INSTITUTO SUPERIOR DE ENGENHARIA DO PORTO

MESTRADO EM BIORRECURSOS

Revisão sobre nanopartículas lipídicas para a encapsulação de extratos e compostos neuroprotetores

FILIPE JORGE RIBEIRO FERNANDES

Novembro de 2020

instituto superior de engenharia do porto

2019/2020

Review on lipid-based nanoparticles for the encapsulation of neuroprotective extracts and compounds

Master's in Bioresources

Supervision: Professor Cristina Delerue-Matos, PhD

Doctor Clara Grosso, PhD

Professor Doctor Mónica Teixeira, PhD

Dissertação submetida como requisito parcial para a obtenção do grau de Mestre em Biorrecursos

Author:

Filipe Jorge Ribeiro Fernandes, n° 1150615

03/11/2020

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porto

2019/2020

Acknowledgments

To Professor Cristina Matos, Dr. Clara Grosso and Professor Mónica Teixeira for all the help and availability in the guidance of my thesis.

To my colleagues for all the moments and memories shared through these 5 years.

To my family for all the help provided during my academic path.

To my girlfriend, for all the support and motivation provided, to help me persevere during this complicated period of pandemic.

This Thesis was developed under the framework of the EU and FCT funding through the project PTDC/OCE-ETA/30240/2017- SilverBrain - From sea to brain: Green neuroprotective extracts for nanoencapsulation and functional food production (POCI-01-0145-FEDER-030240).

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Abstract

The biggest obstacle to the treatment of diseases that affect the central nervous system (CNS) is the passage of drugs across the blood-brain barrier (BBB), a physical barrier that regulates the entry of substances into the brain and assures the CNS's homeostasis.

In this dissertation, a review was done on the lipid-based nanoparticles for the nanoencapsulation of neuroprotective compounds, thus increasing their bioavailability and capacity to cross the BBB.

The gastrointestinal absorption was also discussed, as it may pose some obstacles into the absorption of the neuroprotective compounds into the bloodstream. The BBB's composition was explained, as well as the transport mechanisms through which the compounds may cross into the brain parenchyma.

A survey on the different types of lipid-based nanoparticles - nanoemulsions (NEs), nanoliposomes / nanophytosomes and solid lipid nanoparticles (SLNs) / nanostructured lipid carriers (NLCs) - was performed in this thesis, giving particular emphasis for their unique characteristics, the most important parameters influencing the formulation of each one, and some examples of studies that have been carried out in the encapsulation of neuroprotective compounds with the use of these nanoparticles.

Keywords: Blood-brain barrier, central nervous system, lipids, nanoparticles, nanoemulsions, nanoliposomes, nanophytosomes, natural products, solid lipid nanoparticles, nanostructured lipid carriers

Resumo

O maior obstáculo ao tratamento de doenças que afetam o sistema nervoso central é a passagem do medicamento através da barreira hematoencefálica, uma barreira física que regula a entrada de substâncias para o cérebro e assegura a homeostasia do sistema nervoso central.

Nesta dissertação foi feita uma revisão bibliográfica sobre nanopartículas baseadas em lípidos para a nanoencapsulação de compostos neuroprotetores, aumentando a sua biodisponibilidade e capacidade de atravessar a barreira hematoencefálica.

Foi feita uma abordagem à absorção gastrointestinal, que pode ser um obstáculo à absorção dos compostos neuroprotetores para a corrente sanguínea. A composição da barreira hematoencefálica foi descrita, assim como os mecanismos de transporte através dos quais os compostos neuroprotetores atravessam a barreira para o parênquima cerebral.

Os diferentes tipos de nanopartículas lipídicas, nanoemulsões, nanolipossomas / nanofitossomas, nanopartículas lipídicas sólidas / transportadores lipídicos nanoestruturados, foram abordados. Foi feita uma descrição de cada tipo de partícula, os parâmetros mais importantes na formulação das mesmas e uma compilação de alguns estudos que foram realizados sobreo encapsulamento de compostos neuroprotetores recorrendo a este tipo de nanopartículas.

Palavras-chave: Barreira hematoencefálica, lípidos, nanopartículas, nanoemulsões, nanolipossomas, nanofitossomas, nanopartículas lipídicas sólidas, produtos naturais, sistema nervoso central, transportadores lipídicos nanoestruturados

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Abbreviations

16:0 PEG-2-PE - 1,2-Dipalmitoylsn-glycero-3-phosphoethanolamine-N-[methoxy (poly (ethylene glycol)-2000]

2000PEG-DSPE - N-carbamyl-poly-(ethylene glycol methyl ether)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine triethyl ammonium salt

AAS – Atomic Absorption Spectroscopy

AFM – Atomic Force Microscopy

AMT - Adsorptive Mediated Transcytosis

BACE1 siRNA – Beta-site APP-cleaving enzyme 1 small interfering RNA

BBB - Blood-Brain Barrier

BBD - Box-Behnken Design

BHT – Butylhydroxytoluene

Chol - Cholesterol

CLSM – Confocal Laser Scanning Microscopy

CNS – Central Nervous System

CO_2 – Carbon Dioxide

DBV - Double Bilayer Vesicle

DCM – Dichloromethane

DHDP – Dihexadecyl Phosphate

DLS – Dynamic Light Scattering

DOTAP - (N-[1-(2, 3-dioleoyloxy) propyl]-N, N, N-trimethylammoniumsalt)

DPPC – Dipalmitoylphosphatidylcholine

DPPH - 2,2-diphenyl-1-picrylhydrazyl

DSC – Differential Scanning Calorimetry

DSPC - 1,2-distearoyl-sn-glycerol-3-phosphatidylcholine

DSPE-PEG2000 - 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000]

DSPE-PEG2000-Biotin - 1,2-distearoyl-sn-glycerol-3-phosphoethanolamineN-[biotinyl (polyethyleneglycol)-2000]

DSPE-PEG-maleimide - 1,2-stearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(-poly(ethylene glycol)-2000)]

E – Extract

ECs - Endothelial Cells

EE – Encapsulation Efficiency

EFSA - European Food Safety Authority

ELISA – Enzyme-Linked Immunosorbent Assay

EMSA – Electrophoretic Mobility Shift Assay

EPR – Electron Paramagnetic Resonance

EtOH – Ethanol

EU – European Commission

FCM – Flow Cytometry

FDA – Food and Drug Administration

FITC – Fluorescein Isothiocyanate

FLM – Fluorescence Microscopy

FLS – Fluorescence Spectroscopy

FMPR – Fluorescence Microplate Reader

Folin – Folin-Ciocalteu reagent

FRAP – Ferric Reducing Antioxidant Power

FTIR – Fourier-transform Infrared Spectroscopy

GC – Gas Chromatography

GIT - Gastrointestinal Tract

GLUT-1 - Glucose Transporter Isoform

H-NMR – Proton Nuclear Magnetic Resonance

HPLC – High-Performance Liquid Chromatography

HPLC-DAD – High-Performance Liquid Chromatography coupled to Diode Array Detection

HPLC-FLD – High Performance Liquid Chromatography with Fluorescence Detection

HPTLC – High-Performance Thin Layer Chromatography

HSPC – Hydrogenated Soy Phosphatidylcholine

IgG – Immunoglobulin G

LAT - Large Amino acid Transporter

LC-MS – Liquid Cromatography–Mass Spectrometry

LUV - Large Unilamellar Vesicle

mal-PEG-PE - 1,2-stearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(poly(ethylene glycol)-2000)]

MAO-A – Monoamine oxidase A

MCT – Medium-chain Triglycerides

MetOH – Methanol

mPEG-OH - Methoxypolyethylene glycol

mPEG-MAL - Methoxypolyethylene glycol maleimide

MVV - Multivesicular vesicle

NEs – Nanoemulsions

NLCs – Nanostructured Lipid Carriers

NP – Nanoparticle

O/W – Oil-in-Water

O/W/O – Oil-in-Water-in-Oil

OX-26 – Anti-Transferrin Monoclonal Antibody

PA – Phosphatidic Acid

PBS – Phosphate Buffer Solution

PC – Phosphatidylcholine

PCS – Photon Correlation Spectroscopy

PE - phosphatidylethanolamine

PE-PEG - 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000]

PEG - Poly(ethylene glycol)

PEG2000 PE - 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]

PEG-400 – Polyethylene glycol 400

Phm – Photomicroscopy

PP – Phospholipid

PS - phosphatidylserine

PXRD – Powder X-Ray Diffraction

QbD – Quality by Design

RA – Rosmarinic Acid

RMT - Receptor Mediated Transcytosis

ROS – Reactive Oxygen Species

SCF – Supercritical Fluids

SEDS - Solution Enhanced Dispersion by Supercritical Fluids

SEM – Scanning Electron Microscopy

siRNA – Small interfering RNA

SLNs – Solid Lipid Nanoparticles

SMF 111 - cis,trans,cis $[PtCl_2\{O_2C(CH_2)_4CH_3\}_2(cis\text{-}1,4\text{-}diaminocyclohexane)]$

SMF 144 - cis,trans,cis$[PtCl_2\{O_2C(CH_2)_8CH_3\}_2(cis\text{-}1,4\text{-} diaminocyclohexane)]$

SMF 196 - cis,- trans,cis$[PtCl_2\{O_2CCH_3)_2CH_3\}_2(cis\text{-}1,4\text{-}diaminocyclohexane)]$

SMF 200 - cis,trans,cis$[PtCl_2\{O_2C(CH_2)_2CH_3\}_2(cis\text{-}1,4\text{-}diaminocyclohexane)]$

S_{mix} – Surfactants mixture

SUV – Small Unilamellar Vesicle

T – Temperature

TEER - Transendothelial Electrical Resistance

TEM – Transmission Electron Microscopy

TGA – Thermogravimetric Analysis

THF – Tetrahydrofuran

UHPLC – Ultra-High-Performance Liquid Chromatography

UHPLC-DAD – Ultra-High-Performance Liquid Chromatography coupled to Diode Array Detection

ULV - Unilamellar Vesicles

UPLC – Ultra Performance Liquid Chromatography

UPLC-PDA – Ultra Performance Liquid Chromatography coupled with Photodiode Array Detector

UV-Vis – Ultraviolet-visible spectroscopy

VDDS - Vesicular Drug Delivery Systems

W/O – Water-in-Oil

W/O/W – Water-in-Oil-in-Water

W_F – Amount of free bioactive

W_T -Total amount of bioactive compound

XPS – X-Ray Photoelectron Spectroscopy

XRD – X-Ray Diffraction

1. Introduction

Neurological disorders are the leading cause of disability and the second highest cause of death worldwide, with the numbers being expected to rise in the next decades. This has led to an increase in both the number of deaths and the years lived with disability (Feignin *et al.*, 2020). The main obstacle in treating these disorders is the administration of pharmaceuticals to the brain parenchyma. Three direct routes can be considered: delivery across the blood-brain barrier (BBB), intranasal delivery (which bypasses the BBB via direct absorption through the olfactory and trigeminal nerves) and intrathecal delivery (to the cerebral spinal fluid), of which delivery across the BBB is the most studied and appealing due to its minimal invasive nature (Copeland & Stabenfeldt, 2020).

Encapsulation can be defined "as the technology of encasing bioactive compounds in solid, liquid or gaseous states in matrices, which can be released under particular circumstances with a controlled rate". Recent research has shown different material properties and reactions at the nanoscale, which resulted in the evolution of research towards nanoencapsulation. These nanoencapsulates generate higher bioavailability, solubility and permeation and a better interaction due to a higher surface area. They also allow for a targeted release and protection against process conditions and environmental stresses (Jafari, 2019).

Nanoencapsulation is, by definition, the encapsulation of materials in the nanoscale, below 1 μm in size. The regulatory authorities have, in recent years (Food and Drug Administration (FDA) in the United States, European Union (EU) commission in Europe), introduced regulatory definitions for nanoparticles (NPs) and nanomaterials. These definitions are not uniform, they are enforced differently across different sectors. When it comes to food applications, the European Food Safety Authority (EFSA) defines NPs as an engineered nanomaterial, with a size between 1 and 100 nm. If such a material is present in a certain product, it must go through an extensive and thorough risk assessment to determine if it is non-hazardous (Jafari, 2016; Quintanilla-Carvajal *et al.*, 2009; Uskoković, 2008). In terms of pharmaceutical science, particles up to 1000 nm can still be classified as nanoparticles due to their unique physicochemical properties when compared to bulk materials (Li *et al.*, 2015).

Some of these materials, such as fish oils, essential oils or some other nutraceuticals have particularly unpleasant flavours that limit their addition to food formulations. Nanoencapsulation can be useful in masking unwanted flavours in foods (Ghorbanzade *et al.*, 2016).

Working with plant-derived natural compounds poses some challenges, such as low stability and low bioavailability, chemical degradation during storage, high molecular weight/size, poor plasma membrane permeability and sensitivity to ultraviolet light and oxygen. Nanocarrier systems can be useful ways to improve the delivery of bioactive molecules present in plants, such as antioxidants, vitamins, fatty acids, minerals, phenolic compounds, carotenoids and essential oils (Bayraktar *et al.*, 2017). Many of these compounds are hydrophobic or poorly soluble, which is another problem that can be addressed using nanoencapsulation (Rezaei *et al.*, 2019).

Nanoencapsulation has been the subject of a variety of studies in the last few years, thus resulting in the development of various techniques for the encapsulation of different carriers. These include nanoemulsions (NEs), nanostructured lipid carriers (NLCs), nanosuspensions, solid-lipid nanoparticles (SLNs), nanosized liposomes, biopolymer NPs and micelles made of proteins, polysaccharides, along with their complexes or conjugates (Jafari, 2016).

Jafari (2017) classified nanoencapsulation technologies into five different groups, based on the main mechanism/ingredient. These five groups are then divided into sub-groups, as following:

- Lipid-based nanoencapsulation techniques:
  - Encapsulation by nanoemulsions (NE)
  - Encapsulation by nanoliposomes/ nanophytosomes
  - Encapsulation by nanostructured lipid carriers (NLC)

- Nanoencapsulation techniques based on natural nanocarriers
  - Nanocapsule formation by caseins
  - Nanocapsule formation by cyclodextrins
  - Nanocapsule formation by amylose nanostructures

- Nanoencapsulation techniques based on specialized equipment
  - Nanocapsule formation by electrospinning
  - Nanocapsule formation by electrospraying
  - Nanocapsule formation by nanospray dryer

- Nanoencapsulation technologies based on biopolymer nanoparticles
  - Nanocapsule formation by individual biopolymers nanoparticles
    - Protein nanoparticles
    - Carbohydrate nanoparticles
    - Chitosan nanoparticles
    - Poly-(lactic-co-glycolic acid) nanoparticles
  - Nanocapsule formation by complexation of biopolymers
  - Nanoencapsulation by protein nanotubes
  - Nanoencapsulation by carbohydrate nanogels
- Other nanocapsule technologies
  - Nanocapsule formation by nanocrystals

Lipid-based techniques have been extensively studied and developed for a wide variety of applications. They allow for the entrapment of bioactive compounds with diverse solubilities, separate or simultaneous encapsulation of hydrophobic and hydrophilic compounds, scalable production, and targetability in the human body or food matrices (Rostamabadi *et al.*, 2019). Other nanocarriers, composed of other building blocks, namely carbohydrates and protein-based, have a very high difficulty associated to the industrial scaling up due to the usage of intricate chemical and heat processes that cannot be completely monitored. Besides, an availability of digestible lipids aids in the intestinal absorption of the bioactive compounds (Akhavan *et al.*, 2018; Katouzian *et al.*, 2017).

Therefore, this thesis will focus on lipid-based nanoencapsulates.

1.1. Gastroinstestinal absorption of nanoparticles

Lipid digestion takes place in the gastrointestinal tract (GIT). It is a complex combination of biochemical and physiochemical processes. Absorption can happen either by enterocyte-based transport or via intestinal lymphatic system. The lymphatic system has an extensive network throughout the body and thus allows for the avoidance of a first-pass metabolism and the targeting of certain diseases known to spread through the lymphatics, such as lymphomas and HIV (Kalepu *et al.*, 2013). Some orally administered drugs display low bioavailability due to a pre-systemic, or first-pass, metabolism. This can happen in both the intestinal mucosa and the liver. Any foreign molecule absorbed in the small or large intestine must pass through the liver via the hepatic portal vein before gaining access to other parts of the body. This occurs because the drug absorbed in the GIT enters the portal circulation before entering the systemic circulation. Via the portal circulation it enters the liver where undergoes extensive biotransformation leading to a decrease of drug concentration that after reach the bloodstream (Thummel *et al.*, 1997).

Digestion starts in the mouth, with food being mechanically broken down to smaller pieces. Further mechanical processes lead to the formation of a coarse emulsion. The main lipid digestion is carried out by pancreatic lipases within the duodenum, the upper part of the small intestine, especially for liposomes and phytosomes, because gastric lipases have no activity on phospholipids (Liu *et al.*, 2020).

Lipases adsorb onto the surface of the formed emulsions and hydrolyse the outer ester bonds of the triglycerides, leading to the formation of a monoglyceride and two free fatty acids (Salentining, 2019). These are solubilized by bile salts, biliary-derived phospholipids and cholesterol and generate a series of colloidal structures such as micelles and unilamellar and multilamellar vesicles. The encapsulated compound is resolubilized into these structures, substantially increasing the drug solubilization capacity of lipid-based nanoparticles compared to the free drug (Chakraborty *et al.*, 2009; Kalepu *et al.*, 2013). The extremely fine particle size of the micelles allows them to permeate and be absorbed between cells through membrane internalization and lymphatic transportation (Liu *et al.*, 2020).

Iwanaga *et al.* (1999) studied the effects of coating a liposome’s surface on the gastrointestinal transit of insulin in male Wistar rats. The liposomes were coated with poly(ethylene glycol) (PEG) or the sugar chain of mucin, with increased resistance to bile salts, and the authors observed that the coating increased their overall stability in the gastrointestinal tract. The results also indicated that the behaviour of the coated liposomes is highly dependent on the properties of the coating materials, with PEG coated liposomes being retained longer in the small intestine and mucin coated liposomes being retained longer in the stomach. The PEG coated liposomes displayed a 2-fold increase in mean retention time compared to the other types of liposomes (mucin coated and uncoated). Mucin coated liposomes displayed the highest recovery ratio of insulin (80.71 ± 5.29) and PEG coated and uncoated liposomes had lower ratios (64.54 ± 1.19 and 52.57 ± 4.67, respectively). These results showed that surface coating of nanoparticles can lead to an increase in the bioavailability of orally delivered peptides.

Liu *et al.* (2012) studied the stability of liposomes and nanoliposomes prepared from milk and soybean-derived phospholipids during digestion. During digestion in simulated gastric fluid there was no significant change in the diameter of the NPs. However, for simulated intestinal fluid, there was a marked increase in diameter (approximately 400 to 1000 nm for milk derived liposomes and 800 to 1700 nm for soybean derived liposomes; 75 ± 13 to 114 ± 14 nm for soybean nanoliposomes and from 87 ± 15 to 152 ± 5 nm for milk nanoliposomes). The structure of the liposomes was observed with confocal laser scanning microscopy (CLSM) before and after digestion, and a clear degradation was observed. A large proportion of liposomes, initially smooth and spherical in shape, were elliptical or irregular in shape. For the nanoliposomes similar results were obtained, with the particles changing from smooth surfaces and spherical shapes to rough surfaces and irregular structures after digestion. Lastly, the leakage of calcein from the NPs was assessed in simulated gastric fluid. The soybean liposomes and nanoliposomes released 8 and 12 % calcein, respectively, while the milk liposomes and nanoliposomes released 6 and 8 % calcein, respectively. For simulated intestinal fluid, significantly higher releases were visualized, with all formulations displaying significant calcein release during the first 20 min and the release rate increasing gradually to about 75% after 240 minutes.

Liu *et al.* (2013) developed a polyelectrolyte delivery system based on alginate and chitosan which was used to coat the surface of nanoliposomes. The coating increased the mean diameter of the NPs from 89.3 ± 11.8 nm to 330.6 ± 37.3 nm, the polydispersity index from 0.26 ± 0.05 to 0.37 ± 0.12 and the zeta potential decreased from – 6.34 ± 0.62 to -15.79 ± 0.70. The changes in simulated gastric fluid were negligible, while in simulated intestinal fluid, the particle size of the polyelectrolyte delivery system increased from 335 to 620 nm over the first 15 minutes, and eventually decreased to 530 nm at the end of digestion. This can be explained by a decrease in the number of cationic groups, which results in a decrease in electrostatic interactions between alginate and chitosan, with the medium being able to enter the particles and increasing particle size. A subsequent decrease can be explained by an affinity of chitosan for ions in bile salts. The coating also resulted in a lower amount of medium-chain fatty acids released in simulated gastric fluid, 13.8% for uncoated nanoliposomes and 13.1% for the polyelectrolyte delivery system, after 15 minutes, and 29.8% and 20.4% after 120 minutes, respectively. In simulated intestinal fluid, the release rate, after 120 minutes, was 79.5% for uncoated nanoliposomes and 56.9% for the polyelectrolyte delivery system.

The exchange of molecules between the blood and the brain is limited by the blood-brain barrier (BBB) and controlled by endothelial transport systems in the brain (Ding *et al.*, 2020).

2. Blood-Brain Barrier

The Blood-Brain Barrier (BBB) is a complex cellular network, responsible for limiting the free diffusion and penetration of unwanted drugs or other compounds from the bloodstream to the brain while providing the needed nutrients for proper brain functioning (Bhowmik & Ghosh, 2015; Sánchez-Navarro *et al.*, 2017).

It is composed by three main components: endothelial cells, pericytes and astrocytes, along with some other components, such as basement membranes and microglia, represented in Figure 2.1. The endothelial cells (ECs) lining the cerebral blood vessels are the basic building blocks of the BBB, connected by tight, adherens and gap junctions. This grants them great resistance (transendothelial electrical resistance (TEER) of 100-150 $\Omega.cm^2$ in human cells (Pinheiro *et al.*, 2020)) and restricts transport across the barrier. They are also fundamentally different from other ECs located in different parts of the body, containing as much as six times more mitochondria per capillary section, which is thought to be necessary to provide energy required for active transport across the BBB. The pericytes envelop the ECs, determining the permeability of the BBB and some of its functions, such as the strengthening of tight junctions, specific gene expression and polarization of astrocytes end-feet. The pericytes play a key role in the regulation of development and maintenance of the BBB. The astrocytes completely cover the cerebral blood cells with their end-feet and contain several proteins, indispensable to the proper functioning of the BBB. They also link up the ECs with microglia and neurons. The basement membranes, a complex layer of extracellular matrix proteins, provide an anchor for the cellular components of the BBB. The microglia are monocyte lineage cells, with two main functions: immune defense and CNS maintenance. The tight junctions, formed by several transmembrane and cytoplasmic proteins, are mainly responsible for regulating endothelium permeability, cell polarity and leukocyte migration. The adherens junctions are formed by transmembrane glycoproteins, cadherins, and allow for the formation of the tight junctions and the maintenance of the BBB characteristics. The gap junctions are located between the other two junctions and allow for the transfer of ions and small molecules between ECs, an essential step for maintaining tissue homeostasis. Gap junctions also transduce metabolic signals and regulate BBB permeability by interacting with scaffolding proteins (Copeland & Stabenfeldt, 2020; Pandit *et al.*, 2019; Ding *et al.*, 2020; Harilal *et al.*, 2020).

The BBB is integral to the proper functioning of the brain, serving a number of functions, namely (Harilal *et al.*, 2020):

- Brain nutrition
- Regulation of ionic composition
- Regulating the entry of macromolecules into the brain
- Protection against neurotoxins
- Regulation of neurotransmitters

It has been estimated that the BBB can exclude up to 98% of the small-molecule available drugs from entry into the brain, while only approximately 0.1% of intravenously administered therapeutic antibodies enter the brain. This demands a significantly higher concentration to be administered, which can in turn lead to systemic toxicity (Pandit *et al.*, 2019).

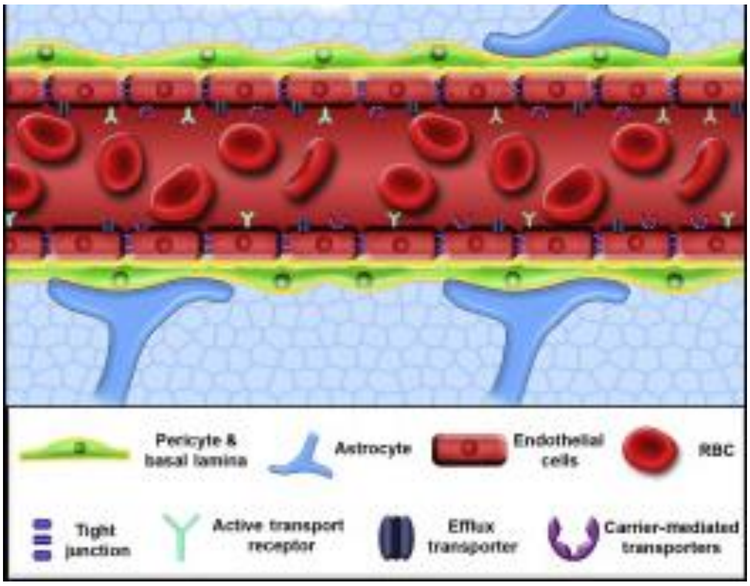

Figure 2.1 - Key components of the blood-brain barrier. Retrieved from (Copeland & Stabenfeldt, 2020)

Despite the BBB acting as a barrier for the passing of molecules between the blood and the brain parenchyma, there are a few transport routes available to allow for the delivery of molecules essential to the maintenance of the brain's homeostasis. These include diffusional transport (paracellular and transcellular transcytosis), transporter proteins mediated transcytosis, receptor-mediated transcytosis, adsorptive mediated transcytosis and cell-mediated transcytosis (Chen & Liu, 2012; Ding *et al.*, 2020). These mechanisms are represented in Figure 2.2.

Paracellular diffusion (Figure 2.2a) is the passage of small water-soluble molecules (molecular weight <500 Da) through a space between two ECs. The negative concentration gradient from blood to brain is the driving force for this transport. Transcellular diffusion (Figure 2.2b) is the diffusion of solute particles through the ECs. The particles transported through this route are small lipid soluble substances that penetrate through the cells by dissolving in their lipid plasma. The driving force for this transport is the same as for paracellular diffusion. Both types of transport are non-specific approaches (Chen & Liu, 2012; Ding *et al*., 2020).

An active transport mechanism is the transporter protein mediated approach (Figure 2.2c), with the usage of glucose transporter isoform (GLUT-1), large amino acid transporter (LAT), or others. Glucose or the amino acids bind with the protein at the blood side of the BBB and the following conformational change allows for the transport to the brain, from a higher to a lower concentration. ATP can provide energy required for the process to be performed in an opposite gradient, flowing from a lower to a higher concentration. The application of this mechanism for drug transport is limited by the fact that these transporter proteins carry only specific substances (glucose for GLUT-1, amino acids for LAT) (Chen & Liu, 2012; Ding *et al.*, 2020).

The efflux pumps (Figure 2.2d) are a set of proteins such as P-glycoproteins and multidrug resistant proteins, responsible for limiting the accumulation of various potentially toxic molecules and afterwards for expelling these molecules from the brain. These proteins are a limiting factor for the delivery of bioactive compounds to the brain (Chen & Liu, 2012; Ding *et al.*, 2020).

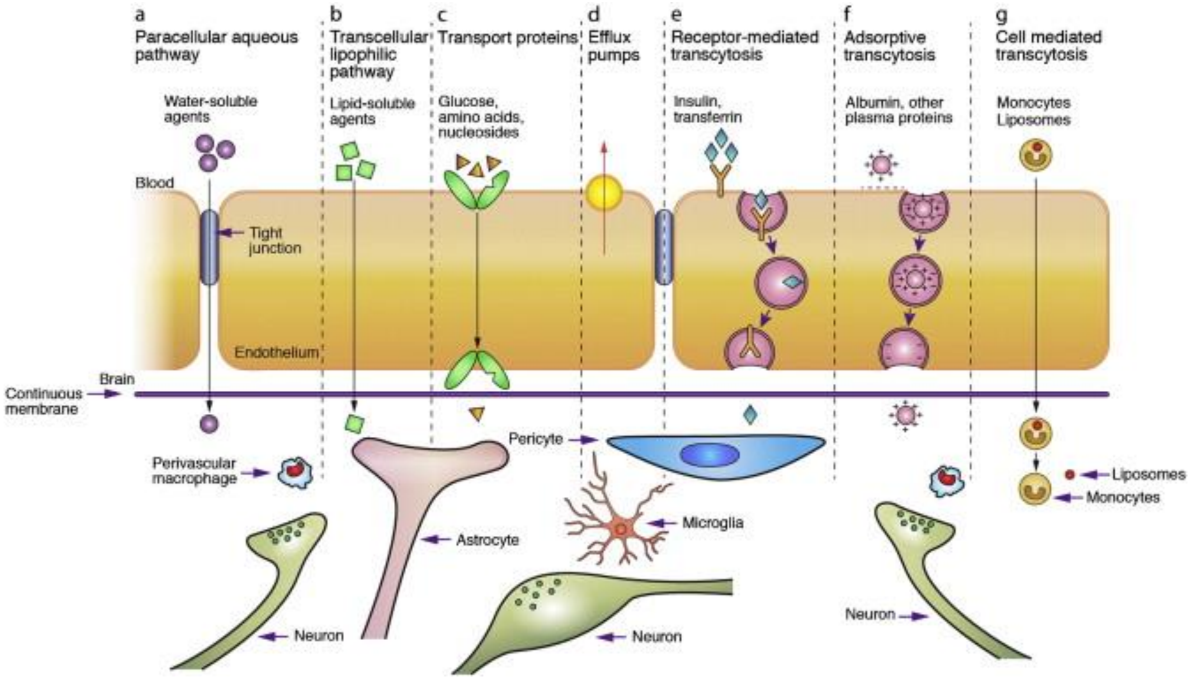

Figure 2.2 - Transport mechanisms across the blood-brain barrier. Retrieved from (Chen & Liu, 2012)

Receptor mediated transcytosis (RMT) (Figure 2.2e) relies on receptors present on the cell surface and is widely used nowadays for NP drug delivery. This type of transport relies on endocytosis, where the substance binds with the receptor and an intracellular vesicle is formed through membrane invagination. The most targeted receptors in RMT are transferrin, lactoferrin, insulin, diphtheria toxin and low-density lipoprotein receptors (Chen & Liu, 2012; Ding *et al.*, 2020).

The adsorptive mediated transcytosis (AMT) (Figure 2.2f) method is used for the transport of charged particles, by taking advantage of the electrostatic interactions between positively charged drug carriers and negatively charged microdomains on the cytoplasmatic membrane surface. This transport method has lower affinity but higher capacity than RMT (Chen & Liu, 2012; Ding *et al.*, 2020).

Cell-mediated transcytosis (Figure 2.2g) relies on immune cells (neutrophils, monocytes and macrophages) with the capability to cross the BBB in both healthy and disease conditions. In this technique, drugs are encapsulated in a liposome, which are in turn absorbed by immune cells that cross the BBB and migrate towards the inflammation sites in the brain. This is a more recent approach, and, unlike the previously mentioned methods which can only carry molecules with specific properties, cell-mediated transcytosis can be used for any type of molecule (Chen & Liu, 2012; Ding *et al.*, 2020).

Due to the BBB's tightness, much research has been done in the last few years to develop ways to effectively carry drugs across the barrier. Of these, lipid-based techniques are the most studied, with citicoline liposomes being studied for the treatment of cerebral ischemia (Fresta *et al.*, 1994), neuroprotection by quercetin (Phachonpai *et al.*, 2010) and epilepsy treatment by phenytoin (Mori *et al.*, 1995). Phytosomes with *Ginkgo biloba* L. extract were also developed to protect the brain and vascular system of people over 50 years old (Purkayastha & Ghosh, 2018). Another technique broadly researched is the NLCs, e.g. for anticancer activity (Emami *et al.*, 2018) and chemotherapy with drugs encapsulated in SLNs (Wong *et al.*, 2007).

Lipid-based nanoencapsulation techniques are the most used techniques for the targeted delivery of drugs across the BBB.

Hu *et al.* (2019) developed glutathione PEGylated nanoliposomes based on either egg yolk or hydrogenated soy phosphatidylcholine to deliver methotrexate to the brain. Compared to the free drug, the liposomes resulted in a concentration in the plasma 717-4330 times higher in rats. However, glutathione does not always result in an increase in brain uptake. Although in hydrogenated soy nanoliposomes, glutathione-PEG coating resulted in a 4-fold increase in brain uptake when compared to simple PEG coating, in egg yolk nanoliposomes it did not show any meaningful increase.

In a study by Chen *et al.* (2016), nanoliposomes encapsulating α-mangostin, a potential candidate for the treatment of Alzheimer's disease, were modified by transferrin. The uncoated NPs and the transferrin coated NPs displayed an average particle size, polydispersity index and zeta potential of 188 ± 5.29 and 196.3 ± 7.09 nm, 0.201 ± 0.019 and 0.211 ± 0.034 and -17.85 ± 6.0 and -22.23 ± 2.87 mV, respectively. *In vitro* studies, in which a membrane with a TEER of 210 Ω/cm^2 was used, showed that the liposomes were able to penetrate the BBB without significant changes to the NPs' morphology and *in vivo* studies demonstrated an improvement in the brain delivery of α-mangostin, increasing the half-life from 0.76 to 0.82 hours and the mean residence time from 0.55 to 0.77 hours.

3. Lipid-based nanoencapsulation techniques

There are several different types of nanoformulations which allow for the encapsulation of bioactive compounds. Most are based on different building blocks, namely carbohydrates, proteins or lipids. These have usually similar characteristics, namely being biocompatible and biodegradable, but differ in other capacities, such as the production method, particle size, structure, morphology, pharmacodynamics, and therapeutic properties (Montes *et al.*, 2019).

The advantages of lipid-based nanoencapsulation are the possibility of industrial production, higher encapsulation efficiency (%EE) and low toxicity. In addition to that, taking into account that compounds usually encapsulated, such as flavonoids, polyphenols, carotenoids and fatty acids, have different polarities, lipid-based NPs also allow for the separate or simultaneous encapsulation of both hydrophilic and hydrophobic substances, due to their amphiphilic behaviour. The usage of digestible lipids facilitates the intestinal absorption of the bioactives since they solubilize and carry the hydrophilic compounds (Akhavan *et al.*, 2018; Rostamabadi *et al.*, 2019).

The main types of lipid-based nanoencapsulation techniques are nanoemulsions (NEs), NLCs, nanoliposomes and nanophytosomes, which will be explained in the following chapter.

3.1. Encapsulation by nanoemulsions

NEs are isotropic colloidal systems composed of two immiscible liquids, stabilized by amphiphilic surfactant molecules. NEs can be in the form of an emulsion of oil-in-water, O/W, or water-in-oil, W/O (Benita & Levy, 1993). Both types of NEs are exemplified in Figures 3.1 and 3.2, respectively. The mean droplet diameter covers a size of 50-200 nm in a transparent nanoemulsion and up to 500 nm when it has a milky appearance (Jafari, 2006). Double NEs have also been developed, either as W/O/W or O/W/O (Montes *et al.*, 2019).

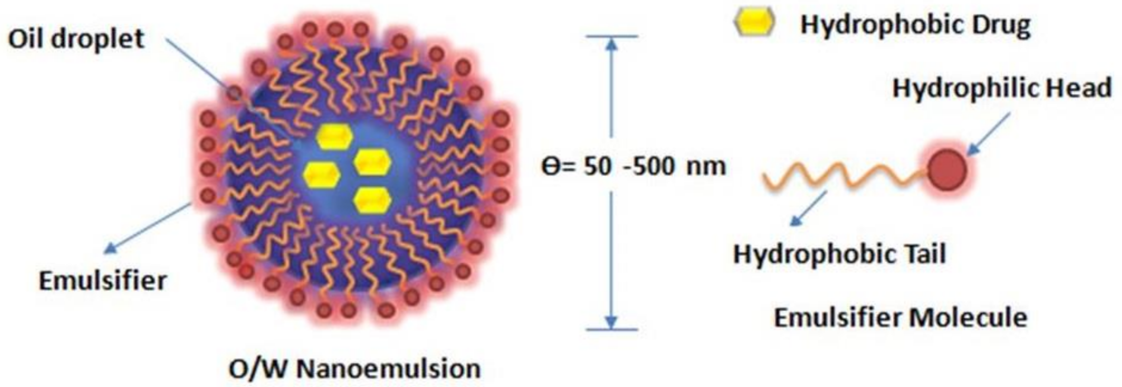

Figure 3.1 - Structure of an O/W nanoemulsion (Franco et al., 2017)

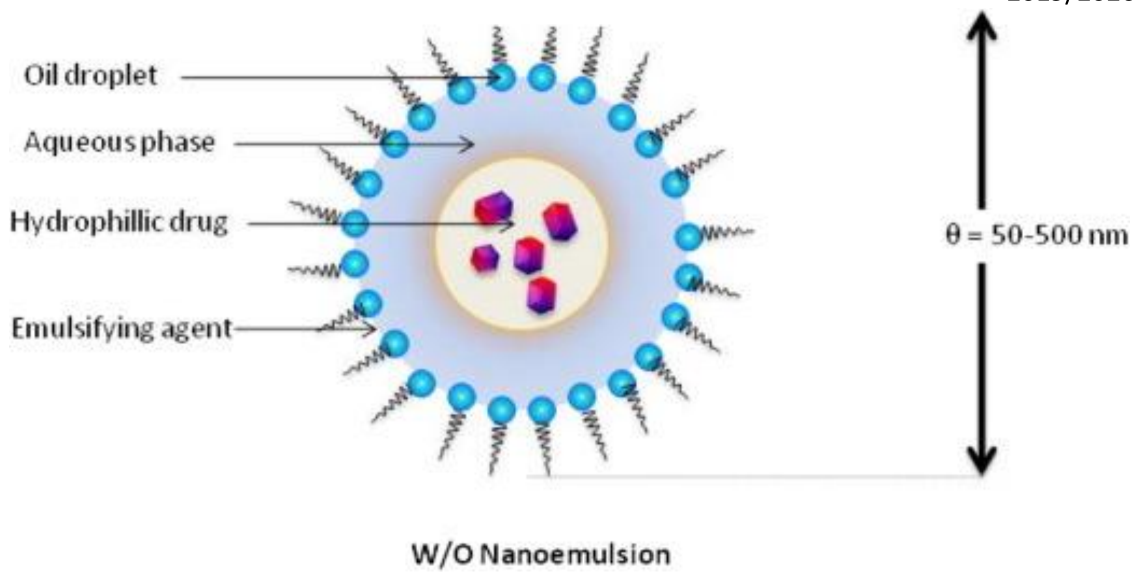

Figure 3.2 - Structure of a W/O nanoemulsion (Qadir et al., 2016)

Unlike microemulsions, which are transparent and thermodynamically stable, NEs cannot be formed spontaneously, as they are non-equilibrium systems. This makes NEs have a higher solubilization capacity for lipophilic drugs and better resistance toward droplet collisions, which makes them have great kinetic colloidal stability (McClements, 2011).

They are thermodynamically unstable since their free energy of formation is greater than that of their separated states. Despite this, due to their nanosized droplets, NEs have long-term physical stability without apparent flocculation during storage. NEs are sometimes referred to as "Approaching Thermodynamic Stability" (Tadros *et al.*, 2004; Sivakumar *et al.*, 2014).

NEs are widely used in the food and nutraceutical industries (Jafari, 2016) and for drug delivery in the cases of neurological disorders such as Alzheimer's, Parkinson's and Prion's (Nirale *et al.*, 2020) due to several advantages, such as a very small droplet size that causes a large reduction in gravity force, which may allow Brownian motion to be enough to overcome gravity. This means that no sedimentation will occur in storage. The small droplet size also denies flocculation of the drops, which enables the system to remain dispersed without the need for separation (Walker *et al.*, 2015). The small droplets are non-deformable, which prevents surface fluctuations. Also, a significant surfactant film thickness, relative to the droplet radius, prevents thinning or disruption of the liquid film between the droplets. Nanoemulsions have a large surface area, which makes them effective in delivering active ingredients through the skin. They require a considerably lower concentration of surfactant when compared to microemulsions, which typically are in the region of 20% or higher. For nanoemulsions, surfactant concentrations in the range of 5-10% may be enough (Tadros, 2014).

Although they have many advantages, they also have several factors that limit their application: the preparation may require special application techniques, such as high-pressure homogenizers or ultrasounds; they are expensive to produce, due to requiring expensive equipment and high concentrations of emulsifiers; there is a lack of understanding of the mechanism of the production of submicron droplets or the role of surfactants and cosurfactants;

there is a lack of demonstration of the benefits of nanoemulsions over conventional macro-emulsion systems and the lack of knowledge on mechanisms that affect and cause problems during the production of nanoemulsions (such as the Ostwald ripening, i.e., the oil diffusion in the aqueous phase) (Tadros *et al.*, 2004).

The typical main components involved in the formulation of NEs are oil, surfactant and co-surfactant, the surfactants mixture (S_{mix}) and an aqueous phase at appropriate ratios. Nevertheless, multiple other ingredients can be used in both the internal and external phases (Qadir *et al.*, 2016).

3.1.1. Oil

Several types of oils can be used in the formulation of NEs, depending on which drug is to be incorporated. Therefore, a previous screening into the drug solubility in oil is often made. The oil represents one of the most important components of NEs, given its ability to solubilize lipophilic drugs, but also for improving the fraction of drug transported through the intestinal lymphatic system, therefore increasing GIT drug absorption (Qadir *et al.*, 2016).

Hydrolyzed vegetable oils, medium-chain and modified long triglycerides are usually used in NE formulation, generally containing 5 to 20% oil/lipids in the case of O/W NEs, although this number can go as high as 70% sometimes. Edible oils are not usually selected for NE formulation due to their poor ability to dissolve large amounts of lipophilic drugs (Harwansh *et al.*, 2019; Qadir *et al.*, 2016).

Arora *et al.* (2020) screened 4 different oils (Capmul MCM, soybean oil, grape seed oil and vitamin E) for the formulations of a tetrabenazine NE. The calculated solubilities for the different oils were 2.50 ± 0.26, 1.00 ± 0.09, 1.50 ± 0.17 and 0.50 ± 0.04 mg/ml, respectively, with Capmul MCM being selected as the optimum oil for the NE formulation.

Đorđević *et al.* (2015) attempted to solubilize the antipsychotic risperidone in medium-chain triglycerides (MCT) and soybean oil, as well as their mixtures, but could not achieve the target concentration of 1 mg/g risperidone in the NE. Lecithin was added to the oil/mixture of oils, and an increase in temperature was applied, without success. The target concentration was achieved by dissolving risperidone in a co-solvent, benzyl alcohol, with the final oil ratio chosen being 4:1 w/w MCT:soybean oil.

3.1.2. Surfactant

Surfactants are amphiphilic molecules used to stabilize the NEs by reducing the interfacial tension between the oil and the aqueous phases. They adsorb onto the oil droplets, forming a flexible film that can deform around the droplets. Surfactants consist of a hydrophilic head and a hydrophobic tail and act as emulsifiers in the production of the NEs. A wide range of surfactants can be used in the formulation of NEs (Singh *et al.*, 2017; Harwansh *et al.*, 2019).

Haider *et al.* (2018) screened different surfactants (Cremophor EL, Cremophor RH 40, Capryol 90, Labrafil M, Labrasol and Tween 80) for the production of rivastigmine hydrochloride

NEs. Tween 80 (45 ± 2 mg/ml) and Capryol 90 (35 ± 2 mg/ml) displayed the highest solubility rates, with Tween 80 being chosen as the surfactant.

3.1.3. Co-surfactants

If a single surfactant is used, the film formed around the droplets is usually highly rigid, thus leading to the production of NEs over a very limited range of compositions. Therefore, a co-surfactant is usually utilized to efficiently lower the surface tension and grant the interfacial film flexibility to guarantee NE formation at higher ranges of compositions. These co-surfactants are usually used at low concentrations due to severe side effects that appear at high concentrations (Qadir *et al.*, 2016; Harwansh *et al.*, 2019).

Haider *et al.* (2018) screened 4 different co-surfactants (Captex 200-P, Polyethylene glycol 400, Sorbitan sesquioleate and Transcutol-P) for rivastigmine hydrochloride NE formulation. Transcutol-P displayed the highest solubility (60 ± 1.5 mg/ml) and was selected as the co-surfactant.

In order to screen the appropriate ratios, a ternary phase diagram (represented in Figure 3.3) is usually constructed to determine the water:oil:S_{mix} better suited for the NE formulation (Harwansh *et al.*, 2019).

Despite their disadvantages, a few studies have been conducted to produce NEs containing neuroprotective drugs. Table 3.1 reports several studies that have been published into the production of NEs.

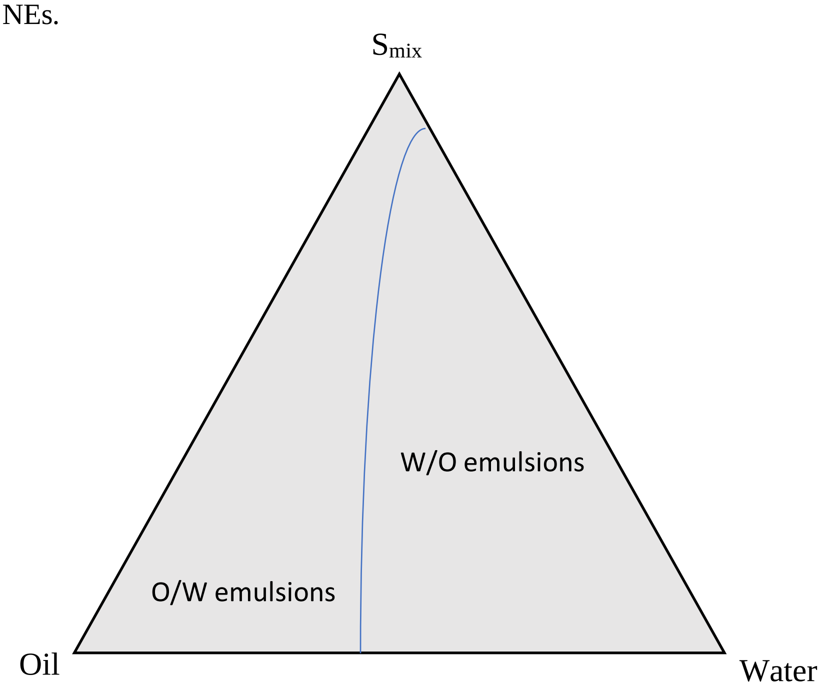

Figure 3.3 - Hypothetical ternary phase diagram of oil, water and S_{mix}

Table 3.1 - Nanoemulsions produced to encapsulate neuroprotective compounds

Compound	Oil	Surfactant: co-surfactant	Oil: Surfactant ratio v/v	T	Oil phase	Aqueous phase	Ultrasound	Rotation	Time	Analysis	Ref
Natural compounds											
Carvacrol	n-hexane	Tween 80			Oil, drug	Deionized water, surfactant	20 kHz, 100 W, 1h	1 h	2 h	TEM, DLS, *in vivo* studies, ELISA, HPLC	Hussein *et al.* (2017)
Kaempferol	16% w/w MCT	1% w/w Polysorbate 80			Oil, 0.1 % drug, 5% w/w lecithin	Distilled water, surfactant		9500 rpm	1 min	PCS, TEM, viscometry, refractometry, HPLC, DPPH, *ex vivo* diffusion, *in vivo* studies	Colombo *et al.* (2018)
Lycopene	Sesame, linseed, walnut oil				Oil, drug	Deionized water, lactoferrin		10000 rpm	3 min	DLS, TEM, UV-Vis, *in vitro* digestion	Zhao *et al.* (2020)
Naringenin	Capryol 90	Tween 20: EtOH			Oil, drug, surfactant	Distilled water		Continuous	> 1 h	PCS, TEM, ROS activity, *in vitro* studies	Md *et al.* (2018)
Rutin	Sefsol 218: Tocopheryl polyethylene glycol 1000 succinate	Solutol HS15: Transcutol P	1:9; 1:8; 1:7; 1:6; 1:5; 1:4; 1:3.5; 1:3; 3:7; 1:2; 4:6; 5:5; 6:4; 7:3; 8:2; 9:1		Oil, drug, surfactant	Double distilled water		Continuous		TEM, HPLC, DLS, *in vitro* drug release, *in vivo* studies	Sharma *et al.* (2020)

siRNA	Flaxseed oil	Tween 80			DOTAP dissolved in EtOH, siRNA, oil	Water, Lipoid E80®, surfactant	10 minutes 22% amplitude 50% duty cycle	6000 rpm	2 min	DLS, TEM, EMSA, CLSM, FCM, *in vitro* gene silencing, *in vivo* studies	Yadav *et al.* (2016)
Thymoquinone	Oleic acid	Tween 20: carbitol (0:3; 1:3; 2:3; 3:3; 3:2; 3:1; 3:0)	1:9; 2:8; 3:7; 4:6; 5:5; 6:4; 7:3; 8:2; 9:1		Oil, drug, surfactants	Purified water		Continuous		DLS, DPPH, FTIR, refractometry, *in vitro* drug release, *ex vivo* permeability, *in vivo* studies, UPLC-PDA	Ahmad *et al.* (2016)
Synthetic compounds											
Asenapine maleate	Capmul PG-8	Kolliphore RH40:Transcutol HP (3:1; 2:1; 1:1; 1:2; 1:3)	1:9; 2:8; 3:7; 4:6; 5:5; 6:4; 7:3; 8:2; 9:1		Oil, drug, surfactant	Double distilled water		Continuous		DLS, TEM, viscometry, *in vitro* drug release, *in vivo* studies, HPLC	Kumbhar *et al.* (2020)
Darunavir	Soybean oil	Tween 80 (0.5; 0.75; 1; 1.25; 1.5% w/v)		55-60 °C	8% w/v oil, surfactant, drug	Distilled water, 1.2% egg lecithin		8000 rpm	20 min	DLS, UV-Vis, conductivity, TEM, *in vitro* drug release, *in vivo* studies	Desai & Thakkar (2018)
Gabapentin	Capmul MCM	Tween 80: PEG-400	1:9; 2:8; 3:7; 4:6; 5:5; 6:4		Oil, surfactants, drug	Double distilled water, 0.3% oleic acid, 2.25% glycerol	70% amplitude		10 min	UV-Vis, FTIR, DLS, TEM, viscometry, *in vitro* diffusion, *ex vivo* permeation	Mallick *et al.* (2020)

Indinavir	10% w/v soybean oil	0.2, 0.6, 1% w/v Tween 80		70°C	Oil, surfactant, 0.25% w/v α-tocopherol, 1.2% w/v egg PC		750 W, 20 min at 50% amplitude	15000 rpm	3 min	DLS, *in vitro* drug release, HPLC, FLM, *in vivo* studies	Prabhakar *et al.* (2013)
Letrozole	Triacetin	Tween 80:PEG-400 1:1; 2:1; 3:1; 4:1	1:9; 1:8; 1:7; 1:6; 1:5; 2:8; 1:3.5; 1:3; 3:7; 1:2; 4:6; 5:5; 6:4; 7:3; 8:2; 9:1		Oil, drug, surfactant	14.66% co-surfactant, 48% distilled water		Yes		UV-Vis, DLS, TEM, *in vitro* drug release, *ex vivo* permeation, DSC, *in vivo* studies	Iqbal *et al.* (2019)
Risperidone	MCT: soybean oil (4:1 w/w)	2% w/w Polysorbate 80, Kolliphor® P 188 or Solutol® HS 15		25 or 50 °C	20% Oil, 2% emulsifier (lecithin), 0.05% antioxidant (BHT). 2% benzyl alcohol	Surfactants dissolved in 0.01M PBS pH 9 or double distilled water		10000 rpm	3 min	PCS, laser diffraction, viscometry, conductivity, AFM, DSC, FTIR, *in vivo* studies	Đorđević *et al.* (2015)
Risperidone (follow-up study)	MCT: soybean oil (4:1 w/w)	2% w/w Polysorbate 80		50°C	20% Oil, 2% emulsifier (lecithin), 0.05 % antioxidant (BHT). 2 % benzyl alcohol	Double-distilled water, Polysorbate 80, sodium oleate, glycerol		10000 rpm	3 min	PCS, laser diffraction, DLS, viscometry, conductivity, *in vivo* studies	Đorđević *et al.* (2017)
Risperidone	Capmul MCM	Tween 80: (transcutol: propylene glycol (1:1 w/w))		35°C	8% oil, 29.33% surfactant, drug	0.5, 0.7% Carbopol 934		Continuous		UV-Vis, PCS, *in vivo* studies	Kumar *et al.* (2008)

Rivastigmine hydrochloride	Capmul MCM	Tween 80:Transcutol P (1:1; 1:2; 2:1; 3:1; 4:1; 5:1)	1:9; 1:8; 1:7; 1:6; 1:5; 2:8; 1:3.5; 1:3; 3:7; 1:2; 4:6; 5:5; 6:4; 7:3; 8:2; 9:1		Oil, drug, surfactant	Distilled water		Continuous		HPLC, UV-Vis, DLS, TEM, *in vitro* drug release, *in vivo* studies	Haider *et al.* (2018)
Saquinavir	Flaxseed or safflower oil	Egg PC: deoxycholic acid		60°C	Oil, drug, ethanol		10 min, 21% amplitude	Continuous		DLS, TEM, *in vivo* studies	Vyas *et al.* (2008)
Tetrabenazine	Capmul MCM	Tween 80: Transcutol P 1:0; 1:1; 1:2; 1:3; 2:1; 3:1; 4:1 v/v	1:9; 1:8; 1:7; 1:6; 1:5; 1:4; 3:7; 1:2; 4:6; 5:5; 6:4; 7:3; 8:2; 9:1	25 °C	Oil, drug, surfactant	HPLC-grade water		High speed		HPLC, UV-Vis, DLS, refractometry, *ex vivo* nasal permeation, *in vivo* studies	Arora *et al.* (2020)
Topiramate	Capmul MCM C8	2:1 Tween 20:Carbitol			Oil, surfactant, drug	Water		100 rpm	30 min	DLS, TEM, *in vivo* studies	Patel & Parikh (2020)
Valproic acid	MCT: safflower seed oil (1:3 w/w)	Tween 80		60ºC	Oil, drug, 1% w/w lecithin		24 kHz, 240 W	Yes	15 min	DLS, TEM	Tan *et al.* (2016)

Abbreviations: AFM – Atomic Force Microscopy; BHT – Butylhydroxytoluene; CLSM – Confocal Laser Scanning Microscopy; DLS – Dynamic Light Scattering; DOTAP - (N-[1-(2, 3-dioleoyloxy) propyl]-N, N, N-trimethylammoniumsalt); DPPH - 2,2-diphenyl-1-picrylhydrazyl; DSC – Differential Scanning Calorimetry; ELISA – Enzyme-Linked Immunosorbent Assay; EMSA – Electrophoretic Mobility Shift Assay; EtOH – Ethanol; FCM – Flow Cytometry; FLM – Fluorescence Microscopy; FTIR – Fourier-transform Infrared Spectroscopy; HPLC – High-Performance Liquid Chromatography; MCT – Medium-chain Triglycerides; PC – Phosphatidylcholine; PEG-400 – Polyethylene glycol 400; PCS – Photon Correlation Spectroscopy; ROS – Reactive Oxygen Species; siRNA – Small interfering RNA; T – Temperature; TEM – Transmission Electron Microscopy; UPLC-PDA – Ultra Performance Liquid Chromatography coupled with Photodiode Array Detector; UV-Vis – Ultraviolet-visible spectroscopy.

3.2. Encapsulation by nanoliposomes/ nanophytosomes

Liposomes have been defined as "closed, continuous bilayered structures made mainly of lipid and/or phospholipid molecules" (Mozafari *et al.*, 2002). Liposomes arrange in a polar head group and a long hydrophobic tail. Phospholipid molecules arrange in a bilayer form, with the heads of one layer contacting the outside media and the heads of the other layer surrounding an interior aqueous phase, which makes the liposome have a general amphiphilic behaviour. This allows liposomes to serve as a storage and carrier of drugs with different lipophilicities (Briuglia *et al.*, 2015).

Nanoscale versions of liposomes are referred to as nanoliposomes. Despite having many similarities to conventional liposomes, they bring the added benefits associated with nanoparticles such as increased surface area and a better penetration potential (Jafari, 2017).

Liposomes can be divided into different groups according to their size, lamellarity and vesicularity characteristics (Mozafari *et al.*, 2008). These include:

- Unilamellar vesicles (ULV), which contain one single lipidic bilayer and can be a small unilamellar vesicle (SUV, when less than 100 nm) or a large unilamellar vesicle (LUV);
- Multilamellar vesicles, composed of multiple concentric bilayers;
- Multivesicular vesicle (MVV), which is composed of many small non-concentric vesicles encapsulated within a single lipid bilayer;
- A double bilayer vesicle (DBV), consisting of two bilayer membranes.

Nanoliposomes can be easily adapted to industrial production, due to the easily available and relatively low prices of materials, such as crude lecithin's. These are a rich source of phospholipids, including phosphatidylcholine (PC), the most used phospholipid (Jafari, 2016).

Nanoliposomes are not thermodynamically stable. Researchers have been trying to address their instability, the degradation of the encapsulated materials and the influence of environmental variables, such as the composition, storage temperature, pH, exposure to light and oxygen (Liu *et al.*, 2015).

Some disadvantages related to the production of nanoliposome are their low encapsulation efficiency (especially for highly water-soluble substances), a wide variation of liposome diameter between batches, difficulties associated with the scale-up, and the use of organic solvents that may impart toxicity to the products (Campardelli *et al.*, 2016). Despite this, nanoliposomes have been prepared without the use of organic solvents, which makes them non-toxic to cells (Gülseren *et al.*, 2012).

In Table 3.2 are summarized several studies where liposomes where produced to incorporate neuroprotective compounds.

Table 3.2 - Nanoliposomes produced to encapsulate neuroprotective compounds

Compound/ extract	PP	Solvent	Molar ratio (E: PP)	T	Chol	Ultrasound	Rotation	Time	Rehydration film	Analysis	Ref
Natural compounds											
7,8-dihydroxy-flavone	Soybean PC	Absolute EtOH	1:40, 1:30, 1:15, 1:10, 1:5 w/w	25 ºC	5:1 PC:Chol	40 kHz, 5 min, 10 s on/off	Yes	30 min	PBS pH 7.4	DLS, UPLC, TEM, FTIR, DSC, DPPH, *in vitro* drug release	Chen *et al.* (2020)
β-carotene	Marine PP or egg PC	5 mL absolute EtOH	0.01, 0.02, 0.03, 0.04 g to 1g PP	55 °C		9 min 240 W 8 s on/off	Yes	60 min	100 mL PBS pH 7.4	DLS, TEM, DSC, UV-Vis	Hamadou *et al.* (2020)
Baicalein	DPPC	Chloroform for DPPC, MetOH for baicalein	1:3 1:5 1:10	37 °C	15% Chol, 10% PEG2000 PE	30 sec	150 rpm	2 h	PBS pH 7.4	TEM, DLS, UV-Vis, HPLC, DSC, DPPH, CLSM, cellular uptake	Aliakbari *et al.* (2018)
Basic fibroblast growth factor	Hydrogenated soy phosphatide	1 mL Poloxamer 188-grafted heparin copolymer 5% w/v, then emulsified in 2 mL 2% w/v acid gelatin solution		5 °C		110W, 20 s	2500 rpm	5 h	Double distilled water	TEM, DLS, viscometry, ELISA, UV-Vis, *in vivo* studies	Zhao *et al.* (2016)

Curcumin	Soy, rapeseed, salmon lecithin	48.5 mL distilled water	10 mg curcumin to 1.5 g lecithin			40 kHz 40% full power, 120 s, 1 s on/off	Yes	5 h		DLS, HPLC, TEM, *in vitro* anti-cancer activity evaluation	Hasan *et al.* (2014)
Curcumin	DPPC	Chloroform: MetOH (2:1 v/v)	1:5	45 °C	2:1 PP:Chol	Yes			PBS pH 7.4	DLS, FCM, *in vivo* studies, brain cell studies	Lazar *et al.* (2013)
Curcumin	DSPC	Chloroform: MetOH (2:1 v/v)	(0.375-0.75):2:1 E:PP:Chol	60 °C	Yes	Yes	Yes	1 h	PBS pH7.4 or FITC-dextran	DLS, *post mortem* brain tests, cellular uptake	Mourtas *et al.* (2014)
Docosa-hexaenoic, eicosa-pentaenoic acid (2:3) w/w	Soybean PP	Deionized water and glycerol (2% v/v)	0.4:2	30 °C		20 kHz 120 W (10, 15, 20 min)	600, 800, 1000 rpm	30, 45, 60 min		DLS, GC	Rasti *et al.* (2013)
OX-26 or IgG	PC, DSPC			37 °C for PC, 53 °C for DSPC	20:10:0.8:(0.002-0.02) DSPC:Chol: DSPE-PEG_{2000}:DSPE-PEG_{2000}-Biotin	Probe sonication		1 h	Excess solution of biotin-OX-26 or biotin-IgG	ELISA, DLS, cellular uptake, TEM, CLSM	Markoutsa *et al.* (2011)
Quercetin, rosmarinic acid (RA)	PA, DPPC, DHDP	MeOH for quercetin, chloroform for PP, PBS for RA		25 °C	5:4:1 DPPC:Chol: DHDP	46 kHz		50 min	1 mL aqueous solution containing RA	HPLC, ELISA, DLS, SEM, TEM, XPS, *in vivo* studies	Kuo *et al.* (2018)
Synthetic compounds											
Cisplatin	Soy PC	Silver nitrate for cisplatin,	(9:1:1.5 mg) PC, PE-	25 °C			Yes		5 mL PBS pH 7.4 and	AAS, AFM, DLS, *in vitro* drug	Saesoo *et al.* (2016)

		chloroform: diethyl ether (3:1 v/v) for PP	PEG, QCS-modified PP						aqueous cisplatin (100 µg/ 1 mg lipid)	release, cellular uptake	
Cytarabine, beclomethasone dipropionate	Lipoid E80	EtOH		Room T	Yes		700 rpm	15 min		PCS, HPLC, TEM, *in vitro* drug release, stability and cell uptake, CLSM	Jaafar-Maalej *et al.* (2009)
Flucytosine	Soybean PC	Chloroform: MetOH 3:2 v/v			1:1:1:(0, 0.25, 0.5 or 0.75) PC: Chol: Span 65: Glutathione	Ultrasonic bath 30 min	90 rpm	1 h	15 mL aqueous solution of flucytosine in PBS pH 7.4	UV-VIS, DLS, TEM, *in vitro* drug release, cellular uptake, *in vivo* studies	Salem *et al.* (2015)
Galantamine	DSPC, DSPE	Chloroform: MetOH (9:1 v/v)		65 °C	Yes	Probe sonicator	60 rpm	30 s	PBS pH 7.4 or 5 % w/v dextrose	FTIR, UV-Vis, DSC, DLS, TEM, *in vitro* drug release	Mufamadi *et al.* (2013)
Lamotrigine	Lipoid 90G	Chloroform: MetOH (2:1 v/v)	Various	35 °C	Yes	Yes	Yes	1 h	Tween 80, nasal saline buffer pH 6.5	PCS, UV-Vis, TEM, DSC, XRD, *in vitro* drug release, CLSM	Praveen *et al.* (2019)
Metmorfin Hydrochloride	PS	Pure EtOH		50 °C		2 min mild frequency	800 rpm		Distilled water	DLS, TEM, UV-Vis, *in vivo* studies	Saffari *et al.* (2020)
Phosphatidic acid, cardiolipin	Bovine brain sphingomyelin	Chloroform: MetOH (2:1 v/v)	5% either extract	55 °C	1:1 molar ratio Chol:PP mixed with			3 h	PBS	DLS, CLSM, cellular uptake	Re *et al.* (2011)

					2.5% mal-PEG-PE						
Quetiapin fumarate	Egg PC	10 mL MetOH: chloroform 2:1	1:1; 1:2; 1:3	37 ºC	1:1; 1:2; 1:3 Chol:PC	2 min 80% amplitude	90 rpm	2 h	10 mL nasal saline buffer pH 6.8	FTIR, SEM, TEM, DLS, *ex vivo* drug diffusion, *in vitro* drug release, *in vivo* studies	Upadhyay *et al.* (2017)
Tempamine	Egg PC or HSPC	Tert-butanol for lipids, 70% EtOH for drug	54:41:5 PP:Chol: ^{2000}PEG-DSPE	60 ºC for HPSC, room T for egg PC					250 mM ammonium sulfate	EPR, *in vivo* studies	Kizelsztein *et al.* (2009)
Teriflunomide	Lipoid S100	10 mL chloroform: MetOH(95:5 v/v)	5, 7.5 or 10 mg drug to 75, 100 or 125 mg PP		Yes	70% amplitude 2-5 min	Yes		PBS pH 7.4	DLS, TEM, SEM, DSC, PXRD, UV-Vis, *in vitro* drug release, *in vivo* studies	Mahtab *et al.* (2019)

Abbreviations: 2000PEG-DSPE - N-carbamyl-poly-(ethylene glycol methyl ether)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine triethyl ammonium salt; AAS – Atomic Absorption Spectroscopy; AFM – Atomic Force Microscopy; Chol – Cholesterol; CLSM – Confocal Laser Scanning Microscopy; DHDP – Dihexadecyl Phosphate; DLS – Dynamic Light Scattering; DPPC – Dipalmitoylphosphatidylcholine; DPPH - 2,2-diphenyl-1-picrylhydrazyl; DSC – Differential Scanning Calorimetry; DSPC: 1,2-distearoyl-sn-glycerol-3-phosphatidylcholine; DSPE-PEG2000 - 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000]; DSPE-PEG2000-Biotin - 1,2-distearoyl-sn-glycerol-3-phosphoethanolamineN-[biotinyl (polyethyleneglycol)-2000]; E – Extract; ELISA – Enzyme-Linked Immunosorbent Assay; EPR – Electron Paramagnetic Resonance; EtOH – Ethanol; FCM – Flow cytometry; FITC – Fluorescein Isothiocyanate; FTIR – Fourier-transform Infrared Spectroscopy; GC – Gas Chromatography; HPLC – High-Performance Liquid Chromatography; HSPC – Hydrogenated Soy Phosphatidylcholine; IgG – Immunoglobulin G; OX-26 – Anti-Transferrin Monoclonal Antibody; mal-PEG-PE - 1,2-stearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(poly(ethylene glycol)-2000)]; MetOH – Methanol; PA – Phosphatidic Acid; PBS – Phosphate Buffer Solution; PC – Phosphatidylcholine; PCS – Photon Correlation Spectroscopy; PE-PEG - 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000]; PEG2000 PE - 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N- [methoxy(polyethylene glycol)-2000]; PP – Phospholipid; PXRD – Powder X-Ray Diffraction; RA – Rosmarinic Acid; SEM – Scanning Electron Microscopy; T – Temperature; TEM – Transmission Electron Microscopy; UPLC – Ultra Performance Liquid Chromatography; UV-Vis – Ultraviolet-visible spectroscopy; XPS – X-Ray Photoelectron Spectroscopy; XRD – X-Ray Diffraction.

Phytosomes, also referred in literature as herbosomes or phyto-phospholipid complexes, are a more enhanced nanoliposomal delivery of bioactive compounds. The term combines “phyto”, the bioactive portion of the complex derived from a plant and “some”, the cell like part of the complex (Ghanbarzadeh *et al.*, 2016). Phytosome is a patented technology, created by the Italian company Indena, that defines phytosomes as “a proprietary 100% food-grade delivery system to optimize bioavailability and pharmacokinetic profile of natural actives by formulating them with a dietary ingredient (lecithin).”

The main interaction occurring in phytosomes is between the active compound and phospholipids, with the polar head (the phosphate and ammonium groups) of the phospholipid being bound to the polar groups (the -OH group of phenolic rings) of the bioactive compound. This link is usually described as an H bond, although some researchers have suggested they may also be Van der Waals forces (Khan *et al.*, 2013).

In a water medium, phytosomes assume a micellar shape, forming a liposome-like structure (Semalty *et al.*, 2010). Despite having similar structures, liposomes and phytosomes have some key differences. In nanoliposomes, the hydrophilic compounds are dispersed in the internal space of the particle and the lipophilic compounds are dispersed within the lipid bilayer, with no H-bonding happening, as can be seen in Figure 3.4b. In nanophytosomes (Figure 3.4a), the compounds are chemically linked to the polar head of the phospholipid, being an integrant part of the lipid bilayer (Lu *et al.*, 2018). Due to these chemical bonds, phytosomes are more stable and allow for higher compound loading capacity (Ting *et al.*, 2014).

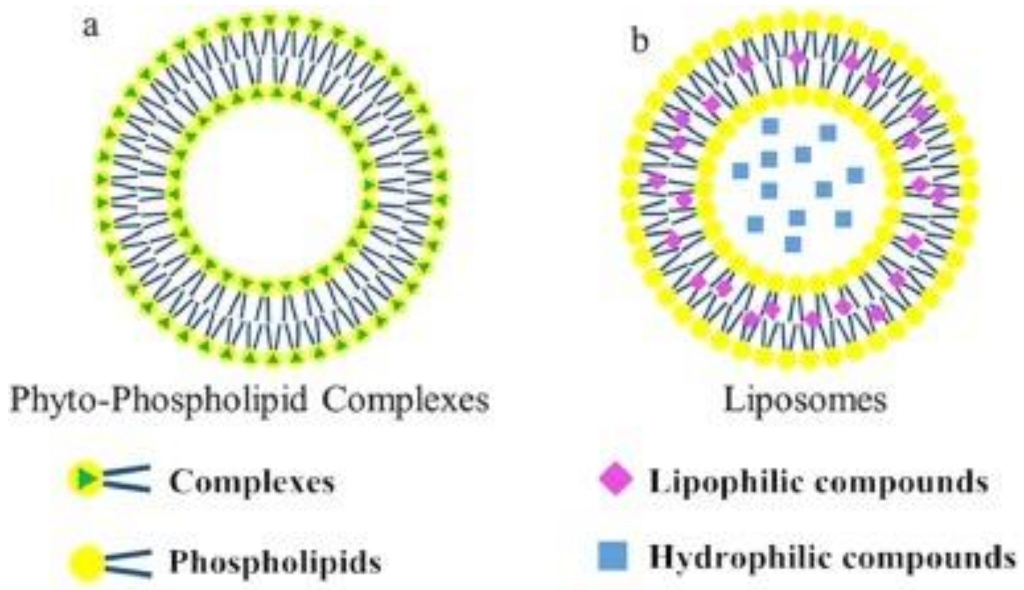

Figure 3.4 - Structure of phytosomes and liposomes (Lu et al., 2018)

The four essential components needed for the production of phytosomes are: phospholipids, an active compound, a solvent and an appropriate stoichiometric ratio of active compound to phospholipid (Ghanbarzadeh *et al.*, 2016).

3.2.1. Phospholipids

Phospholipids can be of natural or synthetic source and are abundant in animal tissues such as egg yolk and bovine brain and plants, such as soy, sunflower and rapeseed (Gnananath *et al.*, 2017). They are amphiphilic molecules, with considerable solubility in both aqueous and lipid mediums. Phospholipids are composed of a glycerol backbone, linked to two fatty acids, with the third linked molecule being the phosphate group. Variations in the head group lead to different phospholipids, with the most used being phosphatidylcholine (PC), phosphatidylserine (PS) and phosphatidylethanolamine (PE) (Froiio *et al.*, 2019). Of these, PC (Figure 3.5) is the most abundantly used. PC possesses two neutral tail groups, the fatty acids, with a polar head group, which contains an oxygen atom with a tendency to gain electrons and a nitrogen atom that loses electrons. This makes PC miscible in both aqueous and lipid environments (Ghanbarzadeh *et al.*, 2016).

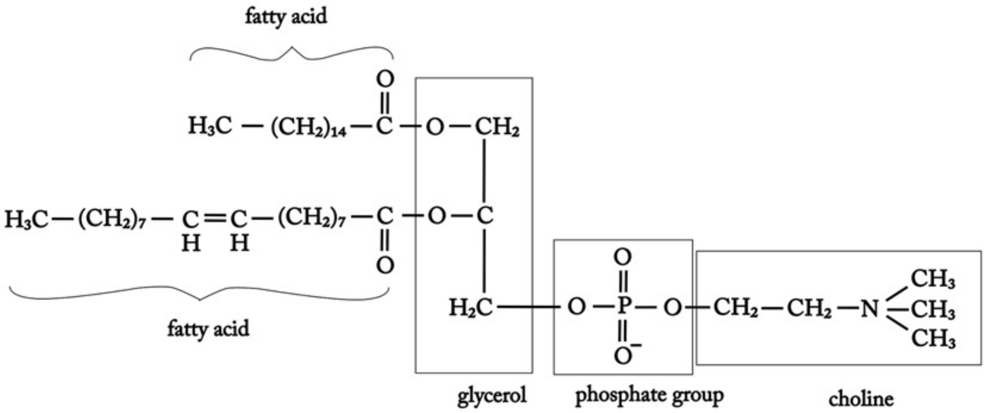

Figure 3.5 - General structure of phosphatidylcholine. Adapted from (Khan et al., 2013)

In addition, it has been reported that PC has several beneficial and therapeutic benefits, such as hepatoprotective activity, nutritional supplement for brain health, role in membrane fluidity, excellent emulsifying activity, precursor for acetylcholine, improving the perception of smell and taste, aiding in the recuperation of fatigue and nourishing skin (Gnananath *et al.*, 2017).

3.2.2. Active compound

The phyto-active compounds utilized are either the active constituents or a standardized extract of a plant. Some natural products lose a part or all their biological activity upon isolation and purification, hence the need for the usage of whole plant extracts at times (Gnananath *et al.*, 2017).

Of these compounds, the majority are polyphenols, divided into flavonoids and phenolic compounds, among others. Flavonoids are themselves divided in the following subtypes: flavones (e.g. luteolin), flavonols (e.g. rutin, quercetin), flavanols (e.g. catechin), flavanones (e.g. naringenin, hesperetin), isoflavones (e.g. puerarin, daidzein), proanthocyanidins and anthocyanins (e.g. cyaniding and pelargonidin). Some of these are lypophilic, and able to diffuse through the biological membranes but cannot dissolve in aqueous gastrointestinal fluids, while others have hydrophilic properties, showing affinity for aqueous phases but being unable to surpass the biological membranes. Phytosomes can remedy both of these cases, while also protecting these compounds from other factors, such as hydrolysis, photolysis and oxidation (Ghanbarzadeh *et al.*, 2016).

Other compounds than polyphenols have been utilized in the production of phytosomes, such as evodiamine (Tan *et al.*, 2012), oxymatrine (Yue *et al.*, 2010), celastrol (Freag *et al.*, 2018), andrographolide (Maiti *et al.*, 2010), ursodeoxycholic acid (Yue *et al.*, 2008), nimesulide (Semalty & Tanwar, 2013), gymnemic acid (Pathan *et al.*, 2012), emodin (Singh *et al.*, 2012a), oleanolic acid (Cao *et al.*, 2012), 20(S)-Protopanaxadiol (Pu *et al.*, 2016), berberine (Yu *et al.*, 2016) and embelin (Pathan & Bhandari, 2011), It has been reported that any compound with π electrons can be used in the formulation of phytosomes.

3.2.3. Solvents

The choice of solvent used in the formulation of phytosomes depends on the solubility of both the phospholipid and the active compound. Initially, mostly aprotic solvents were used, such as dichloromethane (Semalty *et al.*, 2011), dioxane (Telange *et al.*, 2017), tetrahydrofuran (Mancini *et al.*, 2018) and chloroform (El-Fattah *et al.*, 2017). These organic solvents have a high impact on the environment and have been somewhat replaced by protic solvents, such as ethanol (Saoji *et al.*, 2016; Direito *et al.*, 2019; Nazari *et al.*, 2019) and methanol (El-Gazayerly *et al.*, 2013), which are safer. More recently, supercritical fluids (SCF) have shown up as a potential replacement to other solvents (Lu *et al.*, 2018).

The same active compound can be successfully formulated into phytosomes with the addition of different solvents, which will grant the phytosome different characteristics. For example, Babazadeh *et al.* (2017) prepared three rutin nanophytosome formulations (1:1, 1:2 and 1:3 rutin to phospholipid ratio) by dissolving the components in absolute ethanol. All formulations resulted in NPs with mean particle size below 100 nm, and although the formulation with a 1:3 ratio displayed the highest particle size, it also had the highest EE, approximately 99%, and the highest stability. Therefore, rutin nanophytosomes displayed potential in masking undesirable flavors in foods, while maintaining their functionality and increasing stability. Vankudri *et al.* (2016) utilized dichloromethane as the solvent and reported an increase in solubility in both *n*-octanol and water (approximately 2.7-fold and 25.5-fold, respectively). It was also reported an increase in the peak concentration of rutin in rat serum (13.2 µg/ mL for the phytosomes against 10.47 µg/ mL for free rutin), with a higher concentration maintained for a longer period of time, which resulted in an enhancement of the therapeutical efficacy for antidiabetic activity. (Hooresfand *et al.* (2015) prepared three nanophytosome formulations (1:1, 1:2 and 1:4 rutin to phospholipid ratio) with a mixture of methanol and chloroform (1:4) and assessed the optimal ratio by evaluating the mean particle size during a 7-day storage. While on the first day the 1:1 formulation had the lowest particle size (99 ± 6 nm), followed by the 1:2 and 1:3 ratios (119 ± 7 and 123 ± 10 nm, respectively), after 7 days of storage the particle size of formulations 1:1 and 1:3 increased dramatically (14610 ± 326 and 14651 ± 538 nm, respectively). Only the 1:2 formulation displayed an acceptable stability, displaying a particle size of 403 ± 30 nm. The same method was used to evaluate the addition of cholesterol to the optimal ratio of rutin to phospholipid. Three different ratios were used (1:2:0.2, 1:2:0.5 and 1:2:1 rutin to phospholipid to cholesterol) and particle size was assessed through a 21 day storage period. The optimal ratio was discovered to be 1:2:0.2, with particle size of 164.5 ± 11 nm on the first day and 582.5 ± 43 nm after 21 days. This formulation displayed a -45.2 mV zeta potential, therefore having high phyisical stability and an encapsulation efficiency of 80.4 ± 1.3 %.

Maiti *et al.* (2007) developed a phytosome formulation for the encapsulation of rutin by dissolving it in 20 mL dichloromethane, while Tung *et al.* (2017) utilized the same phospholipid and dissolved it in 30 mL dichloromethane. Both experiments had an approximate 88% yield w/w when the molar ratio was 1:1, although the later arrived at higher yields when the ratio was 1:2 and 1:4. Despite having very similar yields, the drug content in the phytosomes differed, with the results being 32 and 26%, respectively.

Li *et al.* (2008) published the first paper where puerarin phytosomes were produced using supercritical carbon dioxide ($ScCO_2$) as an antisolvent. The method utilized was solution enhanced dispersion by supercritical fluids (SEDS), which led to amorphous, partially agglomerated spheres comprised of particles of about 1 µm in size. The usage of $ScCO_2$ has a distinct advantage in the fact that it is done in a single step, while utiziling a "green" solvent in CO_2, which has a critical temperature near room temperature. This diminishes the environmental impact associated with the whole process. Besides, SEDS allows for a control of several parameters, such as the mean particle size and its distribution, surface coating, particle morphology and allows for an easy downstream processing. In this study, several parameters were studied: temperature (30 to 40º C), pressure (8 to 12 MPa), CO_2 flow rate (25 to 65 mL/min), proportion of CO_2 to puerarin solution (1 to 5%) and puerarin concentration in ethanol (60 to 150 mg/mL). The optimal conditions assessed were 35º C, 10 MPa, 45 mL/min CO_2 flow rate, 1% proportion of CO_2 to puerarin solution and 100 mg/mL puerarin concentration.

3.2.4. Stoichiometric ratio of active compound to phospholipid

Different ratios have been studied in the production of phytosomes. It was considered that 1:1 was the ratio that produced the best results, but some studies have disproved this theory. When different compounds are utilized, the optimal ratios differ.

Kalita & Patwary (2019) produced hesperidin phytosomes with different ratios of hesperidin to phospholipid (1:0.5, 1:1, 1:2 and 1:3) and reported that a 1:1 ratio was found to display the best results in terms of solubility in distilled water and PBS at pH 2.5 and 7.4, partition coefficient n-octanol/distilled water and n-octanol/PBS pH 7.4 and drug content (92.54 ± 4.01%). The *in vitro* drug release was also increased from 46.9% after 8h in free hesperidin to 78.2% released by the phytosome formulation.

Telange *et al.* (2017) applied a full factorial design (3^2) to the production of apigenin phytosomes and studied 3 different ratios of phyto-active compound to phospholipid (1:1, 1:2 and 1:3) and reaction temperature (40, 50 and 60 ºC), with the % of apigenin incorporated as the dependent variable. The optimal values were found to be a 1:2 ratio and 60°C, which corresponded to the highest apigenin incorporation, 93.26 ± 0.82 % w/w.

Saoji *et al.* (2016) applied a quality by design approach (QbD) to optimize the production of phytosomes containing standardized *Centella asiatica* (L.) Urban extract, investigating the optimal values of plant extract to phospholipid ratio, reaction temperature and time. The optimal results were 1:3, 60°C and 3 h, respectively, which corresponded to an entrapment efficiency of 95% w/w.

Jain *et al.* (2012) developed rutin phytosomes with three different molar ratios and investigated their free radical scavenging activity via the DPPH radical scavenging assay. The study reported the antioxidant activity increased concomitantly with the increase of a 1:1 to a 1:3 rutin to phospholipid ratio

3.2.5. Other factors affecting phytosome production

Several other factors can affect the production and yield of phytosomes, namely production temperature and time, the usage of cholesterol, ultrasound and agitation.

Matias *et al.* (2015) produced *Plectranthus madagascariensis* (Pers.) Benth.acetonic extract phytosomes and investigated the influence of the variation of three different parameters, the type of solvent (acetone, dichloromethane and ethanol), reaction time (1, 2 or 4 hours) and the molar concentration of cholesterol used (0, 2.5 and 5%). The optimal parameters, corresponding to particles with a mean size of 107.2 ±16.55 nm and a 93% %EE, were considered to be the usage of acetone, a 2 hour reaction time and a concentration of cholesterol of 2.5%, although no statistical difference was found between the usage of 2.5 and 5% cholesterol.

Saoji *et al.* (2017) applied a QbD approach to optimize the production of standardized *Bacopa monnieri* (L.) Wettst. extract phytosomes regarding the molar ratio of extract to phospholipid, reaction temperature and time, with the optimal conditions having been found to be 3:1, 60°C and 3 hours, respectively, with an 87.09 %EE. This formulation was then analysed, having a 395 ± 11 nm mean particle size and -37.6 ± 1.1 mV. The NPs' *in vitro* drug release and antidepressant activity in rats were also assessed. Over 11 hours, 97% of the extract had been released from the phytosomes, compared to 42% in the pure extract. *In vivo* studies were performed, where rats were submitted to the *Tail Suspension Test* (TST) and *Forced Swim Test* (FST), in which their immobility is measured and a decrease in the time of immobility used to assess the efficacy of an antidepressant. The rats were treated with imipramine (10 mg/kg), pure extract (40 mg/kg) and the phytosomes (equivalent to 40 mg/kg). The decrease in immobility time were44.8%, 23.4% and 46.9% for TST, respectively. For the FST, the immobility times were reduced by 43.9%, 22.8% and 45.6%, respectively.

Rasaie *et al.* (2014) produced quercetin phytosomes with different molar ratios of cholesterol, evaluating their particle size and %EE. The lowest particle sizes were achieved with molar ratios of 1:2:0 and 1:2:0.2 (quercetin, phospholipid and cholesterol), 79 and 82 nm, respectively. They also evaluated the physical stability of the produced phytosomes. The phytosomes without cholesterol displayed a 6-fold increase in size over a 7-day period, while the phytosomes with cholesterol displayed physical stability over a 21-day period, with little increase in size.

Nazari *et al.* (2019) produced garlic essential oil phytosomes as a food preservative and evaluated three different methods for size reduction: homogenization, probe sonication and their combination. Both methods could produce particles with size below 200 nm (161 ± 15 and 135 ± 17 nm, respectively). The combination of the methods produced even smaller particles (115 ± 21 nm) with lower turbidity, which is good for food applications. The encapsulation efficiencies obtained for the different methods were 91, 74 and 85%, respectively. The combination of methods also resulted in particles with higher stability after a 30-day storage period, with the particles' size increasing to approximately 200 nm, while for the homogenization method they were increased to approximately 400 nm. They also displayed a zeta potential of -12.36 ± 1.8 mV, and a polydispersity index of 0.34 ± 0.05, both being the lowest results of the three methods used. The antioxidant activity was also measured by DPPH scavenging activity, with the essential oil having a slightly higher antioxidant activity than the prepared nanophytosomes. The *in vitro* release was also assessed, with the essential oil releasing approximately 90% while the nanophytosomes released only 66%. Therefore, the use of nanophytosomes may lead to a durable preservative in food applications.

One of the reasons for the increased attention given lately to phospholipid-based drug delivery systems is the use of natural occurring phospholipid molecules. Their structural components, like the lipid contents of mammalian cell membranes, make them highly compatible with the human physiological system. Phytosomes can penetrate the lipoidal membrane of cells without the need for energy usage and in a non-cytotoxic manner. The most used phospholipids are derived from soybean and have been shown to be free from any acute or chronic effects on laboratory animals, even at doses higher than the recommended (Khan *et al.*, 2013).

Phytosomes have gained increased attention lately, with a variety of research being carried out on their use as nanocarriers. In Table 3.3 some examples of the research that has been conducted and its procedures are represented.

. Cholesterol usage in the formulation grants further stabilization to the phytosomes, making them less likely to break and therefore enabling better crossing of the BBB (Mancini *et al.*, 2018). The use of ultrasounds aids in the arrangement of the lipid membrane into lipid bilayer vesicles, although the fusion of two or more phytosomes may through sonication lead to aggregation resulting in heterogeneous formulations, with both small and large vesicles (Karthivashan *et al.*, 2016). The rehydration is done in order to ensure complete removal of organic solvents (Rasaie *et al.*, 2014)..

Table 3.3 – Phytosomes produced to encapsulate neuroprotective compounds

Extract/ compound	PP	Solvent	Molar ratio (E: PP)	T	Chol	Ultrasound	Rotation	Time	Rehydration film	Analysis	References
Plant extracts											
Annona muricata L. aqueous extract	PC	3 mL THF	1:4	75 °C	32.5%	15 min	Yes	4h	PBS	DLS, HPLC-DAD, FLS, MAO-A inhibition	Mancini *et al.* (2018)
Moringa oleifera aqueous extract	Soy lecithin	10 mL DCM	Various	Room T	Yes	60% amplitude for 15 min, 15 s on / 30 s off	Yes	3h	10 mL n-hexane	DLS, LC-MS, TEM	Lim *et al.* (2019)
Plectranthus madagasca-riensis (Pers.) Benth.acetonic extract	PC	20 mL acetone, DCM or EtOH	1:1	50 °C	0, 2.5 or 5%		Yes	1, 2 or 4h	40 mL reverse osmosis water	HPLC-DAD, SEM, DLS	Matias *et al.* (2015)
Standardized *Bacopa monnieri* (L.) Wettst. extract	Phospholipon ®90H	40 mL EtOH	1:0.5; 1:1; 1:1.75; 1:2.5; 1:3	40, 44, 50, 56, 60 °C			Continuous at rehydration	1, 1.4, 2, 2.6, 3h	n-hexane	HPLC, Phm, SEM, DLS, FTIR, DSC, TGA, PXRD, *in vitro* drug release, *ex vivo* permeation, *in vivo* studies	Saoji *et al.* (2017)
Standardized *Centella asiatica* extract	Phospholipon ®90H	40 mL EtOH	1:0.5; 1:1.01; 1:1.75; 1:2.49;	40, 44, 50			Continuous at rehydration	1; 1,4; 2;	n-hexane	HPLC, Phm, SEM, PCS, FTIR, DSC, PXRD, *in vitro* drug release, *in vivo* studies	Saoji *et al.* (2016)

			1:3	56, 60 °C				2,6; 3h			
Natural compounds											
Apigenin	Phospholipon 90H (Hydrogenated soy PC)	1,4-dioxane: MetOH (14:6)	1:1; 1:2; 1:3	40, 50, 60°C				2h	100 mL n-hexane	UV-Vis, DLS, DSC, FTIR, H-NMR, PXRD, *in vitro* drug release, *in vivo* studies	Telange *et al.* (2017)
Catechin	PC	DCM	1:1					3 h	30 mL n-hexane	UV-Vis, SEM, FTIR, DSC, PXRD, H-NMR, DPPH	Semalty *et al.* (2011)
Celastrol	Soy PC	Anhydrous EtOH	1:1; 1:2; 1:3	40 °C		Brief (2 min)	100 rpm	3 h	6 mL deionized water	UV-Vis, FTIR, DSC, PXRD, DLS, TEM, *in vitro* drug release, *in vivo* studies	Freag *et al.* (2018)
Chrysin	Soy or egg PC	12.5 mL THF	1:2; 1:3	40 ºC			Yes	4 h	12 mL distilled water	DLS, HPLC, AFM, FTIR, XRD, SEM, *in vitro* drug release	Kim *et al.* (2019)
Curcumin	Hydrogenated soy PC	20 mL DCM	1:1	≤ 60 ºC				2 h	10 mL n-hexane	HPLC, DSC, HPTLC, *in vivo* studies	Maiti *et al.* (2007)
Curcumin	PC	30 mL DCM	1:1; 1:2; 1:4	40°C			Yes	2h	50 mL n-hexane	SEM, TEM, HPLC, DSC, H-NMR, FTIR, PCS, *in vivo* studies	Tung *et al.* (2017)
Embelin	Phospholipon ®90H	250 mL EtOH	1:0.5; 1:1; 1:2; 1:3	≤ 60 ºC				2 h		UHPLC, UV-Vis, DSC, FTIR, PXRD, H-NMR, *in vitro* drug release	Pathan & Bhandari (2011)
Hesperidin	Soy lecithin	40 mL DCM	1:0.5; 1:1; 1:2; 1:3	≤ 60 ºC				2 h		UV-Vis, DSC, SEM, *in vitro* drug release	Kalita & Patwary (2019)

Persimmon	PC	20 mL EtOH	1:1; 1:2	25 °C			300 rpm	2 h	40 mL 2% acetic acid solution	HPLC, DPPH, DLS, Folin, UV-Vis, UHPLC-DAD, *in vivo* studies	Direito *et al.* (2019)
Quercetin	PC	MetOH: Chloroform (1:1 v/v)	1:2	45 °C	2:0.2 PC:Chol	Probe sonicator for 5 min	80 rpm		Glucose 50% solution	TEM, DLS, UV-Vis, DPPH	El-Fattah *et al.* (2017)
Rutin	PC	Absolute EtOH	1:1; 1:2; 1:3	45 ºC				30 min	5 mL distilled water	DLS, SEM, UV-Vis, FRAP, FTIR, HPLC	Babazadeh *et al.* (2017)
Rutin	Soy PC	MetOH: Chloroform (1:4)	1:1; 1:2; 1:4	45 °C	Yes				Distilled water	DLS, DSC, FTIR, UV-Vis	Hooresfand *et al.* (2015)
Rutin	PC	20 mL DCM	1:1	45-50°C					100 mL n-hexane	HPLC, FTIR, DSC, PXRD, SEM, DPPH, *in vitro* drug release	Singh *et al.* (2012b)
Silymarin	Soy and egg yolk lecithin	100 mL MetOH	1:0.25; 1:0.5; 1:1; 1:2	Room T			Yes		300 mL petroleum ether	SEM, TEM, H-NMR, DSC, FTIR, HPLC, *in vivo* studies	El-Gazayerly *et al.* (2013)
Silymarin	Soy PC	20 mL absolute EtOH	1:5; 1:10; 1:15	25°C		4min, 5 seconds on/off, 60% amplitude	180 rpm	2 h	PBS, pH 7.4	UV-Vis, FTIR, DSC, TEM, DLS	Maryana *et al.* (2016)

Abbreviatures: AFM – Atomic Force Microscopy; Chol – Cholesterol; DCM – Dichloromethane; DLS – Dynamic Light Scattering; DSC – Differential Scanning Calorimetry; DPPH - 2,2-diphenyl-1-picrylhydrazyl; DSPE-PEG-maleimide - 1,2-stearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(-poly(ethylene glycol)-2000)]; E – Extract; EtOH – Ethanol; FLS – Fluorescence Spectroscopy; Folin – Folin-Ciocalteu reagent; FRAP – Ferric Reducing Antioxidant Power; FTIR – Fourier-transform Infrared Spectroscopy; H-NMR – Proton Nuclear Magnetic Resonance; HPLC – High-Performance Liquid Chromatography; HPLC-DAD - High-Performance Liquid Chromatography coupled to Diode Array Detection; HPTLC – High-Performance Thin Layer Chromatography; LC-MS – Liquid Cromatography–Mass Spectrometry; MAO-A – Monoamine oxidase A; PBS – Phosphate Buffer Solution; PC – Phosphatidylcholine; Phm – Photomicroscopy; PP – Phospholipid; PXRD – Powder X-Ray Diffraction; SEM – Scanning Electron Microscopy; T – Temperature; TGA – Thermogravimetric Analysis; THF – Tetrahydrofuran; TEM – Transmission Electron Microscopy; UHPLC – Ultra-High-Performance Liquid Chromatography; UHPLC-DAD – Ultra-High-Performance Liquid Chromatography coupled to Diode Array Detection; UV-Vis – Ultraviolet-visible spectroscopy; XRD – X-Ray Diffraction.

In some of the studies, the extract was characterized via HPLC prior to the phytosome formation and the capability of the produced phytosomes to encapsulate the compounds present in the extract. Direito *et al.* (2019) determined the concentration of the 9 phenolic compounds present in the persimmon extract. Gallic acid was the most abundant compound, with a concentration of 2.794 ± 0.263 mg/ 100 g fresh weight, followed by epicatechin with a concentration of 0.401 ± 0.045 mg/ 100 g fresh weight. It was also concluded that the produced phytosomes were able to encapsulate 97.4% of the total phenolic compounds and 99.3% of gallic acid present in the extract.

Mancini *et al.* (2018) incorporated *Anonna muricata* L. into phytosomes and used HPLC-DAD to evaluate the efficiency in the incorporation of the compounds present in the plant extract. The initial analysis detected 22 compounds, namely epicatechin (6.23 ± 0.01 mg/ g extract, dry weight), quercetin-pentosyl-rhamnoside (5.33 ± 0.05 mg/ g extract, dry weight) and rutin (5.11 ± 0.01 mg/ g extract, dry weight). All 22 compounds were also found in the analysis of the phytosomes, which displayed and effective incorporation of the plant extract.

Lim *et al.* (2019) detected several compounds in a *Moringa oleifera* aqueous extract, namely chlorogenic acid, kaempferol, quercetin, rosmarinic acid, rutin and vicenin-2. Along with these target compounds, another 122 compounds were identified. Upon the analysis of the phytosomes, rutin and vicenin-2 had not been encapsulated, while the other compounds were. Quercetin (82.8%) and kaempferol (52.2%) appeared to have the higher affinity for the phytosomes.

In Table 3.4 are depicted some examples of phytosome formulations currently or previously available in the market.

Table 3.4 - Commercially marketed phytosomes

Product name	Phytoactive coumpounds	Biological source	Therapeutic indication	References
Ubiqsome®	Coenzyme Q10		Cellular energy	Indena (2020)
Quercefit®	Quercetin	*Sophora japonica* L.	Sports nutrition, allergy seasons' discomforts control, antioxidant activity	
Vazguard®	Bergamot flavanones	*Citrus bergamia* Risso et Poiteau	Cardiovascular health	
Casperome®	Boswellic acids	*Boswellia serrata* Roxb. ex Colebr.	Healthy inflammatory response, joint health, Gut health	
Greenselect®	(-)-Epigallocatechin gallate, polyphenols	*Camellia sinensis* (L.) O. Kuntze	Body weight balance, antioxidant activity	
Leucoselect®	Proanthocyanidins	*Vitis vinifera* L.	Cardiovascular health, antioxidant activity	
Meriva®	Curcuminoids	*Curcuma longa* L.	Joint health, healthy inflammatory response, soothing, anticancer	
Siliphos®	Silybin	*Silybum marianum* (L.) Gaertn.	Hepatoprotective, hepatitis, cirrhosis and inflammation	
Ginkgoselect®	Ginkgoflavonglucosides, ginkgoterpenes, ginkgolides and ginkgolic acids	*Ginkgo biloba* L. - Leaf	Cognition and circulation health, antioxidant activity, vasokinetic	
Rexatrol®	Resveratrol	*Polygonum cuspidatum* Sieb. e Zucc	Antioxidant activity	
Glycyrrhetinic acid Phytosome™	18-β-glycyrrhenitic acid	*Glycirrhiza glabra* L.	Soothing, lenitive, anti-inflammatory	Azeez *et al.* (2018); Froiio *et al.* (2019); Patel *et al.* (2009); Kumar *et al.* (2020); Anjana *et al.* (2017); Singh *et al.* (2020)

Centella triterpenoid Phytosome™	Asiatic acid, madecassic acid	*Centella asiatica* (L.) Urban	Collagen restructurant, antiulcer, wound healing, anti-wrinkles	Azeez *et al.* (2018); Froiio *et al.* (2019); Kumar *et al.* (2020); Singh *et al.* (2020)
Escin β-sitosterol Phytosome™	Saponins	*Aesculus hippocastanum* L.	Anti-oedema and vasoactive properties	Froiio *et al.* (2019); Kumar *et al.* (2020); Anjana *et al.* (2017); Singh *et al.* (2020)
Visnadex™	Visnadine	*Ammi visnaga* (L.) Lam.	Microcirculation improver, vasokinetic, anticellulite	Azeez *et al.* (2018); Froiio *et al.* (2019); Kumar *et al.* (2020); Anjana *et al.* (2017); Singh *et al.* (2020)
Naringenin Phytosome™	Naringenin	*Citrus aurantium* L.	Antioxidant	Froiio *et al.* (2019); Kumar *et al.* (2020); Singh *et al.* (2020)
Hawthorn Phytosome™	Hyperin, quercetin	*Crataegus oxyacanthoides* Thuill.	Anti-hypertensive, cardioprotective, antioxidant	Froiio *et al.* (2019); Patel *et al.* (2009); Kumar *et al.* (2020); Anjana *et al.* (2017); Singh *et al.* (2020)
Echinacea Phytosome™	Echinacosides and high molecular weight polysaccharides	*Echinacea angustifolia* DC	Immunomodulatory, nutraceuticals	Kumar *et al.* (2020); Anjana *et al.* (2017); Singh *et al.* (2020)
Esculoside Phytosome™	Esculin	*Fraxinus ornus* L.	Vasoactive, micro circulation improver, anticellulite	Froiio *et al.* (2019); Kumar *et al.* (2020); Anjana *et al.* (2017); Singh *et al.* (2020)
Lymphaselect™	Melilotoside, flavonoids and terpenoids	*Melilotus officinalis* (L.) Pall.	Hypotensive, indicated in insomnia, thrombophlebitis, anti-inflammatory, venous disorders	Patel *et al.* (2009); Kumar *et al.* (2020); Anjana *et al.* (2017); Singh *et al.* (2020)
Oleaselect Phytosome™	Verbascoside, tyrosol, hydroxytyrosol	*Olea europaea* L.	Anti-hyperlipidemic, anti-inflammatory, anticancer, antioxidant	Patel *et al.* (2009); Anjana *et al.* (2017); Singh *et al.* (2020)
Ginseng Phytosome®	Ginsenosides	*Panax ginseng* C.A.Mey.	Immunomodulator, skin elasticity improver, nutraceutical	Azeez *et al.* (2018); Froiio *et al.* (2019); Patel *et al.* (2009); Kumar

				et al. (2020); Anjana *et al.* (2017); Singh *et al.* (2020)
Millet Phytosome™	Mineral salts, vitamins, unsaturated fatty acids, aminoacids	*Panicum miliaceum* L.	Beauty food for skin, nails and hairs, anti-stress	Froiio *et al.* (2019); Kumar *et al.* (2020); Anjana *et al.* (2017); Singh *et al.* (2020)
Ruscogenin Phytosome™	Steroid saponins	*Ruscus aculeatus* L.	Anti-inflammatory, improves skin circulation, anti-aging, sunscreen agent	Froiio *et al.* (2019); Kumar *et al.* (2020); Anjana *et al.* (2017); Singh *et al.* (2020)
Ximilene and Ximenoil Phytosome™	Ximenynic acid, ethyl xymenynate	*Santalum album* L.	Microcirculation improver	Kumar *et al.* (2020); Anjana *et al.* (2017); Singh *et al.* (2020)
Sabalselect®	Phytosterols	*Serenoa repens* (Bartram) Small	Antioxidant, benign prostatic hyperplasia	Patel *et al.* (2009); Kumar *et al.* (2020); Anjana *et al.* (2017); Singh *et al.* (2020)
Sericoside Phytosome™	Sericoside	*Terminalia serica* Burch. ex DC.	Anti-aging, anti-wrinkles, anti-inflammatory, skin restructuring	Azeez *et al.* (2018); Kumar *et al.* (2020); Anjana *et al.* (2017); Singh *et al.* (2020)
VitaBlue Phytosome™	Anthocyanosides, tocotrienol complex, citrus bioflavonoid, alpha lipoic acid	*Vaccinium angustifolium* Aiton	Antioxidant, improves vision, memory enhancer	Kumar *et al.* (2020); Anjana *et al.* (2017); Singh *et al.* (2020)
Mirtoselect®	Anthocyanosides	*Vaccinum myrtillus* L.	Antioxidant, improvement of capillary tone, reduce abnormal blood vessel permeability, antioxidant	Patel *et al.* (2009); Kumar *et al.* (2020); Anjana *et al.* (2017); Singh *et al.* (2020)
Zanthalene Phytosome™	Zanthalene	*Zanthoxylum bungeanum* Maxim.	Soothing, anti-irritant, anti-itching, anti-reddening	Patel *et al.* (2009); Anjana *et al.* (2017); Singh *et al.* (2020)
Cucurbita Phytosome™	Tocopherols, steroids, carotenoids	*Cucurbita pepo* L.	Anti-inflammatory, benign prostatic hyperplasia	Kumar *et al.*, (2020); Singh *et al.* (2020)
Soyselect Phytosome™	Genistein and daidzein	*Glycine max* (L.) Merr.	Antiangiogenic, anticancer, cardioprotective, immunostimulatory	Kumar *et al.*, (2020); Singh *et al.* (2020)

Pycnogenol Phytosome™	Procyanidins	*Pinus pinaster* Ait.	Anti-inflammatory, anti-wrinkles, anti-allergic	Kumar *et al.* (2020)
Swertia Phytosome™	Xanthones	*Swertia alternifolia* Royle	Antidiabetic	Kumar *et al.*, (2020); Singh *et al.* (2020)
Madeglucyl Phytosome™	Tannins	*Syzygium cumini* (L) Skeels	Antihyperglicemic, anti-inflammatory, antioxidant	Kumar *et al.*, (2020); Singh *et al.* (2020)
Biovin and leucoselect Masquiliers Phytosome™	Resveratrol, catechin, quercitin, epicatechin	*Vitis vinifera* L.	Cardioprotective, systemic antioxidant, nutraceutical	Kumar *et al.*, (2020); Singh *et al.* (2020)

3.3. Encapsulation by nanostructured lipid carriers

NLCs, are an upgrade of the SLNs, the first-generation lipid nanocarriers. SLNs are colloidal drug carriers, where the drugs are carried inside a matrix of lipids that are solid at body temperature (He *et al.*, 2019).

Emulsions and liposomes are greatly limited in their ability to stabilize chemically labile bioactive compounds and are also characterized by the lack of controlled release. This is due to the low viscosity of the oils, that allows the bioactive to diffuse into the aqueous phase. This can be overcome by SLNs, where the solid matrix prevents the bioactive from being degraded (Jafari, 2017).

SLNs have shown promising results against yeasts and dermatophytes due to their deep cellular penetration, longer retention times and higher concentrations (Svetlichny *et al.*, 2014). Besides, they use physiological lipids, avoid organic solvents and can be easily applicable to large-scale production (Fang *et al.*, 2013). However, these nanocarriers displayed some disadvantages, namely the low loading capacity because of their perfect crystalline structure and the expulsion of the bioactive compounds due to the crystallization process during the storage conditions. Another drawback is the initial burst release which usually occurs with these formulations (Carvalho *et al.*, 2013).

In order to overcome these disadvantages, a new lipid carrier was created. The NLCs were developed by replacing a fraction of the solid lipids with liquid lipids to form the drug incorporating matrix (Salvi & Pawar, 2019). The presence of the liquid lipids provides a better solubility of the bioactive ingredients, enhancing the loading efficiency while preserving the physical stability of the nanocarriers (Beloqui *et al.*, 2016).

Both SLNs and NLCs are very similar, both in their production processes and in the composition of their cores/matrix. In SLNs, the solid lipids form a spatially stable composition, with very little imperfections capable of entrapping the bioactive ingredient. With the addition of a liquid lipid, imperfections are expected to appear in the core of the nanocarrier, allowing for a higher loading efficiency (Fang *et al.*, 2013). These differences are represented in Figure 3.6.

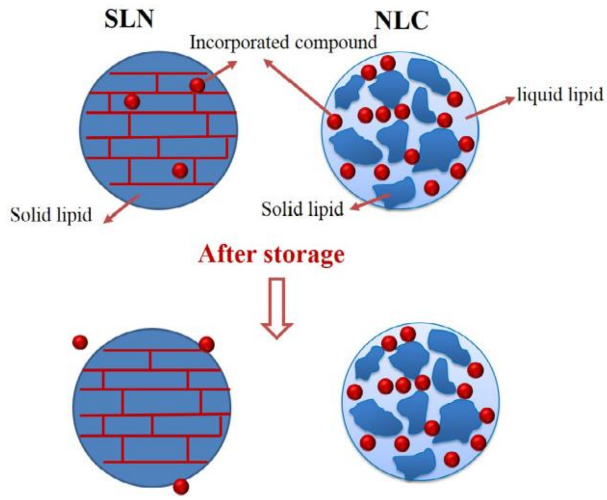

Figure 3.6 - Structure of NLCs and SLNs. Adapted from Rezaei et al., 2019

The main components involved in the preparation of SLNs and NLCs are solid lipids, liquid lipids (in the case on NLCs), surfactant, water, and the compound to be incorporated. Other compounds, such as additives and microbial preservation agents can also be added (Gordillo-Galeano & Mora-Huertas, 2018).

3.3.1. Lipids

Lipids are the main structural material of lipid nanoparticles, up to 30% w/w, being responsible for the main component of the matrix and being largely responsible for the properties of these colloidal systems, namely size, polydispersity, surface charge, short and long-term stability, drug loading and release profile. The main lipids used are free fatty acids, fatty alcohols, glycerol esters and waxes. Some of these lipids have surfactant properties that favour the NPs formation (Tapeinos *et al.*, 2017; Gordillo-Galeano & Mora-Huertas, 2018).

Unlike NEs, in which the lipid phase is composed of oils, in SLNs and NLCs a proportion of the oil is replaced by solid lipids, 100% in the case of SLNs and 70:30 to 99.9:0.1 in NLCs (Khosa *et al.*, 2018).

Yasir & Sara (2014) tested the solubility of haloperidol in solid lipids by evaluating the amount of melted lipid required to dissolve 20 mg haloperidol. The tested lipids were glyceryl monostearate, Compritol 888 ATO, Precirol ATO 5, stearic acid and palmitic acid and the amount required was 47.66 ± 0.95, 49.51 ± 0.83, 55.34 ± 2.24, 82.89 ± 2.10 and 142.37 ± 2.06 mg, respectively. Therefore, glyceryl monostearate was chosen as the solid lipid to produce haloperidol SLNs.

Hady *et al.* (2020) screened a levofloxacin and doxycycline's solubility in five different lipids (stearic acid, Compritol 888 ATO, glyceryl monostearate, Gelucire 50/13 and Carnauba wax). The compounds displayed a higher solubility in stearic acid (approximately 110 mg/g and 90 mg/g for levofloxacin and doxycycline, respectively), followed by Compritol 888 ATO (approximately 80 mg/g and 65 mg/g for levofloxacin and doxycycline, respectively). Both lipids were selected for the development of SLNs to increase the brain uptake of a levofloxacin-doxycycline mixture.

Devkar *et al.* (2014) performed solid and liquid lipids screening to determine which were the best for the production NLCs for nose to brain delivery of ondansetron hydrochloride NLCs. The screened solid lipids were Compritol 888 ATO, glyceryl monostearate and Precirol ATO 5, while the liquid lipids tested were Capryol 90 and oleic acid. The solubilities obtained were 10 ± 2, 52.66 ± 3.05 and 24 ± 1 mg/g, 10 ± 1 and 7.33 ± 1.52 mg/mL, respectively. The chosen lipids were therefore glyceryl monostearate and Capryol 90.

Annu *et al.* (2019) assessed the solubility of lurasidone hydrochloride in different solid and liquid lipids with the goal of optimizing the production of NLCs for brain targeting. The tested solid lipids were Gelot 64, Emulcire, Cremophor, Precirol ATO 5, Compritol 888 ATO and Cutina CP, while the liquid lipids were Capryol 90, castor oil, Labrafac CC and oleic acid. The chosen solid and liquid lipids, which displayed the highest solubility for the drug, were Gelot 64 (approximately 60 mg/g) and Capryol 90 (approximately 35 mg/mL), respectively.

3.3.2. Surfactants

Surfactants and sometimes co-surfactants are used when producing SLNs and NLCs, to diminish the interfacial tension between the lipid and the aqueous phase and prevent aggregation. It has been reported that these substances influence the crystalline structure of the particles and determine their electrokinetic behaviour (Gordillo-Galeano & Mora-Huertas, 2018; Nasirizadeh & Malaekeh-Nikouei, 2020).

Martins *et al.* (2012) produced camptothecin SLNs using three different lipids (cetyl palmitate, Dynasan 14 and Witepsol E85) and 4 different surfactants (polysorbate 20, 40, 60 and 80) at different concentrations (5% and 15% for lipids, 0.8% and 2.0% for surfactant), for a total of 27 formulations and studied their characteristics on the day of production and after a one year storage. The authors, based on the following characteristics of SLNs (having a mean size ≤ 200 nm, homogenous size distribution, low number of microparticles, good storage stability and EE above 90%), considered seven formulations as most suitable. The findings confirmed that the most suitable formulations are composed of cetyl palmitate as lipid (six of the seven formulations), and polysorbate 20, 60 or 80 as surfactant (one, three and three of the formulations, respectively).

Salem *et al.* (2020) attempted to optimize an almotriptan maleate NLC formulation by a D-optimal design with 4 variables: ratio of solid to liquid lipid (50:50, 70:30 or 90:10), type of solid lipid (Compritol 888 ATO, Precirol ATO 5 and stearic acid), type of co-surfactant (Labrasol, Lauroglycol 90 and Transcutol HP) and the effect of chitosan coating. A total of 13 formulations were produced and the formulation with a 70:30 ratio, Compritol 888 ATO as solid lipid and Lauroglycol 90 as co-surfactant (2:1 ratio polysorbate 80:Lauroglycol 90) displayed the lowest particle size and highest %EE, 285.61 ± 3.32 nm and 79.41 ± 0.14 %, respectively. By means of a Design expert software, an optimal formula was found, consisting of Compritol as solid lipid in a 50:50 ratio, lauroglycol as co-surfactant, choated with chitosan. This formula displayed a 254.93 ± 1.85 nm particle size and 80 ± 0.32 %EE.

In Tables 3.5 and 3.6 are represented some published studies where SLNs and NLCs were produced to incorporate several neuroprotective compounds.

Table 3.5 - SLNs produced to encapsulate neuroprotective compounds

Compound	Lipids	Solvent	T	Oil phase	Aqueous phase	Ultrasound	Rotation	Time	Analysis	Ref
Natural compounds										
Andrographolide	Compritol 888 ATO	5 mL acetone for lipids	50 ºC oil phase, 75 ºC aqueous phase	Drug, lipids, fluorescein isothiocyanate	30 mL water, Brij 78		Continuous		DLS, TEM, DSC, HPLC-DAD, HPLC-FLD, *in vitro* drug release, *in vivo* studies	Graverini *et al.* (2018)
BACE1 siRNA	200 mg Witepsol E 85 solid triglycerides	2 mL DCM for lipids	Room T	Drug, lipids, RVG-9R (to increase intracellular pathway), 10 mL polyvinyl alcohol (2% w/v)	Chitosan (1% w/v), water containing 1% v/v acetic acid and PVA (2% w/v)	30 s 70% amplitude	Yes		DLS, SEM, FMPR	Rassu *et al.* (2017)
Camptothecin	5 or 15% Cetyl palmitate, Dynasan 114 or Witepsol E85		5-10 °C above lipid melting point	Drug, lipid	Water, 0.8 or 2% surfactant (Polysorbate 20, 40, 60 and 80)		Yes		PCS, DSC, HPLC, *in vitro* drug release, *in vivo* studies	Martins *et al.* (2012)
Curcumin	Compritol 888 ATO (7.27%)		82-85 ºC	Lipid	Water, polysorbate 80 (45.45%), soy lecithin (0.58%), drug		5000 rpm	1.5h	CLSM, *in vivo* studies	Kakkar *et al.* (2013)

Noscapine	Stearic acid (0.70 mM), egg PC (0.14 mM)		70 ºC	Drug, lipids, sodium glycocholate (0.69 mM)	Distilled water (111.10 mM)		Yes		DLS, TEM, AFM, UV-Vis, FTIR, DSC, PXRD, *in vitro* drug release, *in vivo* studies	Madan *et al.* (2013)
Resveratrol	Compritol 888 ATO	EtOH: Chloroform (20:80% v/v)		Drug, 5 mL lipids	20 mL aqueous solution 3% w/v Tween 80 or 2.5% Tween 80 and 0.5% w/v polyvinyl alcohol	Probe sonicator	15000 rpm		DLS, FTIR, XRD, SEM, UV-Vis, *in vitro* drug release, *in vivo* studies	Jose *et al.* (2014)
Synthetic compounds										
Docetaxel	Soy lecithin, monostearin	3 mL chloroform		10 mg drug, 40 mg lecithin, 100 mg monostearin, 40 mg vitamin E	10 mL deionized water, 150 mg tween 80	15 min	11000 rpm 3 min	3 hours with stirring	XPS, PCS, HPLC, PXRD, *in vitro* drug release, cytotoxicity, *in vivo* studies	Venishetty *et al.* (2013)
Haloperidol	Glyceryl monostearate, Compritol ATO 888, precirol ATO 5, stearic acid or palmitic acid	2.5 mL chloroform: EtOH 1:1 v/v		43.75 - 50 mg Drug, 87.5 - 100 mg lipid	22.5 mL aqueous solution of Tween 80 (1.5 - 1.625% w/v)	5 min 100% amplitude	3000 rpm	30 min	PCS, TEM, UV-Vis, XRD, DSC, HPLC, *in vitro* drug release, *in vivo* studies	Yasir & Sara (2014)
Levofloxacin-Doxycycline	Compritol 888 ATO 2.5-4.5%, stearic acid 1-2%			Drug, lipids, 1.75-2.5% Span 60 (emulsifier)	Distilled water, 0.5% Pluronic F127 (emulsifier)	10 cycles 1 min on/off	24000 rpm		DLS, *in vitro* drug release,	Hady *et al.* (2020)

									UV-Vis, TEM, FTIR, DSC, CLSM, *ex vivo* nasal permeation, HPLC, *in vivo* studies	
Lipophilic Kiteplatin Pt(IV) Prodrugs (SMF 111, 196, 200, 144)	Cetyl palmitate (lipid matrix), 16:0 PEG-2-PE (surface-modifier)	1 mL chloroform	65 °C	Drug, lipids	3 mL ultrapure water, Tween 80 3% p/V (surface modifier)	Probe-tip 0.27 W	Gently stirred	15 min	H-NMR, AAS, *in vitro* drug release, FLM, UV-Vis, DLS, TEM	Arduino *et al.* (2020)

Abbreviations: 16:0 PEG-2-PE - 1,2-Dipalmitoylsn-glycero-3-phosphoethanolamine-N-[methoxy (poly (ethylene glycol)-2000]; AAS – Atomic Absorption Spectroscopy; AFM – Atomic Force Microscopy; BACE1 siRNA – Beta-site APP-cleaving enzyme 1 small interfering RNA; CLSM – Confocal Laser Scanning Microscopy; DCM – Dichloromethane; DLS – Dynamic Light Scattering; DSC – Differential Scanning Calorimetry; EtOH – Ethanol; FMPR – Fluorescence Microplate Reader; FTIR – Fourier-transform Infrared Spectroscopy; H-NMR – Proton Nuclear Magnetic Resonance; HPLC – High-Performance Liquid Chromatography; HPLC-DAD –High-Performance Liquid Chromatography coupled to Diode Array Detection; HPLC-FLD – High Performance Liquid Chromatography with Fluorescence Detection; PCS – Photon Correlation Spectroscopy; PXRD – Powder X-Ray Diffraction; SEM – Scanning Electron Microscopy; SMF 111 - cis,trans,cis $[PtCl_2\{O_2C(CH_2)_4CH_3\}_2$(cis-1,4-diaminocyclohexane)]; SMF 144 - cis,trans,cis$[PtCl_2\{O_2C(CH_2)_8CH_3\}_2$(cis-1,4- diaminocyclohexane)]; SMF 196 - cis,-trans,cis$[PtCl_2\{O_2CCH_3)_2CH_3\}_2$(cis-1,4-diaminocyclohexane)]; - SMF 200 - cis,trans,cis$[PtCl_2\{O_2C(CH_2)_2CH_3\}_2$(cis-1,4-diaminocyclohexane)]; T – Temperature; TEM – Transmission Electron Microscopy; UV-Vis – Ultraviolet-visible spectroscopy; XPS – X-ray Photoelectron Spectroscopy; XRD – X-Ray Diffraction.

Table 3.6 - NLCs produced to encapsulate neuroprotective compounds

Extract	Lipids	Solvent	T	Oil phase	Aqueous phase	Ultrasound	Rotation	Time	Analysis	Ref
Natural compounds										
Baicalin, Salvianolic acid B	Lecithin, Compritol 888 ATO	EtOH for drug and lecithin, chloroform for compritol 888 ATO, mPEG-MAL, mPEG-OH and MCT 812	75°C	Drug, lipids, mPEG-MAL, mPEG-OH, MCT 812	Myrj 52 dissolved in deionized water		Yes	2h	PCS, HPLC, *in vitro* drug release, UPLC, *in vivo* studies	Wu *et al.* (2019)
Resveratrol	Cetyl palmitate, Capmul MCM		5 ºC above solid lipid melting point	Drug, lipids, Acrysol K150	Distilled water, Poloxamer 188, Tween 80	Probe 2 min 30% amplitude, 3s on, 2 off	Yes	2 min	DLS, HPLC, TEM, DSC, FTIR, *in vivo* studies	Rajput & Butani (2019)
Synthetic compounds										
Almotriptan maleate	Compritol 888 ATO, Precirol ATO 5 or stearic acid as solid lipid,		77°C	Drug, lipids	Water, Tween 80: (Lauroglycol, Labrasol or	15 min	Yes	10 min	PCS, UV-Vis, DSC, *in vitro* drug release, *ex vivo* drug permeation,	Salem *et al.* (2020)

	Labrafil M2125CS as liquid lipid				Transcutol) 2:1 (3.5% w/v)				*in vivo* studies	
Asenapine	Glyceryl monostearate (800 mg), oleic acid (160 mg)		70°C	80 mg drug, lipids	50 mL aqueous solution 1.5% w/v Tween 80	5 min 60% amplitude, on/off 0.5 s	16000 rpm		DLS, HPLC, DSC, XRD, FTIR, TEM, AFM, *in vitro* drug release, *in vivo* studies	Singh *et al.* (2018)
Carbamazepine	Trilaurin, oleic acid	DMSO for drug	70°C	Drug, lipids, surfactants (Tween 80, Span 80 and Poloxamer 188)	Deionized water		800 rpm	30 min	DLS, UV-Vis, TEM, DSC, PXRD, FTIR, HPLC, *in vitro* drug release, *in vivo* studies	Khan *et al.* (2020)
Efavirenz	Precirol ATO 5, Captex P 500 (8:2; 7:3)		66°C	Drug, lipids	Deionized water, MYS-25 (1; 2% w/v)	30 s on, 5 off, 75 or 90% amplitude		4 min	HPLC, DLS, TEM, FTIR, DSC, PXRD, *in vitro* drug release, *in vivo* studies	Pokharkar *et al.* (2017)
Lopinavir	Compritol 888, oleic acid (60:40 to 80:20)		80ºC	Drug, lipids	Water, Tween 80	50% amplitude 5 min	1200 rpm	15 min	DLS, UV-Vis, *in vitro* drug release, TEM, *in vivo* studies	Garg *et al.* (2019)
Lurasidone hydrochloride	Gelot 64, Capryol 90	EtOH: acetone 1:1		Drug, lipids	Distilled water, Tween 80, Transcutol P	Probe 6 min	Continuous	2h	PCS, UV-Vis, TEM, SEM, DSC, HPLC, *in vitro* drug release, *in vivo* studies	Annu *et al.* (2019)
Ondansetron hydrochloride	Glyceryl monostearate,		85°C	Drug, glyceryl monostearate, capryol 90	Poloxamer 188, soy lecithin, water		1500 rpm	10 min	DLS, UV-Vis, *in vitro* drug release, *ex vivo* permeation,	Devkar *et al.* (2014)

Rivastigmine	Capryol 90, soy lecithin Glyceryl monostearate, Capmul MCM C8 3:2	1 mL EtOH	70°C	Drug, lipids, 0.1 % stearylamine, lecithin	Double distilled water, Tween 80	180 W, 2 min, 4s pulses, 3s off	Yes	2h	SEM, DSC, XRD, *in vivo* studies UV-Vis, HPLC, *in vivo* studies	Wavikar *et al.* (2017)

Abbreviations: AFM – Atomic Force Microscopy; DLS – Dynamic Light Scattering; DSC – Differential Scanning Calorimetry; FTIR – Fourier-transform Infrared Spectroscopy; HPLC – High-Performance Liquid Chromatography; MCT – Medium Chain Triglycerides; mPEG-OH - Methoxypolyethylene glycol; mPEG-MAL - Methoxypolyethylene glycol maleimide; PCS – Photon Correlation Spectroscopy; PXRD – Powder X-Ray Diffraction; SEM – Scanning Electron Microscopy; TEM – Transmission Electron Microscopy; UPLC – Ultra Performance Liquid Chromatography; UV-Vis – Ultraviolet-visible spectroscopy; XRD – X-Ray Diffraction.

4. Methods for lipid-based nanoparticles characterization

Following the production of nanoparticles, it is essential to perform a characterization of the produced NPs through appropriate and accurate analytical techniques, in order to know their nature, structure and properties, and also the risks associated with the use of said that NPs.

Morphology, size, surface properties and physicochemical properties are important characterization parameters that can affect the stability and functionality of the NPs. These parameters are evaluated via several different techniques, that can be divided into microscopic and spectroscopic techniques, along with techniques from diverse nature for nanostructure characterization (Montes *et al.*, 2019).

4.1. Microscopic techniques

The microscopic techniques used to characterize NPs are usually scanning electron microscopy (SEM), transmission electron microscopy (TEM), atomic force microscopy (AFM) and confocal laser scanner microscopy (CLSM). These techniques provide information on the particles' morphology, size, shape, surface properties, etc (Jafari, 2016).

4.1.1. Electron microscopy

While regular microscopes are based on beams of light, electron microscopes utilize electron beams to form an image. Electrons have a wavelength that can be up to 100,000 times shorter than visible light photons, which in turn leads to an increase of up to 1,000 times in resolving power. The two types of electron microscopes existing are SEM and TEM (Jafari, 2016; Rozo *et al.*, 2020; Fan *et al.*, 2021).

SEM is an electrical conductivity-based technique and is distinguished by having a large depth of field, although it has lower resolution power and does not provide internal details. The electrical beam is focused on the surface of the sample, scanning it line by line, with a detector simultaneously counting the scattered secondary electrons (Jafari, 2016; Rozo *et al.*, 2020; Fan *et al.*, 2021).

TEM is an electron emission-based technique for thin samples (approximately 100 nm), where the beam of electrons passes through the sample, with the detector being present beneath the sample to retrieve the electron providing structural information (Jafari, 2016; Rozo *et al.*, 2020; Fan *et al.*, 2021).

Tung *et al.* (2017) developed nanophytosomes in different curcumin to PC ratios (1:1, 1:2 and 1:4) to protect against paracetamol-induced liver injury and utilized both SEM and TEM to characterize the produced NPs. The particles of the 1:1 ratio under SEM displayed a spherical shape and TEM showed that the particles were uniform and homogeneous. The higher ratios produced particles considered stickier and clammier, possibly explained by an increased presence of PC in the samples. However, a higher ratio resulted in a higher yield in the incorporation of curcumin, 94.64 ± 2.64 % w/w at 1:4 to only 87.74 ± 1.73 % w/w in the 1:1 ratio. This may be also explained by an augmented presence of PC, leading to a bigger interaction between curcumin and the phospholipids, forming more complexes. The 1:1 ratio also displayed higher curcumin content in the complex, 25.64 ± 2.01 to only 8.97 ± 1.25 for the 1:4 ratio. Therefore, the 1:1 formulation was chosen for further studies.

Mahtab *et al.* (2019) applied a QbD approach to the encapsulation of teriflunomide in nanoliposomes and evaluated the NPs via TEM and SEM. The studied variables were the lipid amount (75, 100 or 125 mg), drug amount (5, 7.5 or 10 mg) and sonication time (2, 3.5 or 5 minutes). The optimized formulation had a composition of 100 mg lipid, 7.5 mg teriflunomide and was sonicated for 3.5 min. This formulation led to the formation of nanoliposomes with a mean particle size of 128.92 ± 5.42 nm, 73.94 ± 4.32% EE and *in vitro* drug release of 85.33 ± 8.86% after 24 hours. Also, a polydispersity index of 0.206 ± 0.06 and a zeta potential of 12.6 ± 1.2 mV was obtained by dynamic light scattering (see topic 4.2.1). TEM indicated that the particles were uniformly dispersed and detached from each other while SEM revealed the vesicles were discrete, nanometric, with spherical shape and with no signs of aggregation.

Annu *et al.* (2019) optimized NLCs encapsulating lurasidone hydrochloride through a Box-Behnken design (BBD) and evaluated the particles with both SEM and TEM. The independent variables chosen were the % of total lipid (1, 3 or 5%), the % of surfactant (1, 3 or 4.5%) and the sonication time (2, 6 or 10 minutes), with the optimal formulation having a composition of 5%, 4.5% and 6 min, respectively. The electronic microscopy techniques revealed the spherical shape of the NLCs, with no signs of particle aggregation, with particle size of 200 nm, well correlated with the analysis by another technique (mean particle size of 205.4 ± 2 nm, polydispersity index of 0.371 ± 0.06). The intranasal application of the NLCs led to a max concentration in the brain of 4.90 ± 0.32 μg/mL, compared to 2.54 ± 0.15 and 0.62 ± 0.21 μg/mL of the drug solution delivered intranasally or orally, respectively.

4.1.2. Atomic force microscopy

Several physical properties can be qualitative and quantitatively evaluated by AFM, such as size, morphology, surface texture and roughness. AFM measures the interactions between the surface of the sample and the tip of the probe. These interactions will produce a signal that allow for the image of the structure to be built. AFM grants the possibility to obtain images of the sample from both lateral and vertical positions without significant sample preparation (Jafari, 2017; Rozo, 2020).

Saesoo *et al.* (2016) developed a chitosan surface modified nanoliposome for delivery of cisplatin, an anti-cancer drug. Researchers produced and characterized two different types of nanoliposomes, some with the surface modified by chitosan and others without surface modification. Upon characterization by AFM, it was reported that the particles had discrete and spherical shapes, with no significant differences between the size and shape of different types of NPs. (control blank liposomes had a mean particle size of 141.7 ± 1.8 nm). However, there was a shift in the zeta potential assessed by dynamic light scattering (see topic 4.2.1). The control liposomes had a zeta potential of -7.3 ± 0.5 mV and an incorporation of 2% chitosan resulted in a change from negative to positive. Intracellular uptake was evaluated, with the chitosan modified liposomes displaying an 89.6% uptake, compared to 76.6% from non-modified liposomes, explained by the surface charge. The positive charged chitosan coated liposomes induced electrostatic interactions with the membrane surface, while the negative charged liposomes induced electrostatic repulsion, resulting in a lower rate of endocytosis.

4.1.3. Confocal laser scanner microscopy

CLSM can yield images with high resolution and depth selectivity. It is based on the identification of concentrated light generated by the interaction of an illuminating beam with the small sample. It is dependent on the fluorescent wavelength of the dye utilized. Its main use is the determination of deposition of wall materials (such as oil droplets in O/W nanoemulsions) (Jafari, 2016).

Hady *et al.* (2020) developed SLNs through a BBD approach to increase the brain uptake of a levofloxacin-doxycycline combination and studied the particles formed through CLSM. The independent variables were the concentration of lipids Compritol 888 ATO (2.5, 3.5 or 4.5%) and stearic acid (1, 1.5 or 2%) and the concentration of an emulsifier, Span 60 (1.75, 2.125 or 2.5%). It was reported that the optimized NPs (4.18% Compritol 888 ATO, 1.609% stearic acid and 2.11% Span 60, assessed through a Stat-Ease Design Expert ®), with a mean particle size of 29.33 nm and 100% release rate for levofloxacin and 99.998% for doxycycline, were able to penetrate deeper into the nasal mucosa with high fluorescence intensity, while the free solution had a stronger intensity in the epidermal layers and did not penetrate deeper into the mucosa.

4.2. Spectroscopic techniques

Microscopic techniques are vastly used to determine particle morphology and structure, but they can only provide a semi-quantitative analysis from representative images and may not be able to reveal ultra-small particles or ultra-large aggregates. Therefore, spectroscopic techniques can be used to measure particle size distribution, by using techniques such as dynamic light scattering (DLS), zeta potential, X-ray diffraction (XRD) and nuclear magnetic resonance (NMR) (Fan *et al.*, 2021).

4.2.1. Dynamic light scattering

DLS, also known as photon correlation spectroscopy (PCS), is one of the most utilized and simpler methods for the analysis of particle size distribution. It measures time-dependent fluctuations of the scattered light from particles undergoing Brownian motions (random collisions with solvent molecules that cause diffusion). The particles' hydrodynamic diameter can then be calculated as a function of the diffusion coefficient, which is inversely proportional to particle size. It has been reported that larger particles (≤ 5 mm) are taken up via the lymphatics, while smaller particles (≤ 500 nm) are taken up via endocytosis (Montes *et al.*, 2019; Fan *et al.*, 2021).

DLS is also typically used to measure the surface charge properties, described by the zeta potential of NPs. Zeta potential is usually defined as the distance away from the particle surface below which any counterions remain strongly attached to the particle while it moves in an electrical field. Thus, zeta potential is a measure of the stability of the particles, with the theoretical value to evaluate said stability usually being ± 30 mV. Particles with zeta potential above 30 mV and below – 30 mV will usually have repulsion forces stronger than attraction forces, which prevents aggregation. Particles in the interval between – 30 and 30 mV will usually have attraction forces stronger than repulsion forces, which leads to lower stability and possibility of aggregation (Montes *et al.*, 2019; Fan *et al*., 2021).

Mancini *et al.* (2018) developed four nanoformulations of liposomes and phytosomes to enhance the delivery of an aqueous extract of *Annona muricata* to the brain. All formulations were functionalized by apolipoprotein E to increase BBB crossing. All formulations were evaluated via DLS, with phytosomes having a mean particle size of 106 ± 9 nm and 161 ± 12 nm and polydispersity index (PDI) of 0.245 and 0.275, respectively. The first formulation had cholesterol in the components and the second did not. The liposome formulations were similar, with the results reported being a size of 138 ± 29 nm and 195 ± 33 nm, with PDI of 0.193 and 0.261, respectively. These resulted in a permeability through a BBB model between 4 and 9 times higher.

Saoji *et al.* (2016) developed a phytosome formulation to encapsulate standardized *Centella asiatica* extract, which contains phytoconstituents that act as neuroprotectors against Alzheimer's disease. After characterization through DLS, the mean particle size obtained was 450.1 ± 20.0 nm, with a zeta potential of – 35.0 ± 1.9 mV.

4.2.2. X-Ray Diffraction

XRD is used to characterize the particles' crystallinity. It is based on the passing of an X-ray beam through a crystal of the analysed NP. The beam collides with the electrons of the atoms and is scattered in several directions. This dispersion is due to the symmetry of the set of atoms in the sample. The obtained intensities are interpreted as a function of the geometric ordering of the atoms in the crystal (Montes *et al*., 2019).

Praveen *et al*. (2019) optimized lamotrigine encapsulated nanoliposomes for epilepsy treatment and evaluated their crystalline nature through XRD. The optimization was done by the application of a 11 variable Plackett-Burman design, to assess which variables are significant, followed by a BBD approach to the three variables (sonication time, phospholipid and cholesterol contents) to optimize the formulation. The optimized formulation was as follows: 10 minutes sonication time, 98.5 μmol phospholipid, 50.27 μmol cholesterol, with a mean particle size of 88.90 ± 1.56 nm, polydispersity index of 0.247 ± 0.04, EE of 68.75 ± 0.02% and *in vitro* release of 79.41 ± 1.15%. While pure lamotrigine displayed a crystalline nature, the nanoliposomes showed a disappearance of the peak, representing the reduction of cristallinity and conversion into an amorphous form.

4.2.3. Nuclear Magnetic Resonance

NMR is a tool used for chemical structure analysis. It is based on the measurement of the magnetic properties of the nuclei of the atoms. A pulse of electromagnetic radiofrequency is projected onto the NPs, which causes the alternation of the resonance frequency by the hydrogen nuclei and provide information about the electronic structure of the sample. Its main disadvantage is the significant complexity of the spectra obtained due to many signals present (Jafari, 2017; Montes *et al*., 2019).

Telange *et al.* (2017) applied a full factorial design (3^2) to optimize the production of apigenin-loaded phytosomes. The two variables evaluated were the apigenin to phospholipid ratio (1:1, 1:2 and 1:3) and the reaction temperature (40, 50 and 60 ºC). The optimized NPs (1:2 ratio, 60 ºC), with a mean particle size of 107.8 ± 1.30 nm, zeta potential of -22.35 ± 0.30 mV and polydispersity index of 0.37 ± 0.012, were then characterized via ^{1}H-NMR, by comparing the spectra obtained of pure apigenin and the prepared phytosomes. When comparing the spectra, differences emerged in the upfield and downfield aromatic region, which signal the formation of molecular aggregates with apigenin, through interaction of the charged head of the phospholipids with the phenolic regions of apigenin by weak intermolecular interactions such as hydrogen bonds or ion-dipole interactions. This confirmed the production of apigenin-loaded phytosomes.

4.3. Encapsulation efficiency

The %EE of a nanoformulation is an essential factor in evaluating its successfulness. It measures the amount of encapsulated bioactive compound compared to the amount of bioactive compound used in the NP's production. First, the free bioactive is removed from the medium via techniques such as chromatography, dialysis, centrifugation, etc. Afterwards, the measurement is made via UV-Vis spectrometer or HPLC (Montes *et al.*, 2019).

The encapsulation efficiency is usually calculated by the following formula (Babazadeh *et al.*, 2017):

$$\%EE = \frac{W_T - W_F}{W_T} \, x \, 100$$

Where W_T is the total amount of bioactive compound applied in the formulation and W_f is the amount of free bioactive compound removed.

4.4. *In vitro* drug release

While it is very important to verify if the encapsulation of the bioactive compound is successful, it is also necessary to verify if it is released in the targeted zones. One of the commonly utilized methods is dialysis. The NPs are placed inside a dialysis bag which has pores smaller than the NP's diameter but larger than the bioactive compound's diameter. The released ingredient is measured over time and a release profile is obtained (Jafari, 2017; Fan *et al.*, 2021).

Jaafar-Maalej *et al.* (2009) developed several nanoliposome formulations by modifying phospholipid (20, 40 and 60 mg/mL) and cholesterol (0, 4, 7, 8 and 12 mg/mL) amounts to incorporate beclomethasone dipropionate, a hydrophobic compound, and cytarabine, a hydrophilic compound. The team then evaluated the formulations' drug release by utilizing a dialysis tube on five different formulations of each compound. They reported that no significant differences were verified in the different cytarabine formulas, that showed an initial burst release during the first 3 hours, followed by a steady release, with 80% of the bioactive being released after 7h. As for the beclomethasone dipropionate formulas, phospholipid and cholesterol concentrations had an influence on the release profile. An increase in phospholipid concentrations, while maintaining cholesterol, resulted in a decrease in the amount of bioactive released. Over the first 6 days, less than 10% of beclomethasone dipropionate was released, followed by a following quicker leakage, reaching a maximum over 14 days.

Since the hydrophobic compound is encapsulated within the lipid bilayer, its diffusion through the liposome wall is slow, while the hydrophilic compound, located in the aqueous core, has a rapid efflux to the external medium.

4.5. *In vivo* analysis

An *in vivo* analysis is often made to evaluate the neuroprotective effects of the lipid-based NPs. This is mostly done by resorting to rats which are afterwards subjected to different tests and analysis to assess the NPs' effect.

Saoji *et al.* (2016) developed a phytosome formulation to encapsulate standardized *Centella asiatica* extract , which showed a 12-fold increase in aqueous solubility when compared to the free extract, a 99.2 ± 4.7 to 39.2 ±, % w/w efficiency in the drug release and a 82.8 ± 3.7 to 26.8 ± 2.4, % w/w *ex vivo* permeation through intestine tissue. The efficacy of the prepared phytosomes was also evaluated *in vivo*, by application in Swiss albino mice. The rats were subjected to the *Morris Water Maze Test*, which is used to assess spatial learning and memory in mice by testing the amount of time the mice remain in a target quadrant. The animals were divided into seven groups with six mice each, with 2 control groups, one with young mice and one with aged mice, one group treated with Phospholipon® 90H (900 mg/kg), one group treated with piracetam (200 mg/kg), one group with the free extract (300 mg/kg), one group with the physical mixture of phospholipids and the extract (equivalent to 300 mg/kg of bioactive compound) and one final group with the phytosomes (equivalent to 300 mg/kg of bioactive compound).It was reported that both the phytosome and the free extract show an improvement in the time spent in the target quadrant, although the phytosome displayed a significantly higher efficacy. The phytosomes showed similar results to piracetam, a standard drug. While it has been suggested that phospholipids play a role in memory improvement, no improvement was found in the rats treated with Phospholipon® 90H. The authors suggested that for phospholipids to have a substantial effect in memory improvement, a long-term treatment has to be applied, while in this study the results were assessed after a 4-day treatment period.

Lazar *et al.* (2013) assessed a curcumin nanoliposome formulation's effect on Aβ deposits, an early hallmark lesion of Alzheimer's disease. The analysis were done on human brain tissue, post-mortem mice tissue and live mice. The produced NPs had a mean diameter of 207.2 ± 8.0, a polidispersity index of 0.255 and a zeta potential of – 10.5 ± 1.2. Although the zeta potential is not in the interval in which the particles are considered to exhibit higher stability, it was reported that the particles displayed good stability over a 30 day storage period, while also showing no cell toxicity and partly preventing Aβ-induced cell death. They showed high affinity for the Aβ deposits in both human brain tissue and *post-mortem* mice tissue, and also in living mice tissue. Curcumin is linked to the polar head of the phospholipids, thereby being located in the surface of the nanoliposome, which can explain its high affinity for the Aβ deposits.

5. Comparison of lipid-based nanoparticles with other types of nanoparticles for brain-delivery

Lipid-based, polymeric and inorganic nanoparticles have been extensively studied for the effective delivery of drugs across the BBB via a non-invasive way (Kim *et al.*, 2019).

The polymers used in NP formulation can be natural or synthetic. Some of the most used natural polymers are chitosan, cellulose and gelatine. As for synthetic polymers, polylactic acid and poly-(lactide-coglycolide) are commonly used. These NPs have been vastly researched due to the possibility of high versatility and biocompatibility, depending on the chosen polymer (Kumar *et al.*, 2012; Poovi & Damodharan, 2018; Kim *et al.*, 2019).

The use of polymer NPs has a few critical disadvantages, which has hindered their market application, such as a toxicity of some of the polymers, the presence of solvent residues during production and purification, high cost, degradability, difficulty in the application of large-scale production and the requirement for high quality and high purity polymers (Poovi & Damodharan, 2018).

Inorganic NPs display some advantages over the polymeric NPs, namely in the control of size and shape, the ease of production and functionalization and are easier to track by microscopy or analytic techniques. They also provide a stronger support for the nanostructure than organic-based structures. These NPs are made using heavy metals or semi-conductive metals such as gold, silica or carbon. These NPs have some disadvantages as well, as they might not be degraded or eliminated through the kidneys or display undesired long-term toxicity (Saraiva *et al.*, 2016; Kim *et al.*, 2019).

Lipid-based NPs have several advantages that justify an increased investment in their development. These types of particles display protection against chemical and enzymatic degradation, lipid compatibility, gradual release of active compound from the lipid matrix, diminished adverse side effects and chronic toxicity. Furthermore, they hold the possibility of production without the usage of organic solvents (e.g. in high-pressure homogenization method) and easy scale-up (Kumar *et al.*, 2012; Poovi & Damodharan, 2018).

Of the many NPs being investigated by scientists, the lipid-based NPs have taken the lead, due to the previously mentioned advantages, along with their biocompatibility and versatility. These NPs can be formulated in different ways, to meet a wide range of product requirements, namely disease condition, administration route, as well as other parameters, such as cost, stability, toxicity and efficacy. Their proven safety and efficacy have made them attractive candidates for the formulation of pharmaceuticals (Attama *et al.*, 2012).

Therefore, lipid-based NPs are considered to be at the forefront of the quickly evolving field of nanotechnology, potentially being developed for drug delivery and clinical medicine (Attama *et al.*, 2012).

A lot of formulations have displayed the efficacy of nanocarriers in transporting therapeutic molecules across the BBB at the cellular and animal levels. Despite this, few clinically approved therapeutics across the BBB have yet been reported, while for other diseases (i.e. cancers and cardiovascular diseases) these have already been marketed or clinically approved (Kim *et al.*, 2019).

One of the difficulties associated with the study of BBB crossing are the models utilized. Differences may arise between different species brain microenvironment, hampering the ability to predict the behaviour in humans of a formulation tested in other animals. The appearance of innovative models, organs-on-chips, may help overcome this (Kim *et al.*, 2019).

6. Conclusions

Lipid-based nanoparticles have shown tremendous upside in the prevention and treatment of neurological diseases, which have a large impact on the life of an increasingly higher number of people.

One of the biggest obstacles to the treatment of these diseases is the ability to deliver the drug directly to the brain, mainly due to the BBB's tightness to most drugs. Nanoencapsulation shows up as a possible way to circumvent the BBB.

Lipid-based NPs show some upside when compared to polymeric and inorganic NPs. This has jumpstarted them to the forefront of investigation, with a vast research being done into ways of optimizing formulations in ways that allow for a higher brain uptake.

In this dissertation, the different types of lipid-based NPs were discussed, along with the critical parameters in the formulation of each NP. Furthermore, a summary of existing literature on each type of NP was done, with the formulation parameters being presented and main results discussed.

Moving forward, further research is necessary into the subject, both into optimizing the formulations to improve the bioavailability of the active compounds, and into the brain uptake. The development of models closely resembling the *in vivo* human brain environment may be a critical step in optimizing said particles. This may in turn allow for the appearance of clinically approved therapeutics.

References

Ahmad, N., Ahmad, R., Alam, A. M., Samim, M., Iqbal, Z., & Ahmad, F. J. (2016). Quantification and Evaluation of Thymoquinone Loaded Mucoadhesive Nanoemulsion for Treatment of Cerebral Ischemia. *International Journal of Biological Macromolecules, 88*, pp. 320-332. doi:https://doi.org/10.1016/j.ijbiomac.2016.03.019

Akhavan, S., Assadpour, E., Katouzian, I., & Jafari, S. M. (2018). Lipid Nano Scale Cargos for the Protection and Delivery of Food Bioactive Ingredients and Nutraceuticals. *Trends in Food Science & Technology, 74*, pp. 132-146. doi:https://doi.org/10.1016/j.tifs.2018.02.001

Aliakbari, F., Shabani, A., Bardania, H., Mohammad-Beigi, H., Marvian, A., Esmatabad, F., Vafaei, A., Shojaosadati, S., Saboury, A., Christiansen, G., Otzen, D. & Morshedi, D. (2018). Formulation and Anti-neurotoxic Activity of Baicalein-incorporating Neutral Nanoliposome. *Colloids and Surfaces B: Biointerfaces, 161*, pp. 578-587. doi:https://doi.org/10.1016/j.colsurfb.2017.11.023

Anjana, R., Sunil, K., Hitender, S., & K., K. R. (2017). Phytosome Drug Delivery of Natural Products: A Promising Technique for Enhancing Bioavailability. *International Journal of Drug Delivery Technology, 7*, pp. 157-165. doi:https://doi.org/10.25258/ijddt.v7i03.9559

Annu, I. J., Nabi, B., Moolakkadath, T., Alam, T., Baboota, S., & Ali, J. (2019). Optimization of Nanostructured Lipid Carriers of Lurasidone Hydrochloride Using Box-Behnken Design for Brain Targeting: *In Vitro* and *In Vivo* Studies. *Journal of Pharmaceutical Sciences, 108*, pp. 3082-3090. doi:https://doi.org/10.1016/j.xphs.2019.05.001

Arduino, I., Depalo, N., Re, F., Magro, R. D., Panniello, A., Margiotta, N., Fanizza, E., Lopalco, A., Laquintana, V., Cutrignelli, A., Lopedota, A. A., Franco, M. & Denora, N. (2020). PEGylated Solid Lipid Nanoparticles for Brain Delivery of Lipophilic Kiteplatin Pt(IV) Prodrugs: An *In Vitro* Study. *International Journal of Pharmaceutics, 583*. doi:https://doi.org/10.1016/j.ijpharm.2020.119351

Aroonsri, P., Jintanaporn, W., Saengrawee, S., & Wathita, P. (2008). Anxiety and Cognitive Effects of Quercetin Liposomes in Rats. *Nanomedicine, 4*, pp. 70-78. doi:10.1016/j.nano.2007.12.001

Arora, A., Kumar, S., Ali, J., & Baboota, S. (2020). Intranasal Delivery of Tetrabenazine Nanoemulsion via Olfactory Region for Better Treatment of Hyperkinetic Movement Associated with Huntington's Disease: Pharmacokinetic and Brain Delivery Study. *Chemistry and Physics of Lipids, 230*. doi:https://doi.org/10.1016/j.chemphyslip.2020.104917

Attama, A. A., Momoh, M. A., & Builders, P. F. (2012). Lipid Nanoparticulate Drug Delivery Systems: A Revolution in Dosage Form Design and Development. In A. D. Sezer, *Recent*

Advances in Novel Drug Carrier Systems (pp. 107-109). InTech. doi:http://dx.doi.org/10.5772/50486

Azeez, N. A., Deepa, V. S., & Sivapriya, V. (2018). Phytosomes: Emergent Promising Nano Vesicular Drug Delivery System for Targeted Tumor Therapy. *Vietnam Academy of Science & Technology, 9*. doi:https://doi.org/10.1088/2043-6254/aadc50

Babazadeh, A., Ghanbarzadeh, B., & Hamishehkar, H. (2017). Phosphatidylcholine-Rutin Complex as a Potential Nanocarrier for Food Applications. *Journal of Functional Foods, 33*, pp. 134-141. doi:https://doi.org/10.1016/j.jff.2017.03.038

Bayraktar, O., Erdoğan, İ., Köse, M. D., & Kalmaz, G. (2017). *Nanostructures for Antimicrobial Therapy.* Elsevier.

Beloqui, A., Solinís, M. Á., Rodriguez-Gascón, A., Almeida, A. J., & Préat, V. (2016). Nanostructured Lipid Carriers: Promising Drug Delivery Systems for Future Clinics. *Nanomedicine: Nanotechnology, Biology and Medicine, 12*(1), pp. 143-161. doi:10.1016/j.nano.2015.09.00

Benita, S., & Levy, M. Y. (1993). Submicron Emulsions as Colloidal Drug Carriers for Intravenous Administration: Comprehensive Physicochemical Characterization. *Journal of Pharmaceutical Sciences, 82*(11). doi:10.1002/jps.2600821102

Bhattacharya, S. (2009). Phytosomes: Emerging Strategy in Delivery of Herbal Drugs and Nutraceuticals. *Pharma Times, 41*(3), pp. 9-12.

Bhowmik, A., & Ghosh, M. K. (2015). Blood Brain Barrier: A Challenge for Effectual Therapy of Brain Tumors. *BioMed Research International*, pp. 1-20. doi:10.1155/2015/320941

Campardelli, R., Santo, I. E., Albuquerque, E. C., de Melo, S. V., Porta, G. D., & Reverchon, E. (2016). Efficient Encapsulation of Proteins in Submicron Liposomes using a Supercritical Fluid Assisted Continuous Process. *The Journal of Supercritical Fluids, 107*, pp. 163-169. doi:https://doi.org/10.1016/j.supflu.2015.09.007

Cao, F., Gao, Y., Yin, Z., & Ping, Q. (2012). Enhanced Oral Bioavailability of Oleanolic Acid in Rats with Phospholipid Complex. *Letters in Drug Design & Discovery, 9*, pp. 505-512. doi:10.2174/157018012800389340

Carvalho, S M., Noronha, C. M., Floariani, C. L., Lino, R. C., Rocha, G., Bellettini, I. C., Ogliari, P. J. & Barreto, P. L. M. (2013). Optimization of α-tocopherol Loaded Solid Lipid Nanoparticles by Central Domposite Design. *Industrial Crops and Products, 49*, pp. 278-285. doi:https://doi.org/10.1016/j.indcrop.2013.04.054

Chakraborty, S., Shukla, D., Mishra, B., & Singh, S. (2009). Lipid – An Emerging Platform for Oral Delivery of Drugs with Poor Bioavailability. *European Journal of Pharmaceutics and Biopharmaceutics, 73*, pp. 1-15. doi:https://doi.org/10.1016/j.ejpb.2009.06.001

Chen, Y., & Liu, L. (2012). Modern Methods for Delivery of Drugs Across the Blood–Brain Barrier. *Advanced Drug Delivery Reviews, 64*, pp. 640-665. doi:https://doi.org/10.1016/j.addr.2011.11.010

Chen, Y., Xia, G., Zhao, Z., Xue, F., Gu, Y., Chen, C. & Zhang, Y. (2020). 7,8-Dihydroxyflavone Nano-Liposomes Decorated by Crosslinked and Glycosylated Lactoferrin: Storage Stability, Antioxidant Activity, *In Vitro* Release, Gastrointestinal Digestion and Transport in Caco-2 Cell Monolayers. *Journal of Functional Foods, 65*. doi:https://doi.org/10.1016/j.jff.2019.103742

Chen, Z.-L., Huang, M., Wang, X.-R., Fu, J., Han, M., Shen, Y.-Q., Xia, Z. & Gao, J.-Q. (2016). Transferrin-Modified Liposome Promotes α-Mangostin to Penetrate the Blood-Brain Barrier. *Nanomedicine: Nanotechnology, Biology and Medicine, 12*, pp. 421-430. doi:https://doi.org/10.1016/j.nano.2015.10.021

Colombo, M., Figueiró, F., Dias, A., Teixeira, H. F., Battastini, A. M. & Koester, L. S. (2018). Kaempferol-Loaded Mucoadhesive Nanoemulsion for Intranasal Administration Reduces Glioma Growth *In Vitro*. *International Journal of Pharmaceutics, 543*, pp. 214-223. doi:https://doi.org/10.1016/j.ijpharm.2018.03.055

Copeland, C. & Stabenfeldt, S. E. (2020). Leveraging the Dynamic Blood-Brain Barrier for Central Nervous System Nanoparticle-based Drug Delivery Applications. *Current Opinion in Biomedical Engineering*. doi: https://doi.org/10.1016/j.cobme.2020.04.001

Desai, J. & Thakkar, H. (2018). Enhanced Oral Bioavailability and Brain Uptake of Darunavir using Lipid Nanoemulsion Formulation. *Colloids and Surfaces B: Biointerfaces, 175*, pp. 143-149. doi:https://doi.org/10.1016/j.colsurfb.2018.11.057

Devkar, T. B., Tekade, A. R. & Khandelwal, K. R. (2014). Surface Engineered Nanostructured Lipid Carriers for Efficient Nose to Brain Delivery of Ondansetron HCl using Delonix Regia Gum as a Natural Mucoadhesive Polymer. *Colloids and Surfaces B: Biointerfaces, 122*, pp. 143-150. doi:https://doi.org/10.1016/j.colsurfb.2014.06.037

Ding, S., Khan, A. I., Cai, X., Song, Y., Lyu, Z., Du, D., Dutta, P. & Lin, Y. (2020). Overcoming Blood–Brain Barrier Transport: Advances in Nanoparticle-based Drug Delivery Strategies. *Materials Today*. doi:https://doi.org/10.1016/j.mattod.2020.02.001

Direito, R., Reis, C., Roque, L., Gonçalves, M., Sanches-Silva, A., Gaspar, Maria M., Pinto, R., Rocha, J., Sepodes, B., Bronze, M. M. & Figueira, M. E. (2019). Phytosomes with Persimmon (Diospyros kaki L.) Extract: Preparation and Preliminary Demonstration of In Vivo Tolerability. *Pharmaceutics, 11*. doi:https://dx.doi.org/10.3390/pharmaceutics11060296

Đorđević, S. M., Cekić, N. D., Savić, M. M., Isailović, T. M., Ranđelović, D. V., Marković, B. D., Savić, S. R., Stamenić, T. T., Daniels, R. & Savić, S. D. (2015). Parenteral Nanoemulsions as Promising Carriers for Brain Delivery of Risperidone: Design, Characterization and *In Vivo* Pharmacokinetic Evaluation. *International Journal of Pharmaceutics, 493*, pp. 40-54. doi:https://doi.org/10.1016/j.ijpharm.2015.07.007

Đorđević, S. M., Santrač, A., Cekić, N. D., Marković, B. D., Divović, B., Ilić, T. M., Savić, M. M. & Savić, S. D. (2017). Parenteral Nanoemulsions of Risperidone for Enhanced Brain Delivery in Acute Psychosis: Physicochemical and *In Vivo* Performances. *International Journal of Pharmaceutics, 533*, pp. 421-430. doi:https://doi.org/10.1016/j.ijpharm.2017.05.051

El-Fattah, A. I., Fathy, M. M., Ali, Z. Y., El-Garawny, A. E.-R. & Mohamed, E. K. (2017). Enhanced Therapeutic Benefit of Quercetin-loaded Phytosome Nanoparticles in Ovariectomized Rats. *Chemico-Biological Interactions, 271*, pp. 30-38. doi:https://doi.org/10.1016/j.cbi.2017.04.026

El-Gazayerly, O. N., Makhlouf, A. I., Soelm, A. M., & Mohmoud, M. A. (2013). Antioxidant and Hepatoprotective Effects of Silymarin Phytosomes Compared to Milk Thistle Extract in CCl_4 Induced Hepatotoxicity in Rats. *Journal of Microencapsulation, 31*, pp. 23-30. doi:10.3109/02652048.2013.805836

El-Samaligy, M. S., Afifi, N. N., & Mahmoud, E. A. (2006). Evaluation of Hybrid Liposomes-Encapsulated Silymarin Regarding Physical Stability and *In Vivo* Performance. *International Journal of Pharmaceutics, 319*, pp. 121-129. doi:10.1016/j.ijpharm.2006.04.023

Emami, J., Yousefian, H., & Sadeghi, H. (2018). Targeted Nanostructured Lipid Carrier for Brain Delivery of Artemisinin: Design, Preparation, Characterization, Optimization and Cell Toxicity. *Journal of Pharmacy & Pharmaceutical Sciences, 21*, pp. 225-241. doi:10.18433/jpps30117

Fan, Y., Marioli, M., & Zhang, K. (2021). Analytical Characterization of Liposomes and Other Lipid Nanoparticles for Drug Delivery . *Journal of Pharmaceutical and Biomedical Analysis, 192*. doi:https://doi.org/10.1016/j.jpba.2020.113642

Fang, C.-L., Al-Suwayeh, S. A., & Fang, J.-Y. (2013). Nanostructured Lipid Carriers (NLCs) for Drug Delivery and Targeting. *Recent Patents on Nanotechnology, 7*(1), pp. 41-55. doi:https://doi.org/10.2174/187221013804484827

Feignin, V. L., Vos, T., Nichols, E., Owolabi, M. O., Carroll, W. M., Dichgans, M., Deuschl, G., Parmar, P., Brainin, M. & Murray, C. (2020). The Global Burden of Neurological Disorders: Translating Evidence into Policy. *The Lancet Neurology, 19*, pp. 255-265. doi:https://doi.org/10.1016/S1474-4422(19)30411-9

Franco, M. K., Araújo, D. R., Paula, E. D., Cavalcanti, L., & Yokaichiya, F. (2017). X-Ray Scattering Techniques Applied in the Development of Drug Delivery Systems. *X-Ray Scattering*. doi:10.5772/65326

Freag, M. S., Saleh, W. M., & Abdallah, O. Y. (2018). Self-assembled Phospholipid-based Phytosomal Nanocarriers as Promising Platforms for Improving Oral Bioavailability of the Anticancer Celastrol. *International Journal of Pharmaceutics, 535*, pp. 18-26. doi:https://doi.org/10.1016/j.ijpharm.2017.10.053

Fresta, M., Puglisi, G., Di Giacomo, C., & Russo, A. (1994). Liposomes as *In Vivo* Carriers for Citicoline: Effects on Rat Cerebral Post-ischaemic Reperfusion. *Journal of Pharmacy and Pharmacology, 46*(12), pp. 974-981. doi:10.1111/j.2042-7158.1994.tb03252.x

Froiio, F., Gagliardi, A., Fresta, M., Cosco, D., & Paolino, D. (2019). Phytosomes as Useful Drug Delivery Systems for Cosmeceutical Application. In M. Gupta, D. N. Chauhan, V. Sharma, & N. S. Chauhan, *Novel Drug Delivery Systems for Phytoconstituents* (pp. 105-115).

Garg, B., Beg, S., Kumar, R., Katare, O. P., & Singh, B. (2019). Nanostructured Lipidic Carriers of Lopinavir for Effective Management of HIV-associated Neurocognitive Disorder. *Journal of Drug Delivery Science and Technology, 53*. doi:https://doi.org/10.1016/j.jddst.2019.101220

Ghanbarzadeh, B., Babazadeh, A., & Hamishehkar, H. (2016). Nano-Phytosome as a Potential Food-grade Delivery System. *Food Bioscience, 15*, pp. 126-135. doi:https://doi.org/10.1016/j.fbio.2016.07.006

Ghorbanzade, T., Jafari, S. M., Akhavan, S., & Hadavi, R. (2016). Nano-encapsulation of Fish Oil in Nano-Liposomes and its Application in Fortification of Yogurt. *Food Chemistry, 216*. doi:https://doi.org/10.1016/j.foodchem.2016.08.022

Gnananath, K., Nataraj, K. S., & Rao, B. G. (2017). Phospholipid Complex Technique for Superior Bioavailability of Phytoconstituents. *Advanced Pharmaceutical Bulletin*, pp. 35-42. doi:10.15171/apb.2017.005

Gordillo-Galeano, A. & Mora-Huertas, C. E. (2018). Solid Lipid Nanoparticles and Nanostructured Lipid Carriers: A Review Emphasizing on Particle Structure and Drug Release. *European Journal of Pharmaceutics and Biopharmaceutics, 133*, pp. 285-308. doi:https://doi.org/10.1016/j.ejpb.2018.10.017

Graverini, G., Piazzini, V., Landucci, E., Pantano, D., Nardiello, P., Casamenti, F., Pellegrini-Giampietro, D. E., Bilia, A. R., Bergonzi, M. C. (2018). Solid Lipid Nanoparticles for Delivery of Andrographolide across the Blood-Brain Barrier: *In Vitro* and *In Vivo* Evaluation. *Colloids and Surfaces B: Biointerfaces, 161*, pp. 302-313. doi:https://doi.org/10.1016/j.colsurfb.2017.10.062

Gülseren, I., Guri, A., & Corredig, M. (2012). Encapsulation of Tea Polyphenols in Nanoliposomes Prepared with Milk Phospholipids and Their Effect on the Viability of HT-29 Human Carcinoma Cells. *Food Digestion, 3*, pp. 36-45. doi:10.1007/s13228-012-0019-8

Hady, M. A., Sayed, O. M., & Akl, M. A. (2020). Brain Uptake and Accumulation of New Levofloxacin-Doxycycline Combination Through the Use of Solid Lipid Nanoparticles: Formulation; Optimization and *In Vivo* Evaluation. *Colloids and Surfaces B: Biointerfaces, 193*. doi:https://doi.org/10.1016/j.colsurfb.2020.111076

Haider, M. F., Khan, S., Gaba, B., Alam, T., Baboota, S., Ali, J., & Ali, A. (2018). Optimization of Rivastigmine Nanoemulsion for Enhanced Brain Delivery: *In Vivo* and Toxicity Evaluation. *Journal of Molecular Liquids, 255*, pp. 384-396. doi:https://doi.org/10.1016/j.molliq.2018.01.123

Hamadou, A. H., Huang, W.-C., Xue, C., & Mao, X. (2020). Comparison of β-carotene Loaded Marine and Egg Phospholipids Nanoliposomes . *Journal of Food Engineering, 283*. doi:https://doi.org/10.1016/j.jfoodeng.2020.110055

Harilal, S., Jose, J., Parambi, D. J., Kumar, R., Unnikrishnan, M. K., Uddin, M. S., Mathew, G. E., Pratap, R., Marathakam, A. & Mathew, B. (2020). Revisiting the Blood-Brain Barrier: A Hard Nut to Crack in the Transportation of Drug Molecules. *Brain Research Bulletin, 160*, pp. 121-140. doi:https://doi.org/10.1016/j.brainresbull.2020.03.018

Harwansh, R. K., Deshmukh, R., & Rahman, M. A. (2019). Nanoemulsion: Promising Nanocarrier System for Delivery of Herbal Bioactives. *Journal of Drug Delivery Science and Technology, 51*, pp. 224-233. doi:https://doi.org/10.1016/j.jddst.2019.03.006

Hasan, M., Belhaj, N., Benachour, H., Barberi-Heyob, M., Kahn, C. J., Jabbari, E., Linder, M. & Arab-Tehrany, E. (2014). Liposome Encapsulation of Curcumin: Physico-chemical Characterizations and Effects on MCF7 Cancer Cell Proliferation. *International Journal of Pharmaceutics, 461*, pp. 519-528. doi:https://doi.org/10.1016/j.ijpharm.2013.12.007

He, H., Yao, J., Zhang, Y., Chen, Y., Wang, K., Lee, R. J., Yu, B. & Zhang, X. (2019). Solid Lipid Nanoparticles as a Drug Delivery System to Cross the Blood-Brain Barrier. *Biochemical and Biophysical Research Communications*. doi:https://doi.org/10.1016/j.bbrc.2019.09.017

Hooresfand, Z., Ghanbarzadeh, S., & Hamishehkar, H. (2015). Preparation and Characterization of Rutin-loaded Nanophytosomes. *Pharmaceutical Sciences, 21*, pp. 145-151. doi:10.15171/PS.2015.29

Hu, Y., Gaillard, P. J., Lange, E., & Hammarlund-Udenaes, M. (2019). Targeted Brain Delivery of Methotrexate by Glutathione PEGylated Liposomes: How Can the Formulation Make a Difference? *European Journal of Pharmaceutics and Biopharmaceutics, 139*, pp. 197-204. doi:10.1016/j.ejpb.2019.04.004

Hussein, J., El-Bana, M., Refaat, E., & El-Naggar, M. E. (2017). Synthesis of Carvacrol-based Nanoemulsion for Treating Neurodegenerative Disorders in Experimental Diabetes. *Journal of Functional Foods, 37*, pp. 441-448. doi:https://doi.org/10.1016/j.jff.2017.08.011

Indena. (2020, 07 26). *Product List.* Retrieved from Indena: www.indena.com

Iqbal, R., Ahmed, S., Jain, G. K., & Vohora, D. (2019). Design and Development of Letrozole Nanoemulsion: A Comparative Evaluation of Brain Targeted Nanoemulsion With Free Letrozole Against Status Epilepticus and Neurodegeneration in Mice. *International*

Journal of Pharmaceutics, 565, pp. 20-32. doi:https://doi.org/10.1016/j.ijpharm.2019.04.076

Iwanaga, K., Ono, S., Narioka, K., Kakemi, M., Morimoto, K., Yamashita, S., Namba, Y. & Oku, N. (1999). Application of Surface-Coated Liposomes for Oral Delivery of Peptide: Effects of Coating the Liposome's Surface on the GI Transit of Insulin. *Journal of Pharmaceutical Sciences, 88*. doi:10.1021/js980235x

Jaafar-Maalej, C., Diab, R., Andrieu, V., Elaissari, A., & Fessi, H. (2009). Ethanol Injection Method for Hydrophilic and Lipophilic Drug-loaded Liposome Preparation. *Journal of Liposome Research, 20*, pp. 228-243. doi:https://doi.org/10.3109/08982100903347923

Jafari, S. M. (2006). Nano-Emulsion Production by Sonication and Microfluidization—A Comparison. *International Journal of Food Properties, 9*. doi:10.1080/10942910600596464

Jafari, S. M. (2016). *Nanoencapsulation Technologies for the Food and Nutraceutical Industries.* London: Elsevier.

Jafari, S. M. (2017). *Nanoencapsulation of Food Bioactive Ingredients: Principles and Applications.* London: Elsevier.

Jafari, S. M. (2019). *Lipid-Based Nanostructures for Food Encapsulation Purposes: Volume 2 in the Nanoencapsulation in the Food Industry series.* Cambridge: Academic Press.

Jafari, S. M., He, Y., & Bhandari, B. (2007). Encapsulation of Nanoparticles of d-Limonene by Spray Drying: Role of Emulsifiers and Emulsifying Techniques. *Drying Technology, 25*(6), pp. 1069-1079. doi:https://doi.org/10.1080/07373930701396758

Jain, S., Dhanotiya, C., & Malviya, N. (2012). Physicochemical Characterization and Determination of Free Radical Scavenging Activity of Rutin-Phospholipid Complex. *International Journal of Pharmaceutical Sciences and Research, 3*, pp. 909-913.

Jose, S., Anju, S. S., Cinu, T. A., Aleykutty, N. A., Thomas, S., & Souto, E. B. (2014). *In Vivo* Pharmacokinetics and Biodistribution of Resveratrol-loaded Solid Lipid Nanoparticles for Brain Delivery. *International Journal of Pharmaceutics, 474*, pp. 6-13. doi:https://doi.org/10.1016/j.ijpharm.2014.08.003

Kakkar, V., Mishra, A. K., Chuttani, K., & Kaur, I. P. (2013). Proof of Concept Studies to Confirm the Delivery of Curcumin Loaded Solid Lipid Nanoparticles (C-SLNs) to Brain. *International Journal of Pharmaceutics, 448*, pp. 354-359. doi:https://doi.org/10.1016/j.ijpharm.2013.03.046

Kalepu, S., Manthina, M., & Padavala, V. (2013). Oral Lipid-based Drug Delivery Systems – An Overview. *Acta Pharmaceutica Sinica B, 3*, pp. 361-372. doi:https://doi.org/10.1016/j.apsb.2013.10.001

Kalita, B. & Patwary, B. N. (2019). Formulation and *In Vitro* Evaluation of Hesperidin-Phospholipid Complex and its Antioxidant Potential. *Current Drug Therapy, 14*, pp. 1-10. doi:10.2174/1574885514666190226155933

Karthivashan, G., Masarudin, J., M., Kura, A. U., Abas, F., & Fakurazi, S. (2016). Optimization, Formulation, and Characterization of Multiflavonoids-loaded Flavanosome by Bulk or Sequential Technique. *International Journal of Nanomedicine, 11*, pp. 3417-3434. doi:https://doi.org/10.2147/IJN.S112045

Katouzian, I., Esfanjani, A. F., Jafari, S. M., & Akhavan, S. (2017). Formulation and Application of a New Generation of Lipid Nano-carriers for the Food Bioactive Ingredients. *Trends in Food Science & Technology, 68*, pp. 14-25. doi:https://doi.org/10.1016/j.tifs.2017.07.017

Khan, J., Alexander, A., Ajazuddin, Saraf, S., & Saraf, S. (2013). Recent Advances and Future Prospects of Phyto-Phospholipid Complexation Technique for Improving Pharmacokinetic Profile of Plant Actives. *Journal of Controlled Release, 168*, pp. 50-60. doi:https://doi.org/10.1016/j.jconrel.2013.02.025

Khan, N., Shah, F. A., Rana, I., Ansari, M. M., Din, F. u., Rizvi, S. Z., Aman, W., Lee, G.-Y., Lee, E.-S., Kim, J.-K. & Zeb, A. (2020). Nanostructured Lipid Carriers-mediated Brain Delivery of Carbamazepine for Improved *In Vivo* Anticonvulsant and Anxiolytic Activity. *International Journal of Pharmaceutics, 577*. doi:https://doi.org/10.1016/j.ijpharm.2020.119033

Khosa, A., Reddi, S., & Saha, R. S. (2018). Nanostructured Lipid Carriers for Site-specific Drug Delivery. *Biomedicine & Pharmacotherapy, 103*, pp. 598-613. doi:https://doi.org/10.1016/j.biopha.2018.04.055

Kim, J., Ahn, S. I., & Kim, Y. T. (2019). Nanotherapeutics Engineered to Cross the Blood-Brain Barrier for Advanced Drug Delivery to the Central Nervous System. *Journal of Industrial and Engineering Chemistry, 73*, pp. 8-18. doi:https://doi.org/10.1016/j.jiec.2019.01.021

Kim, S.-M., Jung, J.-I., Chai, C., & Imm, J.-Y. (2019). Characteristics and Glucose Uptake Promoting Effect of Chrysin-Loaded Phytosomes Prepared with Different Phospholipid Matrices. *Nutrients, 11*. doi:doi:10.3390/nu11102549

Kizelsztein, P., Ovadia, H., Garbuzenko, O., Sigal, A., & Barenholz, Y. (2009). Pegylated Nanoliposomes Remote-loaded With the Antioxidant Tempamine Ameliorate Experimental Autoimmune Encephalomyelitis. *Journal of Neuroimmunology, 213*, pp. 20-25. doi:https://doi.org/10.1016/j.jneuroim.2009.05.019

Kumar, M., Misra, A., Babbar, A. K., Mishra, A. K., Mishra, P., & Pathak, K. (2008). Intranasal Nanoemulsion Based Brain Targeting Drug Delivery System of Risperidone. *358*, pp. 285-291. doi:https://doi.org/10.1016/j.ijpharm.2008.03.029

Kumar, S., Baldi, A., & Sharma, D. K. (2020). Phytosomes: A Modernistic Approach for Novel Herbal Drug Delivery - Enhancing Bioavailability and Revealing Endless Frontier of Pharmaceuticals. *Journal of Developing Drugs, 9*(195). doi:10.4172/2329-6631

Kumar, S., Dilbaghi, N., & Saharan, R. (2012). Nanotechnology as Emerging Tool for Enhancing Solubility of Poorly Water-Soluble Drugs. *BioNanoScience, 2*, pp. 227-250. doi:10.1007/s12668-012-0060-7

Kumbhar, S. A., Kokare, C. R., Shrivastava, B., Gorain, B., & Choudhury, H. (2020). Preparation, Characterization, and Optimization of Asenapine Maleate Mucoadhesive Nanoemulsion Using Box-Behnken Design: *In Vitro* and *In Vivo* Studies for Brain Targeting. *International Journal of Pharmaceutics, 586*. doi:https://doi.org/10.1016/j.ijpharm.2020.119499

Kuo, Y.-C., Chen, I.-Y., & Rajesh, R. (2018). Use of Functionalized Liposomes Loaded With Antioxidants to Permeate the Blood–Brain Barrier and Inhibit β-amyloid-induced Neurodegeneration in the Brain. *Journal of the Taiwan Institute of Chemical Engineers, 87*, pp. 1-14. doi:https://doi.org/10.1016/j.jtice.2018.03.001

Lazar, A. N., Mourtas, S., Youssef, I., Parizot, C., Dauphin, A., Delatour, B., Antimisiaris, S. & Duyckaerts, C. (2013). Curcumin-conjugated Nanoliposomes With High Affinity for Aβ Deposits: Possible Applications to Alzheimer Disease. *Nanomedicine: Nanotechnology, Biology, and Medicine, 9*, pp. 712-721. doi:https://doi.org/10.1016/j.nano.2012.11.004

Li, Y., Yang, D.-J., Chen, S.-L., Chen, S.-B., & Chan, A. S.-C. (2008). Process Parameters and Morphology in Puerarin, Phospholipids and their Complex Microparticles Generation by Supercritical Antisolvent Precipitation. *International Journal of Pharmaceutics, 359*, pp. 35-45. doi:https://doi.org/10.1016/j.ijpharm.2008.03.022

Li, Z., Jiang, H., Xu, C., & Gu, L. (2015). A Review: Using Nanoparticles to Enhance Absorption and Bioavailability of Phenolic Phytochemicals. *Food Hydrocolloids, 43*, pp. 153-164. doi:https://doi.org/10.1016/j.foodhyd.2014.05.010

Lim, A.-W., Ng, P.-Y., Chieng, N., & Ng, S.-F. (2019). Moringa Oleifera Leaf Extract–loaded Phytophospholipid Complex for Potential Application as Wound Dressing. *Journal of Drug Delivery Science & Technology, 54*. doi:https://doi.org/10.1016/j.jddst.2019.101329

Lira, M. C., Ferraz, M. S., Silva, D. G., Cortes, M. E., Teixeira, K. I., Caetano, N. P., Sinisterra, R. D., Ponchel, G. & Santos-Magalhães, N. S. (2009). Inclusion Complex of Usnic Acid With b-cyclodextrin: Characterization And Nanoencapsulation into Liposomes. *Journal of Inclusion Phenomena and Macrocyclic Chemistry, 64*, pp. 215-224. doi:10.1007/s10847-009-9554-5

Liu, W., Hou, Y., Wang, Y., Xu, X., & Han, J. (2020). Research Progress on Liposomes: Application in Food, Digestion Behavior and Absorption Mechanism. *Trends in Food Science & Technology*. doi:https://doi.org/10.1016/j.tifs.2020.08.012

Liu, W., Liu, J., Li, T., & Liu, C. (2013). Improved Physical and *In Vitro* Digestion Stability of a Polyelectrolyte Delivery System Based on Layer-by-Layer Self-Assembly Alginate−Chitosan-Coated Nanoliposomes. *Journal of Agricultural and Food Chemistry, 61*, pp. 4133-4144. doi:dx.doi.org/10.1021/jf305329n

Liu, W., Ye, A. L., Liu, C., Han, J., & Singh, H. (2015). Behaviour of Liposomes Loaded With Bovine Serum Albumin During *In Vitro* Digestion. *Food Chemistry, 175*, pp. 16-24. doi:https://doi.org/10.1016/j.foodchem.2014.11.108

Liu, W., Ye, A., Liu, W., & Singh, H. (2012). Structure and Integrity of Liposomes Prepared from Milk- or Soybean-derived Phospholipids During *In Vitro* Digestion. *Food Research International, 48*, pp. 499-506. doi:https://doi.org/10.1016/j.foodres.2012.04.017

Lu, M., Qiu, Q., Luo, X., Liu, X., Sun, J., Wang, C., Lin, X., Deng, Y. & Song, Y. (2018). Phyto-Phospholipid Complexes (Phytosomes): A Novel Strategy to Improve the Bioavailability of Active Constituents. *Asian Journal of Pharmaceutical Sciences, 14*, pp. 265-274. doi:https://doi.org/10.1016/j.ajps.2018.05.011

Madan, J., Pandey, R. S., Jain, V., Katare, O. P., Chandra, R., & Katyal, A. (2013). Poly (ethylene)-glycol Conjugated Solid Lipid Nanoparticles of Noscapine Improve Biological Half-Life, Brain Delivery and Efficacy in Glioblastoma Cells. *Nanomedicine: Nanotechnology, Biology and Medicine, 9*, pp. 492-503. doi:https://doi.org/10.1016/j.nano.2012.10.003

Mahtab, A., Rizwanullah, M., Pandey, S., Leekha, A., Rabbani, S. A., Verma, A. K., Aqil, M. & Talegaonkar, S. (2019). Quality by Design Driven Development and Optimization of Teriflunomide Loaded Nanoliposomes for Treatment of Rheumatoid Arthritis: An *In Vitro* and *In Vivo* Assessments. *Journal of Drug Delivery Science and Technology, 51*, pp. 383-396. doi:https://doi.org/10.1016/j.jddst.2019.03.008

Maiti, K., Mukherjee, K., Gantait, A., Saha, B. P., & Mukherjee, P. K. (2007). Curcumin–Phospholipid Complex: Preparation, Therapeutic Evaluation and Pharmacokinetic Study in Rats. *International Journal of Pharmaceutics, 330*, pp. 155-163. doi:https://doi.org/10.1016/j.ijpharm.2006.09.025

Maiti, K., Mukherjee, K., Murugan, V., Saha, B. P., & Mukherjee, P. K. (2010). Enhancing Bioavailability and Hepatoprotective Activity of Andrographolide from Andrographis Paniculata, a Well-known Medicinal Food, Through its Herbosome. *Journal of The Science of Food and Agriculture, 90*, pp. 43-51. doi:10.1002/jsfa.3777

Mallick, A., Gupta, Archita, Hussain, A., Aparajay, P., Singh, S., Singh, S. K. & Dev, A. (2020). Intranasal Delivery of Gabapentin Loaded Optimized Nanoemulsion for Augmented Permeation. *Journal of Drug Delivery Science and Technology, 56*. doi:https://doi.org/10.1016/j.jddst.2020.101606

Mancini, S., Nardo, L., Gregori, M., Ribeiro, I., Mantegazza, F., Delerue-Matos, C., Masserini, M. & Grosso, C. (2018). Functionalized Liposomes and Phytosomes Loading Annona Muricata L. Aqueous Extract: Potential Nanoshuttles for Brain-delivery of Phenolic

Compounds. *Phytomedicine, 42*, pp. 233-244. doi:https://doi.org/10.1016/j.phymed.2018.03.053

Markoutsa, E., Pampalakis, G., Niarakis, A., Romero, I. A., Weksler, B., Couraud, P.-O., & Antimisiaris, S. G. (2011). Uptake and Permeability Studies of BBB-targeting Immunoliposomes Using the hCMEC/D3 Cell Line. *European Journal of Pharmaceutics and Biopharmaceutics, 77*, pp. 265-274. doi:https://doi.org/10.1016/j.ejpb.2010.11.015

Martins, S., Tho, I., Reimold, I., Fricker, G., Souto, E., & Ferreira, D. B. (2012). Brain Delivery of Camptothecin by Means of Solid Lipid Nanoparticles: Formulation Design, *In Vitro* and *In Vivo* Studies. *International Journal of Pharmaceutics, 439*, pp. 49-62. doi:https://doi.org/10.1016/j.ijpharm.2012.09.054

Maryana, W., Rachmawati, H., & Mudhakir, D. (2016). Formation of Phytosome Containing Silymarin Using Thin Layer-Hydration Technique Aimed for Oral Delivery. *Materials Today: Proceedings, 3*, pp. 855-866. doi:https://doi.org/10.1016/j.matpr.2016.02.019

Matias, D., Roque, L., Simões, M. D., Diaz-Lanza, A., Rijo, P., & Reis, C. P. (2015). Plectranthus Madagascariensis Phytosomes: Formulation Optimization. *Biomedical and Biopharmaceutical Research, 12*, pp. 223-231.

McClements, D. J. (2011). Edible Nanoemulsions: Fabrication, Properties, and Functional Performance. *Soft Matter, 7*(6), pp. 2297-2316. doi:10.1039/c0sm00549e

Md, S., Gan, S. Y., Haw, Y. H., Liang, H. C., Sinrun, W., & Choudhury, H. (2018). *In Vitro* Neuroprotective Effects of Naringenin Nanoemulsion Against β-amyloid Toxicity Through the Regulation of Amyloidogenesis and Tau Phosphorylation. *International Journal of Biological Macromolecules, 118*, pp. 1211-1219. doi:https://doi.org/10.1016/j.ijbiomac.2018.06.190

Montes, C., Villaseñor, M. J., & Ríos, Á. (2019). Analytical Control of Nanodelivery Lipid-based Systems for Encapsulation of Nutraceuticals: Achievements and Challenges. *Trends in Food Science & Technology, 90*, pp. 47-62. doi:https://doi.org/10.1016/j.tifs.2019.06.001

Mori, N., Kurokouchi, A., Osonoe, K., Saitoh, H., Ariga, K., Suzuki, K., & Iwata, Y. (1995). Liposome-entrapped Phenytoin Locally Suppresses Amygdaloid Epileptogenic Focus Created by db-cAMP/EDTA in Rats. *Brain Research, 703*, pp. 184-190. doi:10.1016/0006-8993(95)01095-5

Mourtas, S., Lazar, A. N., Markoutsa, E., Duyckaerts, C., & Antimisiaris, S. G. (2014). Multifunctional Nanoliposomes With Curcumin-lipid Derivative and Brain Targeting Functionality With Potential Applications for Alzheimer Disease. *European Journal of Medicinal Chemistry, 80*, pp. 175-183. doi:https://doi.org/10.1016/j.ejmech.2014.04.050

Mozafari, M. R., Khosravi-Davani, K., Borazan, G. G., Cui, J., Pardakhty, A., & Yurdugul, S. (2008). Encapsulation of Food Ingredients Using Nanoliposome Technology. *International Journal of Food Properties, 11*(4), pp. 833-844. doi:10.1080/10942910701648115

Mozafari, M. R., Reed, C. J., Rostron, C., Kocum, C., & Piskin, E. (2002). Construction of Stable Anionic Liposome-Plasmid Particles using the Heating Method: A Preliminary Investigation. *Cellular & Molecular Biology Letters, 7*, pp. 923-927.

Mufamadi, M. S., Choonara, Y. E., Kumar, P., Modi, G., Naidoo, D., Vuuren, S. v., Ndesendo, V., Toit, L., Iyuke, S. & Pillay, V. (2013). Ligand-functionalized Nanoliposomes for Targeted Delivery of Galantamine. *International Journal of Pharmaceutics, 448*, pp. 267-281. doi:https://doi.org/10.1016/j.ijpharm.2013.03.037

Nasirizadeh, S. & Malaekeh-Nikouei, B. (2020). Solid Lipid Nanoparticles and Nanostructured Lipid Carriers in Oral Cancer Drug Delivery. *Journal of Drug Delivery Science and Technology, 55*. doi:https://doi.org/10.1016/j.jddst.2019.101458

Nazari, M., Ghanbarzadeh, B., Kafil, H. S., Zeinali, M., & Hamishehkar, H. (2019). Garlic Essential Oil Nanophytosomes as a Natural Food Preservative: Its Application in Yogurt as Food Model. *Colloid and Interface Science Communications, 30*. doi:https://doi.org/10.1016/j.colcom.2019.100176

Nirale, P., Paul, A., & Yadav, K. S. (2020). Nanoemulsions for Targeting the Neurodegenerative Diseases: Alzheimer's, Parkinson's and Prion's. *Life Sciences, 245*. doi:https://doi.org/10.1016/j.lfs.2020.117394

Pandit, R., Chen, L., & Götz, J. (2019). The Blood-Brain Barrier: Physiology and Strategies for Drug Delivery. *Advanced Drug Delivery Reviews*. doi:https://doi.org/10.1016/j.addr.2019.11.009

Patel, J., Patel, R., Khambolja, K., & Patel, N. (2009). An Overview of Phytosomes as an Advanced Herbal Drug Delivery System. *Journal of Pharmaceutical Sciences, 4*, pp. 363-371.

Patel, R. J. & Parikh, R. H. (2020). Intranasal Delivery of Topiramate Nanoemulsion: Pharmacodynamic, Pharmacokinetic and Brain Uptake Studies. *International Journal of Pharmaceutics, 585*. doi:https://doi.org/10.1016/j.ijpharm.2020.119486

Pathan, R. A. & Bhandari, U. (2011). Preparation & Characterization of Embelin–Phospholipid Complex as Effective Drug Delivery Tool. *Journal of Inclusion Phenomena and Macrocyclic Chemistry, 69*, pp. 139-147. doi:10.1007/s10847-010-9824-2

Pathan, R. A., Bhandari, U., Javed, S., & Nag, T. C. (2012). Anti-Apoptotic Potential of Gymnemic Acid Phospholipid Complex Pretreatment in Winstar Rats With Experimental Cardiomyopathy. *Indian Journal of Experimental Biology, 50*, pp. 117-127.

Phachonpai, W., Wattanathorn, J., Muchimapura, S., Tong-Un, T., & Preechagoon, D. (2010). Neuroprotective Effect of Quercetin Encapsulated Liposomes: A Novel Therapeutic Strategy against Alzheimer's Disease. *American Journal of Applied Sciences, 7*(4), pp. 480-485. doi:10.3844/ajassp.2010.480.485

Pinheiro, R. G., Granja, A., Loureiro, J. A., Pereira, M. C., Pinheiro, M., Neves, A. R., & Reis, S. (2020). Quercetin Lipid Nanoparticles Functionalized With Transferrin for Alzheimer's Disease. *European Journal of Pharmaceutical Sciences, 148*. doi:https://doi.org/10.1016/j.ejps.2020.105314

Pokharkar, V., Patil-Gadhe, A., & Palla, P. (2017). Efavirenz Loaded Nanostructured Lipid Carrier Engineered for Brain Targeting Through Intranasal Route: *In Vivo* Pharmacokinetic and Toxicity Study. *Biomedicine & Pharmacotherapy, 94*, pp. 150-164. doi:https://doi.org/10.1016/j.biopha.2017.07.067

Poovi, G. & Damodharan, N. (2018). Lipid Nanoparticles: A Challenging Approach for Oral Delivery of BCS Class-II Drugs. *Future Journal of Pharmaceutical Sciences, 4*, pp. 191-205. doi:https://doi.org/10.1016/j.fjps.2018.04.001

Prabhakar, K., Afzal, S. M., Surender, G., & Kishan, V. (2013). Tween 80 Containing Lipid Nanoemulsions for Delivery of Indinavir to Brain. *Acta Pharmaceutica Sinica B, 3*, pp. 345-353. doi:https://doi.org/10.1016/j.apsb.2013.08.001

Praveen, A., Aqil, M., Imam, S. S., Ahad, A., Moolakkadath, T., & Ahmad, F. J. (2019). Lamotrigine Encapsulated Intra-Nasal Nanoliposome Formulation for Epilepsy Treatment: Formulation Design, Characterization and Nasal Toxicity Study. *Colloids and Surfaces B: Biointerfaces, 174*, pp. 553-562. doi:https://doi.org/10.1016/j.colsurfb.2018.11.025

Pu, Y., Zhang, X., Zhang, Q., Wang, B., Chen, Y., Zang, C., Wang, Y., Dong, T. & Zhang, T. (2016). 20(S)-Protopanaxadiol Phospholipid Complex: Process Optimization, Characterization, *In Vitro* Dissolution and Molecular Docking Studies. *Molecules, 21*. doi:10.3390/molecules21101396

Purkayastha, J., & Ghosh, J. (2018). Phytosomes: The Novel Drug Delivery System for Phytomedicine. *New Age Herbals*, pp. 455-464. doi:https://doi.org/10.1007/978-981-10-8291-7_21

Qadir, A., Faiyazuddin, M. D., Hussain, M. D., Alshammari, T. M., & Shakeel, F. (2016). Critical Steps and Energetics Involved in a Successful Development of a Stable Nanoemulsion. *Journal of Molecular Liquids, 214*, pp. 7-18. doi:https://doi.org/10.1016/j.molliq.2015.11.050

Quintanilla-Carvajal, M. X., Camacho-Díaz, B. H., Meraz-Torres, L. S., Chanona-Pérez, J. J., Alamilla-Beltrán, L., Jimenéz-Aparicio, A., & Gutiérrez-López, G. F. (2009). Nanoencapsulation: A New Trend in Food Engineering Processing. *Food Engineering Reviews, 2*, pp. 39-50. doi:10.1007/s12393-009-9012-6

Rajput, A. P. & Butani, S. B. (2019). Resveratrol Anchored Nanostructured Lipid Carrier Loaded *In Situ* Gel via Nasal Route: Formulation, Optimization and *In Vivo* Characterization. *Journal of Drug Delivery Science and Technology, 51*, pp. 214-223. doi:https://doi.org/10.1016/j.jddst.2019.01.040

Rasaie, S., Ghanbarzadeh, S., Mohammadi, M., & Hamishehkar, H. (2014). Nano Phytosomes of Quercetin: A Promising Formulation for Fortification of Food Products with Antioxidants. *Pharmaceutical Sciences, 20*, pp. 96-101.

Rassu, G., Soddu, E., Posadino, A. M., Pintus, G., Sarmento, B., Giunchedi, P., & Gavini, E. (2017). Nose-to-Brain Delivery of BACE1 siRNA Loaded in Solid Lipid Nanoparticles for Alzheimer’s Therapy. *Colloids and Surfaces B: Biointerfaces, 152*, pp. 296-301. doi:https://doi.org/10.1016/j.colsurfb.2017.01.031

Rasti, B., Jinap, S., Mozafari, M. R., & Abd-Manap, M. Y. (2013). Optimization on Preparation Condition of Polyunsaturated Fatty Acids Nanoliposome Prepared by Mozafari Method. *Journal of Liposome Research, 24*, pp. 99-105. doi:10.3109/08982104.2013.839702

Re, F., Cambianica, I., Sesana, S., Salvati, E., Cagnotto, A., Salmona, M., Couraud, P.-O., Moghimi, S. M., Masserini, M. & Sancini, G. (2011). Functionalization With ApoE-derived Peptides Enhances the Interaction With Brain Capillary Endothelial Cells of Nanoliposomes Binding Amyloid-beta Peptide. *Journal of Biotechnology, 156*, pp. 341-346. doi:https://doi.org/10.1016/j.jbiotec.2011.06.037

Rezaei, A., Fathi, M., & Jafari, S. M. (2019). Nanoencapsulation of Hydrophobic and Low-soluble Food Bioactive Compounds Within Different Nanocarriers. *Food Hydrocolloids*, pp. 146-162. doi:https://doi.org/10.1016/j.foodhyd.2018.10.003

Rostamabadi, H., Falsafi, S. R., & Jafari, S. M. (2019). Nanoencapsulation of Carotenoids Within Lipid-based Nanocarriers. *Journal of Controlled Release, 298*, pp. 38-67. doi:https://doi.org/10.1016/j.jconrel.2019.02.005

Rozo, A. J., Cox, M. H., Devitt, A., Rothnie, A. J., & Goddard, A. D. (2020). Biophysical Analysis of Lipidic Nanoparticles. *Methods*. doi:https://doi.org/10.1016/j.ymeth.2020.05.001

Saesoo, S., Bunthot, S., Sajomsang, W., Gonil, P., Phunpee, S., Songkhum, P., Laohhasurayotin, K., Wutikhun, T., Yata, T., Ruktanonchai, U. R. & Saengkrit, N. (2016). Phospholipid-Chitosan Hybrid Nanoliposomes Promoting Cell Entry for Drug Delivery Against Cervical Cancer. *Journal of Colloid and Interface Science, 480*, pp. 240-248. doi:https://doi.org/10.1016/j.jcis.2016.06.071

Saffari, P. M., Alijanpour, S., Takzaree, N., Sahebgharani, M., Etemad-Moghadam, S., Noorbakhsh, F., & Partoazar, A. (2020). Metformin Loaded Phosphatidylserine Nanoliposomes Improve Memory Deficit and Reduce Neuroinflammation in Streptozotocin-induced Alzheimer's Disease Model. *Life Sciences, 255*. doi:https://doi.org/10.1016/j.lfs.2020.117861

Salem, H. F., Ahmed, S. M., Hassaballah, A. E., & Omar, M. M. (2015). Targeting Brain Cells With Glutathione-modulated Nanoliposomes: *In Vitro* and *In Vivo* Study. *Drug, Design, Development and Therapy, 9*, pp. 3705-3727. doi:10.2147/DDDT.S85302

Salem, L. H., El-Feky, G. S., Fahmy, R. H., El Gazayerly, O. N., & Abdelbary, A. (2020). Coated Lipidic Nanoparticles as a New Strategy for Enhancing Nose-to-Brain Delivery of a Hydrophilic Drug Molecule. *Journal of Pharmaceutical Sciences, 109*, pp. 2237-2251. doi:https://doi.org/10.1016/j.xphs.2020.04.007

Salentining, S. (2019). Supramolecular Structures in Lipid Digestion and Implications for Functional Food Delivery. *Current Opinion in Colloid & Interface Science, 39*, pp. 190-201. doi:https://doi.org/10.1016/j.cocis.2019.02.002

Salvi, V. R. & Pawar, P. (2019). Nanostructured Lipid Carriers (NLC) system: A Novel Drug Targeting Carrier. *Journal of Drug Delivery Science and Technology, 51*, pp. 255-267. doi:https://doi.org/10.1016/j.jddst.2019.02.017

Sánchez-Navarro, M., Giralt, E., & Teixidó, M. (2017). Blood–Brain Barrier Peptide Shuttles. *Current Opinion in Chemical Biology, 38*, pp. 134-140. doi:https://doi.org/10.1016/j.cbpa.2017.04.019

Saoji, S. D., Dave, V. S., Dhore, P. W., Bobde, Y. S., Mack, C., Gupta, D., & Raut, N. A. (2017). The Role of Phospholipid as a Solubility- and Permeability-Enhancing Excipient for the Improved Delivery of the Bioactive Phytoconstituents of Bacopa Monnieri. *European Journal of Pharmaceutical Sciences, 108*, pp. 23-35. doi:https://doi.org/10.1016/j.ejps.2016.08.056

Saoji, S. D., Raut, N. A., Dhore, P. W., Borkar, C. D., Popielarczyk, M., & Dave, V. S. (2016). Preparation and Evaluation of Phospholipid-Based Complex of Standardized Centella Extract (SCE) for the Enhanced Delivery of Phytoconstituents. *American Association of Pharmaceutical Sciences, 18*. doi:10.1208/s12248-015-9837-2

Saraiva, C., Praça, C., Ferreira, R., Santos, T., Ferreira, L., & Bernardino, L. (2016). Nanoparticle-mediated Brain Drug Delivery: Overcoming Blood–Brain Barrier to Treat Neurodegenerative Diseases. *Journal of Controlled Release, 235*, pp. 34-47. doi:https://doi.org/10.1016/j.jconrel.2016.05.044

Semalty, A. & Tanwar, Y. S. (2013). Nimesulide-Phosphatidylcholine Complex for Improvement of Solubility and Dissolution. *American Journal of Drug Discovery and Development, 3*, pp. 225-234. doi:10.3923/ajdd.2013.225.234

Semalty, A., Semalty, M., & Singh, D. (2011). Phyto-phospholipid Complex of Catechin in Value Added Herbal Drug Delivery. *Journal of Inclusion Phenomena and Macrocyclic Chemistry, 73*, pp. 377-386. doi:10.1007/s10847-011-0074-8

Semalty, A., Semalty, M., Rawat, M. S., & Franceschi, F. (2010). Supramolecular Phospholipids–Polyphenolics Interactions: The PHYTOSOME® Strategy to Improve the Bioavailability of Phytochemicals. *Fitoterapia, 81*, pp. 306-314. doi:10.1016/j.fitote.2009.11.001

Sharma, S., Rabbani, S. A., Narang, J. K., Pottoo, F. H., Ali, J., Kumar, S., & Baboota, S. (2020). Role of Rutin Nanoemulsion in Ameliorating Oxidative Stress: Pharmacokinetic and Pharmacodynamics Studies. *Chemistry and Physics of Lipids, 228*. doi:https://doi.org/10.1016/j.chemphyslip.2020.104890

Singh, A., Ray, A., Mishra, R., Biswal, P. K., Yadav, R., & Ghatuary, S. K. (2020). Phyto-Phospholipid Complexes: Innovative Approach to Enhance the Bioavailability and Therapeutic Efficacy of Herbal Extract. *Pharmaceutical and Biosciences Journal, 8*, pp. 1-9.

Singh, D., Rawat, M. S., Semalty, A., & Semalty, M. (2012). Emodin–Phospholipid Complex. *Journal of Thermal Analysis and Calorimetry, 108*, pp. 289-298. doi:10.1007/s10973-011-1759-3

Singh, D., Rawat, M. S., Semalty, A., & Semalty, M. (2012). Rutin-Phospholipid Complex: An Innovative Technique in Novel Drug Delivery System- NDDS . *Current Drug Delivery, 9*, pp. 305-314. doi:10.2174/156720112800389070

Singh, S. K., Hidau, M. K., Gautam, S., Gupta, K., Singh, K. P., Singh, S. K., & Singh, S. (2018). Glycol Chitosan Functionalized Asenapine Nanostructured Lipid Carriers for Targeted Brain Delivery: Pharmacokinetic and Teratogenic Assessment. *International Journal of Biological Macromolecules, 108*, pp. 1092-1100. doi:https://doi.org/10.1016/j.ijbiomac.2017.11.031

Singh, Y., Meher, J. G., Raval, K., Khan, F. A., Chaurasia, M., Jain, N. K., & Chourasia, M. K. (2017). Nanoemulsion: Concepts, Development and Applications in Drug Delivery. *Journal of Controlled Release, 252*, pp. 28-49. doi:https://doi.org/10.1016/j.jconrel.2017.03.008

Sivakumar, M., Tang, Y., Siah, T., & Khang, W. (2014). Cavitation Technology – A Greener Processing Technique for the Generation of Pharmaceutical Nanoemulsions. *Ultrasonics sonochemistry, 21*(6), pp. 2069-2083. doi:https://doi.org/10.1016/j.ultsonch.2014.03.025

Svetlichny, G., Kulkamp-Guerreiro, I. C., Cunha, S. L., Silva, F. E., Bueno, K., Pohlmann, A. R., Fuentefria, A. M. & Guterres, S. S. (2014). Solid Lipid Nanoparticles Containing Copaiba Oil and Allantoin: Development and Role of Nanoencapsulation on the Antifungal Activity. *Pharmazie, 70*. doi:10.1691/ph.2015.4116

Tadros, T. F. (2014). *An Introduction to Surfactants.* Berlin, Germany: De Gruyter.

Tadros, T., Izquierdo, P., Esquena, J., & Solans, C. (2004, May 20). Formation and Stability of Nano-emulsions. *Advances in Colloid and Interface Science, 108*, pp. 303-318. doi:https://doi.org/10.1016/j.cis.2003.10.023

Tan, Q., Liu, S., Chen, X., Wu, M., Wang, H., Yin, H., He, D., Xiong, H. & Zhang, J. (2012). Design and Evaluation of a Novel Evodiamine-Phospholipid Complex for Improved Oral

Bioavailability. *American Association of Pharmaceutical Scientists, 13*. doi:10.1208/s12249-012-9772-9

Tan, S. F., Masoumi, H. R., Karjiban, R. A., Stanslas, J., Kirby, B. P., Basri, M., & Basri, H. B. (2016). Ultrasonic Emulsification of Parenteral Valproic Acid-loaded Nanoemulsion With Response Surface Methodology and Evaluation of its Stability. *Ultrasonics Sonochemistry, 29*, pp. 299-308. doi:https://doi.org/10.1016/j.ultsonch.2015.09.015

Tapeinos, C., Battaglini, M., & Ciofani, G. (2017). Advances in the Design of Solid Lipid Nanoparticles and Nanostructured Lipid Carriers for targeting Brain Diseases. *Journal of Controlled Release, 264*, pp. 306-322. doi:https://doi.org/10.1016/j.jconrel.2017.08.033

Telange, D. R., Patil, A. T., Pethe, A. M., Fegade, H., Anand, S., & Dave, V. S. (2017). Formulation and Characterization of an Apigenin-Phospholipid Phytosome (APLC) for Improved Solubility, *In Vivo* Bioavailability, and Antioxidant Potential. *European Journal of Pharmaceutical Sciences, 108*, pp. 36-49. doi:http://dx.doi.org/10.1016/j.ejps.2016.12.009

Thummel, K. E., Kunze, K. L., & Shen, D. D. (1997). Enzyme-catalyzed Processes of First-pass Hepatic and Intestinal Drug Extraction. *Advanced Drug Delivery Reviews, 27*, pp. 99-127. doi:https://doi.org/10.1016/S0169-409X(97)00039-2

Ting, Y., Jiang, Y., Ho, C.-T., & Huang, Q. (2014). Common Delivery Systems for Enhancing *In Vivo* Bioavailability and Biological Efficacy of Nutraceuticals. *Journal of Functional Foods, 7*, pp. 112-128. doi:https://doi.org/10.1016/j.jff.2013.12.010

Tung, B. T., Hai, N. T., & Son, P. K. (2017). Hepatoprotective Effect of Phytosome Curcumin against Paracetamol-induced Liver Toxicity in Mice. *Brazilian Journal of Pharmaceutical Sciences, 53*. doi:http://dx.doi.org/10.1590/s2175-97902017000116136

Upadhyay, P., Trivedi, J., Pundarikakshudu, K., & Sheth, N. (2017). Direct and Enhanced Delivery of Nanoliposomes of Anti Schizophrenic Agent to the Brain Through Nasal Route. *Saudi Pharmaceutical Journal, 25*, pp. 346-358. doi:https://doi.org/10.1016/j.jsps.2016.07.003

Uskoković, V. (2008). Nanomaterials and Nanotechnologies: Approaching the Crest of this Big Wave. *Current Nanoscience, 4*, pp. 119-129. doi:10.2174/157341308784340903

Vankudri, R., Habbu, P., Hiremath, M., Patil, B. S., & Savant, C. (2016). Preparation and Therapeutic Evaluation of Rutin-Phospholipid Complex for Antidiabetic Activity. *Journal of Applied Pharmaceutical Science, 6*, pp. 90-101. doi:10.7324/JAPS.2016.600116

Venishetty, V. K., Samala, R., Komuravelli, R., Kuncha, M., Sistla, R., & Diwan, P. V. (2013). β-Hydroxybutyric Acid Grafted Solid Lipid Nanoparticles: A Novel Strategy to Improve Drug Delivery to Brain. *Nanomedicine: Nanotechnology, Biology and Medicine, 9*, pp. 388-397. doi:https://doi.org/10.1016/j.nano.2012.08.004

Vyas, T. K., Shahiwala, A., & Amiji, M. M. (2008). Improved Oral Bioavailability and Brain Transport of Saquinavir upon Administration in Novel Nanoemulsion Formulations. *International Journal of Pharmaceutics, 347*, pp. 93-101. doi:https://doi.org/10.1016/j.ijpharm.2007.06.016

Walker, R., Decker, E. A., & McClements, D. J. (2015). Development of Food-grade Nanoemulsions and Emulsions for Delivery of Omega-3 Fatty Acids: Opportunities and Obstacles in the Food Industry. *Food & Fucntion, 6*(1), pp. 41-54. doi:10.1039/c4fo00723a

Wavikar, P., Pai, R., & Vavia, P. (2017). Nose to Brain Delivery of Rivastigmine by *In-situ* Gelling Cationic Nanostructured Lipid Carriers: Enhanced Brain Distribution and Pharmacodynamics. *Journal of Pharmaceutical Sciences, 106*, pp. 3613-3622. doi:https://doi.org/10.1016/j.xphs.2017.08.024

Wong, H. L., Bendayan, R., Rauth, A. M., Li, Y., & Wu, X. Y. (2007). Chemotherapy With Anticancer Drugs Encapsulated in Solid Lipid Nanoparticles. *Advanced Drug Delivery Reviews, 59*(6), pp. 491-504. doi:https://doi.org/10.1016/j.addr.2007.04.008

Wu, Y., Song, X., Kebebe, D., Li, X., Xue, Z., Li, J., Du, S., Pi, J. & Liu, Z. (2019). Brain Targeting of Baicalin and Salvianolic Acid B Combination by OX26 Functionalized Nanostructured Lipid Carriers. *International Journal of Pharmaceutics, 571*. doi:https://doi.org/10.1016/j.ijpharm.2019.118754

Yadav, S., Gandham, S. K., Panicucci, R., & Amiji, M. M. (2016). Intranasal Brain Delivery of Cationic Nanoemulsion-Encapsulated TNFα siRNA in Prevention of Experimental Neuroinflammation. *Nanomedicine: Nanotechnology, Biology and Medicine, 12*, pp. 987-1002. doi:https://doi.org/10.1016/j.nano.2015.12.374

Yanyu, X., Yunmei, S., Zhipeng, C., & Qinen, P. (2006). The Preparation of Silybin-Phospholipid Complex and the Study on its Pharmacokinetics in Rats. *International Journal of Pharmaceutics, 307*, pp. 77-82. doi:10.1016/j.ijpharm.2005.10.001

Yasir, M. & Sara, U. V. (2014). Solid Lipid Nanoparticles for Nose to Brain Delivery of Haloperidol: *In Vitro* Drug Release and Pharmacokinetics Evaluation. *Acta Pharmaceutica Sinica B, 4*, pp. 454-463. doi:https://doi.org/10.1016/j.apsb.2014.10.005

Yu, F., Li, Y., Chen, Q., He, Y., Wang, H., Yang, L., Guo, S., Meng, Z., Cui, J., Xue, M. & Chen, X. (2016). Monodisperse Microparticles Loaded with the Self-assembled Berberine-Phospholipid Complex-based Phytosomes for Improving Oral Bioavailability and Enhancing Hypoglycemic Efficiency. *European Journal of Pharmaceutics and Biopharmaceutics, 103*, pp. 136-148. doi:https://doi.org/10.1016/j.ejpb.2016.03.019

Yue, P.-F., Yuan, H.-L., Li, X.-Y., Yang, M., & Zhu, W.-F. (2010). Process Optimization, Characterization and Evaluation *In Vivo* of Oxymatrine–Phospholipid Complex. *International Journal of Pharmaceutics, 387*, pp. 139-146. doi:https://doi.org/10.1016/j.ijpharm.2009.12.008

Yue, P.-F., Yuan, H.-L., Xie, H., Xiao, X.-H., Yang, M., Liao, M.-X., Zhu, W.-F. & Cai, P.-L. (2008). Preparation, Characterization, and Bioavailability of Ursodeoxycholic Acid–Phospholipid Complex In Vivo. *Drug Development and Industrial Pharmacy, 34*, pp. 708-718. doi:10.1080/03639040701842477

Zhao, C., Wei, L., Yin, B., Liu, F., Li, J., Liu, X., Wang, J. & Wang, Y. (2020). Encapsulation of Lycopene Within Oil-in-Water Nanoemulsions Using Lactoferrin: Impact of Carrier Oils on Physicochemical Stability and Bioaccessibility. *International Journal of Biological Macromolecules, 153*, pp. 912-920. doi:https://doi.org/10.1016/j.ijbiomac.2020.03.063

Zhao, Y.-Z., Lin, M., Lin, Q., Yang, W., Yu, X.-C., Tian, F.-R., Mao, K.-L., Yang, J.-J., Lu, C.-T. & Wong, H. L. (2016). Intranasal Delivery of bFGF With Nanoliposomes Enhances *In Vivo* Neuroprotection and Neural Injury Recovery in a Rodent Stroke Model. *Journal of Controlled Release, 224*, pp. 165-175. doi:https://doi.org/10.1016/j.jconrel.2016.01.017