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Ciências Farmacêuticas – Especialidade em Tecnologia Farmacêutica

Functionalized lipid nanocarriers to overcome the blood-brain barrier in the treatment of amyotrophic lateral sclerosis

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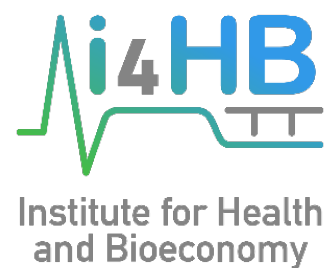
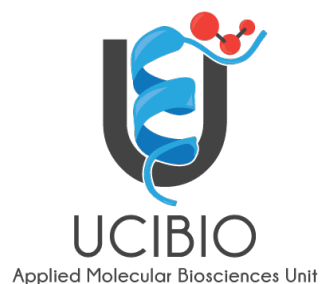
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FACULDADE DE FARMÁCIA
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Functionalized lipid nanocarriers to overcome the blood-brain barrier in the treatment of amyotrophic lateral sclerosis

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É AUTORIZADA A REPRODUÇÃO PARCIAL DESTA TESE, APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE.

To my parents

Publications and Presentations

Peer-reviewed publications and presentations in scientific events:

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- Teixeira MI, Lopes CM, Amaral MH, Costa PC. Chapter 10 - Silver nanoparticles for the management of neurological diseases. In: Kesharwani P, editor. Silver Nanoparticles for Drug Delivery: Academic Press; 2024. pp. 209-39. DOI: 10.1016/B978-0-443-15343-3.00002-4.
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- Teixeira MI, Lopes CM, Gonçalves H, Catita J, Silva AM, Rodrigues F, Amaral MH, Costa PC. Optimization of lipid nanoparticles to improve brain drug delivery in amyotrophic lateral sclerosis (ALS). 12th International Colloids Conference, June 2023, Palma, Mallorca, Spain (Poster communication).
- Teixeira MI, Lopes CM, Amaral MH, Costa PC. Brain drug delivery of riluzole by means of lactoferrin functionalized nanostructured lipid carriers. Nanotech France 2022, June 2022, Paris, France (Poster Communication).
- Teixeira MI, Lopes CM, Amaral MH, Costa PC. Lactoferrin functionalized NLC for amyotrophic lateral sclerosis. EUFEPS Annual Meeting 2021, June 2021, Virtual Meeting (Poster Communication).
- Teixeira MI, Lopes CM, Amaral MH, Costa PC. Riluzole-loaded lipid nanoparticles for brain targeted delivery. 9th International Colloids Conference, June 2019, Sitges, Spain (Poster communication).
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Resumo

A esclerose lateral amiotrófica (ALS, do inglês *Amyotrophic Lateral Sclerosis*) é uma doença neurodegenerativa fatal, caracterizada pela degeneração irreversível dos neurónios motores no cérebro e na medula espinhal. Esta doença é altamente incapacitante e progride num período de 3 a 5 anos, conduzindo a atrofia muscular generalizada. A maioria dos doentes morre de insuficiência respiratória. Apesar do elevado impacto socioeconómico, atualmente, não existe cura. Os tratamentos disponíveis são escassos e, até ao momento, apenas foram aprovadas quatro terapias: três fármacos (riluzol, edaravone e AMX0035, um combinado de fenilbutirato de sódio e de ácido tauroursodesoxicólico) e uma quarta terapia, recentemente autorizada, a qual se baseia num oligonucleótido antisense direcionado a um subgrupo específico de doentes com mutações patogénicas no gene superóxido dismutase 1 (SOD1, do inglês *Superoxide Dismutase 1*). Todavia, os benefícios destes tratamentos são muito limitados.

Não obstante os avultados investimentos na procura de terapias mais eficazes para a ALS, a maioria dos ensaios clínicos têm tido resultados dececionantes. Vários fatores podem ter contribuído para este insucesso, incluindo a heterogeneidade clínica, a presença da barreira hematoencefálica (BBB, do inglês *Blood-Brain Barrier*), ou até mesmo as propriedades físico-químicas inadequadas dos fármacos (e.g., baixa solubilidade em água ou insuficiente biodisponibilidade e estabilidade), o que afeta a sua permeação e atividade no sistema nervoso central (CNS, do inglês *Central Nervous System*). Dado que grande parte dos compostos estudados não consegue alcançar o cérebro em concentrações terapêuticas relevantes, há a necessidade de criar estratégias para o direcionamento e libertação de fármacos no CNS.

A presente tese constitui uma reflexão e resposta a este desafio. Primeiramente, é apresentada uma revisão da BBB, da fisiopatologia da ALS e das estratégias terapêuticas existentes. Seguidamente, são destacadas diferentes abordagens utilizadas para melhorar a distribuição de fármacos no CNS, com foco especial na utilização de nanossistemas, fornecendo também uma visão sobre os esforços mais recentes na aplicação destes nanotransportadores no tratamento da ALS. No que concerne ao trabalho experimental, foi proposto o desenvolvimento de nanopartículas lipídicas contendo riluzol, funcionalizadas com lactoferrina, tendo como objetivo promover o direcionamento do fármaco para o local de ação alvo, aumentar a sua permeação através da BBB e maximizar a quantidade que atinge o cérebro.

Na primeira parte, realizou-se um estudo para otimização da formulação de dois tipos de nanopartículas lipídicas, nomeadamente, nanopartículas de lípido sólido (SLN, do inglês *Solid Lipid Nanoparticles*) e transportadores lipídicos nanoestruturados (NLC, do inglês *Nanostructured Lipid Carriers*), compostas por Precirol® ATO5, Miglyol® 812 (no caso dos NLC), poloxamer 188 e polissorbato 80, preparadas por ultrasonicação e homogeneização a alta pressão (HPH, do inglês *High pressure homogenization*). A seleção dos excipientes efetuou-se de acordo com o *lipid screening*, e também pelo facto de aqueles lípidos e tensioativos serem reconhecidos como seguros (GRAS, do inglês *Generally recognized as safe*) e serem comumente utilizados em estudos de direcionamento de fármacos para o cérebro. O efeito de diferentes variáveis experimentais no tamanho, índice de polidispersão (PDI, do inglês *Polydispersity index*) e potencial zeta (ZP, do inglês *Zeta potential*) das nanopartículas foi avaliado, e com base na caracterização físico-química dos nanossistemas vazios, quatro formulações (duas SLN e duas NLC) foram escolhidas para encapsular o riluzol.

As nanopartículas contendo fármaco apresentaram características apropriadas para vetorização cerebral, incluindo um diâmetro adequado (< 250 nm), alta eficiência de encapsulação (86-94%) e boa estabilidade ao longo de 3 meses de armazenamento em condições controladas. Diferentes técnicas de caracterização, como análise térmica e estrutural, corroboraram estes resultados, demonstrando que o riluzol se encontrava incorporado na matriz lipídica das nanopartículas. Adicionalmente, efetuaram-se estudos morfológicos por microscopia eletrónica, bem como modelação matemática dos perfis de libertação do fármaco, indicando um mecanismo de libertação predominantemente por difusão do tipo não-Fickiana. De forma a estabelecer a biocompatibilidade das formulações, foi realizado um ensaio de citotoxicidade em células endoteliais microvasculares cerebrais humanas (linha celular hCMEC/D3), o qual revelou que os nanossistemas desenvolvidos não comprometiam a integridade celular até uma concentração de 10 µM de riluzol. As SLN e os NLC apresentaram desempenho semelhante.

Na etapa seguinte funcionalizou-se um dos nanossistemas de NLC contendo riluzol com um ligando, a lactoferrina, dado que os recetores para esta proteína estão amplamente presentes na BBB. Uma vez mais, as nanopartículas apresentaram atributos favoráveis como sistema de veiculação de fármacos para o cérebro. O sucesso da conjugação foi confirmado por espectroscopia de infravermelhos, bem como por um método de quantificação de proteínas, o ensaio de Bradford. Numa monocamada de células hCMEC/D3 (modelo *in vitro* da BBB), o fármaco livre permeou mais do que os NLC

funcionalizados, o que foi consistente com os resultados do ensaio de imunoabsorção enzimática (ELISA, do inglês *Enzyme-linked immunosorbent assay*), com o riluzol a desencadear uma maior produção de interleucina-1 alfa (IL-1 α , do inglês *Interleukin-1 alpha*). Porém, as nanopartículas demonstraram boa biocompatibilidade, uma vez que não exibiram efeitos citotóxicos significativos, tanto em células endoteliais microvasculares cerebrais humanas, como em células semelhantes a neurónios motores (linha celular NSC-34). De um modo geral, o nanossistema funcionalizado desenvolvido pode ser considerado um sistema seguro de veiculação de moléculas terapêuticas direcionadas ao cérebro, embora seja necessário realizar trabalho adicional para melhorar o desempenho e a viabilidade clínica da formulação.

Abstract

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease, characterized by irreversible degeneration of motor neurons in the brain and spinal cord. It is extremely debilitating, and within a span of 3 to 5 years, this condition progresses, leading to generalized muscle weakness. Most people die from respiratory failure. Despite its high socioeconomic impact, ALS currently has no cure. Therapies are scarce, with only four therapies approved to date: three pharmacological treatments (riluzole, edaravone and AMX0035, a combination of sodium phenylbutyrate and tauroursodeoxycholic acid) and a fourth (tofersen), recently authorized, which is an antisense oligonucleotide directed to a specific subset of patients with pathogenic mutations in the superoxide dismutase 1 (SOD1) gene. Moreover, the benefits of these treatments are very limited.

Despite major investment in ALS therapeutic research, most clinical trials have had disappointing results. Several factors could have contributed to these outcomes, including clinical heterogeneity, the presence of the blood-brain barrier (BBB), or even inadequate drug physicochemical properties (e.g., low aqueous solubility or insufficient bioavailability and biostability), which affect their penetration and activity in the central nervous system (CNS). As most pharmaceuticals are unable to reach the brain at effective therapeutic concentrations, there is a need to develop strategies for CNS drug targeting and delivery.

The present thesis is a reflection and a response to this challenge. Initially, the BBB, ALS pathogenesis, and corresponding therapeutic strategies are reviewed. Afterwards, different approaches employed to improve CNS targeting, especially nanocarriers, are highlighted, while also providing an insight on the most recent efforts in the application of those nanocarriers for the management of ALS. For the experimental work, the development of lipid nanoparticles loaded with riluzole and functionalized with lactoferrin, to improve targeting efficiency, enhance BBB penetration and increase drug delivery to the brain, was proposed.

In the first part of the experimental work, a formulation optimization study was conducted, wherein two types of lipid nanoparticles, namely, solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC), composed of Precirol® ATO5, Miglyol® 812 (in the case of NLC), poloxamer 188 and polysorbate 80, were prepared by ultrasonication and high-pressure homogenization (HPH). The selection of the excipients was made according to the lipid screening, as well as because those lipids and surfactants have generally recognized as safe (GRAS) status and are commonly used in brain targeting studies. The

effect of different formulation and preparation variables on the size, polydispersity index (PDI), and zeta potential (ZP) of the nanoparticles was investigated and based on the physicochemical characterization of the empty nanocarriers, four formulations (two SLN and two NLC) were chosen to further incorporate riluzole.

The riluzole-loaded nanoparticles exhibited suitable characteristics for brain delivery, including particle mean diameter (< 250 nm), high encapsulation efficiency (86-94%), and good stability over 3 months of storage under controlled conditions. Other characterization techniques, such as thermal and structural analysis, seemed to corroborate these results, showing that the drug was successfully entrapped inside the nanoparticles' lipid matrix. Furthermore, microscopic morphological analysis and mathematical modeling of the drug release profiles were considered, indicating a release mechanism predominantly dominated by non-Fickian diffusion. To establish the safety of the formulations, an *in vitro* cytotoxicity assay was performed in brain microvascular endothelial cells (hCMEC/D3 cell line), which revealed that they did not compromise cell integrity up to a concentration of 10 μ M of riluzole. Thus, SLN and NLC had similar performance.

The next step consisted of functionalizing the riluzole-loaded NLC with a targeting moiety, namely, lactoferrin, since lactoferrin receptors are ubiquitously present in the BBB. Once again, the NLC displayed favorable attributes for brain drug delivery, as demonstrated by the physicochemical characterization. The success of the conjugation was seemingly confirmed by infrared spectroscopy, as well as a protein quantification method, the Bradford assay. In a monolayer of hCMEC/D3 cells (i.e., an *in vitro* model of the BBB), free riluzole permeated more than the functionalized NLC, which was consistent with the ELISA kit results, with the free drug eliciting higher interleukin-1 alpha (IL-1 α) production. Nevertheless, the nanoparticles showed good biocompatibility, as they did not display any significant cytotoxic effects in both hCMEC/D3 and NSC-34 motor neuron-like cells. Overall, the developed functionalized nanocarrier could be considered a safe brain targeting drug delivery system, even though additional work is needed to enhance the performance and viability of the formulation.

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List of Acronyms, Abbreviations and Symbols

ΔH – Melting enthalpy

α -SMA – Alpha-smooth muscle actin

A_{2A}R – Adenosine 2A receptor

AAV9 – Adeno-associated virus serotype 9

A β – Amyloid beta

ABC – ATP binding cassette

AD – Alzheimer's disease

AF – Asymmetry factor

AJs – Adherens junctions

AKT1 – V-akt murine thymoma viral oncogene homolog 1

AKT3 – V-akt murine thymoma viral oncogene homolog 3

ALS – Amyotrophic lateral sclerosis

ALSFRS-R – Revised amyotrophic lateral sclerosis functional rating scale

AMPA – α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid

AMT – Adsorptive-mediated transcytosis

ApoE – Apolipoprotein E

AQP-4 – Aquaporin 4

ASO – Antisense oligonucleotide

ATP – Adenosine 5'-triphosphate

ATR – Attenuated total reflectance

ATXN2 – Ataxin-2

Au NPs – Gold nanoparticles

BBB – Blood-brain barrier

BCECs – Brain capillary endothelial cells

BCS – Biopharmaceutical Classification System

BCSFB – Blood-cerebrospinal fluid barrier

C1q – Component 1q

C9orf72 – Chromosome 9 open reading frame 72

CMT – Carrier-mediated transport

CNM-Au8 – Gold nanocrystal oral suspension

CNS – Central nervous system

CPPs – Cell-penetrating peptides

CRISPR/Cas9 – Clustered regularly interspaced short palindromic repeats-associated protein 9

Cryo-SEM – Cryo-Scanning Electron Microscopy

CSF – Cerebrospinal fluid

CV – Coefficient of variation

DLS – Dynamic Light Scattering

DMEM – Dulbecco's Modified Eagle Medium

DMSO – Dimethyl sulfoxide

DSC – Differential Scanning Calorimetry

DSPC – Distearoylphosphatidylcholine

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

DTPA – Diethylenetriaminepentaacetic acid

EAAT2 – Excitatory amino acid transporter 2

EAE – Experimental allergic encephalomyelitis

ECM – Extracellular matrix

EDC – N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride

EDV-AM – Edaravone-encapsulated agonistic micelle

EE – Encapsulation efficiency

ELISA – Enzyme-linked immunosorbent assay

ELS – Electrophoretic Light Scattering

EMA – European Medicines Agency

epSPCs – Ependymal stem progenitor cells

EVOM – Epithelial voltohmmeter

fALS – Familial amyotrophic lateral sclerosis

FBS – Fetal bovine serum

FDA – Food and Drug Administration

FTIR – Fourier transform infrared

FUS – Fused in sarcoma

FVC – Forced vital capacity

g – Gravitational acceleration

GDNF – Glial cell line-derived neurotrophic factor

GLUT1 – Glucose transporter 1

GRAS – Generally recognized as safe

GSH – Glutathione

HBGF – Heparin-binding growth factors

HBSS – Hank's balanced salt solution

HD – Huntington's disease

HEWL – Hen egg white lysozyme

HPH – High-pressure homogenization

HPLC – High Performance Liquid Chromatography

HPLC-MS – High Performance Liquid Chromatography - Mass Spectrometry

ICH – International Conference on Harmonization

IgG – Immunoglobulin G

IL-1 α – Interleukin-1 alpha

IL-2 – Interleukin-2

IV – Intravenous

JAMs – Junctional adhesion molecules

k' – Retention factor

K_0 – Zero-order release constant

K_H – Higuchi release constant

K_{KP} – Korsmeyer-Peppas release constant

Keap1 – Kelch-like ECH-associated protein 1

LDL – Low-density lipoprotein

$\log P$ – Partition coefficient

LPS – Lipopolysaccharides

mAb – Monoclonal antibody

MAEHP – 2-methyl-N-(2-aminoethyl)-3-hydroxyl-4-pyridinone

MMPs – Matrix metalloproteinases

mRNA – Messenger RNA

MS – Multiple sclerosis

MSCs – Mesenchymal stem cells

MSP – Mesoporous silica nanoparticles

mTOR – Mechanistic target of rapamycin

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

n – Drug release exponent for the Korsmeyer–Peppas model

N – Number of theoretical plates

N2a – Neuro-2a

NDA – New drug application

NF- κ B – Nuclear factor kappa B

NfL – Neurofilament light chain

NIPAAM – *N*-isopropylacrylamide

NHS – N-hydroxysuccinimide

NLC – Nanostructured lipid carriers

NMDA – N-methyl-D-aspartate

NPs – Nanoparticles

Nrf2 – Nuclear factor erythroid 2-related factor 2

NVU – Neurovascular unit

OCT4 – Octamer-binding transcription factor 4

PBS – Phosphate-buffered solution

PD – Parkinson's disease

PDI – Polydispersity index

PECAM-1 – Platelet endothelial cell adhesion molecule-1

PEG – Polyethylene glycol

P-gp – P-glycoprotein

Ph. Eur. – European Pharmacopoeia

PLA – Poly(D, L-lactide)

PLGA – Poly(lactic-co-glycolic acid)

PMA – Polymethacrylate

PMAA – Poly(methacrylic acid)

pNFH – Phosphorylated neurofilament heavy chain

PNS – Peripheral nervous system

POE – Polyoxyethylene

PPO – Polyoxypropylene

PTFE – Polytetrafluoroethylene

PVP – Polyvinylpyrrolidone

PXRD – Powder X-Ray Diffraction

QbD – Quality by design

q.s. – Quantum satis

R – Pearson correlation coefficient

R^2 – Coefficient of determination

R_{adj}^2 – Adjusted coefficient of determination

RBP – RNA-binding proteins

RES – Reticuloendothelial system

RLZ – Riluzole

RMT – Receptor-mediated transcytosis

R_t – Retention time

ROS – Reactive oxygen species

sALS – Spinal-onset amyotrophic lateral sclerosis

SD – Standard deviation

SEM – Scanning Electron Microscopy

SLC – Solute carriers

siRNAs – Small interference RNAs

SLN – Solid lipid nanoparticles

SOD1 – Superoxide dismutase 1

SOX2 – SRY-Box transcription factor 2

SPTLC1 – Serine palmitoyl transferase long chain base subunit 1

STMN2 – Stathmin-2

TAT – Transactivator of transcription

TDP-43 – TAR DNA-binding protein 43

TEER – Transendothelial electrical resistance

TEM – Transmission Electron Microscopy

TJs – Tight junctions

TMAO – Trimethylamine N-oxide

Treg – Regulatory T cells

TUDCA – Tauroursodeoxycholic acid

UCP2 – Uncoupling protein 2

US – Ultrasonication

UV – Ultraviolet

VC – Vital capacity

VE – Vascular endothelial

VEGF – Vascular endothelial growth factor

XRD – X-ray Diffraction

γ_{sl} – Interfacial tension at the solid-liquid interface

ZO – Zonula occludens

ZP – Zeta potential

Chapter I

Contextualization and Structure

1. Scope of the Thesis

Neurodegenerative diseases are debilitating, largely incurable illnesses that are the leading cause of physical and cognitive disability worldwide. Fifty million individuals (about 15% of the world's population) are believed to be affected by these disorders, which include Alzheimer's disease (AD), Parkinson's disease (PD), Amyotrophic lateral sclerosis (ALS), Multiple sclerosis (MS), Huntington's disease (HD), among others. This number is anticipated to increase two-fold over the next two decades, due to the expansion of aging population. Consequently, the economic and social burden associated with these chronic diseases will rise significantly, posing a threat to accessible neurological care [1, 2].

ALS, in particular, is characterized by progressive motor neuron degeneration in the brain and spinal cord, evolving to muscle atrophy, paralysis, and death by respiratory failure within 2 to 5 years after the onset of the first symptoms [3]. Despite technological advances, no direct mechanism for ALS has been identified. Research indicates that its physiopathology is highly complex and multifactorial (e.g., toxic protein aggregates, genetic mutations, mitochondrial dysfunction, excitotoxicity, oxidative stress, inflammation), presenting significant challenges in the quest for innovative therapies. Moreover, the presence of blood-brain barrier (BBB), along with clinical heterogeneity within the patient population, lack of well-defined biological targets, absence of reliable biomarkers, inappropriate animal models, flawed clinical trial design, and insufficient drug bioavailability and biostability, also contribute to the limited translational progress observed in ALS clinical trials [4].

Case in point, only four therapies have ever received approval for ALS treatment. Riluzole (RLZ) is globally approved, while edaravone is authorized in select countries. These molecules have demonstrated a limited impact on extending survival or improving functional status, respectively. A third option, a combination of sodium phenylbutyrate and ursodoxicoltaurine, was greenlighted in both Canada and the United States in 2022. Nevertheless, disappointing topline results from a Phase III clinical trial prompted the manufacturer to make the decision to voluntarily withdraw the drug from the market in early 2024, after it failed to prove efficacy. Additionally, patients with a superoxide dismutase 1 (SOD1) mutation have recently gained access to a targeted gene therapy, Tofersen, an antisense oligonucleotide (ASO) [5]. Hence, there is a need for the development of suitable and effective formulations that can operate within the brain to maximize the pharmacological ability of both existing medications and new or off-label compounds.

In recent years, notable progresses have occurred in the field of nanotechnology, ushering in opportunities for the development of nano-based approaches for ALS. Encouraging results in the treatment of AD and PD provide hope that such strategies may also be applied to ALS and revitalize numerous therapeutic options that are currently ineffective. Employing specially designed nanostructures, such as nanocarriers, can be a viable strategy to tackle certain limitations of the available treatments, as nanosystems show great potential for delivering single or multiple therapeutic agents, while overcoming biological barriers, enhancing targeting efficiency to disease sites, increasing drug bioavailability and stability, enabling real-time tracking, and minimizing systemic and off-target effects [6].

Among different nanocarriers, lipid-based nanocarriers like liposomes, solid lipid nanoparticles (SLN), nanostructured lipid carriers (NLC), and micro and nanoemulsions, have been widely exploited to facilitate central nervous system (CNS) delivery and circumvent the factors that hinder transport across the BBB. These nanosystems have several advantages over other nanocarriers that make them particularly appealing for brain delivery, including better biodegradability and biocompatibility due to the use of generally recognized as safe (GRAS) excipients; natural tendency to permeate the BBB given their lipophilic nature; and easy manufacture. Furthermore, their surface can be functionalized with ligands to interact with specific receptors and transporters in the BBB, thereby enhancing their capability to target the brain [7].

Considering the above remarks, nanocarriers can serve as a crucial therapeutic option that may facilitate patients' access to disease-modifying treatments while also enabling ongoing treatment, irrespective of their functional status.

2. Objectives

The main purpose of this thesis is to shed light on the challenges of drug delivery to the brain in the treatment of ALS, which are posed not only by the BBB, acting as a gatekeeper of the CNS, but also by disease physiopathology. Therefore, a summary of BBB structure and transport systems, BBB dysfunction in neurodegeneration, and ALS pathogenesis is provided. Additionally, this work reviews therapeutic strategies for ALS (including results of clinical trials) and delves into the various approaches employed to facilitate CNS targeting, with an emphasis on nanocarriers.

In response to the identified concerns, the research goal of this thesis was the development of a functionalized lipid nanocarrier formulation that can improve targeting efficiency, enhance BBB permeation, and increase RLZ delivery to the brain.

3. Structure of the Thesis

The thesis has a total of six chapters, divided as follows:

- **Chapter I – Contextualization and Structure.** The scope and motivation of this thesis are presented, along with its objectives and structure.
- **Chapter II – Brain Drug Delivery: The Potential of Nanocarriers in CNS Targeting for the Treatment of ALS.** This chapter is a literature review, providing an overview on the current state of knowledge about the anatomy of the BBB, its dysfunction in CNS diseases, and the physiopathology of ALS. Different pharmacological and genetic/cellular tools used in ALS treatment, as well as the general limitations that hinder their CNS delivery and application, are also discussed. Likewise, several approaches employed to enhance brain drug delivery and clinical outcomes are addressed, with a particular focus on nanocarriers, wherein the most relevant studies reporting on the use of nanoparticles for ALS management are presented and their main findings reviewed.
- **Chapter III – Development of Riluzole-loaded Lipid Nanoparticles.** SLN and NLC, produced either by ultrasonication or high-pressure homogenization (HPH) techniques, and using GRAS lipids and surfactants, were prepared, and optimized to facilitate RLZ uptake. The effect of different experimental variables on the particle size, polydispersity index (PDI), and zeta potential (ZP) of the nanoparticles is described, as well as other fundamental features of the nanocarriers, including their morphology, stability, thermal behavior and crystallization, and *in vitro* biocompatibility.
- **Chapter IV – Development of Lactoferrin-Functionalized NLC for Riluzole Delivery to the Brain.** Focuses on the functionalization of the previously developed RLZ-loaded NLC with a ligand, lactoferrin, to improve BBB targeting efficiency. The physicochemical characterization and biocompatibility of these nanoparticles are displayed. Moreover, the permeation profiles and secretion of interleukin-1 α (IL-1 α) elicited by the nanocarriers on brain microvascular endothelial cells (hCMEC/D3 cell line) are also presented.

- **Chapter V – Final Conclusions and Future Perspectives.** This chapter presents the main conclusions and final observations of the developed work, as well as prospects of the field.
- **Chapter VI – Bibliography**
- **Chapter VII - Appendices**

The text enclosed in quotation marks is transcribed from published papers by the author of this thesis.

Chapter II

Brain Drug Delivery: The Potential of Nanocarriers in CNS Targeting for the Treatment of ALS

Sections in this chapter have been published as:

Teixeira MI, Lopes CM, Amaral MH, Costa PC. Current insights on lipid nanocarrier-assisted drug delivery in the treatment of neurodegenerative diseases. *European Journal of Pharmaceutics and Biopharmaceutics*. 2020; 149:192-217.

1. Blood-Brain Barrier (BBB): Concept and Functions

The nervous system is a highly specialized and integrated network that regulates the organism's response to stimuli, being divided into two parts: the CNS, comprised of the brain and spinal cord, and the peripheral nervous system (PNS), which includes cranial and peripheral nerves. Through its vast neuronal circuits, it detects and processes sensory inputs, collaborating with the endocrine system to mount a suitable response by transmitting signals throughout the body [8]. The nervous system is responsible for the most basic human body activities (such as breathing, heartbeat, and digestion) and for highly complex functions, including feeling, thinking, learning, remembering, and others.

Such processes are made possible, within the CNS, because neural cells and the spinal cord closely communicate with each other through a combination of chemical and electrical signals, namely the release of neurotransmitters and modulators, as well as ionic fluxes that generate and sustain synaptic potentials [9]. To maintain efficient neural signaling, the delicate CNS internal microenvironment must remain strictly regulated [7, 9]. This is accomplished by three physical barriers (Figure II.1), which collectively protect the neural tissue and preserve homeostasis at the blood-brain tissue interfaces [7, 10]: the BBB, a semipermeable barrier primarily formed by the endothelium lining the luminal walls of cerebral microvessels, separating the blood from the brain interstitial fluid; the choroid plexus epithelium, located between the blood and the ventricular cerebrospinal fluid (CSF), which forms the blood-CSF barrier; and the arachnoid barrier, partitioning the blood from the subarachnoid CSF. Among these, the BBB wields the greatest control over the surrounding cerebral microenvironment [10].

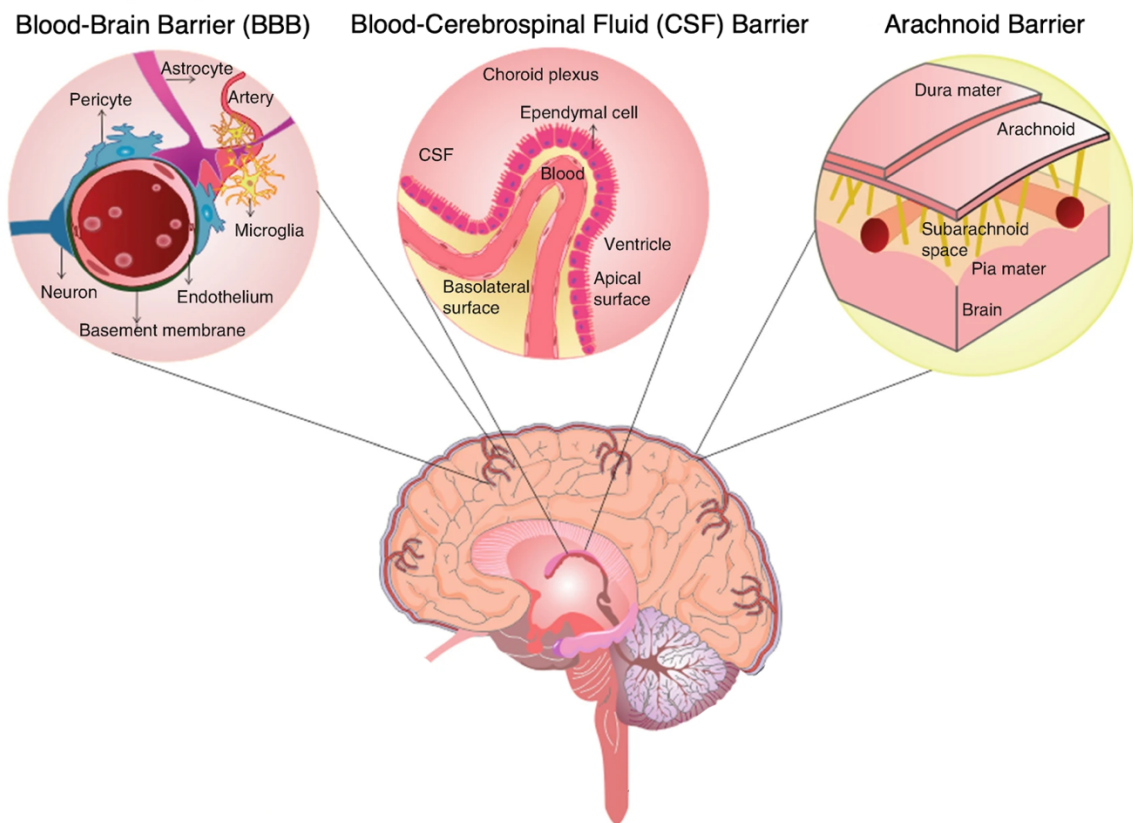


Figure II.1. Different CNS barriers and their relative localization inside the brain: blood-brain barrier (BBB); blood-cerebrospinal fluid (CSF) barrier; and arachnoid barrier. Adapted with permission from [11].

The existence of a unique barrier in the brain was first noticed in the late 19th century by Paul Ehrlich, using chemical dyes that stained certain body tissues but were excluded from the brain [12]. In 1900, Max Lewandowsky coined the term “blood-brain barrier” (*Bluthirnschranke*) [13]. Nevertheless, it was not until a few decades later, in 1960, that its ultrastructure was elucidated, thanks to the invention of electron microscopy [14]. The understanding of the importance of the brain microenvironment is contemporary, leading to the emergence, in 2001, of a new concept of the BBB as a complex multicellular system, known as the neurovascular unit (NVU) [15]. Nowadays, the BBB is regarded as more than a merely static, physical barrier, being deemed as a modulating entity with constant fluctuations in energy demand and neuronal activity, performing a set of essential functions, including transport, metabolism, and immunoregulation [7, 9].

The major function of the BBB is to keep brain homeostasis. This is achieved through a series of mechanisms, including maintaining the composition of the interstitial fluid finely controlled, and strictly regulating ionic and molecular exchange into and out of the CNS [7]. The BBB is vital to sustain brain nutrition. It has a low passive permeability to

small metabolites and water-soluble nutrients required by the nervous tissue. Moreover, the presence of specific transporters enables the selective passage of other essential molecules, such as glucose and amino acids, which cannot enter via passive diffusion. By supplying the brain parenchyma with these nutrients, the BBB guarantees that the neurons can fulfill their complex integrative roles [7, 10].

On the other hand, the BBB confers neuroprotection [7], since it possesses several efflux pumps (e.g., P-glycoprotein (P-gp)), as well as metabolizing enzymes (such as aminopeptidases, endopeptidases, cholinesterases, and monoamine oxidases) that can efficiently pump out and chemically modify neurotoxic substances, including xenobiotics and CNS metabolic waste products [9, 10, 16]. Blood cells, plasma components, and pathogens are also prevented from entering the CNS [17]. In adult mammalian brains, active neurogenesis is restricted to the subgranular zone within the dentate gyrus of the hippocampus, and the subventricular zone of the lateral ventricles. As neuronal replacement is exceptionally low, it is imperative to shield the CNS from any foreign element that may impair neuronal activity or promote cell death. Any increase in neuronal death will accelerate existing neurodegenerative pathologies [7, 18].

Additionally, the BBB has a significant immunoregulatory purpose by keeping the central and peripheral neurotransmitter pools separate, reducing crosstalk and limiting their proliferation, while also secreting substances such as cytokines, prostaglandins, or nitric oxide, which are relevant to neuroimmunity [7, 18, 19]. The uncontrolled release of neurotransmitters, especially excitatory ones like glutamate, has been shown to cause neurodegeneration [20, 21]. This barrier is unique because it can receive stimulation from one compartment and simultaneously respond with secretions into another, allowing non-synaptic signaling in the CNS [7]. Thus, the BBB is a dynamic and extremely selective interface that plays a key role not only in regulating the brain's microenvironment but also in neuroprotection.

1.1. BBB Structure: An Overview

The highly organized structure of the BBB, frequently referred as NVU (Figure II.2), is made up of non-fenestrated brain capillary endothelial cells (BCECs), surrounded by mural cells (pericytes and vascular smooth muscle cells), a basement membrane, astrocytes, neurons, and microglia [15, 22].

A healthy, functional BBB implies that all these components are interacting correctly and working together to maintain appropriate barrier function and guarantee proper CNS activity [16, 17]. The concept of NVU is constantly evolving. Heterogeneity in its cellular composition has been noted between different brain regions and along the vascular tree. BBB phenotype is affected by differential cues from the surrounding vascular microenvironment, therefore leading to variations in the expression of receptors, transporters, and adhesion molecules [23].

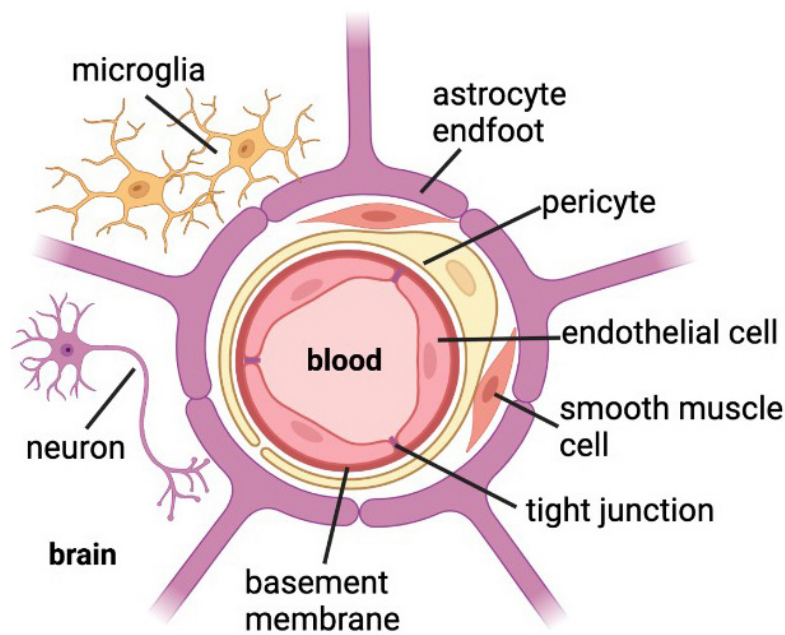


Figure II.2. Cross-section view of the neurovascular unit (NVU) and its main components. Adapted with permission from [22], under the terms of the Creative Commons Attribution 4.0 International License (<https://creativecommons.org/licenses/by/4.0/>).

1.1.1. Endothelial Cells and Junctional Complex

Endothelial cells constitute the core anatomical and functional element of the NVU, and they are supported by a specialized basement membrane [15]. BBB endothelial cells form a monolayer structure lining cerebral blood vessels and have a distinct phenotype when compared to cells from other vascular regions [17]. They are characterized by a lack of fenestrations; presence of continuous intercellular tight junctions (TJs) and adherens junctions (AJs), which form the junctional complex, sealing contact sites between cells and limiting paracellular transport; low rates of transcytosis; negligible pinocytotic trafficking; and luminal/abluminal polarization due to asymmetric distribution of transmembrane

transporters [10, 17, 24]. Moreover, they contain more mitochondria (8-11%), which suggests a higher metabolic activity [7].

TJs are transmembrane protein complexes that extend from adjacent BBB cells and are linked to the actin cytoskeleton by associated scaffold cytoplasmatic proteins, namely the zonula occludens (ZO) family (ZO-1, -2, -3). TJs strands behave dynamically, repeatedly breaking and reannealing to create transitory openings that allow gradual diffusion of solutes. The complexity and dynamic mobility of TJs strands, as well as the molecular size that can pass through the junctions, are determined by the composition of TJs proteins. [25]. The main TJs proteins are claudins, occludins, and junctional adhesion molecules (JAMs) (Figure II.3) [17, 26].

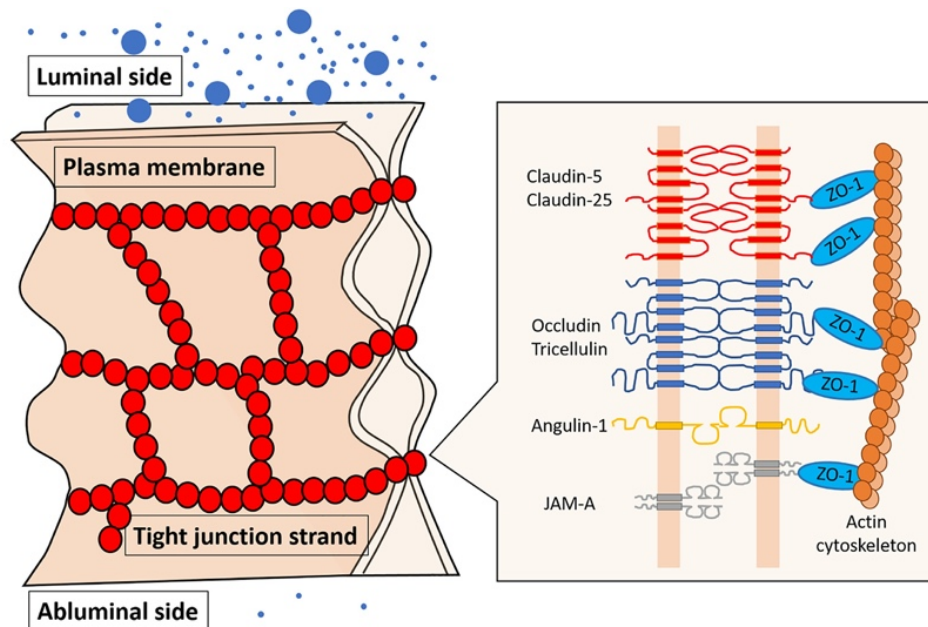


Figure II.3. Configuration of tight junctions (TJs) strands in blood-brain barrier (BBB) endothelial cells, and a closer look at the main TJs proteins. Reproduced with permission from [25].

Claudins are considered the backbone and primary sealing component of TJs, as they can dimerize and polymerize among themselves via *trans*-interaction [25, 26]. Claudin-5, specifically, has been recognized as the dominant TJs protein at the BBB, being an important determinant of paracellular tightness and BBB integrity [27]. Claudin expression enhances BBB endothelial barrier function [28]. Occludins are also present in the filaments of TJs. These proteins are important in regulating adhesion properties between cells, contributing to the stabilization of TJs [17, 29]. Like claudins, occludins can modulate the BBB permeability [30, 31]. On the other hand, JAMs (e.g., JAM-A) intervene in barrier function against molecules larger than 4 kDa [32], and in the regulation of

leukocyte adhesion and transmigration [33]. Additional proteins, such as tricellulin and angulin-1, further improve and stabilize barrier function by increasing the complexity of TJs strands [25].

AJs are another set of junction proteins that participate in cell-cell interactions and support the integrity of the vascular tube. They form a continuous belt that anchors neighboring cells, mainly through the expression of vascular endothelial (VE)-cadherins and platelet endothelial cell adhesion molecule-1 (PECAM-1). AJs are localized to the abluminal (basolateral) side of the BBB, while TJs are located towards the luminal (apical) side [9, 16].

Pathological changes in TJs and AJs result in junctional complex disruption, increasing BBB permeability and exacerbating or possibly initiating neurological dysfunction [34-36]. A deeper knowledge of how junctional complex expression, localization, and function change during disease states may lead to new ways to protect the CNS from the negative consequences of BBB disruption [26].

1.1.2. Basement Membrane

The basement membrane is an important component of the BBB, being a specialized extracellular matrix (ECM) secreted by different NVU cells, including endothelial cells, astrocytes, and pericytes. The four major proteins that constitute the ECM are collagen type IV, laminin, perlecan, and nidogen, which are supplemented by fibronectin, glycosaminoglycans, osteonectin, vitronectin, and agrin. They all bind receptors (integrins and dystroglycan), activating signaling cascades that mediate barrier function and influence BBB permeability [16]. This is supported by experimental results that show a strong correlation between diminished deposition of ECM proteins/receptors and increased vascular leakage, impacting cerebrovascular integrity [37-39]. Hence, the basement membrane contributes significantly to maintaining the BBB's tightness.

1.1.3. Neurons

Neurons can be thought of as the NVU's "pacemaker", being specialized cells that receive and transmit electrical and chemical signals [9]. Neural progenitors promote early brain vascularization/maturation and BBB formation through different signaling pathways, such as the vascular endothelial growth factor (VEGF) and Wnt/ β -catenin pathways [40]. When CNS homeostasis is disrupted, neurons modulate the activity of neighboring cells to restore the physiological state [9].

Indeed, different types of neurons, including cholinergic, serotonergic, dopaminergic, noradrenergic, and GABAergic neurons, directly innervate brain endothelial cells for neurovascular coupling, a crucial mechanism through which cerebral blood flow changes in response to alterations in neuronal activity and brain microenvironment, regulating microvascular permeability and adjusting substrate delivery [9, 41, 42].

Several reports suggest that besides the endothelium, neurons can activate other NVU cells, influencing BBB's properties [43, 44]. More recently, evidence emerged that neuronal activity regulates efflux transporter expression and function, which has considerable clinical implications for CNS drug delivery [45]. Other functions of the neurons involve secreting survival signals for endothelial and pericytic cells, stimulating angiogenesis, maintaining synapse density, and supporting cognition [46, 47].

1.1.4. Pericytes

Pericytes are cells embedded in the basement membrane, covering 90% of the abluminal side of the cerebral blood vessels' endothelium [48]. They form a diverse pool, although the contribution of each subclass to the NVU function is still uncertain [49]. Pericyte morphology is influenced by their position along the vascular system, manifesting the existence of distinct subsets with various functions in blood vessel formation, maintenance, and permeability, cerebral blood flow regulation, immune cell entry, and cellular debris clearance [7, 50].

Owing to the presence of contractile proteins, including myosin, alpha-smooth muscle actin (α -SMA), and tropomyosin, pericytes possess the ability to actively relax and contract, regulating cerebral vascular flow in response to localized changes in neuronal activity [51]. These cells predominantly maintain vascular integrity, being crucial for BBB formation and maturation. They contribute to the assembly of the basement membrane in the NVU, synthesizing the major proteins of its ECM through the secretion of signaling factors [52], a process that likewise supports BBB integrity by stimulating TJs formation and fibronectin expression [53].

Pericytes themselves respond to signaling cues by migrating to new vessels to stabilize the vascular wall [51], and their depletion has been linked to an increase in vascular permeability and endothelial barrier dysfunction, revealing their importance in vascular development and stabilization [54, 55]. At the same time, pericytes regulate the polarization of astrocyte endfeet and coordinate the function of endothelial cells by controlling their gene

expression patterns, cell cycle progression, and sprouting [55-57]. This has been demonstrated *in vitro*, with co-culture of endothelial cells and pericytes increasing TJs expression in endothelial cells when compared to endothelial cell monoculture [58]. It is noteworthy that pericytes carry out immune functions as well, including cell phagocytosis, antigen presentation, recruitment of immune cells, and support of microglia survival and proliferation [51, 59].

In pathological contexts, pericytes produce inflammatory molecules and matrix metalloproteinases (MMPs) which disrupt TJs and the binding of astrocyte endfeet to the blood vessels, leading to an abnormal increase in BBB permeability [60, 61]. Moreover, a reduction in pericyte number/coverage is associated with neuronal loss and acute BBB breakdown [62, 63]. BBB compromise was shown in transgenic pericyte-deficient mice, causing leakage and accumulation of serum proteins and other neurotoxic substances in the brain [64]. Presently, it is recognized that pericyte degeneration occurs in a variety of neurological diseases [51].

1.1.5. Astrocytes

Astrocytes are a type of glial cells that interact with the BBB through its perivascular endfeet, covering almost entirely the cerebral vascular walls [65]. The endfeet are rich in the water channel aquaporin 4 (AQP-4) and the potassium channel Kir4.1, both of which are crucial for water and ion homeostasis at the NVU [66, 67]. The absence of Kir4.1 and AQP-4 channels originate a water-ion imbalance, disrupting the BBB and resulting in an aberrant capillary permeability [68, 69]. Astrocytic endfeet does not make a major contribution to the physical barrier; however, it is widely assumed that astrocytes have an important role in BBB regulation and maintenance [70].

Although the processes by which astrocytes support BBB integrity are not entirely understood, several mechanisms have been proposed, including secreting ECM glycoproteins for the basement membrane [71]; producing astrocyte-derived protective factors of the NVU [71]; upregulating the expression and polarized localization of transporters such as glucose transporter 1 (GLUT1) and P-gp [65, 72], thus ensuring greater charge selectivity of endothelial cells; promoting apolipoprotein E (ApoE) synthesis to control cholesterol homeostasis in the brain [16]; and regulating the expression levels of cell adhesion molecules in endothelial cells, which limits leukocyte infiltration into the CNS [73, 74]. Astrocytes are also important in replenishing neurotransmitter pools for synaptic activity and recognition memory [75].

Like pericytes, it is reported that astrocytes have dual effects on BBB modulation, promoting or disrupting barrier integrity, depending on the signal they receive from neurons and other cells [76]. Bidirectional communication between astrocytes, neurons, and microglia, determines the type of immune response [16, 77]. For instance, astrocytes secrete glial cell line-derived neurotrophic factor (GDNF) and angiopoietin-1 to promote barrier function through cytoskeleton reorganization [78, 79]. However, in some circumstances (e.g., acute injury, inflammation, and neurodegenerative diseases), astrocytes become reactive, undergoing molecular, morphological, and functional changes. These changes impair the astrocytes' ability to maintain brain homeostasis, which has a significant impact on the survival and recovery of other CNS cells [73]. Upon activation, astrocytes release neurotoxic amounts of neurotransmitters nitric oxide and glutamate, as well as MMPs that break down the BBB; decrease secretion of trophic support molecules; and allow leukocytes to infiltrate, eventually leading to neuronal and oligodendrocyte death, and neurogenerative disease progression [73, 77].

1.1.6. Microglia

Microglia are resident immune cells in the brain. BBB microglia actively survey the brain parenchyma through highly mobile processes and interactions with other cells, enabling rapid responses when alterations arise [80]. These cells have multiple functions in the CNS, such as regulating immune response by presenting antigens and secreting cytokines and chemokines; phagocytosing pathogens that enter the brain; clearing cellular debris and aberrant proteins; shaping neuronal development by synaptic pruning; and providing trophic support to sustain myelin integrity as well as remyelination [10, 16, 81].

Microglia form direct connections with distinct areas of neurons, including synapses, affecting synaptic and dendritic structural plasticity [82]. Microglia can detect adenosine 5'-triphosphate (ATP) generated by neuronal activation, metabolizing ATP into adenosine. This adenosine then blocks adenosine receptors present in neurons, leading to the suppression of excessive neuronal activation and inducing a negative feedback loop to impart precise control in local neuronal responses [83, 84]. Conversely, neural circuits modulate microglial motility via neurotransmitter release [82]. As a result, there is an important interplay between microglia and neurons, which is essential for CNS homeostasis.

In physiological conditions, microglia remain quiescent. However, under certain stimuli, they can become activated, adopting either a pro- or anti-inflammatory phenotype, M1 or M2 phenotype, respectively [81]. M1 phenotype, which is frequent in neurodegeneration, contributes to BBB dysfunction due to the increased permeability, disrupted TJs, release of pro-inflammatory cytokines, oxidative stress, and increased neutrophil recruitment [85, 86]. Aging is also associated with increased activation of pro-inflammatory microglia and decreased regulatory status, owing to natural immunosenescence [81]. In this case, the monitoring functions of microglia become compromised, leading to a loss in phagocytic ability and accumulation of aberrant proteins, such as α -synuclein, ApoE, or amyloid beta (A β), which drive neurodegenerative diseases. Aging-related declines in phagocytic activity may thus have a direct link to an increased susceptibility to neurodegenerative progression [87]. Accordingly, to better understand BBB disruption across different neurological disorders, it is imperative to identify the underlying mechanisms behind the activation of microglia cells, and how they exert their deleterious effects [10].

1.2. Transport Across the BBB ¹

1.2.1. Factors Influencing BBB Transport

“The endothelial cell lipid bilayer and the TJs control the movement of molecules into and out of the brain, regulating the permeability across the BBB. This unique structure impacts clinical practice since the BBB does not allow most neurotherapeutics to penetrate the cerebral parenchyma [88]. Many factors influence the capability of the molecules to penetrate the BBB, namely [18]:

- Drug-related factors, including the difference of concentration between compartments, or the physicochemical characteristics of the molecule, such as its conformation, size, lipophilicity, partition coefficient, and ionization (non-ionized form passes better through the BBB), as well as the potential to establish hydrogen bonds. Moreover, the affinity for efflux and carrier-mediated transport must also be considered.

¹ This section is transcribed from previous work by the author of this thesis: Ref. 7. Teixeira MI, Lopes CM, Amaral MH, Costa PC. Current insights on lipid nanocarrier-assisted drug delivery in the treatment of neurodegenerative diseases. *European Journal of Pharmaceutics and Biopharmaceutics*. 2020;149:192-217.

- Peripheral factors, such as plasma protein binding affinity, systemic enzymatic stability, clearance rate, uptake of neurotherapeutics to non-target tissues, and effects of existing pathological conditions.

During transport across the BBB, all molecular properties should be regarded together, instead of considering just one isolated feature [89]. Yet, seeing that the membrane of the endothelial cells acts as a continuous lipid boundary, lipid solubility has long been used to determine the rate at which compounds are capable of entering the brain [90]. The partition coefficient ($\log P$) is generally the parameter used to assess the lipophilicity of a molecule [91]. A $\log P$ of around 1-3 is recommended for CNS penetration [9]. Blocking functional groups that can establish hydrogen bonds or covalently binding lipidic moieties such as long chain fatty acids to the drug, leads to an increase in its lipophilicity. On the other hand, the presence of hydroxyl groups or amides promotes hydrogen bonding with water, decreasing the $\log P$ and also the membrane permeability, therefore limiting drug transfer to the brain [92]. In general, only very small and lipophilic molecules that have a molecular weight smaller than 500 Dalton (Da) can easily diffuse across the BBB. Conversely, larger hydrophilic or lipid-insoluble molecules have a very limited crossing capacity [93, 94]. Even if a molecule is highly lipophilic, its transport through the BBB is limited when its molecular weight exceeds 600 Da [95]. Adding to this, most CNS diseases are resistant to small molecule therapy [94].”

1.2.2. BBB Transport Systems

“According to the literature, six transport pathways by which molecules are physiologically conveyed across the BBB can be distinguished [96-98]” (Figure II.4).

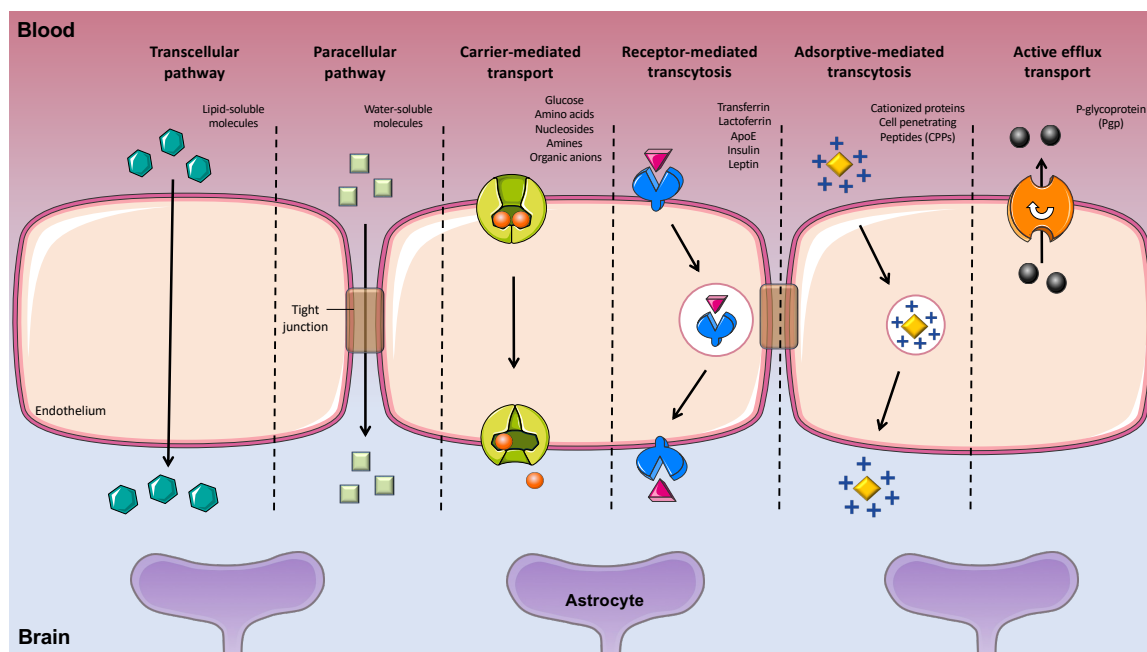


Figure 11.4. Schematic representation of the main blood-brain barrier (BBB) transport systems: transcellular pathway, paracellular pathway, carrier-mediated transport (CMT), receptor-mediated transcytosis (RMT), adsorptive-mediated transcytosis (AMT), and active efflux transport.

The main transport systems in the BBB are:

“(1) **Passive paracellular pathway:** This pathway refers to the mechanism of passage between endothelial cells [99]. It is mainly reserved for small water-soluble molecules [98, 100]. However, the existence of TJs between cells constitutes a limiting obstacle to the passage of these molecules [101]. Therefore, paracellular diffusion does not represent a significant pathway in the targeted delivery of therapeutic agents to the brain [102].

(2) **Passive transcellular pathway:** Since the paracellular pathway is very restrictive, many molecules cross by the transcellular route to access the brain, which involves the passage through the endothelial cells [99]. Small lipophilic molecules can pass through the endothelial cells of the BBB by free diffusion [96, 98]. Nevertheless, high lipid solubility leads to sequestration in the microvessels of the brain, as well as increased uptake by peripheral tissues, both of which reduce CNS drug delivery [96].

(3) **Carrier-mediated transport (CMT):** Several metabolic essential substances are polar molecules, having reduced permeability through the endothelium membrane. Such molecules cross the BBB using saturable, bi-directional transport systems in which the particles attach to protein carriers present in the membrane, being subsequently moved

along concentration gradients [95]. Typically, these carriers are highly stereospecific, selectively transporting small molecules, namely, nucleosides, nucleotides, peptides, vitamins, glucose, amino acids, and amines, among others [98, 103]. The use of these systems might be an appealing strategy for delivering therapeutic proteins and peptides that would otherwise have limited access to CNS [100].

(4) **Receptor-mediated transcytosis (RMT):** It is a saturable process that occurs via energy-dependent endocytic mechanisms [104]. RMT comprises the binding of molecules to specific receptors that are present in the membrane of BCECs, leading to the formation of an endocytic vesicle that contains the ligand attached to its receptor. The vesicle crosses the cytoplasm of the cells and when the ligand dissociates from the receptor, it is released via exocytosis at the basolateral membrane and into the interstitium of the brain [105]. Nevertheless, several vesicles fail to successfully pass through the BBB, given that some of them fuse with lysosomes and have their content broken down due to enzymatic degradation [106]. RMT is considered the pathway used to transport larger molecules across the BBB, including peptides or proteins such as immunoglobulin G (IgG), transferrin, lactoferrin, insulin, insulin-like growth factors, low-density lipoprotein (LDL), ApoE, and leptin [105]. Given that the receptors responsible for RMT are highly expressed on the BCECs, and the shape or size of what is being internalized does not limit the transport too much, small drug molecules, whether lipophilic or not, can also pass through the BBB by this via, providing an appealing strategy for brain delivery [105]. These transport systems can be exploited to target and deliver therapeutics that usually fail to cross the BBB. To this end, the molecules are conjugated with natural or artificial ligands (such as monoclonal antibodies - mAb) of the BBB receptors involved in RMT [105, 107].

(5) **Adsorptive-mediated transcytosis (AMT):** It is a mechanism that depends on nonspecific electrostatic interactions among a positively charged substance and negatively charged surface regions on the endothelial wall of the BBB [107-109]. Given that it is used by many other cells within the body and may lead to widespread absorption, AMT is not typically a specific target for brain delivery [107]. The transcytosis events of AMT are likely to be comparable to those RMT [110]. The difference between these two pathways lies in their respective capacity and affinity [100]. Although AMT has a lower affinity, it has a higher capacity when compared to RMT, as it is not affected by membrane receptor saturation [110]. AMT-based drug delivery usually involves both basic oligopeptides and cationic proteins such as cell-penetrating peptides (CPPs) (e.g., protamine, histone, avidin, or cationized albumin) [108, 111]. Conferring a positive charge to proteins that typically do not cross the BBB, enhances their uptake through BCECs via adsorptive mechanisms [112].

(6) **Active efflux transport:** The BBB possesses a certain number of efflux transporters that exclude from the brain several highly lipophilic molecules whose CNS penetration would be significantly predictable, but do not achieve relevant pharmacological concentrations [113, 114]. These efflux transporters are distributed between the two domains of the endothelial membrane of the cells. They belong to two large families, the outwardly directed energy-dependent ATP binding cassette (ABC) transporters and the solute carriers (SLC) [114]. The P-gp represents one of the most studied ABC transporters in the BBB [112]. It is now clear that many conventional neuroactive drugs designed to fulfill adequate criteria (i.e. size below 500 Da and sufficient lipid solubility) serve as substrates for P-gp, being expelled from the BCECs and into the blood against a concentration gradient [113]. To counteract the activity of those transporters, two strategies are used [113]: (a) developing specific inhibitors, thus facilitating their substrate's access to the CNS; (b) designing analogs of neurotherapeutics that have recognized effectiveness but low penetration through the BBB due to activity of P-gp. Said analogs are designed in a way that will not have reactivity with the efflux transporter."

1.3. BBB Dysfunction in CNS Diseases

BBB dysfunction is characterized by functional changes in the structural and cellular components of the NVU [10]. Factors including aging, systemic diseases, or sustained peripheral inflammation, lead to ion dysregulation, edema, and production of cytokines and chemokines [47]. These mechanisms operate together to foster pathology in the brain, triggering or amplifying other pathogenic processes that lead to BBB breakdown and hasten neuronal degeneration and cognitive decline due to neuronal malfunction [47]. BBB disruption is a key hallmark in a range of neurological conditions, such as brain stroke, brain tumors, AD, PD, MS, or ALS [115-118]. However, it remains unclear whether these diseases arise due to BBB impairment or if BBB disruption occurs because of the conditions. Nevertheless, barrier dysfunction is commonly observed, which can worsen and contribute to disease progression [10, 65].

During BBB breakdown, its integrity becomes compromised at various levels (Figure II.5). The alterations are reflected in the degradation or shrinkage of endothelial cells [119, 120], as well as changes to paracellular transport pathways due to decreased expression or translocation of TJs and AJs proteins [121]. Moreover, the expression or function of receptors and transport mechanisms can be impaired, leading to dysregulated molecular transport and increased transcytosis rates [120, 122]. It is important to note that BBB

breakdown can involve cellular elements beyond brain endothelial cells, including activation of microglia [65, 123], pericyte degeneration or decreased pericytic coverage [124-126], and swollen or detached astrocytic end feet [127]. Activated microglia and astrocytes produce cytokines and chemokines and upregulate luminal adhesion molecules on endothelial cells, allowing leukocyte infiltration from the blood into the brain [128]. Additionally, ECM/basement membrane thinning or thickening [65, 129], extravasation of plasma proteins, perivascular accumulation of toxic products, and an overall increase in immune and inflammatory responses are also common [120, 130].

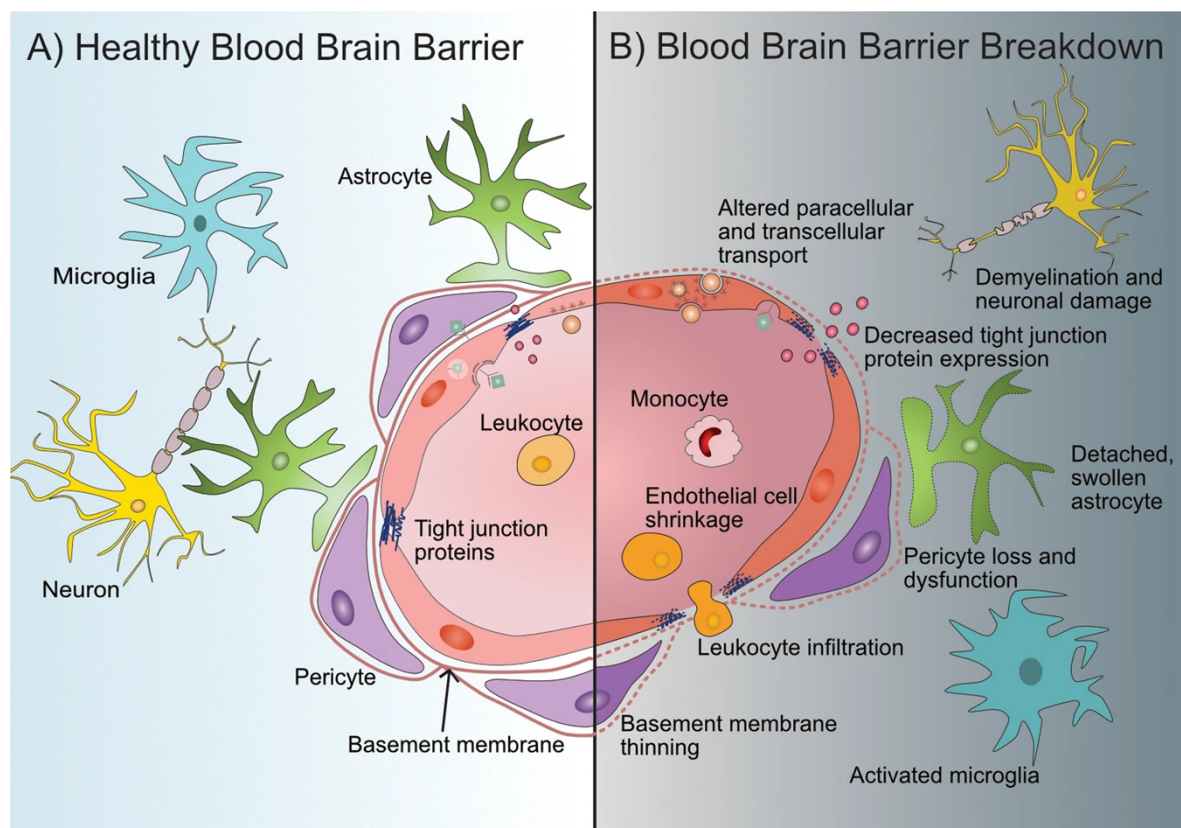


Figure II.5. Comparison of the blood-brain barrier (BBB) in a healthy state (A), and in a state of dysfunction (B). Reproduced from [65], under the terms of the Creative Commons Attribution 4.0 International License (<https://creativecommons.org/licenses/by/4.0/>).

“Lately, major emphasis has been given to the association between the gut and the brain (the so-called “gut-brain axis”). There appears to be a bidirectional crosstalk, with gut microbiota proposed to contribute to BBB disruption and the pathogenesis of neurodegenerative diseases. Braniste *et al.* [131] demonstrated that the BBB of mice deprived of gut microbiome is more permeable compared to conventional-specific pathogen-free mice, with this process being mediated by decreased expression of TJs proteins. Furthermore, compelling clinical and epidemiological data shows that people suffering from neurological disorders not only have a significant imbalance in the

composition of gut microbiota (dysbiosis), with the prevalence of several pro-inflammatory bacterial taxa, but also that histopathological changes in their gastrointestinal tract are present decades before neurodegeneration occurs [132, 133].

Several mechanisms are hypothesized to explain how the gut-brain axis can regulate the BBB's integrity and contribute to the development of neurodegeneration, such as (1) upregulating the release of inflammatory cytokines through bacterial components (e.g., lipopolysaccharides, LPS) that induce molecular mimicry [134], (2) activating the differentiation and brain infiltration of peripheral immune cells (e.g., T cells) by immune signaling [135], (3) directly modulating BBB's TJs and microglia cells via microbial metabolites (e.g., tryptophan metabolites) [136-138], (4) via direct neuronal connections, i.e. the vagus nerve [139]. Numerous studies seem to confirm the plausible validity of the mechanisms mentioned above. For instance, Vogt *et al.* [140] reported that trimethylamine N-oxide (TMAO), a metabolite of dietary choline produced by the gut microbiome and associated with biomarkers of AD, can be detected in higher levels within the CSF of patients with this condition.

Given the crucial role that the intestinal microenvironment has as a modulator of the brain and BBB function, modification of the gut microbiome should be considered as a preventive/therapeutic approach when tackling neurodegenerative diseases [139]. Probiotic supplementation and fecal microbiota transplantation, for instance, have already proven its worth in combating AD and MS, respectively [141, 142].”²

2. Amyotrophic Lateral Sclerosis

ALS, also known as Lou Gehrig's disease, is a progressive and incurable neurodegenerative illness characterized by deterioration of both upper and lower motor neurons in the brain and spinal cord, leading to gradual wasting of the bulbar, limb, trunk, and respiratory muscles (Figure II.6). Neurodegeneration and muscle atrophy result in paralysis and, ultimately, death from common terminal events, such as respiratory failure or aspiration pneumonia, which usually occurs within 3-5 years of diagnosis [6]. Nevertheless, a small fraction of patients experiences less rapidly advancing forms of the disease [143]. The global prevalence is estimated to be 4-5 cases per 100,000 people, with an incidence of 2-3 per 100,000 people [144]. Approximately 10% of ALS cases are familial (fALS),

² This section is transcribed from previous work by the author of this thesis: Ref. 7. *ibid.*

relating to genetic autosomal dominant inheritance pattern, whereas the remaining 90% are sporadic (sALS) [145].

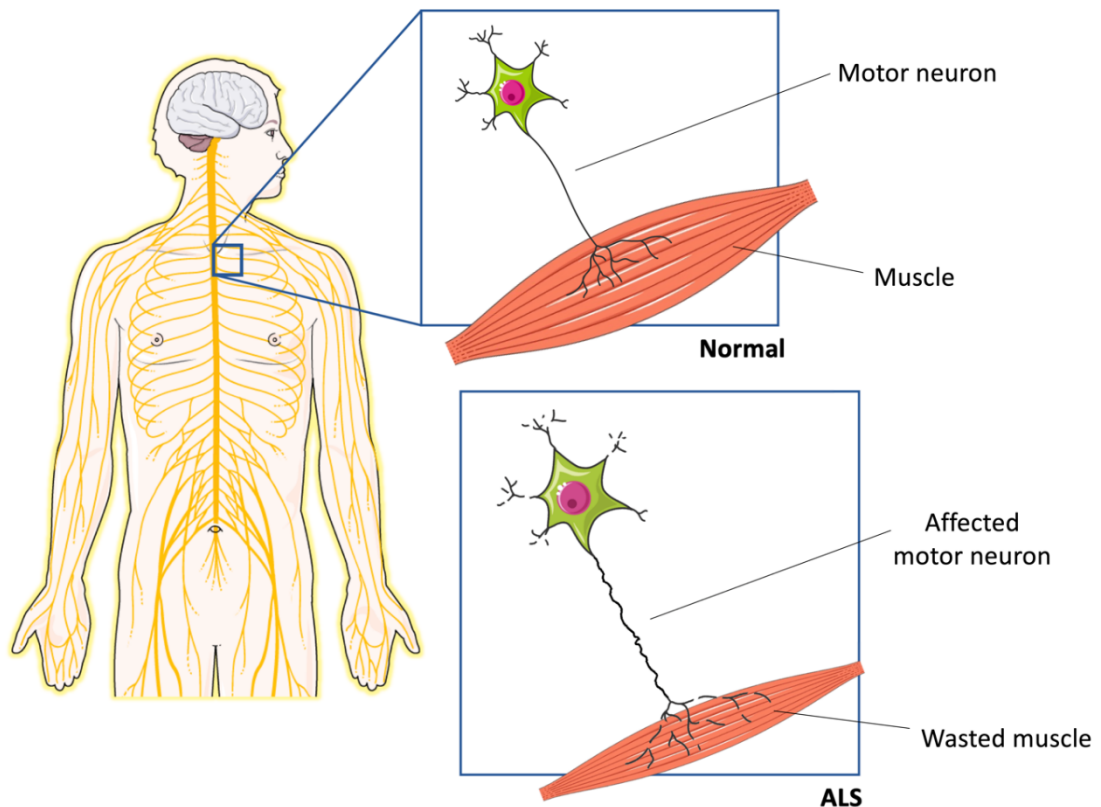


Figure II.6. Schematic diagram showing the effects of amyotrophic lateral sclerosis (ALS) on motor neurons and muscle tissue morphology. When muscle tissue is innervated by normal motor neurons, it contracts, maintaining proper function (upper panel). However, in ALS, neurodegeneration occurs, leading to sclerotic nerve cells, diminished muscle contraction, and consequently, progressive muscle atrophy (lower panel).

This is a condition that exhibits substantial heterogeneity at the genetic, clinical, and neuropathological levels [143]. The first symptoms generally manifest in the mid-to-late 50s and consist of progressive muscle weakness/atrophy accompanied by fasciculations, cramps, stiffness, and slowness of movements. A dependable indicator of disease progression is the initial site of paralysis. Spinal-onset ALS is the most common (75 – 80% of cases), beginning with localized weakness in the limbs, which is typically distal and asymmetric, spreading over time to include most muscles. In bulbar-onset ALS (25 – 30% of cases), paralysis begins in the head and neck, manifesting as dysarthria (difficulty speaking) and dysphagia (difficulty swallowing), along with facial weakness. Individuals with this subtype have faster disease progression, shorter life expectancy, and worse overall prognosis [146, 147]. Despite its original classification as a pure motor neuron disease 150

years ago by French neurologist Jean-Martin Charcot, ALS is now recognized as a multisystem disorder [147]. Up to 50% of patients present extra-motor manifestations, including neurocognitive alterations, language impairments, executive dysfunction, and behavioral and psychological problems. In some instances, frontotemporal dementia is also present [148].

No single diagnostic test is available for ALS [149]. Existing criteria, including the revised El Escorial criteria [150] and the Awaji algorithm [151], indirectly rate the degree of diagnostic certainty, lacking sensitivity. Moreover, they were developed for research purposes, primarily to choose patients who are eligible to participate in clinical trials [147]. To address these limitations, new simplified criteria (Gold Coast Criteria) have recently been proposed, requiring only combined upper and lower motor neuron dysfunction in one body region, or lower motor neuron dysfunction in at least two locations, in the absence of any other diseases that can explain symptomatology [152].

An integrative approach is therefore used, considering the patient's medical history, clinical findings (namely, upper, and lower motor neuron signs), physical examination (e.g., testing strength and reflexes), and confirmatory tests (e.g., electromyography, magnetic resonance imaging, etc.). Genetic testing may be done as well, in cases where fALS is suspected [143, 153]. Nevertheless, diagnosis remains suboptimal, and it can take up to one year after disease onset [147]. Continued efforts into emerging techniques, such as proteomics and the detection of new biomarkers (e.g., neurofilament light chain (NfL)), could contribute to better-predicting disease progression and prognosis [143, 149].

2.1. Etiology and Pathogenesis

Like other neurodegenerative disorders, ALS is believed to arise from a complex interaction of genetic, environmental, and age-related factors [154]. Male sex and aging increase the likelihood of developing the disease. Following the discovery, in 1993, of the initial gene related to ALS – SOD1 [155] –, more than 40 mutations have been identified and associated with ALS pathogenic mechanisms, including fused in sarcoma (FUS), TAR DNA-binding protein 43 (TDP-43) and chromosome 9 open reading frame 72 (C9orf72) [156-159]. Those four genes are the most well-studied, accounting for the majority of fALS cases [160]. Environmental and occupational risk factors, such as smoking, chronic exposure to industrial chemicals, pesticides, heavy metals, viral infections, and head trauma, have also been suggested. Nevertheless, it has yet to be determined whether there is a causal relationship between these factors and ALS [154, 161].

Notwithstanding continued efforts, a cohesive theory explaining the pathophysiology remains elusive. This could be largely attributed to the heterogeneity of the disease, hampering clinical and therapeutic breakthroughs that improve the patients' quality of life [154]. Several mechanisms for neurodegeneration in ALS have been proposed, pointing to a multifactorial pathogenesis (Figure II.7) [6, 162]. Most clinicians agree that a combination of processes may lead to acquiring the disease. Understanding the underlying regulation of those mechanisms is crucial for developing specific drug targets.

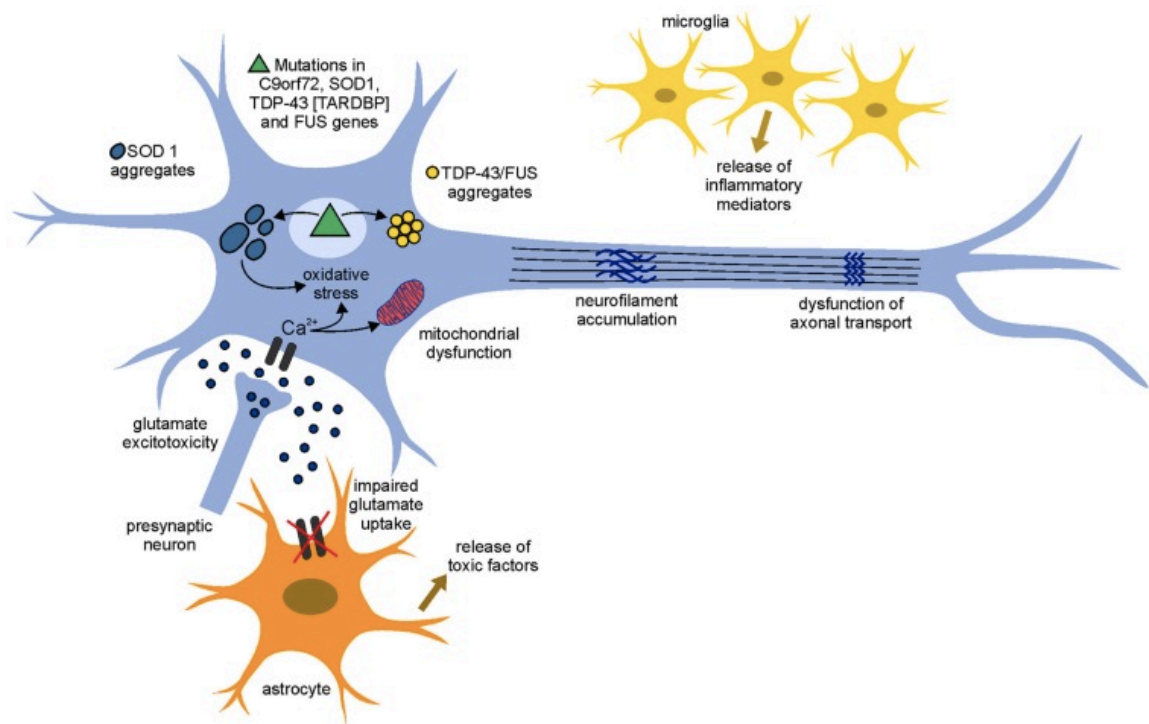


Figure II.7. Proposed cellular and molecular mechanisms involved in amyotrophic lateral sclerosis (ALS) pathogenesis. Reproduced from [162], under the terms of the Creative Commons Attribution 4.0 International License (<https://creativecommons.org/licenses/by/4.0/>).

ALS pathogenic mechanisms include:

(1) **Protein aggregation and altered proteostasis:** the presence of cytoplasmic inclusions in motor neurons, composed of mutant/misfolded protein aggregates (e.g., SOD1, TDP-43, FUS, etc.), is one of the hallmarks of ALS, being seen in up to 97% of cases. These aggregates are neurotoxic, inducing proteostasis (i.e., protein homeostasis) dysfunction and cellular stress. Over time, oligomeric forms overload key components of protein degradation pathways, namely chaperones, the ubiquitin-proteasome system, and autophagy, disrupting their functions and decreasing their ability to degrade the aggregates [163, 164].

(2) **Disrupted RNA metabolism:** related to protein aggregation, several critical RNA-binding proteins (RBPs) (e.g., TDP-43, FUS, ataxin-2 (ATXN2) proteins) [6], which serve important functions in the transcriptome, are displaced from their usual location within the nucleus to the cytoplasm. Nuclear depletion is detrimental, leading to defective microRNA transcription and splicing, impaired stress granule disassembly, and aberrant liquid-liquid phase separation, altering the state of RBPs from soluble to insoluble and further promoting aggregation [165, 166]. Recent data suggests that increased expression of aberrant intron-retaining transcripts is behind nuclear depletion [167].

(3) **Switch genes:** Apart from genetic mutations that cause misfolded proteins, there are also key genes (termed “switch genes”) that are responsible for drastic transcriptional changes and that affect biological pathways associated with ALS [168].

(4) **Glutamate excitotoxicity:** Another significant ALS pathological process seems to be glutamate excitotoxicity [169]. Inadequate uptake of glutamate, due to loss or inactivation of excitatory amino acid transporter 2 (EAAT2) in astrocytes, causes hyperactivation of glutamate receptors and abnormal calcium intake into postsynaptic motor neurons [170, 171]. A sustained increase in intracellular calcium levels triggers the overproduction of reactive oxygen species (ROS), disturbs proteostasis, and leads to mitochondrial and enzymatic dysfunction, ultimately occasioning apoptotic machinery activation and neuronal degeneration [172-175].

(5) **Lipid dysmetabolism:** Deficits in lipid metabolism have also been postulated as a pathway in motor neuron disease. Consistent with this view, mutations in variants of the serine palmitoyl transferase long chain base subunit 1 (SPTLC1) gene, which is important for sphingolipid biosynthesis, have recently been identified in juvenile ALS [176]. Moreover, several studies indicate that lipid peroxidation, as well as elevated levels of arachidonic acid [177], ceramides [178], and lysophosphatidylcholine [179] are detrimental to motor neurons, contributing to neurodegeneration and cell death in ALS.

(6) **Oxidative stress:** Motor neurons are distinguished by their high metabolic requirements, resulting in intense energy production and more susceptibility to oxidative damage. Excessive oxidative stress occurs when the production rate of free radicals or ROS exceeds the neutralization capacity of endogenous radical scavenging molecules in neurons, causing permanent damage to cell structures, inefficient cellular processes, inflammation, and neuronal death [6, 180]. In many cases, antioxidant enzymes (e.g., SOD1, catalase, and glutathione peroxidases) and antioxidant systems (e.g., Kelch-like ECH-associated protein 1 (Keap1)-nuclear factor erythroid 2-related factor 2 (Nrf2) system)

are mutated or impaired [181, 182]. Indeed, most ALS patients show reduced antioxidant enzymatic activity [183, 184], concomitantly with increased markers of peroxidation and oxidative damage in plasma [185], CSF [186], urine [187], and postmortem neuronal tissue [188].

(7) **Mitochondrial dysfunction:** structural/morphological mitochondrial defects, and bioenergetic malfunction, have been described [189-191]. Collective evidence reveals that mitochondria and energy metabolism play critical roles in disease development [192], with early mitochondrial dysfunction seen in both ALS patients and ALS animal models [193, 194]. Mutant misfolded proteins (SOD1 [195] and TDP-43 [196, 197], for instance) can translocate and attach to the mitochondrial outer membrane, intermembrane space, and matrix, where they stimulate mitochondrial DNA release and innate immune activation [198]; increase ROS production [199]; reduce calcium buffering capacity and induce cytochrome-C release [200, 201]; impair electron transport chain activity [202, 203]; disrupt fission-fusion balance [204]; and damage mitophagy and autophagy [205, 206]. All of these processes promote the production of pro-apoptotic proteins and increase mitochondrial permeability, ultimately giving rise to mitochondrial imbalance and motor neuron degeneration [191, 192].

(8) **Neuroinflammation:** Persistent neuroinflammation can cause neurodegeneration due to the excessive production of cytokines and pro-inflammatory growth factors, and the secretion of fewer neuroprotective/neurotrophic substances [6, 165]. Multiple neuroinflammatory markers are elevated in individuals suffering from ALS, correlating with disease progression [207, 208].

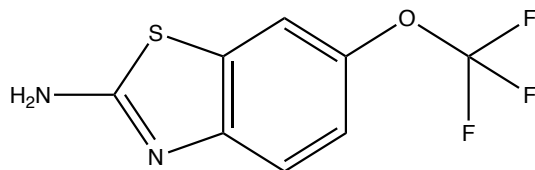
(9) **Defective cytoskeletal organization and axonal transport:** several mutations cause disorganization of cytoskeletal proteins and accumulation of hyperphosphorylated neurofilaments in neuronal cell bodies and proximal axons, disrupting axonal transport integrity [6, 209, 210]. One theory, the “dying-back hypothesis”, postulates that ALS is a distal axonopathy, wherein disease pathology starts in the axon of motor neurons and progresses retrogradely towards the soma. Therefore, axonal retraction and interruption of neuromuscular connections would precede neuron cell death. In contrast, the “dying-forward hypothesis” suggests that denervation starts in the cell body and progresses to the nerve endings [211]. The literature is mixed, leading scientists to believe that these processes may be two independent mechanisms that happen simultaneously [212, 213].

(10) **Non-cell autonomous mechanisms:** compelling findings support the notion that activation of non-neuronal glial cells, such as microglia [214], astrocytes [215], and oligodendrocytes [216], directly contribute to the demise of motor neurons in ALS, either through a loss of beneficial effects (reduced glutamate uptake, reduced lactate efflux; impaired responses to molecular injury, etc.) or through gain of toxic function (e.g., mounting a neuroinflammatory response), both of which exert deleterious effects [217]. Moreover, changes in peripheral immunity and signaling, including monocytes and T cells, have been reported [218, 219].

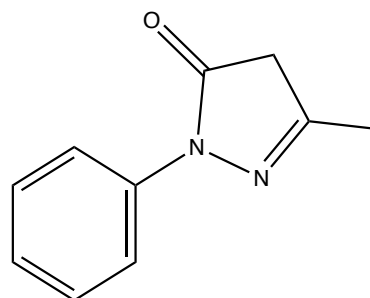
2.2. Therapeutic Strategies for ALS

2.2.1. Pharmacological Strategies

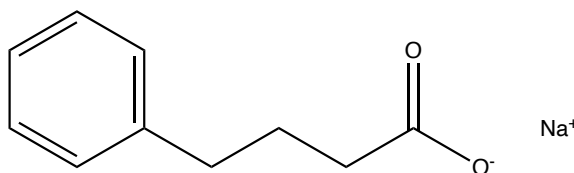
ALS is a disease with an urgent and unmet need for effective disease-modifying drugs. Only three active substances (Figure II.8) have ever been licensed for clinical use: RLZ, edaravone and AMX0035 [220].



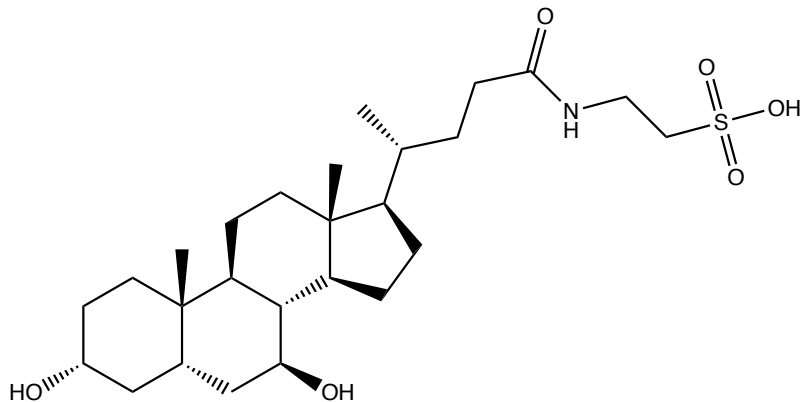
Riluzole



Edavarone



Sodium phenylbutyrate



Tauroursodeoxycholic acid (TUDCA)

AMX0035

Figure II.8. Chemical structures of approved drugs for amyotrophic lateral sclerosis (ALS): riluzole (RLZ), edaravone, and AMX0035 (co-formulation of sodium phenylbutyrate and tauroursodeoxycholic acid).

RLZ (6-(trifluoromethoxy)-2-aminobenzothiazole) was the first therapeutic agent to be approved for ALS by the Food and Drug Administration (FDA) in 1995, and by the European Medicines Agency (EMA) in 1996, under the name Rilutek® [221-223]. It acts as an anti-glutamatergic agent, via suppression of glutamate release from presynaptic terminals by blocking sodium (Na^+) channels and preventing exaggerated calcium (Ca^{2+}) influx (Figure II.9). In addition, it increases extracellular glutamate reuptake in glial cells, and also inactivates the α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor and N-methyl-D-aspartate (NMDA) postsynaptic receptors, thus ameliorating excitotoxicity [224, 225].

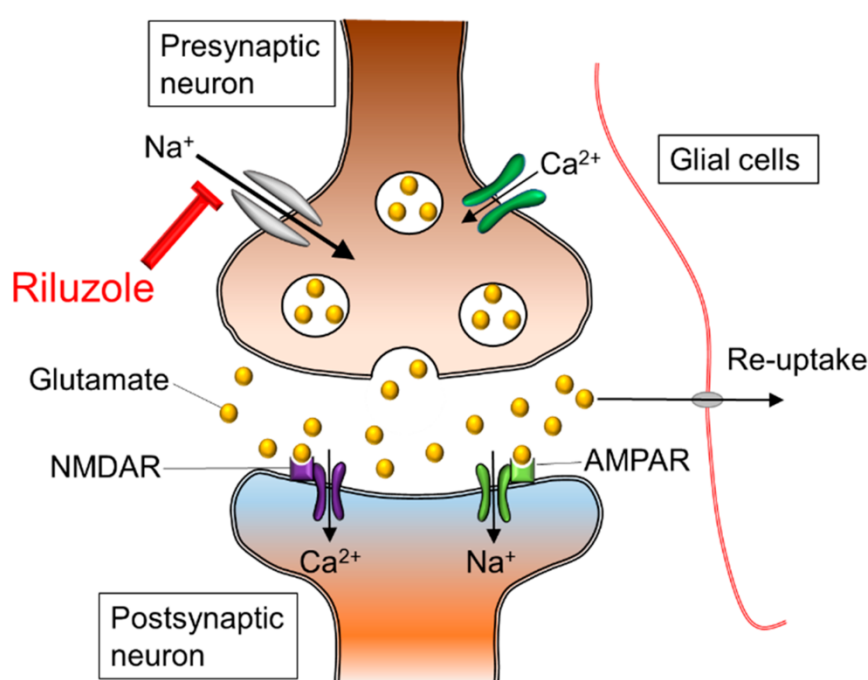


Figure II.9. Mechanism of action of riluzole (RLZ). The neuroprotective effects of RLZ result from the blockade of sodium (Na^+) and calcium (Ca^{2+}) channels in presynaptic neurons. Moreover, RLZ increases reuptake of extracellular glutamate in glial cells, and inactivates the N-methyl-D-aspartate (NMDA) and α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) postsynaptic receptors, decreasing excitotoxicity. Reproduced from [225], under the terms of the Creative Commons Attribution 4.0 International License (<https://creativecommons.org/licenses/by/4.0/>).

The initial trial results revealed that the increase in survival time was modest, prolonging it by only 3 months on average. However, when comparing patients who received RLZ treatment to those who did not, populational studies showed much larger survival improvements, ranging from 6 to 19 months [226]. RLZ is commercially available in the form of tablets (50 mg), as well as a suspension for oral administration (5 mg/mL in 300 mL bottles). The recommended daily dosage for RLZ tablets is 50 mg every 12 h. The primary limitation of the tablets is that individuals with dysphagia may cease treatment due

to their inability to swallow the medication, resulting in deteriorating therapeutic outcomes because of suboptimal adherence to prescribed drugs [227, 228]. On the other hand, the RLZ suspension, which can be swallowed or administered through percutaneous endoscopic gastrostomy tubes, seeks to enhance treatment adherence by ensuring adequate bioavailability and effectiveness. However, this formulation poses several challenges: it has an unpleasant local anesthetic effect and metallic taste, managing multiple-dose glass bottles is more cumbersome than handling tablets, and there is a perception that oral liquids, with their light cream consistency, are meant 'for children' [228]. RLZ continues to be regarded as the benchmark treatment in clinical trials, where novel compounds are required to be compared to RLZ or used as a supplementary therapy alongside RLZ [227].

The second therapeutic agent, edaravone (3-methyl-1-phenyl-2-pyrazoline-5-one), was first developed by Mitsubishi Tanabe Pharma (Japan) to treat acute ischemic stroke. It was licensed for the treatment of ALS in Japan and South Korea, in 2015 [224]. Based on the findings of a pivotal Phase III randomized, double-blind clinical trial (NCT01492686) in a Japanese cohort, in which it significantly slowed disease progression over the 24-week treatment period and reduced physical decline by 33% as measured by the Revised Amyotrophic Lateral Sclerosis Functional Rating Scale (ALSFRS-R) [229], it received further approval by the FDA (2017), as well as regulatory agencies in Canada (2018) and several other Asian nations, including China (2019) [230]. Nonetheless, treatment efficacy remained a subject of debate, and it was not approved by EMA. Factors contributing to this decision were the study's brief duration and stringent eligibility criteria, which only permitted enrollment of individuals in the early stages of the illness. Additionally, an Italian multicenter study of edaravone's effects on ALS patients yielded unfavorable results [231], suggesting that regulatory clearance in Europe will need further proof of its effectiveness, such as long-term survival benefits and efficacy in different cohorts of patients [232].

The precise mechanism of edaravone in ALS is unknown. It is thought to be a potent antioxidant and free radical scavenger drug, possessing both neuroprotective properties against oxidative stress, as well as anti-inflammatory effects against activated microglia cells. Edaravone has shown to inhibit the nuclear factor κ B (NF- κ B)-driven inflammatory pathway (via p50/p65), while enhancing the Nrf2 antioxidant pathway (via Keap1) [233]. This complementary action promotes the expression of antioxidant proteins and decreases the expression of pro-inflammatory proteins and cytokines, leading to an overall reduction in oxidative stress and inflammation (Figure II.10).

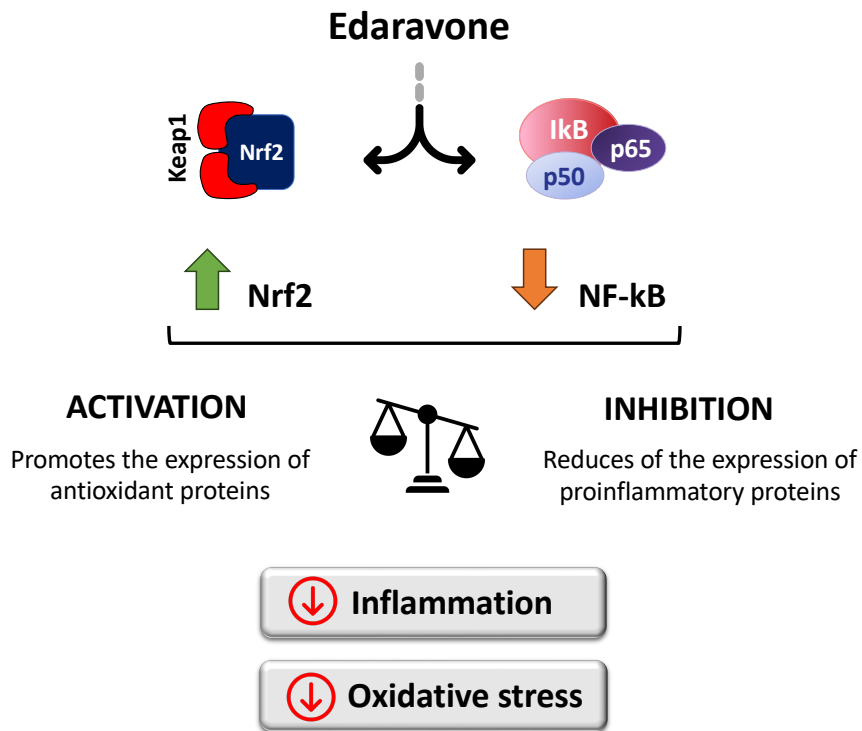


Figure II.10. Proposed mechanism of action of edaravone in amyotrophic lateral sclerosis (ALS). The drug acts in the nuclear factor erythroid 2-related factor 2 (Nrf2) and nuclear factor κ B (NF- κ B) pathways (via Keap1 and p50/p65, respectively), promoting the expression of antioxidant proteins and inhibiting pro-inflammatory ones. This leads to an overall reduction in inflammation and oxidative stress, which in turn can ameliorate ALS pathogenesis.

Edaravone has been used mainly as an intravenous (IV) therapy (Radicava[®], Radicut[®]), at a dosage of 60 mg/day. The first administration takes place over a period of 14 days, with following monthly cycles happening for ten days [224, 234]. The burden and inconvenience of daily infusions led to a lot of patients postponing treatment using the IV formulation. Therefore, an oral suspension of 105 mg/day (Radicava ORS[®]) was developed and approved in the United States in May 2022, and an international Phase III clinical trial (NCT04577404) is being conducted to evaluate its long-term tolerability and safety over 96 weeks. If the results are positive, it is likely that the oral form of edaravone will supersede the IV therapy for ALS management [230, 235].

The remaining drug is AMX0035, a fixed-dose combination of sodium phenylbutyrate and tauroursodeoxycholic acid (TUDCA), also known as ursodocoltaurine, from Amylyx Pharmaceuticals, to mitigate endoplasmic reticulum stress and mitochondrial dysfunction [235]. It was designed as an oral suspension containing 3 g of sodium phenylbutyrate and 1 g of TUDCA, being initially administered once a day for three weeks, and then twice a day. This medicine can be used alone or in conjunction with

existing authorized therapies [234]. The results of the large Phase II CENTAUR trial (NCT03127514) demonstrated that the co-formulation significantly decreased functional decline and disease progression rate, as well as extended survival by 5 to 6 months [236]. In a subsequent open-label extension trial (NCT03488524), survival also improved in patients who had previously received the placebo, conferring a 6.9-month long-term survival advantage [237]. This led to the co-formulation receiving conditional approval in Canada in June 2022 (Albrioza™), and full approval in the United States in September of the same year (Relyvrio™). Full approval in Canada was dependent on the results of the confirmatory Phase III PHOENIX clinical trial (NCT05021536), which were released in March 2024. In a surprise to patients and biotech industry watchers, the drug failed to meet its primary goal of improving function, and it did not meet secondary goals related to physical mobility, independence, eating and drinking, communication, and emotional reactions. Amylyx Pharmaceuticals therefore decided to voluntarily discontinue the marketing authorization in both countries [238]. In Europe, an advisory committee of EMA had recommended against Relyvrio™ for ALS based on the earlier data; the therapy was never approved for use in the European Union. Relyvrio™ is now being developed as a potential treatment for other neurodegenerative conditions, including Wolfram syndrome and progressive supranuclear palsy.

It is important to note that all these treatment options have short-term, limited benefits on disease course and cannot reverse damage to motor neurons [220]. Apart from the approved drugs, several therapeutic agents have been or are being tested in late-stage Phase III and Phase IV clinical trials to tackle different pathogenic mechanisms, with mixed results. Moreover, other compounds have also shown beneficial effects in Phase II trials, including off-label drugs and new investigational drugs described below [239]:

- **Rapamycin** (for protein aggregation): It is a mechanistic target of rapamycin (mTOR) inhibitor, which through autophagy stimulation, counteracts the aggregation and accumulation of pathological inclusions. It was shown to increase regulatory T (Treg) cells, decrease CD8 T-lymphocytes and inflammasome, and improve the quality of life of people with ALS (NCT03359538) [220, 240].

- **Pyrimethamine** (for protein aggregation): It is an antimalarial drug that can offset SOD1 levels, although the molecular mechanism is unknown. A study demonstrated that it was safe and well-tolerated, decreasing total mutant SOD1 levels in the CSF of ALS patients [241].

- **Retigabine** (for glutamate excitotoxicity): It is a neuronal Kv7.2/7.3 channel opener. Chronic retigabine administration decreased the excitability of upper and lower motor neurons in a dose-dependent manner (NCT02450552) [242].

- **Talampanel** (for glutamate excitotoxicity): It is an AMPA receptor blocker. Subjects treated with talampanel had reduced declines in ALSFRS-R score and muscle strength by 30% and 15%, respectively [243].

- **PrimeC - Ciprofloxacin/Celecoxib combination** (for glutamate excitotoxicity and neuroinflammation): Ciprofloxacin is an antibiotic, while celecoxib is an anti-inflammatory agent. It is thought that this combination inhibits several mechanisms that contribute to ALS cell death, including faulty RNA processing, inflammation, and iron accumulation, among others. Fifteen patients took PrimeC three times a day (daily dosage: 909 mg) for 1 year, with results from the initial Phase IIa study (NCT04165850) showing that the formulation was safe and markedly reduced key ALS biomarkers, particularly the amount of TDP-43. The combo therapy has earned Orphan Drug Designation in both the United States (2020) and Europe (2021) [244, 245]. Moreover, an upgraded, extended-release formulation of PrimeC was recently tested in healthy adults and exhibited a favorable pharmacokinetic profile (NCT05436678), which supports its chances in the ongoing Phase IIb PARADIGM study (NCT05357950) [246].

- **ILB[®]** (for glutamate excitotoxicity, mitochondrial dysfunction, and neuroinflammation): It is a patented modified dextran sulfate from Tikomed (Sweden), which exerts inhibitory effects on glutamate excitotoxicity, mitochondrial dysfunction, and neuroinflammation through the release of heparin-binding growth factors (HBGF). It also limits apoptosis, oxidative stress, and fibrosis [247]. Over a 5-week treatment period, ILB[®] subcutaneous injections significantly improved functional decline, as measured by 2.5 points increase in ALSFRS-R score, eliciting a decrease in muscle atrophy biomarkers. Furthermore, the slowing of disease progression was maintained up to 2 months after the last dose (EudraCT 2017-005065-47). These results could represent a breakthrough, with ILB[®] poised to become one of the first safe and well-tolerated disease-modifying treatments, with the potential to halt or reverse ALS symptomatology [248].

- **Bromocriptine** (for oxidative stress): It acts as a free radical scavenger. In a randomized, double-blind trial in Japanese patients (UMIN000008527), bromocriptine therapy preserved motoneuronal activity, which is promising for ALS management [249].

- **EPI-589** (for oxidative stress): It is a quinone derivative, which acts as an oxidoreductase enzyme inhibitor. Data from a recent study (NCT02460679) demonstrated that EPI-589 administered orally at a dose of 500 mg twice a day, was safe, well-tolerated, and clinically effective in slowing ALS progression, associated with reductions in oxidative stress biomarkers, as well as improvements in the ALSFRS-R score, grip strength and swallowing [250].

- **RNS60** (for mitochondrial dysfunction and neuroinflammation): It is a novel immunomodulatory molecule that activates intracellular signaling pathways that enhance mitochondrial biogenesis and anti-inflammatory effects. The drug had no effects on inflammation and neurodegeneration biomarkers, nor in functional decline. However, it did demonstrate positive impacts on respiratory and bulbar functions, namely, a slower decline rate in forced vital capacity (FVC) and eating and drinking domains over 24 weeks of treatment, with the lead researchers acquiescing that it warrants further investigation (NCT03456882) [251].

- **ANX005** (for neuroinflammation): It is a humanized mAb against complement component 1q (C1q) of the complement cascade [252]. According to preliminary data from an ongoing open-label Phase IIa trial (NCT04569435), during the first 12 weeks of ANX005 therapy, there was a decrease in NfL levels, a biomarker of nerve damage. Moreover, all patients exhibited a deceleration or cessation of disease progression. Full study data is expected in 2024 [253].

- **Aldesleukine** (for neuroinflammation): It is a recombinant human interleukin-2 (IL-2) that promotes Treg cell expansion, attenuating neuroinflammation. Results from the Phase IIa IMODALS study (NCT02059759) determined that a low dose of IL-2 was well-tolerated and immunologically effective [254]. In the Phase IIb MIROCALS trial (NCT03039673), aldesleukine led to a statistically significant 40% decrease in the risk of death, as measured by the phosphorylated neurofilament heavy chain (pNFH) levels in the CSF of patients [255, 256].

- **Tegobrupart** (for neuroinflammation): It is a therapeutic antibody that inhibits CD40 transmembrane protein, which is linked to increased neuroinflammation in ALS. Topline results from a Phase IIa trial (NCT04322149) demonstrated that tegobrupart was safe and engaged with its intended target, significantly reducing up to 23 pro-inflammatory

biomarkers in a dose-dependent manner. Moreover, there was a positive trend in the slowing of disease progression, as measured by the ALSFRS-R score [257].

- **NP001** (for neuroinflammation and microglia activation): It downregulates NF-κB expression, decreases the production of pro-inflammatory cytokines, and transforms microglia back to a nonactivated state. A post hoc analysis of a Phase IIb trial (NCT02794857) showed that in individuals aged between 40 and 65 years old, NP001 significantly reduced the decline in ALSFRS-R score by 36%, thus slowing and even halting ALS progression in this age group. Additionally, the molecule led to a 51% reduction in vital capacity (VC) loss, rendering it the first-ever treatment to influence VC preservation [258].

2.2.2. Genetic and Cellular Therapy Strategies

Clinical research has recently shifted its focus to experimental strategies targeting genes and cells, offering potential therapeutic benefits for ALS patients. In fact, advancements in genetic testing, more sophisticated animal models, and an expanding range of treatments have invigorated the pursuit of genetic and cellular solutions for ALS management [259]. These strategies include technologies such as small interference RNAs (siRNAs), antisense oligonucleotides (ASO), *ex vivo* and *in vivo* methods using viral and non-viral vectors, and gene editing, which are currently being tested in preclinical or clinical studies. Encouraging results using an ASO (Nusinersen®) [260] and a viral vector that expresses human survival motor neuron protein (Zolgensma®) [261] for spinal muscular atrophy have increased optimism that these approaches will eventually result in approved ALS treatments very soon. In the cases of fALS, it is especially important to target the expression of mutated genes, including SOD1, FUS, or C9orf72 [235].

ASO are short oligonucleotides that block messenger RNA (mRNA) translation or induce mRNA degradation through RNase H1, which in turn decreases protein synthesis [259]. Tofersen, by Biogen, which is the most sophisticated ASO-based therapy, aims to inhibit SOD1 protein production. The findings of the Phase III VALOR trial (NCT02623699), which registered 108 fALS individuals with a SOD1 mutation for a 28-week treatment period, were disappointing, as the primary goal of slowing functional decline was not met [262]. Nevertheless, Biogen applied for a New Drug Application (NDA) through the accelerated approval pathway, based on data showing a significant decrease in NfL and SOD1 protein levels, related to a deceleration in the disease progression, which was accepted in July 2022 [262]. In April 2023, the FDA approved Tofersen (branded as Qalsody™), making it

the fourth drug available for ALS treatment, and the first ever targeted therapy specifically for SOD1-associated ALS. Continued FDA approval hinges on the confirmation of clinical benefits in subsequent trials. In this regard, as therapy seems to be more successful at early stages of neuronal degeneration, a study (Phase III ATLAS trial, NCT04856982) is underway in people who are SOD1 mutation carriers but are still presymptomatic. Scheduled to finish in August 2026, researchers will investigate whether the drug can delay disease onset [263, 264]. Participants are eligible if their NfL levels rise beyond a predetermined threshold, which has been demonstrated to predict the emergence of symptoms within 1 to 2 years [264, 265].

Other companies have taken a similar approach, developing several ASO to modulate other ALS pathogenic genes. For instance, WVE-004, an experimental ASO designed by Wave Life Sciences, is undergoing a Phase Ia/Ib trial (NCT04931862) for C9orf72-associated ALS and frontotemporal dementia. Preliminary results demonstrated a durable 34% reduction in poly-GP (C9orf72 biomarker) in the CSF of subjects following treatment with low, single doses of WVE-004 [266]. Tadnersen (BIIB078), a joint experimental ASO drug of Ionis and Biogen that likewise targets hexanucleotide repeat expansions in C9orf72, was also tested. Unfortunately, it failed to show any substantial benefit compared with the placebo, along with a challenging safety profile, and so clinical development was abandoned [235].

A Phase III trial, initiated in August 2021, is currently evaluating the impact of Jacifusen (ION363) on the survival and clinical function of patients with FUS gene mutations, which are associated with aggressive juvenile-onset ALS [267]. In contrast, BIIB105, an ASO against toxic ATXN2, is being investigated for its long-term safety and tolerability in patients with sporadic ALS (Phase I/II ALSpire trial, NCT04494256). ATXN2 gene mutations are a risk factor for ALS, as they enhance TDP-43 protein aggregation and toxicity [235].

Nonetheless, the use of these technologies remains limited, as ensuring effectiveness and safety is still challenging. The main limitations of antisense-based therapeutics include the inability to cross the BBB, requiring intrathecal administration; short half-life; nonselective inactivation of the normal copy of the mutated allele, thus reducing the physiological function of the wild-type protein; direct engagement with mRNA being limited by complexities in intracellular processing; risk of immunogenicity, meningitis, and other off-target deleterious effects. For example, severe neurological complications were recorded in 4.8% of patients treated with Tofersen, including two episodes of myelitis (2.0%)

[232, 235, 268]. Moreover, since ASO molecules function downstream to stop protein synthesis, lifelong maintenance dosing would likely be necessary to counteract mRNA transcription from the active ALS gene. Soon, peripheral administration or targeting of specific cell types in the CNS could become possible through the use of novel delivery methods, such as conjugating ASO with cholesterol or lipid nanocarriers [259].

Apart from ASO, rapid advances in genome editing bring up the prospect of precisely targeting ALS genetic mutations *in vivo* using tools such as clustered regularly interspaced short palindromic repeats-associated protein 9 (CRISPR/Cas9) [232, 235]. CRISPR/Cas9 editing involves cleaving double-stranded DNA and subsequently leveraging the inherent DNA repair processes. This approach aims to rectify faulty DNA, which would eliminate any abnormal downstream pathways, making it a potentially one-time intervention. However, few studies have been undertaken in the field of ALS [259]. The effectiveness of CRISPR/Cas9 to target the SOD1 gene was investigated in SOD1^{G93A} neonatal mice. The treated mice had a reduction in SOD1 expression in their spinal cords, which was accompanied by an increase in motor neurons, improved motor function, delayed disease onset, and improved survival compared to control animals [269]. In 2020, two subsequent reports demonstrated a comparable reduction in SOD1 expression in the spinal cord and an improvement in survivability [270, 271]. The primary constraint of these studies lies in the administration of treatment to mice at a young age, before the onset of ALS symptoms. Therefore, it remains uncertain how effective the treatment would be in older mice that have already exhibited clinical signs [259]. Recently, researchers were able to restore stathmin-2 (STMN2) protein levels in TDP-43 deficient human motor neurons and delay paralysis in an ALS mouse model, using a CRISPR effector dCasRx that suppressed cryptic pre-mRNA splicing. STMN2 is crucial for axonal regeneration and maintenance of neuromuscular junctions [272].

On the other hand, therapies that may restore lost function are highly valued. Stem cells, due to their pluripotency and self-renewal ability, have for a while been touted as a regenerative approach to directly replace the damaged motor neurons in ALS. However, this is not a viable option, given the immense complexity of preexisting neuronal circuits into which the transplanted cells would need to integrate while enduring a diseased microenvironment. As a result, the emphasis of stem cell therapy is on generating a neuroprotective milieu to halt neurodegeneration [232, 273]. Mesenchymal stem cells (MSCs), in particular, can secrete neurotrophic factors, stimulate neurogenesis, and modulate inflammatory processes, changing the neural microenvironment from a pro-inflammatory to an anti-inflammatory state [235].

Multiple research studies examining the impact of utilizing MSCs in ALS mouse models have demonstrated that the groups that received MSCs treatment exhibited a decelerated motor neuron decline [274, 275]. Several clinical trials were subsequently conducted to explore the therapeutic effect of intrathecal administration of bone marrow-derived MSCs or neural stem cells in ALS patients [276, 277]. A recent Phase III trial (NCT03280056) did not achieve its primary endpoint of altering ALS decline, although individuals with milder disease preserved greater function than the placebo group [278]. This indicates that there is still substantial work to be done regarding the origin of stem cells, how they are administered, identifying patients who may respond better, determining clinical endpoints, and ensuring safety [235].

Table II.1 and Table II.2 provide a summary of the therapeutic agents that have undergone or are currently undergoing advanced Phase III and Phase IV clinical trials for ALS, and if available, the respective results.

Table II.1. Therapeutic agents in completed Phase III and Phase IV clinical trials for ALS treatment. Results retrieved from the U.S. National Library of Medicine online database (<https://clinicaltrials.gov>).

Therapeutic Agent	Targeted pathogenic mechanism	Mechanism of action	Development stage	Results	Trial identifier	Reference
Tofersen (BIIB067)	Protein aggregation	SOD1 antisense oligonucleotide	Phase III	Failed. Primary endpoint (ALSFRS-R) score was not statistically significant. However, there was a reduction in disease progression, as emphasized by a decrease in mutant SOD1 and in NfL. NDA submitted in the United States.	NCT02623699	[279]
Arimoclomol		Enhances the expression of heat shock protein	Phase III	Failed. Was safe and well-tolerated but lacked efficacy.	NCT03491462	[280]
					NCT03836716	[281]
					NCT00706147	[282]
IGF-1 (Myotrophin™)	Apoptosis	Neurotrophic factor. Blocks cell death, promotes muscle innervation and axonal growth/regeneration	Phase III	Failed. No significant beneficial effect in terms of daily functioning, muscle strength, or survival.	NCT00035815	[279]
Olesoxime	Mitochondrial dysfunction	Modulates the opening of mPTP	Phase III	Failed.	NCT00868166	[283]

Table II.1. (continued – 2/5).

Therapeutic Agent	Targeted pathogenic mechanism	Mechanism of action	Development stage	Results	Trial identifier	Reference
Mexiletine	Glutamate excitotoxicity	Blocks sodium channels	Phase IV	Effectively reduced frequency and severity of muscle cramps.	NCT01811355	[284]
Ceftriaxone		Increases EAAT2 activity	Phase III	Failed.	NCT00349622	[285]
Memantine		NMDA receptor antagonist	Phase III	Failed.	NCT00353665	[286]
Mecobalamin		Increases astrocytic glutamate transporter activity	Phase III	Failed. Showed a decrease in ALSFRS-R scores but not significant results. However, the trial suggested a greater benefit in earlier disease stages, with a higher dose (50 mg).	NCT00444613	[287]
				Ultra-high dose of mecobalamin was safe to use for a 16-week treatment period, effectively decreasing functional deterioration in individuals with early-stage ALS.	NCT03548311	[288]

Table II.1. (continued – 3/5).

Therapeutic Agent	Targeted pathogenic mechanism	Mechanism of action	Development stage	Results	Trial identifier	Reference
Ravulizumab (Ultomiris™)	Neuroinflammation	Anti-C5 monoclonal antibody. Blocks the activation of the complement cascade	Phase III	Failed. Lack of efficacy.	NCT04248465	[289]
NurOwn®		BM-MCSs that are manipulated <i>ex vivo</i> to secrete neurotrophic and neuroprotective factors	Phase III	Failed. Subsequent analysis revealed promising results on less severe disease forms.	NCT03280056	[278]
Acthar® Gel		Mix of ACHT analogs and other pituitary peptides. Stimulates the release of anti-inflammatory hormones	Phase III	Terminated due to pneumonia concerns.	NCT03068754	[290]
Masitinib		Tyrosine kinase inhibitor. Suppresses expression of inflammatory mediators and proliferation of activated microglia	Phase III	Oral masitinib improved ALSFRS-R score, slowed the rate of functional decline with acceptable safety, and prolonged survival by over 2 years. NDA submitted in Canada.	NCT02588677	[291]

Table II.1. (continued – 4/5).

Therapeutic Agent	Targeted pathogenic mechanism	Mechanism of action	Development stage	Results	Trial identifier	Reference
Edaravone	Oxidative stress	Antioxidant and free radical scavenger	Phase III	Failed, but a post-hoc analysis suggested a cohort with milder symptoms might benefit.	NCT00330681	[292]
				Prolonged edaravone therapy can offer a substantial, long-term benefit in ALS patients with greater baseline functionality.	NCT00424463	[293]
				Slowed the rate of functional decline by 33%, as measured by ALSFRS-R score. No serious adverse effects reported.	NCT01492686	[294]
		Antioxidant and free radical scavenger	Phase III	Failed. Patients with more advanced forms of ALS did not benefit.	NCT00415519	[295]
Dexpramipexole	Oxidative stress	Reduces free radical production	Phase III	Failed. Initial phase III trial did not meet primary efficacy endpoint. Open label extension study was terminated.	NCT01281189	[296]
					NCT01622088	[297]

Table II.1. (continued – 5/5).

Therapeutic Agent	Targeted pathogenic mechanism	Mechanism of action	Development stage	Results	Trial identifier	Reference
Minocycline	Oxidative stress, Neuroinflammation	Inhibits the activity of iNOS and caspase enzymes	Phase III	Failed. Not safe.	NCT00047723	[298]
G-CSF	Neuroinflammation, Apoptosis, etc.	Inhibits apoptosis and reduces inflammation	Phase III	Failed. May accelerate disease progression in females.	NCT01825551	[299]
Lithium	ER stress; Autophagy; Excitotoxicity	Inhibitor of glycogen synthase kinase	Phase III	Failed. Combination of lithium with riluzole did not slow ALS progression more than riluzole alone.	NCT00818389	[300]
Tirasemtiv	Muscle abnormalities	FSTA. Improves muscle contraction	Phase III	Failed. No significant difference in disease progression. Treatment did not improve muscle strength, nor change SVC. Program discontinued.	NCT02496767	[301]
Levosimendan		Calcium sensitizer that improves the contractile function of the diaphragm	Phase III	Failed. Oral levosimendan was well-tolerated but showed lack of efficacy on SVC (primary endpoint).	NCT03505021	[302]

Abbreviations: ALS: Amyotrophic lateral sclerosis; ALSFRS-R: Revised Amyotrophic Lateral Sclerosis Functional Rating Scale; ACHT: Adrenocorticotrophic hormone; BM-MCSs: Bone marrow mesenchymal stem cells EAAT2: Excitatory amino acid transporter 2; ER: Endoplasmatic reticulum; FSTA: Fast skeletal troponin activator; G-CSF: Granulocyte colony-stimulating factor; IGF-1: Insulin-like growth factor-1; iNOS: Inducible nitric oxide synthase; mAb: Monoclonal antibody; mPTP: Mitochondrial permeability transition pore; NDA: New drug application; NfL: Neurofilament light chain; NMDA: N-methyl-D-aspartate; SOD1: Superoxide dismutase 1; SVC: Slow vital capacity.

Table II.2. Therapeutic agents in ongoing Phase III and Phase IV clinical trials for ALS treatment. Results retrieved from the U.S. National Library of Medicine online database (<https://clinicaltrials.gov>).

Therapeutic Agent	Targeted pathogenic mechanism	Mechanism of action	Development stage	Results	Trial identifier	Reference
ION363 (Jacifusen)	Protein aggregation	FUS antisense oligonucleotide	Phase III	N/A. Ends in March 2028.	NCT04768972	[303]
Tofersen (BIIB067)		SOD1 antisense oligonucleotide	Phase III	N/A. ATLAS trial in pre-symptomatic patients ends in August 2026.	NCT04856982	[264]
					NCT03070119	[304]
SLS-005 (Trehalose)		Activates autophagy	Phase III	N/A. Part of the HEALEY ALS trial (regime E).	NCT05136885	[305]
					NCT04297683	
ABBV-CLS-7262		EIF2B activator	Phase III	N/A. Part of the HEALEY ALS trial (regime F).	NCT05740813	[305]
	NCT04297683					
Pridopine	Protein aggregation; Neuroinflammation	Sigma-1 receptor agonist	Phase III	N/A. Part of the HEALEY ALS trial (regime D).	NCT04615923	[305]
					NCT04297683	
Memantine and trazodone	Protein aggregation; Glutamate excitotoxicity	Memantine: NMDA receptor antagonist. Trazodone: Slows the production of protein aggregates	Phase III	N/A.	NCT04302870	[306]
Deferiprone	Oxidative stress	Chelator of iron ions	Phase III	N/A.	NCT03293069	[307]

Table II.2. (continued – 2/5).

Therapeutic Agent	Targeted pathogenic mechanism	Mechanism of action	Development stage	Results	Trial identifier	Reference
Edaravone (Oral formulation)	Oxidative stress	Antioxidant and free radical scavenger	Phase III	N/A.	NCT04577404	[308]
					NCT05178810	[309]
					NCT05568615	[310]
					NCT04569084	[311]
					NCT05151471	[312]
Verdiperstat	Microglia activation	Myeloperoxidase enzyme inhibitor	Phase III	Part of the HEALEY ALS trial (regime B). Still active on ClinicalTrials.gov, but officials announced in September 2022 that the study's primary endpoint (ALSFRS-R) was not met.	NCT04436510	[305]
					NCT04297683	
AMX0035 (TUDCA and sodium phenylbutyrate)	Endoplasmatic reticulum stress; Mitochondrial dysfunction	TUDCA is a Bax inhibitor that reduces cell death by apoptosis, while sodium phenylbutyrate acts as a molecular chaperone	Phase III	Results from the PHOENIX trial (NCT05021536) were disappointing. Primary and secondary endpoints were not met. Drug will be discontinued.	NCT05021536	[313]
					NCT05619783	[314]
					NCT04516096	[315]

Table II.2. (continued – 3/5).

Therapeutic Agent	Targeted pathogenic mechanism	Mechanism of action	Development stage	Results	Trial identifier	Reference
Triumeq®	Viral infections	Combination antiretroviral therapy of dolutegravir, abacavir and lamivudine that inhibits HERV	Phase III	N/A. Previous results from the Phase II LIGHTHOUSE trial indicate that the drug is safe, well-tolerated, suppresses HERV activation and may slow ALS progression.	NCT05193994	[316]
MediCabilis CBD Oil (cannabis-based medicine extract)	Oxidative stress; Excitotoxicity; Protein aggregation	Free radical scavenger action; Activation of cannabinoid receptors	Phase III – IV	N/A.	NCT03690791	[317]
					NCT04997954	[318]
Lenzumestrocel (Neuronata-R® Injection)	Neuroinflammation	Autologous BM-MSCs cell therapy. Expression of anti-inflammatory and neuroprotective factors; immunoregulation	Phase III	N/A. Orphan cell therapy conditionally approved in South Korea (2014).	NCT04745299	[319]

Table II.2. (continued – 4/5).

Therapeutic Agent	Targeted pathogenic mechanism	Mechanism of action	Development stage	Results	Trial identifier	Reference
Masitinib	Neuroinflammation	Tyrosine kinase inhibitor. Suppresses the expression of inflammatory mediators and proliferation of activated microglia	Phase III	N/A.	NCT03127267	[320]
Zilucoplan		C5 complement inhibitor	Phase III	Part of the HEALEY ALS trial (regime A). Study still active on ClinicalTrials.gov, but officials from the HEALEY ALS trial announced Zilucoplan regime was stopped early due to futility.	NCT04436497	[305]
					NCT04297683	
TUDCA		Bax inhibitor that incorporates itself into the mitochondria membrane and increases apoptotic threshold	Phase III	N/A.	NCT03800524	[321]
					NCT05753852	[322]

Table II.2. (continued – 5/5).

Therapeutic Agent	Targeted pathogenic mechanism	Mechanism of action	Development stage	Results	Trial identifier	Reference
MN-166 (Idubilast)	Neuroinflammation	Inhibitor of PDE-4 and PDE-10 enzymes and macrophage migration inhibitory factor, blocking the activity of pro-inflammatory signaling molecules	Phase III	N/A. Ends in December 2026. Phase II results (NCT02238626) indicate that the administration of idubilast along with riluzole improved disease progression measures, especially in early-stage ALS patients (less than 520 days from onset).	NCT04057898	[323, 324]
Reldesemtiv		FSTA. Increases muscle contractility	Phase III	Still active on ClinicalTrials.gov, but Cytokinetics announced in March 2023 that the Phase III COURAGE-ALS trial and extension study will conclude earlier than planned due to lack of efficacy of reldesemtiv.	NCT04944784 NCT05442775	[325]

Abbreviations: BM-MSC: Bone marrow mesenchymal stem cells; CBD: Cannabidiol; EIF2B: Eukaryotic translation initiation factor 2B; FSTA: Fast skeletal troponin activator; FUS: Fused in sarcoma; HERV: Human endogenous retroviruses; PDE: Phosphodiesterase; N/A: Not available; NMDA: N-methyl-D-aspartate; SOD1: Superoxide dismutase 1; TUDCA: Tauroursodeoxycholic acid.

3. Challenges of ALS Drug Delivery to the Brain

Despite the development of numerous therapeutic agents to treat ALS, their effectiveness in clinical trials has been largely underwhelming, mainly due to inadequate *in vivo* properties. These limitations comprise poor stability in the biological environment, limited BBB permeability, rapid enzymatic degradation, elimination by the immune system, unfavorable pharmacokinetic properties, and suboptimal release profile. Any of these hindrances can prevent a particular molecule from effectively targeting the brain and achieving clinical efficacy [6]. The next section outlines the major challenges involved in delivering ALS therapeutics to the CNS.

3.1. Blood-Brain Barrier

As previously mentioned, the BBB has a protective function that aims to stop the entry of macromolecules, pathogens, and neurotoxins into the CNS. However, it also poses a significant barrier to the delivery of therapeutic agents to their intended target inside the brain. The BBB blocks the passage of more than 90% of small-molecule drugs and virtually all biologics (e.g., polypeptides, recombinant proteins, mAb, ASO, DNA, siRNA, etc.) [7, 326]. The presence of efflux proteins, especially P-gp, is a major reason why several promising treatments for ALS fail to achieve therapeutic levels in the CNS, as they actively recognize and pump out the medicines, leading to drug resistance. A prime example is RLZ, which exhibits limited CNS accumulation due to active efflux [326]. Furthermore, BCECs contain a variety of degrading enzymes, which contribute to metabolic degradation and limit bioavailability and drug efficiency [6, 326].

3.2. Bioavailability and Biostability

Apart from inadequate BBB permeability, the effectiveness of ALS treatments is also limited by insufficient drug bioavailability and biostability [6]. A significant proportion of newly developed therapeutics have very low aqueous solubility. For instance, olesoxime, a mitochondrial-targeted neuroprotective compound, has limited solubility in water due to its cholesterol-like structure [327]. Similarly, P7C3A20, an aminopropyl carbazole, delayed disease progression in a SOD1^{G93A} ALS mice model, but its efficiency was hindered by limited water solubility, requiring further improvements [328, 329]. Currently, most CNS drugs in clinical use are small, lipid-soluble molecules, such as edaravone and RLZ, which

can cross the BBB through active diffusion. However, for therapeutic purposes, these molecules must be transported to the brain's interstitial fluid after being absorbed by BCECs. Excessively lipophilic drugs risk being sequestered at the capillaries, failing to reach the brain tissue beyond the BBB. Consequently, designing drugs with appropriate lipid solubility is crucial to ensure sufficient drug accumulation within the CNS. Lately, new types of biologics have demonstrated efficacy in treating ALS, including neurotrophic factors, nucleic acids, and therapeutic proteins [6]. One notable example involves using an adeno-associated virus serotype 9 (AAV9) carrier to transport anti-SOD1 RNA, which extended the survival of SOD1 ALS mice [330]. However, due to its susceptibility to biological enzymes and lack of BBB permeability, this biomolecule, and others with similar characteristics, have short half-lives, which limits their efficacy as potential ALS therapies [6, 331].

3.3. Systemic Distribution and Clearance

Another significant obstacle to CNS drug delivery is the tendency of lipophilic drugs to undergo systemic distribution and clearance, thereby weakening their on-target effects. Nonspecific distribution requires that higher drug dosages be given to achieve therapeutic levels in the CNS, increasing the risk of systemic toxicity [6]. Additionally, hydrophilic drugs are often quickly cleared by the kidneys due to poor reabsorption after glomerular filtration, while lipophilic drugs are transformed into hydrophilic metabolites in the liver before undergoing biliary or renal excretion [332]. As a result, the therapeutic efficacy of compounds such as RLZ or edaravone may be compromised by their systemic distribution and rapid clearance, as seen in the oral suspension form of RLZ (Tiglutik™), limiting its bioavailability [333]. Nanotechnology-based approaches, namely, nanocarriers, provide a feasible solution to all these obstacles, enhancing drug targeting to the brain, increasing bioavailability, and decreasing systemic excretion, thus improving therapeutic efficacy.

4. Approaches to Facilitate Drug Transport to the CNS

In the past decades, significant progress has been made towards enhancing the uptake of therapeutic and diagnostic agents into the CNS. To this end, and in conjunction with advancements in BBB research, various methods have been employed, which can be classified into two main categories [7, 41]:

(1) **Invasive techniques**, such as direct CNS administration, transient BBB disruption, intracerebral implants, and biological tissue delivery.

(2) **Non-invasive techniques**, including drug chemical modifications, alternative administration routes (e.g., intranasal delivery), and novel delivery systems such as nanocarriers.

Table II.3 summarizes the benefits and limitations of the different strategies used to enhance the targeted delivery of therapeutic agents to the brain.

Table II.3. Advantages and drawbacks of different strategies for therapeutics delivery to CNS [334-336].³

Approach	Advantages	Drawbacks
Invasive Methods		
Intracerebral, intraventricular, or intrathecal administration	▪ High concentration of therapeutics in the CNS ▪ Administration of a broad range of compounds, including both large and small molecules ▪ Minimal systemic exposure	▪ Possible CNS infection ▪ Catheter obstruction ▪ Inadequate therapeutic distribution
Intracerebral implants	▪ Controlled/sustained therapeutic release profile which restricts tissue damage ▪ Adjustable release	▪ Limited penetration and distribution of therapeutic agents ▪ Highly traumatic technique

³ This table is transcribed from previous work by the author of this thesis: 7. *ibid.*

Table II.3. (continued – 2/3).

Approach	Advantages	Drawbacks
Biological tissue delivery	–	Transplanted tissue cannot live long because of the absence of neurovascular innervation
Osmotic solutions	Disruption of interendothelial TJ increases BBB permeability for several hours	- Possible disturbance of brain functions - Unfavorable therapeutic/toxic ratio frequently observed
Vasoactive substances	Less invasive and more reliable technique for BBB disruption, since it mainly affects the diseased vasculature	- Possible neurotoxicity - Possible disturbance of brain functions
Ultrasounds	Targeted drug delivery	Limited penetration of the ultrasounds through the skull
Non-Invasive Methods		
Chemical modifications	Pharmacokinetic parameters, toxicity and efficacy may be modulated	- Lipidization increases the rate of oxidative metabolism, the volume of distribution and the uptake into other tissues - Poor selectivity and tissue retention of some molecules
Intranasal delivery	- Direct BBB bypass - Simple administration	- Irritation of nasal mucosa - Decreased penetration and efficiency of drugs with larger molecular weight

Table II.3. (continued – 3/3).

Approach	Advantages	Drawbacks
Colloidal carriers	▪ Encapsulation of drugs with varying solubilities ▪ Controlled or targeted drug release ▪ High biocompatibility ▪ Biodegradable ▪ High drug loading ▪ Protection of drugs from enzymatic degradation	▪ Increased risk of systemic toxicity ▪ Cost ▪ Scaling-up ▪ Regulatory hurdles

4.1. Invasive Techniques

One approach to circumvent the BBB involves injecting ALS medicines into the brain parenchyma and ventricles (intracerebral or intraventricular administration) or the lumbar subarachnoid space (intrathecal administration) [334]. Although direct injection enables the delivery of large drug doses directly into the CNS, it remains challenging in the clinic due to its high invasiveness, in addition to limited tissue diffusion of drugs, insufficient pharmacokinetics, as well as other serious risks, such as infection, edema, and neuronal damage [6]. Temporarily disrupting the structural integrity of the BBB in a reversible manner, also facilitates the penetration of molecules into the brain. To achieve barrier disruption, various mechanisms can be employed, including the use of vasoactive substances, osmotic solutions, and physical stimuli (e.g., ultrasound) [337].

On the other hand, biodegradable polymeric matrices impregnated with drugs that control the release of neurotherapeutics over an extended period, are known as intracerebral implants. These implants are particularly advantageous in the treatment of brain tumors [338]. Another strategy involves transplanting biological tissue that secretes the desired therapeutic agent into the brain, which has mainly been applied to the management of PD [339].

4.2. Noninvasive Techniques

Chemical modifications involve altering the molecular structure of a drug to improve its pharmaceutical properties, such as water solubility or lipophilicity [335]. Prodrugs are an example of compounds that arise from chemical modifications, being inactive conjugates of a conventional drug that are covalently attached to a moiety. They can easily pass through the BBB, and once inside the brain, they are metabolized into the active (parent) molecule by cleavage of the moiety. Therefore, prodrugs improve the therapeutic index of the parent drug [340]. The quest to improve CNS drug delivery has led to the rational exploration of alternative administration routes that consider both brain physiology and drug properties. Intranasal delivery is one such route, enabling neurotherapeutics to bypass the BBB and access the brain directly through the olfactory and trigeminal nerve pathways. Despite limitations, such as the limited dosing volume permitted by the nasal cavity or short residence time due to mucociliary clearance, intranasal delivery can be an effective method for delivering potent neurotherapeutic agents to the brain [341]. Finally, nanocarriers also hold great potential as a means of targeting the CNS and have shown great effectiveness in treating a variety of brain diseases.

5. Nanocarriers for CNS Targeting

Despite all the progress in neuroscience, delivering therapeutics to the CNS remains a major challenge, compromising the effective treatment of ALS. Although conventional dosage forms offer the possibility to modify and improve certain physicochemical properties of neurotherapeutic agents, they are still restricted by the BBB [7]. In this sense, overcoming the hurdle of traversing the BBB is a critical factor in achieving successful therapy. Nanotechnology has played a vital role in the development of different nanocarriers, revolutionizing the realm of drug delivery. These systems currently account for 17% of the global pharmaceutical market, totaling a remarkable \$203.4 billion in 2021 [342].

The use of nanocarriers as a strategy to access the CNS has garnered substantial interest, due to their noteworthy properties. Their ability to improve the solubility/stability and bioavailability of active agents, enhance drug transport across the BBB, protect from environmental factors (e.g., enzymatic degradation), adjust biodistribution, and reduce systemic clearance, is of particular significance. These attributes contribute to minimizing adverse side effects and improving the overall therapeutic efficacy [343]. Nanocarriers can

be defined as particulate systems that range from 1 to 1000 nm in size, with a natural or synthetic origin [99].

Distinct types of nanocarriers, including organic (polymeric nanoparticles (NPs), liposomes, lipid NPs, dendrimers, etc.) and inorganic nanocarriers (silica and gold (Au) NPs, magnetic NPs, carbon nanotubes, quantum dots, etc.) have been employed [343]. The suitability of a nanosystem to convey drugs to the brain relies on various properties and desirable features that the nanocarrier should possess, which include: small particle size; biocompatibility and biodegradability; being physically stable in the systemic circulation; minimal immune and inflammatory response; and possibility of surface modification [7].

Among the different nanocarriers, lipid-based nanocarriers, such as SLN, NLC, liposomes, or nanoemulsions, are widely exploited for CNS targeting [344]. Despite the long-standing presence of lipid matrices in literature, the increasing availability of lipophilic excipients with well-established safety profiles and flexibility to modulate drug release has greatly contributed to lipid nanocarriers gaining prominence in the field of therapeutic brain delivery [7]. Moreover, they have several advantages that make them more appealing than other nanosystems for overcoming the challenges associated with CNS drug delivery, including formulation simplicity; inherent ability to cross the BBB by their lipophilic nature and nano dimensions; better biocompatibility and biodegradability, due to the use of GRAS excipients; lower cytotoxicity; possibility to encapsulate both lipophilic and hydrophilic drugs; easy manufacturing and scale-up with the desired reproducibility; and easy surface modification [7, 344, 345].

Described in 1965 by Bangham *et al.* [346], liposomes were among the first nanocarriers employed in the clinic. These are spherical vesicles composed of one or more concentric lipid bilayers surrounding an aqueous core (Figure II.11A). The amphiphilic properties of lipids cause them to spontaneously organize into this unique bilayer structure, enabling liposomes to encapsulate both hydrophilic and lipophilic molecules, in their internal aqueous core and lamellae, respectively [7]. Nevertheless, liposomes have many drawbacks, such as production methods that usually involve the use of organic solvents, difficulty in scaling-up, and low encapsulation efficiency, which led to the emergence of lipid nanoparticles (SLN and NLC) [347].

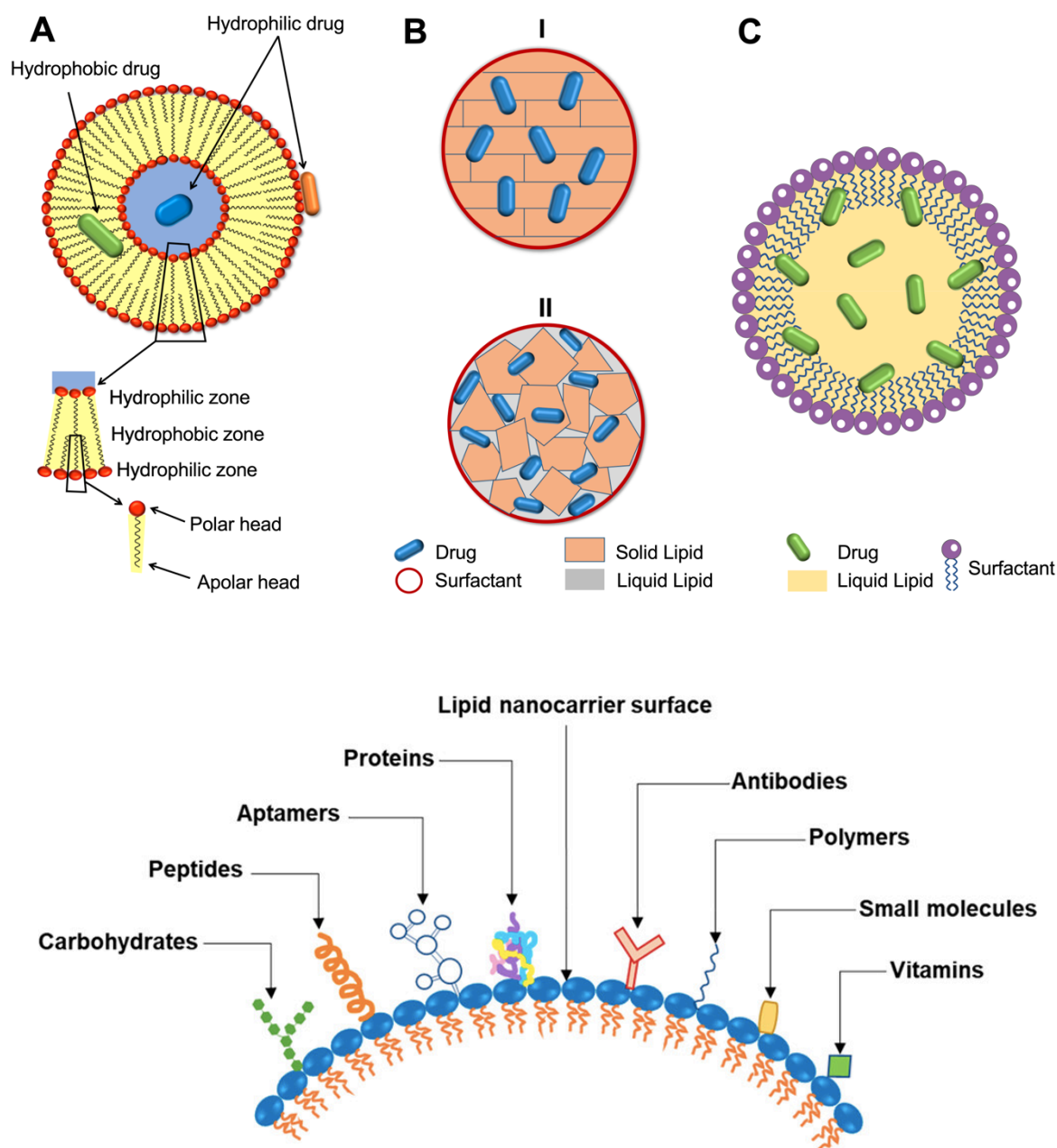


Figure II.11. (A–C) Schematic illustration of the structure of several lipid-based nanosystems, namely, (A) liposomes, (B-I) solid lipid nanoparticles (SLN), (B-II) nanostructured lipid carriers (NLC), and (C) micro and nano emulsions. Reproduced with permission from [7]. (D) Representation of the different types of ligands used for functionalizing the surface of nanocarriers. Reproduced with permission from [342].

SLN are colloidal particles composed of lipids that remain solid at both room and physiological temperatures, dispersed in an aqueous medium, and stabilized by surfactants (typically between 0.5% and 5% (w/w)) (Figure II.11B-I) [344]. Commonly used solid lipids include fatty acids (e.g., palmitic acid, stearic acid, myristic acid, dodecanoic acid), waxes (e.g., cetyl palmitate, beeswax, carnauba wax), and mono/di/triglycerides (e.g., glycerol

monostearate, glyceryl behenate, tripalmitin, tristearin, trimyristin), making up to 30% (w/w) of the composition [344, 348]. Like liposomes, SLN can accommodate both lipophilic and hydrophilic compounds, providing sustained and controlled drug release. Certain limitations, including drug expulsion and limited loading space, were successfully addressed by the second generation of lipid nanoparticles, the NLC [7].

NLC are prepared with a mixture of solid and liquid lipids. The inclusion of the liquid lipid results in an amorphous, less ordered lattice compared to the SLN, characterized by spatially distinct molecules with empty spaces between them (Figure II.11B-II). Hence, NLC exhibit enhanced drug loading capacity, as well as improved physical and storage stability. By regulating the liquid lipid levels, NLC remain solid at body and room temperatures. The selection of the liquid lipid, which typically ranges from 0.1% to 30% of the overall lipid mixture, must be done considering its solubility properties, compatibility with the solid lipids, as well as physiological tolerability [7, 344]. The physical stability of NLC is significantly influenced by both the ratio of solid to liquid lipids and the concentration of the total lipid phase. As noted by Loo *et al.* [349], NLC with the highest lipid concentration (30% w/w), and the highest solid-to-liquid lipid ratio (90:10) demonstrate superior physical stability compared to NLC with lower total and solid lipid concentrations.

Apart from liposomes and lipid nanoparticles, micro and nanoemulsions (Figure II.11C) are also promising lipid-based nanosystems for brain targeting. Although their composition is similar, microemulsions are thermodynamically stable, whereas nanoemulsions are not [350].

Factors such as composition, size, shape, charge, and surface modification influence the stability and *in vivo* behavior of nanocarriers, and ultimately, their ability to cross biological barriers [6, 344]. By tailoring and optimizing these properties, it is feasible to design nanocarriers that can cross the BBB, while enhancing the safety and efficacy of the respective cargos [345]. Nanocarrier size plays a crucial role as it allows interactions with cells at the appropriate magnitude, thereby eliciting a cellular response [99]. Additionally, mean diameter and size distribution patterns substantially affect other parameters, including physical stability and encapsulation efficiency. The "optimal" size range for brain delivery remains a topic of debate [344]. It is commonly reported that nanocarriers should be at least 10 nm to avoid renal excretion. However, regarding the upper limit, different studies suggest diameters ranging from 100 nm to 300 nm [343, 351, 352]. Nanocarriers within this interval (i.e., 100 - 300 nm) have successfully penetrated the BBB and reached the brain parenchyma [353].

Shape and surface charge are other physicochemical properties impacting BBB permeability and integrity [344, 354]. Shape controls not only the BBB uptake mechanism, but also how fast cells internalize nanocarriers [355, 356]. Among the various shapes that nanocarriers can exhibit (cubic, spherical, rod-like, etc.), spheres are commonly chosen in the context of brain delivery, as they allow for easier reproducibility and surface modification [357]. Concerning surface charge, cationic nanocarriers usually have higher internalization rates than their anionic or neutral counterparts, due to electrostatic interactions with the negatively charged BBB cell membrane, facilitating their uptake [354]. Nonetheless, nanocarriers with positive charge exhibit a faster clearance rate from the bloodstream, resulting in a reduced residence time within brain microvessels and diminished drug delivery [358]. Furthermore, their toxicity should be considered, as it has been demonstrated that they can induce acute BBB toxicity and disrupt cell membranes during adsorption, whereas neutral and anionic nanocarriers generally have no effect, being well-tolerated [358, 359]. Hence, the majority of nanocarriers described in the literature for CNS targeting exhibit either moderate [360-362] or high negative ZP [363].

Functionalization is frequently used to enhance targeting efficiency and therapeutic response (Figure II.11D). A frequent strategy consists of coating the nanocarriers with hydrophilic polymers, such as chitosan or polyethylene glycol (PEG), granting them “stealth” properties to bypass immune recognition and clearance. PEGylation reduces opsonization and minimizes phagocytosis by the reticuloendothelial system (RES), prolonging circulation time and indirectly favoring translocation to the brain [7, 345]. Moreover, the protective shield contributes to sterically stabilizing the nanocarrier [344]. Several research groups have shown that PEGylated nanocarriers not only have improved stability and decreased systemic clearance, but also display successful BBB penetration and enhanced cellular uptake compared to free drugs [364, 365].

Likewise, surfactants like polysorbates and poloxamers may also be employed as coating materials to avoid opsonization, while increasing CNS targeting. Polysorbate 80 (Tween[®] 80) and poloxamer 188, for instance, induce the adsorption of serum proteins from the bloodstream onto the nanocarrier’s surface, facilitating their transport across the BBB. In these cases, the nanocarriers are synthesized to selectively interact with certain plasma proteins (e.g., ApoE), which naturally propels the nanocarriers toward the intended target site [366]. Poloxamer 188-coated and polysorbate 80-coated polymeric NPs demonstrated 2- and 19-fold greater uptake and accumulation in the brain than plain polymeric NPs, with subsequent internalization to the neurons and microglia [367]. The same was evidenced by

polysorbate-80 coated SLN, which improved temozolomide bioavailability and brain uptake for glioblastoma treatment compared to plain SLN and free drug [368].

Nevertheless, when it comes to delivering drugs to the brain, the primary focus lies in active targeting [344]. Active targeting entails functionalizing the surface of nanocarriers with ligands that selectively recognize and bind to specific receptors and transporters expressed in the BBB, thereby mediating transcytosis across the brain endothelium via physiological mechanisms, particularly RMT and CMT. This process directs the nanocarriers to the brain (i.e., site-specific delivery), further increasing uptake. Moreover, by engaging different endocytic pathways than those that are used by BBB efflux pumps, it partially avoids premature CNS drug expulsion [342, 345].

Several molecules are used to functionalize the NPs, including glycoproteins (e.g., transferrin, lactoferrin), antibodies, peptides (e.g., CPPs, angiopeps, glutathione (GSH)), aptamers, vitamins (e.g., folate, thiamine), carbohydrates (e.g., mannose) [342, 345]. The ligand must be carefully chosen, considering the target cell, the nanosystem, and the available molecules. While ApoE, transferrin, or lactoferrin, are commonly used as moieties, their high endogenous levels often result in saturation and reduced specificity. On the other hand, antibodies, although effective, are larger, prone to instability, immunogenic, and require proper orientation, which can hinder brain uptake. It appears that peptides, such as CPPs, angiopeps, and others, may offer a promising approach. These peptides are smaller, more stable, and easier to produce compared to proteins. Most times, to target both the BBB and the cell of interest, nanocarriers functionalized with two or more ligands are needed. Multifunctionality increases the complexity of nanocarrier design and production, thereby presenting challenges for scaling up and clinical translation [345].

Pre-clinical evidence consistently indicates that surface-modified nanocarriers enhance BBB penetration, increase targeted drug delivery and controlled release, reduce neurodegeneration, and improve therapeutic efficacy [369-375]. Soon, these findings should be exploited in fully-fledged clinical trials to help bridge the gap between bench to clinic and encourage the commercialization of functionalized nanosystems, which may advance the current therapy for CNS disorders [343, 345].

5.1. Designing Advanced Nanomaterials for ALS Treatment

As explored in Section 5, rapid advancements in nanotechnology present potential solutions to various limitations that currently hinder therapeutic efficacy in ALS. In this subsection, the most relevant studies reporting on the use of NPs for ALS management will be presented, and their main findings discussed.

Over the past few years, numerous nanocarriers have been developed to increase the neuroprotective capabilities of several compounds. Bondi *et al.* [376] produced RLZ-loaded SLN, which enhanced RLZ delivery to the brain and significantly increased its CNS concentration compared to the free drug (1.3 times higher after IV administration in rats). The superiority of the nanocarrier was evidenced by the fact that SLN-treated rats developed clinical signs of experimental allergic encephalomyelitis (EAE) later than those treated with an aqueous RLZ dispersion. Moreover, biodistribution studies revealed that SLN had a lower distribution in other organs (heart, kidneys, liver, spleen, and lungs), lessening systemic toxicity [376]. To overcome pharmacoresistance, verapamil, a P-gp efflux pump inhibitor, was co-encapsulated with RLZ in liposomes. Verapamil efficiently silenced P-gp in an *in vitro* BBB model of bEND.3 mouse brain endothelial cells, as well as in astrocytic C8D1A cells, both under normal conditions and TNF- α stimulation, improving RLZ uptake and biodistribution (Figure II.12A). The authors suggested that this nanoplatform could be used to advance ALS therapy, although its long-term potential and safety need to be further investigated in animal models [377].

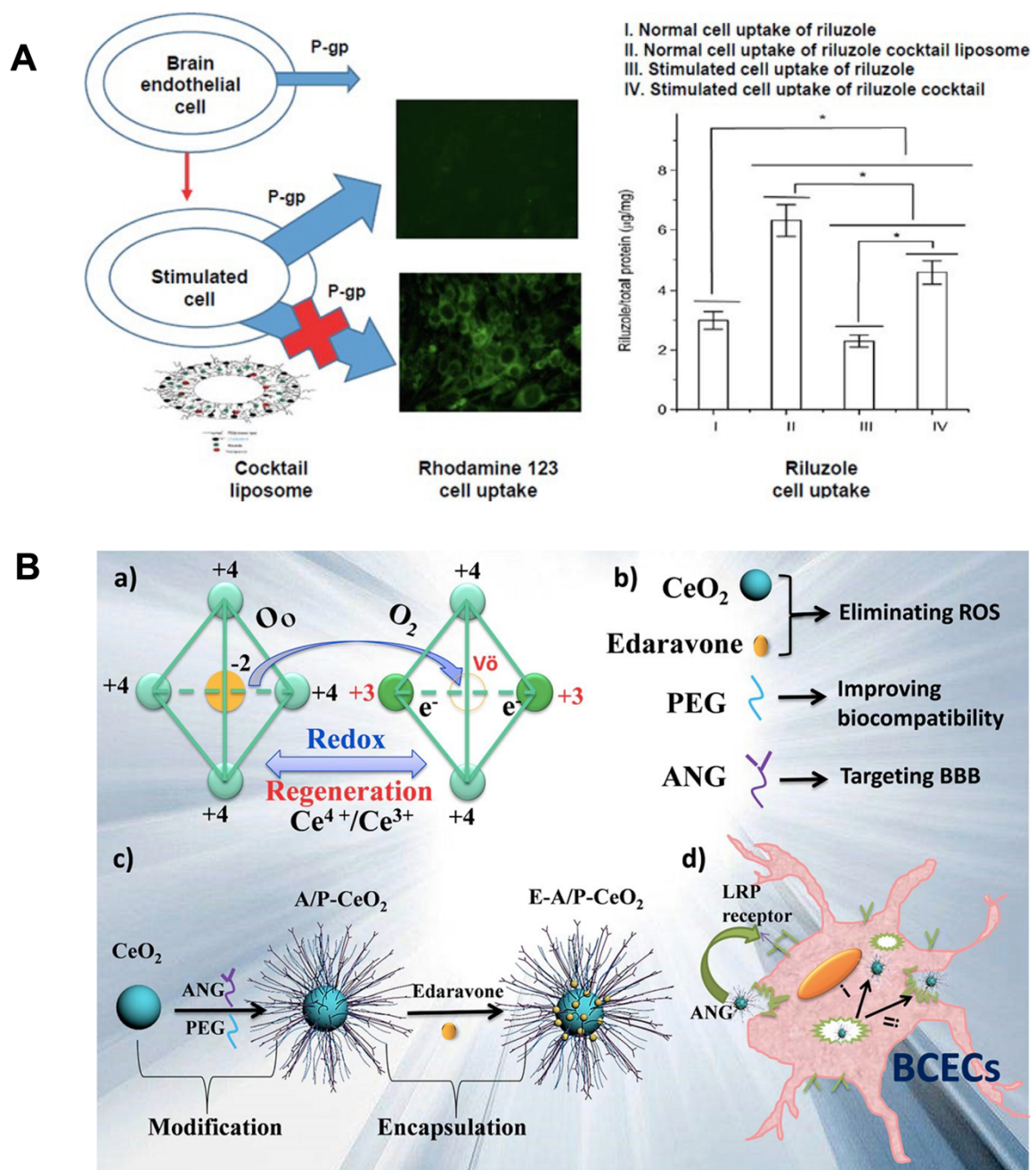


Figure II.12. (A) Liposomes containing riluzole (RLZ) and verapamil efficiently inhibited the P-gp efflux pump, increasing RLZ uptake in an in vitro BBB model (bEnd.3 cells). Reproduced with permission from [377]. (B) Edaravone-loaded ceria nanoparticles (NPs), functionalized with polyethylene glycol (PEG) and angiopep-2, permeated the blood-brain barrier (BBB), and promoted neuroprotection in the brains of ischemic rats. Reproduced with permission from [378].

Using a different lipid-based nanosystem, Parikh *et al.* [379] prepared a nanoemulsion to address RLZ's poor aqueous solubility and improve its bioavailability. Intranasal delivery of the nanoemulsion resulted in a significantly higher brain uptake than through the oral route in Wistar rats, as well as a better neuroprotective effect in

SH-SY5Y695 cells compared to free RLZ. According to this research, pairing this kind of nanocarrier with intranasal administration could lower the dosage of RLZ needed, minimizing the risk of side effects [379]. This was proven by Verma *et al.* [380], which evaluated the therapeutic activity of RLZ-loaded chitosan *N*-isopropylacrylamide (NIPAAAM)-conjugated NPs. Even at very low concentrations (10 µg/kg), NPs treatment led to a profound reduction in the expression of inflammatory markers (NF-κB, NOS-2, and COX-2) in a rat model of cerebral ischemia, conferring significant neuroprotection with no side effects. Behavioral and motor deficits, including spontaneous motor activity, grip strength, and flexion, were restored [380]. Another group encapsulated the same drug in transferrin-functionalized chitosan NPs [381]. The use of transferrin proved advantageous, resulting in superior targeting efficiency, higher BBB permeability, and greater RLZ accumulation in the brains of rats compared to plain NPs. Direct nose-to-brain delivery enabled continuous RLZ release for 24 h and greater therapeutic effect, with the NPs attenuating oxidative stress, reversing memory and learning loss, and improving hippocampal neuronal damage induced by haloperidol [381].

Violatto *et al.* [382] highlighted the importance of cargo release to achieve therapeutic efficacy. The group engineered poly-avidin NPs functionalized with ApoE that transiently crossed the BBB in SOD1^{G93A} ALS transgenic mice but were unable to release the covalently linked drug dexamethasone, with its levels below the detection limit in CNS tissues. This study indicated that NPs penetration into the CNS alone is insufficient for effective drug targeting to the brain and spinal cord. The authors endorsed the exploitation of different strategies for drug targeting that do not require cell internalization for cargo release, such as taking advantage of parenchyma-specific enzymatic activities, as well as the need for drug measurement *in vivo* when developing a nanocarrier for brain delivery [382].

In some instances, drugs can retain their functionality even without being released from nanocarriers [6]. For example, Bao *et al.* [378] absorbed edaravone onto the shell of PEG/angiopep-2 modified ceria NPs (E-A/P-CeO₂ NPs), which exhibited minimal release (less than 4%) after 24 h. Nonetheless, the reduced density of the organic shell allowed the absorbed edaravone to still be effective at scavenging ROS, especially free radicals not close to the ceria core. PEG and angiopep-2 were used to facilitate systemic circulation and BBB crossing into the brain of ischemic rats, respectively (Figure II.12B). Selective brain accumulation of ceria NPs was achieved via RMT, with the synergistic effect between edaravone and the NPs enhancing ROS elimination. Moreover, the Evans Blue extravasation assay demonstrated that the functionalized NPs prevented BBB damage

more efficiently than non-loaded functionalized NPs (P-CeO₂ NPs and A/P-CeO₂ NPs). The NPs had excellent hemo/histocompatibility and low cytotoxicity, coupled with improved targeting efficiency, being therefore a potential asset for ALS therapy [378]. Due to catalase and SOD1 mimetic activity, ceria nanocarriers also counteract oxidative stress on their own, prolonging survival and reducing disease severity in SOD1^{G93A} mice when given intravenously twice a week (20 mg/kg), even when treatment was started late at the onset of muscle weakness. Compared to control animals, NP-treated groups showed considerably greater cumulative survival and mean latency to fall from a hanging wire, which serves as an assessment of muscle strength [383].

Edaravone's properties against neurodegeneration have been explored in other nanosystems, such as a GSH-conjugated poly(methacrylic acid) (PMAA) nanogel [384]. The conjugation of GSH as a targeting ligand increased site-specific drug delivery to brain tissue. The simultaneous presence of edaravone and GSH amplified the antioxidant capacity, prompting a decrease in lipid peroxidation and an increase in intracellular antioxidant levels, which in turn improved cognitive function, spatial memory, and learning in ischemic Wistar rats, at three different concentrations (20, 40, and 250 µg/kg), and with no discernable toxicity. The findings demonstrated that the neuroprotective efficacy of the edaravone nanogel is more pronounced than that of GSH-PMAA nanogel, making it a suitable nanosystem to reduce oxidative stress-induced cell damage in ALS [384].

Additionally, co-administration of edaravone with agents that induce temporary opening of the BBB has been shown to be remarkably effective in facilitating drug delivery to the brain. For instance, Jin *et al.* [385] devised a strategy in which edaravone was encapsulated within the hydrophobic core of an organic micelle (designated as edaravone-encapsulated agonistic micelle (EDV-AM)), while the hydrophilic shell was conjugated with an agonist for the adenosine 2A receptor (A_{2A}R) (Figure II.13). This approach enabled the targeted delivery of edaravone for brain ischemia treatment by temporarily triggering the opening of TJ in the BBB through EDV-AM-A_{2A}R signaling. The release of edaravone from the micelles prompted a significant reduction in ROS production by microglia, astrocytes, endothelium, as well as infiltrated inflammatory cells. Importantly, the quick restoration of the TJ minimized irreversible BBB damage.

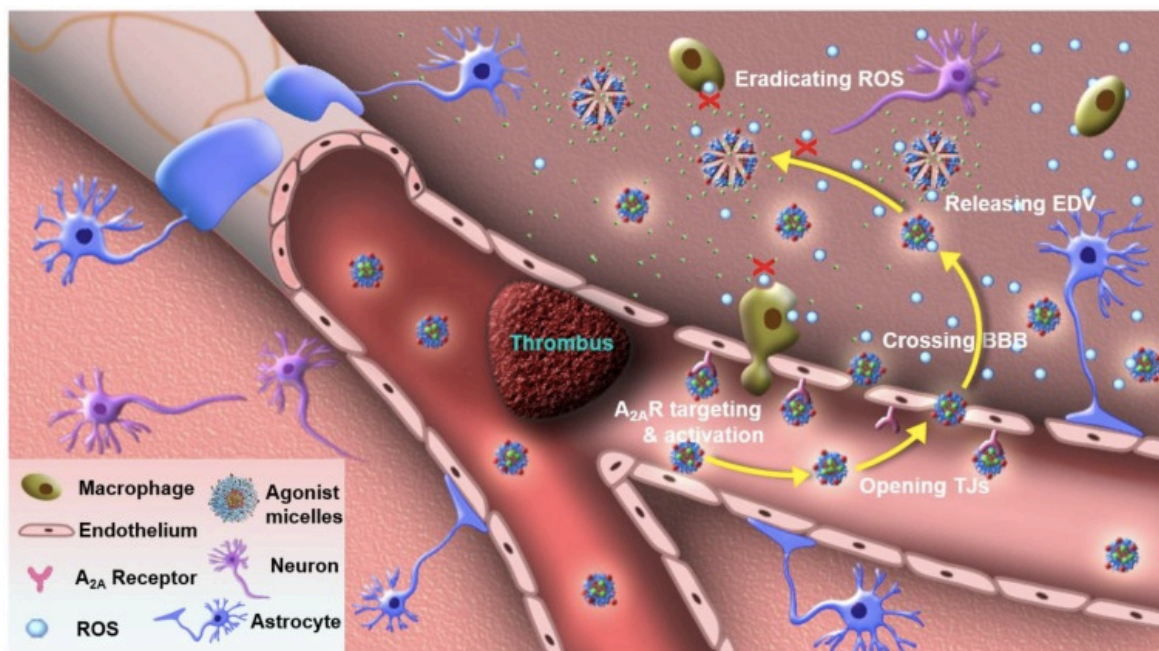


Figure II.13. Agonist micelles functionalized with an adenosine 2A (A_{2A}) receptor agonist triggered the temporary opening of tight junctions (TJs), enhancing edaravone's central nervous system (CNS) delivery to eradicate the production of reactive oxygen species (ROS) production, and rescue ischemic brain tissue. Reproduced with permission from [385].

Other compounds, such as fullerol encapsulated into hydroxypropyl- β -cyclodextrin NPs [386], or 4-amino-TEMPOL loaded into self-assembling PEG nanomicelles [387] or into PEG/poly(methylstyrene) NPs [388], showed even better ROS scavenging activity than edaravone. Fullerol NPs had a protective effect against H₂O₂-induced cytotoxicity, increasing the viability of HeLa and HepG2 cells [386], while the 4-amino-TEMPOL nanocarriers protected zebrafish embryos and larvae from oxidative stress inducers [387] and inhibited BBB disruption, supported M2 microglia polarization, and improved NVU impairment and neurological deficits in C57BL/6J mice [388]. These encouraging results raise the possibility of their future application as nanomedicine treatments for ALS.

A common ALS pathogenic mechanism is reactive microglia. Diapocynin, an apocynin dimer, is beneficial in mitigating microgliosis. However, this compound is hydrophilic, being rapidly released when physically loaded into polymeric NPs. To overcome this problem, Auriemma *et al.* [389] proposed the synthesis of a diapocynin prodrug, which was afterwards incorporated into biodegradable polymeric NPs. The incorporation avoided premature drug expulsion, with the diapocynin prodrug being continuously released for more than 9 days. The strategy could benefit not only diapocynin's therapeutic efficiency but also patient compliance, as the prolonged release would require fewer repeated

administrations. Nevertheless, this was a preliminary study, and as such, the formulation should be further evaluated to establish its physiological biocompatibility and biodistribution [389].

Likewise, intravenously administered poly(D, L-lactide) (PLA)/PEG NPs encapsulating adapalene were effective in reducing microgliosis, by activating the retinoic acid signaling pathway [390]. NPs extended the lifespan of SOD1^{G93A} mice by 7 days and improved their motor function (Figure II.14A and B), which was correlated with a reduction in neuromuscular degeneration markers and a 50% reduction in motor neuron loss at both the early and late stages of the disease. Groups treated with adapalene-loaded NPs had more neuromuscular innervation and larger fiber diameter than the control group (Figure II.14C and D). Moreover, there were no indicators of toxicity related to the therapy at any point during the study. These data simultaneously substantiate retinoid signaling as a therapeutic target and underscore the potential of the developed nanocarriers to alleviate ALS pathology and delay disease progression due to their anti-inflammatory and neuroprotective actions [390].

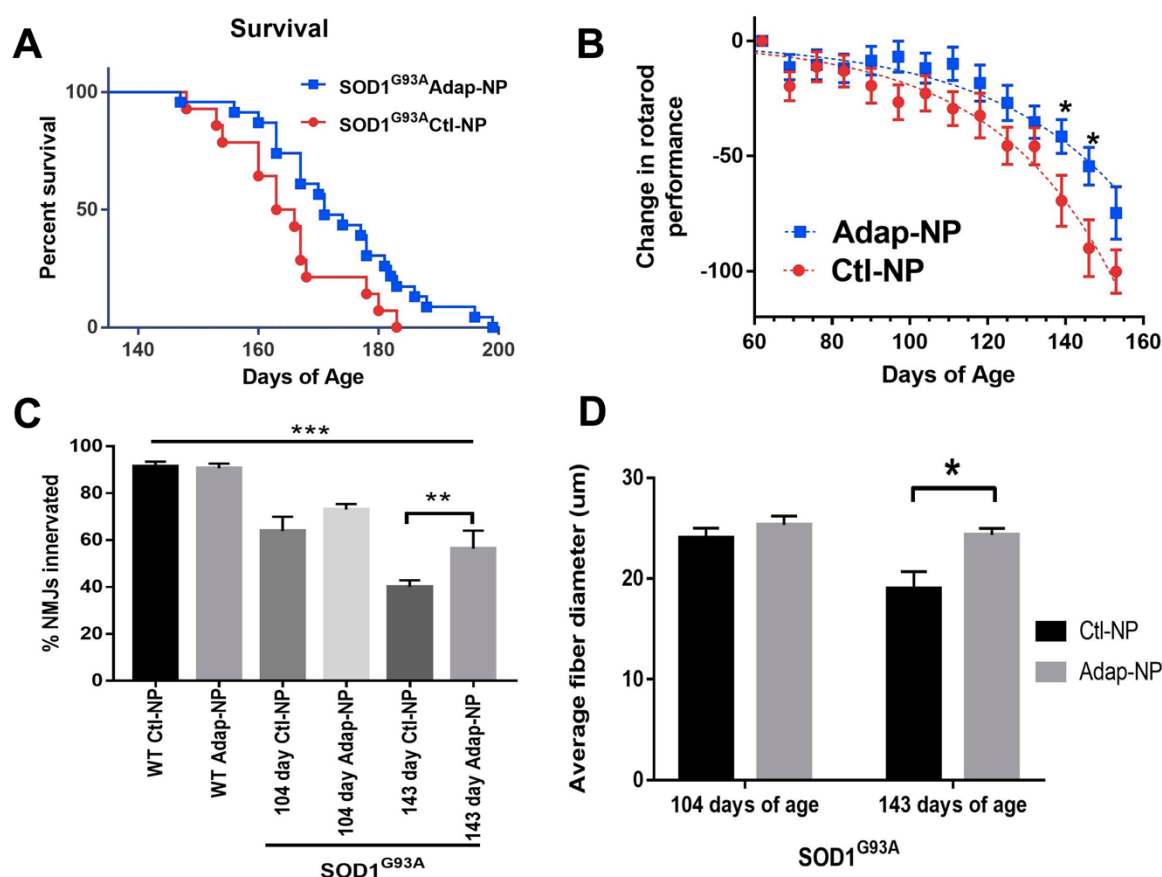


Figure II.14. Application of adapalene-loaded polymeric nanoparticles (NPs) to alleviate amyotrophic lateral sclerosis (ALS) pathology in transgenic SOD1^{G93A} mice. NPs treatment resulted in (A) extended lifespan; (B) improved motor function; (C) increased neuromuscular innervation and (D) increased fiber diameter. Reproduced with permission from [390].

In another work [391], biodegradable poly(lactic-co-glycolic acid) (PLGA) NPs were obtained by nanoprecipitation and loaded with PHA-767491, a CDC7 kinase inhibitor that reduces TDP-43 aggregation. The results showed that PHA-767491 permeability across porcine brain lipids, which were used to mimic the human BBB, increased 4-fold when loaded into the PLGA NPs. Moreover, TDP-43 pathology in SHSY5Y cells was better modulated by the NPs than with PHA alone, as evidenced by the substantial reduction in cytosolic TDP-43 and significantly higher neuronal cell viability after NPs treatment [391].

Excessive metal ion accumulation in the CNS, such as copper (Cu²⁺) and iron (Fe³⁺), has been proposed as another contributing factor to ALS pathophysiology, by catalyzing ROS formation and increasing oxidative stress [6, 392]. Enhancing the efficacy of metal chelators through nanotechnology can lead to improved outcomes by promoting BBB crossing and increasing site selectivity [6]. As an illustration, Liu *et al.* [393] devised NPs conjugated to an iron chelator, 2-methyl-N-(2-aminoethyl)-3-hydroxyl-4-pyridine (MAEHP),

and coated their surface with polysorbate 80 to promote BBB penetration, providing safer and more effective chelation therapy for neurodegenerative diseases.

Aside from iron chelators, some distinct inorganic nanostructures also exhibit metal-chelating abilities. For instance, black phosphorous nanosheets displayed not only efficacy in capturing excessive Cu^{2+} to safeguard neuronal cells against neurotoxicity induced by transition metals but also the capability to traverse the BBB when exposed to near-infrared laser irradiation, leveraging their inherent photothermal effect. This attribute represents a significant advantage over traditional chelators, eliminating the need for drug loading and release. Furthermore, their biostability and biocompatibility further underscore their favorable profile. Nonetheless, additional studies are required in animal models of ALS [394]. Mazibuko *et al.* [395] developed innovative PLA/polymethacrylate (PMA) nanospheres functionalized with diethylenetriaminepentaacetic acid (DTPA), a chelating ligand that can remove metal ions. As DTPA is highly ionizable, the nanospheres were suspended in a thermoresponsive hydrogel to reduce environmental reactions and increase its stability. Cellular imaging denoted an appreciable uptake of the nanosphere-hydrogel composite in mouse motor neuron-like hybrid NSC-34 cells, with no significant cytotoxicity. Nevertheless, since the authors did not test the effect of the nanosystem on metal homeostasis, its utility as a chelation therapy is questionable [395].

As already mentioned, ASO-based therapy is one of the most advanced experimental treatments currently being tested for ALS. However, ASO warrants the use of a drug carrier to improve circulation time and BBB penetration. Bearing this in mind, Chen *et al.* [396] designed calcium phosphate lipid-coated NPs to increase the delivery of an ASO targeting SOD1 in motor neurons. The NPs were rapidly taken up by NSC-34 cells and downregulated SOD1 expression in HEK293 cells. SOD1 knockdown was 8-fold higher upon incubation with the NPs than with free ASO. *In vivo* application was demonstrated after microinjections in transgenic zebrafish. Due to the PEG in the formulation, the nanocarriers exhibited prolonged circulation up to 29 h after injection, accumulating throughout the brain and spinal cord, including inside of neurons and astrocytes, suggesting they could be a safe tool to increase gene therapy delivery in ALS [396].

The potential of gold and silica NPs in ALS management has also been investigated. PEGylated gold NPs showed significant therapeutic potential in $\text{SOD1}^{\text{G93A}}$ mice, as evidenced by the moderate extension of survival (approximately 1 week), the substantial delay in symptom onset and disease progression, and improved performance in rotarod tests. It is known that pathogenic SOD1 disrupts microtubule motility, affecting its

conformation and flexural plasticity in motor neurons. *In silico* studies demonstrated that the developed nanosystem can act as a chaperon to A4V SOD1, stabilizing kinesin motor binding in the microtubules and thus improving ALS motor symptoms (Figure II.15) [397]. The same was verified in the work of Kim *et al.* [398], wherein citrate-coated gold NPs repaired the defective kinesin motor stepping by inhibiting SOD1, thus restoring motor dynamics.

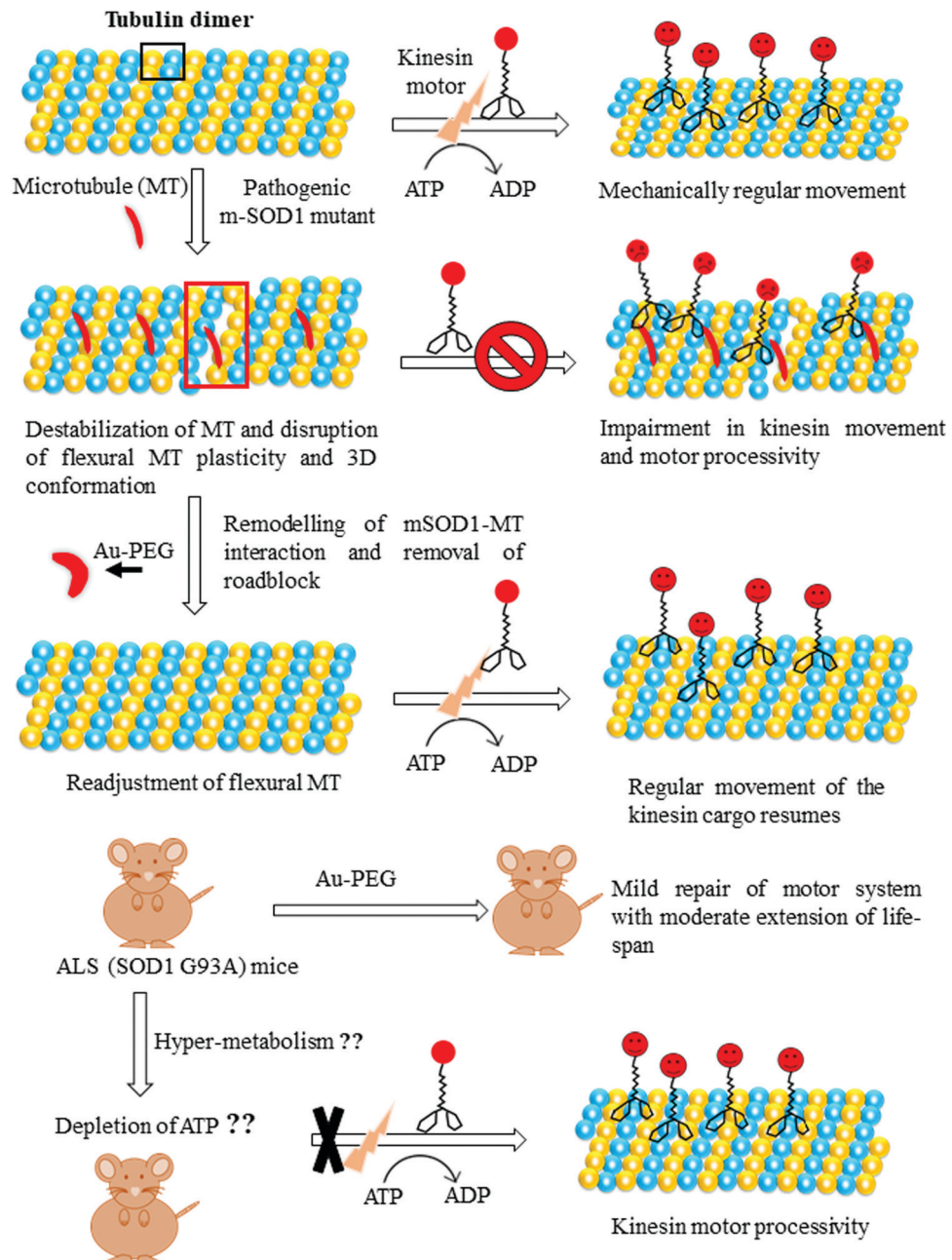


Figure II.15. Gold nanoparticles (NPs) can restore amyotrophic lateral sclerosis (ALS) motor dynamics. Microtubules (MT) are comprised of repeated tubulin dimers, with protein kinesin motor using ATP hydrolysis to move along the MT. Pathogenic mutant superoxide dismutase 1 (SOD1) disrupts MT structure and motor stepping. PEGylated gold (Au-PEG) NPs act as a chaperon for SOD1, leading to the resumption of motor movement by stabilizing the binding between MT and kinesin. This correlated well with the *in vivo* study, wherein gold NPs treatment improved motor symptoms and extended the lifespan of SOD1^{G93A} transgenic mice. Reproduced with permission from [397].

On the other hand, ependymal stem progenitor cells (epSPCs) isolated from SOD1^{G93A} mice responded favorably to FM19G11-loaded gold NPs in terms of self-renewal and proliferation [399]. EpSPCs are important in the context of neurodegeneration because they can differentiate into neurons and astrocytes *in vivo*, as well as rescue dying motor neurons. V-akt murine thymoma viral oncogene homolog 1 (AKT1), V-akt murine thymoma viral oncogene homolog 3 (AKT3), SRY-Box transcription factor 2 (SOX2) and octamer-binding transcription factor 4 (OCT4) were among the pluripotency-related genes whose expression was increased by FM19G11, a hypoxia-inducible factor modulator of epSPCs proliferation. Furthermore, there was an activation of mitochondrial uncoupling protein 2 (UCP2), inducing mitochondrial biogenesis. These findings imply that the neuroprotective effects of FM19G1 were not compromised by encapsulation and that its delivery in gold NPs can have a favorable impact in slowing neurodegeneration [399]. Additionally, polyvinylpyrrolidone (PVP)-functionalized gold NPs showed anti-amyloidogenic activity towards hen egg white lysozyme (HEWL) fibrils, making them a good candidate to tackle ALS protein aggregation [400].

Díaz-García *et al.* [401] developed mesoporous silica NPs (MSP) using a drug cocktail that included pioglitazone (anti-inflammatory) and leptin (neuroprotective). X-ray diffraction (XRD) confirmed the incorporation of both drugs into the NPs. Afterward, their therapeutic effect was tested in a TDP-43 proteinopathy murine model (TDP-43^{A315T} mice). MSP-treated TDP-43^{A315T} mice had improved motor performance and slower disease progression than wild-type mice, indicating the usefulness of the system in treating ALS [401]. Leyton-James *et al.* [402] also employed these nanocarriers to target neural crest stem cells and neurotrophic factors (mimetics of glial-derived neurotrophic factor (gliadin), ciliary neurotrophic factor (cintrofin), and VEGF) to the spinal cord, promoting neuronal survival and regeneration. Intraspinal administration of the MSP led to long-term bioavailability of the encapsulated molecules, attenuating the decline of muscle function in SOD1 mice, delaying median disease onset, and extending median disease progression and survival (Figure II.16 A-E). One month after injection, the nanocarriers could still be found in the spinal cord, remaining associated with microglia (Figure II-16F). Surprisingly, the best therapeutic outcome was achieved with empty MSN. A possible explanation is that they can absorb harmful molecules, thereby indirectly maintaining functional neuromuscular connections [402].

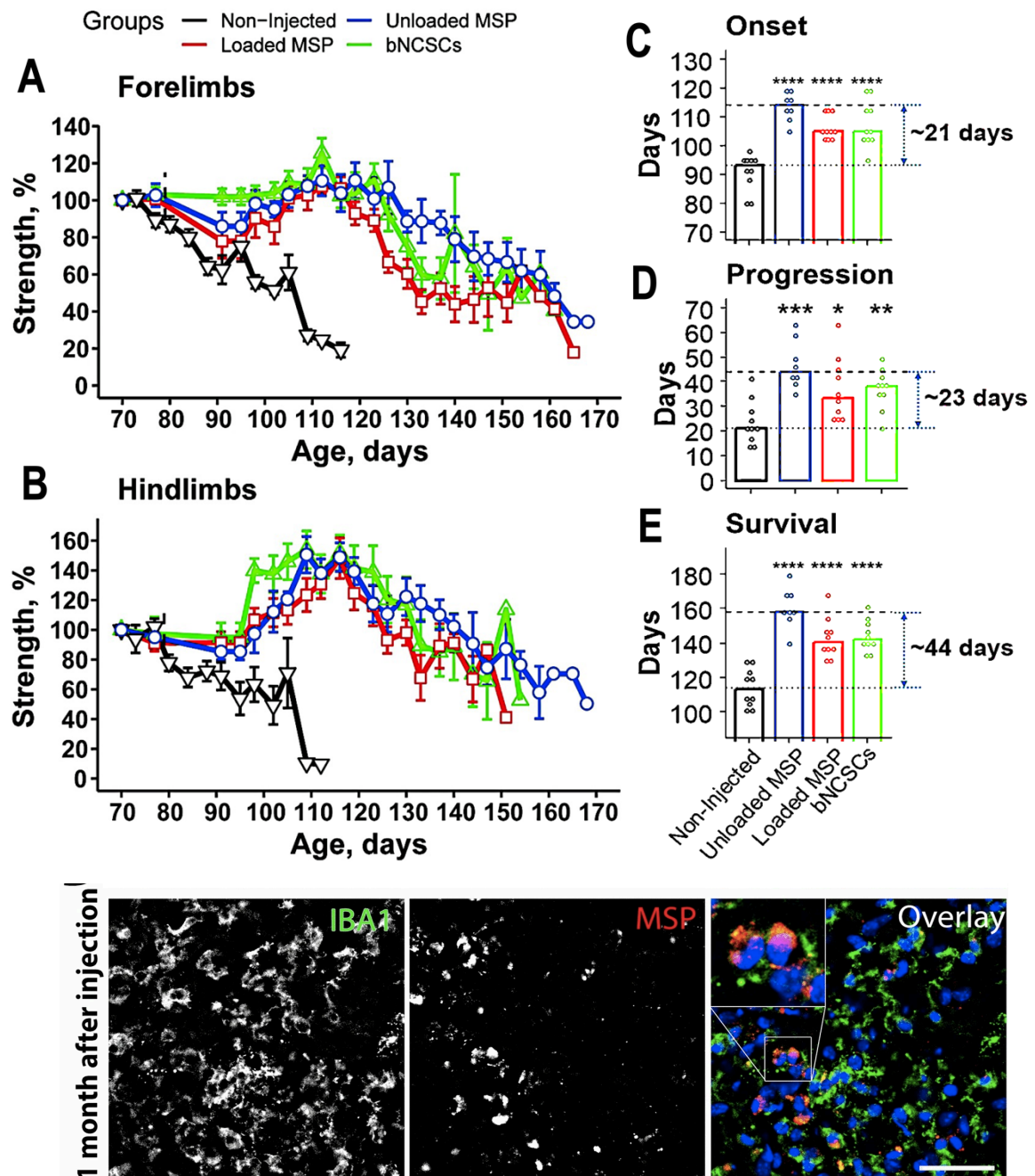


Figure II.16. Intraspinal delivery of empty mesoporous silica nanoparticles (MSP) and mimetic-loaded MSP significantly improved the condition of amyotrophic lateral sclerosis (ALS) mice, improving forearm and hindlimb grip strength (A and B), delaying onset (C), and extending disease progression (D) and survival (E). One month after injection, MSP (red) remained present in the spinal cord, associated with ionized calcium-binding adapter molecule 1 (IBA1) microglia (green). Reproduced with permission from [402].

In a 2019 study [403], the co-delivery of curcumin and charged plasmid RhoG-DsRed in MSP provided an appealing strategy to target simultaneously ROS production and actin cytoskeletal deficiencies. The NPs efficiently decreased neuron cell damage and inflammation in neuro-2a (N2a) cells, as phosphorylated p38 protein expression, an important biomarker of intracellular oxidative stress, was significantly downregulated. N2a cellular uptake was promoted through surface modification with a transactivator of transcription (TAT) peptide, which also facilitated nuclear delivery of the plasmid, leading to enhanced RhoG expression and neurite growth [403]. Collectively, silica NPs offer a remarkably innovative approach to effectively delaying ALS progression, either alone or in combination with other treatments, including neurotrophic factors and stem cells.

So far, the only nanosystem that has ever been tested for ALS in clinical trials is a gold nanocrystal oral suspension (CNM-Au8). CNM-Au8 aims to restore neuronal health by supporting bioenergetic cellular reactions while lowering oxidative stress [6]. Newly presented data from the open-label extension of the Phase II RESCUE-ALS trial (NCT04098406) shows the nanosuspension preserved motor neuron function and slowed disease progression. Moreover, CNM-Au8 was well-tolerated, with no safety concerns, decreasing the risk of death by 70% compared with the placebo and improving patient quality of life throughout 120 weeks [404]. As a result, this nanosystem provides significant benefits and an outstanding safety profile, being anticipated to become the first commercial NPs formulation for ALS therapy. Furthermore, it is not unreasonable to assume that surface modification with targeted ligands could boost its potential even further.

Overall, the studies reviewed in this subsection show that nanocarriers can improve the efficacy and therapeutic action of drugs and other compounds. Among the different nanosystems, lipid-based nanocarriers offer numerous benefits that render them exceptionally appealing as colloidal vehicles for drug delivery to the brain, outperforming alternative nanocarriers due to their excellent biocompatibility, ease of functionalization, simple manufacturing, and sustained therapeutic effect, particularly for poorly-water soluble active substances such as RLZ. This is why the experimental work of this thesis focused on this type of NPs.

Despite the promising results regarding brain targeting, there are pressing issues that still need to be tackled. An important aspect is nanomaterial biosafety, which is frequently overlooked during preclinical stages, as most of the investigation has primarily focused on demonstrating proof-of-concept. Delivery systems should not have a significant impact on brain processes, whether short or long-term, and this becomes even more crucial

when prolonged therapy is required, as in the case of ALS. As a result, standardized *in vivo* assays that can be correlated with *in vitro* experiments are needed [6, 7]. Other additional concerns that must be addressed to help the clinical translation of NPs include understanding individual variability in the context of BBB disruption and how aging affects this process; refining nanocarrier design and development (e.g., quality by design (QbD) approach); improving pharmacokinetic/pharmacodynamic analytical tools; and reinforcing the regulatory framework [345].

Chapter III

Development of Riluzole-loaded Lipid Nanoparticles

This chapter has been published as follows, with some minor modifications:

Teixeira MI, Lopes CM, Gonçalves H, Catita J, Silva AM, Rodrigues F, Amaral MH, Costa PC. Riluzole-loaded lipid nanoparticles for brain delivery: Preparation, optimization and characterization. *Journal of Molecular Liquids*. 2023; 388:122749. DOI: 10.1016/j.molliq.2023.122749

1. Introduction

Public health is presently facing a significant problem in the area of neurodegenerative disorders, which require effective disease-modifying therapies and preventive measures [405]. ALS treatment is extremely difficult to implement using conventional strategies, as the limited permeability of the BBB usually leads to subtherapeutic concentrations in the brain [406]. Drug development for CNS diseases has the lowest success rate when compared to other therapeutic areas [4, 220, 407]. RLZ, a sodium-blocking channel from the benzothiazole class, has been the mainstay drug for ALS management, showing a strong neuroprotective action. It also exhibits antiepileptic and sedative activity, being mainly administered by the oral route [408]. However, RLZ is a Biopharmaceutical Classification System (BCS) class II drug (lipophilic compound), having high absorption, but poor aqueous solubility and low bioavailability. Invasive methods, such as direct intraventricular or intrathecal administration, are not always feasible in settings requiring continuous treatment, as there are concerns pertaining to patient compliance and risk of infection. Thus, innovative, noninvasive formulations that are easy to administer and capable of targeting RLZ to the CNS in a safe and controlled manner are required [7, 407].

In this regard, NPs have been extensively investigated since the 90s to improve drug delivery and penetration into the brain, showing very promising results regarding sustained therapeutic effect, especially for poorly-water soluble active substances like RLZ [409]. Lipid-based formulations, such as SLN or NLC, are considered highly attractive colloidal systems for CNS targeting over other nanocarriers because of their advantages. Despite recent reports on the benefits of employing nanosystems to deliver ALS drugs to the brain [381, 397, 401], the use of lipid nanocarriers for this disease remains underexplored. In fact, only a few studies have been published so far [376, 377, 379, 396, 410].

Given the current state of the art of ALS management, and the urgent need for therapeutic innovation in this biomedical sector, this section aimed to develop lipid NPs suitable for brain delivery that could overcome potential drawbacks of conventional RLZ administration. The work presented in this chapter is a preformulation study that was designed to evaluate the best system for RLZ brain targeting. To the best of our knowledge, the composition and the experimental conditions used to prepare the formulations in this study are unique, whereas in previous literature different excipients and techniques were used. Moreover, we present a rational development and optimization, analyzing in detail

the effect of several formulation and preparation parameters on the physicochemical properties of the NPs.

Accordingly, empty SLN and NLC were prepared by ultrasonication and HPH, and optimized based on their physicochemical characteristics, namely, particle size, PDI and ZP. The optimized empty nanocarriers were then converted into RLZ-loaded lipid NPs and further characterized, regarding their encapsulation efficiency (EE), loading capacity (LC) long-term stability, morphology, *in vitro* drug release, assessment of crystallinity and thermal behavior, and biocompatibility.

2. Materials and Methods

2.1. Materials

Precirol® ATO 5 (glyceryl distearate/glyceryl palmitostearate), Compritol® 888 ATO (glyceryl dibehenate), cetyl palmitate, Witepsol® E85 (triglycerides of C10-C18 saturated fatty acids), Suppocire® D (triglycerides of fatty acids), Gelucire® 43/01 (glycerol esters of saturated C12-C18 fatty acids esters), Gelucire® 50/13 (stearoyl polyoxyl-32 glycerides), Lipocire® DM (hydrogenated palm glycerides), Labrasol® (caprylocaproyl polyoxyl-8 glycerides), Labrafil® M 1944 CS (oleoyl polyoxyl-6 glycerides), Labrafil® M 2125 CS (linoleoyl polyoxyl-6 glycerides), Capryol® 90 (propylene glycol monocaprylate type II) and Capryol® PGMC (propylene glycol mono and dicaprylate NF) were kindly provided by Gattefossé SAS (Nanterre, France). Dynasan® 114 (trimyristin), Dynasan® 118 (tristearin), Imwitor® 491 (glyceryl monostearate) and Imwitor® 900K (glycerol monostearate 40 – 55%, Type II) were provided by IOI Oleochemical (Hamburg, Germany).

Kolliphor® P 188 micro Geismar (Poloxamer 188) was donated by BASF (Ludwigshafen am Rhein, Germany). RLZ (purity > 98%) was obtained from BeanTown Chemical (Hudson, NH, USA). Tween® 80 (polysorbate 80), Miglyol® 812 (triglycerides of capric/caprylic acids) and oleic acid were purchased from Acofarma (Madrid, Spain). All the other reagents were of analytical grade. The purified water was obtained from a Milli-Q® Direct 3 UV-R Water Purification system (Millipore®; Darmstadt, Germany).

Cell reagents were provided by Life Technologies, S.A. (Madrid, Spain), Merck (Darmstadt, Germany) and Biowest (Nuaillé, France). Immortalized human cerebral microvascular endothelial cells (hCMEC/D3 cell line) were obtained from Cedarlane (Hornby, ON, Canada). Dimethyl sulfoxide (DMSO) and Triton X-100 were acquired from Sigma-Aldrich (Steinheim, Germany). All the other reagents were of analytical grade and used as supplied.

2.2. Screening of Excipients

Prior to developing lipid NPs, it is imperative to select a suitable lipid blend that allows to obtain a high EE/LC of the active compound and ensures long-term stability. For this, not only the solubility of the drug in the lipids that compose the lipid matrix of the nanocarrier should be considered, but also the compatibility between solid and liquid lipids, in the case of NLC [411, 412].

The solubility of RLZ at four concentration levels (0.1%, 0.5%, 1% and 1.3%) (% w/w) was studied in different solid lipids (Cetyl palmitate, Compritol® 888 ATO, Dynasan® 114, Dynasan® 118, Gelucire® 43/01, Gelucire® 50/13, Imwitor® 491, Imwitor® 900K, Lipocire® DM, Precirol® ATO 5, Suppocire® D and Witepsol® E85), as well as in various liquid lipids (Capryol® 90, Capryol® PGM, Labrafil® M 1944 CS, Labrafil® M 2125 CS, Labrasol®, Miglyol® 812 and oleic acid). Increasing amounts of the drug were added to the lipids melted 5–10 °C above the melting point of the solid lipid for 1 h, under continuous stirring (200 rpm), using an orbital incubator shaker (Unimax 1010, Heidolph; Germany). The mixtures were visually checked every 15 min to assess the solubility of RLZ in the molten lipid, by macroscopic observation. Transparent and clear solutions without traces of drug crystals or powder were considered negative (i.e., drug dissolved) [413].

The results of the lipid screening were complemented through Differential Scanning Calorimetry (DSC) of the pure solid lipids and respective mixtures with RLZ (see Appendix I). The thermal program comprised three heating/cooling cycles, under constant nitrogen flushing (40 mL/min). The first cycle included a heating from 10 to 160 °C (10 K/min), followed by cooling to 0 °C. In the second and third cycles, there was a heating from 0 to 160 °C (10 K/min), and a cooling to 0 °C. An isotherm was done between cycles, for 5 min. Data was analyzed with Proteus Software (Version 6.1, NETZSCH-Gerätebau GmbH, Germany). The DSC cell was calibrated (sensitivity and temperature) with Hg (m.p. -38.8 °C), In (m.p. 156.6 °C), Sn (m.p. 231.9 °C), Bi (m.p. 271.4 °C), Zn (m.p. 419.5 °C), and CsCl (m.p. 476.0 °C) as standards.

Afterwards, and regarding the NLC, the physical compatibility of the solid and liquid lipids with the best solubilizing potential for RLZ was evaluated in different ratios, namely, 50:50, 60:40, 70:30, 80:20 and 90:10 (% w/w), respectively. Briefly, the lipid mixture was heated at 80.0 ± 1.0 °C for 1 h under stirring (200 rpm) and cooled down to room temperature (25.0 ± 1.0 °C). After 24 h, a small portion of each solidified mixture was

smear in a filter paper. Based on the absence of oil droplets in the filter, which is indicative of the miscibility between the lipids, the best proportion was selected [414, 415].

In terms of lipid NPs composition, it has been reported that a combination of at least two surfactants is usually preferred, since it results in lower particle sizes and greater storage stability when compared to formulations with only one surfactant [412, 416]. The choice of the surfactant and co-surfactant (Tween[®] 80 and Poloxamer 188, respectively), and their respective proportions, was based on the literature, considering several factors, such as the intended application, desired administration route and compatibility with the selected lipids.

2.3. Preparation of the Lipid Nanoparticles

SLN and NLC formulations were produced by two different techniques: ultrasonication [369] and HPH [417]. SLN were prepared using Precirol[®] ATO 5 as the solid lipid, whereas NLC were produced using Precirol[®] ATO 5 as the solid lipid and Miglyol[®] 812 as the liquid lipid. Both techniques were preceded by high-shear homogenization, to obtain a pre-emulsion [418].

For the ultrasonication, lipid and aqueous phases were heated separately, approximately 10 °C above the melting point of the solid lipid, with the aqueous phase containing Tween[®] 80 and Poloxamer 188 being added to the molten lipid phase. Afterwards, the mixture was emulsified at either 5000, 8000 or 13500 rpm for 5 min with an Ultra-Turrax T25 with S 25 N-18 G dispersing element (Janke and Kunkel IKA-Labortechnik; Staufen, Germany), and subjected to ultrasonication at an amplitude of 50% or 70% for 15 min with a probe sonicator (Vibro Cell VCX 130, 6 mm probe, Sonics & Materials; Newtown, CT, USA). Finally, the nanoemulsion was transferred to glass vials and cooled down in an ice bath for about 20 min, with the lipid NPs being formed by lipid recrystallization [369].

Concerning HPH, the first part of the method was similar to the previously mentioned above for ultrasonication, in that the lipid and aqueous phases were heated separately, added to each other, and emulsified with an Ultra-Turrax T25 with S 25 N-18 G dispersing element at 8000 rpm, for 5 min. The pre-emulsion was then passed through a high-pressure homogenizer (Stansted Pressure Cell Homogenizer, Stansted Fluid Power Ltd.; Harlow, Essex, UK) at 70.0 ± 1.0 °C for 3 homogenization cycles, at a constant pressure of 1750 bar. Once again, the nanoemulsion was cooled in an ice bath for the formation of lipid NPs [417].

The composition of the developed lipid NPs is described in Table III.1.

Table III.1. Composition (% w/w) and experimental conditions used to prepare the solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC).

Formulation								Experimental Conditions ¹
	Precirol	Miglyol	RLZ	Tween 80	Pol. 188	Water		
SLN	F1	7.50	-	-	2.00	1.00	q.s. 100	A
	F2	7.50	-	-	2.00	1.00	q.s. 100	B
	F3	7.50	-	-	2.00	1.00	q.s. 100	C
	F4	7.40	-	0.10	2.00	1.00	q.s. 100	C
	F5	7.50	-	-	2.00	1.00	q.s. 100	D
	F6	7.50	-	-	2.00	1.00	q.s. 100	E
	F7	7.50	-	-	2.00	0.50	q.s. 100	C
	F8	7.50	-	-	2.00	0.50	q.s. 100	E
	F9	7.50	-	-	1.75	0.75	q.s. 100	C
	F10	7.50	-	-	1.75	0.75	q.s. 100	E
	F11	7.50	-	-	1.50	0.50	q.s. 100	C
	F12	7.50	-	-	1.50	0.50	q.s. 100	E
	F13	10.00	-	-	2.00	1.00	q.s. 100	C
	F14	9.90	-	0.10	2.00	1.00	q.s. 100	C
	F15	10.00	-	-	2.00	1.00	q.s. 100	D
	F16	10.00	-	-	2.00	1.00	q.s. 100	E
NLC	F17	5.25	2.25	-	2.00	1.00	q.s. 100	C
	F18	5.25	2.15	0.10	2.00	1.00	q.s. 100	C
	F19	5.25	2.25	-	2.00	1.00	q.s. 100	D
	F20	7.00	3.00	-	2.00	1.00	q.s. 100	C
	F21	7.00	2.90	0.10	2.00	1.00	q.s. 100	C
	F22	7.00	3.00	-	2.00	1.00	q.s. 100	D

¹ Experimental Conditions: (A) Ultrasonication: 5000 rpm, 5 min; 70% amplitude, 15 min; (B) Ultrasonication: 8000 rpm, 5 min; 50% amplitude, 15 min; (C) Ultrasonication: 8000 rpm, 5 min; 70% amplitude, 15 min; (D) HPH: 8000 rpm, 5 min; 3 homogenization cycles; (E) Ultrasonication: 13500 rpm, 5 min; 70% amplitude, 15 min.

2.4. Physicochemical Characterization of the Lipid Nanoparticles

Physicochemical characterization of the lipid NPs included evaluating their appearance, particle size, PDI and ZP at the production day. To study the stability of the RLZ-loaded nanocarriers, the same parameters were also monitored at different time points (day 0, day 7, day 15, 1 month, 2 months and 3 months), after storage in closed glass vials at both 5 ± 1 °C and 25 ± 1 °C (long-term stability study). Furthermore, EE and LC were also assessed.

2.4.1. Visual Inspection

Visual inspection is a rapid way to evaluate formulation quality, as differences in the appearance of the lipid NPs can be a sign of issues during production and storage [418]. Phenomena such as creaming, coalescence, flocculation, and sedimentation, point towards dispersion instability [419].

2.4.2. Particle Size, PDI and ZP Analysis

The mean hydrodynamic diameter (Z-average) and PDI were determined by dynamic light scattering (DLS) (Nanobrook Omni, Brookhaven Instruments; Holtsville, NY, USA). ZP was estimated by electrophoretic light scattering (ELS), using the same equipment. The samples were previously diluted in Milli-Q[®] water (dilution 1:100). The analyses were done in triplicate (mean $\pm$ standard deviation, SD) at a temperature of 25.0 ± 1.0 °C, and with a fixed light incidence angle of 90° [420].

2.4.3. Encapsulation Efficiency (EE) and Loading Capacity (LC)

The EE and LC of RLZ-loaded lipid NPs were assessed through High-Performance Liquid Chromatography (HPLC) (Dionex UltiMate™ 3000, Thermo Scientific; Waltham, MA, USA), following a method that was previously developed in-house and validated according to the International Conference on Harmonization (ICH) guideline Q2(R1) (see Appendix II) [421]. System suitability was evaluated by considering the values of the retention time (R_t), capacity factor (k'), resolution (R_s), asymmetry (A_s) and number of theoretical plates (N) [422]. Moreover, graphical residual analysis and a RIKILT test were done to infer about the linearity of the method.

The chromatographic analysis was performed at $25 \pm 1^\circ\text{C}$, using a BDS Hypersil™ C18 column as stationary phase (150 mm × 4.60 mm internal diameter, 5 µm particle size; Thermo Fisher Scientific; Waltham, MA, USA). Mobile phase consisting of 70:30 (v/v) methanol/water acidified with acetic acid to pH 3 was used for the elution in isocratic mode, with a flow rate of 1 mL/min. The ultraviolet (UV) detector was set to 263 nm, since it was the maximum absorption wavelength in the RLZ spectra obtained previously by spectrophotometry (Jasco V-650 UV-Vis. spectrophotometer; Tokyo, Japan). EE (%) was assessed using three replicates for each formulation ($n = 3$), with the results presented as mean ± SD. Injections were performed in triplicate, in a sample volume of 10 µL. Before sample injection, the mobile phase was left to run for at least 20 min, to ensure proper equilibration of the system. A calibration curve was plotted by analyzing independent standard solutions of RLZ in ethanol (3.36 – 40.27 µg/mL) and fitting the respective data to the least squares linear regression, which gave a coefficient of determination (R^2) of 0.9988. The data was analyzed with the Chromeleon™ 7.2 SR4 Chromatography Data System Software (Thermo Fisher Scientific; Waltham, MA, USA).

Briefly, 2 mL of freshly prepared formulation was diluted in 10 mL of Milli-Q® water and filtered through a 5 µm cellulose nitrate membrane (Sartorius, Gottingen, Germany) to remove unencapsulated drug crystals. Afterwards, 2 mL of the diluted formulation was added to 8 mL of ethanol and thoroughly mixed to extract the RLZ from the lipid matrix. The mixture was centrifuged for 15 min at 5000 rpm (Thermo Scientific™ Heraeus™ Multifuge X1R Refrigerated Benchtop Centrifuge, Waltham, MA, USA), and the supernatant was collected, filtrated using a 0.45 µm polytetrafluoroethylene (PTFE) syringe filter (Millipore®, Germany) [423], and analyzed by HPLC. The EE and LC were determined as follows:

$$\text{EE (\%)} = \frac{\text{Amount of RLZ in the filtered formulation}}{\text{Total RLZ amount}} \times 100 \quad \text{Equation III.1}$$

$$\text{LC (\%)} = \frac{\text{Amount of RLZ encapsulated}}{\text{Total lipid amount}} \times 100 \quad \text{Equation III.2}$$

2.5. Cryo-Scanning Electron Microscopy (Cryo-SEM)

The shape and surface morphology of the RLZ-loaded SLN and NLC and corresponding empty lipid NPs were examined by Cryo-SEM. A small amount of the undiluted dispersions was dropped onto a grid, rapidly cooled in slush nitrogen ($-210\text{ }^{\circ}\text{C}$) and transferred under vacuum to the cold stage of the preparation chamber. Here, the samples were fractured, sublimated (2 min, $-90\text{ }^{\circ}\text{C}$) to reveal greater detail, and coated with gold and palladium for 45 s. Finally, the specimens were moved under vacuum into the SEM chamber (JEOL JSM-6301F; Tokyo, Japan), cooled to $-150\text{ }^{\circ}\text{C}$ and analyzed [420].

2.6. *In Vitro* Drug Release

In vitro release studies were performed through the dialysis bag diffusion technique [11,12], using a dissolution apparatus type 2 (EDT-08Lx, Electrolab; Mumbai, India). To simulate bloodstream conditions and mimic the release of RLZ from the nanocarriers until they reach the BBB, phosphate-buffered solution (PBS) with a pH 7.4 was selected as dissolution medium [13], being prepared beforehand according to the European Pharmacopoeia (Ph. Eur.) [14].

Briefly, 10 mL of each of the formulations was placed inside a dialysis membrane (3.5 kDa, CelluSep[®]; France), clamped and immersed in 500 mL of PBS heated at $37.0 \pm 0.5\text{ }^{\circ}\text{C}$, while being continuously stirred through paddle rotation at 100 rpm. At regular intervals (0.25, 0.50, 0.75, 1, 2, 4, 6, 8, 10, 12, 25, 29 and 30 h), 5 mL of sample was withdrawn, without dissolution medium replacement. Afterwards, the collected samples were filtered using a $0.45\text{ }\mu\text{m}$ PTFE syringe filter (Millipore[®]; Germany), and the amount of released RLZ was quantified by spectrophotometry, using an UV-VIS spectrophotometer (Jasco-V650; Germany) set to 263 nm. The absorbances were acquired in Spectra Manager[™] software (Jasco Inc; Germany). The release studies were conducted in triplicate ($n = 3$) and the results are reported as the mean $\pm$ SD. A calibration curve was previously built by analyzing independent standard RLZ solutions in ethanol (2.06 – 24.42 $\mu\text{g/mL}$) and fitting the respective data to the least squares linear regression, which gave a R^2 of 0.999 (see Appendix III).

The release profiles were fitted to various kinetic models, including zero-order (Equation III.3), Higuchi (Equation III.4), and Korsmeyer-Peppas (Equation III.5) [424]:

$$Q_t = Q_0 + K_0 t \quad \text{Equation III.3}$$

$$Q_t = Q_0 + K_H t^{0.5} \quad \text{Equation III.4}$$

$$Q_t = Q_0 + K_{KP} t^n \quad \text{Equation III.5}$$

where Q_t is the amount of RLZ dissolved at time t , Q_0 is the amount of RLZ in the dissolution medium at time 0, K_0 is the zero-order release constant, K_H is the Higuchi dissolution constant, K_{KP} is the Korsmeyer-Peppas release constant, and n is the latter's release exponent.

R^2 is commonly used to assess the best fit for the release data. Nonetheless, as more parameters are added, R^2 is likely to increase. As a result, when comparing kinetic models with varying numbers of parameters, the adjusted coefficient of determination (R_{adj}^2) is preferable [425]. R_{adj}^2 can be calculated from Equation III.6:

$$R_{adj}^2 = 1 - \frac{(1 - R^2)(q - 1)}{q - p - 1} \quad \text{Equation III.6}$$

where q is the number of experimental data points, and p is the number of parameters. Experimental data were analyzed by nonlinear regression using IBM SPSS Statistics (Version 24.0, International Business Machines; Armonk, NY, USA).

2.7. Fourier Transform Infrared (FTIR) Spectroscopy

Interactions between the drug and the lipids were assessed by FTIR spectroscopy [426]. For freeze-drying, the aqueous dispersions of lipid NPs were previously frozen overnight at $-85\text{ }^{\circ}\text{C}$ and then dehydrated under vacuum at $-75\text{ }^{\circ}\text{C}$ and 0.4 mBar using a LyoQuest freeze dryer (Telstar; Terrassa, Spain). Afterwards, the infrared spectra were obtained by placing the samples (pure compounds – RLZ and Precirol[®] ATO5 –, SLN and NLC drug–lipid melt, and the empty and RLZ-loaded lyophilized lipid NPs – formulations F3, F4, F13, F14, F17, F18, F20 and F21) on a FTIR Spectrometer (PerkinElmer Frontier[™]; Waltham, MA, USA) equipped with a universal attenuated total reflectance (ATR) accessory fitted with a diamond crystal. For each measurement, 32 scans at a resolution of 4 cm^{-1} were accumulated at wavenumbers between 4000 to 600 cm^{-1} .

2.8. Differential Scanning Calorimetry (DSC)

Thermal analysis was performed to investigate thermal behavior, namely recrystallization and melting behavior of the lipid NPs [427]. Thermograms were obtained using a DSC 200 F3 Maia® (NETZSCH-Gerätebau GmbH, Germany). Pure compounds, SLN and NLC drug-lipid melts, and lyophilized NPs were accurately weighed for approximately 10-20 mg and sealed in aluminum pans. An empty sealed aluminum pan was used as reference.

The thermal program comprised two heating/cooling cycles, under constant flushing with nitrogen (40 mL/min). The first cycle included one cooling to 0 °C, an isotherm for 5 min, a heating from 0 to 160 °C, and finally, a second cooling to 0 °C (scan rate: 10 K/min). An isotherm at 10 °C for 5 min was done between cycles. On the second cycle, there was a heating from 10 to 160 °C, followed by cooling to 15 °C (10 K/min). Data was analyzed with Proteus Software (Version 6.1, NETZSCH-Gerätebau GmbH, Germany). The DSC cell was calibrated (sensitivity and temperature) with Hg (m.p. -38.8 °C), In (m.p. 156.6 °C), Sn (m.p. 231.9 °C), Bi (m.p. 271.4 °C), Zn (m.p. 419.5 °C), and CsCl (m.p. 476.0 °C) as standards.

2.9. Powder X-ray Diffraction (PXRD)

The physical structure of RLZ, Precirol® ATO5, as well as the SLN and NLC drug-lipid melts and the lyophilized nanocarriers was determined by PXRD [428]. The diffraction patterns were acquired with a Miniflex 600 X-ray diffractometer (Rigaku; Tokyo, Japan) equipped with Cu-K α radiation, in the range of 3°- 60° (2 θ), using a scanning rate of 5 °/min and a step size of 0.01°. The measurements were performed at a current of 15 mA and a voltage of 40 kV.

2.10. Biocompatibility Study

To establish the safety of the formulations for brain targeting, it is crucial to examine their potential ability to cause cytotoxicity in the BBB. Accordingly, the hCMEC/D3 cell line was selected to assess the biocompatibility, through the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [429].

BBB cell models are useful in both basic research and drug permeability testing [430]. The hCMEC/D3 cell line is the best characterized and one of the most widely used *in*

vitro models to mimic the human BBB, given its ease of culture and similar morphological and functional properties to human brain endothelial cells, including expression of junctional proteins, uptake and efflux transporters, and paracellular permeability values [431, 432]. Nevertheless, this cell line is costly, and its preparation requires time and technical expertise [430].

2.10.1. Cell Culture

Immortalized human cerebral microvascular endothelial cells (hCMEC/D3 cell line) were grown in EndoGRO™ basal medium supplemented with fetal bovine serum (FBS) (5%, *v/v*), rhEGF (5 ng/mL), L-glutamine (10 mM), ascorbic acid (50 µg/mL), EndoGRO-LS supplement (0.2%, *v/v*), hydrocortisone hemisuccinate (1.0 µg/mL), and heparin sulfate (0.75 U/mL). Cells were maintained in an incubator (Cell Culture® CO₂ incubator, ESCO GB Ltd., Barnsley, England, UK) at 37 °C in a water-saturated atmosphere with 5% CO₂ and subcultured every 2–3 days using trypsin–EDTA to detach them from the flasks. The culture medium was replaced every other day.

2.10.2. Cytotoxicity Assay

A MTT assay was performed to determine the number of viable cells after exposure to the different formulations. hCMEC/D3 cell line was treated as described by Teixeira *et al.* [433]. Passages 50–54 were used (*n* = 5).

Briefly, after confluence, cells were seeded in 96-well plates (25 × 10³ cells/mL) and incubated for 24 h in the absence or presence of the samples (0.1–10 µM) dissolved in cell culture medium. Following the removal of the formulations from each well, cells were washed with Hank's balanced salt solution (HBSS). The number of viable cells was determined by adding MTT reagent and incubating for 3 h at 37 °C. DMSO was used to solubilize the crystals. Triton X-100 1% (*w/v*) and EndoGRO™ basal medium were used as negative and positive controls, respectively. The absorbance was read at 590 nm with background subtraction at 630 nm. Results were expressed as percentages of cell viability.

3. Results and Discussion

3.1. Lipid Screening

The results of the lipid screening studies for the maximum amount of RLZ tested (1.3%, w/w) are presented in Table III.2. Moreover, the respective DSC results for the first cycle are shown in Table III.3.

Table III.2. Results of the riluzole (RLZ) solubility (1.3%, w/w) in the tested solid lipids and liquid lipids. At each time point, “+” indicates the presence of visible drug crystals, and “–” indicates the absence of crystals.

	Lipid	Time (min)			
		15	30	45	60
Solid lipid	Cetyl palmitate	–	–	–	–
	Compritol® 888 ATO	–	–	–	–
	Dynasan® 114	–	–	–	–
	Dynasan® 118	–	–	–	–
	Gelucire® 43/01	–	–	–	–
	Gelucire® 50/13	–	–	–	–
	Precirol® ATO5	–	–	–	–
	Suppocire® D	–	–	–	–
	Witepsol® E85	–	–	–	–
	Lipocire® DM	+	–	–	–
	Imwitor® 491	+	–	–	–
	Imwitor® 900K	+	+	–	–
Liquid lipid	Capryol® 90	–	–	–	–
	Capryol® PGMC	–	–	–	–
	Labrafil® M 1944 CS	–	–	–	–
	Labrafil® M 2125 CS	–	–	–	–
	Labrasol®	–	–	–	–
	Miglyol® 812	–	–	–	–
	Oleic acid	–	–	–	–

Table III.3. DSC results (onset temperature) for the lipid screening.

Solid Lipid	Solid Lipid Onset (°C)	Solid Lipid/Drug Mixture Onset (°C)
Cetyl palmitate	50.5	49.6
Compritol® 888 ATO	69.9	70.4
Dynasan® 114	54.9	55.1
Dynasan® 118	70.2	51.6
Gelucire® 43/01	41.1	39.0
Gelucire® 50/13	35.4	31.2
Precirol® ATO5	53.0	47.0
Suppocire® D	43.1	37.0
Witepsol® E85	42.7	36.8
Lipocire® DM	39.5	35.3
Imwitor® 491	72.0	69.2
Imwitor® 900K	56.2	57.3

As reported in Table III.2, after 60 min, the maximum amount of RLZ tested was soluble in all solid and liquid lipids. DSC was done to complement the lipid screening results and evaluate the polymorphic behavior of the lipids, facilitating the selection of the most appropriate excipient. As can be seen in Table III.3, some pure solid lipids (namely, Gelucire® 43/01, Gelucire® 50/13, Suppocire® D, Witepsol® E85 and Lipocire® DM) or their mixture with RLZ had onset temperatures below 40 °C. Using lipids with suitable melting temperatures (i.e., above 40 °C) is critical to prevent supercooled melts, ensuring that the NPs remain solid at room and body temperatures [415, 434]. In fact, it has been shown that lipid NPs tend to aggregate and gel when the storage temperature is around the melting temperature of the lipid excipients, due to partial coalescence, resulting in appreciable changes in particle size and morphology [435]. Destabilization phenomena could thus arise if the nanocarriers are subjected to higher temperatures. To avoid such issues, those lipids were excluded from further optimization studies.

Imwitor® 491 presented a small shoulder of decreased energy at about 38°C, dislocated from the main peak at 80 °C, which became apparent during the second and third DSC cycles (data not shown). This also points to possible destabilization phenomena, and so Imwitor® 491 was likewise discarded. The remaining lipids (cetyl palmitate, Compritol® 888 ATO, Dynasan® 114, Dynasan® 118, and Imwitor® 900K) have adequate onset and melting peak temperature but have several disadvantages when compared to Precirol® ATO5. Cetyl palmitate and Compritol® 888 ATO tend to form NPs with higher mean sizes as demonstrated by Nascimento *et al.* [436] and Bahari *et al.* [437]. On the other hand,

the highly crystalline nature of Dynasan[®] 114 and Dynasan[®] 118, along with their fatty acids of the same chain length, is anticipated to result in lower drug loading than Precirol[®] ATO5, which incorporates fatty acids of varying chain lengths (mixture of mono, di and triglycerides of palmitic and stearic acid). Drug molecules are embedded within the crystal defects of the lipid lattice, and lipids containing fatty acids of different chain lengths exhibit imperfections that create more space for accommodating the drug [438]. Moreover, lipids with a higher percentage of monoglycerides, such as Precirol[®] ATO5, have greater solubilization capacity [439]. Therefore, Precirol[®] ATO5 is expected to better accommodate RLZ. Finally, regarding Imwitor 900k, this excipient has not been used significantly as a solid lipid in lipid formulations for brain delivery.

Given what was mentioned above, Precirol[®] ATO5 and Miglyol[®] 812 were chosen as solid and liquid lipids, respectively. Miglyol[®] 812, a medium chain triglyceride of capric and caprylic acids, has high resistance against oxidation, increasing the stability of the formulation; easily solubilizes lipophilic molecules; and when mixed with a solid lipid such as Precirol[®] ATO5, has the ability to form imperfect matrices, providing higher drug loading capacity [434, 439]. Both lipids are commonly used in lipid NPs for brain targeting, not least because they are biodegradable and have GRAS status [434]. Lipids with GRAS status are preferable as they are less likely to cause acute and chronic toxicity, thus granting the NPs high biocompatibility and low cytotoxicity, which is favorable for CNS targeting [440, 441].

Regarding the evaluation of the miscibility between the solid and the liquid lipid, the best proportion for the preparation of the NLC dispersions was shown to be 70:30 (% w/w), due to the absence of oil droplets on the filter paper [415].

3.2. Physicochemical Characterization

After preparation, the empty dispersions of lipid NPs had a homogeneous, milky white aspect, without any phase separation. RLZ-loaded nanocarriers had the same post-production aspect as the empty NPs (Figure III.1), maintaining their initial appearance and integrity after 3 months, independent of the storage temperature. No caking, creaming or flocculation were observed, suggesting that the dispersions had good stability. Moreover, all samples presented the same color pattern and turbidity.

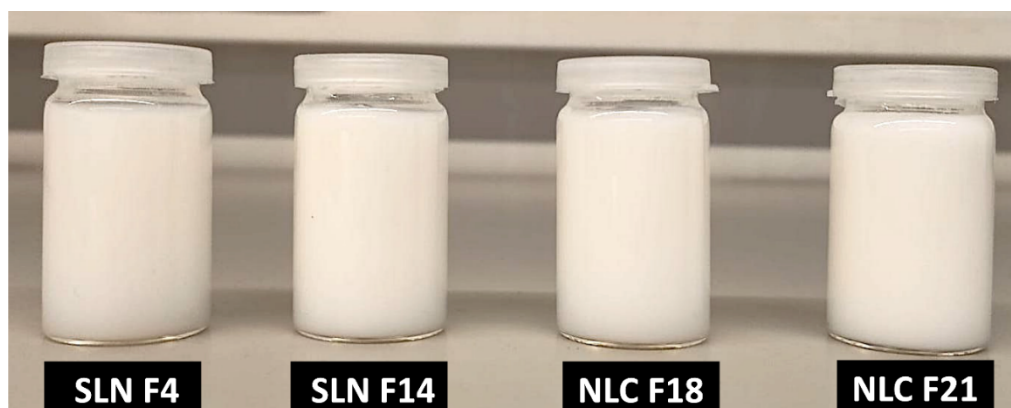


Figure III.1. Appearance of RLZ-loaded lipid nanoparticles after production.

The characterization of the lipid NPs can be seen in Table III.4, where the mean diameter (Z-average), PDI, ZP, EE and LC are summarized.

Table III.4. Mean diameter, polydispersity index (PDI), zeta potential (ZP), encapsulation efficiency (EE) and loading capacity (LC) of the lipid nanoparticles, evaluated on preparation day. Values are expressed as the mean $\pm$ SD ($n = 3$).

Formulation	Z-average (nm)	PDI	ZP (mV)	EE (%)	LC (%)
F1	154.1 $\pm$ 2.7	0.231 $\pm$ 0.004	-22.0 $\pm$ 3.4	–	–
F2	181.7 $\pm$ 0.8	0.231 $\pm$ 0.011	-15.2 $\pm$ 4.5	–	–
F3	152.9 $\pm$ 3.3	0.229 $\pm$ 0.012	-24.2 $\pm$ 0.9	–	–
F4	149.6 $\pm$ 1.1	0.206 $\pm$ 0.005	-22.5 $\pm$ 1.2	87.07 $\pm$ 1.83	0.84 $\pm$ 0.02
F5	124.4 $\pm$ 2.2	0.200 $\pm$ 0.012	-20.0 $\pm$ 1.9	–	–
F6	154.5 $\pm$ 4.3	0.222 $\pm$ 0.003	-22.3 $\pm$ 1.3	–	–
F7	184.0 $\pm$ 2.9	0.219 $\pm$ 0.012	-24.7 $\pm$ 2.0	–	–
F8	181.7 $\pm$ 0.4	0.213 $\pm$ 0.006	-21.5 $\pm$ 2.9	–	–
F9	179.5 $\pm$ 5.7	0.230 $\pm$ 0.007	-27.1 $\pm$ 2.2	–	–
F10	178.6 $\pm$ 2.3	0.232 $\pm$ 0.003	-21.4 $\pm$ 3.9	–	–
F11	213.7 $\pm$ 1.9	0.229 $\pm$ 0.009	-24.2 $\pm$ 2.1	–	–
F12	220.3 $\pm$ 5.0	0.248 $\pm$ 0.009	-24.3 $\pm$ 0.9	–	–
F13	215.5 $\pm$ 5.1	0.259 $\pm$ 0.008	-22.0 $\pm$ 2.5	–	–
F14	203.1 $\pm$ 0.6	0.244 $\pm$ 0.004	-27.2 $\pm$ 3.7	86.22 $\pm$ 1.89	0.67 $\pm$ 0.01
F15	157.7 $\pm$ 4.3	0.184 $\pm$ 0.006	-21.5 $\pm$ 4.2	–	–
F16	221.0 $\pm$ 6.1	0.256 $\pm$ 0.004	-21.9 $\pm$ 1.5	–	–
F17	144.9 $\pm$ 3.5	0.169 $\pm$ 0.003	-20.6 $\pm$ 1.0	–	–
F18	147.2 $\pm$ 0.0	0.176 $\pm$ 0.004	-23.0 $\pm$ 3.4	94.09 $\pm$ 2.62	0.90 $\pm$ 0.03
F19	120.1 $\pm$ 1.5	0.161 $\pm$ 0.003	-20.8 $\pm$ 1.7	–	–
F20	194.4 $\pm$ 2.6	0.192 $\pm$ 0.005	-21.1 $\pm$ 3.7	–	–
F21	199.2 $\pm$ 3.1	0.192 $\pm$ 0.016	-27.5 $\pm$ 3.0	94.26 $\pm$ 1.81	0.74 $\pm$ 0.01
F22	150.2 $\pm$ 0.8	0.152 $\pm$ 0.017	-21.6 $\pm$ 0.1	–	–

Particle size is one of the most significant determinants for successful permeation across the BBB [442]. All the developed formulations presented an initial mean diameter below 220 nm, suitable for brain delivery (see Appendix IV). Of interest, the RLZ-loaded nanocarriers exhibited mean diameters between 147.2 and 203.1 nm. According to the literature, sizes ranging from 100 to 300 nm are best for NPs drug delivery to the brain [343, 443]. Researchers have shown that lipid nanocarriers in this size range can efficiently transpose the BBB. For instance, Neves *et al.* [444] produced SLN functionalized with ApoE with mean diameters of 151–192 nm; Juffri *et al.* [445] formulated valproic acid-loaded liposomes for the treatment of seizures, with sizes ranging between 80 and 250 nm; Khan *et al.* [446] optimized NLC with atazanavir, with a mean particle size of 227.6 ± 5.4 nm, for mitigating neuroAIDS; and Migliore *et al.* [447] developed cationic liposomes for brain delivery of proteins with a mean particle size of 299 ± 26.4 nm. Nevertheless, during the production of the lipid NPs, it was decided to set 250 nm as the limit value for mean particle size [369].

As shown in Table III.4, PDI values were lower than 0.3, indicating uniformity in particle size, with high homogeneity among particles and narrow size distribution [446, 448]. ZP is also an important element for predicting the stability of colloidal formulations such as SLN and NLC, since dispersions with ZP values equal or greater than $|30|$ mV are considered highly stable and with low propensity to aggregate [446, 449]. The lipid NPs showed ZP ranging from -15.2 ± 4.5 to -27.5 ± 3.7 mV. These intermediate values could be due to the presence of the nonionic surfactants, rendering low magnitude of surface charge to the NPs [446].

Nanocarriers can be stabilized by two types of mechanisms: steric stabilization and electrostatic repulsion [450]. The backbone structure of Tween[®] 80 consists of a tetrahydrofuran ring surrounded by polyoxyethylene (POE) chains, whereas Poloxamer 188 is a triblock copolymer of a central hydrophobic polyoxypropylene (PPO) chain flanked by two hydrophilic POE segments. The dense POE tails of the surfactants provide steric hindrance, preventing particle aggregation [451, 452]. Moreover, Tween[®] 80 and Poloxamer 188 also impart negative surface charge to the NPs, which causes electrostatic repulsion [451]. Thus, the chosen surfactants have a synergic effect, and the developed lipid nanocarriers will be stabilized by both steric and electrostatic mechanisms, minimizing particle aggregation, and ensuring long-term stability.

Previous works have shown the compatibility of these surfactants with the lipids used [415, 451, 453], being routinely employed in the development of lipid NPs for brain delivery [369, 429, 454]. Furthermore, since they are nonionic, they have a very low toxic potential, being less hemolytic and irritant to cellular surfaces when compared to their anionic, cationic, or amphoteric counterparts [455], which is also advantageous in the neurodegeneration context [412]; provide rapid brain uptake, and produce smaller particles with narrow size distribution [446, 456].

Tween[®] 80 and Poloxamer 188 possess additional benefits, including facilitating the adsorption of serum proteins (e.g., ApoE) onto the surface of the NPs, which promote and increase nanocarrier uptake by interacting with receptors of BBB endothelial cells; and P-gp modulation, thus inhibiting drug efflux from the brain [456, 457]. Several research studies have documented the preparation of lipid nanocarriers for brain delivery using similar components. For instance, Vitorino *et al.* [458] developed fluoxetine-loaded NLC for depression treatment, through intranasal administration, using Precirol[®] ATO5 and Tween[®] 80. Another recent study reported the preparation and application of transferrin-modified NLC to improve rapamycin transport to the CNS, wherein Precirol[®] ATO5, Tween[®] 80 and Poloxamer 188 were used [459]. Also, Precirol[®] ATO5 was used as solid lipid by Youssef *et al.* [460] to optimize almotriptan malate-loaded SLN for the management of migraine.

3.3. Effect of Formulation and Preparation Parameters on Particle size, PDI and ZP

The effect of different variables on the size, PDI and ZP of the lipid NPs is reported in the next section. Statistical analysis was performed with SPSS software (v 28.0, IBM; Armonk, NY, USA), using an independent samples Student's t-test with a significance level of 95% ($\alpha = 0.05$). p values lower than 0.05 ($p < 0.05$) were considered statistically significant (see Appendix V).

3.3.1. High-Shear Homogenization Speed

High-shear homogenization was applied as a first step on the preparation of the lipid NPs, before ultrasonication and HPH, to obtain a pre-emulsion [418]. Three high-shear homogenization speeds were used: 5000 rpm, 8000 rpm and 13500 rpm. Overall, variation of the homogenization speed did not have an influence on either of the three measured

parameters, as $p > 0.05$ (comparison between F1/F3, F1/F6, F3/F6, F7/F8, F9/F10, F11/F12, and F13/F16). The only statistically significant difference was seen between F1 and F6, for PDI ($p = 0.036$). An increase in homogenization speed often results in a decrease in particle size, due to the higher energy supplied by the homogenizer [461-463]. In this case, the difference in shear stress produced at 13500 rpm may not have been enough to overcome the viscous forces that lead to the production of smaller lipid NPs than those that are obtained at 8000 rpm and 5000 rpm [461]. Hence, the homogenization speed was fixed at the intermediate speed of 8000 rpm.

3.3.2. Preparation Method (Ultrasonication vs HPH)

As can be seen in Table III.4, comparing formulations F3/F5, F13/F15, F17/F19 and F20/F22, the HPH technique resulted in particles with statistically significant smaller mean sizes and PDI ($p < 0.001$) than ultrasonication. Regarding ZP, the only statistically significant difference was noted for F3 and F5 ($p = 0.026$). These results are in accordance with several reports, where HPH yielded lipid NPs with lower sizes and PDI [464-466]. Nevertheless, since all the nanocarriers had sizes in the intended range (< 250 nm) and given that ultrasonication is an easier and faster production method [418], this technique was selected to prepare the RLZ-loaded lipid NPs.

3.3.3. Ultrasonication Amplitude

Increasing the sonication amplitude from 50% to 70%, led to a significant decrease in particle size ($p < 0.001$) (comparison between F2 and F3). As for PDI and ZP, no differences were noted. The same was observed by Sidiqqi *et al.* [467]. Therefore, a pulse frequency of 70% was selected as optimal sonication parameter in subsequent studies.

3.3.4. Total Lipid Concentration

Evaluating the effect of total lipid phase concentration using either 7.5 or 10% (w/w), revealed that increasing the lipid concentration caused a significant increase in the obtained size and PDI ($p < 0.05$) in both SLN and NLC, whereas there was no impact in the ZP (comparison between F3/F13, F4/F14, F17/F20 and F18/F21). Similar results were observed by Mura *et al.* [468] for SLN and NLC containing Precirol® ATO5 as solid lipid, with a progressive increase in lipid content. This finding could be attributed to the concomitant decrease in the surfactant-to-lipid ratio [469].

3.3.5. Surfactant Concentration

Increasing the concentration of both Tween[®] 80 and Poloxamer 188 resulted in a decrease in particle size (comparison between F7/F11 and F3/F7, respectively; $p < 0.001$). No differences were found for PDI, nor for ZP. When the percentage of surfactants is higher, more hydrophilic chains are available to cover the emulsion droplets, forming a more prominent steric barrier, and preventing the approach and coalescence of those droplets more efficiently [470, 471]. Concurrently, the interfacial tension between the aqueous and lipid phases is reduced, which protects the NPs from aggregation phenomena [471]. These results are analogous to those reported by Salem *et al.* and Khalil *et al.* [471, 472].

3.3.6. Type of Lipid Nanosystem (SLN vs NLC)

The type of lipid nanocarrier also impacts the physicochemical properties of the particles [437]. Under identical conditions (i.e., the same total lipid and surfactant concentrations, as well as preparation parameters), empty NLC had a smaller size than empty SLN. Likewise, there was also a statistically significant difference in PDI, with NLC showing a decrease in size distribution (comparison between F3/F17, F5/F19, F13/F20 and F15/F22). No differences were observed for the ZP. This is in line with other works [473, 474], and may be explained by improved emulsification of the lipid matrix when oil is added to the solid lipids [437]. Regarding the RLZ-loaded formulations, the only statistically significant difference between loaded SLN and NLC was related to the PDI (comparison between F4/F18 and F14/F21).

3.3.7. Drug Incorporation

Based on the results of the physicochemical characterization of the empty lipid NPs, four optimized formulations – two SLN (F3 and F13) and two NLC (F17 and F20) – with either 7.5% or 10% of lipid phase, were chosen to further incorporate the drug (formulations F4, F14, F18 and F21).

The incorporation of RLZ did not seem to directly influence either of the physicochemical properties of the lipid NPs, as for the most part, no differences were found between empty and loaded lipid NPs (comparison between F3/F4, F17/F18 and F20/F21). The only statistically significant difference was noted in formulations F13 and F14, for size and PDI ($p = 0.014$ and $p = 0.048$, respectively). These results translated in the high EE

(86-94%) seen for all RLZ-loaded formulations, suggesting that both types of nanocarriers can efficiently incorporate the drug. Moreover, the LC was between 0.7-0.9%, similar to the values that have been reported in other studies for lipid NPs incorporating hydrophobic drugs, such as loperamide [475].

3.4. Stability study

Figure III.2 presents the results of the stability study for the RLZ-loaded lipid NPs over 3 months of storage at 5 °C and 25 °C.

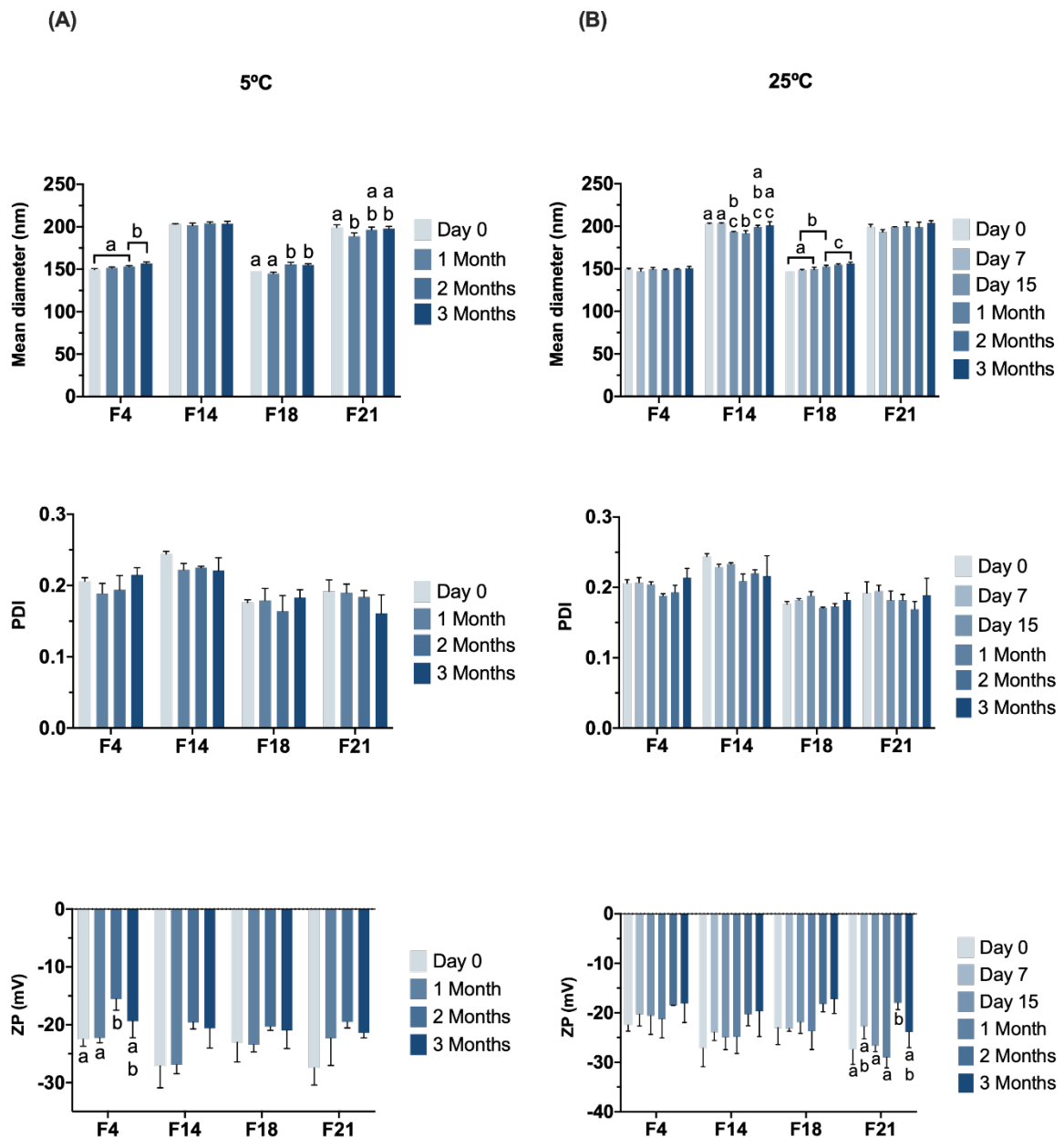


Figure III.2. Effect of storage time on particle size, polydispersity index (PDI), and zeta potential (ZP) of riluzole (RLZ)-loaded solid lipid nanoparticles (SLN) (formulations F4 and F14) and nanostructured lipid carriers (NLC) (formulations F18 and F21) over 3 months at 5 °C (A) and 25 °C (B). Values are expressed as the mean $\pm$ SD ($n = 3$). Data were analyzed using Tukey's HSD test, with different letters (a, b, c) representing statistically significant differences ($p < 0.05$) for the same formulation at different time points (see Appendix VI).

SLN and NLC sustained their characteristics throughout the storage period, at both temperatures. For the most part, particle size exhibited no tendency to increase. After 3 months, sizes remained below 205 nm for all formulations. There was a slight increase in the mean sizes of NLC F18 at 5 °C (155.1 ± 1.4 nm) and 25 °C (156.1 ± 1.6 nm), and of SLN F4 at 5 °C (157.0 ± 1.6 nm), compared to initial values. Nevertheless, in both cases, the increase was minimal (only around 8 nm), and the values remained well within the established range for brain delivery (i.e., between 100 and 300 nm). As for PDI and ZP, no significant changes over time at either temperature were observed ($p > 0.05$). This is consistent with the findings of the visual analysis, with RLZ-loaded nanocarriers preserving their initial appearance and integrity during storage, as no signs of instability phenomena, such as formation of drug crystals or coloration changes, were detected.

At the end of the storage period, the influence of the different temperatures (5 °C vs. 25 °C) on the various physicochemical parameters was also evaluated. The size of SLN F4 was the only statistically significant variation ($p = 0.03$). Altogether, the findings of the stability study seem to indicate that the lipid NPs remain stable for a minimum of 3 months.

3.5. Morphology

Cryo-SEM images of the optimized empty and drug-loaded SLN and NLC are presented in Figure III.3 and Figure III.4, respectively. As depicted, the NPs showed a uniform and rounded shape, with a smooth surface. The images confirm the nanometric sizes provided in the previous sections, validating the results obtained with the DLS technique. Moreover, there was no visible aggregation between the NPs, indicating the good stability of the developed formulations.

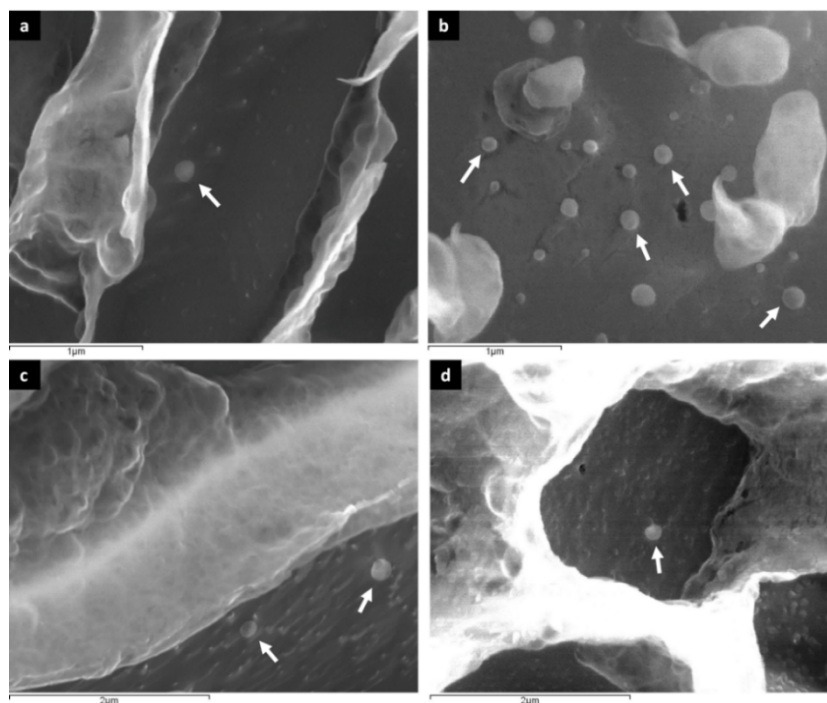


Figure III.3. Cryo-SEM images of optimized empty and riluzole (RLZ)-loaded solid lipid nanoparticles (SLN) (white arrows): (a) Formulation F3 ($\times 40000$ magnification); (b) Formulation F4 ($\times 40000$ magnification); (c) Formulation F13 ($\times 30000$ magnification); (d) Formulation F14 ($\times 30000$ magnification).

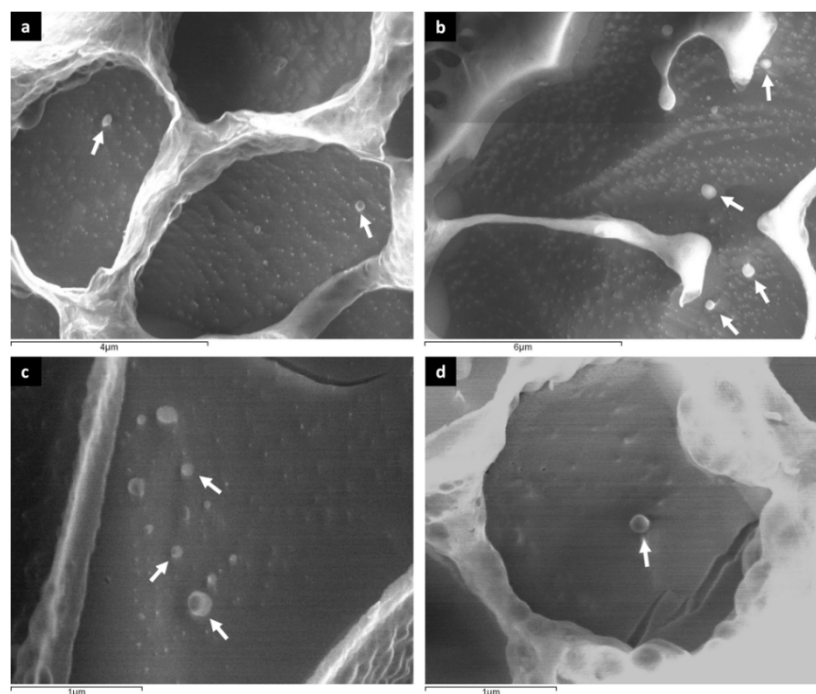


Figure III.4. Cryo-SEM images of optimized empty and riluzole (RLZ)-loaded nanostructured lipid carriers (NLC) (white arrows): (a) Formulation F17 ($\times 15000$ magnification); (b) Formulation F18 ($\times 10000$ magnification); (c) Formulation F20 ($\times 40000$ magnification); (d) Formulation F21 ($\times 40000$ magnification).

3.6. *In Vitro* Release Studies

The *in vitro* release profiles of the RLZ-loaded lipid NPs are shown in Figure III.5.

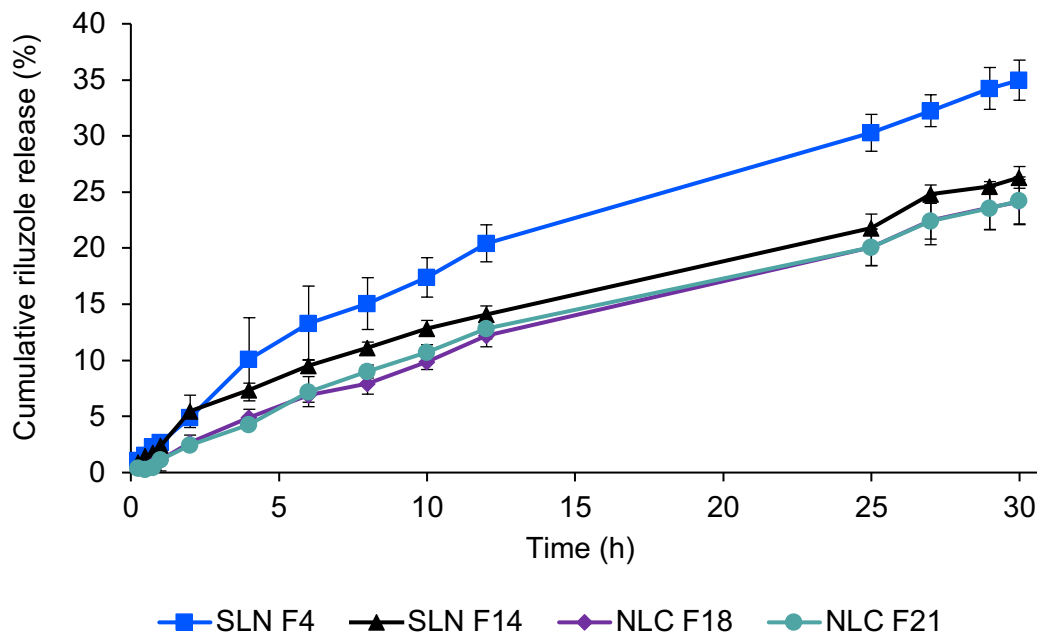


Figure III.5. Cumulative drug release from riluzole (RLZ)-loaded solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC), in phosphate-buffered solution (PBS) medium pH 7.4. Data is presented as mean $\pm$ SD ($n = 3$) (see Appendix VII).

A similar pattern of slow and sustained RLZ release was achieved for both SLN and NLC, suggesting that the drug is homogeneously dispersed within the lipid matrices [476]. However, after 30 h, SLN had a higher drug release compared to NLC, with the highest maximum cumulative value being for SLN F4 (ca. 35% vs ca. 25% for both NLC formulations). This could be related to the structural differences between SLN and NLC [477]. In NLC, the mixture of solid and liquid lipids leads to an imperfect, less ordered lattice, resulting in enhanced drug loading and less expulsion during storage [477]. On the other hand, in SLN, the crystallization of the solid lipid(s) gives way to the formation of a more densely packed crystalline matrix, where fewer molecules can be incorporated, thus promoting a higher release of the encapsulated drug [477]. Other research groups have reported comparable results. For example, Pinheiro *et al.* [478] developed rifabutin-loaded NLC which showed a release of approximately 20% after 25 h, using the same release medium. Analogously, resveratrol and docetaxel encapsulated in SLN achieved a maximum release of about 30% within 24 h and 28 h, respectively [479, 480].

Furthermore, no burst release was detected, indicating that both types of lipid NPs are indeed able to efficiently prolong the release of RLZ [481]. Although the crystallization degree of the lipids, and consequently, the type of matrix, is crucial to understanding the release behavior, other factors are known to also impact the kinetics, including the nature of the drug [482]. RLZ is a lipophilic drug, having poor aqueous solubility and a high affinity for the lipids that comprise the lipid nanocarriers [483, 484]. Its lipophilic nature hints at a preferential location/accumulation inside the core of the NPs, and not on their surface [479], which could help to explain the lack of initial burst release that otherwise often occurs with these types of systems [485].

The profiles demonstrate that the developed NPs can prevent the premature release of the encapsulated compound, implying that the majority of RLZ will be released in a controlled and sustained way only after the nanocarriers reach the brain [479]. However, it is important to underline that in a clinical setting the formulations would be exposed to a biological system, and thus, a number of variables (e.g., formation of protein corona, enzymatic degradation, different pH microenvironment) could possibly alter the modulated release profile [345, 486, 487]. Indeed, the entire delivery process should be considered, not simply BBB transcytosis. Having a thorough grasp of those influencing variables is key for a more rational design and successful translation of lipid nanocarriers in brain drug delivery [345].

The results of the kinetic data modeling of the RLZ release profiles from the lipid NPs are presented in Table III.5.

Table III.5. Estimated kinetic parameters of the different mathematical models fitted to the riluzole (RLZ) release from solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) formulations.

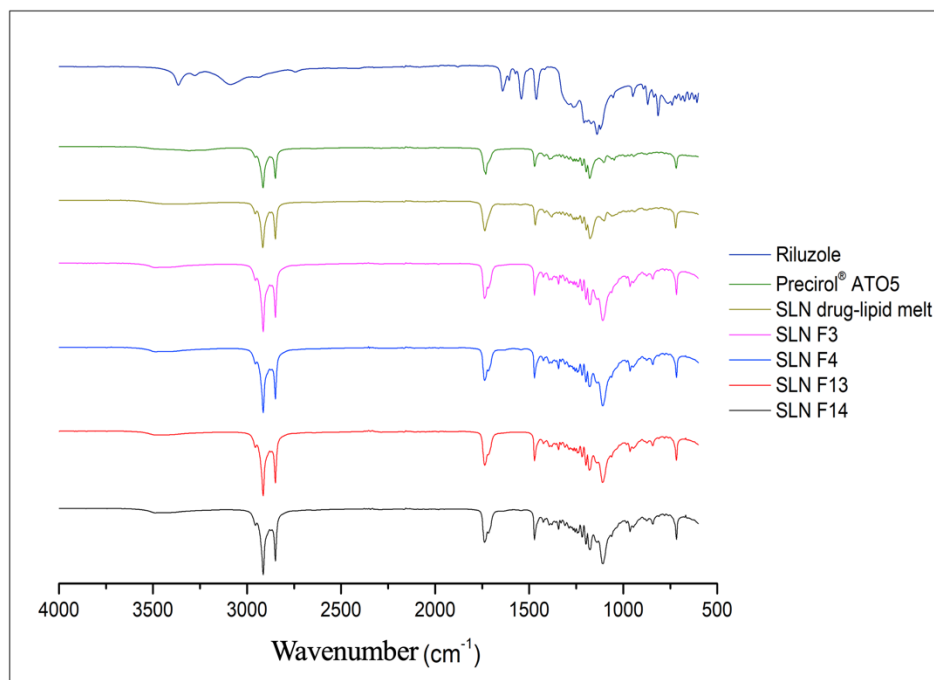
Kinetic model	Parameters	SLN F4	SLN F14	NLC F18	NLC F21
Zero-order	K_0	0.0278	0.0190	0.0166	0.0182
	R^2	0.9778	0.9614	0.9906	0.9954
	R_{adj}^2	0.9714	0.9537	0.9887	0.9945
Higuchi	K_H	0.8371	0.5930	0.5071	0.5541
	R^2	0.9918	0.9951	0.9817	0.9756
	R_{adj}^2	0.9895	0.9941	0.9781	0.9707
Korsmeyer-Peppas	K_{KP}	0.3239	0.5921	0.0622	0.0397
	R^2	0.9950	0.9951	0.9949	0.9970
	R_{adj}^2	0.9926	0.9926	0.9939	0.9964
	n	0.6830	0.5002	0.8027	0.8830

The Korsmeyer-Peppas was chosen as the most suitable model to describe the RLZ release (highest R_{adj}^2). Additionally, the drug release exponents (n) were between $0.45 < n < 0.89$, indicating an anomalous or non-Fickian diffusion, where the transport mechanism is due to a combination of molecular drug diffusion and lipid erosion [438]. Interestingly, the n values for SLN were closer to the lower range of the interval (i.e., to 0.45), whereas for NLC, the values approximate more the upper range (i.e., to 0.89). Therefore, it can be considered that for SLN the release is mainly governed by diffusion, in contrast with NLC, where the release will be primarily controlled by an erosion process.

3.7. FTIR Spectroscopy

FTIR was conducted to assess the intermolecular interactions in the formulations [419]. The absorption spectra of RLZ, Precirol® ATO5, SLN and NLC drug-lipid melts, and lipid NPs are displayed in Figure III.6.

(A)



(B)

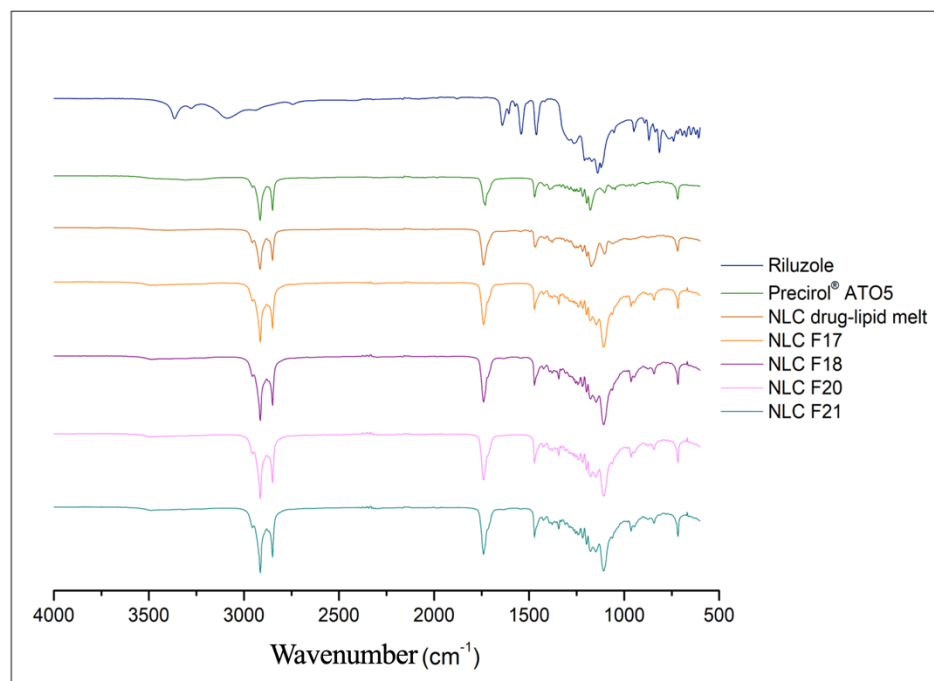


Figure III.6. FTIR spectra of (A, B) pure compounds (riluzole (RLZ) and Precirol® ATO5); (A) solid lipid nanoparticles (SLN) drug-lipid melt, empty SLN (formulations F3 and F13) and RLZ-loaded SLN (formulations F4 and F14); and (B) nanostructured lipid carriers (NLC) drug-lipid melt, empty NLC (formulations F17 and F20) and RLZ-loaded NLC (formulations F18 and F21). All graphs are plotted with the same scale.

The IR spectrum of pure RLZ had distinctive bands at 3266 and 3360 cm^{-1} (N–H stretching), due to the presence of the primary amine group, as well as another band around 3090 cm^{-1} , corresponding to O–H stretching of the carboxylic acids [488]. Furthermore, the peaks at 814 and 868 cm^{-1} (C–H bend), at 1460 cm^{-1} (C=C stretch), 1550 cm^{-1} (C–H in plane bend) and 1640 cm^{-1} (C=N stretch) are all attributable to various vibrations of its benzothiazole aromatic ring [489, 490]. Precirol® ATO5 displayed several absorption peaks, as presented elsewhere [458, 491, 492]: 1470 cm^{-1} (C=C stretch), 1200 cm^{-1} and 1730 cm^{-1} (C–O stretch), and 2850 and 2914 cm^{-1} (C–H stretch). It is possible to discern that most absorption bands from the drug were not found in the spectra of either drug-lipid melts or the lipid NPs, and those that were present, had a lower intensity. This demonstrates that RLZ was successfully entrapped in the lipid nanocarriers. Instead, their molecular fingerprints reflect the FTIR spectrum of Precirol® ATO5, given the high lipid content compared to the drug.

3.8. DSC

The thermal behavior and crystallization of NPs are critical variables that impact their effectiveness as drug delivery systems [493]. The results of the thermal analysis for the first DSC cycle are shown in Table III.6, and Figures III.7 and III.8.

Table III.6. Thermal properties of Precirol® ATO 5, drug-lipid melts and the lipid nanoparticles.

Sample	Onset (°C)	Peak (°C)	Offset (°C)	Enthalpy (J/g)
Precirol® ATO 5	53.0	67.8	70.5	-162.80
SLN drug-lipid melt	47.0	56.7	62.3	-127.70
SLN F3	38.1	64.0	66.2	-139.50
SLN F4	38.1	64.4	66.4	-136.40
SLN F13	38.5	63.4	65.5	-137.90
SLN F14	38.0	65.1	68.3	-134.76
NLC drug-lipid melt	51.2	56.7	59.1	-116.10
NLC F17	41.2	61.9	65.6	-93.53
NLC F18	37.5	62.3	64.1	-93.64
NLC F20	39.5	64.0	64.4	-97.99
NLC F21	39.6	61.8	64.8	-91.41

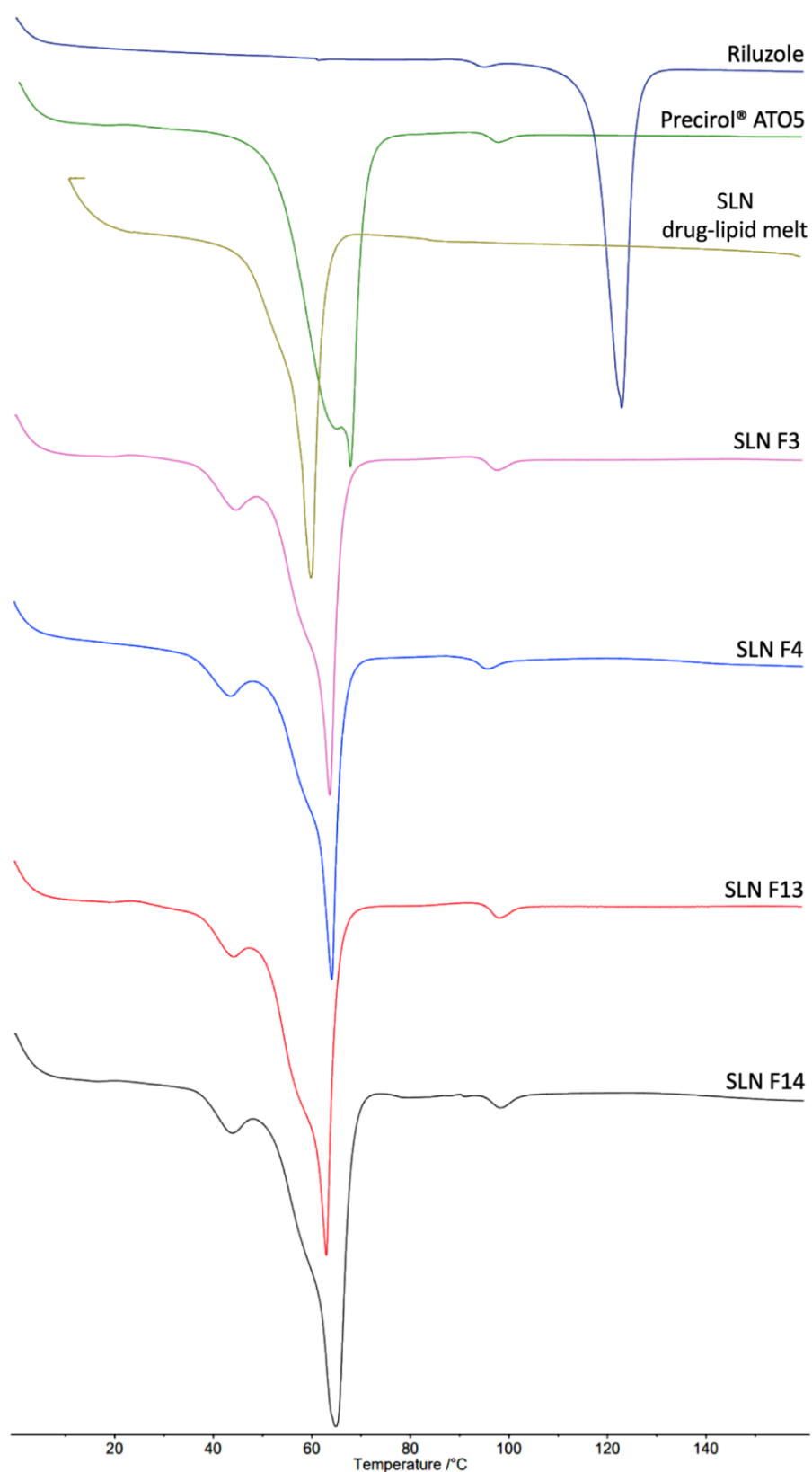


Figure III.7. DSC thermograms of riluzole (RLZ), Precirol® ATO5, solid lipid nanoparticles (SLN) drug-lipid melt (Precirol® ATO5+RLZ), and lyophilized SLN (formulations F3, F4, F13 and F14). All graphs are plotted with the same scale.

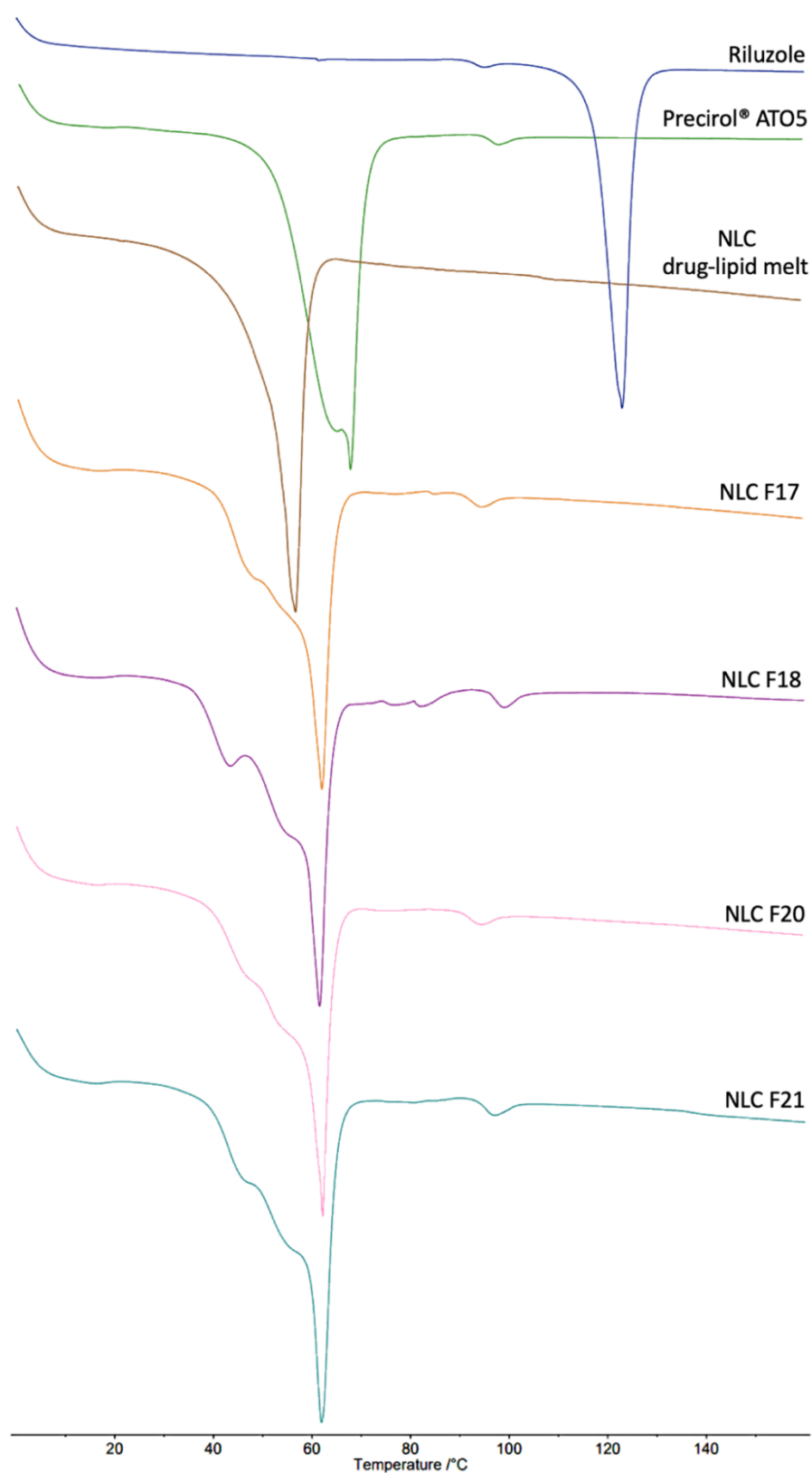


Figure III.8. DSC thermograms of riluzole (RLZ), Precirol® ATO5, nanostructured lipid carriers (NLC) drug-lipid melt (Precirol® ATO5+Miglyol® 812+RLZ), and lyophilized NLC (formulations F17, F18, F20 and F21). All graphs are plotted with the same scale.

The pure drug displayed a sharp peak at 121.3 °C, related to its melting temperature [488]. On the other hand, Precirol® ATO5 exhibited an endothermic event at 67.8 °C, and a small but well-defined shoulder of decreased energy at 65 °C, which is in agreement with the diffuse melting in complex glycerides due to the presence of different polymorphs [494, 495]. As observed in Figures III.7 and III.8, the thermograms of the drug-lipid melts presented a single endothermic peak at about 60 °C, corresponding to a left shift of the Precirol® ATO 5 peak. Similar behavior was apparent in the heating curves of all lyophilized SLN and NLC, where the onset and melting points were depressed, showing a significant shift towards lower temperatures compared to those of the bulk solid lipid. This also translated into a reduction of the enthalpy values (Table III.6), denoting the formation of disordered structures in the inner cores of the nanocarriers [496]. Interestingly, NLC had even lower fusion enthalpies and temperatures than SLN, as the addition of the liquid lipid creates an imperfect matrix wherein the energy requirements for overcoming lattice force and melting of NLC structures are lower than SLN [497]. The free spaces created by the imperfect matrix of NLC allow to accommodate more RLZ molecules, enhancing drug loading and minimizing potential expulsion of the active compound during storage [441, 497].

Other factors that may have contributed to the differences include interactions between the lipids and surfactants that take place during the preparation process and affect the crystallization, or the higher surface area resulting from the smaller particle size of the NPs [493, 497]. The high aspect ratio of the particles increases surface energy and creates an energetically suboptimal state, resulting in a reduction of the melting point [419]. In fact, those phenomena conform to the behavior predicted by the Gibbs-Thomson equation (Equation III.7) for nanodispersed materials [497, 498]:

$$\ln \frac{T}{T_0} = - \frac{2\gamma_{sl}V_s}{r\Delta H_{fus}} \quad \text{Equation III.7}$$

where T and T_0 represent, respectively, the melting temperature of a particle with radius r and of the bulk material, γ_{sl} is the interfacial tension at the solid-liquid interface, V_s is the specific volume of the solid, and ΔH_{fus} is the specific heat fusion. The equation determines that as particle size decreases, so will the melting temperature.

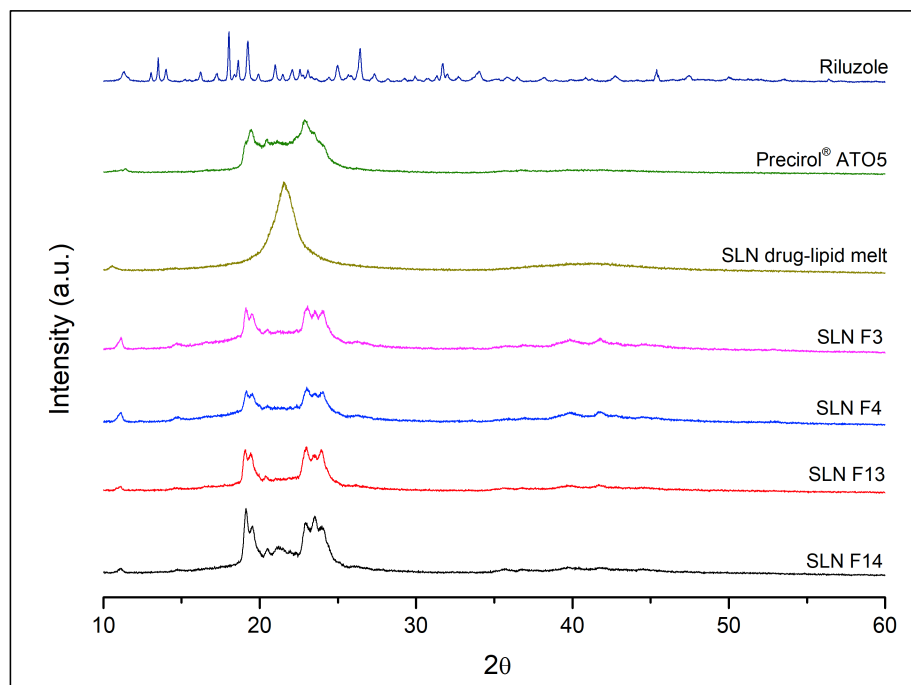
More importantly, the drug peak was completely absent from the thermograms of both drug-lipid melts and the RLZ-loaded lipid NPs (formulations F4, F14, F18 and F21), suggesting that (1) the drug was successfully solubilized and included in the lipid matrix and (2) the encapsulated RLZ was homogeneously dispersed in an amorphous or molecular

state within the nanocarriers [497, 498]. Overall, it can be assumed that the developed NPs might offer a greater bioavailability of RLZ, as the melting point, an indicator of intermolecular attractive forces, is usually lower for non-crystalline substances.

3.9. PXRD

To supplement the data acquired from FTIR and DSC, PXRD was performed (Figure III.9).

(A)



(B)

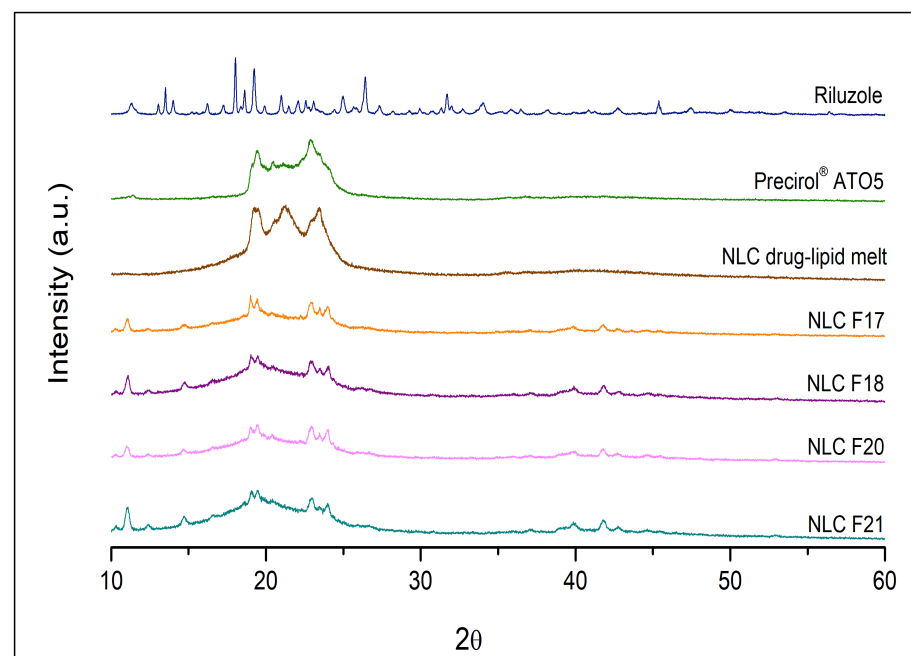


Figure III.9. X-ray diffractograms of (A, B) riluzole (RLZ) and Precirol® ATO5; (A) solid lipid nanoparticles (SLN) drug-lipid melt, empty SLN (formulations F3 and F13) and RLZ-loaded SLN (formulations F4 and F14); and (B) nanostructured lipid carriers (NLC) drug-lipid melt, empty NLC (formulations F17 and F20) and RLZ-loaded NLC (formulations F18 and F21). All graphs are plotted with the same scale.

The diffractogram of RLZ was similar to what is presented in the literature, with typical peaks (2θ of 13.50° , 18.03° , 18.62° , 19.3° , 21.06° , 24.98° , 26.41° , 31.70° , 34.02° , and 45.36°) revealing the highly crystalline nature of the drug [490]. The diffractogram of pure Precirol[®] ATO5 presented several peaks between 15° and 25° , which are in agreement with diffractograms for aged Precirol[®] ATO5 [495]. In the case of the SLN drug-lipid melt, the diffractogram is compatible with that of freshly solidified Precirol[®] ATO5, presenting a single peak at approximately 21° [495]. For the NLC drug-lipid melt, the diffractogram showed a different pattern, evidencing the influence of the liquid lipid (Miglyol[®] 812). The characteristic peaks of RLZ were absent from the diffractograms of the drug-lipid melts and the lyophilized NPs, suggesting the amorphous state of RLZ in the drug-loaded nanocarriers [499]. In turn, the PXRD patterns of the drug-lipid melts and of the lyophilized lipid NPs essentially exhibited crystalline aspects ascribed to the presence of the solid lipid. Overall, their peaks were broader and weaker than that of the bulk lipid, which suggests that Precirol[®] ATO5 was partially recrystallized or with less order [499].

The PXRD spectral analyses were therefore in accordance with the FTIR and DSC results, reiterating not only the successful encapsulation of RLZ, but also a decrease in the crystalline nature of the drug and the lipid when formulating the nanocarriers, giving rise to an amorphous stabilized structure [500].

3.10. Cell Viability Assay

Figure III.10 shows the results of the cell viability assessed by the MTT assay, after exposition of hCMEC/D3 cells to the nanocarriers and free RLZ samples, with increasing concentrations.

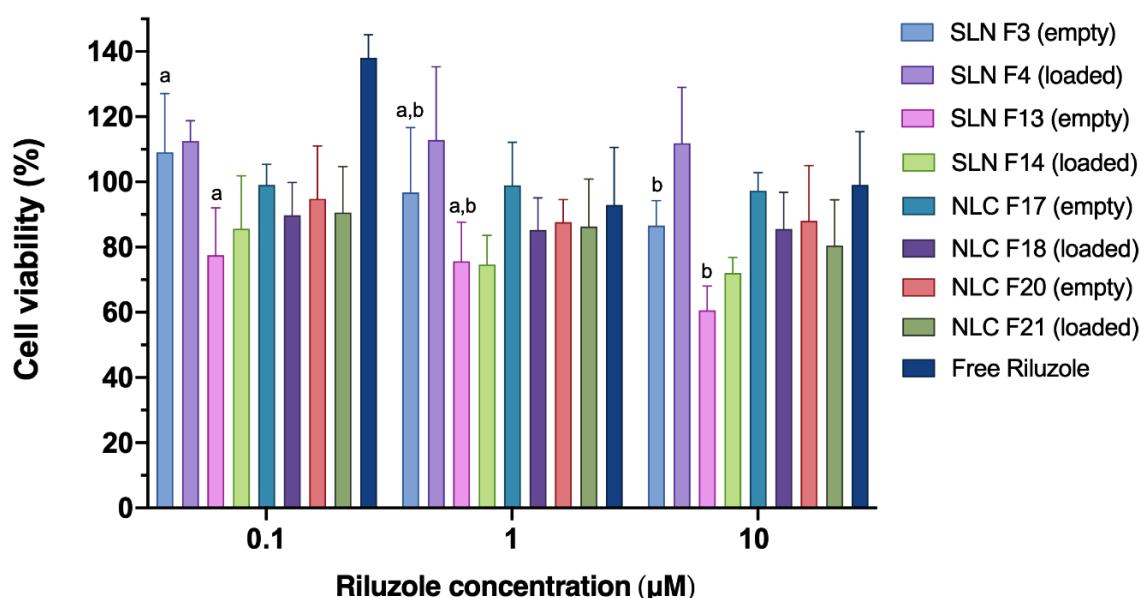


Figure III.10. Cell viability of hCMEC/D3 cells after 24 h of incubation with the optimized empty and riluzole (RLZ)-loaded lipid nanoparticles, as well as free RLZ, at different concentrations (0.1–10 μM). Values are expressed as mean ± SD (n = 3). Different letters (a, b) represent significant differences between different concentrations of the same sample, according to Tukey's HSD test.

As can be observed, the free drug was not toxic for the cells. The cells incubated with either type of lipid nanoparticle showed reasonable viability in the studied range, as even at the highest concentration tested (10 μM), the viability remained equal or above 70% within the error for all formulations, which is the threshold that is usually considered when testing biomedical materials and devices [501]. As a result, the encapsulation of RLZ inside the NPs did not appear to compromise cell integrity, showing that the formulations have a good biocompatibility.

Herein, a preformulation study was designed to evaluate the best lipid nanosystem for RLZ brain targeting, involving the rational development of a diverse range of formulations that were thoroughly characterized in terms of physicochemical properties, thermodynamic behavior, morphology, and colloidal stability. The composition (mixture of lipids and surfactants, and respective proportions) and experimental conditions employed to prepare the selected formulations, as well as their physicochemical characterization through specific techniques (FTIR, PXRD, DSC), constitute a novelty compared to previous studies reporting the use of lipid-based nanosystems for ALS, and more specifically, RLZ administration. Such characterization is of the utmost importance since the physicochemical properties of a nanosystem can greatly impact drug performance, both *in vitro* and *in vivo*.

For instance, Bondi *et al.* [376] used Compritol® 888 (solid lipid), Epikuron 200 and taurocholate (surfactant and co-surfactant, respectively) to produce RLZ-loaded SLN through the warm oil-in-water microemulsion technique, enhancing RLZ delivery to the brain and significantly increasing its CNS concentration compared to free drug after IV administration in rats. On the other hand, Yang *et al.* [377] co-encapsulated RLZ with verapamil, a P-gp efflux pump inhibitor, inside liposomes composed of distearoylphosphatidylcholine (DSPC), cholesterol, and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(poly ethylene glycol)-2000] (DSPE-PEG2000) that were prepared using a hydration method, and which efficiently silenced P-gp in bEND.3 mouse brain endothelial cells, as well as in astrocytic C8D1A cells. Also, Parikh *et al.* [379] prepared a RLZ nanoemulsion for intranasal delivery, using Sefsol-218 as the lipid, Tween® 80 as surfactant and Carbitol as co-surfactant, resulting in a significantly higher brain uptake than through the oral route in Wistar rats, as well as better neuroprotective effect in SH-SY5Y695 cells compared to free RLZ. Moreover, to the best of our knowledge, no other group has ever tested the cytotoxicity of RLZ-loaded lipid NPs intended for ALS treatment in the specific cell line that was used, namely, the hCMED/D3 cell line.

4. Conclusions

In this chapter, SLN and NLC were prepared and optimized by two different techniques (ultrasonication and HPH), to be used as a viable and efficient tool to circumvent the selective BBB and facilitate RLZ uptake into the CNS. Following an initial assessment, four formulations (two SLN and two NLC) were chosen to further incorporate the drug. Both RLZ-loaded SLN and NLC displayed comparable performance, with physicochemical features suitable for brain application (sizes < 250 nm, PDI < 0.3, and intermediate ZP values between -22.5 and -27.5 mV). Stability studies under storage conditions proved the colloidal stability of the formulations over 3 months, at both 5 °C and 25 °C. FTIR, DSC and PXRD results seemed to demonstrate the successful encapsulation of the drug within the core of the NPs, which translated in high EE (86-94%). Additionally, the dissolution profiles showed the achievement of a prolonged RLZ release from the formulations over 30 h. As for the cytotoxicity assay, the NPs did not significantly affect the viability of an hCMEC/D3 cell monolayer at a RLZ concentration up to 10 µM, indicating they were biocompatible.

Overall, the findings of this study suggest that the selected optimized lipid nanocarriers (either SLN or NLC) can be exploited as an alternative delivery system to target RLZ to the brain. Nonetheless, additional studies are warranted to confirm this application, including assessing permeability in cells of the BBB (e.g., hCMEC/D3 model), *in vivo* brain distribution and toxicity studies, ranging from intraarticular to intravenous, and evaluation of the therapeutic efficacy in an ALS animal model. Moreover, stability tests should also be conducted in plasma or cell medium, as the presence of proteins and enzymes in plasma could potentially compromise the quality and applicability of the NPs.

Chapter IV

Development of Lactoferrin-Functionalized NLC for Riluzole Delivery to the Brain

This chapter has been published as follows, with some minor modifications:

Teixeira MI, Lopes CM, Gonçalves H, Catita J, Silva AM, Rodrigues F, Amaral MH, Costa PC. Formulation, Characterization, and Cytotoxicity Evaluation of Lactoferrin Functionalized Lipid Nanoparticles for Riluzole Delivery to the Brain. *Pharmaceutics*. 2022;14(1).

Teixeira MI, Lopes CM, Reguengo H, Oliveira JC, Silva AM, Rodrigues F, Amaral MH, Costa PC. Permeability assay and inflammatory marker quantification of lactoferrin functionalized lipid nanoparticles intended for brain delivery. *Proceedings of the Nanotech France/ NanoMatEn/ Nanometrology 2022 International Conference*. DOI: 10.26799/cp-nanotechfrance2022

1. Introduction

The following chapter describes the development of lactoferrin-functionalized NLC to enhance RLZ delivery to the brain and improve targeting efficiency. As previously mentioned, passive targeting alone falls short in terms of efficiency when compared to active targeting. Active targeting involves the attachment of ligands to the surface of nanocarriers, enabling them to engage with specific receptors and transporters of the BBB. This interaction facilitates the process of transcytosis through the brain endothelium, ultimately enhancing drug uptake and delivery to the CNS. Nanocarrier functionalization has been shown to be the most crucial factor for improving BBB permeation [343, 358]. Moreover, by directing nanocarrier delivery to the BBB, active targeting reduces the occurrence of side effects linked to the accumulation of particles in peripheral organs, a common issue with non-functionalized nanosystems [502].

Lactoferrin is a glycoprotein that belongs to the transferrin family. Lactoferrin receptors are overexpressed on brain endothelial cells of the BBB, intervening in RMT [503, 504]. Some authors have already demonstrated the superiority of nanocarriers functionalized with lactoferrin when compared to non-functionalized ones, improving drug uptake through the BBB [505, 506].

Taking this into account, and based on the results of the previous chapter, one of the developed riluzole-loaded formulations (NLC F18) was functionalized with lactoferrin. The functionalization was confirmed by FTIR spectroscopy and protein quantification methods. Afterwards, the NLC were characterized regarding their physicochemical characteristics and stability. To assess their potential efficacy for ALS treatment, *in vitro* cell viability assays on hCMEC/D3 cell line and on motor neuron-like NSC-34 cell line were also performed. To the best of our knowledge, this is the first study that reports the use of functionalized NLC for ALS.

2. Materials and Methods

2.1. Materials

Precirol®ATO5 (glyceryl distearate/glyceryl palmitostearate) was kindly provided by Gattefossé (Nanterre, France), and Kolliphor® P188 micro Geismar (Poloxamer 188), by BASF (Ludwigshafen am Rhein, Germany). Miglyol® 812 (triglycerides of capric/caprylic acids), Tween® 80 (polysorbate 80), and stearic acid were acquired from Acofarma (Madrid, Spain), and RLZ (purity > 98%), from Beantown Chemical (Hudson, NH, USA). N-hydroxysuccinimide (NHS), N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), and recombinant human lactoferrin were purchased from Sigma-Aldrich (Steinheim, Germany).

Dulbecco's Modified Eagle Medium (DMEM), FBS, HBSS, nonessential amino acids, penicillin, streptomycin, and trypsin–EDTA were obtained from Invitrogen Corporation (Life Technologies, S.A., Madrid, Spain). EndoGRO™ basal medium and EndoGRO-MV Complete Culture Media Kit were obtained from Merck (Darmstadt, Germany). DMSO and Triton X-100 were purchased from Sigma-Aldrich (Steinheim, Germany). All the other reagents were of analytical grade and used as supplied. The water used in all experiments was purified as obtained from a Milli-Q® Direct 3 UV-R system (Millipore®, Darmstadt, Germany).

2.2. Preparation of the Lipid Nanoparticles

NLC were produced by a hot homogenization technique followed by ultrasonication [369, 429]. Precirol® ATO5 and Miglyol® 812 were used as solid and liquid lipids, respectively. Both lipids have GRAS status [446]. A blend of mono-, di- and triglycerides, Precirol® ATO5 leads to the formation of a lattice with an imperfect arrangement, thus providing more space to incorporate the drug and avoid its expulsion [446]. Polysorbate 80 and poloxamer 188 were chosen as nonionic surfactants. Compared with ionic surfactants, stabilizers of this type have a very low toxic and irritation potential [412]. Moreover, they produce particles with smaller sizes, also having the advantage of enhancing brain uptake [446]. Indeed, polysorbate 80 and poloxamer 188 have been repeatedly demonstrated to be able to increase drug transport to the brain through different administration routes, by coating on the surface of the nanocarriers [507]. To facilitate the posterior functionalization of the NLC with lactoferrin, stearic acid was also incorporated as excipient.

Briefly, the lipid and aqueous phases were first heated separately at about 80 °C, with the aqueous phase being added to the molten lipid mixture. The pre-emulsion was then homogenized with an Ultra-Turrax T25 with S 25 N–18 G dispersing element (Janke and Kunkel IKA-Labortechnik, Staufen, Germany) at 8000 rpm for 5 min; this was followed by sonication for 15 min at an amplitude of 70% with a probe sonicator (Vibro Cell VCX 130, 6 mm probe, Sonics & Materials, Newtown, CT, USA). The obtained oil-in-water nanoemulsion was transferred to glass vials and cooled down in an ice bath for about 20 min, with the NLC being formed by lipid recrystallization. The composition of the different NLC is described in Table IV.1.

Table IV.1. Composition of the developed nanostructured lipid carriers (NLC) (% , w/w).

Excipient	NLC Placebo	NLC Riluzole^a
Precirol® ATO5	5.250	5.250
Miglyol® 812	2.250	2.150
Stearic acid	0.125	0.125
Riluzole	—	0.100
Tween® 80	2.000	2.000
Poloxamer 188	1.000	1.000
Ultra-purified water	q.s. 100	q.s. 100

^a The same composition was applied to produce the functionalized NLC.

2.3. Functionalization of NLC with Lactoferrin

Functionalization of NLC with lactoferrin was achieved by carbodiimide chemistry, following a previously reported method with some minor modifications [480]. Lactoferrin was covalently coupled by its amino group to the carboxylic acid group of the stearic acid present on the surface of RLZ-loaded NLC (NLC RLZ), with EDC and NHS serving as activators of the carboxylic group [508].

Briefly, 10 mL of RLZ-loaded NLC was incubated with 22.5 mL of a lactoferrin solution (1 mg/mL of lactoferrin in PBS, pH 7.4) at room temperature for 4 h with gentle stirring, in the presence of EDC and NHS. To remove excessive unbound lactoferrin and any other by-products, the functionalized NLC were then centrifuged (Allegra® X-15R

Centrifuge, Beckman Coulter, Brea, CA, USA) at 5000× *g* for 1 h using Amicon® Centrifugal Filter Units with a cutoff of 100 kDa.

2.4. Physicochemical Characterization and Stability of NLC

To characterize and study the stability of NLC, the particle size, PDI, and ZP were monitored at different time points (day 0, 1 month, 2 months, and 3 months) after production and storage in closed glass vials at both 5 ± 1 °C and 25 ± 1 °C. Furthermore, EE and LC were also assessed.

2.4.1. Particle Size, PDI, and ZP Analysis

The mean hydrodynamic diameter and PDI were determined by DLS using a particle size analyzer (Brookhaven Instruments, Holtsville, NY, USA). ZP was estimated using the same equipment by ELS. All samples were previously diluted in Milli-Q® water (dilution 1:100), and measurements were performed with a light incidence angle of 90°.

2.4.2. Encapsulation Efficiency and Loading Capacity

The EE and LC of NLC RLZ and functionalized NLC were assessed by HPLC (Dionex UltiMate™ 3000, Thermo Scientific, Waltham, MA, USA), following a method that was developed in-house. The chromatographic analysis was performed at 263 nm, using a BDS Hypersil™ C18 column (150 mm × 4.60 mm internal diameter, 5 µm particle size; Thermo Scientific, Waltham, MA, USA), and methanol/water pH 3, 70:30 (v/v), as the mobile phase, eluted at a flow rate of 1 mL/min. EE (%) was determined by analyzing three replicates of each formulation ($n = 3$) and the results are reported as the mean ± SD. Injections were made in triplicate, with a sample volume of 10 µL. A calibration curve was previously built up by analyzing independent RLZ standard solutions in ethanol and fitting the respective data to the least squares linear regression, which gave an R^2 of 0.999.

Afterwards, the dispersions of lipid NPs were diluted with Milli-Q® water, mixed with ethanol, centrifuged, filtered, and the supernatant analyzed in the HPLC unit to determine the EE and LC as follows:

$$EE (\%) = \frac{\text{Amount of RLZ in the filtered formulation}}{\text{Total RLZ amount}} \times 100 \quad \text{Equation III.1}$$

$$LC (\%) = \frac{\text{Amount of RLZ encapsulated}}{\text{Total lipid amount}} \times 100 \quad \text{Equation III.2}$$

2.5. Transmission Electron Microscopy (TEM)

The shape and morphology of the functionalized NLC were examined by transmission electron microscopy (TEM). First, 10 µL of the NLC dispersion was mounted on Formvar/carbon film-coated mesh nickel grids (Electron Microscopy Sciences, Hatfield, PA, USA) and left standing for 2 min. The liquid in excess was removed with filter paper, and afterwards, 10 µL of 1% uranyl acetate was added on to the grids for 10 s for negative staining. Visualization was carried out on a JEOL JEM 1400 TEM at 80 kV (Tokyo, Japan). Images were digitally recorded using a CCD digital camera Orious 1100W (Tokyo, Japan).

2.6. *In Vitro* Drug Release Studies

The *in vitro* release profiles of NLC RLZ and functionalized NLC were determined using the dialysis bag technique [509]. The dialysis membrane (molecular weight cutoff: 3.5 kDa; Cellu•Sep®, Montluçon, France) was soaked in distilled water for 12 h prior to use. Briefly, 5 mL of either NLC RLZ or functionalized NLC was put in the dialysis membrane, clamped, and incubated in 200 mL of PBS pH 7.4 in a water bath at 37.0 ± 0.5 °C, under continuous magnetic stirring (450 rpm). At specified time intervals (0.25, 0.50, 1, 2, 4, 6, 8, 10, 24, 27, 29, 30, 34, 48, and 60 h), 2 mL of aliquot was withdrawn, with no medium replacement. The samples were filtered through a 0.45 µm membrane filter, and the amount of RLZ released was quantified by HPLC as stated before. Measurements were carried out in triplicate ($n = 3$), and the results were expressed as the percentage of cumulative drug released at each time point (mean ± SD).

To characterize the transport mechanisms, the release profiles were fitted to various kinetic models, namely, zero-order (Equation III.3), Higuchi (Equation III.4), and Korsmeyer-Peppas (Equation III.5) [424]:

$$Q_t = Q_0 + K_0 t \quad \text{Equation III.3}$$

$$Q_t = Q_0 + K_H t^{0.5} \quad \text{Equation III.4}$$

$$Q_t = Q_0 + K_{KP} t^n \quad \text{Equation III.5}$$

where Q_t is the amount of RLZ dissolved in time t , Q_0 is the amount of RLZ in the dissolution medium in the time 0, K_0 is the zero-order release constant, K_H is the Higuchi dissolution constant, K_{KP} is the Korsmeyer-Peppas release constant, and n is the latter's release exponent.

For model fitting, only the first 60% of the drug release was considered [510]. The best fit for the release data is usually evaluated by the R^2 [425]. However, R^2 tends to increase with the addition of more parameters. Therefore, when comparing kinetic models that have different numbers of parameters, the R_{adj}^2 is more appropriate [425]. R_{adj}^2 can be calculated from Equation III.6:

$$R_{adj}^2 = 1 - \frac{(1 - R^2)(q - 1)}{q - p - 1} \quad \text{Equation III.6}$$

where q is the number of experimental data points and p is the number of parameters. The model with the highest R_{adj}^2 was considered as the best fitting model [425].

2.7. Lactoferrin Conjugation Efficiency

The Bradford assay was employed to quantify the lactoferrin conjugated on the surface of the NLC [480]. After production, the NPs were centrifuged (Allegra® X-15R Centrifuge, Beckman Coulter, Brea, CA, USA) at $5000 \times g$ for 1 h, using Amicon® Centrifugal Filter Units (cutoff of 100 kDa). Following incubation with the Bradford reagent, the amount of unbound lactoferrin within the supernatant was measured by reading the absorbance at 595 nm using a UV-Vis spectrophotometer (Jasco V-650 spectrophotometer, Easton, MD, USA) [480]. The experiments were performed in triplicate ($n = 3$), and conjugation efficiency was expressed as percentage of lactoferrin bound to the NLC.

2.8. FTIR Spectroscopy

To confirm the functionalization of the NLC with lactoferrin and infer about possible drug-lipid interactions, FTIR spectroscopy was performed. The functionalized NLC were previously frozen overnight at $-85\text{ }^{\circ}\text{C}$ and lyophilized at $-75\text{ }^{\circ}\text{C}$ and 0.4 mBar using a LyoQuest freeze dryer (Telstar, Terrassa, Spain).

The infrared spectra were obtained by placing the samples (RLZ, Precirol[®] ATO5, molten mixture of the lipids and drug – drug-lipid melt, and functionalized NLC) on a PerkinElmer Frontier[™] FTIR Spectrometer (Waltham, MA, USA) equipped with a universal ATR attachment and a diamond crystal. For each measurement, 32 scans at a resolution of 4 cm^{-1} were accumulated at frequencies between 4000 to 600 cm^{-1} .

2.9. DSC

To study crystallinity and melting behavior, DSC analysis was performed using a DSC 214 Polyma[®] (NETZCH, Selb, Germany). Mygliol[®] 812, Precirol[®] ATO5, RLZ, drug-lipid melt, and freeze-dried functionalized NLC (10–20 mg) were put into aluminum crucibles that were perforated on top. An empty aluminum crucible was used as reference.

The thermal program included two cycles of heating/cooling. The first cycle, recorded with a scan rate of $10\text{ }^{\circ}\text{C}/\text{min}$, involved one cooling to $10\text{ }^{\circ}\text{C}$ followed by an isotherm for 5 min, a heating from 10 to $160\text{ }^{\circ}\text{C}$, and another cooling to $10\text{ }^{\circ}\text{C}$. Between cycles, an isotherm at $10\text{ }^{\circ}\text{C}$ for 5 min was performed. The second cycle comprised a heating from 10 to $160\text{ }^{\circ}\text{C}$ (at a rate of $10\text{ }^{\circ}\text{C}/\text{min}$) followed by cooling to $25\text{ }^{\circ}\text{C}$ (at a rate of $20\text{ }^{\circ}\text{C}/\text{min}$). Data were obtained using the Proteus[®] 8.0.1 software (NETZCH, Selb, Germany).

2.10. PXRD

X-ray diffractograms for Precirol[®] ATO5, RLZ, drug-lipid melt, and freeze-dried functionalized NLC were acquired using a Miniflex 600 X-ray diffractometer (Rigaku, Tokyo, Japan) equipped with Cu-K α radiation. The instrument was set at a current of 15 mA and a voltage of 40 kV. The samples were scanned from 3 to 60° (2θ) with a scanning rate of $5^{\circ}/\text{min}$ and a step size of 0.01° .

2.11. *In Vitro* Cell studies

2.11.1. Cell Culture

Mouse motor neuron-like hybrid cells (NSC-34 cell line) were obtained from Cedarlane (Hornby, ON, Canada). The cells were grown in DMEM supplemented with inactivated FBS (10%, v/v), L-glutamine (1%, v/v), nonessential amino acids (1%, v/v) and antibiotic–antimycotic mixture (1%, v/v; final concentration of 100 U/mL penicillin and 100 U/mL streptomycin).

Immortalized human cerebral microvascular endothelial cells (hCMEC/D3 cell line) were purchased from Cedarlane (Hornby, ON, Canada). The cells were grown in EndoGRO™ basal medium supplemented with FBS (5%, v/v), rhEGF (5 ng/mL), L-glutamine (10 mM), ascorbic acid (50 µg/mL), EndoGRO-LS supplement (0.2%, v/v), hydrocortisone hemisuccinate (1.0 µg/mL), and heparin sulfate (0.75 U/mL).

Cells were maintained in an incubator (Cell Culture® CO₂ incubator, ESCO GB Ltd., Barnsley, England, UK) at 37 °C in a water-saturated atmosphere with 5% CO₂ and subcultured every 2–3 days using trypsin–EDTA to detach them from the flasks. The culture medium was replaced every other day.

2.11.2. Cytotoxicity Assay

A MTT assay was performed following the methodology described by Pinto *et al.* [511], in order to evaluate the cytotoxic effect of the different formulations on the two cell lines. Passages 44–45 and passages 4–6 were used for the hCMED/D3 and NSC-34 cell lines, respectively.

Briefly, cells were seeded in 96-well plates (25×10^3 cells/mL) and exposed to different concentrations (0.1, 1, and 10 µM) of NLC Placebo, NLC RLZ, functionalized NLC, and free RLZ for 24 h. Following the removal of the formulations from each well, cells were washed with HBSS. The number of viable cells was determined by adding MTT reagent and incubating for 3 h at 37 °C. DMSO was used to solubilize the crystals. Triton X-100 1% (w/v) and culture medium were used as negative and positive controls, respectively. The absorbance was read at 590 nm with background subtraction at 630 nm. Results were expressed as percentages of cell viability.

2.11.3. Permeability Assay

hCMEC/D3 cells were seeded at a density of 2.5×10^4 cells per insert in transwell devices (6-well polyester inserts, effective area: 4.2 cm^2 , pore size: $0.4 \mu\text{m}$ and pore density: $100 \pm 10 \times 10^6/\text{cm}^2$) pre-coated with $50 \mu\text{g/mL}$ collagen I rat protein in 0.02 M acetic acid [512]. The apical and basolateral chambers of the inserts were filled with supplemented EndoGRO™ basal medium, and cells were incubated until confluence (8 days), replacing the medium each 2 days. To ensure the integrity of the hCMEC/D3 monolayer, the transendothelial electrical resistance (TEER) was measured with an epithelial voltohmmeter (EVOM) (World Precision Instruments, Sarasota, FL, USA) every time the culture medium was changed, as well as during the permeability studies [512].

Permeability studies were performed after 8 days of seeding. The culture medium was previously replaced by HBSS, as isotonic buffer solution. The RLZ-loaded NLC with $10 \mu\text{M}$ concentration and free RLZ samples in the same concentration were incubated in the apical donor compartment for 24 h at 37°C in $5\% \text{ CO}_2$, and the total amount of RLZ in the basolateral acceptor compartment was quantified after 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 8 and 24 h by High Performance Liquid Chromatography – Mass Spectrometry (HPLC-MS).

The concentration of RLZ in the basolateral compartment of the transwell device was quantified by HPLC-MS, following a method that was developed in-house. A reversed-phase C18 column (BDS Hypersil™ C18 column, $150 \text{ mm} \times 4.60 \text{ mm}$ internal diameter, $5 \mu\text{m}$ particle size; Thermo Fisher Scientific, Waltham, MA, USA) was connected to an HPLC-MS system (LCMS-8050 model, Shimadzu®, Kyoto, Japan). Chromatographic separation was achieved by isocratic mode consisting of methanol/water pH 3 80:20 (v/v) as the mobile phase, eluted at a flow rate of 1 mL/min . Injections were made in triplicate, with a sample volume of $10 \mu\text{L}$. The oven and sampler temperature were set at 45°C and 15°C , respectively. A calibration curve was made by using independent standard solutions of RLZ in ethanol ($0.375\text{--}2.4 \mu\text{g/mL}$) and fitting the respective data to the least squares linear regression, which gave an R^2 of 0.9994 (see Appendix VIII).

2.11.4. Enzyme-linked Immunosorbent Assay (ELISA)

The secretion of IL-1 α after 24 h of incubation with the NLC dispersions and free RLZ from the hCMEC/D3 cell model to the culture medium was quantified using the Human IL-1 α /IL-1F1 Quantikine® immunoassay kit, according to the protocol provided by the manufacturer [513, 514].

Briefly, 200 μ L of cell culture supernatant samples collected after 24 h of exposure during the transwell permeability assay, and 50 μ L of assay diluent were added to anti-IL-1 α monoclonal antibody-precoated microplate wells and incubated for 2 h at room temperature [514]. Afterwards, the horseradish peroxidase-conjugated secondary antibodies were added to the pre-washed test samples and the plate was incubated for 1 h at room temperature. Then, the substrate solution was added, and the plate was incubated for another 20 min. The chemical reaction was stopped by adding 50 μ L of 2 N sulfuric acid solution. UV-visible spectrophotometric measurements were performed at 450 nm using a microplate reader (Synergy HT, Bio-Tek Instruments, VT, USA), with wavelength correction at 570 nm. A calibration curve was used to calculate the IL-1 α concentration ($R^2 = 0.991$).

3. Results and Discussion

3.1. Physicochemical Characterization and Stability Studies

Assessing the physicochemical properties of the NPs is essential to determine their biological fate, as well as their passage mechanism across the BBB [515]. The characterization of NLC is summarized in Table IV.2, where the mean diameter, PDI, ZP, EE and LC are presented.

Table IV.2. Size, polydispersity index (PDI), zeta potential (ZP), encapsulation efficiency (EE) (%) and loading capacity (LC) (%) of nanostructured lipid carriers (NLC) formulations on day 0. Values are expressed as the mean $\pm$ SD ($n = 3$).

	Size (nm) ¹	PDI ¹	ZP (mV) ¹	EE (%) ²	LC (%)
NLC Placebo	180.3 $\pm$ 6.3 ^a	0.216 $\pm$ 0.023 ^b	16.97 $\pm$ 4.98 ^b	–	–
NLC Riluzole	208.5 $\pm$ 5.0 ^{a, b}	0.154 $\pm$ 0.023 ^a	20.67 $\pm$ 2.11 ^b	98.70 $\pm$ 0.96 ^a	0.93 $\pm$ 0.01
Functionalized NLC	221.9 $\pm$ 18.1 ^b	0.219 $\pm$ 0.027 ^b	–16.41 $\pm$ 0.31 ^a	94.21 $\pm$ 4.35 ^a	0.89 $\pm$ 0.50

¹ Data were analyzed using Tukey's HSD test, with different letters (a, b) representing statistically significant differences ($p < 0.05$) between the different formulations. ² Data were analyzed using the *t*-Student's test (statistical significance was considered for $p < 0.05$).

Stability studies were also performed by monitoring variations in the size, PDI, and ZP of the NLC, which were stored at 5 °C and 25 °C, for 3 months (Figure IV.1).

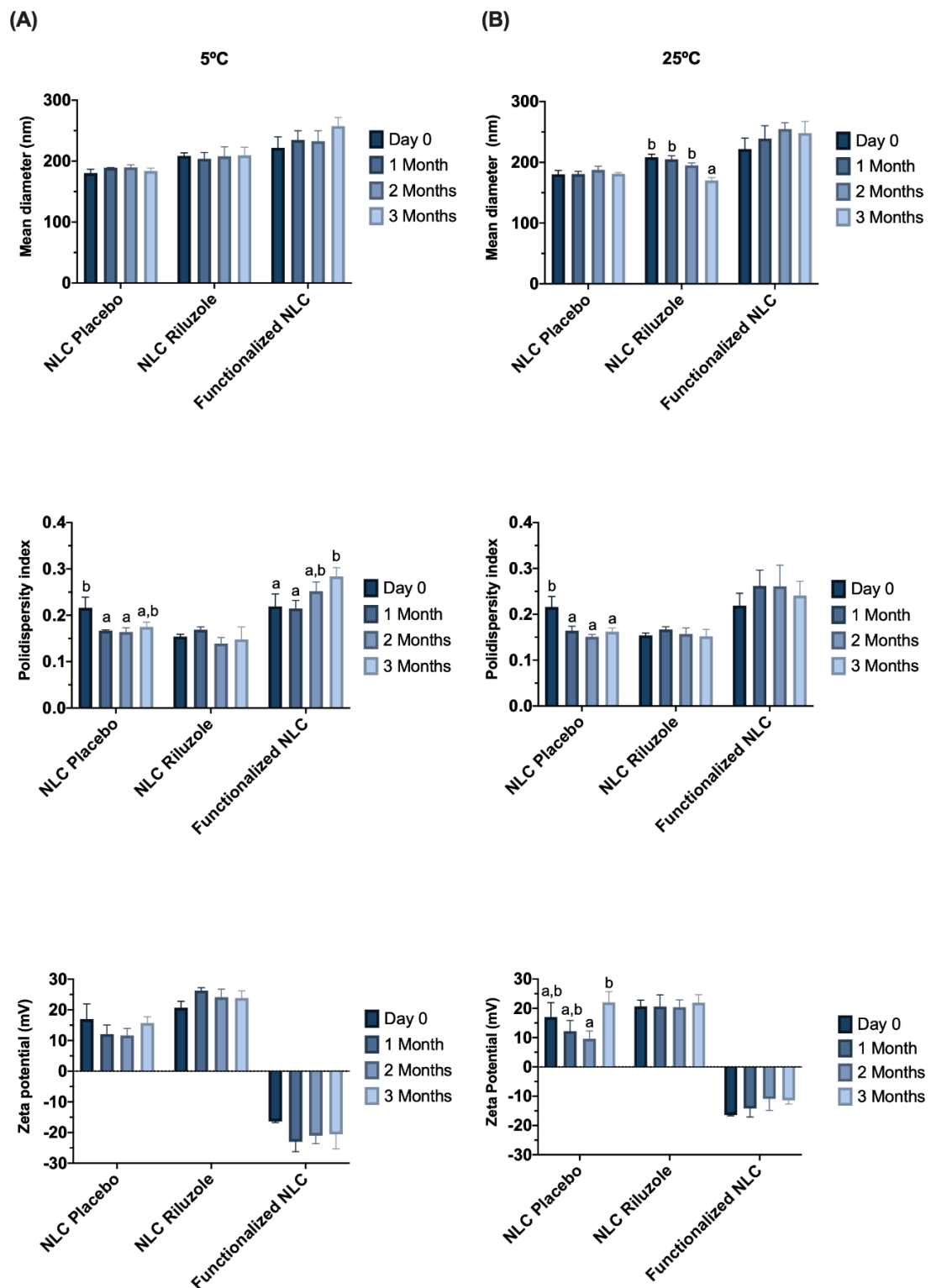


Figure IV.1. Effect of storage time on particle size, polydispersity index (PDI), and zeta potential (ZP) for nanostructured lipid carriers (NLC) Placebo, NLC Riluzole, and functionalized NLC over 3 months at 5 °C (A) and 25 °C (B). Values are expressed as the mean $\pm$ SD ($n = 3$). Data were analyzed using Tukey's HSD test, with different letters (a, b) representing statistically significant differences ($p < 0.05$) for the same formulation at different time points.

Although for functionalized nanoparticles the surface coating seems to be the most crucial determinant for BBB crossing [358], size should always be taken into consideration, given that smaller particles tend to penetrate better into the brain [515]. NLC showed an initial mean diameter lower than 220 nm (Table IV.2), with the particle size remaining below 250 nm for all formulations (Figure 1), which is an important characteristic that enables permeation through the BBB [369]. Several studies have demonstrated that different nanocarriers in this size range can effectively cross the BBB. For instance, Pinheiro *et al.* developed quercetin-loaded SLN and NLC functionalized with transferrin [369] or RVG29 [429] (all particles with sizes below 250 nm); Zensi *et al.* [516] formulated albumin NPs attached with apolipoprotein A-I, with an average size of 250–270 nm; Tosi *et al.* [361] produced PLGA polymeric NPs modified with a mutated form of diphtheria toxin (CRM197), with sizes around 220 nm; and Gu *et al.* [517] developed antibody-modified chitosan NPs for the delivery of siRNA with a mean size of 235.7 nm.

The Incorporation of RLZ and functionalization with lactoferrin did not directly influence the size of the lipid NPs, since no statistically significant differences were found between NLC Placebo and NLC RLZ, nor between NLC RLZ and functionalized NLC (Table IV.2). Furthermore, all the formulations remained stable throughout the storage period at each analyzed temperature (Figure IV.1), given that the average particle size had no tendency to increase.

As previously mentioned, PDI is a measure of the uniformity of particle size distribution. The formulations showed PDI values below 0.3 (Table IV.2), which for lipid NPs suggests a narrow and homogeneous size distribution with low variability [369, 518]. Notably, there was an increase in the PDI of the functionalized NLC over time at 5 °C ($p = 0.026$) (Figure IV.1). Nevertheless, the value remained below 0.3, implying that any aggregation phenomena that may occur are minimal.

The ZP refers to the overall surface charge of a particle and is an indicator to predict stability over time. It is known that NPs exhibit electrostatic stability and low propensity to aggregate when ZP is equal or greater than $|30|$ mV [449]. NLC Placebo and NLC RLZ presented positive ZP values between 16 and 20 mV. The presence of the drug did not have any impact in this parameter ($p = 0.505$) (Table IV.2). However, the addition of lactoferrin led to an inversion of the ZP, with the NLC acquiring a negative charge of about -16 mV. This inversion in the charge is an indicator that the functionalization occurred. Despite the initial values not being as high as desired, the ZP of the formulations did not change significantly over time at either temperature (Figure IV.1).

The EE was very high (94–98%) for all formulations, suggesting that functionalization did not affect the ability to efficiently incorporate the drug within the lipid NPs. LC was between 0.8-0.9%, which is in line with the results from Chapter III. Finally, the effect of the different temperatures (5 °C vs. 25 °C) on the various physicochemical properties was assessed at the end of the storage period. The only statistically significant difference regarded the size of the NLC RLZ ($p = 0.017$). Overall, the results of the stability studies appear to suggest that the NLC remained stable for at least 3 months.

3.2. In Vitro Release Studies

The release profiles of NLC RLZ and functionalized NLC are shown in Figure IV.2.

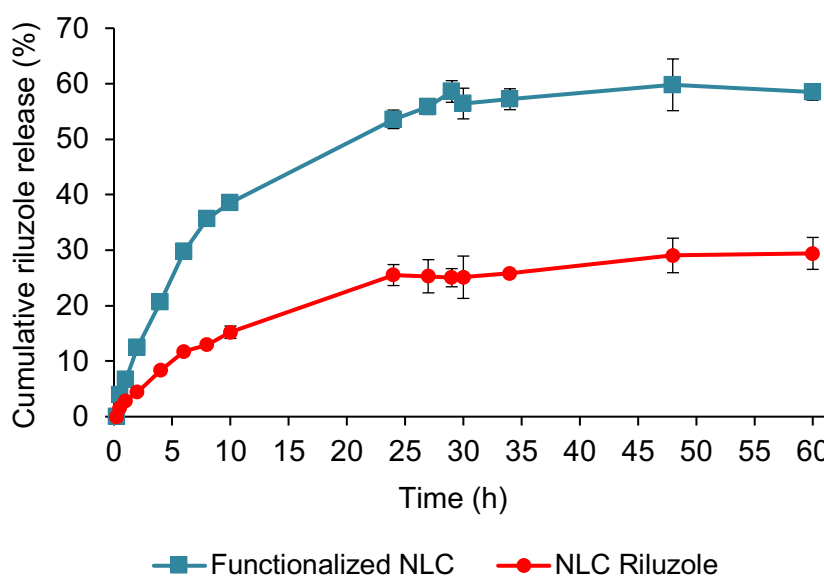


Figure IV.2. In vitro dissolution profiles of functionalized nanostructured lipid carriers (NLC) and NLC Riluzole in phosphate buffer solution (PBS) pH 7.4. Values are expressed as the mean $\pm$ SD ($n = 3$) (see Appendix VII).

Both formulations exhibited a gradual and sustained release of RLZ for 10 h, followed by a plateau phase. At the end of the assay ($t = 60$ h), only about 30% of the drug was released from NLC RLZ. This could be explained by the nature of the drug since it is a BCS class II drug, being a lipophilic compound with very low aqueous solubility and a high affinity for the lipids that constitute the NLC [488, 519].

In contrast, the functionalized NLC released twice as much, with a cumulative release around 58% after 60 h. Sebastiani *et al.* [520] demonstrated that the binding of proteins on the surface of lipid NPs can induce a redistribution of the lipids at the shell and the core, which also impacts the internal structure of the NPs, causing the release of the encapsulated compound. Therefore, the incorporation of lactoferrin could have resulted in a structural rearrangement of the lipids within the lipid matrix, leading to a higher and faster drug diffusion to the PBS medium. Moreover, lactoferrin has amphiphilic domains, possessing both hydrophilic and hydrophobic regions [521-523]. It is possible that the hydrophilic regions of lactoferrin exposed on the functionalized NLC also facilitated the interaction with the solvent molecules and released the RLZ molecules entrapped closer to the surface [524].

The results for the kinetic modeling of the RLZ release profiles from the NLC are presented in Table IV.3.

Table IV.3. Estimated kinetic parameters of the different mathematical models fitted to the riluzole (RLZ) release from nanostructured lipid carriers (NLC) Riluzole and Functionalized NLC.

Kinetic Model	Parameters	NLC Riluzole	Functionalized NLC
Zero-order	K_0	0.0254	1.2734
	R^2	0.9681	0.9689
	R^2_{adj}	0.9617	0.9626
Higuchi	K_H	0.7353	1.9151
	R^2	0.9945	0.9951
	R^2_{adj}	0.9933	0.9941
Korsmeyer-Peppas	K_{KP}	0.4999	1.2734
	R^2	0.9950	0.9957
	R^2_{adj}	0.9925	0.9935
	n	0.5548	0.5579

Both formulations showed good correlation to the Higuchi and Korsmeyer-Peppas equations ($R^2_{adj} > 0.99$). Ultimately, the Higuchi model provided the best fit for the NLC (highest R^2_{adj}). As shown in Table IV.3, the n was 0.5548 and 0.5579 for NLC RLZ and functionalized NLC, respectively. Given that $0.45 < n < 0.89$, the release can be categorized as anomalous or non-Fickian diffusion, where a combined pattern of both diffusion and lipid erosion may be involved in the transport mechanism [438, 525]. Nonetheless, since the

value of n is closer to the lower range of the interval (i.e., to 0.45), it can be considered that the release is mainly governed by diffusion.

3.3. Morphology Determination

TEM images (Figure IV.3A and B) showed that the functionalized NLC have a size around 200 nm, seemingly confirming the results obtained by DLS. Furthermore, the micrographs also revealed that the NLC were spheric and uniform in shape, with a smooth and round surface, and that there was no visible aggregation of NPs, which is a good indicator of the stability of the formulations.

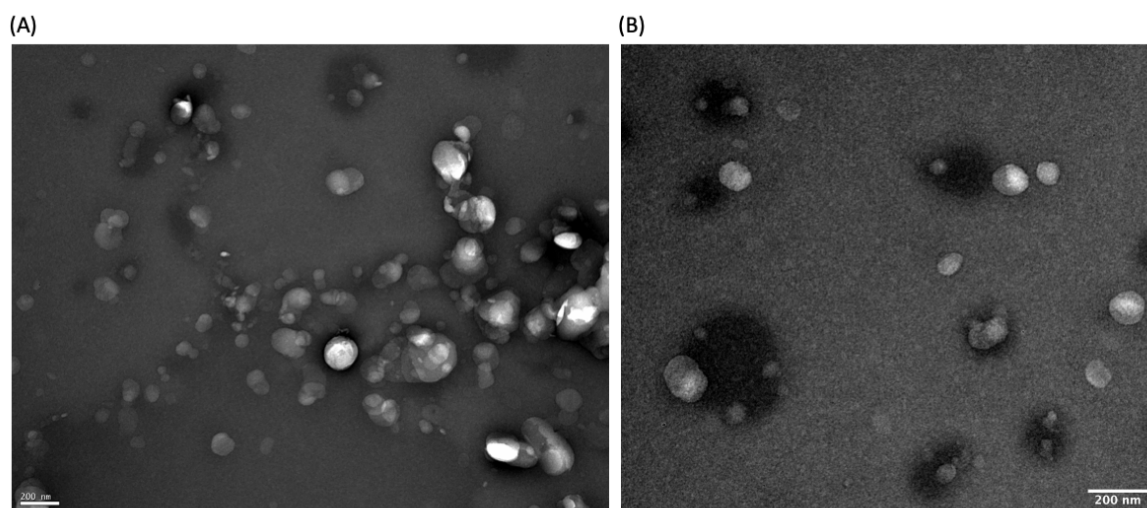


Figure IV.3. (A, B) High-resolution transmission electron microscopy (TEM) images of different sections of freshly prepared functionalized nanostructured lipid nanocarriers (NLC). Magnification of 50.000 $\times$.

3.4. Lactoferrin Conjugation Efficiency

The conjugation efficiency was assessed by the Bradford assay. Previous works with lactoferrin-functionalized nanocarriers have generally had conjugation efficiencies around 70% or lower. For instance, Singh *et al.* [480] reported SLN with a conjugation efficiency of 71%, and Huang *et al.* [526] produced liposomes with a protein conjugation efficiency of 74%. Both systems were intended for brain delivery.

The functionalized NLC obtained in the present study surpassed these values, with a coupling efficiency estimated at $95.3 \pm 0.3\%$. This indicates that the conjugation process was very efficient and that the developed NLC could, in theory, lead to a more efficient

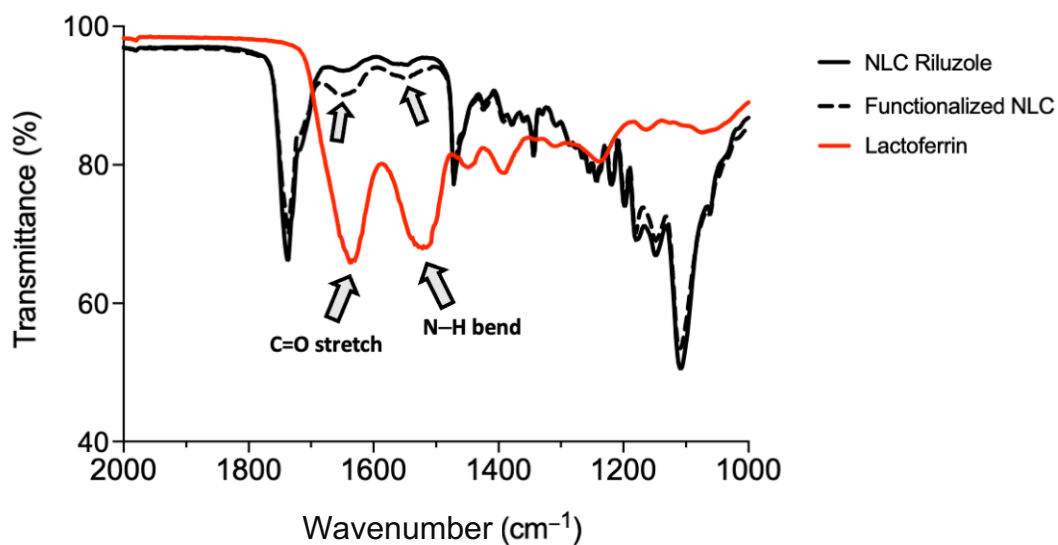
targeting and brain uptake, since more lactoferrin is available to interact with BBB endothelial cell receptors.

3.5. FTIR Spectroscopy

The functionalization of NLC was assessed by FTIR analysis. Figure IV.4A depicts two main bands in the spectrum of lactoferrin, which are also present in the functionalized NPs, specifically the bands at around 1630 and 1510 cm^{-1} , corresponding to the C=O stretching and N–H bending vibrations of the amide I and amide II functional groups of the lactoferrin protein, respectively [506, 527]. The amide I bond of lactoferrin was recorded at 1628 cm^{-1} and underwent a shift to 1640 cm^{-1} in the functionalized NLC, whereas the amide II bond shifted from 1510 to 1538 cm^{-1} . These findings seem to confirm that lactoferrin was conjugated on the surface of the NPs. Such peaks appear as well in the spectrum of NLC RLZ, although in this case, they can be attributed to the presence of the liquid lipid Miglyol® 812, which has a chemical structure containing N–H and C=O bonds [369]. Moreover, in comparison with those of the functionalized NLC, the intensity of the peaks was lower, which further corroborates that the functionalization of the NPs with lactoferrin was indeed successful [369].

To gain a better understanding on the state of the drug in the NLC, in Figure IV.4B is displayed the full FTIR spectrum of bulk RLZ, Precirol® ATO5, drug–lipid melt, and the functionalized NLC. Precirol® ATO5 presented several characteristic peaks, identified at 1470 cm^{-1} (C–C stretching), 1730 cm^{-1} (C=O stretching), and 2850 and 2914 cm^{-1} (C–H stretching), as reported elsewhere [458, 491]. RLZ showed two bands at 3266 and 3360 cm^{-1} (N–H stretching), confirming the presence of the primary amine group [488]. Additionally, the peaks at 814 and 868 cm^{-1} (C–H bending), as well those at 1460 cm^{-1} (C=C stretching), 1550 cm^{-1} (C–H in plane bending) and 1640 cm^{-1} (C=N stretching), can all be attributed to different vibrations of its benzothiazole aromatic ring [489, 490]. The molecular signatures of the drug–lipid melt and the functionalized NLC were comparable to that of Precirol® ATO5. This was to be expected, given the high lipid content compared to the drug. This also demonstrates the successful entrapment of the drug in the lipid NPs, since most absorption bands ascribed to RLZ were not visible in the NLC spectrum, and those that were, were of lower intensity.

(A)



(B)

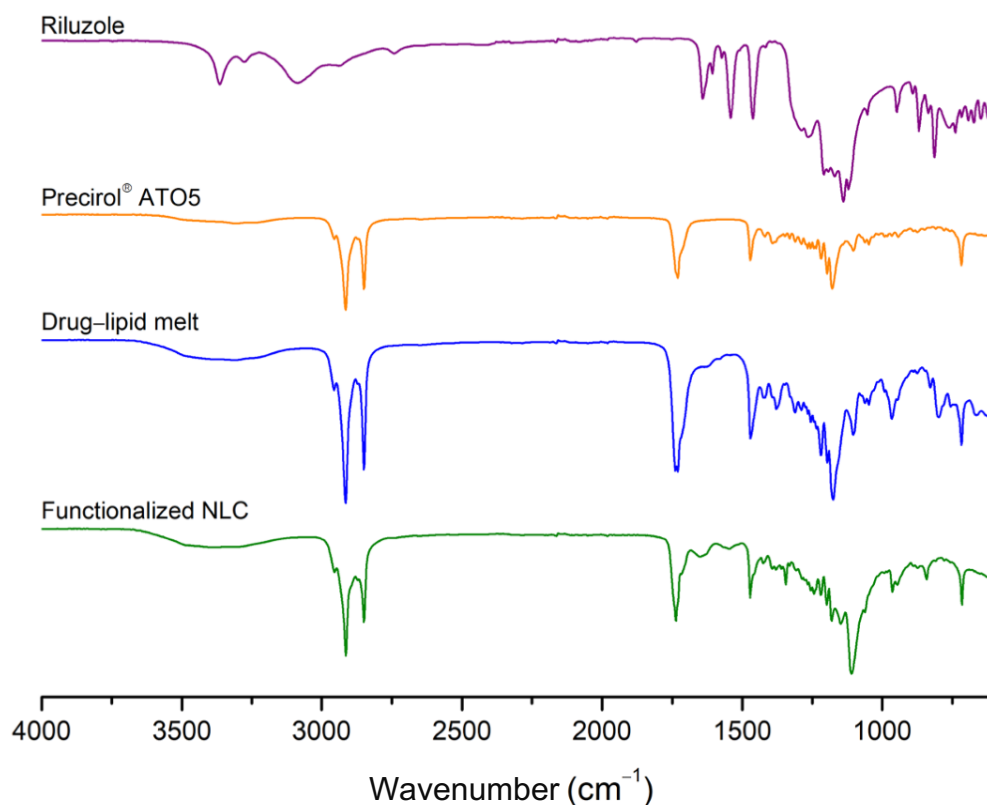


Figure IV.4. (A) Infrared spectra for nanostructured lipid carriers (NLC) before and after functionalization. Note: Lactoferrin was used as a reference to compare with the functionalized nanoparticles. To show the bands of interest, the wavenumber scale was limited to the range of 1000 to 2000 cm^{-1} ; (B) full infrared spectra of RLZ, Precirol® ATO5, drug-lipid melt, and functionalized NLC.

3.6. DSC

The overlay of the thermograms for the first DSC cycle is presented in Figure IV.5.

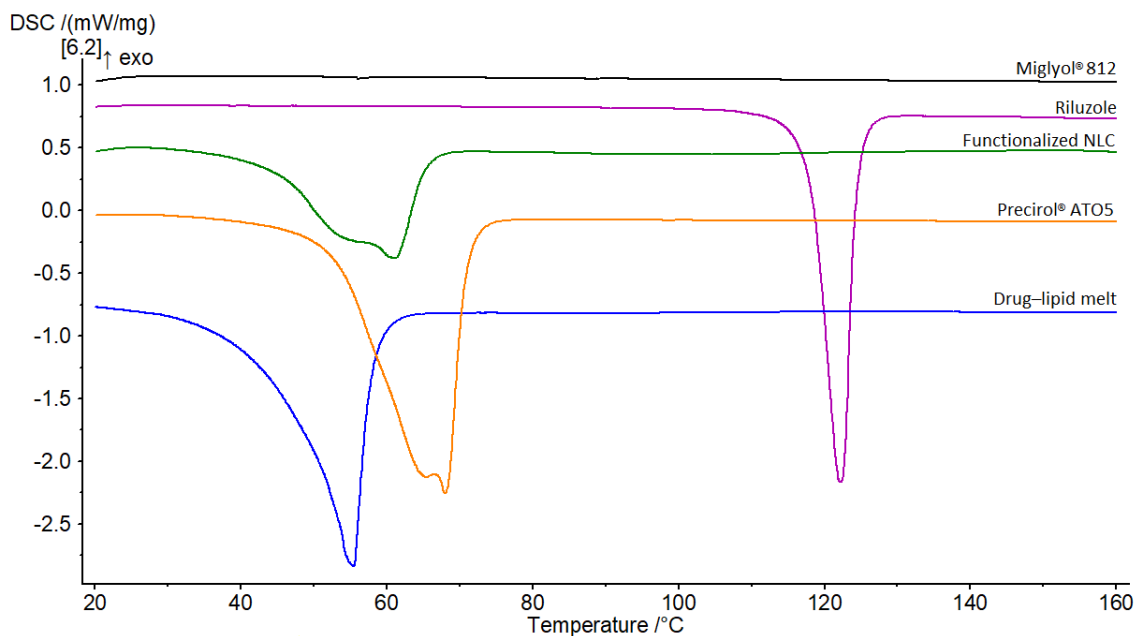


Figure IV.5. DSC thermograms of Miglyol® 812, riluzole, functionalized NLC, Precirol® ATO5 and drug-lipid melt.

RLZ demonstrated a single sharp peak indicative of a crystalline compound. It occurred at 122.1 °C, which corresponds to its melting point [488]. Bulk solid lipid Precirol® ATO5 displayed a peak at 68 °C, with a small shoulder of lower enthalpy at around 65 °C. These two distinct melting events are attributable to the presence of different polymorphic forms that occur in complex mixtures of glycerides such as Precirol® ATO5 [494]. This phenomenon has been well reported in literature, with the melting event of lower temperature corresponding to the metastable α -polymorph of the lipid and the major peak to its stable β -form [528]. Concerning Miglyol® 812, its thermogram did not show any thermal event, since this excipient is an oil at room temperature [415].

The RLZ peak was not observed in the thermogram of the melt mixture, nor in that of the functionalized NLC. This suggests that (1) the drug was well solubilized in the molten lipids and (2) it was encapsulated in an amorphous or molecularly dispersed state within the NPs [529, 530]. For both thermograms, the endothermic peaks were broader, accompanied by a shift of the onset and the melting point to lower temperatures. The shift was slightly more pronounced in the case of the drug-lipid melt, with the melting point demonstrating a 12 °C drop compared to that of the solid lipid (from 68 °C to 56 °C).

Moreover, there was a sharp decline in the melting enthalpies (ΔH). These differences are mainly due to the interactions of the solid lipid with the liquid lipid and the surfactants during the preparation process [528], which lead to a less ordered lattice that is favorable for encapsulating greater amounts of drug [491, 530]. However, a certain effect due to the nanometric size of the NLC should be taken into account as well [528]. Bunjes *et al.* [531] demonstrated that particles with smaller sizes lead to a high surface area, creating an energetically suboptimal state that causes depression of the melting point [531].

3.7. PXRD

To further investigate possible changes in the crystalline structure of the NPs, PXRD experiments were carried out. The diffraction patterns of RLZ, Precirol® ATO5, drug–lipid melt, and functionalized NLC are displayed in Figure IV.6.

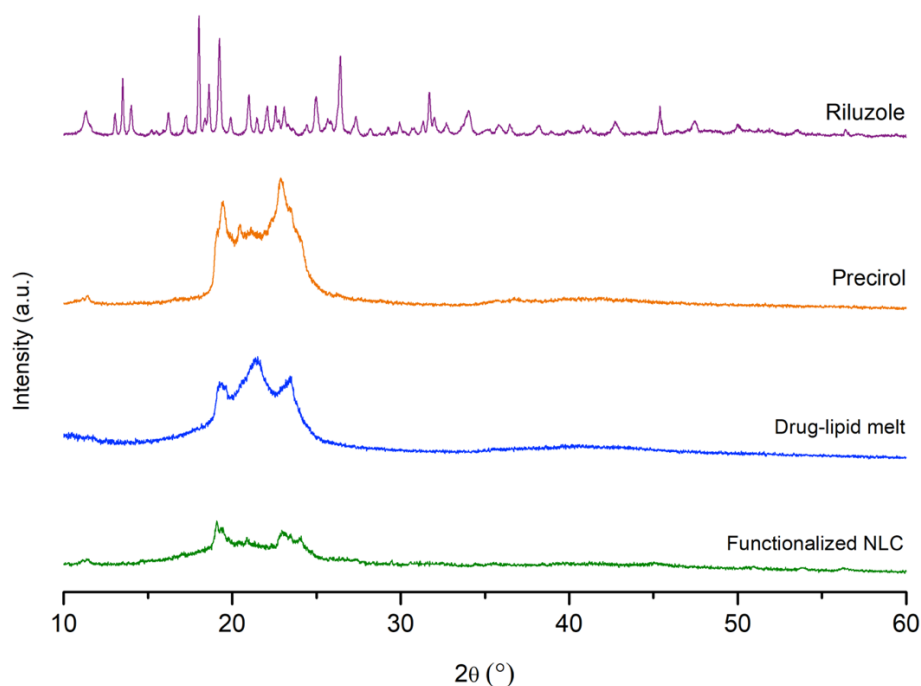


Figure IV.6. X-ray diffractograms of riluzole (RLZ), Precirol® ATO5, drug–lipid melt, and functionalized NLC.

RLZ exhibited several characteristic sharp peaks at different diffraction angles (2θ of 13.5° , 18.0° , 19.3° , 21° , 25° , 26.4° , 31.7° , 35° , and 45.4°), revealing the highly crystalline nature of the drug [532]. These peaks were absent in the diffractograms of the drug–lipid melt and of the functionalized NLC. In turn, the diffractograms essentially garnered aspects ascribed to the solid lipid, namely, the peaks at 19.5° and 23° . However, these peaks were wider and less intense compared to those of the lipid alone. Such findings indicate not only

the successful encapsulation of the drug within the lipid matrix, but a reduction in the crystalline nature of RLZ and the lipid, forming a stabilized amorphous structure [533]. The results were in agreement with the FTIR and DSC data.

3.8. Cytotoxicity Assay

To validate the nanocarriers as a safe strategy for brain drug delivery, a MTT assay was undertaken to test the cell viability after exposure to the NLC formulations [429]. As the nanosystems were developed for brain targeting aiming at the treatment of ALS, it was important to assess their potential cytotoxicity not only in a BBB model, but also in the disease-affected cells, which in ALS are the motor neurons. Therefore, two cell lines were selected: NSC-34 and hCMEC/D3. NSC-34 is a hybrid cell line generated by the fusion of motor neurons from the spinal cords of mouse embryos with mouse neuroblastoma N18TG2 cells. These cells express many motor neuron-like properties and are the most used in ALS research [534, 535]. On the other hand, hCMEC/D3 cell monolayer is one of the most reliable human BBB models, since it is easy to grow, reproducible and closely mimics the *in vivo* phenotype and permeability values [536, 537].

Figure IV.7 shows the viability, expressed as percentage of the positive control, induced in NSC-34 cells (A) and hCMEC/D3 cells (B) after 24 h of incubation in the presence of different concentrations (0.1, 1, and 10 μ M) of NLC Placebo, NLC RLZ, functionalized NLC, and free drug. Free drug was not toxic for the cell lines. It is possible to observe that the NLC did not produce any relevant cytotoxic effect on either cell line in the studied range, with viability remaining above 70% even at the highest concentration tested (10 μ M). Therefore, the incorporation of RLZ in the nanocarriers and their functionalization did not seem to affect the cell integrity, indicating that the formulations have good biocompatibility.

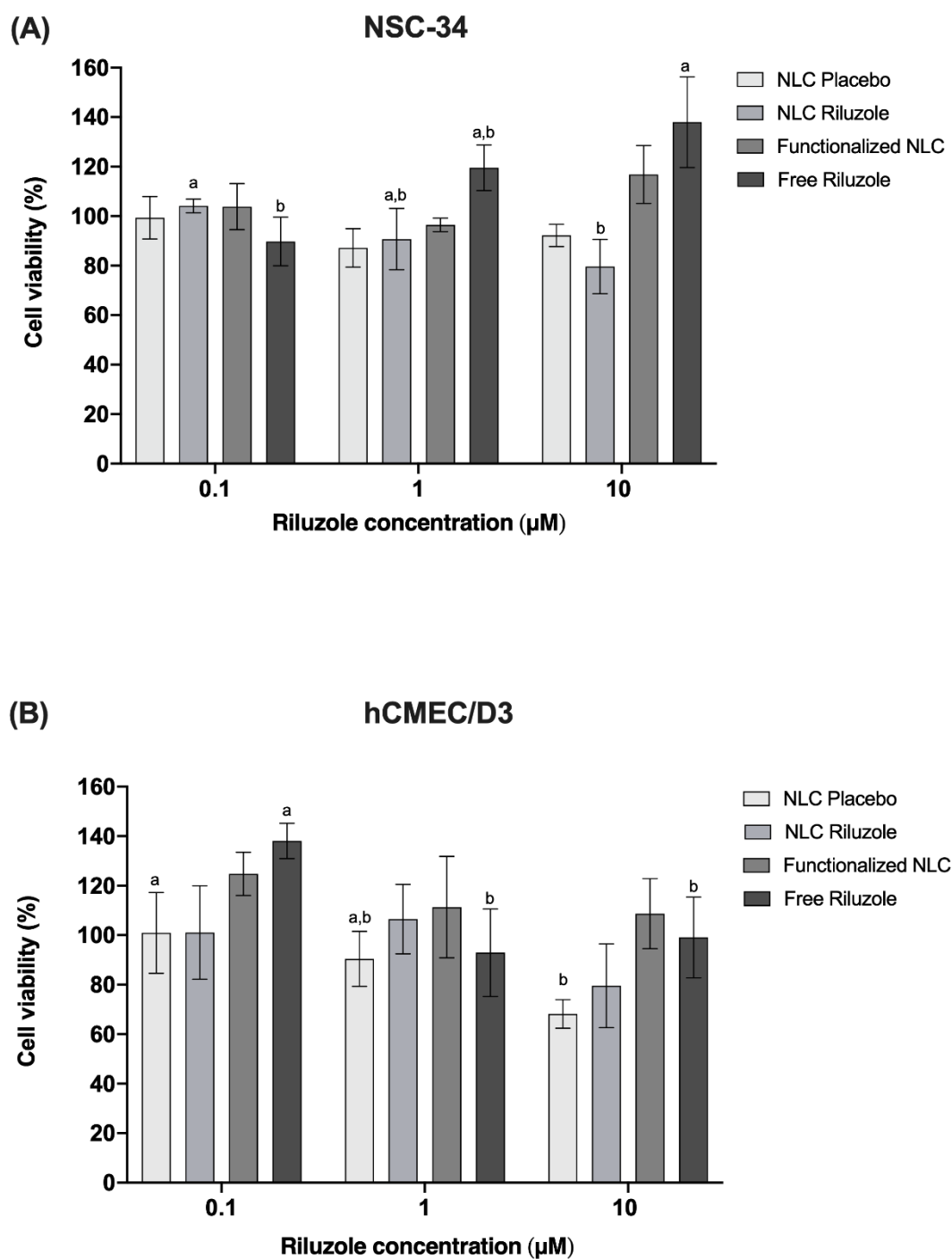


Figure IV.7. Effects of nanostructured lipid carriers (NLC) Placebo, NLC Riluzole, functionalized NLC, and free RLZ exposure on the viability of NCS-34 (A) and hCMEC/D3 (B) cells at different concentrations (0.1–10 µM), as measured by the MTT assay. Values are expressed as mean $\pm$ SD ($n = 6$). Different letters (a, b) mean significant differences between concentrations of the same sample ($p < 0.05$) according to Tukey's HSD test.

3.9. Permeability Assay

The permeation profiles of RLZ-loaded NLC (functionalized and non-functionalized) and the free drug are shown in Figure IV.8.

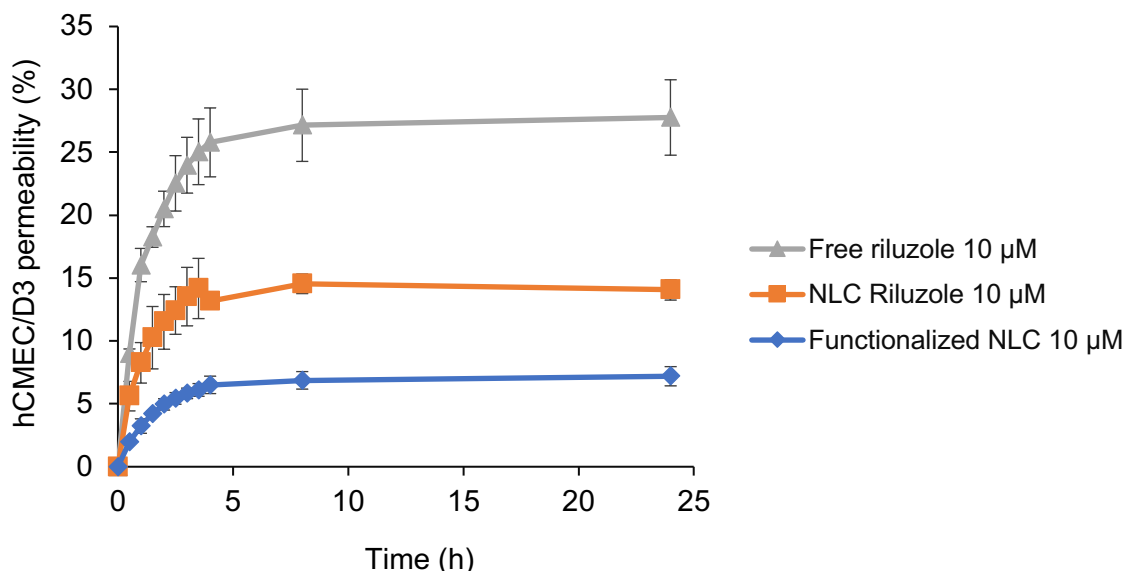


Figure IV.8. Permeation profiles of the nanostructured lipid carriers (NLC) and free riluzole (RLZ) (10 μ M) through the hCMEC/D3 monolayer over 24 h. All values represent the mean $\pm$ standard deviation ($n = 2$) (See Appendix IX).

At first glance, it is possible to observe that free RLZ had a higher permeability than the functionalized and non-functionalized NPs. BBB permeability is dependent upon the physicochemical characteristics of the molecule, such as its conformation, lipophilicity, or size [7]. RLZ is a BCS class II drug, being a lipophilic compound with a high affinity for the phospholipids that constitute the cellular membranes of the BBB [488]. Furthermore, free RLZ is molecularly dispersed, having comparatively smaller dimensions than the NLC, which had sizes between 208.5 and 221.9 nm [433]. Both aspects could have contributed to the higher free drug permeation through the BBB.

On the other hand, the BBB permeation profile of the functionalized NLC was lower in comparison to NLC RLZ. The functionalized NLC had a ZP of -16.41 ± 0.31 mV, while the NLC RLZ achieved a value of 20.67 ± 2.11 mV [433]. The overall surface charge of BBB is highly negative, due to the phospholipids in the plasma membrane and the glycocalyx [538]. The negative surface charge of the BBB may have repelled the functionalized NLC, limiting their entrance into the brain, while facilitating the penetration in the case of the NLC RLZ. Moreover, it has been shown that high concentrations of endogenous proteins (e.g.,

transferrin) can result in saturation of the binding sites on the BBB receptors, and thus of uptake mechanisms [369, 539]. It is possible that the high amount of lactoferrin used to prepare the NLC induced receptor desensitization, leading to decreased responsiveness and hindering RMT of the lactoferrin-functionalized NLC [369].

3.10. ELISA

After 24 h of incubation, free RLZ elicited IL-1 α secretion and release (2 pg/mL) in the hCMEC/D3 cell model, while for both NLC dispersions, no IL-1 α was detected. These results are in agreement with the data from the BBB permeation profiles, where the free drug had the highest penetration, and also with the literature, which has shown that interleukin release is related to increased BBB permeability [540].

4. Conclusions

The advent of nanotechnology has thrust lipid NPs such as NLC into the spotlight for the treatment of neurodegenerative diseases. Surface-modified nanocarriers can curtail the inherent constraints imposed by the BBB, enhancing drug delivery and uptake into the brain. Within this context, NLC were functionalized with a specific ligand, lactoferrin, to facilitate RLZ diffusion through the BBB and improve ALS treatment.

NLC exhibited physicochemical properties compatible with brain application (mean sizes below 250 nm, PDI below 0.3, and intermediate ZP values between $[-16]$ and $[-20]$ mV), having remained stable throughout at least 3 months of storage, allowing drug protection from adverse conditions of the physiological environment. DSC and PXRD confirmed the encapsulation of the drug inside the NPs, which was corroborated by the high encapsulation efficiency achieved (94–98%). FTIR and protein quantification studies seemed to demonstrate that the conjugation with lactoferrin was successful. RLZ permeated the BBB more than both NLC (functionalized and non-functionalized), which was also consistent with the ELISA kit results, with the free drug eliciting a higher IL-1 α production. Despite these findings, the developed NPs showed good biocompatibility, as they did not display any significant cytotoxic effects up to 10 μ M of RLZ concentration in both hCMEC/D3 and NSC-34 cells. Therefore, the nanocarrier could be considered a safe brain targeting drug delivery system.

Further research should be carried out to assess the associated transport mechanisms and internalization pathways across the BBB, as well as to test *in vivo* performance and efficacy in an ALS animal model.

Chapter V

Final Conclusions and Future Perspectives

The burden of neurodegenerative diseases has increased exponentially in the last decades. Among these, ALS stands out for its rapid progression and significant decrease in patients' quality of life. Historically, the development of effective treatments has posed a significant challenge. Numerous new molecules and off-label drugs have been investigated in clinical trials, with the majority failing to demonstrate clinical benefits or yielding conflicting data.

The clinical heterogeneity and complex molecular mechanisms of the disease complicate experiment design and data interpretation, often precluding the identification of appropriate outcomes for a given therapy. The application of enrichment designs, individual risk stratification or even prognostic modeling could mitigate the diversity of the populations studied and enhance the reproducibility and efficacy of ALS clinical trials. Moreover, the establishment of validated biomarkers, especially in scenarios where multiple targeted therapies are assessed simultaneously, may also accelerate drug development. These biomarkers can shed light on the pharmacodynamics and bioavailability of drugs, offer insights into treatment effectiveness, enable the monitoring of disease progression, and expedite the diagnostic process. Early diagnosis is vital since the initial stages of ALS are when therapies are most able to diminish or halt disease progression. Preventing motor neuron degeneration is crucial, as once these cells degenerate, they cannot be restored. In recent years there has been a significant surge in this field, driven by new technological platforms, including genetic and proteomic strategies, poised to facilitate such breakthroughs.

Beyond patient heterogeneity and poor trial design, inadequate drug properties (e.g., insufficient bioavailability/biostability), along with the existence of biological barriers such as the BBB, further hinder translational progress. Within this context, nanocarriers can significantly improve the pharmacokinetics and BBB penetration of drugs and other macromolecules (e.g., siRNA and ASO), as well as enable the simultaneous delivery of multiple compounds, potentially facilitating more effective synergistic therapeutic outcomes. Moreover, ligands can be grafted onto the nanocarrier's surface to enhance BBB targeting and achieve specific delivery to diseased cells such as motor neurons or astrocytes.

As a response to the above matters, and considering the pharmacological trends and ALS physiopathology, a lactoferrin-functionalized lipid nanocarrier drug delivery system was developed. This strategy was proposed for ALS for the first time in this work. The goal was to overcome several of the limitations associated with the currently used formulations. The nanosystem was designed to target RLZ to the BBB, protect it from adverse conditions

of the physiological environment, enhance its permeation, and gradually release it within the brain. RLZ was selected as the drug since it continues to be regarded as the benchmark treatment in ALS.

The NLC exhibited physicochemical properties compatible with brain application, having remained stable throughout at least 3 months of storage. While the functionalization process was successful, the permeability assay in brain microvascular endothelial cells (hCMEC/D3 cell line) showed that free RLZ permeated more than the functionalized lipid NPs, which could be due to the substantial amount of lactoferrin used during production, inducing receptor desensitization and reduced responsiveness. This issue could potentially be solved by reducing the lactoferrin concentration. Other general functionalization challenges that may have contributed to this outcome include improper ligand orientation or unstable ligand attachment influenced by the pH and ionic strength of the microenvironment during experimental assays. Proteins, with their intricate and delicate structures featuring numerous reactive residues, typically do not withstand harsh reaction conditions and are prone to non-specific adsorption. These parameters impact ligand bioactivity, binding capacity, and uptake efficiency. Nevertheless, the NLC demonstrated excellent biocompatibility in both hCMEC/D3 and NSC-34 cells, with no substantial cytotoxicity observed even at a concentration of up to 10 μM of RLZ and could therefore be considered a safe brain targeting drug delivery system.

Although the developed nanosystem showed some promising results *in vitro*, any extrapolation to an *in vivo* setting needs to be approached with caution. Additional studies are warranted, including evaluating the transport mechanisms and internalization pathways of the functionalized NLC across the BBB and assessing their performance and efficacy in an ALS animal model. Another key concern to be addressed is the overall biosafety of the nanocarriers and bulk materials. While lipid nanocarriers typically display a low cytotoxicity profile, understanding the cellular fate and clearance mechanisms of the NPs within the brain, as well as their systemic toxicity and elimination by the RES is crucial to mitigate clinical failure. Accordingly, a comprehensive analysis of batch to-batch variability, including sterility, endotoxin quantification, interaction with biological components, and *in vivo* immunotoxicology, should be conducted. The adoption and adherence to standardized guidelines for obtaining nanotoxicological profiles are recommended. Resorting to other functionalization strategies (i.e., using other ligands) could also be an interesting option to improve the likelihood of clinical translation.

Advances in conjugation techniques have facilitated the development of surface-modified nanocarriers for brain targeting. However, none of these formulations have reached the market yet. Several factors contribute to the slow clinical translation of targeted nanomedicines, such as preclinical models capable of closely mimicking the complex nature of the human brain, including neurodegenerative processes; inadequate clinical endpoints; vague regulatory framework; high production costs and scale-up challenges. These issues need to be tackled to streamline the number of effective and reliable functionalized lipid nanosystems. Moreover, concerted efforts between academia and industry will open new avenues to hasten clinical development and validation. Notwithstanding all constraints, the swift expansion of nanotechnology suggests that groundbreaking CNS targeting strategies are likely to emerge, thereby enhancing the prospects of functionalized lipid nanosystems making a significant contribution to ALS treatment in the future.

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Appendices

Appendix I – DSC for the Lipid Screening Studies

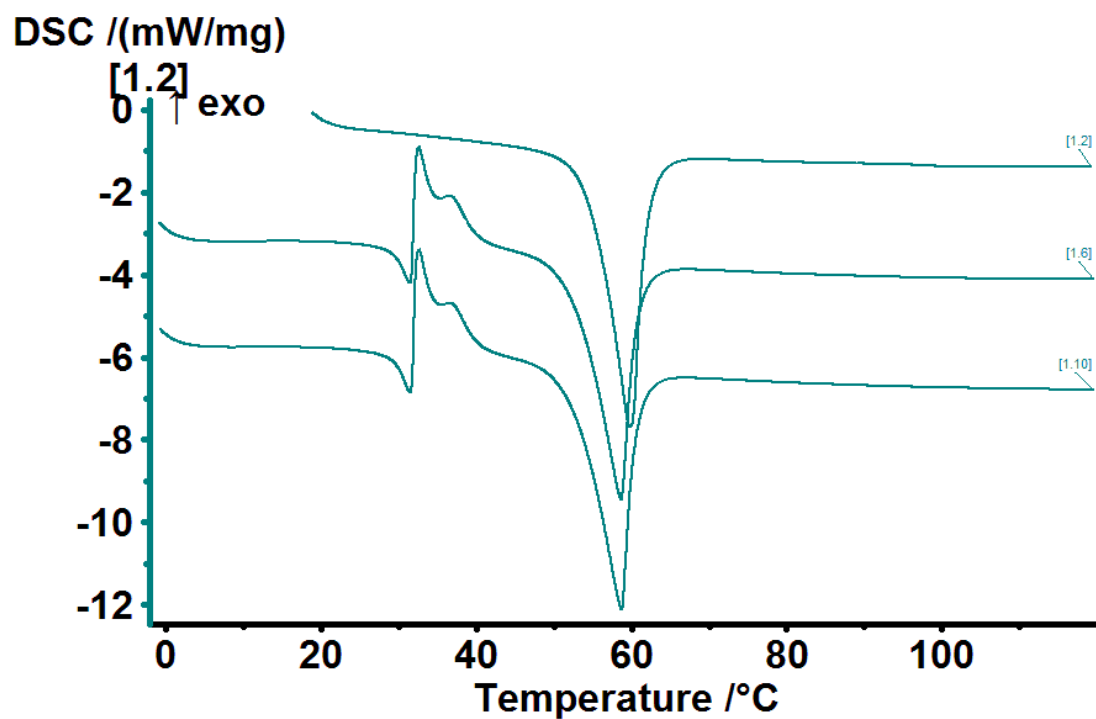


Figure S1. DSC thermogram comprising three cycles for Dynasan® 114 as a complement to the lipid screening studies.

Appendix II – HPLC Method

1. HPLC Method Validation

An HPLC method for RLZ quantification was developed in accordance with the ICH guideline Q2(R1) for the validation of analytical procedures [421]. The assessed parameters were system suitability, linearity, specificity, accuracy, and precision.

1.1. System suitability

Testing system suitability is crucial to ensure that the performance of the chromatographic system meets the required quality standards and produces acceptable results. System suitability was assessed by doing an elution gradient and evaluating its impact on several parameters, including the retention time (R_t), retention factor (k'), asymmetry factor (AF) and number of theoretical plates (N). Using a fixed stationary phase, the ratio of organic/aqueous phase was changed by gradually increasing the acidified aqueous phase from 20% to 40%. Ratios higher than 40% aqueous phase were not considered, as the drug peak took too long to elute.

The results for the variation of the elution gradient on the selected chromatographic parameters are displayed in Figure S2. Moreover, the corresponding acceptance limits are presented in Table S1.

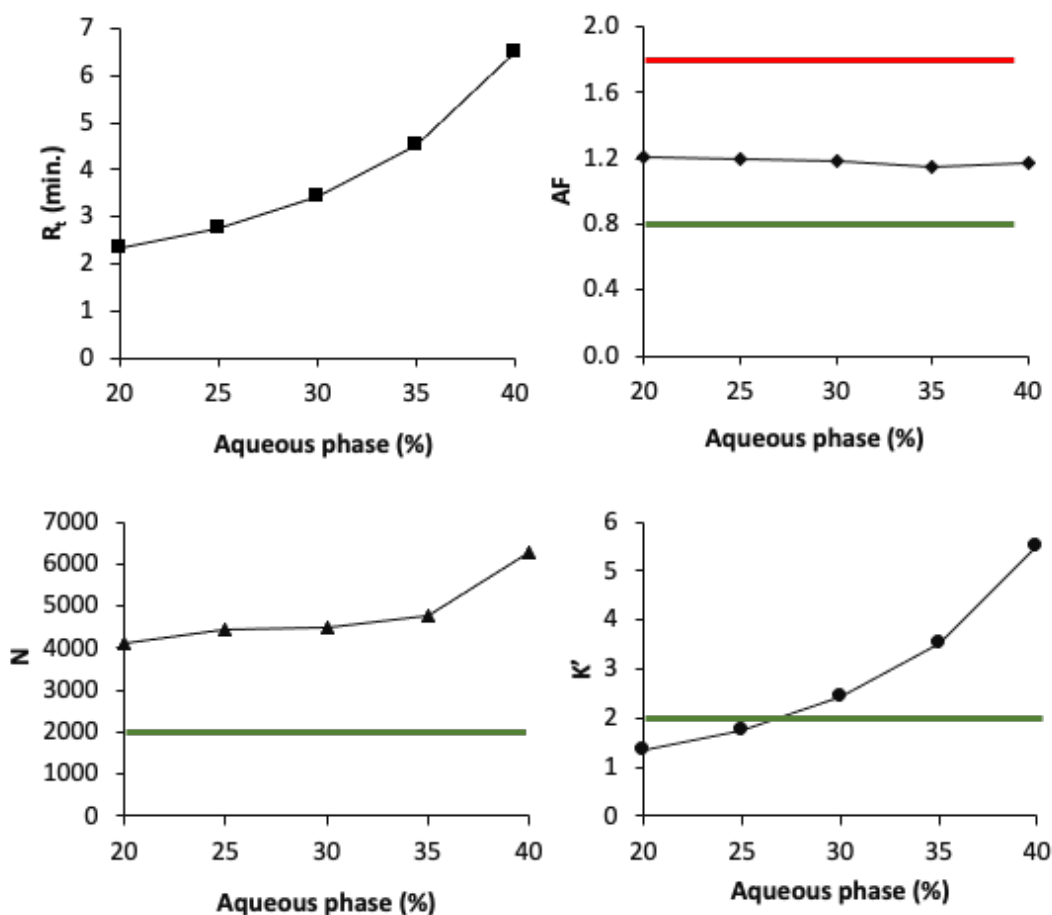


Figure S2. Chromatographic parameters selected to assess system suitability. The green lines indicate the minimal ideal values, whereas the red line in the AF graph indicates the value below which peak asymmetry should remain.

Table S1. Acceptance limits for the chromatographic parameters related to system suitability.

Parameter	Acceptance limits [422, 541]
Retention time (R_t)	–
Retention factor (k')	> 2
Asymmetry factor (AF)	0.8 – 1.8
Theoretical plate number (N)	> 2000

The theoretical plate number (N) is a measure of column efficiency. Columns with higher plate number are more efficient, providing narrower peaks and better separation among them [542]. Generally, it is recommended that N should be > 2000. As can be seen in Figure S2, any of the tested ratios permitted values almost double of that.

Regarding AF , this parameter was not affected by the polarity of the mobile phase, with values ranging between 1.15 and 1.20. Achieving symmetric peaks ($AF = 1.0$) is advantageous, as fronting or tailing behavior (i.e., asymmetric peaks) may cause problems regarding resolution, quantification, and reproducibility. For all the tested ratios, AF remained within the accepted limits (0.8 – 1.8, Table S1). Furthermore, it is possible to observe that as the percentage of aqueous phase increased, so did the R_t and k' values. This outcome was anticipated due to the nonpolar nature of the stationary phase and the drug's low aqueous solubility. As the mobile phase's polarity increases, the drug is less prone to interact with it, demonstrating a stronger affinity for the column. Excessive retention wastes valuable analysis time and leads to higher costs [543]. Therefore, the selection of the composition of the mobile phase should be such as to achieve a reasonable balance between all these parameters.

Typically, the requirement is for k' to be greater than 2. This ensures effective separation of early-eluting peaks from the solvent front. As depicted in Figure S2, acceptable k' were achieved when the percentage of aqueous phase was 30% or higher. Given the assurance of peak AF and the number of theoretical plates at any ratio, the choice of the final proportions of the eluents were determined by enabling minimal elution time with good k' values. Thus, the ratio of 70:30 (v/v) methanol/water acidified with acetic acid to pH 3 was selected.

1.2. Linearity

Linearity is the method's capability to achieve results that exhibit a direct proportionality to the concentration of the analyte within a specified range [544]. The linearity was assessed by plotting a calibration curve for RLZ quantification, which was developed by analyzing independent standard drug solutions (3.36 – 40.27 $\mu\text{g/mL}$) and fitting the resulting data to the least squares linear regression. Additionally, a residual analysis was conducted. The calibration curve for the HPLC method is presented in Figure S3.

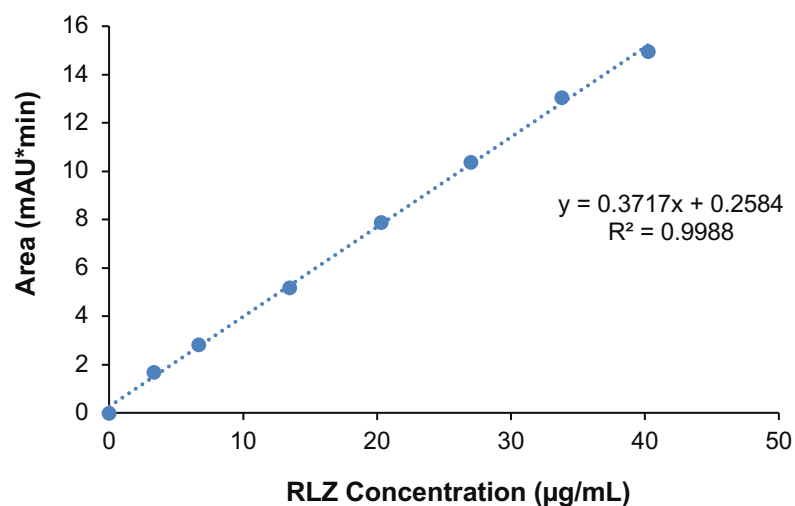


Figure S3. Calibration curve for the quantification of RLZ with HPLC ($n = 3$).

The R^2 was 0.9988, denoting a strong linear fit and confirming that the attained linearity is suitable for quantifying RLZ within the specified concentration range. Moreover, the residual analysis did not show any tendency, with a random pattern being observed (Figure S4). A summary output of the regression and residuals analysis for this method is depicted in Figure S5.

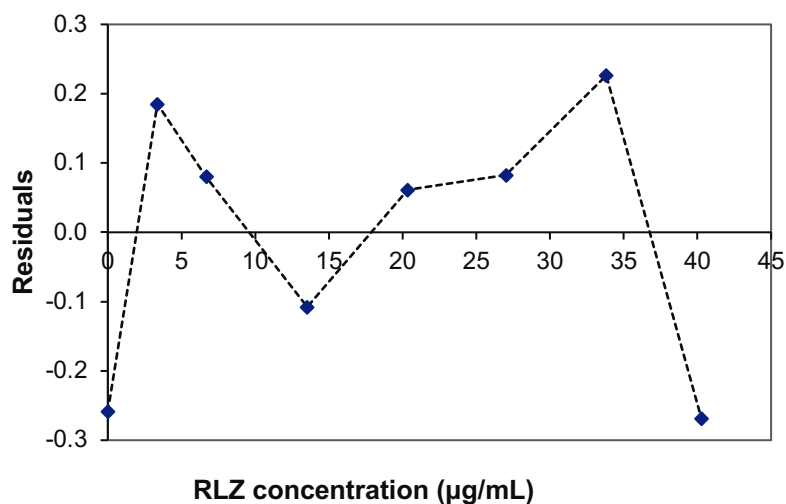


Figure S4. Graph of residuals for the HPLC method.

Regression Statistics	
Multiple R	0.99939774
R Square	0.998795842
Adjusted R Square	0.998595149
Standard Error	0.205334306
Observations	8

ANOVA					
	<i>df</i>	<i>SS</i>	<i>MS</i>	<i>F</i>	<i>Significance F</i>
Regression	1	209.8300249	209.8300249	4976.736012	5.45879E-10
Residual	6	0.252973062	0.042162177		
Total	7	210.082998			

	<i>Coefficients</i>	<i>Standard Error</i>	<i>t Stat</i>	<i>P-value</i>	<i>Lower 95%</i>	<i>Upper 95%</i>	<i>Lower 95.0%</i>	<i>Upper 95.0%</i>
Intercept	0.2583897	0.119943223	2.15426691	0.07466525	-0.035101	0.55188021	-0.03510078	0.551880209
X Variable	0.3716968	0.005268858	70.5459851	5.4588E-10	0.358804	0.38458921	0.358804349	0.384589212

RESIDUAL OUTPUT				
<i>Observation</i>	<i>Predicted Y</i>	<i>Residuals</i>	<i>Standard Residuals</i>	
1	0.258389716	-0.258389716	-1.359211704	
2	1.507290899	0.185042435	0.973381786	
3	2.74132421	0.080009123	0.420873318	
4	5.280013221	-0.108013221	-0.568183735	
5	7.818702232	0.061297768	0.322445666	
6	10.29791976	0.082413575	0.433521496	
7	12.8217409	0.226259102	1.190194505	
8	15.22661907	-0.268619068	-1.413021332	

Figure S5. Summary output of the regression and residual data analysis for the HPLC method.

1.3. Specificity

Specificity refers to the ability to precisely measure the response of the analyte of interest in the presence of other compounds [544]. This parameter was evaluated by injecting supernatant from empty NLC and SLN formulations and comparing their respective chromatograms with the chromatogram obtained for a RLZ standard solution, to identify potential interferences, including matrix components (lipids and surfactants), degradation products or impurities.

The chromatograms in Figure S6 revealed that the mobile phase and the excipients of the lipid nanoparticles did not interfere with the drug analysis. RLZ eluted at a retention time of 3.547 min, and its peak did not appear in the chromatograms of either the empty NLC nor the empty SLN formulations. Therefore, the method was deemed specific for RLZ quantification.

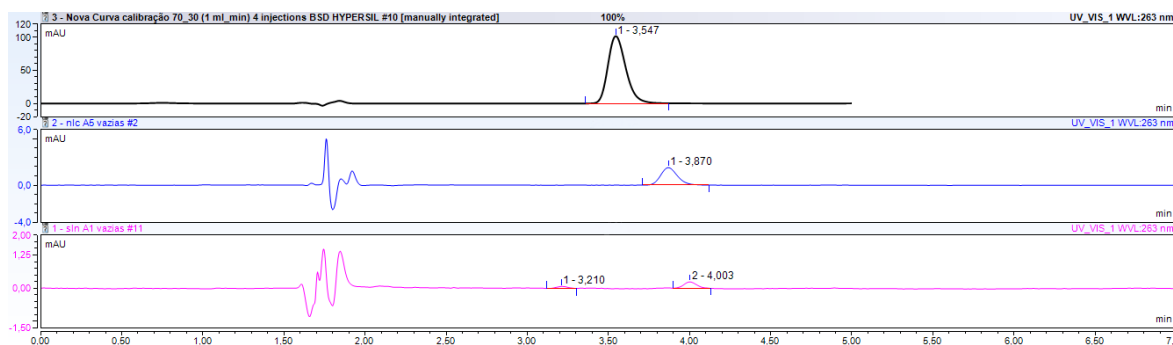


Figure S6. Chromatogram of the RLZ standard (upper panel, black line), of the empty NLC formulation (middle panel, blue line) and of the empty SLN formulation (lower panel, pink line).

1.4. Accuracy

Accuracy reports to the closeness between test results and accepted reference values [544]. This parameter was assessed by analyzing three independent replicates of three concentrations (6.68, 20.34 and 33.80 µg/mL) representing low, medium, and high concentration levels within the observed range of the calibration curve. The measured concentrations were then compared to theoretical values, and the RLZ percentage recovery was calculated as follows:

$$\text{Recovery (\%)} = \frac{\text{Concentration found}}{\text{Theoretical concentration}} \times 100 \quad \text{Equation S1}$$

The results were expressed in terms of mean percentage recovery (mean ± SD), and coefficient of variation (CV) (Table S2).

Table S2. Determination of accuracy of the HPLC method (mean ± S.D., n=3).

Theoretical RLZ concentration (µg/mL)	Mean Recovery (%)	CV (%)
6.68	101.55 ± 3.76	3.70
20.34	97.94 ± 2.09	2.14
33.80	99.39 ± 1.54	1.55

The mean recovery was between 97 and 101%. While the ICH guidelines lack acceptance criteria for accuracy and precision, both González *et al.* [544] and Stewart *et al.* [545] propose that the CV should not surpass 15%. As shown in Table S2, for all tested concentrations, the CV was less than 4%, which is below the recommended limit. This suggests minimal variability between theoretical and experimental values, establishing a high level of accuracy for the developed method.

1.5. Precision

Precision indicates the closeness between values in a series of measurements carried out under identical analytical conditions for multiple replicates of the same sample [544]. It can be assessed in the same day (intra-day precision or repeatability) or in multiple days (inter-day or intermediate precision). Intra-day precision was determined by analyzing three independent replicates at low, medium, and high concentrations (6.68, 20.34 and 33.80 µg/mL) in a single day. Inter-day precision was determined using one replicate of each of the previous concentrations, over three consecutive days. The results are presented in Table S3.

Table S3. Intra and inter-day precision of the HPLC method (mean $\pm$ S.D., $n=3$).

Theoretical RLZ concentration (µg/mL)	Intra-day Precision		Inter-day Precision	
	Mean concentration (µg/mL)	CV (%)	Mean concentration (µg/mL)	CV (%)
6.68	6.54 $\pm$ 0.07	1.05	6.41 $\pm$ 0.32	1.51
20.34	20.08 $\pm$ 0.44	2.18	20.19 $\pm$ 0.21	1.03
33.80	34.36 $\pm$ 1.35	3.93	33.17 $\pm$ 0.33	1.00

The results demonstrate the existence of intra and inter-day precision, as evidenced by CV values below 4%. Therefore, the method is compliant with the previously mentioned recommendation.

Appendix III – Spectrophotometry Method

The calibration curve for the spectrophotometry method is presented in Figure S7. The R^2 obtained was almost 1, showing that the linearity is adequate for RLZ quantification. Moreover, the residuals graph (Figure S8) did not show any discernible pattern, confirming the randomness of the data. The response's linearity was further confirmed by a RIKILT test. No data point exhibited a response factor ratio (Absorbance/RLZ concentration) deviation exceeding 5% (Figure S9), supporting the proper linearity of the signal response. A summary output of the regression and residuals analysis for this method is also depicted (Figure S10).

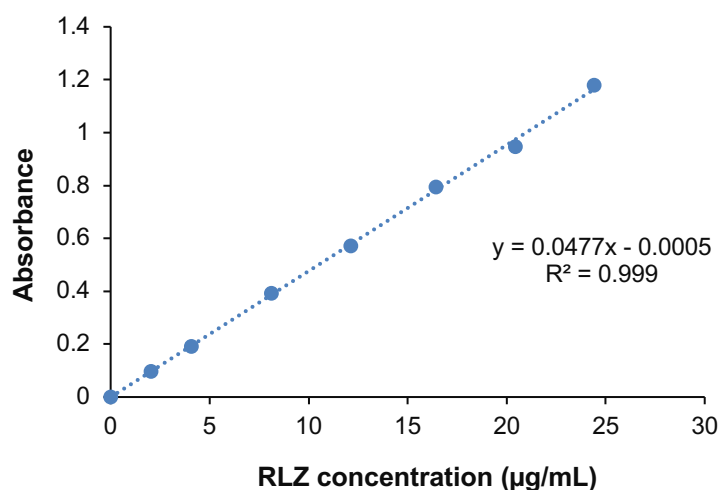


Figure S7. Calibration curve for the quantification of RLZ with spectrophotometry ($n = 3$).

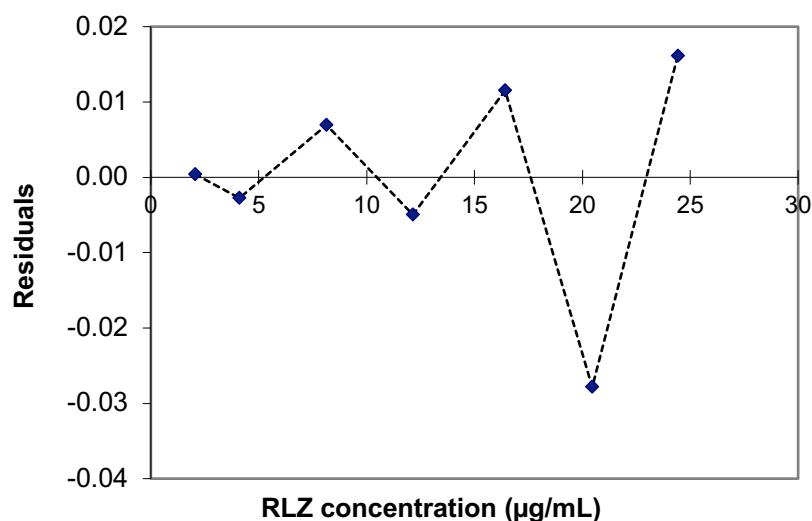


Figure S8. Graph of residuals for the spectrophotometry method.

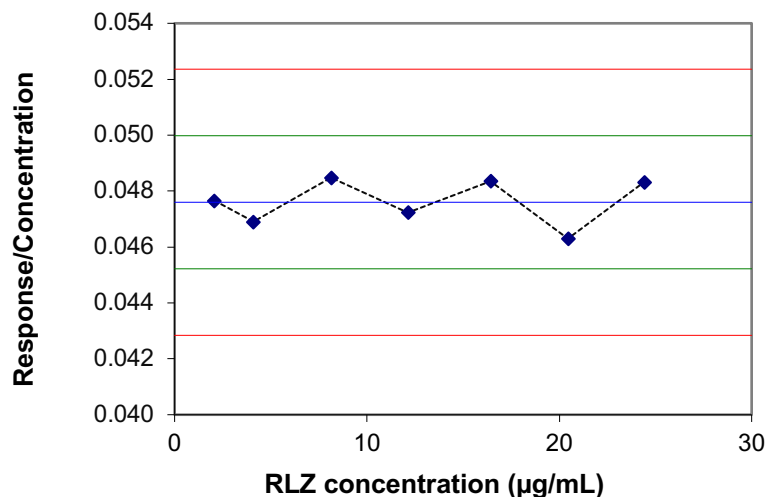


Figure S9. Graph of the response factor ratio for the spectrophotometry method.

Regression Statistics	
Multiple R	0.999507
R Square	0.999014
Adjusted R Square	0.99885
Standard Error	0.014433
Observations	8

ANOVA					
	<i>df</i>	<i>SS</i>	<i>MS</i>	<i>F</i>	<i>Significance F</i>
Regression	1	1.266457	1.266457	6079.412	2.99636E-10
Residual	6	0.00125	0.000208		
Total	7	1.267707			

	<i>Coefficients</i>	<i>Standard Error</i>	<i>t Stat</i>	<i>P-value</i>	<i>Lower 95%</i>	<i>Upper 95%</i>	<i>Lower 95.0%</i>	<i>Upper 95.0%</i>
Intercept	-0.0004898	0.008426318	-0.05813	0.955535	-0.021108253	0.02012866	-0.0211083	0.020128664
X Variable 1	0.0476845	0.000611571	77.97058	3E-10	0.046188053	0.04918097	0.04618805	0.049180971

RESIDUAL OUTPUT			
<i>Observation</i>	<i>Predicted Y</i>	<i>Residuals</i>	<i>Standard Residuals</i>
1	-0.0004898	0.000489794	0.036654
2	0.0977403	0.000426367	0.031907
3	0.1945208	-0.00272079	-0.20361
4	0.3875477	0.00695231	0.520281
5	0.5781331	-0.00493315	-0.36918
6	0.7826043	0.011495665	0.860286
7	0.9745631	-0.02786311	-2.08516
8	1.1640804	0.016152903	1.208814

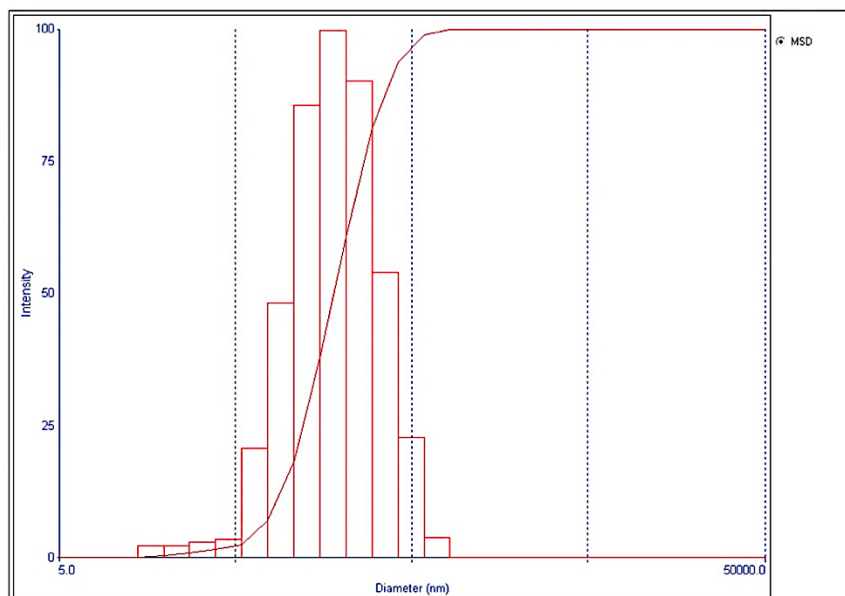
Figure S10. Summary output of the regression and residual data analysis for the spectrophotometry method.

Appendix IV – DLS data

Figures S11 to S35 demonstrate the DLS graphs obtained for one batch of each of the SLN and NLC formulations that were developed in the experimental work.

Formulation F1

Effective Diameter: **151.7 nm**
Polydispersity: **0.229**
Avg. Count Rate: **365.8 kcps**
Baseline Index: **9.3/ 97.80%**
Elapsed Time: **00:12:00**

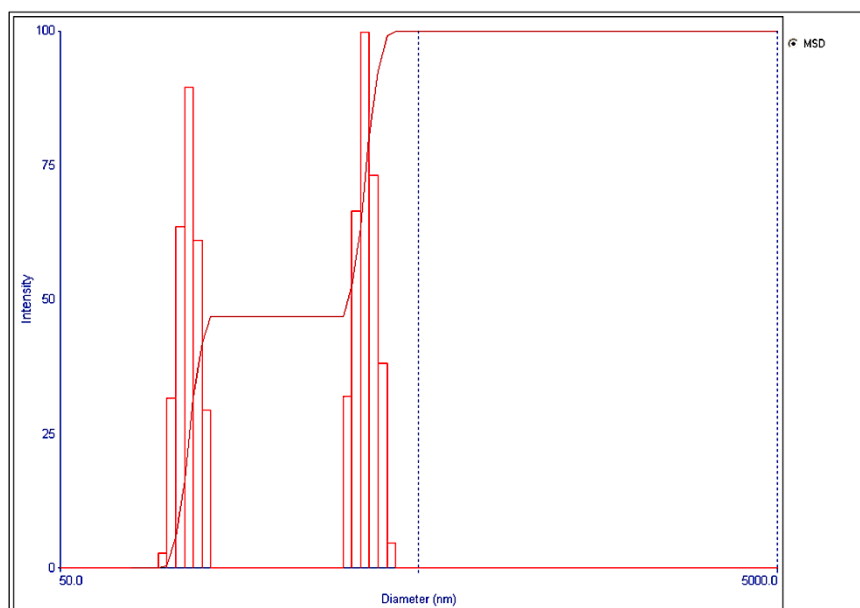


Run	Eff. Diam. (nm)	Half Width (nm)	Polydispersity	Baseline Index
1	149.8	70.1	0.219	7.3/ 96.64%
2	152.8	70.4	0.212	9.0/100.00%
3	149.7	75.0	0.251	9.9/ 97.76%
4	154.4	71.7	0.216	8.8/ 95.75%
5	152.3	73.9	0.235	9.3/ 97.76%
6	151.0	73.5	0.237	9.2/ 98.88%
Mean	151.7	72.4	0.228	9.0/ 97.80%
Std. Error	0.7	0.8	0.006	0.3/ 0.62
Combined	151.7	72.5	0.229	9.3/ 97.80%

Figure S11. DLS graph for formulation F1.

Formulation F2

Effective Diameter: **181.3 nm**
Polydispersity: **0.224**
Avg. Count Rate: **494.3 kcps**
Baseline Index: **7.3/ 99.26%**
Elapsed Time: **00:03:00**

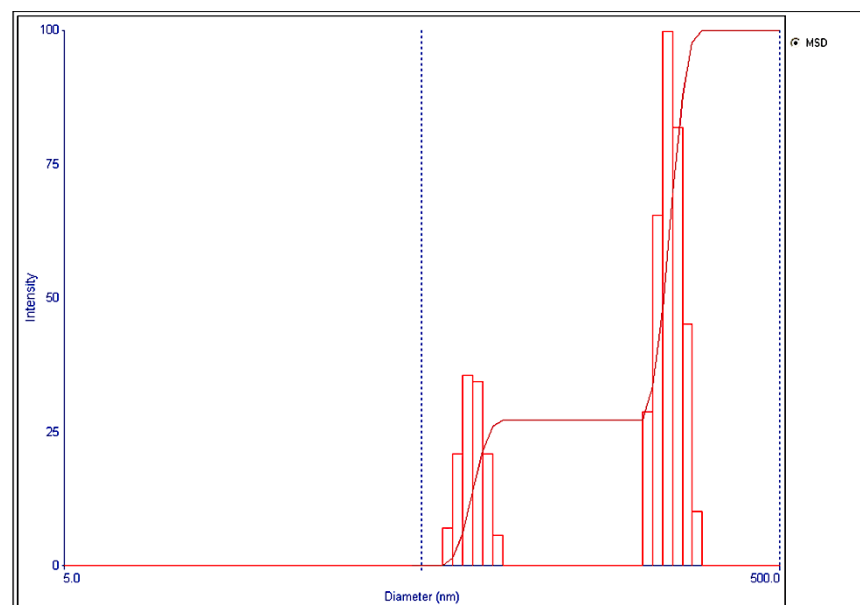


Run	Eff. Diam. (nm)	Half Width (nm)	Polydispersity	Baseline Index
1	178.5	82.7	0.214	8.6/100.00%
2	178.7	78.0	0.191	3.9/100.00%
3	188.1	95.1	0.255	9.3/ 97.79%
Mean	181.7	85.3	0.220	7.3/ 99.26%
Std. Error	3.2	5.1	0.019	1.7/ 0.74
Combined	181.3	85.9	0.224	7.3/ 99.26%

Figure S12. DLS graph for formulation F2.

Formulation F3

Effective Diameter: **152.8 nm**
Polydispersity: **0.242**
Avg. Count Rate: **399.9 kcps**
Baseline Index: **9.6/ 99.07%**
Elapsed Time: **00:12:00**

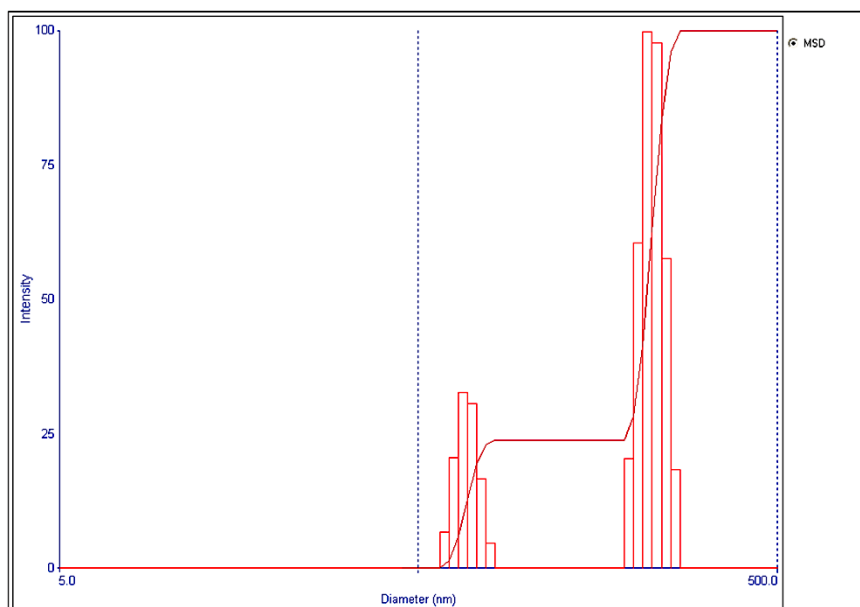


Run	Eff. Diam. (nm)	Half Width (nm)	Polydispersity	Baseline Index
1	146.8	71.3	0.236	9.6/ 98.65%
2	152.2	74.3	0.238	9.5/ 98.88%
3	152.6	74.1	0.236	8.6/100.00%
4	155.8	78.3	0.252	9.5/ 99.88%
5	154.3	77.0	0.249	8.9/100.00%
6	155.3	76.7	0.244	9.3/100.00%
Mean	152.8	75.3	0.242	9.2/ 99.07%
Std. Error	1.3	1.0	0.003	0.2/ 0.53
Combined	152.8	75.2	0.242	9.6/ 99.07%

Figure S13. DLS graph for formulation F3.

Formulation F4

Effective Diameter: **150.7 nm**
Polydispersity: **0.214**
Avg. Count Rate: **384.7 kcps**
Baseline Index: **9.7/ 98.92%**
Elapsed Time: **00:12:00**

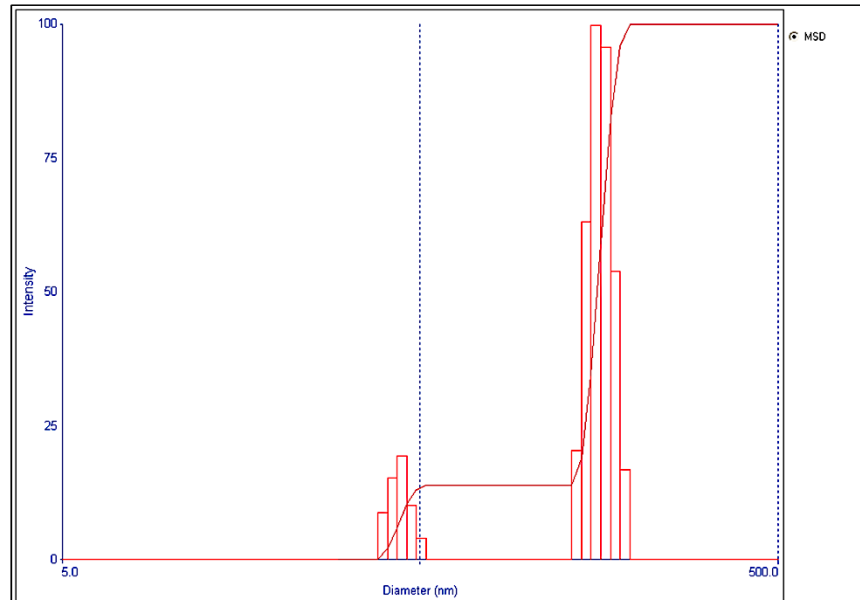


Run	Eff. Diam. (nm)	Half Width (nm)	Polydispersity	Baseline Index
1	146.3	61.7	0.178	8.6/100.00%
2	150.3	69.2	0.212	9.1/ 95.54%
3	150.8	68.8	0.208	9.8/100.00%
4	151.4	74.0	0.239	7.2/100.00%
5	155.5	70.9	0.208	9.0/ 99.11%
6	152.6	74.0	0.235	8.8/ 98.88%
Mean	151.1	69.8	0.213	8.6/ 98.92%
Std. Error	1.2	1.9	0.009	0.4/ 0.71
Combined	150.7	69.7	0.214	9.7/ 98.92%

Figure S14. DLS graph for formulation F4.

Formulation F5

Effective Diameter: **122.3 nm**
Polydispersity: **0.186**
Avg. Count Rate: **460.5 kcps**
Baseline Index: **9.7/ 96.92%**
Elapsed Time: **00:12:00**

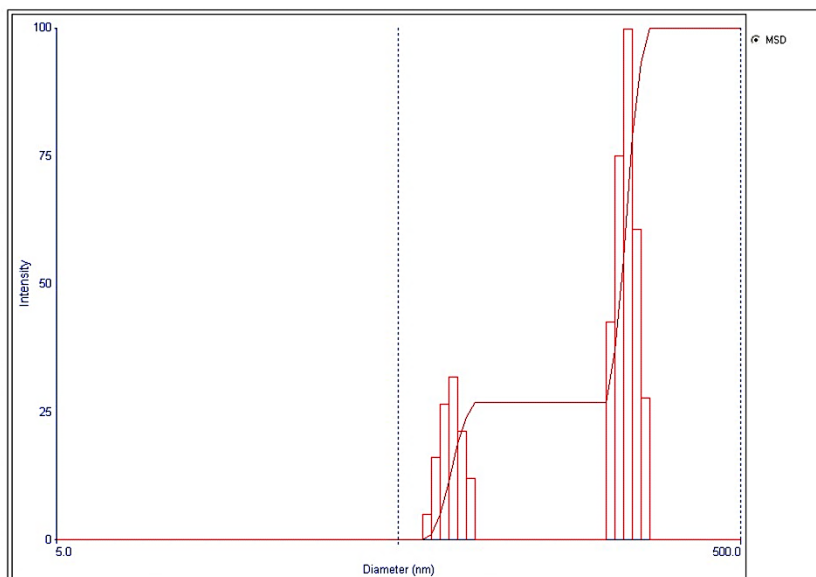


Run	Eff. Diam. (nm)	Half Width (nm)	Polydispersity	Baseline Index
1	117.0	51.6	0.194	9.4/ 98.89%
2	121.8	52.5	0.186	8.6/ 96.00%
3	124.1	54.3	0.192	9.4/ 98.89%
4	124.2	65.3	0.198	8.7/ 95.56%
5	123.7	51.5	0.173	7.4/ 94.43%
6	123.0	51.7	0.177	8.8/ 97.77%
Mean	122.3	52.8	0.187	8.7/ 96.92%
Std. Error	1.1	0.7	0.004	0.3/ 0.76
Combined	122.3	52.8	0.186	9.7/ 96.92%

Figure S15. DLS graph for formulation F5.

Formulation F6

Effective Diameter: **150.2 nm**
Polydispersity: **0.223**
Avg. Count Rate: **446.2 kcps**
Baseline Index: **9.7/ 98.59%**
Elapsed Time: **00:12:00**

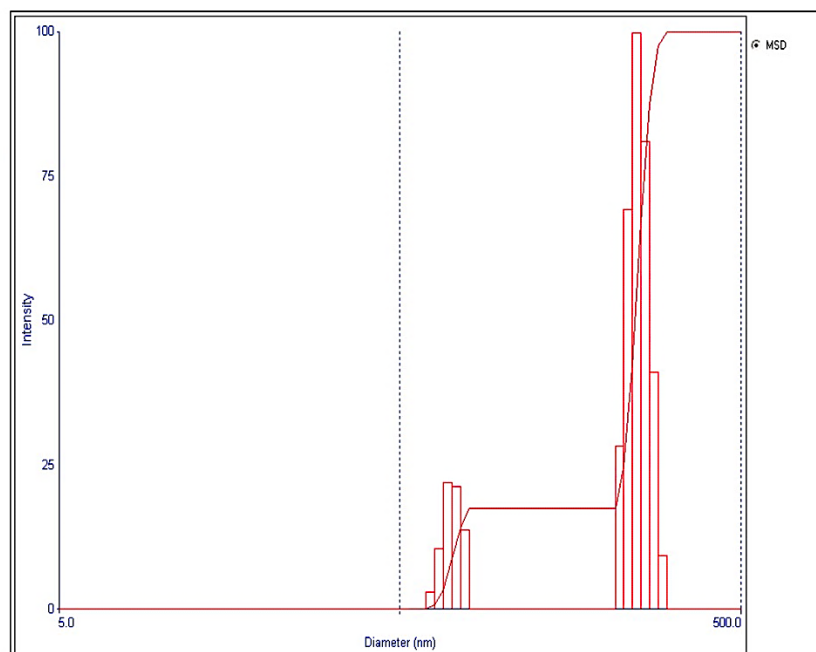


Run	Eff. Diam. (nm)	Half Width (nm)	Polydispersity	Baseline Index
1	149.8	68.9	0.212	9.9/ 98.89%
2	150.6	74.1	0.242	8.9/ 97.77%
3	150.7	68.9	0.209	9.9/ 98.89%
4	148.5	72.4	0.238	9.9/ 98.22%
5	149.6	69.6	0.217	8.3/ 87.77%
6	151.5	72.9	0.232	9.6/100.00%
Mean	150.1	71.2	0.225	9.4/ 98.59%
Std. Error	0.4	0.9	0.006	0.3/ 0.35
Combined	150.2	71.0	0.223	9.7/ 98.59%

Figure S16. DLS graph for formulation F6.

Formulation F7

Effective Diameter: **181.2 nm**
Polydispersity: **0.206**
Avg. Count Rate: **401.2 kcps**
Baseline Index: **8.0/ 98.18%**
Elapsed Time: **00:12:00**

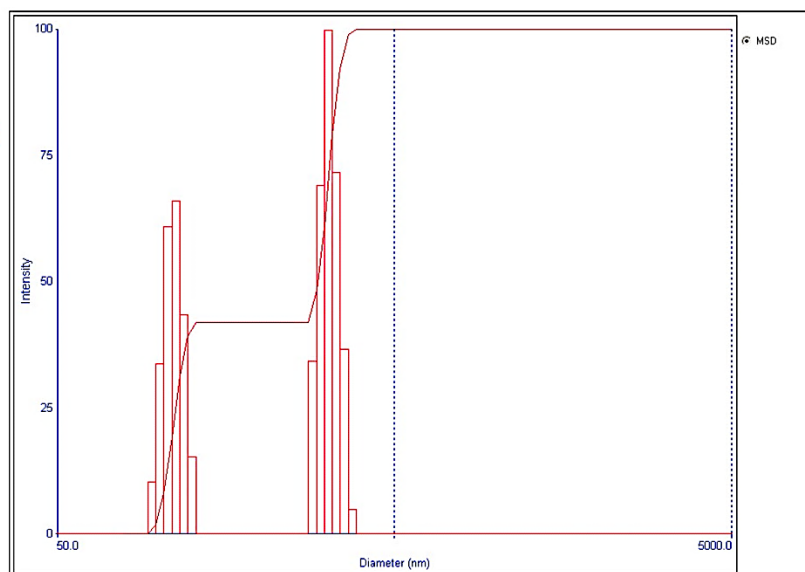


Run	Eff. Diam. (nm)	Half Width (nm)	Polydispersity	Baseline Index
1	179.2	79.9	0.199	7.5/ 98.88%
2	180.9	85.2	0.222	8.2/ 97.77%
3	180.4	71.9	0.159	5.6/100.00%
4	181.2	85.3	0.222	6.4/ 94.64%
5	183.2	81.4	0.197	8.5/ 97.77%
6	185.3	89.0	0.231	9.1/100.00%
Mean	181.7	82.1	0.205	7.5/ 98.18%
Std. Error	0.9	2.4	0.011	0.6/ 0.82
Combined	181.2	82.3	0.206	8.0/ 98.18%

Figure S17. DLS graph for formulation F7.

Formulation F8

Effective Diameter: **180.4 nm**
Polydispersity: **0.209**
Avg. Count Rate: **413.9 kcps**
Baseline Index: **9.9/ 99.18%**
Elapsed Time: **00:12:00**

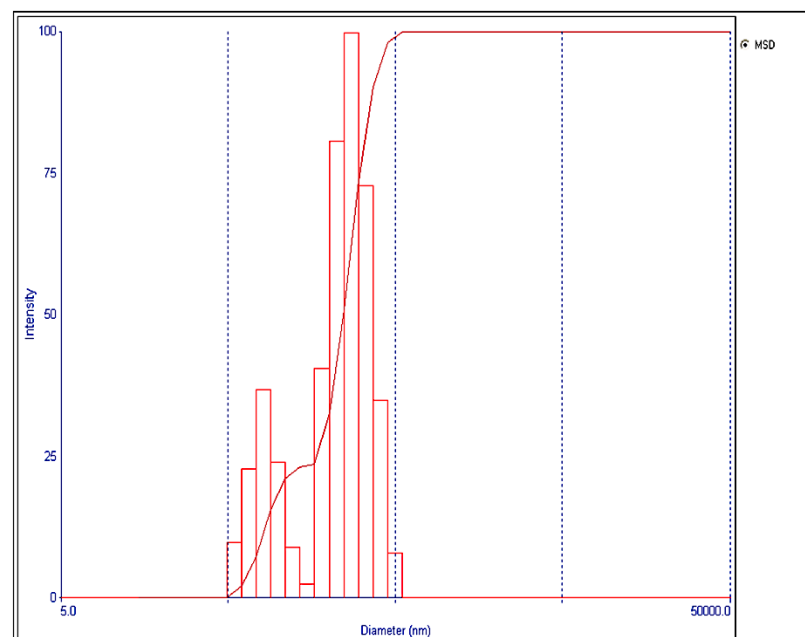


Run	Eff. Diam. (nm)	Half Width (nm)	Polydispersity	Baseline Index
1	169.7	78.2	0.212	9.7/100.00%
2	175.7	78.1	0.195	9.1/ 98.89%
3	182.3	85.2	0.218	6.4/ 97.77%
4	189.6	79.9	0.177	8.8/100.00%
5	185.5	90.6	0.239	9.2/ 99.11%
6	185.7	91.4	0.242	8.5/ 99.33%
Mean	181.6	83.9	0.214	8.6/ 99.18%
Std. Error	3.0	2.5	0.010	0.5/ 0.34
Combined	180.4	82.6	0.209	9.9/ 99.18%

Figure S18. DLS graph for formulation F8.

Formulation F9

Effective Diameter: **179.5 nm**
Polydispersity: **0.233**
Avg. Count Rate: **399.8 kcps**
Baseline Index: **9.9/ 98.55%**
Elapsed Time: **00:12:00**

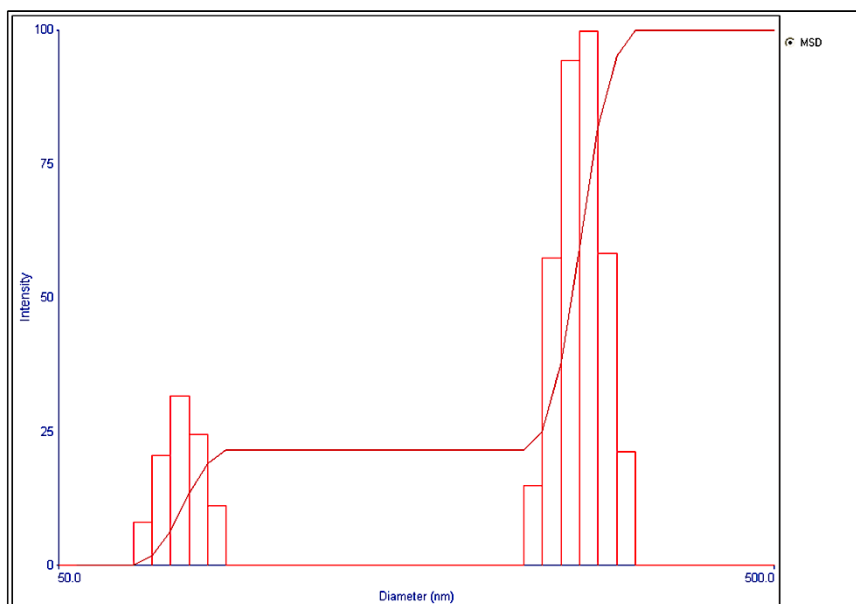


Run	Eff. Diam. (nm)	Half Width (nm)	Polydispersity	Baseline Index
1	182.3	90.7	0.248	10.0/ 95.54%
2	175.4	87.0	0.246	7.4/100.00%
3	175.5	78.2	0.199	7.2/100.00%
4	182.8	92.3	0.255	8.7/ 98.88%
5	183.9	87.2	0.225	9.9/ 98.88%
6	179.1	85.7	0.229	9.4/ 97.99%
Mean	179.8	86.9	0.233	8.8/ 98.55%
Std. Error	1.5	2.0	0.008	0.5/ 0.34
Combined	179.5	86.7	0.233	9.9/ 98.55%

Figure S19. DLS graph for formulation F9.

Formulation F10

Effective Diameter: **180.9 nm**
Polydispersity: **0.232**
Avg. Count Rate: **472.7 kcps**
Baseline Index: **9.5/ 98.89%**
Elapsed Time: **00:12:00**

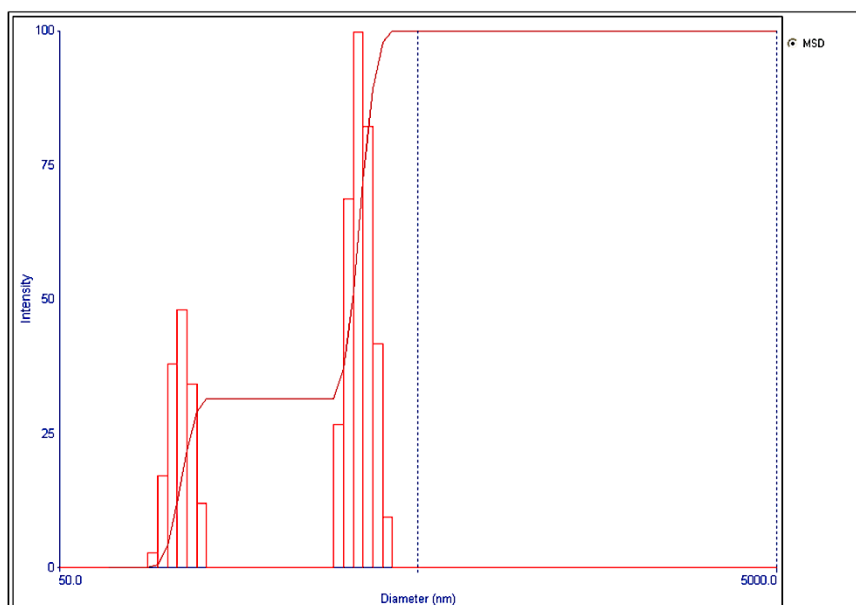


Run	Eff. Diam. (nm)	Half Width (nm)	Polydispersity	Baseline Index
1	177.8	88.4	0.247	9.9/100.00%
2	181.7	86.8	0.228	9.1/ 95.55%
3	182.5	86.7	0.226	8.7/ 98.89%
4	183.5	86.6	0.222	9.4/100.00%
5	178.6	87.1	0.238	6.8/100.00%
6	181.4	87.0	0.230	8.9/ 98.89%
Mean	180.9	87.1	0.232	8.8/ 98.89%
Std. Error	0.9	0.3	0.004	0.4/ 0.70
Combined	180.9	87.2	0.232	9.5/ 98.89%

Figure S20. DLS graph for formulation F10.

Formulation F11

Effective Diameter: **211.5 nm**
Polydispersity: **0.226**
Avg. Count Rate: **380.0 kcps**
Baseline Index: **8.0/ 97.95%**
Elapsed Time: **00:12:00**

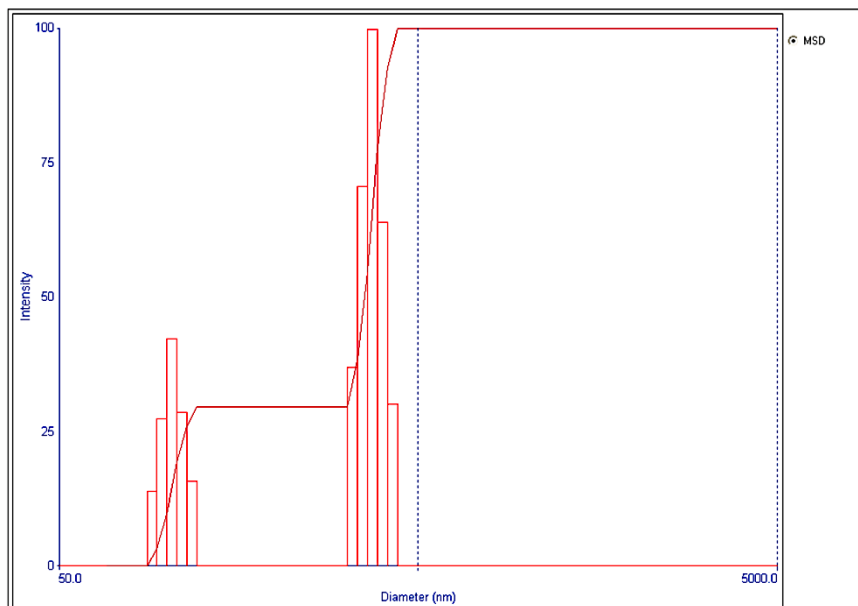


Run	Eff. Diam. (nm)	Half Width (nm)	Polydispersity	Baseline Index
1	199.2	93.5	0.220	9.8/ 96.64%
2	208.3	99.7	0.229	9.1/100.00%
3	214.2	96.0	0.201	8.1/ 96.64%
4	213.7	102.8	0.232	8.0/ 95.53%
5	217.8	105.1	0.233	7.9/ 98.88%
6	215.9	106.9	0.245	5.9/100.00%
Mean	211.5	100.7	0.227	8.1/ 97.95%
Std. Error	2.8	2.1	0.006	0.5/ 0.79
Combined	211.5	100.5	0.226	8.0/ 97.95%

Figure S21. DLS graph for formulation F11.

Formulation F12

Effective Diameter: **220.7 nm**
Polydispersity: **0.252**
Avg. Count Rate: **405.2 kcps**
Baseline Index: **8.4/ 98.25%**
Elapsed Time: **00:12:00**

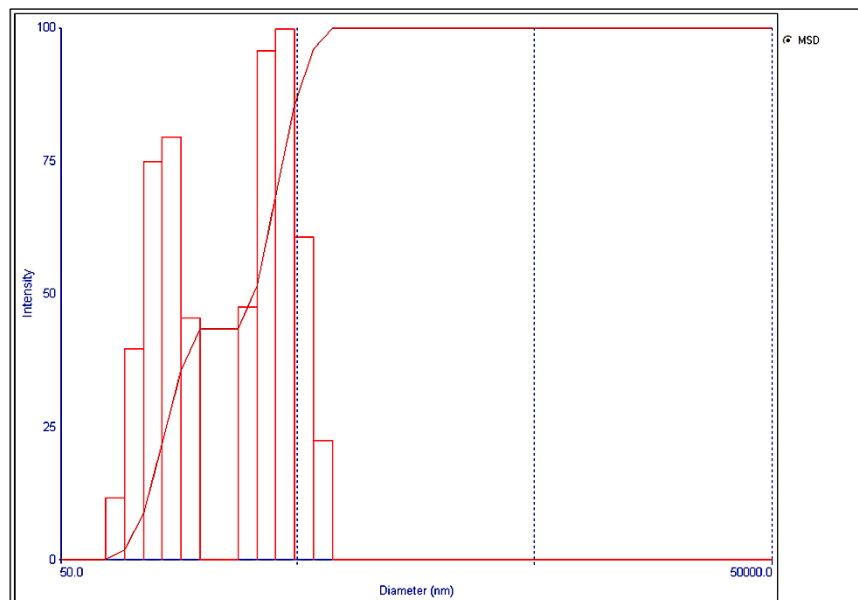


Run	Eff. Diam. (nm)	Half Width (nm)	Polydispersity	Baseline Index
1	214.1	98.9	0.213	7.7/ 95.76%
2	220.9	115.4	0.273	9.0/ 98.88%
3	220.0	113.2	0.265	9.3/ 96.88%
4	221.9	115.2	0.259	9.4/100.00%
5	227.1	109.2	0.231	8.7/ 97.98%
6	223.5	112.8	0.255	6.0/100.00%
Mean	221.3	110.8	0.251	8.4/ 98.25%
Std. Error	1.8	2.6	0.010	0.5/ 0.70
Combined	220.7	110.7	0.252	8.4/ 98.25%

Figure S22. DLS graph for formulation F12.

Formulation F13

Effective Diameter: **214.1 nm**
Polydispersity: **0.252**
Avg. Count Rate: **424.6 kcps**
Baseline Index: **7.7/ 98.22%**
Elapsed Time: **00:12:00**

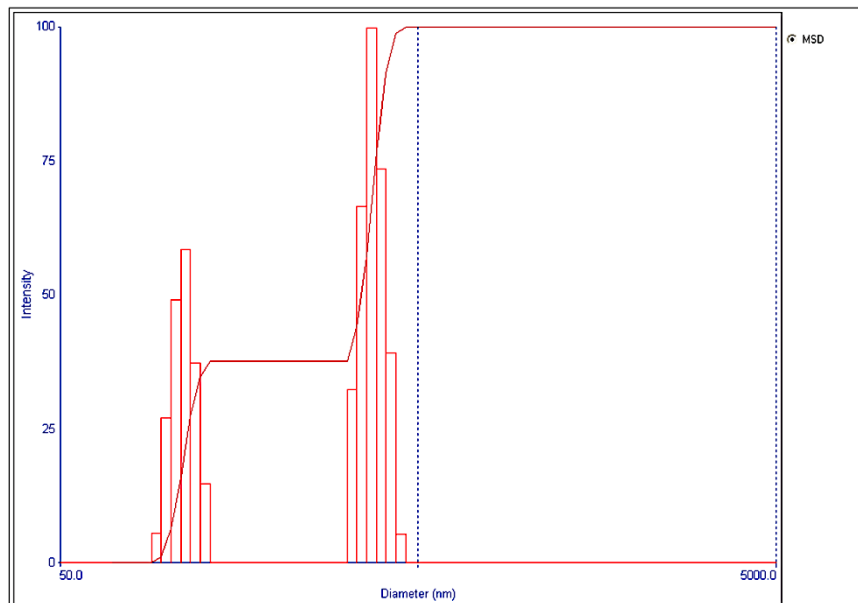


Run	Eff. Diam. (nm)	Half Width (nm)	Polydispersity	Baseline Index
1	209.4	103.0	0.242	10.0/100.00%
2	210.7	107.5	0.260	6.5/ 97.10%
3	212.2	108.3	0.261	4.0/ 96.88%
4	220.2	112.0	0.259	9.7/ 98.66%
5	214.5	105.4	0.242	9.5/ 96.66%
6	217.3	110.1	0.257	9.3/100.00%
Mean	214.1	107.7	0.253	8.0/ 98.22%
Std. Error	1.7	1.3	0.094	1.0/ 0.63
Combined	214.1	107.5	0.252	7.7/ 98.22%

Figure S23. DLS graph for formulation F13.

Formulation F14

Effective Diameter: **203.9 nm**
Polydispersity: **0.242**
Avg. Count Rate: **428.7 kcps**
Baseline Index: **9.3/ 97.81%**
Elapsed Time: **00:12:00**

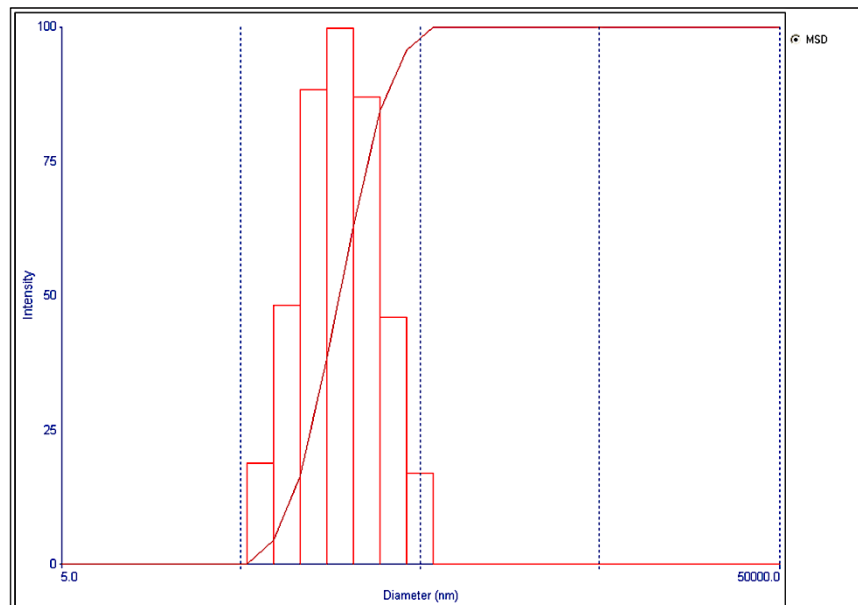


Run	Eff. Diam. (nm)	Half Width (nm)	Polydispersity	Baseline Index
1	196.6	92.3	0.220	9.0/ 97.77%
2	201.0	100.4	0.249	9.4/ 97.99%
3	203.5	98.9	0.236	8.3/ 98.44%
4	207.4	99.2	0.229	8.8/ 98.21%
5	208.1	105.7	0.258	5.9/ 98.89%
6	205.6	105.8	0.285	6.6/ 95.54%
Mean	203.7	100.4	0.243	8.0/ 97.81%
Std. Error	1.8	2.1	0.007	0.6/ 0.46%
Combined	203.9	100.3	0.242	9.3/ 97.81%

Figure S24. DLS graph for formulation F14.

Formulation F15

Effective Diameter: **154.7 nm**
Polydispersity: **0.190**
Avg. Count Rate: **448.0 kcps**
Baseline Index: **9.4/ 99.48%**
Elapsed Time: **00:12:00**

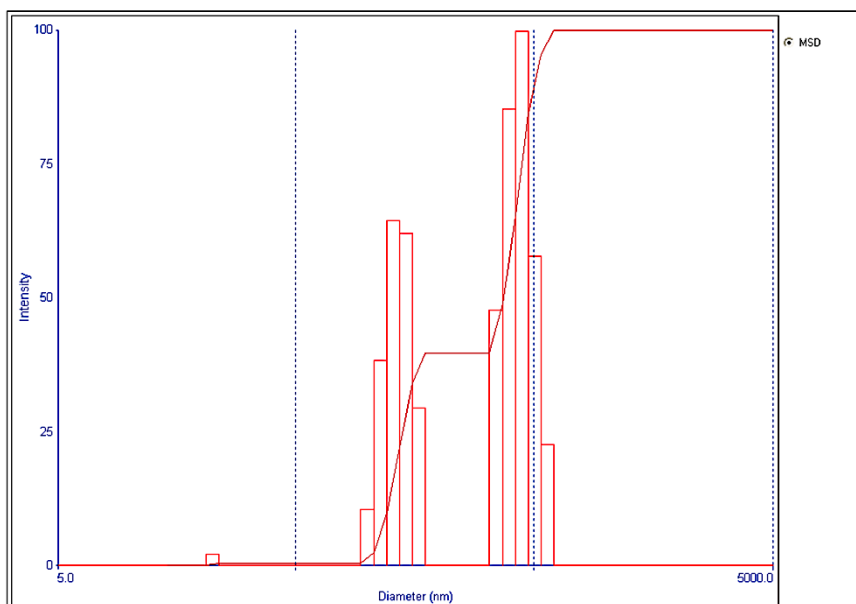


Run	Eff. Diam. (nm)	Half Width (nm)	Polydispersity	Baseline Index
1	153.3	55.9	0.133	8.4/ 100.00%
2	153.6	75.6	0.243	6.7/ 99.11%
3	155.5	64.1	0.170	7.0/ 100.00%
4	155.3	65.4	0.177	6.9/ 100.00%
5	151.9	69.2	0.208	8.1/ 98.89%
6	158.7	72.8	0.210	6.7/ 98.89%
Mean	154.7	67.2	0.190	7.3/ 99.48%
Std. Error	1.0	2.9	0.016	0.3/ 0.23%
Combined	154.7	67.5	0.190	9.4/ 99.48%

Figure S25. DLS graph for formulation F15.

Formulation F16

Effective Diameter: 225.6 nm
Polydispersity: 0.256
Avg. Count Rate: 412.0 kcps
Baseline Index: 6.8/ 98.40%
Elapsed Time: 00:12:00

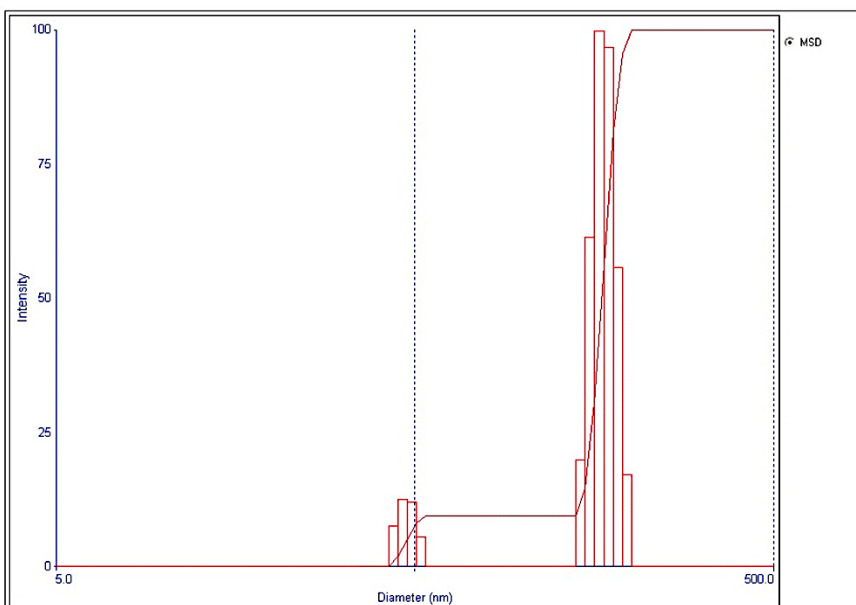


Run	Eff. Diam. (nm)	Half Width (nm)	Polydispersity	Baseline Index
1	218.3	108.3	0.246	4.2/ 97.77%
2	228.9	117.7	0.264	2.9/ 97.99%
3	231.4	119.5	0.267	5.3/100.00%
4	226.7	115.2	0.258	7.4/ 98.88%
5	227.8	119.3	0.274	9.7/ 95.76%
6	222.6	106.4	0.228	6.1/100.00%
Mean	225.9	114.4	0.256	5.9/ 98.40%
Std. Error	1.9	2.3	0.007	1.0/ 0.66
Combined	225.6	114.1	0.256	6.8/ 98.40%

Figure S26. DLS graph for formulation F16.

Formulation F17

Effective Diameter: 140.4 nm
Polydispersity: 0.166
Avg. Count Rate: 431.1 kcps
Baseline Index: 9.7/ 97.25%
Elapsed Time: 00:12:00

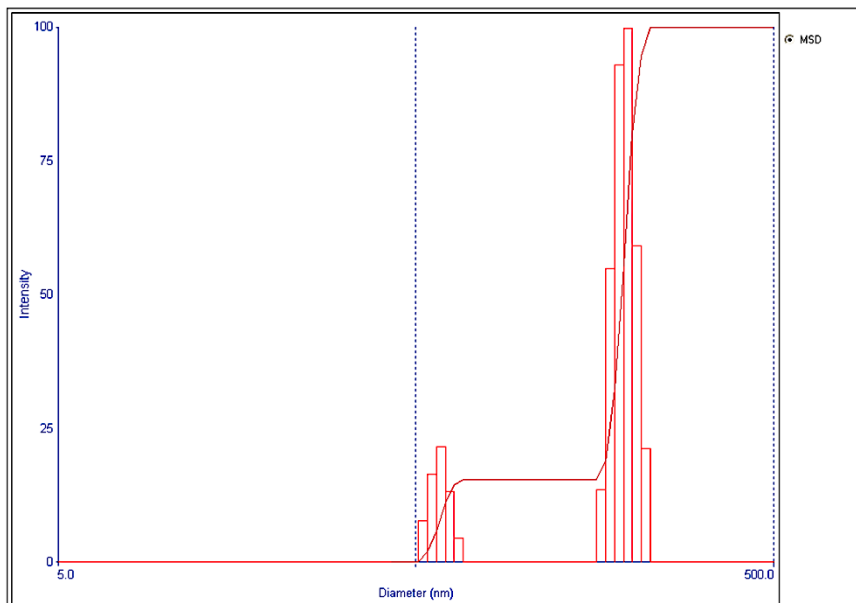


Run	Eff. Diam. (nm)	Half Width (nm)	Polydispersity	Baseline Index
1	130.5	56.0	0.184	9.9/ 93.32%
2	135.9	49.3	0.132	8.6/ 97.77%
3	141.5	58.6	0.171	8.6/ 96.88%
4	144.7	62.5	0.186	7.4/ 98.88%
5	147.4	57.0	0.150	9.8/ 96.66%
6	146.7	61.5	0.176	8.7/100.00%
Mean	141.1	57.5	0.166	8.8/ 97.25%
Std. Error	2.7	1.9	0.009	0.4/ 0.94
Combined	140.4	57.1	0.166	9.7/ 97.25%

Figure S27. DLS graph for formulation F17.

Formulation F18

Effective Diameter: **146.6 nm**
Polydispersity: **0.173**
Avg. Count Rate: **477.2 kcps**
Baseline Index: **9.9/ 98.33%**
Elapsed Time: **00:12:00**

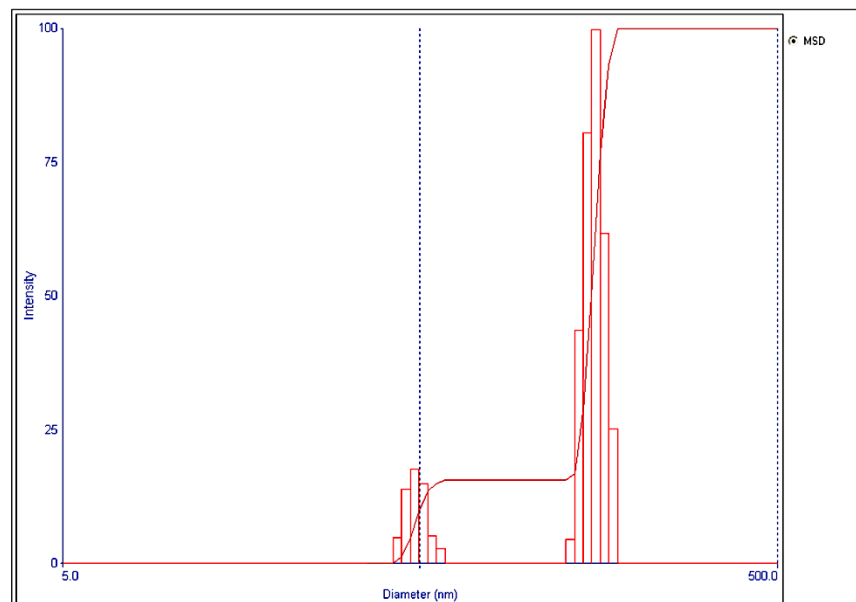


Run	Eff. Diam. (nm)	Half Width (nm)	Polydispersity	Baseline Index
1	140.8	52.8	0.141	9.6/ 98.89%
2	146.8	69.8	0.226	7.1/ 96.66%
3	145.0	60.3	0.173	9.4/ 98.89%
4	154.6	57.6	0.139	9.0/ 96.67%
5	146.3	56.3	0.148	7.0/ 98.89%
6	146.8	66.2	0.203	9.4/ 100.00%
Mean	146.7	60.5	0.172	8.6/ 98.33%
Std. Error	1.8	2.6	0.015	0.5/ 0.56%
Combined	146.6	61.1	0.173	9.9/ 98.33%

Figure S28. DLS graph for formulation F18.

Formulation F19

Effective Diameter: **119.1 nm**
Polydispersity: **0.162**
Avg. Count Rate: **405.5 kcps**
Baseline Index: **9.6/ 98.18%**
Elapsed Time: **00:12:00**

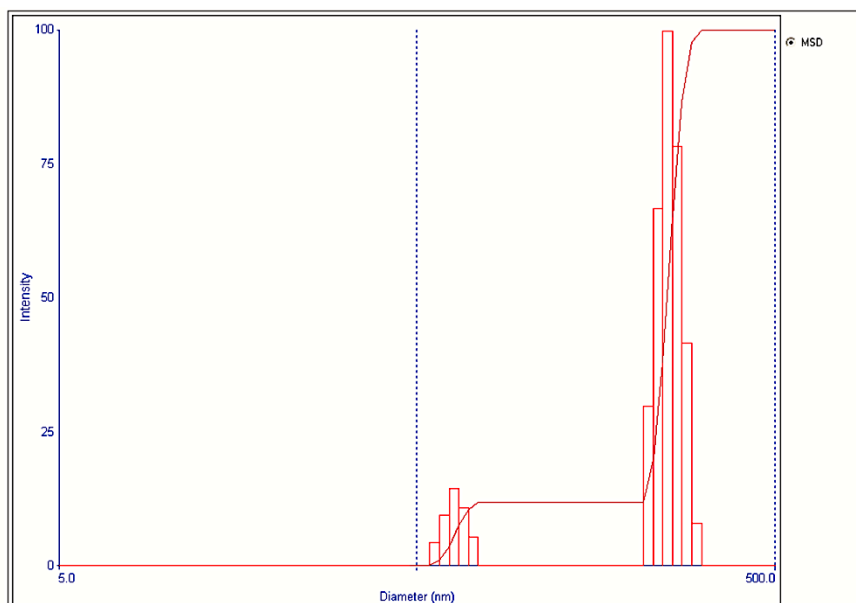


Run	Eff. Diam. (nm)	Half Width (nm)	Polydispersity	Baseline Index
1	116.0	45.8	0.156	8.0/ 97.77%
2	117.4	48.8	0.173	9.6/ 98.88%
3	119.4	50.0	0.179	7.0/ 95.88%
4	120.3	44.7	0.138	9.1/ 98.88%
5	120.6	50.0	0.172	9.8/ 97.77%
6	121.0	48.4	0.160	8.6/ 98.88%
Mean	119.0	48.0	0.163	8.7/ 98.18%
Std. Error	0.8	0.9	0.006	0.4/ 0.34%
Combined	119.1	47.9	0.162	9.6/ 98.18%

Figure S29. DLS graph for formulation F19.

Formulation F20

Effective Diameter: **197.5 nm**
Polydispersity: **0.191**
Avg. Count Rate: **366.1 kcps**
Baseline Index: **8.5/ 98.22%**
Elapsed Time: **00:06:00**

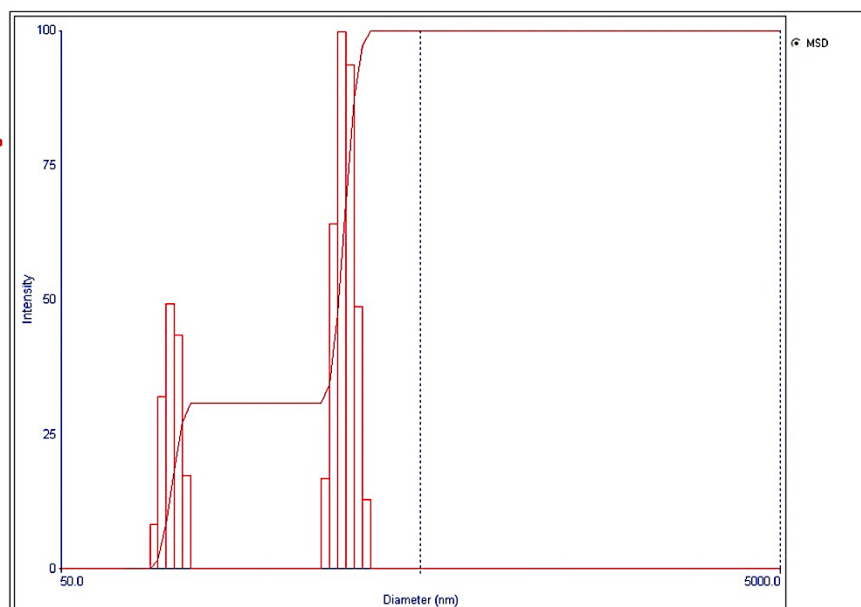


Run	Eff. Diam. (nm)	Half Width (nm)	Polydispersity	Baseline Index
1	192.5	77.7	0.163	7.4/100.00%
2	195.6	86.9	0.198	8.4/100.00%
3	195.9	78.8	0.162	4.2/96.00%
4	199.7	87.5	0.194	3.2/97.73%
5	194.2	91.4	0.222	9.1/97.78%
6	206.1	93.5	0.206	7.7/97.78%
Mean	197.3	86.0	0.191	6.7/98.22%
Std. Error	2.0	2.6	0.010	1.0/0.63
Combined	197.5	86.4	0.191	8.5/98.22%

Figure S30. DLS graph for formulation F20.

Formulation F21

Effective Diameter: **195.9 nm**
Polydispersity: **0.205**
Avg. Count Rate: **360.2 kcps**
Baseline Index: **10.0/ 98.25%**
Elapsed Time: **00:12:00**

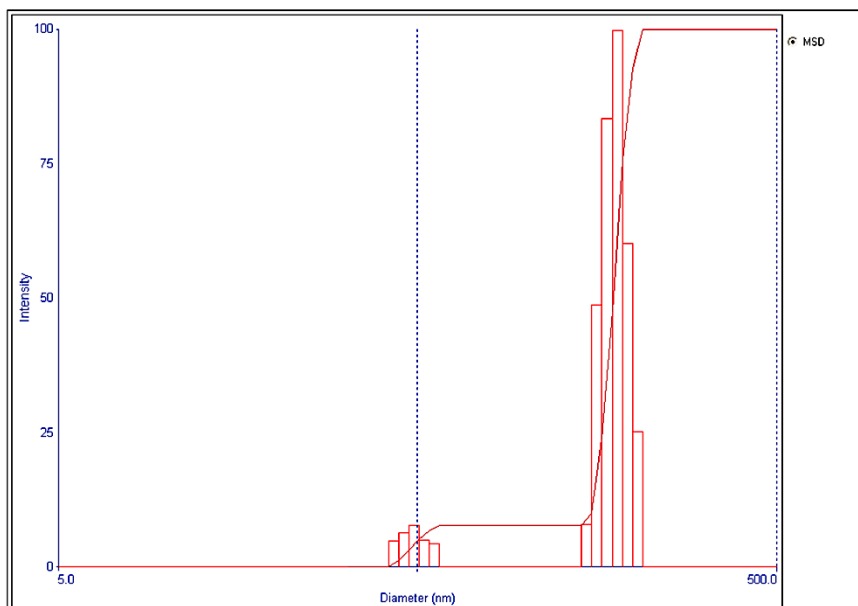


Run	Eff. Diam. (nm)	Half Width (nm)	Polydispersity	Baseline Index
1	184.2	80.4	0.190	9.4/99.11%
2	195.2	92.1	0.223	8.0/99.55%
3	203.5	83.7	0.169	7.9/96.87%
4	196.4	90.3	0.211	9.8/99.55%
5	195.7	95.6	0.238	9.8/97.76%
6	199.5	88.2	0.195	9.7/96.64%
Mean	195.8	88.4	0.205	9.1/98.25%
Std. Error	2.6	2.3	0.010	0.4/0.54
Combined	195.9	88.7	0.205	10.0/98.25%

Figure S31. DLS graph for formulation F21.

Formulation F22

Effective Diameter: **149.5 nm**
Polydispersity: **0.137**
Avg. Count Rate: **412.9 kcps**
Baseline Index: **9.0/ 98.21%**
Elapsed Time: **00:12:00**

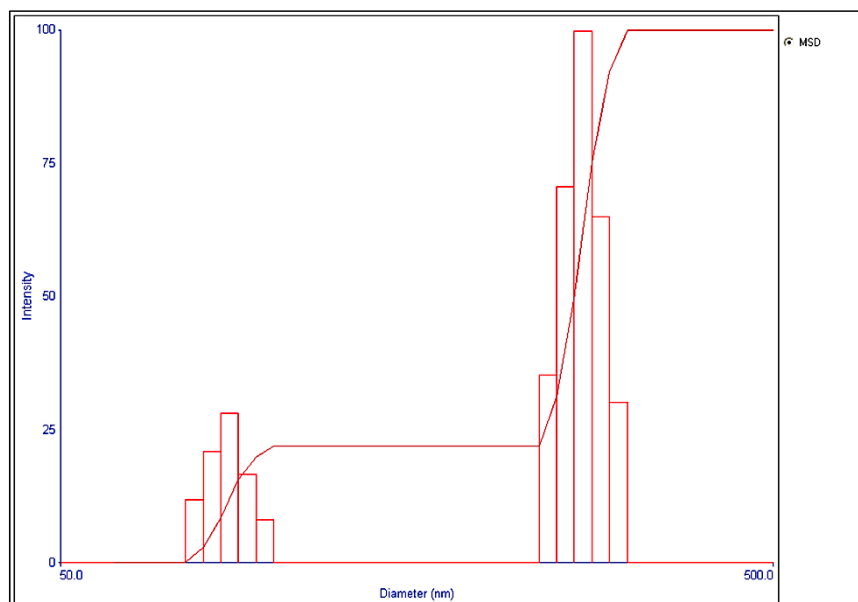


Run	Eff. Diam. (nm)	Half Width (nm)	Polydispersity	Baseline Index
1	144.7	46.8	0.105	8.9/100.00%
2	145.6	57.3	0.155	8.9/100.00%
3	147.5	59.9	0.165	9.4/ 96.88%
4	152.1	54.9	0.130	5.3/ 97.77%
5	150.9	60.8	0.162	9.2/ 94.64%
6	154.7	52.5	0.115	7.9/100.00%
Mean	149.3	55.4	0.139	8.3/ 98.21%
Std. Error	1.6	2.1	0.010	0.6/ 0.90
Combined	149.5	55.4	0.137	9.0/ 98.21%

Figure S32. DLS graph for formulation F22.

NLC Placebo

Effective Diameter: **189.8 nm**
Polydispersity: **0.196**
Avg. Count Rate: **412.2 kcps**
Baseline Index: **9.5/ 98.70%**
Elapsed Time: **00:12:00**

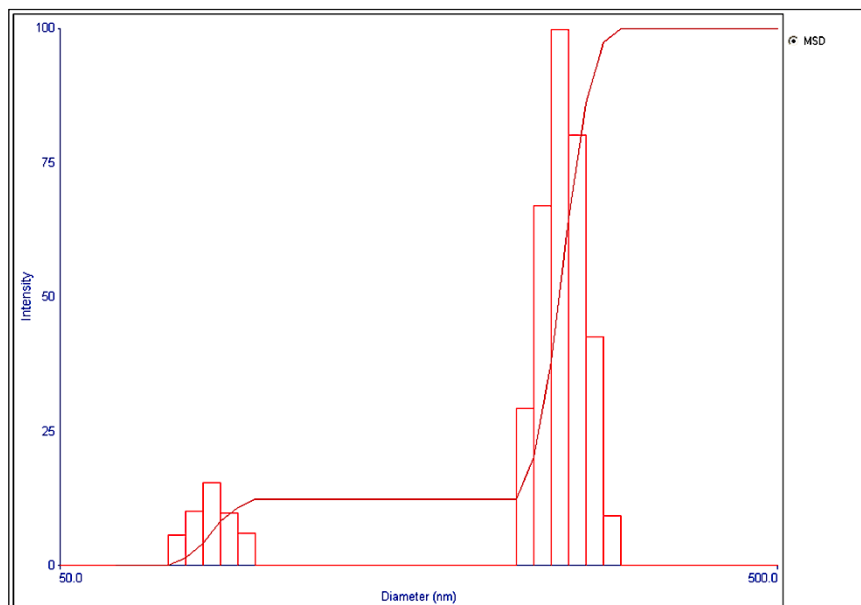


Run	Eff. Diam. (nm)	Half Width (nm)	Polydispersity	Baseline Index
1	197.1	88.9	0.203	9.8/100.00%
2	196.1	78.1	0.159	9.9/ 98.88%
3	188.2	88.5	0.221	7.9/ 97.77%
4	184.7	82.5	0.199	8.2/100.00%
5	184.7	95.2	0.213	9.3/ 98.88%
6	184.3	79.8	0.188	9.2/ 96.66%
Mean	189.2	83.8	0.197	9.0/ 98.70%
Std. Error	2.4	1.8	0.009	0.3/ 0.53
Combined	189.8	83.9	0.196	9.5/ 98.70%

Figure S33. DLS graph for NLC Placebo.

NLC Riluzole

Effective Diameter: **205.2 nm**
Polydispersity: **0.144**
Avg. Count Rate: **491.6 kcps**
Baseline Index: **9.5/ 99.11%**
Elapsed Time: **00:12:00**

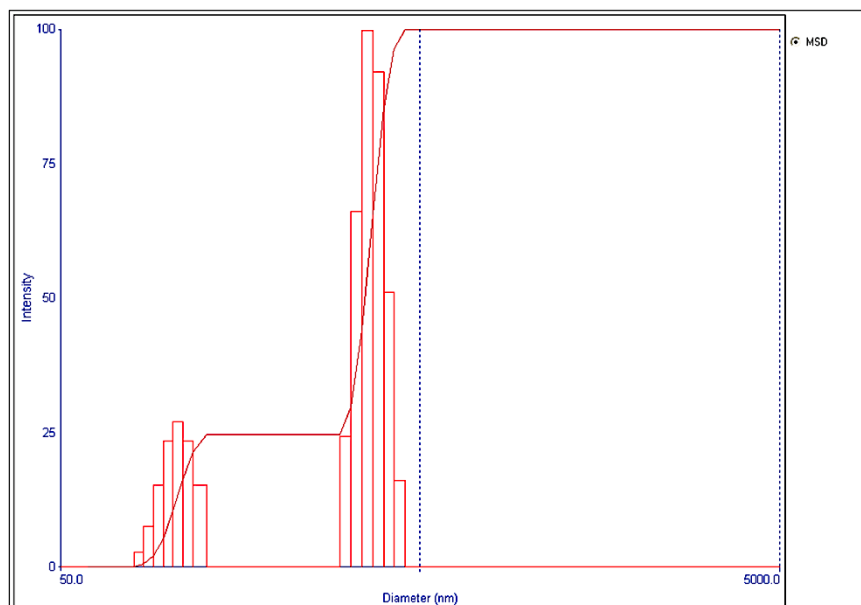


Run	Eff. Diam. (nm)	Half Width (nm)	Polydispersity	Baseline Index
1	215.8	87.3	0.163	8.7/100.00%
2	211.3	85.1	0.162	8.8/100.00%
3	204.2	81.7	0.160	8.4/100.00%
4	200.1	73.7	0.136	7.1/ 97.78%
5	198.3	76.7	0.150	7.9/ 96.89%
6	200.6	68.8	0.118	7.4/100.00%
Mean	205.1	78.9	0.148	8.1/ 99.11%
Std. Error	2.9	2.9	0.007	0.3/ 0.57%
Combined	205.2	77.7	0.144	9.5/ 99.11%

Figure S34. DLS graph for NLC Riluzole.

Functionalized NLC

Effective Diameter: **235.3 nm**
Polydispersity: **0.240**
Avg. Count Rate: **413.7 kcps**
Baseline Index: **9.2/ 99.18%**
Elapsed Time: **00:12:00**



Run	Eff. Diam. (nm)	Half Width (nm)	Polydispersity	Baseline Index
1	232.4	113.4	0.238	8.5/100.00%
2	233.9	116.6	0.249	6.8/ 97.32%
3	240.7	118.1	0.241	7.3/100.00%
4	231.1	113.1	0.239	6.3/100.00%
5	239.4	125.8	0.276	5.6/ 97.77%
6	235.1	106.7	0.206	7.4/100.00%
Mean	235.5	115.6	0.241	7.0/ 99.18%
Std. Error	1.6	2.6	0.009	0.4/ 0.52%
Combined	235.3	115.3	0.240	9.2/ 99.18%

Figure S35. DLS graph for Functionalized Riluzole.

Table S4 contains the DLS data related to the initial size, PDI and ZP of the lipid nanoparticles, obtained on the day of production (day 0).

Table S4. DLS data for the initial mean diameter, PDI and ZP of the lipid nanoparticles (day 0).

Formulation	Batch	Size	PDI	ZP
F1	1	151.7	0.228	-18.45
	2	153.7	0.235	-25.23
	3	157.0	0.229	-22.38
F2	1	182.5	0.231	-20.28
	2	181.0	0.241	-13.14
	3	181.7	0.220	-12.11
F3	1	152.8	0.242	-24.63
	2	149.7	0.220	-24.81
	3	156.2	0.225	-23.13
F4	1	148.9	0.202	-23.63
	2	151.1	0.213	-20.82
	3	148.8	0.204	-23.01
F5	1	122.3	0.187	-21.53
	2	126.7	0.210	-20.60
	3	124.1	0.203	-17.93
F6	1	158.7	0.222	-23.05
	2	150.1	0.225	-20.84
	3	154.8	0.219	-23.13
F7	1	181.7	0.205	-22.61
	2	185.2	0.229	-26.67
	3	185.0	0.222	-24.86
F8	1	182.1	0.218	-21.60
	2	181.6	0.214	-18.62
	3	181.4	0.207	-24.38
F9	1	173.7	0.235	-24.55
	2	179.8	0.233	-28.56
	3	185.0	0.222	-28.15
F10	1	180.9	0.232	-22.12
	2	178.6	0.229	-17.23
	3	176.4	0.234	-24.85
F11	1	211.5	0.227	-24.97
	2	214.5	0.222	-21.84
	3	215.1	0.239	-25.92
F12	1	214.8	0.238	-23.77
	2	221.3	0.251	-25.25
	3	224.7	0.254	-23.75

F13	1	214.1	0.253	-22.94
	2	221.2	0.268	-19.17
	3	211.2	0.256	-23.97
F14	1	203.7	0.243	-21.88
	2	202.3	0.249	-29.66
	3	203.2	0.239	-29.91
F15	1	162.7	0.179	-26.10
	2	155.8	0.183	-17.99
	3	154.7	0.190	-20.35
F16	1	214.1	0.253	-22.79
	2	225.9	0.256	-22.94
	3	222.9	0.260	-20.77
F17	1	141.1	0.166	-19.53
	2	145.6	0.172	-20.89
	3	148.0	0.169	-21.36
F18	1	146.7	0.172	-26.71
	2	146.7	0.18	-19.94
	3	148.2	0.188	-22.40
F19	1	119.0	0.163	-22.74
	2	119.4	0.158	-19.99
	3	121.8	0.163	-19.56
F20	1	197.3	0.191	-25.36
	2	192.1	0.198	-18.67
	3	193.9	0.188	-19.21
F21	1	202.0	0.173	-27.48
	2	195.8	0.205	-31.07
	3	199.7	0.199	-23.81
F22	1	149.3	0.139	-21.50
	2	150.9	0.147	-21.69
	3	150.4	0.171	-21.63
NLC Placebo	1	189.2	0.197	19.52
	2	175.2	0.248	10.01
	3	176.5	0.202	21.39
NLC Riluzole	1	205.1	0.148	23.22
	2	215.6	0.160	18.05
	3	204.8	0.155	20.75
Functionalized NLC	1	196.3	0.188	-16.19
	2	235.5	0.241	-16.19
	3	234.0	0.228	-16.85

Appendix V – Student's t-test

In the case of independent samples Student's t-test the procedure was the following:

Descriptives					Statistic	Std. Error
Formulation						
Size	F3	Mean			152.9000	1.87705
		95% Confidence Interval for Mean	Lower Bound		144.8237	
			Upper Bound		160.9763	
		Median			152.8000	
		Variance			10.570	
		Std. Deviation			3.25115	
		Minimum			149.70	
		Maximum			156.20	
		Range			6.50	
		Skewness			0.138	1.225
	F6	Mean			154.5333	2.48618
		95% Confidence Interval for Mean	Lower Bound		143.8361	
			Upper Bound		165.2305	
		Median			154.8000	
		Variance			18.543	
		Std. Deviation			4.30620	
		Minimum			150.10	
		Maximum			158.70	
		Range			8.60	
		Skewness			-0.278	1.225

Tests of Normality

Formulation		Statistic	Shapiro-Wilk df	Sig.
Size	F3	0.999	3	0.949
	F6	0.997	3	0.898

Independent Samples Test

Independent Samples Test

Levene's Test for Equality of Variances				t-test for Equality of Means							
		F	Sig.	t	df	Significance		Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
						One-Sided p	Two-Sided p			Lower	Upper
Size	Equal variances assumed	0.196	0.681	-0.524	4	0.314	0.628	-1.633	3.1152	-10.2825	7.0158
	Equal variances not assumed			-0.524	3.721	0.315	0.630	-1.633	3.1152	-10.5445	7.2778

Appendix VI – Tukey’s HSD Test

In the case of ANOVA with Tukey’s HSD test the procedure was the following:

Descriptives		Statistic		Std. Error
Day				
0	Mean		203.0667	0.40961
	95% Confidence Interval for Mean	Lower Bound	201.3043	
		Upper Bound	204.8291	
	Median		203.2000	
	Variance		0.503	
	Std. Deviation		0.70946	
	Minimum		202.30	
	Maximum		203.70	
	Range		1.40	
	Skewness		-0.816	1.225
7	Mean		203.5000	1.18462
	95% Confidence Interval for Mean	Lower Bound	198.4030	
		Upper Bound	208.5970	
	Median		203.6000	
	Variance		4.210	
	Std. Deviation		2.05183	
	Minimum		201.40	
	Maximum		205.50	
	Range		4.10	
	Skewness		-0.219	1.225
15	Mean		193.5667	.28480
	95% Confidence Interval for Mean	Lower Bound	192.3413	
		Upper Bound	194.7921	
	Median		193.8000	
	Variance		0.243	
	Std. Deviation		.49329	
	Minimum		193.00	
	Maximum		193.90	
	Range		0.90	
	Skewness		-1.652	1.225
30	Mean		191.7667	2.16205
	95% Confidence Interval for Mean	Lower Bound	182.4641	
		Upper Bound	201.0692	
	Median		191.1000	
	Variance		14.023	
	Std. Deviation		3.74477	
	Minimum		188.40	
	Maximum		195.80	
	Range		7.40	
	Skewness		0.776	1.225
60	Mean		199.1667	1.34825
	95% Confidence Interval for Mean	Lower Bound	193.3656	
		Upper Bound	204.9677	
	Median		198.7000	
	Variance		5.453	
	Std. Deviation		2.33524	
	Minimum		197.10	
	Maximum		201.70	
	Range		4.60	

	90	Range	4.60	
		Skewness	0.863	1.225
		Mean	201.0667	3.02783
		95% Confidence Interval for Mean	Lower Bound	188.0389
			Upper Bound	214.0944
		Median	198.5000	
		Variance	27.503	
		Std. Deviation	5.24436	
		Minimum	197.60	
		Maximum	207.10	
		Range	9.50	
		Skewness	1.675	1.225

Tests of Normality

	Day	Shapiro-Wilk		
		Statistic	df	Sig.
Size	0	0.974	3	0.688
	7	0.998	3	0.919
	15	0.832	3	0.194
	30	0.976	3	0.704
	60	0.970	3	0.668
	90	0.820	3	0.164

a. Lilliefors Significance Correction

Tests of Homogeneity of Variances

		Levene Statistic	df1	df2	Sig.
Size	Based on Mean	3.833	5	12	0.026
	Based on Median	0.701	5	12	0.634
	Based on Median and with adjusted df	0.701	5	3.806	0.654
	Based on trimmed mean	3.455	5	12	0.036

ANOVA

	Sum of Squares	Size df	Mean Square	F	Sig.
Between Groups	367.044	5	73.409	8.481	0.001
Within Groups	103.873	12	8.656		
Total	470.918	17			

Multiple Comparisons

Dependent Variable: Size
Tukey HSD

(I) Day	(J) Day	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0	7	-0.43333	2.40224	1.000	-8.5023	7.6356
	15	9.50000*	2.40224	0.018	1.4311	17.5689
	30	11.30000*	2.40224	0.005	3.2311	19.3689
	60	3.90000	2.40224	0.600	-4.1689	11.9689
	90	2.00000	2.40224	0.955	-6.0689	10.0689
7	0	0.43333	2.40224	1.000	-7.6356	8.5023
	15	9.93333*	2.40224	0.014	1.8644	18.0023
	30	11.73333*	2.40224	0.004	3.6644	19.8023
	60	4.33333	2.40224	0.498	-3.7356	12.4023
	90	2.43333	2.40224	0.905	-5.6356	10.5023
15	0	-9.50000*	2.40224	0.018	-17.5689	-1.4311
	7	-9.93333*	2.40224	0.014	-18.0023	-1.8644
	30	1.80000	2.40224	0.971	-6.2689	9.8689
	60	-5.60000	2.40224	0.254	-13.6689	2.4689
	90	-7.50000	2.40224	0.074	-15.5689	0.5689
30	0	-11.30000*	2.40224	0.005	-19.3689	-3.2311
	7	-11.73333*	2.40224	0.004	-19.8023	-3.6644
	15	-1.80000	2.40224	0.971	-9.8689	6.2689
	60	-7.40000	2.40224	0.079	-15.4689	0.6689
	90	-9.30000*	2.40224	0.021	-17.3689	-1.2311
60	0	-3.90000	2.40224	0.600	-11.9689	4.1689
	7	-4.33333	2.40224	0.498	-12.4023	3.7356
	15	5.60000	2.40224	0.254	-2.4689	13.6689
	30	7.40000	2.40224	0.079	-.6689	15.4689
	90	-1.90000	2.40224	0.964	-9.9689	6.1689
90	0	-2.00000	2.40224	0.955	-10.0689	6.0689
	7	-2.43333	2.40224	0.905	-10.5023	5.6356
	15	7.50000	2.40224	0.074	-0.5689	15.5689
	30	9.30000*	2.40224	0.021	1.2311	17.3689
	60	1.90000	2.40224	0.964	-6.1689	9.9689

*. The mean difference is significant at the 0.05 level.

Tukey HSD^a

Day	N	Subset for alpha = 0.05		
		1	2	3
30	3	191.7667		
15	3	193.5667	193.5667	
60	3	199.1667	199.1667	199.1667
90	3		201.0667	201.0667
0	3			203.0667
7	3			203.5000
Sig.		0.079	0.074	0.498

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.

Appendix VII – *In vitro* release assays

Table S5. Data for the cumulative RLZ release (%) from SLN F4.

Time (h)	SLN F4		
	Batch 1	Batch 2	Batch 3
0.25	0.95	1.13	1.16
0.50	1.47	1.75	1.39
0.75	2.13	2.67	2.04
1	2.44	3.03	2.53
2	4.57	5.30	4.89
4	7.47	14.33	8.48
6	11.00	17.11	11.86
8	12.90	17.49	14.80
10	15.52	18.99	17.68
12	18.59	21.78	20.92
25	28.39	31.34	31.12
27	30.64	33.32	32.80
29	32.09	35.43	35.20
30	32.91	36.08	35.94

Table S6. Data for the cumulative RLZ release (%) from SLN F14.

Time (h)	SLN F14		
	Batch 1	Batch 2	Batch 3
0.25	0.98	0.95	0.84
0.50	1.38	1.46	1.53
0.75	1.65	1.76	1.81
1	2.31	2.53	2.34
2	5.09	4.23	7.05
4	7.06	7.03	8.06
6	9.47	9.00	10.09
8	10.76	10.88	11.71
10	12.66	12.29	13.64
12	13.60	13.78	14.96
25	20.68	21.50	23.16
27	23.89	25.25	25.33
29	25.13	25.48	25.95
30	25.53	26.00	27.39

Table S7. Data for the cumulative RLZ release (%) from NLC F18.

Time (h)	NLC F18		
	Batch 1	Batch 2	Batch 3
0.25	0.00	0.25	0.99
0.50	0.00	0.00	1.06
0.75	0.43	0.17	1.66
1.00	0.49	0.65	2.31
2.00	2.30	2.31	3.44
4.00	4.25	4.87	5.67
6.00	6.34	6.79	7.61
8.00	7.01	7.87	8.84
10.00	9.13	10.01	10.48
12.00	11.10	13.01	12.49
25.00	18.21	20.96	21.05
27.00	20.13	22.91	24.46
29.00	21.52	23.90	25.49
30.00	22.05	24.41	25.97

Table S8. Data for the cumulative RLZ release (%) from NLC F21.

Time (h)	NLC F21		
	Batch 1	Batch 2	Batch 3
0.25	0.17	0.48	0.58
0.50	0.53	0.07	0.22
0.75	0.34	0.37	0.61
1.00	0.89	1.30	1.16
2.00	2.48	2.79	2.05
4.00	3.93	4.54	4.38
6.00	5.77	7.47	8.41
8.00	8.35	9.42	9.26
10.00	9.98	11.27	10.94
12.00	11.79	13.52	13.22
25.00	18.18	20.89	21.16
27.00	20.56	23.28	23.37
29.00	21.40	24.40	24.89
30.00	21.80	25.09	25.78

Table S9. Data for the cumulative RLZ release (%) from NLC Riluzole.

Time (h)	NLC Riluzole		
	Batch 1	Batch 2	Batch 3
0.25	0.00	0.00	0.00
0.50	1.69	1.58	1.29
1.00	2.84	3.09	2.72
2.00	4.23	4.92	4.12
4.00	8.11	8.93	7.93
6.00	11.61	12.22	11.37
8.00	13.22	13.04	12.58
10.00	15.28	16.29	14.07
24.00	25.18	27.56	23.82
27.00	21.85	26.97	27.08
29.00	25.35	26.50	23.27
30.00	23.28	29.51	22.58
34.00	25.99	25.97	25.43
48.00	27.24	32.64	27.27
60.00	29.67	32.16	26.42

Table S10. Data for the cumulative RLZ release (%) from Functionalized NLC.

Time (h)	Functionalized NLC		
	Batch 1	Batch 2	Batch 3
0.25	0.00	0.00	0.00
0.50	3.96	3.86	4.03
1.00	7.14	6.34	6.45
2.00	13.01	12.92	11.52
4.00	21.17	21.10	19.57
6.00	29.60	29.95	29.69
8.00	36.44	35.31	35.09
10.00	39.78	37.51	38.29
24.00	54.90	54.14	51.71
27.00	56.71	56.59	54.24
29.00	58.88	60.40	56.53
30.00	59.55	55.44	54.30
34.00	57.73	58.77	55.11
48.00	64.89	55.75	58.77
60.00	58.97	59.53	56.84

Appendix VIII – HPLC-MS Method

The calibration curve for the HPLC-MS method is shown in Figure S36. As evidenced by an R^2 of 0.9994, as well as the fact that the residual analysis graph (Figure S37) did not show any tendency, proper linearity was achieved. Figure S38 presents the summary output of the regression and residuals analysis for the developed method.

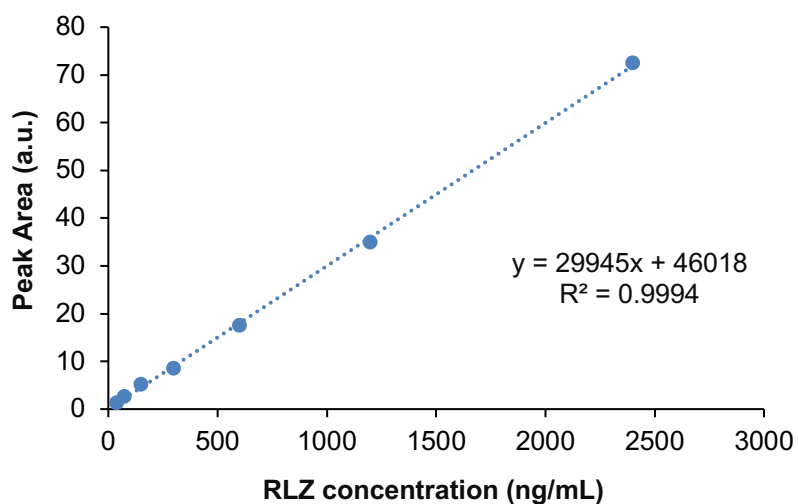


Figure S36. Calibration curve for the quantification of RLZ with HPLC-MS ($n = 3$).

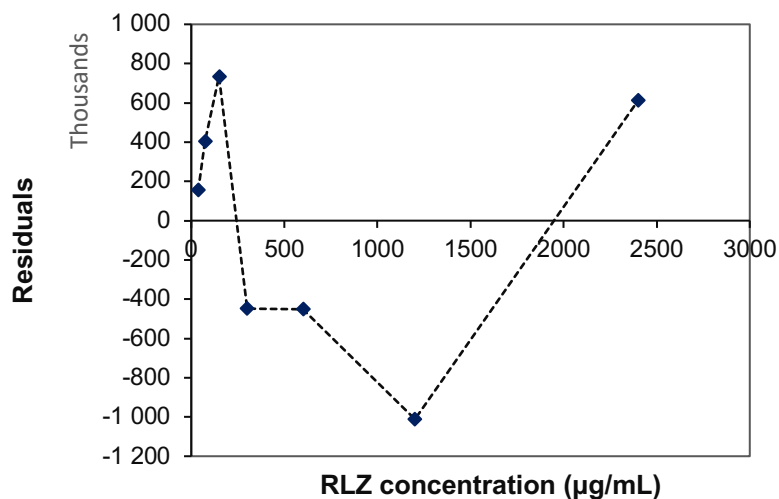


Figure S37. Graph of residuals for the HPLC-MS method.

Regression Statistics	
Multiple R	0.9997
R Square	0.9994
Adjusted R Square	0.9992
Standard Error	711320.342
Observations	7

ANOVA					
	<i>df</i>	<i>SS</i>	<i>MS</i>	<i>F</i>	<i>Significance F</i>
Regression	1	3.98071E+15	3.98071E+15	7867.37717	3.45253E-09
Residual	5	2.52988E+12	5.05977E+11		
Total	6	3.98324E+15			

	<i>Coefficients</i>	<i>Standard Error</i>	<i>t Stat</i>	<i>P-value</i>	<i>Lower 95%</i>	<i>Upper 95%</i>	<i>Lower 95.0%</i>	<i>Upper 95.0%</i>
Intercept	46017.9483	353610.2395	0.130137488	0.90153118	-862966.1102	955002.007	-862966.11	955002.007
X Variable 1	29944.8045	337.6031549	88.69823654	3.4525E-09	29076.96795	30812.641	29076.9679	30812.641

RESIDUAL OUTPUT				
<i>Observation</i>	<i>Predicted Y</i>	<i>Residuals</i>	<i>Standard Residuals</i>	
1	1168948.12	156581.8835	0.241138696	
2	2291878.28	404430.7153	0.622830004	
3	4537738.62	734874.3789	1.131718722	
4	9029459.29	-447343.294	-0.688916086	
5	18012900.6	-450357.64	-0.693558228	
6	35979783.3	-1011357.33	-1.55750705	
7	71913548.7	613171.2866	0.944293943	

Figure S38. Summary output of the regression and residual data analysis for the spectrophotometry method.

Appendix IX – Permeability assay

Table S11. Data for the cumulative % of RLZ 10 μ M that permeated through the hCMEC/D3 monolayer.

Time (h)	Free Riluzole 10 μ M	
	Sample 1	Sample 2
0.0	0.00	0.00
0.5	9.25	8.74
1.0	16.97	15.10
1.5	18.84	17.69
2.0	21.49	19.50
2.5	24.09	20.98
3.0	25.54	22.41
3.5	26.88	23.19
4.0	27.72	23.85
8.0	29.18	25.12
24.0	29.89	25.65

Table S12. Data for the cumulative % of NLC Riluzole 10 μ M that permeated through the hCMEC/D3 monolayer.

Time (h)	NLC Riluzole 10 μ M	
	Sample 1	Sample 2
0.0	0.00	0.00
0.5	6.44	4.78
1.0	9.40	7.13
1.5	12.01	8.50
2.0	13.06	9.98
2.5	13.76	11.08
3.0	15.17	11.88
3.5	15.86	12.48
4.0	13.12	13.16
8.0	15.09	13.99
24.0	13.49	14.63

Table S13. Data for the cumulative % of Functionalized RLZ 10 μ M that permeated through the hCMEC/D3 monolayer.

Time (h)	Functionalized NLC 10 μ M	
	Sample 1	Sample 2
0.0	0.00	0.00
0.5	1.89	2.08
1.0	2.83	3.64
1.5	4.06	4.35
2.0	5.28	4.64
2.5	5.76	5.11
3.0	6.13	5.53
3.5	6.46	5.75
4.0	7.00	6.02
8.0	7.36	6.38
24.0	7.73	6.65