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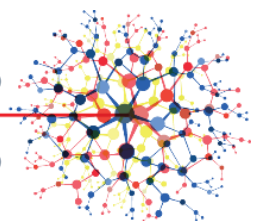
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**Development and evaluation of
cannabidiol formulations for delivery to
the central nervous system to treat
neuropathic pain**

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degree of Doctor of Philosophy*

**CDT in Advanced Therapeutics
and Nanomedicines**



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Abstract

Neuropathic pain is a great societal burden estimated to affect approx. 40% of the general population, being accompanied by a significant decline in normal functioning and quality of life. Moreover, it is a major economic burden, costing the NHS £5 billion annually. Pain has complex pathophysiology and is currently poorly managed due to the ineffectiveness of many therapies. The drawback associated with the development of novel analgesics is multifaceted. One of the limitations is linked to the lack of effective delivery of therapeutics to the site of action - the central nervous system (CNS). Effective drug delivery to the CNS is extremely challenging due to the presence of the blood-brain barrier (BBB) and the blood-cerebrospinal fluid-barrier (BCSFB), however a technique involving a direct intrathecal (IT) injection into the lumbar segment of the spinal cord by-passes these barriers. Additionally, numerous patients are also resistant to traditional opioids (i.e., morphine) used in the treatment of pain, leaving many with no alternative therapies. Nonetheless, cannabidiol (CBD) has been emerging as efficacious in treating pain, amongst other disease states such as depression, anxiety or inflammation. Therefore, in order to tackle neuropathic pain in patients resistant to traditional opioid therapies and by-pass the protective CNS barriers, the development of novel formulations coupled with effective interventional treatments is required.

CBD is non-psychoactive and has been shown to be efficacious in decreasing pain, however, it is limited in therapeutic action due to its poor pharmacokinetic profile observed in both humans and rodents after oral and intravenous administration. In order to overcome this drawback, I developed two different CBD formulations, a stable lipid based nanoemulsion (286 nm diameter and 0.135 PDI) and polymer-coated nanoparticles (PCNPs) (121 nm diameter and 0.079 PDI), with high CBD association and loading (1.28 mg/mL (65.1%) and 0.46 mg/mL (28.52%) respectively), for administration directly at the site of action via an IT injection. Furthermore, I also aimed to investigate how the biodistribution of CBD and its anti-nociceptive effects within the CNS were influenced by these two distinct formulations.

I found that both formulations exhibited anti-nociceptive effects within 10 mins of IT administration in rats, upon the stimulation with noxious von Frey hairs, in comparison to the blank vehicles. Both formulations were preferentially retained in the spinal cord with high

concentrations reaching the brain immediately after injection. The CBD nanoemulsion reached $C_{\max}$ in the brain at 210 ng/g within 120 mins ($T_{\max}$), whereas the CBD PCNPs had a $C_{\max}$ of 94 ng/g at 30 mins ($T_{\max}$), indicating that rapid delivery can be achieved through the use of PCNPs. Moreover, the $AUC_{0-4 \text{ hrs}}$ of CBD in the brain was increased 3.7-fold through the delivery of the nanoemulsion as opposed to the PCNPs, indicating higher retention of CBD at this site. These data demonstrate that the concentration of therapeutics delivered to the brain can be increased through the use of a nanoemulsion formulation.

No CBD was detected in peripheral tissues or in circulating blood, suggesting that the drug was localised to the CNS throughout the time-points tested. These findings pose critical considerations for the development of nanotechnology for small lipophilic molecules for IT delivery. They allow the optimisation of colloidal formulation properties to attain rapid, sustained and widespread distribution within the spinal cord, with successful delivery to the brain, whilst being localised to the CNS.

Keywords: cannabidiol, drug delivery, intrathecal, pain, central nervous system, nanoparticles, nanoemulsion

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Abbreviations

5HT _{1A} – serotonin 1A receptor	EMG – electromyography
ACN – acetonitrile	ESEM – environmental scanning microscopy
AUC – area under the curve	FDA – Food and drug administration
BBB – blood-brain barrier	GPC – gel permeation chromatography
BCNU - 1,3-bis(2-chloroethyl)-2-nitrosourea	H&E – haemotoxylin and eosin
BCSFB – blood cerebrospinal fluid barrier	HPLC – high performance liquid chromatography
BSU – bio-support unit	ICM – intracisternal magna
CB1 – cannabinoid receptor 1	ICV – intracerebroventricular
CB2 – cannabinoid receptor 2	IFN α – interferon α
CBD – cannabidiol	IFN γ – interferon γ
CED – convection enhanced delivery	IP – intraperitoneal
C _{max} – maximum concentration reached	IS – internal standard
CNS – central nervous system	ISF – interstitial fluid
CPZ – capsazepine	IT – intrathecal
CSF – cerebrospinal fluid	ITDD – intrathecal drug delivery
DDT – dichlorodiphenyltrichloroethane	IV – intravenous
DEPG – dielaidoyl phosphatidylglycerol	IVC – individually ventilated cages
DL – drug loading	KBr – potassium bromide
DL – dioleoyl lecithin	logP – partition coefficient
DLS – dynamic light scattering	MLV -multilamellar vesicles
DMPC: dimyristoylphosphatidylcholine	MNPs – magnetic nanoparticles
DOE – design of experiments	MRI – magnetic resonance imaging
DPPC – dipalmitoyl phosphatidylcholine	NaCl – sodium chloride
DPPG – dipalmitoyl phosphatidylglycerol	nanoemulsion – nanoemulsion
EDX – energy dispersive X-ray	NMR – nuclear magnetic resonance
EE – encapsulation efficiency	NPs- nanoparticles

NRW – nociceptive withdrawal reflex
 OCT – optimal cutting temperature
 PBS – phosphate buffered solution
 PC – phosphatidylcholine
 PDI – polydispersity index
 PE - phosphatidylethanolamine
 PEG – poly(ethylene glycol)
 PEI – polyethylenimine
 PFA – paraformaldehyde
 PG – phosphatidylglycerol
 PLA – poly(lactic acid)
 PLGA – poly(lactic-co-glycolic acid)
 PMAM – polyamidoamine
 PPAR γ - peroxisome proliferator-activated receptor γ
 RI – refractive index
 ROP – ring-opening polymerization
 SAS – subarachnoid space

SCI – spinal cord injury
 SLN – solid lipid particles
 SNR – signal-to-noise ratio
 SPIONs – superparamagnetic iron oxide nanoparticles
 SUV – unilamellar vesicles
 TBI – traumatic brain injury
 TEM – transmission electron microscopy
 THC – tetrahydrocannabinol
 $T_{\max}$ – time of maximum concentration
 TNF α – tumour necrosis factor α
 tPEG - 3star-PEG₁₀₁₄-PLA₁₀₀
 TRPA₁ – transient receptor potential ankyrin 1
 TRPV₁ – transient receptor potential cation channel subfamily V member 1
 vFh – von Frey hairs
 ZP – zeta-potential

1. Chapter 1 – Introduction

1.1. Neuropathic pain

Pain affects millions of people worldwide, is presented as a symptom of various common medical conditions and has been shown to be the main reason patients pursue medical assistance (McAteer *et al.*, 2011). It is a great societal and economic burden (costing the NHS approx. £5 billion annually), being accompanied by a decline in normal functioning and quality of life (britishpainsociety.org). The most widely occurring type of pain is tension-type headache, with musculoskeletal and neuropathic pain also very widespread (Rice *et al.*, 2016). Chronic pain is estimated to affect 11% to 40% of the general population, with as many as 10% of patients suffering from severe pain (Dahlhamer *et al.*, 2016). Neuropathic pain is a sub-type of chronic pain and is described by the International Association for the Study of Pain (IASP) as “Pain caused by a lesion or disease of the somatosensory nervous system” (IASP, iasp-pain.org; accessed on 15/10/21). It is associated with a significant decrease in quality of life and is frequently very poorly managed due to current ineffectiveness of drug therapies (Murnion, 2018). Approximately 25% of patients suffering from diabetes and 35% patients suffering from HIV exhibit neuropathic pain (Murnion, 2018), with an estimated 7%-8% of the total population affected (Mondello *et al.*, 2018). It is more widely reported in women (8% in comparison to 5.7% in men) and also more prevalent in patients exceeding 50 years of age (reported 8.9% in > 50-year-olds compared to 5.6% in patients below 49 years old) (Bouhassira *et al.*, 2005).

The pathophysiology of neuropathic pain is complex; however, the current understanding is that it is caused by a lesion or disease of the somatosensory system (Colloca *et al.*, 2017). Sensory information gathered from the periphery is integrated in the dorsal horn of the spinal cord, and passes through to supraspinal sites within the brain through ascending tracts (Finnerup *et al.*, 2016) (Figure 1.1). The majority of nociceptive neurons in the ascending systems are conveyed in three different pathways: the spinothalamic, spinoreticular and spinomesencephalic tracts (Schug *et al.*, 2011). The dorsal horn also receives input from the higher centres in the brain, which modulate the response to nociceptive input. This inhibitory

modulation, also known as the descending control pathway, synapses in the dorsal horn (Figure 1.2) and is controlled by key neurotransmitters such as noradrenaline and serotonin (Schug *et al.*, 2011).

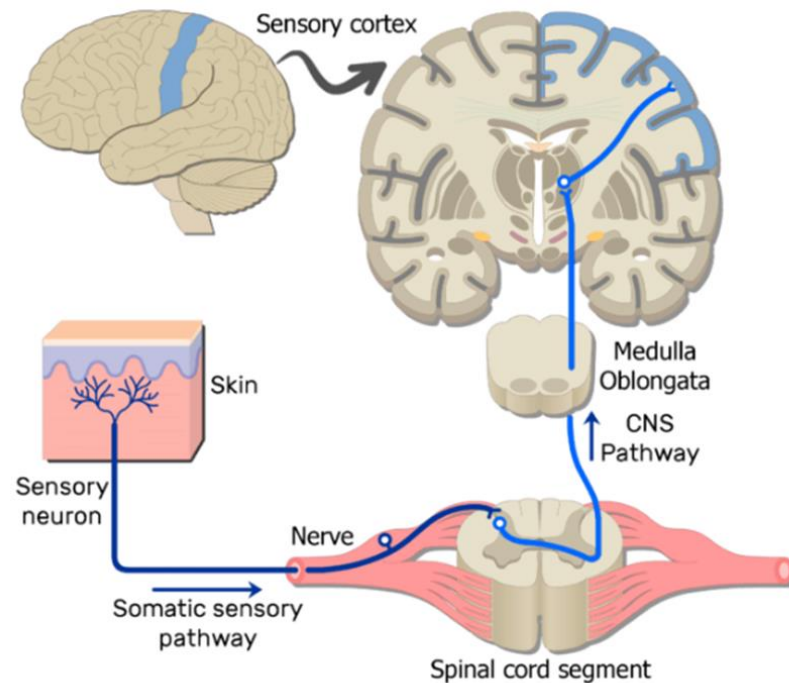


Figure 1.1. The nociception pathway (diagram reproduced from GBS.com, accessed on 23/09/ 2021); showing external stimuli passed through sensory neurons, integrated within the dorsal horn of the spinal cord and sent through the ascending pathways to higher pain centres in the brain.

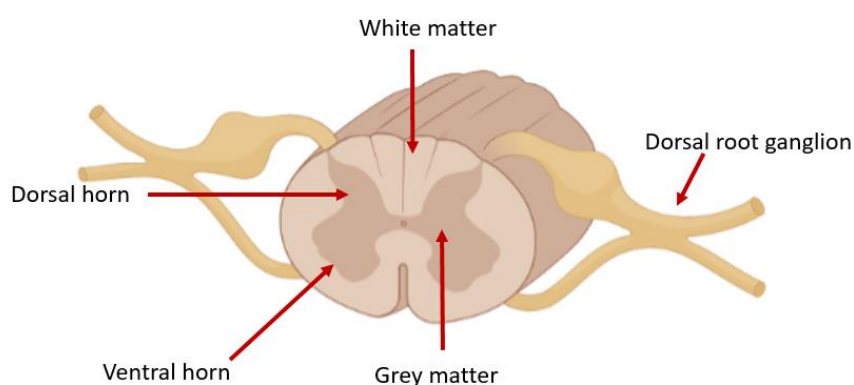


Figure 1.2. A cross-section of the spinal cord (constructed on Biorender.com) showing the location of the dorsal horn, the white matter and grey matter.

The dorsal horn is therefore the site of action of endogenous opioids (endorphins and enkephalins), where they are involved in the descending inhibition of pain (e.g., these neurotransmitters are released during stress and lead to increased analgesia). The central mechanisms involved in the occurrence of neuropathic pain are thought to involve neuroplastic changes in the CNS. In this process, neurons are sensitised and changed in the way they respond to inputs, and the pathways thought to be involved are primarily in the dorsal horn. Most often this dysregulation leads to spontaneous and continuous pain (Schug *et al.*, 2011).

Symptoms of neuropathic pain include allodynia, which is pain resulting from non-noxious stimuli (such as touch) and hyperalgesia (heightened response to painful stimuli) alongside feeling burning and electrical – like sensations (Colloca *et al.*, 2017). Typically, these types of symptoms are not responsive to pain medication leading to increased burden on health care providers and increased prescriptions of ineffective therapeutics (Attal *et al.*, 2011).

Research in this field has been carried out to identify the structural changes and anomalies in the nervous system in order to determine new potential therapeutic targets and facilitate translational research from *in vivo* models to clinical trials. Central neuropathic pain is caused by either a lesion or disease of the CNS such as spinal cord injury, multiple sclerosis or demyelinating disease (Watson *et al.*, 2016). In contrast, the pathology of peripheral neuropathies involves C and A sensory fibres (Finnerup *et al.*, 2016). This sub-type of neuropathic pain is likely to increase in the future due to the rise of the ageing population, greater rates of diabetes mellitus and higher incidence of cancer (as a result of chemotherapy which impacts sensory fibres) (Colloca *et al.*, 2017). It is therefore imperative to develop novel ways of treating neuropathic pain, as current therapeutics are sub-standard and many patients are unresponsive to their effects.

1.1.1. Clinical treatment

The first line of treatment for neuropathic pain are generally antidepressants and antiepileptics; however numerous negative trials have emerged in the last few years (Luo *et al.*, 2001; Yalcin *et al.*, 2009). Results have been shown to be inconsistent and weak or have many adverse side-effects (Moore *et al.*, 2015). The second line of treatment includes lidocaine, a local anaesthetic with sodium-channel blocking effects (Binder *et al.*, 2009) and

capsaicin, which acts on transient receptor potential cation channel subfamily V member 1 (TRPV₁). These are ligand-gated channels localised on nociceptive fibres and their activation demonstrated efficacy following single applications of capsaicin patches in cases of neuralgia and diabetic / non-diabetic neuropathies (Burness *et al.*, 2006). Tramadol, an opioid, is also widely used and has displayed efficacy mostly in peripheral neuropathies (Finnerup *et al.*, 2015). Other opioids such as morphine and oxycodone are also efficacious, however there are obvious limitations in their long-term use in regards to adverse-effects, misuse, addiction and opioid-associated overdoses (Ray *et al.*, 2016). Moreover, numerous patients are also unresponsive to opioids, and this most commonly occurs in conditions such as cancer, AIDS, pancreatitis or post herpetic neuralgia (Portenoy *et al.*, 1990). It was estimated that as many as 20% of all cancer patients are resistant to opioids administered for pain management (Patel *et al.*, 2017). This therefore leaves a major gap in the effective treatment of this patient sub-group, considering that the use of opioids is the final line of defence against pain, and the limited efficacy of other widely available therapies.

1.1.2. Patient population resistant to opioids

Patients are regarded as resistant to opioid treatment when they are completely unresponsive to the administration of very large doses of opiates. Moreover, this is also exacerbated by the inherent pre-existing variation in the response to morphine, typically as a result of type of pain, genetic predisposition and gender (McCormick & Schreiner, 2001). Furthermore, changing the type of opiate administered does not have an effect on analgesia in this patient sub-group. When this occurs in the clinic, the patients are subsequently put on a therapy of psychotropic medications such as barbiturate and / or neuroleptic agents, in a last attempt to offer them some relief from the pain they are experiencing (Bell, 2010; Breitbart, 1992)

Overall, most clinically available drugs exhibit weak and inconclusive analgesia for neuropathic pain and their effects are rather inconsistent. Moreover, opioid therapy for the terminally ill can also sometimes fail to reduce pain, leaving patients with no other alternative therapy available. This highlights the need for the development of novel therapeutics and formulations, coupled with effective interventional treatments capable of delivering drugs

directly to the site of action, which in the case of neuropathic pain is the spinal cord (Cruccu *et al.*, 2007).

1.2. Cannabidiol

Numerous preclinical studies have suggested that cannabinoids, which exhibit complex pharmacological activity, act as analgesics and thus play a potential role in pain management (Rice *et al.*, 2002). The cannabis plant consists of over 450 compounds, with approximately 100 categorised as phytocannabinoids. The most widely studied compounds have been Δ^9 -tetrahydrocannabinol (THC) which is the main psychoactive constituent of cannabis, and cannabidiol (CBD), the non-psychoactive component. Various studies have shown that cannabinoids have therapeutic effects in several disease states including anxiety, depression, nausea, inflammation and pain (McPartland *et al.*, 2014).

1.2.1. Pharmacological targets

CBD is capable of acting on a variety of molecular targets that mediate the feeling of pain. Some of the receptors which CBD interacts with include the serotonin 1A receptor (5-HT_{1A}), TRPV₁, transient receptor potential ankyrin 1 (TRPA₁) and peroxisome proliferator-activated receptor γ (PPAR γ) (Fisher *et al.*, 2019). The activity of CBD on TRPV₁ and TRPA₁ leads to receptor desensitisation which causes analgesia (Maione *et al.*, 2011). The TRPV₁ receptor is a non-selective cation permeable channel which mediates hyperalgesia, allodynia, inflammation and pain (Jara-Oseguera *et al.*, 2006). This receptor can be activated by chemical ligands (such as CBD) alongside heat and an acidic pH. TRPV₁, TRPA₁ and 5-HT_{1A} are expressed in the CNS and on the dorsal root ganglia, but also in the skin and somatic organs (Zádor & Wollemann, 2015a). Nonetheless, the full pharmacodynamics profile of CBD has been difficult to elucidate due its complex activity on multiple receptors, far beyond its activity on analgesia.

Initial reports have suggested that CBD is a poor ligand at the orthosteric site of cannabinoid receptors (CB₁ and CB₂), suggesting that its therapeutic action is independent of the direct activity on cannabinoid receptors (Thomas *et al.*, 1998). Further studies have later demonstrated that this conclusion was only partially accurate, and that CBD directly and indirectly interacted with the endocannabinoid system, alongside its complex activity on the

various receptors and ion channels mentioned above (Tham *et al.*, 2019). Studies have also shown that CB₁ and CB₂ are co-localised with TRPV₁ in the CNS, and their simultaneous activation leads to intracellular crosstalk (Zádor & Wollemann, 2015b). All of these interactions between receptors in pain modulation suggest that CBD has multiple targets, leading to improved efficacy (Roche & Finn, 2010).

1.2.2. Synthetic cannabinoids

There are currently two synthetic cannabinoids on the market: dronabinol (Marinol®) and nabilone (Cesamet®). They are oral formulations of synthetic THC (nabilone mimics THC action but does not have the same structure) and have been approved for the treatment of chemotherapy-associated nausea and vomiting. However, off-label, they are also being used for the treatment of pain (Abrams & Guzman, 2015). Nonetheless, regardless of their effectiveness, the psychoactive side effects of THC are limiting its medical use in the treatment of chemotherapy induced nausea and for off-label pain treatment. However, CBD exhibits none of the psychoactive side effects of THC, but retains therapeutic efficacy as outlined in Section 1.3.1. Nevertheless, the pharmacokinetic profile of CBD (Figure 1.3) makes oral and intravenous (IV) administration problematic (Huestis, 2007) which is a major limiting factor in its effective administration.

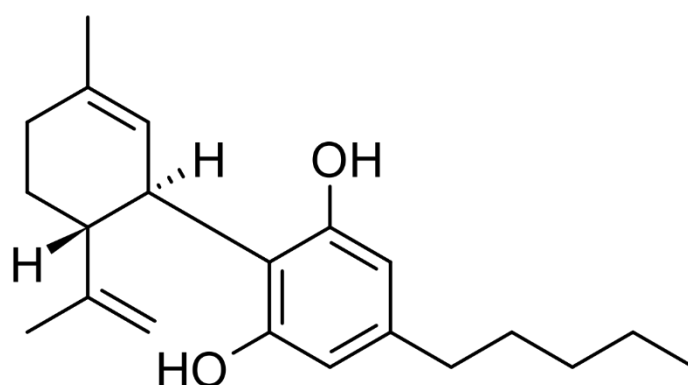


Figure 1.3. Cannabidiol chemical structure (Mw 314.469 g/mol, PubChem Chemistry Database).

1.2.3. Pharmacokinetics

The main drawback associated with the use of cannabinoids in analgesia are the side-effects observed in most trials and extensive first-pass metabolism (Burns *et al.*, 2006). Following the oral administration of CBD, its absorption is slow and variable, with plasma concentrations

usually achieved within 60-120 minutes. Moreover, the extensive-first pass metabolism (primary and side-chain oxidation) taking place in the liver, significantly reduces the bioavailability of CBD after oral administration (Bruni *et al.*, 2018).

A study by Nadulski *et al.*, 2005 reported that an oral intake of 5.4 mg CBD leads to an average plasma concentration of 0.95 ng/mL 1 hr after administration. Moreover, the drug remained within the detection capabilities of mass spectrometry for 3-4 hrs after dosing (Vázquez *et al.*, 2021). In another study involving the oral administration of 800 mg CBD, the reported C_{max} (maximum concentration reached) was 77.9 ng/mL and T_{max} (time of maximum concentration) was 3 hrs. It was also demonstrated that C_{max} was dose dependant up until 400 mg, where a saturation effect was observed (Millar *et al.*, 2018)

Intravenously, an injection of 20 mg deuterated CBD, led to an average of 48.4 ng/mL CBD detected 1 hr after administration (C_{max} of 686 ng/mL was observed 3 mins post-administration). In contrast, smoking a cigarette containing 19.2 mg deuterated CBD led to an average of 10.2 ng/mL detected in the blood of the participants, with a C_{max} of 110 ng/mL within 3 mins of smoking (Ohlsson *et al.*, 1986). The bioavailability of CBD exhibited also varied, with up to 4-fold differences in concentrations detected between participants, suggesting that there are other individual, patient specific characteristics leading to such variability.

Overall, in human studies it was reported that T_{max} was generally reached between 1 and 4 hrs, and that C_{max} was dose-dependent, up to a point of saturation. There is limited data in the literature describing the elimination rate and clearance or the biodistribution of CBD in humans (Millar *et al.*, 2018), warranting further studies in this area. No accumulation of CBD in plasma was observed after repeated dosing, however there is a possibility that tissue accumulation was occurring, as a result of the lipophilicity. The accumulation of CBD in fat would therefore act as a depot, slowly releasing the drug, causing differences in bioavailability between participants (Gunasekaran *et al.*, 2009). A summary of pharmacokinetic parameters observed in human participants after CBD administration alongside references can be referred to in Table 1.1. These examples have been selected to highlight the variability of the CBD PK profile in humans, and more specifically the low C_{max} achieved following administration of high CBD doses through oral, inhalation and intravenous delivery.

There is limited data on CBD delivery to the brain, with even less data on delivery to the spinal cord, where CBD is predicted to have some of its analgesic activity. Orally administered CBD reached T_{max} in the brain at 6 hrs (Deiana *et al.*, 2012). When inhaled, CBD enters the brain via the nose-to-brain route resulting in T_{max} of <10 min (Huestis *et al.*, 2005). However, this type of administration lacks efficiency and does not allow for CBD to reach its therapeutic dose within the entirety of the CNS. These data suggest that conventional administration techniques (oral and IV) do not allow for CBD to have a long-acting and sustained effect, as a result of the extensive and rapid first-pass metabolism, and inability to penetrate the CNS at therapeutic concentrations because of its rapid elimination.

Table 1.1. Summary of CBD pharmacokinetic parameters after administration in humans through various delivery routes and via different vehicles. A few selected examples of CBD administered through different delivery routes to highlight the variability in detection times, T_{max} and C_{max} (i.e., the variability of the PK profile in humans).

Administration route	Dose given	Delivery vehicle	T_{max}	C_{max}	Detectable for	Reference
Oral	5.4 mg	Capsules	1 hr	0.93 ng/mL	3-4 hrs	Nadluski <i>et al.</i> , 2005
Oral	800 mg	Capsules	3 hrs	77.9 ng/mL	-	Hanley <i>et al.</i> , 2016
Inhalation	19.2 mg	Smoking	3 mins	119 ng/mL	10.2 ng/mL detected 1 hr later	Ohlsson <i>et al.</i> , 1986
Inhalation	20 mg	Nebuliser	36 min	9.49 ng/mL	-	Guy and Flint, 2004
Inhalation	20 mg	Aerosol	2.35 hr	2.61 ng/mL	-	Guy and Flint, 2004
Intravenous	20 mg	Ethanol solution	3 mins	686 ng/mL	48 ng/mL detected 1 hr later	Ohlsson <i>et al.</i> , 1986

1.2.4. Formulation challenges

CBD is a highly lipophilic molecule ($\log P$ 7.03 ± 0.37 ; ACD/Labs, Advanced Chemistry Development) with very low aqueous solubility (0.0126 mg/mL; ALOGPS; <http://www.vcclab.org/lab/alogps>, accessed on 11/09/2021), and therefore its formulation is critical in improving its solubility and bioavailability. It was reported that the terminal half-life

of CBD was 1.09 hrs after the oral administration of 10 mg CBD and 2.95 hrs after the oral administration of CBD lipid capsules (Guy & Flint, 2008). The oral co-administration of CBD with lipids also increased systemic bioavailability by 3-fold in rats (Zgair *et al.*, 2016). Furthermore, C_{max} was reached faster and was increased if CBD was administered as a nanoliposphere formulation (bioavailability was increased 6-fold), suggesting that the formulation of CBD has the potential to improve its pharmacokinetic profile (Cherniakov *et al.*, 2017). Lipid-based carrier systems are frequently used and have been vastly studied for the delivery of lipophilic compounds, whereby the solubility, absorption and bioavailability of therapeutics are increased.

CBD is soluble in the lipid content of foods due to its high lipophilicity, thus increasing its absorption after its oral administration with food. It has been shown that delivery of CBD with lipids increases bioavailability by 3-fold in rodents (Zgair *et al.*, 2016).

It is therefore evident that the main limitation of CBD use for the treatment of neuropathic pain in the clinic, is the poor pharmacokinetic profile, which can be improved by formulation strategies. Another aspect to be considered is a different route of administration, whereby first-pass metabolism and enzymatic degradation can be avoided, in order to ultimately enhance its analgesic effect and lead to sustained therapeutic concentrations at the site of action. These drawbacks make it necessary to utilise an administration strategy suitable for locally delivery of CBD to the CNS, the site where most CBD receptors involved in pain are located. One such administration technique is IT delivery, an injection directly in the lumbar area of the spinal cord, which is covered in detail in Section 1.4.2. Moreover, this technique can also be used in combination with different nanocarrier formulations to aid the drug solubilisation and enhance its retention, resulting in minimal loss of the initial dose, thus allowing the therapeutic to remain at the site of action for prolonged periods of time.

1.3. CNS targeting with systemic administration of therapeutics

Systemic drug administration via parenteral routes usually leads to very low concentrations of the active therapeutic agent within the CNS (Fowler *et al.*, 2020), as is the case with CBD. This is therefore a major disadvantage in the development of novel treatments for treating CNS diseases and disorders, including neuropathic pain. The confounding issue lies in the fact that the majority of the receptors which need to be targeted to lead to analgesia are found

within the CNS, and are therefore very well protected against compounds freely circulating in peripheral venous system. This is due to the various CNS barriers protecting the spinal cord and the entire brain parenchyma from the molecules within the circulation. These complex biological barriers are the blood-brain barrier (BBB) and the blood-CSF barrier (BCSFB) (Calias *et al.*, 2014). The BBB and the BCSFB have multiple functions, however their main role is the prevention of movement of unregulated substances from the systemic circulation into the CNS. This is of utmost importance for the maintenance of CNS function, as extremely well-regulated concentrations of neurotransmitters, proteins and ions are required.

1.3.1. Barriers of the CNS

The BBB and the BCSFB are composed of endothelial and choroid plexus epithelial cells respectively. These cells are joined together by tight junctions and express influx and efflux pumps on their membranes. The tightly packed cells, amongst many other transporters and enzymes comprise a highly impermeable barrier to the delivery of therapeutics to the brain and the spinal cord (Garg *et al.*, 2015). Thus, the passage of most, but a fraction of small and uncharged molecules is impeded (Daneman & Prat, 2015), making it extremely difficult for therapeutics to reach the intended site of action in the CNS.

1.3.1.1. The blood-brain barrier

The BBB effectively separates the CNS from the systemic circulatory system. The endothelial cells lining the brain micro-vessels, alongside the astrocytes and pericytes form the “neurovascular unit” (McCarthy *et al.*, 2015). Freely circulating therapeutics in the blood must pass through the endothelial cells lining the BBB in order to reach the CNS (Figure 1.4). The BBB is in part permeable to a few lipophilic compounds smaller than 400 – 500 Da, which have the ability of crossing through transmembrane diffusion (Banks *et al.*, 2009). Nonetheless, the drawback associated with this is that lipophilic drugs are sequestered by the capillary bed as a result of their lipid solubility. This in turns reduce their bioavailability within the aqueous environment of the CNS, thus significantly impacting their therapeutic effects (Banks *et al.*, 2009). On the other hand, larger molecules (i.e., neuropeptides or hydrophilic drugs) which cannot penetrate the BBB through diffusion alone can employ influx transporters expressed on the endothelial cells of the BBB for their passage across into the CNS (McCarthy *et al.*, 2015; Pardridge, 2002). Nonetheless, this mechanism of transport can

only work if the therapeutics are a substrate for the particular influx transporter. Moreover, the BBB also expresses efflux transporters which have the opposing function of expelling molecules back into the systemic circulation. The function of these efflux transporters is to maintain brain homeostasis and clear away metabolites and potential neurotoxins (Ohtsuki *et al.*, 2007). There are also a variety of other transport mechanisms across the BBB including transcytosis and endocytosis (Smith *et al.*, 2006). However, it is currently quite complex to hijack these biological processes for the movement of therapeutics into the CNS. Overall, the BBB is vital for the maintenance of CNS health, however due to its effectiveness, the delivery of therapeutics to the CNS is greatly hindered.

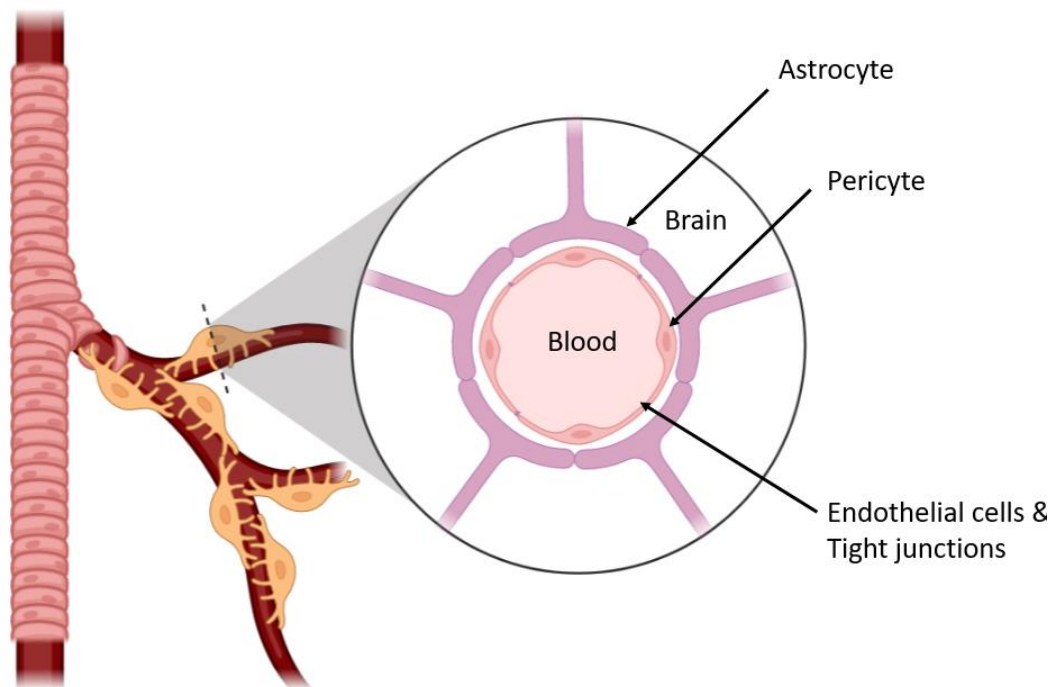


Figure 1.4. The neurovascular unit of the BBB. Schematic displaying the barrier (endothelial cells joined by tight junctions) that therapeutics must cross to pass from the systemic circulation into the CNS (schematic made using Biorender.com).

1.3.1.2. The blood-CSF-barrier

The BCSFB is located at the choroid plexus (the location of CSF origin) and the arachnoid membrane (Figure 1.5) and has an important role of controlling the entry of molecules into the CSF (Ueno *et al.*, 2016). The choroid plexus is essential in the homeostasis of the CNS, as it is involved in secretion of CSF and brain interstitial fluid (ISF) (Bartanusz *et al.*, 2011). The

CSF is involved in ion regulation, pH buffering for the brain and the drainage of brain metabolites with constant exchange between blood and CSF at the interface of the choroid plexus (Gherzi-Egea *et al.*, 2018; Warren, 2018). The CSF serves many purposes, including the protective cushioning of the brain, providing buoyancy, removing waste products and compensating for changes in blood volume (Lehtinen *et al.*, 2013).

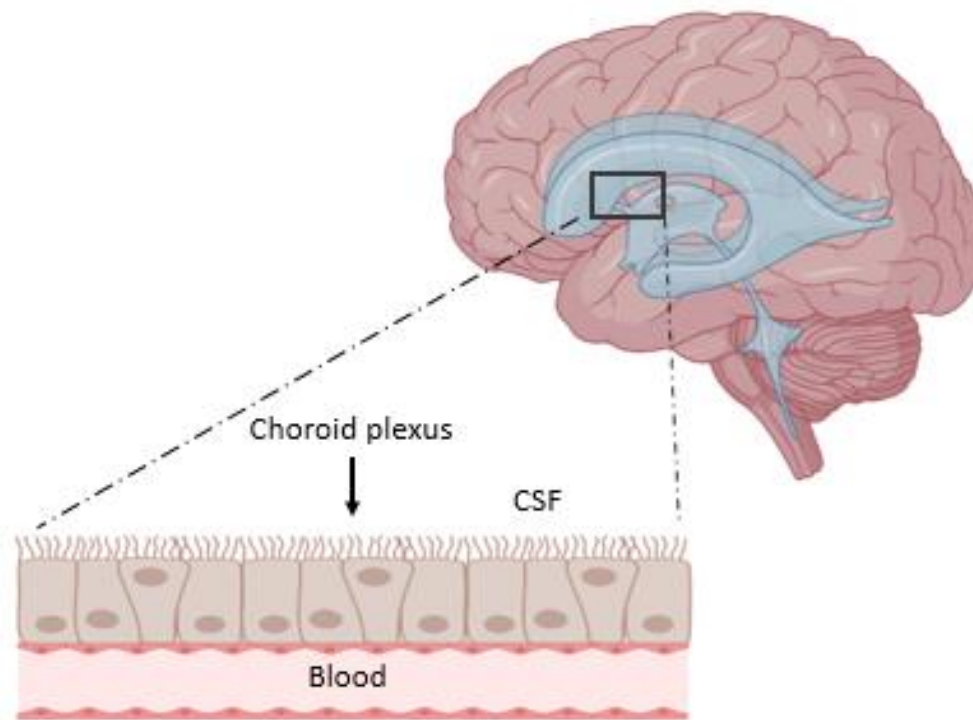


Figure 1.5. A schematic of the BCSFB at the choroid plexus. Diagram denoting the monolayer of epithelial cells preventing the entry of molecules from the blood into the CSF. The choroid plexus contains villi which increase the surface area and subsequently the number of transporters which can be expressed on the membranes (diagram constructed on Biorender.com).

Altogether these barriers act to protect the CNS and prevent any unwanted molecules from entering and disrupting homeostasis. An overview of their anatomical location alongside the barriers of the CNS is displayed in Figure 1.6.

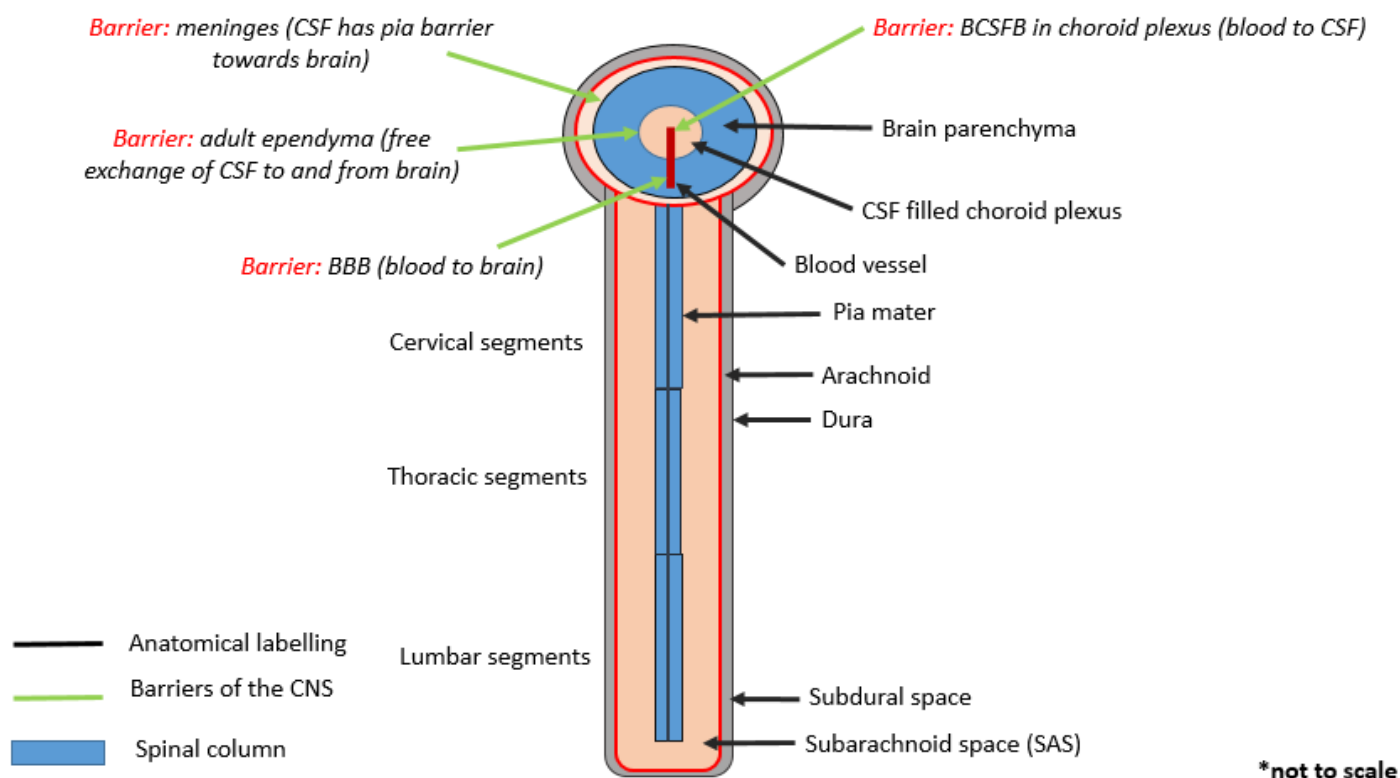


Figure 1.6. Schematic showing the different barriers of the CNS which protect and regulate homeostasis from systemic venous circulation. The BBB is at the interface of brain to blood, the BCSFB is at the interface of blood–CSF, the pia mater enclosing the brain at the CSF interface in the meninges, and the adult ependyma facilitating free exchange of CSF to and from the brain. The spinal cord is enclosed by three membranes, the dura, arachnoid and the pia membranes, all forming barriers around the CNS.

These barriers also have different permeabilities, as evidenced by IV administration of deuterated compounds. Preclinical experiments in a rabbit model have shown that there was significant increase in the uptake of [^3H]-D-mannitol and [^{14}C]-carboxyl-inulin in the spinal cord in comparison to the brain (Prockop *et al.*, 1995) after their IV delivery. There are also several studies highlighting an increase in permeability to radiolabelled cytokines (IFN α , IFN γ and TNF α) following IV administration in mice (Bartanusz *et al.*, 2011). All cytokines had the highest permeability into the cervical segment of the spinal cord, followed by the lumbar and the thoracic segments, with the least permeability in the brain (Pan *et al.*, 1997). This highlights the differences in permeability across these membranes and the barrier they pose to therapeutics administered systemically into the venous circulation.

One way of overcoming these barriers is by delivery through convection enhanced delivery (CED), which uses a positive pressure gradient to infuse agents directly into the brain

parenchyma (Mehta *et al.*, 2017). This technique works by improving the volume of distribution of therapeutics by dilating the extracellular space. Even though this method has shown promise in preclinical models, it has been very challenging to extend its use for clinical application (Fowler *et al.*, 2020). Moreover, CED is not applicable for the treatment of diseases encompassing the entirety of the CNS, therefore would lack clinical utility in certain disorders disseminated through the surfaces of the brain and spinal cord (i.e., leptomeningeal metastasis) (Mehta *et al.*, 2017). Fortunately, an alternative technique whereby the CNS can be reached directly is via an IT injection, which will be the focus of Section 1.4.2.

1.3.2. Intrathecal drug delivery

The intrathecal drug delivery (ITDD) route refers to the administration of therapeutics directly to the CSF within the subarachnoid space via a lumbar puncture in the spinal cord (Figure 1.7). It is widely estimated that approximately 100% of large molecules and 98% of small molecules do not achieve CNS penetration upon systemic delivery (Papisov *et al.*, 2013). This is also the case with CBD (discussed in Section 1.3), where its penetration into the brain is very poor when administered orally and intravenously. Many studies in the field of anaesthesia have focused on IT delivery, and several significant developments demonstrated that hydrophobic anaesthetic drugs induced a rapid segmental anaesthetic effect, whereas the delivery of hydrophilic anaesthetics lead to a more gradual onset with effects observed further away from the injection site (Papisov *et al.*, 2013), indicating different pharmacokinetic effects in the CNS according to drug's physicochemical properties.

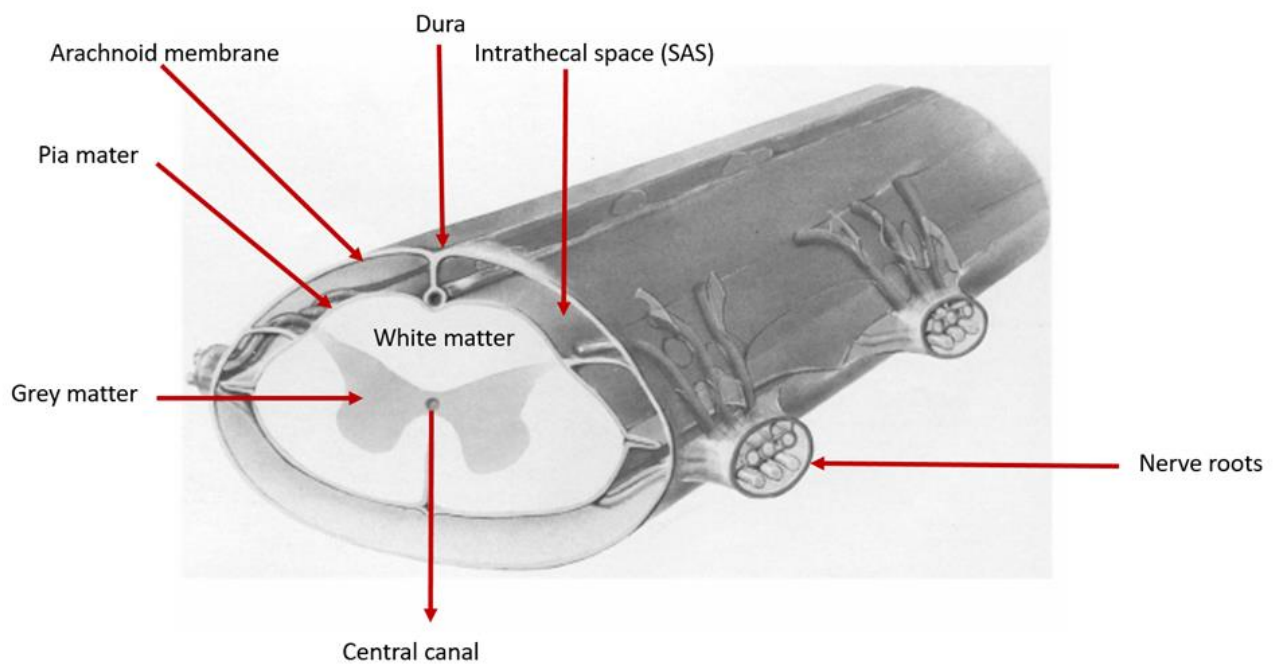


Figure 1.7. Internal structure of the IT space, reproduced from Nauta et al., 1983. Schematic shows the spinal cord enveloped by dura, subarachnoid and pia mater. The dorsal and ventral nerve roots are shown on the sides of the spinal cord. The trabeculae which make up the mesh of ligaments within the subarachnoid space are omitted in this diagram.

Anatomically, the spinal cord consists of the most extensive network of nervous pathways within the body. There are a total of 21 pairs of spinal nerves innervating the spinal cord: 1 coccygeal, 5 sacral (S1 – S5), 5 lumbar (L1 – L5), 12 thoracic (T1 – T12) and 8 cervical (C1 – C8). Most commonly IT injections are administered to the lumbar segments at the base of the spinal column, between L4 - L5, and this will be the method pursued in this thesis. There are other routes of administering therapeutic or diagnostic agents to the CSF, which include intracerebroventricular (ICV) and intracisternal magna (ICM) routes (Figure 1.8). However, these routes are highly invasive and are only considered as a last resort, as the penetration of catheters into the brain can result in parenchymal inflammation. The associated literature suggests that ICV drug delivery results in 33% of non-infectious complications and 27% of infectious complications, highlighting the need for a delivery system that reaches the brain without a direct injection into the parenchyma (Slavc et al., 2018).

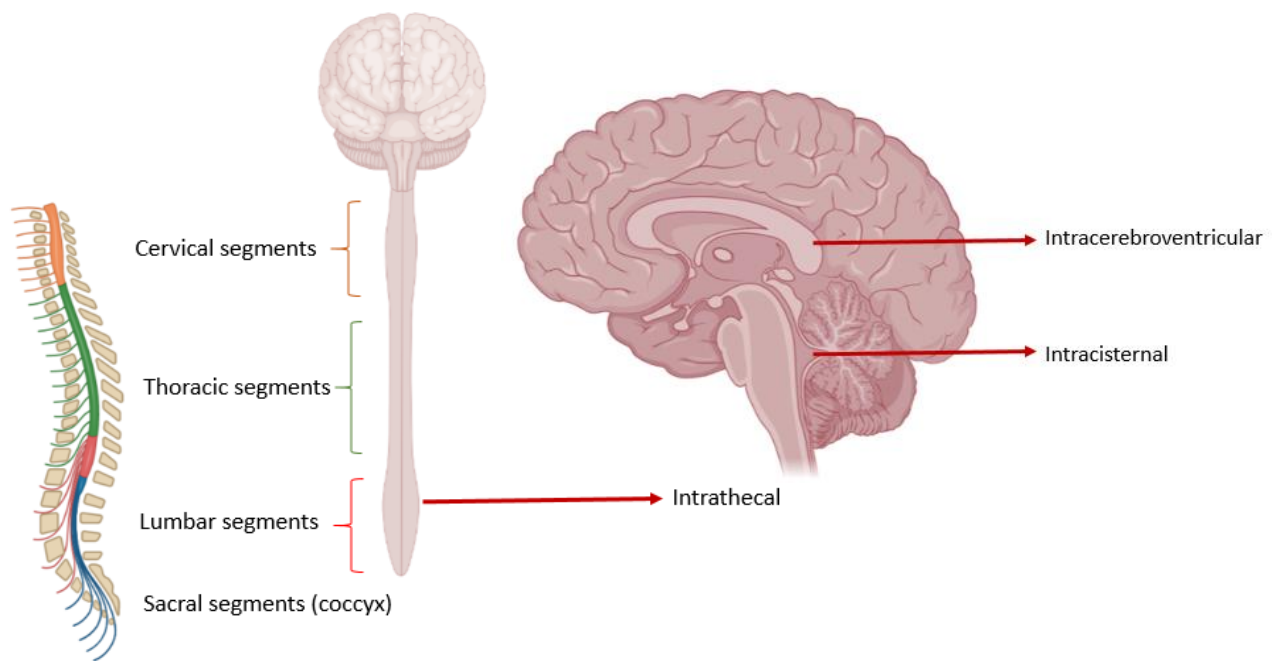


Figure 1.8. Schematic of the CNS and the spinal cord vertebrae displaying the cervical, thoracic, lumbar and sacral segments (not to scale). The different ways of CNS delivery are highlighted as intrathecal to the lumbar segment of the spinal cord and ICM and ICV for delivery to the brain (diagrams constructed on Biorender.com).

IT administration in rodents is challenging due to the small size of the spine and its structural complexity. The spinal cord is wrapped in three meningeal membranes: the pia mater, the arachnoid and the dura mater (Figure 1.9). The pia mater is the innermost membrane and it directly wraps onto the spinal cord and the brain. The arachnoid is the densest membrane surrounding the pia mater, with the space in between these two membranes consisting of the subarachnoid space (SAS) which is filled with CSF. The dura is the outermost membrane covering the arachnoid, creating the subdural space in between these two layers (Fowler *et al.*, 2020). The arachnoid and pia mater form the leptomeninges, and the space between these two membranes is known as the SAS, which is filled with CSF. The SAS also contains a mesh-like system of collagen fibrous trabeculae, blood vessels and nerves (Figure 1.9). It has been reported that these trabeculae have a mechanical support role between the pia and arachnoid membranes, and that they aid CSF mixing (Mortazavi *et al.*, 2018; Reina *et al.*, 2002)

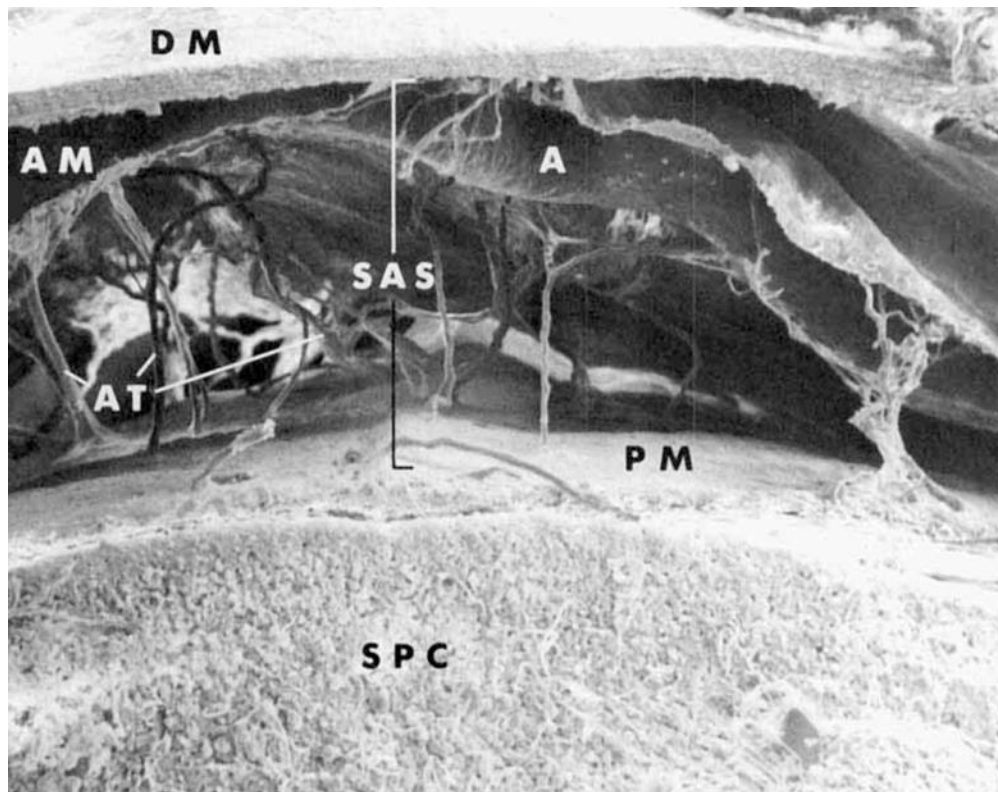


Figure 1.9. A microscopy image showing the SAS of the spinal cord (SPC) of a dog. The pia mater (PM) is enclosing the surface of the spinal cord, with the arachnoid trabeculae (AT) extending throughout the SAS and to the surface of the arachnoid mater (AM). A represents an artery and DM is the dura mater (reproduced from Mortazavi *et al.*, 2018).

The administration of therapeutics to the CSF in *in vivo* models is relatively challenging, considering the small size of SAS. There are however advantages in performing a lumbar puncture as opposed to ICV and ICM administration, as the needle does not have to pass through healthy brain tissue (ICV) or through the neck muscle (ICM). A lumbar puncture is comparatively easier and faster than the alternative methods, has a relatively easy surgical recovery and there is a possibility of carrying out multiple injections or infusions by using an osmotic pump (Ommaya reservoir) (Ineichen *et al.*, 2017). There is also the possibility of utilising appropriate drugs and formulation strategies to reach the brain from a lumbar injection, negating the need for intrusive direct brain administration (such as ICM or ICV).

There is however an associated technical challenge with carrying out the IT injection technique, especially in adult rats, due to the ossification of the spinal cord, which makes it inherently difficult for a needle to penetrate into the SAS. In order to overcome this, *in vivo* experiments would either need to be carried out in very young rats (1-2 weeks old) as bone ossification has yet to occur, or via a laminectomy during non-recovery surgery, where the

spinal vertebrae are removed in order to expose the spinal column for a direct lumbar injection, which is the technique explored in this thesis.

In a clinical setting, IT injections are also usually carried out via a lumbar puncture or ICV infusion. The lumbar injection is feasible in humans, albeit it is rather painful and requires a high degree of skill, and therefore must be carried out by trained professionals under anaesthesia (and sedation in children) (Fowler *et al.*, 2020). If repeated administrations are required, a mini pump implanted subcutaneously must be used (discussed in Section 1.5.3). This is most common in patients suffering from spasticity and who are administered baclofen, or to alleviate chronic pain by the use of narcotics. The use of narcotics in the treatment of unretractable pain is associated with a multitude of side effects which highlights the need of novel therapies for the treatment of pain. Additionally, as previously discussed, a high proportion of patients are also resistant to opioids, leaving them with no viable option to treat the pain they are suffering with. As a results of this, novel analgesics, coupled with other interventional drug delivery strategies must be investigated to target the patient population unresponsive to current treatments.

1.4. The IT space

1.4.1. Clearance and biodistribution of drugs in the CNS

A pharmacologically relevant effect in the CNS requires the distribution of drugs reaching therapeutic concentrations throughout the targeted CSF compartments, whilst concurrently overcoming clearance into the blood (Belov *et al.*, 2021). Nonetheless, many aspects of the CNS can influence the biodistribution of drugs after IT administration; one being the presence of epidural fat, which is unevenly distributed along the spinal canal. A systematic review of the literature carried out by Reina *et al.*, has described that there is no fat in the cervical segments, whilst in the lumbar segment there are two unconnected fat structures in the posterior and anterior sides. There is also an abundance of fat cells in the dura forming the sleeves around the spinal nerve roots, meaning that drugs could be stored in this space and act as a depot, within short distances from the nerves of the spinal cord (Reina *et al.*, 2009). Lipophilic drugs injected in the CNS have the ability to quickly diffuse across membranes such as the dura and the arachnoid encompassing the IT space, and therefore may move into

neighbouring structures of the nerve root fat sleeves. Lipophilic drugs are then slowly released, allowing for prolonged pharmacological action. It was also shown that the administration of lipophilic fentanyl and alfentanil by an epidural injection leads to a 20 to 30-fold increased accumulation in fatty tissue as opposed to the water-soluble morphine (Hindle *et al.*, 2008). Moreover, the fat components also compete for the absorption of the drug with other tissues such as the spinal cord, blood vessels and nerve root axons, therefore having an impact on the pharmacological effect of a drug, especially if it is lipophilic (Lynch, 2014).

Drug movement within the SAS is also facilitated by CSF convective mechanisms, including the rate of CSF turnover and the pulsatile flow through the IT space, the cardiac and respiratory thoracic motion and lastly, body movement (gravity effect) (Hettiarachchi, 2011; Hladky, 2014). For example, in rodents, the IT injection procedure itself can be leveraged to increase the spread of therapeutics across the spinal cord by adjusting the bolus volume to be relative to the animal's total CSF volume, and by enhancing the binding affinity of the administered drug (Wolf *et al.*, 2016). Moreover, in cynomolgus monkeys it has also been shown that an externally applied percussive force and increasing the bolus volume can improve cranial distribution of imaging agents and antisense oligonucleotide therapeutics in the treatment of Spinal Muscular Atrophy (Sullivan *et al.*, 2020)

The flow of CSF within the CNS affects movements of therapeutics injected intrathecally. CSF has a pulsatile movement around the cervical area and undergoes longitudinal oscillations throughout the entire SAS. Various MRI studies have allowed for the evaluation of *in vivo* CSF movement and have demonstrated that it follows the arterial cardiac cycle, with variations in pulsatile flows (from 120 mL/min to 360 mL/min in humans) (Linninger *et al.*, 2009).

Moreover, the biodistribution of drugs and formulations in the CNS can be affected by the trabeculae network in the SAS, which forms a porous mesh connecting the arachnoid and pia mater (Mortazavi *et al.*, 2018). These collagen fibrils can therefore restrict the movement of molecules with a larger size, in particular within the small anatomy of a rat. This was reported by Householder *et al.*, who established that the distribution of 10 μ m particles after IT administration was not as widespread as the smaller, 100 nm nanoparticles (NPs) (Householder *et al.*, 2019).

1.4.2. Injection parameters

The volume of the injected formulations is a critical parameter for IT delivery, as it has a significant effect on the therapeutic effect due to drug diffusion, toxicity and the residence time in the SAS (Rieselbach *et al.*, 1962). In contrast, rapid addition of liquids to the CSF leads to elevated intracranial pressure and can result in fatal consequences if the safe threshold is exceeded (Belov *et al.*, 2021). Generally, the injectable volume is restricted to about 10% of the total CSF volume, which is approx. 15 mL in humans and 15 μ L in rats (Papisov *et al.*, 2013; Vuilleminot *et al.*, 2016). An imaging study carried out in primates revealed that increasing the volume of a dose can significantly increase the distribution of imaging agents and therapeutic molecules (Sullivan *et al.*, 2020). If a smaller volume is injected, exogenous convective forces can be used to achieve the same distribution as achieved by the larger volume injected (Sullivan *et al.*, 2020). The authors described that externally applied percussive forces/wraps influenced convective forces within the CSF and thus the spread of therapeutics alongside the neuraxis (Sullivan *et al.*, 2020; Wolf *et al.*, 2016).

There are many other considerations which should be taken into account for IT injections. The CSF volume in humans ranges from 100 to 150 mL in total, therefore very small doses of hydrophilic molecules are required to reach therapeutic levels. The CSF is cleared 5 times a day on average, and care must be taken to avoid reaching toxicity levels (Artru, 1999; Kroin, 1999). However, in the rat the average CSF volume is 150 μ L and the CSF turnover is significantly higher at 28.8 times per day. This indicates that *in vivo* evaluations of CNS therapeutics in the rat might be vastly different in humans due to a slower CSF turnover rate, leading to potential drug accumulation in the SAS (Vuilleminot *et al.*, 2016).

Various reports have also demonstrated that the CSF movement leads to mixing within the SAS, which is thought to enhance the distribution of IT administered therapeutics (Bulat, 1993; Kwon *et al.*, 2018; Mestre *et al.*, 2018). The injection of indocyanine green in humans showed that it immediately distributed rostrally and caudally through the spine, entering in the SAS and the perivascular spaces and was eventually cleared through the cervical and lumbar lymph nodes (Kwon *et al.*, 2018).

It is therefore evident from the literature to date that intrathecally administered therapeutics are affected by various factors such as CSF clearance and dynamics, the physicochemical

properties of the drugs / formulations, the amount of fat encompassing the CNS, and the precise location of the injection followed by the bolus volume (Wolf *et al.*, 2016).

1.4.3. Intrathecal devices

To overcome the need for performing repeated injections for short-acting therapeutics, reservoir pumps connected to catheters implanted in the IT space can be used. These devices can control the infusion rate during the course of treatment, or as the patient requires, according to their pain symptoms. The use of such devices is a major advantage over the need for IT injections in the clinic, especially if repeated injections are required, as once the reservoir is implanted, there is limited clinical intervention required. For infusions in the lumbar area, a catheter can be inserted in the lumbar cistern and connected to a pump (Ommaya reservoir) which is usually inserted in the subcutaneous layer of the abdomen. At present in the clinic, most pumps are used for patients suffering with chronic pain or spasticity (Fowler *et al.*, 2020). Alternatively, a catheter can also be inserted into the lateral ventricle of the brain, in a similar approach to ICV delivery as previously depicted in Figure 1.8. This method is however highly invasive as it requires the penetration of the brain parenchyma through the cerebral cortex, which could unfortunately lead to leukoencephalopathy. A more recent technique developed by Sandberg *et al.*, 2020 is an infusion into the 4th ventricle, which does not require brain parenchyma penetration, thus preventing the aforementioned side-effects. This method is currently used for infusions of 5-Azacytidine and methotrexate in the resection cavity of paediatric patients suffering from posterior fossa tumours (Sandberg *et al.*, 2015; Sandberg & Kerr, 2016). Nonetheless, there are various disadvantages associated with the use of Ommaya reservoirs, such as the occurrence of infections and inflammation, or in several cases catheter dysfunction (Bejjani *et al.*, 1997; Byers *et al.*, 1995; Sampson *et al.*, 2002). It is therefore imperative that further research is carried out in the area to advance our understanding of therapeutics in the CNS, prolonging their pharmacological effects, and improving the safety of the devices associated with their use.

1.5. Nanomedicines for intrathecal delivery

In spite of an increasing interest in the field of IT delivery, and the many advantages it poses in regard to treating neuropathic pain in opioid-resistant patient populations, there has been relatively little consensus on the use of nanomedicines for IT administration. It is therefore

evident that a better understanding of nanocarriers and their subsequent influence on drug release, biodistribution and therapeutic efficacy will shed light on the further optimisation of colloidal nanomedicines for IT delivery. This has already been the case with the understanding of CSF flow dynamics and clearance, as it has allowed for further developments in the distribution of nanomedicines through fluid convection (Fowler *et al.*, 2020; Iliff *et al.*, 2012). It is therefore unsurprising that this delivery technique has so far been used in numerous preclinical models of CNS diseases including neurovascular disease, cancer, neuroinflammation and metabolism (Chiari *et al.*, 1999; Westin *et al.*, 2010; Yaksh *et al.*, 2002).

1.5.1. Nanocarrier formulations

Nanocarriers possess many advantages, including improved drug half-life within the CNS when compared to the free drug form. As a result, numerous nanomedicines have been investigated preclinically for IT administration (Table 1.2), with some reaching the clinic and being approved for human use (discussed in Section 1.6.2). The issues of poor drug solubility and inefficient pharmacokinetic profiles alongside limited CNS distribution are yet to be tackled for IT administration (Nutt, 2003). Hydrophilic agents are rapidly cleared away from the CSF (through CSF flow turnover as previously discussed), therefore their effects are limited and require frequent administration to maintain therapeutic levels, which is not ideal for this delivery route. In contrast, lipophilic / hydrophobic drugs such as CBD have very poor solubility and therefore their administration is also problematic (Gherzi-Egea, 1996; Pardridge, 2011; Shapiro, 2006). Moreover, smaller molecules also have the tendency to bind to matrix proteins, partition into cell membranes thus further restricting their movement and dissemination across the CNS. As a result of these drawbacks, the encapsulation of molecules within nanocarriers is widely used in overcoming poor pharmacokinetic profiles, solubility issues and biophysical limitations (Sandberg, 2012, 2015; Shapiro, 2006). Due to the versatility of nanocarrier vehicles, their properties can be easily tuned and optimised for the appropriate administration route, taking into account all the biological parameters (i.e., CSF total volume and rate of turnover, site of action, size of SAS trabeculae).

Colloidal formulations have received much attention in the last few decades because of their potential to enhance and improve drug delivery. Formulating poorly soluble lipophilic

therapeutics in nanoscale colloidal systems has been shown to improve drug diffusion through tissues and slow down drug degradation (through protection from enzymatic breakdown and interactions with other surrounding tissues) (Nance *et al.*, 2014). For example, it was demonstrated that hydrophobic interactions and ionic forces hinder the movements of therapeutics through tissues, therefore encapsulating these in colloidal formulations can protect molecules from such interactions (Blank *et al.*, 2015; Wang *et al.*, 2016). NPs can consist of different sizes, surface charges and physical properties, which can all affect their behaviour *in vivo* (Wolak *et al.*, 2013; Sousa *et al.*, 2019). NPs with a positive charge have been demonstrated to rapidly extravasate into tumours, whereas negative surface charges are thought to enhance penetration into brain parenchyma. It was also shown that a dense poly(ethylene glycol) coating on the surface of NPs improves brain penetration for particles 114 nm in diameter (Nance *et al.*, 2012). A multitude of different nanomedicines have been described in the literature, mostly categorised based on the materials used in their formulation (i.e., polymeric NPs, liposomes, nanoemulsions, metallic NPs). These formulations all exhibit different properties and are chosen according to the drug of interest, the administration route and the formulation technology available. Usually, various formulations will be referred to as nanocarriers or NPs interchangeably, as these are umbrella terms for multiple types of colloidal formulations.

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Table 1.2. Several preclinical formulations for IT delivery selected to showcase the variety of therapeutics and types of vehicles used to date. Formulations described according to their composition, the therapeutic encapsulated, route of administration to the CNS and the disease application (partially reproduced from Fowler *et al.*, 2020).

Composition	Type of formulation	Therapeutic	Route	Species	Application	Reference
PMAM^a (hyperbranched polymer)	Polyamidoamine dendrimer	Trimcinolone	Not spec.	Mouse	Pain	Kim <i>et al.</i> , 2017
PEI-PEG^b (linear polymer)	Polycations (co-polymers)	pDNA	IT-lumbar	Rat	None	Tang <i>et al.</i> , 2003
PLA^c (solid polymer)	Composites	Fentanyl	IT-lumbar	Rat	Pain	Okuda <i>et al.</i> , 1999
PLGA^d (solid polymer)	Microspheres	Baclofen	IT-lumbar	Rabbit	Pain	Lagarce <i>et al.</i> , 2005
DEPG^e, DPPG^f (lipid)	Liposomes	Bupivacaine	IT-lumbar	Dog	Pain	Joshi <i>et al.</i> , 2015
DPPC^g (lipid)	Liposomes	Alfentanil	IT-lumbar	Rat	Pain	Bernards <i>et al.</i> , 1992
DSPG^h, PGⁱ (lipid)	Liposomes	Amphotericin-B	IT-lumbar	Mouse	Neuroimmunity	Capilla <i>et al.</i> , 2013
PC^j (lipid)	Liposomes	Methotrexate	ICV	Primate	Cancer	Kimelberg <i>et al.</i> , 1978
DL^k (lipid / multivesicular liposomes)	Liposomes	Cytarabine	IT-lumbar	Rats	Cancer	Kim <i>et al.</i> , 1987
DMPC^l (lipid)	Liposomes	BCNU	ICM	Dogs	Cancer	Kitamura <i>et al.</i> , 1996

Polymers: ^apolyamidoamine, ^bpolyethylenimine polyethylene glycol, ^cpoly(lactic acid), ^dpoly(lactic-co-glycolic acid). **Lipids:** ^edielaidoyl phosphatidylglycerol, ^fdipalmitoyl phosphatidylglycerol, ^gdipalmitoyl phosphatidylcholine, ^hDistearoyl-sn-glycero-3-phosphoglycerol, ⁱdimyristoylphosphatidylcholine, ^jphosphatidylglycerol, ^kphosphatidylcholine. ^ldioleoyl lecithin.

Many nanomedicines have been investigated so far preclinically for IT administration (Table 1.2), in various early studies researching treatments for cancer metastasis such as methotrexate, cytarabine (Ara C) and 1,3-bis(2-chloroethyl)-2-nitrosourea (BCNU). Methotrexate was encapsulated within [¹⁴C] cholesterol – labelled liposomes and injected (lateral cerebroventricles) in the CSF of primates, resulting in a 4-fold increase in drug concentration found in tissue compared to the free drug form (Kimelberg *et al.*, 1978). Cytarabine was encapsulated within multilamellar liposomes and led to sustained high levels of cytotoxic drug concentrations in the CSF for several weeks post infusion (Kim *et al.*, 1987). The encapsulation resulted in the half-life of cytarabine increasing from 2.7 hrs to 148 hrs, leading to an extended maintenance of therapeutic drug levels, thus improving efficacy and possibly decreasing toxicity by flattening the peak of cytotoxicity. BCNU was also encapsulated in liposomes and administered intracisternally to dogs to treat malignant glioma. This formulation led to the increased in median survival times from 24.5 and 26 days in the control groups to 32 and 45 days when injected with liposomal BCNU formulation (Kitamura *et al.*, 1996). These are just a few examples of nanocarriers being used to extend the half-life of therapeutic agents and thus enhance their pharmacokinetic profile within the SAS and thus improve their efficacy.

The SAS can also serve as a sustained release depot for formulations, which has been evidenced by studies investigating the plasma concentration of nimodipine PLGA microparticles in canines following IT injection (Hanggi *et al.*, 2016). This led to nimodipine being detected in plasma 21 days after administration, which is also indicative of the microparticles crossing from the CSF into the systemic circulation (Hanggi *et al.*, 2016). Sustained concentrations of the drug were also detected in the CSF 29 days post administration, indicating the possibility of a long-term release formulation acting locally within the CNS.

Non-degradable, silica-lipid NPs (with a 230 nm diameter) were also administered IT by a lumbar injection, and they were found to reside within the meninges of the CNS for at least 8

weeks post-administration (Dengler *et al.*, 2013; Hagihara *et al.*, 2000). Moreover, it was also demonstrated that NPs were also able to transfect cells in the CNS, which has tremendous impact in the treatment of spinal cord injury (Cerqueira *et al.*, 2013; Donaghue *et al.*, 2015; Tan *et al.*, 2013). Furthermore, a rat model of meningeal gliomatosis had shown increased survival after the delivery of chemotherapy-loaded liposomes (Kitamura *et al.*, 1999). There have been various studies investigating the potential of this administration route, however little is known about the impact of formulations on the biodistribution of lipophilic drugs within the CNS, and their effect on therapeutic efficacy, which is what I sought to investigate in this thesis.

1.5.2. Intrathecal nanomedicines in the clinic

Since the first reports of IT administration of nanomedicines in the late 80s and 90s, numerous other studies have been undertaken investigating a variety of colloidal systems including lipids, polymers and metals (Table 1.2). A few of these have been so far approved for use in the clinic: bupivacaine liposome (DepoFoam®) for pain, PLGA encapsulated nimodipine (Hanggi *et al.*, 2019) for neurotrauma, cytarabine liposomes for cancer (DepoCyt®) and blank hydroxypropyl- β -cyclodextrin for neurodegeneration (Ory *et al.*, 2017; Fowler *et al.*, 2020) (Table 1.3).

DepoCyt® was approved for use in the clinic by the FDA in 1999, for the treatment of Lymphomatous Meningitis (Fowler *et al.*, 2020). The pharmacokinetic profile of the therapeutic was significantly improved through its formulation in multilamellar liposomes, maintaining cytotoxic levels of the drug up to 7 days after administration (Peryl *et al.*, 2014). This formulation was specifically designed to prolong release of cytarabine within the SAS, in order to act as a depot in the CNS. The formulation strategy was ultimately successful in achieving the need for less frequent IT injections (therapeutic levels were maintained for 7 days as opposed to 3.4 hrs for the free drug) (Zimm *et al.*, 1984). Unfortunately, DepoCyt® was discontinued for use in the clinic in 2017, as it was reported to have unspecified manufacturing problems (Salehi *et al.*, 2019).

Table 1.3. Clinical formulations for IT delivery according to their composition, therapeutic used in the formulation, route of administration and the application they are licenced for.

Composition	Therapeutic	Formulation specifics	Route	Application	Reference
DepoCyt (MLV liposomal formulation)	Cytarabine	3 – 30 µm diameter, honeycomb arrangement of aqueous chambers	IT - lumbar or ICM	Cancer	Kripp <i>et al.</i> , 2008
DepoFoam	Bupivacaine	30 µm diameter, extended release multivesicular liposomal formulation	IT – lumbar	Pain	Bergese <i>et al.</i> , 2011
Hydroxypropyl-β-cyclodextrin	Blank	HPβCD formulated as a 20% solution and diluted in saline	IT – lumbar	Neurodegeneration (Niemann-Pick Type C)	Ory <i>et al.</i> , 2017
PLGA microparticles	Nimodipine	1.3 – 2.4 µm diameter, drug dissolved in PLGA matrix	IT lumbar	Neurotrauma (subarachnoid haemorrhage) *clinical trials terminated early	Hanggi <i>et al.</i> , 2019

For the treatment of pain, bupivacaine was also formulated in a DepoFoam® multivesicular liposomal formulation, and was approved by the FDA in the treatment of postoperative pain in 2015. When the free-drug was compared to the liposomal formulation, it was shown that the formulation had extended the release profile of bupivacaine. In cases of epidural administration, the therapeutic effect was extended from 3.2 hrs to 6.25 hrs (Chahar *et al.*, 2012). In another case in patients who had undergone aortic surgery, they had extended their pain relief from 2.4 hrs to 10.6 hrs upon the use of liposomal bupivacaine (Bergese *et al.*, 2011). This significant increase in the period of analgesia demonstrates that the formulations can enhance pharmacokinetic effects of therapeutics whilst decreasing the need of opioids in

pain management. This is especially critical in patients who are unresponsive or resistant to opioids.

An important aspect of successful IT delivery of nanomedicines is their widespread dissemination through the CNS, and penetration of brain parenchyma. This is specifically important in the treatment of cancers of the CNS which have embedded tumours deep into the brain (Brigger *et al.*, 2002). Recent evidence suggests that not all nanocarriers (i.e., solid NPs and microparticles) are able to penetrate the parenchyma when circulating in the CSF (Pardridge *et al.*, 2002). On the other hand, ultra-small colloids, hyper-branched and linear polymers have demonstrated the ability to enter the brain (Thorne *et al.*, 2006). Nonetheless, there are not enough studies out there to generate conclusions as to what colloidal constituents and size are essential to therapeutic penetration of the parenchyma from the CSF. One report has suggested that colloidal size is essential, due to the extracellular space in the brain which ranges between 40 and 60 nm (Nance *et al.*, 2012). However, another study has shown that particles with a diameter of 70 nm were able to diffuse through the parenchyma, albeit with low binding (Nance *et al.*, 2014). Nonetheless, there are not enough reports in this area in order to make any generalised statements on what the optimum characteristics are for IT delivery, subsequent parenchymal penetration, and diffusion across the neuraxis and clearance from the SAS. It is therefore imperative to elaborate on current knowledge and further develop our understanding for formulations for the IT space.

1.5.3. Safety of formulations in the CNS

The toxicity exhibited by formulations in the CNS is of utmost importance, and is therefore a major challenge in the delivery to the spinal tissue. Following administration, the drugs and excipients delivered are retained in very close contact with the spinal tissues, due to the small volume of the CSF space. As a result of slow diffusion due to volumetric constraints, very high concentrations of drugs and excipients at the site of injection might lead to toxicity (Lagarce *et al.*, 2004). It is therefore imperative that the formulations injected are compatible with the CNS. Unfortunately, few studies have investigated the toxicity profiles of therapeutics / formulations injected intrathecally, and only the safety of 0.9% NaCl, glucose, β -cyclodextrin and polysorbate 80 have been reported (Grouls *et al.*, 1999; Yaksh *et al.*, 1995; Jang *et al.*,

1992). Thus, it is crucial that any novel formulations developed for intrathecal delivery are free of excipients which might induce toxicity.

Despite most studies carrying out research to investigate molecules with a therapeutic end-goal (proteins, genes, small molecules), papers have also focused on investigating the basic science of IT delivery coupled with imaging studies to elucidate how formulations behave in the SAS. In this thesis I aim to bring these two aspects together, by exploring how different formulations of a novel analgesic compound impact the movement of the molecule within the CNS, whilst concurrently exploring its therapeutic potential. We hope that this investigation will elucidate the structure-function relationship of nanocarriers within the CNS, and aid the development of future clinical treatments for neuropathic pain.

1.6. Thesis rationale

The goal of this thesis is to develop and evaluate formulations for the localised delivery of cannabidiol (CBD) to the spinal cord via an intrathecal (IT) injection, for the treatment of neuropathic pain. The target population for this treatment are patients resistant to conventional opioid therapy. My ultimate aim is to investigate how two fundamentally distinct formulations of the same drug impact its biodistribution profile in the central nervous system (CNS), and its subsequent clearance away from the site of injection. We also set out to evaluate whether the formulations affect the anti-nociceptive efficacy of the drug immediately after injection. Furthermore, I also investigate the IT delivery route in rats through various imaging approaches, to ensure that this challenging *in vivo* approach was effective in the administration of therapeutics to the CNS. My animal model consists of a laminectomy surgery to dissect the spinal vertebrae and expose the spinal cord, therefore establishing the correct administration to this space prior to evaluating the formulations *in vivo* was essential.

The contents of the over-arching introductory chapter include neuropathic pain characteristics and current clinical treatments, cannabidiol as an emerging therapy and difficulties in systemic administration due to pharmacokinetic challenges. It will also discuss potential routes for CNS targeting with a focus on overcoming the blood-brain barrier (BBB) and blood-cerebrospinal fluid-barrier (BCSFB) and ultimately focusing on IT administration. Advantages in the administration of therapeutics to the CNS will be evaluated, alongside the

existing medical devices which allow for long-term, sustained release of formulations at the site of action and at therapeutic doses, with minimal invasiveness following their insertion. Moreover, current clinical and preclinical nanocarrier drug delivery systems developed for IT administration will also be discussed, to establish the current field in CNS delivery.

1.7. Aims and hypotheses of the thesis

The overarching hypothesis of this PhD is that the two CBD formulations will exhibit different biodistribution profiles following injection into the IT space.

The sub-hypotheses of this thesis are:

- CBD polymer-coated nanoparticles (PCNPs) will spread further away from the site of injection as a result of the PEGylated coating which would decrease interactions with the SAS components.
- The CBD nanoemulsion would be retained at the site of injection due to the hydrophobicity of the lipid, which would preferentially be absorbed in the fat surrounding the SAS.
- Both formulations are efficacious in eliciting an anti-nociceptive effect in rats after lumbar administration, according to the CBD release from the formulations.

In order to overcome the pharmacokinetic profile of orally and intravenously administered CBD and to test the previously formulated hypotheses, the overarching aim of the PhD was **to develop and evaluate intrathecally compatible CBD formulations to treat neuropathic pain**. This therapeutic strategy is mostly aimed at the patient population suffering from neuropathic pain and is resistant to conventional opioid therapy. I aimed to investigate the biodistribution of a single bolus injection into the CNS, however upon further investigation these formulations can be extended for use in an Ommaya reservoir, to allow for a constant supply of pain relief in patients. This proposed project aims to establish an *in vivo* model for IT injections utilising a non-recovery laminectomy surgery technique, allowing the evaluation of immediate electrophysiological anti-nociceptive effects of the formulations. I also intended to investigate the IT injection method through the use of imaging techniques, in order to establish and confirm that the injection was effective in penetrating the SAS. Therefore, the biodistribution profiles of the different CBD formulations in the CNS, should

help determine the impact of nanocarrier formulations on the fate of therapeutics after lumbar injections, alongside their effect on drug efficacy within the CNS.

In order to test my hypotheses, I have outlined several objectives:

- Incorporate CBD in a stable and safe nanoemulsion, compatible for IT delivery.
- Formulate CBD in stable and homogenous PCNPs.
- Evaluate IT injections as a technique *in vivo* and establish the successful injection through imaging approaches.
- Administer CBD formulations and evaluate their anti-nociceptive effects *in vivo*.
- Evaluate the biodistribution profiles in the CNS and the clearance of CBD after IT injection .

To the best of my knowledge, this is the first time two different formulations of the one drug are administered and evaluated in an *in vivo* IT model, with the aim of inducing analgesia.

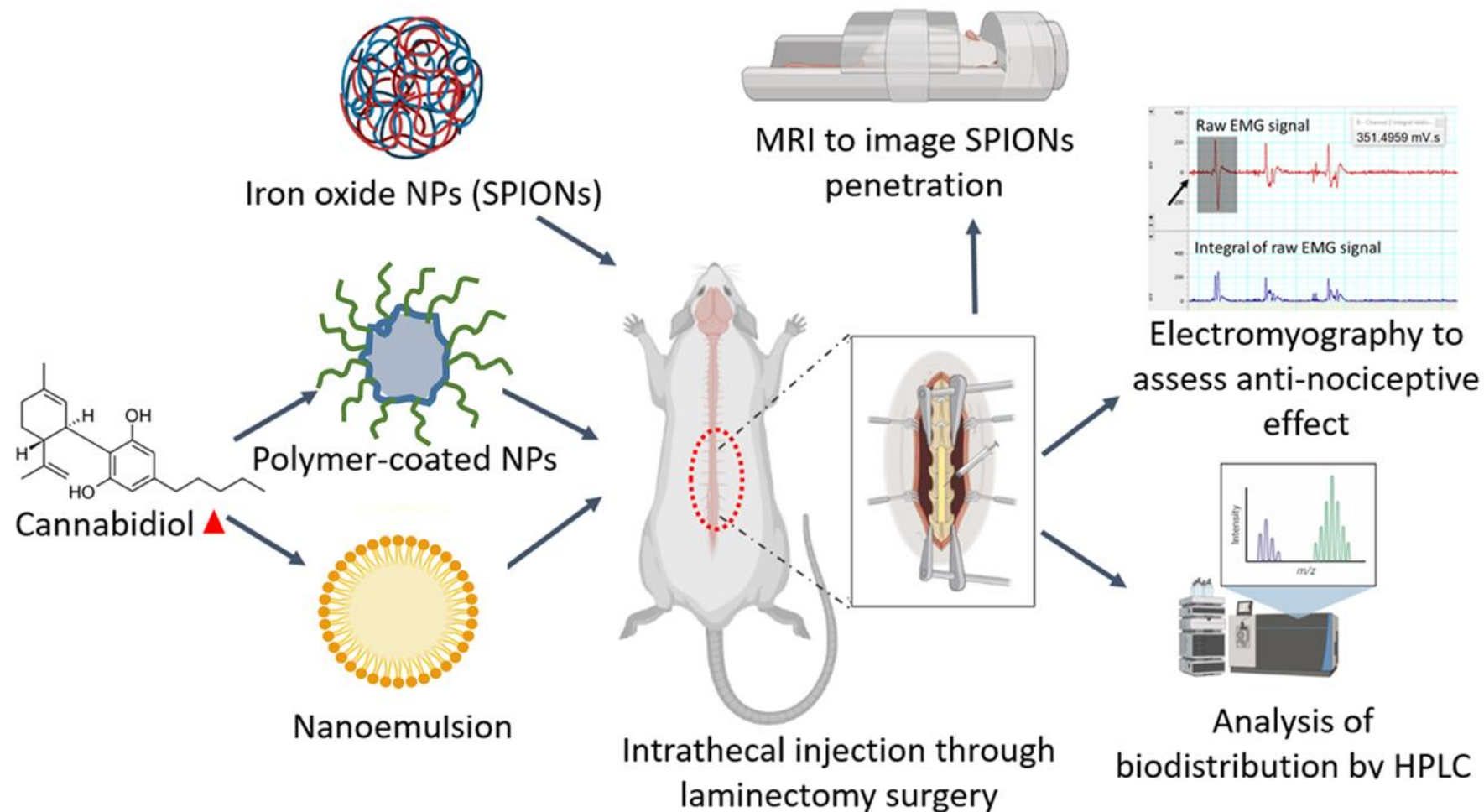


Figure 1.10. Thesis workflow. The development of CBD formulations followed by their evaluation after IT delivery post-laminectomy surgery in rats. The IT injection technique was investigated by imaging methods (MRI) after the injection of iron oxide nanoparticles to visualise whether they distribute in the CNS. After the administration of CBD formulations, their electrophysiology effect was analysed to establish if CBD had an anti-nociceptive effect. Lastly, HPLC was used to determine biodistribution of CBD within the CNS, peripheral tissues and serum.

2. Chapter 2 – Materials and Methods

2.1. Materials

20% Intralipid® (CAS-No. 68890-65-3), 4,4'-DDT (CAS-No. 50-29-3), propylene glycol (CAS No. 57-55-6), glycerol (CAS-No. 56-81-5) potassium bromide (CAS. No 77580-02-3) were purchased from Sigma-Aldrich. CBD was purchased from THC Pharm (Frankfurt, Germany). Isotonic saline (9 g NaCl per 1 L) was purchased from Thistle Scientific. Iron oxide (II, III) magnetic nanoparticle solution (CAS-No. 1317-61-9 and 1317-61-9) was purchased from Sigma-Aldrich. Egg-derived phospholipids and soybean oil were kindly donated by Lipoid (Ludwigshafen, Germany).

All organic solvents were procured from Fisher Scientific unless otherwise stated (solvents used for HPLC were of analytical grade). All other chemicals used were purchased from Sigma-Aldrich unless otherwise stated. Sprague Dawley rats were procured from Charles River.

2.2. Formulation of Intralipid nanoemulsion

Intralipid nanoemulsion was formulated using a previously reported method (Washington and Davis, 1987) with slight modifications. 1.2 g of natural egg-derived phospholipids (Lipoid E80) was dispersed in 77.5 mL distilled water whilst stirring and heating at 50 °C until phospholipids were fully dissolved. The formulation was then mixed using an Ultra-Turax (T 25, IKA, UK) homogeniser at 10 000 rpm. Purified soybean oil (20 mL) was then slowly added and the formulation was homogenised for a further 10 mins at a constant speed of 10 000 rpm. This was followed by the addition of 2.25 mL glycerol, which required homogenisation for a further 2 mins. This process generated a coarse unstable emulsion which was subsequently processed by a microfluidizer (LM-20, Microfluidics, US) to form a fine emulsion. This emulsion will be called microfluidized Intralipid throughout this thesis, and will be distinguished from the emulsion acquired from Sigma-Aldrich, UK (commercial Intralipid®)

An alternative emulsion was also formulated, consisting of the same components as Intralipid, with the addition of sodium oleate B, an anionic surfactant composed of oleic acid of vegetable origin and sodium salt. The same formulation steps as previously described were followed with 0.03 g sodium oleate B being added during the same step as the phospholipids. This procedure generated a coarse emulsion which was also subsequently processed by the microfluidizer to generate a nanoemulsion (which I will call Lipoid).

Table 2.1. Components of the Lipoid nanoemulsion, the microfluidized Intralipid nanoemulsion and the commercially available Intralipid® formulation.

Lipoid	
Components	Percentage composition (w/v)
Soybean oil	20 %
Egg-derived phospholipids	1.2 %
Glycerol	2.25 %
Sodium oleate B	0.03 %
Water	76.52 %

Microfluidized Intralipid	
Components	Percentage composition (w/v)
Soybean oil	20 %
Egg-derived phospholipids	1.2 %
Glycerol	2.25 %
Water	76.55 %

Commercially available Intralipid®	
Components	Percentage composition (w/v)
Soybean oil	20 %
Egg-derived phospholipids	1.2 %
Glycerol	2.25 %
Water	76.55 %

2.2.1. Microfluidizer optimisation

Previously prepared coarse emulsions were passed through the microfluidizer for processing in order to reduce their particle size and polydispersity. The microfluidizer consisted of an inlet reservoir, an intensifier pump and a Z-type diamond interaction chamber (Figure 2.1). The pump was able to generate pressures up to 30 000 PSI (2 068 bar) and the minimum formulation volume for processing was 14 mL. Emulsions were processed through the microfluidizer for 4 cycles at different pressures to investigate optimum processing parameters. The interaction chamber was cooled down using an ice bucket to alleviate temperature fluctuations as a result of the energy generated. For each cycle, 50 mL of sample was fed through the inlet reservoir at a set pressure. Processing variables tested were the

operating pressure (range 10 000 to 21 000 PSI) and number of cycles required for optimum size and polydispersity. All experiments were carried out with interaction chamber submerged in ice.

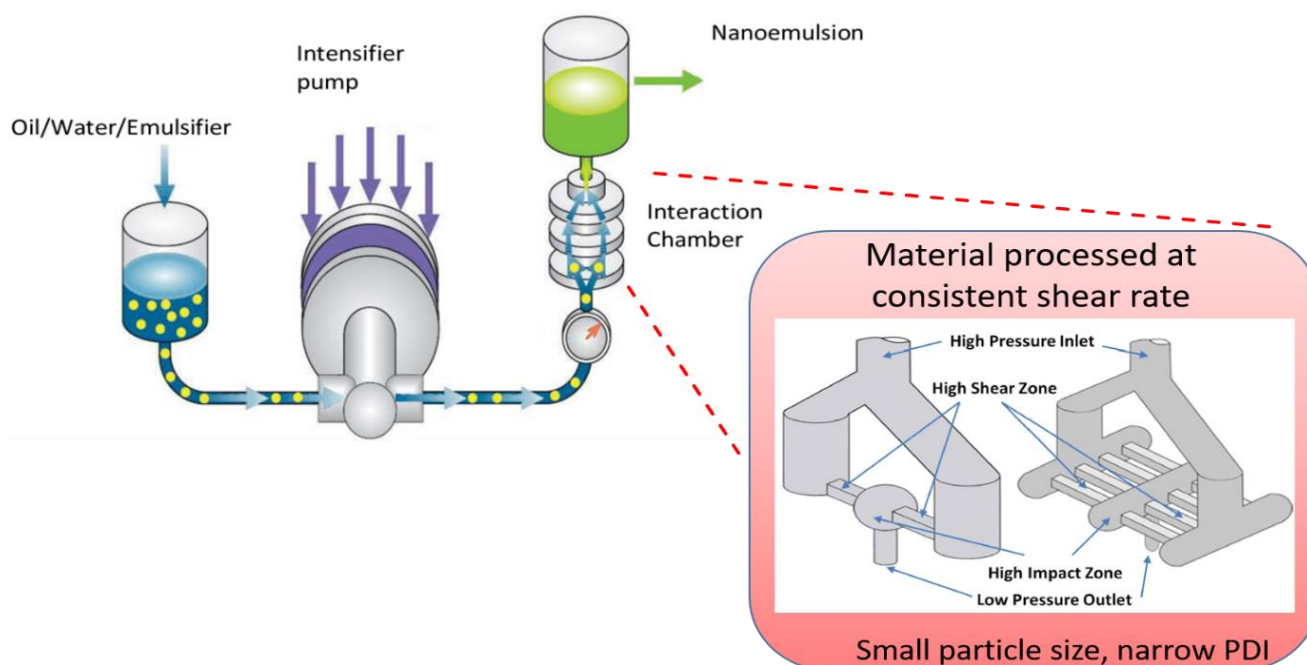


Figure 2.1. Overview of the microfluidizer processor used for the emulsion processing (Figure adapted from Panagiotou et al., 2012). Formulations were fed through the reservoir, pushed through the intensifier pump and subsequently fed through the interaction chamber. At this point two different streams are impinging towards each other at very high velocities in order to shear oil droplets. These were broken down and re-formed whilst stabilised by phospholipids present in the aqueous phase.

2.2.2. Incorporation of CBD in Intralipid® nanoemulsion by density gradient ultracentrifugation

Stock solutions of CBD (100 mg/mL) were prepared in propylene glycol – ethanol (9:1, v/v), and further diluted in Intralipid® nanoemulsion to 2 mg/ml final CBD concentration. Control samples were prepared with nanoemulsion spiked with blank propylene glycol – ethanol (9:1, v/v). Formulations were incubated at 37 °C for 1 hr (stirring at 170 rpm). Thereafter, the density of the nanoemulsion was modified to 1.1 g/mL with potassium bromide (KBr). Saline solution (0.9% NaCl) served as a standard solution with a density of 1.0046 g/mL. Gradient solutions with densities of 1.019 and 1.063 g/mL were prepared from the 0.9% NaCl solution by the addition of KBr. A density gradient (Figure 2.2) was then built and the CBD-containing

nanoemulsion was separated from free drug by ultracentrifugation (Sorvall® TH-641 Rotor, Thermo Fisher Scientific, 20 000 rpm, 15 °C for 30 mins). The top 1 mL layer was collected and vortexed immediately. Samples were stored at 4 °C until further analysis.

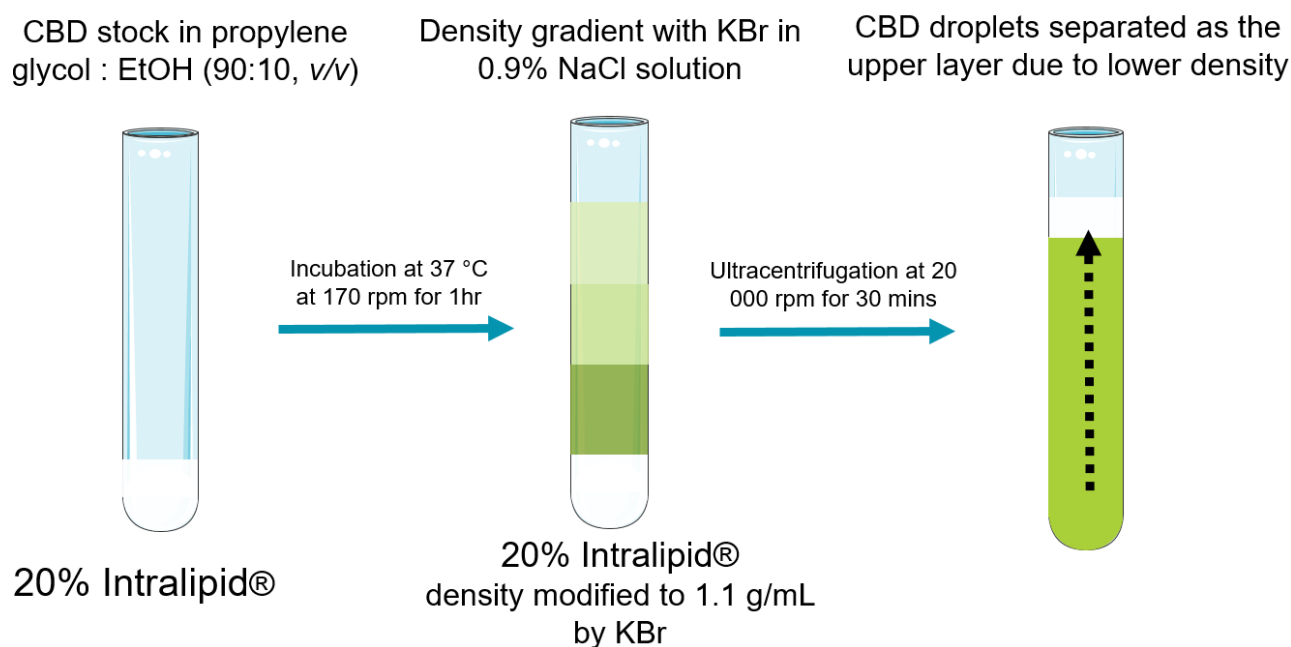


Figure 2.2. Graphical schematic for association of CBD with lipid phase of nanoemulsions by density gradient ultracentrifugation protocol. This was used to separate CBD loaded oil droplets from freely dispersed drug in aqueous phase.

2.2.3. Optimisation of the association procedure

The association of CBD with emulsions was optimised due to phase separation encountered with some samples. It was observed that the oil phase was separating from the aqueous phase following ultracentrifugation resulting in higher PDI. This process was therefore optimised using Design-Expert® (DOE) software (Version 11, Stat-Ease Inc, US). A factorial design was developed encompassing gradient solutions, ultracentrifugation speed and run time as the critical factors to be investigated (Table 2.2). Following association of CBD with nanoemulsions under different conditions described below, the samples were analysed for particle size, PDI, zeta-potential and concentration of CBD.

Table 2.2. Factorial design for emulsion optimisation procedure. Investigated factors and analysed responses displayed below, alongside the variable ranges investigated.

Factors	Variable ranges investigated	Responses
Ultracentrifugation speed	20 000 to 39 600 rpm	Particle size
Ultracentrifugation run time	10 to 30 mins	PDI
Gradient solutions	PBS or saline	Zeta-potential
		CBD concentration

2.3. Formulation of CBD PCNPs by nanoprecipitation

2.3.1. Polymer synthesis and characterisation

2.3.1.1. 3star-PEG-initiated ring-opening polymerization of lactide block copolymer

3 star-PEG₁₀₁₄-PLA₁₀₀ (tPEG) was synthesised and characterised as per methods reported by Vasey *et al.*, 2019, via PEG-initiated ring-opening polymerization (ROP) of lactide block copolymer. Briefly, a predetermined amount of 3 star-PEGylated chain initiator and DL-LA were weighed out (2.00 g of LA and 140 mg of 3 star-PEG) in a glass vial (kept in dry conditions in an oven at 100°C for 24 hrs) and capped. Dry dichloromethane (Sigma-Aldrich, UK) (10 mL) was added and the mixture was allowed to dissolve. 1,8-Diazabicyclo[5.4.0]undec-7-ene (Sigma-Aldrich, UK) (3% w/w with respect to the amount of LA) was directly added to the reaction mixture as a catalyst for the ROP. After 15 min, the polymerization was quenched by adding the solution dropwise into cold hexane (30 mL) and the polymer was purified via multiple precipitation steps in hexane and diethyl ether. The final polymer was dried in vacuum (conversion of monomer into polymer ~85-90% w/w). A final NMR spectrum was recorded of the products post purification. ¹H NMR (400 MHz, CDCl₃, ppm): δ 5.19 (broad m, 202H, circa 101 units), 3.67 (broad s, 84H), 1.70–1.50 (2 asymmetric broad s, 620H). %. Polymer synthesis, NMR and GPC were carried out by Dr Vincenzo Taresco (School of Chemistry, University of Nottingham).

2.3.1.2. Nuclear magnetic resonance (NMR)

Bruker AV400 NMR spectrometers operating at 400 MHz (^1H) was used to perform nuclear magnetic resonance (NMR) analysis in deuterated solvents. Chemical shifts were assigned in parts per million (ppm). Spectra were obtained at ambient temperature ($22\text{ }^\circ\text{C} \pm 1\text{ }^\circ\text{C}$) and analysed by MestReNova (Mestrelab Research S. L.).

2.3.1.3. Gel permeation chromatography (GPC)

Gel permeation chromatography (GPC) was used for determination of number average molecular weight (M_n) and molecular weight distribution (dispersity, $\mathcal{D}$). The analysis was performed using a PL50+ Polymer Laboratories system equipped with a refractive index detector (dRI), employing 2 mixed bed (D) columns maintained in an oven at $50\text{ }^\circ\text{C}$ and using a solution of DMF 0.1% LiCl as mobile phase at a flow rate 1 mL min^{-1} . Poly (methyl methacrylate) standards were used for calibration.

2.3.2. Double nanoprecipitation procedure

CBD PCNPs were formulated by a double nanoprecipitation technique (Figure 2.3; Fessi *et al.*, 1989), based on a previously developed procedure by Styliari *et al.*, with some additional modifications (Styliari *et al.*, 2020). The previously developed tPEG (Vasey *et al.*, 2019) and synthesised herein was used to coat the surface of CBD nanocrystals. CBD was dissolved in 1 mL acetone (5 mg/mL stock concentration) and then added to 5 mL distilled water (1:5 solvent to aqueous ratio) in a dropwise manner over 90 secs. The 1 mg/mL CBD solution was left stirring for 4 hrs at 550 rpm to allow acetone evaporation. Subsequently, tPEG dissolved in 1 mL acetone (5 mg/mL stock concentration) was added to water containing CBD in a similar dropwise manner over 90 secs. The final formulation containing a CBD to tPEG ratio of 1:1 was allowed a minimum of 4 hrs stirring at 550 rpm for acetone to evaporate and CBD PCNPs to form. Samples were stored at $4\text{ }^\circ\text{C}$ until further analysis.

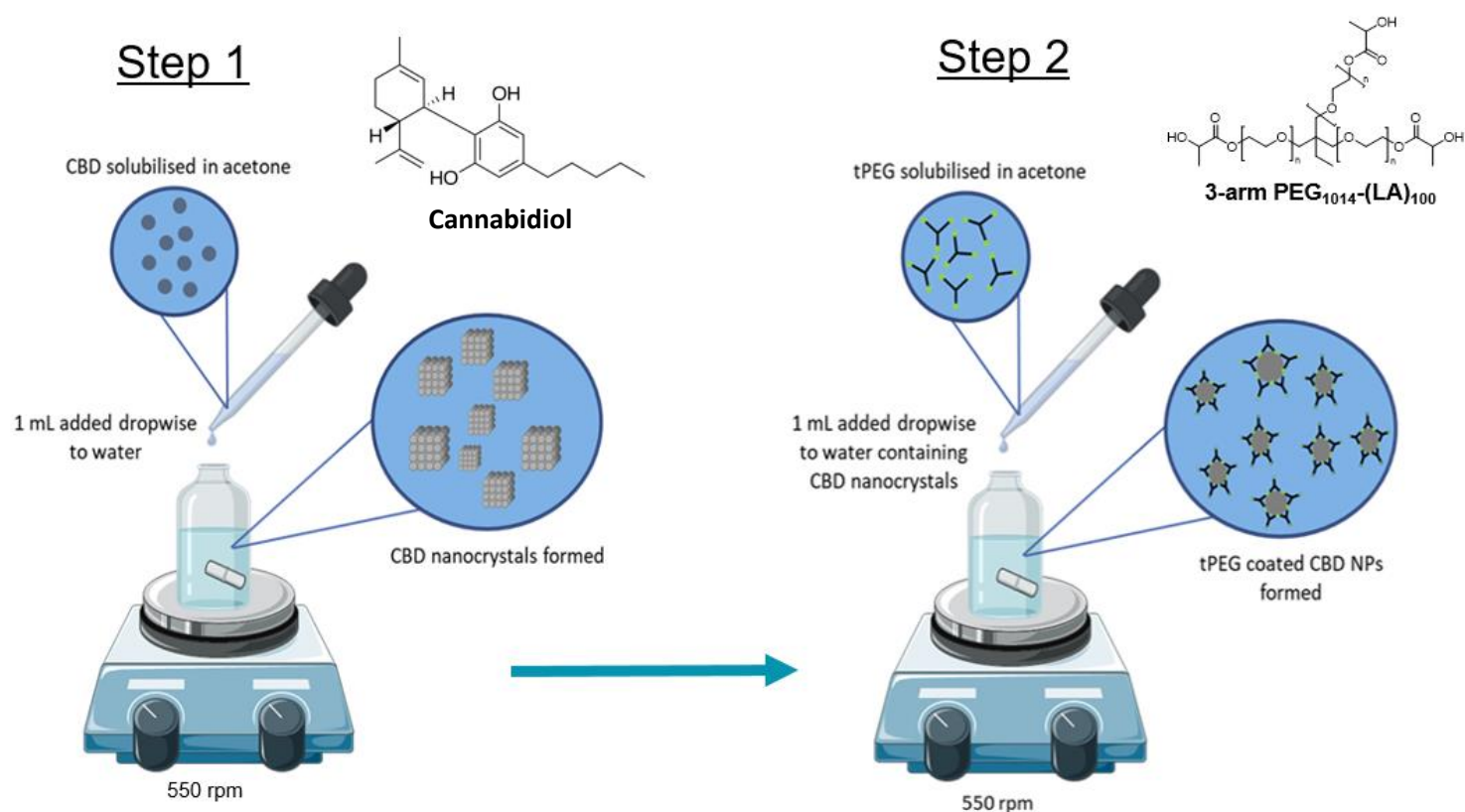


Figure 2.3. Steps involved in the double nanoprecipitation procedure for CBD PCNPs formulation. Step 1 consists of creating CBD nanocrystals through the addition of CBD solubilised in acetone to water in a drop-wise manner under constant stirring at 500 rpm. Step 2 then consists of tPEG solubilised in acetone added to coat the CBD nanocrystals through a similar drop-wise addition method (schematic made on BioRender.com).

2.3.2.1. Separation of uncoated crystals from PCNPs

Larger uncoated CBD crystals were initially separated from the PCNPs using PD10 columns, which work by separating molecules based on molecular weight. The effective separation of larger crystals was investigated by particle sizing. Briefly, 2 mL PCNPs were added to an equilibrated PD10 column. Once the solution started passing through, aliquots of 1 mL were collected into separate Eppendorf tubes, until a total of 12 mL was collected (distilled water was added to the PD10 column to wash out all of the nanoparticle formulation). Each sample was analysed for size and PDI (according to methods in section 2.4), and only the aliquots consistent in size and PDI with the initial PCNPs were kept for further analysis. As a result of this optimisation PCNPs samples were collected between 3 mL and 10 mL of eluent.

Due to PCNPs resulting in very low drug loading after passing through the PD10 columns, the larger uncoated crystals were separated from the PCNPs via a different centrifugation (Eppendorf centrifuge 5430, Germany) method (4 000 g for 5 mins at room temperature). The larger crystals will sediment during centrifugation, allowing for their separation. The resulting formulation was then analysed by DLS.

2.3.2.2. Optimisation of PCNPs

Optimisation of CBD PCNPs was carried out to investigate optimum formulation variables (CBD and tPEG concentrations ranges tested between 1 and 2.5 mg/mL, and ratio of acetone to water, range between 1:4 to 1:6). This process was therefore optimised using Design-Expert® (DOE) software (Version 11, Stat-Ease Inc, US). A factorial design with 2 centre points was used to test the variables enumerated above. The samples made for the DOE were subsequently analysed for their particle size, PDI, zeta-potential and drug loading.

2.3.2.3. Concentrating PCNPs

The stable PCNPs formulation was then concentrated to the desired CBD concentration with gaseous N₂ gas at 37 °C. 2 mL PCNP formulation was evaporated down to a pre-determined volume and physical characterisation was carried out (as described in section 2.4) to investigate whether this process had an effect on stability in comparison to controls. This method was determined successful if no significant change in size and PDI was observed as compared to the control formulations which did not undergo this process.

2.4. Physical characterisation of formulations

The particle size (d. nm) and polydispersity index (PDI) were determined by dynamic light scattering (DLS) on Malvern Zetasizer Nano ZS series (Malvern Instruments, UK) using back-scatter detection at 173° scattering angle (He-Ne laser wavelength of 633 nm). Samples were diluted by a factor of 1/1000 with deionised water and measured at 25 °C. The refractive index of materials was set to 1.47 and 1.46 for soybean oil and tPEG respectively and 1.330 for the dispersant. The zeta potential (ZP) was also measured on Malvern Zetasizer Nano ZS at 25 °C. This was carried out through determination of electrophoretic mobility. All measurements

were performed in triplicate (ZP measurements consisted of 12 runs each). Data was processed using Malvern DTS software to calculate the mean diameter and PDI.

2.4.1. Stability of nanoemulsion and PCNPs

Nanoemulsion and PCNPs stability was analysed by DLS periodically following storage at 4 °C. Briefly, nanoemulsion formulations were vortexed vigorously then diluted 1/1000 in distilled water. PCNPs were analysed in a similar manner, by diluting at a factor of 1/1000 in distilled water if formulations were turbid.

2.5. Quantification of CBD in nanoemulsion and PCNPs

The concentration of CBD in formulations was determined by reverse-phase HPLC (Waters Alliance 2695 separations module) coupled to a photodiode array ultraviolet (UV) detector (Waters 996). Analytical conditions used were previously published (Zgair *et al.*, 2015) and applied with slight modifications. Briefly, the mobile phase consisted of acetonitrile : water (62:38, v/v), flow rate 1 mL/min and oven temperature of 55 °C. The stationary phase was comprised of ACE C18-PFP column (150 x 4.6 mm, 3 µm). Dichlorodiphenyltrichloroethane (DDT) was used as an internal standard.

2.5.1. Quantification of CBD in nanoemulsion

CBD was extracted by ethanol dilutions which solubilised the nanoemulsion. Samples were then diluted in mobile phase spiked with internal standard DDT and 40 µL injected into the HPLC for analysis. Calibration curves were linear in the range of 0.5 µg/mL to 10 µg/mL and the limit of quantification for these analytical conditions was reported to be 10 ng/mL (Zgair *et al.*, 2015).

2.5.2. Quantification of CBD in PCNPs

Samples were flash frozen in liquid nitrogen and freeze-dried for 12 hours. CBD was quantified by reverse-phase HPLC to calculate encapsulation efficiency and drug loading. Methanol was used to reconstitute 1 mg of lyophilised material. Samples were vortexed and centrifuged (Eppendorf 5430, 14 000 rpm for 5 mins) through filtered Eppendorf tubes to separate precipitated polymer. Filtrate was collected and diluted further in methanol then in mobile phase. Following filtration through 0.22 µm syringe filters, 40 µL of sample was injected into

HPLC for quantification. Calibration curves were linear ($r^2 > 0.99$) in the range of 0.5 µg/mL to 50 µg/mL.

The following equations were used to calculate encapsulation efficiency (EE%) in Equation 1 and drug loading (DL%) in PCNPs in Equation 2.

$$1. EE\% = \frac{\text{Drug present in NPs (mg)}}{\text{Initial drug added (mg)}} \times 100$$

$$2. DL\% = \frac{\text{Drug present in NPs (mg)}}{\text{Polymer and drug after freeze drying (mg)}} \times 100$$

2.6. *Ex vivo* imaging techniques for intrathecal injection investigation

Commercially available superparamagnetic iron oxide nanoparticles (SPIONs) were used to investigate the IT injection technique by utilising magnetic resonance imaging (MRI), environmental scanning microscopy (ESEM) and immunohistochemistry.

MRI images can be classified into longitudinal relaxation time weighted images (T_1 contrast) and transverse relaxation time weighted images (T_2 contrast). SPIONs with a diameter > 4 nm are considered to be T_2 contrast agents (Javed *et al.*, 2017). They are thus able to provide clear images for diagnosis purposes, and were used in this work to investigate the IT injection technique. ESEM was also utilised on *ex vivo* spinal cord sections after the administration of SPIONs, to investigate whether the distribution of NPs can be assessed in the grey / white matter. Furthermore, immunohistochemistry was carried out to examine whether an acute inflammatory reaction was triggered after the injection.

2.6.1. Characterisation of SPIONs

SPIONs (Sigma Aldrich, UK) were physically characterised by DLS following the methods outlined in section 2.4. Briefly, the commercially available formulations (5 and 1 mg/mL starting concentrations for iron oxide and PEGylated iron oxide NPs respectively) were diluted to 0.01 mg/mL in deionised water and their size and ZP was determined via DLS. The RI was set to 2.343 for the iron oxide NPs and 1.467 for the PEGylated iron oxide NPs.

2.6.2. Administration of SPIONs *in vivo*

SPIONs were diluted in water to a pre-determined concentration (15 μ L, 1 mg/mL) and were administered intrathecally following the surgery procedures outlined in section 2.6.

2.6.3. Perfusion and tissue collection

10 mins after the administration of SPIONs intrathecally, the animals were sacrificed by an intraperitoneal pentobarbital overdose (Dolethal, 200 mg/kg). Following areflexia and cessation of breathing, the thoracic cavity was opened via an incision using scissors to reveal the heart (still beating to aid the perfusion of solutions throughout the circulation). A needle connected to a 50 mL syringe was used to puncture the left ventricle, whilst a small incision was made in the right atrium. 150 mL of 0.9 % saline were used for perfusion, followed by 200 mL of 4 % paraformaldehyde (PFA, Sigma Aldrich) solution. The animals were then placed on ice and taken to the magnetic resonance imaging (MRI) apparatus for *ex vivo* scanning. *Ex vivo* MRI analysis took place over the course of a couple of hours after the completion of the experiments.

For alternate imaging experiments, the spinal cords were quickly dissected and submerged in 4% PFA for post-fixing over-night. The following day the 4% PFA solution was exchanged for 30% sucrose in 0.1 M PBS and 0.01% azide (Sigma Aldrich, UK) for 24 hrs for cryoprotection. The tissues were then sectioned using a freezing microtome for immunohistochemistry experiments (section 2.8.8) and a cryostat for SEM and histological staining (section 2.8.7 and 2.8.9 respectively).

2.6.4. Tissue sectioning for immunohistochemistry

The spinal cord was sectioned using a freezing microtome (Leica, SM2010R) at a set thickness of 40 μ m. Dry ice was used to maintain freezing temperatures and to mount the tissue onto the cutting stage. The slices were then collected and stored in 96 well plates submerged in 30% sucrose in 0.1 M PBS and 0.01% azide solution at 4 $^{\circ}$ C.

2.6.5. Tissue sectioning for histology and ESEM

The spinal cord was sectioned using a cryostat (Leica, CM3050S) at a set thickness of 20 μ m. The base of the spinal cord samples was adhered to the cryostat chucks using a Tissue-Tek

Optimal Cutting Temperature (OCT) compound. The samples were cooled to -23 °C and sectioned on the cryostat. They were then collected and placed on gelatinised microscope slides for Haematoxylin and Eosin (H&E) staining and on plain slides for ESEM.

2.6.6. Magnetic resonance imaging (MRI)

Rats were scanned by MRI as soon as possible after sacrifice. Two rats scanned were not perfused (one saline injection and one iron oxide NPs) and 3 rats were perfused and fixed transcardially with PFA (one saline injection and two iron oxide NPs).

Ex vivo MRI (scans were performed and constructed by Dr Peter Harvey at the Imaging Beacon, UoN) was acquired using a 12 cm outer diameter birdcage transceiver for imaging in a 20 cm bore Bruker 7 T Avance III MRI scanner. The rats were placed in two different positions to allow full coverage of the spine and brain and scanned with a number of different sequences, with the field of view of 8 x 4 cm and data matrices of 512 x 256 points (see Table 2.3 for details of analysis parameters).

ImageJ (with MRI Analysis Calculator plugin) was used to reconstruct the images and compute relaxation time constants by fitting image intensity data to exponential decay curves.

Table 2.3. Details of MRI analysis parameters.

Analysis	Sequence	TR	TE (eff)	RARE factor
T₂ – weighted	Turbo-RARE sequence	2500 ms	48.4 ms	8 and 6 averages over 12 slices of 11 mm thickness and 0 mm gap.
T₁ – weighted	RARE sequence	1300 ms	12.3 ms	4 and 1 average over 12 slices of 1 mm thickness and 0 mm gap
Contrast – weighted	MDEFT sequence	4000 ms	4.2 ms	2 averages over 12 slices of 1 mm thickness and 0 mm gap
T₁ / T₂ maps	RARE sequence	5000, 3000, 1500, 800, 400, 200 ms	16.4, 49.2, 82.0, 114.8, 147.6 ms	1 average over a single slice of 2 mm thickness

2.6.6.1. Relaxivity measurements by MRI

Phantom MRI data were acquired in a 12 cm outer diameter birdcage transceiver for imaging in a 20 cm bore Bruker 7 T Avance III MRI scanner. Samples at varying concentrations (0 up to 10 μ M) in PBS buffer were loaded into the wells of a 384-well clear polystyrene plate (Thermo Scientific Nunc), which had been pre-cut in half to optimally fit the coil. Unused wells were filled with PBS buffer. 2 mm slices were imaged through the samples with the field of view of 5 x 5 cm and the data matrices were 256 x 256 points. Longitudinal (R_1) and transverse (R_2) relaxivity measurements were acquired using a RARE sequence with multiple TR and TE values; TR = 5000, 3000, 1500, 800, 400, 200 ms. TE = 11, 33, 55, 77, 99 ms. ImageJ (with MRI Analysis Calculator plugin) was used to reconstruct the images and compute relaxation time constants by fitting image intensity data to exponential decay curves. The relaxivity

measurements were calculated by analysing the increase in relaxation rate as a function of [Fe] concentration ($R_x = R_{x0} + r_x[Fe]$; $x = 1,2$).

2.6.7. Environmental SEM

The slides with the sections were imaged in a FEI Quanta 650 ESEM using Low Vac mode and water vapour as the imaging gas, with the chamber pressure set at 55 Pa (ESEM analysis was carried out by Ms Nicola Weston, NMRC). This enabled both SE detection using LFD detector and also back scattered electron detection using BSED, allowing for identification of any contamination particles (heavier atomic number elements appeared brighter). EDX analysis was then used to identify the elements present.

2.6.8. Immunofluorescent staining

Spinal cord tissue sections were rinsed in 0.1 M PBS (pH 7) to remove any remaining cryoprotectant solution. Sections were then transferred into 10% citrate buffer solution and incubated at 90 °C for 20 mins for antigen retrieval. After cooling, the sections were subsequently incubated with 0.3% Triton X-100 and 5% serum from the species of the secondary antibody for 1 hr. Primary antibodies Caspase-1 (1:500), DAPI (1:1000) and IBA1 (1:1000) diluted in 5% serum and PBS were used for overnight incubation at 4 °C. The next day, sections were washed three times in 0.1 M PBS for 10 mins to ensure all unbound primary antibodies were rinsed. Secondary antibodies at 1:500 concentration (Alexa-Fluor conjugates; Invitrogen; DaG488, DaRb594; DaM647) were then used to incubate the sections at RT for 2 hrs (in the dark). The tissue sections were then rinsed and mounted onto microscopy slides. Following air drying, a cover slip was placed on top with fluoromount (Sigma Aldrich, UK), and after drying clear nail polish was used to seal the edges.

2.6.8.1. Microscopy

The immunofluorescent sections were analysed by an M200 Leica fluorescent microscope, captured with Volocity 6.1 software (Perkin Elmer, UK). 10x and 20x images were taken of each section and ImageJ was utilised for the image acquisition (the same brightness and contrast was used for each section).

2.6.9. Haematoxylin and Eosin (H&E) staining

Previously sectioned spinal cord tissues (section 2.8.5) were baked at 60 °C overnight and deparaffinised in xylene three times for 10 mins. The slides were then treated sequentially in ethanol (starting with 100, 95, 80, 70 % concentrations), followed by water, Harris haematoxylin and then eosin (Surgipath, UK). Subsequently, the sections were rehydrated and mounted using DPX mounting medium. Sections were then observed by light microscopy (H&E staining was carried out by Denise Mclean, NMRC and microscope analysis carried out by myself).

2.7. *In vivo* experimentation

2.7.1. Experimental animals

Sprague-Dawley male adult rats obtained from Charles River (Kent, UK) were housed in the Bio-Support Unit (BSU) at the University of Nottingham. They were maintained on 12 hr light/day cycle at ambient temperature with free access to food and water. They were provided with cage enrichment (cardboard tubes), sawdust and nesting material. All animals were exposed to the same diet throughout the experiments, housed as 4 rats per cage in individually ventilated cages (IVC) and handled 1-2 days prior to surgery. Rat weights ranged from 200 – 225 g upon delivery and were housed for 5-7 days in the BSU before experiments. On the day of the experiments the animals were weighed and transferred to Home Office approved laboratories for surgeries. All animal experiments were carried out in line with the Home Office Animals (Scientific Procedures) Act 1986 adopting the principles of the 3Rs (replacement, reduction and refinement). All studies were carried out under approved Home Office project (PB3DA999F) and personal licences (IC03AC971).

2.7.2. Anaesthesia

Rats were anaesthetised with 3% isoflurane (Baxter, UK) delivered in 100% oxygen at 1 L/min rate after placed in a plexiglass induction chamber. This level of anaesthesia was maintained by mechanical ventilation (model number 50-9703, Harvard Apparatus), which was set to 80 breaths / minute and lung volume of 1 mL / 100 g. The animal's state of anaesthesia was assessed after 5 minutes by checking the loss of righting, corneal and pedal reflex. If no

reflexes were observed, the rat was then removed from the induction chamber and placed in a supine position. Anaesthetic level was continually maintained at 3% isoflurane via a glass nose-cone.

2.7.3. Tracheostomy surgery

Cannulation of trachea to allow very precise mechanical ventilation was carried out via a tracheostomy surgery (Figure 2.4). This was essential in order to ensure complete control over anaesthetic delivery, as any fluctuations (which could take place when using the nose-cone) have an impact on the quality of electrophysiology recordings (Rampil and King, 1996). The fur covering the throat of the rat was removed using scissors. The skin and the muscle layers on top of the trachea were then bluntly dissected using rat-tooth forceps to expose the trachea underneath. The trachea was then elevated by placing a pair of sharp forceps beneath it to separate it from other surrounding layers of tissue. Two loose ligatures (2.0 suture silk) were then placed underneath the trachea and tied 1 cm apart. The nose-cone was taken off the rat briefly during the next step. The tracheotomy was carried out in the middle of the ligatures with a no. 11 blade and scalpel. Once a cut was made, a cannula was inserted with the bevel facing upwards, at a 3 mm depth into the tracheal opening. The cannula was made of 5-6 cm of curved tubing (1.57 mm interior and 2.08 mm exterior; Portex). Once the cannula was inserted, the silk ligatures tightened at the bottom and top of the trachea. After the cannula was secured on the trachea, it was connected to anaesthetic tubing.

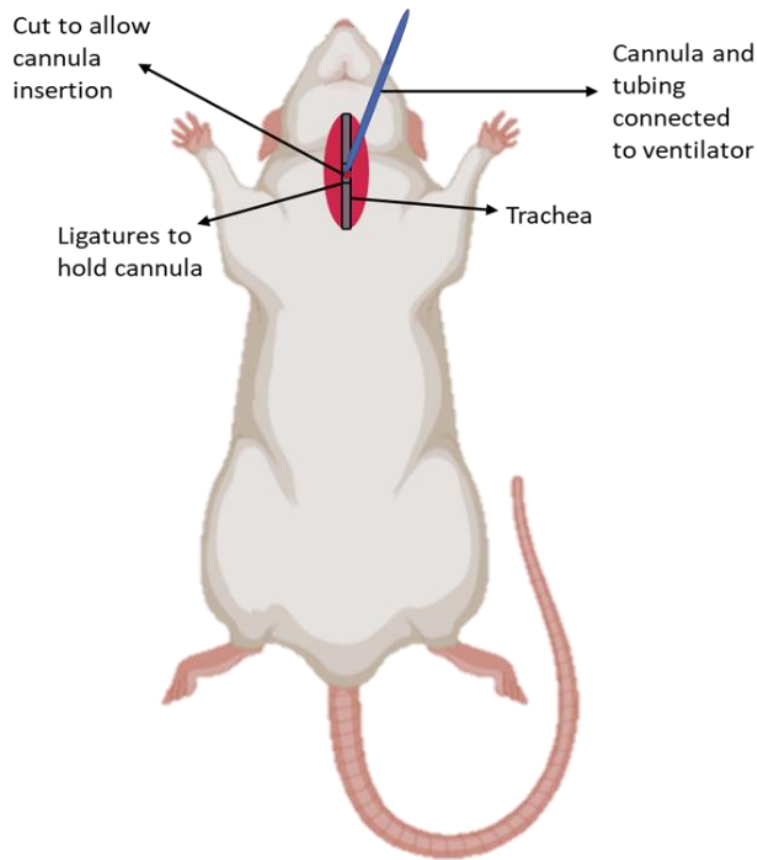


Figure 2.4. Diagram representation of the tracheostomy surgery for cannulation to anaesthetic (schematic made using Biorender.com).

2.7.4. Stereotaxic placement

All rats were moved onto the stereotaxic frame in a prone position, fitted in conventional ear bars and bite bars after the trachea was cannulated. This was done to ensure there was no movement of the head throughout the procedures. A rectal temperature probe connected to a thermostatically controlled heat blanket was inserted to maintain a core body temperature of 37 ± 1 °C.

2.7.5. Laminectomy surgery

Once the animal was placed on the frame, the isoflurane was reduced to 2% for the remainder of the surgery. An incision through the fur and skin alongside the spinal cord from the middle of the shoulder-blades all the way to the hip was made using a no. 11 blade and scalpel. After the musculature beneath the skin was exposed and connective tissue was removed with

scissors, incisions on either side of the spinal column starting from the shoulder blades were made (approx. 1 mm from the spinal column and 6 - 7 cm in length). Once the incisions (approx. 5 mm in depth) were performed, the column was elevated using rat-tooth forceps and subsequently clamped into position for the laminectomy. A scalpel was used to make an incision in the muscle layer overlaying the spinal cord (T - 13 and L - 1 vertebrae). This layer of tissue was then removed by rongeurs, which then exposed two spinal processing underneath. The remaining muscle covering the vertebrae was cleared away using the rongeurs, which were then used to insert into the space between L1 - L2 vertebrae. The vertebrae were then carefully removed with rongeurs in order to expose the L4 – L5 region of the spinal cord for the intrathecal injection (Figure 2.5). Anaesthetic level was then reduced to 1.1 or 1% isoflurane over the course of 45 minutes to allow for anaesthetic stabilisation.

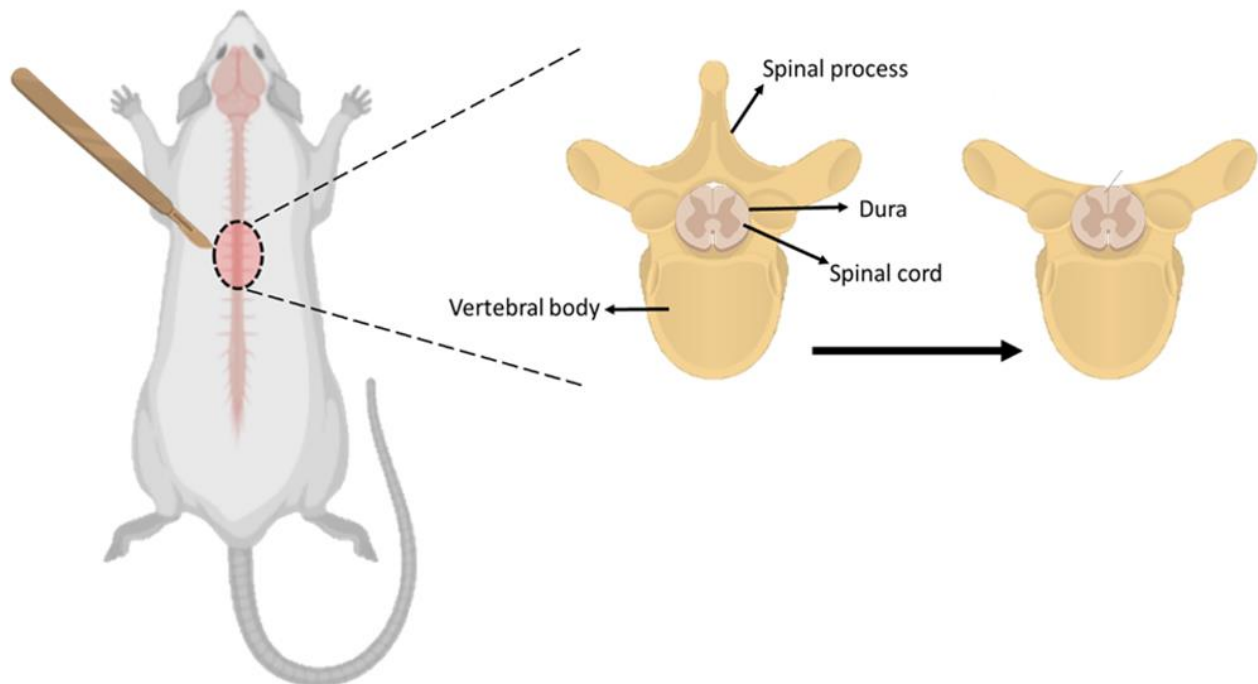


Figure 2.5. Schematic of the laminectomy surgery involving the removal of the spinal processes encompassing the spinal cord (schematic made using Biorender.com).

2.7.6. Electromyography (EMG) recordings

Following the application of hibiscrub, the fur was shaved off the biceps femoris muscle on the right thigh. A bipolar EMG electrode (modified 27-gauge needle with two wires running through; Ainswork, UK) was then inserted into the biceps femoris muscle. The electrode was connected to a NeuroLog head-stage (module NL100AK; Digitimer, UK) and the signals were amplified x2000 (module NL104A) before being filtered through a band-pass at 10 -1000 Hz

(module NL125). Data was analysed by LabChart software through a PowerLab acquisition unit (AD Instruments, UK).

2.7.7. Mechanical stimulation

Once anaesthetic level was stabilised at 1.1 or 1%, von Frey hairs (vFh) were used to evoke mechanical stimulation of the plantar hind paw. The vFh are used as a technique to measure mechanical sensitivities and threshold (Chaplan *et al*, 1994). This was achieved by applying vFh, a series of plastic monofilaments, to the plantar surface of the hind paw for 3 seconds in order to record the EMG response to the mechanical stimulation. The filaments used had logarithmically increasing bending force of 100, 180 and 300 grams, and were applied sequentially, from the lowest force to the highest. Each filament application was repeated 3 times during 1 min, with 1 min rest in between increasing filament bending forces. The EMG responses were analysed using LabChart (PowerLab, AD Instruments), and were displayed as the raw signal and the integral of the root mean square (RMS) of the raw EMG response. The area under the curve (AUC) of the RMS was then calculated and plotted against the Von Frey filament used to establish the reflex response of both the baseline (before drug administration via IT injection) and the measurements after injection. Following IT injection, EMG recordings were carried out every 10 mins for 30 mins, by stimulating plantar surface of the hind paw with 100, 180 and 300 g vFhs.



Figure 2.6. An example of the EMG activity in response to vFh stimulation. Represented as the raw signal (red) and the integral (RMS) of the raw signal (blue). The AUC of the RMS response is taken for each stimulation and the means and SEM are calculated.

2.7.8. Intrathecal injection

After baseline EMG responses were established, a Hamilton syringe coupled with 30 g needle was used to inject 15 μ L of formulations in the IT space on the contralateral side of the spinal cord, beneath the L4 – L5 segments. To minimise injection variability, a micromanipulator was used and the formulation was injected over the course of 1-2 minutes to reduce back-flow. The successful injection technique was subsequently investigated through *ex vivo* imaging techniques (described in section 2.8) or for biodistribution analysis and EMG (section 2.7 and 2.6.6).

2.8. Biodistribution analysis

2.8.1. Tissue collection

Rats were sacrificed by pentobarbital overdose (intraperitoneal injection, 200 mg/kg). Death was confirmed by exsanguination and tissues were collected for biodistribution. The spinal cord was exposed and cut into cervical, thoracic and lumbar segments according to their morphological neuroanatomy (Toossi *et al.*, 2021). The other tissues collected included the brain, liver, spleen and deep cervical lymph nodes. They were placed on ice straight away and stored at -80 °C until analysis. Blood was collected during exsanguination in an Eppendorf tube (1 mL) and was immediately centrifuged at 4 000 g for 5 minutes to separate out the serum. The upper serum layer was then collected and stored at -80 °C until analysis.

2.8.2. Analytical methods

Biodistribution of the CBD following injection was assessed by reverse-phase HPLC-UV. Tissues were weighed and diluted with HPLC water (1/4 dilution) and homogenised (Cole-Parmer, UK). 100 μ L of sample was spiked with 10 μ L internal standard (DDT, final concentration 5 μ g/mL). Calibration curve samples were prepared with blank homogenised tissues spiked with 10 μ L internal standard (DDT, final concentration 5 μ g/mL) and CBD calibration curve stock solutions (1 000 ng/mL to 10 ng/mL range, minimum of 6 samples per tissue).

450 µl of acetonitrile was added to samples to precipitate tissue proteins, followed by 450 µl HPLC water. Samples were placed in a vortex (IKA, Ultra-Turax, 1200 rpm) for 5 minutes to allow thorough mixing. 3 mL hexane was added for liquid-liquid extraction, after which samples were placed in a vortex again followed by centrifugation at 4 000 g for 10 mins. Samples were reconstituted in 100 µL mobile phase (62% ACN : 38% water), and 40 µL was injected in the HPLC and analysed with ACE C18-PFP (150 x 4.6 mm, 3 µm) coupled with an ACE C18-PFP 3 µm guard cartridge (Hichrom Ltd, UK). All samples were run for 30 mins at isocratic conditions with the mobile phase consisting of 62% ACN and 38% water at a flow rate of 1 mL/min. The column oven was set to 55 °C and the autosampler to 4 °C. The absorbance of analytes was monitored at 220 nm.

2.9. Statistical analyses

Statistical analysis of the data was carried out by t-tests, one-way ANOVA or two-way ANOVA with Sidak's or Tukey post hoc analysis, using GraphPad Prism v9.00. Significance was found when * $p < 0.05$. Data expressed as mean $\pm$ SD or SEM.

3. Chapter 3 – Development of CBD formulations for IT delivery

3.1. Introduction

Drug delivery to the IT space poses numerous advantages in the treatment of neuropathic pain, as extensively discussed in Chapter 1. However, despite many benefits attained by this administration technique, IT delivery can be limited by low drug bioavailability (due to enzymatic degradation), retention of drugs at the site of injection, high clearance and restrictions due to the anatomical constraints of the CNS. These issues can be addressed through the development of suitable IT formulations. In this chapter I discuss the development of different nanocarrier systems for CBD, for subsequent administration to the IT space to treat neuropathic pain. We focused on two different systems, a lipid-based nanoemulsion (nanoemulsion) and PCNPs, evaluating how the formulations perform as drug delivery system for the IT space. The oily droplets of the nanoemulsion means that the CBD, being a lipophilic drug, is fully dissolved within the particles, and subsequently stabilised by phospholipids. On the other hand, within the PCNPs, CBD is in a nanocrystal form surrounded by a polymeric shell, which controls the release profile of the drug from the core of the NP. These two formulations can be recognised as a reservoir system (lipid-based nanoemulsion) or as a polymer-coated CBD nanocrystal. The aims of this chapter are to develop and optimise these two different CBD formulations, investigate the optimum processing parameters and subsequently create stable formulations ready to be tested *in vivo*.

3.2. Nanoemulsions

Parenteral injectable lipid emulsions (intravenous fat) have been used in the clinic as an energy source for patients during the past 5 decades (Hippalgaonkar *et al.*, 2010). These emulsions represent a nutrition method for the critically ill, who are unable to be fed orally using triglycerides. Once administered by an intravenous (IV) catheter, the triglycerides within the emulsions undergo metabolism into essential fatty acids. The safety aspect of the starting materials utilised in the formulation of emulsions alongside their metabolism and utilisation

as a source of energy in the body is the main advantage of this type of vehicle in the field of drug delivery (Hörmann & Zimmer, 2016a). Another important aspect, which I will be focusing on in this Chapter, is the capability of acting as a drug delivery vehicle and dissolving water-insoluble drugs. Therefore, the solubilisation of drugs which readily dissolve in oil, such as CBD, makes their administration using a nanoemulsion possible (Zgair *et al.*, 2016).

3.2.1. Safety and toxicity

For safe IV delivery (according to USP <729>) the emulsion droplets must be below 5 µm, the size of the smallest blood vessels in the lung in order to avoid pulmonary embolisms. Moreover, no more than 0.05% of the emulsion must be above 5 µm in size (Hörmann & Zimmer, 2016b), hence why homogeneity and long-term stability of these formulations are essential. In order to abide by these safety regulations, emulsions are most often processed to create droplet sizes in the sub-micron size range, which are represented as nanoemulsions (Sutradhar & Amin, 2013). Nanoemulsions typically have an upper size limit of 300 nm as reported by various studies (Anton *et al.*, 2008). Therefore, the small lipid droplet size of nanoemulsions means that this type of formulation has a high surface area and good long-term stability. The emulsifiers on the surface of the nanoemulsion droplets act to reduce interfacial tension resulting in the formation of small formulations, which in turn increase their surface areas (Aswathanarayan *et al.*, 2019).

Nanoemulsions pose various advantages which indicate they are an ideal drug delivery system for IT administration. Numerous studies have demonstrated reduced pain and irritation upon injection and lower toxicity. An example is diazepam, which formulated using propylene glycol, ethanol and benzyl alcohol as vehicle gives pain and tissue irritation upon administration (Levy *et al.*, 1989). However, when formulated in an emulsion form (Diazemuls®) only 0.4% patients were reported to experience pain (von Dardel *et al.*, 1983). Reduced toxicity is also observed when marketed therapeutics are formulated in emulsion form. An example includes Paclitaxel (Taxol®) and Cyclosporine (Sandimmune® Injection) which are formulated in Cremophor® EL and ethanol. These excipients lead to bronchospasms and nephrotoxicity amongst many other side-effects (Constantinides *et al.*, 2000). On the other hand, when cyclosporine is in emulsion form no nephrotoxicity is observed in the clinic (Tibell *et al.*, 1993). It is also essential to note that emulsions allow for tolerability of higher therapeutic doses,

which is the case with paclitaxel. Mice were observed to tolerate a dose of paclitaxel 3.5-fold higher than Taxol® (Constantinides *et al.*, 2000). Another crucial factor is the increased stability and solubility of drugs in emulsion formulations, as this is the problem associated with most lipophilic therapeutics. It is also suggested that their stability is improved as a result of a lower potential for oxidation or hydrolysis in the oily core (Eccleston *et al.*, 2007). This is achieved through preventing the drugs from freely dissolving in the aqueous environment of the body, thus being exposed to enzymatic degradation for longer periods of time (Lovelyn *et al.*, 2011).

3.2.2. Formulation processes

Various high-energy processes can be utilised to decrease the size of emulsions, including ultrasonication, microfluidization and high-pressure homogenisation. Alternatively, low-energy processes can also be employed, most specifically, low-energy emulsification (phase inversion temperature, solvent diffusion, phase inversion composition) through which the chemical potential of the surfactants is used for creating small droplet sizes (Hippalgaonkar *et al.*, 2010; Maali & Mosavian, 2013; Sutradhar & Amin, 2013). The process employed in this thesis was the use of microfluidization, due to previous reports in the literature regarding the effective generation of homogenous formulations, high reproducibility and efficiency in decreasing particle sizes (Silva-Avellaneda *et al.*, 2021).

3.2.3. Nanoemulsions as drug delivery systems

The first experiment involving the administration of oil parenterally, took place in the 17th century, when William Courten injected 1 g of pure olive oil / kg to a dog, who later died, undoubtedly as a result of a pulmonary embolism (Raman *et al.*, 2017). Subsequent research led to the development of the first approved IV emulsion, Intralipid® (Tamilvanan, 2009). This oil-in-water (O/W) nanoemulsion contains either 10 or 20% soybean oil as the lipid phase, 1.2% egg phospholipids acting as the surfactant, 2.5% glycerol for formulation tonicity and sodium hydroxide to adjust the pH (Figure 3.1). The soybean oil has a high content of C18 fatty acids and is therefore a long chain triglyceride (LCT). In an O/W nanoemulsion, the oil phase is dispersed in the continuous water phase by an amphiphilic emulsifier, which is localised at the oil and water interface protecting the droplets from coalescing, and thus leading to instability in the system (Figure 3.2). Many other ingredients can be added to

nanoemulsions, such as co-emulsifiers and / or synthetic PEGylated surfactants, other oil mixtures containing both medium and long chain triglycerides), stabilisers such as EDTA or antioxidants (Hörmann & Zimmer, 2016b; Maali & Mosavian, 2013).

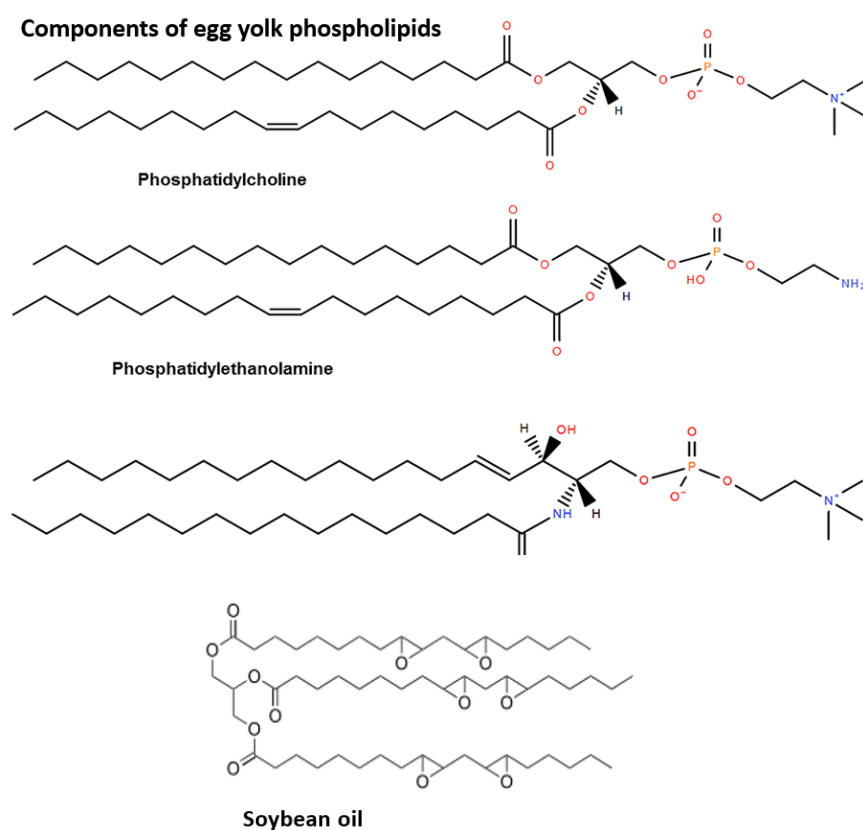


Figure 3.1. Chemical structures of egg yolk phospholipids components (phosphatidylcholine, phosphatidylethanolamine and sphingomyelin) and soybean oil. These are the lipid phase and emulsifier components of the commercially available Intralipid® nanoemulsion.

Recently, there has been an added interest in using lipid emulsions as a drug delivery platform for lipophilic therapeutic agents as a result of their biocompatibility and low-toxicity, which is imperative for IT delivery, as extensively discussed in Chapter 1. These types of formulations have been previously used to aid the delivery of insoluble lipophilic drugs or therapeutics which are prone to chemical instability (enzymatic degradation, hydrolysis) (Bonferoni *et al.*, 2019). Furthermore, the dispersion of therapeutics in lipids is also a known method of enhancing their delivery by increasing their subsequent residence at the site of injection. This is achieved by decreasing the elimination of drugs through their sequestration within the

formulation, in addition to binding to fatty tissues within the body (Leal-Calderon & Cansell, 2012). Moreover, the biodistribution can also be impacted, because if the lipophilicity of the drug is high, it will be preferentially retained in the oil droplet preventing its fast release into the blood (Ganta *et al.*, 2008), or into the CSF in the case of IT administration. Takino *et al.*, suggested that a $\log P > 9$ is necessary to retain the drug within the lipid phase. It would therefore be interesting to further explore the biodistribution profile exhibited by CBD, especially in the CNS, when formulated in a nanoemulsion.

On the other hand, possible disadvantages of nanoemulsion formulations are the short circulation times in the body when administered systemically, due to the fast metabolism of the formulation components. This can however be tackled through the PEGylation of the lipid droplets surface and / or the size reduction of the particles (Hörmann & Zimmer, 2016b). Nonetheless, in comparison to solid NPs, the half-time of nanoemulsions is still short due to these issues.

Considering the potential benefits of nanoemulsions outlined above, and the safety profiles of these types of formulations, I aimed to optimise microfluidizer processing parameters to create a stable and homogenous nanoemulsion formulation, recreating the commercially available Intralipid® nanoemulsion. I then associated CBD to the oil droplets of microfluidized Intralipid nanoemulsion, through the optimisation of the association process based on high-speed ultracentrifugation. Considering the physico-chemical properties ($\log P$ 6.3) of CBD, its association with the soybean lipids droplets of the nanoemulsion will lead to it being solubilised in the oil core, which will then be stabilised by phospholipids (Figure 3.2).

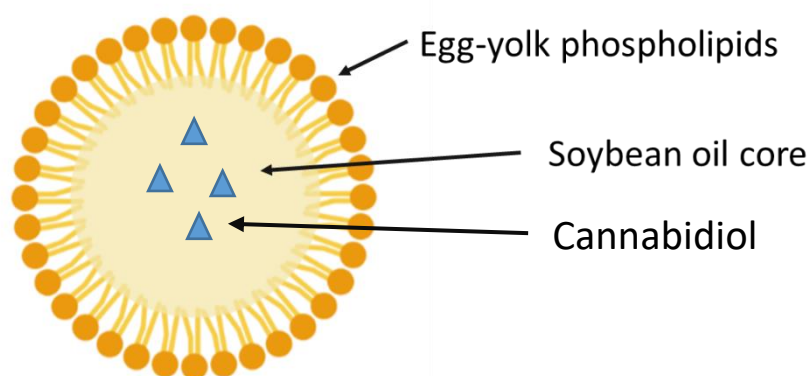


Figure 3.2. A schematic of the structure of an O/W nanoemulsion droplet, representing the soybean oil core surrounded by phospholipids on the outer layer, acting to stabilise the lipid. Cannabidiol is localised to the soybean oil core (Image created with Biorender.com).

3.3. Polymer-coated nanoparticles (PCNPs)

A completely different drug delivery system in comparison to nanoemulsions are NPs. These also possess some of the advantages highlighted in the section above, such as the ability to enhance bioavailability and increase the therapeutic efficacy of water-insoluble drugs. However, they have different physico-chemical properties, in terms of composition and surface chemistry. NPs are usually smaller in size but can range between 10-500 nm in diameter (Nishiyama & Kataoka, 2006). PCNPs, most specifically those based on amphiphilic block copolymers, have become a very popular drug delivery system in the last few decades (Abushrida *et al.*, 2020; Fessi *et al.*, 1989). This type of formulation consists of a pure drug nanocrystal within the core, surrounded by a polymeric coating (Figure 3.3). As amphiphilic polymers have both a hydrophobic and hydrophilic block, it is assumed that the hydrophobic block would interact with the drug core (Styliari *et al.*, 2020). In contrast, the hydrophilic outer layer would allow for biodistribution within an aqueous environment (Kataoka *et al.*, 2001), and provide protection from degradation. This in turn further promotes the drug's bioavailability and increases its therapeutic effect (Soppimath *et al.*, 2001; Owens *et al.*, 2006).

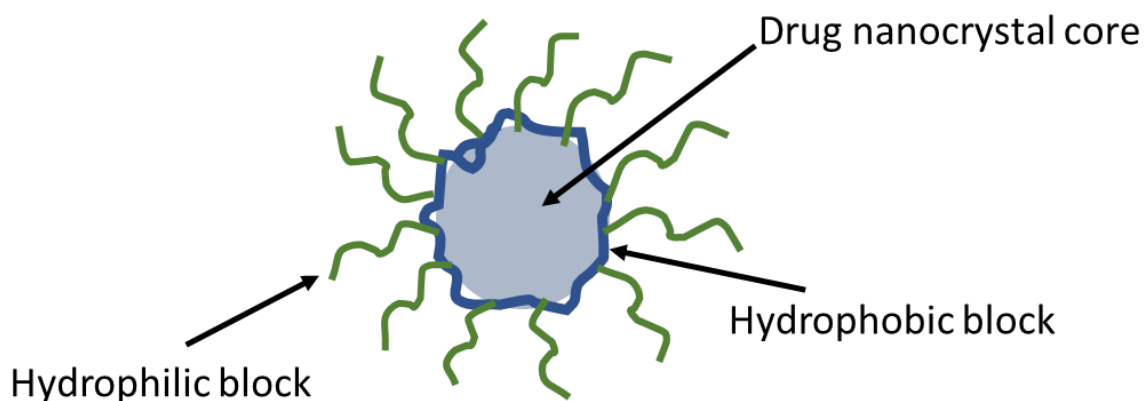


Figure 3.3. A schematic of a polymer coated nanocrystal made using the double nanoprecipitation technique. Diagram shows the drug nanocrystal core (light blue) in the middle surrounded by the hydrophobic PLA chains (dark blue) and the hydrophilic PEG chains (dark green) on the outer layer of the NP providing steric stabilisation.

One such polymer is poly(lactic acid) (PLA), which is ideal for delivery due to its biocompatible and biodegradable properties. Many studies involving PEG₅₀₀₀-PLA with various PLA chain lengths have been reported, in order to evaluate how this influences the self-assembly but not deposition of a polymer coating for a PCNP. For example, Riley *et al.*, have found that the NP size was dependant on copolymer architecture and scaled linearly following $N^{\frac{1}{3}}PLA$ (number of monomeric units in the PLA block) (Riley *et al.*, 2001). It is therefore evident that the NPs can be optimised based on the choice of polymer coatings, an aspect which will be evaluated in this Chapter.

3.3.1. Formulation techniques

The production of PCNPs can be achieved in various ways, with the most popular technique being nanoprecipitation (also known as the interfacial deposition method) (Fessi *et al.*, 1989). The first step in this method involves the solubilisation of the drug in an organic solvent (this solvent must be chosen based on full solubility of drug and the amphiphilic polymer). The second step involves an aqueous phase, in which both the drug and the polymer are insoluble (water is most commonly used). At this stage it is essential to select an organic solvent which is miscible with the aqueous phase.

The most common nanoprecipitation method is co-precipitation, where the organic phase (containing the drug and polymer) is added to the aqueous phase. During this process, the hydrophobic forces lead to the polymer collapsing around the drug forming an NP. This method is called interfacial deposition and was initially introduced by Fessi *et al.*, using acetone as the organic phase and PLA as the polymer, in order to prepare indomethacin NPs (Fessi *et al.*, 1989). Through this process, the drug molecules are encapsulated in a polymeric matrix, thereby forming NPs. An alternative method is the double nanoprecipitation technique, where the first step involves the creation of drug nanocrystals by precipitating the therapeutic in the aqueous phase, followed by a second step in which the polymer, previously dissolved in organic phase, is added to the aqueous phase already containing the nanocrystals. Therefore, this process also allows for the hydrophobic forces to lead to the polymer collapsing around the drug forming an NP (Valdeperez *et al.*, 2017).

3.3.2. Formulation optimisation

There are various critical parameters in this formulation process, which have been extensively discussed by Oliviera *et al.*, where they reportedly used PLGA to form NPs, assessing different polymer concentrations, organic solvents and temperatures. They found that the size of the NPs can be optimised by adjusting the processing parameters, such as changes in organic solvents or polymer concentrations (Oliviera *et al.*, 2013). This highlights the fact that the final formulation is easily tuneable by processing parameters, which can be investigated to establish which factors generate the most reproducible and stable NPs, with the highest drug loading. The variables investigated through DOE were CBD and tPEG concentrations (range between 1 – 2.5 mg/mL), solvent to aqueous ratio (from 1:4 to 1:6).

3.3.3. Safety and toxicity

The safety of these NPs is imperative, especially for administration to the IT space, as previously discussed. The PEG and PLA synthetic polymers have been approved by the US FDA and EMA as a result of their safety profiles and confirmed biocompatibility, non-immunogenicity and biodegradability during *in vivo* studies (Chenthamara *et al.*, 2019; Lombardo *et al.*, 2019). Moreover, the same polymeric backbone comprised of PEG-PLA has been used for the formulation of Genexol®, a therapeutic formulation approved in South Korea for the treatment of breast cancer (Lee *et al.*, 2017).

We decided to focus on the formulation of polymer coated CBD NPs, as the basis of this drug delivery system is completely different from the other developed CBD formulation, the nanoemulsion. Moreover, the nanoprecipitation technique is an attractive method of developing novel NP formulations due to its scalability, cost-effectiveness and speed. Additionally, the nanoprecipitation process parameters can be easily tuned to produce personalised NP formulations according to the drug of interest. Therefore, I set out to trial three PEG-PLA amphiphilic block polymers synthesised by Vasey *et al.*, in order to formulate polymer-coated CBD NPs: PLA₅₀-PEG₄₀₀₀-PLA₅₀, mPEG₅₀₀₀-PLA₁₀₀ and 3-armPEG₁₀₁₄-PLA₁₀₀ (tPEG) (Vasey *et al.*, 2019).

In this chapter, the two different methodologies for formulating drug delivery systems for CBD for IT administration are discussed. We evaluate how a thermodynamically stable CBD nanoemulsion has been developed, alongside the optimisation of the nanoprecipitation technique to form polymer-coated CBD NPs.

3.4. Chapter objectives

- Define microfluidizer parameters necessary for the processing of Intralipid® emulsion into an nanoemulsion
- Associate CBD with the nanoemulsion by “extemporaneous” addition and optimise the ultracentrifugation association procedure to develop a stable formulation
- Identify amphiphilic polymers which create a stable and homogenous polymer coating for CBD NPs
- Optimise nanoprecipitation procedure for the development of CBD NPs
- Quantify amount of CBD incorporated in nanoemulsion and NPs
- Evaluate the stability of both CBD nanoemulsion and NPs formulations

3.5. Results and discussion

3.5.1. Nanoemulsion formulation

Commercially available Intralipid® (Sigma-Aldrich, UK) was used for incorporation of CBD (THC Pharm, Germany) to create a lipid-based delivery system for IT administration. This emulsion has been proven to have a very safe profile as it has been used as a parenteral nutrition method for patients since the 1970s. I decided to recreate the emulsion utilising microfluidizer technology and investigated the optimisation process parameters. This method would have allowed us to associate CBD with the lipid droplets by the “*de novo*” incorporation method, through the addition of the drug directly to the oil phase prior to homogenisation with the phospholipids and the aqueous phase. The development of my own nanoemulsion processing methodology offered us an alternative for the association procedure with CBD in case the ultracentrifugation protocol (discussed in section 2.2.2) utilising the “*extemporaneous addition*” method would have not created a stable and homogenous nanoemulsion. Alongside the commercially available nanoemulsion formulation Intralipid® I also generated an additional emulsion containing an anionic surfactant (sodium oleate B), which I named Lipoid, to investigate whether this can increase the stability of the formulation.

The goal in optimising the processing parameters was to manufacture a monodisperse nanoemulsion with small mean droplet size of 100 – 500 nm, as this improves physical stability (Eccleston *et al.*, 2007; Tadros *et al.*, 2004). Prior to the processing of formulations by microfluidization, the droplet size of emulsion was large with high standard deviations, the oil and aqueous phases separated and the formulation was physically unstable (Table 3.1).

Table 3.1. *The particle size and PDI of emulsions after benchtop homogenisation and before microfluidization. Prior to processing these emulsions were coarse and unstable leading to their phase separation (microfluidized nanoemulsions covered in Section 3.5.1.1.).*

Formulation	Particle size (d.nm)	St. Dev	PDI
20% Intralipid	1 280	210	0.736
Lipoid	1 662	160	0.989

Following microfluidization the two coarse and unstable emulsions which phase separated (Figure 3.4) were shown to be stable with homogenous particle population ranging in size between 148 and 274 nm (data for microfluidized emulsions shown in Figure 3.5).

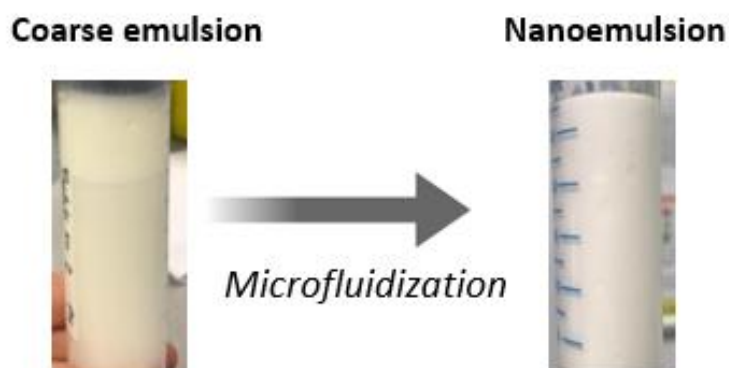


Figure 3.4. Emulsion physical stability after microfluidization. Coarse emulsion particle size decreases after microfluidization and this was demonstrated by the stability of the formulations over time and the lack of phase separation.

3.5.1.1. Microfluidizer optimisation

Microfluidizers are high-pressure homogenisers composed of a high-pressure pump connected to an interaction chamber. Typically, coarse emulsions are fed through the system by entering an inlet reservoir, and then accelerated by the pump into an interaction chamber. Once emulsions enter the interaction chamber, they are split into two streams which are impinged towards each other causing shearing of oil particles (Jafari *et al.*, 2007). During this process, oil droplets are disrupted and their specific surface area is increased (Jafari *et al.*, 2008). These newly formed oil droplets are subsequently stabilised by emulsifiers / phospholipids. Various factors influence the properties of nanoemulsions including the type of interaction chambers, microfluidization cycles, pressure and temperature. Typically, an increase in pressure and cycles leads to smaller particle size as a result of the disruption and reformation of the lipid droplets (Clements *et al.*, 2011). In this case, 4 cycles were carried out for each processing pressure, as the literature suggests this is when Intralipid reaches a minimum particle size and no reduction in droplet diameter is observed with further processing (Washington *et al.*, 1988). For the processing of the coarse emulsions, it was required to identify the optimum microfluidizer pressure. A range between 10 000 to 21 000

PSI was tested followed by characterisation of emulsion for particle size and polydispersity. I found that 21 000 PSI generated the smallest particle size of 228 nm and a PDI of 0.107 (Figure 3.5). The size of Intralipid nanoemulsion processed at 21 000 PSI was significantly smaller than the size of the nanoemulsion processed at 12 000 PSI, ($p < 0.0001$) and there were no significant differences observed in the PDIs of the formulations.

Considering that a 21 000 PSI processing pressure resulted in the smallest particle size and PDI of the Intralipid nanoemulsion, this parameter was used for further processing of the Lipoid nanoemulsion. After microfluidization, the Lipoid nanoemulsion had a particle size of 148.9 nm and PDI of 0.12. The smaller particle size as compared to Intralipid was probably due to the addition of sodium oleate B, an anionic surfactant, which stabilises particles by electrostatic repulsion mechanisms in addition to stabilisation by steric repulsion (Araujo – Neto *et al.*, 2014). It is also possible that this was due to the ability of this surfactant to rapidly adsorb onto the surface of the lipid droplets during microfluidization (Jafari *et al.*, 2011).

Reports in the literature suggested that nanoemulsion particle size tends to decrease with an increase in the number of microfluidization cycles, however, in the case of certain emulsifiers such as biopolymers, this can also lead to an increase in particle size due to “over-processing”. This can happen because of the high shear or turbulence leading to mechanical damage (Clements *et al.*, 2011; Jafari *et al.*, 2006). Nonetheless, this effect was not observed in this case of the Intralipid nanoemulsion, indicating that the emulsifiers used in this process are effective at stabilising the lipid droplets and preventing coalescence at the pressures tested. To ensure that this stability remained within the colloidal nanoemulsion systems, physical characterisation was carried out 7 days later, after storage at 4 °C. No significant changes in size nor PDI were observed in the Intralipid nanoemulsions (Figure 3.5). The PDI of all formulations remained well below 0.2, which is indicative that all nanoemulsions processed through the microfluidizer were stable upon short term storage.

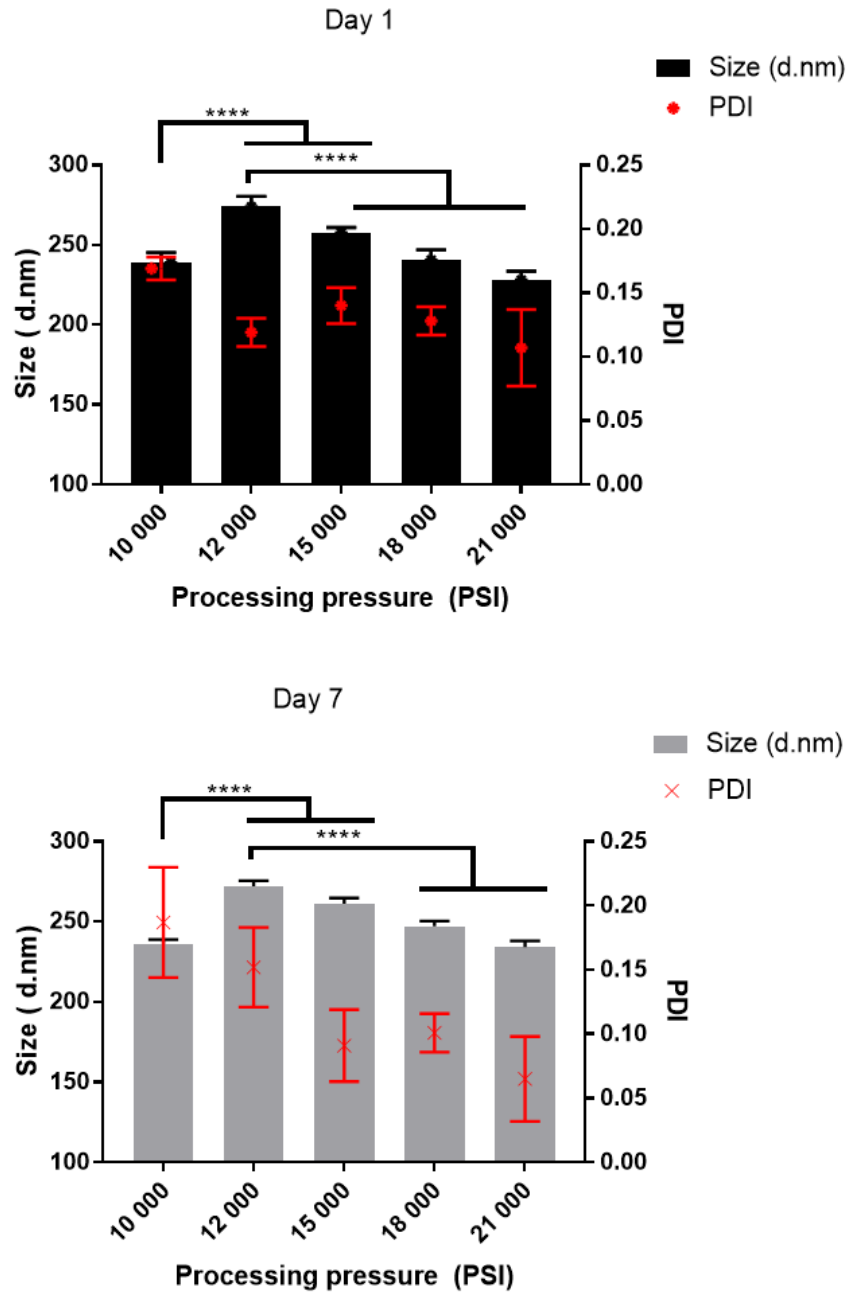


Figure 3.5. The particle size and PDI of the Intralipid nanoemulsion processed by the microfluidizer. Processing was carried out at various pressures until the smallest particle size and PDI similar to data in the literature was achieved (Washington and Davis, 1987), means $\pm$ SD (n=3), analysed by one-way ANOVA followed by Tukey's multiple comparisons test.

3.5.1.2. Zeta-potential

Zeta-potential (ZP) measurements of microfluidized nanoemulsions were carried out in order to ascertain their physical stability. Generally, ± 30 mV is indicative of good stability as a result of resistance to aggregation and interaction with proteins in vivo (Freitas *et al.*, 1998).

The ZP of the microfluidized formulations were compared against the commercially available Intralipid® nanoemulsion. Overall, the microfluidized nanoemulsions exhibited a significantly lower ZP (ranging between -31.9 to -37.3 mV) in comparison to the commercially available Intralipid® which measured at -51.4 mV. The Intralipid nanoemulsions processed at 21 000 and 18 000 PSI had a significantly lower ZP than the nanoemulsion processed at 10 000 PSI (Figure 3.6). This could indicate that a higher processing pressure might lead to non-uniform coating of the lipid droplets by the egg-yolk phospholipids during the re-formation process as previously reported by Jafari *et al.*, 2006. A lack of emulsifier on the surface of the lipid droplets may lead to stability issues, especially after long-term storage. The Lipoid nanoemulsion was not significantly different to the Intralipid nanoemulsion processed at the same pressure (21 000 PSI) demonstrating that the anionic surfactant did not influence the surface charge of this formulation.

These results indicated that the commercially available Intralipid® nanoemulsion was most stable at the point of testing, as a more negative charge is indicative of increased repulsion between lipid droplets, successfully preventing coalescence and aggregation over time. This is particularly important for the use of this formulation as a drug delivery system to solubilise CBD using my ultracentrifugation association protocol, during which the formulation undergoes very rapid centrifugation, which may lead to phase separation.

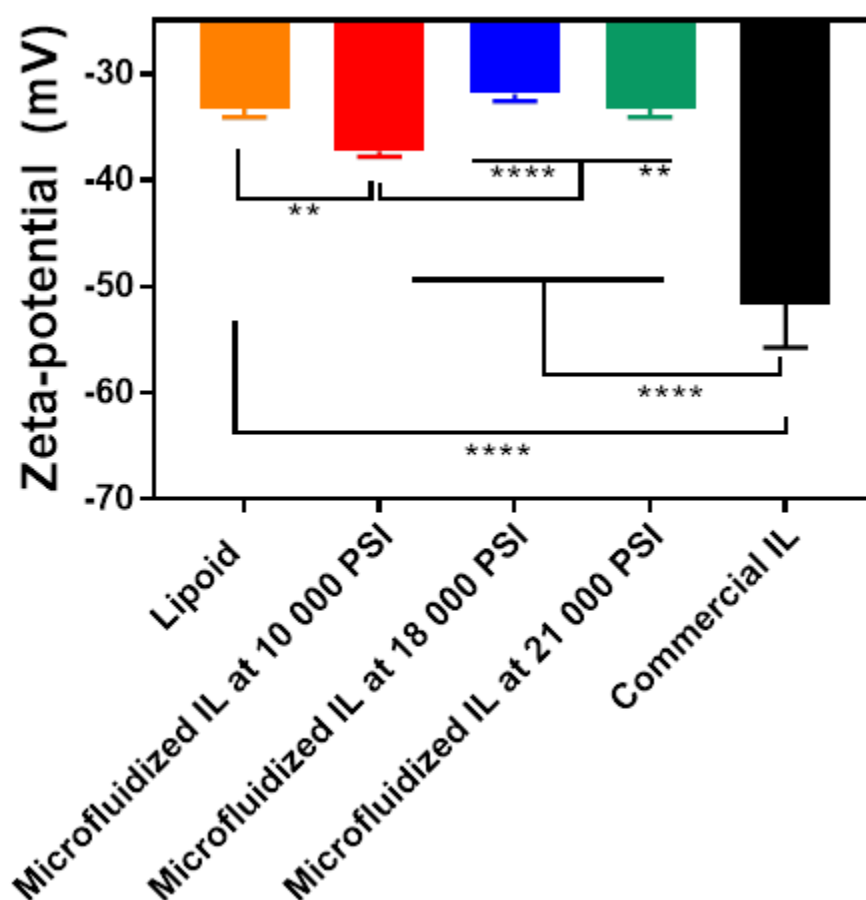


Figure 3.6. The ZP measurements of microfluidized Intralipid (IL) and Lipoid nanoemulsions alongside the commercially available Intralipid nanoemulsion. Results shown as intensity distribution traces of ZP measurements and as mean values represented as bar charts. Means $\pm$ SD (n=3), analysed by one-way ANOVA followed by Tukey's multiple comparisons test.

3.5.1.3. Optimisation of CBD association with Intralipid nanoemulsion

I decided to utilise the “extemporaneous addition” method to incorporate CBD in the lipid droplets of the nanoemulsion. I initially trialled this procedure with the commercially available Intralipid® nanoemulsion and assessed the association efficiency and stability of the formulations after separation of free drug through density gradient ultracentrifugation. This procedure is an effective separation and purification technique for nanoparticles and chylomicrons (Li *et al.*, 2018), whereby the gradient aids the preservation of the particle structures (Fernandez *et al.*, 2016).

Density gradient ultracentrifugation separated the lipid droplets (containing CBD) from freely dispersed drug in the aqueous phase. Initially, the density of emulsions was modified to 1.1 g/mL to allow the build-up of gradient solutions with subsequent lower densities layered on top. Following ultracentrifugation, the soybean oil particles, with a reported density of 0.917 g/mL (Noureddini, 1992) would rise to the top of the ultracentrifugation tubes allowing for easy sampling of CBD containing lipid droplets.

Initially I used PBS as the gradient solution for the association protocol. In order to ensure the isotonicity of the formulation for later *in vivo* testing, the solutions were swapped for 0.9% sodium chloride (Saline; Sigma-Aldrich, UK) with the uppermost layer being isotonic saline. This ensured that upon collection of nanoemulsion droplets containing CBD, the aqueous phase is composed of isotonic saline and is ready for injection.

I initially associated CBD with the nanoemulsion at 39 600 rpm for 35 mins, and observed the occurrence of phase separation during ultracentrifugation. We then optimised the association parameters using DOE (Design expert, Stat-Ease, USA). DOE results indicated that the optimum ultracentrifugation parameters in order to achieve PDI < 0.2 (homogenous population) and particle size < 300 nm (no significant increase in particle size compared to blank control) with CBD loading of around 1 mg/mL were 20 000 rpm, run time of 30 mins and saline used as the gradient solution.

At 39 600 rpm ultracentrifugation speed, the PDI of emulsions were above 0.2 for all conditions tested at various run times and gradient solutions (Figure 3.7). However, when the speed was reduced to 20 000 rpm, the PDI decreased to 0.2 for saline at 10- and 20-mins ultracentrifugation time. At 30 mins run time, the PDI of emulsions was 0.18 which was lower than emulsions processed at 39 600 rpm which had a PDI of 0.28. The increase in PDI at a higher ultracentrifugation speed was probably due to the higher energy at which the formulations are subjected leading to the oil phase separating from the aqueous phase.

The concentration of CBD in emulsions was highest when the ultracentrifugation time was 30 mins as opposed to 20 and 10 mins when using both saline and PBS. This could be due to the longer time allowed for lipid droplets containing CBD to rise to the top thus increasing the chance of separation. As a result of this, an ultracentrifugation time of 30 mins was selected for further experiments and for the preparation of formulation for *in vivo* testing. Under these

conditions, the particle size of the nanoemulsion was 286.8 ± 13.46 nm which was not significantly different from the particle size of Intralipid® prior to ultracentrifugation 291.5 ± 10.5 nm.

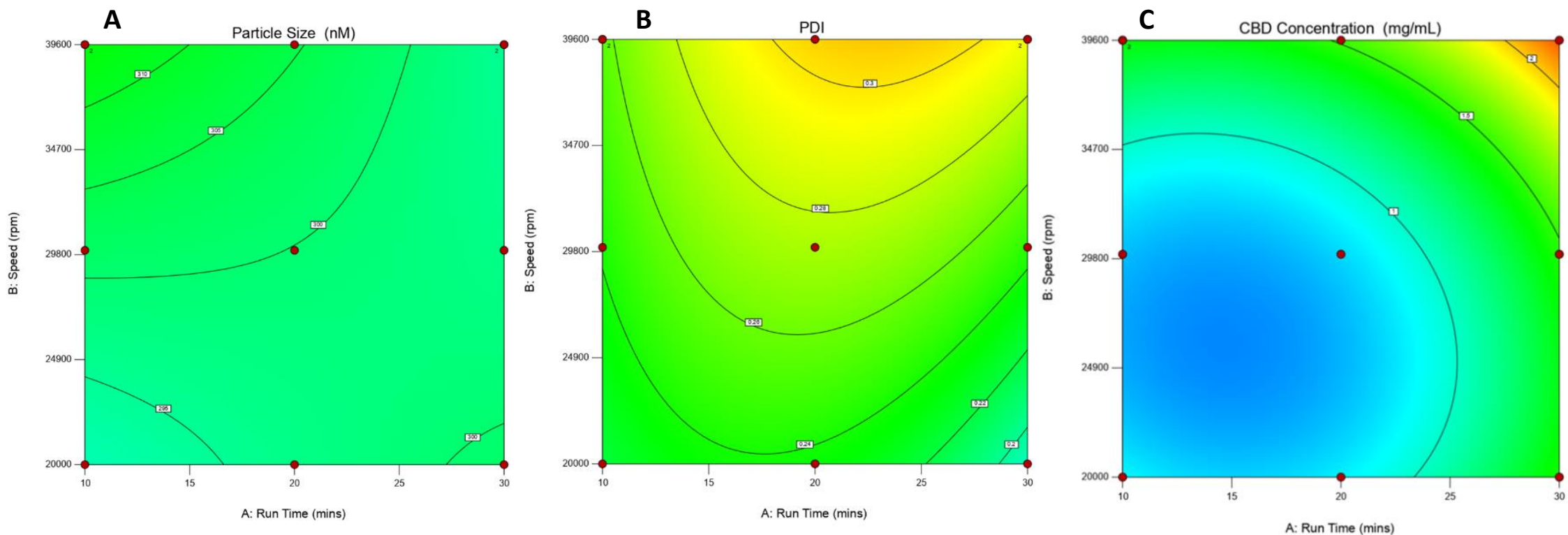


Figure 3.7. DOE optimisation for the association of CBD with Intralipid nanoemulsion through density gradient ultracentrifugation. Contour plots of A. particle size, ultracentrifugation speed and run time, B. PDI, ultracentrifugation speed and run time, C. CBD concentration, ultracentrifugation speed and run time. Lowest particle size and PDI of the nanoemulsion was observed at 20 000 rpm with a run time of 30 mins, at which the concentration of CBD quantified was 1.28 mg/mL (values of the variables investigated are referred to in the Methods section 2.3.3).

3.5.1.4. Quantification of CBD in nanoemulsion

Following the density gradient centrifugation procedure, the amount of CBD associated with oil droplets within the nanoemulsions was quantified by reverse-phase HPLC. Briefly, the samples were diluted in ethanol to completely solubilise the nanoemulsion, followed by dilutions in mobile phase (68% ACN , 32% water).

Dichlorodiphenyltrichloroethane (DDT) was used as an internal standard (IS) and results were analysed at 220 nm absorbance. Linear regression was determined based on the area ratio of CBD peaks and the IS peaks vs the concentration of the CBD calibration standards. CBD eluted at minute 8.7 and the IS at minute 20.4 (Figure 3.8). The calibration curve for CBD was shown to be linear with an r^2 coefficient of > 0.99 in the range of 0.5 to 10 $\mu\text{g/mL}$ (Figure 3.9).

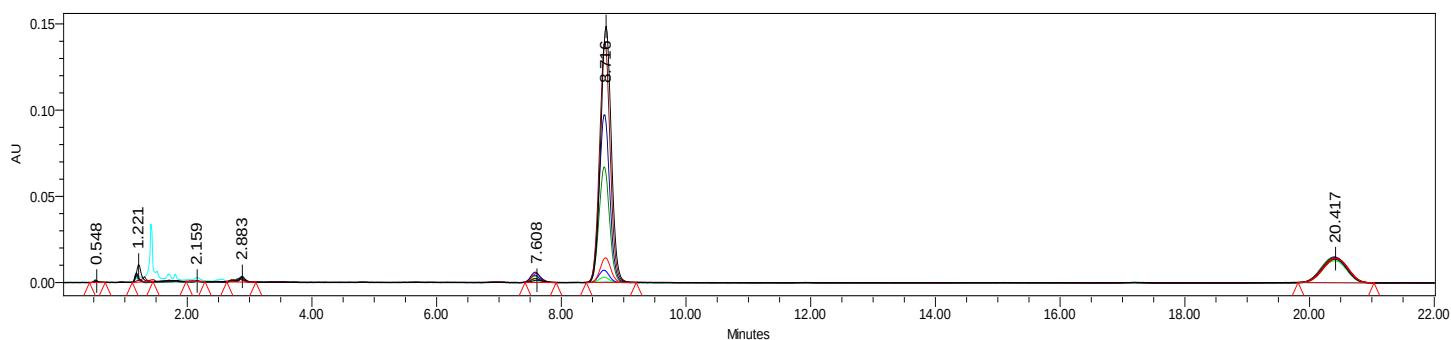


Figure 3.8. Chromatogram of CBD calibration curve for reference. The elution of CBD at minute 8.7 and the IS (DDT) at min 20.4. Total run time for the quantification of CBD in formulation samples was 22 mins.

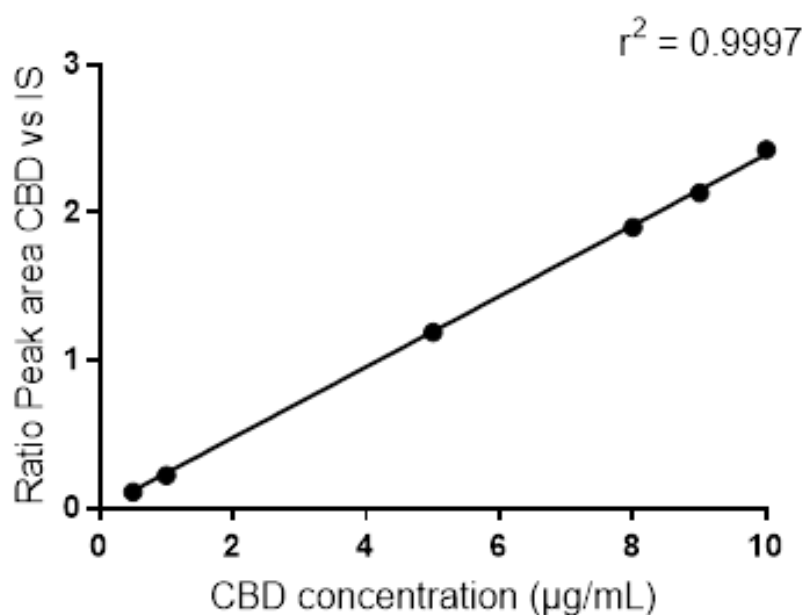


Figure 3.9. Calibration curve of CBD plotted as area ratio of CBD vs IS against the nominal concentration of CBD injected in the HPLC system (r^2 coefficient of 0.9997). The equation of the line of the calibration curve was used to calculate unknown concentrations of CBD.

The concentration of CBD detected in commercially available Intralipid® nanoemulsion was 1.28 mg/mL resulting in a 65.1% association with lipid phase (from a starting concentration of 2 mg/mL CBD in nanoemulsions). The concentration of CBD in the microfluidized Intralipid nanoemulsion was not significantly different at 1.62 mg/mL, with an association of 80.95%. On the other hand, the amount of CBD quantified in the Lipoid nanoemulsion was significantly lower than both the other formulations, with 0.28 mg/mL (14%) CBD quantified (Figure 3.10). It was observed that with Lipoid, the oil phase tended to separate more from the aqueous phase as a coarse emulsion prior to microfluidization. This then made it difficult to microfluidize the entire oil component in with the emulsion due to the soybean floating at the top of the inlet reservoir during feeding. To ascertain whether this is the reason for poor loading in this formulation triglyceride measurement should be considered so the total amount of the oil phase in the fine emulsion is quantified.

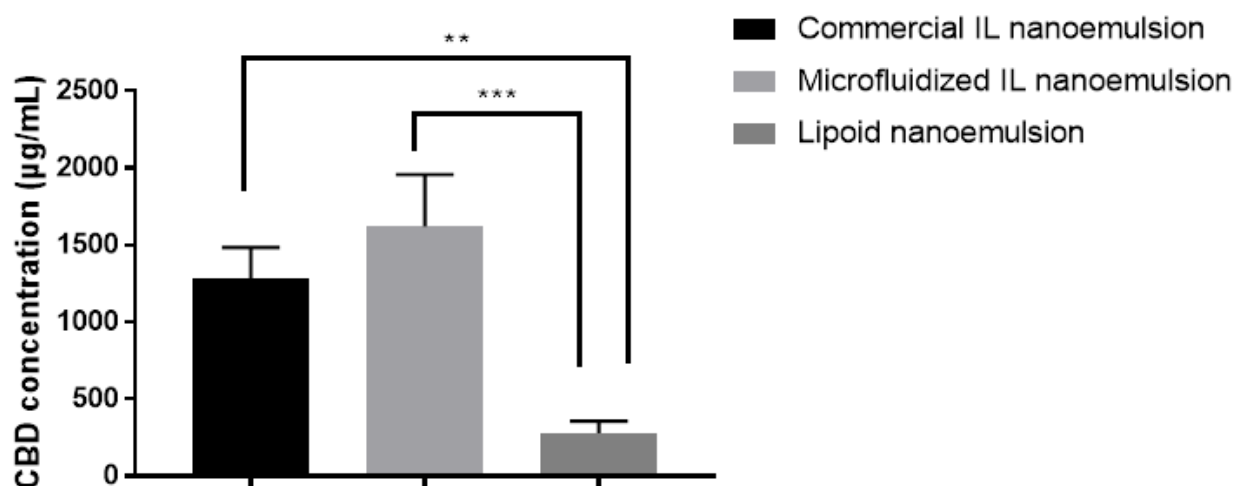


Figure 3.10. Concentrations of CBD quantified in formulations after the ultracentrifugation association protocol ($n=3$, means $\pm$ SD). The concentration of CBD quantified in the Lipoid nanoemulsion was significantly lower than the concentration of CBD in the commercial IL nanoemulsion (** $p < 0.01$) and the microfluidized IL nanoemulsion (** 0.001).

Overall, CBD association with lipid phase of both commercial and microfluidized Intralipid is in the desired concentration range (Figure 3.10), as the literature suggests that 1 mg/mL CBD is required for therapeutic potency, where an injection of dehydroxyl-CBD lead to a significant suppression of inflammatory pain after plantar administration of CFA in rats (Xiong *et al.*, 2012).

Other nanoemulsion formulations have been reported in the literature, with similarly high drug loading capabilities. Sumatriptan, a poorly soluble drug, was formulated in copaiba oil stabilised by biopolymers (xanthan, pectin and alginate) nanoemulsion, for intranasal delivery, resulting in 41-69% encapsulation efficiency (Ribeiro *et al.*, 2020). In another study by Sandip *et al.*, gadolinium was loaded in nanoemulsions based on pine-nut oil phase for the purpose of anti-cancer delivery and magnetic resonance imaging, leading to a 20% encapsulation efficiency (Sandip *et al.*, 2006). Achyrocline satureioides were also loaded in nanoemulsions for the treatment of methicillin-resistant *Staphylococcus aureus*, leading to > 94% encapsulation efficiency through high pressure homogenisation using flaxseed oil and Tween 80 as a stabiliser (Machado *et al.*, 2020). These studies show that there is quite a variation in encapsulation efficiencies in nanoemulsion formulations, most probably due to a variety of processing parameters and mixtures / ratios of oils and surfactants. Nonetheless, the encapsulation / association efficiency % of CBD in the Intralipid® nanoemulsions

presented in this work are within an acceptable range for progression to *in vivo* studies for the evaluation of their analgesic potential and biodistribution after IT administration.

3.5.1.5. CBD nanoemulsion physical characterisation

I established that adding CBD to the commercially available nanoemulsion through the ultracentrifugation association protocol did not have a significant effect on particle size nor PDI (Figure 3.11), after the optimum processing parameters had been screened through DOE. The addition of CBD to nanoemulsion did not cause a significant change in particle size nor PDI as analysed by DLS. The size of CBD nanoemulsion was 286.8 ± 13.46 nm with a PDI of 0.135, indicative of a homogenous formulation.

A single particle size population was shown by DLS analysis, indicating that the CBD was fully solubilised within lipid droplets and the formulation was not coalescing or aggregating. Nonetheless, when the same ultracentrifugation parameters were used for the association of CBD with the other nanoemulsion formulations (microfluidized Intralipid and Lipoid), the addition of the drug led to a significant increase in particle size for both formulations ($p < 0.001$) and PDI for Lipoid ($p < 0.001$) (Figure 3.11). This indicated that the size of the lipid droplets aggregated in the presence of CBD, suggesting instability of the colloidal system. As a result of these data, only the commercial Intralipid® nanoemulsion loaded with CBD was taken forward for particle size stability testing and *in vivo* administration discussed in Chapter 5.

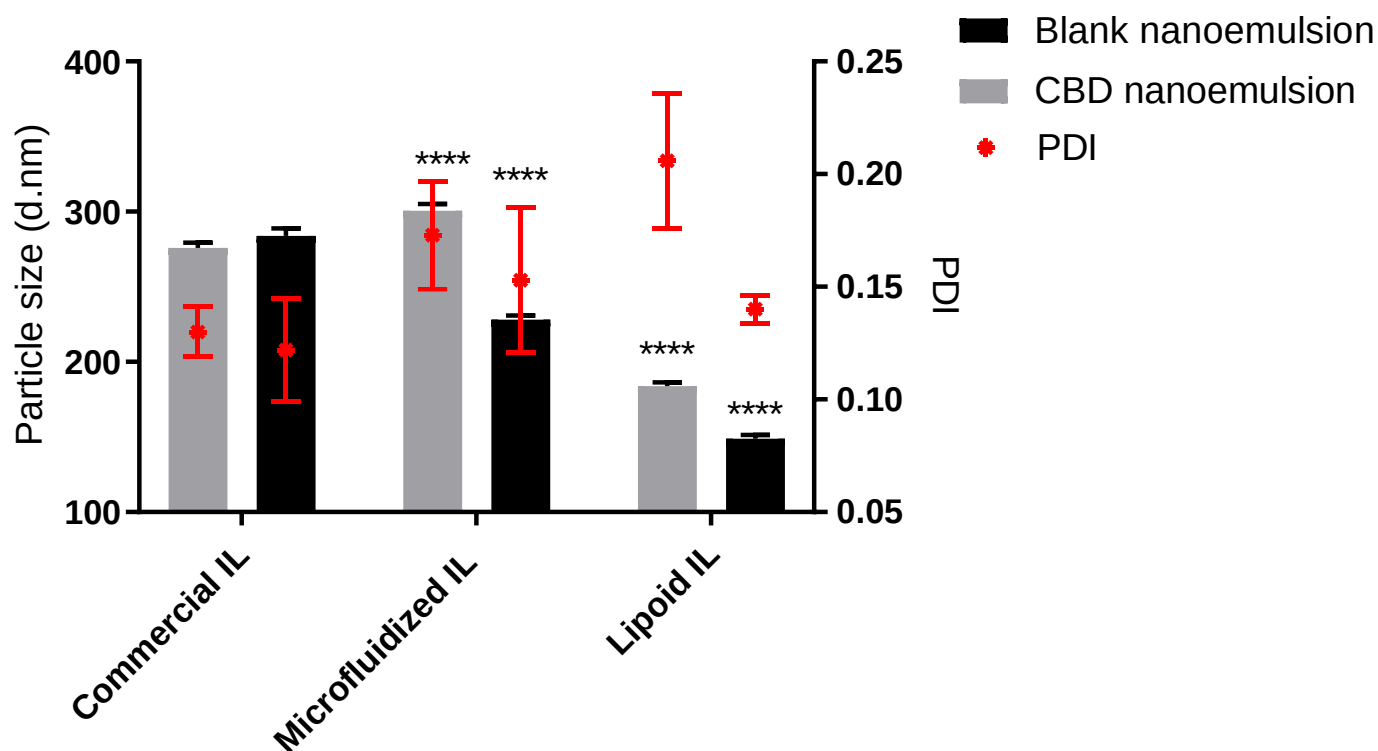


Figure 3.11. Particle sizes of the nanoemulsion formulations before and after the association with CBD. The drug addition led to a significant increase in particle size of the Lipoid and microfluidized nanoemulsions (**** $p < 0.0001$), and PDI of Lipoid (**** $p < 0.0001$) ($n=3$, mean $\pm$ SD).

3.5.1.6. Nanoemulsion shelf stability

USP <729> stipulates that during the shelf-life of an emulsion, its mean droplet size should not exceed 500 nm. It is therefore important that the nanoemulsion does not undergo creaming, flocculation or coalescence during storage at 4 °C, therefore stability studies are critical. Nanoemulsions stored at 4 °C were characterised for their particle size, polydispersity and ZP.

3.5.1.6.1. Particle size

The size of the oil droplets in a nanoemulsion may impact drug release, stability and toxicity of formulations. Consequently, the increase in droplet size and changes in size distribution are essential factors in determining the potential of an injectable emulsion. To monitor nanoemulsion stability, DLS measurements were carried out to determine droplet size over time. The commercial Intralipid® nanoemulsion was stable over the 70 days tested (stored at 4 °C), both before and after the addition of CBD (Figure 3.12). There were no significant

differences in neither PDI (which remained < 0.2) nor particle size over the time-course tested, with no phase separation observed.

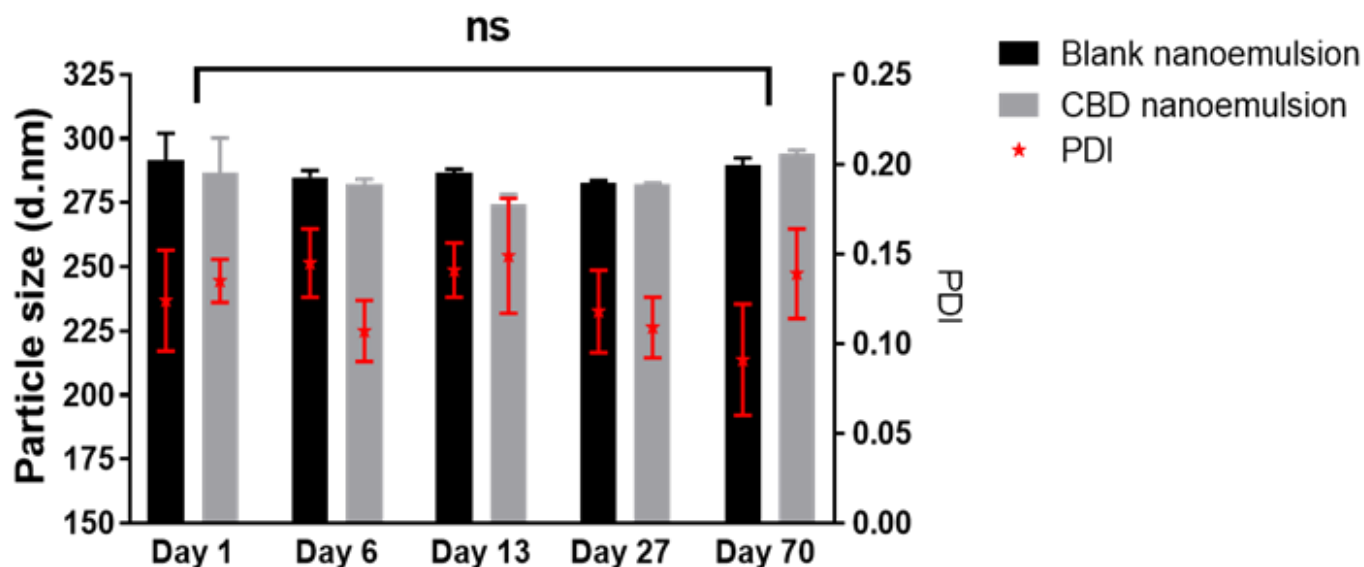


Figure 3.12. Size and PDI of nanoemulsions before and after CBD association over the course of 70 days after storage at 4 °C. No significant difference in size nor PDI was observed after the association of CBD with the commercial IL nanoemulsion ($n=3$, means $\pm$ SD).

3.5.1.6.2. Zeta-potential

Zeta-potential is the electrical potential at the interface of lipid droplets and is a good indicator of emulsion stability. This is due to the electrostatic forces provided by the emulsifier, in this case egg-yolk phospholipids, which form an interfacial film at the O/W interface (Washington, 1996). The interfacial film allows for a barrier to protect against coalescence of oil droplets destabilising the emulsion system. Natural egg-derived lecithin (80% phosphatidylcholine PC, 8% phosphatidylethanolamine PE) was used as an emulsifier in this system due to its low toxicity. Following incorporation of CBD, the ZP of the Intralipid® nanoemulsion did not significantly change over time, ranging between -54.1 to -53.2 mV (Figure 3.13), and was not significantly different from the blank nanoemulsion (which ranged between -58.5 to -54.0 mV)

The ZP of the Lipoid and microfluidized nanoemulsion formulations were not measured over-time, as initial recordings indicated that the addition of CBD lead to a significant increase in particle size. As a result of this, these formulations were not taken forward.

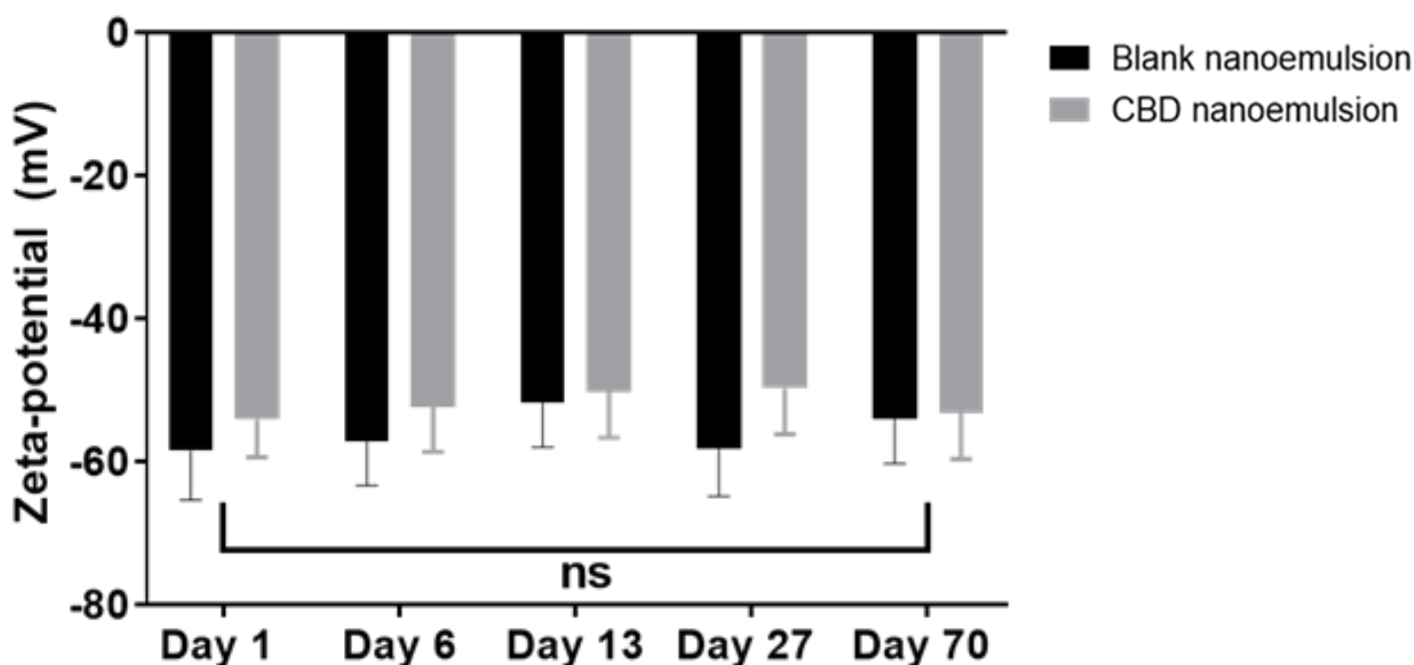


Figure 3.13. Zeta-potential of the blank and CBD nanoemulsion over the course of 70 days after storage at 4 °C. Formulations were stable and no phase separation occurred, with no significant differences between the ZP of the blank nanoemulsion and CBD nanoemulsion across the days tested ($n=3$, mean $\pm$ SD).

3.5.1.7. Conclusions

I have successfully identified microfluidizer processing parameters for the re-creating of the commercially available Intralipid® nanoemulsion. Nonetheless, upon association of CBD with both microfluidized formulations, it was apparent that the addition of the drug lead to a significant increase in the particle size of formulations. This could have been as a result of slight instability in the colloidal systems, as it was evident from ZP measurements that the surface charge of these formulations was less negative than the commercially available nanoemulsion. Following the optimisation of the CBD ultracentrifugation association protocol, successful solubilisation of the drug in the commercially available Intralipid® nanoemulsion was achieved, with a 65% association efficiency. This formulation was proven to be stable after storage at 4 °C, and the CBD concentration was in the optimum range for therapeutic efficacy as demonstrated by prior *in vivo* experiments (Xiong *et al.*, 2012).

3.5.2. Polymer-coated nanoparticles formulation

In order to develop an alternative CBD formulation to be tested in the IT space, a double nanoprecipitation protocol was trialled using PEG (poly(ethylene glycol)) – PLA (poly(D,L-lactic)) polymers which generate NPs consisting of a hydrophobic core nanocrystal core with a polymer coating (Figure 3.3). This particular formulation allows for the delivery of lipophilic drugs to a hydrophilic environment (i.e., the CSF), by coating insoluble drug nanocrystals with amphiphilic polymers. The double nanoprecipitation method allows for the deposition of polymers on top of drug nanocrystals through the formation of non-covalent bonds between the PLA chains and the drug nanocrystal. This is achieved through methyl groups on the PLA chains which allow for hydrophobic interactions with the methyl groups and the benzene rings in the structure of CBD. On the other hand, the hydrophilic PEG chains interact with the aqueous environment as a result of their hydrophilicity, forming hydrogen interaction with water (Figure 3.14) (Shimoyama *et al.*, 2006). Through this method, the polymer adsorbs onto the surface of the nanocrystal (which is formed in the first nanoprecipitation step), as opposed to being entangled within the core of the NPs (Rabanel *et al.*, 2014). Therefore, this process provides the possibility of increasing the density of PEG on the surface of the NPs.

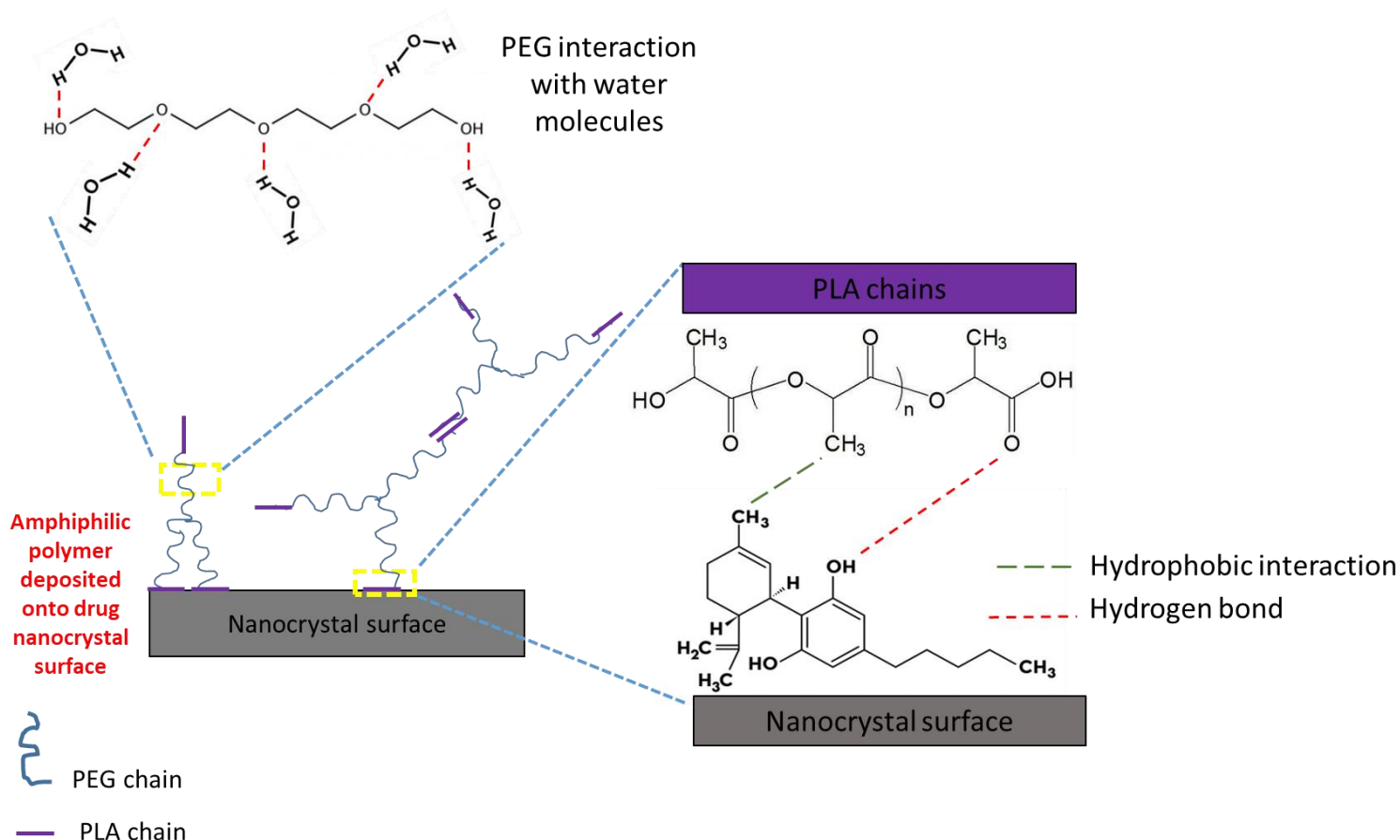


Figure 3.14. Schematic showing the polymer deposition on the surface of a nanocrystal forming polymer coated NPs. The hydrophobic PLA (purple) block adsorbs onto the surface of the CBD nanocrystals (grey) through non-covalent interactions (hydrogen bonds and hydrophobic interactions). The PEG chains of the polymer interact with the water molecules of the aqueous phase through hydrogen bonds (Rabanel *et al.*, 2014). The amphiphilic nature of the polymer stabilises the drug nanocrystals within an aqueous environment.

I utilised polymers of different PEG molecular weights, as studies have demonstrated that there is a correlation between PEG chains and NP penetration, due to the PEG shell decreasing corona formation and opsonisation (Nance *et al.*, 2012; Shalguov *et al.*, 2017). We hypothesised that polymers with a higher PEG molecular weight (i.e., mPEG₅₀₀₀-PLA₁₀₀) would be more efficient at penetrating the spinal cord and brain parenchyma in the IT space, as opposed to polymers with a lower molecular weight (i.e., tPEG₁₀₁₄-PLA₁₀₀). Nonetheless, a balance between NP stability, drug loading and preferred polymers for tissue penetration must be identified, therefore I set out to determine which polymer was most effective at coating CBD nanocrystals and creating stable NPs.

3.5.2.1. Identification of polymers able to coat CBD nanocrystals

I first trialled three different polymers synthesised by Dr Vincenzo Taresco (School of Chemistry, University of Nottingham). These polymers were based on a PEG backbone of different chain lengths and structures (2 linear polymers and a 3-arm star shape), with lactide monomers attached to the PEG chain. The polymers had different PLA to PEG ratios and different arrangements and structures: PLA₅₀-PEG₄₀₀₀-PLA₅₀, mPEG₅₀₀₀-PLA₁₀₀ and 3-armPEG₁₀₁₄-PLA₁₀₀ (Figure 3.15).

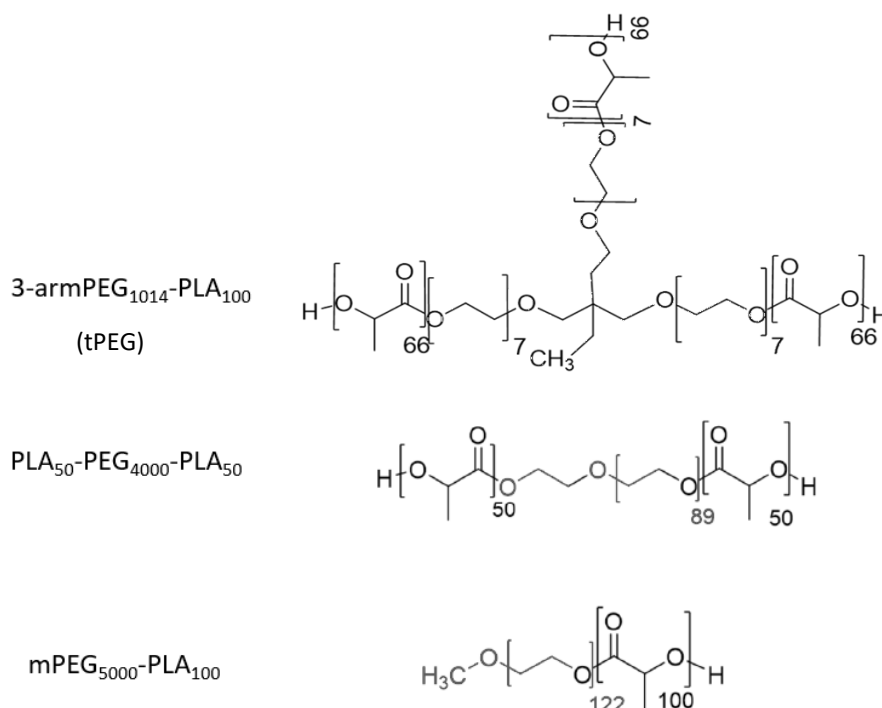


Figure 3.15. Chemical structures of the polymers used in these studies, with different PEG chain lengths and structures. The number of PEG and PLA units are given next to the structures and the abbreviated names for each polymer is associated with each polymer structure.

Different PLA chain unit lengths of 15, 25, 50 and 100 were tested with a PEG backbone of either mPEG₂₀₀₀ or mPEG₅₀₀₀ molecular weight. It was found that an increase in the hydrophobic block (PLA) from 15 to 100 units decreased NP size and PDI whilst improving physical stability. This was likely due to the steric effects enhanced by the LA (as a result of the improved packing inside the NP core). Moreover, the encapsulation efficiency of

hydrophobic drugs (such as etoposide, a chemotherapeutic used in the clinic) was shown to increase with hydrophobic polymer chain (poly (caprolactone)) length in another study (Kuo *et al.*, 2019). These results suggest that an increase in the hydrophobicity of the polymer used in nanoprecipitation increases drug loading within the NP if the drug used is hydrophobic. Based on these results it was therefore decided that a hydrophobic chain of 100 lactide units will be used in polymers for the formulation of CBD NPs.

Interestingly the linear polymers (mPEG₅₀₀₀-PLA₁₀₀ and PLA₅₀-PEG₄₀₀₀-PLA₅₀) were the least effective in coating the CBD nanocrystals through the double nanoprecipitation technique (Appendix 8.1). All NPs formulated with mPEG₅₀₀₀-PLA₁₀₀ had a PDI higher than 0.2, indicative of a non-homogenous particle size population (Table 3.2). Various ratios of CBD to polymer were trialled, however this polymer was ineffective at coating the drug at any ratio and concentration tested. The best PDI (0.226) was exhibited at a 1:2 ratio of drug to polymer, which resulted in a particle size of 37.69 nm. However, it was apparent that a bimodal particle size populations was created, which was not ideal, as it is indicative that the polymer coating process was unsuccessful. However, in the absence of CBD, mPEG₅₀₀₀-PLA₁₀₀ effectively self-assembled to create polymeric micelles with a 33.2 nm diameter and a PDI of 0.135.

PLA₅₀-PEG₄₀₀₀-PLA₅₀ produced similar results using the double nanoprecipitation technique, leading to ineffective coating of CBD, with PDIs of > 0.2. The smallest PDI observed was at a 1:1 ratio of CBD to polymer, with NPs created exhibiting a size of 193.9 nm and PDI of 0.214. Nonetheless, these measurements were not reliable as samples were aggregating during measurements as indicated by DLS, with a mean size and count rate increasing by 48.3%. In the absence of CBD, PLA₅₀-PEG₄₀₀₀-PLA₅₀ self-assembled to produce polymeric micelles with a size of 53.02 nm and PDI of 0.283.

A CBD only control, without any addition of polymers was included in the experiments and the physical characterisation data demonstrated that in the absence of a polymeric coating, CBD nanocrystals are unstable and aggregated leading to a size of 2222 nm ± 537.5 nm and PDI of 0.412. However, DLS measurements were deemed unreliable due to the constant aggregation and sedimentation of particles in this sample (Figure 3.16).

On the other hand, the 3-arm polymer, 3-armPEG₁₀₁₄-PLA₁₀₀ successfully coated CBD nanocrystals at a 1:1, 2:1 and 10:1 drug to polymer ratios, all experimental conditions

producing homogenous NPs (with sizes of: 121.8 nm, PDI of 0.079, 123.7 nm, PDI of 0.104, and 89.42 nm, PDI of 0.189 respectively). DLS characterisation showed only one peak indicative of a single population (Appendix 8.2) therefore confirming that this polymer was able to coat the CBD nanocrystals, without creating two or more different sized populations. In the absence of CBD, tPEG self-assembled into polymeric micelles with a size of 85.44 and a PDI of 0.123 (Figure 3.16).

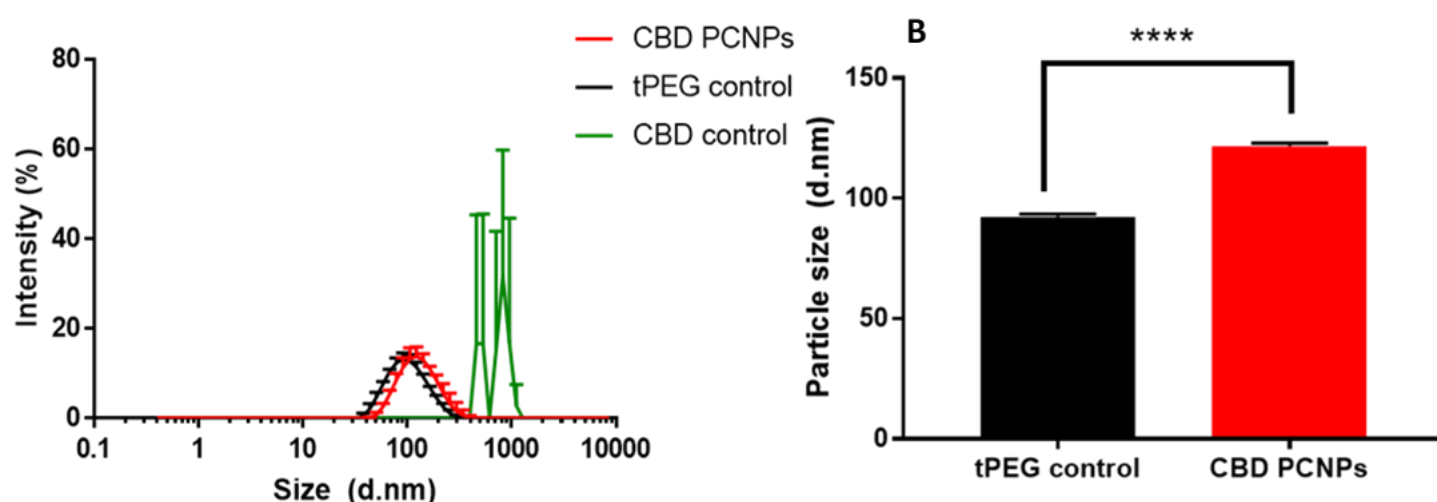


Figure 3.16. A. DLS traces of CBD PCNPs coated with tPEG in a double nanoprecipitation technique, alongside the traces for tPEG only control (polymeric micelles) and CBD nanocrystals with no polymeric coating. The presence of a single population after the coating of CBD by tPEG is indicating of successful drug coating. **B.** CBD PCNPs were significantly larger in particle size than the blank tPEG control micelles ($n=3$, mean $\pm$ SD, *** $p<0.001$) (tPEG control particle size was 85.44 ± 1.16 nm and CBD PCNPs were 121.8 ± 1.1 nm; there were no significant differences in PDI).

As the lengths of the PLA hydrophobic blocks were maintained across these 3 different polymers, it is assumed that the difference in coating efficiency of CBD was due to the different PEG molecular weight or the different architectures (i.e., linear vs 3-arm structure) of the polymers. The literature suggests that the molecular weight of PEG is closely correlated to the effective surface shielding of NPs (Suk *et al.*, 2016). Miteva *et al.*, have demonstrated that increasing the molecular weight of PEGylated micelles (from 2 to 20 kDa) prevents aggregation and improves subsequent blood-circulation time *in vivo* (Miteva *et al.*, 2015). Interestingly, in my work, I found that the amphiphilic polymer with a lower PEG molecular

weight (1014 instead of 4000 or 5000) was the most efficient in coating of CBD nanocrystals. On the other hand, it was possible that the presence of three PLA hydrophobic arms of this polymer (i.e., different polymeric architecture) aided the encapsulation of the lipophilic CBD nanocrystals through its improved structural stabilisation (Figure 3.17), as opposed to the linear polymers consisting of only one or two PLA side-chains which did not end up effectively coating the nanocrystals. Moreover, it is also likely that the hydrophobic blocks form bonds with other hydrophobic blocks on adjacent polymer molecules, further stabilising the PCNPs (Shi *et al.*, 2016).

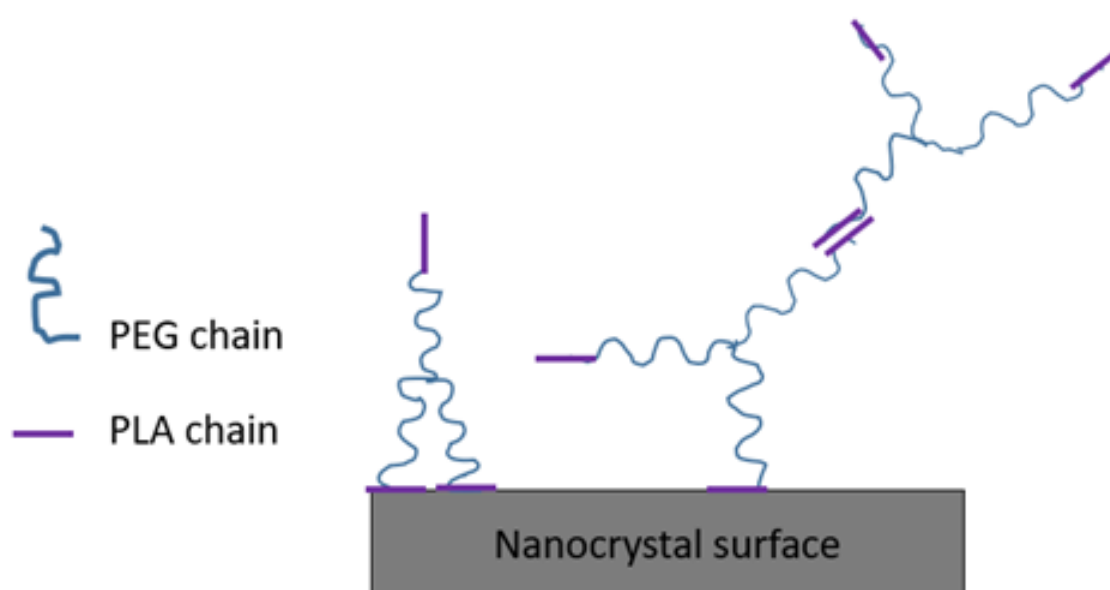


Figure 3.17. Schematic of the tPEG hydrophobic block (PLA chains) interacting with the CBD nanocrystal surface and adjacent PLA chains on other polymer molecules. Diagram showing the possible increased structural stability of the polymeric coating through the adsorption of 2 PLA blocks to the surface of CBD nanocrystals and hydrophobic interactions with other adjacent tPEG molecules.

Nonetheless, the 1:1 ratio at 1 mg/mL CBD and 1 mg/mL tPEG concentrations were deemed to be the most reliable and reproducible experimental conditions, thus they were taken forward for further characterisation. Moreover, it was thought that a 1:1 ratio of drug to polymer would produce a more uniform coating of the CBD nanocrystals as opposed to 2:1 ratio and a 10:1 ratio, which might lead to stability issues over time due to the aggregation of the drug if the NPs are not fully coated.

Table 3.2. Physical characterisation of CBD NPs made by using PLA₅₀-PEG₄₀₀₀-PLA₅₀, mPEG₅₀₀₀-PLA₁₀₀ and 3-armPEG₁₀₁₄-PLA₁₀₀. The concentration of CBD was kept constant at 1 mg/mL, and the polymer concentration was varied between 2 to 0.01 mg/mL to test different drug to polymer ratios.

Polymer used: 3-armPEG ₁₀₁₄ -PLA ₁₀₀					
CBD concentration (mg/mL)	Polymer concentration (mg/mL)	Drug : polymer ratio	Size (nm)	PDI	ZP (mV)
1	1	1:1	121.8	0.079	-39.9
1	0.5	2:1	123.7	0.104	-57.1
1	0.25	4:1	161.5	0.261	-
1	0.1	10:1	89.42	0.189	-57.1
1	0.05	20:1	1660	0.553	-
1	0.01	100:1	2955	0.343	-
1	-	1:0	1032	0.59	-79.9
-	1	0:1	85.44	0.123	-

Polymer used: PLA ₅₀ -PEG ₄₀₀₀ -PLA ₅₀					
CBD concentration (mg/mL)	Polymer concentration (mg/mL)	Drug : polymer ratio	Size (nm)	PDI	ZP (mV)
1	2	1:2	53.1	0.489	-
1	1.5	1:1.5	67.64	0.375	-
1	1.2	1:1.2	56.68	0.285	-63
1	1	1:1	193.9	0.214	-
1	0.75	1.3:1	184.3	0.472	-
1	0.5	2:1	-	-	-
1	0.25	4:1	-	-	-
1	0.1	10:1	-	-	-
1	0.05	20:1	-	-	-
1	0.01	100:1	-	-	-
-	1	0:1	53.02	0.283	-58.3

Polymer used: mPEG ₅₀₀₀ -PLA ₁₀₀					
CBD concentration (mg/mL)	Polymer concentration (mg/mL)	Drug : polymer ratio	Size (nm)	PDI	ZP (mV)
1	2	1:2	37.69	0.226	-75.5
1	1.5	1:1.5	209.9	0.497	-76.2
1	1.2	1:1.2	85.52	0.753	-70.6
1	1	1:1	2252	0.852	-72
1	0.5	2:1	344.7	0.65	-73.6
1	0.1	10:1	1135	0.618	-78.8
1	0.05	20:1	2837	0.303	-84.9
1	0.01	100:1	1999	0.435	-85.8
-	1	0:1	33.2	0.135	-28.5

The organic phase used in the double nanoprecipitation technique was acetone, at a molar fraction pre-determined by previous work on polymer-coated iron oxide NPs by Abushrida *et al.* Solvent choice is essential in the development of NPs by nanoprecipitation, as its miscibility

with water can determine the final size of the formulation (Oliveira *et al.*, 2013; Abushrida *et al.*, 2020). Slight variations in the ratios of acetone to water were trialled in a DOE experimental set up, to investigate how the organic and aqueous phases impact the production of PCNPs through nanoprecipitation (discussed in section 3.5.2.4).

3.5.2.2. CBD PCNPs stability

3.5.2.2.1. Particle size

I carried out stability studies of the CBD PCNPs after storage of the formulations at 4 °C and periodically analysing them by DLS (Figure 3.18). I found that the size of the CBD PCNPs remained constant throughout the 31-day period, with no significant changes in particle size nor PDI. The PCNPs were stable at 4 °C for at least 31 days, with no significant change in particle size (121.8 ± 1.08 nm on the day of formulation to 116.9 ± 0.24 nm 31 days later) nor PDI (0.079 ± 0.014 to 0.137 ± 0.036).

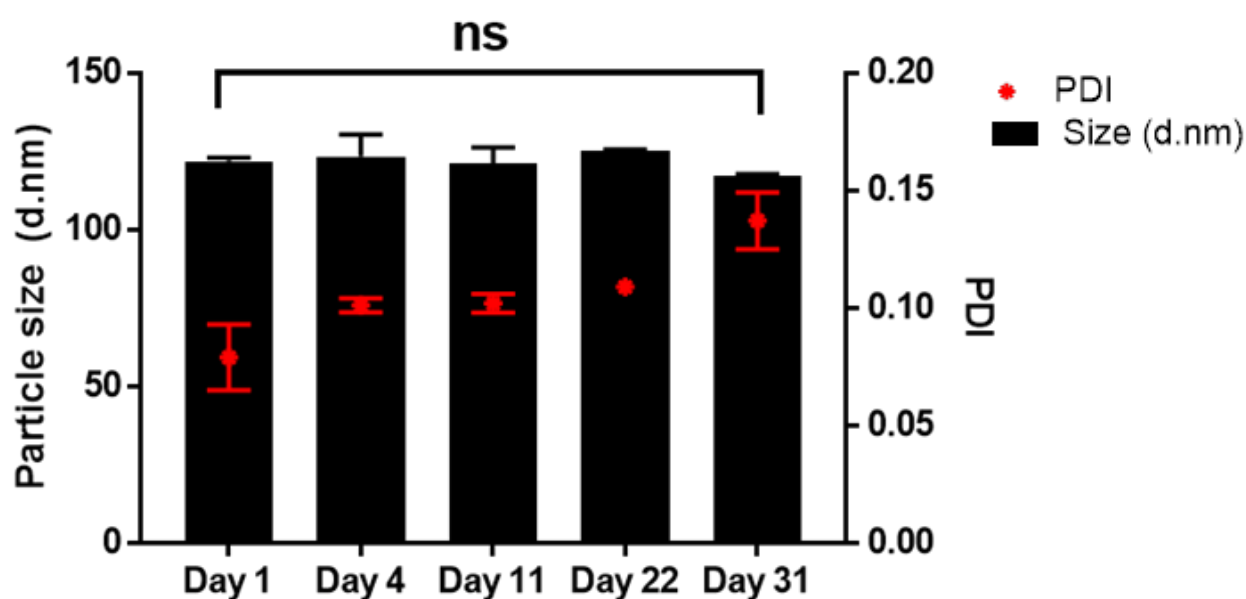


Figure 3.18. The physical stability of CBD PCNPs after double nanoprecipitation, with storage at 4 °C. 1 mg/ml CBD : 1 mg/ml tPEG PCNPs were stable at 4 °C for at least 31 days, with no significant change in particle size (121.8 ± 1.08 nm on the day of formulation to 116.9 ± 0.24 nm 31 days later) nor PDI (0.079 ± 0.014 to 0.137 ± 0.036) ($n=3$, mean $\pm$ SD).

3.5.2.2.2. Zeta-potential

The zeta-potential of these NPs were all negative, due to the PEG outer shell (and the lone-pair cation interactions at the oxyethylene units with their corresponding association of anions forming the outer layers) (Vasey *et al.*, 2019). The ZP of tPEG coated CBD NPs was -

39.9 ± 1.22 mV and that of CBD nanocrystals control with no tPEG coating was -79.1 ± 10.2 mV, with high variability in measurements resulting in a poor-quality report (mean ZP trending by 15.4%) as per DLS software, due to sample being unstable (Figure 3.19). Although the addition of tPEG significantly increased the surface charge (more positive than CBD nanocrystals alone), the control formulation with no polymer coating was highly unstable as demonstrated by ZP variability coupled with high PDI and particle size (previously depicted in Figure 3.16).

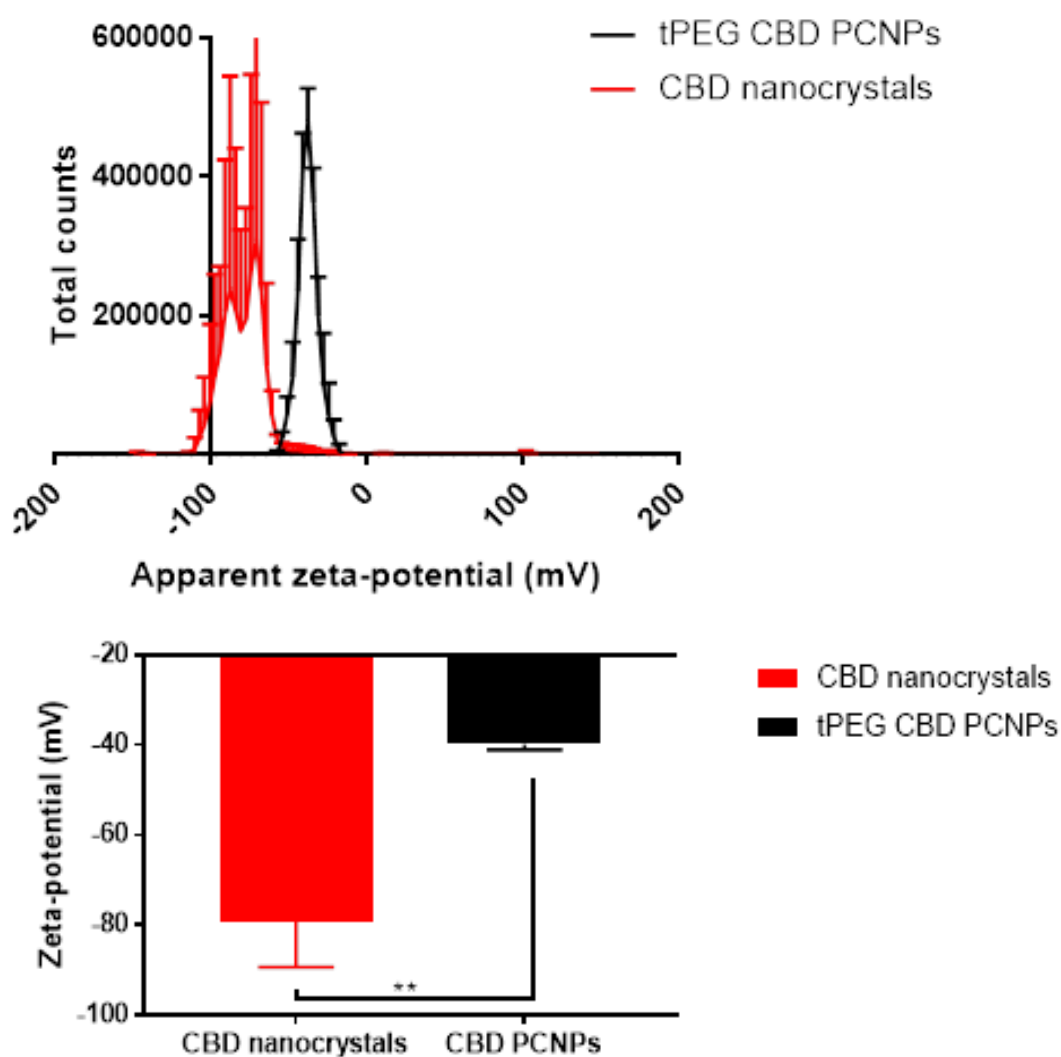


Figure 3.19. The ZP traces of CBD nanocrystals with no polymer coating and after coating with tPEG after double nanoprecipitation (A). The coating of CBD nanocrystals with tPEG significantly increased the ZP (** $p < 0.01$) of the PCNPs (statistical analysis carried out by a t-test; $n=3$; mean ± SD).

3.5.2.3. Drug loading and encapsulation

I investigated the amount of CBD loaded in the NPs made at a starting concentration of 1 mg/mL CBD and 1 mg/mL 3-armPEG₁₀₁₄-PLA₁₀₀ using the double nanoprecipitation technique. I initially determined DL% and EE% of freeze-dried formulations after the removal of free drug by PD10 columns. PD10 columns work by separating molecules based on molecular weight and gravity, thereby heavier components elute earlier than lighter / smaller ones. I obtained 500 μ L fractions of NP formulations after their passing through the separation column, and physically characterised each to determine how much volume was required to separate the free drug from the NP formulation. I observed that the first 1.5 mL fraction contained very large particles with heterogeneous populations, thus I determined this fraction to contain the free drug (Figure 3.20). When CBD nanocrystals are uncoated, they tend to aggregate as a result of the absence of a steric barrier provided by the PEG-PLA polymer. This aggregation leads to increases in particle size, which can then be used to separate the free drug from the rest of the stabilised NPs by a PD10 column.

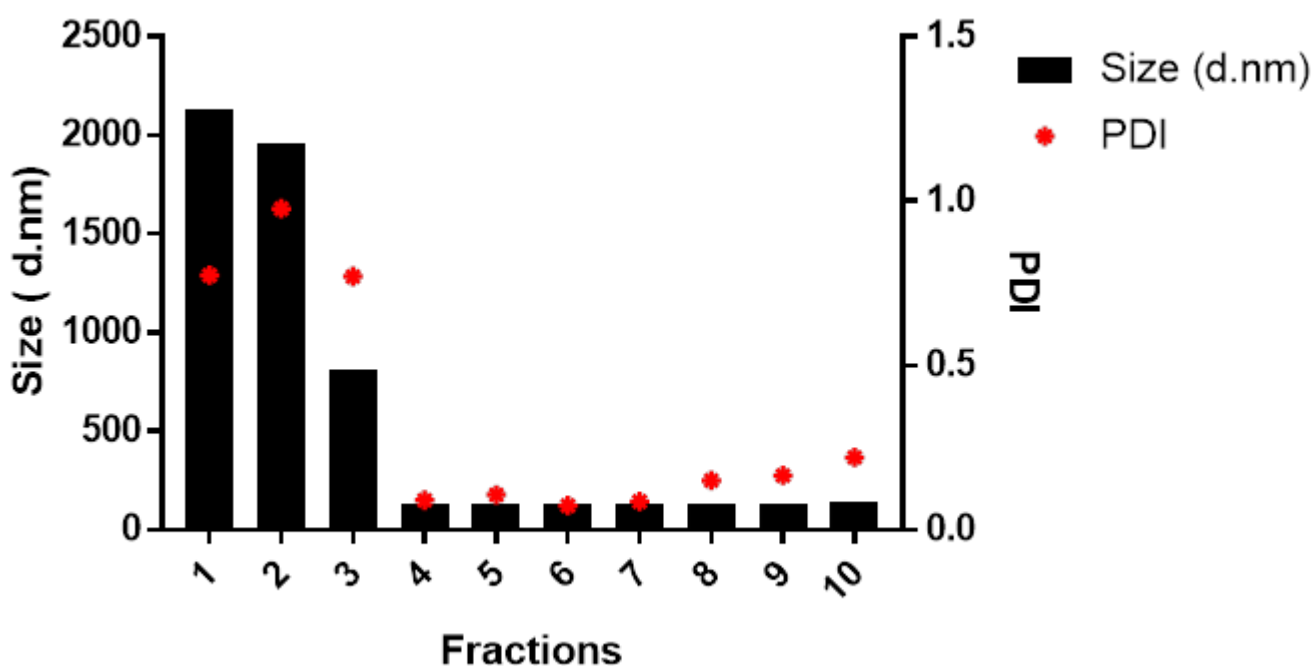


Figure 3.20. PD10 fractions. Size and PDI of fractions (0.5 mL each) eluted from a PD10 column for the separation of free drug from CBD NPs.

Fractions 4-10 were therefore collected and freeze-dried overnight for DL% and EE% analysis. Unfortunately, this technique resulted in a low DL of 9% and EE of 33%, possibly due to the some of the polymer coating interacting with the surface of the PD10 column, resulting in the NP being destabilised. This destabilisation would have resulted in particle aggregation, which would in turn be separated out and discarded. An alternative method of free drug removal I trialled was gentle centrifugation. This works by sedimentation of aggregates as a result of the centrifugation force, resulting in a NP suspension in the upper layer of the sample. This method resulted in a DL of 29% and EE of 46%, which is very good for my NP system as compared to the literature. For example, Lobato *et al.*, 2014 prepared THC NPs coated with PEG-chitosan and achieved an EE of 96% and DL of 4.8%. Ossa *et al.*, made poly- ϵ -caprolactone CBD microspheres (20 – 50 μm size, for anticancer treatment) achieving EE ranging between 90 – 100 % and DL% between 3-26% depending on the polymer to drug ratio used (Ossa *et al.*, 2012).

I therefore decided to use the centrifugation method of free drug separation when preparing formulations for further studies. For preparation of CBD PCNPs for *in vivo* testing, the formulations were evaporated under N_2 air for 2-3 hours in order to concentrate CBD to a pre-determined volume to reach 1 mg/mL. This was done to ensure that it matched the previously developed CBD nanoemulsion formulation. Physical characterisation was carried out before and after evaporation to ensure these processes did not affect PCNPs stability (Appendix 8.3).

The advantage of this NP manufacturing method is the high DL% achieved, which is similar to other reported nanoprecipitation studies achieving DL of around 25% (Papadimitriou *et al.*, 2009; Thirumala *et al.*, 1999; Kumar *et al.* 2008). Nonetheless, significantly higher amounts (86%) were reported by Matteuci *et al.*, where itraconazole NPs averaging 300 nm in diameter were stabilised by poloxamer 407 (Matteuci *et al.*, 2006). However, the drawbacks associated with this method were that all experiments were performed at 3 °C to prevent aggregation and physical characterisation was performed within 30 seconds of nanoprecipitation. In my experiments, formulation was carried out at room temperature and stability measurements were taken at several points over a longer time period.

3.5.2.4. Fine-tuning of nanoprecipitation parameters by DOE

I carried out a 2-level factorial DOE consisting of 10 runs to investigate how changing CBD and tPEG concentrations, alongside the ratio of aqueous to organic solvents influence PCNP parameters such as particle size, PDI, encapsulation efficiency and drug loading capabilities of my colloidal system. I tested CBD and tPEG concentrations ranging from 1 to 2.5 mg/mL, and acetone to water ratios from 1:4 to 1:6.

I found that significant factors in influencing the particle size of PCNPs were tPEG concentrations and aqueous to organic solvent ratios (Figure 3.21; Figure 3.22; statistical analysis ANOVA for factorial model, $p < 0.05$). A decrease in acetone to water ratio from 1:4 to 1:6 led to larger PCNPs particle sizes (157.22 nm). Moreover, at any aqueous to solvent ratios, if polymer concentration was increased from 1 mg/mL to 2.5 mg/mL, particle size of PCNPs also increased. For example, at a CBD concentration of 2.5 mg/mL, the tPEG concentration ranging from 1 to 2.5 mg/mL led to a particle size increase from 130.27 nm to 164.38 nm (at a set 1:6 organic to aqueous ratio) (Figure 3.21). This effect has also been observed in the preparation of PLGA NPs by Sharma *et al.*, where they suggested that an increase in PLGA concentration leads to higher organic phase viscosity, and decrease in stirring efficiency, thus increasing NP size (Sharma *et al.*, 2014). Moreover, increasing the amount of organic solvent in the system increases drug solubility which in turn creates lower supersaturation levels, restricting drug nucleation induction thus leading to smaller particle sizes (Tao *et al.*, 2019). On the other hand, Hernandez-Giottonini *et al.*, reported that cefixime loaded PLGA NPs decrease in size when a lower organic solvent fraction is used (Hernandez-Giottonini *et al.*, 2020). They attributed this size reduction to the coalescence prevention taking place in the system. Moreover, a similar result has also been observed by Oliveira *et al.*, who hypothesised that this is due to the formation of a higher number of nucleation sites (Oliveira *et al.*, 2013). It is therefore interesting that different views have been reported in the literature, highlighting that this might be as a result of differences in the drugs and polymers used, the nature of the organic and aqueous phases, and their respective solubilities (Hernandez-Giottonini *et al.*, 2020).

In terms of influencing changes in the PDI of PCNPs, I found that CBD and polymer concentrations were significant factors. Interestingly, an increase in both factors lead to a decrease in PDI from 0.16 to 0.08 at all organic to aqueous ratios tested. It could be that at

lower concentrations of polymer in the colloidal system there are some CBD nanocrystal aggregates present, however when tPEG proportion increases, no nanocrystals remain uncoated thus decreasing PDI.

Interestingly, in terms of drug loading, the most influential factor was the polymer concentration. Increasing polymer concentrations lead to lower DL%, indicating that although this can improve stability in the system, as I have observed a decrease in PDI with increases in tPEG, it might also lead to lower amounts of CBD loaded in NPs. This was possibly caused due to the formation of empty polymeric micelles, increasing weight of freeze-dried NPs, thus decreasing DL%.

Overall, these experiments have demonstrated that physical characteristics alongside DL% can be optimised to a certain degree through the fine-tuning of drug and polymer concentrations. It was also shown that the ratio of aqueous to solvent phases is important in determining particle size, however care must be taken when increasing the amount of acetone in the system, especially when formulations are due to be tested *in vivo*.

Nonetheless, the nanoprecipitation parameters were not changed for the formulations taken forward for *in vivo* studies. This process was investigated by DOE to explore the robustness of the process and the possible parameters might could be changed in the future, to fine-tune the nanoprecipitation process.

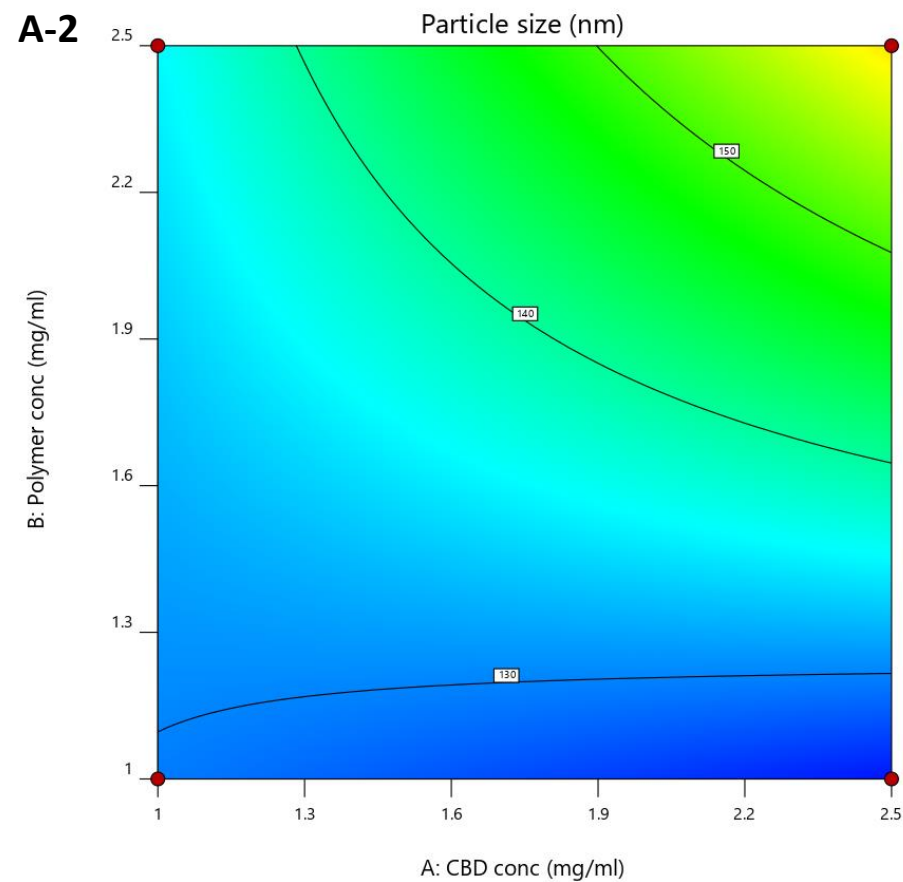
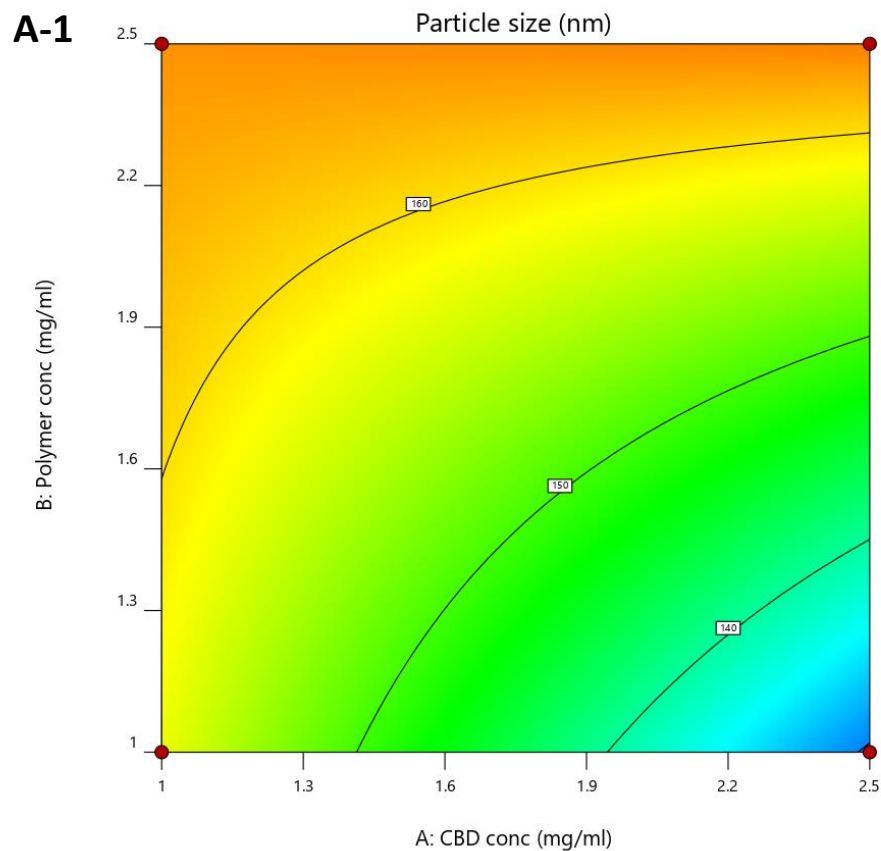


Figure 3.21. DOE optimisation for the manufacture of CBD PCNPs through double nanoprecipitation. Contour plots of **A**. Particle sizes at various CBD and polymer concentrations, with (1) being at 1:6 and (2) 1:4 acetone to water ratio. tPEG concentration and acetone to water ratios were significant factors in influencing the particle size of PCNPs created (* $p < 0.05$, ANOVA for factorial models, DOE). The ranges of CBD and polymer concentrations are referred to in the Methods section (3.3.2).

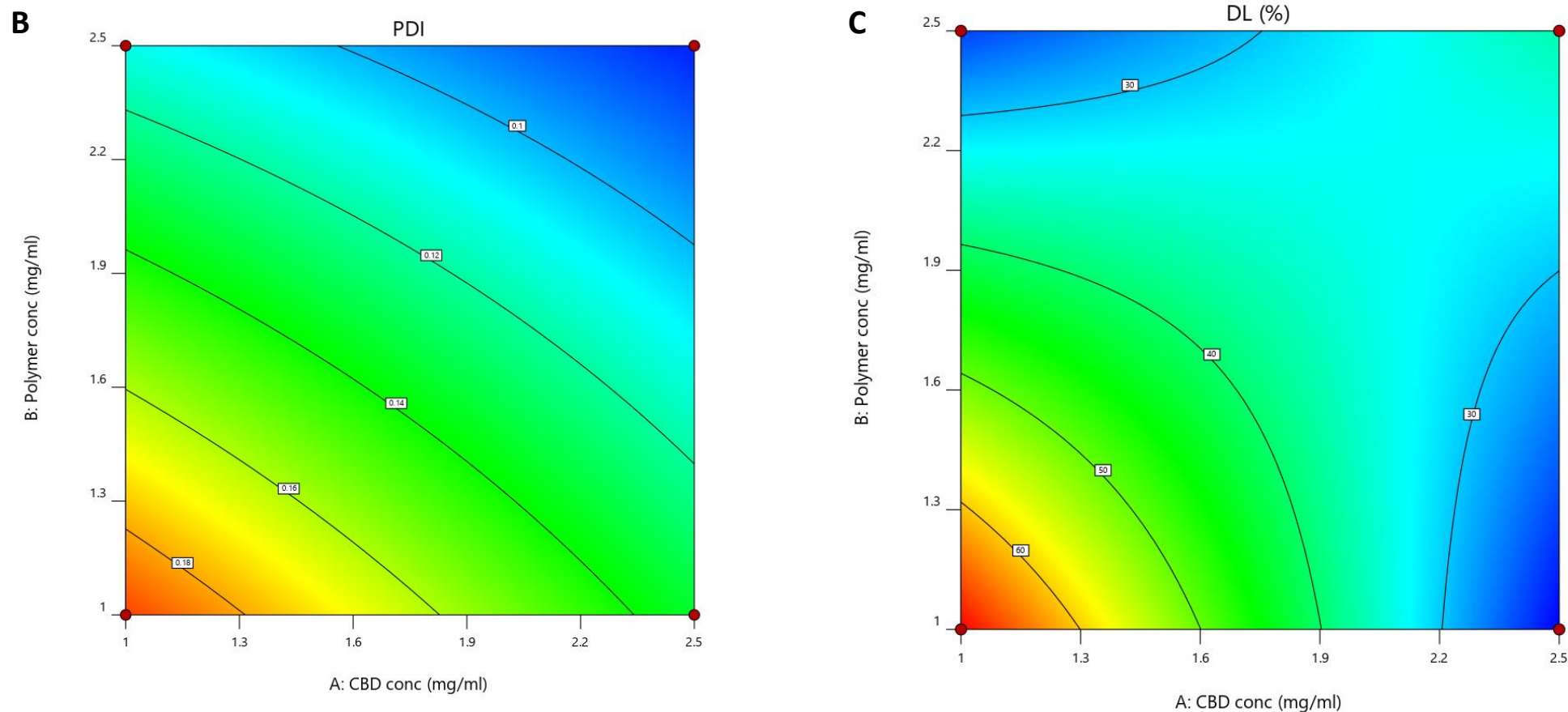


Figure 3.22. DOE optimisation for the manufacture of CBD PCNPs through double nanoprecipitation. Contour plots of **B**. PDI at various CBD and polymer concentrations, at 1:6 and acetone to water ratio. Both CBD and tPEG concentrations are significant factors in influencing PDI, with increases in both leading to smaller PDIs ($*p < 0.05$). **C**. Contour plot of drug loading (%) at various CBD and polymer concentrations, at 1:6 acetone to water ratio. Polymer concentration led to a significant change in DL%, with increases from 1 to 2 mg/mL leading to lower DL% of the PCNPs ($*p < 0.05$, ANOVA for factorial models, DOE). The ranges of CBD and polymer concentrations are referred to in the Methods section (3.3.2).

3.5.2.5. Conclusions

The double nanoprecipitation technique I have used to develop CBD PCNPs has proved to be a fast, reproducible and cost-effective method. I used acetone as an organic phase to solubilise a lipophilic drug and create unstable nanocrystals, which were then coated by a solubilised polymer upon its addition to the aqueous phase. Once the organic phase was evaporated, separation of coated NPs from free drug was carried out through gentle centrifugation, resulting in a stable CBD PCNP population. This formulation was also shown to be stable after undergoing 60% evaporation of the aqueous phase, to concentrate the amount of CBD within the system to a pre-determined concentration, to match the previously developed nanoemulsion formulation. This process poses many advantages in the pharmaceutical industry, as it may be applicable to other poorly soluble APIs, once the appropriate coating polymers have been identified.

3.6. Summary

I have developed two distinct novel CBD formulations, a lipid-based nanoemulsion and a polymer coated NP system (Table 3.3). I have optimised the manufacturing and formulation processes, evaluated their drug loading and stability, to ensure that they have the same drug concentration i.e., mg/ml, ready for *in vivo* dosing.

Table 3.3. Summary of both CBD formulations developed.

Formulation	Type	Size (d.nm)	PDI	ZP (mV)	Concentration of CBD (mg/mL)
CBD nanoemulsion	Lipid	286.8	0.135	-54.1	1.28
CBD PCNPs	Polymer-coated nanocrystal	121.8	0.079	-39.9	0.46

4. Chapter 4 – *Ex vivo* imaging analysis

investigation into the IT injection technique

4.1. Introduction

In order to investigate the biodistribution of the CBD nanoemulsion and PCNPs within the CNS, it is necessary that these formulations are correctly injected at the site of interest - within the SAS through the IT delivery route (not durally or epidurally). The first technique for administering drugs into the SAS of rats was developed by Yaksh and Rudy (1976), who initially carried it out through the use of chronic catheters. However, the drawback associated with this method was the preparative surgery involved prior to catheterisation and the side effects involving spinal lesions. Hylden and Wilcox then developed a technique involving a lumbar puncture in un-anesthetised mice, with a high degree of accuracy and reproducibility (Hylden and Wilcox., 1980). They inserted a needle transcutaneously straight through the groove between the spinal processes, injecting either methylene blue or [³H] morphine. They found that methylene blue was consistently distributed in the CNS, with higher concentrations around the site of injection. When radio-labelled morphine was administered, around 3% reached the brain within 10 mins, with estimated anti-nociceptive effects occurring in the spinal cord (Hylden and Wilcox., 1980). The transcutaneous technique which they used relies of the lack of ossification in the spinal cord, to allow for the needle to easily pass through the groove between the spinal processes. Generally, in rats, ossification of the spinal cord occurs immediately after weaning and rapidly progresses, taking place between 21 and 23 days of age (Uchida *et al.*, 2011). Therefore, in order to be able to perform transcutaneous IT injections in rats, very young pups would be required. However, the pain pathways in young rat pups are different from adults as they are not yet fully developed (Moss *et al.*, 2007). This is because the physiological response to acute pain in neonatal rat pups is age-specific, following milestones in the development of the pain system over the course of several weeks (Anand *et al.*, 1999). Therefore, the use of young rat pups would not be applicable for testing

anti-nociceptive effects of CBD formulations for my study, due to the incompletely developed pain pathways. As a result of this, a partial laminectomy removing the spinal processes and uncovering the spinal cord is required, in order to achieve successful penetration of the needle into the IT space. This technique involves preparative micro-surgery, thus it is extremely challenging; moreover, the anatomy associated with the SAS in rodents is very small, making needle penetration to this space complicated. Therefore, I decided to pursue investigative imaging analysis to evaluate the delivery of therapeutics in the IT space of the spinal cord prior to the administration of CBD formulations. Alongside *ex vivo* imaging, I also utilised methylene blue due to its ability to dye certain tissues (Nguyen *et al.*, 2018). In the clinic, methylene blue used to be administered through an IT injection in order to track CSF leaks, therefore I administered it to rodents to assess my injection technique.

4.2. Imaging of the CNS after IT injections

4.2.1. Magnetic resonance imaging

Magnetic resonance imaging is one of the most widely used techniques for clinical diagnosis as a result of its high tissue contrast and spatial resolution (Stephen *et al.*, 2011). Moreover, images can be acquired without the use ionising radiation or radiotracers which can lead to a variety of harmful complications in the body. The contrast achieved between soft tissues can also be adjusted by modifying the data acquisition parameters, providing pronounced soft-tissue contrast for *in vivo* imaging. Water molecules present in the body are the source of protons, which are detected by the MRI, however lipids can also have this functionality (Ozdemir *et al.*, 2016).

Substantial research has been undertaken into the development of novel contrast agents, including magnetic nanoparticles (MNPs). Superparamagnetic iron oxide nanoparticles (SPIONs) are a type of MNPs which have contrast agent properties alongside excellent biodegradability and biocompatibility (Sun *et al.*, 2010; Weissleder *et al.*, 1989). The safety aspect associated with SPIONs is critical, especially because many other NP based contrast agents are linked to various toxicity concerns *in vivo* (Stephen *et al.*, 2011). Nonetheless, the FDA has approved SPION formulations for diagnostic uses in the clinic, particularly for bowel,

liver, spleen and lymph node metastases imaging (Bonnemain *et al.*, 1998; Wang *et al.*, 2001; Harisinghani *et al.*, 2003).

Traditional SPION contrast agents include gadolinium and manganese-based molecules, which act by lowering the T_1 relaxation time. This in turn creates a bright signal on MR images (Ozdemir *et al.*, 2016). On the other hand, iron oxide based SPIONs are negative contrast agents, which act by shortening the T_2 relaxation time, thus darkening the contrast seen on MR images. Much research is currently undergoing in the development of novel contrast agents, due to the side-effects and accumulation observed with the use of traditional gadolinium and manganese SPIONs based contrast agents (Bertin *et al.*, 2009). The alternative iron oxide SPIONs have much lower toxicity in the body and higher biocompatibility, as previously mentioned, due to the natural occurrence of iron in blood (Yu *et al.*, 2008; Gupta *et al.*, 2005).

Briefly, MRI works through the use of powerful magnets which force the protons within the body to align with that field. When a radiofrequency pulse is passed through the body, the protons become stimulated and spin out of equilibrium. Once the radiofrequency pulse is removed, the protons relax to equilibrium again. The time it takes for the protons to reach the equilibrium state (relaxation time) depends on the tissue (Berry *et al.*, 2009; Edelman *et al.*, 1996). The MRI contrast achieved in soft tissue is due to differences in proton density, spin-lattice (T_1) and spin-spin (T_2) relaxation times of protons (Stephen *et al.*, 2011). Nearly all MRI contrast agents have an effect of both T_1 and T_2 , however they are usually more pronounced for one of them. The effect of T_1 probes results in increased signal intensities leading to positive contrasts, whereas T_2 probes lead to lowered signal intensities causing negative contrasts. Iron oxide based SPIONs mainly have an effect on T_2 values of water protons around the particles, leading to a negative contrast enhancement surrounding the areas where high concentrations are present (appear darker on MR images) (Holz, 1993). The magnetic properties of SPIONs and thus their subsequent MRI is affected by the crystallinity and size of the magnetite core and polymeric coating. Nonetheless, there are various drawbacks in terms of current detection limits which are constantly being improved upon through developments in MRI acquisition methods and SPION synthesis procedures (Stephen *et al.*, 2011).

IT delivery confers an evident solution to the direct delivery of therapeutics directly to the CNS, however there are a variety of gaps in our knowledge as to how molecules behave within

this unique environment. Optimum drug delivery through this route requires the efficient transport from the site of injection in the lumbar segment alongside brain parenchymal penetration through the glymphatic system (Dyke *et al.*, 2020). The glymphatic system has been investigated through MRI after the injection of gadolinium contrast in healthy volunteers (Watts *et al.*, 2019). They observed that entry of contrast agents into the brain parenchyma is faster than what would be expected due to diffusion alone, demonstrating that the IT delivery is capable of achieving penetration into the entirety of the brain. Illiff *et al.*, have also found similar results in rodents, whereby MRI was used to determine the exchange of CSF in the CNS, allowing for the definition of kinetic parameters to explain the exchange of solutes from the brain (Illiff *et al.*, 2013). Verma *et al.*, have demonstrated that upon an injection of ^{99m}Tc -DTPA in humans, increasing the volume injected from 5 mL to 15 mL led to a stronger brain signal, whereas a subsequent increase to 30 mL had no other significant effect in comparison to the other two doses investigated (Verma *et al.*, 2020). Another key finding was that the entry of DTPA into the brain was recognised to be through ISF, considering that this molecule does not readily enter cells. Bearing in mind that SPIONs are NPs and more easily resemble the formulations investigated in this thesis, I decided to utilise them in investigating the IT injection in my rat model.

4.2.2. Scanning electron microscopy

An alternative method for the detection of SPIONs in the CNS is through environmental scanning electron microscopy (ESEM) coupled with energy dispersive X-ray (EDX) micro-analysis. This technique can be carried out on *ex vivo* tissue sections to confirm the presence of SPIONs in a sample, and therefore may be coupled with MR scanning. The EDX detector is capable of determining the X-ray spectrum of an element within a sample (through representation of a histogram plot of number of counts vs X-ray energy) (Khan *et al.*, 2020), which in turn allows us to identify the elements present on the surface of a thin sample (i.e., tissue sections). I therefore decided to employ this technique to confirm the presence of SPIONS in the spinal cord sections after IT injection.

4.3. Inflammation of the spinal cord

A variety of innate neuroinflammatory responses occur within the immune privileged CNS space as a result of either injury or disease. However, these processes, can be either beneficial

through supporting regenerative events, or deleterious through their amplification of destructive pathways (Mortaezaee *et al.*, 2018; Zendedel *et al.*, 2017). The initiation of these pathways in the CNS involves various multi-protein complexes known as ‘inflammasomes’, which have been defined by Tschopp *et al.*, in the early 2000s. The upregulation of the inflammasome cascade in disease states involving traumatic brain injury (TBI) and spinal cord injury (SCI) renders this research critical (McKee and Lukens, 2016; Zendedel *et al.*, 2016).

Vaccari *et al.*, have demonstrated that the inflammasome cascade consisting of caspase-1 and caspase-11 among a few others are involved in the rat innate CNS inflammatory response following SCI. The inflammasome cascade proteins are generally found in the motor neurons of the spinal cord, however alterations in the expression patterns are observed after SCI. Inflammasomes have the ability to sense pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) (Mortaezaee *et al.*, 2016). These are in turn recognised by pattern recognition receptors (PRRs), which are activated through post-traumatic neuroinflammation (Wallisch *et al.*, 2017; Liu *et al.*, 2013).

In naïve animals, caspase-1 expression is typically in neuronal nuclei. Within 6 hrs of injury, caspase-1 staining increases within the nuclei and extends into the cell cytoplasm and the processes close to the plasma membrane (Vaccari *et al.*, 2008). Moreover, immunoblot analysis of caspase-1 demonstrated that as soon as 3 hrs after injury, the relative density units of pro-caspase-1 exhibits a significant increase in comparison to naïve uninjured animals. Based on these data, I decided to investigate whether increased caspase-1 expression occurs 3 hrs after IT injections in my animal models (6 hrs was not possible due to experimental time constraints). This is to determine whether an acute inflammatory response is elicited as a result of the IT injection in a healthy animal model, which might be detrimental in the clinic, especially if repeated injections would be required to achieve a sustained, anti-nociceptive effect.

Therefore, the aim of this chapter was to investigate the IT injection method in my animal models through *ex vivo* imaging techniques. This was done to evaluate whether my technique correctly and successfully delivers therapeutics to the SAS. I also aimed to investigate whether the SPIONs penetrated the spinal cord after injection through *ex vivo* EDX analysis following MR scanning. I subsequently set out to also evaluate whether the injections were causing an acute inflammatory response in the spinal cord, as this could be detrimental in a clinical

setting, meaning repeated injections must be avoided and an indwelling catheter should be pursued for chronic delivery of nanomedicines.

4.4. Chapter objectives

- Establish whether a direct injection into the spinal cord is successful through delivery of methylene blue dye
- Investigate distribution of SPIONs in the CNS through MRI
- Determine localisation of SPIONS in the spinal cord through environmental SEM coupled with H&E staining
- Identify whether an IT injection triggers an acute inflammatory response

4.5. Results and Discussion

4.5.1. Injection of methylene blue dye into spinal cord

Methylene blue is commonly administered intravenously during surgical procedures because of its ability to dye certain tissues (Nguyen *et al.*, 2018). It also used to be administered intrathecally to patients in the clinic to track sources of CSF leaks, however this is no longer performed as a result of its toxicity (Liu *et al.*, 2016). In one case, a patient ended up developing paraplegia, therefore this practice is no longer carried out in the clinic. I decided to utilise the dyeing properties of methylene blue to evaluate out IT injection technique, however due to its toxicity, I administered it post-mortem.

Methylene blue was injected intrathecally in the lumbar segment after a laminectomy surgery. This was carried out for an initial assessment of the injection technique. Following a full laminectomy of the spinal processes in order to display the spinal cord, I observed that even with the lack of CSF circulation post-mortem, methylene blue diffused through the neuraxis reaching the cervical segments of the spinal cord (Figure 4.1) within a couple of minutes. These results were satisfactory and confirmed the success of my IT injection technique.

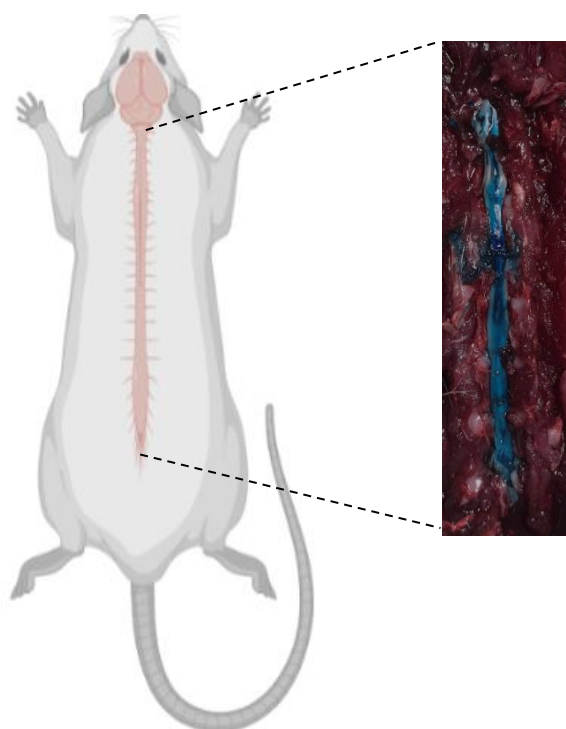


Figure 4.1. Methylene blue (15 μ L) injected into the lumbar segment of a rat post-mortem, to evaluate the success of the IT injection technique.

4.5.2. Magnetic resonance imaging (MRI)

I performed IT injections of SPIONs *in vivo* and carried out post-mortem MRI to confirm successful IT injections (rats were sacrificed 10 mins after injection to allow for immediate transport in the SAS). The advantage of post-mortem imaging is the ability to carry out long scanning sessions, which would be very difficult to perform in my animal model, as a result of the stereotaxic frame, anaesthetic supply and the open laminectomy surgery.

A total of 5 animals were used for the MRI experiments. One saline injection control and two SPIONs injections (one PEGylated SPIONs formulation and one without PEG functionalisation on the surface) which were fixed and perfused with 4% paraformaldehyde (PFA) prior to scanning. Tissue fixation was carried out to immediately cross-link molecules and precipitate proteins so the movement of SPIONs in the CNS was minimised after animals were sacrificed and during scanning.

I also scanned two animals which were not fixed with PFA – one un-injected control and one animal injected with SPIONs (without PEG functionalisation).

The iron core of the NPs acts by enhancing proton relaxation of specific tissues, therefore regions in which these are present are visible in MR images (Hahn *et al.*, 2011; Mandal *et al.*, 2005). The SPIONs provide a negative contrast in T₂-weighted images, by decreasing the MR signal intensity as previously discussed. Moreover, they also lead to hypointense signals, producing darker regions thus making their distribution distinguishable from the rest of the images. The degree of magnetism elicited by iron oxide SPIONs is dependent on the size, magneto-crystalline phase and composition.

4.5.2.1. Physical characterisation of SPIONs

Two different formulations of commercially available iron oxide (II, III) magnetic NPs solution (M_w 231.53) were acquired from Sigma-Aldrich and characterised by DLS. The main difference between the NPs was the PEG functionalisation of the surface of the NPs for one formulation, whereas this was absent in the other. It was stated that both NP formulations had an average particle size of 10 nm as characterised by TEM, were dispersed in water at a 5 mg/mL and 1 mg/mL (for the PEG functionalised NPs) initial concentration.

For my measurements, both NP formulations were diluted to a concentration of 0.01 mg/mL and the RI was set to 2.343 (magnetite) for the iron oxide NPs and 1.467 (PEG) for the iron oxide NPs with a PEG coating. The size of the NPs with a PEG coating was 44.87 ± 0.5 nm, PDI of 0.45 and ZP of -24.4 mV. In contrast, the size of the NPs without a PEG coating was 89.33 ± 1.33 nm, PDI of 0.25 and ZP of -34.6 mV (Figure 4.2).

As ZP measurements show the potential at the shear plane of the NPs in solution, it was expected that SPIONs had a negative charge, as they have been previously reported to be around -35 mV (Meisel *et al.*, 2020). In my case, the coating with a more neutral PEG raised the surface charge to -24.4 mV. In the literature PEG has been shown to raise the ZP of SPIONs closer to 0 mV, due to the masking effect PEG has on the negative iron core (Meisel *et al.*, 2020). Nonetheless, the degree of “masking effect” is influenced by the PEG chain length, which might vary, but also by the size of the iron core which has a counteracting effect on the overall charge.

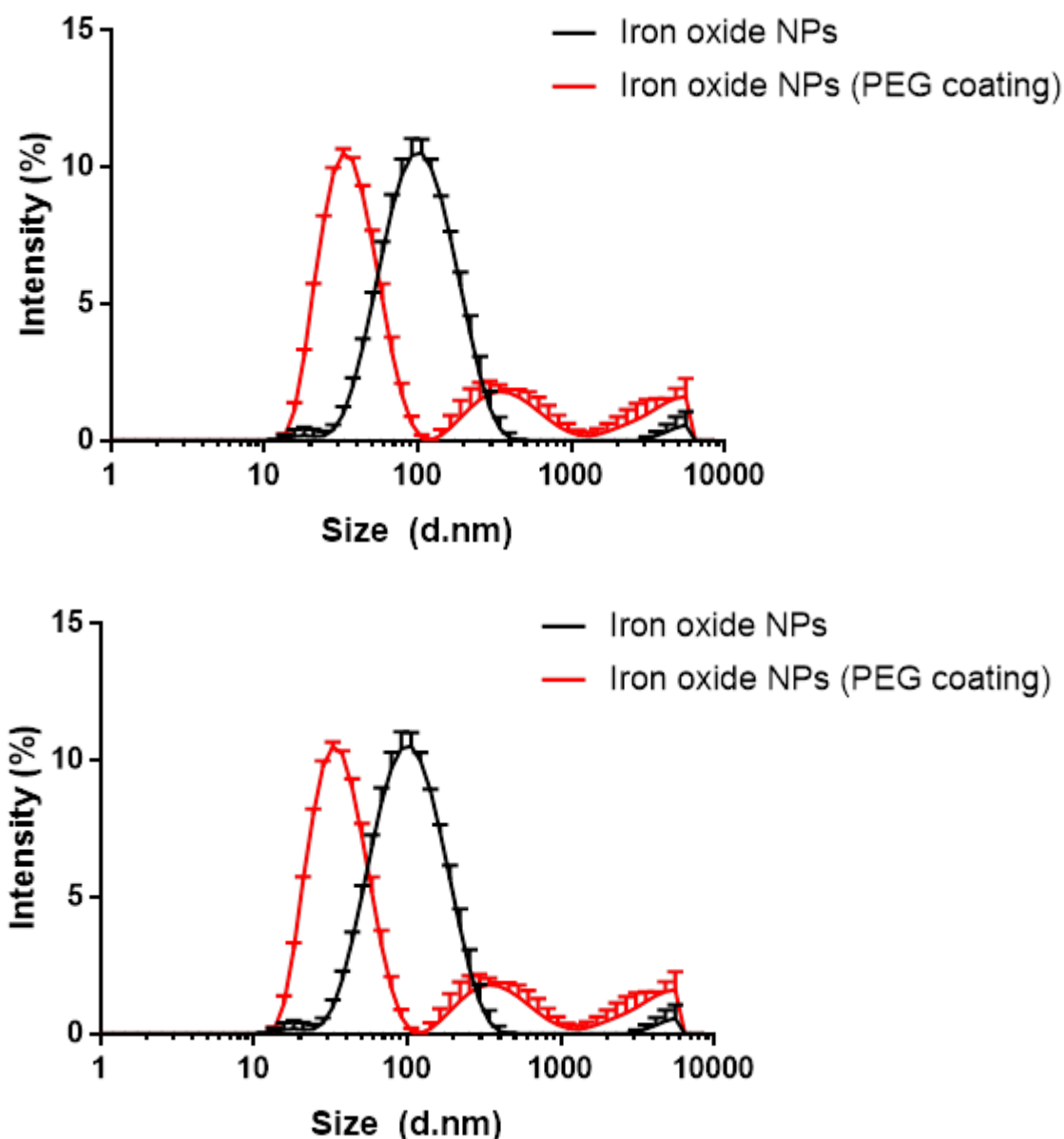


Figure 4.2. A. Particle size analysis and **B.** ZP characterisation of the commercially available iron oxide NP formulations. The particle size of the iron oxide NPs without a PEG coating was $89.33 \text{ nm} \pm 1.33$ with a PDI of 0.25. The particle size of the iron oxide NPs with a PEG coating was $44.87 \text{ nm} \pm 0.5$ with a PDI of 0.45. The ZP of the formulations was -34.6 mV and -24.4 mV respectively.

These results are very different from the particle size data (10 nm average size) reported by Sigma-Aldrich. The PDI of the formulations were not reported, however there was an expectation that this would be a homogenous population of NPs given the commercial availability of the products, whereas both formulations exhibited multiple peaks indicative of aggregation. Nonetheless, the difference in size could be attributed to the analysis method, which in my case was carried out by DLS as opposed to the reported TEM results. Because

there is variation in the size of the NPs, DLS measurements give more weight to larger particle sizes as they scatter light intensity more strongly. Moreover, TEM analysis might also size a proportion of the particles which might not have been representative of the entire sample.

It is important to note that the PEGylated iron oxide NPs exhibited a more heterogeneous population in comparison to the non-PEGylated formulation. This might have been the outcome of the coating procedure carried out during synthesis, as it sometimes leads to coating clusters of NPs as opposed to coating individual ones (Dagata *et al.*, 2008). Nonetheless, the difference in particle size and ZP of these formulations were noted and progressed to *in vivo* experiments.

4.5.1.2. SPION relaxivities

The standard way of highlighting contrast agent uptake prior to imaging animals is through evaluating their relaxation rates (relaxivity) over a range of concentrations. This is due to the fact that the relationship between relaxivity and concentrations follows zero order kinetics (Hobson *et al.*, 2019), therefore the slope (gradient) of the line is indicative of relaxivity.

There was good correlation between the concentration and relaxivity of both SPION formulations, with the relaxivities of SPIONs and PEGylated SPIONs being $52.8 \text{ mM}^{-1} \text{ s}^{-1}$ and $79.9 \text{ mM}^{-1} \text{ s}^{-1}$ respectively (Figure 4.3). In the clinic, higher MR relaxivity leads to higher signal-to-noise ratio (SNR), thus increasing the resolving power (Meisel *et al.*, 2020). Various physicochemical properties have an effect on SPION relaxivity measurements, such as the iron oxide core (size / material), NP coating (density / thickness) and chemical composition (Meisel *et al.*, 2020). It has been previously shown that the R_2 relaxivity of SPIONs increases with magnetization and total core size, indicating that larger SPIONs (i.e., SPIONs without surface PEG functionalisation, in my case, as discussed in section 4.5.2.1) should in theory display higher relaxivity measurements.

Nonetheless, a higher relaxivity measurement of $79.93 \text{ mM}^{-1} \text{ s}^{-1}$ was displayed by the smaller PEGylated NPs (Figure 4.3). It has however been highlighted in the literature that polymer coating (density, thickness, chemical composition) has a strong effect on SPION relaxivity. However exact correlations between these factors have not been established. One theory as to why PEGylated SPIONs have higher relaxivity is to do with the larger corona which slows the movement of water molecules surrounding the particles. This in turn would lead to an

increase in the amount of time protons spend in proximity to the core, causing higher relaxivity measurements (Meisel *et al.*, 2020). It is also possible that the larger clusters of PEGylated SPIONs (as demonstrated by the high PDI of 0.45 in Figure 4.2) might be causing the superior relaxivity observed, even though the average particle size was 44.87 nm. This has been previously demonstrated by Hobson *et al.*, who showed that the aggregation of 8 nm SPIONs into 150 nm clusters increased r_2 relaxivity (Hobson *et al.*, 2019). This is as a result of the cluster behaving as one magnetic entity, as opposed to single crystal SPIONs.

Nonetheless, further investigation is required in elucidating the effect of PEG chains on the surface of SPIONs and their size on relaxivity measurements for MR imaging, however this is outside the scope of this thesis.

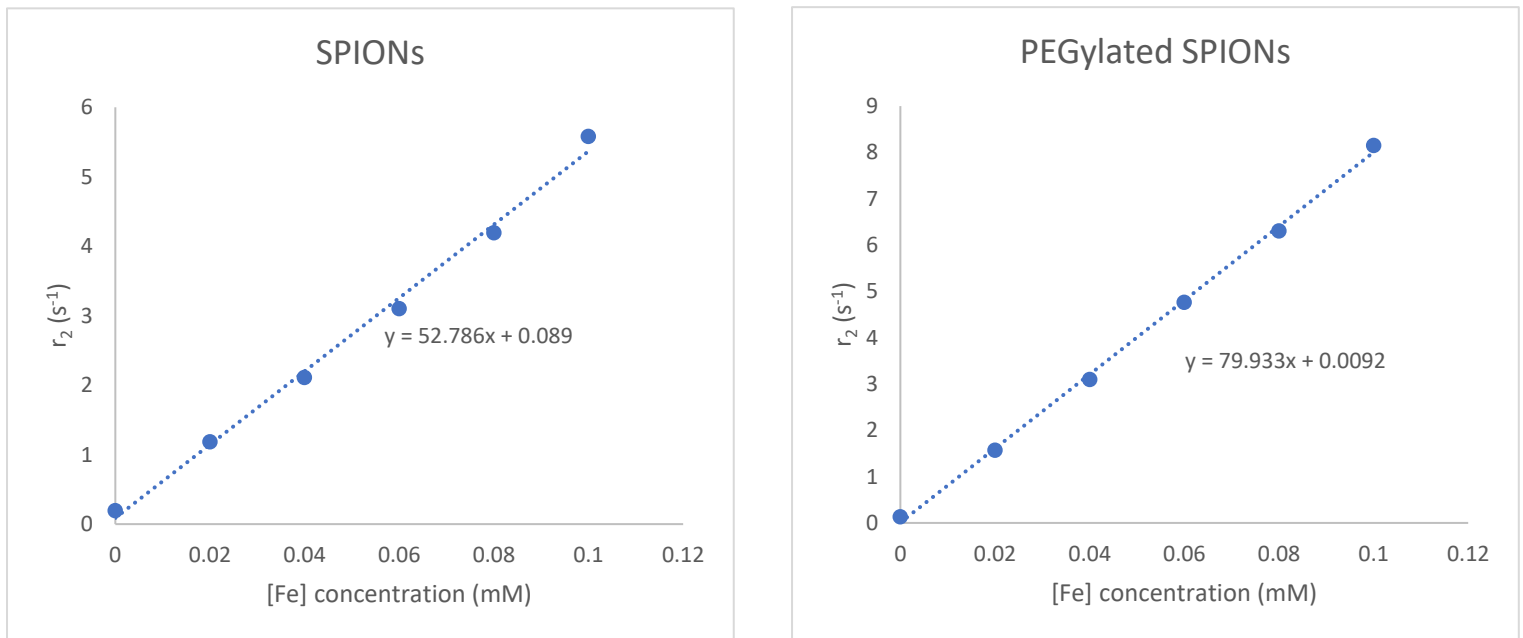


Figure 4.3. Relaxivity plots of SPIONs (A) and PEGylated SPIONs (B) were $52.8 \text{ mM}^{-1} \text{ s}^{-1}$ and $79.9 \text{ mM}^{-1} \text{ s}^{-1}$ respectively between a concentration of 0 and 0.10 mM [Fe]. The relaxivities can be determined from the slope of the plots.

4.5.1.3. Post-mortem MRI of rats after IT injection of SPIONs

Post-mortem MRI of animals allows examination with much higher spatial resolution and SNR in comparison to *in vivo*. Moreover, there are no time constraints in regards to data acquisition, alongside no motion artefacts during scanning. Nonetheless, acquisition should be performed as soon as possible after the experiment is finalised, and should be kept consistent between animals (Shatil *et al.*, 2018). In order to prevent tissue degradation and

post-mortem diffusion of the injected formulations, I decided to preserve the tissues with a chemical fixative (4% PFA) through transcatheter perfusion after sacrifice.

Unfortunately, the post-mortem perfusion and fixation with 4% PFA led to a substantial increase in R_2 intensities across all animals, with the highest signal emitted by the saline injected control (Appendix 8.10 and 8.11). Upon further investigation of the literature, it was apparent that PFA leads to shortening of T_1/T_2 relaxation times occurring immediately after fixation, leading to an R_2 increase over-time (Dawe *et al.*, 2009). The shortening of the T_2 signal also leads SNR loss, demonstrating that the use of a formalin-based fixative is detrimental in successful MR imaging (Ericsson *et al.*, 1992; Dawe *et al.*, 2009). The exact mechanism as to how this occurs is currently unknown, however the protein-cross linking resulting in tissue rigidity and interactions between myelin lipids and water might be one of the causes (Laule *et al.*, 2007). Moreover, it was also suggested that the replacement of water from tissues with formalin also has an effect on R_2 relaxivity (Richardson *et al.*, 2013), and that even small differences between fixation times can affect MRI results.

As a result of this, I discarded the scans acquired from the animals fixed with PFA and carried out the analysis on the animal injected with one SPION formulation (without PEG functionalisation) and compared it to an un-injected control which had not been fixed with PFA. Injection of SPIONs to the IT space of rats did not result in visible hypointense zones around the site of injection on T_2 -weighted MR images, as would have been expected (Figure 4.4) upon the scanning of the front and back of the animals.

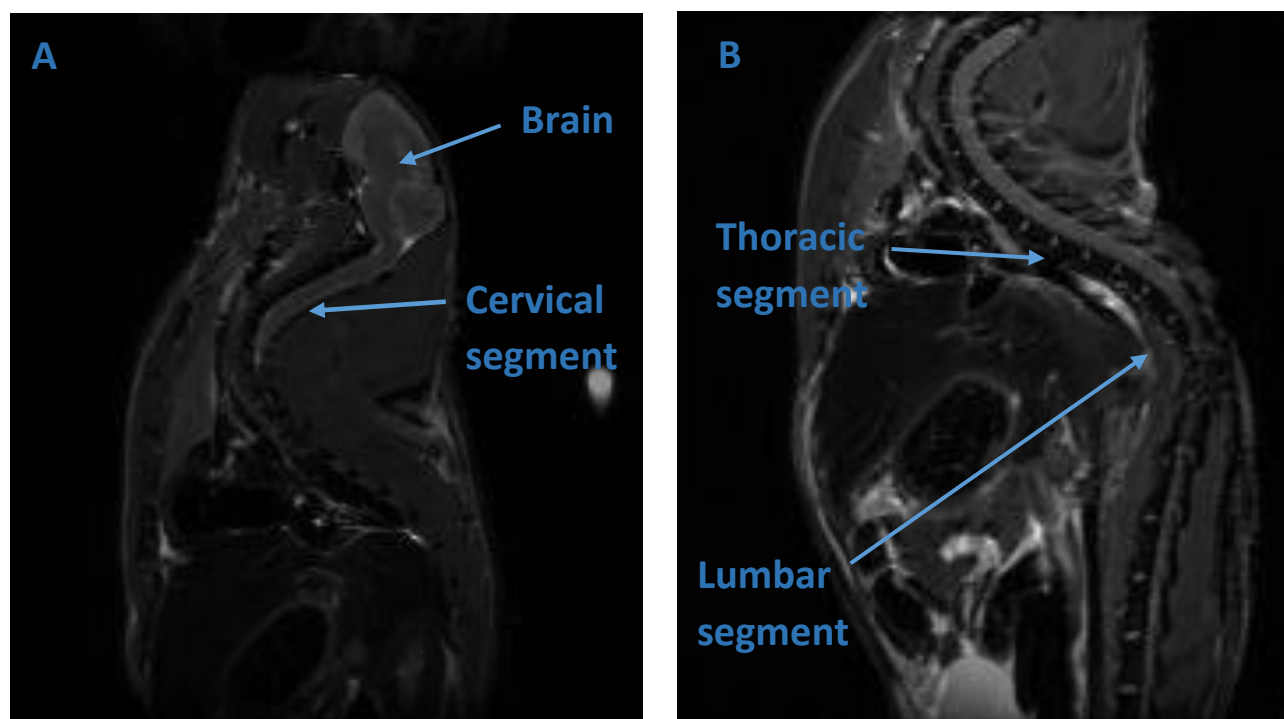


Figure 4.4. T_2 -weighted MR images of a rat injected with SPIONs in the IT space (**A.** front and **B.** back). Images were acquired post-mortem, over-night, following the IT administration procedure.

Nonetheless, upon analysis of the R_2 maps, it seemed that R_2 intensity was increased alongside the spinal cord in the SPION injected animal as compared to the control (Figure 4.5). ImageJ was used to measure the pixel intensity around the lumbar segment spinal cord area to compare the SPION injected animal vs the control. A pixel intensity mean of 0.222 ± 0.0441 was shown across the lumbar segment for the SPION injected animal, whereas average pixel intensity of 0.178 ± 0.023 was observed in the control un-injected animal (calculated as mean pixel intensity over sampled area). These results therefore demonstrate that a 19.57% increase in intensity around the lumbar spinal cord is evident upon post-mortem MR imaging 10 mins after IT injection of SPIONs. The higher intensity seems to be localised at the site of injection with no distribution higher up the spinal cord or in the brain as detected by MRI (Figure 4.5). Unfortunately, no statistical analysis could be carried out as a result of an $n=1$ for each treatment group. Moreover, the MR images of the other animals perfused by PFA cannot be used due the alteration of R_2 signals as a result of the fixation.

Higher R_2 intensity was also observed in peripheral organs in the abdominal cavity, however as this was also present in the control, un-injected animal, it was thought to be an artefact. When SPIONs are injected IV *in vivo*, they have been shown to accumulate and eliminated

through the kidney, liver and spleen (Ashraf *et al.*, 2019). During experiments, the highest signal was observed as soon SPIONs were injected, with reduction over the course of the following days (Ashraf *et al.*, 2019). Nonetheless, the SPIONs accumulated in the liver did not fully clear by day 14, suggesting the possibility of toxicity, especially if this is carried out in the clinic. Consistent high levels of Fe ions in the body might lead to homeostasis imbalance and irregular cellular responses in regards to oxidative stress, DNA damage or inflammatory responses (Singh *et al.*, 2010). On the other hand, the persistence of SPIONs *in vivo* might be advantageous, due to the ability to be tracked over longer periods of time.

The widespread utility of SPIONs is currently limited, due to fairly low detection limits *in vivo*. The lack of significant detection of SPIONs in my study might be due to a couple of reasons: aggregation in the body due to the high PDI exhibited when analysed *in vitro*, the low detection limit of MRI or extremely rapid clearance after injection. These negative results were exacerbated by the use of PFA fixation, which rendered the other animals unusable due to the negative effects formalin had on the MR images.

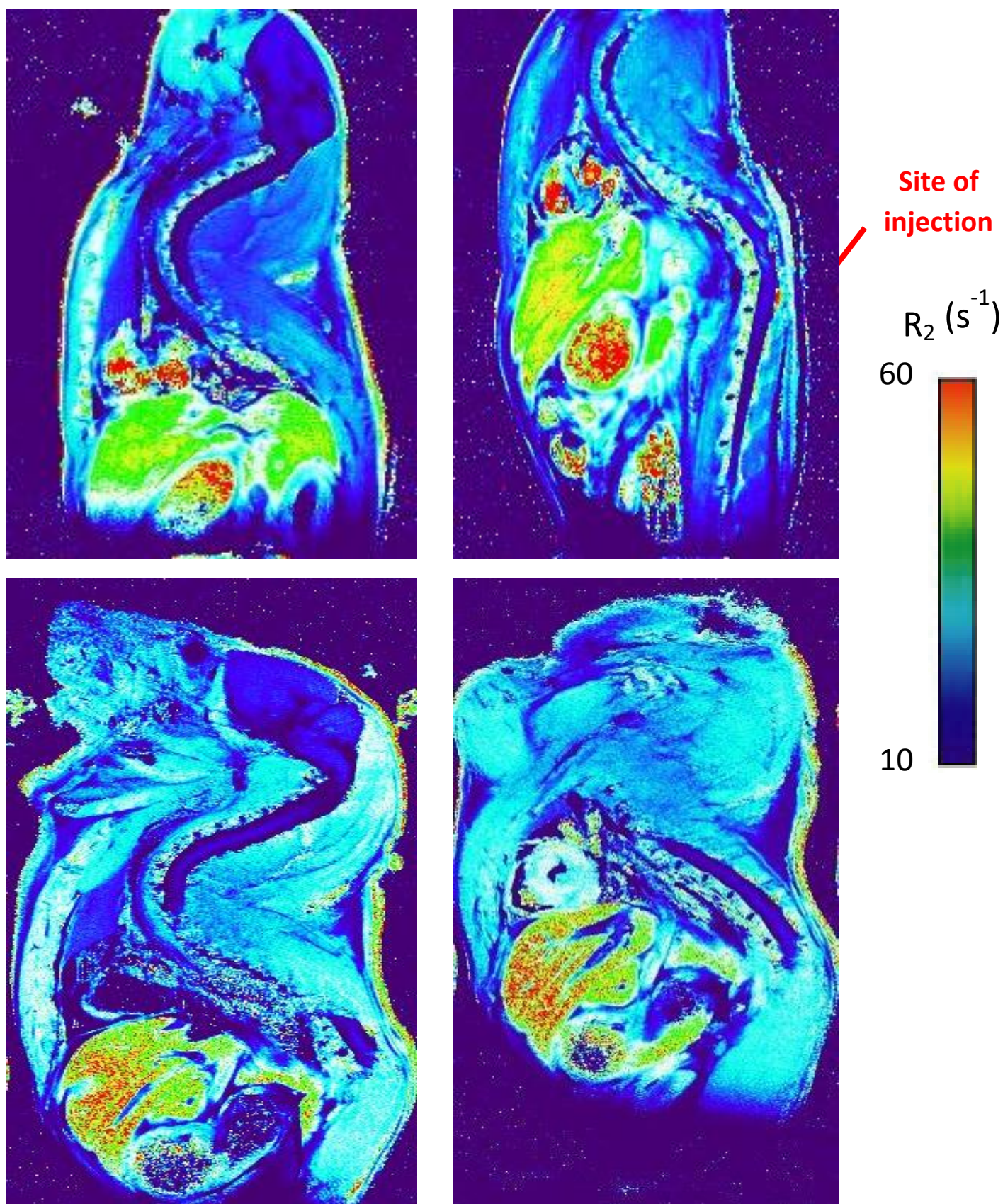


Figure 4.5. R_2 post-mortem MRI maps. **A** and **B** are front and back images of the SPION injected rat. **C** and **D** are front and back images of the un-injected control rat. Higher R_2 intensity (red) can be seen in the lumbar area of the SPION injected animal (**B**).

4.5.3. Histological analysis of the rat spinal cord

I carried out histological analysis of the lumbar spinal cord by H&E staining to utilise this concurrently with environmental SEM data to identify the site of distribution of SPIONs after injection. My aim was to explore whether the SPIONs diffused into the grey or white matter of the spinal cord after injection. Evidence of the SPIONs in spinal cord might be indicative of whether the injection technique was correctly penetrating the SAS and whether the NPs diffuse into the spinal cord tissue at the site of injection.

Sequential serial axial sections were cut from the lumbar segment between L4 and L5, at the site of IT injection. H&E staining was used to indicate the structures of the spinal cord (haematoxylin stains the cell nucleus (purple / blue) whereas eosin stains cytoplasmic proteins (red / pink)). Magnification was set to 2.5x

The differences between grey and white matter can be distinguish in the spinal cord sections, with grey matter being in the centre denoted by the darker purple colour (Figure 4.6). Several tears were observed in the sections, however the origin of these is uncertain, as the damage could have happened during sample preparation (i.e., sectioning, staining, and dissecting). Nonetheless, it is also possible that the tears into the spinal cord occurred during IT injections, which might have penetrated into the tissue of the spinal cord.

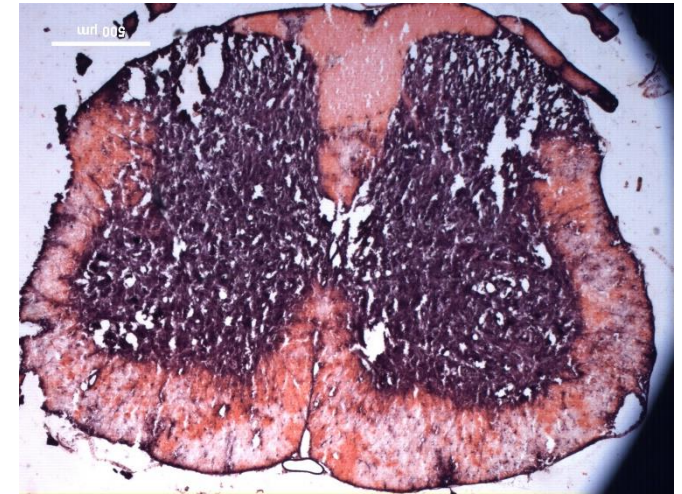
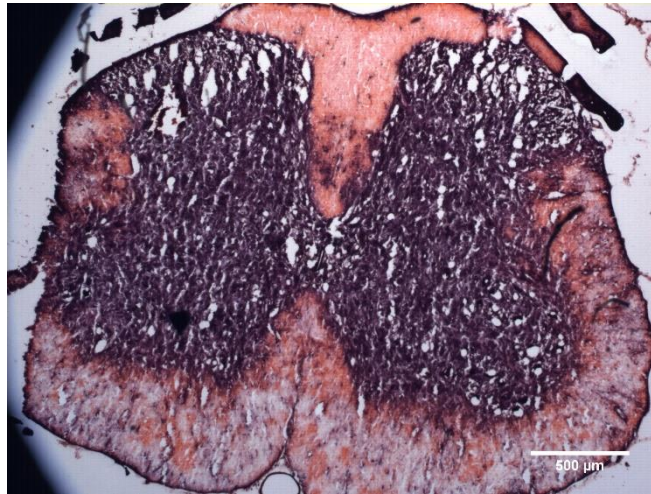
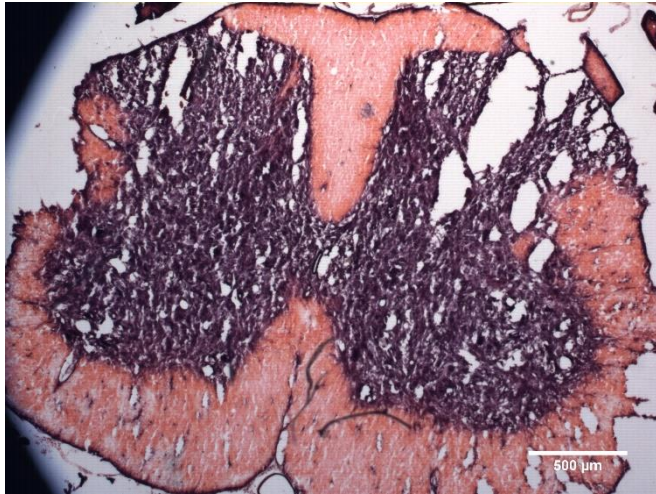


Figure 4.6. H&E staining of spinal cord sections (L5 segment) cut after injection of iron oxide NPs. various tears were apparent in the sections denoting either section damage during preparation or sectioning of the tissues or as a result of the injection force.

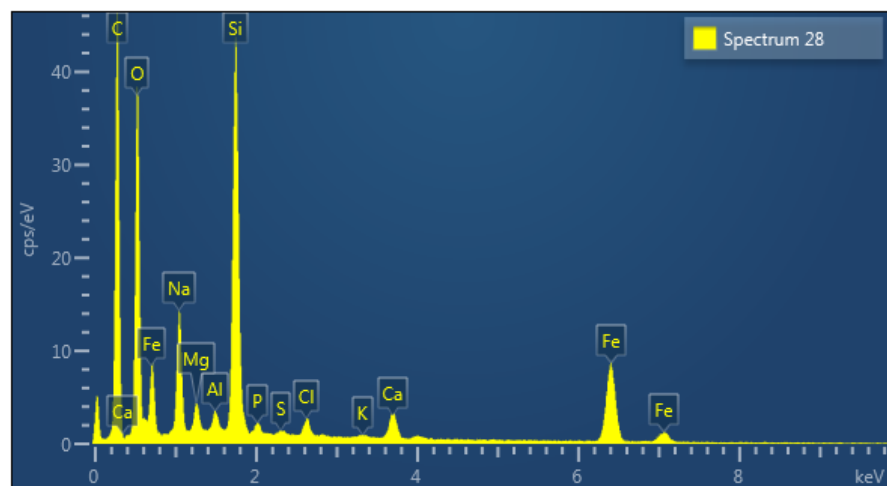
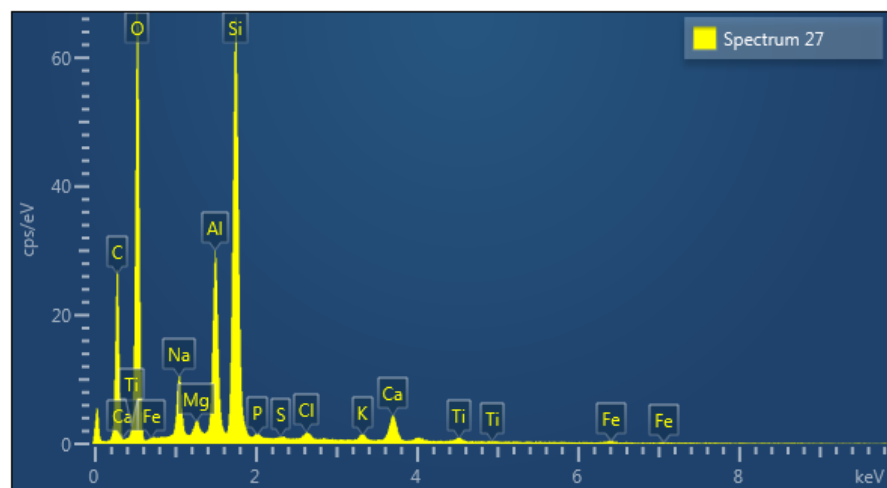
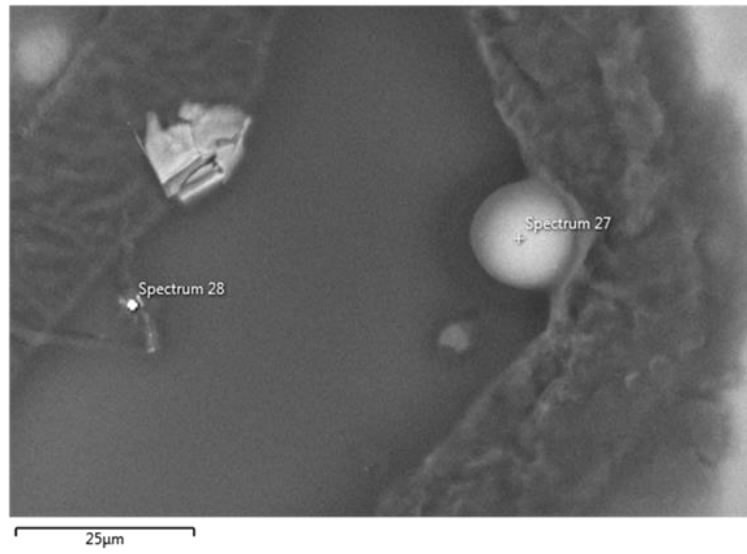
4.5.4. Environmental scanning electron microscopy

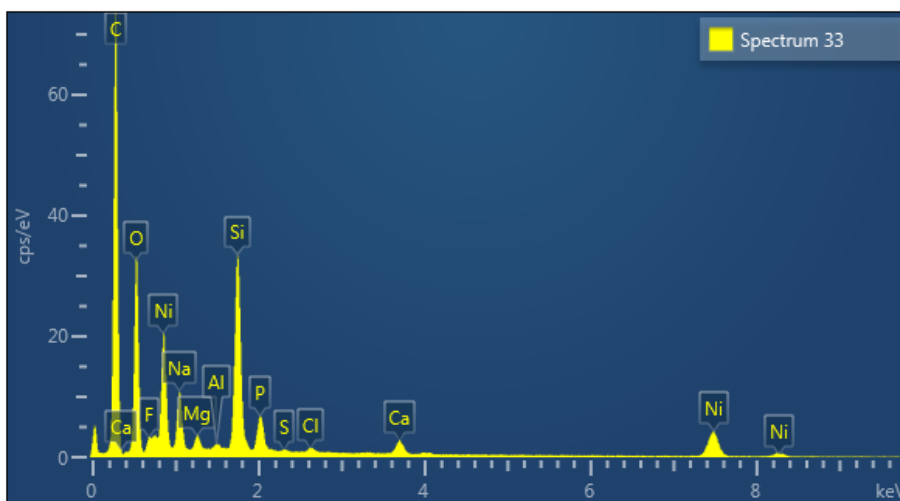
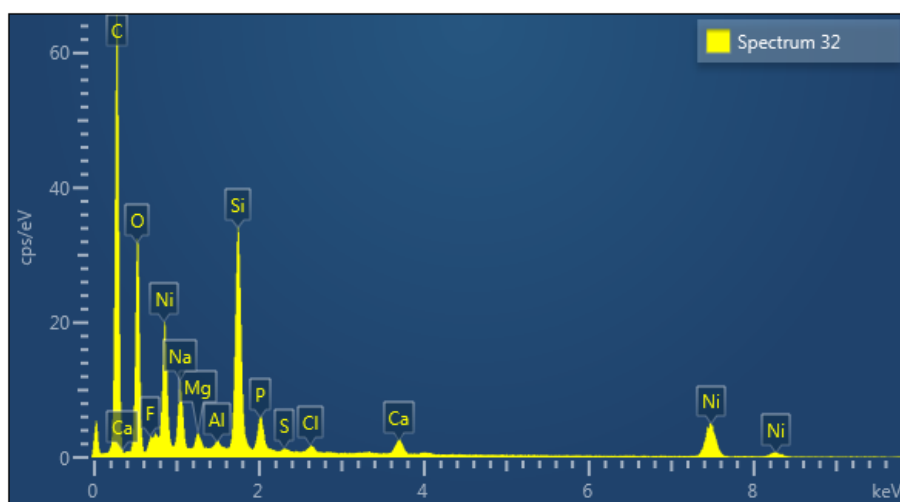
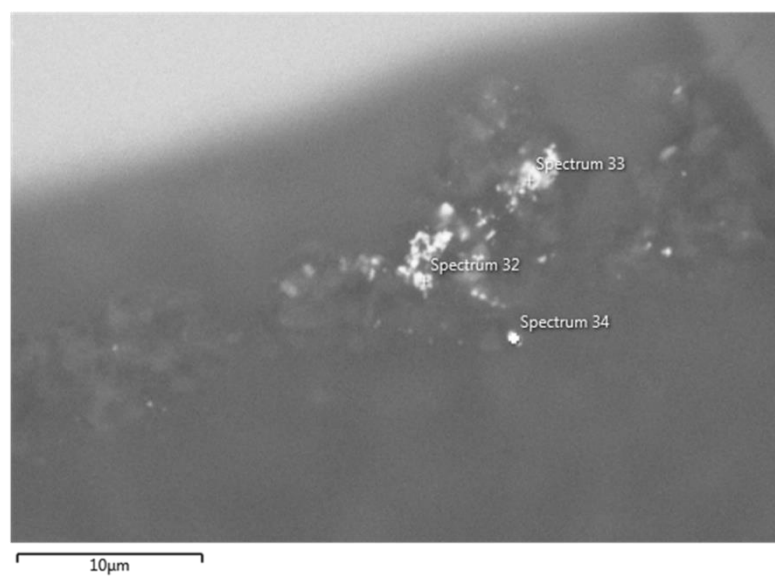
EDX analysis is a method used for the analysis of NPs through SEM. This technique is used for the detection of elements present in the sample through the use of an X-ray spectrophotometer coupled to an SEM. A method for the detection of iron (Fe) in samples has previously been developed by Hala *et al.*, who demonstrated that peaks around 6.39 keV in EDX spectra are related to the binding energies of Fe (Hala *et al.*, 2016).

I carried out SEM-EDX analysis of L5 spinal cord sections injected with SPIONs. EDX analysis identified the presence of iron in the sections, alongside other elements including nickel and zinc (Figure 4.7, spectra 32, 33, 34). Figure 4.7 spectra 27 and 28 show the iron peaks around 0.9, 6.1 and 7 keV, which are related to the binding energies of iron. This has also been reported in the literature by Rahman *et al.*, who characterised iron oxide NPs *in vitro* after their synthesis. (Rahman *et al.*, 2017). In comparison to data in the literature showing EDX spectra of iron NPs, the counts (cps/ev) shown in my tissue sample are 600-fold lower (Hala *et al.*, 2016). This might be due to the very small quantities detected *ex vivo*, or the lower limit of detection of my EDX system.

Unfortunately, the precise exact location of the SPIONs within the spinal cord could not be exactly determined due to the high resolution of this technique. Many other images taken had no iron detected on the spinal cord section (data not shown), indicating the scarcity of the iron elements in mysections. The largest EDX peak corresponded to carbon and / or oxygen in most samples, closely followed by silicon. The presence of silicon could be attributable to contamination of samples through handling (i.e., the use of tweezers) or the Si-Li detector in the EDX system. Nevertheless, the presence of nickel in Figure 4.7, spectra 32-34 is interesting, and it was possibly due to the sectioning of the tissue on the cryostat or the injection needle itself.

Nonetheless, these data confirm that there is some presence of SPIONs in the spinal cord after my IT injection. Unfortunately, the exact location of these particles cannot be established, however it is a good indication that some NPs diffuse into the spinal cord at the lumbar cord level, after the administration into SAS.





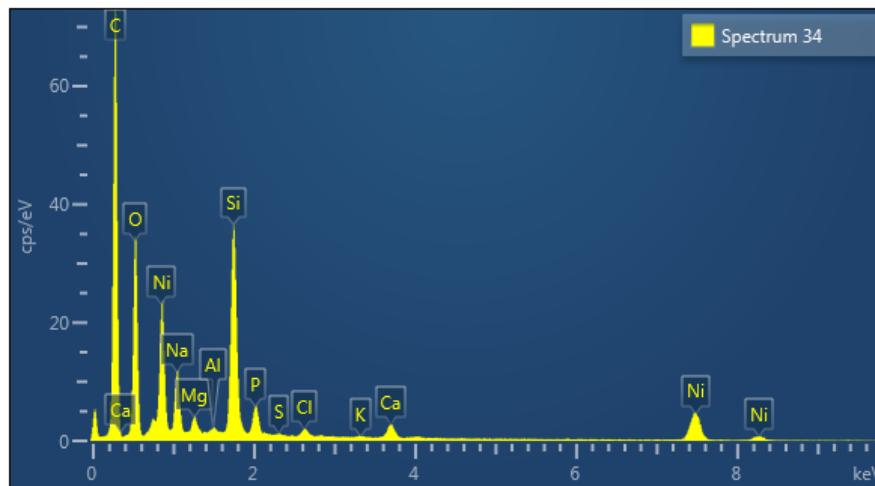


Figure 4.7. EDX spectra of SPIONs in spinal cord tissue sections after IT administration. Fe elements can be observed in spectra 27 and 29, peaks 0.9, 6.1 and 7 keV. No Fe detected in spectra 32, 33 or 34.

4.5.5. Immunofluorescent staining of inflammatory markers in the spinal cord

Acute inflammation associated with the mechanical trauma of an IT injection could lead to serious consequences in the clinic. For this reason, I sought out to investigate whether an upregulation of inflammatory markers immediately after injection was observed. Vaccari *et al.*, found that spinal cord injury induced caspase-1 immunoreactivity in the spinal cord of animals as early as 3 hrs after injury (Vaccari *et al.*, 2008). Caspase-1 is a cysteine protease involved in the inflammasome cascade. In the rat, the activation of the inflammasome cascade leads to secretion of pro-inflammatory IL-1 β and IL-18, triggering an inflammatory response. Moreover, this activation can also lead to pro-inflammatory cell death (McKenzie *et al.*, 2018).

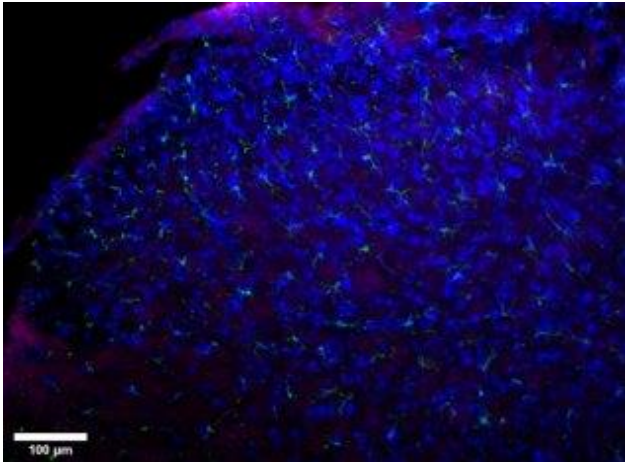
Immunohistochemistry studies demonstrated that within a lesioned section of the lumbar segment of the spinal cord there were notably higher levels of fluorescence (purple) pertaining to caspase-1 labelling inside and surrounding the lesion (Figure 4.8). IBA1, a marker of microglia expression (green) was also observed, which seemed show elongated microglial projections around the lesion as compared to an un-lesioned section (Figure 4.8). Minimal caspase-1 was observed in the un-lesioned section, where there also seemed to be no signs of elongated microglial processes.

DAPI

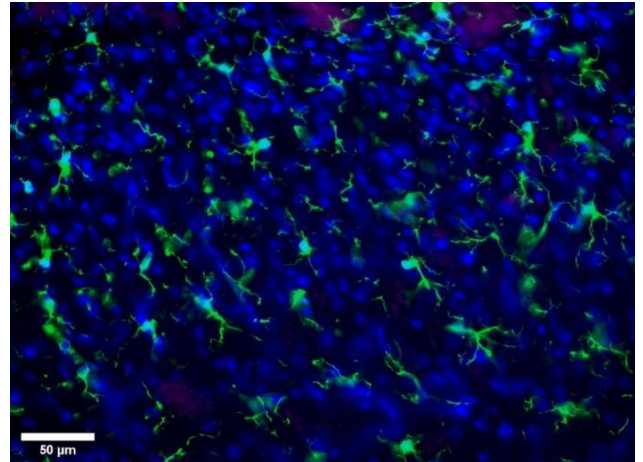
IBA1

Caspase1

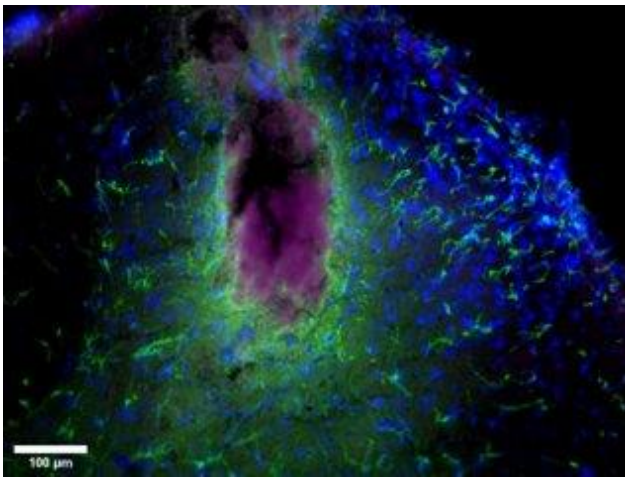
L5 section 10 x magnification



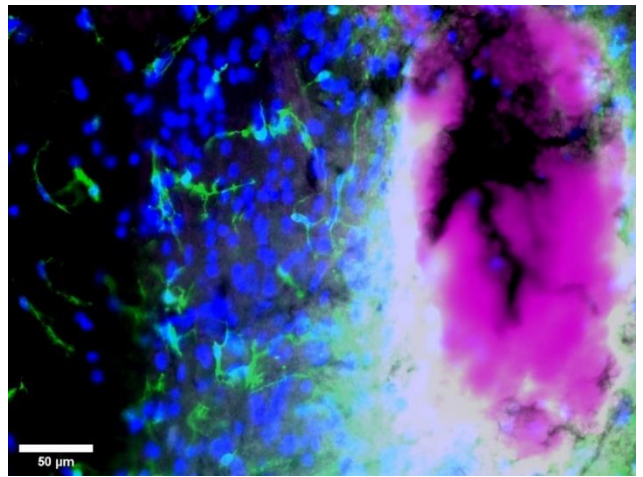
L5 section 20 x magnification



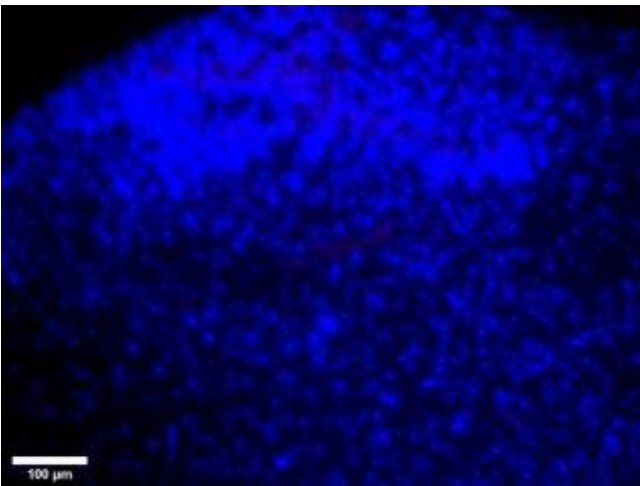
L2 section with lesion 10 x magnification



L2 section with lesion 20 x magnification



L2 control section 10 x magnification



L2 control section 20 x magnification

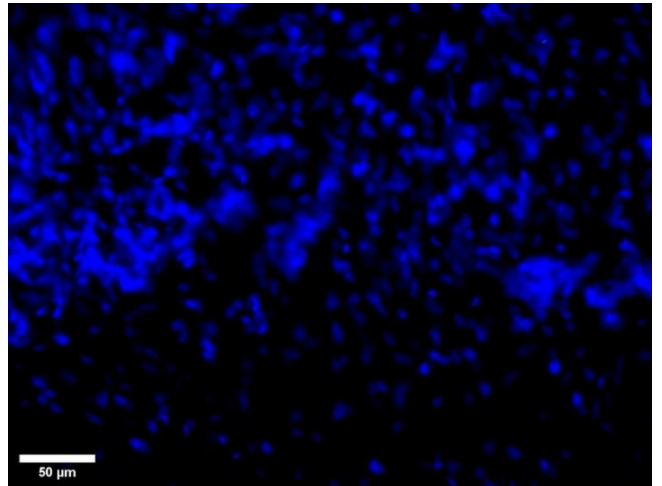


Figure 4.8. Immunofluorescent images of lumbar segment sections 3 hrs after the administration of SPIONs. Caspase-1 expression was observed surrounding a lesioned section of the spinal cord, with increased microglial processes extending towards the lesion. IBA1 (green) is a marker of microglia and DAPI (blue) is a marker for nuclei. Caspase-1 (purple) is a marker for the inflammasome cascade. Microscopy images taken by Dr Stephen Woodhams.

Higher levels of fluorescence also extend into the healthy tissue surrounding the lesion; however, this might be an artefact. Future work would need to include a lesioned section in control sections in order to evaluate whether this is an artefact or not.

These data provide interesting evidence of caspase-1 and thus inflammasome activation surrounding a lesioned area of the lumbar spinal cord after IT injection in my *in vivo* animal model. Moreover, extended microglial processes were observed surrounding this area. It therefore appears that the IT injection might damage the spinal cord and thus trigger the activation of the inflammasome cascade surrounding the lesion site. Nonetheless, a control of un-injected lumbar spinal cords is required to ascertain whether this occurs due to the injection. The fact that the lesion (tear) is surrounded by caspase-1 inflammatory markers suggests that this took place whilst the animal was alive, therefore it could be concluded that the tears present in the H&E sections were also resultant from the same cause (i.e., penetration of the needle into the spinal cord).

4.6. Conclusions

I carried out various imaging techniques to evaluate whether my IT injection in healthy rats was successful. I utilised iron oxide based SPIONs as contrast agents for MR imaging to locate where if the formulations were successfully injected into the CNS. Initial MRI scans suggested that SPIONs are localised at the site of injection in the lumbar segment, leading to a 20% increase in pixel intensity after administration in post-mortem scans. Unfortunately, other MR images were unsuccessful due to the effect PFA fixation had on the R_2 relaxation times, leading to the control animal emitting most signal throughout.

I then sought out to investigate whether SPIONs were detectable in the spinal cord through EDX analysis. I identified some iron elements in some lumbar cord sections; however, I was not able to determine the exact location through the corroboration with histology sections. Moreover, I identified a variety of other metallic elements present in the samples such as nickel or zinc, which are probably contaminants from the needle, dissection or sectioning equipment. Finally, I aimed to evaluate the acute inflammatory response in the spinal cord, after injection, to determine whether repeated administration of nanomedicines would be feasible in the clinic. In one lesioned section of the lumbar cord, I observed increased fluorescence after staining with a caspase-1 antibody, indicative of the activation of the

inflammasome cascade. Moreover, microglial processes seemed to be elongated around the lesion, whereas this was not observed in a section with the absence of a lesion. This suggests that the injection triggers an acute inflammatory cascade within 3 hrs of administration, however further investigation of other sites of the spinal cord are required to establish this.

Overall, the work carried out in this Chapter highlights the technical difficulties associated with this delivery technique – the requirement of preparative micro-surgery involved and the small anatomical dimensions of the IT space in rodents. Nonetheless, the methylene blue dye injection and the MRI of unfixed animals suggests that the technique I have used was successful in delivery to the CNS. The post-mortem dye injection demonstrated that methylene blue diffused alongside the entire neuraxis, even with the absence of CSF flow, and injection of SPIONs in one rat led to an increase in pixel intensity at the lumbar site. Moreover, I have also detected iron elements in spinal cord sections through the use of EDX analysis. Therefore, this animal model set-up will be used for the *in vivo* evaluation of the previously developed CBD formulations.

5. Chapter 5 – *In vivo* biodistribution and anti-nociceptive evaluation of CBD formulations

5.1. Introduction

Systemic administration of therapeutics lacks effective access into the CNS. Many delivery routes (IV, IP, oral) do not lead to efficacious entry into the spinal cord and / or the brain as a result of the BBB and the BCSFB. IT administration has arisen as a route to bypass these pharmacokinetic challenges, administering therapeutics directly into the areas expressing receptors which modulate pain states affected in patients suffering from neuropathic pain.

Although much progress has been made in the delivery of therapeutics to this area, this procedure remains vastly complex as a result of the intricacies associated with the injection, anatomical structures, CSF flow and clearance of analytes, to name a few. Recent discoveries in drug delivery systems have shown that nanomedicines can be utilised to overcome the drawbacks associated with conventional dosage forms, leading to higher physical stability, solubility, long-term release, permeation and partitioning into tissues. A few different colloidal systems injected intrathecally have been utilised so far, such as magnetic drug targeting, dendrimers, lipid nanocarriers and gold NPs. Nonetheless, there has been no direct comparison on the effects of formulation strategies in the biodistribution of drugs in the IT compartment, coupled with an investigation into the clearance of therapeutics from the CNS. Investigating this route of delivery and establishing how formulations affect the effectiveness of therapeutics and their biodistribution in the CNS will help improve treatment for patients suffering from neuropathic pain. Moreover, this technology can also be advanced in the near future through the use of Ommaya reservoirs, providing a constant localised influx of stable formulations whenever required by patients.

5.2. Biodistribution in the IT space

A variety of formulations have been investigated in the IT space so far. One paper looked at lipid formulations in a rabbit model investigating tetracaine and meperidine (Langerman *et al.*, 1991 and 1990). The drugs were formulated in a hyperbaric lipid (iophendylate) in a 10% glucose solution or an aqueous solution. Langerman *et al.*, has demonstrated that the lipid formulations accumulated in the dural sac of the spinal cord acting as a depot, and their spinal analgesic effects were enhanced as opposed to their lipid free controls. The enhanced analgesic activity observed was hypothesised by the authors as being the result of higher specific gravity of the lipid carrier. However, the use of the vehicle had to be ceased due to its causation of meningeal irritation (Mehta *et al.*, 1992). These data suggest that the therapeutic efficacy of drugs can be impacted upon by the formulation, however it is essential that a non-toxic vehicle is utilised, as I previously emphasised in Chapters 1 and 3.

The distribution of formulations injected intrathecally is thought to be facilitated by diffusion and / or fluid exchange. This process is very complex due to the SAS not being homogenous. After administration, the therapeutics diffuse into a variety of micro-environments including the grey and white matter of the spinal cord, the CSF, arachnoid cell membranes and extra-cellular fluids. However, immediate distribution across the neuraxis and into brain parenchyma has also been described in the literature, suggesting that CSF flow plays a role in facilitating the movement of formulations in the CNS. This has been demonstrated by early preclinical work involving the delivery of ^{99m}Tc -labeled multilamellar and small unilamellar lipid vesicles (MLV and SUV respectively) to the IT space of rats via a lumbar puncture (Umbrain *et al.*, 1997). Rapid biodistribution according to particle size was observed alongside the neuraxis within a few minutes following injection. The SUV possessed a size of approximately 50 nm in diameter whereas the MLV were approx. 3.5 μm . The smaller lipid vesicles cleared away into the brain and thoracic spinal cord, whereas the MLV displayed slower distribution profiles away from the spinal cord and into the brain over time. These different biodistribution profiles observed in relation to nanocarrier size, highlight the possibility of optimising drug delivery to specific parts of the CNS by simply changing the particle size.

Another group has investigated the biodistribution of fluorescently labelled polystyrene nano- and micro-particles within the SAS after an ICM injection in mice (Householder *et al.*, 2019). They showed that there was wide distribution of both formulations within the entire CNS (brain and spinal cord) with limited clearance from the SAS, indicating the feasibility of a sustained localised effect. It is also important to mention that the NPs achieved widespread sustained biodistribution within the CNS 2 hrs post injection and persisted for at least 3 weeks. These data highlight the advantages of NP delivery systems and their feasibility for a long term sustained release formulation with rapid action and biodistribution within the SAS of the CNS (Householder *et al.*, 2019).

It is widely accepted that drugs encapsulated within nanocarriers have also shown to have improved half-lives within the CNS in comparison to their free form. Examples include the previously discussed cytotoxic agent methotrexate, cytarabine (Ara C) and BCNU. Ara C displayed cytotoxic levels within the CSF lasting for a few weeks following injection when administered in a MLV formulation (Kim *et al.*, 1993). This example demonstrates the possibility of utilising nanocarriers to achieve sustained release within the SAS. Ara C was subsequently progressed into the clinic as DepoCyt®, used to treat lymphomatous meningitis through IT delivery. Since its release onto the market, it has been used for the treatment of various cancers, considering that the pharmacokinetic properties of Ara C have a notable improvement when its delivery is facilitated by the use of DepoCyt®.

A different study evaluated encapsulating minocycline and ciprofloxacin in solid lipid particles (SLNs) for the treatment of arthritis induced pain (Vlades *et al.*, 2018). Their hypothesis was that SLNs would work by providing protection for the drug, whilst also increasing the absorption surface area in order to improve drug efficacy. Subsequent results demonstrated that the antibiotics encapsulated within SLNs had increased efficacy of approximately 50% over the free drug, and their anti-nociceptive activity lasted up to 7 days. Various other studies investigating liposomal morphine (Nishiyama *et al.*, 2000) and PLGA nanocarriers encapsulating baclofen (Lagarce *et al.*, 2005) and ketamine (Han *et al.*, 2015) have showed constant improvement in pharmacokinetics, lower toxicity resulting in fewer side effects and extended periods of efficacy in the treatment of pain.

These examples provide evidence that nanocarriers enhance the pharmacokinetic profiles of drugs administered to the CNS, however the impact different formulations have on the same

therapeutic and their biodistribution has not been thoroughly evaluated. I therefore aim to investigate the fate of CBD in the CNS after its formulation within two physically distinct formulations.

5.3. IT administered therapeutics for anti-nociception

Many therapeutics have been administered systemically through IV injections in the treatment of pain, however various side-effects and a short therapeutic window led to a problematic use in the clinic. One example being neostigmine, an acetylcholinesterase inhibitor which presents with numerous side effects and unpredictable incidences of nausea, leg weakness, hallucinations, increased blood pressure to name a few. Neostigmine was formulated as an MLV formulation by Grant *et al.*, with the aim to compare it to a solution of pure drug after IT administration in healthy rats. Results demonstrated that liposomal neostigmine prolonged spinal anti-nociception from 0.312 to 6 hrs, providing a sustained release depot within the CNS for the gradual release of the drug (Grant *et al.*, 2002). Similar results were also shown with the use of liposomes to deliver morphine and bupivacaine, where a significant prolongation of anaesthetic response and decreased toxicity were observed (Grant *et al.*, 1995; Grant *et al.*, 1997). Altered pharmacokinetic profiles have been observed with the use of liposomal opioids, more specifically bupivacaine which was assessed in canine animal models (Joshi *et al.*, 2015). When administered in a liposomal form, bupivacaine demonstrated extended half-life in comparison to the free drug. This was also associated with lower toxicity which was observed even in instances where higher doses of liposomal bupivacaine were administered.

Many other drugs have been used intrathecally or epidurally for the treatment and the control of pain. Preclinical examples of this includes small molecules such as bupivacaine, morphine, alfentanil, ketamine and ketoprofen encapsulated within lipids or polymers. These drug delivery systems work by increasing the drugs' half-life within the CNS, minimising adverse effects and increasing their efficacy. Reducing adverse effects is essential, especially in the case of morphine which can very easily lead to respiratory depression, by affecting the hypoxic ventilatory response in the brainstem, which can be fatal (Boom *et al.*, 2012).

Taken together, this evidence strongly suggests an advantage in using nanocarrier systems in governing analgesic effects following IT administration. It is however unclear whether these

differences are attributed to the changes in the release rate of therapeutics or due to their differential biodistribution as a result of the nanocarriers' material composition (Fowler *et al.*, 2020). Carrying out further investigation into the properties governing the efficacy and biodistribution of analgesics following delivery to the SAS would provide a state-of-the-art insight into this drug delivery method. One way of assessing the anti-nociceptive effects of IT administered therapeutics is through measurements of the paw withdrawal reflex via electromyography recordings, which will be discussed in the next section.

5.4. Electrophysiology

The mammalian withdrawal reflex is a sensory behaviour which protects animals from surrounding danger, and is also known as the nociceptive withdrawal reflex (NWR). This NRW has a high stimulus threshold which normally lies within the noxious range, and is therefore widely used in pain research in both rodents and humans (Le Bars *et al.*, 2001). This phenomenon was at first extensively researched in the early 1900s, when Sherrington spinalised cats and demonstrated that noxious stimuli in the afferent nerves of the skin, hip, knee or ankles lead to widespread flexion of the limbs (Sherrington, 1910). This took place as a result of the excitation of the flexor muscles concurrent with the inhibition of the extensor muscles of the limbs. It was subsequently also shown to also take place in rodents (Schouenborg and Kalliomaki 1990; Thelin and Schouenborg 2008). In infants, it has been previously demonstrated that noxious skin stimuli lead to withdrawal reflexes, which consequently evoke measurable electrophysiological activity which can be recorded by inserting an electrode directly in the contracting muscle (Fitzgerald *et al.*, 2013). Also, good correlations between the intensity of the stimulus and amplitude of the muscular reflex have been established (Andrews and Fitzgerald 2000), therefore this technique may be employed in measuring anti-nociceptive potential upon mechanical stimulation of the withdrawal response.

5.4.1. Mechanism of the nociceptive withdrawal reflex

Nociceptors consist of a single processing which originates in the dorsal root ganglion (DRG) and extends into the periphery to innervate the skin, whilst a central axon extends to synapse in the dorsal horn of the spinal cord (Dubin and Patapoutian 2010). These axons then transmit cutaneous information originating in the skin to the CNS through C and A δ fibres. In rodents

these fibre nociceptive units are evenly distributed on the plantar surface, ready to be activated by noxious stimulation of the paw (Leem *et al.* 1993). These nociceptive fibres which innervate the paw and the legs of rodents terminate in the dorsal horn of the spinal cord spanning from L5 to L2 segments (Woolf and Swett 1983).

5.4.2. Mechanical stimulation by von Frey hairs

The paw withdrawal reflex evoked as a response to mechanical stimulation from vFhs is widely used in the field of pain research, both in preclinical and clinical settings (Cornelissen *et al.*, 2013). Each vFh provides a standardised method of evaluating allodynia and hypersensitivity to direct mechanical stimulation of the paw (Chaplan *et al.*, 1994). Through the combination of both vFh stimulation and EMG measurements of the paw withdrawal reflex, the muscle activity corresponding to the nociceptive stimuli can be accurately and reliably quantified (Figure 5.1). Drawbacks associated with this technique is that it only takes into consideration mechanical stimulation (as opposed to thermal or chemical) which were not pursued in this thesis. Difficulties with this method also included variability observed between animals, due to differences in anaesthetic sensitivities and / or anaesthetic baseline. Therefore, for ease of comparison between individual animals, each EMG response after the injection of a formulation must be initially standardised to the baseline response of the respective rat.

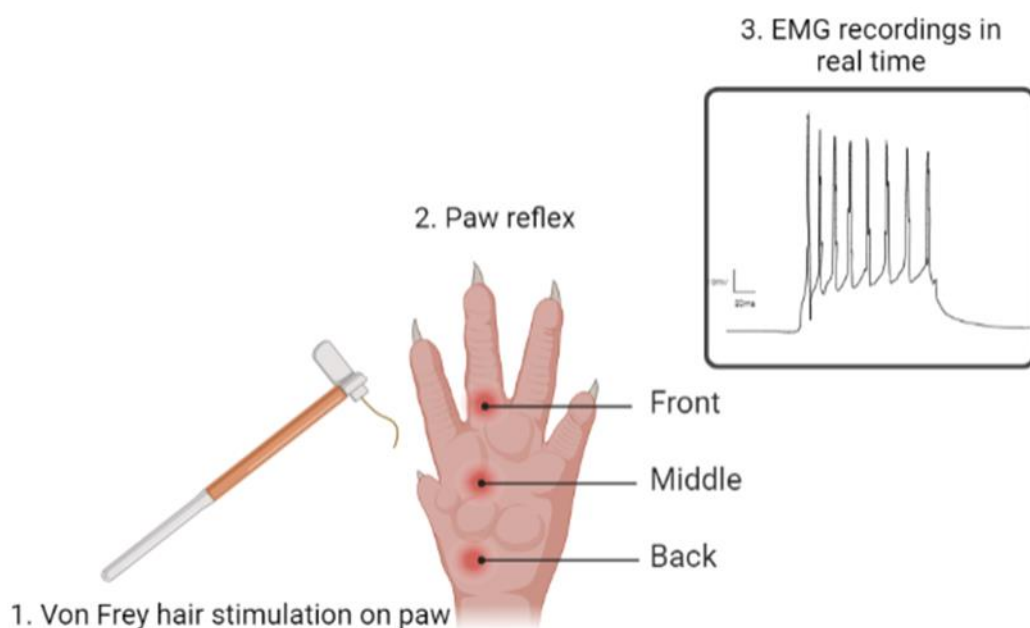


Figure 5.1. Diagram of the EMG procedure including the vFh stimulation in the middle of the rat paw leading to a withdrawal reflex, which is quantified in real time by a bipolar electrode inserted into the biceps femoris of the thigh muscle (diagram made with Biorender.com).

5.4.3. Anti-nociceptive effects of formulations

To investigate whether the CBD formulations I previously developed and characterised (Chapter 3) were effectively injected into the spinal cord SAS and to assess their anti-nociceptive effects, I carried out mechanical allodynia testing with vFhs whilst measuring the paw withdrawal EMG response as previously discussed. In this Chapter I also evaluated how the two distinct CBD formulations distribute after IT administration in healthy rats.

5.5. Chapter objectives

- Investigate the anti-nociceptive potential of CBD formulations through EMG measurements in comparison to the respective blank formulations
- Evaluate CBD biodistribution in the CNS at various time-points after injection
- Evaluate CBD biodistribution in peripheral tissues and serum after injection
- Investigate the potential of CBD delivery to the brain through IT injection in the lumbar segment

5.6. Results and discussion

5.6.1. Electromyography measurements after injection of CBD

formulations

EMG recordings coupled with the application of vFhs has also been used in the literature to assess hyperalgesia after treatment with mustard oil and capsaicin (Fitzgerald *et al.*, 2007). We therefore employed this technique to assess the excitability of the dorsal horn and thus evaluate EMG activity to noxious mechanical stimuli after the IT administration of the CBD formulations.

During measurements, I took the area under the curve (AUC) for the integral of the EMG signal as a response to paw stimulation with vFhs of various strengths (100 g, 180 g and 300 g) after IT injection and compared it to the AUC generated prior to injection. To control for the different sensitivities to isoflurane in rats and variations in the anaesthetic cycle over time, I established the EMG baseline prior to injection and set this as 100% (Tremoleda *et al.*, 2012). All subsequent measurements after injections were calculated as a percentage change from each individual baseline, as previously described (Hathaway *et al.*, 2006; Koch *et al.*, 2008). The AUC for all vFhs was calculated and plotted against the time after injection of each formulation.

The AUC of the EMG response (represented as a change relative to baseline) elicited after the injection of both CBD formulations showed a significant decrease within 10 mins of administration as compared to the respective blank controls (Figure 5.2). There was a clear anti-nociceptive effect of both CBD formulations as measured by the significant decrease in EMG AUC after mechanical stimulation with all VF filaments 10 mins after injection. These results confirm the successful IT injection in my *in vivo* model and also indicate that CBD has an immediate therapeutic effect within the CNS after IT administration into the lumbar segment of healthy rats. No significant effects were observed beyond the 10 mins effect when the AUC of all vFhs was analysed.

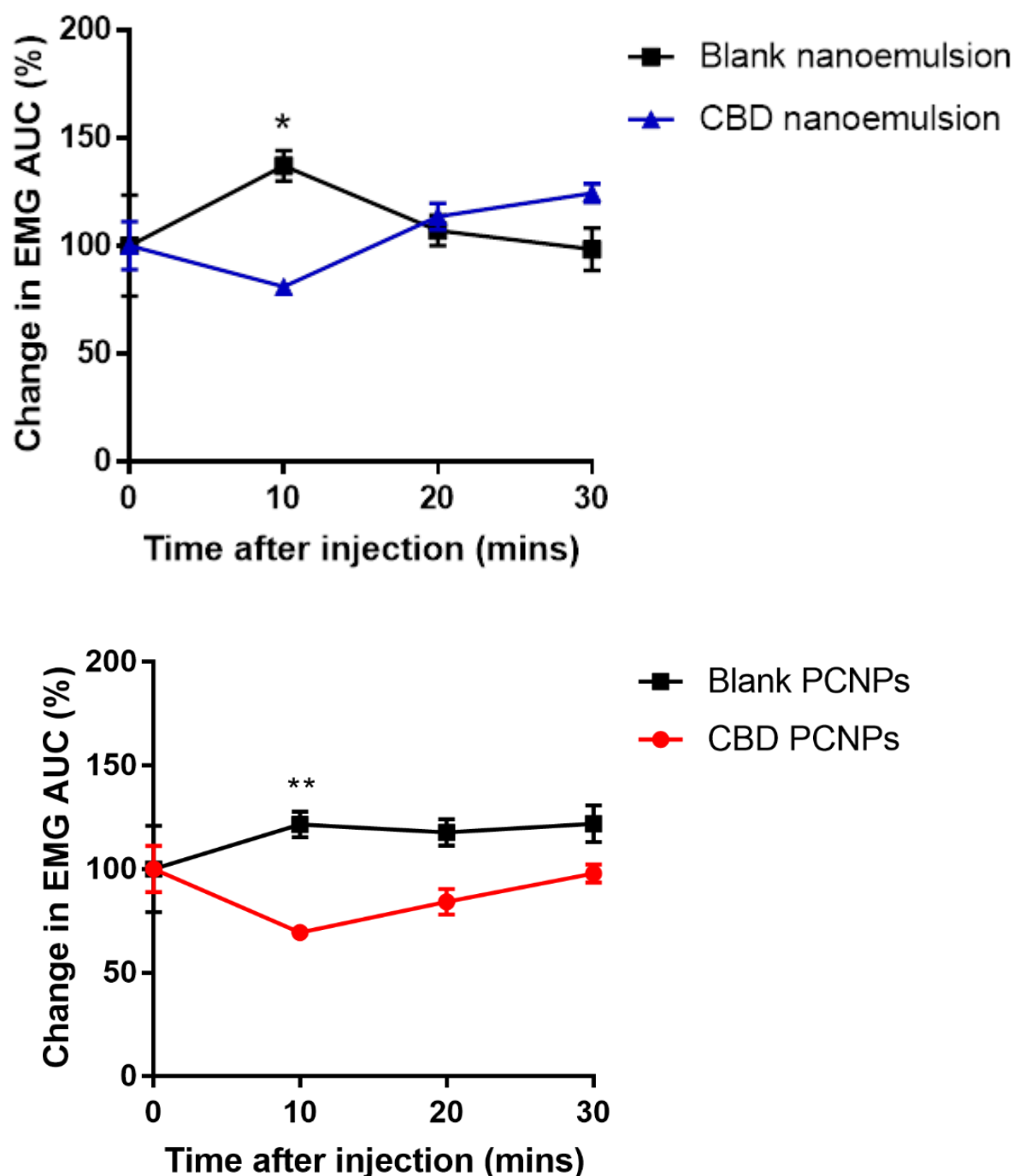


Figure 5.2. EMG recordings calculated as area under the curve (AUC) of the 100, 180 and 300 g vFhs at 10, 20 and 30 mins after injection of formulations (CBD nanoemulsion and CBD PCNPs) plotted against their respective controls. **A.** AUC of EMG response after injection of CBD (n=5) nanoemulsion compared to blank nanoemulsion (n=4) (mean $\pm$ SEM). Injection of CBD nanoemulsion significantly decreased EMG AUC at 10 in comparison to blank (* p < 0.05). **B.** AUC of EMG response after injection of CBD PCNPs (n=5) compared to blank PCNPs (n=5) (means $\pm$ SEM). Injection of CBD PCNPs significantly decreased EMG AUC at 10 mins in comparison to blank (** p < 0.01). No significant effects in AUC of EMG response were observed with the injection of either formulation in comparison to the respective blanks beyond the 10 min time-point. Statistical analysis done by 2-way ANOVA with Sidak's multiple comparisons test.

The increase in EMG signals after injection of blank nanoemulsion and PCNPs in comparison to baseline could be indicative of sensitisation as a result of the needle penetrating the SAS, which might also lead to a shallower level of anaesthesia. Either way, this data provides evidence that CBD has an immediate anti-nociceptive effect in the dorsal horn of healthy rats in both formulations, indicating its partitioning from the vehicle and its subsequent activity on pain receptors in the spinal cord (Urits *et al.*, 2020). In previous studies, Genaro *et al.*, injected CBD (10 to 50 nmol in 0.25 μ L total volume; dissolved in 100% grape seed oil) directly to the rostral anterior cingulate cortex in rats. They showed a dose-dependent decrease in mechanical allodynia which lasted 120 mins (Genaro *et al.*, 2017). Interestingly, this decrease in mechanical allodynia started 20 mins after injection, which possibly indicated a slower therapeutic onset when injected directly to the brain. However, this could also be due to the fact that CBD in that study was dissolved in pure oil as opposed to a nanoemulsion or PCNPs. Taken together, previous literature indicates that CBD is effective in reducing pain (Xiong *et al.*, 2012; Costa *et al.*, 2007; Izzo *et al.*, 2009), and my study also demonstrates its inhibition of EMG activity to noxious mechanical stimuli within 10 mins of IT delivery.

CBD has previously been investigated for its modulation of anti-nociception and anxiety-like behaviour in rodent neuropathic models. Gregorio *et al.*, have shown that cumulative IV dosing of CBD for 7 days (0.05 to 2 mg/kg) leads to decreased serotonergic firing in the dorsal root neurons, causing decreased anxiety-like behaviour (Gregorio *et al.*, 2019). Similarly, TRPV₁ channels were desensitised by cumulative dosing of CBD preventing mechanical allodynia in rodents (through the increase in paw withdrawal threshold in response to mechanical stimulation). This effect was prevented through the treatment of a TRPV₁ antagonist, capsazepine (CPZ), but not by a CB₁ antagonist AM251, indicating that CBD's anti-nociceptive effects are mediated in part by its activity of TRPV₁ receptors (Gregorio *et al.*, 2019). Another study has shown that oral administration of CBD (20 mg/kg dose) leads to anti-hyperalgesic effects in both models of neuropathic and inflammatory pain in rodents. This effect was also blocked by the TRPV₁ antagonism of CPZ (Costa *et al.*, 2007). These studies confirm that CBD has anti-nociceptive effects in pain models, demonstrated by repeated cumulative IV dosing at much higher concentrations (0.05 to 2 mg/kg) over the course of a week, than what I administered in my study (0.05 mg/kg) in a single bolus injection. This was

carried out in order to achieve constant therapeutic plasma concentrations overcoming the on-going metabolism of the first-pass effect.

I have also investigated the effect of my CBD formulations on each individual vFh, as opposed to taking the AUC of all vFhs. Considering that the strengths of each hair is on a logarithmic scale ranging from 100 g, which could be associated with a light touch (innocuous), to 300 g which reaches a maximal noxious stimulus, the differences elicited by these may be indicative to the level of anti-nociception of the CBD formulations.

Interestingly, I found that both CBD formulations led to a significant decrease in EMG signal at the 10 min time point after injection at all vFhs tested, with the exception of 300 g after the administration of CBD nanoemulsion (Figures 5.3; 5.4). I observed similar trends in responses between CBD formulations at 100 g and 180 g, however when more noxious 300 g vFhs were applied, the CBD PCNPs exhibited sustained anti-nociceptive effects over the course of 30 mins (Figure 5-3), as opposed to the CBD nanoemulsion which had no anti-nociceptive effect at any time-point (Figure 5.4). This is a very interesting observation, and it suggests that when noxious mechanical stimuli are applied, the CBD PCNPs are able to still maintain an anti-nociceptive effect, whereas the CBD nanoemulsion does not. On the other hand, when lighter and innocuous mechanical stimuli are applied (100 and 180 g), the effects of both formulations are similar. A hypothesis as to why this happens could be linked to the biodistribution data (discussed in section 5.6.2), where within 10 mins of injection the mass of CBD found in the spinal cord is significantly higher after the administration of CBD PCNPs as opposed to the nanoemulsion. The lower concentration of CBD found in the spinal cord (and thus ready to act on TRPV₁ receptors in the dorsal horn) might be because when formulated as a nanoemulsion, it immediately partitions into fatty tissues of the CNS after administration into the IT space. Thus, CBD is “trapped” and accumulated within fatty tissues of the CNS, and is not immediately freely available to act on TRPV₁ receptors. On the other hand, this might not happen with the PCNPs which are immediately distributed within the SAS and possibly CSF after injection, ready for CBD to act on the receptor binding sites.

Taken together, my results suggest that CBD has an immediate anti-nociceptive effect after IT injection in healthy rats, when stimulated by all vFhs (from innocuous to noxious mechanical stimulation). Upon investigation of individual vFhs, it became apparent that both formulations elicit similar anti-nociceptive effects when stimulated with innocuous hairs,

however this response was lost when stimulated by noxious vFhs after injection of the CBD nanoemulsion. Nonetheless, the CBD PCNPs maintained their anti-nociceptive effects upon stimulation with noxious vFhs over the course of 30 mins, highlighting the improved effectiveness of this formulation.

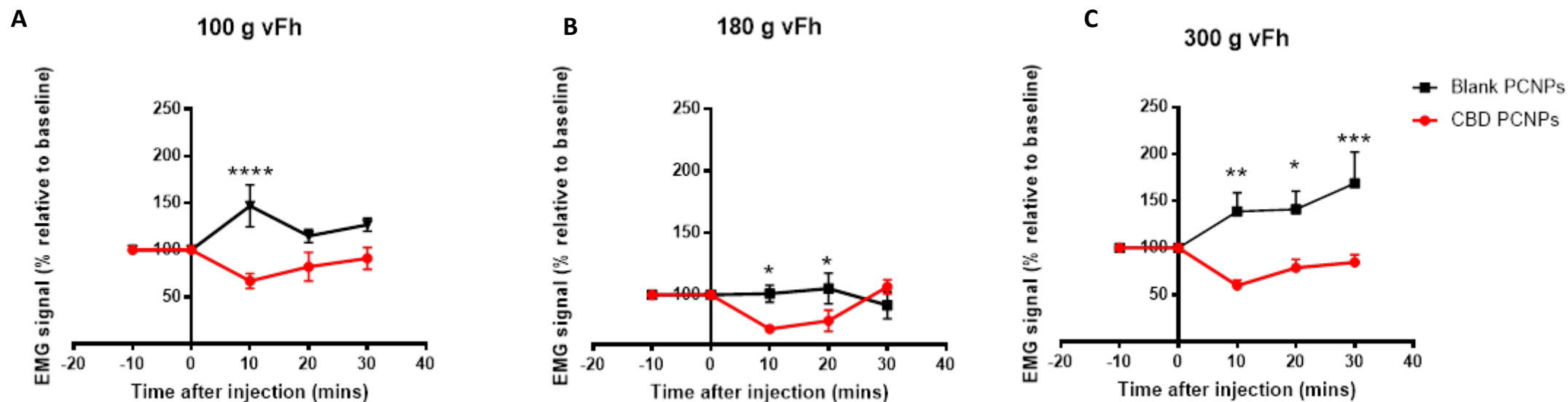


Figure 5.3. EMG recordings following the application of 100 g (A), 180 g (B), 300 g (C) vFhs after CBD PCNPs (n=5) and blank PCNPs (n=5) IT injection. Data displayed as mean percentage change ($\pm$ SEM) normalised to baseline prior to injection (set as 100%). All measurements following injection represent a percentage increase / decrease (relative to baseline) in EMG signal evoked by the respective vFh over 30 mins. Within 10 mins of CBD PCNPs administration, there was a significant decrease in EMG activity in comparison to blank PCNPs at 100 (**** $p < 0.0001$), 180 (* $p < 0.05$) and 300 g (** $p < 0.01$) vFhs. A significant decrease after administration of CBD PCNPs was also observed 20 mins after injection at 180 and 300 g (* $p < 0.05$) and 30 mins after injection only at 300 g vFh (*** $p < 0.001$).

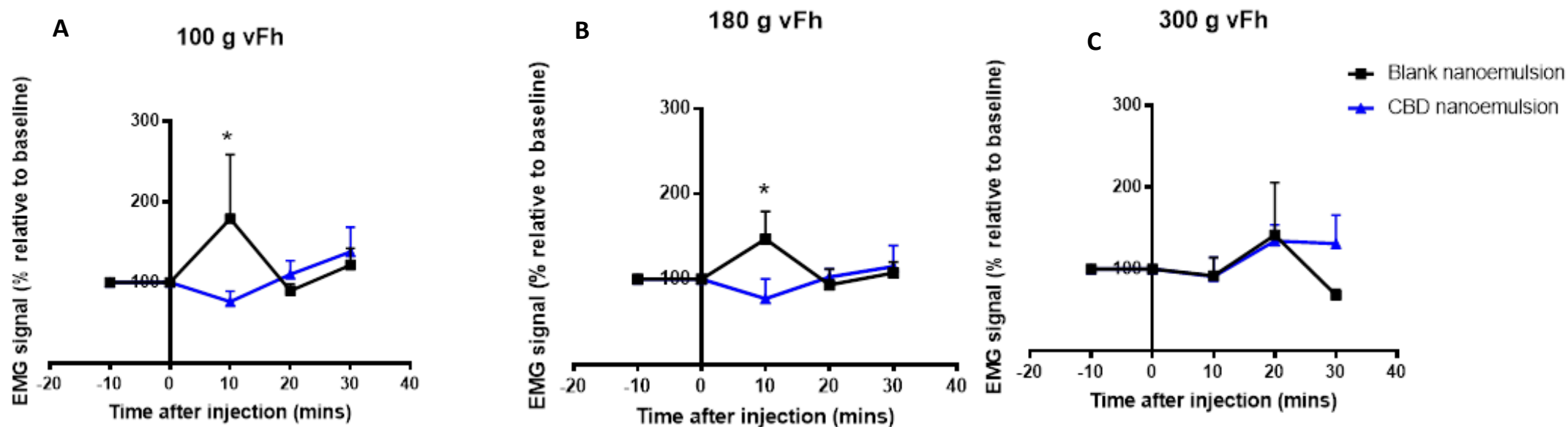


Figure 5.4. EMG recordings following application of 100 g (A), 180 g (B), 300 g (C) vFhs after CBD nanoemulsion ($n=5$) and blank nanoemulsion IT injection ($n=4$). Data displayed as mean percentage change ($\pm$ SEM) normalised to baseline prior to injection (set as 100%). All measurements following injection represent a percentage increase/decrease (relative to baseline) in EMG signal evoked by the respective vFhs over 30 mins. Within 10 mins of CBD nanoemulsion administration, there was a significant decrease in EMG activity in comparison to blank nanoemulsion ($*p < 0.05$) at 100 and 180 g vFhs. No significant change was observed upon the stimulation with 300 g vFh.

5.6.1.1. EMG technique challenges

There are many difficulties associated with EMG experiments because they are carried out in live animals, with significant biological variations between individuals. The most evident aspect of this variation is the anaesthetic level which must be adjusted based on each animal's paw withdrawal response and the consistency of the EMG signals, rather than a set, pre-described value (i.e., 1.3% isoflurane for all animals). Due to these variations, it is therefore complicated to achieve stable recordings, and thus the sample sizes are small. Also, general cyclic variations in the anaesthetic plane (depth) over time make long-term EMG measurements variable, especially when anaesthetic depth is maintained within a very shallow and narrow window to allow for a paw withdraw reflex (Tremoleda *et al.*, 2012). Large variabilities in EMG responses beyond the 30 min time-point were the reason why measurements past that specific time-point were not pursued (Figure 5.5).

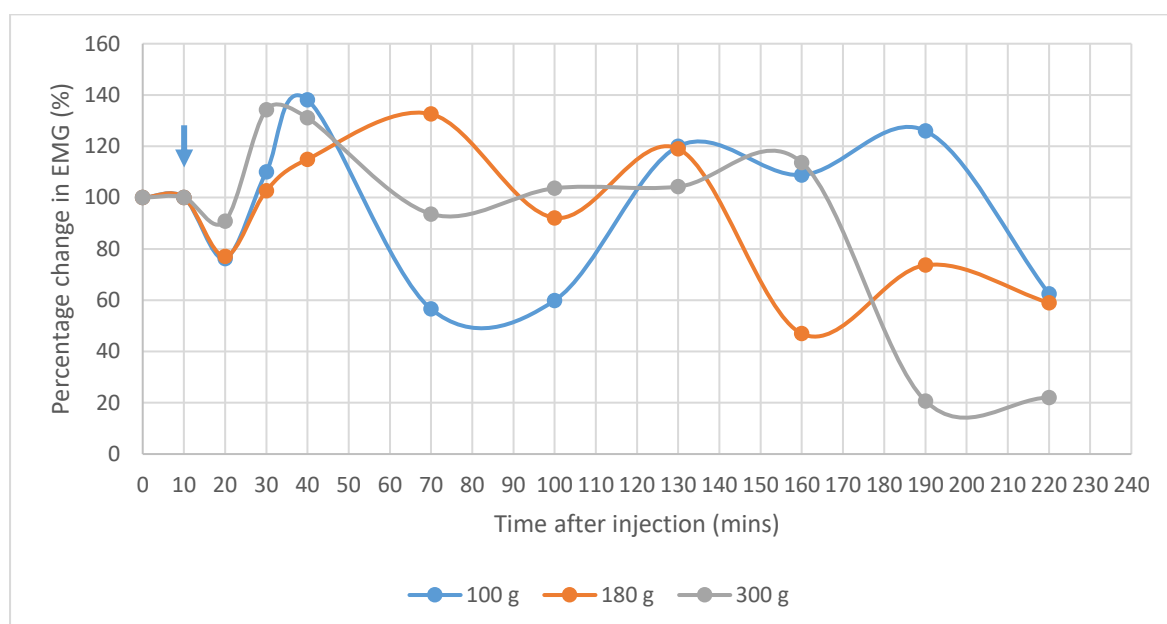


Figure 5.5. An example of EMG responses (percentage change relative to baseline) after injection of CBD nanoemulsion (denoted by an arrow) over the course of 240 mins. This graph demonstrates the variability in EMG responses when analysed over a long period of time, as a result of methodology challenges due to anaesthetic plane variations. For this reason, the anti-nociceptive evaluation of formulations was performed over the course of 30 mins after injection.

Moreover, the fact that anaesthetic levels need to be low in order to maintain a shallow level of unconsciousness means that these experiments are tricky to carry out due to the risk of

animals becoming conscious during the experiments, which has happened a few times when repeated vFhs are too noxious for the animals, especially at 300 g.

The EMG recordings are also limited to the bicep femoris muscle meaning that the measurements are localised to just one limb. Moreover, there is also damage to the skin and the muscle during the insertion of the needle electrode (which can be minimised through the use of small needle gauge). Another important aspect which cannot be overlooked is the variability in anatomy between animals, such as the precise location of the peripheral nerves (L4 - L6) in association with the respective spinal segments (Zhao *et al.*, 2014). Nonetheless, if great care is taken during surgical preparation alongside handling of tissues, coupled with the baseline correction for each animal, it allows for the greatest chance of success with EMG measurements.

5.6.2. Biodistribution of CBD formulations after IT injection

Following the investigation into the anti-nociceptive effect of the CBD formulations when injected intrathecally, I assessed the biodistribution of the CBD formulations in the CNS and peripheral organs by HPLC. I sampled tissues over the course of 4 hrs, as previous CBD pharmacokinetic data suggests that T_{max} (time of maximum concentration) is achieved between 1 and 4 hrs (Millar *et al.*, 2018). Moreover, this was also coupled with non-recovery experimental time constraints. I found that there was no CBD detected in peripheral tissues (liver, spleen or deep cervical lymph nodes) and no detectable CBD in serum at any time-point tested after the administration of the nanoemulsion and PCNPs.

The highest concentration of CBD found in the spinal cord after the administration of the nanoemulsion was at the 2 hrs time-point (10133 ng/g), indicating that the formulation was acting as a depot upon injection with CBD being released over time (Figure 5.6, A). A significantly lower concentration of CBD was found 10 mins after injection with the administration of the nanoemulsion (874 ng/g) in contrast to PCNPs (15469 ng/g), possibly indicative of lipid droplets partitioning into fatty tissues of the CNS immediately after delivery. Bujedo *et al.*, 2017 have found that hydrophilic drugs such as morphine reached the CSF at higher concentrations in comparison to lipophilic opioids which were sequestered in the epidural fat (Bujedo *et al.*, 2017). Moreover, fentanyl and alfentanil had accumulated in epidural fat at 32- and 20-fold higher concentrations than morphine, respectively, leading to

decreased quantities reaching the more distal spinal segments (Bujedo *et al.*, 2017). This effect might also be observable with the CBD nanoemulsion, given the fact that the lipid droplets may display an affinity towards the fatty tissues of the CNS. Ummenhofer *et al.*, have also demonstrated that opioids in the IT space of pigs are largely governed by their lipid solubility, with sufentanil rapidly partitioning out of the CSF into more hydrophobic environments (i.e., epidural fat) (Ummenhofer *et al.*, 2000).

The highest concentration of CBD in the spinal cord after the administration of PCNPs was within 10 mins of delivery, after which it shows significant decreases at 30 mins, with the lowest concentration at 4 hrs (Figure 5.6, A). This could be indicative of clearance of the drug from the CNS over time, especially if the PCNPs were solubilised in the freely circulating CSF, as opposed to the nanoemulsion partitioning into fatty tissues. The rapid CSF turn-over in rats 9 - 11 times a day leads to fast clearance of therapeutics from the CNS space unless the drug partitions into epidural fat (Chiu *et al.*, 2012). However, no CBD was detected in the serum samples of any rats. It is possible that the systemic circulation acts as a sink for CBD clearance from the CNS and that this was not detected due to the very low concentrations once diluted in blood. Both formulations lead to CBD delivery into the brain within 10 mins of IT administration in the lumbar segment (Figure 5.6, B). The pharmacokinetic parameters of both formulations in the brain are discussed in section 5.6.2.2.

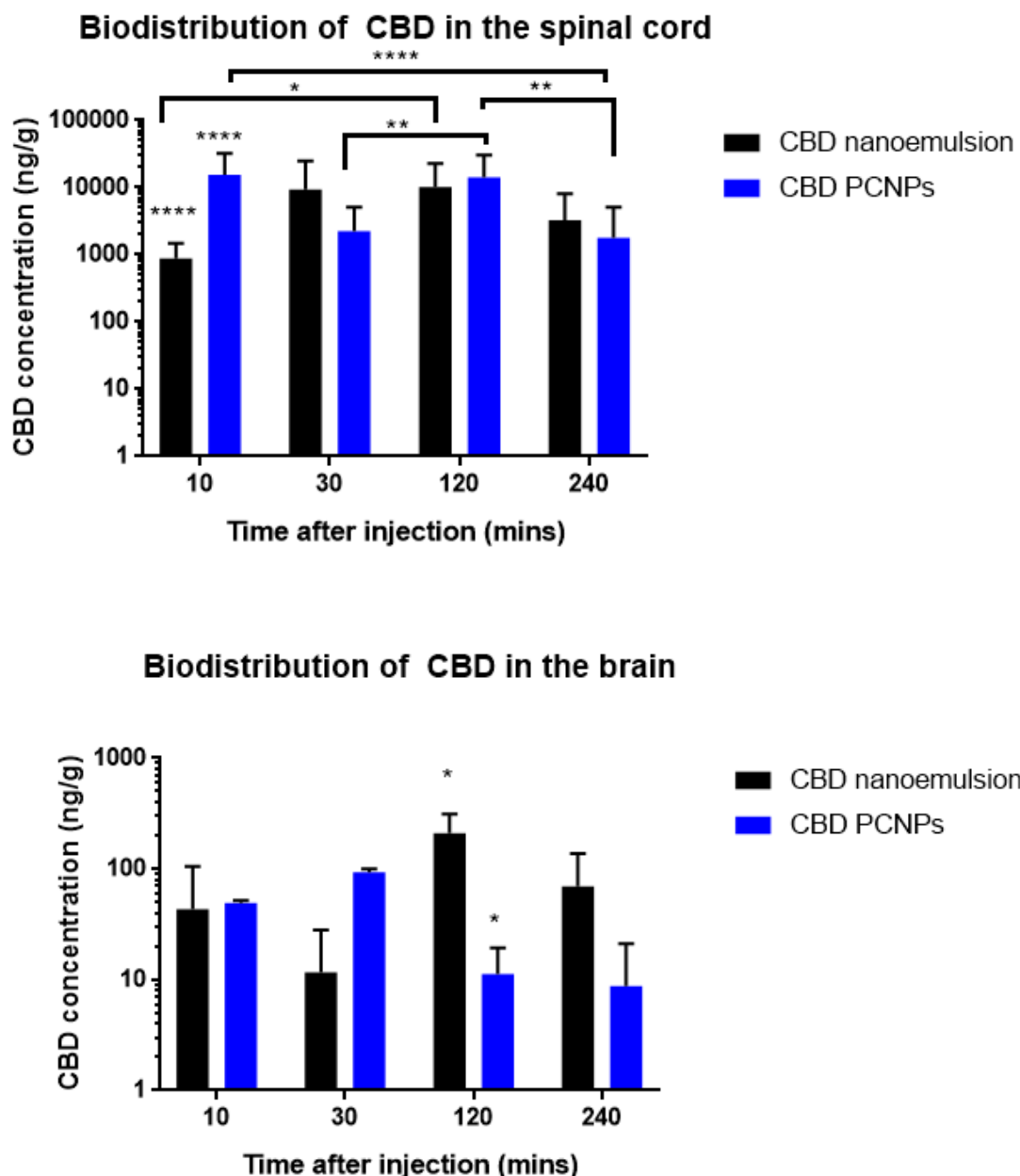


Figure 5.6. A. Biodistribution of CBD in the spinal cord (dose of CBD formulations administered 0.05 kg/mg or 15 μ g in 15 μ L injection volume for both formulations). CBD concentration was significantly higher in the spinal cord after administration of CBD PCNPs within 10 mins (**** $p < 0.001$). CBD concentration after injection of PCNPs was highest at the 10 mins time-point (15469 ng/g), significantly higher than at 4 hrs (**** $p < 0.001$) and lowest at 4 hrs after injection (1789 ng/g). The concentration of CBD after administration of the CBD nanoemulsion was the lowest 10 mins after administration (874 ng/g), peaking with a significantly higher concentration at 2 hrs (10133 ng/g) (* $p < 0.05$). **B.** Biodistribution of the CBD concentration in the brain after the administration of CBD nanoemulsion and PCNPs. There was a significantly higher concentration of CBD detected in the brain 2 hrs after injection of CBD nanoemulsion (210 ng/g) as compared to CBD PCNPs (11 ng/g) (* $p < 0.05$). No other significant differences were observed, with CBD concentration found in the brain ranging from 8 ng/g to 210 ng/g over the course of 4 hrs.

5.6.2.1. Biodistribution of CBD across the spinal segments

Following the administration of CBD formulations, I aimed to establish biodistribution through sectioning the spinal cord into segments, as previously discussed in Chapter 1. Briefly, I dissected the spinal cord and cut it into the lumbar, thoracic and cervical segments. My aim was to try and understand how CBD moves through the neuraxis after injection into the lumbar area, and whether this is affected by the formulations.

I found it difficult to establish consistent trends in the biodistribution of CBD in spinal segments over time, as it was apparent that the concentration of CBD in tissues was changing over time. I hypothesised that this was due to the oscillatory nature of the CSF, flowing into various directions in the CNS space. Therefore, this would have resulted in CSF mixing back and forth in the CNS, making it challenging to establish a reliable trend within 10 and 30 mins of injection.

Nonetheless, I observed that 10 mins after administration of CBD PCNPs, there was a significantly higher concentration of CBD localised in the lumbar segment compared to the cervical segment and the brain (Figure 5.7). No significant differences were observed between the lumbar segment and the adjacent thoracic segment of the spinal cord, indicating an immediate spread across the next segment after administration of CBD PCNPs, but with significantly lower quantities reaching the more distal sites. No significant differences observed in the CBD mass 10 mins after the administration of CBD nanoemulsion, with similar amounts of CBD detected in all CNS tissues. The significantly lower proportion of CBD with the administration of the nanoemulsion in contrast to PCNPs was possibly indicative of lipid droplets partitioning into fatty tissues of the CNS immediately after delivery, as I previously discussed (section 5.6.2).

At the 30 mins time point after administration of CBD PCNPs, no significant differences were observed across the spinal segments, indicating that the spread of CBD away from the lumbar segment was occurring. However, after the administration of CBD nanoemulsion, the amount of CBD in the lumbar segment was significantly higher ($p < 0.05$) than in the thoracic and cervical segments, indicating retention at the site of injection occurring at this time-point. Subsequently, at the 2 hr time-point I observed no other significant differences in the amounts of CBD found across the CNS.

Finally, at the 4 hr time-point I observed a significant retention of CBD at the lumbar segment of the spinal cord in comparison to the cervical and thoracic segments ($p < 0.0001$) after administration of the nanoemulsion (Figure 5.7). No significant differences were observed in the distribution of CBD across the spinal cord segments after PCNPs were administered. These results might be indicative of CBD retention at the site of injection over-time when formulated as a nanoemulsion, however this significant accumulation at the site of injection was not observed at the earlier time-points tested (i.e., 10 mins, 30 mins and 2 hrs) (Figure 5.7), suggesting that the oscillatory nature of the CSF leads to back and forth mixing of therapeutics immediately after injection.

Nonetheless, overall, I found that there was widespread CBD distribution in the CNS after administration of both my formulations at all time-points with immediate delivery to the brain within 10 mins of injection.

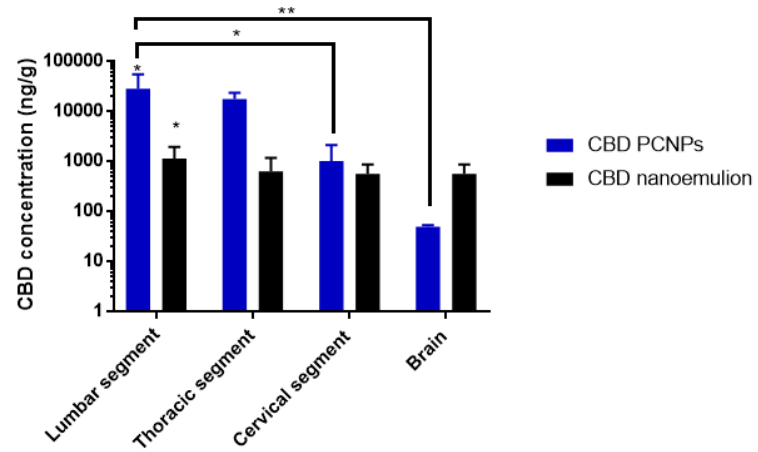
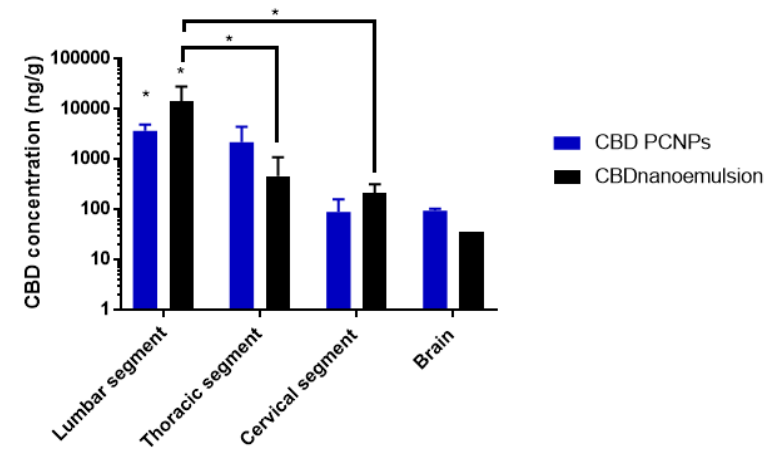
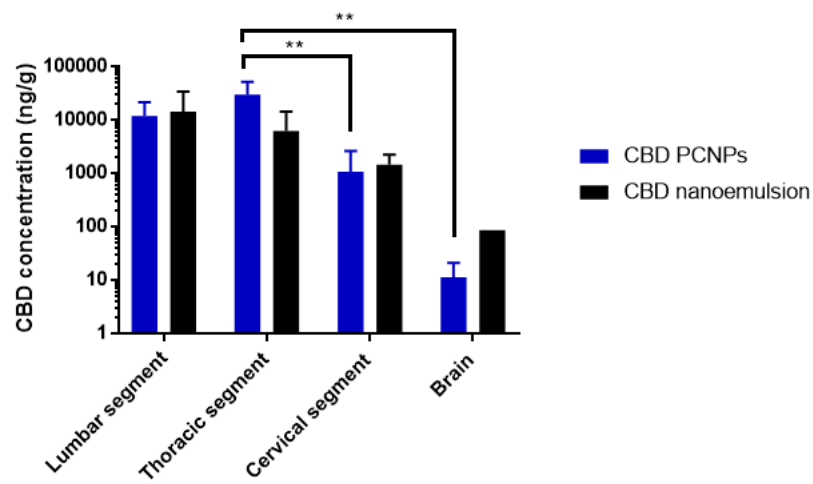
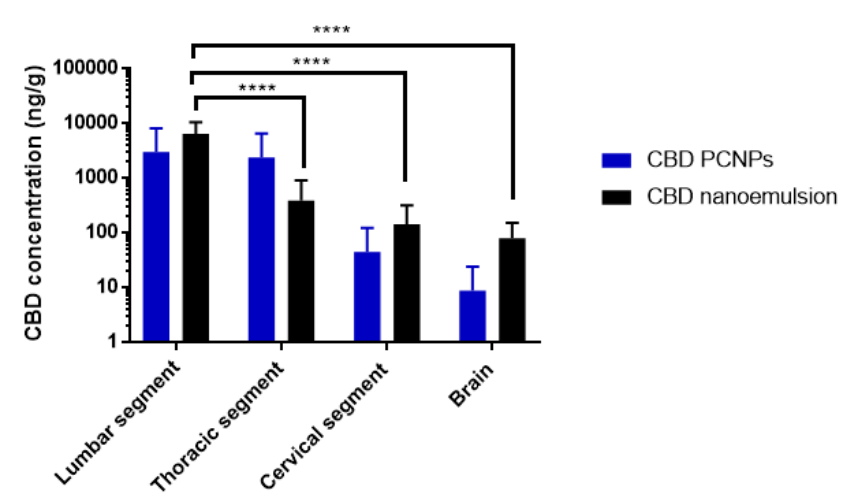
A**Biodistribution of 10 mins after administration****B****Biodistribution 30 mins after administration****C****Biodistribution 2 hours after administration****D****Biodistribution 4 hours after administration**

Figure 5.7. Biodistribution of CBD in the rat CNS following injection of CBD nanoemulsion and CBD PCNPs at 10 mins (A), 30 mins (B), 2 hrs (C) and 4 hrs (D). CBD concentration in the lumbar segment was significantly higher than the concentration in the cervical segment and the brain at 10 mins (* $p < 0.05$) after administration of CBD PCNPs. CBD concentration was also significantly higher at the site of injection (lumbar) within 10 mins after administration of CBD PCNPs in comparison to the nanoemulsion (* $p < 0.05$). Within 30 mins, CBD concentration was significantly higher in the lumbar segment compared to the thoracic and cervical segments after the administration of CBD nanoemulsion (* $p < 0.05$). No significant differences were observed at the 2 hrs time point. The concentration of CBD in brain, cervical segment and thoracic segment was significantly lower (**** $p < 0.0001$) than the concentration of CBD in the lumbar segment 4 hrs after the administration of formulation ($n=6$, tissues analysed in duplicated by HPLC). No significant differences in the concentration of CBD analysed in different CNS tissues were observed after the administration of CBD PCNPs ($n=3$, tissues analysed in duplicates by HPLC). Values are expressed as mean $\pm$ SEM (2way ANOVA with Sidak's multiple comparisons test).

5.6.2.2. Brain delivery and pharmacokinetics

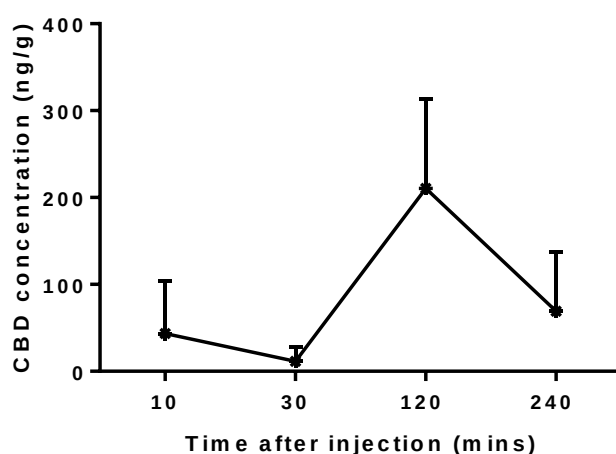
CBD was detected in the brain 10 mins after the injection of both formulations, ranging in concentrations from 8 – 210 ng/mL, and was detected up to 4 hrs after administration (Figure 5.8). The fact that no CBD was detected outside of the CNS indicates that the IT administration technique achieves localised drug delivery with the highest concentrations of drug in the spinal cord and a fraction of the drug reaching the brain within 10 mins of injection of both formulations. No detectable CBD concentrations were found clearing from the CNS into the periphery (spleen, liver, deep cervical nodes or serum) at any time-points tested.

The dose administered in my study was 0.05 mg/kg for both formulations, with the highest C_{max} in the brain achieved upon administration of the CBD nanoemulsion at T_{max} 120 mins (Table 5.1). Deiana *et al.*, have administered CBD in a cremophor and solutol formulation at a 120 mg/kg dose, through oral gavage and IP administration. The highest C_{max} achieved in the brain of rats was 12.6 $\mu\text{g/g}$ through oral delivery. Equivalence of the dose I administered intrathecally to the one in this study results in a 2,400-fold difference, therefore, assuming linear pharmacokinetics, the C_{max} achieved through oral administration would be 5.25 ng/g at the same dose as in the current work. My study therefore demonstrates that significantly higher brain concentrations can be achieved through IT delivery, and the C_{max} was enhanced through the use of a nanoemulsion. On the other hand, T_{max} was reached earlier when PCNPs were administered (30 mins as opposed to 120 mins), indicating that the use of PCNPs leads to the faster distribution of CBD from the lumbar segment of the spinal cord to the brain, whereas the interactions of the nanoemulsion lipid droplets with the SAS components (and epidural fat) might hinder the movement through the IT space. This has also been shown by Householder *et al.*, 2019 where fluorescent 100 nm PEGylated NPs administered to the cisterna magna of healthy mice rapidly distributed alongside the entire neuraxis, whilst being retained in the leptomeninges for over 3 weeks (Householder *et al.*, 2019). They also investigated larger 10 μm microparticles which did not exhibit as widespread distribution as the NP counterparts. Nonetheless, my CBD nanoemulsion leads to a higher C_{max} in the brain in comparison to PCNPs (210 vs 94 ng/g), which might be attributable to the clearance of the smaller NPs via perivascular spaces in the CSF or through the cribriform plate into the nasal mucosa (Fowler *et al.*, 2020; Household *et al.*, 2019).

Table 5.1. Pharmacokinetic parameters of CBD formulations in the brain.

<i>PK parameter</i>	CBD nanoemulsion	CBD PCNPs
C_{max} (ng/g)	210	94
T_{max} (min)	120	30
$AUC_{0-4 h}$ (ng/g)	27337	7341

A CBD nanoemulsion concentration in the brain



B CBD PCNPs concentration in the brain

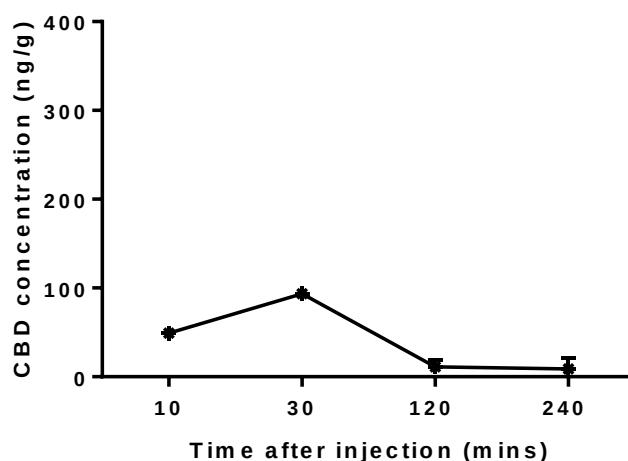


Figure 5.8. Area under the curve (AUC) of CBD concentration (means $\pm$ SEM) in the brain over time after injection of the nanoemulsion (**A**) and PCNPs (**B**) in the lumbar segment. T_{max} after administration of CBD nanoemulsion is reached at 120 mins, with a C_{max} of 210 ng/g, whereas after injection of CBD PCNPs the T_{max} is reached at 30 mins, with a C_{max} of 94 ng/g.

In other studies, brain delivery after IV administration of carbamazepine nanoemulsions was observed in mice, with the emulsifier 1-O-decylglycerol leading to 2.37-fold higher brain

uptake than all the other formulations tested. Nonetheless, most tissue accumulation was demonstrated to be in the lung, followed by the liver, spleen, brain, kidney then heart (Madhusudhan *et al.*, 2008). This suggests that although nanoemulsions can be utilised to improve CNS targeting after IV administration, most of the drug is still distributed in peripheral tissues (lung) and quickly metabolised (liver). Another study investigating the uptake of therapeutics in the brain was carried out by Wen *et al.*, aimed to compare solid lipid NPs, nanostructured lipid carriers and nanoemulsions. After the IV administration of formulations (squalene and cetyl palmitate as the lipid phase and forestall as the cationic surfactant) encapsulating sulforhodamine B dye, *in vivo* imaging in rats revealed that the retention time in the brain was extended from 20 mins to 50 mins when using nanoemulsions (Wen *et al.*, 2011). The later study therefore demonstrates that lipid based nanoemulsion formulations have the potential to improve biodistribution and retention in the brain after IV administration. This was also observed in my work, where the AUC_{0-4h} of CBD in the brain after IT administration was extended 3.7-fold by the use of a nanoemulsion as opposed to PCNPs. This is the first time such results are reported for the IT drug delivery route, to the best of my knowledge.

Therefore, in this study I have shown that CBD reaches the brain with the delivery of both formulations, demonstrating that IT delivery is a potential administration technique for efficacious CNS targeting. Interestingly, a much higher C_{max} and AUC of CBD in the brain was reached after delivery of the nanoemulsion, as compared to the PCNPs.

A drawback associated with the HPLC method utilised in these experiments was the requirement that whole tissue was homogenised, in order to be able to extract the drug from the lipid / protein matrix. As a result of this procedure, I cannot establish as to whether CBD deeply penetrates the brain parenchyma, diffuses a few mm inside or simply adheres to the outer surface layers which are surrounded by the freely floating CSF in the CNS. Moreover, I also unable to determine whether CBD enters brain cells or is simply circulating in the brain micro-circulation system. The drawbacks associated with the use of traditional analytical systems as the HPLC may be overcome by high resolution mass spectrometry, such as the ToF or Orbi SIMS. These results may therefore supplement my existing data and establish whether therapeutics do indeed deeply penetrate the brain parenchyma. This would be extremely

beneficial for other diseases states, such as brain tumours, which lack effective therapeutics with the ability to reach deeply embedded cancerous cells.

5.6.2.3. Clearance of CBD formulations from the CNS

To gather a broader picture of the fate of nanoparticulate formulations in the CNS, I also investigated the clearance of CBD from each segment after the administration of formulations, over time (Figure 5.9). I observed that immediately after administration of CBD PCNPs, the amount of CBD detected in the lumbar segment (site of injection) was significantly higher ($*p < 0.05$) than the cervical segment and the brain. The amount of CBD at the site of injection was also significantly higher at 10 mins in comparison to 30 mins, 2 hrs and 4 hrs, indicating an immediate clearance of CBD NPs away from the site of injection over time. The CBD mass identified in the thoracic segment was the highest 2 hrs after administration of NPs, significantly higher than at 30 mins and 4 hrs ($*p < 0.05$). At the 2 hr time-point the amount of CBD in the thoracic segment was also equivalent to the amount in the adjacent lumbar segment, indicating its movement across the spinal cord.

On the other hand, after the administration of CBD nanoemulsion, the amount of CBD detected in the lumbar segment was significantly higher at 2 hrs and 30 mins in comparison to 10 mins, indicating that there was an immediate partitioning of CBD after injection, into fatty tissues of the CNS as previously discussed. There is an indication of CBD retention at the lumbar site between 30 mins and 4 hrs, whereas the loss observed within 10 mins of injection could be explained by distribution to the sacral region of the spinal cord, or adjacent fatty tissues in the CNS which were not included in the HPLC analysis.

A study investigating CNS drug distribution in an *in vitro* model of the CNS space has highlighted that drug injection flow rate and catheter angle can affect drug spread direction and subsequent concentrations (Haga *et al.*, 2017). Although I tried to maintain these factors as constant as possible throughout experiments, any slight variations in injection technique / speed / angle might have an effect on the drug distribution and clearance from the IT space. It is therefore imperative that these aspects are taken into consideration when any major conclusions are drawn from these data. Haga *et al.*, have suggested that C_{max} can be altered by the drug injection flow rate, whereby higher injection speeds increase drug distribution and also reduce peak concentrations, observations which were also demonstrated through

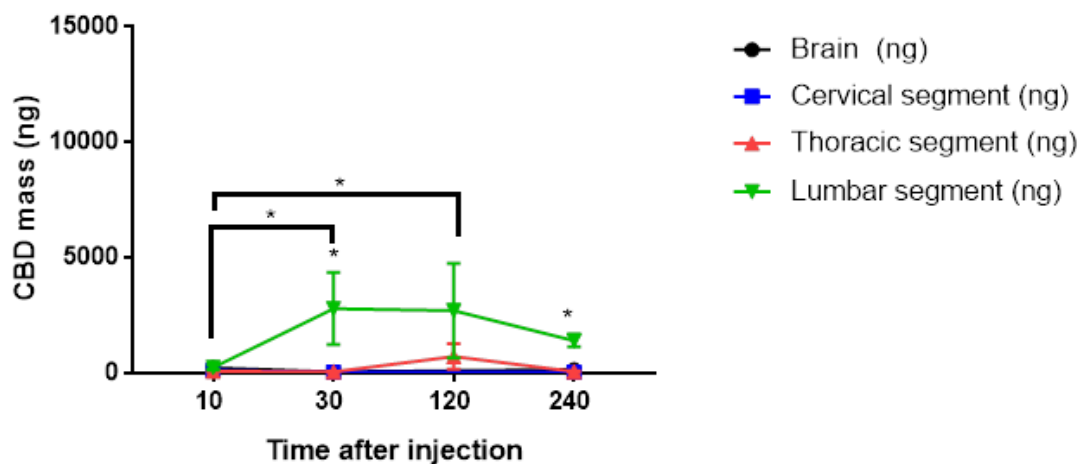
experimental set-up (Robinson *et al.*, 1994). On the other hand, distribution can also be altered through variation in needle angle (i.e., dorsal or dorsolateral), where a more upwards angle leads to increased drug concentrations moving towards the brain (Haga *et al.*, 2017). Although a micromanipulator was used throughout these experiments in order to maintain a constant set-up in regards to the IT injection, an exact replication of the injection angle and speed cannot be definite from one animal to the next.

I also carried out an investigation into the amounts of CBD in the CNS tissues as a percentage of the total amount of drug identified in the tissues by HPLC (Figure 5.10). This was done to allow for a better idea of the biodistribution of all the detectable CBD in the CNS. I found that most CBD was located in the lumbar segment with the administration of CBD nanoemulsion, whereas with CBD PCNPs this amount was divided between the lumbar and the thoracic segments, indicating a further spread from the injection site, as I previously highlighted.

A drawback in this study was the fact that I cannot discern between the drug found in the CSF and in tissue, as whole spinal segments were homogenised and analysed by HPLC. It would have been very interesting to differentiate between the two, and investigate whether CBD is actually retained in tissue or freely circulating in the CSF. Nonetheless, drawing CSF from a live rat is a very difficult procedure which has not been approved on the animal licence these studies were performed under. Although this study offers us a good indication of CBD distribution in different formulations within a short-term period after injection, it would be extremely interesting to have a long-term investigation into how biodistribution and clearance changes over time. From my initial data, the indication is that there is CBD remaining within the CNS past the 4 hrs' time point after injection. Partridge suggests a drug injected into the CSF would rapidly leave the SAS aided by bulk flow and end up exported into the blood (Partridge, 2011). This does not seem to be the case with either of my formulations, as CBD was still found in the lumbar segment 4 hrs post injection, and no CBD was found in the serum at any of the time points tested. Moreover, no CBD above the limit of detection of the HPLC was found in any of the peripheral tissues tested (spleen, liver, deep cervical lymph nodes). Whether this is still the case long term would be essential for use of this delivery system in the clinic.

Another disadvantage pertaining to this particularly challenging technique is the complexity of the anatomy. Therefore, without appropriate real-time guiding imaging techniques to help aid the precise injection of the needle into the rodent spinal cord, inherent variability can take place. This was also noted by Shapiro *et al.*, when they injected ^{131}I -labelled human serum albumin alongside methotrexate, and found that on three occasions the drug was injected in the epidural or subdural space, as opposed to the SAS (Shapiro *et al.*, 1975). This has also happened in the clinic, where experienced clinicians and radiologists were aware of this possibility and sought to prevent the complication. Nonetheless, 8 out of 30 patients (27%) still had an instance of a sub-dural injection as opposed to a correct and desired IT injection (Rugoff *et al.*, 1974). Based on my current experimental set up, I am unable to know whether this also occurred during my experiments.

A Clearance after CBD nanoemulsion administration



B Clearance after CBD PCNPs administration

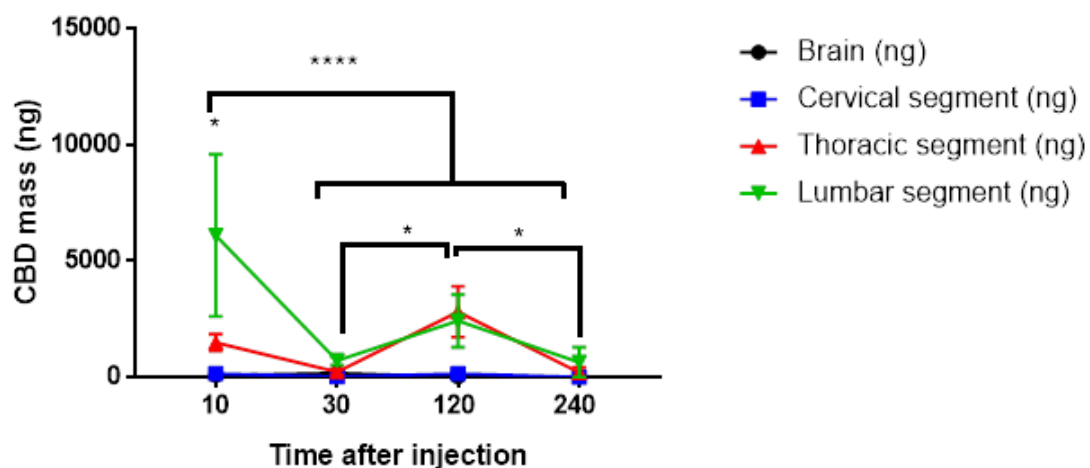


Figure 5.9. Clearance of CBD mass from the CNS over the course of 4 hrs, after injection of **A.** CBD nanoemulsion and **B.** CBD PCNPs. **A.** The mass of CBD in the lumbar segment of the spinal cord was significantly lower 10 mins after injection in comparison to 30 mins and 2 hrs ($*p<0.05$). The mass of CBD in the lumbar segment was significantly higher than the thoracic and cervical segments 30 mins and 4 hrs after injection ($*p<0.05$). **B.** The mass of CBD was significantly higher 10 mins after injection in the lumbar segment compared to all the other segments ($*p<0.05$), and also higher than the mass in the lumbar segments at the other time points tested ($****p<0.001$).

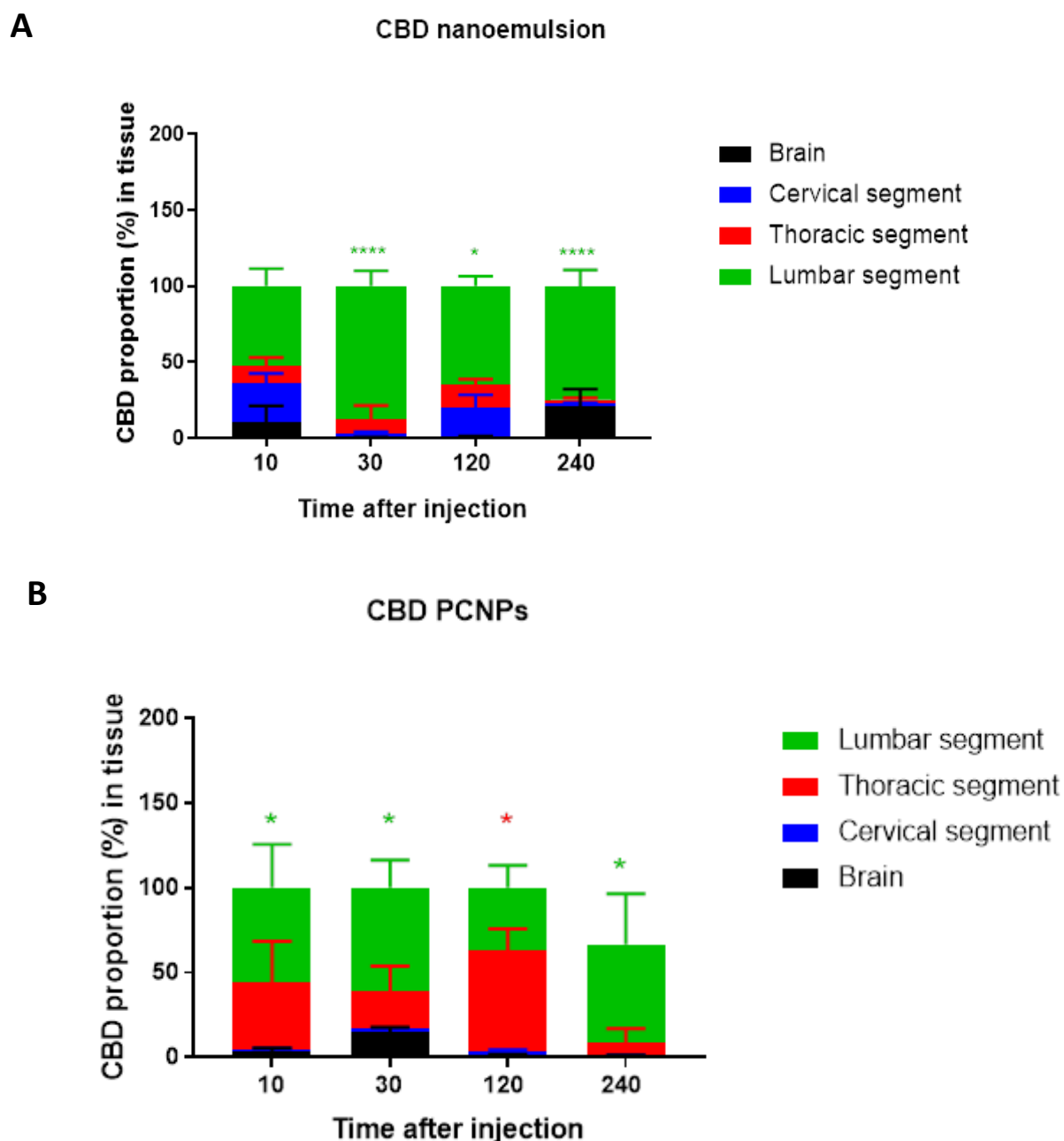


Figure 5.10. CBD mass proportion (%) in each segment of the spinal cord and the brain over time after injection of CBD nanoemulsion (**A**) and CBD PCNPs (**B**). The total amount of CBD detected at a particular time point was set as 100%, with the amount of CBD found in each segment set as percentage of the total amount. Significantly higher proportions of CBD found in the lumbar segment after administration of the nanoemulsion at 30 mins, 2 hrs and 4 hrs after injection (**** $p < 0.0001$, * $p < 0.05$, **** $p < 0.0001$ respectively). Whereas no significant differences between lumbar and thoracic segments after injection of CBD PCNPs.

5.7. Conclusions

In this work, I have demonstrated the successful injection of two different CBD formulations into the IT space of healthy rats and subsequently evaluated their anti-nociceptive effects and biodistribution in the CNS and peripheral tissues.

Upon evaluation of immediate anti-nociceptive effects to assess the success of the injection technique coupled with the effectiveness of my formulations, I demonstrated that both formulations were effective in significantly decreasing EMG responses to all vFhs, taken as the AUC of all stimulations. Nonetheless, when investigating each vFhs individually, in separate categories, ranging from innocuous to noxious mechanical stimulation, it was apparent that CBD PCNPs were more effective than the CBD nanoemulsion when more noxious stimulation was applied to the rat paw. Taking into consideration the biodistribution data of the formulations, this result could be attributed to the fact that a significantly lower proportion of CBD was found in the spinal cord 10 mins after the injection of the nanoemulsion. My hypothesis was that this was as a result of an immediate partitioning into the fatty tissues of the CNS, thus trapping CBD and delaying its binding to TRPV₁ receptors in the dorsal horn. On the other hand, the PCNPs would be freely available within the CSF / SAS of the IT space, ready for CBD to partition out and bind to receptors.

I have also shown that immediate (within 10 mins) brain delivery can be achieved through IT injections. A significantly higher amount of CBD was detected in the brain (calculated per dose injected) than what has been previously demonstrated in the literature with alternative methods of delivery (oral, IP). Most importantly, I have shown that although T_{max} can be increased through the use of PCNPs, C_{max} and AUC in the brain were higher when a nanoemulsion was administered.

Taken together, these results suggest that I can accept my initial hypothesis that both formulations elicit an anti-nociceptive response after lumbar administration. On the other hand, my initial hypothesis that CBD nanoemulsion administration leads to CBD being retained at the site of injection, and more spread will be observed with the delivery of CBD PCNPs will be rejected. Although I have observed that within 4 hrs a significantly higher amount of CBD was retained in the lumbar segment of the spinal cord, the use of a nanoemulsion leads to a higher C_{max} and AUC in the brain as opposed to the PCNPs. This

demonstrates that much more CBD is delivered to the most distal site of the CNS (the brain) when a nanoemulsion is used as opposed to PCNPs, and this still occurs even though a significant amount of CBD was retained at the site of injection. I suggest that although PCNPs reach the brain very quickly (lower T_{max}) as a result of their small size and PEGylated nature, they are also quickly cleared away from this site. Whereas, on the other hand, a much higher amount of CBD was detected in the brain with the delivery of the nanoemulsion, which I did not hypothesise to be the case initially. I can therefore also accept my hypothesis that CBD PCNPs spread further away from the site as demonstrated by the higher mass proportion reaching the thoracic segment in contrast to the CBD nanoemulsion. Nonetheless, the uptake of CBD in the brain after delivery of CBD PCNPs was lower than what I achieved after delivery of the CBD nanoemulsion, which I did not anticipate initially.

In conclusion, this study has highlighted the complexity of the IT space, and the difficulties associated with both the injection technique and the EMG measurements. Nonetheless, I have demonstrated that this is an effective method of attaining significant drug delivery to the brain, with a higher C_{max} when formulated as a nanoemulsion. On the other hand, its anti-nociceptive effectiveness with noxious stimulation is improved upon formulation as PCNPs, possibly as a result of the availability of CBD immediately after injection. Therefore, these data clearly demonstrated that drug delivery strategies can be manipulated through formulations in order to benefit the particular disease state, alongside the targeted biodistribution pattern. Nonetheless, this experimental model only allows for an initial (acute) biodistribution and electrophysiological investigation, as a result of the open laminectomy surgery. Therefore, in order to obtain a larger picture of the pharmacokinetic parameters, longer-term studies must be carried out. Moreover, animal models of neuropathic pain need to be utilised in order to further investigate the analgesic potential of CBD formulations for this therapeutic need.

6. Chapter 6 – Conclusions and future work

6.1. Aims and hypotheses of the thesis

The overarching aim of this project was to develop two physically distinct CBD formulations and investigate their biodistribution and anti-nociceptive effects after direct administration into the CNS through an IT injection. I hypothesised that:

- CBD PCNPs will spread further away from the site of injection as a result of the PEGylated coating which would decrease interactions with the SAS components.
- The CBD nanoemulsion would be retained at the site of injection due to the hydrophobicity of the lipid, which would preferentially be absorbed in the fat surrounding the SAS.
- Both formulations will be efficacious in eliciting an anti-nociceptive effect in rats after lumbar administration, according to the CBD release from the formulations.

6.2. Overall discussion

The work carried out in this thesis has demonstrated the successful development and optimisation of two distinct CBD formulations for IT delivery for the treatment of pain. As previously discussed, the treatment of neuropathic pain in the clinic is extremely complex and difficult, with many therapeutics unable to reach their site of action, the CNS, due to unfavourable physico-chemical characteristics and poor pharmacokinetic profiles. Previous literature has shown that CBD is efficacious in acting on molecular targets mediating pain, with most of its activity on receptors in the CNS (i.e., TRPV₁ and TRPA₁, 5-HT_{1A} and PPAR γ) (Fisher *et al.*, 2019; Maione *et al.*, 2011; Jara-Oseguera *et al.*, 2006). Nonetheless, the use of CBD for the treatment of pain is difficult, mostly due to the first pass metabolism observed in clinical trials (Burns *et al.*, 2006). This is mostly caused by the slow and variable absorption of CBD after oral administration and extensive liver metabolism following IV delivery (Bruni *et al.*, 2018). Nonetheless, these drawbacks can be overcome through different formulation strategies. The lipophilicity of CBD makes it an attractive option for formulation in lipid-based

nanocarriers, whereby its pharmacokinetic profile may be improved, and first-pass metabolism can be avoided. Moreover, in order to enhance analgesic activity and lead to sustained concentrations at the site of action, formulations can be coupled with the local delivery directly CNS, via an IT injection.

As outlined in the overarching hypothesis, I hypothesised that two distinct CBD formulations will exhibit different biodistribution profiles following delivery directly to the CNS. In order to test my hypothesis, I incorporated CBD in two formulations, a commercially available nanoemulsion and in PCNPs. I optimised the manufacturing and formulation processes, leading to a nanoemulsion with an average diameter of 286.8 nm and a PDI of 0.135 and CBD PCNPs with a diameter of 121.8 nm and PDI of 0.079. The double-nanoprecipitation technique I employed for the formulation of CBD PCNPs was fast and reproducible, leading to stable PCNPs. The association protocol I utilised for the incorporation of CBD in the lipid-phase of the nanoemulsion achieved high association efficiency, leading to a stable formulation. Both processes were extensively optimised through DOE, in order to establish the optimum processing parameters for each particular formulation, ready for *in vivo* testing. Prior to injecting the formulations through an IT injection in rats, I sought to establish this technique through imaging methods. The successful injection of methylene blue directly into the spinal cord of a rat post-mortem has demonstrated that it diffuses across the entirety of the neuraxis. Moreover, the injection of iron oxide NPs has also led to an increase in pixel intensity in the lumbar segment, suggesting that this administration technique was efficient in my animal model. As it has been extensively discussed in the previous Chapters, other methods of delivering therapeutics to the CNS may be used in the clinic and in *in vivo* experiments (i.e., Omayya reservoirs, indwelling catheters), however these methods were not available to us under the Home Office *in vivo* project licence. Nonetheless, the data acquired in this thesis, through a single bolus injection via a laminectomy surgery, demonstrate how different formulations have a pharmacological effect in terms of analgesia immediately after delivery, and that formulation strategies can significantly impact biodistribution profiles.

The distribution of intrathecally injected formulations is extremely complex and is thought to be influenced by diffusion and / or fluid exchange led by the movements and dynamics of the CSF (Umbrain *et al.*, 1997). The particle size of the formulations can also dictate how rapidly the therapeutics distribute through the CNS after administration. Moreover, clearance from

the SAS and the ability to have a long-term sustained effect may also be modulated (Householder *et al.*, 2019). In this work I have demonstrated that, indeed, formulations do have an effect on the biodistribution of CBD after IT injection. I have shown that immediate brain delivery can be achieved, with a higher T_{max} reached through the use of PCNPs, whereas an increased C_{max} and AUC achieved in the brain after the administration of a nanoemulsion. Moreover, a significantly higher amount of CBD was detected in the brain (calculated per dose injected) than what has previously been shown in the literature through oral and IP injections (Deiana *et al.*, 2012). This work has also demonstrated that both formulations led to immediate anti-nociceptive effects, significantly decreasing the EMG responses to all vFhs, upon analysis of the AUC of all stimulations. When analysing the data individually, in separate categories ranging from light to heavy mechanical stimulation, it was shown that the PCNPs were more efficacious than the CBD nanoemulsion. I hypothesised that this was due to the different biodistribution profiles exhibited by the formulations, with the nanoemulsion leading to partitioning in the fatty tissues of the CNS immediately after injection, thus delaying binding to TRPV₁ receptors in the dorsal horn.

Overall, these data have shown that CNS delivery through a single bolus intrathecally is extremely complex. This is because of the associated difficulties with the EMG measurements, coupled with the complicated laminectomy surgery. The IT injection itself is challenging, due to the variability achieved in terms of injection angle, speed, and precise location. Nonetheless, this work has demonstrated that IT delivery is efficacious in attaining therapeutically significant doses in the CNS, and formulation strategies can be employed to modulate biodistribution profiles and furthermore influence the level of brain delivery achieved through a lumbar injection. Moreover, analgesic effects can also be improved upon formulation in PCNPs, as opposed to lipid-based nanoemulsions which, presumably, have a tendency to partition into fatty tissues. This work has therefore demonstrated the novelty of modulating drug delivery in the IT space using different formulation strategies for the effective treatment of pain. Going forwards, a longer-term *in vivo* investigation in animal models of neuropathic pain (i.e., induced through sciatic nerve ligation) must be carried out, to effectively ascertain the analgesic effects of CBD for this therapeutic area. This could be achieved through the installation of an IT indwelling catheter, with repeated administration of formulations over a longer period of time, or the installation of an Ommaya reservoir.

Moreover, other pain assays could be employed to test the effectiveness of the formulations, alongside their long-term pharmacokinetics through tissue and serum sampling. This is particularly essential as previous work by Householder et al., 2019 has demonstrated that NPs administered to the CNS can last at the site of delivery for as long as 3 weeks, which would be essential in the area of pain management. Moreover, examples of other pain assays include the Hargreaves test (assesses thermal pain sensations in the paw), the tail-flick test or the hot plate test. Nonetheless, the experiments performed in this thesis have demonstrated how formulations are effective in modulating the effects of therapeutics in the CNS, which is essential in advancing the drug delivery field.

6.3. General conclusions:

I successfully generated two distinct and stable CBD formulations, physically characterised them and administered them *in vivo*. Moreover, I also evaluated the IT injection technique through imaging approaches to evaluate its feasibility in successfully reaching the CNS space.

Through the utilisation of SPIONs and MRI, I demonstrated that an IT injection leads to increased R_2 signal intensity in the lumbar area of the spinal cord. Moreover, I have also shown that iron elements were present on spinal cord sections upon EDX analysis and that methylene blue dye diffused through the entirety of the neuraxis after injection. Therefore, I determined that my method of performing IT injections was successful in delivering formulations to the spinal cord, and could be used for the testing of my CBD formulations.

Upon *in vivo* administration, I demonstrated that both CBD formulations were retained in the CNS and no CBD was detected in peripheral tissues (spleen, liver, cervical lymph nodes or serum) at any-time point tested. I also accepted my hypothesis that both had an anti-nociceptive effect, within 10 min of IT administration. As this was the case when I analysed the AUC of the EMG response to all vFhs stimuli. Nonetheless, upon analysis of each vFhs individually, in different categories ranging from innocuous to noxious mechanical stimulation, I found that CBD PCNPs were more effective in eliciting anti-nociceptive effects than the CBD nanoemulsion upon application of noxious stimuli to the rat paw. I hypothesised that this was attributed to the biodistribution data, as there were significantly lower quantities of CBD in the spinal cord 10 mins after the injection of the nanoemulsion, due to

its partitioning into the fatty tissues of the CNS, thus preventing CBD from binding to TRPV₁ receptors in the dorsal horn.

On the other hand, my initial hypothesis that the CBD nanoemulsion will be preferential retained at the site of injection will be rejected. This is because I have observed that a higher $C_{\max}$ and AUC between 0-4 hrs in the brain was achieved with the delivery of the nanoemulsion as opposed to the PCNPs. These data suggest that more CBD was delivered to the sites furthest away from the site of injection (i.e., the brain) when a nanoemulsion was used, and this still occurred even though a significant amount of CBD was retained at the site of injection (lumbar segment). I also observed that $T_{\max}$ in the brain is reached earlier with the delivery of PCNPs, which is probably resulting from the smaller, and PEGylated nature of this formulation. Nonetheless, the PCNPs were quickly cleared away from this site, therefore the accumulation of CBD in the brain was reduced in comparison to the nanoemulsion. Interestingly, these results go against my initial hypothesis, thus, leading to its rejection. In contrast, I can accept my hypothesis that CBD PCNPs led to more spread from the site of injection, as I have observed that a higher mass proportion of CBD reached the adjacent thoracic segment in comparison to the CBD nanoemulsion. Nonetheless, this spread was only applicable to the spinal cord, as when PCNPs reached the most distal site, the brain, their accumulation was hindered, possibly due to their rapid clearance of the smaller PCNPs, as previously discussed.

This work has therefore demonstrated the complexity of the IT space and the difficulties associated with the injection technique and EMG measurements. Nonetheless, I showed that the IT delivery method is effective in achieving significant delivery to the brain, which can be further improved with the use of a nanoemulsion. The concentration of CBD reached in the brain in my current work is 2,400-fold higher than those achieved through oral or IP administration (Deiana *et al.*, 2012), assuming linear PK and dose equivalence. These results demonstrate the capabilities of this delivery route, which can be further enhanced through formulation optimisation. Both formulations achieved rapid widespread biodistribution within the CNS, whilst remaining localised in the spinal cord and the brain without diffusion into the systemic venous circulation. Moreover, their analgesic effect was almost immediate, demonstrating the importance of effectively reaching the site of action and by-passing first pass metabolism.

6.4. Future work

The MR imaging studies carried out were not as informative as anticipated owing to the formalin fixation of the animals. This was due to be repeated in order to increase the sample size with no fixation, however it was not possible due to Covid-19 restrictions and the closure of the University which severely shortened the time available.

Future work would have also included real-time MRI of live animals after injection of both SPIONs and fluorinated CBD (F-CBD) to investigate the biodistribution after IT injection. I planned to utilise the F-CBD properties which render it visible on MR imaging, and formulate it as a nanoemulsion and PCNPs in order to investigate its biodistribution in the CNS.

Furthermore, I also wanted to carry out more immunohistochemistry experiments of various sections of the spinal cord and include more control animals (injected rats, un-injected rats and various time-points) to evaluate the inflammasome cascade after injections.

6.5. Emerging hypotheses

The data acquired from this work could be used to test future hypotheses:

- The CBD nanoemulsion provides a sustained release depot in the lumbar segment and achieves delivery in the brain over a couple of weeks.
- CBD achieves deep parenchymal penetration in the brain through the PCNPs formulation.

The first hypothesis could be tackled through the use of an alternative IT injection which does not require an open laminectomy surgery, therefore allowing for a recoverable procedure. An injection through the ossified spinal cord could be carried out by an experienced veterinary consultant (successful injection determined through a tail-flick response). This procedure would allow for the rats to recuperate, and biodistribution studies to be carried out weeks after the injection. It might also be possible to carry out repeated dosing of the formulation to investigate the cumulative CBD concentrations in the CNS.

The second hypothesis could be tested with alternative mass-spectrometry instrumentation, with higher resolution than the traditional HPLC system. Furthermore, this would also by-pass the drawback of homogenising the tissues, which is required for HPLC sample preparation.

For example, ToF-SIMS would allow the detection of CBD in brain parenchymal tissues, determining whether the drug penetrates into the brain or is simply bound to the surface within the CSF circulating spaces (i.e., meninges or choroid plexus).

References

- Abrams, D. & Guzman, M. Cannabis in cancer care. *Clinical Pharmacology & Therapeutics* 97, 575–586 (2015).
- Abushrida, A. *et al.* A simple and efficient method for polymer coating of iron oxide nanoparticles. *Journal of Drug Delivery Science and Technology* 55, 101460 (2020).
- Anand, K. J. S. *et al.* Long-Term Behavioral Effects of Repetitive Pain in Neonatal Rat Pups. *Physiology & behavior* 66, 627 (1999).
- Angst, M. S. & Drover, D. R. Pharmacology of Drugs Formulated with DepoFoam™. *Clinical Pharmacokinetics* 2006 45:12 45, 1153–1176 (2012).
- Ashby, L. S. *et al.* Gliadel wafer implantation combined with standard radiotherapy and concurrent followed by adjuvant temozolomide for treatment of newly diagnosed high-grade glioma: a systematic literature review. *World Journal of Surgical Oncology* 2016 14:1 14, 1–15 (2016).
- Aswathanarayan, J. B. *et al.* Nanoemulsions and Their Potential Applications in Food Industry. *Frontiers in Sustainable Food Systems* 3, 95 (2019).
- Araújo-Neto, R.P. *et al.* Monodisperse sodium oleate coated magnetite high susceptibility nanoparticles for hyperthermia applications. *Journal of Magnetism and Magnetic Materials*. 1;364:72–9. (2014).
- Banks, W. A. Characteristics of compounds that cross the blood-brain barrier. *BMC Neurology* 9, 1–5 (2009).
- Bartanusz, V. *et al.* The blood-spinal cord barrier: morphology and clinical implications. *Ann Neurol*. 70(2):194–206.(2011).
- Bell, R. F. Cancer Pain: Analgesics and Co-Analgesics. *Evidence-Based Chronic Pain Management* 327–336 (2010).
- Bejjani, G. K. *et al.* Intrathecal granuloma after implantation of a morphine pump: case report and review of the literature. - *Surg Neurol.*, 48, 288-91, (1997).
- Belov, V. *et al.* Large-Volume Intrathecal Administrations: Impact on CSF Pressure and Safety Implications. *Frontiers in Neuroscience* 15, (2021).
- Benoit, A. *et al.* Design and production of nanoparticles formulated from nano-emulsion templates—A review. *Journal of Controlled Release* 128, 185–199 (2008).
- Bernards, C.M. *et al.*, Liposome encapsulation prolongs alfentanil spinal analgesia and alters systemic redistribution in the rat. *Anesthesiology*, 77(3): p. 529–35 (1992).
- Bergese, S. D. *et al.* Evaluation of DepoFoam® bupivacaine for the treatment of postsurgical pain . *Pain Management* 1, 539–547 (2011).

- Binder, A. *et al.* Topical 5% lidocaine (lignocaine) medicated plaster treatment for post-herpetic neuralgia: results of a double-blind, placebo-controlled, multinational efficacy and safety trial. *Clin Drug Investig.* 29:393–408. (2009).
- Bonferoni, M. C. *et al.* Nanoemulsions for “Nose-to-Brain” Drug Delivery. *Pharmaceutics* 11, (2019).
- Bota, D. A. *et al.* Interstitial chemotherapy with biodegradable BCNU (Gliadel®) wafers in the treatment of malignant gliomas. *Therapeutics and Clinical Risk Management* 3, 707 (2007).
- Boom, M. *et al.* Non-analgesic effects of opioids: opioid-induced respiratory depression. *Curr Pharm Des.* 18(37):5994–6004. (2012).
- Branca, J. J. V. *et al.* Targeting cannabidiol to specific areas of the brain: an ultrasound-based strategy. *Neural Regeneration Research* 15, 2247 (2020).
- Breitbart, W. Psychotropic adjuvant analgesics for cancer pain. *Psycho-Oncology* 1, 133–145 (1992).
- Bruni, N. *et al.* Cannabinoid Delivery Systems for Pain and Inflammation Treatment. *Molecules* 2018, Vol. 23, Page 2478 23, 2478 (2018).
- Blanco, E., Shen, H. & Ferrari, M. Principles of nanoparticle design for overcoming biological barriers to drug delivery. *Nat. Biotechnol.* 33, 941–951 (2015).
- Bujedo, B. Physiology of Spinal Opioids and its relevance for Pain Management Selection. *Open Journal of Pain Medicine* 1, 021–026 (2017).
- Bulat, M. Dynamics and Statics of the Cerebrospinal Fluid: The Classical and a New Hypothesis. *Intracranial Pressure VIII* 726–730 (1993).
- Burness, C. B. *et al.* Capsaicin 8% patch: a review in peripheral neuropathic pain. *Drugs.* 76:123–134 (2016).
- Byers, K.P. *et al.* Infections complicating tunneled intraspinal catheter systems used to treat chronic pain. - *Clin Infect Dis.*, 21, 403-8 (1995).
- Capilla, J. *et al.*, Efficacy of intrathecal liposomal amphotericin B plus oral posaconazole in the treatment of acute meningeal cryptococcosis in a murine model. *Int J Antimicrob Agents*, 42(3): p. 282–3 (2013).
- Calias, P. *et al.* Intrathecal delivery of protein therapeutics to the brain: A critical reassessment. *Pharmacology & Therapeutics* 144, 114–122 (2014).
- Campbell, J. N. & Meyer, R. A. Mechanisms of Neuropathic Pain. *Neuron* 52, 77 (2006).
- Cerqueira, R. *et al.* Microglia Response and In Vivo Therapeutic Potential of Methylprednisolone-Loaded Dendrimer Nanoparticles in Spinal Cord Injury. *Small* 9, 738–749 (2013).
- Chahar, P. *et al.* Liposomal bupivacaine: a review of a new bupivacaine formulation. *Journal of Pain Research* 5, 257 (2012).
- Cherniakov, I. *et al.* The effect of Pro NanoLipospheres (PNL) formulation containing natural absorption enhancers on the oral bioavailability of delta-9-tetrahydrocannabinol (THC) and

cannabidiol (CBD) in a rat model. *European Journal of Pharmaceutical Sciences* 109, 21–30 (2017).

Chiari, A. *et al.* Preclinical Toxicity Screening of Intrathecal Adenosine in Rats and Dogs. *Anesthesiology* 91, 824–824 (1999).

Chiu, C. *et al.* Temporal course of cerebrospinal fluid dynamics and amyloid accumulation in the aging rat brain from three to thirty months. *Fluids and Barriers of the CNS* 9, (2012).

Chung, J.K. *et al.* Biodistribution of Idursulfase Formulated for Intrathecal Use (Idursulfase-IT) in Cynomolgus Monkeys after Intrathecal Lumbar Administration. *PLOS ONE* 11, e0164765 (2016).

Dagata, J. A. *et al.* Physical characterization methods for iron oxide contrast agents encapsulated within a targeted liposome-based delivery system. *Nanotechnology* 19, 305101 (2008).

Daneman, R. & Prat, A. The Blood–Brain Barrier. *Cold Spring Harbor Perspectives in Biology* 7, (2015).

Dawe, R. J., *et al.* Postmortem MRI of human brain hemispheres: T2 relaxation times during formaldehyde fixation. *Magnetic Resonance in Medicine* 61, 810–818 (2009).

Dahlhamer, J. *et al.* Prevalence of chronic pain and high impact chronic pain among adults—United States, 2016. *Morbidity Mortality Weekly Rep.* 67:1001–6. (2018).

de Gregorio, D. *et al.* Cannabidiol modulates serotonergic transmission and reverses both allodynia and anxiety-like behavior in a model of neuropathic pain. (2018)

de Oliveira, A.M. *et al.* Physicochemical aspects behind the size of biodegradable polymeric nanoparticles: A step forward. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* 436, 1092–1102 (2013).

Deiana, S. *et al.* Plasma and brain pharmacokinetic profile of cannabidiol (CBD), cannabidivarin (CBDV), Δ^9 -tetrahydrocannabivarin (THCV) and cannabigerol (CBG) in rats and mice following oral and intraperitoneal administration and CBD action on obsessive–compulsive behaviour. *Psychopharmacology* 219:3 219, 859–873 (2011).

DeSesso, J. M. *et al.* Bone development in laboratory mammals used in developmental toxicity studies. *Birth Defects Research* 110, 1157–1187 (2018).

Dengler, E. C. *et al.* Mesoporous silica-supported lipid bilayers (protocells) for DNA cargo delivery to the spinal cord. *J. Control. Release Off. J. Control. Release Soc.* 168, 209–224 (2013).

Donaghue, I. E. H. *et al.* Sustained delivery of bioactive neurotrophin-3 to the injured spinal cord. *Biomater. Sci.* 3, 65–72 (2015).

Eccleston, G.M. Emulsions and Microemulsions. *Encyclopedia of Pharmaceutical Technology*. New York: Informa Healthcare USA. Inc; 1548–65. (2007).

Fisher, E. *et al.* Cannabinoids, cannabis, and cannabis-based medicine for pain management: a protocol for an overview of systematic reviews and a systematic review of randomised controlled trials. *Pain Reports*.4(3):e741 (2019).

Finnerup, N.B. *et al.* Pharmacotherapy for neuropathic pain in adults: a systematic review and meta-analysis. *Lancet Neurol.* 14:162–173. (2015).

Feng, W. *et al.* Natural sesame oil is superior to pre-digested lipid formulations and purified triglycerides in promoting the intestinal lymphatic transport and systemic bioavailability of cannabidiol. *European journal of pharmaceutics and biopharmaceutics* :162, 43–49 (2021).

Fessi, H. *et al.* Nanocapsule formation by interfacial polymer deposition following solvent displacement. *International Journal of Pharmaceutics* 55, (1989).

Fowler, M. *et al.* Intrathecal Drug Delivery in the Era of Nanomedicine. *Advanced drug delivery reviews* 165–166, 77 (2020).

Freitas, C. *et al.* Effect of light and temperature on zeta potential and physical stability in solid lipid nanoparticle (SLN (TM)) dispersions. *Int J Pharm.* 168(2):221e9. (1998).

Garg, T. *et al.* Current strategies for targeted delivery of bio-active drug molecules in the treatment of brain tumor. *Journal of Drug Targeting.* 26;23(10):865–87 (2015).

Ganta, S. *et al.* Pharmacokinetics and pharmacodynamics of chlorambucil delivered in parenteral emulsion. *International Journal of Pharmaceutics* 360, 115–121 (2008).

Gherzi-Egea, J. Fate of cerebrospinal fluid-borne amyloid beta-peptide: rapid clearance into blood and appreciable accumulation by cerebral arteries. *J. Neurochem.* 67, 880–883 (1996).

Gherzi-Egea, J.-F. *et al.* Molecular anatomy and functions of the choroidal blood-cerebrospinal fluid barrier in health and disease. *Acta Neuropathologica* 2018 135:3 135, 337–361 (2018).

Grant, G. *et al.* Intrathecal administration of liposomal neostigmine prolongs analgesia in mice. *Acta Anaesthesiologica Scandinavica* 46, 90–94 (2002).

Grant, G. *et al.* Wound infiltration with liposomal bupivacaine prolongs analgesia in rats. *Acta Anaesthesiologica Scandinavica* 41:204–207.17 (1997).

Grant, G. *et al.* Prolonged analgesia and decreased toxicity with liposomal morphine in a mouse model. *Anesth Analg.* 79: 706–709 (1994).

Gunasekaran, N. *et al.* Reintoxication: the release of fat-stored Δ^9 -tetrahydrocannabinol (THC) into blood is enhanced by food deprivation or ACTH exposure. *British Journal of Pharmacology* 158, 1330–1337 (2009).

Guy, G. W. & Flint, M. E. A Single Centre, Placebo-Controlled, Four Period, Crossover, Tolerability Study Assessing, Pharmacodynamic Effects, Pharmacokinetic Characteristics and Cognitive Profiles of a Single Dose of Three Formulations of Cannabis Based Medicine Extracts (GWPD9901 EXT), 35–77 (2008).

Haga, P. T. *et al.* A numerical investigation of intrathecal isobaric drug dispersion within the cervical subarachnoid space. *PLOS ONE* 12, e0173680 (2017).

Hagihara, Y. *et al.* Widespread gene transfection into the central nervous system of primates. *Gene Ther.* 7, 759–763 (2000).

Hala, E.-K. *et al.* Green synthesis of iron oxide nanoparticles using two selected brown seaweeds: Characterization and application for lead bioremediation (2016).

- Han, F.Y. *et al.* Novel Polymeric Bioerodable Microparticles for Prolonged-Release Intrathecal Delivery of Analgesic Agents for Relief of Intractable Cancer-Related Pain. *Journal of Pharmaceutical Sciences*. 2015 Jul 1;104(7):2334–44.
- Hänggi, D. *et al.* A Site-Specific, Sustained-Release Drug Delivery System for Aneurysmal Subarachnoid Hemorrhage. *Neurotherapeutics*.1;13(2):439–49.(2016).
- Hernan Perez De La Ossa, D. *et al.* Poly-ε-caprolactone microspheres as a drug delivery system for cannabinoid administration: Development, characterization and in vitro evaluation of their antitumoral efficacy. *Journal of Controlled Release* 161, 927–932 (2012).
- Hettiarachchi, Y.H. *et al.* The effect of pulsatile flow on intrathecal drug delivery in the spinal canal. *Ann Biomed Eng* 39, 2592–2602 (2011).
- Hindle, A. Intrathecal opioids in the management of acute postoperative pain. doi:10.1093/bjaceaccp/mkn016.
- Hippalgaonkar, K. *et al.* Injectable lipid emulsions-advancements, opportunities and challenges. *AAPS PharmSciTech* 11, 1526–1540 (2010).
- Hladky, M. B. Mechanisms of fluid movement into, through and out of the brain: evaluation of the evidence. *Fluids Barriers CNS* 11, 26 (2014).
- Hobson, N. J. *et al.* Facile aqueous, room temperature preparation of high transverse relaxivity clustered iron oxide nanoparticles. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* 570, 165–171 (2019).
- Holz, M. L. *et al.* Nuclear and electron relaxation. The magnetic nucleus-unpaired electron coupling in solution. VCH, Weinheim, New York, Basel, Cambridge, 1991, pp. 216, DM 118. ISBN 3-527-28306-4. *Magnetic Resonance in Chemistry* 31, S154–S154 (1993).
- Hörmann, K. & Zimmer, A. Drug delivery and drug targeting with parenteral lipid nanoemulsions — A review. *Journal of Controlled Release* 223, 85–98 (2016).
- Householder, K. T. *et al.* Fate of nanoparticles in the central nervous system after intrathecal injection in healthy mice. *Scientific Reports* 2019 9:1 9, 1–11 (2019).
- Huestis, M. A. Human Cannabinoid Pharmacokinetics. *Chemistry & Biodiversity* 4, 1770–1804 (2007).
- Huestis, M.A. Pharmacokinetics and metabolism of the plant cannabinoids, delta9-tetrahydrocannabinol, cannabidiol and cannabinol. *Handb. Exp. Pharmacol.* 168, 657–690 (2005).
- Hylden, J. L. K. *et al.* Intrathecal morphine in mice: A new technique. *European Journal of Pharmacology* 67, 313–316 (1980).
- Ineichen, B.V. *et al.* Direct, long-term intrathecal application of therapeutics to the rodent CNS. *Nature Protocols*. 12(1):104–21. (2017).
- Iliff, J. J. *et al.* A Paravascular Pathway Facilitates CSF Flow Through the Brain Parenchyma and the Clearance of Interstitial Solutes, Including Amyloid β. *Science translational medicine*. 4. (2012).

- Jafari, S.M. *et al.* Nano-particle encapsulation of fish oil by spray drying. *Food Res. Int.* 41 (2), 172–183 (2008)
- Jafari, S.M. *et al.* Nano-emulsion production by sonication and microfluidization—a comparison. *Int. J. Food Prop.* 9, 475–485 (2006)
- Javed, Y. *et al.* MRI based on iron oxide nanoparticles contrast agents: effect of oxidation state and architecture. *Journal of Nanoparticle Research* 2017 19:11 19, 1–25 (2017).
- Joshi, G.P. *et al.* The safety of liposome bupivacaine following various routes of administration in animals. *J Pain Res.* 30;8:781–9. (2015).
- Joshi, K. *et al.* Involvement of the TTX-resistant sodium channel Nav 1.8 in inflammatory and neuropathic, but not post-operative, pain states *Pain*, 123 . 75-82 (2006).
- Karimy, J. K. *et al.* A novel method to study cerebrospinal fluid dynamics in rats. *Journal of neuroscience methods* 241, 78 (2015).
- Kathmann, M., *et al.* Cannabidiol is an allosteric modulator at mu- and delta-opioid receptors. *Naunyn-Schmiedeberg's Archives of Pharmacology* 2006 372:5 372, 354–361 (2006).
- Khan, M. S. I., *et al.* Power of Scanning Electron Microscopy and Energy Dispersive X-Ray Analysis in Rapid Microbial Detection and Identification at the Single Cell Level. *Scientific Reports* 2020 10:1 10, 1–10 (2020).
- Kim, B. *et al.* Tuning payload delivery in tumour cylindroids using gold nanoparticles. *Nature Nanotechnology* 2010 5:6 5, 465–472 (2010).
- Kim, S. *et al.* Prolongation of Drug Exposure in Cerebrospinal Fluid by Encapsulation into DepoFoam. *Cancer Research* 53, (1993).
- Kim, S. *et al.* Multivesicular Liposomes Containing 1- β -d-Arabinofuranosylcytosine for Slow-Release Intrathecal Therapy. *Cancer Research.* 1;47(15):3935–7 (1987).
- Kim, H. *et al.*, Polyamidoamine dendrimer-conjugated triamcinolone acetonide attenuates nerve injury-induced spinal cord microglia activation and mechanical allodynia. *Mol Pain*, 13: p. 1744806917697006. (2017).
- Kimelberg, H. K. *et al.* Distribution of Free and Liposome-entrapped [3H]Methotrexate in the Central Nervous System after Intracerebroventricular Injection in a Primate. *Cancer Research* 38, (1978).
- Kitamura, I. *et al.* Intrathecal Chemotherapy with 1,3-Bis(2-chloroethyl)-1-nitrosourea Encapsulated into Hybrid Liposomes for Meningeal Gliomatosis: An Experimental Study. *Cancer Research* 56, (1996).
- Koch, S. C., *et al.* Midazolam Potentiates Nociceptive Behavior, Sensitizes Cutaneous Reflexes, and Is Devoid of Sedative Action in Neonatal Rats. *Anesthesiology* 108, 122–129 (2008).
- Kohrs, N.J *et al.* Drug delivery systems and controlled release. *Encyclopedia of Biomedical Engineering* 1–3, 316–329 (2019).
- Kwon, S. *et al.* Near-infrared fluorescence lymphatic imaging in vascular endothelial growth factor-C overexpressing murine melanoma. *Biomedical Optics Express* 9, 4631 (2018).

- Kuo, Y. C. *et al.* Targeted delivery of etoposide, carmustine and doxorubicin to human glioblastoma cells using methoxy poly(ethylene glycol)-poly(ϵ -caprolactone) nanoparticles conjugated with wheat germ agglutinin and folic acid. *Mater. Sci. Eng. C* 96, 114–128 (2019).
- Kroin, J.S. Intrathecal drug administration. Present use and future trends. - *Clin Pharmacokinet.*, 22, 319-26 (1992).
- Lallemant, F., *et al.* Successfully Improving Ocular Drug Delivery Using the Cationic Nanoemulsion, Novasorb. *Journal of Drug Delivery* 2012, 1–16 (2012).
- Laun, A. S. *et al.* GPR3, GPR6, and GPR12 as novel molecular targets: their biological functions and interaction with cannabidiol. *Acta Pharmacologica Sinica* 2018 40:3 40, 300–308 (2018).
- Lagarce, F. *et al.* Baclofen-loaded microspheres: preparation and efficacy testing in a new rabbit model. *European Journal of Pharmaceutics and Biopharmaceutics*. 1;59(3):449–59. (2005).
- Laird, J.M. *et al.* Deficits in visceral pain and referred hyperalgesia in Nav1.8 (SNS/PN3)-null mice *J. Neurosci.* 8352-8356 (2002).
- Langerman, L. *et al.* Prolongation of the pharmacologic effect of intrathecal meperidine by the use of a lipid solution of it. - *Anesth Analg.*, 72, 635-8, (1991).
- Langerman, L. *et al.* A rabbit model for evaluation of spinal anaesthesia: chronic cannulation of the subarachnoid space. - *Anesth Analg.*, 71, 529-35, (1990).
- Laule, C, *et al.* Magnetic resonance imaging of myelin. *Neurotherapeutics* 4:460–84.05.004 (2007).
- Leal-Calderon, F. & Cansell, M. The design of emulsions and their fate in the body following enteral and parenteral routes. *Soft Matter* 8, 10213–10225 (2012).
- Lee, S. W., *et al.* An open-label, multicenter, phase I trial of a cremophor-free, polymeric micelle formulation of paclitaxel combined with carboplatin as a first-line treatment for advanced ovarian cancer: a Korean Gynecologic Oncology Group study (KGOG-3016). *Journal of Gynecologic Oncology* 28, 26 (2017).
- Leem, J.W. *et al.* Cutaneous sensory receptors in the rat foot. *J Neurophysiol* 69:1684-1699 (1993).
- Lemberger, L. *et al.* Comparative pharmacology of Δ^9 tetrahydrocannabinol and its metabolite, 11 OH Δ^9 tetrahydrocannabinol. *Journal of Clinical Investigation* 52, 2411–2417 (1973).
- Lehtinen, M.K. *et al.* Expression of the NLRP3 inflammasome in cerebral cortex after traumatic brain injury in a rat model. *Neurochemical research* 38(10):2072-2083 (2013).
- Li, F., *et al.* Fluorescent Graphene Quantum Dots for the Determination of Metal Ions. *Novel Nanomaterials for Biomedical, Environmental and Energy Applications* 215–239 (2018).
- Li, P. *et al.* Density gradient ultracentrifugation for colloidal nanostructures separation and investigation. *Science Bulletin* 63, 645–662 (2018).

Linninger, A. A. *et al.* A mathematical model of blood, cerebrospinal fluid and brain dynamics. *Journal of Mathematical Biology* 2009 59:6 59, 729–759 (2009).

Liu, H. *et al.* Inadvertent intrathecal injections and best practice management. doi:10.1111/aas.12821. (2016)

Luo, Z. D. *et al.* Upregulation of dorsal root ganglion $\alpha 2\delta$ calcium channel subunit and its correlation with allodynia in spinal nerve-injured rats. *J Neurosci.* 21:1868–1875 (2001).

Lovelyn, C. *et al.* Current State of Nanoemulsions in Drug Delivery. *Journal of Biomaterials and Nanobiotechnology* 2, 626–639 (2011).

Lynch, L. Intrathecal drug delivery systems. *Continuing Education in Anaesthesia Critical Care & Pain* 14, 27–31 (2014).

Maali, A. & Mosavian, M. T. H. Preparation and Application of Nanoemulsions in the Last Decade (2000-2010). *Journal of Dispersion Science and Technology* 34, 92–105 (2013).

Machado, G. T. P., *et al.* Formulation and evaluation of anti-MRSA nanoemulsion loaded with Achyrocline satureioides: a new sustainable strategy for the bovine mastitis. *Nano Express* 1, 030004 (2020).

Madhusudhan, B. *et al.* 1-O-alkylglycerol stabilized carbamazepine intravenous o/w nanoemulsions for drug targeting in mice. 15, 154–161 (2008).

Maione, S. *et al.* Non-psychoactive cannabinoids modulate the descending pathway of antinociception in anaesthetized rats through several mechanisms of action. *British Journal of Pharmacology* 162, 584–596 (2011).

Mastorakos, P. *et al.* Highly PEGylated DNA Nanoparticles Provide Uniform and Widespread Gene Transfer in the Brain. *Advanced healthcare materials* 4, 1023 (2015).

Mandal, M. *et al.* Magnetite nanoparticles with tunable gold or silver shell. *J. Colloid Interface Sci.* 286(1), 187–194 (2005).

McCarthy, D. J. *et al.* Nanoparticles and the blood-brain barrier: Advancing from in-vitro models towards therapeutic significance. *Pharmaceutical Research* 32, 1161–1185 (2015).

McCormick, W. C. & Schreiner, R. L. Evidence-Based Case Reviews: Diagnosis and treatment of opiate-resistant pain in advanced AIDS. *Western Journal of Medicine* 175, 408 (2001).

McKenzie, B. A. *et al.* Caspase-1 inhibition prevents glial inflammasome activation and pyroptosis in models of multiple sclerosis. *Proceedings of the National Academy of Sciences of the United States of America* 115, E6065–E6074 (2018).

McClements, D. J. *et al.* Food-grade nanoemulsions: formulation, fabrication, properties, performance, biological fate, and potential toxicity. *Crit. Rev. Food Sci. Nutr.* 51 (4), 285–330. (2011).

Media Resources | British Pain Society. <https://www.britishpainsociety.org/media-resources/>.

Mehta, A. M., *et al.* Convection-Enhanced Delivery. *Neurotherapeutics* 14, 358 (2017).

- Meisel, C. L., *et al.* Assessment of Superparamagnetic Iron Oxide Nanoparticle Poly(Ethylene Glycol) Coatings on Magnetic Resonance Relaxation for Early Disease Detection. *IEEE open journal of engineering in medicine and biology* 1, 116 (2020).
- Mestre, H. *et al.* Flow of cerebrospinal fluid is driven by arterial pulsations and is reduced in hypertension. *Nature Communications* 9, (2018).
- Millar, S. A. *et al.* A Systematic Review on the Pharmacokinetics of Cannabidiol in Humans. *Frontiers in Pharmacology* 0, 1365 (2018).
- Miteva, M. *et al.* Tuning PEGylation of mixed micelles to overcome intracellular and systemic siRNA delivery barriers. *Biomaterials*. 38(2015):97–107 (2015).
- Mondello, E. *et al.* Cannabinoids and spinal cord stimulation for the treatment of failed back surgery syndrome refractory pain. *Journal of Pain Research* 11, 1761 (2018).
- Mortazavi, M. M. *et al.* Subarachnoid Trabeculae: A Comprehensive Review of Their Embryology, Histology, Morphology, and Surgical Significance. *World Neurosurgery* 111, 279–290 (2018).
- Mortezaee, K. *et al.* A. Inflammasome: Its role in traumatic brain and spinal cord injury. *Journal of Cellular Physiology* 233, 5160–5169 (2018).
- Nakano, Y. *et al.* Development of a Novel Nano-emulsion Formulation to Improve Intestinal Absorption of Cannabidiol. *Medical Cannabis and Cannabinoids* 2, 35–42 (2019).
- Nance, E. A. *et al.* A dense poly(ethylene glycol) coating improves penetration of large polymeric nanoparticles within brain tissue. *Science Translational Medicine* 4, (2012).
- Nance, E. *et al.* Brain-Penetrating Nanoparticles Improve Paclitaxel Efficacy in Malignant Glioma Following Local Administration. *ACS Nano* 8, 10655–10664 (2014).
- Nauta, H. J. W. *et al.* Microsurgical anatomy of spinal subarachnoid space. *Surgical Neurology* 19, 431–437 (1983).
- Nishiyama, N. & Kataoka, K. Current state, achievements, and future prospects of polymeric micelles as nanocarriers for drug and gene delivery. *Pharmacology and Therapeutics* 112, 630–648 (2006).
- Nishiyama, T. The Effects of Intrathecal Morphine Encapsulated in l- and d-Dipalmitoylphosphatidyl Choline Liposomes on Acute Nociception in Rats. *Anesthesia & Analgesia*. 91(2):423–428. (2000).
- Nutt, J. Randomized, double-blind trial of glial cell line-derived neurotrophic factor (GDNF) in PD. *Neurology* 60, 69–73 (2003).
- Ohtsuki, S. *et al.* Contribution of carrier-mediated transport systems to the blood-brain barrier as a supporting and protecting interface for the brain; importance for CNS drug discovery and development. *Pharm Res.* 24(9):1745–58. (2007).
- Okuda T, *et al.* Prolonged antinociceptive effect of poly (DL-lactic acid)-fentanyl composites after their intrathecal injection in rats. *Masui*. 48(2): p. 141–5 (1999).

Ory, D. S. *et al.* Intrathecal 2-hydroxypropyl- β -cyclodextrin decreases neurological disease progression in Niemann-Pick disease, type C1: a non-randomised, open-label, phase 1–2 trial. *The Lancet* 390, 1758–1768 (2017).

Ozdemir, A. *et al.* Amphiphilic peptide coated superparamagnetic iron oxide nanoparticles for: In vivo MR tumor imaging. *RSC Advances* 6, 45135–45146 (2016).

Pablo, J. *et al.* Development/Plasticity/Repair A Molecular Platform in Neurons Regulates Inflammation after Spinal Cord Injury (2008).

Papisov, M. I., *et al.* Physiology of the intrathecal bolus: the leptomeningeal route for macromolecule and particle delivery to CNS. *Molecular pharmaceutics* 10, 1522 (2013).

Pardridge, W. Drug transport in brain via the cerebrospinal fluid. *Fluids Barriers CNS* 8, 7 (2011).

Pardridge W. Drug and gene targeting to the brain with molecular Trojan horses. *Nat Rev Drug Discov.* 1(2):131–9. (2002).

Pan, W. *et al.* Permeability of the blood-brain and blood-spinal cord barriers to interferons. *J Neuroimmunol.* 76:105–111. (1997).

Peyrl, A. *et al.* Pharmacokinetics and Toxicity of Intrathecal Liposomal Cytarabine in Children and Adolescents Following Age-Adapted Dosing. (2013) .

Phan, H. *et al.* Role of self-assembly conditions and amphiphilic balance on nanoparticle formation of PEG-PDLLA copolymers in aqueous environments. *J. Polym. Sci. Part A Polym. Chem.* 57, 1801–1810 (2019).

Phuphanich, S. *et al.* A pharmacokinetic study of intra-CSF administered encapsulated cytarabine (DepoCyt®) for the treatment of neoplastic meningitis in patients with leukemia, lymphoma, or solid tumors as part of a phase III study. *Journal of Neuro-Oncology* 2006 81:2 81, 201–208 (2006).

Prockop, L.D. *et al.* Selective permeability of [3H]-D-mannitol and [14C]-carboxyl-inulin across the blood-brain barrier and blood-spinal cord barrier in the rabbit. *J Spinal Cord Med.* 18:221–226. (1995).

Rabanel, J. M. *et al.* Assessment of PEG on polymeric particles surface, a key step in drug carrier translation. *Journal of Controlled Release* 185, 71–87 (2014).

Ray, W.A. *et al.* Prescription of long-acting opioids and mortality in patients with chronic noncancer pain. *JAMA.* 315:2415–2423 (2016).

Rahman, S. S. U. *et al.* Single step growth of iron oxide nanoparticles and their use as glucose biosensor. *Results in Physics* 7, 4451–4456 (2017).

Raman, M. *et al.* Parenteral Nutrition and Lipids. *Nutrients* 9, (2017).

Reina, M. A., *et al.* A. Electron microscopy and the expansion of regional anesthesia knowledge. *Techniques in Regional Anesthesia and Pain Management* 6, 165–171 (2002).

Reina, M. A. *et al.* Clinical implications of epidural fat in the spinal canal. A scanning electron microscopic study. *Acta Anaesthesiol Belg.* 60(1):7-17 (2009).

Ribeiro, L. N. M. *et al.* Functional Hybrid Nanoemulsions for Sumatriptan Intranasal Delivery. *Frontiers in Chemistry* 8, (2020).

Richardson, S. *et al.* A viable isolated tissue system: a tool for detailed MR measurements and controlled perturbation in physiologically stable tissue. *Magn Reson Med.* 69:1603–10 (2013).

Riley, T. *et al.* Physicochemical evaluation of nanoparticles assembled from poly(lactic acid)-poly(ethylene glycol) (PLA-PEG) block copolymers as drug delivery vehicles. *Langmuir* 17, 3168–3174 (2001).

Roche, M. & Finn, D. P. Brain CB2 Receptors: Implications for Neuropsychiatric Disorders. *Pharmaceuticals* 2010, Vol. 3, Pages 2517-2553 3, 2517–2553 (2010).

Robinson, R.A. *et al.* In vitro modeling of spinal anesthesia. A digital video image processing technique and its application to catheter characterization. *Anesthesiology.* Oct;81(4):1053–1060. (1994).

Sandberg, D. *et al.* Chemotherapy administration directly into the fourth ventricle in a nonhuman primate model. *J. Neurosurg. Pediatr.* 9, 530–541 (2012).

Sandberg, D. *et al.* Methotrexate administration directly into the fourth ventricle in children with malignant fourth ventricular brain tumors: a pilot clinical trial. *J. Neurooncol.* 125, 133–141 (2015).

Sandberg, D. *et al.* Pharmacokinetic analysis of etoposide distribution after administration directly into the fourth ventricle in a piglet model. *J. Neurooncol.* 97, 25–32 (2010).

Sandberg, D. I. & Kerr, M. L. Ventricular access device placement in the fourth ventricle to treat malignant fourth ventricle brain tumors: technical note. *Child's Nervous System* 32, 703 (2016).

Sampson, F.C. *et al.* Functional benefits and cost/benefit analysis of continuous intrathecal baclofen infusion for the management of severe spasticity. - *J Neurosurg.*, 96, 1052-7, (2002)

Schug, S. A. *et al.* Pathophysiology of Pain. *Mechanisms of Vascular Disease: A Reference Book for Vascular Specialists* (2011).

Shapiro, W.R. *et al.* A randomized phase III/IV study to determine benefit and safety of cytarabine liposome injection for treatment of neoplastic meningitis. *J. Clin. Oncol.* 24, 1528–1528 (2006).

Shapiro, W. R. *et al.* Methotrexate : Distribution in Cerebrospinal Fluid in Man, *N. Engl. J. Med.* 293:161-166 (1975)

Sharma, D. *et al.* Formulation and optimization of polymeric nanoparticles for intranasal delivery of lorazepam using Box-Behnken design: In vitro and in vivo evaluation. *BioMed Research International* 2014, (2014).

Shalgunov, V. *et al.* Comprehensive study of the drug delivery properties of poly(L-lactide)-poly(ethylene glycol) nanoparticles in rats and tumor-bearing mice. *J. Control. Release* 261, 31–42 (2017).

- Shatil, A. S., *et al.* Quantitative Ex Vivo MRI changes due to progressive formalin fixation in whole human brain specimens: Longitudinal characterization of diffusion, relaxometry, and myelin water fraction measurements at 3T. *Frontiers in Medicine* 5, 31 (2018).
- Shimoyama, M. *et al.* Influence of Cu Electroplating Solution on Self-Assembled Porous Silica Low-k Films. *Journal of The Electrochemical Society* 153, G870 (2006).
- Shi, K. *et al.* Synthesis, characterization and application of reversible PDLLA-PEG-PDLLA copolymer thermogels in vitro and in vivo. *Sci. Rep.* 6, 19077. (2016).
- Schouenborg, J. *et al.* Functional organization of the nociceptive withdrawal reflexes. I. activation of hindlimb muscles in the rat. *Experimental Brain Research* 83:67-78 (1990).
- Silva-Avellaneda, E. *et al.* The effect of composition, microfluidization and process parameters on formation of oleogels for ice cream applications. *Scientific Reports* 2021 11:1 11, 1–10 (2021).
- Singh, N., *et al.* Potential toxicity of superparamagnetic iron oxide nanoparticles (SPION). <http://dx.doi.org/10.3402/nano.v1i0.5358> 1, 5358 (2010).
- Slavc, I. *et al.* Best practices for the use of intracerebroventricular drug delivery devices. *Molecular Genetics and Metabolism* 124, 184–188 (2018).
- Smith, M.W. *et al.* Endocytosis at the blood-brain barrier: from basic understanding to drug delivery strategies. *J Drug Target*.14(4):191–214. (2006).
- Sousa, F. *et al.* Enhanced antiangiogenic effects of bevacizumab in glioblastoma treatment upon intranasal administration in polymeric nanoparticles. *J. Control. Release* 309, 37–47 (2019).
- Stephen, Z. R., *et al.* Magnetite Nanoparticles for Medical MR Imaging. *Materials today* (Kidlington, England) 14, 330 (2011).
- Styliari, I. D. *et al.* Nanoformulation-by-design: an experimental and molecular dynamics study for polymer coated drug nanoparticles. *RSC Advances* 10, 19521–19533 (2020).
- Suk, J. S. *et al.* PEGylation as a strategy for improving nanoparticle-based drug and gene delivery. *Advanced drug delivery reviews* 99, 28 (2016).
- Sullivan, J. M. *et al.* Convective forces increase rostral delivery of intrathecal radiotracers and antisense oligonucleotides in the cynomolgus monkey nervous system. *Journal of Translational Medicine* 2020 18:1 18, 1–17 (2020).
- Sun, C. *et al.* PEG-Mediated Synthesis of Highly Dispersive Multifunctional Superparamagnetic Nanoparticles: Their Physicochemical Properties and Function In Vivo. *ACS nano* 4, 2402 (2010).
- Sutradhar, K. B. & Amin, L. Nanoemulsions: Increasing possibilities in drug delivery. *European Journal of Nanomedicine* 5, 97–110 (2013).
- Sutradhar, K. B. & Amin, Md. L. Nanoemulsions: increasing possibilities in drug delivery. *European Journal of Nanomedicine* 5, 97–110 (2013).

Takino, T. *et al.*. Long Circulating Emulsion Carrier Systems for Highly Lipophilic Drugs. *Biological and Pharmaceutical Bulletin* 17, 121–125 (1994).

Tamilvanan, S. Formulation of multifunctional oil-in-water nanosized emulsions for active and passive targeting of drugs to otherwise inaccessible internal organs of the human body. *International Journal of Pharmaceutics* 381, 62–76 (2009).

Tadros, T. *et al.* Formation and stability of nano-emulsions. *Adv Colloid Interface Sci.* 20; 108-109():303-18. (2004).

Tang, G.P. *et al.*, Polyethylene glycol modified polyethylenimine for improved CNS gene transfer: effects of PEGylation extent. *Biomaterials*, 24(13): p. 2351–62.(2003).

Tan, J. *et al.* Changes in compressed neurons from dogs with acute and severe cauda equina constrictions following intrathecal injection of brain-derived neurotrophic factor-conjugated polymer nanoparticles. *Neural Regen. Res.* 8, 233–243 (2013).

Tao, J., *et al.* Application of flash nanoprecipitation to fabricate poorly water-soluble drug nanoparticles. *Acta Pharmaceutica Sinica B* 9, 4–18 (2019).

Teale, J. D., *et al.* Plasma cannabinoids measured by radioimmunoassay in rabbits after intravenous injection of tetrahydrocannabinol, 11-hydroxy-tetrahydrocannabinol, cannabinol and cannabidiol. *Research Communications in Chemical Pathology and Pharmacology* 11, 339–342 (1975).

Tibell, A. *et al.* Dissolving intravenous cyclosporin A in a fat emulsion carrier prevents acute renal side effects in the rat. *Transpl Int.* 6(2):69-72. (1993).

The Spinal Cord: A Christopher and Dana Reeve Foundation Text and Atlas - Google Books.

Thorne, R. G. & Nicholson, C. In vivo diffusion analysis with quantum dots and dextrans predicts the width of brain extracellular space. *Proceedings of the National Academy of Sciences of the United States of America* 103, 5567 (2006).

Tovi, M. *et al.* Measurements of T1 and T2 over time in formalin-fixed human whole-brain specimens. *Acta Radiol* ;33(5):400-404 (1992).

Thelin, J. *et al.* Spatial encoding in spinal sensorimotor circuits differs in different wild type mice strains. *BMC Neurosci.* 9:45-2202-9-45 (2008).

Thirumala, G. *et al.* PLGA nanoparticles prepared by nanoprecipitation: drug loading and release studies of a water-soluble drug. *Journal of Controlled Release* 57.2. 171– 185 (1999).

Thormählen, I. *et al.* Refractive Index of Water and Its Dependence on Wavelength, Temperature, and Density. *Journal of Physical and Chemical Reference Data.* 1;14(4):933–45 (1985).

Tiwari, S., *et al.* Preparation and In Vitro Characterization of Multifunctional Nanoemulsions for Simultaneous MR Imaging and Targeted Drug Delivery. *Journal of Biomedical Nanotechnology* 2, 217–224 (2006).

Toossi, A. *et al.* Comparative neuroanatomy of the lumbosacral spinal cord of the rat, cat, pig, monkey, and human. *Scientific Reports* 2021 11:1 11, 1–15 (2021).

Tremoleda, J. L. *et al.* Anaesthesia and physiological monitoring during in vivo imaging of laboratory rodents: considerations on experimental outcomes and animal welfare. *EJNMMI Research* 2012 2:1 2, 1–23 (2012).

Uchida, K. *et al.* Initiation and progression of ossification of the posterior longitudinal ligament of the cervical spine in the hereditary spinal hyperostotic mouse. *European Spine Journal* 2011 21:1 21, 149–155 (2011).

Ueno, M. *et al.* Blood–brain barrier and blood–cerebrospinal fluid barrier in normal and pathological conditions. *Brain Tumor Pathology* 33, 89–96 (2016).

Urits, I. *et al.* Use of cannabidiol (CBD) for the treatment of chronic pain. *Best Practice & Research Clinical Anaesthesiology* 34, 463–477 (2020).

Valdeperez, D. *et al.* Polymer-coated nanoparticles: Carrier platforms for hydrophobic water- and air-sensitive metallo-organic compounds. *Pharmacological Research* 117, 261–266 (2017).

Vasey, C. *et al.* Amphiphilic tri- and tetra-block co-polymers combining versatile functionality with facile assembly into cytocompatible nanoparticles. *Biomaterials Science* 7, 3832–3845 (2019).

Vázquez, M., *et al.* Clinical Pharmacokinetics of Cannabinoids and Potential Drug-Drug Interactions. *Advances in Experimental Medicine and Biology* 1297, 27–42 (2021).

Verma, A. *et al.* Intrathecal ^{99m}Tc-DTPA imaging of molecular passage from lumbar cerebrospinal fluid to brain and periphery in humans. *Alzheimer's & Dementia: Diagnosis, Assessment & Disease Monitoring* 12, e12030 (2020).

Vuilleminot, B. R. *et al.* Safety Evaluation of CNS Administered Biologics—Study Design, Data Interpretation, and Translation to the Clinic. *Toxicological Sciences* 152, 3–9 (2016).

Valdes, C. *et al.* Antinociceptive antibiotics-loaded into solid lipid nanoparticles of prolonged release: Measuring pharmacological efficiency and time span on chronic monoarthritis rats. *PLoS One* . (2018).

Von Dardel, O. *et al.* Fat emulsion as a vehicle for diazepam. A study of 9492 patients. *Br J Anaesth.* 55(1):41-7. (1983).

Warren, K. E. Beyond the Blood:Brain Barrier: The Importance of Central Nervous System (CNS) Pharmacokinetics for the Treatment of CNS Tumors, Including Diffuse Intrinsic Pontine Glioma. *Frontiers in Oncology* 0, 239 (2018).

Watts, R., *et al.* Measuring Glymphatic Flow in Man Using Quantitative Contrast-Enhanced MRI. *AJNR: American Journal of Neuroradiology* 40, 648 (2019).

Wang, H. X. *et al.* Surface charge critically affects tumor penetration and therapeutic efficacy of cancer nanomedicines. *Nano Today* 11, 133–144 (2016).

Wallisch, J. S. *et al.* Cerebrospinal fluid NLRP3 is increased after severe traumatic brain injury in infants and children. *Neurocritical care*:1-7 (2017).

Watson, J.C. *et al.* Central neuropathic pain syndromes. *Mayo Clin Proc.* 91:372–385. (2016).

Washington, C. Stability of lipid emulsions for drug delivery. *Adv Drug Deliv Rev.* 20:131–45. (1996).

Wen, C.-J. *et al.* In vivo real-time fluorescence visualization and brain-targeting mechanisms of lipid nanocarriers with different fatty ester:oil ratios. *6*, 1545–1559 (2011).

Westin, B. D. *et al.* Validation of a preclinical spinal safety model: effects of intrathecal morphine in the neonatal rat. *Anesthesiology* 113, 183–199 (2010).

Wolf, D. A. *et al.* Dynamic dual-isotope molecular imaging elucidates principles for optimizing intrathecal drug delivery. *JCI Insight* 1, (2016).

Woolf, C.J. *et al.* The cutaneous contribution to the hamstring flexor reflex in the rat: An electrophysiological and anatomical study. *Brain Res* 303:299-312. (1984).

Wolak, D. J. & Thorne, R. G. Diffusion of macromolecules in the brain: Implications for drug delivery. *Molecular Pharmaceutics* vol. 10 1492–1504 (2013).

Wretling, A. Development of Fat Emulsions. *Journal of Parenteral and Enteral Nutrition* 5, 230–235 (1981).

Xiong, W. *et al.* Cannabinoids suppress inflammatory and neuropathic pain by targeting $\alpha 3$ glycine receptors. *J Exp Med.* 4;209(6):1121–34. (2012).

Yaksh, T. L. *et al.* Analgesia mediated by a direct spinal action of narcotics. *Science* 192, 1357–1358 (1976).

Yaksh, T. L. *et al.* Inflammatory masses associated with intrathecal drug infusion: A review of preclinical evidence and human data. *Pain Medicine* 3, 300–312 (2002).

Yang, Y. *et al.* Mutations in SCN9A, encoding a sodium channel alpha subunit, in patients with primary erythralgia. *J Med Genet.* 41:171–174 (2004)

Yalcin, I. *et al.* $\beta 2$ -Adrenoceptors are critical for antidepressant treatment of neuropathic pain. *Ann Neurol.* 65:218–225.(2009).

Yesenia Hernández-Giottonini, K. *et al.* PLGA nanoparticle preparations by emulsification and nanoprecipitation techniques: effects of formulation parameters. *RSC Advances* 10, 4218–4231 (2020).

Zádor, F. & Wollemann, M. Receptome: Interactions between three pain-related receptors or the “Triumvirate” of cannabinoid, opioid and TRPV1 receptors. *Pharmacological Research* 102, 254–263 (2015).

Zendedel, A. *et al.* Activation and regulation of NLRP3 inflammasome by intrathecal application of SDF1a in a spinal cord injury model. *Molecular neurobiology* 53(5):3063-3075 (2016)

Zgair, A. *et al.* Dietary fats and pharmaceutical lipid excipients increase systemic exposure to orally administered cannabis and cannabis-based medicines. *American Journal of Translational Research* 8, 3448 (2016).

Zgair, A. *et al.* Oral administration of cannabis with lipids leads to high levels of cannabinoids in the intestinal lymphatic system and prominent immunomodulation. *Scientific Reports*. 7:17, 1–12 (2017).

Zgair, A. *et al.* Development of a simple and sensitive HPLC–UV method for the simultaneous determination of cannabidiol and Δ^9 -tetrahydrocannabinol in rat plasma. *Journal of Pharmaceutical and Biomedical Analysis*. 10;114:145–51. (2015).

Zhao, F.-Y. *et al.* In Vivo Electrophysiological Recording Techniques for the Study of Neuropathic Pain in Rodent Models. *Curr. Protoc. Pharmacol* 66 (2014)

Zhang, X. F. *et al.* A-887826 is a structurally novel, potent and voltage-dependent Nav1.8 sodium channel blocker that attenuates neuropathic tactile allodynia in rats. *Neuropharmacology*.1;59(3):201–7.(2010).

Zimm, S. *et al.* Cytosine arabinoside cerebrospinal fluid kinetics. *Clinical Pharmacology & Therapeutics* 35, 826–830 (1984).

Appendix A

A.1. CBD PCNPs nanoprecipitation with linear polymers mPEG₅₀₀₀-PLA₁₀₀ and PLA₅₀-PEG₄₀₀₀-PLA₅₀

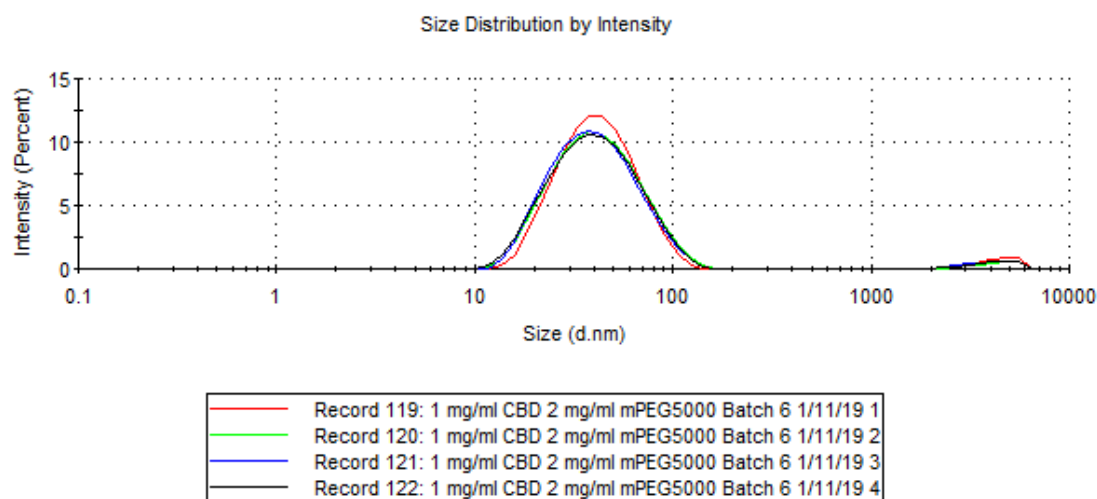


Figure A.1. Coating of CBD nanocrystals by mPEG₅₀₀₀-PLA₁₀₀ through the double nanoprecipitation procedure creates a dual sized NP population.

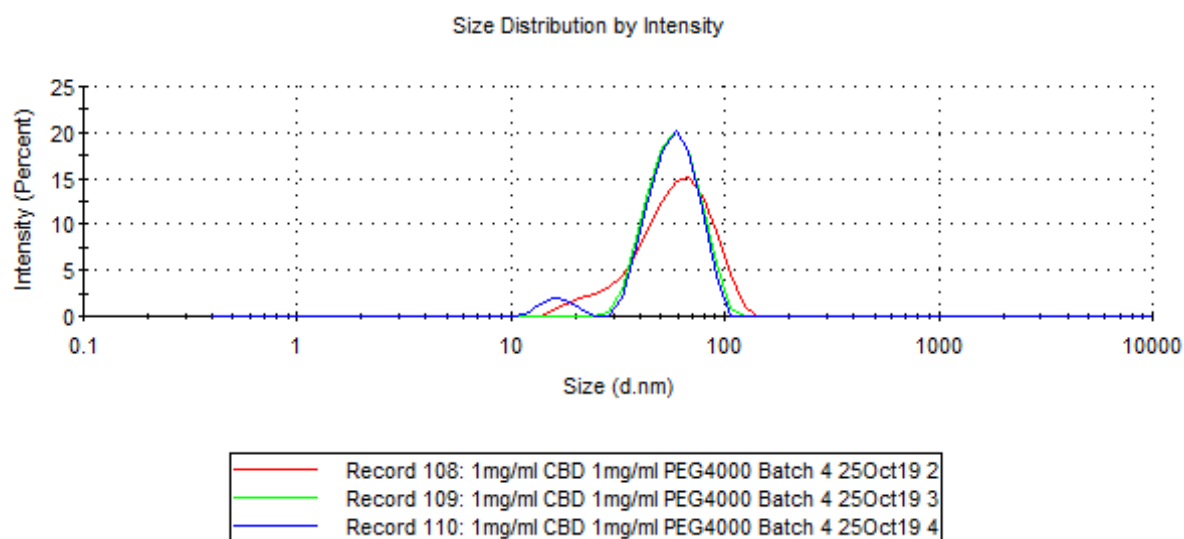


Figure A.2. Coating of CBD nanocrystals by PLA₅₀-PEG₄₀₀₀-PLA₅₀ through the double nanoprecipitation procedure creates a dual sized NP population.

A.2. CBD PCNPs nanoprecipitation with 3-arm polymer, 3-armPEG₁₀₁₄-

PLA₁₀₀ at 1:1 ratio

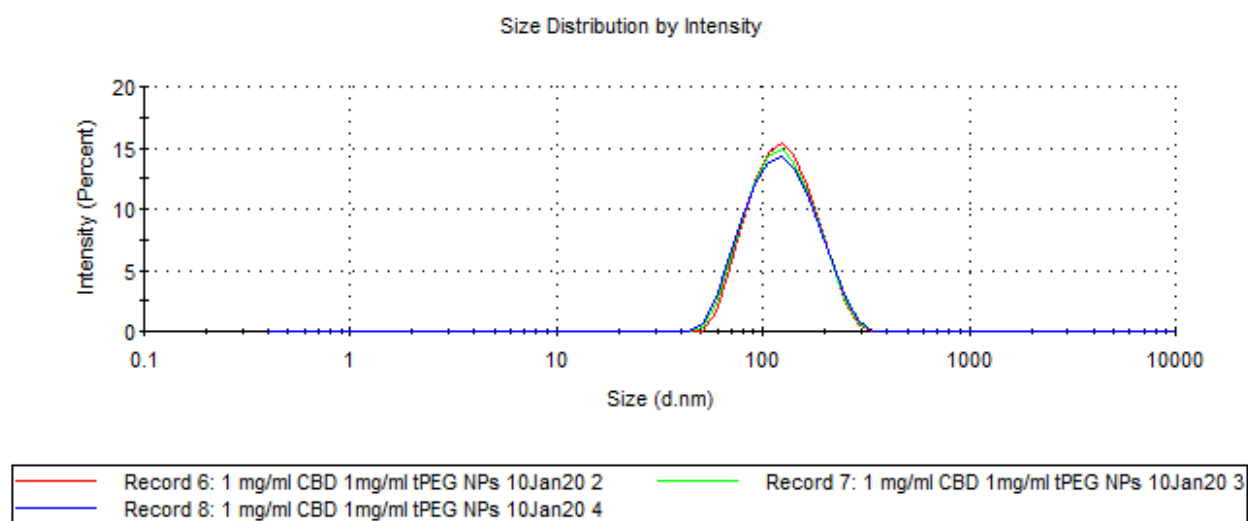


Figure A.3. Coating of CBD nanocrystals by 3-armPEG₁₀₁₄-PLA₁₀₀ through the double nanoprecipitation procedure creates a single sized NP population.

A.3. CBD PCNPs (coated with 3-armPEG₁₀₁₄-PLA₁₀₀) after N₂ air

evaporation

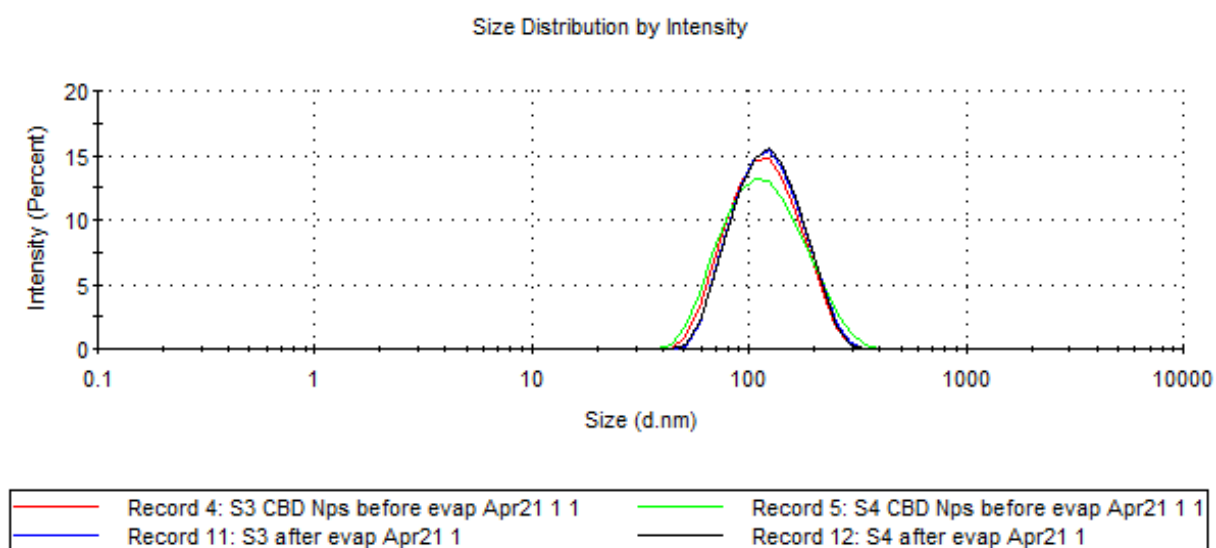


Figure A.4. 3-armPEG₁₀₁₄-PLA₁₀₀ CBD PCNPs were not affected by evaporation to concentrate CBD.

A.4. CBD nanocrystals with no polymer coating

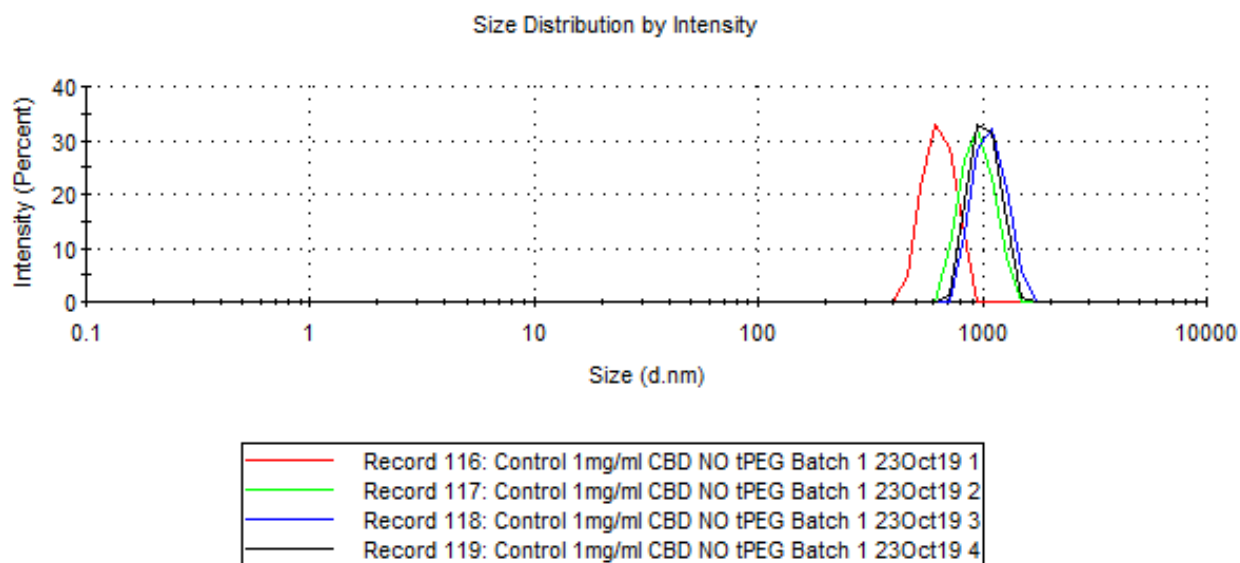


Figure A.5. CBD nanocrystals only control without any polymeric coating.

A.5. NMR spectrum of 3-armPEG₁₀₁₄-PLA₁₀₀

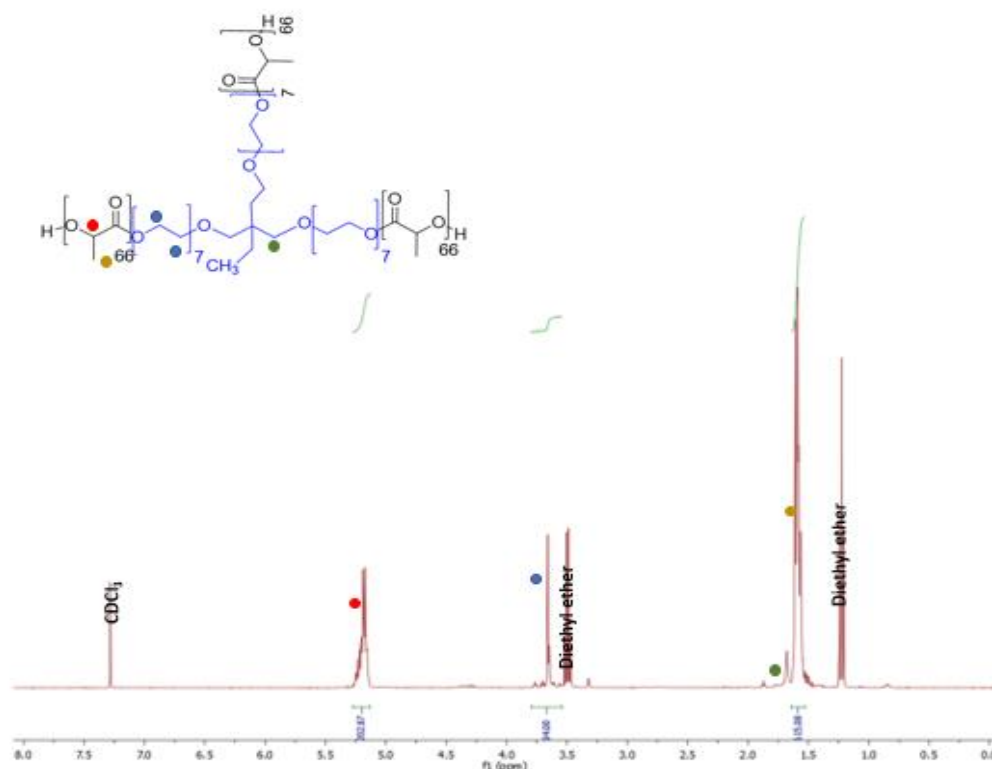
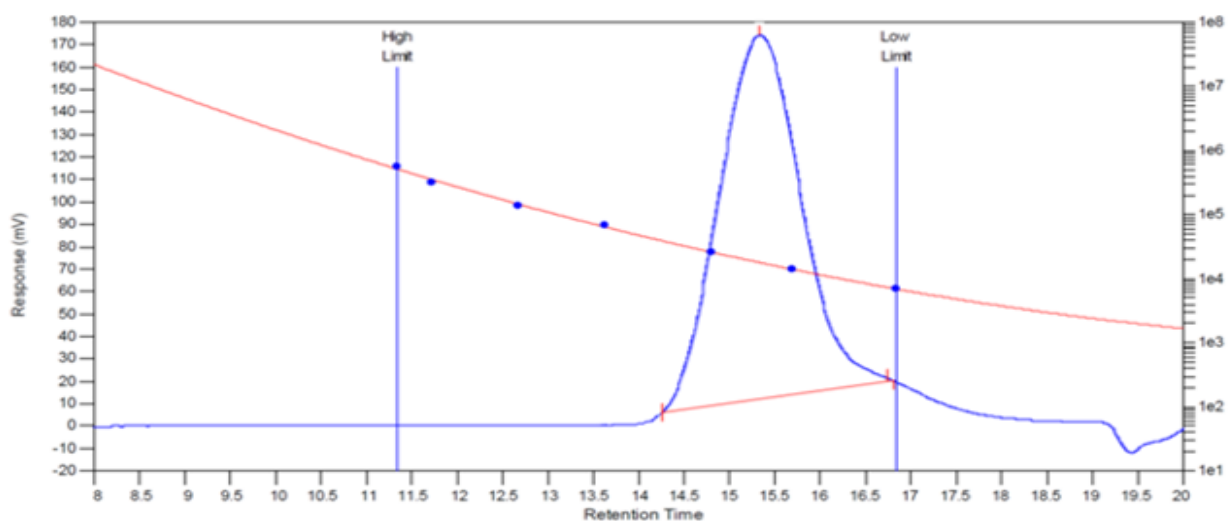


Figure A.6. The ¹H-NMR spectra of 3-armPEG₁₀₁₄-PLA₁₀₀ and structural formula alongside corresponding proton.

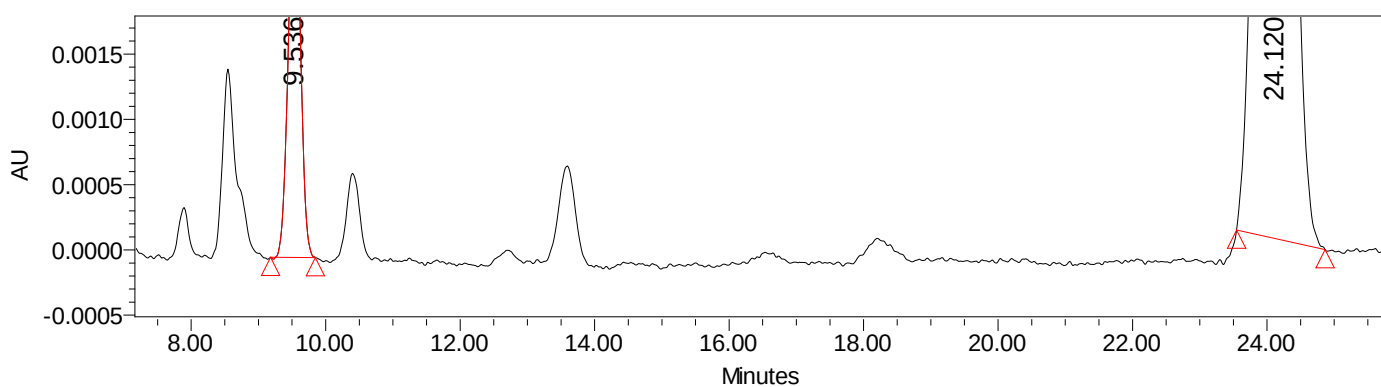
A.6. GPC of 3-armPEG₁₀₁₄-PLA₁₀₀



M_n = 17900 g/mol
Đ = 1.10

Figure A.7. GPC of 3-armPEG₁₀₁₄-PLA₁₀₀.

A.7. Chromatogram of CBD calibration curve detected by HPLC



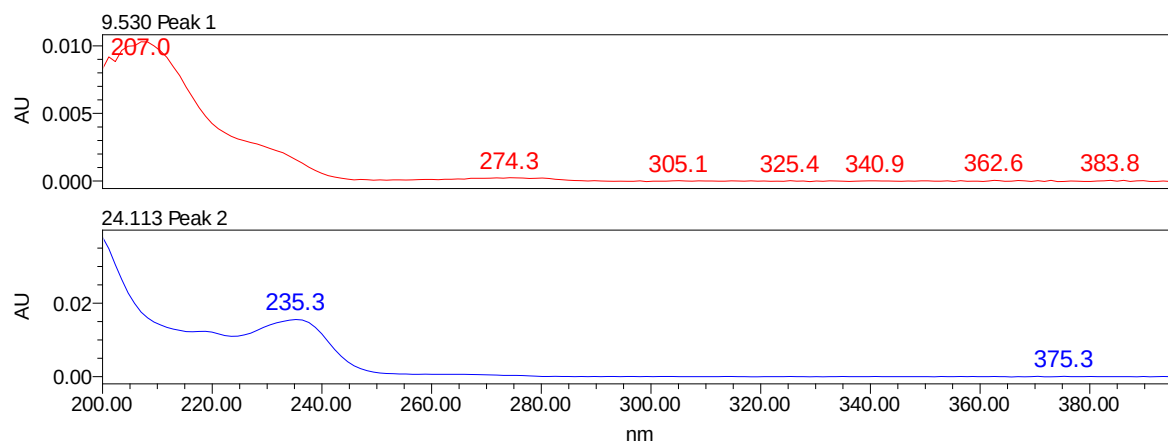


Figure A.8. Chromatogram of CBD calibration curve. CBD retention time 9.5 min (Peak 1) and DDT internal standard 24.00 min (Peak 2). Associated UV spectra of CBD and DDT (Peak 1 and Peak 2 respectively).

A.8. Representative chromatogram of CBD detected in Intralipid®

nanoemulsion

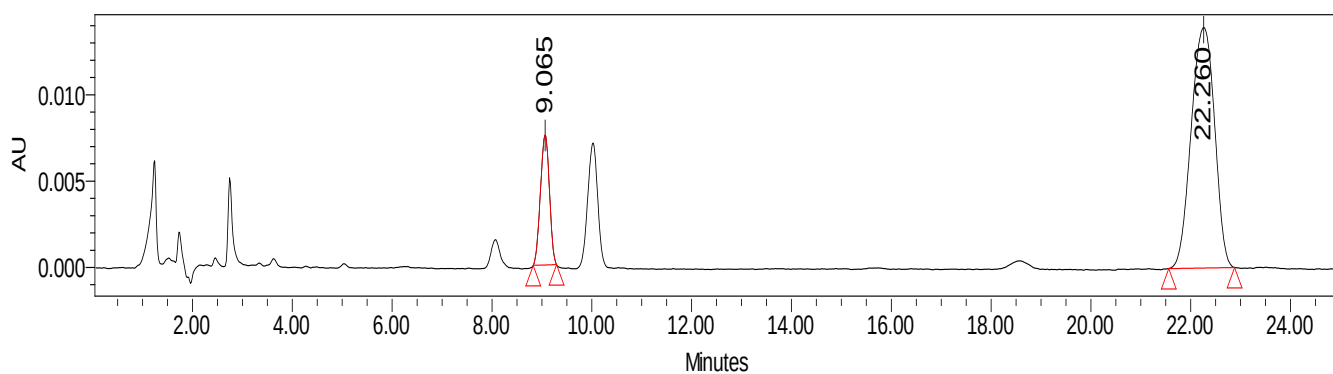


Figure A.9. Representative HPLC chromatogram of CBD detected and quantified in Intralipid® nanoemulsion after the association protocol (retention time of CBD 9 min and DDT 22.2 min)

A.9. Representative chromatogram of CBD detected in PCNPs

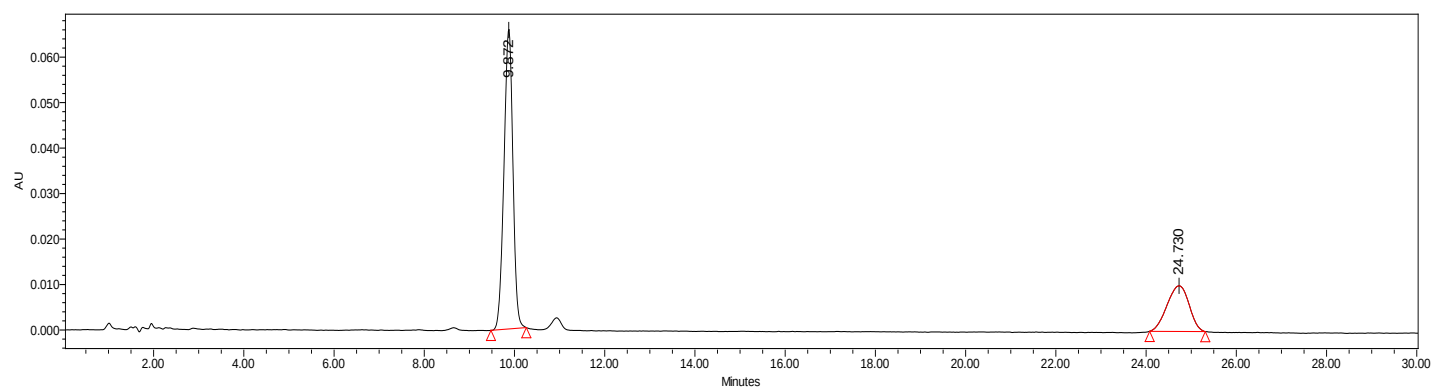


Figure A.10. Representative HPLC chromatogram of CBD detected and quantified in CBD PCNPs after double nanoprecipitation and removal of free nanocrystals (retention time of CBD 9.8 min and DDT 24.7 min)

A.10. MRI R_2 maps (front) of PFA fixed animals

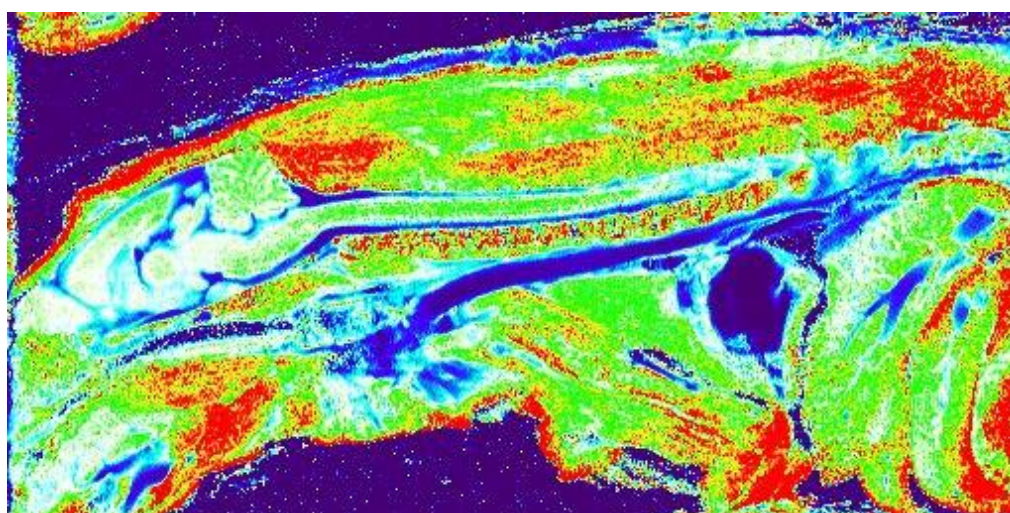
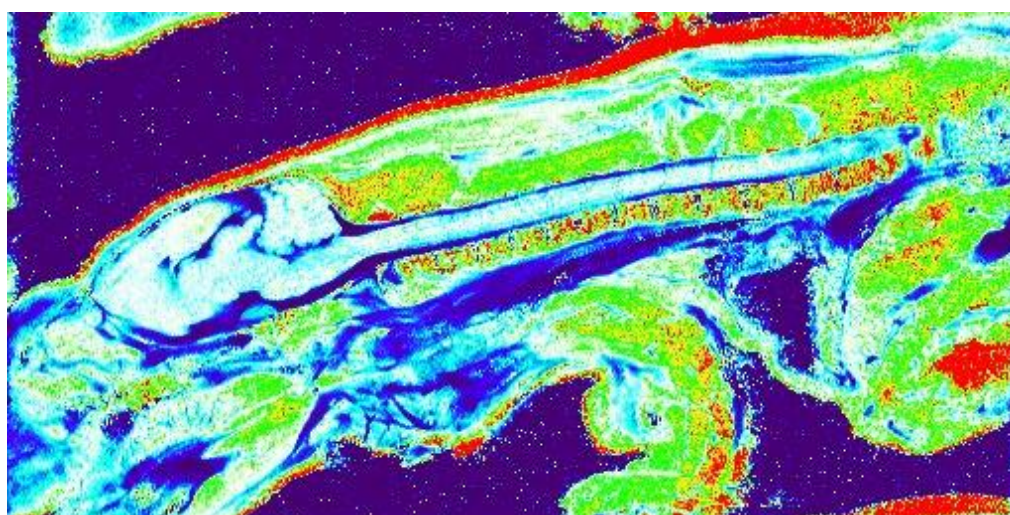
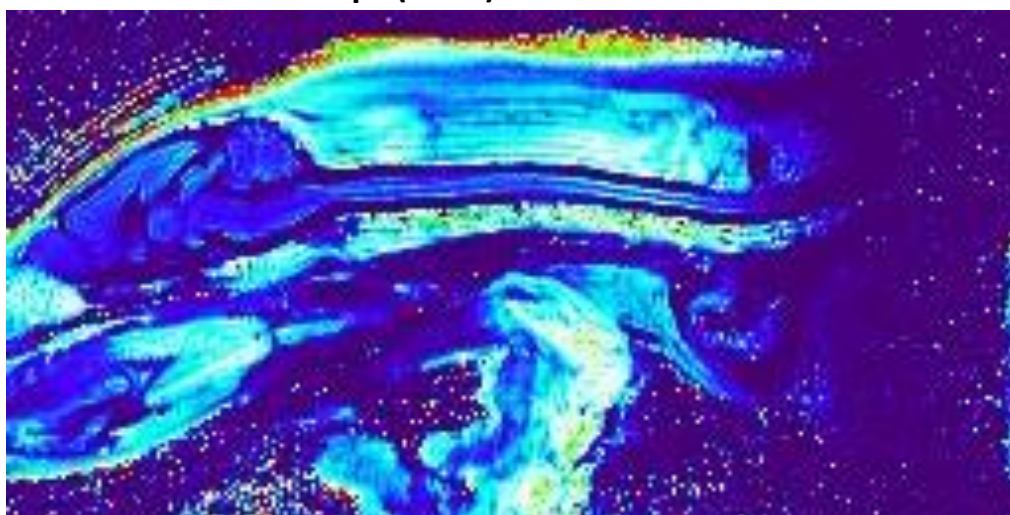


Figure A.11. R_2 maps of PFA fixed animals injected with: 1=SPIONs, 2= PEG SPIONs, 3=saline. PFA fixative lead to an overall increase in signal throughout the tissues rendering analysis unusable for the purpose of SPION detection in the body.

A.11. R_2 maps (back) of PFA fixed animals

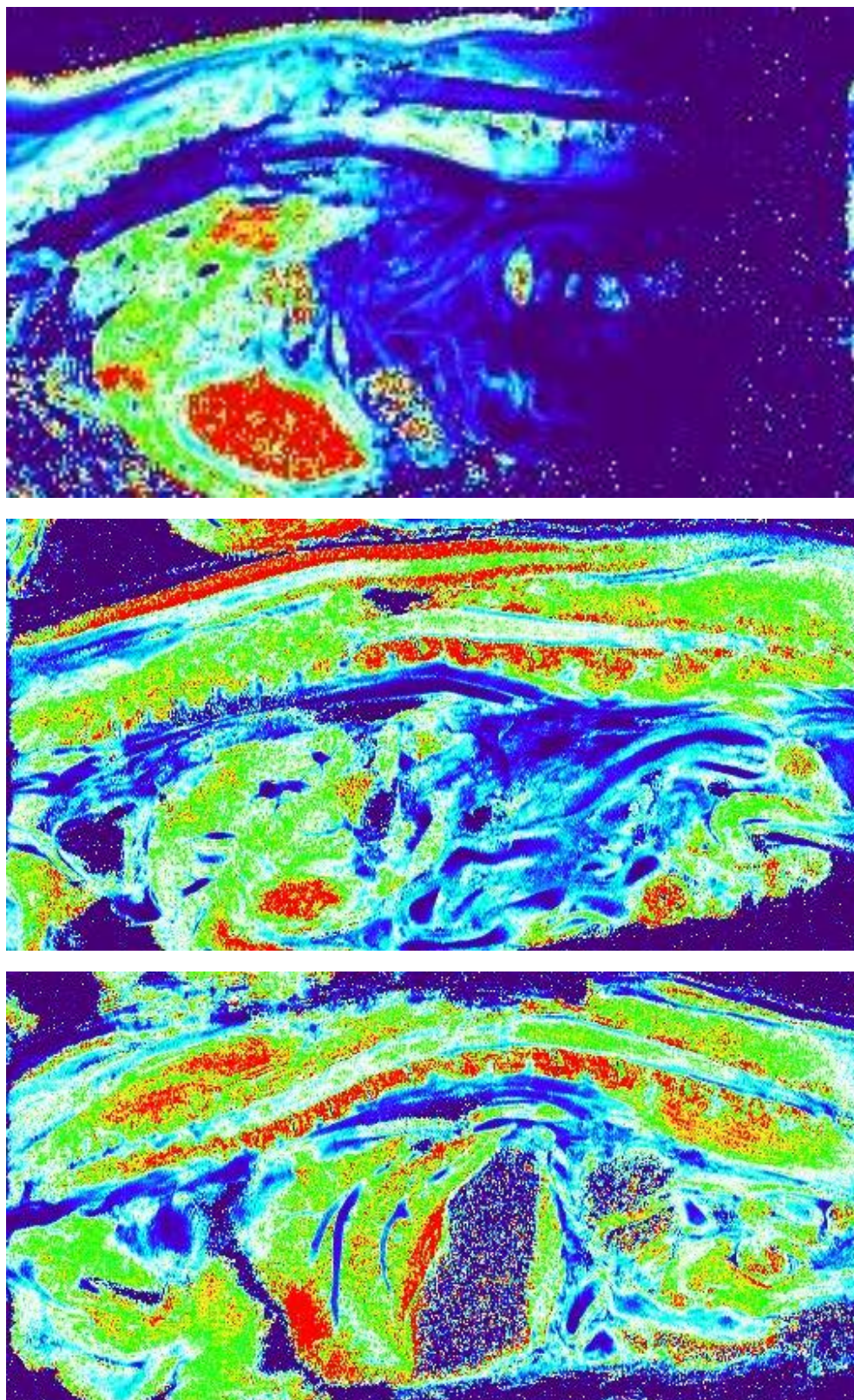


Figure A.12. R_2 maps of PFA fixed animals injected with: 1=SPIONs, 2= PEG SPIONs, 3=saline. PFA fixative lead to an overall increase in signal throughout the tissues rendering analysis unusable for the purpose of SPION detection in the body.

A.12. SEM image of a spinal cord section

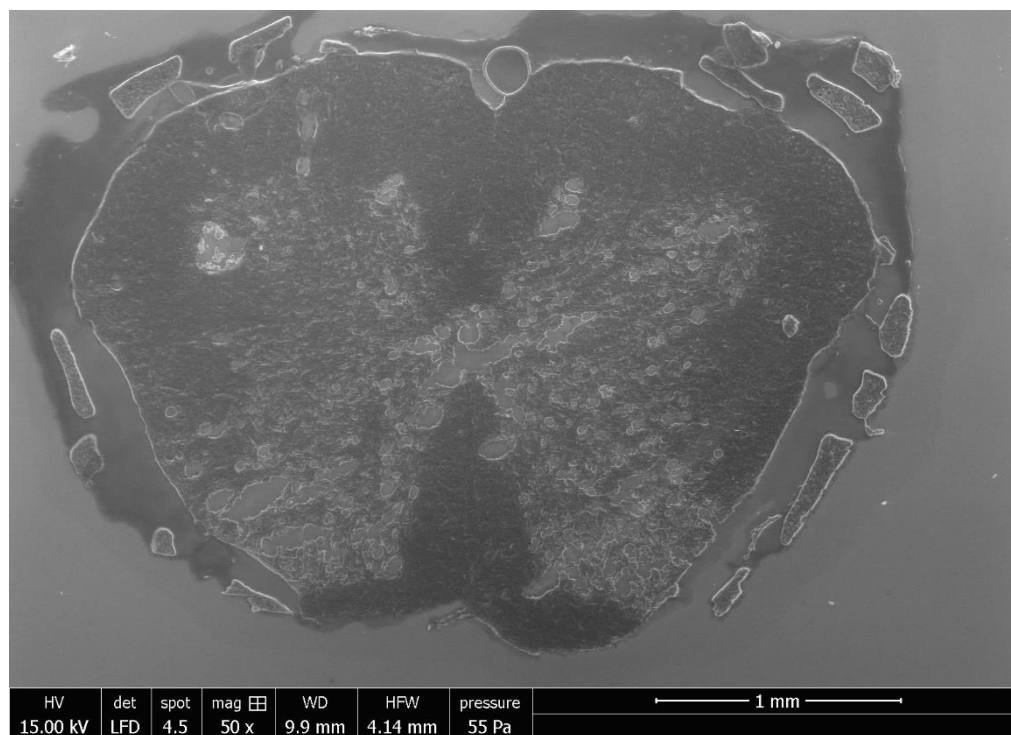
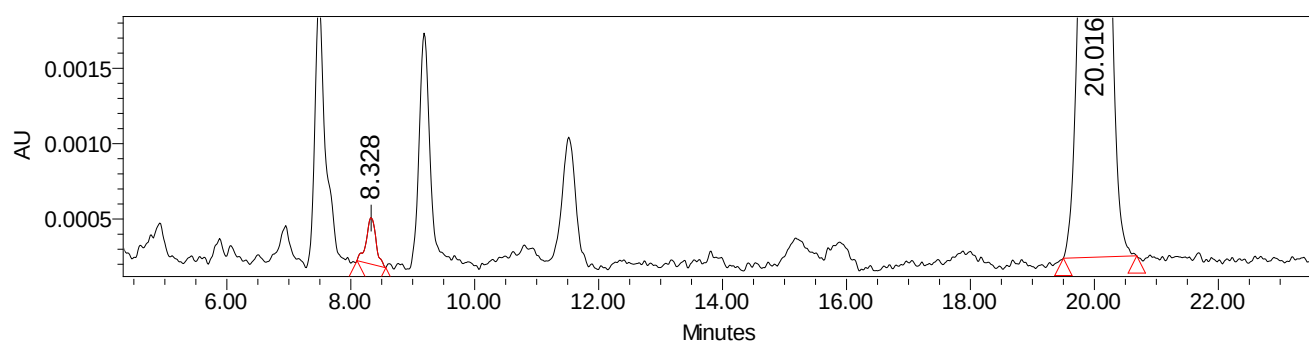


Figure A.13. SEM image of the spinal cord section. No Fe can be detected at this resolution. If Fe could have been detected, the H&E sections would have been transposed with SEM – EDX data to map the distribution of SPIONs in the sections.

A.13. Representative chromatograms for CBD detection in tissues after administration of CBD Intralipid® nanoemulsion *in vivo*



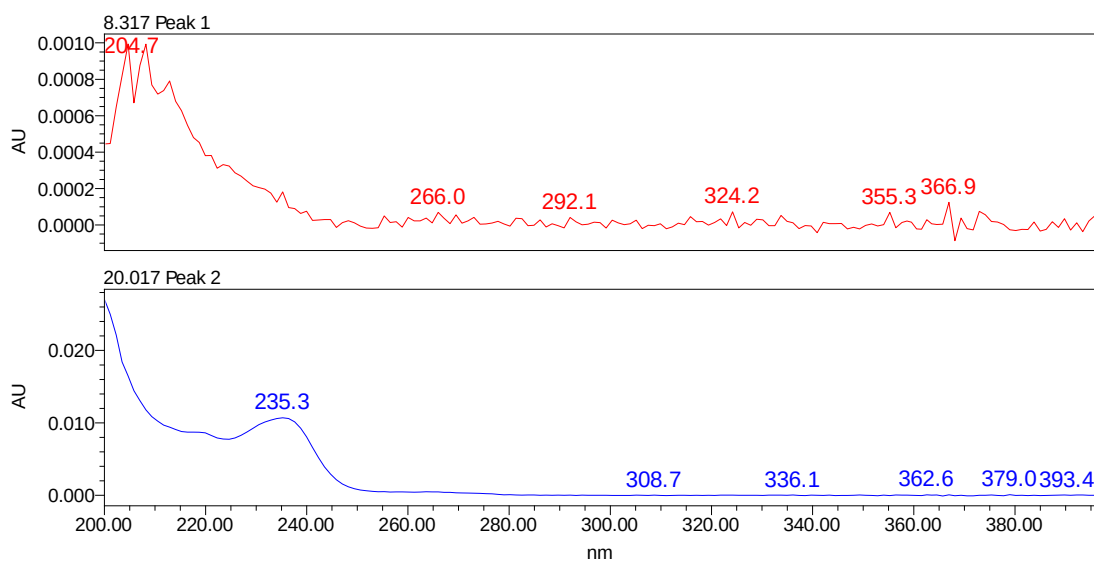


Figure A.14. Representative HPLC chromatogram of the lumbar segment of the spinal cord after the IT administration of CBD nanoemulsion in vivo. CBD retention time 8.32 mins and DDD (IS) 20.02 mins. UV spectra of both peaks associated for reference.

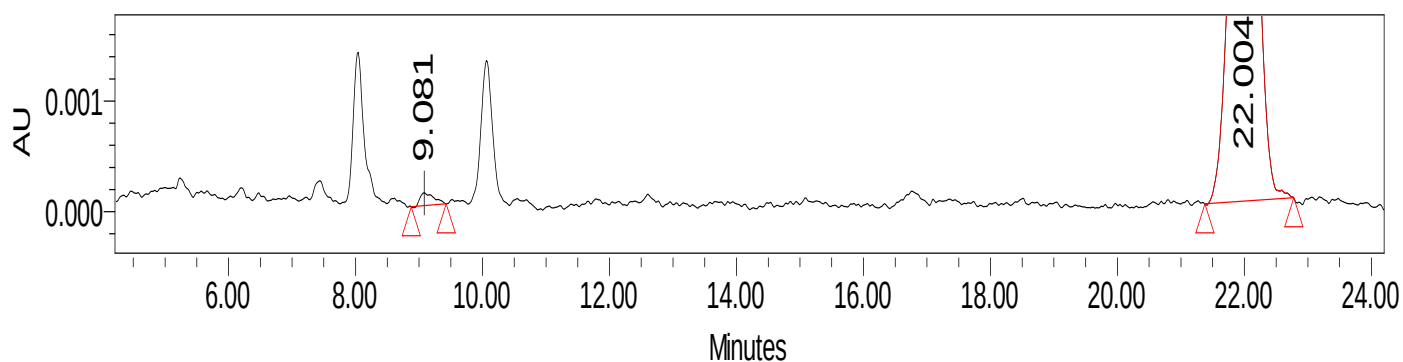


Figure A.15. Representative HPLC chromatogram of the thoracic segment of the spinal cord after the IT administration of CBD nanoemulsion in vivo. CBD retention time 9.08 mins and DDD (IS) 22.0 mins.

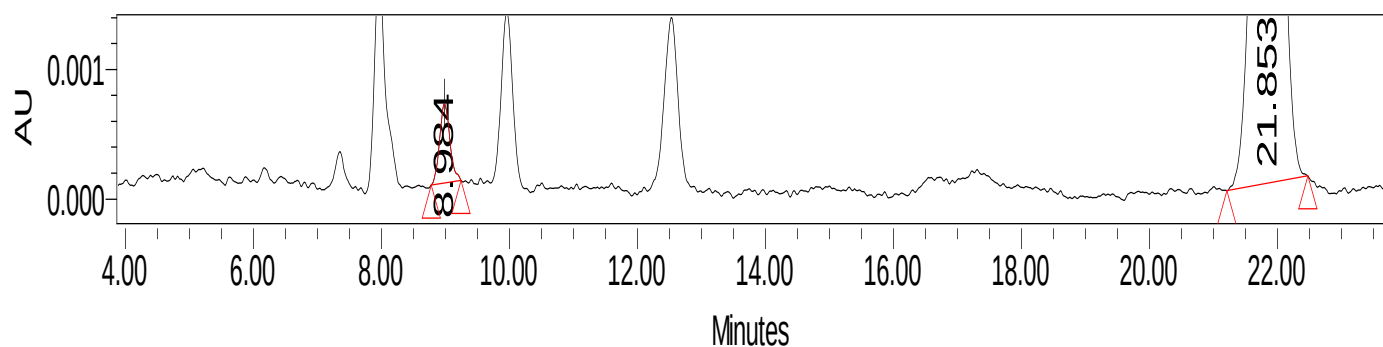


Figure A.16. Representative HPLC chromatogram of the cervical segment of the spinal cord after the IT administration of CBD nanoemulsion *in vivo*. CBD retention time 8.98 mins and DDD (IS) 21.85 mins.

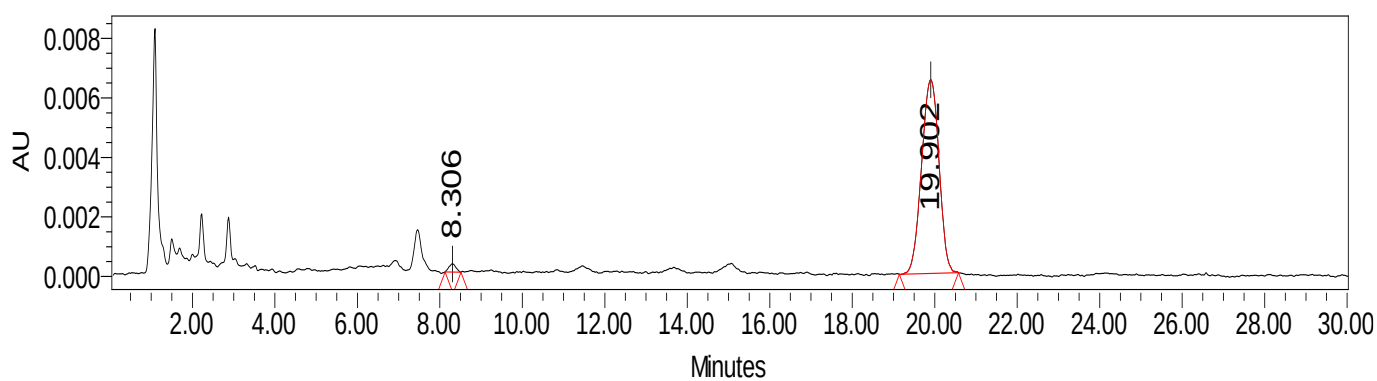


Figure A.17. Representative HPLC chromatogram of the brain after the IT administration of CBD nanoemulsion *in vivo*. CBD retention time 8.3 mins and DDD (IS) 19.9 mins.

A.14. Representative chromatograms for CBD detection in tissues **after administration of CBD PCNPs *in vivo***

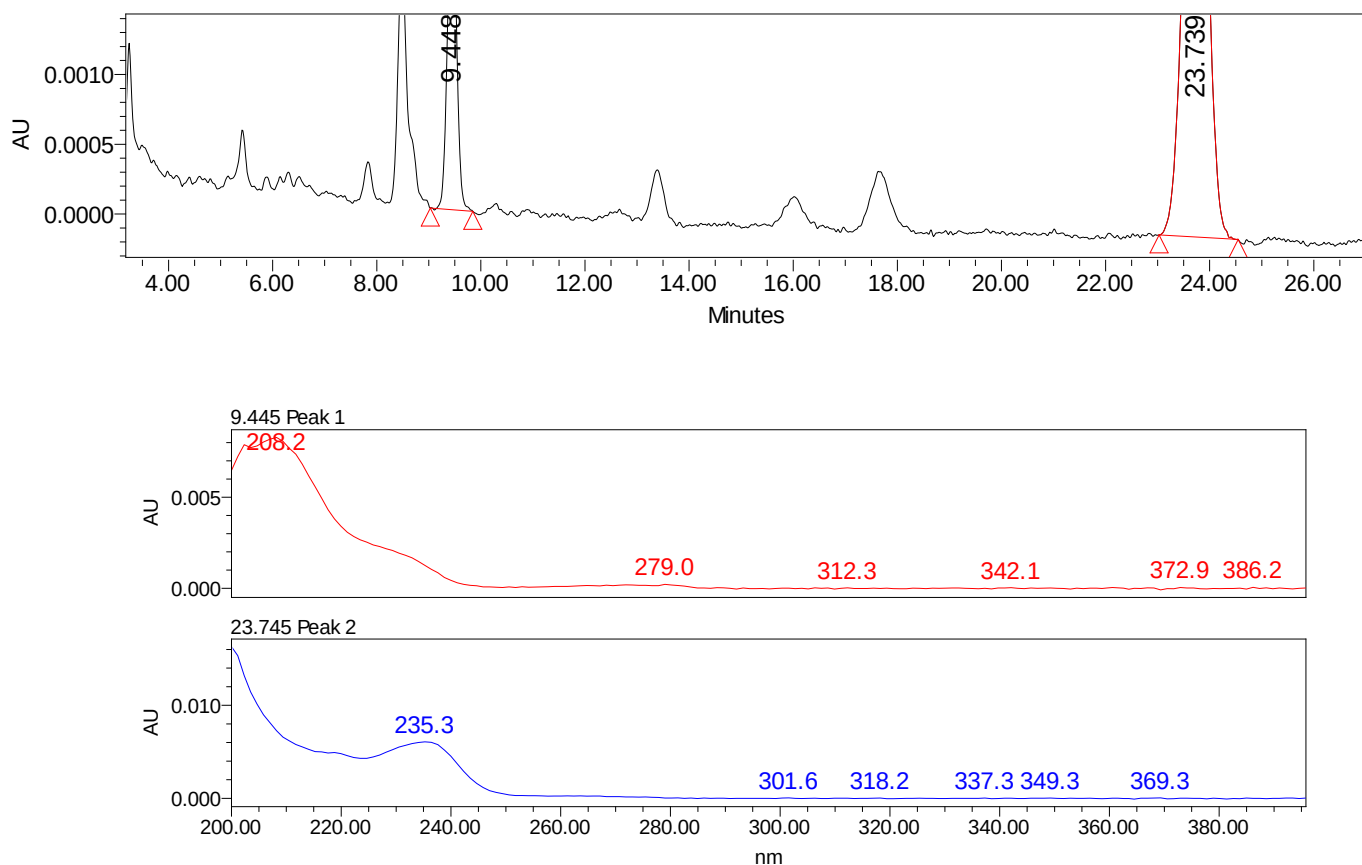


Figure A.18. Representative HPLC chromatogram of the lumbar segment of the spinal cord after the IT administration of CBD PCNPs *in vivo*. CBD retention time 9.44 mins and DDD (IS) 23.73 mins. UV spectra of both peaks associated for reference.

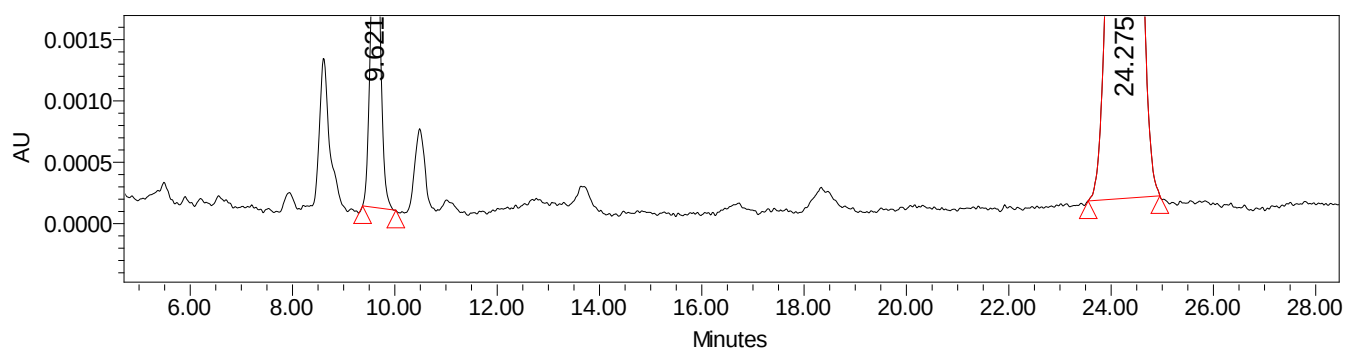


Figure A.19. Representative HPLC chromatogram of the thoracic segment of the spinal cord after the IT administration of CBD PCNPs in vivo. CBD retention time 9.62 mins and DDD (IS) 24.27 mins.

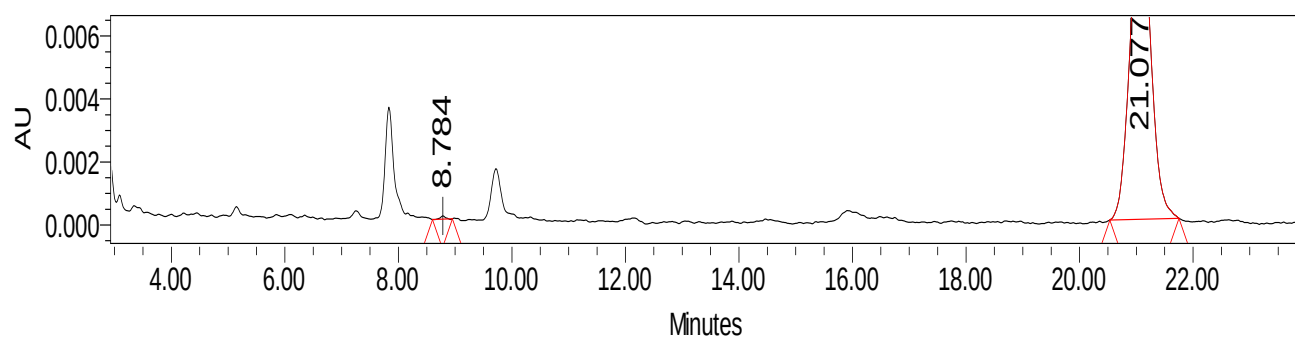


Figure A.20. Representative HPLC chromatogram of the cervical segment of the spinal cord after the IT administration of CBD PCNPs in vivo. CBD retention time 8.78 mins and DDD (IS) 21.07 mins.

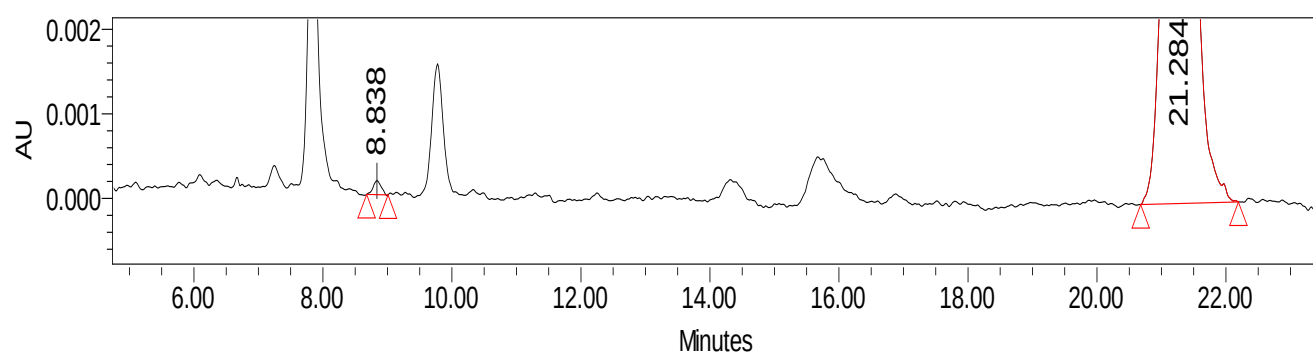
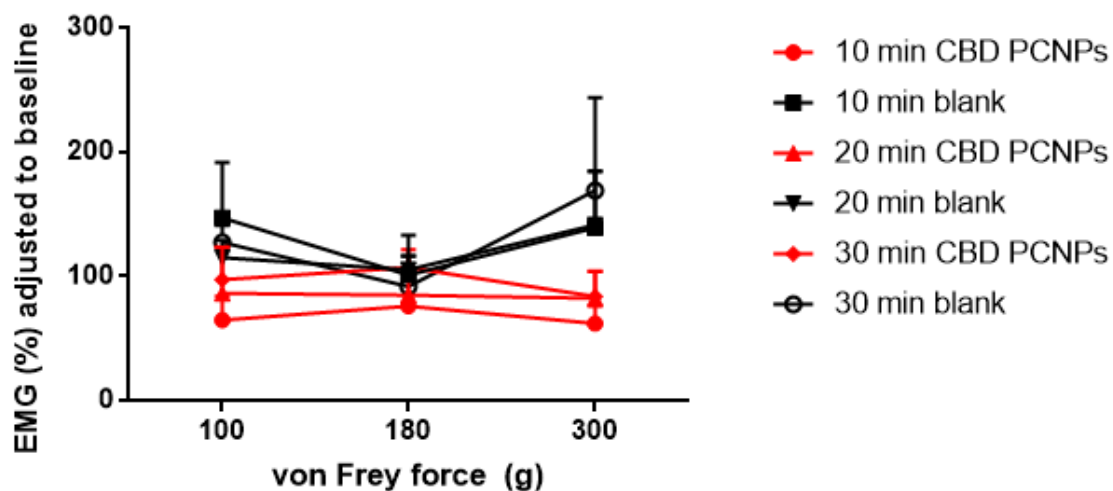


Figure A.21. Representative HPLC chromatogram of the brain after the IT administration of CBD PCNPs in vivo. CBD retention time 8.83 mins and DDD (IS) 21.28 mins.

A.15. EMG AUC data after administration of CBD formulations

EMG AUC data after administration of CBD PCNPs



EMG AUC data after administration of CBD nanoemulsion

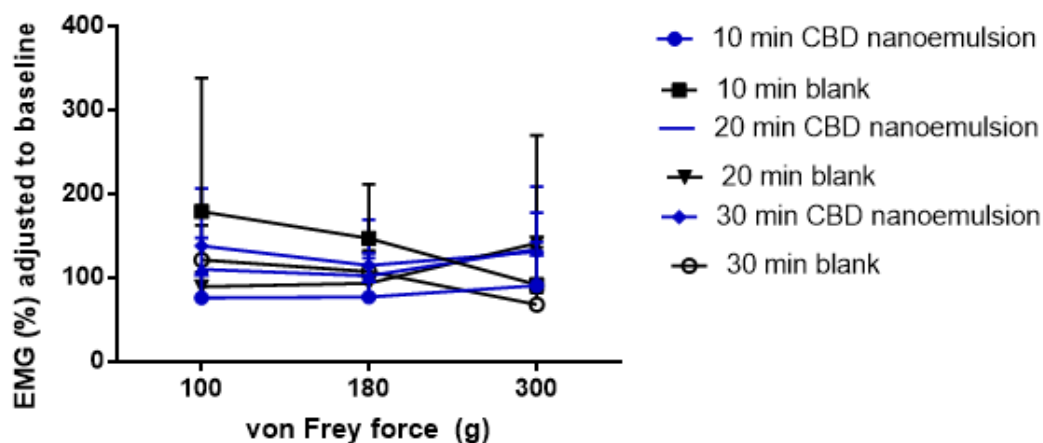


Figure A.22. The EMG AUC data used for the EMG plots. The AUC after the administration of each formulation (CBD NPs, blank NPs, CBD nanoemulsion or blank nanoemulsion) was calculated through the GraphPad software and plotted as percentage change.

Scientific outputs

Muresan. P, Woodhams. S, Smith. F, Taresco. V, Shah. J, Wong. S, Smith. S, Chapman. V, Hathaway. G, Rahman. R, Gershkovich. P, Marlow. M. Development and evaluation of cannabidiol nanoemulsion and nanoparticles for intrathecal administration to treat pain. JCR. **In submission**.2021.

Muresan. P*, McCrorie. P*, Smith. F, Vasey. C, Taresco. V, Scurr. D, Kern. S, Smith. S, Gershkovich. P, Rahman. R, Marlow. M. Development of nanoparticle loaded microneedles for drug delivery to a brain tumour resection site. Eur. J. Pharm. Sci. **Manuscript in preparation**. 2021.

Muresan. P. Development and evaluation of cannabidiol formulations for the treatment of pain. **Poster**. APS (online) 2021.

Muresan. P. Development and evaluation of cannabidiol formulations for the treatment of pain. **Presentation**. CRS. (Las Vegas, US – online) 2020.

Muresan. P. Development and evaluation of cannabidiol formulations for the treatment of pain. **Presentation**. UKICRS. (Liverpool, UK) 2020.

Muresan. P. Development and evaluation of cannabidiol formulations for the treatment of pain. **Poster**. The British Society for Nanomedicine (Liverpool, UK) 2019.

Muresan. P. Development and evaluation of cannabidiol formulations for the treatment of pain. **Poster**. Formulation Days (Lyon, France) 2019.

Sekhar. G, Georgian. A, Sanderson. L, Vizcay-Barrena. G, Brown. R, **Muresan. P**, Fleck. R, Thomas. S. Organic cation transporter 1 (OCT1) is involved in pentamidine transport at the human and mouse blood-brain barrier (BBB). PLoS ONE. 12(3): e017347. 2017.

*authors contributed equally

Covid-19 impact on doctoral research

I was in my 3rd year of my 4-year CDT course when the Covid-19 pandemic started, leading to the subsequent University closure. This has had an effect on the research data I generated, firstly as a result of the closure which impacted my experiments, and secondly as a result of the severe lab restrictions imposed following the opening of the research facilities.

I had planned to finish my first *in vivo* study testing the CBD nanoemulsion by June 2020, prior to my attendance of the CRS conference in the US. As a result of Covid-19, this study was only finalised in December 2020. Owing to the multi-disciplinary aspect of my PhD, re-starting my work required access to 3 different buildings at UoN (BSB, BDI, and QMC). Initially, intra-departmental work was not allowed, in order to minimise being in contact with too many people, thus further affecting my research. A summary of the impact COVID-19 pandemic had on my PhD is listed below:

- *In vivo* work was only restarted based on an approval system according to a list of priority projects (a few weeks delay before approval was granted by the BSU) – *in vivo* work approval was granted at the end of Sept 2020, with *in vivo* work procedures resuming at the end of Oct 2020
- *In vivo* lab occupancy limit of 2 people – due to collaborative aspect of this work, the project was not necessarily a priority within the Pain Group (QMC)
- Due to the lengthy University closure a refresher was required in regards to surgical techniques (trained with a postdoctoral fellow)
- 2 people required for surgeries (one person assisting needed to wear an airflow helmet, and due to excessive demand, the delivery of this equipment was delayed)
- Only allowed back in the lab in August 2020 (labs were not ready for research – i.e., no liquid nitrogen available)
- Initially the formulation labs were only open 8 am – 4 pm leading to shorter working days (no weekend work – impacting the use of the HPLC for longer runs of tissue samples)

- Access to instruments was only granted after Covid-19 risk assessments were submitted and reviewed (long wait times due to all students submitting at the same time)
- Various different labs were required for sample preparation (limited occupancy and instrument booking slots) thus slowing down work
- HPLC was needed to analyse tissues stored at -80 °C. Due to long closure time, numerous students required access to the instrument at the same time, thus leading to very long wait times to use the instrument
- Only 6 hrs a week was allocated to myself in BDI due to a limited lab occupancy and rota-based system
- Severe delays in ordering consumables (finance department approval of orders took 1 week, whereas approval used to be done daily before Covid-19 lockdown)
- Fixing broken instruments took significantly longer due to Finance Dept. not processing purchase orders in a timely manner

Additional points:

- Flat-mate was shielding so I had to assist with personal matters (picking up prescriptions, grocery shopping, etc.)
- Had COVID-19 in October 2020 and lost an additional 2 weeks lab work
- Graduate school CRS travel grant was not awarded due to circumstances