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Liposomal encapsulation of levodopa to improve current Parkinson's disease treatment

Predicting retention kinetics of liposomal encapsulated levodopa in relevant biological media with innovative drug-retention method

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Abstract

Parkinson's disease (PD) is the second most prevalent neurodegenerative disease known. PD is characterised by the progressive degeneration of dopaminergic neurons in the *substantia nigra pars compacta* (SNpc) due to the formation of Lewy Bodies (LB). LB disturb essential cellular pathways and induce neural cell death, resulting in dopamine (DA) deficiencies which manifests itself in (non-)motor symptoms including tremors, rigidity and loss of smell. At the time of writing, no treatment exist that cures or slows down the progressivity of PD. Current treatments aim at the delivery of L-3,4-dihydrophenylalanine (L-DOPA), a DA precursor, to the brain. L-DOPA is generally taken orally as this is convenient for patients, but is poorly taken up systemically, resulting in inefficient drug delivery. Moreover, rapid enzymatic and oxidative conversion of L-DOPA systemically cause a low half-life (50 minutes), leading to the need of high and frequent drug intake. As PD progresses, the SNpc lose the ability to store L-DOPA which further increases the need of excessive L-DOPA intake. As 1% of the administered L-DOPA reaches the brain, the majority is present systemically resulting in severe side effects including dyskinesia.

To improve current treatments, L-DOPA is encapsulated in liposomes to protect against rapid degradation, lead to sustained drug release, lower the systemic concentration and result in increased (indirect) targeting. The preparation, characterisation and L-DOPA loading of the liposomes have been formulated by previous students. This report continues on the project by demonstrating the L-DOPA retention kinetics in liposomes in relevant biological media to mimic *in vivo*. These retention studies have been performed with an innovative method, based on the biotin and streptavidin interaction.

The retention kinetics in blood plasma demonstrated the presence of a burst release in the first 2h of incubation in which close to 40% of the original content is released. Incubation of ≥6h revealed a discontinuation in drug release. Retention study in HEPES buffered saline (HBS) (pH 6.5) revealed a smaller burst release of 27% in the first 2h, followed by a 1%/h drug release, indicating the cruciality of blood components on liposomal drug release. Unfortunately, the retention studies performed in whole blood were unconclusive as molecules closely resembled L-DOPA were present in measured samples, making the quantification of solely L-DOPA unattainable. Moreover, the results concluded that applying HBS or dialyse cassettes for retention studies can be used to create an initial indication, but no *in vivo* retention kinetics can be derived due to the lack of protein corona formulation.

In conclusion, the liposomal encapsulation of L-DOPA is promising for future treatments as pharmacological properties including half-life and sustained drug release are improved. Moreover, the use of the biotin-streptavidin method has shown to be advantageous in predicting the drug retention kinetics *in vivo.* Yet additional research is needed to improve the method.

Layman's summary

Parkinson's disease (PD) is a brain disease that has a high occurrence in which the neurons that produce dopamine (DA) break down in time. As DA is essential for the control of body movements, the insufficient production of DA in PD patients eventually lead to motor dysfunctions symptoms, such as shaking. However, the loss of PD also leads to non-motor symptoms including sleeping problems and the loss of smell. As PD is more present in elderly, this disease will have a higher impact later in time as people generally grow older due to modern healthcare. But, to this day, there is no drug that stops or inhibits the progression of PD. There are multiple treatments available and most are based on increasing the amount of DA in the brain. The most used treatment is L-3,4-dihydrophenylalanine (L-DOPA), which is taken orally. As L-DOPA can be made into DA, increasing the L-DOPA levels indirectly leads to a rise in the DA levels as well. However, this treatment has many down-sides. Most of these are caused by the high instability of the drug. It takes 50 minutes for the human body to remove 50% of the L-DOPA concentration. This results in the need of frequent and high L-DOPA dosages administration. Moreover, because the drug is taken orally, a big fraction of the drug is removed by the stomach, intestine and liver in a process called first-pass metabolism before the drug even reaches the bloodstream. The combination of the instability and first-pass metabolism result in only 1% of the taken drug actually reaches the brain. The other 99% of the drug is present elsewhere in the body, leading to side effects such as unvoluntary movements of the face or arms/legs. Therefore, a new treatment is needed.

In this report, the use of liposomes that are able to envelop L-DOPA is described. Liposomes are small vesicles that are able to carry and protect L-DOPA, thus increasing the stability. Moreover, this treatment can be given intravenously, so the first-pass metabolism does not apply as the drug is injected directly in the bloodstream. As a small portion of L-DOPA leaks out of the liposomes at a time, also known as drug release, the exposed amount of drug can be regulated better compared to the oral drug. However, how much and how fast the drug is released out of the liposomes is unknown. Today's techniques are not able to follow that drug release precisely. Therefore, this report describes a newly developed method to analyse the L-DOPA release.

The results of the experiments that were done to determine the release of L-DOPA from the liposomes were positive. In blood plasma, it was revealed that half of the original L-DOPA concentration leaked out of the liposomes in the first 6h. That was followed by a hold in drug leaking. This is an improvement on the orally taken version of L-DOPA. Unfortunately, the results were difficult to analyse when the liposomes were placed in blood, so further development on the method is needed.

In conclusion, enveloping L-DOPA in liposomes has shown to be promising for improving the treatment for PD. Moreover, the method that is used to measure the leaking speed has shown to be convenient. However, additional research is needed to improve the method.

List of Abbreviations (alphabetical)

AUC	Area under the curve
BBB	Blood-brain-barrier
CPDA-1	Citrate phosphate dextrose adenine
DA	Dopamine
CF	Continuous flow
DDC	DOPA Decarboxylase
DI	Decarboxylase inhibitors
DiD	1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine-4-chlorobenzenesulfonate salt
DLS	Dynamic light scattering
DMF	Dimethylformamide
DPPC	Dipalmitoyl phosphatidylcholine
DSPE-PEG(2000)	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000]
ECD	Electrochemical Determination
EDTA	ethylenediaminetetraacetic acid
EE%	Encapsulation Efficiency
ELS	Electrophoretic light scattering
HBS	HEPES buffered saline
IV	Intravenously
LB	Lewy Bodie
L-DOPA	L-3,4-dihydrophenylalanine
MSB	Magnetic streptavidin beads
PBS	Phosphate buffered saline
PC	Protein Corona
PD	Parkinson disease
PDI	Polydispersity index
RBC	Red blood cell(s)
SNpc	*Substantia nigra pars compacta*
SS	Sample and separate
$T^{1/2}$	Half-life
TL	Total lipids
UPLC	Ultra-performance liquid chromatography

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Inhoud

1. Introduction

Parkinson's disease (PD) is the second most prevalent neurodegenerative disorder, affecting 1-2 per 1000 of the total world population[1,2]. PD predominantly occurs in elderly, increasing its prevalence from 1% in 60-year-olds to 2% for the population with the age of 80[1,3]. As the world population is ageing due to modern medicine, the number of PD patients will rise in time, increasing the economic and social burden[1,3,4]. Besides advanced age, European ancestry and the male gender are epidemiological factors that increase the liability of PD[3].

The progressive pathology of PD is largely based on the degeneration of dopaminergic neurons in the *substantia nigra pars compacta* (SNpc)[5]. This small, yet dense, area of neurons in the mid-brain is predominantly responsible for the dopamine (DA) production, making DA deficiency the main characteristic of PD[5]. The degeneration of the dopaminergic neurons is thought to be mostly caused by the presence of α-synuclein aggregations called Lewy Bodies (LB). These clusters of proteins induce microtubule regression, leading to disrupted cellular transport in neurons which impairs neurological signaling[6]. Moreover, LB are associated with nuclear degradation, leading to cell death[7]. As DA is crucial for motor control and LB are known to disrupt brain functions in the lower brain stem, these pathological changes lead to numerous motor (tremor, akinesia, rigidity, etc.) and nonmotor (fatigue, depression, dementia, etc) symptoms[1,5,7]. How this disease originates is often unknown. However, mutations in several genes correlate to the susceptibility of PD[8]. Environmental factors correlated with increased risk of developing PD are exposure to pesticides and herbicides[9].

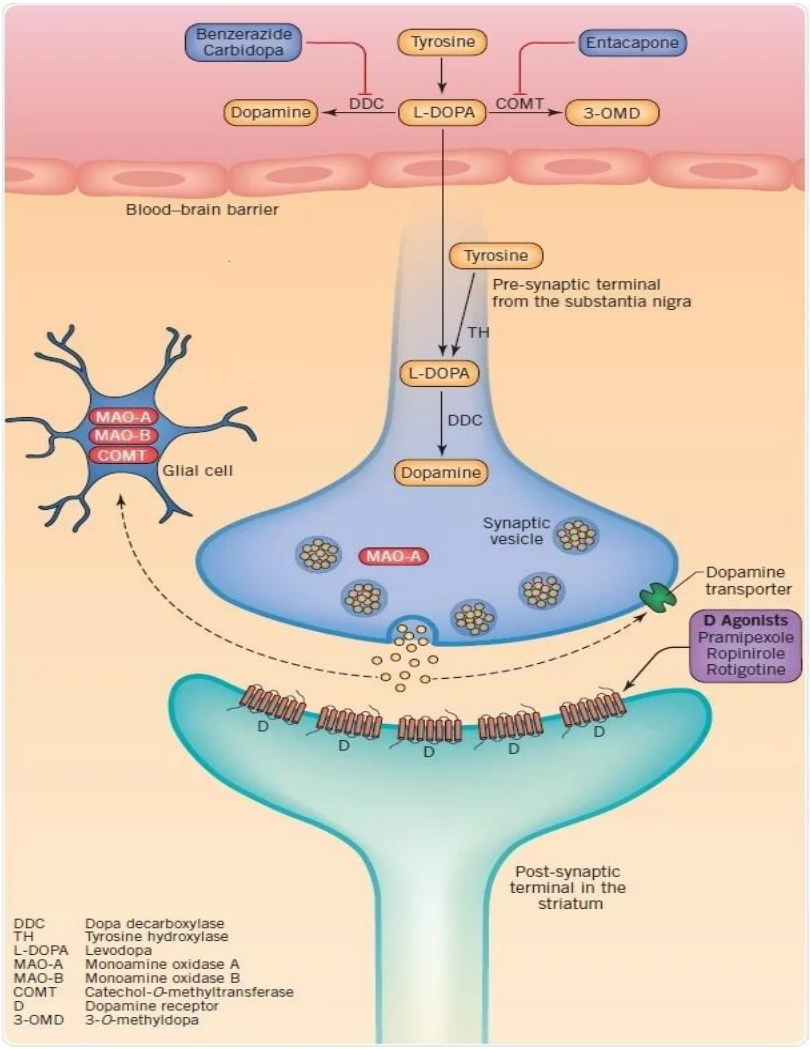


Figure 1: A schematic overview of the dopamine production in dopaminergic neurons. Also, PD treatments are depicted. Figure from: News Medical, 2019[10].

In Figure 1, the DA production in dopaminergic neurons is shown[10]. Systemically present L-3,4-dihydroxyphenylala-nine (L-DOPA), also known as levodopa, is able to cross the blood-brain-barrier (BBB)[11]. L-DOPA is a DA precursor and is converted to DA in the dopaminergic neurons[11,12]. Subsequently, DA can be released in the synaptic gap to create a signal post-synaptically. These DA producing neurons progressively break down in PD patients, leading to DA deficiencies. Several early-stage treatments are available, such as monoamine-oxidase (MAO) inhibitors (lowers breakdown DA by MAO enzymes), DOPA decarboxylase (DDC) and catechol-*O*-methyltransferase inhibitors (decreases systemic L-DOPA conversion and competition for BBB transport) and DA agonists (mimicking DA in synaptic gap)[10,13]. However, due to the progressive nature of PD, the increasing shortcoming in DA production eventually grows beyond the efficacy of these treatments. At that point, physicians generally switch to the combination of the earlier treatment and L-DOPA[12].

L-DOPA is the golden standard treatment for PD for over 60 years as the treatment is extensively researched and is effective in relieving PD symptoms. The pharmacological foundation of the treatment is raising the systemic L-DOPA concentration, resulting in more L-DOPA crossing the BBB reaching the SNpc. L-DOPA is generally taken orally and the drug is mainly absorbed in the upper intestine. A fraction of the absorbed L-DOPA concentration crosses the BBB and is converted to DA. Therefore, L-DOPA is considered a prodrug[11].

Even though L-DOPA is the golden standard, PD patients suffer from the many flaws this treatment contains. The primary issue is the half-life ($T^{1/2}$), which is close to 50 minutes[14]. This can be increased to 1.5h with DDC inhibitors such as carbidopa or benserazide[14,15]. Due to this short $T^{1/2}$, patients need L-DOPA administrations 3-5 times a day with a high dosage up to 2450 mg/day[16]. Surprisingly, close to 1% of the taken drug reaches the brain unconverted, of which only a part reaches the SNpc[11]. The high dosage and poor targeting result in high systemic L-DOPA concentrations that lead to L-DOPA caused side-effects such as dyskinesia, the unvoluntary movement of the face and limbs[12]. A secondary problem with the L-DOPA treatment, caused by the progressive nature of PD, is that the effect of L-DOPA wears off. The so called “honeymoon phase” describes the first 3-5 years in which L-DOPA treatment is effective (Figure 2)[13,17,18]. Early-stage PD patients have enough dopaminergic neurons that partly control the release and storage of DA after the administration of L-DOPA. Later-stage patients have suffered more degeneration of dopaminergic neurons and lack the L-DOPA buffer capacity so more frequent and higher dosage administrations of L-DOPA are needed to meet the minimal concentration of DA to relieve patients from PD symptoms[19,20]. However, increases in L-DOPA are correlated to higher risk of dyskinesia[12,21].

To conclude, no treatment exists that can stop or slow down the progression of PD. The current available treatments are aimed at increasing and maintaining L-DOPA concentration systemically, indirectly leading to higher DA concentrations in the brain[10–12,15,19]. The high systemic L-DOPA concentration leads to side-effects that are often considered worse than PD itself[21]. To improve the unfavourable $T^{1/2}$ and side-effects of the L-DOPA treatment, there is a need for an innovative treatment that increases the bioavailability, specifically in the SNpc.

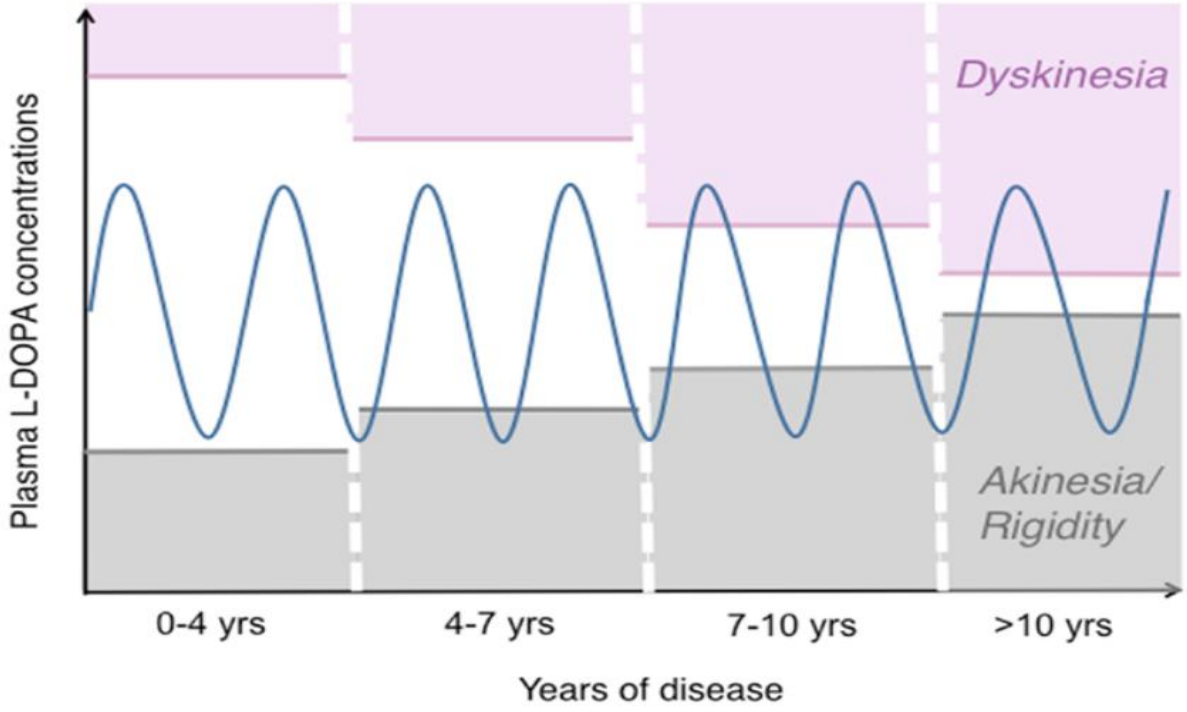


Figure 2: The progressive nature of PD is shown by the bars indicating akinesia and rigidity in time. To treat the increasingly prominent symptoms, higher L-DOPA dosages are taken, which is shown by the equally growing dyskinesia bar. At late-stage PD, L-DOPA concentrations in plasma has a small effective range that is closely bordered by PD symptoms and L-DOPA caused side-effects. Figure from: A Cenci, 2014[18].

1.2 The project

The goal of this project is increasing the bioavailability of L-DOPA at the SNpc aiming to improve the unfavourable $T^{1/2}$ and the side-effects, ultimately leading to better therapeutic possibilities. The use of liposomes to protect the encapsulated drug from conversion and delay the systemic clearance is a viable method to improve the $T^{1/2}$ of L-DOPA[22]. Moreover, by intravenous injection (IV) of L-DOPA loaded liposomes, the gastrointestinal tract and fist-pass-metabolism are avoided. Studies have shown that the BBB, which normally is a strong impediment for substances including liposomes for entering the brain, is more permeable due to angiogenesis that is caused by PD associated inflammation at the brain[23,24]. Therefore, using liposomes that facilitate long systemic circulation time could theoretically lead to an accumulation of encapsulated L-DOPA at the brain[22–24]. Furthermore, the occurrence of side-effects in the L-DOPA treatment is predominantly caused by the high systemic concentration[12]. This problem will partly be resolved with the use of liposomes as the drug release will be sustained, leading to a lower plasma level and less fluctuation compared to the plasma L-DOPA line shown in Figure 2[18,22].

Liposomes have been extensively used as drug delivery systems to pharmacologically enhance encapsulated molecules. The phospholipid bilayer of liposomes surrounds an aqueous centre[25]. In consequence of this composition, liposomes are efficient in encapsulating hydrophobic and hydrophilic compounds residing the lipid bilayer and aqueous pool respectively[26]. Furthermore, the components of liposomes can be functionalised in order to increase viability, including polyethylene glycerol (PEG) on lipids, as can be seen in Figure 3[25].

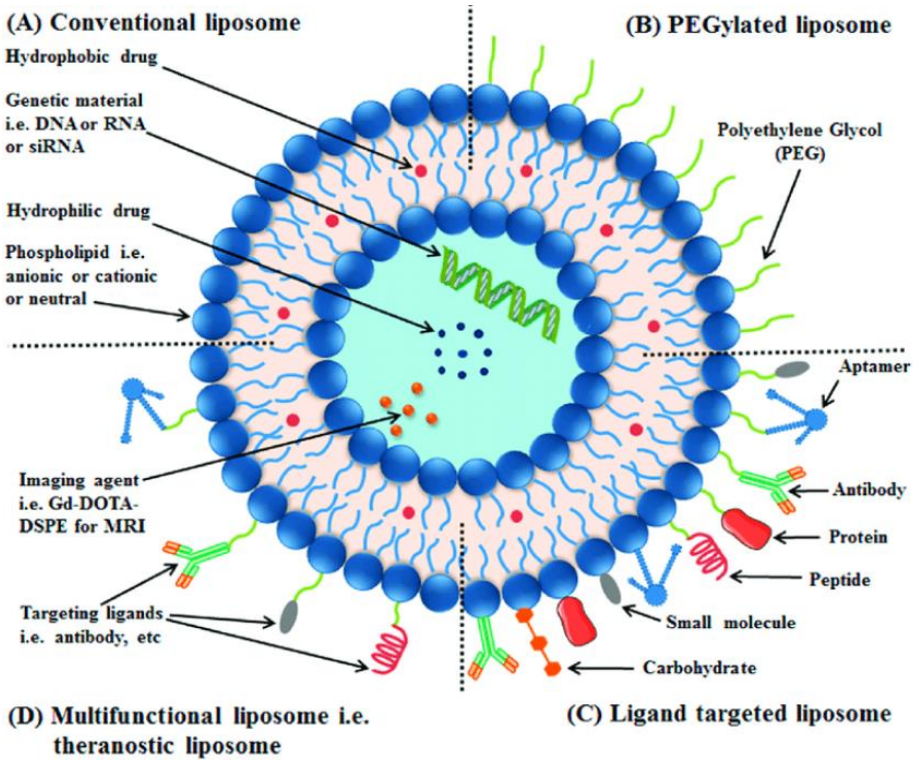


Figure 3: Different classes of liposomes are shown: general phospholipid-based liposomes (A), stealthy PEGylated liposomes (B), target-specific liposomes (C) and multifunctional liposomes (D). Figure from: J. Tao et al., 2016[25]

This project is aimed to deliver L-DOPA more precisely to the brain. The concept behind the indirect selective delivery to the brain is based on the prolonged circulation of liposomes and the higher permeability of the BBB in PD patients[23,24]. A lot has been done by predecessors on this project, such as designing and characterizing liposomes, investigating loading techniques, performing cellular uptake studies and even doing the first *in vivo* experiments. With the current report, I show the continuation on the project. The research questions that was answered in the current study was: What are the release/retention kinetics of liposomal encapsulated L-DOPA incubated in relevant biological media (HEPES buffered saline (HBS), blood plasma and whole blood)? Drug retention is the fraction of liposomal encapsulated drug still enclosed after predetermined incubation times in media. Drug release is the opposite, stating the drug fraction that leaked out of the liposomes during incubation[27]. The primary motivation behind this research question was aiming to predict *in vivo*. However, current *in vitro* release techniques, including flow-through cell, dialysis and separation techniques, show difficulty in studying drug stability and release kinetics in complex media due to the inability of distinguishing retained and released drug[28,29]. Therefore, an innovative retention method was developed in the present study based on the interaction between biotin and streptavidin, facilitating an approach to distinguish liposomal content from the environment[29]. Using labelled, biotinylated, L-DOPA loaded liposomes and magnetic streptavidin beads (MSB), liposomes were incubated in- and removed from biological media at predetermined times and the liposomal L-DOPA concentration was measured. This method has recently been developed and published by Y. Wang et al., 2022 for the determination of drug release from- and retention in polymeric micelles[29]. Therefore, additionally to the results of the L-DOPA retention studies in biological media, this report also describes the methods and results used to validate the biotin-streptavidin application in liposomes. Moreover, to directly analyse the possible benefit that the biotin-streptavidin method has over current release methods, L-DOPA retention data obtained using biotin-streptavidin and dialysis cassettes were compared.

2. Material and Methods

A detailed description of the methods can be found in Appendix A. For a more detailed description of the used buffers, calculations and mixtures, see Appendix B.

2.1 Materials

Dipalmitoyl phosphatidylcholine (DPPC) (Avanti Polar Lipids, USA), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethyleneglycol)-2000] (DSPE-PEG(2000)) (Avanti Polar Lipids, USA), cholesterol (Sigma Aldrich, USA), 1,1'–dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine-4-chloroben-zenesulfonate salt (DiD)-label (ThermoFisher Scientific Inc., USA), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[biotinyl(polyethylene glycol)-2000] (DSPE-PEG(2000)-biotin) (NanoCS, USA), Rotavapor® R210 (Buchi, The Netherlands), LIPEX® Extruder (Transferra Nanosciences Inc., Canada), Whatman® nucleopores™ (Merck KGaA., Germany), Slide-A-Lyzer™ Dialysis Cassettes 0.5-3 mL 10k MWCO (ThermoFisher Scientific Inc., USA), 0.90 x 40 mm BL/LB 20 G x 1.5 needle (B. Braun, Germany), L-3,4-di-hydroxy-phenylalanine (Sigma-Aldrich, USA), 10 mL polycarbonate bottle (Analis, Belgium), the Beckmann Ultra-Centrifuge Optima® L-90K (Beckmann Coulter, USA), ZetaSizer Nano-S (Malvern Instruments, UK), ZetaSizer Nano-Z (Malvern Instruments, UK), ZetaSizer Nano software v3.30 (Malvern Instruments, UK), ultra-performance liquid chromatography system (Acquity™ Ultra-Performance LC, Waters, USA), Empower Chromatography Data System (Acquity™, Waters, USA), HSS T3 1.8 µm, 2.1 mm X 50 mm (Waters, USA), SPECTROstar Nano (BMG LABTECH, Germany), MARS Data Analysis Software (BMG LABTECH, Germany), Transparent F-bottom 96-Well plate (Greiner bio-one, Germany). GraphPad Prism 9.4.1 (Dotmatics, UK). Streptavidin-coated magnetic beads (BioMag®, USA), dynaMag™-2 Magnet rack (Invitrogen, USA), SRT6 roller mixer (Stuart Equipment, UK), black F-bottom 96-Well plate (Greiner bio-one, Germany), FP-8300 Fluorescence Spectrometer (JASCO, Japan), Spectra Manager™ (JASCO, Japan), human plasma (mini donor service UMC Utrecht, The Netherlands), 18,0 mg Vacutainer® ethylenediaminetetraacetic acid (EDTA) tubes (BD, USA), human whole blood (mini donor service UMC Utrecht, The Netherlands).

2.2 Preparation of the liposomes

See Figure 4 for a schematic overview of the techniques used to prepare liposomes.

2.2.1 Thin-film hydration

The thin-film hydration method was used to prepare different types of (biotinylated) DiD-labelled liposomes.

- **DiD-labelled non-biotinylated liposomes** were made with DPPC, DSPE-PEG(2000) and cholesterol at a 1.85: 0.08: 1 ratio, respectively. The DiD consisted of 0.2 mol % of the total lipid concentration.
- **DiD-labelled biotinylated (1% of PEG lipids) liposomes** were made with DPPC, DSPE-PEG(2000), cholesterol and DSPE-PEG(2000)-biotin at a 1.85: 0.1485: 1: 0.0015 ratio, respectively. The DiD consisted of 0.2 mol % of the total lipid concentration.
- **DiD-labelled biotinylated (1% of all lipids) liposomes** were made with DPPC, DSPE-PEG(2000), cholesterol and DSPE-PEG(2000)-biotin at a 1.85: 0.12: 1: 0.03 ratio, respectively. The DiD consisted of 0.2 mol % of the total lipid concentration.

Lipids were dissolved in 1 mL chloroform and methanol (2:1 v/v) in a 50 mL round-bottom flask. The organic solvent was evaporated using the Rotavapor (200 mbar, 65 °C, maximum rotation for 30 minutes) to form a thin-film lipid layer. A 30 minutes N_2 flush was done to remove remaining traces of solvents, followed by re-hydration of the lipid film by adding hydration buffer (Ammonium sulfate, 300 mM; pH 4) (see Appendix B, Hydration buffer). For obtaining liposomes, the flask was connected to the Rotavapor (65 °C, maximum rotation) until dispersion of the thin layer was completed. The content of the flask was pipetted to a 15 mL tube and stored at 4°C.

2.2.2 Extrusion

To prepare liposomes in predetermined sizes, extrusion was performed. The LIPEX® Extruder was used with 0.6 µm, 0.4 µm, 0.2 µm, 0.1 µm and 0.05 µm Whatman® nucleopores™ filters to filter liposomes with a diameter of 80nm and 120nm. The combinations of nucleopore filters were 0.6-0.4 µm (2 times), 0.4-0.2 µm (5 times), 0.2-0.2 µm (10 times), 0.2-0.1 µm (20 times) and 0.1-0.1 µm (15 times) to create 120 nm liposomes. 20 additional extrusions cycles with 0.1-0.05 µm nucleopore filters were performed to prepare 80 nm liposomes. Dynamic Light Scattering (DLS) was done to determine liposomal size (see chapter 2.3.1). After extrusion and DLS, liposomes were stored at 4°C in 15 mL tubes.

2.2.3 Dialysis 1

Liposome samples were dialysed to remove excess unloaded ammonium sulfate present from the hydration buffer. The liposomes were injected in a Slide-A-Lyzer™ Dialysis Cassettes 0.5-3 mL 10k MWCO with a 5 mL syringe and 0.90 x 40 mm BL/LB 20 G x 1.5 needle. The cassettes were transferred in 2 L dialysis 1 buffer (tris(hydroxymethyl)-aminomethane, 4.5 mM; HEPES, 20 mM; pH 6.5) (See Appendix B, Dialysis 1) for 2h at 4°C. The buffer was refreshed with 2 L dialysis 1 buffer and was dialysed overnight at 4°C. The liposomes were transferred to a 15 mL tube and stored at 4°C after dialysis.

2.2.4 Remote loading of the liposomes and Dialysis 2

By mixing L-DOPA, sodium bisulfite (anti-oxidant) (10:1, w/w) and HCL (1.6M), a 0.5 M L-DOPA stock solution was made (see Appendix B, Levodopa stock solution 0.5 M). The L-DOPA stock solution was diluted with 20 mM HEPES (1:5.67, v/v) to form 75 mM L-DOPA stock solution (see Appendix B, Levodopa stock solution 75 mM). For remote loading, 75 mM L-DOPA stock solution, unloaded liposomes and absolute ethanol were mixed (2:1:0.3, v/v) with the Rotavapor® (at 60°C, maximum rotation speed for 2h). Absolute ethanol was used to increase liposomal permeability, resulting in enhanced L-DOPA loading. After L-DOPA loading, the mixture was incubated at 4°C for 30 minutes to stop the loading. To remove unloaded L-DOPA, the mixture was dialysed against HBS (pH 6.5) (See Appendix B, Dialysis 2) at 4°C using Slide-A-Lyzer™ Dialysis Cassettes 0.5-3 mL 10k MWCO as described in 2.2.3. The liposomes were stored in a 15 mL at 4°C.

2.2.5 Ultracentrifugation (optional)

To increase the L-DOPA loaded liposomes concentration, ultracentrifugation was performed by transferring liposomes in a 10 mL polycarbonate tube in the Beckmann Ultra-Centrifuge Optima® for 1h at 55000x*g* at 4°C. After centrifugation, supernatant was removed and HBS (pH 6.5) was added to the pellet to obtain the final concentration. The vials were transferred to the fridge and left overnight to loosen and resuspend the pellet.

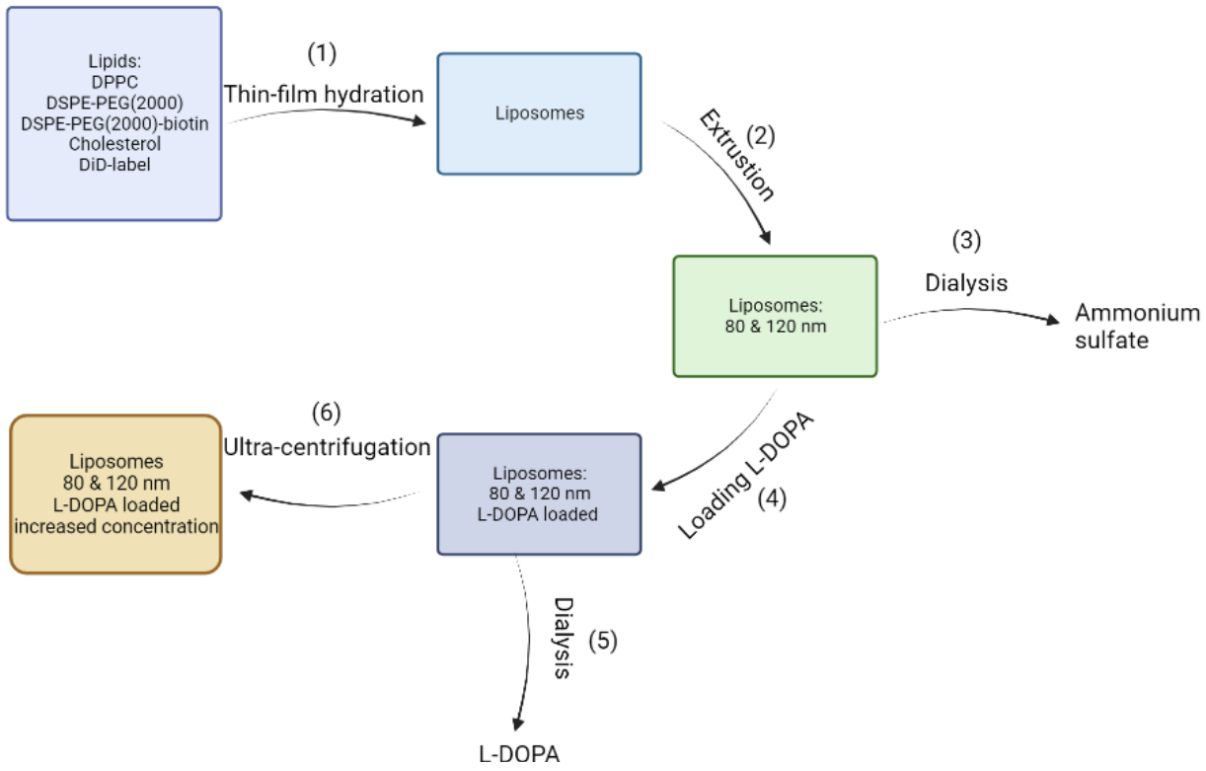


Figure 4: A schematic overview of the methods used to prepare the (non-)biotinylated, DiD-labelled and L-DOPA loaded liposomes. The numbering system indicate the order of the techniques.. Made with: BioRender.

2.3 Characterisation of the liposomes

Techniques used to analyse the prepared liposomes are described below. An overview of these techniques is visually shown in Figure 5.

2.3.1 Size and Zeta potential of the liposomes

The size and polydispersity index (PDI) have been determined using the DLS: He-Ne laser 633 nm, max 4 mW of 100 VA power with the ZetaSizer Nano-S by 3 independent measurements. The Zeta potential (ζ) was measured by electrophoretic light scattering (ELS): He-Ne laser 633 nm, max 4 mW of 100 VA power with the ZetaSizer Nano-Z by 3 independent measurements.

2.3.2 Liposomal L-DOPA concentration

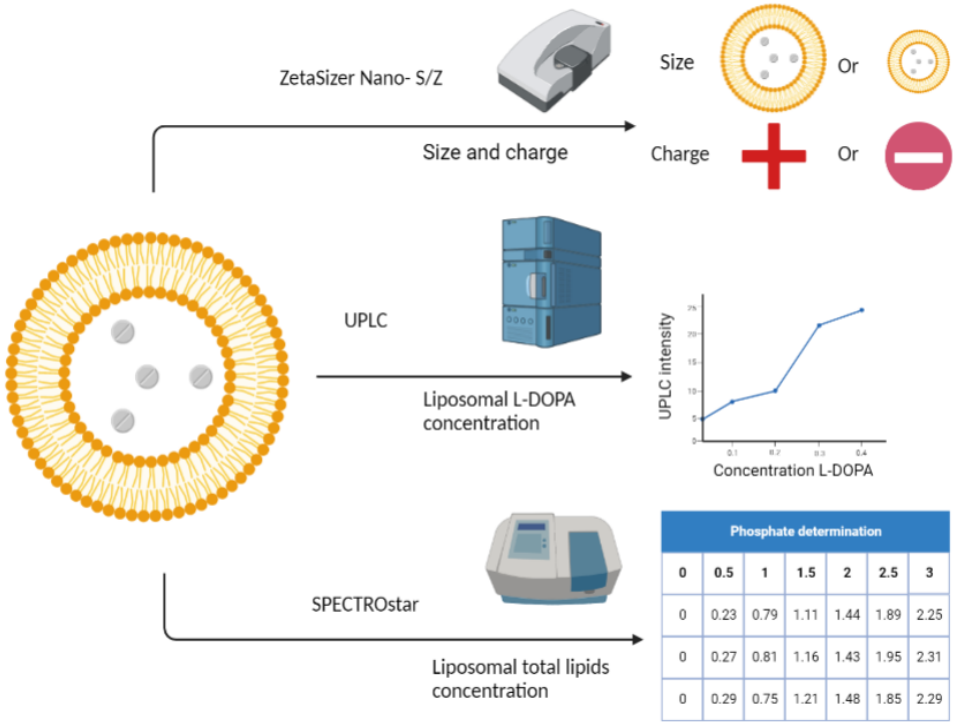
Liposomal L-DOPA concentration was quantified by ultra-performance liquid chromatography (UPLC) containing an UV photodetector array. The stationary phase consisted of the HSS T3 1.8 µm, 2.1 mm X 50 mm column at 50°C. The mobile phase resides Milli-Q containing 0.1% formic acid and 5mM monopotassium phosphate (see Appendix B, UPLC eluent). UPLC parameters: Detection wavelength, 280 nm; injection volume, 5 µL; flow rate, 0.3 mL/minute; sample rate, 40 points/second and; runtime, 2 minutes. Samples and standards were mixed with Milli-Q containing 0.05% triton-X100 to disrupt liposomal bilayer and enhance L-DOPA release. The L-DOPA loading efficiency is indicated with the Encapsulation Efficiency (EE%):

$$EE\% = (C_m/C_i) \times 100\%$$

- C_m = Measured concentration L-DOPA
- C_i = Initial concentration L-DOPA (75 mM)

2.3.3 Liposomal total lipid concentration

Total lipid (TL) concentration was determined by measuring the total phosphorus concentration in liposomal samples. This method was described by Rouser *et al.,* 1970, and is based on colorimetric detection of phospholipids[30]. Prepared liposomes were compared to standards containing sodium biphosphate in Milli-Q. Samples (10-30 µL of liposomes, triplicates) and standards (0, 40, 60, 80, 100, 120 and 140 nM sodium biphosphate, triplicates) were transferred in glass tubes and evaporated in a heat block at 180°C. The contents of the glass tubes were subjected to 0.3 mL perchloric acid and heated for 1-3h at 180°C. Subsequently, 1 mL dH_2O, 0.5 mL 1.25% hexa-ammoniummolybdate and 0.5 mL 5% ascorbic acid were added to the tubes. Next, the glass tubes were placed in warm water bath at 55°C for 5 minutes and were subsequently cooled down with cold water of 10°C for 5 minutes. 200 µL of each tube was transferred in a transparent F-bottom 96-well plate for photometric measurements by the SPECTROstar Nano at absorbance wavelength of 797 nm.



Phosphate determination						
0	0.5	1	1.5	2	2.5	3
0	0.23	0.79	1.11	1.44	1.89	2.25
0	0.27	0.81	1.16	1.43	1.95	2.31
0	0.29	0.75	1.21	1.48	1.85	2.29

Figure 5: A schematic overview of the techniques used for the characterisation of biotinylated, DiD-labelled and L-DOPA loaded liposomes. Made with: BioRender.

2.4 Binding kinetics and efficiency of magnetic streptavidin beads and biotinylated liposomes

(Non-)biotinylated liposomes were prepared, loaded and characterised as described in chapter 2.2 and 2.3 (See Appendix B, Lipid mixture for liposome preparation) to confirm binding of biotinylated liposomes on MSB as shown in Figure 6. 4 aliquots containing 450 µM (non-) biotinylated 80 nm liposomes labelled with DiD were prepared by mixing with 550 µL (5 mg/mL) prewashed MSB in 100 µL phosphate buffered saline (PBS). Non-biotinylated liposomes were used as negative control and to measure nonspecific MSB binding. The mixtures were incubated at 4 °C on a roller mixer for 10 minutes to facilitate the biotin-streptavidin interaction. After the incubation, samples were relocated in the magnetic rack for 1 minute to induce bead sedimentation. Supernatants, containing unbound liposomes, were collected and diluted with 100 µL dimethylformamide (DMF). The samples were washed with 100 µL PBS and DMF 2 and 3 times, respectively, and the supernatants were collected. DMF and PBS were added (1:1, v/v) to the PBS and DMF supernatants, respectively, to ensure equal fluorescence signal originating from extra-liposomal environment. DMF washes were performed to disconnect the bound liposomes. Collected supernatants were transferred in a black F-bottom 96-Well plate and the DiD intensities were measured (excitation wavelength: 648 nm; emission wavelength: 670 nm) with the JASCO FP-8300 Fluorescence Spectrometer. The detected DiD intensities of the DMF supernatants corresponded to the liposomes that bound to the MSB. The unbound fractions of liposomes were demonstrated by the DiD intensities of the PBS supernatants.

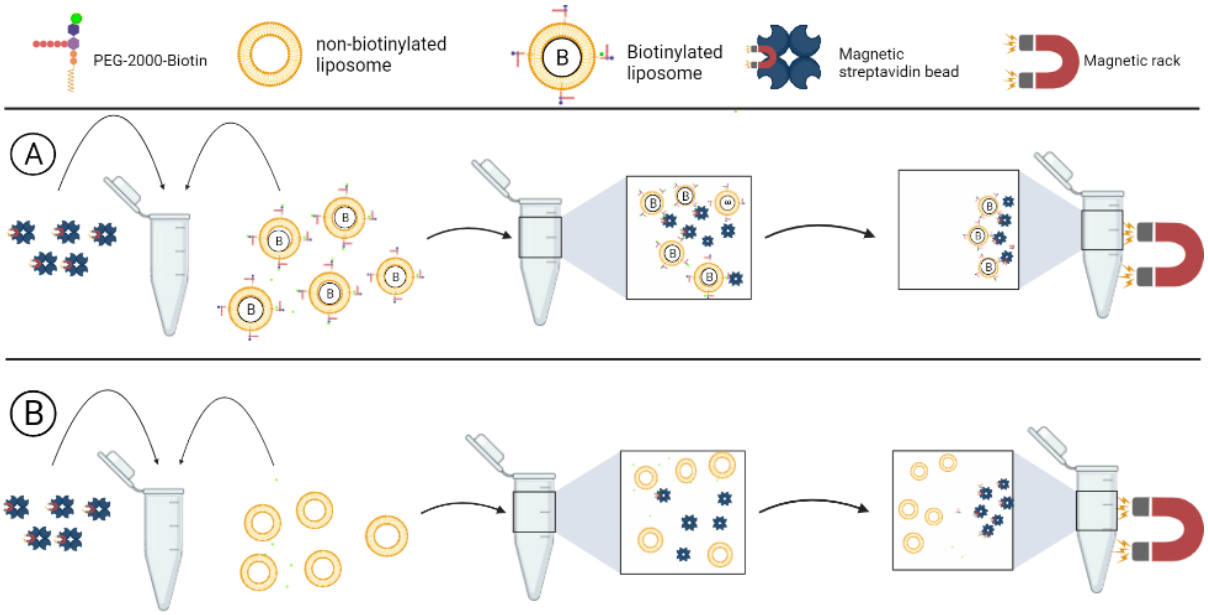


Figure 6: The theory behind using the MSB to separate biotinylated liposomes from media is depicted. The MSB can bind to the biotinylated liposomes (A), but not to the non-biotinylated liposomes (B). Therefore, the magnetic rack is able to indirectly attract only biotinylated liposomes. As the (non-)biotinylated liposomes are DiD-labelled, the fractions of liposomes bound and unbound to the MSB can be measured. Made with: BioRender.

To determine the time required for biotin-streptavidin binding, 6 samples of 350 µM 80 nm DiD-labelled biotinylated liposomes were subjected to 275 µL (5mg/mL) prewashed MSB in 100 µL PBS. The samples were incubated at 4°C for 2.5, 5 or 10 minutes under constant agitation and subjected to identical washing as described above. DiD intensities were determined with the JASCO 8300 Fluorescence Spectrometer at fixed wavelengths (excitation wavelength: 648 nm; emission wavelength: 670nm). DiD intensities of DMF washes corresponded to the liposomes that bound to the MSB. Free liposomes were demonstrated by the DiD levels in PBS washes.

The points of saturation for MSB with 80 and 120 nm biotinylated liposomes were determined to establish the binding efficiency. 20 aliquots consisting of 275 µL (5 mg/mL) prewashed MSB were subjected to predetermined amounts of 80 or 120 nm biotinylated DiD-labelled liposomes (50, 100, 150, 200, 250 and 300 µM). The samples were incubated at 4°C for 5 minutes at constant agitation, followed by placing the samples in a magnetic rack. The DiD intensities of bound and unbound biotinylated liposomes were determined using JASCO 8300 Fluorescence Spectrometer at fixed wavelengths (excitation wavelength: 648 nm; emission wavelength: 670 nm) as previously described.

2.5 Retention studies

Ideally, the quantification of both released and retained L-DOPA was performed. However, the released drug concentration was not measured due to the instability of L-DOPA outside liposomes. As drug release comprises the total drug concentration minus the retained fraction, L-DOPA release was indirectly determined. Biotinylated, L-DOPA loaded, 120 nm liposomes were prepared to conduct retention studies in HBS (pH 6.5), human blood plasma and human whole blood. The motivation for the use of 120 nm liposomes are based on obtained data of the biotinylated liposomes and MSB efficiency study. 3 independent retention studies were conducted in each media. An individual retention study was done with blood or blood plasma from a single donor. Blood was collected in EDTA containing tubes to avoid coagulation. For whole blood retention study samples, the 37°C incubation step was initiated on the same day that blood was withdrawn. Blood plasma was obtained from whole blood by centrifugation for 15 minutes at 4°C and 1000*xg* on the day of withdrawal. Citrate phosphate dextrose adenine (CPDA-1) was used in whole blood. CPDA-1 contains citric acid, sodium citrate, sodium phosphate monohydrate, dextrose and adenine (1:8.8:7.4:10.6:0.092, g/g) in 5 mL Milli-Q and served as anticoagulant and aided in the preservation of whole blood during the 37 °C incubation (see Appendix B, CPDA-1 for blood preservation).

2.5.1 Retention of L-DOPA from liposomes in HBS, blood serum or whole blood

For 1 independent retention study, 14 Eppendorf tubes were prepared by addition of 275 µL (5 mg/mL) prewashed MSB. The 14 tubes were divided into 7 groups:

1. T=0h
2. T=1h
3. T=2h
4. T=4h
5. T=6h
6. T=24h
7. T=48h

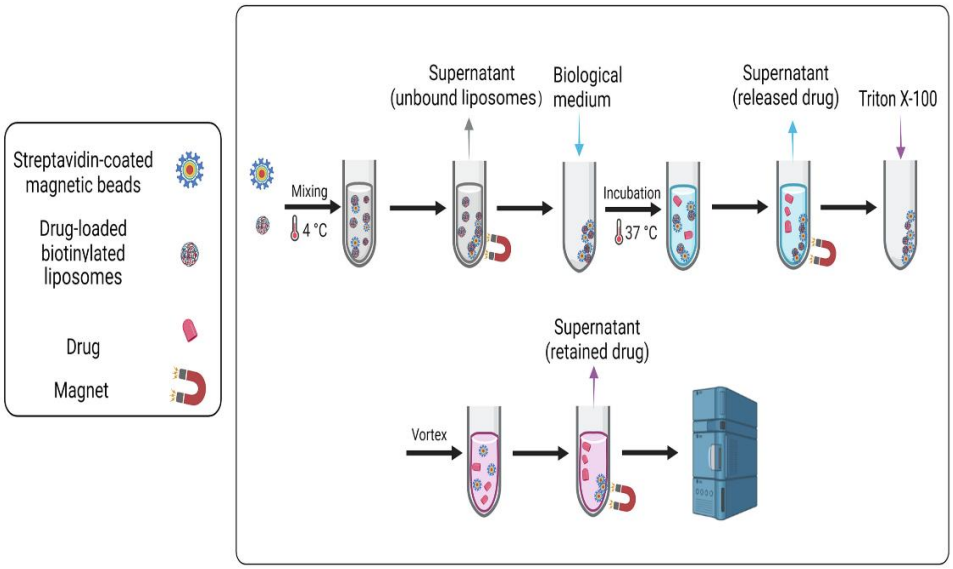


Figure 7: Steps taken to perform the in vitro release study using biotinylated liposomes and magnetic streptavidin coated beads. Adapted from: Y. Wang et al., 2022[29].

The retention assay steps are depicted in Figure 7. 500 µL PBS and 250 µM biotinylated 120 nm liposomes (saturation point for 275 µL MSB) were mixed with 275 µL MSB. The tubes were incubated for 5 minutes at 4°C with constant agitation. Next, the samples were transferred in the magnetic rack and supernatant was removed after pellet formation. An additional wash with 500 µL PBS was performed to remove remaining unbound liposomes. 500 µL biological media was added to groups 2-7, followed by the incubation at 37°C on a roller mixer. After the predetermined timespans ended, the sample tubes were transferred in the magnetic rack. To remove released L-DOPA, supernatant was removed after the bead pellet formed. The pellet was washed with 1 mL PBS twice and finally 200 µL Milli-Q containing 0.05% Triton-X100 was added to disrupt the liposomal bilayer and release encapsulated L-DOPA. The samples were vortexed and the drug concentration was directly quantified by UPLC as released L-DOPA is instable. UPLC quantifications of L-DOPA greater that T = 0 were considered outliers.

2.5.2 Comparing Biotin-streptavidin retention method with dialysis cassette method

The drug retention with dialysis cassettes was performed by a predecessor on this project, Devin Veerman. After the removal of unloaded L-DOPA during dialysis 2, the dialysis cassette containing the L-DOPA loaded liposomes were dialysed at 37°C against 5 L of HBS (pH 6.5) stirred at 250 RPM (See Appendix B, Dialysis 2). At predetermined incubation times, 100 µL of the L-DOPA loaded liposomes was removed from the dialysis cassette and stored at -20°C. The L-DOPA concentration was quantified using UPLC. L-DOPA retention data generated from dialysis cassettes was compared to the results obtained with the biotin-streptavidin method.

2.6 Streptavidin recycling

By heating up Eppendorf tubes containing water and MSB with bound 120 nm biotinylated liposomes to 70°C in a water bath, it was attempted to recycle costly MSB by breaking the high-affinity biotin-streptavidin interaction as allegedly possible according to A. Holmberg et al., 2005[31]. 12 samples containing 150 µL (5 mg/mL) prewashed MSB in 0.5 mL PBS were prepared. The 12 samples were divided in 6 groups: 1, not heated; 2, heated to 70°C (1 second); 3, heated to 70°C (30 seconds); 4, heated to 70°C (60 seconds); 5, heated to 70°C (600 seconds) and; 6, heated before incubation (600 seconds). Group 6 was transferred in a water bath at room temperature, heated to 70°C which was maintained for 600 seconds. Next, group 6 was removed from the water bath. Group 1-6 were subjected to 120 µM DiD-labelled 120 nm and were incubated for 5 minutes on a roller mixer at 4°C to facilitate the interaction between biotinylated liposomes and MSB. The Eppendorf tubes were transferred to the magnetic rack. Similar to 2.4.2 and 2.4.3, the DiD intensities of free and captured biotinylated liposomes were determined, except DMF was replaced by Milli-Q containing 0.05 % Triton-X100 to preserve MSB integrity. Triton-X100 disrupts the integrity of lipid nanoparticles. 500 µL Milli-Q was added to the Eppendorf tubes containing the MSB. Excluding group 1 and 6, the Eppendorf tubes were transferred in a water bath at room temperature and heated to 70°C for the predetermined timespans. Next, the Eppendorf tubes were placed in the magnetic rack in the water bath. The supernatants were removed and the Eppendorf tubes were placed in a rack outside the water bath. Group 1 was transferred in the magnetic rack outside the water bath and the supernatant were removed. 500 µL PBS and 120 µM biotinylated 120 nm liposomes were added to group 1-5 and were incubated for 5 minutes at 4°C on a roller mixer. The DiD intensity that corresponded to the liposomes bound to the MSB were measured and compared to the DiD intensity of the liposomes bound to MSB prior to the heating step.

2.7 Statistical analysis

GraphPad Prism (version 9.4.1) was used to analyse obtained data. Results acquired by studying the binding kinetics and efficiency of MSB and biotinylated liposomes were analysed using (multiple) t-tests. Liposomal L-DOPA retention data was analysed by one-way ANOVA. $P<0.05$ was considered as statistically significant.

3 Results

3.1 The characterisation of the liposomes

Multiple batches DiD-labelled (non-)biotinylated liposomes of approximately 80 or 120 nm were prepared. The corresponding characterisations are shown in Table 1. Each prepared batch contained DiD labelled liposomes. The low PDIs indicate small size distributions in the batches. As the non-biotinylated liposomes (batch 3 and 4) were used as negative control to study the binding between biotinylated liposomes and magnetic beads coated with streptavidin, batch 3 and 4 were not loaded with L-DOPA. Batch 5 and 6 were made with increased biotinylated lipid concentration (see 2.1 or Appendix B, Lipid mixture for liposome preparation). Studying the saturation points of MSB for 80 and 120 nm biotinylated liposomes revealed that higher total L-DOPA levels could be tracked using liposomes of 120 nm. Batch 6, containing 80 nm biotinylated liposomes, was therefore not loaded. The decrease in TL after L-DOPA loading was the results of diluting unloaded liposomes with the L-DOPA 75 mM stock and absolute ethanol (1:2:0.3, v/v). Removal of remaining L-DOPA using dialysis farther diluted the liposome batch.

Batch description	Used for the following results	Liposome sizes (nm)	Biotin-ylated (Y/N)	ζ potential	PDI	TL (mM/mL) Prior/after loading	EE% (L-DOPA concentration in mM)
1: 120 nm, biotinylated (1% of PEGylated lipids) liposomes	3.2, 3.3, and 3,5	111.2 ± 0.9	Y	-3.38 ± 0.26	0.016 ± 0.01	31.5/5.49	1.16 (0.87)
2: 80 nm, biotinylated (1% of PEGylated lipids) liposomes	3.2, 3.3, and 3,5	85.1 ± 2.2	Y	-3.23 ± 0.24	0.056 ± 0.05	24.2/4.29	1.19 (0.89)
3: 120 nm, non-biotinylated liposomes	3.2	116 ± 1.7	N	-4.59 ± 0.31	0.02 ± 0.01	34.5/-	-
4: 80 nm, non-biotinylated liposomes	3.2	84.4 ± 1.4	N	-4.06 ± 0.28	0.019 ± 0.02	33/-	-
5: 120 nm, biotinylated (1% of lipids) liposomes	3.3, 3.4 and 3.6	123.6 ± 2.3	Y	-2.68 ± 1.03	0.07 ± 0.03	45/9.33	1.14 (0.85)
6: 80 nm, biotinylated (1% of lipids) liposomes	3.3, 3.4 and 3.6	81.64 ± 3.42	Y	-3.01 ± 0.48	0.107 ± 0.04	44.7/-	-

Table 1: Characteristics of prepared liposome batches.

3.2 Binding potential of biotinylated liposomes to magnetic streptavidin beads

Fractions of 80 nm (non-)biotinylated liposomes that were free or captured by MSB are shown in Figure 8A. DiD-labelling of the liposomes was utilised to determine the effect that biotinylating liposomes had on the ability to bind to MSB. The results demonstrated that 62.8% of the 450 µM 80 nm biotinylated liposomes bound to 550 µL MSB after 10 minutes incubation at 4°C. No affinity was demonstrated between the non-biotinylated liposomes and MSB, validating the concept that binding of liposomes to the beads occurred through the interaction between biotin and streptavidin.

The time required to establish the bond between the biotinylated liposomes and MSB was researched by measuring the DiD intensities of free and captured 350 µM 80 nm biotinylated liposomes after 2.5, 5 or 10 minutes of incubation at 4°C with 275 µL MSB. The results are demonstrated in Figure 8B. The biotinylated liposomes bound to the beads indicated an equilibrium was reached within 2.5 minutes of incubation.

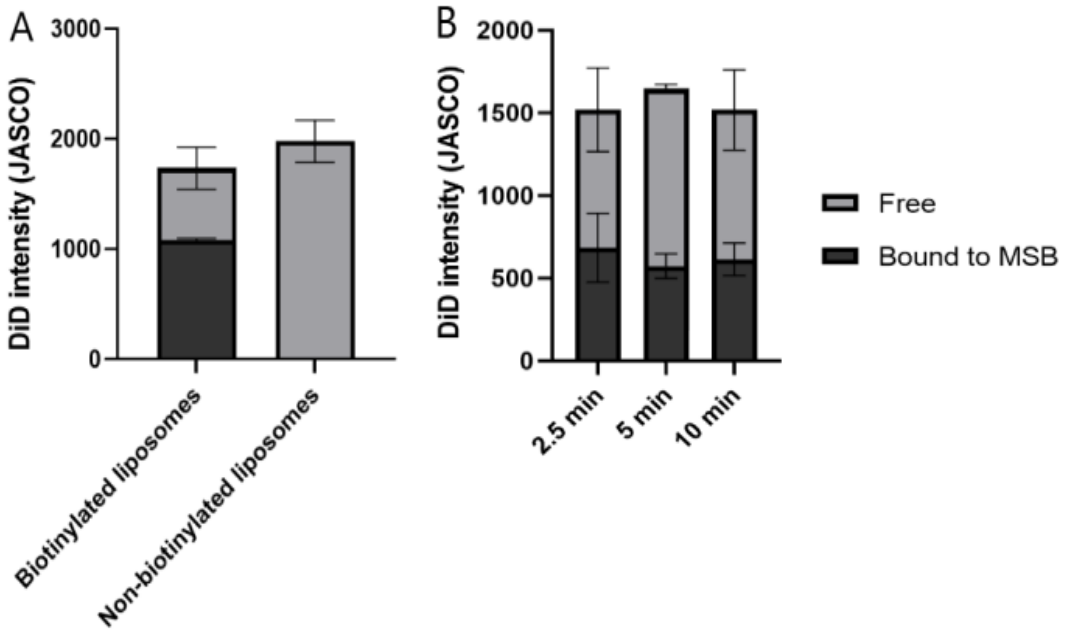


Figure 8: The DiD intensities of MSB-bound and free 80 nm (non-)biotinylated liposomes after 10 minutes (A) and varying incubation times at 4°C (B). The error bars indicate the standard deviation. Made with: GraphPad Prism.

3.3 Determining liposomal concentration for optimal binding to magnetic streptavidin beads

The maximum lipids able to bind to a fixed amount of MSB (275 µL) was determined for 80 and 120 nm biotinylated liposomes by incubating MSB with increasing TL concentrations. As demonstrated in Figure 9, the total DiD intensity for 80 and 120 nm liposomes that bound to the MSB are equal at 200 µM and lower concentration. The saturation points of MSB were indicated by the no further increasing DiD intensities after further TL exposure. Results reveal that the point of saturation for 120 nm liposomes lies slightly higher compared to the 80 nm liposomes. Calculations employing data from Table 2 showed that the maximum TL concentrations of 120 and 80 nm liposomes bound to 275 µL MSB were 223.9 and 195.8 µM, respectively, and are statistically different (P = 0.0108). As result of these outcomes, retention studies were performed using 120 nm liposomes.

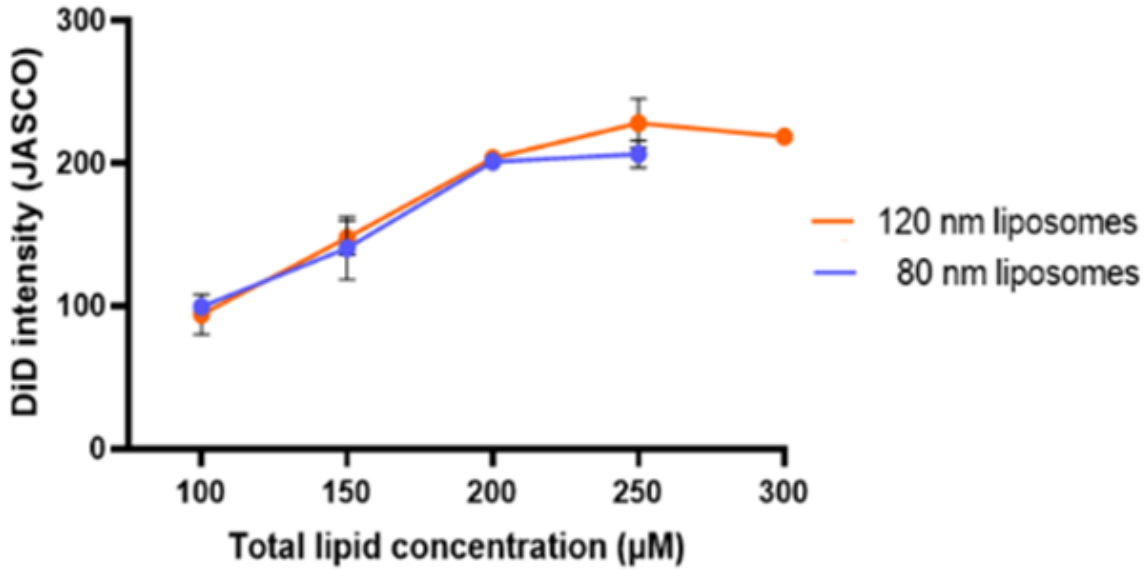


Figure 9: The DiD intensity of predetermined TL concentration of 120 and 80 nm biotinylated liposomes bound to 275 µL MSB are shown. The error bars indicate the standard deviation. Made with: GraphPad Prism.

	µM liposomes	*100*	*150*	*200*	*250*	*300*
120 nm liposomes	Bound to MSB (%)	98	98	96	90	72
		98	98	98	93	74
80 nm liposomes	Bound to MSB (%)	98	96	91	84	X
		98	98	93	82	X

Table 2: The percentage of predetermined TL concentrations of 120 and 80 nm biotinylated liposomes bound to 275 µL MSB are shown. The grey values in Table 2 are used to calculate the maximum TL concentration able to bind MSB.

3.4 Liposomal L-DOPA retention in biological media

3.4.1 Liposomal L-DOPA retention in HBS (pH 6.5)

The results of the 3 independent retention studies of L-DOPA in 120 nm liposomes in HBS are shown in Figure 10, together with the mean. The retained L-DOPA concentration after 4h of incubation value in the 1st L-DOPA retention study was noticeably high, but none of the values in the three studies are considered outliers. No significant difference was found between the retention studies (P = 0.5762). The mean of the 3 retention studies in HBS demonstrated the presence of a burst release in which 27.3% of the L-DOPA content was released from the liposomes in the first 2h. This was followed by a more stable release pattern of approximately 1 %/h, ending with 27.5% L-DOPA retained in liposomes after 48h of incubation in HBS. An increased L-DOPA concentration can be seen at 4h of incubation compared to 2h. This was caused by remarkable high 4h values of the 1st retention study as also revealed in Figure 10.

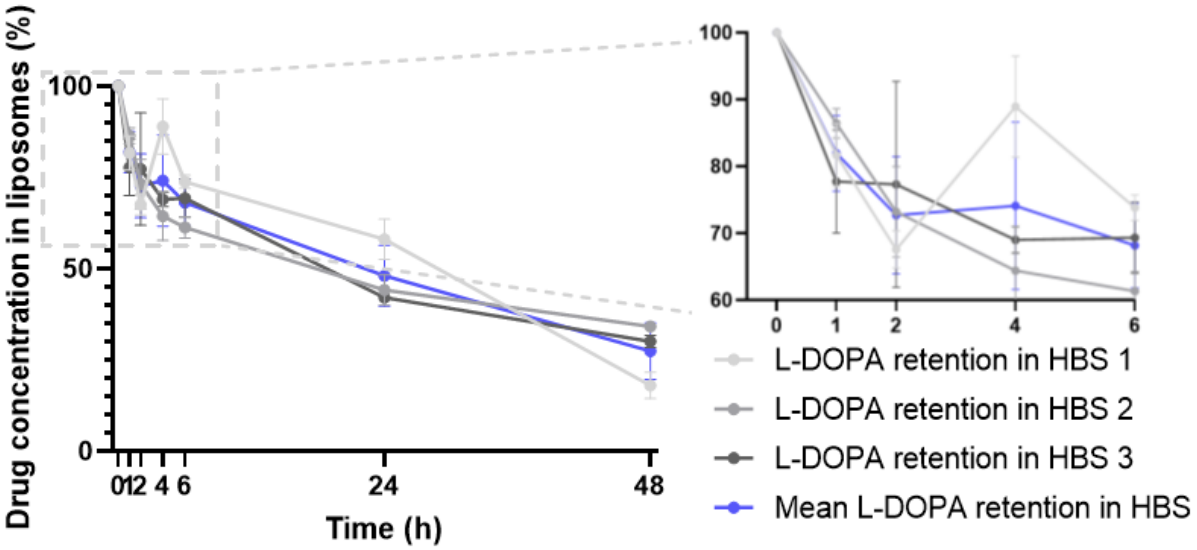


Figure 10:The results of the 3 separate L-DOPA retention studies in HBS (pH, 6.5) and de corresponding mean. The top right highlights the first 6h of incubation. The error bars indicate the standard deviation. Made with: GraphPad Prism

3.4.2 Liposomal L-DOPA retention in human blood plasma

UPLC quantification of retained L-DOPA in 120 nm liposomes after incubation in blood plasma were difficult to analyse as shoulders or additional peaks arose in the spectrum interfering with the peak that represents L-DOPA. Examples of these shoulders and peaks are shown in Figure 11B and C, respectively. Figure 11 A is shown as comparison. Generally, interfering shoulders and peaks arose at 4- and 24h of incubation in blood plasma, respectively. The intensity of the shoulders and peaks increased with longer incubations. The area under the curve (AUC) representing L-DOPA are indicated as red peaks. Due to the interfering shoulders and peaks, analysis of the AUC representing L-DOPA are not exact. Therefore, the following results must be considered as an estimation.

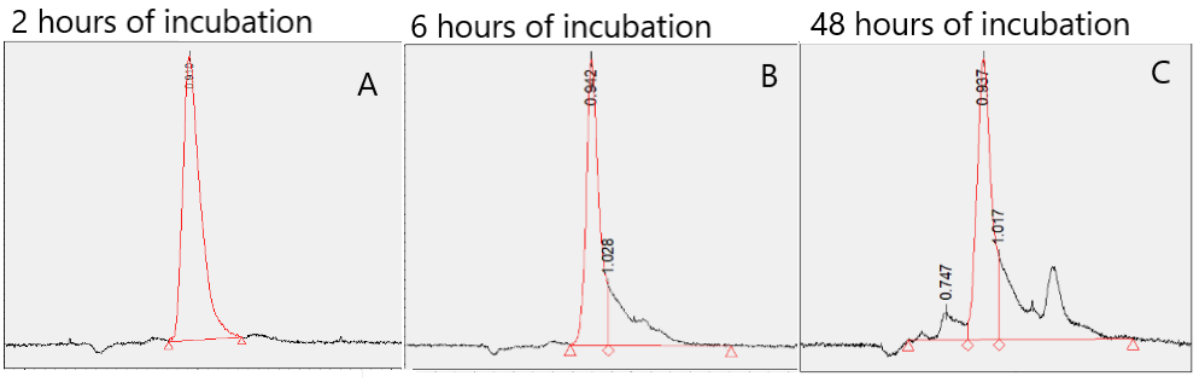


Figure 11: Examples of UPLC data representing the retained L-DOPA in 120 nm liposomes after 2-, 6- and 48h of incubation in human blood plasma at 37°C. The vertical lines shown in B and C indicate the integration lines for the AUC that represent L-DOPA. Data is retrieved with Empower Chromatography Data System (Acquity™, Waters, USA).

As mentioned in 2.6.1, L-DOPA quantifications greater than T = 0 are considered as outliers. Instances of this occurred in the third retention study for a L-DOPA quantification after 4- and 6h incubation in blood plasma. In Figure 12, the individual retention studies and the corresponding mean in blood plasma are shown. Upon incubation in blood plasma, the L-DOPA retained in the liposomes dropped approximately 44% in the first 2h. This was followed by a slight drug release reaching 48% retention at 6h and the formation of a plateau in L-DOPA retention at ≥ 6h. Unexpectedly, the mean of the 4h incubations values lies higher than the 2h values.

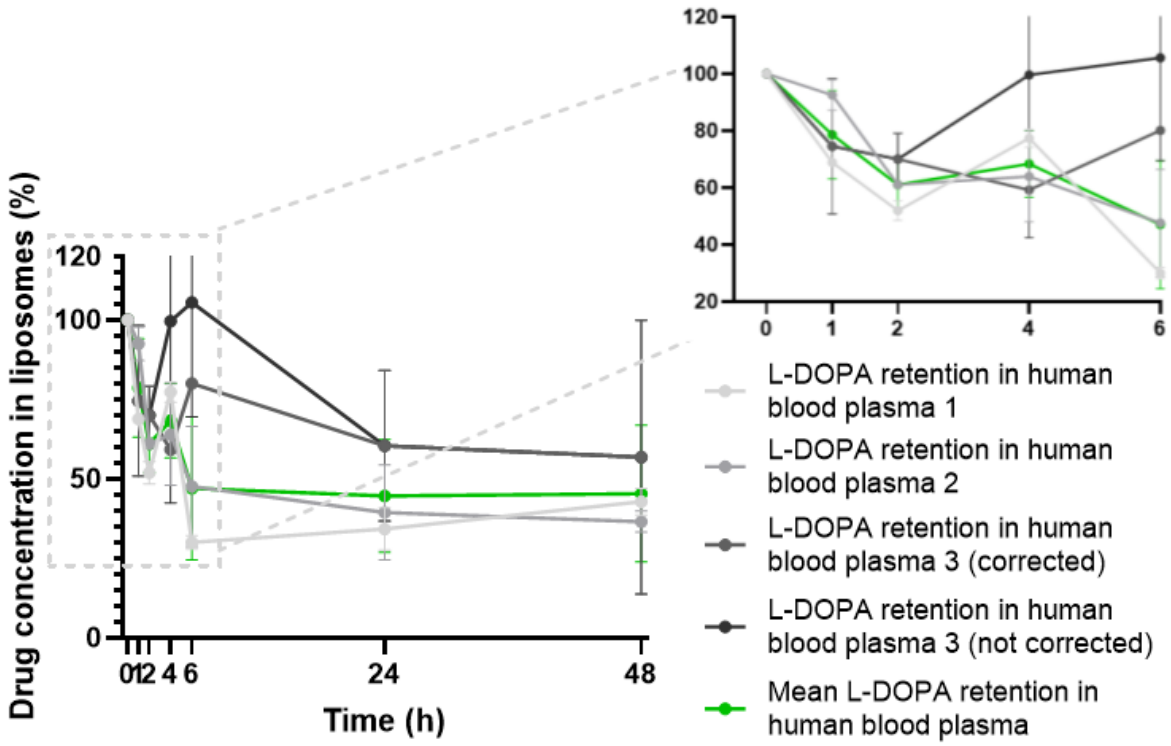


Figure12: The results of the 3 separate L-DOPA retention studies in human blood plasma, and de corresponding mean. The top right highlights the first 6h of incubation. The error bars indicate the standard deviation. Made with: GraphPad Prism.

3.4.3 Liposomal L-DOPA retention in human whole blood

Similar to UPLC quantification of L-DOPA retention after incubation in human blood plasma, shoulders and peaks interfering with L-DOPA integration arose after incubation in human whole blood. In Figure 13, representative UPLC data is shown together with used integration approach. Unlike the UPLC data in blood plasma, the additional shoulders and peaks are present in whole blood results regardless of the incubation time. Moreover, dissimilar to the results in blood plasma, where the L-DOPA peak started at the baseline and was largely distinguishable from the additional shoulders and peaks, UPLC quantification was largely inseparable in whole blood data. Consequently, the data analysis as earlier performed for HBS and human blood plasma were not executed for human whole blood as the results would give an inaccurate indication of the retention kinetics of L-DOPA from liposomes in human whole blood.

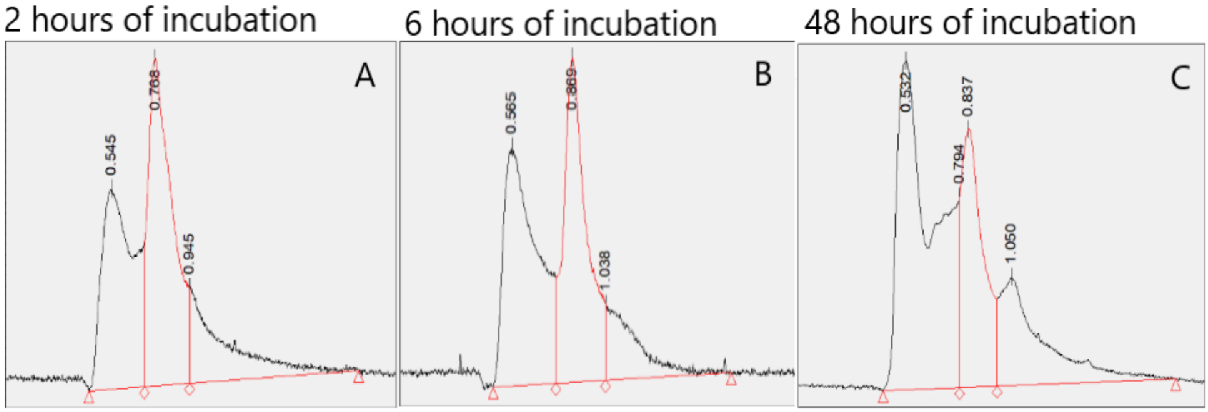


Figure 13: Examples of UPLC data representing retained L-DOPA in 120 nm liposomes after 2-, 6- and 48h of incubation in human whole blood. The vertical lines shown indicate the cut off lines for the AUC that represents L-DOPA. Data is retrieved with Empower Chromatography Data System (Acquity™, Waters, USA).

3.5 Streptavidin recycling.

To analyse the possibility of recycling MSB, the DiD intensity of 120 nm biotinylated liposomes that were bound to 150 µL (5 mg/mL) MSB was measured, followed by heating up the mixture and removing the supernatant, supposedly containing released biotinylated liposomes. An equal TL concentration, 120 µM, of biotinylated liposomes have been added to the MSB again. Subsequently, the DiD intensity of the newly added liposomes that bound was measured and compared to the first liposomes. The data is shown in Figure 14.

Of the two quantities of biotinylated liposomes that were subjected to MSB, the latter demonstrated significantly less MSB binding. The results showed that at least the majority of the initially present DiD-labelling was removed by applying current methods. However, the low DiD-levels found after MSB was subjected to the second quantity of liposomes suggest the presence of DiD-label residues from the first quantity of liposomes or limited newly bound DiD-labelled liposomes.

Not performing the heating step resulted in significantly lower DiD intensities of the second portion of liposomes bound to MSB when compared to heating up the samples ($P = 0.0132$). This demonstrated that the streptavidin released biotin to an extend when heated. The release was further increased when the heating step was prolonged ($P = 0.0297$).

The red bar denoting “heated before incubation” represents the DiD intensity of 120 nm liposomes that were captured on MSB, though the heating step occurred before the first incubation with liposomes. Surprisingly, the DiD intensity was notably lower compared to the blue bars, suggesting that heating the MSB to 70°C partly impairs the capacity for binding biotin.

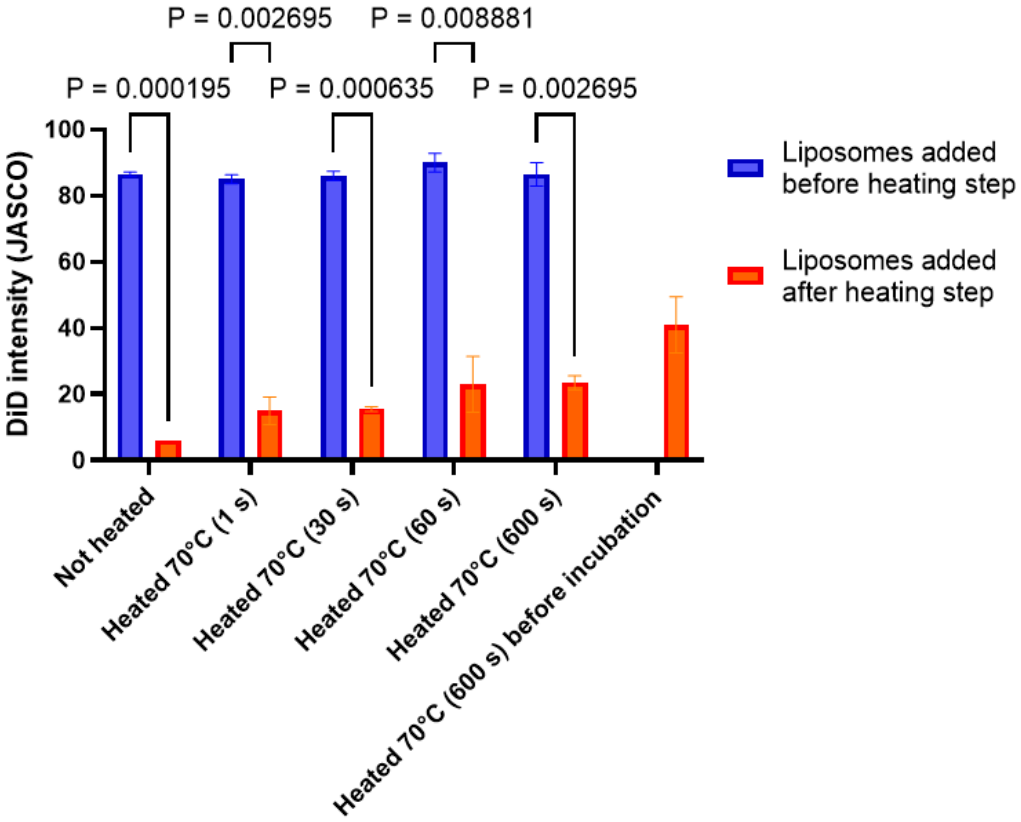


Figure 14: The DiD intensities of 120 nm liposomes that are subjected to MSB before and after a heat step with predetermined timespans are demonstrated. Moreover, results where the heating step was not performed or performed before the first liposomes exposure, are shown. The error bars indicate the standard deviation. Made with GraphPad Prism.

4 Discussion

4.1 Method and results

L-DOPA is the golden standard treatment for PD for over 60 years[20]. Even though L-DOPA is the most efficient PD treatment that is currently available, PD patients suffer from the many downsides of the drug[18,21]. The rapid enzymatic and oxidative degradation lead to a short $T^{1/2}$, resulting in the need of frequent and high dosage of L-DOPA administrations[16]. This is further increased as elevated L-DOPA levels are required as PD progresses[20]. As 1% of administered L-DOPA reaches the brain, the remaining drug is exposed elsewhere the body, leading to side-effects including dyskinesia[11,18]. After approximately 4 years of L-DOPA treatment, also known as the honeymoon phase, the L-DOPA caused relieve of symptoms is often diminishes by the side-effects[21].

The primary goal of the project is improving the current treatment for PD by encapsulating L-DOPA in liposomes. Liposomal encapsulation is aimed at providing prolonged pharmacological activity, enabling a stabler drug release, protection of L-DOPA from rapid conversion/oxidation and improving (indirect) targeting[22]. Several research groups have endeavoured entrapping L-DOPA in nanoparticles in the past, showing promising results *in vitro* and *in vivo* in Parkinsonian mice[32–36]. Y Xiang et al., 2011, demonstrated increased DA concentration in SNpc after intraperitoneal injection of chlorotoxin-modified liposomes encapsulating L-DOPA in PD mice[36]. However, no L-DOPA encapsulated nanoparticle formulations are currently on the market or tested in clinical trials.

The experiments of the present study were aimed at improving present *in vitro* release/retention assays to better analyse L-DOPA release from liposomes in complex media. Current methods used to study liposomal drug release *in vitro* are often based on continuous flow (CF), sample and separate (SS) or dialysis techniques[37,38]. CF simulates the bloodstream by facilitating constant media circulation that passes a nano-particle containing column. Drug release is measured by collecting media samples at predetermined intervals. Drawbacks of CF include adsorption in filter, clogging and high variability in flowrate which affects obtained results. The strategy of SS relies on nano-particle incubation in media at constant temperature, followed by filtration or centrifugation after which drug quantity in the supernatant or filtrate is determined. Due to nano-particle aggregation, filter clogging or forced drug release during separation, SS is considered as a poor release technique. Dialysis is the most popular release technique and can be performed with cassettes or bags with adjustable weight cut offs. Media is able to cross the membrane of the dialysis cassette or bag for nano-particle exposure. Besides leakage and lack of flux over the used membrane when set up incorrectly, important compounds in biological media are not able to diffuse through the membrane, resulting in inaccurate release data[38]. Generally, these techniques are unable to correctly represent *in vivo*, resulting in the need of animal studies to efficiently analyse release kinetics[29,38]. Y. Wang et al., 2022 proposed the biotin-streptavidin approach for “fishing” biotinylated nanoparticles from media using streptavidin coated beads[29]. This article showed promising results using polymeric micelles encapsulating paclitaxel and curcumin. The application of the biotin-streptavidin method facilitates a broad use on nanomedicines and could potentially reduce the number of animal experiments[29]. As the methods Y. Wang et al., 2022 described to determine the release and retention of the encapsulated drugs in complex media were applicable for liposomes, the methods was implemented in the current study. L-DOPA-loaded, DiD-labelled, (non-) biotinylated liposomes were prepared and characterised for the development of an innovative drug retention method. The biotin-streptavidin method facilitated direct liposome-media contact under constant agitation. Moreover, this method simplified the process of separating liposomes from the environment using magnetic attraction without challenging the liposomal integrity, enabling the quantification of time-dependent drug release. Despite the fact that measuring both the liposomal L-DOPA retention and release was preferable, drug release was not quantified due to the instability of released L-DOPA.

Methods used to prepare and analyse L-DOPA loaded liposomes were developed by previous students on this project, Omnia Elsharkasy and Cedric Hustinx. Using their methods, the liposomal formulations of the current study were prepared. As stated in literature, PEGylated liposomes with sizes of 100-150 nm have a long circulation time, but smaller nanoparticles are generally taken up by cells more efficiently[35,39]. To substantiate the use of 120 nm liposomes, prolonged circulation is preferable for this project as (indirect) targeting to the inflamed BBB assumably results in liposomal accumulation at the target site. However, as crossing the BBB is essential for L-DOPA delivery, liposomes of 80 nm were also prepared and the obtained results for both liposomal sizes were compared. To prepare the biotinylated liposomes that could be employed in the retention study using streptavidin beads, the liposomal compositions were slightly adapted (Appendix B, Lipid mixture for liposome preparation). The EE% of L-DOPA in liposomes was approximately 1%, which was poor compared to other studies that encapsulate L-DOPA in liposomes, suggesting that the L-DOPA loading step could be improved[35,40]. E. Estaban et al., 2018 demonstrated increased liposomal L-DOPA EE% in co-encapsulation with ascorbic acid, presumably due to antioxidative characteristics of ascorbic acid[40]. Therefore, liposomal co-loading of L-DOPA with ascorbic acid could improve current future research.

Experiments aimed at defining binding kinetics and efficiency between MSB and the biotinylated liposomes proved that the magnetic beads could be utilised to bind the liposomes and demonstrated that the interaction between biotin and streptavidin was formed under 2.5 minutes of incubation at 4°C. Prior to studying L-DOPA retention, the concentration of 80 and 120 nm liposomes able to bind 275 µL (5 mg/mL) MSB was examined. The use of 275 µL MSB was based on results of Y. Wang et al., 2022 and data obtained during the examination of the binding kinetics of MSB and biotinylated liposomes[29]. Even though UPLC is able to detect small L-DOPA quantities, it contains a detection limit[41]. Therefore, it is beneficial to track a higher concentration of L-DOPA loaded liposomes. Acquired results promoted the use of 120 nm liposomes as 223.9 µM TL bound to 275 µL MSB. This was 12.5% higher compared to 80 nm liposomes, of which 195.8 µM bound. According to Y. Wang et al., 2022, 1 mg of MSB can bind approximately 2 µg biotin, which is in consensus with results found in this study[29]. Regardless of liposomal size, the number of liposomes able to bind MSB is comparable. As larger liposomes are able to encapsulate higher L-DOPA concentrations, 120 nm liposomes are employed in the retention studies. Moreover, 2 biotinylated lipid concentrations were used to determine to define the effect increased biotin concentrations had on MSB binding (Appendix B, Lipid mixture for liposome preparation). Both biotin concentrations demonstrated >90% liposomal binding to MSB, indicating that biotinylation of 1% PEGylated lipids was sufficient for the performance of the retention study.

As previously mentioned, present release methods fail to effectively study *in vivo* as a result of the complexity of biological media[37,38]. The biotin-streptavidin method allows physiological conditions as liposomes were continuously in direct contact with media, under constant agitation, incubated at 37°C and were able to move freely in the media, unlike present release methods. Moreover, the liposomes were easily removed from the environment.

The retention studies in HBS (pH 6.5) showed little sample-to-sample variety in the results, representing good reproducibility. An initial L-DOPA burst release of 27.3% was observed in the first 2h of incubation, followed by a stable release of 1 %/h. After 4h of incubation, an unexpected increase in L-DOPA retention was observed as result of noticeably high L-DOPA quantifications at 4h of incubation in the 3rd retention study. A viable explanation of these elevated L-DOPA values could be a decrease in constant mixing in the Eppendorf tube

during the 37°C incubation. An increased flow inside an Eppendorf tube elevates liposomal exposure to the environment, enhancing drug release. Consequently, a decrease in constant mixing leading to less direct liposomal contact to media could theoretically result in the remarkable 4h L-DOPA quantities. To improve the used method, the volume of used media could be increased as a lower volume in an Eppendorf tube equals a higher relative liquid friction, impairing liquid movement. Therefore, increasing the volume of used media stimulates the flow in the Eppendorf tube during incubation.

32.6% of the liposomal L-DOPA concentration was released during the first 4h of incubation in blood plasma, followed by the occurrence of a plateau in L-DOPA retention at ≥6h of incubation. An unexplainable increase in liposomal L-DOPA retention was seen at the 4h incubation timespan. Unfortunately, due to the presence of interfering shoulders and peaks in the UPLC data of liposomal retained L-DOPA in blood plasma, quantification of L-DOPA were not exact but considered as estimates. Literature research demonstrated that the probable cause for the shoulders and peaks in the UPLC data were Protein Corona (PC)[42]. PC is a multi-layered complex of proteins that are adsorbed in the surface of nanoparticles. The formation of the PC complexes takes seconds and begins immediately after nanoparticles are subjected to blood or plasma[42]. As aromatic amino acids have an optical absorption that is measured at 280 nm wavelength, similar to L-DOPA, the presence of PC was assumably measured during L-DOPA quantification using UPLC, resulting in the interfering shoulders and peaks[43,44]. It is important to realise that the effect PC has on the obtained UPLC data using the streptavidin-biotin method is exclusive for drugs with absorbance wavelengths similar to proteins. Y. Wang et al,. 2022 showed no PC related additional peaks in the curcumin release assay, as the absorbance wavelength lies outside the spectrum of amino acids[29].

To decrease the interference of PC associated shoulders and peaks in UPLC data, various adjustments in the current method could be made. The inclusion of filtration or centrifugation techniques could be performed to separate L-DOPA from formed PC after disruption of liposomal bilayer using Milli-Q containing 0.05% Triton-X100[45]. However, this is presumably difficult due to the instability of L-DOPA, leading to drug loss during the filtration or centrifugation step[14]. Using DDC inhibitors (carbidopa or benserazide) and oxidation preventing actions (co-loading of L-DOPA with ascorbic acid), the rapid conversion might slow down[10]. Furthermore, altering UPLC settings could be used to increase the chromatographic separation of L-DOPA from interfering compounds. Lowering the flow rate and injection volume increases resolution and shrinks AUC in UPLC quantifications, respectively, optimising the quality of chromatography data[46]. Additionally, electrochemical detection (ECD) can be utilised to enhance the UPLC measurements[47]. ECD is based on the measurements of electrical current, caused by reduction and oxidation, and complements UV detection. The ECD is directly connected to the UPLC column so introduced L-DOPA undergoes an electrochemical reaction which results in a detectable electric current. It should not be attempted to inhibit the manifestation of PC on liposomes *in vitro* to avoid the interference in UPLC data. The occurrence of PC is physiological and research has demonstrated that the formation of PC lowers drug release[48]. As a results, inhibition of PC formulation *in vitro* would lead in an overestimation of drug release. Nevertheless, the direct contact of nanoparticles with complex media using the biotin-streptavidin method improves *in vivo* representability by enabling the physiological formation of PC[29].

Similar to UPLC data of incubation in blood plasma, L-DOPA quantification showed interfering peaks in whole blood, yet more intense. The higher intensity was unexpected as the plasma protein concentration in plasma was higher, theoretically resulting in elevated PC formation after liposomal exposure to blood plasma. An important characterisation of red blood

cells (RBC) is the containment of haemoglobin, an iron-based protein. After the incubation of L-DOPA encapsulated liposomes at 37°C in whole blood, the samples were transferred to the magnetic rack. It is possible that RBC were magnetically forced to sediment with MSB and bound liposomes via haemoglobin[49,50]. The high direct contact of liposomes with RBC could indirectly lead to the intense UPLC results. However, more research is needed to investigate this.

The lysis of RBC, also known as haemolysis, could be a consequence of the magnetically forced sedimentation via haemoglobin. The released RBC content caused by haemolysis could have an impact of the PC formation[51]. The presence of haemolysis was researched by observing the colour of blood plasma after 1- and 48h of incubation. A change of colour was noticed, indicating elevated free haemoglobin levels and haemolysis[52]. However, no spectrophotometry was performed.

The L-DOPA retention in liposomes in HBS (pH 6.5) using dialysis cassettes and the biotin-streptavidin methods were compared and shown in Figure 15. Throughout the entire assay, L-DOPA retention was larger using dialysis cassettes. Multiple factors could contribute to the found differences, including adsorption and precipitation of released L-DOPA in the dialysis cassette. Moreover, the created flow in the buffer may not be as efficient as assumed due to dialysis cassettes floating freely. As cassettes eventually rotate at a similar speed as the buffer, the buffer flux over the membrane is reduced, resulting in lower drug release. Because the biotin-streptavidin method facilitates *in vivo* characteristics including direct liposomal contact with the media under constant agitation, more drug release was observed compared to the dialysis technique[29]. By virtue of these results, it is unadvised to convert release kinetics from dialysis methods.

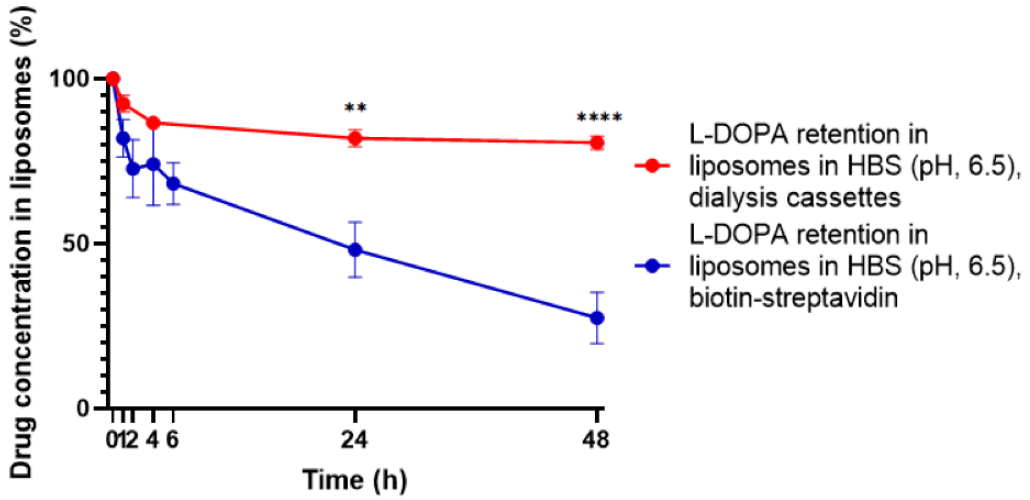


*Figure 15: The mean results of L-DOPA retention studies in HBS (pH, 6.5) using dialysis cassette and biotin-streptavidin methods are depicted. The error bars indicate the standard deviation. P<0.05 = *, P<0.01 = **, P<0.001 = ***, and P<0.0001 = ****. Made with: GraphPad Prism.*

Presumptive causes inducing differences seen in L-DOPA retention using the biotin-streptavidin method in HBS and blood plasma consist predominantly due to the presence of PC in blood plasma[48]. L-DOPA retention data in both media is shown in Figure 16. The burst releases shown in the first hours of incubation are similar, yet the subsequent observed release patterns vary greatly. As stated previously, PC formation inhibits drug release, possibly resulting in the ceased drug release at ≥6h of incubation[48]. As HBS does not contain blood proteins, drug release inhibiting PC are not present, resulting in continuing drug release.

Examination of release profiles of L-DOPA encapsulated in DPPC and cholesterol (8:2, 7:3 and 6:4, m/m) liposomes, PEGylated liposomes (2 and 4 % PEG) and targeted liposomes (0.35 and 7% maltodextrin conjugation) in phosphate buffer (0.1M, pH 7.4) at 38-, 40-, 42- and 44°C using dialysis (MW cut off: 12000 Da) by Z. Gurturk et al., 2017 revealed similar retention kinetics as found in HBS in the present study[35]. The found retention profiles demonstrated a burst release in the first 4h of incubation, followed by slow and stable L-DOPA release[35]. Concluding, the study of drug retention kinetics performed in media unable to formulate PC after exposure of nano-particles provide an overestimation of drug release.

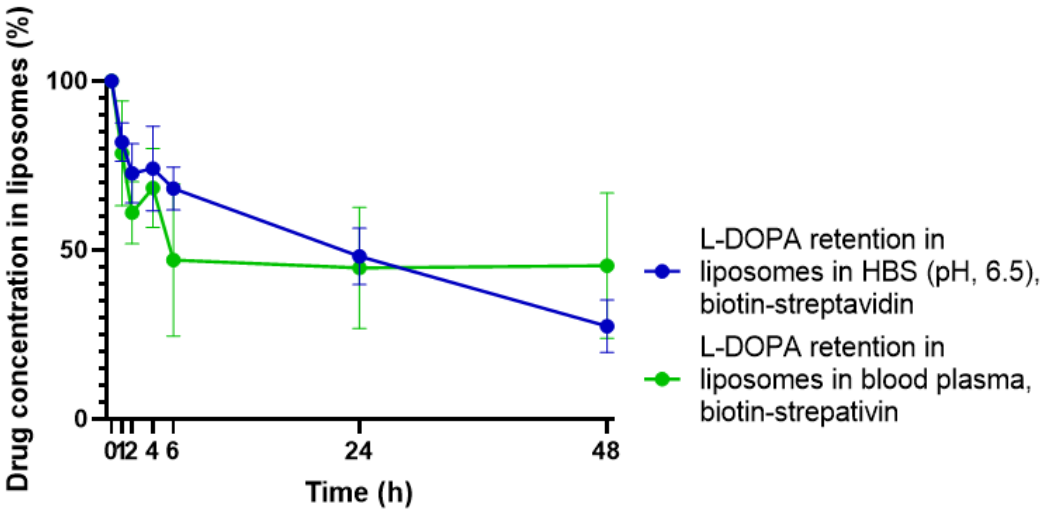


Figure 16: The mean results of L-DOPA retention studies in HBS (pH, 6.5) and blood plasma using the biotin-streptavidin method are depicted. The error bars indicate the standard deviation.

A substantial disadvantage of the biotin-streptavidin method is the high cost of the MSB. To reduce costs, it was attempted to break the interaction between biotin and streptavidin by heating. This method was recently published by A. Holmberg et al., 2005 and claimed that breaking the biotin-streptavidin bond could be done multiple times without affecting the affinity of streptavidin towards biotin[31]. Even though obtained data indicated that heating up biotin-streptavidin did break the interaction slightly, the results clearly demonstrated that heating the MSB up to 70°C harmed the ability to bind biotinylated liposomes.

Moreover, serious batch-to-batch differences were observed among MSB. Results obtained during this study by determining saturation points of 2 MSB batches showed major differences. Consequential, it is crucial to measure the points of saturation for used MSB batches.

4.2 Future prospects

The initial thought administration route for L-DOPA loaded liposomes was an IV injection as this bypasseses the first-pass metabolism and therefore increases the systemic liposomal encapsulated L-DOPA concentration[53]. The use of liposomes to encapsulate L-DOPA contributes to the protection against enzymatic or oxidative conversion, a sustained drug release, lower systemic L-DOPA concentration and theoretically a more precise drug targeting compared to the orally taken version[22]. Even though the combination of liposomes and IV injection implies a good drug delivery system, it has some major drawbacks.

The foremost hindrance is the need of drug administration by health practitioners. Orally taken pills can be ingested by PD patients[16]. Generally, PD patients are not able to administer an IV to themselves, especially PD patients in a later stage. Therefore, PD patients would need to frequently pay a visit to the hospital or have a health care provider at home to administer the IV injection, greatly reducing patient compliance. With the results obtained during the current study, an injection given on alternate days would be possible after further optimalisation. For PD patients, this would have a major effect on the quality of life. For this reason, a different route of administration is preferable.

The main target of both the oral and IV L-DOPA administration is targeting and crossing the BBB, as this is a big impairment of drug delivery to the brain[15,24,54]. Recent research demonstrated a route of administration for L-DOPA treatments that is non-invasive, self-administrable and is able to reach the brain without having to cross the BBB: nose-to-brain delivery through nasal spray[55–57]. Via this promising and direct route, PD symptoms would be lowered in minutes after administration[58]. *In vivo* nasal administration of free L-DOPA (12 mg/kg) in Parkinsonian rats has shown to be effective in 20 minutes after administration but the therapeutic benefit continues for approximately 1h[59]. Due to the Parkinsonian brain being in constant need of DA, the delivery of L-DOPA encapsulated in liposomes would be favourable as this results in prolonged pharmacological activity[22].

A logical next step for the use of nasal spray is repeating the retention study with the use of biotin-streptavidin with cerebrospinal fluid as the biological media[60]. With this, the L-DOPA retention in liposomes in nasal-spray associated relevant biological media can be determined. Research on nasal delivery using lipid-based nanoparticles carrying astaxanthin demonstrated significant systemic bioavailability, indicating multiple routes of drug uptake[61]. These data indicated that a fraction of the nasally administrated L-DOPA loaded liposomes was not taken up in the nasal cavity, ended up systemically through the oral and respiratory route. Therefore, the retention data in cerebrospinal fluid would be a great extension on the data of the current report. The retention data combined with liposome distribution data in animal models, preferably primate to increase translatability towards human use, would result in an indication of liposomal encapsulated and released L-DOPA distribution in time.

In vitro intracellular translocation of formulated liposomes and L-DOPA should be studies in BBB and nasal respiratory epithelium for IV and nasal administration, respectively. This could be done using a trans-well system and labelled L-DOPA, allowing the analysis of the fate of L-DOPA intracellularly. Moreover, cellular changes and cytotoxicity in used tissue due to prolonged exposure to L-DOPA should be investigated. To accentuate pathological variation in (targeted) liposomal distribution, *in vivo* administration of liposomal encapsulated L-DOPA via the nasal and IV route should be examined in healthy and Parkinsonian animal models. If, due to the increased permeability of the BBB in PD patients, (targeted) liposomal accumulation at the brain is (more) apparent in Parkinsonian models, toxicity and therapeutic efficacy should be evaluated. To further study the benefits of nasal drug delivery via spray, L-DOPA administration via nasal, oral and IV routes should be performed. With brain (or SNpc) and systemic L-DOPA concentrations as primary and secondary outcome, respectively, the efficacy and risk of side effects of the administration routes can be determined. Importantly, if accumulation at the brain is evident, liposomal targeting to the brain can be adopted for other neurological disorders including Alzheimer's disease.

Another aspect of liposomes as drug carrier that needs further investigation is the effects that liposomes have on the already overly active immune system of PD patients[62]. The benefit that PEGylation of liposomes entails, is mainly based on increasing the stealthiness. However, research revealed that the repeated exposure of the body to PEGylated liposomes

triggers the formulation of anti-PEG[63]. The enhanced presence of anti-PEG results in the faster elimination of liposomes, decreasing the effectiveness of the treatment[63]. The triggering of the anti-PEG production occurs in both IV and nasal administration of L-DOPA in PEGylated liposomes, making this a crucial topic for further investigation.

5 Conclusion

The research question of the current report was: What are the release/retention kinetics of liposomal encapsulated L-DOPA in relevant biological media (HBS, blood plasma and whole blood)? This was measured with the use of the newly developed biotin-streptavidin method for the removal of liposomes from media. L-DOPA retention data in HBS (pH 6.5) showed a burst release in the first 2h of incubation and was followed by a stable 1%/h release, which was in accordance with found literature[35]. The retention of liposomal encapsulated L-DOPA in blood plasma demonstrated a of 50% L-DOPA release in the first 6h of incubation, accompanied by the discontinuation of drug release after ≥6h incubation. The data indicates the importance of PC formation as this is known to reduce drug release[48]. The results of this report suggest that the biotin-streptavidin is, momentarily, not compatible for measuring the L-DOPA retention in liposomes after incubation in blood. The presence of PC during L-DOPA quantifications after incubation in blood (plasma) is the main culprit for the undesired UPLC data. However, the current method could be optimised with above mentioned adaptations to improve the technique. Notwithstanding, the biotin-streptavidin release/retention method is arguably superior to the momentarily available techniques. The main benefits that the biotin-streptavidin method has over the standard release methods is that the liposomes can roam freely in the media, come in direct contact with the medium specific properties and that the liposomes can be removed from the environment without disrupting the liposomal integrity. Important down-sides to using the biotin-streptavidin method are the costs and batch-to-batch differences in MSB batches.

Summarising, the liposomal encapsulation of L-DOPA is promising for future treatments as pharmacological properties including half-life and sustained drug release are improved. Moreover, the use of the biotin-streptavidin method has shown to be advantageous in predicting the drug retention kinetics *in vivo.* Yet additional research is needed to improve the method.

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