UNIVERSITY OF HELSINKI

Nanotechnology-based therapy for Parkinson's Disease

Optimisation and synthesis of peptide-encapsulated liposome

Master's programme in Neuroscience, Neuroscience study track

Master's thesis

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Abstract:

Parkinson's disease (PD) is the second most common neurodegenerative disorder characterised by motor symptoms such as bradykinesia, rigidity, tremor, and postural instability. Current treatments only relieve symptoms but do not stop disease progression. Neurotrophic factors (NTFs) are promising drug candidates for the treatment of PD: cerebral dopamine neurotrophic factor (CDNF) and mesencephalic astrocyte-derived neurotrophic factor (MANF) are proteins with neurotrophic properties that protect and restore dopamine neurons in animal models of PD. However, a major limitation of NTFs and CDNF and MANF is the need for direct delivery into the brain. Drug delivery across the blood-brain barrier (BBB) is a key unmet need in neurological drug development. Nanoparticles (NPs) can be used for non-invasive delivery of drugs into the brain. Due to the customizability of size, charge, and surface chemistry, NPs display advantages as drug delivery systems posing high drug loading capacity, permeability through biological barriers, and the ability to deliver drugs to specific body parts. Several studies have shown that potential PD drug candidates that do not cross the BBB can be delivered to the brain by NPs. In this project, we optimized the lipid nanoparticle–liposome composition. Using thin-film hydration, the prepared liposomes were sonicated, followed by protein loading, and dialysis, which resulted in homogenous and purified drug-encapsulated liposomes. Before protein loading, the liposomes were characterised for size and charge. After protein loading, the encapsulation efficiency was measured using ELISA analysis.

Table of Contents

Abbreviations:

ALS	Amyotrophic Lateral Sclerosis
BBB	Blood-Brain Barrier
CDNF	Cerebral Dopamine Neurotropic factor
CEA	Carcinoembryonic antigen
COM-T	Catechol O-methyltransferase
CV	Coefficient of Variation
DI	De-Ionised water
DLS	Dynamic Light Scattering
DOPC	Dioleoyl Phosphatidyl Choline
DRS	Diffuse reflectance spectroscopy
DSC	Differential Scanning Calorimetry
DSPE	Di stearoyl Phosphatidyl ethanolamine
DTA	Differential thermal analysis
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic Reticulum
FTIR	Fourier Transform Infrared Spectroscopy
GDNF	Glial cell line-derived neurotrophic factor
GWAS	Genome-Wide Association Studies
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
LUV	Large Unilamellar Vesicles
MANF	Mesencephalic astrocyte-derived neurotrophic factor
MAO-B	Monoamine Oxidase Inhibitors
MLV	Multi-Lamellar Vesicles
MRI	Magnetic Resonance Imaging
MUV	Multilamellar vesicles
NTF	Neurotropic Factor
PD	Parkinson's Disease
PDI	Poly Disperse Intensity
PEG	Polyethylene Glycol
PET	Positron Emission Tomography
RGD	Arginyl-glycyl-aspartic acid
RUK	Rabbit Unknown Antibody
SEM	Scanning Electron Microscopy
SPECT	Single-photon emission computed tomography
SQUID	superconducting quantum interference device
SUV	Single Unilamellar Vesicles
TEM	Transmission Electron Microscopy
UPR	Unfolded Protein Response
UPSIT	The University of Pennsylvania Smell Identification Test
US	Ultrasound
UV-Vis	Ultraviolet-Visible
VFM	Variable Frequency Microwave

1. Introduction:

1.1. Parkinson's disease:

Parkinson's is a progressive neurodegenerative disorder where dopaminergic neurons are degenerated in the nigrostriatal pathway. Levy bodies consisting of alpha-synuclein, are a common neuropathological finding in PD patient brains *(Poewe et al., 2017)*. The exact mechanism behind PD is not known, but pathological events such as oxidative stress and mitochondrial dysfunction, play a role in PD *(Dias et al., 2013)*. It is a progressive disease whose prevalence increases with age, gender, and the industrialised countries *(Sveinbjornsdottir, 2016)*. A systematic review of data from the 2016 Global Burden of Disease research found that while crude prevalence grew by almost 74% between 1990 and 2016, the age-standardized prevalence rise, accounting for demographics, was less apparent, at 22% *(Dorsey et al., 2018)*. PD is characterised by parkinsonism which is featured by motor dysfunction symptoms like rigidity, tremors at rest, bradykinesia and akinesia. *(Kalia & Lang, 2015)*. There are recent studies related to the non-motor symptoms associated with PD such as abnormalities in vision, change in pain sensation and especially olfactory impairment which is seen in about 90% of the patients but not recognised, along with sleep disturbances, autonomic dysfunction, hypotension, constipation, and cognitive decline *(Pfeiffer, 2016) , (Doty et al., 1988)*. Because the symptoms are also not very specific it is always difficult to diagnose based on them even though in most cases they precede the motor symptoms *(Pfeiffer, 2016)*.

There are various risk factors both genetic and non-genetic like environmental and occupational factors associated with the PD, but the exact reason for its prevalence has still not been discovered. In context with the genetic risk factors, the relation was found not earlier than two decades ago. The most common risk factors according to the findings from GWAS and other studies are SNCA, LRRK2, MAPT as well as low-frequency coding variants in GBA *(Billingsley et al., 2018)*. GWAS is a Genome-Wide Association Study used to identify and locate the genome variants associated with a particular disease or trait *(Visscher et al., 2012)*. According to a meta-analysis report, environmental

factors for PD include exposure to pesticides, insecticides like farming chemicals, working with Metals like manganese, smoking, coffee consumption etc *(Noyce et al., 2012)*.

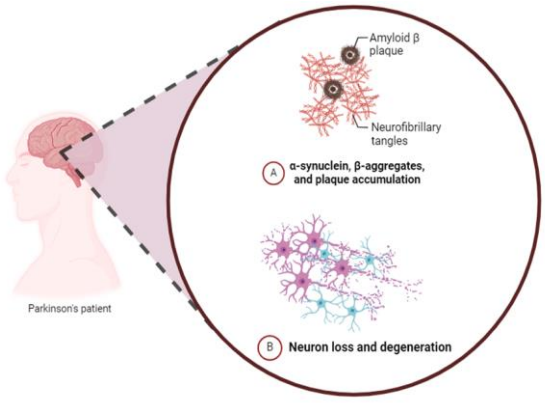


Figure 1: Characteristics of Parkinson's disease. Created with Biorender.com

Various sub-types have been proposed owing to the complexity of the disease. While some of them are based on the symptoms as motor and non-motor, sporadic and familial, age of onset and the progression rate other forms of classification include classic parkinsonism, and high gene risk (Tolosa et al., 2021).

The most important challenge to diagnose PD patient is that when the PD patient visits the doctor (due to motor symptoms) about 60-80 % of brain DA is already lost. There are various classifications used for the diagnosis criteria. One such categorisation is distinguishing three stages in Parkinson's disease development: (i) the preclinical stage—from the onset of neurodegeneration to the appearance of nonmotor symptoms; (ii) the prodromal stage—from the onset of nonmotor symptoms before the appearance of motor symptoms, bradykinesia, rigidity, rest tremor (iii) the clinical stage—from the appearance of motor symptoms to death (Schapira et al., 2017; Ugrumov, 2020). They are diagnosed mostly by clinical symptoms and neurological examinations without particular diagnostic tests. The absence of specific biomarkers to confirm the diagnosis of PD makes it difficult especially in patients with a family history and non-motor symptom scenarios. Recent developments in diagnostics include genetic testing, imaging (Structural, Diffusion-weighted MRI,

Dopamine transporter-SPECT, Fluorodeoxyglucose PET, transcranial Ultrasound), UPSIT and Sniffin Sticks for olfactory function *(Tolosa et al., 2021)*.

There are no existing treatment strategies to cure the disease completely but mostly to alleviate the symptoms, and improve the quality of life. Dopamine-based treatment for PD is a widely used therapeutic strategy. Either oral prescription of levodopa, dopamine agonists, MAO-B inhibitors, or COMT inhibitors to manage the level of endogenous dopamine. Levodopa has the potential to cross the BBB and get converted to dopamine in the brain as dopamine cannot cross the BBB. Also, medications are to be balanced with rehabilitation therapy and other lifestyle changes to increase the quality of life. Because of the complexity and heterogeneity that two people with the disorder may not have the same symptoms, pain and disease progress, it is very important to address individually the symptoms *(Church, 2021)*. For patients with problems in the levodopa or dopamine treatment such as creating more motor complications (Stocchi et al., 2008) or non-responsiveness to medical therapy, deep brain stimulation is the surgery performed to avoid any further complications *(Cernera et al., 2020)*. But Deep brain stimulations also come with its drawbacks like dysarthria, and intracranial haemorrhage *(Nabizadeh, 2022)*. Protein-based treatment is one of the most promising fields of future therapeutics for neurodegenerative diseases.

1.2. Therapeutic Protein Treatment for PD:

Various endogenous proteins have shown promising results in preclinical studies like CDNF and MANF. Neurotrophic factors (NTFs) in the preclinical and clinical studies function in the neurons' development, maturation and survival at different stages of neuronal growth *(Sullivan & Toulouse, 2011)*. In addition to development, they are also prominently involved in protective functions like improving the neurons to recover instead of dying after an injury, and further supporting its repair and regeneration functions *(Eremin et al., 2021)*. NTFs have been used in pre-clinical and clinical studies in specifically retrieving the damaged dopaminergic neurons in disease conditions like Parkinson's. GDNF, CDNF, and MANF are researched deeply in these aspects.

1.2.1. CDNF and MANF:

CDNF and MANF are proteins with neurotrophic factors. CDNF and MANF are composed of two domains namely an amino-terminal saposin-like domain and a carboxy-terminal SAP-domain. CDNF and MANF differ from other known growth factors in having unique modes of action: During cellular homeostasis CDNF and MANF interact in the endoplasmic reticulum (ER) with unfolded protein response (UPR) regulating proteins, protect cells from ER stress, and rescue neurons from ER stress-induced cell death. In other words, CDNF and MANF regulate UPR and ER stress intracellularly. Furthermore, extracellularly, CDNF and MANF act only on ER-stressed and lesioned cells and inhibit the synthesis and release of pro-inflammatory cytokines (Nadella et al., 2014; Zhao et al., 2014). They also have roles to play in regulating neuroinflammation and neuronal death *(Eremin et al., 2021)*. In specific to the research on Parkinson's, Both CDNF and its homologue MANF are involved in protecting and promoting the survival of dopaminergic neurons while also preventing further apoptosis of neurons. Studies suggest that the neuroprotective features of MANF and CDNF are only in stressed conditions and not during normal conditions *(Pakarinen & Lindholm, 2023)*. Apart from the function of neuroprotection, MANF is also seen aiding in the therapeutics of the disease. One of such roles is to have a hand in the growth and development of the axonal terminal of dopaminergic neurons *(Voutilainen et al., 2009)*.

Figure 2: 3D structure of CDNF and MANF from the Protein Data Bank

1.3. Blood-Brain Barrier and Mode of Administration:

Using peptides as treatment must be very much articulated as various other

parameters impact the efficiency. One such factor is the mode of administration. The use of intravenous or subcutaneous injections is less invasive than the other routes, but it has its drawbacks of not enough amount of the drug reaching the brain resulting in the decreased ratio of brain to blood, first-pass metabolism and causing side effects as it can reach the wrong organs. Also, with low circulation time, the half-life of the drug is not enough to overcome all the barriers in the system to reach the brain. One of the most difficult barriers is the blood-brain barrier (BBB) *(Niu et al., 2019)*. BBB protects the brain parenchyma from the periphery thus restricting molecules from entering the brain.

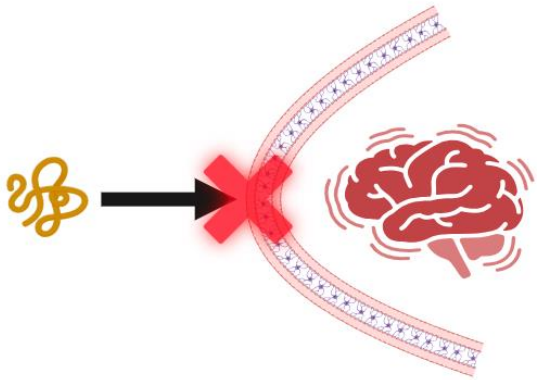


Figure 3: BBB restricts the entry of molecules thus protecting the brain. Created with Biorender.com

The structure of BBB consists of tight junction proteins and adheren junctions through which the endothelial cells are interconnected *(Johnsen et al., 2017)*. The tight junctions contribute to the low paracellular permeability and high electrical resistance of the BBB. They are also involved in various other systemic functions like synaptic transmission, synaptic remodelling, angiogenesis, and neurogenesis in the adult brain. Disruption in the structure and function of BBB will cause problems in homeostasis, inflammatory responses, and neuronal dysfunction, being also one of the hallmarks of neurodegenerative disorders like Alzheimer's, ALS, and PD etc, *(Zlokovic, 2008)*. So one of the main takeaways is to make way for the drugs to reach the site of action inside the brain without actually disrupting the BBB structure. The use of invasive methods like direct cerebral injections, and convection-enhanced diffusion has shown promising effects of the drugs, but the practicality of opening the skull of the patients is only possible in the later stage

of the disease as it is difficult to get ethical permission at early stages.

There are various researches on tackling and bypassing the BBB like transferrin receptors, nanoparticles and the use of other small lipid molecules as they can bypass the BBB *(Johnsen et al., 2017)*. Transferrin receptors are native receptors present in the system which is responsible for the transport of iron molecules to the brain via intracellular trafficking of the iron-binding protein called transferrin *(Ge & Li, 2019)*. There is research showing the inability of these receptors to completely transport the drug molecule into the brain due to various reasons like competition with native ligands, becoming internalised and either transported back to the luminal membrane or sorted to the intracellular stores of residing transferrin receptors *(Johnsen et al., 2017)*.

1.4. Nanoscience and Nanotechnology:

Nanoscience and technology is a prominent blooming field in multiple aspects of engineering, optics, applied science, molecular sciences, pharma research and industry and various other notable fields *(Sahu et al., 2021)*. They are indulged in most of the biological activities making them an important candidate for the present and future where medicine and technology meet to develop new therapeutic strategies *(Sahu et al., 2021)*. The recent advancement in nanotechnology in the medical field specifically in drug delivery is promising owing to its pros of targeted delivery, minimal toxicity, and reduced side effects. Nanoparticles comprise different types, compositions, morphology, and other classifications *(Khan et al., 2019)*. They are usually around 10 to 100 nm in range. Metal nanoparticles are also distinguished based on their colour. Based on their dimensionality, they are divided into 0D (Quantum dots), 1D (Nanorod), 2D (Nanosheets) and 3D (array of nanowires) nanomaterials *(Chenthamara et al., 2019)*. Based on the composition they can be classified as organic nanoparticles (lipids, polymer), inorganic nanoparticles (metal, ceramic), or carbon-based nanoparticles (fullerenes, carbon quantum dots).

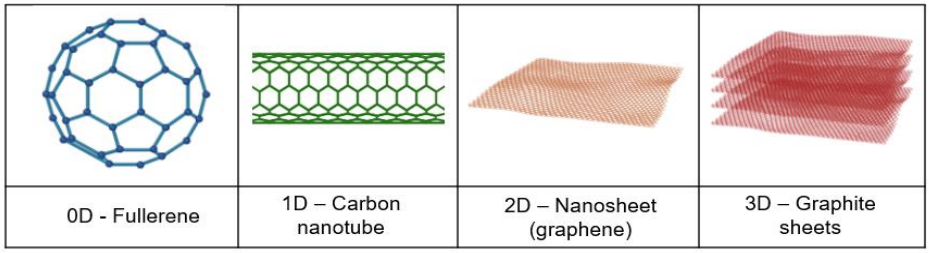


Figure 4: Nanoparticles are classified based on various factors. Based on dimension they are divided into 0D, 1D, 2D, and 3D nanoparticles with examples shown in the figure created with Biorender.com

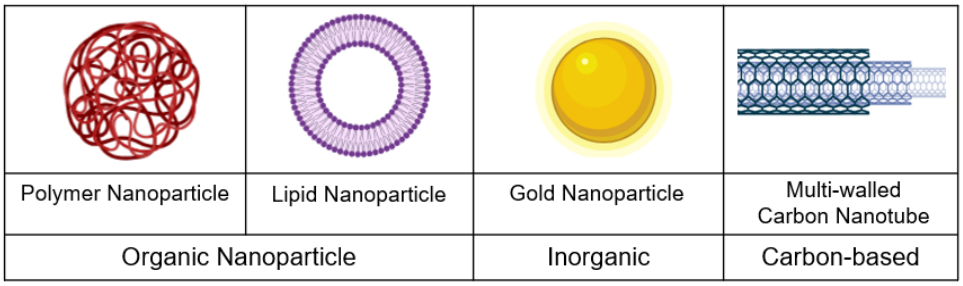


Figure 5: Nanoparticles are classified based on various factors. Based on composition they are classified as organic, inorganic and carbon-based nanoparticles with examples shown in the figure created with Biorender.com

1.4.1. Nanoparticle properties and its characterisation technique:

The nanoparticles are also known for their unique magnetic, mechanical, thermal, electrical, catalytic, and optical properties *(Joudeh & Linke, 2022)*. To start with the magnetic properties, the size of the particle is directly proportional to the magnetic anisotropic energy. At a particular size of the nanoparticle types, it matches the thermal energy leading to the random flipping of the magnetic moments. This is known as superparamagnetism (Jun et al., 2008; Kolhatkar et al., 2013). With optical properties like Surface Plasmon Resonance (SPR) of the nanoparticles, they show characteristics like photoluminescence emission and nonlinear optical properties. Also, this allows the nanoparticles to have a UV-visible excitation band which is absent in the bulk materials (Khlebtsov & Dykman, 2010; Kumbhakar et al., 2014). Owing to the quantum and surface effects of the nanoparticles, they exhibit different mechanical properties like flexibility, shear strength, plasticity, and rigidity *(Wu et al., 2020)*. In the case of thermal and electrical properties, with the decrease in size and increase in the surface area to volume ratio, there is a rise in the electrons for heat transfer than the bulk materials *(Qiu et al., 2020)*. All

these unique and important features of the nanoparticles are also used while designing the therapeutic strategies. For instance, light-sensitive, thermo-sensitive, and PH-sensitive nanoparticles are used for targeted drug release in specific regions in the human system. To analyse and characterise these nanoparticles, quality and their properties, there are various characterisation techniques.

To characterise its morphology, techniques like Scanning Electron Microscopy, Transmission Electron Microscopy, and Dynamic Light Scattering are used. To measure the structure and chemical properties, Zeta analyser, FTIR, X-ray diffraction, Raman Spectroscopy etc, are used. For thermal properties, Differential Thermal Analysis, Differential Scanning Calorimetry, and Thermo Gravimetric Analysis are being used. Techniques like SQUID and VFM for the analysis of magnetic properties and UV-Vis and DRS for the optical and electrical properties *(Joudeh & Linke, 2022)*.

1.4.2. Nanoparticles as carriers in targeted drug delivery:

Nanoparticles are used in various platforms in the medical and research fields. One of the most promising and even successful areas of nanoscience is drug delivery, especially targeted delivery to the sites which are difficult by other means, especially in the treatment of brain tumours, and neurological disorders where the main problem was crossing the BBB. Using the nanoparticles as carriers for the therapeutic moiety to reach the destination reduces the collateral damage in the other sites and reduces dose concentration as it is targeted to the site of action, leading to increased efficacy and decreased toxicity of the drug *(Hong et al)*. Drug delivery using nanoparticles can be classified as targeted, sustained and triggered based on the type of release *(Singh & Lillard, 2009)*.

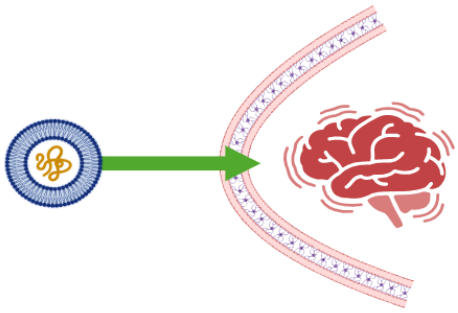


Figure 6: Nanoparticles (liposomes) serve as carriers to permit the entry of drug molecules via BBB. Created with Biorender.com

There are various strategies to overcome the problem of opsonisation which is a process of the host immune system to tag opsonin to the foreign body to signal the macrophages for phagocytosis. Using decoy nanoparticles, in which blank nanoparticles are sent initially to keep the phagocytes busy and distracted while sending the drug-loaded nanoparticles without interference *(Rao et al., 2020)*. But this method also comes with the disadvantage of too much of nanoparticles in the system and compromising the immune system against actual pathogens. Another technique is to modify the surface of the nanoparticle by changing the charge for instance, but too much positive charge will make the nanoparticle more immunogenic and thus more prone to opsonisation *(González-García et al., 2022)*. Too much of a negative charge will make it difficult to enter the cells. Thus, this removes the charge alteration solution from the picture.

The most appreciated solution is the use of Polyethylene Glycol (PEG). Optimising the PEG level in the composition helps modify the molecules' circulation time in the system. Increasing the circulation time will increase the number of drugs reaching the target without getting opsonised or phagocytosed by the native immune system *(Yoo et al., 2010)*. The nanoparticles which are coated by a substance like PEG are known as stealth nanoparticles. They can either be coated via adsorption (it can get sheared off while flowing along the bloodstream because of the blood's shear stress) or use the covalent link (direct or by using a linker/spacer molecule) *(Fam et al., 2020)*. While PEGylating the nanoparticles, there are some factors like molecular weight, density and conformation to be considered to ensure its functional efficiency. An increase in the molecular weight will affect the nanoparticle's speed, thus attracting the macrophages much faster for opsonisation *(Suk et al., 2016)*. According to

Abbasi et al, the chances of getting cleared by the system are higher for a 240nm nanoparticle if compared to a 75nm nanoparticle *(Abbasi et al., 2023)*. Even stealth nanoparticles with higher molecular weight are more prone to be cleared from the system than low molecular weight stealth nanoparticles *(Owens & Peppas, 2006)*. Also, the meagre molecular weight will not show evident modification thus failing to resist opsonisation. It should be at an optimal level. With the density of the coating, low density will lead to incomplete coverage and hence opsonisation, while very high density retards the movement failing to internalise into the cell. The Optimal range is 2- 4 nm. With the conformation, there are four major structures: brush, bunch, loop and star. In the case of the bunch and star, the movement is restricted leading to the alternative pathway of complement activation. With Brush and Loop, they are more flexible with the movement (brush can move left to right and loop can move up and down) and can oppress opsonisation. So, based on the requirement and application of the nanoparticles, they are optimised and synthesised using various techniques like chemical reduction, microemulsion, coprecipitation, and sono-electrodeposition *(Nam & Luong, 2019)*.

1.5. Liposomes as carriers for Brain-related disorders:

Liposomes are lipid nanoparticles which contain different lipid components in various ratios based on the application requirement. Liposomes are special because of their unique properties like self-assembly, ability to encapsulate both hydrophobic and hydrophilic drugs, biocompatible and degradable. Liposomes can be classified as small unilamellar vesicles, large unilamellar vesicles, multilamellar vesicles and multi-vesicular vesicles. There are two types of targeted carrier liposomes; active and passive targeting. Tagged Boron and acylated midocarborane with liposomes for synovium where the synovium to blood ratio was observed to be 3.0 which is very high in this case *(Watson-Clark et al., 1998)*. The use of targeting ligands like immuno-liposomes, transferrin, and RGD-modified liposomes are active drug liposomes. In boronated model immuno-liposomes, PEGylated liposomes are modified with antibodies called anti-CEA when the target is the Carcinogenic Embryonic Antigen in the cancer cell *(Yanagië et al., 1991)*. Targeting transferrin receptors and tagging transferrin with the PEGylated Liposomes resulted in more entry

through BBB than PEGylated Liposomes *(AlSawaftah et al., 2021)*. Targeting overexpressed integrin α(v)β(3) in cancer increased the tumor-to-blood ratio than the untargeted delivery *(Sheikh et al., 2022)*.

Liposomes can be classified based on their structure, size and number of bilayers as small unilamellar vesicles (SUV), large unilamellar vesicles (LUV), multilamellar vesicles (MLV), and multi-vesicular vesicles (MVV). Based on their compositions and applications, liposomes can be classified into conventional, charged, stealth stable, actively targeted, stimuli-responsive (remote and local - light, Redox, Thermal, pH, and enzyme), and bubble liposomes *(Nsairat et al., 2022)*.

These liposomes can be synthesised using various techniques. Some of the techniques are thin-film hydration, solvent injection, detergent removal, Hydration-dehydration, heating, pH jumping method, and microfluidics. These depend on factors like the polarity of the drug, for example, solvent injection is preferred for hydrophobic drugs, where the lipid and organic solvent are added with the aqueous phase solution under a specific temperature and constant stirring followed by evaporation of the organic solvent leading to liposomal suspension and thin film hydration for the hydrophilic drugs where evaporation of the organic solvent where the lipids are dissolved is done primarily and the aqueous phase addition is done in the next step *(Akbarzadeh et al., 2013; Nsairat et al., 2022)*. In the detergent removal method, lipids, high critical micellar concentrated surfactant and organic solvent are mixed and subjected to evaporation followed by the removal of surfactant to retrieve LUV after solvent concentration *(Vemuri & Rhodes, 1995)*. In microfluidics, the organic phase with hydrophobic molecules (lipids) and the hydrophilic molecule in the aqueous phase are made to go through the axial mix of various cycles in microchannels leading to liposomal suspension *(Nsairat et al., 2022; Yu et al., 2009)*. The encapsulation percentage of the drug varies greatly based on the encapsulation technique, the composition of the liposome and the drug's properties *(Lee, 2020)*. The nanoparticles are not homogenised depending on the preparation method and other parameters. To convert the Multi Lamellar Vesicles (MLV) into SUV and LUV, methods like extrusion and sonication (tip and bath) are used *(Mozafari, 2010)*. The liposomal solution is subjected to the ultrasound for a certain time interval, pulses and frequency *(Lombardo &*

Kiselev, 2022). Once immersed in the sample, the tip sonicator emits the US, which creates cavitation and forces. In contrast, in the bath sonicator, the container is filled with water and ultrasound is passed on to the sample through water which is evenly spread throughout.

Once the drug is encapsulated, it is also necessary to remove the unencapsulated drug from the sample. Purification of the sample can be done by various means like dialysis, ultracentrifugation, tangential flow infiltration, ion exchange and size exclusion chromatography. One of the most common methods is dialysis, where the samples in the dialysis bag have pores which will allow the movement of the free drugs outside the bag into the solution leaving behind the drug-encapsulated liposomes *(Lin & Qi, 2021)*.

Characterisation and further analysis of the synthesised drug-encapsulated nanoparticles could be done using various methods. One such characterisation tool is using ELISA – Enzyme-Linked Immunosorbent Assay to calculate the number of peptides that have practically made it inside the prepared structure. Though this method requires lysis of the liposome, it helps identify low limits of peptide detection because of the high specificity of the antibodies *(Aydin, 2015; Edwards & Baeumner, 2006)*. Various methods to perform the lysis include mechanical, enzymatic and chemical methods. The use of force (mechanical means) has its downfall in damaging the peptides as well. So, chemical lysis methods using solvents and surfactants like triton-X 100 are preferred *(Clark et al., 1986; Edwards & Baeumner, 2006)*. ELISA is a technique that detects and quantifies a specific molecule like proteins. There are types of ELISA direct, indirect, sandwich, and competitive ELISA *(Shah & Maghsoudlou, 2016)*. The sample of interest (considered as an antigen) is fixed in the well plate in which the experiment is carried out, followed by the addition of complementary antibodies to form the antigen-antibody complex. In case of the direct ELISA, the enzyme is attached directly to the primary antibody for detection and in indirect ELISA the primary antibody is attached to the secondary antibody which is attached to the enzyme for detection. The introduction of a substrate generates a colour signal, indicating the presence of the antigen in the sample. The optical density measurement is directly proportional to the amount of antigen in the sample *(Shah & Maghsoudlou, 2016)*.

In this project, liposomes are optimised and synthesised followed by peptide encapsulation and characterisation to enhance the efficiency of delivery of the peptides for the treatment of Parkinson's disease

Aims of the study:

- To optimise the liposome composition and its synthesis
- Characterisation of the liposome
- Protein encapsulation
- Characterisation and Analysis - ELISA

2. Methods and materials:

2.1. Optimising Composition

Many study suggest combinations of lipids based on the carrier's requirement for the drug/molecule it carries. As discussed in the introduction, it also depends on the mode of administration. To increase the structural stability and as a neutral lipid, the main component of the liposome is Dioleoyl Phosphatidyl Choline (DOPC). To improve the circulation time, reduce opsonisation and for the stealth layer, Di stearoyl Phosphatidyl Ethanolamine- Poly - (Ethylene Glycol) (DSPE-PEG) is the next component in the liposome. To avoid a very rigid structure, cholesterol is also added to give fluidity to the component. Adding too much cholesterol could cause an increase in the gaps between the structures causing the leak of the drug. The final liposome is a component composed of DOPC-DSPE-PEG-Cholesterol. The molecular ratio of DOPC-DSPE-PEG-Cholesterol was decided as 45:5:50 respectively.

2.2. Synthesis:

2.2.1. Thin film hydration:

Thin film hydration is one of the most used techniques in the preparation of liposomes. It involves two steps, formation of the thin film by evaporation of the organic solvent, followed by hydration of the layer by adding the aqueous solution. To prepare the thin film, rotavapor is used. All the lipids for the preparation of liposomes are in the organic solvent. They are added with the amount calculated by the ratio and the stock solution in a test tube.

Table 1: Liposome formula

Lipids (Mol W)	Molecular Ratio	Stock Soln (mg/ml)	In µl
DOPC	45	25	283
DSPE-PEG	5	20	140
Cholesterol	50	50	77.3

Based on the transition temperature of the lipids, the water bath is pre-heated (10 degrees higher than the transition temperature). The water bath was set to 63°c. The vacuum pump, pressure meter, water faucet and the gas are all turned on. The pressure was set in the range of 800 – 1000 mBar. The test tube with the lipids was placed inside a bigger test tube which was later inserted on the bottom of the bigger rotavapor tube and clipped. The rotation was turned on and the pressure was set to 450 – 500 mBar. The setup was maintained for 45 minutes. Later, to remove the organic solvent (mostly chloroform) the pressure was reduced to 80 -100 mBar for 10 minutes. After 10 minutes, the pressure was increased to 800 - 1000 mBar and the test tube was recovered from the rotavapor, and the setup was turned off in the reverse sequential order.

The hydrating solution was then added to the test tube, put in the water bath for 30 to 60 minutes and vortexed from time to time until no film was seen through the naked eye.

2.2.2. Sonication

The prepared liposomes were subjected to tip sonication to homogenise the solution and convert all the MLV to SUV. The tip was immersed in the liposome solution and the time was set to 15 seconds without changing the temperature settings. To regulate the temperature, the tube with the liposome solution was placed in dry ice.

2.2.3. Characterisation

The liposomes were then characterised for size and charge using zeta sizer ZS (Malvern Instruments). The liposomal solution (10 µl) was added to the HEPES buffer (990 µl) and then added to the cuvette to analyse the size. The pre-designed SOP for size measurement was used to get the results. To check the surface charge and PDI, the liposomal solution (10 µl) was added to De Ionised water (990 µl) and then added to the appropriate cuvette. A specific pre-designed SOP was used for the measurement.

2.2.4. Protein Loading

To encapsulate the peptides in the liposome, the freeze-thaw technique was used. Based on the stability of the peptides the number of cycles was limited to three. The amount of peptides to be loaded was calculated by assuming the encapsulation efficiency of 1%. So to encapsulate 2ng/ml, 0.2µg/ml was added to the liposome solution before the cycle started. The liposomes were subjected to thirty minutes at -80°C and thirty minutes at 60°C. To prevent sudden shock, intermediate steps were added. After thirty minutes at -80°C, 10 minutes at room temperature before putting in 60°C and after thirty minutes at 60°C, 10 minutes at -20°C before putting in -80°C. The cycle was repeated three times.

2.2.5. Dialysis

After protein loading, purification is required to remove the unencapsulated peptides from the liposome solution before further analysis. Without purification, it causes miscalculation of the number of peptides present inside the liposomes and gives information about the free peptides which failed to get loaded in the liposomes. One of the common methods to do purification is dialysis. The liposome solution was filled in the dialysis bag. The bag was cut based on the amount of solution added Based on the dimensions of the bag, the protocol stated 3.2cm/ml. Once cut, one end of the bag was sealed using the dialysis clips. After adding the solution, the other side was also clipped. The bag was then immersed in the container with DI water. The whole set-up was placed in the magnetic stirrer at 4°C for 2-3 days. The DI water was replaced three times a day. After dialysis, the purified liposomes were collected for analysis.

2.2.6. Liposome Lysis

To calculate the number of peptides that actually were encapsulated in the liposomes, Enzyme-Linked Immuno-Sorbent Assay (ELISA) was done. To perform ELISA, Lysis of the liposome structure to release the peptides, the surfactant triton-x 100 was used. 1% Triton-X was added to the solution for a time interval of 1 hour.

2.2.7. ELISA Analysis

A 3-day protocol of ELISA specific for CDNF was followed for the analysis. The plate contained standard duplicates, duplicates of blank liposomes (with a dilution factor of 1/1000, 1/2000, 1/4000, and 1/8000), unpurified liposomes (with a dilution factor of 1/1000, 1/2000, 1/4000, and 1/8000) and purified liposomes (with a dilution factor of 1/2, 1/4, 1/8, and 1/16). The change in the dilution factor for the purified liposomes was because of the calculation made about the encapsulation efficiency as 1%. Day 1 involved coating the ELISA plate with coating buffer and RUK Antibody (Rabbit unknown) (ICOSAGEN; CAT: 300-500). The plate was then incubated overnight. Day 2 involved the steps of blocking and adding the samples and standards. Day 3 involved the addition of respective primary (R and D; REF: DS 98 200) and secondary (Agilent Technologies; REF: P0448) antibodies followed by detection molecule addition and finally reading the absorption results in the BI Victor 3 at 530 – 450 nm.

3. Results:

3.1. Optimisation and Synthesis of liposome

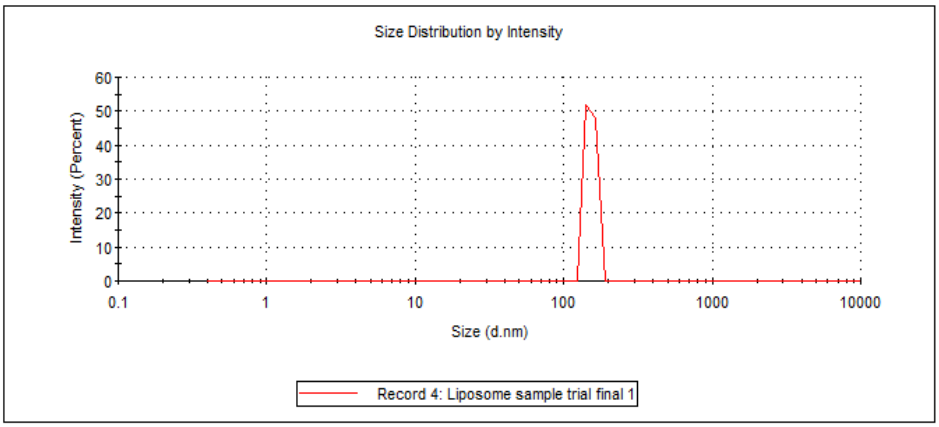
The liposomes were synthesised in the rotavapor to form the thin film. The last 10 minutes were critical as it helped in the complete evaporation of the chloroform. Following the evaporation, in trial 1 of liposome formation, the peptides were added in the hydration step. Since the results were not positive because of heterogeneous and aggregation of the liposomes formed, the other trials were conducted so that the liposomes were prepared without the protein encapsulation step followed by sonication to homogenise the samples to avoid large-sized and multi-lamellar vesicle liposomes.

3.2. Characterisation of liposomes

The importance of sonication was evident in the characterisation results of the liposome samples. The zeta analyser was used for the size and charge values of the liposome samples. Without sonication, the liposome size was very high, making it invalid for the charge analysis. The average size was 7645 d.nm and the Polydisperse Index (PDI) width of 2962.2 d.nm. In the other trial, the size was 4065 d.nm and also with a very high PDI of 0.913. The samples were polydisperse and did not meet the quality criteria and the analysis for the charge. When the samples were sonicated in the tip sonicator and then characterised, the results were promising. The average size of the liposomes was greatly reduced to 171.2 d.nm with a good PDI of 0.349 and PDI width of 101.1 d.nm. The sonicated samples were of good quality and the requirement criteria for testing the zeta potential of the sampled was cleared. The zeta potential was measured as -31.6 mV. The optimal value for zeta potential usually lies > +30 or > -30mV. The higher the value more it repels and the less aggregation of the samples which is a requirement for good liposomes *(Sharma, 2019)*.

		Size (d.nm):	% Intensity:	St Dev (d.nm):
Z-Average (d.nm): 7645	Peak 1:	152.6	100.0	11.20
PdI: 0.150	Peak 2:	0.000	0.0	0.000
Intercept: 0.945	Peak 3:	0.000	0.0	0.000

Result quality : Refer to quality report



		Size (d.nm):	% Intensity:	St Dev (d.nm):
Z-Average (d.nm): 171.2	Peak 1:	216.9	96.0	135.6
PdI: 0.349	Peak 2:	4872	4.0	690.8
Intercept: 0.960	Peak 3:	0.000	0.0	0.000

Result quality : Good

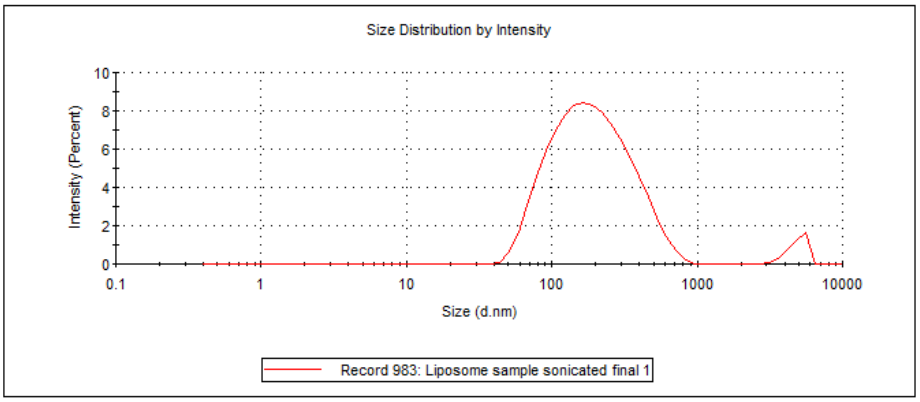


Figure 7: Size Analysis: Liposomes were analysed before and after sonication to calculate their size. (top) Unsonicated Liposome. (bottom) Sonciated liposomes. The graph shows that before sonication the size of the liposomes was 10 folds higher than the size of the sonicated samples. In the left graph, the y-axis value (intensity) is high, meaning the liposome size is larger.

RESULT DOES NOT MEET QUALITY CRITERIA

Multimodal fit error high
 * Data quality too poor for distribution analysis
 * Sample too polydisperse for distribution analysis

Cumulant fit error high
 * Data quality too poor for cumulant analysis
 * Sample too polydisperse for cumulant analysis

zAverage is larger than upper size display limit 7645
> 6000)
 * Wrong size limits used in display range

Figure 8: Quality Report generated by the zeta analyser for the unsonicated liposomes. It clearly states the larger size and poor quality of the results for further analysis

		Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): -31.6	**Peak 1:**	-31.6	100.0	4.19
Zeta Deviation (mV): 4.19	**Peak 2:**	0.00	0.0	0.00
Conductivity (mS/cm): 0.220	**Peak 3:**	0.00	0.0	0.00

Result quality : Good

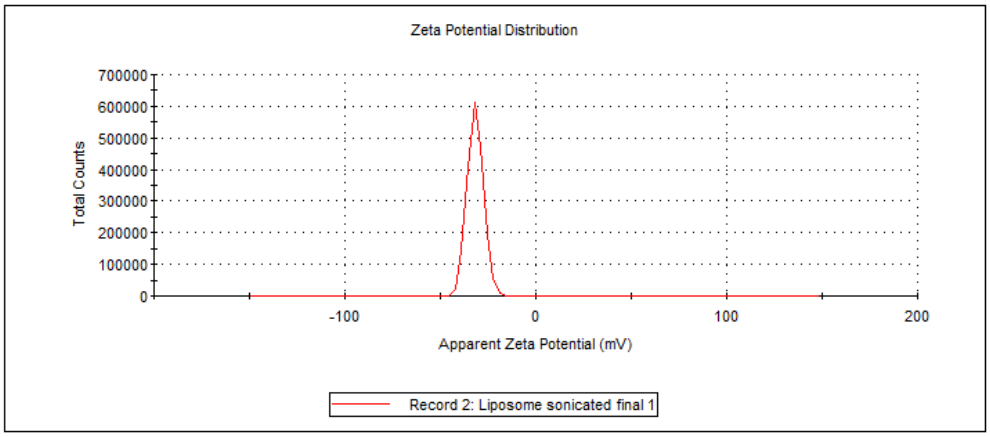


Figure 9: Zeta Potential for Sonicated Liposomes. The X-axis denotes the charge of the liposome is negative (the peak is left of 0) and the single peak represents all the liposomes are in the same range of zeta potential and no subpopulation. The Y-axis represents the total counts of liposomes at specific potential (here -31.6 mV).

		Mean (µmcm/Vs)	Area (%)	St Dev (umcm/Vs)
Mobility (µmcm/Vs): -2.479	Peak 1:	-2.48	100.0	0.329
Mobility Dev. (µmcm/Vs): 0.3285	Peak 2:	0.00	0.0	0.00
Conductivity (mS/cm): 0.220	Peak 3:	0.00	0.0	0.00
Result quality : Good				

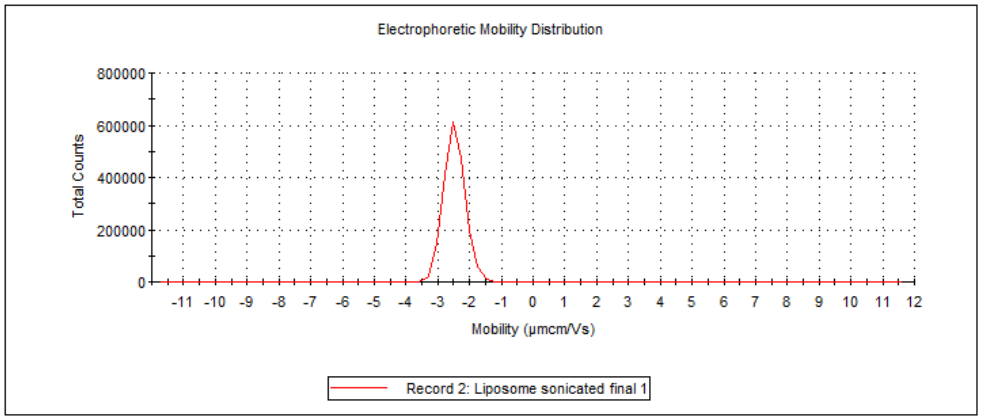


Figure 80: Electrophoretic Mobility Distribution explains how fast the particles move in an electric field. The higher the value, the stronger the liposome's charge. The Y-axis represents the total counts of liposomes at specific mobility value (here -2.479 µmcm/Vs).

3.3. Protein Loading and Dialysis

The addition of the peptides during the hydration was unsuccessful because of the liposome aggregation and the polydisperse nature. The liposome sample post-sonication was loaded with peptides through the freeze-thaw technique. The completion of the three cycles took around four hours, and the liposome solution was stored at -20°C until further use. The dialysis of the samples resulted in the purified liposomes. The free peptides were small enough to pass through the pores of the dialysis bag. The solution with purified liposomes was then collected back in the Eppendorf tube from the dialysis bag for analysis of the number of peptides encapsulated in the liposomes using ELISA.

3.4. ELISA Analysis:

Lysis buffer to degrade the liposomes to free the encapsulated drugs was performed using triton-X 100 for 1 hour. After that ELISA protocol was carried out for 3 days. At the end of day 3, the 96-well plate was subjected to the plate reader for absorption (450/530 nm) measurement. Using the absorption values, the standard curve was plotted using a series of mathematical calculations. It started with finding the average of the duplicates, normalized

values, and then the CV% of the standards. With the obtained standard curve, the R^2 equation was retrieved which was used to calculate the recovery value. Various trials were conducted because of the failure in the results. There could be various factors associated with those results. Some of them could be due to handling errors, antibody inefficacy, lysis buffer interference, and standard solution peptide inefficacy. Troubleshooting the protocol with the use of fresh peptides for the samples resulted in positive results. Changing the antibodies batch in the final analysis resulted in the R^2 value of 0.96 and the recovery values were in the range. The equation values were used to calculate the recovery of the blank liposomes and the peptide-encapsulated liposomes. The recovery value was notably lower for the 1000 picogram but the 500 picogram almost had a 50% recovery with the value of 238 picograms. The blank liposomes with no peptides did not have recovery values as expected.

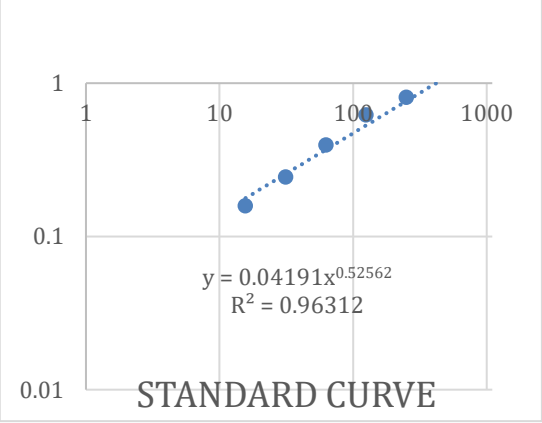


Figure 9: Standard Curve

Expected (pg/ml)	Recovery (pg/ml)
1000	728.7388595
500	537.0915165
250	279.5391643
125	169.3341592
62.5	71.38079173
31.25	28.65845498
15.625	12.56333869

Table 2: Recovery Table

Recovery:

Blank Liposomes (pg)		Peptide + Liposomes (pg)	
Expected	Recovery	Expected	Recovery
0	2.171644	1000	308.121
0	2.617701	500	238.563
0	1.855515	250	155.563
0	2.617701	125	190.104

Table 3: Recovery data.

Failed Standard Curves with its Recovery table:

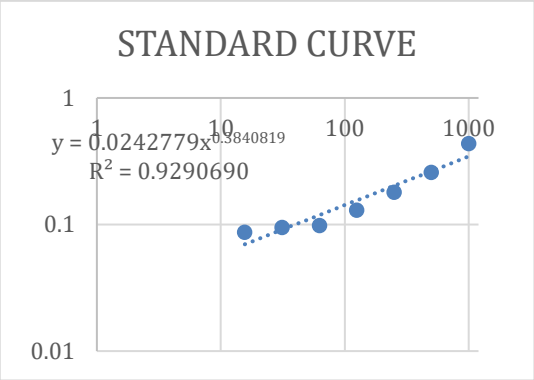


Figure 10: Trial 1

Expected (pg/ml)	Recovery (pg/ml)
1000	1860.069085
500	475.054332
250	184.2051276
125	78.94698098
62.5	38.3337949
31.25	34.88769246
15.625	27.74616461

Table 4: Recovery Table

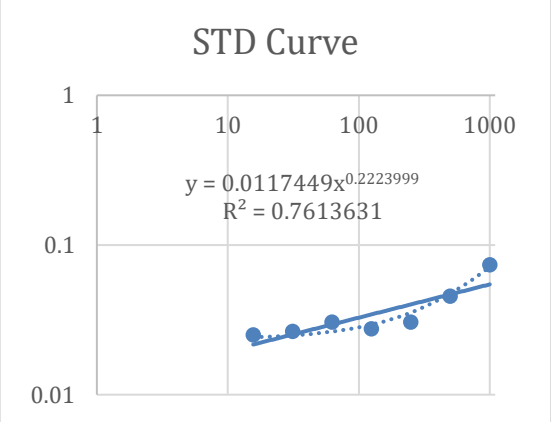


Figure 11: Trial 2

Expected (pg/ml)	Recovery (pg/ml)
1000	3811.604191
500	441.1789958
250	73.03605895
125	45.8507915
62.5	73.03605895
31.25	38.8162862
15.625	29.86954209

Table 5: Recovery Table

4. Discussion:

The ultimate goal of this study is to develop a novel drug candidate combined with liposomes for the treatment of PD. In this master thesis experimental part, I optimized and characterised liposomes which were then loaded with CDNF peptide. As there is a lot of research in the field of peptide treatment for neurodegenerative diseases, the drug delivery part is still struggling because of the immune system attack (opsonisation or phagocytosis) and the blood-brain barrier. The failure of the drug, especially in *vivo* even after successful results in the in-silico and in-vitro studies, is because of the system complexity and its impact on the drug of interest, making it impossible to reach the target. On the other hand, nanotechnology stands as a tool by providing a solution to this problem. The use of nanoparticles as carriers especially in places in the system where naked drugs fail to reach like the brain for the treatment of brain tumours and other neurological diseases is a blooming section of research. In this study, liposomes which are lipid nanoparticles were optimised for the composition based on the requirement and synthesised using the thin film hydration technique with rotavapor. The synthesised liposomes were then sonicated for Single Unilamellar Vesicles and less aggregated liposomes. Using the freeze-thaw technique, peptides were loaded into the liposomes by alternate intense heat-cold treatment. Dialysis was performed for the purification of the liposomes and after the removal of the free peptides, the peptide-encapsulated liposomes were then quantified using the ELISA protocol.

4.1. Limitations of the study

The study involves designing, optimising, and composing the liposomes followed by protein encapsulation. However, some unmet challenges are yet to be addressed. To prove that liposomes are better models for the carriers of peptides, further designing experiments involving cell culture to check how they perform in real-life situations is important. Due to time constraints and other technical withholds, *in vitro* studies were not completed. The ELISA experiment's inconsistent outcome resulted in troubleshooting and increased the number of trials. The study did not address other characterisation methods for the liposomes like Transmission Electron Microscopy (TEM) or the release

profile studies to understand more about the structure and functional properties of the prepared particles.

4.2. Future Perspectives and Conclusion

Successful treatment strategies for neurological diseases like PD is the need of the hour in the research field. The incorporation of the field of nanoscience to enhance the efficacy and functionality of the drugs is one of the important findings in the scientific community. After successful liposome preparation, the bioactivity of the drug-liposome complex should be studied in the in vitro model of PD. After demonstrating the bioactivity of the drug-liposome complex in a cell model of PD, the therapeutic effect in an animal model of PD can be assessed

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