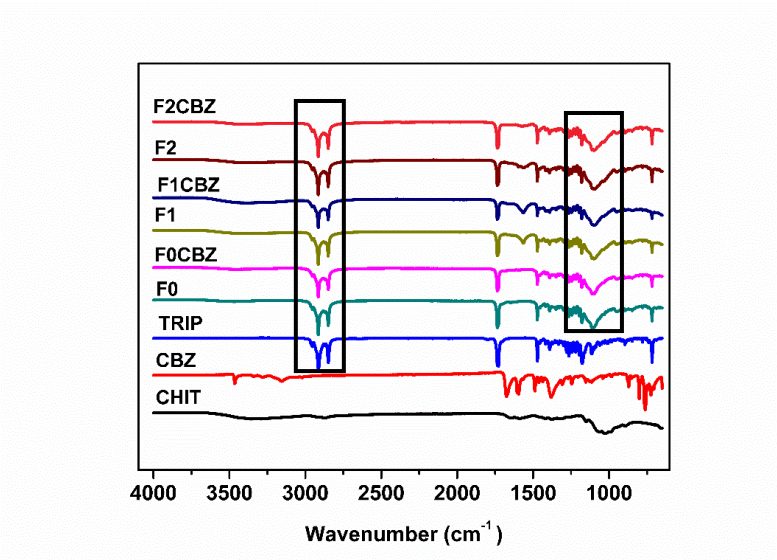

Figure 20: FTIR spectra of pure compounds (Tripalmitin, Carbamazepine and Chitosan), non-loaded formulations (F0, F1, F2) and CBZ loaded formulations (F0CBZ, F1CBZ, F2CBZ).

3.3.5 X-Ray Diffraction

X-Ray diffraction analysis allows to characterize the crystalline behaviour of the formulations in a deeply way. Indeed, as the DSC results suggested, CBZ is presented in its polymorphic phase III, since diffraction peaks appear at 2θ of 6.58°, 15.3°, 19.5°, 24.93° and 27.55° (Aparecida *et al.*, 2014). Tripalmitin and Chitosan peaks (at 2θ of 10.51° and 19.87°, respectively) (Nunthanid *et al.*, 2004) are also according to the literature. The formulation diffractograms are similar to the one corresponding to tripalmitin, as expected. However, the more expressive peak in F0 and F0CBZ is found at 2θ of 6.6°, whereas in F2 and F2CBZ, it is found at 2θ of 19.4°, for the arrangement of nanoparticle surface due to the presence of chitosan. Peaks corresponding to CBZ are not found in the formulation diffractograms, thus, confirming once again the tendency of the last results.

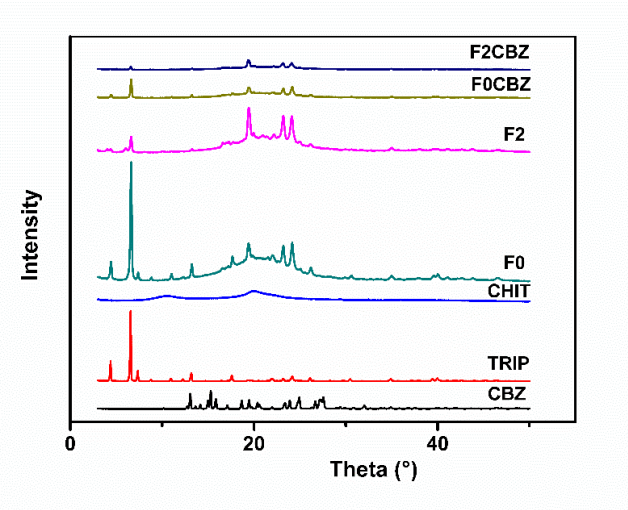

Figure 21: X-Ray spectra of pure compounds (Tripalmitin, Carbamazepine and Chitosan), non-loaded formulations (F0, F2) and CBZ loaded formulations (F0CBZ, F2CBZ). Key: CHIT: chitosan, CBZ: carbamazepine, TRIP: tripalmitin.

3.3.6 Morphology

Lipid nanoparticle formulations analysed by SEM are in agreement with PS values and respective distribution values of DLS findings, also showing different shapes and appearances (Figure 22). Indeed, in formulation F0, it is possible to note some the coalescence of the nanoparticles, a consequence of the lower stability found for this formulation through zeta potential determinations. In turn, the coated nanoparticles seem to be spherical with a homogeneous coating. Note that, comparing both

formulations, is once again possible to confirm the importance of the chitosan coating in the stability and particles characteristics, as described above.

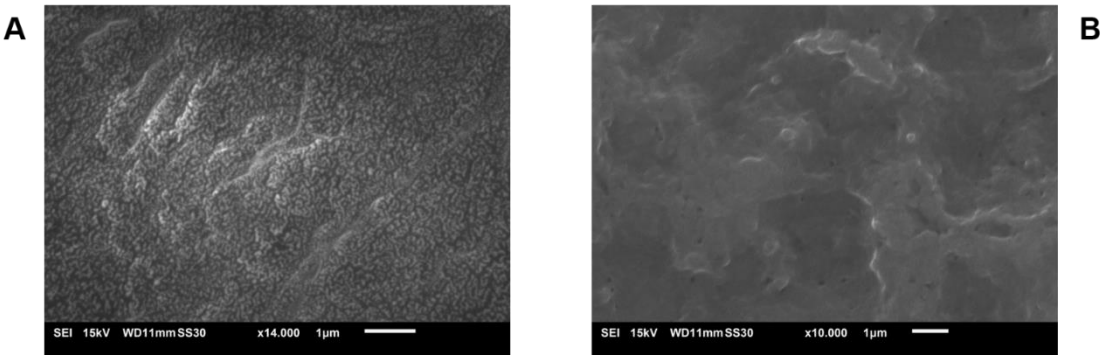

Figure 22: SEM images of F0 (A) and F2 (B) formulations.

3.3.7 Permeability and mucoadhesiveness studies

Drug intestinal absorption can be predicted through *in vitro* permeability studies, across mice small intestine incorporated in ussing chambers. The obtained results are depicted in figure 23 and evidence the substantial difference between both formulations. Indeed, the values of apparent permeability coefficient of non-coated LN formulation (F0) rounds $1x10^{-6}$ cm/s, with standard deviations lower than 0.23, whereas the chitosan coated LN formulation (F2) reached a maximum value of $120x10^{-6}$ cm/s, presenting a profile where the permeability increases during almost all the study time (with exception for 30 min and 180 min). Furthermore, the permeability values observed for F2 are significantly superior, with an enhancement ratio of 7.1 times higher (Table 10) than those reported in the literature, which rounded $20x10^{-6}$ cm/s (Fortuna *et al.*, 2012). Accordingly, a CBZ solution was used and the same methodology of Ussing Chambers was applied. The results herein found suggest that the presence of chitosan strongly increases the permeation of the drug, due to its mucoadhesiveness and penetration-enhancer properties. Chitosan strongly interacts with the epithelium, since it is positively charged, improving in this way the drug transport capacity (Oyarzun-Ampuero *et al.*, 2010). Additionally, chitosan is involved in intercellular tight junctions disruption, thus increasing the permeability of an epithelium (Smith, Wood e Dornish, 2004). For these reasons, it was predictable that F2 would present higher drug amount and better permeation. It is important to emphasize that the membrane integrity was monitored by measuring the TEER and the apparent permeability coefficient sodium fluorescein, a paracellular marker, which values were higher than 636 $\Omega.cm^{-2}$ for TEER and lower than 0.13 $x10^{-6}$ cm/s for sodium fluorescein.

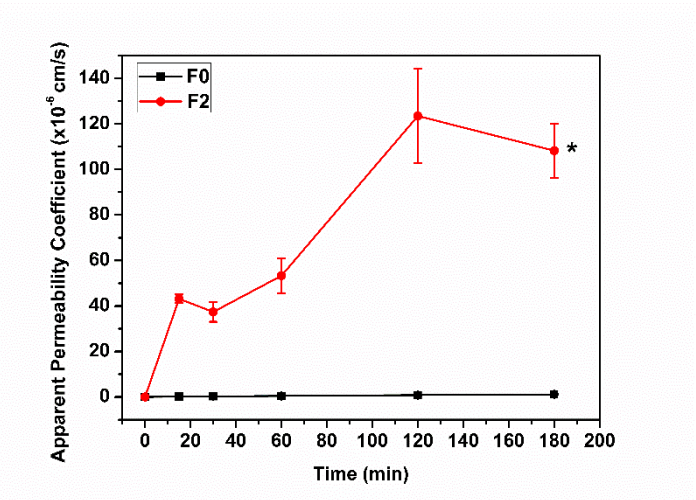

Figure 23: Apparent permeability coefficients for CBZ lipid nanoparticles non-coated (F0) and coated nanoparticles (F2). * F2 apparent permeability coefficient is statistically higher than F0, for a $p < 0.05$.

Table 10: Apparent permeability coefficient of F0, F2 and reference solution (Fortuna *et al.*, 2012) and respective enhancement ratios.

Formulation	Papp values (cm.s) CBZ (x10^{-6})	ER
F0	1.1±0.2	0.07
F2	108±20	7.1
Reference	15.2±3.18	1

Mucoadhesive behaviour of coated LN was evaluated and confirmed through the particle size after the interaction of LN (F0 and F2) with mucin (Figure 24A). It is seen that F2 presents higher PS than F0, which suggests a stronger interaction of mucin with hybrid NLC than with the corresponding uncoated. Note that F2 maintains the positive charge associated to the presence of chitosan (Figure 24B).

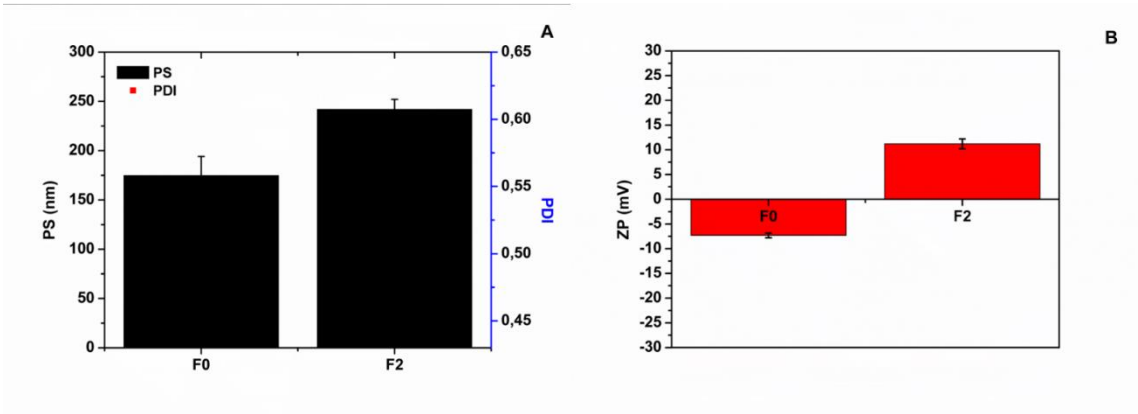

Figure 24: Particle Size (PS), Polydispersity Index (PDI) (A) and Zeta Potential (ZP) (B) of F0 and F2 after interaction with a mucin solution.

In addition, the intestinal fragment was submitted to a cytotoxicity assay, at the final of the Ussing Chamber permeability study. To this end, the MTT method was carried on, and cell viability was compared to that observed for mice membranes immediately after animal dissecation. MTT test results showed a cell viability ca. 100%, thus indicating that both formulations were not considered cytotoxic and present a favourable safety profile.

3.3.8 *In vivo* Study

The *in vivo* studies aimed at characterizing and understanding the pharmacokinetics of CBZ after its oral administration as F2 formulation. The previous *in vitro* studies, prompted us to administrate only the F2 formulation to mice, reducing, hence, the number of animals sacrificed as required by ethical issues involved, that requires less animals to experimentation (*Directive 2010/63/EU on protection of animals used for scientific purposes, caring for animals aiming for better science*, 2010).

The time-variations of plasma and brain concentrations of CBZ and its metabolite after F2 oral administration to mice are represented in Figure 25 and Figure 26; the corresponding pharmacokinetic parameters calculated are presented in Table 11. The results were compared with those reported in literature after a CBZ suspension had been administered to mice (Fortuna *et al.*, 2013). Since the administered doses were different for both studies (0.54 mmol/Kg in the present work and 1.4 mmol/Kg in the mentioned work), the pharmacokinetic parameters were normalized in order to be comparable. Considering the results of table 11, it is possible to admit a slower absorption of the encapsulated drug in relation to the non-encapsulated drug (t_{max} of 2h *versus* 0.5h). This supports a controlled release mediated by the F2 formulation,

probably associated to the lipid nanoparticles by their adhesion and absorption behaviour reproducibility, which is enhanced by the presence of lipids (Muchow, Maincent e Müller, 2008). Controlled release is also supported by the chitosan coating and its characteristics, such as mucoadhesive properties (Hejazi e Amiji, 2003). Moreover, it is also important herein to emphasise that the C_{max} values obtained in brain tissue after F2 oral administration for encapsulated-drug was almost twice of that of the non-encapsulated drug (43.89 µg/g versus 20.01 µg/g). It supports the already mentioned advantages of nanocarriers, especially lipid nanocarriers, since it is known that lipids promote the drug absorption (Muchow, Maincent e Müller, 2008). The results also suggest the existence of a brain targeting of CBZ, as evidenced by the estimated drug target index (DTI) – the ratio between the drug content in brain at a standard time after administration of targeting preparation and the drug content in the same organ, at the same standard time (in this case 24h was considered) after the administration of nontargeting preparation - is higher than 1, which indicates a brain-targeting distribution (Cai *et al.*, 2008). Moreover, drug selectivity index (DSI), which reflects the ratio of the drug content in the target organ (brain) by that one measured in the plasma, at a standard time (24h) is also higher for hybrid CBZ-NLC formulation than for the suspension (2.79 *versus* 0.74). This behaviour, along with the controlled release of F2, as extracted from the dissolution studies, and the high bioavailability reached are still supported by the mean residence time (MRT) values, once that in case of hybrid CBZ-NLC formulation this one was higher than the CBZ suspension, as described in table 11. Elimination half-time is also according with this last parameter, showing higher values for hybrid CBZ-NLC formulation. Regarding the CBZ-epoxide pharmacokinetic analysis, it is important to highlight the great reduction of $t_{1/2}$ (h) observed for this metabolite with F2, comparing with CBZ suspension (3.73 *versus* 12.52 for plasma and 0.14 *versus* 7.78 in brain), and consequently the reduction of MRT (6.75 *versus* 19.73 for plasma and 9.23 *versus* 12.78 in brain). This means that this CBZ metabolite, responsible for antiepileptic activity, but also for the major side effects, is present in human body during lesser time with hybrid NLC. In what regards the T_{max}, the values are similar for both formulations, the encapsulated and non-encapsulated CBZ (3h *versus* 4h). Likewise, no differences were observed between the corresponding results obtained in plasma and brain (Fortuna *et al.*, 2013).

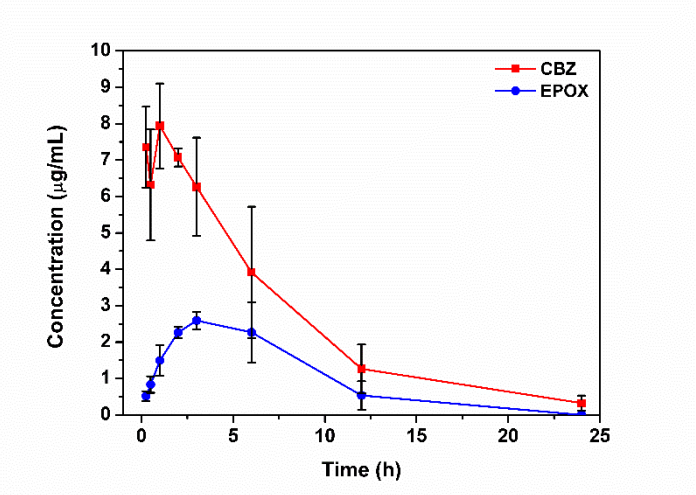

Figure 25: Concentrations of CBZ and EPOX in plasma mice during 24 hours. Results are expressed as mean and SEM (3≤n≤5). Key: CBZ: carbamazepine, EPOX: carbamazepine-10,11-epoxide.

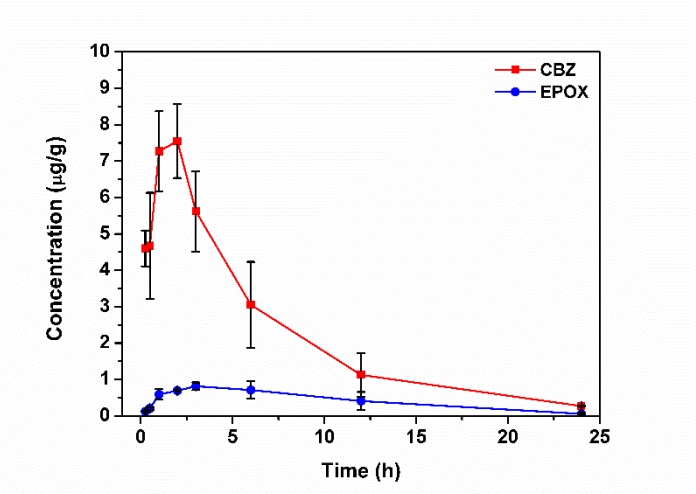

Figure 26: Concentrations of CBZ and EPOX in brain tissue of mice during 24 hours. Results are expressed as mean and SEM (3≤n≤5). Key: CBZ: carbamazepine, EPOX: carbamazepine-10,11-epoxide.

Table 11: Pharmacokinetic parameters obtained for CBZ containing F2 formulation (concentration of 0.54mmol/Kg), in comparison to CBZ solution (concentration of 1.4mmol/Kg) and the respective dose normalization values of F2 formulation.

Key: AUC0-inf, Area under the concentration time-curve from time zero to infinite; AUC0-last, Area under the concentration time-curve from time zero to the last measurable drug concentration; C_{max}, Maximum concentration; ke, Apparent elimination rate constant; MRT, Mean residence time; $t_{1/2}$, Elimination half-life; t_{max}, Time to achieve the maximum concentration; DSI, Drug selectivity index; DTI, Drug targeting index.

	Hybrid CBZ-NLC (F2)		**CBZ solution***	
	Plasma	**Brain**	**Plasma**	**Brain**
T_{max} (h)	2	0.5	0.5	2
C_{max}/Dose	15.704	-	27	-
C_{max} (µg/g)	-	43.89	-	20.01
AUClast/Dose	104.44	-	116.66	-
AUClast (µg*h/g)	-	92.96	-	101.27
AUCinf/Dose	108.33	-	128.71	-
AUCinf (µg*h/g)	-	93.52	-	106.48
AUC % Extrap (%)	3.59	0.60	9.36	4.89
Ke (h^{-1})	0.14	0.21	0.205	0.28
MRT (h)	6.96	4.69	4.85	3.86
DSI	2.79		0.74	
DTI	-	2.19	-	-

*The pharmacokinetic parameters used were based on previous *in vivo* studies (Fortuna *et al.*, 2013).

Other *in vivo* studies, involving intravenous (IV) administration of hybrid CBZ-NLC formulation were further carried out, in order to study the brain targeting of the drug, as suggested by the previous findings. The sampling time points were chosen considering the results obtained in the first study. Once that the T_{max} was 0.5h in brain tissue, and this was the focus of the study, it had a duration of only 1 h. Accordingly, it was not possible to quantify any drug in brain tissue, and the results of drug concentration in plasma also displayed a low concentration of CBZ. Note that the intravenous route requires the use of anaesthetics before the administration. Since

these drugs cause vasoconstriction, the drug distribution can be affected through its decreasing. Moreover, the drug targeting by IV route to support the drug targeting have some problems as the recognition of the injected foreign particles and their uptake by macrophages from the mononuclear phagocytic system (MPS)– previously reticuloendothelial system (RES).

Considering the hybrid CBZ-NLC previously characterized as release controlled system by release studies, it was found a drug release up to 20% for the first hour, what could mean that the concentration of drug released was lower than LCQ. Furthermore, chitosan is insoluble in basic pH, and consequently is insoluble in plasma pH (7.4) (Hejazi e Amiji, 2003), and stuck the drug in the lipid core.

3.4 Solid dosage form approach –hybrid NLC based Tablets

Solid dosage forms are an alternative for oral administration of the aqueous hybrid NLC dispersion, by its transformation in a solid powder. This process was performed by spray-drying, once that previous studies reported the several advantages of this method (Mendes *et al.*, 2016). Atomized powder was introduced in the tabletting powder in order to obtain hybrid-NLC based tablets. (Müller, Mäder e Gohla, 2000). This new approach will allow to increase the stability of the dosage form and of the nanoparticles, as well as to improve the patient compliance.

3.4.1 Spray-dried particles characterization

After atomization by spray-drying (Figure 27), hybrid NLC particle size increased and reached micro size range. Particles exhibited a size between 11 µm and 13 µm, with a span value of 3 (Table 12), which indicates that their distribution seems to be adequate, when compared with previous work developed (Mendes *et al.*, 2016), where similar values for this parameter were obtained. Regarding laser diffraction (LD) values of the distribution by number, they are in clear agreement with those obtained through DLS for the corresponding aqueous dispersions. SEM analysis (Figure 28) confirmed these size results obtained by LD, since by the images is possible to verify particles with ca. 10 µm of diameter. The latter technique also allows to characterize the morphology of hybrid NLC. They present a very well-defined spherical shape, revealing a homogeneous chitosan coating, and regular distribution. It also confirms the results obtained from DSC, and FT-IR, where no drug was evidenced in the external

phase, suggesting that it is encapsulated within the lipid matrix. The integrity of the hybrid LN after spray-dried process is also confirmed, as well as their solid state.

Moreover, in order to evaluate the potential mucoadhesive behaviour of the dried particles, mucoadhesivity tests were performed now using a texture analyser. However, the results obtained were not discriminatory, since the force required to detach nanoparticles from the defined surface, which is a measure of the adhesiveness was similar (0.14 Kg) for both F0 and F2, while the work of adhesion was 100 for F0 and 97 for F2. Such results could be ascribed to the lack of sensitivity of the method carried out under the specified conditions.

Figure 27: Atomized hybrid NLC powder obtained by spray-drying.

Table 12: Particle and respective distribution of formulations F2 and F2CBZ after dispersion in water.

	Distribution by % volume		**Distribution by % number**	
	Particle Size (µm)	**Span Value**	**Particle Size (µm)**	**Span Value**
F2	13 ± 3	3	0.104 ± 0.001	1.25
F2CBZ	11.4 ± 0.8	3	0.102 ±0.009	1.26

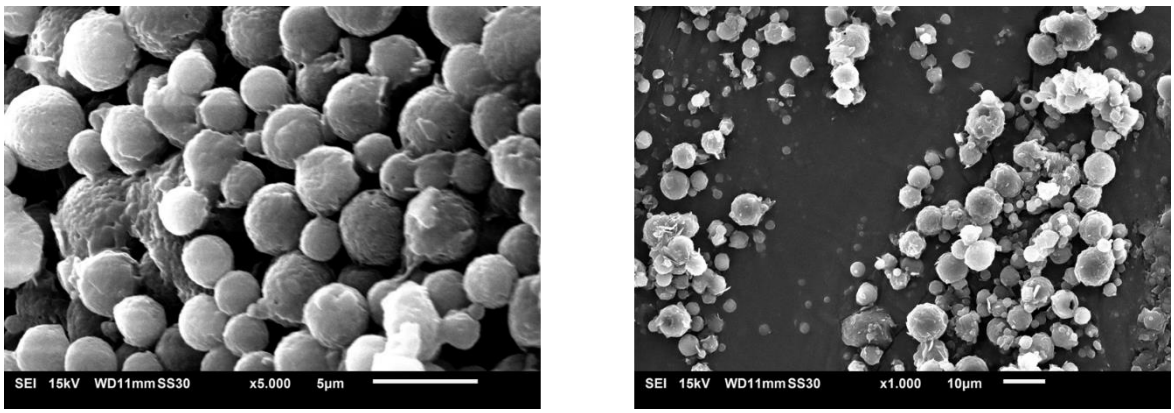

Figure 28: SEM images of spray-dried nanoparticles.

3.4.2 Dissolution studies

Finally, the dissolution studies were carried out with hybrid LN tablets, CBZ tablets with similar composition of hybrid LN tablets, where CBZ hybrid LN were replaced by lactose and CBZ (reference), and commercial controlled release tablets **(Figure 29)**. Through the profiles obtained and displayed in **figure 30,** the release rate can be ordered as follows: the lowest CBZ amount released was observed for the commercial tablets, which is consistent with the claimed prolonged release pattern; in an intermediate position, are found hybrid LN tablets, which still keeps some control over release, and finally the reference tablets. With the latter formulation all the drug is released after 2h in SGF. of the commercial tablets. It should be also noted that an approximate dissolution behaviour is observed between the liquid F2 formulation and the corresponding solid dosage form.

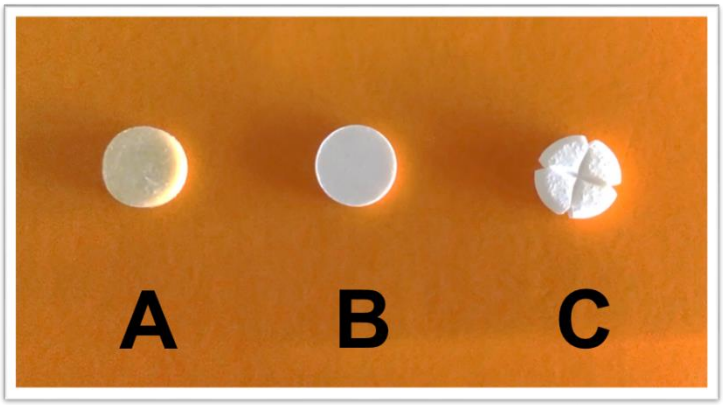

Figure 29: Tablets used for dissolution studies – hybrid NLC based tablet (A), Reference tablet (B) and commercial tablet (C).

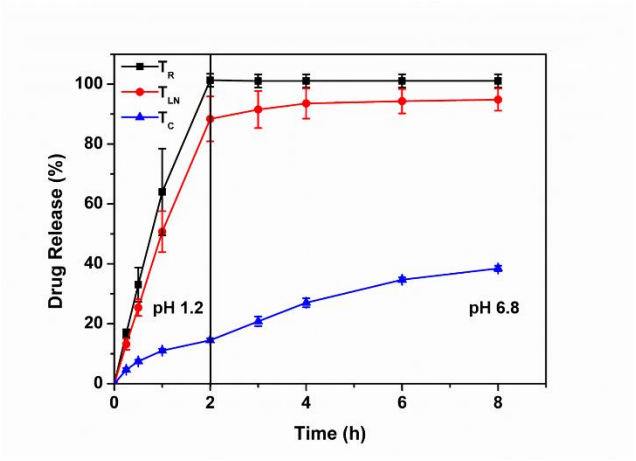

Figure 30: Release profiles of reference CBZ tablets (T_R), lipid nanoparticles tablets (T_{LN}), and commercial tablets (T_C).

Table 13: Regression parameters resulting from the application of the different mathematical functions to dissolution profiles.

Function	Formulations	c_1	c_2	c_3	R^2
Zero order $c_1 t$	T_R	18.4912 ±3.60224	-	-	0.57432
	T_{LN}	17.0104 ±3.03027	-	-	0.63428
	T_C	5.61331 ±0.403788	-	-	0.95843
First Order $c_1(1-\exp(-c_2 t))$	T_R	104.512 ±3.85193	0.968873 ±0.133260	-	0.98393
	T_{LN}	97.8028 ±3.84804	0.800106 ±0.109382	-	0.98538
	T_C	47.1560 ±5.41573	0.212271 ±0.0439024	-	0.98879
Higuchi $c_1 t^{0,5}$	T_R	46.4477 ±4.25472	-	-	0.80168
	T_{LN}	42.2253 ±0.295916	-	-	0.84222
	T_C	13.0525 ±0.480007	-	-	0.98265

Weibull $c_1(1-\exp(-c_2t^{c3}))$	T_R	101.946 ±1.69874	1.04901 ±0.0591788	1.44506 ±0.135226	0.99688
	T_{LN}	94.4653 ±1.55948	0.886630 ±0.0469383	1.44116 ±0.117981	0.99744
	T_C	132.392 ± 252.009	0.0280467 ± 0.099801	0.696822 ±0.0231878	0.99263
Kormeyer-Peppas c_1t^{c2}	T_R	64.9378 ±0.0000082	0.973897 ±0.0000001	-	1
	T_{LN}	50.6766 ±0.312833	0.984520 ±0.0142425	-	0.99993
	T_C	10.7014 ±0.671866	0.630552 ±0.0368133	-	0.99245

For a better understanding of the drug release mechanism, dissolution profiles obtained were submitted to a fitting with different mathematical models and the respective values obtained for the fitting with the mathematical models are shown in table 13. Korsmeyer-Peppas, followed by Weibull were the functions that provided the best regression. Relying on Korsmeyer-Peppas fitting that allows to characterize the type of drug transport mechanism by c_2 parameter, it can be applied a zero-order model for reference tablets and LN tablets, once that c_2 is close to 1 (Peppas, 2014). This model considers that the pharmaceutical dosage forms release the same amount of drug by unit of time. It is used to describe the drug dissolution of some pharmaceutical dosage forms with modified release, such as matrix tablets with low soluble drugs (Costa e Lobo, 2001). The commercial tablets are characterized by a non-Fickian diffusion process, associated to an anomalous drug transport. This model is commonly applied to modified release dosage forms relying on use of polymers (Peppas, 2014), confirming the modified release behaviour of the commercial tablets.

Concluding remarks

The work developed in this dissertation aimed at developing polymer-lipid hybrid nanoparticles, specifically, chitosan coated lipid nanoparticles, for oral delivery of carbamazepine, as antiepileptic model drug. After the optimization of the formulation, hybrid NLC were evaluated, in terms of physicochemical properties, *in vitro* release, cytotoxicity, permeability and *in vivo* performance.

The preparation of hybrid NLC was performed by hot HPH process, and lipids and surfactants were properly selected considering the CBZ solubility in these compounds. The suitable screening of formulation compounds led to the efficient entrapment of CBZ, reflected by the high values of entrapment efficiency and drug loading. The drug entrapment was also confirmed by morphology, crystallinity and structure analysis.

Surface modification of lipid nanoparticles with chitosan coating had as purpose to increase the stability in gastric medium, obtain a controlled drug release, increase bioavailability and enhance the absorption rate, because of its mucoadhesive characteristics. *In vitro* studies suggested a controlled drug release, while permeability studies confirmed the absorption enhancing effect by the high rates achieved.

The improved CBZ absorption rate was supported by *in vivo* studies, whose results also suggested a brain drug targeting by the high concentration of CBZ determined in the brain tissue and the analysis of DTI and DSI parameters. However, this behaviour was not possible to verify with intravenous administration of the formulation, since the tests carried out were not conclusive.

Cytotoxicity studies, as expected because of the use of physiological lipids and a biocompatible, biodegradable and non-toxic polymer (chitosan), showed that the formulation was not toxic for cells.

In this way, it was possible to infer that chitosan coated lipid nanoparticles are an interesting nanosystem for the CBZ delivery in order to improve its bioavailability.

The spray-drying of the optimized hybrid lipid nanoparticles formulation was performed, in order to increase the stability and obtain an intermediate form to produce solid dosage forms, like tablets. Dissolution studies of the hybrid LNC tablets were not totally conclusive, but it was possible to observe that it still keeps some control over release.

The developed work and the main achievements may provide a promising basis to further pre-clinical studies in the treatment of epilepsy.

Future work

As future work, it is desirable to repeat the inconclusive tests, such as the *in vivo* studies via IV, in order to verify the brain targeting effect promoted by the hybrid CBZ-NLC, since this confirmation is essential to prove the potential of this formulation and support the results that were found for oral administration. Moreover, other pre-clinical studies, involving, for example, multiple drug administrations and pharmacodynamic endpoints.

From a technological point of view, the preliminary tablets here presented should be subject of an optimization process, in order to obtain better control over carbamazepine release, supported on a deeper characterization of the spray-dried nanoparticles properties (e.g. flow and mucoadhesiveness).

In what concerns the mechanisms involved in the intestinal absorption, cell culture and fluorescence studies, using e.g. dye-loaded nanoparticles to monitor the uptake pathways could be performed to provide additional information.

These complementary studies could help to refine the developed formulation, which already shows to be interesting. This could be considered an opportunity to improve the epilepsy treatment, thus minimizing the commercial therapeutic issues and offering a better quality of life to the patients.

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