Levodopa-loaded polymeric- and lipid-based nanoparticles for the treatment of Parkinson's Disease

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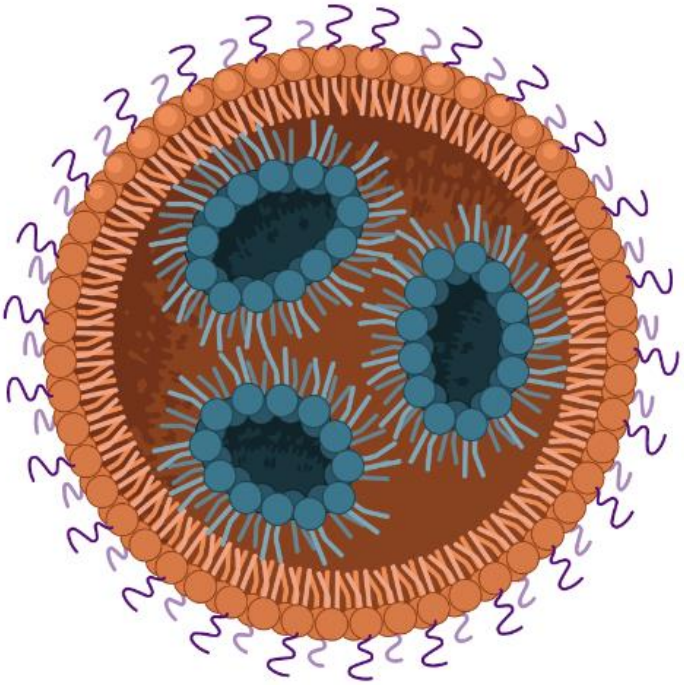

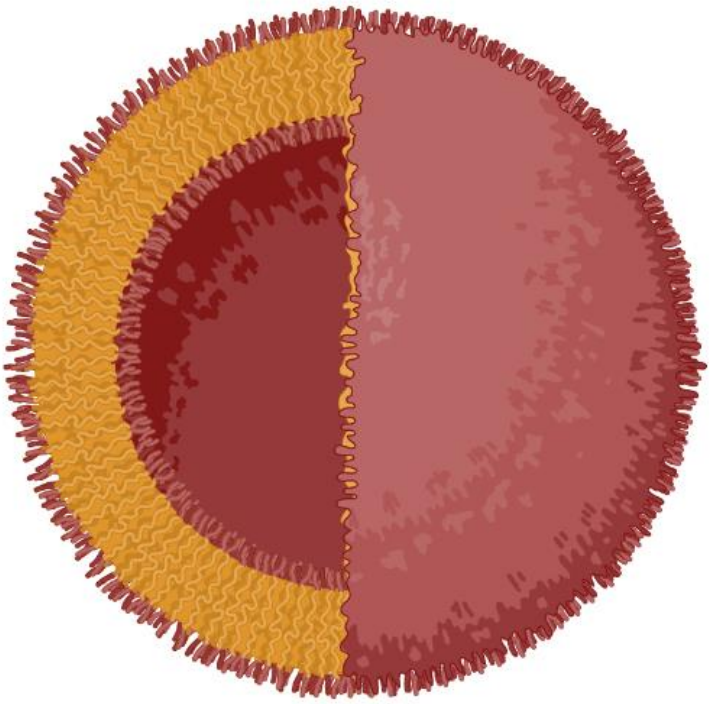

Abstract

Parkinson's disease (PD) is a neurodegenerative disorder where formation of Lewy Bodies (LBs) in the dopaminergic neurons of the substantial nigra pars compacta (SN) cause progressive cell death, resulting in a dopamine (DA) deficiency which manifests itself in motor symptoms including tremors and bradykinesia. Current PD treatments focus on symptom reduction through oral delivery of levodopa (L-DOPA), a precursor of DA. However, L-DOPA delivery to the brain is inefficient due to low oral bioavailability, degradation in the gastrointestinal (GI) tract and the liver, short systemic half-life, systemic L-DOPA decarboxylation into DA and limited brain uptake due to the blood brain barrier (BBB). Additionally, due to the buildup of L-DOPA tolerance by the PD brain, increased dosages are required as the disease progresses, resulting in increased systemic DA concentrations causing serious side effects like dyskinesias. To improve PD treatment efficiency and to reduce side effects, recent research focuses on the encapsulation of L-DOPA in nanoparticles (NPs), the most popular of which include the polymeric- and lipid-based NPs. Both formulations are able to protect L-DOPA from systemic decarboxylation into DA and increase L-DOPA delivery to the central nervous system (CNS). Additionally, these NPs can be modified with proteins and antibodies specifically targeting the BBB, not only improving targeting to-, but also the crossing of the BBB, thereby reducing required dosages and free systemic DA. An alternative treatment strategy is to avoid the BBB altogether through direct intranasal delivery of NP encapsulated L-DOPA to the brain. Through intranasal delivery, L-DOPA can be directly delivered to the brain via the olfactory and trigeminal nerves, reducing free systemic DA and avoiding the BBB-associated problems. These polymeric- and lipid-based NPs can be additionally modified to improve mucoadhesion and cell penetration, resulting in increased therapeutic concentrations of DA in all parts of the brain. In this review I will give an overview of the recent advancements made in the field of PD treatment, regarding NP encapsulated L-DOPA delivery to the brain via either the oral and IV route, as well as the direct intranasal delivery.

Laymen's summary

Parkinson's disease (PD) is a neurodegenerative disease where dopamine (DA) producing neurons of the substantia nigra pars compacta (SN) region of the brain die, resulting in DA deficiency which causes symptoms like tremors. Current PD treatments focus on the reduction of these tremors through the oral delivery of levodopa (L-DOPA), which can be converted into DA, compensating for the loss of DA producing brain cells. However, because of the strict protection of the brain by the blood brain barrier (BBB) and the L-DOPA conversion into DA which also occurs in the blood, PD patients suffer from serious side effects including involuntary movements of limbs and face after long-term oral L-DOPA treatment. To improve these treatments, research focusses on the encapsulation of L-DOPA in nanoparticles (NPs), which are small vesicles (10-200 nm) that usually consist of either lipids or polymers. These NPs are able to protect L-DOPA from enzymatic degradation in the blood, reducing DA levels in the blood and the associated side effects. Additionally, these NPs can be modified with proteins or antibodies which are specific for the BBB, increasing the targeting to the BBB and the transportation into the brain. Different research focusses on the direct delivery of L-DOPA to the brain through the nose, avoiding the BBB and the complications it brings altogether. After intranasal delivery, the NPs containing L-DOPA are able to travel to the brain through the olfactory and trigeminal nerves, reducing blood DA concentrations and the side effects it causes. These polymeric and lipid-based NPs can be additionally modified to increase NP residence time in the nasal cavity as well as NP transport across the nerves, achieving fast delivery and higher drug concentrations in the brain. In this review I will give an overview of the recent advancements made in the field of PD treatment, regarding NP encapsulated L-DOPA delivery to the brain via either the oral and IV route, as well as the direct intranasal delivery.

1. Introduction to Parkinson's disease

1.1 PD epidemiology

Parkinson's disease (PD) is a neurodegenerative disorder where loss of dopaminergic neurons in the substantial nigra pars compacta (SN) results in dopamine (DA) deficiency (Dimiou et al., 2022). After Alzheimer's disease, PD is the most prevalent neurodegenerative disease worldwide, with 35-100 per 100.000 population new PD cases on a yearly basis (Simon, Tanner, & Brundin, 2020), affecting between 100-300 per 100.000 population in total (Tysnes & Storstein, 2017). PD mainly manifests in the elderly population, with its prevalence increasing almost 10-fold when comparing the 50-59 age group with the 70-79 age group, being slightly more prevalent in males compared to females (Tysnes & Storstein, 2017). Due to a general aging of the population, PD prevalence is expected to double by the end of 2030 (Dorsey et al., 2007). The progressiveness of the disease, in combination with the lack of drugs that could potentially lead to a cure, and treatments which do not impair disease progression but mainly focus on symptom reduction, lead to a life expectancy that ranges between 6.9 to 14.3 years, with a median of 12.6 years after PD diagnosis (Macleod, Taylor, & Counsell, 2014; Tysnes & Storstein, 2017).

1.2 PD Symptoms and diagnosis

Patients suffering from PD often present with motor (movement and physical tasks) and non-motor (no movement) symptoms (NMS), which are, together with disease progression, highly variable between different patients (Armstrong & Okun, 2020). Consistent with a progressive disease, the earlier stages of PD are hardly noticeable, where small inconveniences, also called prodromal features, including constipation, complications during rapid eye movement sleep and shoulder pain are the main symptoms (Armstrong & Okun, 2020; Bloem, Okun, & Klein, 2021). However, as the disease progresses, which may take up to 10 years from first symptoms to diagnosis, NMS start to develop, including: olfactory loss (problems with sense of smell), sleep disorders (e.g. daytime sleeping), autonomic dysfunction (e.g. irregularities in urination and blood pressure variability), psychiatric disturbances (e.g. depression and anxiety) and cognitive impairment (e.g. dementia, problems with attention span) (Armstrong & Okun, 2020). During later stages of PD, motor symptoms including bradykinesia (progressive deterioration of speed and size of movements), rigidity (resistance to passive movements of for example joints), tremor (involuntary rapid movement in rest) and postural instability (complications with balance and posture) start to develop (Armstrong & Okun, 2020). In the absence of a diagnostic tool, PD diagnosis criteria include: 1) presence of 2 or more motor symptoms including bradykinesia, tremor at rest and rigidity, 2) presence of 1 or more NMS and 3) response to levodopa (L-DOPA) treatment (Armstrong & Okun, 2020; Bartels & Leenders, 2009; Bloem et al., 2021; Costa, Esteves, Empadinhas, & Cardoso, 2022). When a patient meets all 3 criteria, PD can be diagnosed (Armstrong & Okun, 2020). However, because diagnosis is based on the presence of motor symptoms which develop during the later stages of PD, approximately 60-70% of dopaminergic neurons have already died (Bartels & Leenders, 2009). If patients fail to meet all 3 criteria, other syndromes including vascular parkinsonism and multiple system atrophy are a more likely diagnosis (Bartels & Leenders, 2009).

1.3 PD pathology and risk factors

The initial pathological feature present in PD is a loss of dopaminergic neurons in the SN, resulting in DA deficiency which progressively worsens over time (Simon et al., 2020). This neurodegeneration is associated with accumulation of α-synuclein (α-syn) aggregates within the dopaminergic neurons of the SN (Cavaliere et al., 2017). In PD, already enriched α-syn production in the synapses of the SN is significantly increased due to locus duplications and triplications of the α-synuclein (SCNA) gene

(Simon et al., 2020; Singleton et al., 2003). Under physiological conditions, these α-syn aggregates are cleared, but due to a defect in the ubiquitin proteosome system (UPS) resulting in impaired lysosomal function and autophagy, often caused by mutations in the Leucine-rich repeat kinase 2 (LRRK2) gene, these α-syn aggregates accumulate in intracellular inclusions termed Lewy Bodies (LBs) instead (Reich & Savitt, 2019; Silva, Almeida, & Vale, 2021; Simon et al., 2020; Volpicelli-Daley, Luk, & Lee, 2014). These LBs are known to interfere with cellular functions, increase cellular stress and eventually cause cell death. Additionally, mitochondrial dysfunctions, attributed to mutations in the Parkin RBR E3 Ubiquitin Protein Ligase (PARKIN) and PTEN-induced kinase 1 (PINK1) genes, result in increased reactive oxygen species (ROS) production, which contributes to the increased cellular stress state and neuronal cell death, ultimately leading to DA depletion within the SN (Dias, Junn, & Mouradian, 2013; Shin et al., 2011; Simon et al., 2020; Valente et al., 2004). As DA is essential for motor control through the nigrostriatal pathway, DA depletion results in PD pathology and the beforementioned symptoms (Simon et al., 2020). While cell death during PD is believed to be mainly localized within the dopaminergic neurons of the SN, recent research has illustrated that as PD progresses, LB formation and neurodegeneration spreads to other brain regions, including the cerebral cortex, optic bulb and the autonomic nervous system (Raza, Anjum, & Shakeel, 2019). A schematic overview of the pathological processes present in PD is displayed in Fig. 1, while the 4 most prevalent gene mutations are displayed in Table 1.

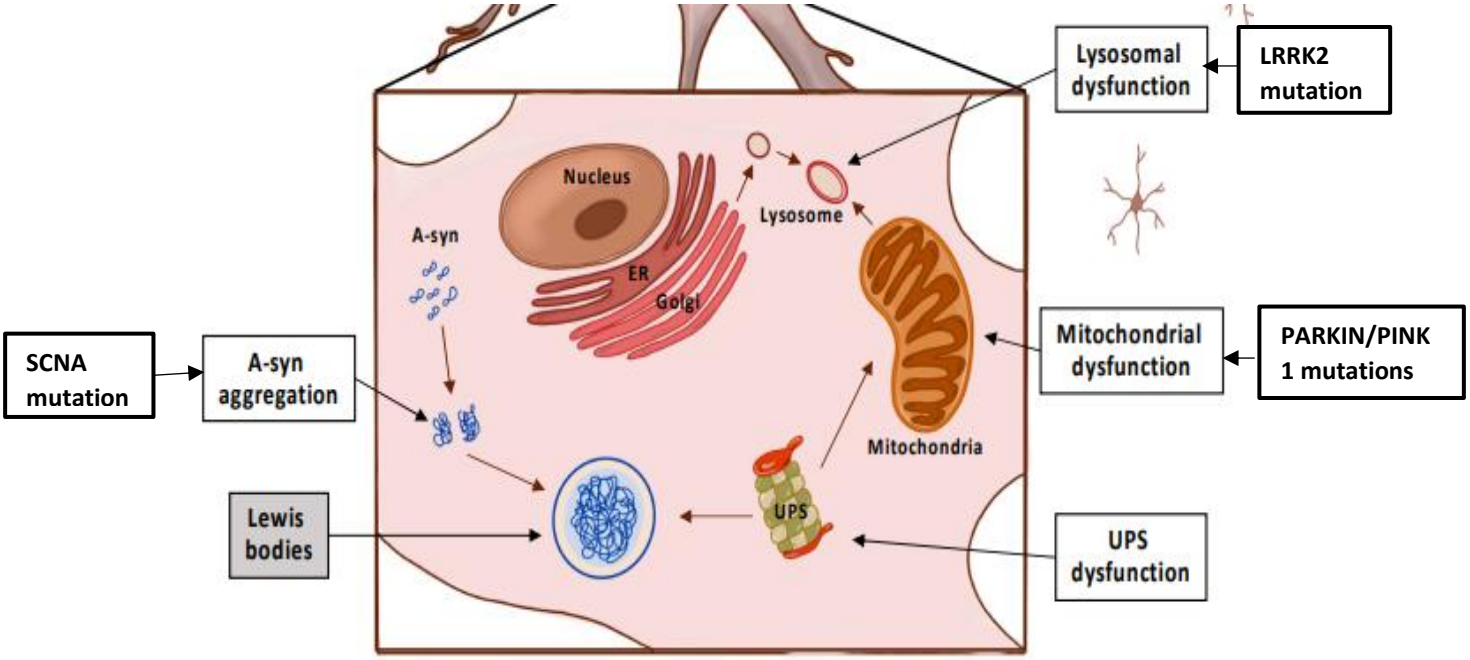

Figure 1. Pathology and risk factors of PD: *The main hallmark of PD pathology is the accumulation of α-syn aggregates forming LBs which increase cellular stress and interfere with normal cellular function, caused by mutations in the SCNA gene. These Lewy bodies are not cleared properly due to UPS defects, resulting in lysosomal dysfunctions and impaired autophagy, caused by mutations in the LRRK2 gene. Additionally, mitochondrial dysfunctions in combination with impaired mitochondria biogenesis as well as clearance caused by mutations in PARKIN and PINK1 contribute to increased cellular stress and increased ROS production, ultimately resulting in dopaminergic cell death and PD pathology. Adapted from: (Silva et al., 2021).*

In addition to the genetic factors displayed in Table 1, which are responsible for 3-41% of familial PD cases, environmental factors like exposure to certain pesticides have also been linked to increased risk of PD development (Lees, Hardy, & Revesz, 2009; Reich & Savitt, 2019). Several studies have demonstrated that increased exposure to the neurotoxin 1-methyl-4-phenyl-1,2,3,6-

tetrahydropyridine (MPTP) resulted in rapid and significant degeneration of dopaminergic neurons, ultimately leading to PD phenotypes (Bloem et al., 2021; Costa et al., 2022; Raza et al., 2019). The mechanisms behind MPTP driven neurodegeneration are irreversible inhibitions of mitochondrial complex I, resulting in mitochondrial-dependent apoptosis within the SN (Simon et al., 2020). These observations support the now common believe that PD is caused by a combination of genetic predisposition and external factors, which mainly impact processes associated with mitochondrial biogenesis and clearance, as well as lysosomal clearance of α-syn (Simon et al., 2020). Additionally, other risk factors including: head trauma, diabetes, hypertension and cancer have been linked with PD, the underlying mechanisms are unknown however (Ascherio & Schwarzschild, 2016).

Table 1. Most prevalent genetic mutations associated with familial PD pathology.

Gene	Cell part impacted	Physiological function	Pathological/mutated function	References
SCNA	α-syn.	Regulation of synaptic activity through chaperone functions during SNARE complex formation.	α-syn duplications and triplications resulting in α-syn aggregates and LB formation.	(Simon et al., 2020; Singleton et al., 2003).
LRRK2	Lysosome.	Lysosome formation and lysosome-mediated autophagy.	Lysosomal dysfunction resulting in reduced α-syn aggregate autophagy and thus LB formation.	(Giaime et al., 2017; Simon et al., 2020).
PARKIN	Mitochondria.	Ubiquitin-E3-ligase function which recognizes proteins on the outer surface of defect mitochondria, marking them for degradation. Additional functions in mitochondria biogenesis through regulation of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1-α).	Impaired clearance of defect mitochondria leading to mitochondria accumulation, as well as impaired mitochondrial biogenesis, resulting in increased cellular stress, ROS production and mitochondria-dependent apoptosis.	(Shin et al., 2011; Simon et al., 2020).
PINK1	Mitochondria.	Serine/threonine kinase localized to mitochondrial membrane, exerting neuroprotective properties through the protection of neurons from stress-induced mitochondrial dysfunction which results in mitochondria-dependent apoptosis.	Impairment of mitochondrial activity and increased stress-induced cell death through ROS production. Also linked with increased α-syn aggregation.	(Simon et al., 2020; Valente et al., 2004).

2. Current treatments for PD

2.1 Treatment of early stage PD

Due to the inability to slow, stop or reverse the progression of dopaminergic neuronal degradation, current PD treatments focus on the reduction of both motor symptoms and NMS (Armstrong & Okun, 2020). The 4 main treatment strategies include: physical therapy (e.g. physiotherapy, treadmill exercise and flexibility training) (Mak, Wong-Yu, Shen, & Chung, 2017; van der Kolk, N. M. & King, 2013), rehabilitating therapy (e.g. speech therapy), pharmacological therapy (e.g. drugs) and surgery (e.g. deep brain stimulation) (Armstrong & Okun, 2020; Silva et al., 2021). Due to the high variability in symptom severity per patient, 1 or more of the above mentioned treatments is applied. Due to the risk of the procedure, surgical treatments like deep brain stimulation are only applied when other

treatment options fail, due to tolerance or severe motor symptom fluctuations (Armstrong & Okun, 2020).

Out of the 4 main treatment strategies, pharmacological therapy is the most effective at treating motor symptoms, and is always included in PD treatment. Although intravenous (IV) delivery is the most effective method for a sustained and constant delivery of therapeutics, due to patient compliance the majority of drugs are delivered via the oral route (He et al., 2019). There are three main classes of drugs which are used in PD therapy (Silva et al., 2021):

1) Levodopa (L-DOPA): L-DOPA is a precursor of DA and focusses on the increase in DA levels within the SN.
2) DA agonists: drugs including apomorphine and ropinirole which focus on the imitation of DA through interaction with DA receptors.
3) Monoamine oxidase type B (MAOB) inhibitors and catechol O-methyltransferase (COMT) inhibitors: MOAB and COMT inhibitors focus on the preservation of available DA, through active inhibition of DA degradation and metabolism by MAOB and COMT.

Early stage PD treatment is usually started with relatively mild drugs including the MOAB and COMT inhibitors (Raza et al., 2019). These drugs are administered daily and are able to cross the blood brain barrier (BBB) and reach the brain where they inhibit DA degradation, thereby preserving available DA storages and increasing overall DA concentrations, resulting in slight motor symptom reduction with little to no side effects (Fox et al., 2018; Lees et al., 2009). However, as PD progresses and motor symptom severity increases, these inhibitors are unable to sufficiently suppress disease symptoms, and therefore DA agonists are added to the treatment regime. DA agonists like apomorphine and ropinirole are small drugs with lipophilic proprieties, allowing them to readily cross the BBB (Silva et al., 2021). DA agonists are able to mimic DA function through activation of D1 like and D2 like receptors in various brain regions, and are therefore moderately effective at the reduction of motor symptoms (Auffret, Drapier, & Vérin, 2018). Dependent on the type of DA agonist prescribed, oral administration (1-3x a day) or transdermal patches are used for delivery (Reich & Savitt, 2019). While DA agonists possess a higher efficacy compared to MAOB and COMT inhibitors, they can cause several side effects including: nausea, hallucinations, sleep disorders, impulse control disorders and psychosis (Arisoy et al., 2020; Armstrong & Okun, 2020; Reich & Savitt, 2019).

2.2 Oral Levodopa treatment

During the later stages of PD, either due to lack of treatment efficacy or severity of side effects caused by DA agonists, PD treatment regime is switched to oral L-DOPA (Silva et al., 2021). Currently, oral L-DOPA is the golden standard for PD treatment and shows the highest efficacy for motor symptom reduction (Tambasco, Romoli, & Calabresi, 2018). After systemic uptake, L-DOPA is able to cross the BBB and reach the brain, where it can be converted to DA by l-aromatic amino acid decarboxylase (AAAD) (Arisoy et al., 2020). This L-DOPA to DA conversion mainly takes place within the presynaptic terminals of the dopaminergic neurons in the SN, resulting in an increased DA concentration in these neurons, significantly reducing motor symptoms caused by PD (Arisoy et al., 2020; Tambasco et al., 2018). However, while L-DOPA displays improved motor symptom reduction compared to the other 2 pharmacological drugs, there are several disadvantages to L-DOPA treatment.

L-DOPA is a hydrophilic compound which is rapidly degraded by enzymes within the gastrointestinal (GI) tract and decarboxylated and metabolized by AAAD during hepatic first pass metabolism as well as in the systemic circulation, resulting in a half-life of approximately 50 minutes (Ahmad et al., 2022; LeWitt, 2015). This rapid conversion of L-DOPA within the systemic circulation does not only result in

a low oral L-DOPA bioavailability (approximately 30%) and low percentages of the L-DOPA reaching the brain (approximately 1%), but also leads to DA exposure to the rest of the body, causing several adverse effects (Palmer, 2011). The most common side effects of short-term L-DOPA treatment include dizziness, headaches, vomiting and insomnia, while long-term L-DOPA treatment often results in involuntary movements and dyskinesia's, severely decreasing quality of life and patient compliance (Calabresi, Di Filippo, Ghiglieri, Tambasco, & Picconi, 2010; Mittur, Gupta, & Modi, 2017). To counteract these systemic adverse side effects and increase L-DOPA bioavailability, the currently prescribed L-DOPA formulations consist of L-DOPA which is co-administered with carbidopa (Haddad, Sawalha, Khawaja, Najjar, & Karaman, 2017). Carbidopa is a peripheral amino acid decarboxylase inhibitor, which inhibits L-DOPA metabolism by AAAD in the systemic circulation, while being unable to cross the BBB and enter the brain (Ahmad et al., 2022; Silva et al., 2021). This L-DOPA/carbidopa co-administration therefore not only results in a reduction of adverse effects due to reduced systemic DA concentrations, but also increases L-DOPA half-life from 50 minutes to 1.5 hours, resulting in increased brain uptake from 1% to approximately 5-10%, without the inhibition of L-DOPA conversion to DA after entering the central nervous system (CNS) (Hauser, 2009; Tambasco et al., 2018).

To further increase L-DOPA half-life, current prescribed PD treatments like RYTARY utilize a combination of carbidopa and levodopa in instant release capsules as well as extended release capsules (Dhall & Kreitzman, 2016; Hauser, Ellenbogen, Khanna, Gupta, & Modi, 2018). While these treatments are very advantageous in the earlier stages of PD, and can be administered in starting doses of 23.75 mg carbidopa/96 mg L-DOPA 3 times a day, the prescribed dose increases as PD progresses and can reach 612.5mg carbidopa/ 2450 mg L-DOPA per day depending on disease severity (Hauser et al., 2018). This necessary increase in treatment dose is a result of progressive neurodegeneration. While the dopaminergic neurons are able to store L-DOPA supplied DA to some extent in the earlier phases, as PD progresses and more dopaminergic neurons die this buffer function is lost, resulting in depleted brain DA storages which cause brain DA concentrations to resemble blood DA concentrations, resulting in so called "L-DOPA tolerance" (Abbott, 2010). Therefore, due to the short systemic L-DOPA half-life, higher and more frequent L-DOPA/carbidopa dosages are required to induce symptom relieve as PD progresses (Abbott, 2010). Due to this dosage increase, the "on-off phenotype" is developed, where DA levels spike just after treatment and are low in between 2 consecutive treatments (Tambasco et al., 2018). It is now believed that these swings in DA concentrations between 2 treatment times are causative for severe adverse effects like dyskinesias, stressing the importance of a continuous DA supply (LeWitt, 2015). The severity of these adverse effects increases with time, ultimately surpassing the beneficial effect of the treatment. Recent research indicated that L-DOPA/carbidopa treatment sustained long-term benefits in only 20% of patients after 2 years, while more than 75% of these patients experienced serious adverse events like dyskinesias (Calabresi et al., 2010; Tambasco et al., 2018).

2.3 The blood brain barrier

To reduce side effects and improve patient compliance, increased L-DOPA bioavailability and brain delivery is required in order to minimize free systemic DA. One major hurdle for the effective and targeted delivery of any therapeutic compound to the brain is the BBB, which is responsible for the discontinuation of approximately 95% of potential therapeutic molecules for drug development (Dong, 2018). The BBB is the gatekeeper of the CNS and maintains a strictly controlled brain microenvironment by selection of molecules that enter the brain (Palmer, 2011). The BBB consists of multiple cell types, including unfenestrated endothelial cells (ECs) connected through tight junctions (TJs), pericytes, astrocytes and microglial cells (Poudel & Park, 2022). While the BBB is highly

selective, certain molecules like nutrients and amino acids (AAs) are able to cross the BBB through 2 main pathways: the transcellular pathway and the intracellular pathway (Poudel & Park, 2022). Due to the tightly packed TJs and adherens junctions (AJs), transcellular transport is mainly utilized by small hydrophobic molecules (MW < 400 Da), while intracellular transport is mainly utilized by the hydrophilic macromolecules (Lee & Leong, 2020; Masserini, 2013). Examples of intracellular transport across the BBB are the carrier mediated transport (CMT) of glucose through glucose transporters GLUT1 and GLUT3, receptor mediated transcytosis (RMT) of larger macromolecules through for example the insulin receptor and the low-density lipoprotein receptor, and adsorptive-mediated transcytosis (AMT), which is used to transport charged proteins through electrostatic interactions between the proteins and the ECs (Lee & Leong, 2020; Poudel & Park, 2022). Additionally, recent research has illustrated that BBB permeability is altered in diseases involving inflammatory, traumatic or degenerative conditions, becoming disrupted and allowing the passage of more and larger molecules (Dong, 2018; Reinhold & Rittner, 2017; Saraiva et al., 2016). While the exact mechanisms of BBB disruption are still unknown, disrupted EC junctions are believed to be at the base of this phenomenon (Dong, 2018). While the high selectivity of the BBB causes problems with effective L-DOPA delivery to the brain, insight in the mechanisms responsible for the facilitation of BBB transport in combination with altered BBB permeability in PD, opens a window for precise L-DOPA targeting to the CNS, with the potential to increase L-DOPA bioavailability and reduce free systemic DA (Palmer, 2011).

3. Nanoparticles for improved L-DOPA brain delivery

3.1 Nanoparticle composition

As mentioned earlier, improved L-DOPA targeting to the CNS as well as protection from systemic conversion by AAAD are required to decrease current oral L-DOPA dose, increase bioavailability and reduce systemic side effects (Saraiva et al., 2016). The use of nanoparticles (NPs) for the encapsulation and targeting of anti-parkinsonian drugs to the BBB and therefore the CNS has been thoroughly explored in the past decades (Saraiva et al., 2016; Singh & Lillard, 2009; Wohlfart, Gelperina, & Kreuter, 2012). NPs are small colloidal nano-sized carriers (usually between 10 and 200 nm in size) which can either be organic, hybrid or inorganic (Jagaran & Singh, 2022). Inorganic NPs, usually consist of metals or quantum dots and display low batch-to-batch variability, are easily controlled in size, are easily functionally modified and are easy to track using different imaging techniques, and are therefore mainly utilized for imaging rather than drug delivery (Saraiva et al., 2016; Silva et al., 2021). Organic NPs can consist of virtually all biological products, but usually contain either lipids, polymers or proteins. Unlike inorganic NPs, the organic variants display high biocompatibility, low toxicity and are easily modified for precise BBB targeting (Silva et al., 2021). Regardless of materials used, organic NPs are able to successfully encapsulate L-DOPA, increasing its circulation time, reducing required L-DOPA dose through sustained release, while preventing its systemic degradation, resulting in increased brain uptake through the BBB at lower dosages (Poudel & Park, 2022). Additionally, organic NP properties can be tailored to increase specific BBB targeting and facilitate targeted uptake into the CNS (Silva et al., 2021). First of all, NP size is an important feature to overcome the BBB, where BBB penetration decreases as NP size increases (Saraiva et al., 2016). Secondly, zeta potential (surface charge) strongly influences the biological fate of NPs, where a negative zeta potential increases NP circulation time while reducing protein absorption, and a positive zeta potential facilitates AMT across cellular barriers like the BBB through interactions with negatively charged plasma membranes (Saraiva et al., 2016; Silva et al., 2021). While positively charged NPs have been associated with increased brain uptake, positive charges which are too high have been linked with immediate BBB toxicity (Lockman, Koziara, Mumper, & Allen, 2004; Saraiva et

al., 2016). Thirdly, NP hydrophobicity impacts the method of NP passage across the BBB, where hydrophobic NPs tend to utilize the receptor/carrier mediated paracellular pathway, and the hydrophilic NPs utilize transcellular diffusion (Jagaran & Singh, 2022; Silva et al., 2021). Therefore, NP biomaterial composition should be carefully considered and optimized to ensure appropriate size, hydrophilicity and zeta potential for BBB penetration.

3.2 Organic NP modifications to increase BBB penetration

In addition to the modification of NP composition to control parameters like size, hydrophobicity and zeta potential, organic NPs are often modified with ligands which aim to increase BBB targeting and penetration (Saraiva et al., 2016). These ligands can be classified into 3 different types based on the mechanism they facilitate:

1. Ligands which directly target receptors or carriers located on the BBB: these ligands often involve antibodies or proteins ligated to the surface of the NP, which specifically target receptors known to be overexpressed on the BBB (Ouyang et al., 2022). Through direct interaction between coupled NP ligand and receptor, either RMT or CMT is facilitated, increasing BBB penetration (Fig. 2C/D) (Silva et al., 2021). Commonly targeted receptors which are upregulated in the BBB and facilitate RMT include transferrin receptors, insulin receptors, leptin receptors, low-density lipoprotein receptors and lactoferrin receptors (Gao & Gao, 2018). Additionally, the brain's requirement for energy can also be exploited to increase NP uptake, through NP modification with glucose specifically targeting GLUT-1 facilitated transport across the BBB into the CNS (Agrawal et al., 2017; Ouyang et al., 2022; Tajes et al., 2014)
2. Ligands which increase NP hydrophobicity and charge: these ligands are either build into the NP or modified at the surface, and increase either hydrophobicity or zeta potential (Saraiva et al., 2016). Examples include NPs coated with amphiphilic peptides to increase hydrophobicity, or altered NP composition/ addition of compounds to increase zeta potential (Guerrero et al., 2010; Saraiva et al., 2016). CNS uptake is then stimulated through either lipophilic transcellular transport (Fig. 2A), hydrophilic paracellular transport (exclusively used for smaller NPs size) (Fig. 2B) or AMT (Fig. 2E) (Saraiva et al., 2016).
3. Ligands which disrupt the BBB: these ligands are either ligated to the NPs or are co-administered, and focus on the temporary disruption of TJs and AJs connecting the ECs of the BBB (Poudel & Park, 2022). These ligands include cell penetrating peptides (CPPs), microbubbles, hyperosmotic agents and surfactants which are able to promote the rapid transport of proteins, AAs, small liposomes and other small NPs into the CNS (Lee & Leong, 2020; Poudel & Park, 2022). While being an effective method, TJs can only be opened to a certain extent, so exclusively small NPs (<20 nm) can utilize this pathway (Masserini, 2013; Poudel & Park, 2022). Additionally, these ligands are aspecific and might become neurotoxic if used on a long term basis, therefore bringing additional limitations and risks compared to the ligands mentioned in 1 and 2 (Masserini, 2013; Poudel & Park, 2022).

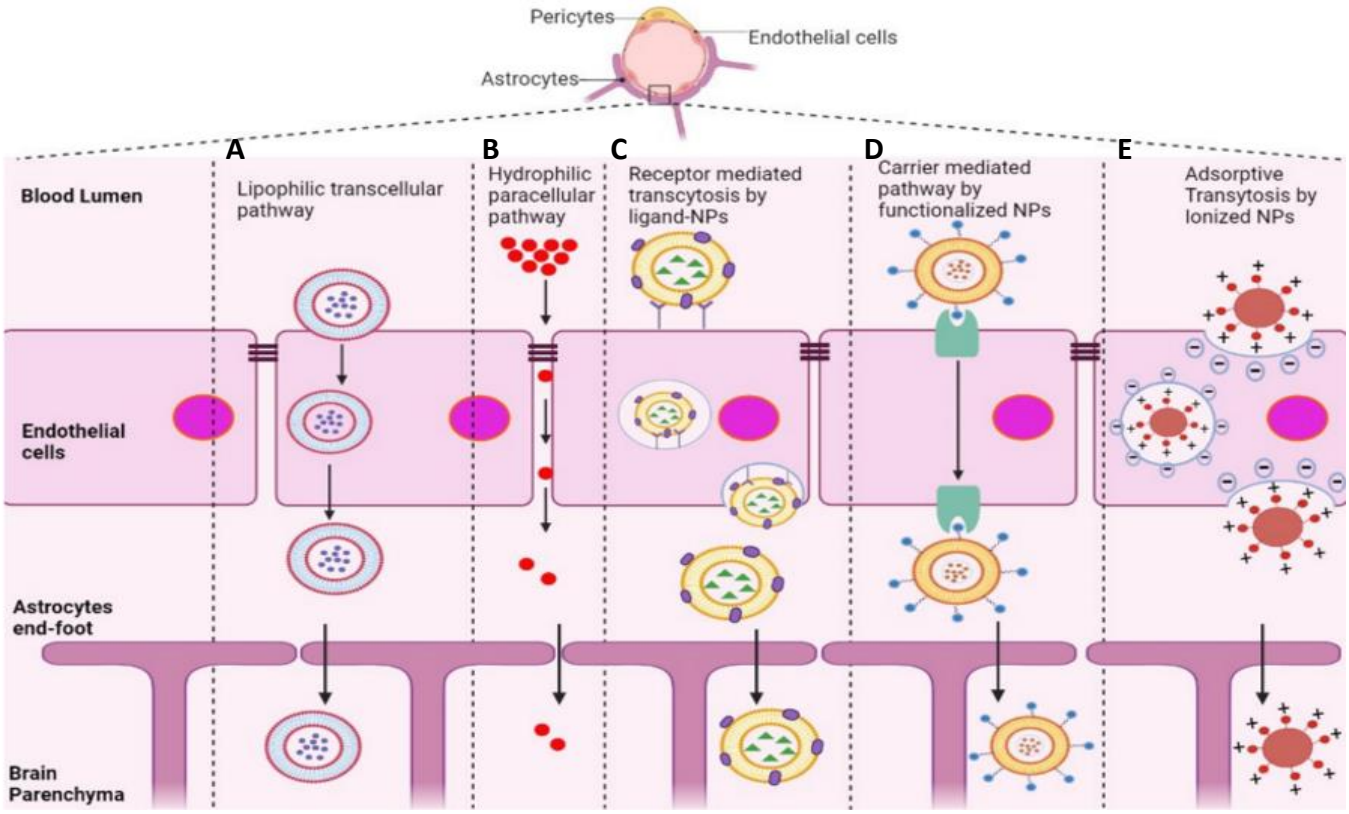

Figure 2. NP modifications which increase BBB penetration. *Different NP modifications aim to increase BBB penetration exploiting several mechanisms. A) NP hydrophilicity can be increased to increase lipophilic transcellular transport of larger NPs or B) hydrophilic paracellular transport of smaller NPs. Protein and antibody modifications specifically targeting receptors and carriers on the BBB facilitate C) RMT or D) CMT across the BBB, while E) increased NP zeta potential improves AMT. Adapted from: (Poudel & Park, 2022)*

3.3 lipid-based NPs and polymeric NPs

While there are virtually endless configurations for organic NPs, lipid nanoparticles (LNPs) and polymer-based NPs are the most popular at the moment, due to their high biocompatibility, low toxicity and potential to customize to control biological fate (e.g. targeting to the BBB), stability and drug release capability (Silva et al., 2021). Due to the nature and composition of these NPs, polymers and polymeric micelles are mostly used for delivery of hydrophobic drugs, while liposomes and LNPs are more suitable for the delivery of hydrophilic drugs (Silva et al., 2021). Despite the fact that there are limited NP-based treatments currently on the market, numerous lipid and polymeric NPs have been investigated and are currently in pipelines. I will give a brief overview of the most common polymeric- and lipid-based NPs, as well as the advantages and disadvantages of each type.

3.3.1 polymeric NPs

Polymeric NPs are composed of 1 or more synthetic or natural polymer(s) which are assembled together to form vesicles that are biocompatible, biodegradable and exhibit controlled and sustained release properties (Baskin, Jeon, & Lewis, 2021; Poudel & Park, 2022). The simplest polymeric NP is the nanocapsule, where a drug is encapsulated by a single polymer vesicle (Baskin et al., 2021). While there are virtually endless polymeric NP configurations, poly(ethylene glycol) (PEG), poly(trimethylene carbonate) (PTMC), poly(lactic-co-glycolic acid) (PLGA) and chitosan are the most widely investigated FDA approved polymers due to their sustained-release properties in combination with their low toxicity and favorable safety profiles (Poudel & Park, 2022; Wang et al., 2022). Examples of currently explored co-polymer NPs for brain delivery of anti-parkinsonian drugs are PEG-PTMC NPs developed by Wang and co-workers (Wang et al., 2022). They demonstrated the generation of PEG-PTMC NPs, 78 nm in size with a surface charge of approximately -10 mV. These NPs could be efficiently loaded with anti-parkinsonian drugs which showed a partial *in vitro* rapid

release over 4 hours, as well as sustained release properties for up to 48 hours. *In vivo* pharmacokinetic experiments in rats demonstrated a significantly increased plasma concentration as well as brain concentration compared to free drug after oral administration, which was sustained for up to 48 hours (Wang et al., 2022). More sophisticated copolymers include the utilization of copolymers which are amphiphilic and which can be assembled into NPs with a hydrophilic shell and a hydrophobic core, called polymeric micelles. These polymeric micelles are stable, enable sustained drug release which can be tailored to respond to external stimuli and can be precisely targeted to the BBB (Masserini, 2013; Wang et al., 2022). Liu et al studied polymeric micelles for the use of increased and sustained drug-brain delivery, through the generation of PEG micelles modified with transcriptional activator TAT peptides (Liu, L. et al., 2008). These micelles self-assembled into NPs of 180 nm or smaller, showed efficient drug loading and illustrated an *in vitro* sustained drug release for 6 hours in PBS at body temperature, as well as increased cellular uptake in an *in vitro* human astrocyte model. Additionally, these PEGylated micelles showed significantly increased BBB targeting and brain delivery after IV delivery in rats, which was visualized by imaging of fluorescein 5-isothiocynate (FITC)-loaded micelles compared to free injected FITC (Liu, L. et al., 2008).

While none of the polymeric NPs have received FDA approval yet in regards to treatment of PD, some of these polymers are already applied in other treatments, where they are used to coat NPs, stabilize proteins and facilitate controlled hormone release in the treatment of for example prostate cancer (Baskin et al., 2021; Bobo, Robinson, Islam, Thurecht, & Corrie, 2016; Tunn, 2011). The main advantage of polymer NPs over other carrier systems is the variety of available polymers in combination with the possibility to modify their surfaces, allowing the fine-tuning of NP composition to precisely control NP properties like size, hydrophilicity, surface charge, circulation time, drug release profile, degradation rate, stimuli to external responses etc. (Baskin et al., 2021; Boyuklieva & Pilicheva, 2022; Wang et al., 2022). However, polymeric NPs and polymeric micelles struggle with drug leakage out of the polymeric NPs after injection into the body, as well as possible toxicity from degradation products, batch-to-batch differences when using natural polymers and difficulty in upscaled production (Baskin et al., 2021; Tapeinos, Battaglini, & Ciofani, 2017; Wang et al., 2022). Additionally, antibody responses against polymers, for example PEG, induce faster clearance after multiple administrations, leading to reduced circulation times and increased premature drug release often referred to as the ABC-effect (Estapé Senti et al., 2022; Sroda et al., 2005).

3.3.2 Lipid-based NPs

In contrast to polymeric NPs, lipid-based NPs are mainly used to encapsulate hydrophilic compounds, and comprise carriers composed of one or more types of lipids. The most commonly used and studied lipid-based NPs are liposomes, consistent of a bilayer of phospholipids with a hydrophilic aqueous core, micelles, consisting of a single layer of phospholipids with an aqueous core and solid lipid NPs (SLNs) with a solid hydrophobic core (Baskin et al., 2021; Masserini, 2013; Tapeinos et al., 2017). Liposomes and micelles are mostly used for the encapsulation and delivery of hydrophilic compounds stored in their water-soluble core, but have been demonstrated to be able to transport hydrophobic and lipophilic payloads to some extent within their hydrophobic lipid outer layer(s) (Poudel & Park, 2022). Compared to polymeric NPs, lipid-based NPs are more biocompatible and show decreased toxicity, and are therefore currently on the market for several applications including drug delivery in specific cancers, delivery of viral vaccines and treatment of fungal diseases (Alam et al., 2014; Bulbake, Doppalapudi, Kommineni, & Khan, 2017). Additionally, as a consequence of their relatively small size and outer layer consisting entirely of lipids, they are able to readily pass the BBB without any functional modifications, through either the hydrophobic transcellular pathway or the lipophilic paracellular pathway (Fig. 2A/B) (Tapeinos et al., 2017). Additionally, these lipid NPs are

more cost effective and are easier to scale up compared to polymer-based NPs, highlighting their economic and practical benefits (Silva et al., 2021; Tapeinos et al., 2017). However, most lipid-based NPs experience difficulties with oxidation and hydrolysis after arrival in the systemic circulation, show increased structural instability and leaking of the cargo compared to polymeric NPs, and are slightly more susceptible for the accumulation of plasma components and proteins after IV injections, creating a so called "protein corona", resulting in their rapid clearance from the body by the reticuloendothelial system (RES) (Baskin et al., 2021; Wohlfart et al., 2012). Functional liposome modifications can aid some of these shortcomings, enabling direct targeting to the BBB through liposome modifications with BBB specific antibodies or ligands to facilitate RMT and CMT as described in section 3.2. (Wang et al., 2022). Examples of surface modifications utilized for liposome targeting the BBB include the mannose and CPPs penetratin and abies virus glycoprotein peptide modified liposomes generated by Arora and coworkers (Arora, Layek, & Singh, 2021). They demonstrated a significant increase in brain delivery of an anti-Alzheimer's drug using these double modified liposomes *in vitro* and *in vivo*, with no noticeable toxicity (Arora et al., 2021)**.** Additionally, the circulation time of lipid-based NPs can be increased through modifications including PEG coating and modifications neutralizing liposome charge, disguising liposomes from the RES system and creating so called "stealth liposomes" (Kang, Jung, Oh, & Song, 2016; Masserini, 2013; Tröster, Müller, & Kreuter, 1990). While these lipid NP modifications are essential for effective L-DOPA brain delivery, they interfere with the cost-effectiveness and up scalability of lipid NPs, and should therefore be carefully considered.

While recent research has demonstrated that encapsulation of anti-parkinsonian drugs in NPs can improve circulation time and targeting to the CNS, thereby reducing both drug dosage and systemic side effects, there are some shortcomings and questions regarding this strategy. First of all, there is an ongoing debate whether the entire NP penetrates the BBB, or the drug is released before reaching the CNS, with various studies reaching contradictory conclusions (Masserini, 2013; Saraiva et al., 2016). Additionally, one of the major hurdles that orally administered NPs face is digestion within the GI tract, extensive first pass metabolism, as well as EC barriers and tightly packed TJs after reaching the circulation (Ensign, Cone, & Hanes, 2012). These natural barriers result in the excretion and degradation of 85-90% of orally administered NPs, with only 2-3% of orally administered drug reaching the bloodstream after 30 minutes, highlighting the inefficiency of this delivery route (Lalatsa et al., 2012). Therefore, modifications to protect NPs in the GI tract and the circulation are required to optimize oral NP delivery, which further complicates production cost and up scalability (He et al., 2019). To circumvent these issues, most polymeric and lipid-based NP formulations are designed for IV administration (Kang et al., 2016). However, current NP formulations show sustained drug release for no more than a few days, which would result in bi-daily hospital visitations for PD patients, thereby drastically reducing patient compliance (Wang et al., 2022). Therefore, alternative strategies are required to enable delivery of anti-parkinsonian drugs to the CNS without significantly interfering with the patient's daily life.

4. Intranasal delivery of anti-parkinsonian drugs to the CNS

4.1 Intranasal delivery routes

While the previously described orally administered and IV injected NPs focus on targeting to – and facilitation of crossing the BBB, another strategy is to avoid the BBB altogether (Silva et al., 2021). Over the past decade, increasing evidence supports the existence of a more direct delivery route between the nose and the CNS (Arisoy et al., 2020; Crowe, Greenlee, Kanthasamy, & Hsu, 2018). Direct nose-to-brain delivery is a non-invasive and easy to self-administer pathway which circumvents some of the major flaws of IV administration or oral delivery as described in the previous

chapter (Boyuklieva & Pilicheva, 2022). These advantages include: 1) evasion of degradation in the GI tract and hepatic first-pass metabolism, which excretes approximately 90% of orally administered NP encapsulated drugs (Lalatsa et al., 2012), 2) reduction of free systemic therapeutics, which significantly decreases side effects, 3) evasion of the BBB, which is one of the biggest hurdles in drug delivery to the CNS (Boyuklieva & Pilicheva, 2022).

After intranasal administration, drugs are deposited on the respiratory and olfactory epithelium, and can be absorbed via either the nose-to-blood-to-brain pathway or via the direct nose-to-brain pathway (Boyuklieva & Pilicheva, 2022; Yarragudi, Kumar, Jain, Tawhai, & Rizwan, 2020). During the nose-to-blood-to-brain pathway, the drug deposited on the respiratory epithelium can be absorbed through the fenestrated nasal epithelial cells of the well vascularized lateral walls of the nasal cavities (Erdő, Bors, Farkas, Bajza, & Gizurarson, 2018). From there, the drug can enter the circulation, after which it may pass to the CNS if the drug is capable of crossing the BBB (Boyuklieva & Pilicheva, 2022; Erdő et al., 2018). Drugs deposited in the olfactory region of the nasal cavity can be directly transported to the CNS in a matter of minutes via the olfactory or trigeminal nerves, which are the only direct connection between the brain and the "outside world" (Djupesland, Messina, & Mahmoud, 2014; Erdő et al., 2018). This direct nose-to-brain route is composed of an intracellular and an extracellular pathway (Djupesland et al., 2014; Erdő et al., 2018). During the intracellular route, therapeutics are internalized within the olfactory and trigeminal neurons via endocytosis, after which they are transported within the endosome towards the neuronal axon where they are released in the olfactory bulb via exocytosis before finally reaching the brain stem (Fig. 3A) (Boyuklieva & Pilicheva, 2022; Erdő et al., 2018). During the extracellular route, drugs penetrate the TJs of the olfactory epithelium towards the lamina propria, after which they travel through the paracellular space of the nasal epithelium along the length of the neuronal axon towards the subarachnoid space before reaching the CNS (Fig. 3B) (Boyuklieva & Pilicheva, 2022; Crowe et al., 2018). After reaching the brain stem, therapeutics are either further distributed to the rest of the brain via the perivascular pump driven by arterial pulsation, transported back to the nasal cavity via P-gp efflux proteins (ATP-binding cassettes present in cell membranes able to export foreign substances) or absorbed in blood/lymphatic vessels (Battaglia et al., 2018; Hadaczek et al., 2006; Lochhead & Thorne, 2012). Unlike the systemic route, direct nose-to-brain delivery facilitates fast transport to the CNS, reaching the target site within minutes (Boyuklieva & Pilicheva, 2022). Salameh and coworkers demonstrated the presence of labeled insulin in and around the olfactory bulb only 5 minutes after nasal administration in rats, with the insulin reaching all parts of the brain within 30 minutes, unlike the IV injected control rats (Salameh et al., 2015). Additionally, Chao and coworkers demonstrated the rapid effect of intranasally administered L-DOPA on PD rats, where intranasal L-DOPA treatment (12 mg/kg) illustrated mild reductions of modeled PD symptoms like turning behavior, footslips and motor asymmetry 10 to 20 minutes after treatment administration, which could be sustained for approximately 60 minutes (Chao et al., 2012).

4.2 Nanoparticles for improved nose-to-brain delivery

While the direct intranasal administration of anti-parkinsonian drugs like L-DOPA have been demonstrated to result in mild symptom relief, these benefits are minor and short-lived due to several problems encountered with this delivery method. First of all, particles and molecules deposited on the olfactory epithelium which are smaller than 10 µm are trapped in the nasal mucosa and are cleared within minutes, giving therapeutics limited time to be absorbed (Djupesland et al., 2014). This rapid mucociliary clearance in combination with the relatively small surface area of the olfactory epithelium only allow small volumes of drug administration (25-200 µL in humans). Moreover, the active enzymatic degradation of deposited compounds by peptidases and proteases

results in limited drug absorption into the CNS, and therefore limited and short-lived therapeutic benefits (Erdő et al., 2018; Lochhead & Thorne, 2012).

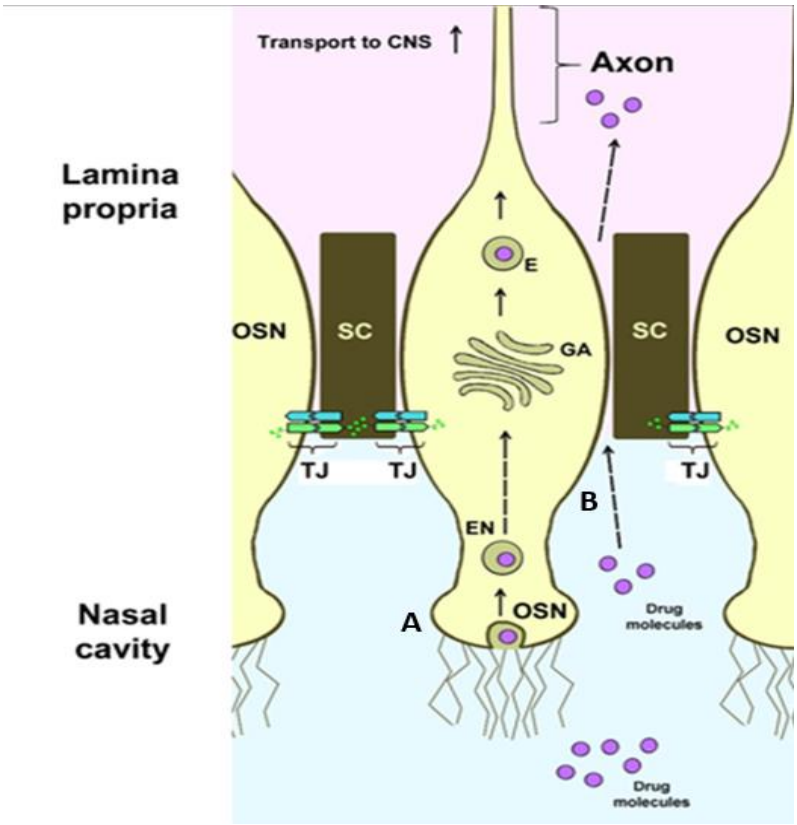

Figure 3. Mechanisms of intracellular and extracellular nose-to-brain transportation. *A) During the intracellular pathway, therapeutics enter the olfactory sensory neurons (OSN) through endocytosis, after which they travel within the endosome, through the Golgi Apparatus (GA), towards the neuronal axon where they are released in the olfactory bulb through exocytosis. B) During extracellular transport, drugs translocate through TJs connecting the OSNs, after which they move through the paracellular space along the OSN axon, through the subarachnoid space before reaching the CNS. Adapted from (Erdő, Bors, Farkas, Bajza, & Gizurarson, 2018).*

Therefore, current research studying intranasal delivery of anti-parkinsonian drugs focusses on the encapsulation of these drugs using NPs, protecting them from enzymatic degradation, increase retention time on the olfactory epithelium, facilitating sustained release, stimulating transport across the olfactory and trigeminal nerves and protecting drug from P-gp efflux proteins after CNS penetration (Erdő et al., 2018; Liu, Z. et al., 2013). To achieve the abovementioned benefits, NPs are used as vehicles to encapsulate drugs and increase brain uptake. As described in section 3, there are many different NP formulations currently under investigation, including the polymeric NPs, lipid nanocarriers and mucoadhesive agents (Erdő et al., 2018). While the pros and cons of the different NP formulations have been described in detail in section 3, there are some additional considerations when using NPs for intranasal delivery. Firstly, an important characteristic for intranasal delivery is NP size, where a smaller size (< 90 nm) is associated with higher brain uptake through the rapid extracellular olfactory and trigeminal nerve pathways (Liu, Z. et al., 2013). pH is an additional important characteristic for intranasal drug absorption and safety, with the human nasal mucosal pH ranging between 5.5 and 6.5, NP pH should be comparable to facilitate improved transportation through nasal mucosa and drug absorption into olfactory and trigeminal nerves, while avoiding cellular damage induced by pH values below 3 and above 10, particularly when designed for repeated administration (Alam et al., 2014).

4.2.1 Polymeric NPs for intranasal delivery

One of the most studied formulations for intranasal delivery are polymeric NPs, who's main function is drug protection from degradation, facilitation of mucoadhesion, increasing drug stability, and enhancing drug absorption (Erdő et al., 2018). The main advantage, however, is the wide range of available polymers to control NP properties, as well as the possibility of polymeric NP modification, which is thoroughly exploited in recent research into intranasal polymeric NP delivery (Boyuklieva & Pilicheva, 2022). Examples of these polymeric NP modifications include: modification of PEG-PCL copolymer micelles with CPP Tat, aiming to increase NP transport through the extracellular and intracellular olfactory and trigeminal neural pathways (Kanazawa, Akiyama, Kakizaki, Takashima, & Seta, 2013)**.** The authors demonstrated a 5-fold increase in drug uptake after intranasal NP delivery compared to IV NP delivery. This significantly increased after Tat modification, showing a peak increase in NP concentration in the olfactory bulb 15 minutes after intranasal administration, and in the entire brain after 1 hour (Kanazawa et al., 2013). Other researchers utilized a similar approach, modifying L-DOPA-loaded PEG-PLGA NPs with the lectin wheat germ agglutinin (WGA), to increase direct NP transport across the neural pathways (Arisoy et al., 2020). The authors demonstrated therapeutic concentrations of DA within the brain with little to no free systemic DA, while showing NP toleration in the mice brain tissue with low short-term toxicity (Arisoy et al., 2020). However, previous research has drawn contradictory conclusions after polymeric NP modification with lectins (like WGA) and CPPs, proving the induction of minor toxicity and oxidative stress after short term treatment, with little to no data on safety of longer term treatment (Liu, Z. et al., 2013; Reynoso-Camacho, de Mejía, & Loarca-Piña, 2003). Therefore, Liu and coworkers. modified PEG-PCL NPs with lactoferrin, which is an iron binding protein mainly expressed in the respiratory epithelial cells and neurons, to enhance endocytosis-mediated brain uptake and direct translocation to the CNS, as well as targeting to all brain regions (Liu, Z. et al., 2013). The authors discovered that lactoferrin modified NPs resulted in increased coumarin-6 delivery in healthy rat: cerebrum (with hippocampus removed) (1.36x), cerebellum (1.53x), olfactory tract (1.70x), olfactory bulb (1.57x) and hippocampus (1.23x), compared to unmodified "naked" NPs, which could be distinguished for up to 8 hours, while showing reduced drug levels in the blood (*Fig. 4A-F*) (Liu, Z. et al., 2013). Tracking of the labeled lactoferrin-modified NPs demonstrated that the entire NP was translocated to the CNS, rather than just the coumarin-6, suggesting protected drug delivery along the entire delivery route (Liu, Z. et al., 2013).

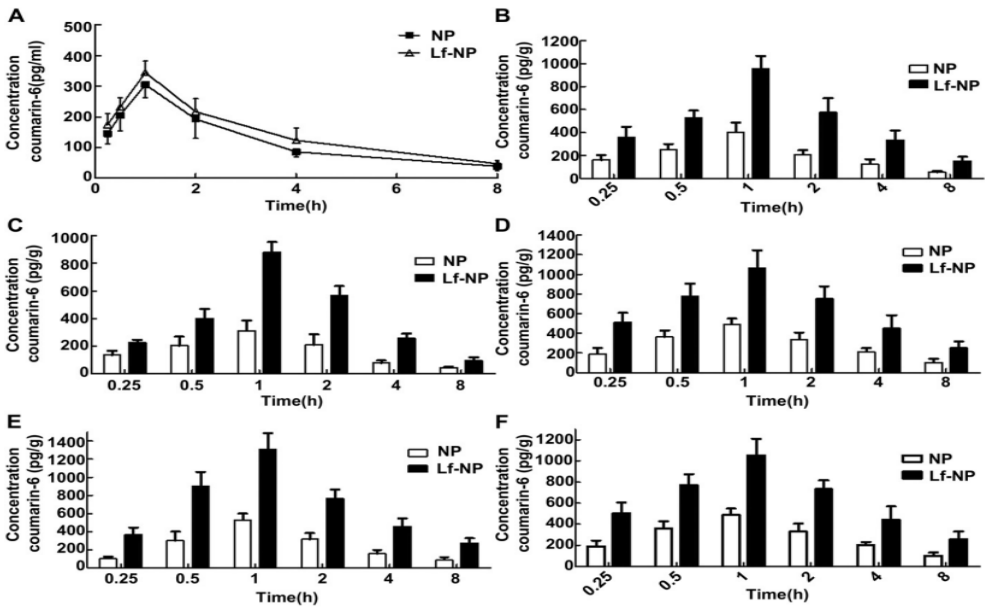

Figure 4. Lactoferrin modified PEG-PCL NPs are targeted to all brain regions which is sustained for up to 8 hours after intranasal delivery. *Concentration of coumarin-6 in pg/ml after intranasal administration of naked NPs or lactoferrin-modified NPs in A) blood, B) olfactory bulb, C) olfactory tract, D) cerebrum w/o hippocampus, E) cerebellum and F) hippocampus. Adapted from (Liu, Z. et al., 2013).*

Most research groups do not specifically focus on increasing NP penetration into the CNS, but rather attempt to increase NP retention and residence time on the olfactory epithelium through NP modification with chitosan, which has been illustrated to display mucoadhesive properties as well as a positive effect on epithelial membrane permeability (Ahmad et al., 2022; Erdő et al., 2018). Dimiou and coworkers designed a self-assembling N-palmitoyl-N-monomethyl-N,N-dimethyl-N,N,N-trimethyl-6-O-glycolchitosan polymeric NP (GCPQ) loaded with L-DOPA, which displayed a significant increase in brain DA levels compared to unmodified L-DOPA after intranasal administration in rats (Dimiou et al., 2022). Remarkably, they discovered that the brain DA concentrations kept increasing 2 hours after intranasal administration, highlighting the benefits of the mucoadhesive properties in combination with sustained drug release from polymeric NPs (Dimiou et al., 2022). Similarly, Ahmad et al. designed chitosan modified PLGA NPs which demonstrated a 2-fold increase in brain L-DOPA concentrations compared to non-modified L-DOPA (Ahmad et al., 2022)

4.2.2 Lipid-based NPs for intranasal delivery

Alternatively, lipid-based NPs are under investigation as vehicles for intranasal drug delivery. Advantages of lipid-based NPs include cost, easiness to scale up, ability to penetrate epithelial cells more easily due to lipophobic properties and their safety, causing no cellular damage and necrosis in the nasal mucosa or the CNS (Battaglia et al., 2018; Cunha, Amaral, Lobo, & Silva, 2017). However, they struggle with efficient drug loading, are harder to modify, and have been illustrated to display major systemic bioavailability after intranasal delivery, most likely due to their lipophilic properties, which is unwanted when treating PD patients with L-DOPA (Battaglia et al., 2018; Cunha et al., 2017). To partially overcome the problems with drug loading and sustained release, SLNs are currently the most popular lipid-based NP for intranasal delivery (Alam et al., 2014; Battaglia et al., 2018). Paradeshi and coworkers generated ropinirole hydrochloride loaded SLNs with surface-modified stearylamine-induced cationic charge for improved SLN loading and stability (Pardeshi, Belgamwar, Tekade, & Surana, 2013). Intranasal delivery in a PD mouse model demonstrated significant reduction in PD symptoms like tremors and immobility compared to oral formulations, even at lower dosages (Pardeshi et al., 2013). Similarly, Prakash Chandra Bhatt et al. described a stearic acid and lecithin (ratio 1:3 and 1:6) coated SLN for the intranasal delivery of astaxanthin (Bhatt, Srivastava, Pandey, Khan, & Panda, 2016). Biodistribution studies in healthy rats elucidated a 2-fold increase in brain astaxanthin levels compared to IV administration, which could be maintained for approximately 4 hours. It is important to note however, that a significant percentage of astaxanthin could be found in the blood, lungs, kidneys, liver and particularly the intestine, highlighting the high systemic bioavailability and excretion (Bhatt et al., 2016). In an attempt to overcome this high systemic bioavailability, Oihane Gartziandia et. al. generated lipid-based NPs consisting of Precirol ATO5, Dynasan 114 and Miglyol lipids, which were coated with chitosan after SLN formation (Gartziandia et al., 2015). Biodistribution studies after intranasal delivery of the chitosan-coated SLNs in mice illustrated the presence of labeled SLNs in the olfactory bulb and the rest of the brain 1 hour after administration, which increased in time and was maintained for 24 hours (Gartziandia et al., 2015)**.** However, a significantly higher percentage of labeled SLNs were distributed to the lungs and thus the circulation when compared to the olfactory tract and olfactory bulb, highlighting the current problems with lipid-based NP targeting to the brain, as well as the need for novel modifications for lipid NPs to improve brain targeting (Gartziandia et al., 2015). In an attempt to improve brain targeting of lipid NPs by improving BBB penetration, Zhen-Zhen Yang and coworkers generated rivastigmine-loaded EPC, Cholesterol (1:1 mol ratio) liposomes, which were surface-modified with PEG-coupled CPPs for intranasal delivery (Yang, Z. et al., 2013). The authors demonstrated increased CPP-liposome penetration in an *in vitro* BBB cell model (Yang, Z. et al., 2013). Additionally, the authors highlighted the significantly increased rivastigmine concentration in the plasma,

hippocampus, cortex and olfactory region of a healthy *in vivo* rat model using CPP-liposomes compared to unmodified liposomes, which peaked 15 min and 60 min after intranasal administration, concluding that both the olfactory pathway as well as systemic the systemic pathway where CPP-liposomes penetrated the BBB contributed to CNS rivastigmine concentration (Yang, Z. et al., 2013). Additionally, increased rivastigmine concentrations could be distinguished in all brain regions as well as the plasma when comparing intranasally administered and IV injected CPP-liposomes with IV injected rivastigmine, demonstrating its superiority over current treatment (Yang, Z. et al., 2013).

Discussion:

PD is a prevalent neurodegenerative disorder defined by progressive cell death of dopaminergic neurons causing DA deficiency, leading to tremors and bradykinesia (Armstrong & Okun, 2020). The current most prescribed PD treatment is comprised of a combination of oral L-DOPA and carbidopa, which significantly decreases PD symptoms, particularly in the earlier stages of PD (Ahmad et al., 2022). Nevertheless, this treatment struggles with low oral bioavailability, extensive L-DOPA degradation in the GI tract, hepatic first pass metabolism, systemic decarboxylation into free DA and low L-DOPA accumulation in the CNS due to the BBB (Ahmad et al., 2022; Palmer, 2011). The progressive dopaminergic degeneration requires increasing L-DOPA dosages as PD progresses, resulting in elevated systemic DA concentrations causing major side effects like dyskinesias after long term treatment (Tambasco et al., 2018). To increase L-DOPA bioavailability and protect L-DOPA from systemic degradation, current research increasingly focusses on the encapsulation of L-DOPA in NPs, the most popular of which are the polymer-based and lipid-based NPs (Jagaran & Singh, 2022; Saraiva et al., 2016). These NPs can be modified with proteins/antibodies to increase targeting to- and penetration of the BBB, thereby increasing drug delivery to the CNS (Arora et al., 2021; Liu, L. et al., 2008). However, orally delivered NPs are still subject to extensive degradation in the GI tract and the liver, and struggle with absorption in the intestines (Ensign et al., 2012), while IV injected NPs reduce patient compliance due to (bi-)daily hospital visitations (Wang et al., 2022). Interestingly, intranasal delivery of NP-encapsulated L-DOPA offers an alternative as a non-invasive delivery strategy, providing a direct pathway between nose and brain via the olfactory and trigeminal nerves, reducing exposure to the systemic circulation and the BBB (Boyuklieva & Pilicheva, 2022; Erdő et al., 2018). An overview of the research into polymeric- and lipid-based NPs to encapsulate L-DOPA and improve brain delivery from the last 10 years is displayed in Table 2.

Intranasal delivery of NP encapsulated L-DOPA holds great potential as an alternative to the currently prescribed oral L-DOPA/carbidopa tablets, as it provides a non-invasive, easily self-administered treatment option with the potential to reduce symptoms within minutes which can be sustained for several hours through direct nose-to-brain delivery, while reducing free systemic DA and the associated side effects (Djupesland et al., 2014). The NP formulation should be carefully considered however, as both polymeric and lipid-based NPs have their strengths and weaknesses. While lipid-based NPs are relatively cheap, easier to scale up and non-toxic, they struggle with efficient L-DOPA entrapment and have so far been illustrated to mainly distribute drugs to the lungs and systemic circulation rather than the brain after intranasal delivery in rodents, which increases systemic drug concentrations compared to for instance polymeric NPs, warranting further research (Battaglia et al., 2018; Cunha et al., 2017). Functional surface modifications including lactoferrins and CPPs, targeting the lipid-based NPs to the BBB and stimulating its penetration can limit some of these flaws and has a definitive edge over oral or IV delivery (Yang, Z. et al., 2013). Polymeric NPs are easier to modify and show improved control over NP characteristics like size, zeta-potential and hydrophobicity, and have been illustrated to efficiently localize to the CNS after intranasal delivery with low systemic exposure

(Erdő et al., 2018). The main problem with polymeric NPs is safety and nasal mucosal/brain toxicity of the co-polymers and their frequently used surface modifications (e.g. lectins) upon frequent administration, which have been illustrated to induce minor toxicity and oxidative stress (Liu, Z. et al., 2013; Reynoso-Camacho et al., 2003). It is important to consider that the parkinsonian brain requires a constant supply of DA and thus L-DOPA to attenuate PD symptoms. While NP encapsulation can protect L-DOPA from degeneration, and sustained release mechanisms are able to increase the time between 2 treatment to roughly 24-48 hours, (bi-)daily intranasally delivered NP-encapsulated L-DOPA will still be required (Wang et al., 2022). Because of the required long-term frequent dosages, research not only into brain toxicity, but toxicity and irritation/inflammation at the nasal level are essential to push NP formulations from animal models to the clinic, which is currently one of the main bottlenecks of intranasal delivery formulations (Djupesland et al., 2014; Illum, 2012). Another aspect which is often ignored during research into intranasal NP delivery is the effect of the immune system in both the nasal cavity and the brain. It is known that later stage PD patients display activated innate and adaptive immune responses, especially at the damaged brain sites like the SN (Tansey & Romero-Ramos, 2019). The (bi-)daily L-DOPA loaded NP treatments should not interfere with/avoid the already active immune system, which could trigger undesirable immunological reactions (Djupesland et al., 2014). For example, polymeric PEGylated NPs have been demonstrated to activate the immune system and trigger PEG antibody formation, resulting in faster degeneration and clearance of Pegylated NPs after repeated administration, reducing treatment efficacy (Eshete, Bailey, Nguyen, Aryal, & Choi, 2017; Estapé Senti et al., 2022; Sroda et al., 2005). Therefore, future research into intranasal delivery of NPs should focus on the establishment of long term toxicity and safety profiles as well as NP interactions with the immune system on the nasal and brain level. Additionally, while several publications discriminated between drug delivery to different brain regions within their biodistribution studies (Kanazawa et al., 2013; Liu, Z. et al., 2013), there are currently no publications discussing L-DOPA loaded NP targeting to its desired destination, the SN. Improved local sustained L-DOPA delivery to specifically the SN could not only improve treatment efficacy, but could also drastically impact treatment toxicity and safety profiles, warranting further research.

While both polymeric – and lipid-based NPs have great potential and could become an improvement on oral L-DOPA/carbidopa treatment, the vast majority of the current NP formulations under investigation only focus on PD symptom reduction by compensating decreased DA production in the SN, ignoring the neurodegeneration and PD disease progression (Tambasco et al., 2018). While this effectively increases quality of life, this treatment strategy will not lead to a definitive PD cure (LeWitt, 2015). Wang and coworkers described the therapeutic benefits of IV injected polymeric NPs containing the neuroprotective drug ginkgolide B (GB) on PD symptom progression in mice, and illustrated the neuroprotective properties against MPTP-induced PD (Wang et al., 2022). Therefore, it would greatly benefit the PD patients if intranasally delivered NPs would contain L-DOPA and DA degradation inhibitors like MOAB and COMT inhibitors to facilitate symptom relief aiming to increase quality of life, in combination with a neuroprotective drug like GB aiming to delay/impair disease progression, which will counteract the need to increase drug dose after long term treatment and might result in increased life expectancy.

Although the intranasal delivery of polymeric- and lipid-based NPs to treat neurodegenerative diseases has been under investigation for over 20 years now, the research field is stagnating and struggles to pass the pre-clinical testing phase (Erdő et al., 2018). There are multiple explanations for this halt in progression. While the vast majority of *in vivo* studies are performed in either mice or rats, research has illustrated significant differences in both brain- and nasal cavity anatomy between humans and rodents (Djupesland et al., 2014). For example, the olfactory epithelium/body mass ratio

is roughly 200x smaller in humans compared to rats, and mucosal clearance takes place at different rates (Djupesland et al., 2014). Additionally, the anatomy and the structural arrangement of the SN also show vast differences when comparing humans to rodents, which results in discrepancies in DA release and distribution within the SN, significantly altering PD modulation in rodents, which might explain the difficulty in the translation of *in vivo* results to the clinic (Eslamboli, 2005). Therefore, the use of animal models which more closely resemble the human anatomy, like marmoset monkey models, could be a valuable intermediate between rodent studies and human clinical trials (Eslamboli, 2005). Currently available marmoset monkey models include MPTP-induced PD monkeys, which model disease characteristics with biochemical, anatomical and behavioral resemblance to the human situation (Eslamboli, 2005). These MPTP-lesioned PD monkey models have already been employed for research into currently approved and available D-1 and D-2 DA agonists like apomorphine as well as L-DOPA treatments, and could be beneficial for the progression of intranasally delivered NPs to the clinic (Maratos, Jackson, Pearce, Cannizzaro, & Jenner, 2003).

Besides the usage of mismatched animal models, lack of consideration to the methods applied for intranasal delivery also hampers the progression of intranasally delivered NP formulations to the clinic (Djupesland et al., 2014). Charlton et al. demonstrated the influence of delivery to specific nasal regions on observed drug biodistribution, where targeted delivery specifically to the olfactory epithelium resulted in significantly increased direct nose-to-brain transport with reduced systemic absorption, compared to conventionally used uncontrolled intranasal delivery, which resulted in significantly increased systemic exposure (Charlton, Davis, & Illum, 2007; Djupesland et al., 2014). Despite the direct link between olfactory epithelium targeting and brain uptake, the majority of *in vivo* studies ignore factors such as olfactory targeting, nasal airflow and lack of sensory reflexes during animal sedation after intranasal administration, limiting clinical relevance of their findings while decreasing reproducibility (Djupesland et al., 2014). Additionally, lacking experimental procedure methodology of intranasal delivery results in inconsistency of experimental data (e.g. biodistribution) when comparing data published by different research groups, which further complicates the understanding of therapeutic benefits of intranasally administered NPs. Therefore, a more systematic, clear and well documented approach to intranasal treatment methodology is required to enable the normalization and comparison of data.

Hence, methods for intranasal delivery in humans are equally important when attempting to move treatments to the clinic. While the olfactory and trigeminal nerves are mainly located in the upper and posterior regions of the nasal cavity, beyond the nasal valve, currently used nasal delivery devices like spray pumps and pressurized metered dose inhalers fail to successfully target this area due to a mismatch in delivery device and nasal anatomy (Djupesland et al., 2014). Instead, the majority of the drug is delivered either to the anterior of the nasal valve or to the lower parts of the nasal canal, which results in drug clearance through the GI tract (Djupesland et al., 2014). To improve intranasal delivery efficiency, different devices have been developed, including the breath powered bi-directional nasal delivery devices (Obaidi et al., 2013), pressurized gas powered bi-directional delivery devices (Warnken et al., 2016) and vortex based nebulizers (Giroux, 2007), which have been demonstrated to significantly increase targeting to the olfactory region in humans, increasing therapeutic benefit of the intranasally delivered drug (Djupesland et al., 2014; Obaidi et al., 2013). Animal studies regarding the optimal delivery of NP encapsulated L-DOPA using the abovementioned delivery devices are required before these can be pushed towards clinical trials. However, due to the vast differences in intranasal drug delivery between humans and rodents, the previously mentioned PD marmoset monkey models could be employed as an appropriate intermediate.

In conclusion, the NP encapsulation of L-DOPA is a promising new treatment strategy for PD which has been demonstrated to improve drug delivery to the CNS while reducing dose, dosing frequency and systemic side effects. Moreover, due to problems with oral delivery and IV injection, intranasal NP delivery offers a non-invasive and easy to self-administer alternative which has the potential to increase therapeutic efficacy through direct brain delivery via the olfactory and trigeminal nerves, which could be of interest for various brain diseases. Nevertheless, before intranasally delivered NPs can be pushed to the clinic, further research into optimal NP composition and characteristics, systemic methodology for intranasal delivery through usage of delivery devices, more representative *in vivo* model systems, improved NP brain targeting and long-term NP safety are required.

Table 2. Research published in the past decade studying polymeric- and lipid-based NP encapsulation of L-DOPA.

Administration route	**NP Type**	**NP Composition**	**Size + Zeta potential**	**Reference**
Unspecified	Polymeric	Glutathione (GSH) coated NH2–Poly (ethylene oxide)(PEO)–PCL	99.5 ± 7.3 nm +25.6 ± 0.5 mV	(Mogharbel et al., 2022)
IV administration	Polymeric	PLGA + PVA	173.1 to 500.6 nm dependent on PLGA and PVA concentration. Zeta potential unspecified	(Zhou, Y. Z., Alany, Chuang, & Wen, 2013)
Subcutaneous administration	Polymeric	PLGA + PA	± 500 nm	(Yang, X. et al., 2012)
IV + subcutaneous administration	Polymeric	PLGA + PEG	43 nm -16 mV	(Nie et al., 2021)
Intranasal administration	Polymeric	PLGA	250 ± 50 nm Zeta potential unspecified	(Gambaryan, Kondrasheva, Severin, Guseva, & Kamensky, 2014)
Intranasal administration	Polymeric	WGA-conjugated PLGA	383.7 ± 66.94 nm -20.8 ± 3.63	(Arisoy et al., 2020)
Intranasal administration	Polymeric	GCPQ	72.0 ± 5.0 nm +40.5 ± 2.1 mV	(Dimiou et al., 2022)
Intranasal administration	Polymeric	PLGA + Chitosan	553 ± 52 nm +46.2 ± 2.3 mV	(Ahmad et al., 2022)
Intranasal administration	Polymeric	Chitosan NPs incorporated in CNLP gel	164.5 ± 3.4 nm Zeta potential unspecified	(Sharma, Lohan, & Murthy, 2014)
IV administration	Lipid-based nanocarriers	Tristearin + Lecithin	161.9 ± 0.8 nm Zeta potential unspecified	(Ravani et al., 2015)
IV administration	Lipid-based nanocarriers	Mesoporous silica NP core coated by a lipid bilayer modified with lactoferrin	± 90 nm + Zeta potential (exact number unspecified)	(Zhou, W. et al., 2021)

Unspecified	Lipid-based: liposomes	phosphatidylethanolamine, cardiolipin and phosphatidic acid/ cholesterol (2:1)	0.5-2 µm based on phospholipid composition Zeta potential unspecified	(Moholkar, Sadalage, Havaldar, & Pawar, 2021)
Unspecified	Lipid-based: liposomes	EPC, Ch, and SA (5:4:1 molar ratio) liposomes	Ranging from 302 nm to 2432 nm based on lipid composition Zeta potential ranging from -27.56 mV to 29.50 mV based on lipid composition	(García Esteban, Cózar-Bernal, Rabasco Álvarez, & González-Rodríguez, 2018)
Intraperitoneal injection	Lipid-based: liposomes	Chlorotoxin-modified HSPC/Chol/DSPE-PEG stealth liposomes	106.8 ± 3.01 nm 0.375 ± 0.09 mV	(Xiang et al., 2012)
Intragastric administration	Lipid-based: liposomes	Chitosan-coated liposomes	unspecified	(Cao et al., 2016)

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